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Pelagitalea pacifica gen. nov., sp. nov., a New Marine Bacterium Isolated from Seawater

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Abstract A strictly aerobic, Gram-negative, beige-pigmented, short-rod-shaped, non-motile and chemoheterotrophic bacteria, designated K2-48^T was isolated from seawater collected in the Western North Pacific Ocean near Japan. Preliminary analysis based on the 16S rRNA gene sequence revealed that the novel isolate was affiliated with the family *Oceanospirillaceae* within the class *Gammaproteobacteria* and that it showed the highest sequence similarity (93.7 %) to *Neptunomonas qingdaonensis* P10-2-4^T. The strain could be differentiated phenotypically from recognized members of the family *Oceanospirillaceae*. The major fatty acids of strain K2-48^T were identified as summed feature 3 (C16:1 ω7c and/or iso-C15:0 2-OH) and C16:0 as defined by the MIDI system. The DNA G+C content was determined to be 43.2 mol%, the major respiratory quinone was identified as ubiquinone 9 and a polar lipid profile was present consisting of phosphatidylethanolamine, a phosphatidylglycerol and an unidentified phospolipid. On the basis of polyphasic taxonomic studies, it was concluded that strain K2-48^T represents a novel genus sp. We propose the name *Pelagitalea pacifica* gen. nov., sp. nov. for this strain; its type strain is K2-48^T (=KCCM 90119^T).

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Introduction

Many culture-independent studies based on the 16S rRNA gene sequences revealed that representatives of the class *Gammaproteobacteria* are ubiquitous in nature such as deep and intertidal sediments, seawater and saline soil [14, 32, 36]. Especially, they are predominant among the marine bacterioplankton, together with members of the *Alphaproteobacteria* and the *Bacteroidetes* [10]. Halophilic and chemoheterotrophic members of this phylogenetic group are considered to represent a large portion of the marine bacteria that are able to thrive and associate with degradation of complex bioorganic molecules [2, 27]. At the time of writing, the genus *Neptunomonas* includes five species, *Neptunomonas naphthovorans*, the type species of the genus [12], *N. japonica* [23], *N. antarctica* [38], *N. concharum* [24] and *N. qingdaonensis* [25], which have been isolated from marine sediments, intertidal sand and ark clam. In 2010, in the course of our study on the diversity of culturable marine bacteria in seawater samples collected from the Western North Pacific near Japan, a bacterium, designated K2-48^T, was isolated. Phylogenetic analysis based on the 16S rRNA gene sequences revealed that the novel strain belongs to the class *Gammaproteobacteria*, with their closest relatives being *Neptunomonas* species (92.5–93.7 % sequence similarity). In this study, we characterized a novel marine *Gammaproteobacteria* strain, K2-48^T, isolated from seawater using polyphasic taxonomic methods, including 16S rRNA gene sequence

analysis, physiological, biochemical and chemotaxonomic analyses. Based on the polyphasic taxonomic data, we suggest that the isolate represents a novel genus and a new species of the family *Oceanospirillaceae* within the class *Gammaproteobacteria*.

Materials and Methods

Isolation of Bacterial Strain and Culture Conditions

Strain K2-48^T was isolated from the seawater samples collected from the Western North Pacific (46°52′ N, 160°01′ E; depth, 50 m; temperature 1.5 °C) during the R/V Mirai (Japan Agency for Marine-Earth Science and Technology [JAMSTEC]) on February 14, 2010 (MR10-01 cruise). The seawater (200 µL) sample was inoculated on medium G [0.5 g of peptone, 0.1 g of yeast extract, 5 g of gelrite in 1 L of water (80 % aged seawater and 20 % deionized water)] and incubated at 10 °C for 30 days. After incubation, colonies were picked and then re-isolated on marine agar 2216 (Difco). This strain was routinely cultured on TYS broth [0.5 % tryptone (Oxoid), 0.1 % yeast extract (Oxoid), artificial seawater (containing 2.75 % NaCl, 0.07 % KCl, 0.54 % $MgCl_2{\cdot}6H_2O$, 0.68 % $MgSO_4{\cdot}7H_2O$, 0.14 % $CaCl_2{\cdot}2H_2O$, 0.02 % $NaHCO_3$ and distilled water [23] or on TYS agar (1.5 % agar) and was stored at −70 °C in TYS broth with 20 % (v/v) glycerol.

Morphological, Physiological and Biochemical Analysis

Cell morphology was observed using transmission electron microscopy (TEM) and motility was measured by phase contrast microscopy (Primo Star; ZEISS) using cells grown in marine agar 2216. Gram-staining was performed using the BD Gram-Staining Kit (Becton, Dickinson and Company, USA). The temperature (4, 10, 15, 20, 25, 30, 35 and 40 °C) ranges for growth were determined by incubating K2-48^T on TYS broth at pH 7.0. The NaCl concentration for growth was determined on TYS broth containing 0–8.0 % (w/v) NaCl (0, 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 %) containing 0.5 % tryptone, 0.1 % yeast extract, 0.5 % $MgCl_2$, 0.2 % $MgSO_4$, 0.05 % $CaCl_2$, 0.1 % KCl, 0.0001 % $FeSO_4$ and distilled water (pH 7.0). Growth condition for pH ranges from pH 5.0 to 9.0 (in increments of 0.5 pH units) was examined at 15 °C in TYS broth with the pH adjusted with hydrochloric acid (pH 5.0–6.5), sodium hydroxide (pH 7.0–9.0). Acid production was performed with API 50CH strips (bioMérieux). Catalase activity was detected by the observation of the formation of bubbles in 3 % (v/v) H_2O_2 solution. Oxidase activity test was performed using commercial dropper oxidase (Becton, Dickinson and Co). Tests for other enzyme activities were performed using API ZYM and API 20E strips (bioMérieux) according to the manufacturer's instructions except that cells were suspended in artificial seawater. Anaerobic growth was tested on marine agar 2216 (Difco) using an anaerobic chamber (Becton, Dickinson and Co).

Determination of G+C Content of DNA, 16S rRNA Gene Sequencing and Phylogenetic Analysis

Genomic DNA was prepared according to the method of Marmur [20] from cells grown on marine agar 2216, and the DNA base composition was determined using the HPLC method of Mesbah et al. [21].

An approximately 1,500-bp fragment of the 16S rRNA gene was amplified from the extracted DNA using bacterial universal primers specific to the 16S rRNA gene: 27F and 1,492R (*Escherichia coli* numbering system [35]). To ascertain the phylogenetic position of the novel isolate, the 16S rRNA gene sequence of strain K2-48^T (GenBank/EMBL/DDBJ accession number AB742372) was compared with sequences obtained from GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov). Multiple alignments of the sequences were performed using CLUSTAL_X (version 1.83) [34]. Alignment gaps and ambiguous bases were not taken into consideration when 1,375 bases of the 16S rRNA gene were compared. Evolutionary distances (distance options according to Kimura's two-parameter model; [17]) were calculated and clustering was performed with the neighbour-joining method [29], maximum-parsimony [9] and maximum-likelihood [8] methods using MEGA5 software [33]. Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1,000 resamplings [8]. The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein [8] with 1,000 replicates.

Chemotaxonomic Analysis

Gas chromatography analysis of the cellular fatty acid methyl esters was performed using a culture grown on marine agar 2216 at 28 °C for 3 days, and fatty acid methyl esters were extracted and prepared according to the standard protocols provided by the MIDI/Hewlett Packard Microbial Identification system Sherlock Version 3.10/TSBA 50 [30]. Polar lipids were extracted according to the procedures described by Minnikin et al. [22]. They were identified by two-dimensional TLC followed by spraying with appropriate detection reagents [18, 22]. Phospholipids were detected with the Zinzadze reagent of Dittmer and Lester [6]. Whole lipid profiles were detected by spraying with molybdatophosphoric acid (5 g molybdatophosphoric acid hydrate in 100 mL ethanol) followed by heating at

150 °C [37]. Determination of the respiratory quinone system was carried out as described previously [5].

Results and Discussion

Morphological, Physiological and Biochemical Characteristics

Cells of strain K2-48^T grown on marine agar 2216 were observed to be straight short rods with 0.8–1.2 μm in width and 1.5–1.8 μm in length, devoid of flagella or cell appendages (Fig. 1) and produced a beige pigment. Gliding motility was not observed by a light microscopy. Strain K2-48^T contained Q-9 as the major respiratory quinone, which is different from the major ubiquinone 8 (Q-8) reported previously in the neighbouring taxa such as *Neptunomonas* sp., *Oceanospirillum* sp. and *Neptuniibacter* sp.

The strain also showed distinct phenotypic, physiological and biochemical features that discriminated it from the closest described members in the family *Oceanospirillaceae* within the class *Gammaproteobacteria* as shown in Table 1.

Molecular Phylogenetic Analysis

The almost complete 16S rRNA gene sequence was determined for strain K2-48^T (GenBank/EMBL/DDBJ accession number AB742372). Comparative phylogenetic analysis based on 16S rRNA gene sequences revealed that strain K2-48^T belongs to the family *Oceanospirillaceae* in the class *Gammaproteobacteria* (Fig. 2). Analysis of the 16S rRNA gene sequence also indicated that that strain K2-48^T showed the highest sequence similarity to the *Neptunomonas qingdaonensis* P10-2-4^T (93.7 %), followed by *Neptunomonas japonica* JAMM 0745^T (93.5 %), *Neptunomonas concharum* LHW37^T (92.9 %). Sequence similarity was less than 92.8 % with all other members of family *Oceanospirillaceae* with validly published names. Thus, on the basis of phylogenetic data presented, we believe that strain K2-48^T should be considered as representative of a novel genus and species of the family *Oceanospirillaceae* within the class *Gammaproteobacteria.*

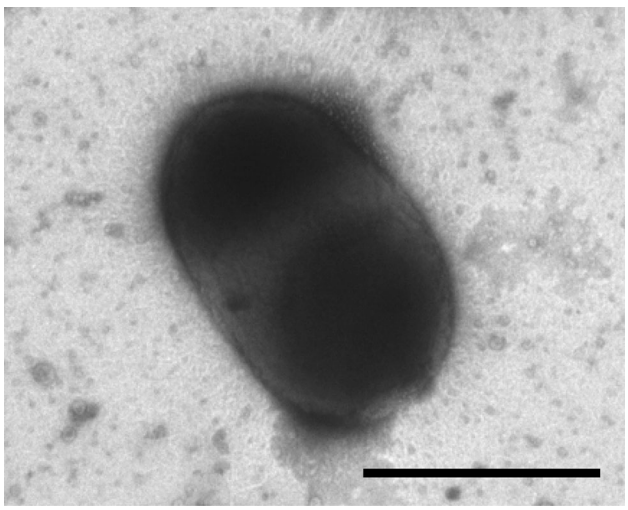

Fig. 1 Transmission electron micrograph of a negatively stained cell of strain K2-48^T. *Bar* 1 μm

Chemotaxonomic Characteristics

As shown in Table 2, the predominant cellular fatty acids of strain K2-48^T were identified as summed feature 3 (C16:1 ω7c and/or iso-C15:0 2-OH) and C16:0 as defined by the MIDI system. On the basis of the fatty acid composition, strain K2-48^T could be differentiated from the phylogenetically closest taxa such as *Neptunomonas qingdaonensis* P10-2-4^T, *Neptunomonas japonica* JAMM 0745^T, *Neptunomonas naphthovorans* NAG-2N-126^T, *Neptuniibacter caesariensis* MED92^T and *Marinobacterium georgiense* KW-40^T as shown in Table 2. Moreover, strain K2-48^T could be distinguished from the neighbouring taxa within the family *Oceanospirillaceae* by the presence of C12:0 3-OH, C14:0 and summed feature 2 (C14:0 3-OH and/or iso-C16:1 I) and the absence of C10:0 3-OH.

The polar lipids of strain K2-48^T were determined to be composed of phosphatidylethanolamine, a phosphatidylglycerol and an unidentified phospolipid (supplementary Fig. 1). From these results, it is strongly suggested that strain K2-48^T represents an independent genus of the family *Oceanospirillaceae* within the class *Gammaproteobacteria*.

Polyphasic Taxonomic Conclusion

From the distinct phylogenetic position and combinations of genotypic and phenotypic characteristics, strain K2-48^T cannot be assigned to any previously recognized bacterial genus and thus can be described as representing a novel species within a new genus, *Pelagitalea pacifica* gen. nov., sp. nov.

Description of *Pelagitalea* gen. nov.

Pelagitalea (Pe.la.gi.ta'le.a. L. n. *pelagus*, the sea; L. fem. n. *talea*, a rod; N.L. fem. n. *Pelagitalea*, a rod of the sea).

A member of the family *Oceanospirillaceae*, class *Gammaproteobacteria*, according to 16S rRNA gene sequence analyses. Cells are short-rod-shaped, Gram-negative and strictly aerobic. Endospores are not formed. Catalase-positive but oxidase-negative. The major respiratory quinone is ubiquinone 9 (Q-9). The predominant cellular fatty acids are summed feature 3 (C16:1 ω7c and/

Table 1 Differential characteristics of strain K2-48^T and other closely related taxa

Characterisitic	1	2	3	4	5	6	7
Source	Seawater	Marine	Marine	Marine	Marine	Marine	Marine
Pigmentation	Beige	White, Beige, Cream	White, Beige	White, Cream	White, Cream, Beige	White	Cream
O_2 requirement	Strictly aerobic	Strictly aerobic, facultatively anaerobic	Strictly aerobic	Strictly aerobic	Strictly aerobic, facultatively anaerobic	Strictly aerobic, facultatively anaerobic	Facultatively anaerobic
Cell size (μm)							
Length	1.5–1.8	1.0–3.0	2.0–50	1.5–1.7	1.0–10	1.0–10	0.9–2.0
Width	0.8–1.2	0.5–1.0	0.4–1.2	0.2–0.4	0.2–1.2	0.2–0.4	0.6–0.9
Motility	−	+, −	+	+	+	+, −	+
Temperature range for growth (°C)	10–35	4–45	10–40	15–37	4–45	22–30	20–24
pH range for growth	5.5–7.5	6.0–10.0	ND	7.0–9.0	5.0–9.0	4.0–9.0	6.5–8.0
NaCl concentration for growth (%, w/v)	0–4	0.5–12	0.5–8	0.5–6	0–10	0–8	2–5
Nitrate reduction	−	+	−	+	+	+, −	+
Oxidase	−	+	+	+	+	+	+
Polar lipids	PE, PG, 2UPL	PE, PG, UAL, 2UL	ND	PE, PG, UAL, DPG	PE, PG, UAL	PE, PG, UAL, DPG	ND
DNA G+C content (mol%)	43.2	43.6–48.2	40–48	46.6–54.2	40–55	41.2–48.7	46.6–52.2

Strains: *1* K2-48^T (*P. pacifica* gen. nov., sp. nov.; present study), *2 Neptunomonas* spp. [12, 24–26, 38], *3 Oceanospirillum* spp. [3, 28, 31], *4 Neptuniibacter* spp. [1, 4], *5 Marinobacterium* spp. [11, 15, 16], *6 Marinomonas* spp. [7, 13, 19], *7 Amphritea* spp. [23]

PE phosphatidylethanolamine, *PG* phosphatidylglycerol, *DPG* diphosphatidylglycerol, *UAL* unidentified aminolipid, *UPL* unidentified phospholipid, *UL* unidentified lipid, + positive, − negative, *W* weakly positive, *ND* no data

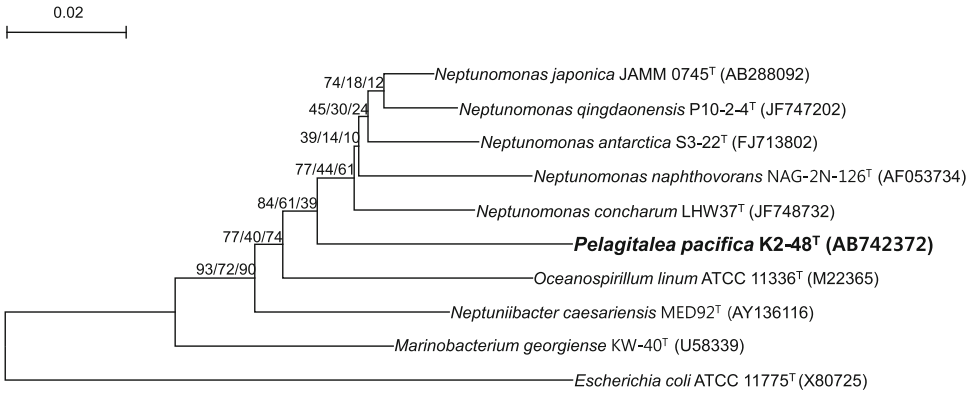

Fig. 2 Neighbour-joining tree of 16S rRNA gene sequence similarity, showing the phylogenetic position of strain K2-48^T and representatives of the family *Oceanospirillaceae*. The sequence of *Escherichia coli* ATCC 11775^T (X80725) was used as an outgroup. The sequence determined in this study is shown in *bold*. Bootstrap values from neighbour-joining, maximum-parsimony and maximum-likelihood analyses are shown (NJ/MP/ML). *Bar* 2 % sequence divergence

or iso-C15:0 2-OH) and C16:0 as defined by the MIDI system. The DNA G+C content of the type strain of the type species is 43.2 mol%.

The type species is *P. pacifica*.

Description of *P. pacifica* sp. nov.

Pelagitalea pacifica (pa.ci'fi.ca. N.L. fem. adj. *pacifica* referring to the Pacific Ocean, from which the type strain was isolated).

The main characteristics are the same as those given for the genus. In addition, cells are rod shape 0.8–1.2 μm in width and 1.5–1.8 μm in length. Cells lack flagella and are non-motile. Gliding motility is not observed. Colonies are circular to slightly irregular, opaque, smooth, low convex, beige-coloured after incubation for a week on marine agar 2216. Temperature range for growth is 10–35 °C, the optimal temperature is between 15 °C, but no growth occurs at 0 or 40 °C. The pH range for growth is 5.5–7.5 (optimum, pH 7.0), while no growth was observed below 5

Table 2 Comparison of cellular fatty acids for strain K2-48^T and other closely related taxa

Fatty acid	1	2	3	4	5	6
C10:0	tr	tr–2.5	–	0.6–3.1	tr–2	tr
C10:0 3-OH	tr	3.7–4.5	7.5–8.6	4.8–6.7	3.9–25	58–81
iso-C:10	–	–	1.2	–	–	–
C:11 2-OH	–	–	1.6	–	–	–
anteiso-C:11:0	–	–	1.8	–	–	
C12:0	7.8	tr–3.6	–	2.1–5.6	tr–4.6	2
C12:1	–	–	–	–	–	3–4
C12:0 3-OH	5	–	–	–	tr–2.5	–
C12:1 3-OH	–	–	–	–	2.4–10.7	19–42
C14:0	3.6	–	1.1	1–1.2	tr–1.6	–
C16:0	18.2	7.9–26.6	15.5–20.2	21–30	8.3–19	14–29
C16:1	–	–	–	–	–	37–47
C16:1 ω7c	–	–	32.6–39.8	–	–	
C18:1 ω9c	7.8	tr–1.8	–	–	–	–
C18:1 ω7c	1.9	7.6–31.6	29.4–33.5	36.2–43.3	30.7–45.4	–
C18:0	tr	tr–1.1	1.8–2.2	tr	1.4–3.9	tr–1
C18:1	–	–	–	–	–	22–35
Summed feature 2[a]	2	–	–	–	tr	–
Summed feature 3[b]	50	40.3–56.3	32.6	22.5	19.9–28.4	–

Strains: *1* K2-48^T (*P. pacifica* gen. nov., sp. nov.; present study), *2 Neptunomonas* spp. [12, 24–26, 38], *3 Neptuniibacter* spp. [1, 4], *4 Marinobacterium* spp. [11, 15, 16], *5 Marinomonas* spp. [7, 13, 19], *6 Amphritea* spp. [23]

tr trace (less than 1.0 %), – not detected. The data were typically obtained by GLC using the MIDI system

[a] Summed feature 2 consists of C14:0 3-OH and/or iso-C16:1 I

[b] Summed feature 3 consists of C16:1 ω7c and/or iso-C15:0 2-OH

or above 8. NaCl is required for growth and can be tolerated at a concentration of up to 4 % (w/v). No growth was occurred above 5 % (w/v) NaCl. Nitrate and nitrite reduction are negative. Urea is hydrolysed but gelatin, agar, casein, starch and tyrosine are not. The reactions for arginine dihydrolase, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), ornithine decarboxylase, Voges–Proskauer test, citrate utilization, hydrogen sulphide production, indole production and lysine decarboxylase activities are negative (API 20E). Acid production tests using API 50CH strips give the following reactions: acid is produced from methyl-β-D-xylopyranoside, rhamnose, sorbitol, D-turanose, D-lyxose, D-tagatose and 5-keto-gluconate but not from D-arabinose, galactose, glucose, fructose, mannose, arbutin, esculin ferric citrate, melibiose, salicin, L-arabitol, amygdalin, maltose, lactose, sucrose, trehalose, starch, glycogen, gentiobiose, L-fucose, ribose, sorbose, methyl-α-D-mannopyranoside, L-arabinose, D-xylose, L-xylose, methyl-α-D-glucopyranoside, *N*-acetyl-glucosamine, cellobiose, melezitose, D-fucose, inulin, raffinose, glycerol, erythritol, adonitol, dulcitol, inositol, mannitol, xylitol, D-arabitol, gluconate and 2-keto-gluconate. In the API ZYM strip, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase and *N*-acetyl-β-glucosaminidase are present but trypsin, naphthol–AS–BI–phosphohydrolase, β-galactosidase, α-glucosidase, β-glucosidase esterase (C4), α-galactosidase, lipase (C4), α-chymotrypsin, β-glucuronidase, α-mannosidase and α-fucosidase are absent. The major fatty acids are summed feature 3 (C16:1 ω7c and/or iso-C15:0 2-OH) and C16:0 as defined by the MIDI system. The major polar lipids are phosphatidylethanolamine, a phosphatidylglycerol and an unidentified phospolipid. The G+C of the genomic DNA of the type strain is 43.2 mol%.

The type strain is K2-48^T (=KCCM 90119^T), which was isolated from Strain K2-48^T was isolated from the seawater samples collected from the Western North Pacific near Japan. The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of strain K2-48^T is AB742372.

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