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TAXONOMIC DESCRIPTION

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Muricauda maritima sp. nov., *Muricauda aequoris* sp. nov. and *Muricauda oceanensis* sp. nov., three marine bacteria isolated from seawater

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Abstract

Three Gram-stain-negative, non-motile, rod-shaped strains, designated 72^T, NH166^T and 40DY170^T, were isolated from seawater samples of the West Pacific Ocean, South China Sea and West Pacific Ocean, respectively. The 16S rRNA gene sequence similarity results revealed that strains 72^{T}and NH166^T were most closely related to *Muricauda antarctica* Ar-22^T, *Muricauda taeanensis* JCM 17757^T, *Muricauda beolgyonensis* KCTC 23501^T, *Muricauda lutimaris* KCTC 22173^T and *Muricauda hadalis* MT-229^T with 97.2–98.0% sequence similarity. 16S rRNA gene sequence analysis also indicated that strain 40DY170^T was most closely related to *Muricauda ruestringensis* DSM 13258^T, *Muricauda aquimarina* JCM 11811^T, *Muricauda lutimaris* KCTC 22173^T and *Muricauda oceani* 501str8^T with 97.6–98.1% sequence similarity. The 16S rRNA gene sequence similarity values among strains 72^T, NH166^T and 40DY170^T were 96.5–99.2%. Phylogenetic analyses indicated that three new isolates represented three novel species by forming two distinctive lineages within the genus *Muricauda*. The DNA G+C contents of strain 72^T, NH166^T and 40DY170^T were 43.4, 43.4 and 42.4 mol%, respectively. The average nucleotide identity and *in silico* DNA–DNA hybridization values between strains 72^T, NH166^T, 40DY170^T and the reference strains were 76.5–93.5% and 19.2–53.5%, respectively. The sole respiratory quinone in all strains was menaquinone-6. Their major fatty acids were iso-$C_{17:0}$ 3-OH, iso-$C_{15:0}$ and iso-$C_{15:1}$ G. The major polar lipids of strains 72^T and NH166^T were phosphatidylethanolamine, one unidentified aminolipid and two unidentified lipids. The major polar lipids of strain 40DY170^T were phosphatidylglycerol, one unidentified phospholipid, one unidentified aminolipid and two unidentified lipids. On the basis of their distinct taxonomic characteristics, the three isolates represent three novel species of the genus *Muricauda*, for which the names *Muricauda maritima* sp. nov. (type strain 72^T=KCTC 62229^T=MCCC 1K03350^T), *Muricauda aequoris* sp. nov. (NH166^T=KCTC 62228^T=MCCC 1K03449^T) and *Muricauda oceanensis* sp. nov. (40DY170^T=KCTC 72200^T=MCCC 1K03569^T) are proposed.

The genus *Muricauda*, belonging to the family *Flavobacteriaceae* in the phylum *Bacteroidetes*, was first proposed by Bruns *et al.* with the type species *Muricauda ruestringensis* [1]. Subsequently, the genus was emended by Yoon *et al.* [2] and Hwang *et al.* [3]. At the time of writing (August 2020), the genus *Muricauda* consisted of 25 species with validly published names (www.bacterio.net/genus/muricauda) [4]. The genus has been isolated from marine and marine-derived habitats, including seawater [5–7], salt lake [2], coastal hot spring [8], tidal flat [9–11], sediment [1, 12, 13], shrimp gill [14] and sponge [15]. Members of the genus *Muricauda* are Gram-stain-negative, facultatively anaerobic or strictly aerobic [16]. The fatty acid profile is characterized by large amounts of branched and hydroxy fatty acids (i.e. iso-$C_{15:0}$, iso-$C_{15:1}$ G and iso-$C_{17:0}$ 3-OH). The DNA G+C contents are 37.6–51.3 mol% [12]. Menaquinone-6 (MK-6) is the major isoprenoid quinone. The aim of the present work was to determine the taxonomic position of strains 72^T, NH166^T

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Abbreviations: ANI, average nucleotide identity; DDH, DNA–DNA hybridization; MA, marine agar; MB, marine broth; MK-6, menaquinone-6.
The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 72^T, NH166^T and 40DY170^T are MF093149, MF093150 and MK318948, respectively. The GenBank accession numbers for the whole genome sequences of strain 72^T, strain NH166^T, *M. taeanensis* JCM 17757^T, *M. beolgyonensis* KCTC 23501^T, *M. lutimaris* KCTC 22173^T, strain 40DY170^T, *M. aquimarina* JCM 11811^T are QXFI00000000, QXFJ00000000, QXFO00000000, RZMY00000000, QXFH00000000, RZNA00000000 and RZMZ00000000, respectively.
Three supplementary figures and four supplementary tables are available with the online version of this article.

and 40DY170^T, which were isolated from the seawater of the West Pacific Ocean, South China Sea and West Pacific Ocean, respectively.

During a scientific investigation of marine bacterial diversity, seawater samples were collected from three different sites. The samples collected with a rosette sampler connected to a CTD system (SBE911 plus, Sea-Bird Electronics) were spread on modified G2216 marine agar plates (0.5 g peptone, 0.1 g yeast extract, 15 g agar and 1 l natural seawater; pH 7.2–7.4) with different materials added immediately on the ship. Aboard the ship, cultivation was done at room temperature (about 28 °C) immediately. Microbial growth was observed after 1 month. A yellow colony on the G2216 agar supplemented with 1 mM Zn^{2+} was picked out, designated as 72^T from water sample collected from the West Pacific Ocean in 2015 (at a depth of 75 m; 130° E, 16° N). A small colony appeared on the G2216 agar supplemented with 1% ornithine (w/v), designated as NH166^T, from the surface water sample collected from the South China Sea in 2014 (118.5° E, 19° N). Another small colony was obtained on the G2216 agar supplemented with 2 mM Cd^{2+}, designated as 40DY170^T, from the water sample collected from the West Pacific Ocean in 2016 (at a depth of 150 m; 158.9° E, 19.6° N). The three strains were purified on marine agar 2216 (MA; BD) by repeated restreaking for further research. The following eight strains were used as reference strains for comparative experiments. *Muricauda antarctica* Ar-22^T was available from our lab, *Muricauda ruestringensis* DSM 13258^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, *Muricauda taeanensis* JCM 17757^T and *Muricauda aquimarina* JCM 11811^T were obtained from the Japan Collection of Microorganisms, *Muricauda beolgyonensis* KCTC 23501^T and *Muricauda lutimaris* KCTC 22173^T were obtained from the Korean Collection for Type Culture, and *Muricauda oceani* 501str8^T and *Muricauda hadalis* MT-229^T were obtained from Chen's [17] and Fang's labs [18], respectively. All strains were cultured in marine broth 2216 (MB; BD) or on MA at 30 °C and preserved at −80 °C with 25% (v/v) glycerol for long-term storage. Unless otherwise stated, cells grown to the exponential phase on MA or MB at 30 °C were used for the physiological tests.

Cell morphology, ultrastructure, size and presence of flagellum were observed by transmission electron microscopy (JEM-1230, JEOL) using cells grown on MA to the late exponential phase. Cell motility was assessed using a semi-solid stab-culture method with incubation at 30 °C for 2 weeks [19]. The temperature range for growth was tested on MA at 4, 10–45 °C (at 5 °C intervals). The pH range for growth was measured in MB adjusted to pH 5.0–10.0 (at 0.5 pH unit intervals) by using the following buffers (at a concentration of 50 mM): MES (pH 5.0–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5) and CAPSO (pH 9.0–10.0) as described by Huang *et al.* [20]. Tolerance to NaCl (0, 0.5, 1.0, 3.0, 5.0, 7.5, 10.0 and 15.0%, w/v) for growth was determined in NaCl-free MB (prepared according to the MB formula, but without Na^+). Requirement of sea salts (0, 0.5, 1.0, 2.0, 3.0, 4.0 4.5 and 5.0 %, w/v; Sigma) was assessed in PY medium (5.0 g peptone, 1.0 g yeast extract and 1 l distilled water; pH 7.2). Optimal cell growth in liquid cultures, under the above various growth conditions, was evaluated by measuring turbidity (OD_{600}) with a UV/visible spectrophotometer (Ultrospec 6300 Pro, Amersham Biosciences) over 3 days' incubation. Upper and lower limits for growth were confirmed when no visible turbidity was observed after 1 month's incubation. To study anaerobic growth ability, sodium nitrate (20 mM) or sodium nitrite (20 mM) was added to MA as a potential electron acceptor with an AnaeroPack (Mitsubishi) to create an anaerobic environment.

Oxidase activity was determined by using the oxidase reagent (bioMérieux), and the result was read according to the manual. Degradation of starch (1.0%, w/v), catalase activities and hydrolysis of Tweens 20, 40 and 80 (0.5%, w/v) were tested according to Dong and Cai *et al.* [21]. Biochemical properties including hydrolysis of aesculin and other enzyme activities were tested using API 20NE and API ZYM (bioMérieux) systems according to the manuals with the addition of 2.0% sea salts (w/v; Sigma) solution for suspension. The pigment absorption spectrum analysis was performed by the methods described by Rainey *et al.* [22] and Hildebrand *et al.* [23], using a Beckman DU800 spectrophotometer (absorption spectrum from 300 to 1000 nm). To detect the utilization of single carbon sources, growth was tested when different organic substrates were added in the basic modified medium (BM) [24] including 0.2% (w/v) sugars or 0.2% (w/v) alcohols, 0.1% (w/v) organic acids or 0.1% (w/v) amino acids. Acid production from the carbohydrates was examined in MOF medium [25] with 1.0% sugars or alcohols supplemented. The utilization of carbohydrates or acid production was observed 1 week after inoculation in the tubes. Susceptibility to antibiotics on MA was tested using the following antibiotic discs (μg per disc unless stated otherwise): amoxicillin (20), ampicillin (10), bacitracin (0.04 IU), carbenicillin (100), cephalexin (30), chloroamphenicol (30), erythromycin (15), gentamycin (10), kanamycin (30), novobiocin (30), nystatin (30), penicillin G (10 U), rifampicin (5), streptomycin (10) and vancomycin (30). The results were read as described by Guo *et al.* [26]. Heavy metal tolerance was studied on MA supplemented with different concentrations of Mn^{2+} (0, 1, 2, 5, 15, 20, 50, 100, 200 and 800 mM), Co^{2+} (0, 0.25, 0.5, 0.75, 1, 2, 4, 5, 10, 15 and 30 mM), Cd^{2+} (0, 0.25, 0.5, 1, 2, 3, 5 and 10 mM), Ni^{2+} (0, 0.25, 0.5, 0.75, 1, 2, 4, 5, 10, 15 and 30 mM) or Zn^{2+} (0, 0.25, 0.5, 1, 2, 3, 5, 10, 15 and 20 mM). Eight reference strains were used for parallel comparisons under identical conditions in the above tests.

For cellular fatty acid analysis, cells of strains 72^T, NH166^T, 40DY170^T and eight reference strains were harvested from quadrant three (at the late-exponential phase) along the streaking axis after cultivation of 3 days at 30 °C on MA plates according to the instructions of the Microbial Identification System (MIDI). The physiological age of the cells was standardized by observing the growth development of colonies on the plates by harvesting them from the same quadrant on the agar plates. After being saponified, methylated and extracted following the standard MIDI protocol (Sherlock Microbial

Identification System, version 6.1) from the biomasses, the fatty acids were analysed and identified using the RTSBA6 database of the Microbial Identification System [27]. To confirm the composition of isoprenoid quinone and lipids, cells of strains 72^T, NH166^T and 40DY170^T were collected from the cultures in 500 ml flasks containing 200 ml MB medium at 30 °C respectively at the exponential phase. Quinones were extracted by the method of Minnikin *et al.* [28], separated on GF254 silica gel plates (Qingdao Haiyang Chemical Co. Ltd.) and identified by an HPLC-MS system (Agilent 1200 and Thermo Finnigan LCQ DECA XP MAX mass spectrometer). Polar lipids were extracted according to the procedure described by Minnikin *et al.* [28], and separated by two-dimensional TLC using chloroform–methanol–water (65:25:3.8, by vol.) for the first dimension and chloroform–methanol–acetic acid–water (40:7.5:6:1.8, by vol.) for the second dimension on silica gel 60 F254 plates (Merck). The further detection and identification of lipids was performed by spraying with the appropriate detection reagents [29].

High-quality genomic DNA was obtained by the Bacterial Genomic DNA Fast Extraction kit (Dongsheng Biotech). The 16S rRNA gene was amplified using the universal primers 27F (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The PCR products were sequenced and the almost-complete 16S rRNA gene sequences of strain 72^T, NH166^T and 40DY170^T were compared with closely related sequences of reference organisms via EzBioCloud's database (www.ezbiocloud.net) and the NCBI-nr database (www. ncbi.nlm.nih.gov) [30]. The genomes of the three novel isolates, as well as those of *M. taeanensis* JCM 17757^T, *M. beolgyonensis* KCTC 23501^T, *M. lutimaris* KCTC 22173^T and *M. aquimarina* JCM 11811^T were sequenced by Illumina PE150 sequencing technology with the HiSeq platform (Beijing Genomics Institute Co. Ltd, Wuhan, PR China). The *de novo* assembly of the reads was performed using ABySS 1.5.2 [31]. The quality of microbial genomes was assessed using CheckM [32]. The genome sequences of *M. antarctica* Ar-22^T (FRAT00000000), *M. ruestringensis* DSM 13258^T (CP002999), *M. oceani* 501str8^T (CP049616) and *M. hadalis* MT-229^T (VNIK00000000) were retrieved from the GenBank database. The genome was annotated by Prokka and the RAST server [33, 34]. Average nucleotide identity (ANI) values were calculated using the OrthoANIu algorithm of the ChunLab's online ANI calculator [35]. *In silico* DNA–DNA hybridization (DDH) values were calculated by the Genome-to-Genome Distance Calculator [36]. Comparative genomic analysis was performed as previously described [37, 38]. Orthologous clusters (OCs) were identified by comparing whole protein sequences translated from coding genes (CDSs) pairwise with execution of Proteinortho version 5. 16b [39]. Protein sequences of filtered single-copy OCs were performed in the phylogenomic analyses. A maximum-likelihood phylogenomic tree was reconstructed based on the whole-genome nucleotide sequences of *Muricauda* species using IQ-TREE 1.6.1 software [40] with the bootstrap value set to 100 replicates [41]. *Bacteroides fragilis* ATCC 25285^T was used as an outgroup.

The complete sequence of the 16S rRNA gene was annotated via the RNAmmer 1.2 Server [42]. CLUSTAL_W software was used to align the corresponding sequences [43]. Based on the above alignment results, 25 *Muricauda* species were selected for phylogenetic analysis and *B. fragilis* ATCC 25285^T was used as an outgroup. A phylogenetic tree was reconstructed with the neighbour-joining method [44] using the MEGA 7.0 program package [45], and a matrix distance was calculated using a Kimura two-parameter correction [46]. Maximum-likelihood [47] and minimum-evolution methods [48] were also used. A bootstrap analysis was performed with 1000 resamplings for assessing the reliability of tree topology [46].

Cells of strains 72^T, NH166^T and 40DY170^T were Gram-stain-negative, obligately aerobic, non-spore-forming, non-motile and rod-shaped. Cells of strain 72^T were 0.2–0.4 μm wide and 1.3–2.9 μm long, cells of strain NH166^T were 0.2–0.3 μm wide and 1.1–2.5 μm long, and cells of strain 40DY170^T were 0.2–0.8 μm wide and 1.2–3.8 μm long (Fig. S1, available in the online version of this article). No flagellum was observed. Colonies of the three novel isolates on MA plate were yellow, circular, convex, smooth and approximately 2 mm in diameter after incubation for 3 days at 30 °C. Strains 72^T, NH166^T and 40DY170^T did not produce flexirubin-type pigment, but showed the presence of carotenoid-like pigments characterized by maximal absorption at 450 nm. Strains 72^T, NH166^T and 40DY170^T could grow at pH 5.5–9.0 (optimum, pH 6.5–7.0), pH 5.5–8.5 (optimum, pH 6.5–7.0) and pH 5.5–8.5 (optimum, pH 6.5–7.0), respectively. Strains 72^T, NH166^T and 40DY170^T could grow at 15–40 °C (optimum, 35 °C), 15–40 °C (optimum, 35 °C) and 10–40 °C (optimum, 25 °C), respectively. No growth occurred at 4 or 45 °C after incubation of 1 month. Strains 72^T, NH166^T and 40DY170^T grew in the presence of 0.5–10.0% NaCl (w/v; optimum, 1.0%), 0.5–7.5% NaCl (w/v; optimum, 3.0%) and 0.5–10.0 % NaCl (w/v; optimum, 3.0%), respectively. Sea salts were necessary for growth. Strains 72^T, NH166^T and 40DY170^T grew in the presence of 1.0–5.0 % sea salts (w/v; optimum, 2.0%), 0.5–5.0% sea salts (w/v; optimum, 2.0%) and 0.5–5.0% sea salts (w/v; optimum, 4.0–4.5%), respectively. Strains 72^T and NH166^T were both susceptible to erythromycin, novobiocin and vancomycin, resistant to bacitracin, gentamycin, kanamycin, nystatin, penicillin G and streptomycin. Strain NH166^T was susceptible to amoxicillin, ampicillin and rifamcin, but strain 72^T was resistant to all of them. Strain 40DY170^T was susceptible to amoxicillin, cephalexin, chloroamphenicol, erythromycin, novobiocin, penicillin G, rifampicin and vancomycin, resistant to ampicillin, bacitracin, gentamycin, kanamycin, nystatin and streptomycin. Detailed phenotypic characteristics are given in the species description and Table 1.

The three new strains were able to grow on MA containing high concerntrations of heavy metals, such as Mn^{2+} (20 mM), Co^{2+} (1 mM), Cd^{2+} (3 mM), Ni^{2+} (5 mM) and Zn^{2+} (5 mM) for strain 72^T, Mn^{2+} (50 mM), Co^{2+} (0.75 mM), Cd^{2+} (0.5 mM), Ni^{2+} (10 mM) and Zn^{2+}(5mM) for strain NH166^T, and Mn^{2+} (20 mM), Co^{2+} (2mM), Cd^{2+} (5 mM), Ni^{2+} (5 mM) and Zn^{2+}(5 mM) for strain 40DY170^T (Table S1). The resistance of strains 72^T and 40DY170^T to Cd^{2+}

Table 1. Differential characteristics of strains 72^T, NH166^T, 40DY170^T and the type strains of *Muricauda* species

Strains: 1, 72^T; 2, NH166^T; 3, 40DY170^T; 4, *M. antarctica* Ar-22^T; 5, *M. taeanensis* JCM 17757^T; 6, *M. beolgyonensis* KCTC 23501^T; 7, *M. lutimaris* KCTC 22173^T; 8, *M. aquimarina* JCM 11811^T; 9, *M. ruestringensis* DSM 13258^T; 10, *M. oceani* 501str8^T; 11, *M. hadalis* MT-229^T. All results were obtained from this study under identical growth conditions, except where indicated otherwise. All species are negative for hydrolysis of Tween 40, and positive for catalase and hydrolysis of aesculin. Based on the API ZYM results, acid and alkaline phosphatases, α-chymotrypsin, cystine arylamidase, α-glucosidase, α-glucuronidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase activities are present, whereas α-fucosidase activity is absent. All strains use D-glucose, lactose, maltose, D-mannose, *N*-acetyl-glucosamine, raffinose, sucrose and trehalose as carbon resources, but not capric acid and trisodium citrate; acid is not produced from inositol and D-mannitol; all species are susceptible to erythromycin and novobiocin, negative for bacitracin, gentamicin, kanamycin, nystatin and streptomycin. +, Positive; –, negative; W, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Colony colour	Yellow	Yellow	Yellow	Cream*	Cream†	Yellow‡	Yellow§	Yellow‖	Yellow¶	Yellow**	Beige††
Temperature for growth (°C):											
Range	15–40	15–40	10–40	15-37*	15–40†	10–40‡	10–38§	10–44‖	8–40¶	8–42**	10–40††
Optimum	35	35	25	35*	30–37†	37‡	30§	30–37‖	20–30¶	25–30**	30††
pH for growth:											
Range	5.5–9.0	5.5–8.5	5.5–8.5	5.4–8.4*	5.5–9.0†	ND	ND	ND	6.0–8.0¶	5.5–9.0**	2.0–11.0††
Optimum	6.5–7.0	6.5–7.0	6.5–7.0	7.5*	7.0–7.5†	7.0–7.5‡	7.0–8.0§	7.0‖	6.0–7.5¶	7.0**	6.0††
NaCl for growth (%, w/v):											
Range	0.5–10.0	0.5–7.5	0.5–10.0	ND	1.0–8.0†	0.5–8.0‡	ND	ND	0.5–9.0¶	0.5–10.0**	0–17††
Optimum	1.0	3.0	3.0	1.0–3.0*	2.0–4.0†	2.0–3.0‡	ND	2.0‖	3.0¶	3.0**	11.0††
Hydrolysis of:											
Gelatin	W	–	–	+	+	+	+	–	+	–	–
Starch	+	–	W	–	–	–	–	–	–	–	–
Tween 20	+	+	+	+	+	–	+	+	+	+	+
Tween 80	+	+	+	–	–	–	–	+	–	–	–
Urease	–	–	–	–	+	–	–	–	–	+	–
Enzyme activities:											
Arginine dihydrolase	–	+	–	+	+	+	–	+	–	+	+
Esterase (C4)	+	W	+	W	W	W	+	+	+	+	+
Esterase lipase (C8)	+	+	+	W	W	+	+	+	+	W	W
α-Galactosidase	+	+	+	+	+	W	W	+	+	+	+
β-Galactosidase	+	+	+	+	+	+	–	+	+	+	+
β-Glucosidase	+	+	–	+	+	+	–	–	–	+	+
β-Glucuronidase	–	–	+	–	–	+	+	+	+	–	–
Indole production	–	–	–	–	–	–	–	+	–	–	–
Lipase (C14)	+	+	–	+	+	–	–	–	–	W	W
α-Mannosidase	+	+	+	+	+	+	–	+	+	+	+
Oxidase	+	W	+	W	+	+	+	+	+	+	+
Reduction of nitrates to nitrites	+	+	–	+	+	+	–	+	W	–	–
Assimilation of:											
Adipic acid	+	W	–	+	W	W	–	–	–	–	–

Continued

Table 1. Continued

Characteristic	1	2	3	4	5	6	7	8	9	10	11
L-Arabinose	+	–	+	+	+	–	+	–	+	+	+
Cellobiose	+	+	+	+	+	+	–	+	+	+	+
D-Galactose	+	+	+	+	+	+	–	+	+	–	+
Fermentation (glucose)	–	–	+	+	+	+	+	+	+	+	+
Malate	+	+	+	–	–	+	+	–	+	–	–
D-Mannitol	–	w	w	–	–	–	–	–	w	–	–
Phenylacetic acid	+	+	w	+	+	w	–	–	–	–	–
Potassium gluconate	+	w	–	w	–	w	–	–	–	–	–
Acid production from:											
Cellobiose	+	+	+	+	+	+	–	+	+	–	–
D-Galactose	+	+	+	+	+	+	–	+	+	–	–
D-Glucose	+	+	+	+	+	+	–	+	+	–	–
Lactose	+	w	+	+	+	+	+	+	+	–	–
Maltose	+	+	+	+	+	+	–	+	+	–	–
D-Mannose, raffinose, sucrose	+	+	+	+	+	+	+	+	+	–	–
Susceptibility to (μg per disc):											
Amoxicillin (20)	–	w	+	–	+	–	–	–	–	–	–
Ampicillin (10)	–	+	–	–	w	–	–	–	–	–	–
Cefalexin (30)	w	+	+	w	+	–	+	–	–	+	–
Chloramphenicol (30)	+	w	+	w	w	+	+	+	w	+	-
Penicillin G (10 U)	–	–	+	–	–	–	–	–	–	+	–
Rifamcin (5)	–	+	+	w	w	+	+	+	+	+	+
Vancomycin (30)	+	+	+	w	+	+	+	+	–	+	w
DNA G+C content (mol%; by genome)	43.4	43.4	42.4	45.2	45.6	43.7	40.2	43.5	41.4	42.8	45.6

*Data was taken from Wu *et al*. [5].
†Data was taken from Kim *et al*. [11].
‡Data was taken from Lee *et al*. [9].
§Data was taken from Yoon *et al*. [10].
||Data was taken from Yoon *et al*. [2].
¶Data was taken from Bruns *et al*. [1].
**Data was taken from Dong *et al*. [17].
††Data was taken from Zhang *et al*. [18].

(3 mM and 5 mM, respectively) is strikingly higher than that of the reference strains (0-1 mM). Strain NH166^T tolerated Ni^{2+} concentrations up to 10 mM on MA plates. None of the reference strains grew on plates containing more than 1 mM Cd^{2+} or 5 mM Ni^{2+}.

The complete 16S rRNA gene sequences of the three novel isolates were obtained from their whole genome sequences. The 16S rRNA gene sequences obtained from the whole genome sequences were identical to the gene sequences obtained from PCR. Based on the NCBI BLAST service and CLUSTAL_W results, strains 72^T(1506 nt) and NH166^T (1506 nt) were most closely related to *M. antarctica* Ar-22^T, *M. taeanensis* JCM 17757^T, *M. beolgyonensis* KCTC 23501^T, *M. lutimaris* KCTC 22173^{T}and *M. hadalis* MT-229^T with 97.2–98.0% sequence similarity. Strain 40DY170^T (1514 nt) was most closely related to *M. ruestringensis* DSM 13258^T, *M. aquimarina* JCM 11811^T, *M. lutimaris* KCTC 22173^T and *M. oceani* 501str8^T with 97.6–98.1% sequence similarity.

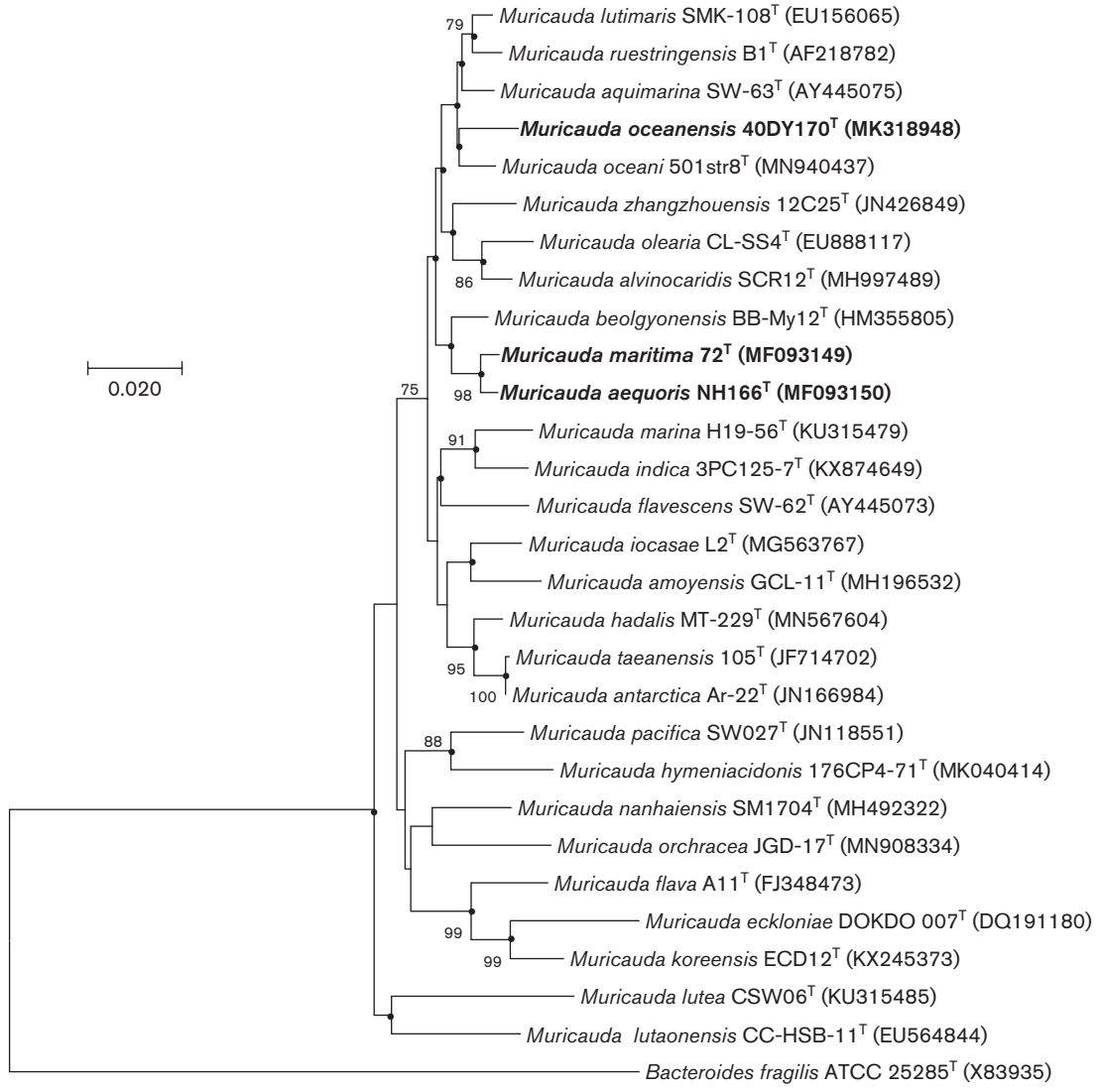

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic relationships of the isolates and related taxa. Bootstrap values (>70%) based on 1000 replications are shown at branch nodes. Dots indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and minimum-evolution algorithms. Bar, 0.02 substitutions per nucleotide position.

The 16S rRNA gene sequence similarity among strains 72^T, NH166^T among 40DY170^T was 96.5–99.2% (Table S2). The topologies of the neighbour-joining, maximum-likelihood and maximum-evolution phylogenetic trees indicated that the genus *Muricauda* formed a monophyletic clade. Strains 72^T and NH166^T were clustered with *M. beolgyonensis* KCTC 23501^T together, and 40DY170^T clustered within the clade containing *M. ruestringensis* DSM 13258^T, *M. aquimarina* JCM 11811^T, *M.lutimaris* KCTC 22173^T and *M. oceani* 501str8^T (Fig. 1). Phylogenetic analysis indicated that strains 72^T, NH166^T and 40DY170^T represented three novel members of the genus *Muricauda*.

The genome completeness of strains 72^T, NH166^T and 40DY170^T were 99.0, 99.5 and 99.3%, with contamination percentages of 1.7, 2.0 and 0.5%, respectively. Genome sequences estimated to be ≥95% completeness, with ≤5% contamination, are considered as excellent reference genomes for deeper analyses [32]. The draft genome sequences of strains 72^T, NH166^T and 40DY170^T were deposited into the NCBI with accession numbers QXFI00000000, QXFJ00000000 and RZNA00000000. The general features of strains 72^T, NH166^T, 40DY170^T, *M. taeanensis* JCM 17757^T, *M. beolgyonensis* KCTC 23501^T, *M. lutimaris* KCTC 22173^T and *M. aquimarina* JCM 11811^{T}are displayed in Table S3. The DNA G+C contents of strains 72^T, NH166^T and 40DY170^T by genome data were 43.4, 43.4 and 42.4 mol%, respectively, values in the range reported for *Muricauda* type strains, i.e. 37.6–51.3 mol% [12]. The ANI values and *in silico* DDH

(the recommended results from formula 2) between strains 72^T, NH166^T, 40DY170^T and the reference strains were 76.5–93.5 and 19.2–53.5%, which are strikingly lower than the threshold values of the species boundary (ANI 94–96% and *in silico* DDH 70%) [49, 50] (Table S4). These values indicated that strains 72^T, NH166^T and 40DY170^T represented three novel *Muricauda* species. The phylogenomic tree showed that they represented three novel species by forming two distinctive lineages within the genus *Muricauda*. This result supports that the three new isolates represent three species-level taxa in agreement with the result of 16S rRNA gene phylogeny (Fig. S2).

Genomic data indicated that the three new isolates contained dozens of genes related to heavy metal resistance. The genome of the three new isolates encode operons of *czcCBA* which can transport metal ions to the extracellular space. CzcA is an inner membrane protein, which acts as a cationic reverse transporter and proton pump. CzcB is a membrane fusion protein with an ion-binding subunit, which can facilitate the transport of metal ion. CzcC is an outer membrane protein, which determines the specificity of the binding metal substrate [51]. In addition, the genome of the three new isolates encode *czcD* genes which act as chemiosmotic ion-proton exchangers and transcriptional regulator gene of the MerR family. The MerR family is a group of transcriptional activators with similar N-terminal helix-turn-helix DNA binding regions and C-terminal effector binding regions that are specific to the effector recognized, which regulates expression of metal resistance operons [52].

The sole respiratory quinone found in three novel isolates was MK-6, which is a typical characteristic of the genus *Muricauda* [13]. Fatty acid analysis revealed that strains 72^T, NH166^T and 40DY170^T contained high values of iso-$C_{17:0}$ 3-OH (29.8–34.7%), iso-$C_{15:0}$ (19.2–19.9 %) and iso-$C_{15:1}$ G (13.5–20.1%) as the predominant fatty acids (Table 2). Interestingly, strains 72^T, NH166^T and 40DY170^T contained high percentages of iso- branched fatty acids (80.7–85.9%), which was similar to the reference strains (75.2–86.7%). The polar lipid profile of strain 72^T mainly consisted of phosphatidylethanolamine, one unidentified aminolipid (AL1), one unidentified glycolipid (GL1), two unidentified lipids (L1–2), and moderate or minor amounts of three unidentified aminolipids (AL2–4) and eight unidentified lipids (L3–10) were also detected (Fig. S3). The polar lipid profile of strain NH166^T mainly consisted of phosphatidylethanolamine, one unidentified aminolipid (AL1), one unidentified glycolipid (GL1), two unidentified lipids (L1–2), and moderate or minor amounts of three unidentified aminolipids (AL2–4) and 11 unidentified lipids (L3-14) were detected (Fig. S3).The polar lipid profile of strain 40DY170^T consisted of phosphatidylethanolamine, two unidentified lipids (L1–2), one unidentified aminolipid (AL1) and one unidentified phospholipid (PL1). The major polar lipids detected in strains 72^T, NH166^T and 40DY170^T were phosphatidylethanolamine, one unidentified aminolipid (AL1) and two unidentified lipids (L1–2), which was in common with those of the reference strains, respectively (Fig. S3).

The chemotaxonomic results also showed some clear differences in major fatty acid compositions (Table 2) and polar lipid profiles (Fig. S3) among the three novel strains and the type strains of the *Muricauda* species. The amount of iso-$C_{15:0}$ (19.5–19.9 %) was higher in strains 72^T and NH166^T than in the reference strains (12.7–16.5%). The amount of iso-$C_{15:1}$ G (13.5–14.9%) was lower in strains 72^T and NH166^T than five reference strains *M. antarctica* Ar-22^T, *M. taeanensis* JCM 17757^T, *M. beolgyonensis* KCTC 23501^T, *M. lutimaris* KCTC 22173^T and *M. hadalis* MT-229^T (16.4–24.3%). The amount of iso-$C_{17:0}$ 3-OH (34.7%) in strain 40DY170^T was higher than in the four reference strains *M. lutimaris* KCTC 22173^T, *M. ruestringensis* DSM 13258^T, *M. aquimarina* JCM 11811^T and *M. oceani* 501str8^T (27.6–32.9%), respectively. Moderate or minor amounts of eight unidentified lipids (L3–10) and four unidentified aminolipids (AL1–4) were present in strains 72^T and NH166^T, which were not found in four reference strains *M. antarctica* Ar-22^T, *M. taeanensis* JCM 17757^T, *M. beolgyonensis* KCTC 23501^{T}and *M. lutimaris* KCTC 22173^T (Fig. S3) [5, 9–11]. In addition, L7–9 and GL1 present in strain 72^T and NH166^T were not found in *M. hadalis* MT-229^T [18]. A moderate account of phospholipid (PL1) was found in 40DY170^T and *M. oceani* 501str8^T, but not in reference strains *M. aquimarina* JCM 11811^T, *M. lutimaris* KCTC 22173^T and *M. ruestringensis* DSM 13258^T. Three unidentified aminolipids (AL2–4) were found in moderate or minor amounts in *M. lutimaris* KCTC 22173^T, *M. ruestringensis* DSM 13258^T, *M. aquimarina* JCM 11811^T and *M. oceani* 501str8^T (Fig. S3) [17], which were not present in strain 40DY170^T. The three strains could also be distinguished from the type strains of the *Muricauda* species by different phenotypic characteristics such as growth temperature, pH, NaCl range and optimum, hydrolysis of starch, enzyme activities, carbohydrate utilization, acid production, and sensitivity to antibiotics (Tables 1 and S1).

Interestingly, even though strains 72^T and NH166^T were highly homogeneous showing the same phylogenetic and chemotaxonomic characteristics, many differences were found between them based on this study (Tables 1, 2, S1 and S4). For example, strain NH166^T had the ability to use adipic acid, D-mannitol and potassium gluconate as a single carbon resource and was weakly sensitive to amoxicillin and chloramphenicol, which was different from strain 72^T (Table 1). Moreover, some differences in the composition of fatty acids could distinguish the two strains (Table 2). $C_{18:0}$, iso-$C_{14:0}$ and iso-$C_{16:0}$ were only detected in one strain for the two novel isolates. In addition, strain NH166^T had four unidentified lipids (L11–14), which were not found in strain 72^T. ANI and *in silico* DDH values between strains 72^T and NH166^{T}were 93.5 and 53.5%, respectively. On the basis of the phylogenetic analysis, genomic data and chemotaxonomic results, as well as phenotypic characteristics, strains 72^T, NH166^T and 40DY170^T represent three novel species of the genus *Muricauda* [53], for which the names *Muricauda maritima* sp. nov., *Muricauda aequoris* sp. nov. and *Muricauda oceanensis* sp. nov are proposed.

Table 2. Comparison of cellular fatty acid compositions of strain 72^T, NH166^T, 40DY170^T and the type strains of *Muricauda* species

Strains: 1, 72^T; 2, NH166^T; 3, 40DY170^T; 4, *M. antarctica* Ar-22^T; 5, *M. taeanensis* JCM 17757^T; 6, *M. beolgyonensis* KCTC 23501^T; 7, *M. lutimaris* KCTC 22173^T; 8, *M. aquimarina* JCM 11811^T; 9, *M. ruestringensis* DSM 13258^T. 10, *M. oceani* 501str8^T; 11, *M. hadalis* MT-229^T. All results were obtained from this study under identical growth conditions. –, Not detectable; TR, traces (<1.0%); The major fatty acids (>10%) are in bold.

Fatty acid (%)	1	2	3	4	5	5	7	8	9	10	11
Straight-chain:											
$C_{16:0}$	1.6	1.7	1.0	1.1	1.3	1.7	TR	TR	1.0	TR	1.6
$C_{18:0}$	–	1.5	TR	TR	TR	TR	–	TR	–	–	TR
Branched-chain:											
iso-$C_{13:0}$	–	–	1.3	–	–	–	–	–	–	TR	TR
iso-$C_{14:0}$	2.9	–	–	–	2.3	–	–	–	–	–	–
iso-$C_{15:0}$	**19.5**	**19.9**	**19.2**	**15.8**	**16.5**	**12.7**	**13.6**	**21.0**	**18.7**	**20.9**	**15.7**
anteiso-$C_{15:0}$	TR	TR	TR	TR	1.2	1.3	TR	2.8	1.4	TR	TR
iso-$C_{15:1}$ G	**13.5**	**14.9**	**20.1**	**18.7**	**16.4**	**20.7**	**24.3**	**18.9**	**22.0**	**24.5**	**22.4**
iso-$C_{16:0}$	1.1	–	–	TR	TR	TR	–	–	–	TR	TR
Unsaturated:											
$C_{15:1}$ *ω6c*	–	TR	–	1.4	2.5	–	–	TR	–	TR	1.2
$C_{17:1}$ *ω6c*	TR	TR	TR	TR	1.8	–	TR	TR	TR	TR	1.2
Hydroxy:											
$C_{15:0}$ 3-OH	2.5	2.7	1.5	1.8	4.0	3.9	4.8	2.1	2.2	1.6	1.8
iso-$C_{15:0}$ 3-OH	9.0	8.9	7.3	6.5	7.6	6.1	10.0	8.4	7.3	5.9	5.4
$C_{16:0}$ 3-OH	1.9	1.7	1.2	1.3	1.4	2.0	1.3	1.4	TR	1.5	1.2
iso-$C_{16:0}$ 3-OH	4.9	3.3	3.3	2.1	2.3	7.6	5.2	7.7	3.0	1.6	2.5
$C_{17:0}$ 2-OH	TR	TR	TR	TR	TR	1.9	1.1	1.8	1.2	TR	TR
$C_{17:0}$ 3-OH	1.6	TR	TR	1.6	2.0	1.9	TR	TR	1.4	1.6	2.4
iso-$C_{17:0}$ 3-OH	**29.8**	**32.3**	**34.7**	**32.4**	**27.3**	**31.8**	**28.5**	**27.9**	**32.9**	**27.6**	**28.9**
Summed features:*											
3	5.6	7.3	4.6	9.9	5.2	5.2	4.9	2.0	5.0	7.6	6.8
9	TR	1.8	TR	1.4	1.6	–	TR	TR	TR	1.3	1.2

*Summed features represent groups of two fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained $C_{16:1}$ *ω7c* and/or $C_{16:1}$ *ω6c*; summed feature 9 contained iso-$C_{17:1}$ *ω9c* and/or 10-methyl $C_{16:0}$.

DESCRIPTION OF *MURICAUDA MARITIMA* SP. NOV.

Muricauda maritima (ma.ri'ti.ma. L. fem. adj. *maritima* of the marine environment).

Cells are Gram-stain-negative, non-spore-forming, non-motile, rod-shaped, 0.2–0.4 μm wide and 1.3–2.9 μm long. No flagellum is observed. Colonies are yellow, circular, smooth, convex and 2 mm in diameter after incubation for 3 days at 30 °C on MA. Does not produce flexirubin-type pigment. Produces a carotenoid characterized by maximal absorption at 450 nm. Requires sea salts for growth. The sea salts concentration range for growth is 1.0–5.0% (w/v; optimum, 2.0%). Grows on NaCl-free MB supplemented with 0.5–10.0% (w/v) NaCl (optimum 1.0%). The pH and temperature ranges for growth are pH 5.5–9.0 and 15–40 °C (optima at pH 6.5–7.0 and 35 °C). No anaerobic growth occurs on MA supplemented with sodium nitrate or sodium nitrite. Positive for oxidase and catalase activities. Negative for nitrate reduction and indole production from tryptophan. Negative for arginine dihydrolase and para-nitrophenyl-*β*-galactpyranosidase activities. Positive for hydrolysis of Tweens 20 and 80, starch and aesculin, weakly positive for gelatin, while Tween 40 and urea are not hydrolysed. The following substances are utilized as single carbon resources for growth: adipic acid, L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose,

lactose, malate, maltose, D-mannose, *N*-acetyl-glucosamine, phenylacetic acid, potassium gluconate, raffinose, sodium acetate, sodium gluconate, sucrose and trehalose, but not L-alanine, capric acid, L-isoleucine, D-mannitol, L-rhamnose, D-sorbitol or trisodium citrate. Acid is produced from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, melezitose, raffinose, sucrose, trehalose and D-xylose, and weakly from L-rhamnose and L-sorbin, while not from D-mannitol, D-ribose and D-sorbitol. Based on the API ZYM results, acid and alkaline phosphatases, *α*-chymotrypsin, cystine arylamidase, esterase (C4), esterase lipase (C8), *α*- and *β*-galactosidases, *α*- and *β*-glucosidase, leucine arylamidase, lipase (C14), *α*-mannosidase, *N*-acetyl-*β*-glucosaminidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase activities are present, whereas *α*-fucosidase and *β*-glucuronidase activities are absent. The sole respiratory quinone is MK-6. The major fatty acids are iso-$C_{17:0}$ 3-OH, iso-$C_{15:0}$ and iso-$C_{15:1}$ G. The major polar lipids of the type strain are phosphatidylethanolamine, one unidentified aminolipid (AL1) and two unidentified lipids (L1–2). Morderate or minor accounts of three unidentified aminolipids (AL2–4), one unidentified glycolipid (GL1) and eight unidentified lipids (L3–10) are also present. The DNA G+C content of strain 72^T is 43.4 mol%.

The type strain, 72^T (=KCTC 62229^T=MCCC 1K03350^T), was isolated from seawater from the West Pacific Ocean. The GenBank accession numbers for the 16S rRNA gene and the whole genome sequences of strain 72^T are MF093149 and QXFI00000000, respectively.

DESCRIPTION OF *MURICAUDA AEQUORIS* SP. NOV.

Muricauda aequoris (ae′quo.ris L. gen. neut. n. *aequoris*, of the sea).

Cells are Gram-stain-negative, non-spore-forming, non-motile, rod-shaped, 0.2–0.3 μm wide and 1.1–2.5 μm long. No flagellum is observed. Colonies are yellow, circular, smooth, convex and 2 mm in diameter after incubation for 3 days at 30 °C on MA. Does not produce flexirubin-type pigment. Produces a carotenoid characterized by maximal absorption at 450 nm. Requires sea salts for growth. The sea salts concentration range for growth is 0.5–5.0% (w/v; optimum with 2.0%). Grows on NaCl-free MB supplemented with 0.5–7.5% (w/v) NaCl (optimum, 3.0%). The pH and temperature ranges for growth are pH 5.5–8.5 and 15–40 °C (optima at pH 6.5–7.0 and 35 °C). No anaerobic growth occurs on MA supplemented with sodium nitrate or sodium nitrite. Positive for catalase activity and weakly positive for oxidase activity. Positive for nitrate reduction, negative for indole production from tryptophan. Positive for arginine dihydrolase and para-nitrophenyl-*β*-galactpyranosidase activities. Positive for hydrolysis of Tween 20 and 80, while Tween 40, starch, gelatin and urea are not hydrolysed. The following substances are utilized as single carbon resources for growth: cellobiose, D-fructose, D-galactose, D-glucose, lactose, malate, maltose, D-mannose, *N*-acetyl-glucosamine, phenylacetic acid, raffinose, sodium acetate, sodium gluconate, sucrose and trehalose, weakly for adipic acid, D-mannitol, potassium gluconate, but not L-arabinose, L-alanine, capric acid, L-isoleucine, L-rhamnose, D-sorbitol or trisodium citrate. Acid is produced from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannose, melezitose, raffinose, sucrose, trehalose and D-xylose, weakly from lactose, L-rhamnose and L-sorbin, while not from D-mannitol, D-ribose or D-sorbitol. Based on the API ZYM results, acid and alkaline phosphatases, *α*-chymotrypsin, cystine arylamidase, esterase lipase (C8), *α*- and *β*-galactosidases, *α*- and *β*-glucosidase, leucine arylamidase, lipase (C14), *α*-mannosidase, *N*-acetyl-*β*-glucosaminidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase activities are present, weakly positive for esterase (C4) activity, whereas *α*-fucosidase and *β*-glucuronidase activities are absent. The sole respiratory quinone is MK-6. The major fatty acids are iso-$C_{17:0}$ 3-OH, iso-$C_{15:0}$ and iso-$C_{15:1}$ G. The major polar lipids of the type strain are phosphatidylethanolamine, one unidentified aminolipid (AL1) and two unidentified lipids (L1–2). Moderate or minor accounts of three unidentified aminolipids (AL2–4), one unidentified glycolipid (GL1) and 11 unidentified lipids (L3–14) are also present. The DNA G+C content of strain NH166^T is 43.4 mol%.

The type strain, NH166^T (=KCTC 62228^T=MCCC 1K03449^T), was isolated from seawater from seawater from the South China Sea. The GenBank accession numbers for the 16S rRNA gene and the whole genome sequences of strain NH166^T are MF093150 and QXFJ00000000, respectively.

DESCRIPTION OF *MURICAUDA OCEANENSIS* SP. NOV.

Muricauda oceanensis (o.ce.a.nen′sis. L. fem. adj. *oceanensis*, belonging to the ocean).

Cells are Gram-stain-negative, non-spore-forming, non-motile, rod-shaped, 0.2–0.8 μm wide and 1.2–3.8 μm long. Colonies are yellow, circular, smooth, convex and 2 mm in diameter after incubation of 3 days at 30 °C on MA. No flagellum is observed. Does not produce flexirubin-type pigment. Produces a carotenoid characterized by maximal absorption at 450 nm. Requires sea salts for growth. The sea salts concentration range for growth is 0.5-5.0 % (w/v; optimum with 4.0–4.5%). Grows on NaCl-free MB supplemented with 0.5–10.0% (w/v) NaCl (optimum 3.0%). The pH and temperature ranges for growth are pH 5.5–8.5 and 10–40 °C (optima at pH 6.5–7.0 and 25 °C). No anaerobic growth occurs on MA supplemented with sodium nitrate or sodium nitrite. Positive for oxidase and catalase activities. Negative for nitrate reduction, indole production from tryptophan and fermentation (glucose). Negative for arginine dihydrolase and para-nitrophenyl-*β*-galactpyranosidase activities. Positive for hydrolysis of Tweens 20 and 80 and aesculin, and weakly positive for

starch, while Tween 40, gelatin and urea are not hydrolysed. The following substances are utilized as single carbon resources for growth: L-arabinose, cellobiose, D-galactose, D-glucose, lactose, malate, maltose, D-mannose, *N*-acetylglucosamine, raffinose and sucrose, but not adipic acid, capric acid, D-mannitol, potassium gluconate or trisodium citrate; weakly positive for phenylacetic acid. Acid is produced from cellobiose, D-galactose, D-glucose, lactose, maltose, D-mannose, raffinose, sucrose and trehalose, while not from inositol and D-mannitol. Based on the API ZYM results, acid and alkaline phosphatases, α-chymotrypsin, cystine arylamidase, esterase (C4), esterase lipase (C8), α- and β-galactosidases, α-glucosidase, α- and β-glucuronidase leucine arylamidase, lipase (C14), α-mannosidase, *N*-acetyl-β-glucosaminidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase activities are present, whereas α-fucosidase and β-glucosidase activities are absent. The sole respiratory quinone is MK-6. The major fatty acids are iso-$C_{17:0}$ 3-OH, iso-$C_{15:1}$ G and iso-$C_{15:0}$. Major polar lipids of the type strain are phosphatidylethanolamine, phospholipid (PL1), one unidentified aminolipid (AL1) and two unidentified lipids (L1–2). The DNA G+C content of 40DY170^T is 42.4 mol%.

Strain 40DY170^T (=KCTC 72200^T=MCCC 1K03569^T) was isolated from seawater from the West Pacific Ocean. The GenBank accession number for the 16S rRNA gene sequence of strain 40DY170^T is MK318948. The GenBank accession number for the whole genome sequences of strain 40DY170^T is RZNA00000000.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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