**Amphritea spongicola** sp. nov., isolated from a marine sponge, and emended description of the genus *Amphritea*

Hani Jang, Sung-Hyun Yang, Hyun-Seok Seo, Jung-Hyun Lee, Sang-Jin Kim and Kae Kyoung Kwon

Marine Biotechnology Research Center, Korea Institute of Ocean Science & Technology, PO Box 29, Ansan 425-600, Republic of Korea

A Gram-stain-negative, rod-shaped (1.2–2.1 μm × 0.8–0.9 μm), flagellated and motile marine bacterium, designated MEBiC05461^T, was isolated from a marine sponge inhabiting Micronesia. Strain MEBiC05461^T was oxidase-negative and catalase-positive. Growth was observed at 8.0–35.6 °C (optimum 30.0 °C), at pH 5.0–9.0 (optimum pH 7.0) and with 1.5–6.0 % (w/v, optimum 2.0–2.5 %) NaCl. 16S rRNA gene sequence analysis revealed that strain MEBiC05461^T showed high similarity to members of the genus *Amphritea* (96.4–96.6 %). The predominant cellular fatty acids were C_{16:0} (23.9 %), summed feature 3 (C_{16:1}ω7c and/or C_{16:0}ω6c; 39.7 %) and summed feature 8 (C_{18:1}ω7c and/or C_{18:1}ω6c; 22.0 %). The DNA G+C content was 48.5 mol%. The major respiratory quinone was Q-8. Phosphatidylethanolamine, phosphatidylglycerol, one unidentified glycolipid, one unidentified aminolipid, one unidentified glycosylphospholipid and two unidentified lipids were detected as the major polar lipids. On the basis of the data from this polyphasic taxonomic study, strain MEBiC05461^T should be classified as a representative of a novel species in the genus *Amphritea*, and the name proposed is *Amphritea spongicola* sp. nov. The type strain is MEBiC05461^T (=KCCM 42943^T=JCM 16668^T). Emendations of the genus *Amphritea* and species *Amphritea atlantica* Gärtner et al. 2008 and *Amphritea balenae* Miyazaki et al. 2008 are also were given.

The genus *Amphritea* Gärtner et al. (2008) was suggested to be a group of rod-shaped Gram-negative bacteria, which are motile by flagella, oxidase- and catalase-positive and accumulate poly-β-hydroxybutyrate (Gärtner et al., 2008). The first member of the genus was isolated from a hydrothermal vent field (Gärtner et al., 2008) and two other members with validly published names were reported to be isolated from marine sediments (Miyazaki et al., 2008). However, the majority of isolates or clones have been obtained mainly from marine animals such as sponges (Esteves et al., 2013; Pike, 2013), ascidians (Dishaw et al., 2009), echinoderms (Becker et al., 2009), corals (Sére et al., 2013; Sunagawa et al., 2009), bone-eating worms (Goffredi et al., 2007; Verna et al., 2010) and algae (Zhang, 2011), but rarely from seawater (D’Ambrosio et al., 2014) or sediments (Li et al., 2009). According to these reports, members of the genus are closely associated with living marine organisms. During a survey of cultivable marine microbial diversity, a novel member of the genus *Amphritea* was isolated from a marine sponge and the taxonomic properties of this novel isolate are reported on here.

Strain MEBiC05461^T was isolated from a marine sponge inhabiting Chuuk State, Federated States of Micronesia (151° 54’ 7” E 7° 27’ 7” N). A small piece of sponge sample was homogenized and diluted with sterilized seawater, spread onto solid marine agar 2216 (MA; BD), and incubated at 25 °C for 3 days. Individual colonies were isolated from MA depending on morphological differences and purified by a series of streaks on new MA. After purification, strain MEBiC05461^T was cultivated at 25 °C on the same medium for biochemical and physiological characterization and stored at −80 °C in marine broth 2216 (MB; BD) supplemented with 20 % (v/v) glycerol. For phenotypic comparisons, *Amphritea balenae* JCM 14781^T (=JAMM 1525^T; Miyazaki et al., 2008) and *Amphritea atlantica* LMG 24143^T (=M41^T; Gärtner et al., 2008) were purchased from JCM (Japan Collection of

**Abbreviations:** PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MEBiC05461^T is GU289646.

Three supplementary figures and one supplementary table are available with the online Supplementary Material.
Microorganisms) and BCCM/LMG (Belgian Co-ordinated Collections of Microorganisms) and cultivated on MA at 25 °C. The cultivation of Amphritea japonica JAMM 1866T in our own laboratory failed; therefore, phenotypic comparisons with this strain could not be conducted.

The extraction of genomic DNA was conducted by using an Exgene cell SV kit (GeneAll), according to the manufacturer’s instructions. Extracted DNA was amplified by using Premix Taq Polymerase PCR solution (T&I) with the bacterial primer set 27F and 1492R (Weisburg et al., 1991). The amplified 16S rRNA gene was sequenced using an ABI 3730 automatic sequencer (ABI) according to the manufacturer’s instructions. The sequences obtained were assembled by using Vector NTI version 9.1 (Life Technologies) and checked by BLAST pair-wise alignment with the EzTaxon-e database (Kim et al., 2012). Strain MEBiC05461T was found to be closely related to members of the genus Amphritea with 96.4–96.6 % 16S rRNA gene sequence similarity. Phylogenetic analysis based on almost complete 16S rRNA gene sequences (1352 bp) of the isolated strain and of closely related members of the family Oceanospirillaceae was conducted using MEGA version 5.2 (Tamura et al., 2011). The neighbour-joining (Saitou & Nei, 1987) tree (Fig. 1) revealed that the isolated strain formed a coherent clade with members of the genus Amphritea that was also recovered in maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) trees with strong bootstrap support (Fig. 1). This result implies that strain MEBiC05461T is a representative of a novel species of the genus Amphritea.

Unless otherwise stated, physiological and morphological characterizations were conducted according to the methods described in Kwon et al. (2005) and Yang et al. (2006).

Transmission electron micrographs were taken using a LIBRA120 (Carl-Zeiss) electron microscope after negative staining of fixed cells with 2 % (w/v) phosphotungstic acid reagent at pH 7.0 and an image of rod-shaped cells with a polar flagellum was obtained (Fig. S1, available in the online Supplementary Material). The temperature for growth was checked firstly in MB at 8.0, 15.5, 19.9, 24.3, 28.0, 31.6, 35.6, 39.5, 53.8 and 60.0 °C, and secondly at 5.0, 12.1, 16.3, 20.2, 23.7, 26.8, 30.2, 33.6, 37.3, 41.1, 45.3 and 50.0 °C in a temperature gradient incubator (TVS126MA; Adaventec) for up to 70 h. Strain MEBiC05461T required components of seawater for growth; therefore, the NaCl tolerance range was tested by incubating with NaCl (Sigma) at 0.0, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0 and 7.0 % (w/v) supplemented with seawater components as described by Bae et al. (2007). The pH range for growth was determined (pH 4.0, 5.0, 5.5, 6.0, 7.0, 7.5, 8.0, 8.5, 9.0 and 10.0) in MB with the pH adjusted using 10 mM MES (pH 4–6), HEPES (pH 6–8) or AMPSO (pH 8–10) as biological buffers. Oxidase and catalase tests were performed using API kits (bioMérieux) according to protocols described by the manufacturer. The DNase test was conducted using commercial medium (DNase test agar, BD). Cellulase activity was evaluated with a method described by Carder (1986). The bacterial suspension used to inoculate API 20E, 20NE, API ZYM kits (bioMérieux) and a Microlog GN2 system (Biolog) was prepared in a 3 % (w/v) sea salt (Sigma) solution with 0.01 % yeast extract (Difco). Experiments to determine the NaCl and pH tolerance range and the biochemical characterization were performed at 25 °C for 2 days. The results of the morphological, cultural, physiological and biochemical characterization of strain MEBiC05461T are given in the species description, Table 1 and Fig. S2.

![Phylogenetic tree based on nearly complete 16S rRNA gene sequences (1352 bp) showing the relationship between strain MEBiC05461T and members of the genus Amphritea with validly published names. The tree is based on the maximum likelihood distances model and the neighbour-joining algorithm. Bootstrap values (>50 %) from neighbour-joining, maximum-likelihood and maximum-parsimony algorithms from 1000 replicated analyses are on the left of nodes. Filled circles, recovered using the three different methods; open circles, recovered using two methods. Bar, 0.01 substitutions per nucleotide position.](http://ijs.sgmjournals.org)
The cellular fatty acid profiles of strain MEBiC05461\textsuperscript{T}, *A. balanee* JCM 14781\textsuperscript{T} and *A. atlantica* LMG 24143\textsuperscript{T} were determined commercially by the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea). The MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990) with Sherlock version 6.2 and the RTSBA6 database was used for analysis (according to the manufacturer’s instructions) on cells grown on MB for 2 days at 25 °C (physiologically at the late-exponential phase). The predominant cellular fatty acids of strain MEBiC05461\textsuperscript{T} were determined to be C\textsubscript{16:0} (23.9 %), summed feature 3 (comprising C\textsubscript{16:1}ω7c and/or C\textsubscript{16:1}ω6c; 39.7 %) and summed feature 8 (comprising C\textsubscript{18:1}ω7c and/or C\textsubscript{18:1}ω6c; 22.0 %), which were similar to those of species belonging to the genus *Amphritea* (Table S1).

Components of the polar lipids were extracted and determined by using a chloroform/methanol solvent system and then separated by two-dimensional TLC using silica gel 60 F\textsubscript{254} aluminium-backed thin-layer plates (Merck) (Minnikin *et al.*, 1984). After two-dimensional development, each component was visualized with an appropriate reagent or treatment. The detailed procedure was described by Yang *et al.* (2013). The predominant polar lipids of strain MEBiC05461\textsuperscript{T} were phosphatidylethanolamine (PE), phosphatidylglycerol (PG), one unidentified glycolipid, one unidentified aminolipid, one unidentified glyco-phospholipid and two unidentified lipids. *A. balenae* JCM 14781\textsuperscript{T} and *A. atlantica* LMG 24143\textsuperscript{T} also contained PG and PE; however, the detailed composition was quite different (Fig. S3). The major isoprenoid quinone was determined commercially by HPLC analysis and found to be Q-8, according to the method of Collins (1985). The DNA G+C content was 48.5 mol%, as determined by HPLC using a symmetry reversed-phase C\textsubscript{18} column (Stackebrandt & Liesack, 1993).

### Table 1. Differential phenotypic characteristics of strain MEBiC05461\textsuperscript{T} and the type strains of the genus *Amphritea*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature range for growth (°C)</td>
<td>8–35.6 (30.0)</td>
<td>4–28 (20–22)*</td>
<td>4–40 (31–34)†</td>
</tr>
<tr>
<td>pH range for growth</td>
<td>5–9 (7.0)</td>
<td>6.5–7.5*</td>
<td>4.6–9.5 (8.0)†</td>
</tr>
<tr>
<td>NaCl concn range for growth (% w/v)</td>
<td>1.5–6 (2–2.5)</td>
<td>2–3 (3)*</td>
<td>0.3–9 (3)†</td>
</tr>
<tr>
<td>Reduction of nitrite to N\textsubscript{2}</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>Production of:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Indole</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Acetoin</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Activities of:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>DNase, esterase lipase</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Acid phosphatase, urease</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Oxidase</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Valine arylamidase</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Fermentation of (API 20E): Sucrose</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Rhamnose</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Assimilation of (API 20NE): Arabinose</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Mannitol, gluconate, phenylacetate</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Malate, citrate</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of (GN2 plate):</td>
<td></td>
<td></td>
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<tr>
<td>Tween 40</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>L-GLutamic acid, D-glucuronic acid, quinic acid, l-alanine</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Sebacic acid</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Itaconic acid, succinamic acid, alaninamide</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>48.5</td>
<td>46.7*</td>
<td>52.2†</td>
</tr>
</tbody>
</table>

*Data from Miyazaki *et al.* (2008).
†Data from Gärtner *et al.* (2008).
The results of the phylogenetic analysis based on 16S rRNA gene sequences suggested that strain MEBiC05461T is affiliated to the genus *Amphritea* (Fig. 1). Strain MEBiC05461T shares common features with members of the genus *Amphritea* such as cell morphology (flagellated rods, Fig. S1), accumulation of storage material, nutrition type (chemo-organotrophic), and activities of catalase, gelatinase, lipase, protease and urease. Additionally, the utilization of methyl-pyruvate and the inability to produce gelatinase, lipase, protease and urease. Furthermore, strain MEBiC05461T could be distinguished from *A. balenae* JCM 14781T and *A. atlantica* LMG 24143T, for example, by DNase and oxidase activities; utilization of Tween 40, 1-l-alanine and some organic acids; fermentation of arabinose, mannitol, sucrose and rhamnose; and nitrate reduction (Table 1). When compared with published data, strain MEBiC05461T could be distinguished from *A. japonica* by its oxidase, DNase and lipase activities; fermentation of glucose and mannitol; and utilization of 1-l-alanine, Tween 40 and of many organic acids in GN2 plates (Miyazaki et al., 2008). On the basis of evidence from this different polyphasic taxonomical study, we suggest that strain MEBiC05461T should be classified as a representative of a novel species in the genus *Amphritea* with the proposed name *Amphritea spongicola* sp. nov.

**Emended description of the genus of *Amphritea* Gartner et al. 2008**

The description is as given by Gartner et al. (2008) with the following emendations. Cells are chemo-organotrophic and motile by monopolar or bipolar flagella. Catalase-positive, but oxidase activity is variable. Mesophilic, slightly halophilic and neutrophilic. Predominant fatty acids are summed feature 8 (C18:1ω7c and/or C18:1ω6c), summed feature 3 (C16:1ω7c and/or C16:1ω6c) and C16:0. The DNA G + C contents range from 46.7 to 52.2 mol%. The major respiratory quinone is Q8. The common polar lipids are PE and PG.

**Emended description of *Amphritea atlantica* Gartner et al. 2008**

The description given by Gartner et al. (2008) remains with the following emendations. The main characteristics are as those given for the genus. Growth occurs at 44 and 40 °C (optimally at 31–34 °C), in the presence of 0.3–9 % (w/v) NaCl (optimally at 3 %, w/v), and at pH 4.6–9.5 (optimally at pH 8.0). Oxidase- and catalase-positive. Valine arylamidase activity is detected (API ZYM) but arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and urase activities are not detected. Reduces nitrate to N₂ and assimilates glucose, mannitol, gluconate, malate, citrate and phenyl-citrace (API 20NE). Fermentation of glucose, mannitol, inositol, sorbitol, rhamnose and sucrose is positive (API 20E). The carbon sources oxidized (Biolog GN2) are β-hydroxybutyric acid, D-lactic acid, propionic acid, sebacic acid and phenylethylamine. The predominant polar lipids are PG, PE, an unidentified lipid and three unidentified aminolipids.

**Emended description of *Amphritea balenae* Miyazaki et al. 2008**

The description given by Miyazaki et al. (2008) remains with the following emendations. Alkaline phosphatase, naphthol-AS-BI phosphohydrolase, esterase (C₄), esterase lipase (C₈) and leucine arylamidase activities are detected (API ZYM). Ferments glucose, mannitol, inositol and sorbitol (API 20E); assimilates glucose and arabinose (API 20NE). Carbon sources oxidized (Biolog GN2) are Tween 80, monomethyl succinate, p-hydroxyphenylacetic acid, itaconic acid, α-ketoglutaric acid, D-lactic acid, propionic acid, sebacic acid and phenylethylamine, but methyl β-D-glucoside, L-alanine, L-asparagine and L-serine are not oxidized. The predominant polar lipids are PG, PE, two unidentified lipids and three unidentified aminophospholipids.

**Description of *Amphritea spongicola* sp. nov.**

*Amphritea spongicola* [spon.gi.co.la. L. fem. spongia a sponge; L. masc./fem. suffix n. -cola (from incola) inhabitant; N.L. nom. n. (in apposition) spongica inhabitant of sponges].

Cells are rod-shaped (1.22.1 μm × 0.8–0.9 μm). Colonies on MA are circular with entire edges, yellow and 0.5–1 mm in diameter after cultivation at 25 °C for 2–3 days. Growth is observed at 8.0–35.6 °C (optimum, 30.0 °C), at pH values of between pH 5.0 and 9.0 (optimum pH 7.0) and in the presence of 1.5–6.0 % (w/v) NaCl (optimum 2.0–2.5 %, w/v). Requires Mg²⁺ or Ca²⁺ in addition to NaCl for growth. DNase and cellulase activities are negative. Catalase-positive, but oxidase-negative. Nitrate is reduced to nitrite. In the API 20E and 20NE systems, assimilates glucose, N-acetylglucosamine, citrate and malate, and ferments glucose, mannitol, inositol, sorbitol and rhamnose.Produces acetoin and indole. When assayed with the API ZYM system the alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase and naphthol-AS-BI phosphohydrolase reactions are positive. Utilizes Tween 40, Tween 80, methyl pyruvate, monomethyl succinate, D-glucoronic acid, β-hydroxybutyric acid, p-hydroxyphenylacetic acid, itaconic acid, α-ketoglutaric acid, D-lactic acid, propionic acid, succinic acid, quinic acid, alaminamide, L-alanine, D-glutamic acid and phenylethylamine, based on Microlog GN2 plates. The major fatty acids are C₁₆:₀ summed feature 3 (comprising C₁₆:₁ω7c and/or C₁₆:₁ω6c) and summed feature 8 (comprising C₁₆:₁ω7c and/or C₁₆:₁ω6c). The predominant polar lipids are PG, PE, glycolipid, aminolipid, glycopospholipid and two unidentified lipids.

The type strain, MEBiC05461T (=KCCM 42943T = JCM 16668T) was isolated from a marine sponge collected in Micronesia. The DNA G+C content of the type strain is 48.5 mol%.

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References


