Amphritea japonica sp. nov. and *Amphritea balenae* sp. nov., isolated from the sediment adjacent to sperm whale carcasses off Kagoshima, Japan

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Two novel species were isolated from the sediment adjacent to sperm whale carcasses off Kagoshima, Japan, at a depth of about 230 m. The isolated strains, JAMM 1866^T, JAMM 1548 and JAMM 1525^T, were Gram-negative, rod-shaped, non-spore-forming and motile by means of a single polar or bipolar flagellum. Phylogenetic analysis based on 16S rRNA gene sequences of strains JAMM 1866^T and JAMM 1548 indicated a relationship to the symbiotic bacterial clone R21 of *Osedax japonicus* (100 % sequence similarity) and all three isolates were closely related to *Amphritea atlantica* (97.7–97.8 % similarity) within the class *Gammaproteobacteria*. The novel isolates were able to produce isoprenoid quinone Q-8 as the major component. The predominant fatty acids were C_{16:0}, C_{16:1} and C_{18:1}, with C_{12:1} 3-OH present in smaller amounts. The DNA G+C contents of the three isolated strains were about 47 mol%. Based on differences in taxonomic characteristics, the three isolated strains represent two novel species of the genus *Amphritea* for which the names *Amphritea japonica* sp. nov. (type strain JAMM 1866^T=JCM 14782^T=ATCC BAA-1530^T; reference strain JAMM 1548) and *Amphritea balenae* sp. nov. (type strain JAMM 1525^T=JCM 14781^T=ATCC BAA-1529^T) are proposed.

Members of the genus *Amphritea* are described as Gram-negative, rod-shaped cells with a monopolar flagellum and have been isolated from a *Bathymodiolus* sp. specimen collected at the Mid-Atlantic Ridge at a depth of about 3000 m (Gärtner *et al.*, 2008). *Amphritea atlantica* is the only recognized species of this genus. The genus *Amphritea* is related to the genera *Oceanospirillum* and *Neptunomonas* and to a symbiotic bacterial clone of *Osedax* (Goffredi *et al.*, 2007). The genus *Osedax* (Polychaeta: Siboglinidae) has recently been discovered in whale carcass environments on the deep-sea floor (Rouse *et al.*, 2004; Glover *et al.*, 2005; Fujikura *et al.*, 2006; Braby *et al.*, 2007). *Osedax* specimens host symbiotic bacteria in their ovisac and root systems. Phylogenetic analysis placed these *Osedax* microbes within a well-supported clade of the *Gammaproteobacteria* that contains heterotrophic members of the order

Oceanospirillales (Goffredi *et al.*, 2007). Most genera of the order *Oceanospirillales* (Garrity *et al.*, 2005) are halotolerant or halophilic and motile. These genera are aerobic, microaerophilic or facultatively anaerobic chemo-organotrophs. Sperm whale carcass ecosystems off Kagoshima, Japan, were investigated over a period of 3 years (Fujiwara *et al.*, 2007). About 800 aerobic bacteria were isolated from sediment adjacent to the sperm whale carcasses and three of these strains were related to members of the family *Oceanospirillaceae* and a symbiotic bacterial clone of *Osedax japonicus*. In this paper, several lines of evidence are presented that indicate that these isolates represent two novel species of the genus *Amphritea*.

The three strains, JAMM 1866^T, JAMM 1548 and JAMM 1525^T, were collected from marine sediment adjacent to the sperm whale carcasses by the unmanned ROV *Hyper Dolphin* off Kagoshima, Japan (dive 457, 31° 20.720' N 129° 59.285' E, at a depth of 228 m; dive 465, 31° 20.730' N 129° 59.314' E, at a depth of 229 m) during cruise NT05-12. The sediment samples were collected with the manipulator of the ROV and placed in the sample holder of the sterilized sampler. A portion of each sample was cultivated on marine agar 2216 (MA; Difco) at 12 °C

Abbreviations: ML, maximum likelihood; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains JAMM 1866^T, JAMM 1548 and JAMM 1525^T are AB330881–AB330883, respectively.

Transmission electron micrographs of negatively stained cells of the two novel species described in this study are available with the online version of this paper.

for approximately 7 days. The bacteria were maintained on MA plates or in marine broth 2216 (MB; Difco) at 20 °C and stored at -80 °C in 15 % (v/v) glycerol. Unless otherwise indicated, physiological tests were performed according to the general procedures described by Barrow & Feltham (1993) and Miyazaki *et al.* (2008b) with the modification that artificial seawater (ASW; 1 × ASW consisting of 2.75 % NaCl, 0.07 % KCl, 0.54 % MgCl₂·6H₂O, 0.68 % MgSO₄·7H₂O, 0.14 % CaCl₂·2H₂O, 0.02 % NaHCO₃) was used. Growth at various temperatures (4–30 °C) was measured in MB for 7 days. Growth at various NaCl concentrations was examined in medium containing 0.5 % peptone (Difco), 0.5 % yeast extract (Difco) and 0.32 % MgSO₄·7H₂O, with NaCl concentrations of 0–6 % (w/v) (pH adjusted to 7.2 at 20 °C). The pH range for growth was determined in 0.5 % peptone, 0.5 % yeast extract, 0.32 % MgSO₄·7H₂O and 3.0 % NaCl (w/v), with the pH adjusted to 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. The test strains retained viability for about 7 days at the optimal temperature. Acid production from sugars was assessed using modified oxidization-fermentation medium (Hugh & Leifson, 1953) containing 0.5 × ASW, 0.1 % (NH₄)₂SO₄, 0.1 % yeast extract (Difco), 0.1 % Tris, 1.4 % NaCl, 1 % sugar and 0.006 % bromothymol blue (pH adjusted to 7.2 at 20 °C) and incubated at the optimum temperature. Oxidase activity was determined by spreading cell pellets on oxidase test paper (Nissui Pharmaceutical). Catalase activity was determined based on bubble production in 3 % (v/v) H₂O₂ solution. Gelatinase, protease, amylase and lipase (hydrolysis of tri-*n*-butyrin) activities were detected on MA plates using substrate concentrations of 1 % (w/v). DNase activity was assessed using DNase test agar (Difco). Hydrogen sulfide production from thiosulfate and indole production were assessed using SIM medium (Nissui Pharmaceutical) slabs prepared with 0.5 × ASW instead of water. Additional phenotypic characteristics were determined using the Biolog GN2 microbial identification system following the protocol provided by the manufacturer. Susceptibility to antimicrobial substances was examined on MA using Sensi-Discs (Becton Dickinson). Any sign of growth inhibition after 72 h incubation at 20 °C was recorded as sensitivity to the respective antimicrobial agent. The following antibiotics (Becton Dickinson) were examined (amounts per disc): ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), neomycin (30 µg), novobiocin (30 µg), penicillin (10 IU), streptomycin (10 µg) and tetracycline (30 µg).

Transmission electron microscopy of negatively stained cells was conducted as described by Nogi *et al.* (1998). Cells of strains JAMM 1866^T and JAMM 1525^T grown on MA at optimal temperature and in the mid-exponential phase of growth were used for electron microscopic observations (JEM-1210; JEOL).

Cellular fatty acids and isoprenoid quinones were analysed. Isolated strains were cultured in MB at optimal growth

temperatures. Cellular fatty acids were analysed by GLC-MS (Miyazaki *et al.*, 2006). Isoprenoid quinones were extracted with chloroform/methanol (2 : 1) from dried cells (200 mg) and purified by TLC. The purified isoprenoid quinones were analysed using reversed-phase HPLC (Komagata & Suzuki, 1987).

Chromosomal DNA was purified using the phenol extraction method (Saito & Miura, 1963). The DNA G + C content was determined using reversed-phase HPLC (Tamaoka & Komagata, 1984). For analysis of relatedness, DNA-DNA hybridization was carried out at 44 °C for 4 h and measured fluorometrically using the method of Ezaki *et al.* (1989).

16S rRNA gene sequences were obtained by direct sequencing of PCR-amplified DNA as described previously (Miyazaki *et al.*, 2008a). Sequences were aligned with the subset of 16S rRNA gene sequences obtained from GenBank using the FASTALIGNER utility of the ARB software (Ludwig *et al.*, 2004). The phylogenetic relationships of each taxon were inferred with the neighbour-joining (NJ) and maximum-likelihood (ML) methods using PAUP* 4.0 beta 10 (Swofford, 1998). ML trees (Felsenstein, 1981) were inferred using the default starting parameters (NJ using a Jukes-Cantor model of evolution). NJ trees (Saitou & Nei, 1987) were inferred using the Hasegawa, Kishino and Yano (HKG85) distance. Statistical support of the branch points was tested by performing 1000 NJ and 500 ML bootstrap replications. The GenBank/DBJ/EMBL accession numbers for the 16S rRNA gene sequences of the isolates are shown in Fig. 1. Other reference sequences were obtained from GenBank.

Cultural, physiological and biochemical characteristics of the isolates are shown in Table 1 and given in the species descriptions below. Physiological and biochemical characteristics of the two groups showed some differences when compared with the type strains of related species and with each other. Strains JAMM 1866^T and JAMM 1548 showed no growth at 30 °C or in 6 % NaCl and did not hydrolyse Tweens 40 and 80; their DNA G + C contents differed from those of related strains. Strain JAMM 1525^T had a bipolar or polar flagellum, did not grow at 30 °C or in 6 % NaCl, was positive for catalase (weak) and gelatinase, and did not hydrolyse Tweens 40 and 80; the DNA G + C content differed from those of related strains.

The whole-cell fatty acid compositions of the isolates and reference strains are shown in Table 2. The major fatty acids of the novel isolates and *A. atlantica* M41^T were C_{16:0}, C_{16:1} and C_{18:1}. Furthermore, these strains contained C_{12:1} 3-OH, a characteristic feature of the genus *Amphritea*. The fatty acid profile showed low levels of similarity to those of reference strains. For example, the isolates produced C_{12:1} and C_{18:2}, but *A. atlantica* M41^T did not produce C_{12:1} and the reference strains did not produce C_{18:2}.

Results of the NJ phylogenetic analyses using 16S rRNA gene sequences are shown in Fig. 1. These results support

Fig. 1. Phylogenetic tree constructed using the NJ method and based on 16S rRNA gene sequences showing the relationship between the three isolates and related members of the class *Gammaproteobacteria*. The bootstrap resampling percentages (NJ, 1000 replicates; ML, 500 replicates) that support branching points above the 50 % confidence level are indicated. Bar, 0.01 nucleotide substitutions per site.

the conclusions described below and further clarify the taxonomic and phylogenetic positions of the three isolated strains and the symbiotic bacterial clone R21 of *Osedax japonicus* within the genus *Amphritea*. The three strains showed high sequence similarities to the following species/genera: *A. atlantica* M41^T (97.7–97.8 %), genus *Neptunomonas* (92.6–94.3 %), genus *Oceanospirillum* (91.3–93.2 %) and *Neptuniibacter caesariensis* MED92^T (94.3–94.5 %).

However, they were more closely related to the 16S rRNA gene sequence of symbiotic bacterial clone R21 of *Osedax japonicus* (99.0–100 %). The level of 16S rRNA gene sequence similarity between strains JAMM 1866^T and JAMM 1525^T was 99.0 %. The generally recommended and accepted criteria for delineating bacterial species state that strains with 16S rRNA gene sequence dissimilarity of greater than 3 % are considered to belong to separate species

Table 1. Differential characteristics of the two novel *Amphritea* species and related type species of the family *Oceanospirillaceae*

Strains: 1, *A. japonica* sp. nov. (strains JAMM 1866^T and JAMM 1548); 2, *A. balenae* sp. nov. (strain JAMM 1525^T); 3, *A. atlantica* M41^T; 4, *Neptunomonas naphthovorans* ATCC 700637^T; 5, *Oceanospirillum linum* NBRC 15448^T. +, Positive; –, negative; w, weak reaction; ND, no data. Data from this study, Garrity *et al.* (2005), Gärtner *et al.* (2008), Hedlund *et al.* (1999), Holt *et al.* (1994), Krieg (1984) and Satomi *et al.* (2002).

Characteristic	1	2	3	4	5
Cell morphology:					
Shape	Rods	Rods	Rods	Rods	Spirillum
Length (μm)	0.9–1.7	1.3–2.0	0.5–2.0	2.0–3.0	1.8–4.0
Flagella	Single polar	Single polar or bipolar	Single polar	Polar	Bipolar
Growth at/in:					
30 °C	–	–	+	–	+
6 % NaCl	–	–	+	+	+
Catalase	+	w	+	+	+
Gelatinase	–	+	–	–	+
DNase	+	+	ND	–	–
Lipase	+	+	–	ND	ND
Nitrate reduction	+	+	ND	–	–
Hydrolysis of:					
Tween 40	–	–	+	ND	ND
Tween 80	–	–	+	–	ND
Acid production from sugars	+	+	+	+	–
DNA G + C content (mol%)	46.6–47.0	46.7	52.2	46.3	48–50

Table 2. Fatty acid contents of the two novel species of the genus *Amphritea* and the type species of related genera

Strains: 1, *A. japonica* sp. nov. JAMM 1866^T (data from this study); 2, *A. balenae* sp. nov. JAMM 1525^T (data from this study); 3, *A. atlantica* M41^T; 4, *Neptunomonas naphthovorans* ATCC 700637^T; 5, *Oceanospirillum linum* NBRC 15448^T. tr, Trace (<1 %); empty cells indicate that the fatty acid was not detected. Data for taxa 3–5 are from Gärtner *et al.* (2008), Miyazaki *et al.* (2008a) and Sakane & Yokota (1994).

Fatty acid	1	2	3	4	5
Non-polar:*					
C _{10:0}			tr		
C _{12:0}			2	7	3
C _{12:1}	3	4		4	2
C _{14:0}					1
C _{15:1}					
C _{16:0}	18	14	29	21	16
C _{16:1}	37	47	43	41	48
C _{18:0}	1	tr	tr	tr	
C _{18:1}	35	34	22	28	30
C _{18:2}	5	2			
C _{20:1}	tr				
3-Hydroxy:†					
C _{10:0}	58	81	64	100	100
C _{12:1}	42	19	36		

*Numbers refer to the percentage of an acid relative to the total non-polar fatty acids.

†Numbers refer to the percentage of an acid relative to the total hydroxy fatty acids.

(Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002). The generally recognized criteria for delineating bacterial species state that strains with a DNA–DNA relatedness of less than 70 %, as measured by hybridization, represent separate species (Wayne *et al.*, 1987). The results of DNA–DNA hybridization analysis of the isolated strains and *A. atlantica* M41^T showed that there was greater than 91 % DNA–DNA relatedness between strains JAMM 1866^T and JAMM 1548, but the relatedness values between these two strains, JAMM 1525^T and *A. atlantica* were less than 18 % and each group was clearly separate, representing distinct species according to the recommendations of Wayne *et al.* (1987).

From the results of phenotypic, genotypic and phylogenetic analyses, it is evident that the three isolated strains represent two novel species within the genus *Amphritea* for which the names *Amphritea japonica* sp. nov. and *Amphritea balenae* sp. nov. are proposed.

Description of *Amphritea japonica* sp. nov.

Amphritea japonica (ja.po'ni.ca. N.L. fem. adj. *japonica* pertaining to Japan, where the isolate originated).

Cells are rod-shaped; cell width ranges from 0.6 to 0.9 µm and cell length ranges from 0.9 to 1.7 µm (see

Supplementary Fig. S1 in IJSEM Online). Cells are Gram-negative, non-spore-forming, chemo-organotrophic and motile by means of a single polar flagellum. Colonies on MA are circular with entire edges, smooth, convex, cream coloured and 0.5–1.0 mm in diameter after 1–2 days incubation at 20 °C. The optimal growth temperature is 22–24 °C. Growth occurs at 4 and 28 °C, but not above 30 °C. Optimal growth occurs in the presence of 3 % NaCl. Growth occurs in the presence of 2 % and 5 % NaCl, but not without NaCl or in the presence of >6 % NaCl. Growth occurs at pH 6.5 and pH 8.0, but not at pH 6.0 or at pH values greater than 8.5. Facultatively anaerobic and capable of respiratory metabolism. Catalase and cytochrome oxidase tests are positive. Does not produce H₂S or indole. Nitrate is reduced to nitrite, but nitrite is not reduced. DNase and lipase (tri-*n*-butyrin) are positive. Gelatinase, protease, amylase, urease and agarase are negative. Acid is formed oxidatively from glycerol. No acid is produced from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, *myo*-inositol, D-lactose, maltose, D-mannitol, D-mannose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose or xylose. Susceptible to ampicillin, chloramphenicol, erythromycin, gentamicin, nalidixic acid, neomycin, novobiocin and penicillin, but resistant to tetracycline. Variable susceptibility to kanamycin and streptomycin. Carbon sources oxidized (Biolog GN2) are methyl α-D-glucoside, xylitol, acetic acid, α-ketovaleric acid, lactamide, L-glutamic acid, glycyl-L-glutamic acid, methyl β-D-glucoside, stachyose, L-malic acid, methyl pyruvate, succinamic acid and L-alanine. The G+C content of the DNA is about 46.6–47.0 mol% (determined using HPLC). The major isoprenoid quinone is Q-8. The cellular fatty acids are C_{16:1}, C_{18:1}, C_{16:0}, C_{18:2}, C_{10:0} 3-OH, C_{12:1} 3-OH, C_{12:1}, C_{18:0} and C_{18:0}; full details are given in Table 2.

The type strain, JAMM 1866^T (=JCM 14782^T=ATCC BAA-1530^T), was isolated from sediment adjacent to a sperm whale carcass off Kagoshima, Japan. A second strain of this species, JAMM 1548, was also isolated from the same environment.

Description of *Amphritea balenae* sp. nov.

Amphritea balenae (ba.le'nae. L. gen. n. *balenae* of a whale).

Cells are rod-shaped; cell width ranges from 0.6 to 0.9 µm and cell length ranges from 1.3 to 2.0 µm (see Supplementary Fig. S1 in IJSEM Online). Cells are Gram-negative, non-spore-forming, chemo-organotrophic and motile by means of a single polar or bipolar flagellum. Colonies on MA are circular with entire edges, smooth, convex, cream coloured and 0.5–1.0 mm in diameter after 1–2 days incubation at 20 °C. The optimal growth temperature is 20–22 °C. Growth occurs at 4 and 28 °C, but not above 30 °C. Optimal growth occurs in the presence of 3 % NaCl. Growth occurs in the presence of 2 % and 3 % NaCl, but not without NaCl or in the presence of >4 % NaCl. Growth occurs at pH 6.5 and at pH 7.5, but

not at pH 6.0 or at pH values greater than 8.0. Facultatively anaerobic and capable of respiratory metabolism. The catalase test is weakly positive and the cytochrome oxidase test is positive. Does not produce H₂S or indole. Nitrate is reduced to nitrite, but nitrite is not reduced. Gelatinase, DNase and lipase (tri-*n*-butyrin) are positive. Protease, amylase, urease and agarase are negative. Acid is formed oxidatively from glycerol. No acid is produced from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, myo-inositol, D-lactose, maltose, D-mannitol, D-mannose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose or xylose. Susceptible to ampicillin, chloramphenicol, kanamycin, nalidixic acid, neomycin, novobiocin and penicillin, intermediately susceptible to erythromycin, gentamicin and streptomycin and resistant to tetracycline. Carbon sources oxidized (Biolog GN2) are β -hydroxybutyric acid, D-malic acid, L-asparagine, L-serine, fructose 6-phosphate, methyl β -D-glucoside, stachyose, L-malic acid, methyl pyruvate, succinamic acid and L-alanine. The major isoprenoid quinone is Q-8. The cellular fatty acids are C_{16:1}, C_{18:1}, C_{16:0}, C_{10:0} 3-OH, C_{12:1}, C_{18:2}, C_{12:1} 3-OH and C_{18:0}; full details are given in Table 2.

The type strain, JAMM 1525^T (=JCM 14781^T=ATCC BAA-1529^T), was isolated from the sediment adjacent to a sperm whale carcass off Kagoshima, Japan. The DNA G + C content of the type strain is about 46.7 mol% (determined using HPLC).

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