Amphritea ceti sp. nov., isolated from faeces of Beluga whale (Delphinapterus leucas)

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A Gram-stain-negative, aerobic, non-spore-forming, non-flagellated and rod-shaped or ovoid bacterial strain, designated RA1T, was isolated from faeces collected from Beluga whale (Delphinapterus leucas) in Yeosu aquarium, South Korea. Strain RA1T grew optimally at 25 °C, at pH 7.0–8.0 and in the presence of 2.0% (w/v) NaCl. Neighbour-joining, maximum-likelihood and maximum-parsimony phylogenetic trees based on 16S rRNA gene sequences revealed that strain RA1T joins the cluster comprising the type strains of three species of the genus Amphritea, with which it exhibited 95.8–96.0% sequence similarity. Sequence similarities to the type strains of other recognized species were less than 94.3%. Strain RA1T contained Q-8 as the predominant ubiquinone and summed feature 3 (C16 : 1ω7c and/or C16 : 1ω6c), C18 : 1ω7c and C16 : 0 as the major fatty acids. The major polar lipids of strain RA1T were phosphatidylethanolamine, phosphatidylglycerol, two unidentified lipids and one unidentified aminolipid. The DNA G+C content of strain RA1T was 47.4 mol%. The differential phenotypic properties, together with the phylogenetic distinctiveness, revealed that strain RA1T is separated from other species of the genus Amphritea. On the basis of the data presented, strain RA1T is considered to represent a novel species of the genus Amphritea, for which the name Amphritea ceti sp. nov. is proposed. The type strain is RA1T (=KCTC 42154T=NBRC 110551T).

The genus Amphritea, a member of the class Gammaproteobacteria, was proposed by Gärtner et al. (2008) with the description of a single species, Amphritea atlantica, isolated from a Bathymodiolus sp. specimen collected from a hydrothermal vent field at the Mid-Atlantic Ridge. Subsequently, two further species of the genus Amphritea, Amphritea balenae and Amphritea japonica (Miyazaki et al., 2008), have been isolated from sediment adjacent to sperm whale carcasses off Kagoshima, Japan. In this study, a novel bacterial strain, designated RA1T, which was isolated from faeces of Beluga whale (Delphinapterus leucas) in Yeosu aquarium, South Korea, is described. Comparative 16S rRNA gene sequence analysis showed that this novel strain is phylogenetically most closely related to members of the genus Amphritea of the class Gammaproteobacteria. The aim of the present work was to determine the exact taxonomic position of strain RA1T by using a polyphasic taxonomic characterization which included determination of the chemotaxonomic and other phenotypic properties and detailed phylogenetic analyses based on 16S rRNA gene sequences.

Faeces were collected from Beluga whale (D. leucas) in Yeosu aquarium, South Korea, and used as a source for the isolation of bacterial strains. Strain RA1T was isolated by the standard dilution plating technique at 20 °C on a mixture (1:1, v/v) of R2A agar (Becton Dickinson) and marine agar 2216 (MA; Becton Dickinson), and cultivated routinely at 25 °C on MA. Amphritea atlantica DSM 18887T, and Amphritea balenae JCM 14781T and Amphritea japonica JCM 14782T, which were used as reference strains for phenotypic characterization and analyses of fatty acids and polar lipids, were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, and the Japan Collection of Microorganisms (JCM), Saitama, Japan, respectively. The cell morphology, Gram reaction, pH range for growth and anaerobic growth

Abbreviations: AL, unidentified aminolipid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RA1T is KJ867528.

Three supplementary figures are available with the online Supplementary Material.
were determined as described by Kim et al. (2013). Motility was assessed under ×1500 magnification by using the hanging drop technique with cells grown for 3–5 days at 25 °C. Growth at 4, 10, 20, 25, 30, 35 and 37 °C was measured on MA to determine the optimal temperature and temperature range for growth. Growth at various concentrations of NaCl (0, 0.5 and 1.0–8.0 %, in increments of 1.0 %) was investigated by supplementing with appropriate concentrations of NaCl in marine broth 2216 (MB) prepared according to the formula of the Becton Dickinson medium except that NaCl was excluded. Requirement of Mg²⁺ ions was investigated by using MB, prepared according to the formula of the Becton Dickinson medium, that comprised all of the constituents except MgCl₂ and MgSO₄. Catalase and oxidase activities were determined as described by Lányi (1987). Hydrolysis of casein, starch, hypoxanthine, L-tyrosine and xanthine was tested on MA using the substrate concentrations described by Barrow & Feltham (1993). Hydrolysis of aesculin and Tween 80 and nitrate reduction were investigated as described by Lányi (1987) with the modification that artificial seawater was used for the preparation of media. The artificial seawater contained (1 l−1 distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂.6H₂O, 5.94 g MgSO₄.7H₂O and 1.3 g CaCl₂.2H₂O (Bruns et al., 2001). Hydrolysis of gelatin and urea was investigated by using nutrient gelatin and urea agar base media (Becton Dickinson), respectively, with the modification that artificial seawater was used for the preparation of media. Utilization of various substrates for growth was tested according to the methods of Baumann & Baumann (1981), using supplementation with 1 % (v/v) vitamin solution (Staley, 1968) and 2 % (v/v) Hutner’s mineral salts (Cohen-Bazire et al., 1957). Susceptibility to antibiotics was determined after incubation on MA plates by using antibiotic discs (Advantec) containing the following (µg per disc unless otherwise stated): ampicillin (10), carbenicillin (100), cefalotin (30), chloramphenicol (100), gentamicin (30), kanamycin (30), lincomycin (15), neomycin (30), novobiocin (5), oleandomycin (15), penicillin G (20 U), polymyxin B (100 U), streptomycin (50) and tetracycline (30). Enzyme activities were determined, after incubation for 8 h at 25 °C, by using the API ZYM system (bioMérieux); the strip was inoculated with cells suspended in artificial seawater (Bruns et al., 2001) from which CaCl₂ was excluded.

Cell biomass of strain RA¹T for DNA extraction and for the analyses of isoprenoid quinones and polar lipids was obtained from cultures grown for 2 days in MB at 25 °C, and cell biomass of A. atlantica DSM 18887T, A. balenae JCM 14781T and A. japonica JCM 14782T for polar lipid analysis was obtained from cultures grown under the same conditions. Chromosomal DNA was extracted and purified according to the protocol of Yoon et al. (1996), with the modification that RNase T1 was used in combination with RNase A to minimize contamination by RNA. The 16S rRNA gene was amplified by PCR as described previously (Yoon et al., 1998) using two universal primers (5'-GAGTTTGATCCTGCTCAG-3' and 5'-ACGTTACCTTGTTACGACTT-3'). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon et al. (2003).

Isoprenoid quinones were extracted and analysed as described by Komagata & Suzuki (1987), using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. The isoprenoid quinones were eluted by a mixture of methanol/2-propanol (2:1, v/v) using a flow rate of 1 ml/min.

Table 1. Differential phenotypic characteristics of strain RA¹T and the type strains of three species of the genus Amphritea

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>RA¹T</th>
<th>RA²T</th>
<th>JCM 14781T</th>
<th>JCM 14782T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility*</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth at:*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 °C</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>30 °C</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Growth with 5 % (w/v) NaCl*</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Fructose</td>
<td>−</td>
<td></td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>d-Glucose</td>
<td>−</td>
<td></td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>d-Xylose</td>
<td>−</td>
<td></td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Acetate</td>
<td>−</td>
<td></td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Benzoate</td>
<td>−</td>
<td></td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Citrate</td>
<td>−</td>
<td></td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>L-Malate</td>
<td>−</td>
<td></td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>−</td>
<td></td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Succinate</td>
<td>−</td>
<td></td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Enzyme activity (API ZYM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Esterase (C4)</td>
<td>−</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Esterase lipase (C8)</td>
<td>−</td>
<td></td>
<td>w</td>
<td>−</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>−</td>
<td></td>
<td>w</td>
<td>−</td>
</tr>
<tr>
<td>Naphthol-AS-BI-phosphohydrolase</td>
<td>−</td>
<td></td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Susceptibility to:</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>−</td>
<td></td>
<td>−</td>
<td>+</td>
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<td>Novobiocin</td>
<td>+</td>
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<td>−</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>+</td>
<td></td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

*Data for A. atlantica DSM 18887T, and A. balenae JCM 14781T and A. japonica JCM 14782T taken from Gärtner et al. (2008) and Miyazaki et al. (2008), respectively.
min⁻¹ at room temperature and detected by UV absorbance at 275 nm. For cellular fatty acid analysis, cell masses of strain RA1ᵀ, *A. atlantica* DSM 18887ᵀ, *A. balenae* JCM 14781ᵀ and *A. japonica* JCM 14782ᵀ were harvested from MA plates after cultivation for 3 days at 25 °C. The physiological age of the cell masses was standardized by observing the growth development of colonies on the agar plates followed by harvesting them from the same quadrant of the agar plates according to the standard MIDI protocol (Sherlock Microbial Identification System, version 6.1). Fatty acids were saponified, methylated and extracted using the standard MIDI protocol (Sherlock Microbial Identification System, version 6.1). The fatty acids were analysed by GC (Hewlett Packard 6890) and identified using the TSBA6 database of the Microbial Identification System (Sasser, 1990). Polar lipids were extracted according to the procedures described by Minnikin *et al.* (1984) 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 as described by Embley & Wait (1994). Individual polar lipids were identified by spraying the plates with 10% (w/v) ethanolic molybdophosphoric acid, molybdenum blue, ninhydrin and ɑ-naphthol reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987) and with Dragendorff’s reagent (Sigma). The DNA G+C content was determined by the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. The nucleotides were eluted by a mixture of 0.55 M NH₄H₂PO₄ (pH 4.0) and acetonitrile (40:1, v/v), using a flow rate of 1 ml min⁻¹ at room temperature and detected by UV absorbance at 270 nm.

Morphological, cultural, physiological and biochemical characteristics of strain RA1ᵀ are given in the species description and in Table 1 or Fig. S1 (available in the online Supplementary Material). The almost complete 16S rRNA gene sequence of strain RA1ᵀ determined in this study comprised 1462 nt, approximately 95% of the

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**Fig. 1.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain RA1ᵀ, the type strains of species of the genus *Amphritea* and some other related taxa of the class Gammaproteobacteria. Bootstrap values (expressed as percentages of 1000 replications) of >50% are shown at branching points. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. *E. coli* ATCC 11775ᵀ (GenBank accession number X80725) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.
Escherichia coli 16S rRNA gene sequence. In the neighbour-
joining phylogenetic tree based on 16S rRNA gene
sequences, strain RA1\textsuperscript{T} joined the cluster comprising the
type strains of *A. atlantica*, *A. balenae* and *A. japonica* with
a bootstrap resampling value of 99.0\% (Fig. 1). The
relationships among strain RA1\textsuperscript{T} and the type strains of
*A. atlantica*, *A. balenae* and *A. japonica* were maintained in the
trees reconstructed using the maximum-likelihood and
maximum-parsimony algorithms (Fig. 1). Strain RA1\textsuperscript{T}
exhibited 16S rRNA gene sequence similarity values of 96.0,
96.0 and 95.8\% to *A. atlantica*, *A. japonica* and *A. balenae*,
respectively, and of less than 94.3\% to the type strains of
other recognized species.

The predominant isoprenoid quinone detected in strain RA1\textsuperscript{T} was ubiquinone-8 (Q-8). The predominant ubiqui-
none of the type strains of *A. balenae* and *A. japonica* was
described to be Q-8 (Miyazaki et al., 2008). In Table 2, the fatty acid profile of strain RA1\textsuperscript{T} is compared with those
of the type strains of three species of the genus *Amphritea*
with validly published names, which were grown and
analysed under identical conditions in this study. The
major fatty acids (>10\% of the total fatty acids) detected
in strain RA1\textsuperscript{T} were summed feature 3 (C\textsubscript{16:1}ω7c and/or
C\textsubscript{16:1}ω6c, 45.6\%), C\textsubscript{18:1}ω7c (26.9\%) and C\textsubscript{16:0} (18.7\%).
The fatty acid profile of strain RA1\textsuperscript{T} was similar to those of
*A. atlantica* DSM 18887\textsuperscript{T}, *A. balenae* JCM 14781\textsuperscript{T} and
*A. japonica* JCM 14782\textsuperscript{T}, although there were differences in the proportions of some fatty acids (Table 2). The major
polar lipids found in strain RA1\textsuperscript{T} were phosphatidylethyla-
nolamine (PE), phosphatidylglycerol (PG), two uniden-
tified lipids (L1–2) and one unidentified aminolipid (AL); significant or minor amounts of one unidentified phos-
pholipid and two unidentified aminophospholipids were
also present (Figs. S2 and S3). The polar lipid profile of
strain RA1\textsuperscript{T} was similar to those of *A. atlantica* DSM 18887\textsuperscript{T},
*A. balenae* JCM 14781\textsuperscript{T} and *A. japonica* JCM 14782\textsuperscript{T} in that
PE, PG and L1 were major polar lipids. However, it was
distinguishable from those of *A. atlantica* DSM 18887\textsuperscript{T}
and *A. japonica* JCM 14782\textsuperscript{T} by the presence of one additional
unidentified lipid (L2), and distinguishable from that of *A.
balenae* JCM 14781\textsuperscript{T} by the presence of one additional
unidentified lipid (L2) and AL (Figs. S2 and S3). The DNA
G+C content of strain RA1\textsuperscript{T} was 47.4 mol\%, a value in the
range reported for members of the genus *Amphritea*
(Gärnter et al., 2008; Miyazaki et al., 2008).

It is reasonable to classify strain RA1\textsuperscript{T} as a member of the
genus *Amphritea* as shown by the phylogenetic data and the
absence of differentiating chemotaxonomic properties
from the type strains of the three species of the genus
*Amphritea* (Figs 1 and S2, Table 2; Gärnter et al., 2008;
Miyazaki et al., 2008). Strain RA1\textsuperscript{T} could be distinguished
from the type strains of *A. atlantica*, *A. balenae* and *A.
japonica* by differences in several phenotypic characteris-
tics, including motility, growth at 4 and 30 °C, utilization
of some substrates, activity of some enzymes and
susceptibility to some antibiotics (Table 1). These differ-
ences, in combination with the phylogenetic distinctiveness

### Table 2. Cellular fatty acid compositions of strain RA1\textsuperscript{T} and the type strains of three species of the genus *Amphritea*

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Straight-chain</th>
<th>Unsaturated</th>
<th>Hydroxy</th>
<th>Summed feature 3*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C\textsubscript{12:0}</td>
<td>C\textsubscript{13:0}</td>
<td>C\textsubscript{12:1}</td>
<td>C\textsubscript{10:0}</td>
</tr>
<tr>
<td>1</td>
<td>7.9</td>
<td>5.2</td>
<td>3.8</td>
<td>2.4</td>
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<tr>
<td>2</td>
<td>8.9</td>
<td>5.2</td>
<td>3.8</td>
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<tr>
<td>3</td>
<td>8.9</td>
<td>5.2</td>
<td>3.8</td>
<td>2.4</td>
</tr>
<tr>
<td>4</td>
<td>8.9</td>
<td>5.2</td>
<td>3.8</td>
<td>2.4</td>
</tr>
</tbody>
</table>

*Summed feature 3 comprised C\textsubscript{16:1}ω7c and/or C\textsubscript{16:1}ω6c. of strain RA1\textsuperscript{T}, suggest that the novel strain is separated
from other species of the genus *Amphritea* (Stackebrandt &
Goebel, 1994). On the basis of the phenotypic, chemotaxonomic and phylogenetic data, therefore, strain RA1\textsuperscript{T}
is considered to represent a novel species of the genus
*Amphritea*, for which the name *Amphritea ceti* sp. nov. is proposed.

### Description of *Amphritea ceti* sp. nov.

*Amphritea ceti* (ce’ti. L. gen. n. ceti of a whale).

Cells are Gram-stain-negative, non-spore-forming, non-
flagellated and rod-shaped or ovoid, approximately 0.2–
0.7 μm in diameter and 0.5–>10.0 μm in length; a few cells
greater than 10 μm in length are observed. Colonies on MA
are circular, slightly convex, smooth, glistening, greyish-
yellow in colour and 1.0–2.0 mm in diameter after
incubation for 3 days at 25 °C. Optimal growth occurs at
25 °C; growth occurs at 10 and 30 °C, but not at 4 or
35 °C. Optimal pH for growth is between pH 7.0 and 8.0;
growth occurs at pH 5.5, but not at pH 5.0. Growth occurs
in the presence of 1.0–5.0 \% (w/v) NaCl with an optimum of
approximately 2.0 \% (w/v) NaCl. Mg\textsuperscript{2+} ions are required
for growth. Anaerobic growth does not occur on MA or on
MA supplemented with nitrate. Catalase- and oxidase-
positive.Nitrate is reduced to nitrite. Hypoxanthine, Tween
80 and L-tyrosine are hydrolysed, but aesculin, casein,
gelatin, starch, urea and xanthine are not. D-Xylose is
utilized as a carbon and energy source, but L-arabinose,
cellobiose, D-fructose, D-galactose, D-glucose, maltose,
D-mannose, sucrose, trehalose, acetate, benzoate, citrate, formate, L-malate, pyruvate, succinate, salicin and L-glutamate are not. In assays with the API ZYM system, alkaline phosphatase and leucine arylamidase activities are present, but esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, \( \sigma \)-chymotrypsin, acid phosphatase, naphthol-AS-BI-phospho- hylidase, \( \sigma \)-galactosidase, \( \beta \)-galactosidase, \( \beta \)-glucuronidase, \( \sigma \)-glucosidase, \( \beta \)-glucosaminidase, \( \sigma \)-mannosidase and \( \sigma \)-fucosidase activities are absent. Susceptible to carbenicillin, cefalotin, chloramphenicol, gentamicin, kanamycin, neomycin, novobiocin, penicillin \( \sigma \) and streptomycin, but not to ampicillin, lincomycin, gentamicin, kanamycin, neomycin, novobiocin, penicillin \( \sigma \) and streptomycin, but not to ampicillin, lincomycin, oleandomycin, polymyxin B and tetracycline. The predominant ubiquinone is Q-8. The major fatty acids (>10% of the total fatty acids) are summed feature 3 (C16:1 \( \alpha 7 \)c and/or C16:1 \( \alpha 6 \)c), C18:1 \( \omega 7 \)c and C16:0. The major polar lipids are PE, PG, two unidentified lipids and one AL.

The type strain, RA1 \(^T\) (=KCTC 42154\(^T\)=NBRC 110551\(^T\)), was isolated from faeces collected from Beluga whale (Delphinapterus leucas) in Yeosu aquarium, South Korea. The DNA G+C content of the type strain is 47.4 mol%.

Acknowledgements

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References


