**Aliikangiella marina** gen. nov., sp. nov., a marine bacterium from the culture broth of *Picochlorum* sp. 122, and proposal of *Kangiellaceae* fam. nov. in the order *Oceanospirillales*

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A Gram-stain-negative, non-motile, non-spore-forming, long rod-shaped bacterium, designated strain GYP-15T, was isolated from the culture broth of a marine microalga, *Picochlorum* sp. 122. Phylogenetic analyses revealed that strain GYP-15T shared 90.6 % 16S rRNA gene sequence similarity with its closest relative, *Kangiella aquimarina* KCTC 12183T, and represents a distinct phylogenetic lineage in a robust clade consisting of GYP-15T and members of the genera *Kangiella* and *Pleionea* in the order *Oceanospirillales*. Chemotaxonomic and physiological characteristics, including major cellular fatty acids, NaCl tolerance and pattern of carbon source utilization, could also readily distinguish strain GYP-15T from all established genera and species. Thus, it is concluded that strain GYP-15T represents a novel species of a new genus, for which the name *Aliikangiella marina* gen. nov., sp. nov. is proposed. The type strain of *Aliikangiella marina* is GYP-15T (=MCCC 1K01163T =KCTC 42667T). Based on phylogenetic results, 16S rRNA gene signature nucleotide pattern and some physiological characteristics, the three genera *Kangiella*, *Pleionea* and *Aliikangiella* are proposed to make up a novel family, *Kangiellaceae* fam. nov., in the order *Oceanospirillales*.

The family *Alcanivoracaceae* was established with the only genus *Alcanivorax* by Golyshin *et al.* (2005), and comprises species capable of degradation of petroleum-derived compounds as their main carbon source (Silveira & Thompson, 2014). Recently, the genus *Kangiella* was assigned to this family based on molecular analyses of 16S rRNA gene sequences (Silveira & Thompson, 2014). However, phylogenetic results of the latest The All-Species Living Tree (Release LTPs119, Munoz *et al.*, 2011) and species identification in the genera *Kangiella* and *Pleionea* do not support this assignment (Yoon *et al.*, 2004, 2012; Romanenko *et al.*, 2010; Ahn *et al.*, 2011; Jean *et al.*, 2012; Fagervold *et al.*, 2013; Lee *et al.*, 2013; Kim *et al.*, 2015; Xu *et al.*, 2015), so the exact taxonomic standings of these *Kangiella*-related organisms are uncertain (Fagervold *et al.*, 2013). In this study, strain GYP-15T, phylogenetically close to the genera *Kangiella* and *Pleionea*, was isolated from the culture broth of a marine microalga, *Picochlorum* sp. 122, and is proposed to represent a novel species of a new genus (*Aliikangiella*) in the order *Oceanospirillales*. Finally, these three genera (*Kangiella*, *Pleionea* and *Aliikangiella*) are proposed to make up a novel family, *Kangiellaceae* fam. nov., in the order *Oceanospirillales* on the basis of their phylogenetic, chemotaxonomic and physiological characteristics.

*Picochlorum* sp. 122 was isolated from the India Ocean, and cultivated outdoors in filtered natural seawater (collected from the coast of Sanya at 18.30°N 109.32°E, in December 2013) emended with (per litre) 1 g urea, 1 g NaHCO3, 8 mg Na2HPO4 and 5 mg FeSO4 .7H2O. The culture broth of *Picochlorum* sp. 122 was collected at the late exponential phase, stored and transported at room temperature. Strain GYP-15T was isolated from this culture broth by series dilution on a GYP plate (2 g tryptone, 1 g yeast extract, 3 ml glycerol, 18 g agar powder and 1 litre aged seawater, pH 8.0, with autoclaving at 121°C for 15 min). Subsequent growth experiments were performed aerobically on marine agar 2216 (MA; BD) or in marine broth 2216 (MB; BD) in the dark at 30°C. Bacterial stocks were stored at −70°C in sterile aged seawater supplemented with...
20 % (v/v) glycerol. For comparative studies, reference strains *Kangiella koreensis* KCTC 12182^T, *Kangiella aquamarina* KCTC 12183^T and *Pleione mediterranea* DSM 25350^T were obtained from the Korean Collection for type Cultures (KCTC) and the German Collection of Microorganisms and Cell Cultures (DSMZ). These bacteria were grown on MA or in MB.

The 16S rRNA gene of strain GYP-15^T was obtained by using PCR amplification with the universal primers 27F and 1492R (Lane, 1991) and sequenced by Shanghai Majorbio Biopharm Technology. 16S rRNA gene sequence similarities were determined in the GenBank and EzTaxon-e (Kim et al., 2012) databases by using the BLAST tool (Altschul et al., 1990) and Global Alignment (Myers & Miller, 1988). Alignment of 16S rRNA gene sequences was performed using the SINA software package (Pruesse et al. 2012) in the SILVA rRNA database. Phylogenetic trees were reconstructed using the maximum-likelihood (Felsenstein, 1981), neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Swofford, 1993) algorithms in the software package MEGA version 5.0 (Tamura et al., 2011). The phylogenetic distance matrices were estimated by using Kimura’s two-parameter model (Kimura, 1980). The topology of the phylogenetic trees was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

The nearly complete 16S rRNA gene sequence (1491 bases) of strain GYP-15^T was obtained. Results of 16S rRNA gene sequence BLAST comparisons revealed that strain GYP-15^T was related most closely to *K. aquamarina* KCTC 12183^T with a similarity of 90.63 %. This low sequence similarity suggested that strain GYP-15^T may represent a novel species of a new genus (Yarza et al., 2014). Phylogenetic analysis based on the maximum-likelihood algorithm indicated that strain GYP-15^T formed a distinct monophyletic lineage in a robust clade consisting of strain GYP-15^T, and members of the genera *Kangiella* and *Pleione* (*Kangiella* group) in the order Oceanospirillales (Fig. 1; see also Fig. S1 in the online Supplementary Material), and this tree topology was also supported by the neighbour-joining and maximum-parsimony algorithms (Figs S2 and S3). Phylogenetic analyses also indicated that *Alcanivorax*, the type genus of the family *Alcanivoracaceae*, does not form a monophyly with the *Kangiella* group (Figs 1, S2 and S3), which contrasts with the results of Silveira & Thompson (2014). The 16S rRNA gene signature nucleotides of the *Kangiella* group were found to consist of characteristic nucleotides at positions 103(T), 134(A), 152(G), 169(T), 235(T), 511–513(C/GC), 538–540(GAG), 589(A), 650(T), 658(A), 681(A), 719(T), 748(T) and 1152(A) (*Escherichia coli* 16S rRNA gene sequence numbering). This signature nucleotide profile readily distinguished members of the *Kangiella* group from those of the genus *Alcanivorax* and all other defined families in the order Oceanospirillales (Table 1). Therefore, the genera *Kangiella*, *Pleionea* and the newly proposed genus herein should be assigned to a new family separate from *Alcanivoracaceae* in the order Oceanospirillales.

Cellular morphology and size were determined by using transmission electron microscopy (Hitachi TEM System-H7650). Cell mobility was tested by optical microscopy (Olympus BX53) using the hanging drop technique (Bernardet et al., 2002). The Gram reaction was determined as described by Gerhardt et al. (1994). Catalase activity was determined by observing bubble production in a 3 % (v/v) hydrogen peroxide solution and oxidase activity was determined by using oxidase test strips (Huankai). NaCl requirement and tolerance were tested at 30 °C for 4 days in reconstituted MB with NaCl concentrations ranging from 0 to 15 %, specifically 0, 0.25, 0.5, 1, 2, 3, 5, 6, 8, 10, 13 and 15 % (w/v). Growth at different pH was tested in MB at 30 °C for 4 days emended with different buffers (at 0.5 pH unit intervals: pH 5–8, 0.1 M KH₂PO₄/K₂HPO₄; pH 8.5–10, 0.1 M NaHCO₃/Na₂CO₃; pH 10.5–11, 0.1 M Na₂CO₃/NaOH). Optimal growth temperature was determined on MA after 4 days of growth at 4, 10, 15, 22, 28, 37, 40 and 45 °C. The ability to form endospores and hydrolysis of starch, casein, chitin, gelatin and Tweens 20, 40 and 80 were tested as described by Dong & Cai (2001). Utilization of sole carbon sources was assayed by addition of trehalose, sucrose, D-glucose, D-mannose, D-xylene, lactose, myo-inositol, nicotinic acid, citric acid, l-malic acid, maleic acid, l-alanine, l-glycine, l-glutamate, l-arginine, l-histidine, l-methionine and glycerol to sterile aged natural seawater, respectively. For alkane degradation, 0.25 % n-dodecane was added to bacterial culture broth (MB amended with 0.5 % Tween 20), and after 7 days of incubation with shaking at 30 °C in the dark, the alkane residue was extracted in n-hexane and analysed by GC (Shimadzu 2014C). Additional carbohydrate metabolism was tested using the API 20NE and Biolog GEN III MicroPlate systems according to the manufacturer’s protocols except that cells were suspended in sterile aged natural seawater. Anaerobic growth was determined by using the nitrate reduction hole of the API 20NE system, which was sealed with mineral oil. Other phenotypic characteristics were tested using standard procedures (Tindall et al., 2007).

Cells of strain GYP-15^T were Gram-stain-negative, non-motile, non-spore-forming, aerobic long rods (Fig. S4). Colonies on MA were pale tyre-like circles 1–3 mm in diameter after 3 days of incubation at 30 °C, which then became smooth, transparent and pale yellow–green after extended periods of incubation. NaCl was essential for growth, although this isolate was less halotolerant than members of the genera *Pleionea* (Fagervold et al., 2013) and *Kangiella* (Kim et al., 2015). There was no decrease in n-dodecane content after incubation, so strain GYP-15^T, *K. koreensis* KCTC 12182^T, *K. aquamarina* KCTC 12183^T and *P. mediterranea* DSM 25350^T appear to have no n-alkane-degrading ability. Only l-arginine was used as sole carbon source. Other cultural, physiological and biochemical properties of strain GYP-15^T are listed in Table 2 and in the genus and species descriptions.
Biomass of strain GYP-15T and reference strains for cellular fatty acid analysis was harvested from MA plates grown at 30°C at late exponential phase (3 days). The fatty acid composition was analysed by GC (Agilent G6890N) and components were identified by using the Sherlock Microbial Identification System (Version 6.0) according to the manufacturer’s instructions. Respiratory lipoquinones were extracted as described by Collins (1994) and analysed using reversed-phase HPLC (Komagata & Suzuki, 1987). Polar lipids were extracted as described by Kamekura (1993) and identified by spraying with ethanoic molybdophosphoric acid, molybdenum blue and ninhydrin after two-dimensional TLC (Tindall, 1990). The G+C content of the genomic DNA was determined by using the HPLC method (Mesbah et al., 1989).

The major cellular fatty acids (>5 % of the total) detected in strain GYP-15T were iso-C₁₅:₀, iso-C₁₇:₁₀, 9c/C₁₆:₁₀ 10-methyl, iso-C₁₇:₀, iso-C₁₆:₁₀, C₁₆:₁₀ 9c/C₁₆:₁₀ 6c and iso-C₁₁:₀ 3-OH (Table S1). Strain GYP-15T and P. mediterranea DSM 25350T (Fagervold et al., 2013) share a high amount of iso-C₁₇:₁₀, 9c/C₁₆:₁₀ 10-methyl (>21 %) in addition to iso-C₁₅:₀, while members of the genus Kangiella are characterized by a dominance of iso-C₁₅:₀ (~50 %, Xu et al., 2015). Strain GYP-15T contained a higher proportion of C₁₆:₁₀ 9c/C₁₆:₁₀ 6c than P. mediterranea DSM 25350T. Meanwhile, members of

Fig. 1. Maximum-likelihood phylogenetic tree based on 1491 positions of the 16S rRNA gene sequence of strain GYP-15T and related Gammaproteobacteria. Numbers at nodes indicate percentages of 1000 bootstrap resamplings; only values above 50 % are shown. Bar, 0.05 substitutions per nucleotide position.
the genus *Alcanivorax* are characterized by a dominance of C_{16} : 0, C_{18} : 1_{\text{v}7\text{c}/\text{v}6\text{c}}, cyclo fatty acids and C_{16} : 1_{\text{v}7\text{c}/\text{v}6\text{c}} (Lai et al., 2013; Rahul et al., 2014; Kwon et al., 2015). Thus, the cellular fatty acid profile not only distinguishes strain GYP-15^T from members of the genera *Alcanivorax*, *Kangiella* and *Pleionea*, but also distinguishes the *Kangiella* group from the genus *Alcanivorax*. Polar lipids in strain GYP-15^T included diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, an unidentified amino phospholipid, an unidentified amino lipid and four unknown lipids (Fig. S5). The presence of phosphatidylethanolamine, phosphatidylglycerol and unidentified amino lipid (near the phosphatidylethanolamine spot) is in line with data for *P. mediterranea* DSM 25350^T (Fagervold et al., 2013), but no diphosphatidylglycerol was detected in *P. mediterranea* DSM 25350^T (Fagervold et al., 2013). An amino phospholipid was detected in GYP-15^T and *P. mediterranea* DSM 25350^T (Fagervold et al., 2013), but not found in members of the genus *Kangiella* (Yoon et al., 2012; Xu et al., 2015). Phosphatidylmonomethylethanolamine was found in some species of the genus *Kangiella* (Yoon et al., 2012; Xu et al., 2015), but was not detected in *P. mediterranea* DSM 25350^T (Fagervold et al., 2013) or strain GYP-15^T. The predominant respiratory lipoquinone was ubiquinone-8 (Q-8), which is also the major isoprenoid quinone of members of the genera *Kangiella* and *Pleionea* (Table 2).

In summary, strain GYP-15^T differs from members of the genera *Kangiella* and *Pleionea*, and also members of the genus *Alcanivorax*, in phylogenetic, chemotaxonomic and numerous metabolic characteristics listed in Table 2. Thus, it is suggested that strain GYP-15^T represents a novel species of a new genus, for which the name *Aliikangiella marina* gen. nov., sp. nov. is proposed. On the basis of phylogenetic distance, and differences in major cellular fatty acids and alkane degradation ability of the *Kangiella* group from members of the genus *Alcanivorax* (Table 2), the *Kangiella* group (genera *Kangiella*, *Pleionea* and *Aliikangiella*) is proposed to make up a new family, *Kangiellaceae* fam. nov., in the order *Oceanospirillales*; the type genus of the family is *Kangiella*.

### Description of *Aliikangiella* gen. nov.

*Aliikangiella* (A.li.i.kang.i.el’lə). L. pronoun. *alius* other, another; N.L. dim. fem. n. *Kangiella*, the name of a bacterial genus; N.L. fem. n. *Aliikangiella* the other *Kangiella*).

Cells are Gram-stain-negative, non-motile, non-spore-forming rods. Catalase- and oxidase-positive. Negative for urease. Nitrate reduction is weakly positive under aerobic conditions, or negative under anaerobic conditions. NaCl is required for growth. The respiratory lipoquinone is Q-8. The major fatty acids (>10%) are iso-C_{15} : 0, iso-C_{17} : 0\text{C}_{16} : 0\text{10-methyl and iso-C}_{17} : 0. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and an unidentified amino phospholipid.
The type species is *Aliikangiella marina*.

**Description of *Aliikangiella marina* sp. nov.**

*Aliikangiella marina* (ma.ri’na L. fem. adj. marina of the sea, marine).

The description is as for the genus with the following additional properties. Cells are usually 0.44–0.67 μm wide and 3.42–5.2 μm long. Colonies are pale tyre-like circles on MA. Growth occurs at pH 6.0–9.0, at 15–37 °C and 3.42–5.2 °C. Additional properties. Cells are usually 0.44–0.67 μm wide and 3.42–5.2 μm long. Colonies are pale tyre-like circles on MA. Growth occurs at pH 6.0–9.0, at 15–37 °C and 3.42–5.2 °C. Cells are usually 0.44–0.67 μm wide and 3.42–5.2 μm long. Colonies are pale tyre-like circles on MA. Growth occurs at pH 6.0–9.0, at 15–37 °C and 3.42–5.2 °C.

**Table 2.** Differential phenotypic properties between strain GYP-15T and members of related genera

<table>
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<tr>
<th>Characteristic</th>
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<tr>
<td>Nitrate reduction</td>
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<td>-</td>
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<tr>
<td>Aerobic</td>
<td>+</td>
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<tr>
<td>Anaerobic</td>
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<td>n-Alkane degradation</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td>Temperature range for growth (°C)</td>
<td>15–37</td>
<td>4–43 (4–49)*</td>
<td>15–37</td>
<td>4–35 (4–45)*</td>
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<td>NaCl range for growth (w/v) (%)</td>
<td>1–5</td>
<td>2–12 (0–16)*</td>
<td>0.5–10</td>
<td>1–12.5 (0–20)*</td>
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<td>Hydrolysis of:</td>
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<tr>
<td>Aesculin</td>
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<td>Gelatin</td>
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<td>Starch</td>
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<td>Casein</td>
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<tr>
<td>Tween 80</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Major fatty acids†</td>
<td>iso-C_{15} : 0</td>
<td>iso-C_{17} : 0</td>
<td>iso-C_{15} : 0</td>
<td>iso-C_{11} : 0</td>
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<tr>
<td>Quinone</td>
<td>Q-8</td>
<td>Q-8</td>
<td>Q-8</td>
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<tr>
<td>DNA G + C content (mol%)</td>
<td>44.7</td>
<td>44 (43.9–48.9)*</td>
<td>44.5</td>
<td>53.4 (53.4–66.4)*</td>
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</table>

*Characteristics for the genera *Kangiella* and *Alcanivorax*, respectively.†

The type strain, GYP-15T (=MCCC 1K01163T=KCTC 42267T), was isolated from the culture broth of a marine microalga, *Picochlorum* sp. 122. The DNA G+C content of the type strain is 44.7 mol %.

**Description of *Kangiellaceae* fam. nov.**

*Kangiellaceae* (Kan.gi.el.lae ‘ae n. L. dim. fem. n. Kangiella, the name of a bacterial genus; suff. -aceae ending to denote the name of a family; N.L. fem. pl. n. Kangiellaceae the Kangiella family).

The family is a member of the order *Oceanospirillales* and encompasses Gram-stain-negative bacteria retrieved from marine environments. The family comprises the genera *Kangiella*, *Pleionea* and *Aliikangiella*. The G+C content of the genomic DNA is 43.7–48.9 mol %. The major fatty acids are iso-C_{15} : 0 and iso-C_{17} : 0 9Ω/C_{16} : 0 10-methyl. The 16S rRNA gene signature nucleotides include 103(T), 134(A), 152(G), 169(T), 235(T), 511–513(CTC), 538–540(GAG), N-acetyl neuraminic acid, D-glucose, D-mannose, D-fructose, L-fucose, L-rhamnose, D-sorbitol, D-mannitol, D-arabitol, myo-inositol, glycerol, glucononamide, mucic acid, quinic acid, D-saccharic acid, citric acid, z-ketoglutaric acid, D-malic acid, L-malic acid and acetoclastic acid are oxidized.
Reference


