Neptunomonas japonica sp. nov., an *Osedax japonicus* symbiont-like bacterium isolated from sediment adjacent to sperm whale carcasses off Kagoshima, Japan

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Novel bacterial species were isolated from sediments adjacent to sperm whale carcasses off Kagoshima, Japan, at a depth of 226–246 m. The isolated strains, JAMM 0745^T, JAMM 1380, JAMM 1475 and JAMM 1610, were Gram-negative, rod-shaped, non-spore-forming and motile by means of a single polar or subterminal flagellum. Phylogenetic analysis based on 16S rRNA gene sequences of the novel isolates indicated a relationship to a symbiotic bacterial clone of the polychaete *Osedax japonicus* (99.6–99.9 % sequence similarity) and these bacteria were closely related to members of the genus *Neptunomonas* (95.6–96.0 % similarity) within the class *Gammaproteobacteria*. The novel strains were able to produce isoprenoid quinone Q-8 as the major quinone component. The predominant fatty acids were C_{16:0}, C_{16:1} and C_{18:1}, with C_{18:2} and C_{20:2} present in smaller amounts. The DNA G + C contents of the four novel strains were about 43.6–43.8 mol%. Based on the taxonomic differences observed, the four isolated strains appear to represent a novel species of the genus *Neptunomonas*. The name *Neptunomonas japonica* sp. nov. (type strain JAMM 0745^T=JCM 14595^T=DSM 18939^T) is proposed for the novel strains.

The type species of the genus *Neptunomonas* was described as a rod-shaped, Gram-negative, facultatively aerobic gammaproteobacterium with a single polar flagellum (Hedlund *et al.*, 1999) and was isolated from creosote-contaminated sediment from Puget Sound. The polycyclic aromatic hydrocarbon-degrading *Neptunomonas naphthovorans* is currently the only species in this genus. The genus *Neptunomonas* is most closely related to the genera *Oceanospirillum* and *Neptuniibacter* and to a symbiotic bacterial clone of the genus *Osedax* (Hedlund *et al.*, 1999; Arahall *et al.*, 2007; Goffredi *et al.*, 2007). The genus *Osedax*, composed of siboglinid polychaeta, has recently been discovered in whale carcasses on the deep-sea floor (Rouse *et al.*, 2004; Glover *et al.*, 2005; Fujikura *et al.*, 2006). Members of the genus *Osedax* host symbiotic bacteria in the ovisac and root systems. Phylogenetic analysis placed the micro-organisms isolated from *Osedax*

sp. within a well-supported clade of *Gammaproteobacteria* associated with heterotrophic members of the order *Oceanospirillales* (Goffredi *et al.*, 2007). *Neptunomonas naphthovorans* is the closest cultured relative (93–94 % 16S rRNA gene sequence similarity) to the *Osedax* symbionts (Goffredi *et al.*, 2005). We investigated a sperm whale carcass ecosystem off Kagoshima, Japan, for three years (Fujiwara *et al.*, 2007). About 800 aerobic bacteria were isolated from the sediment adjacent to the sperm whale carcasses, of which four strains were related to the symbiont-like bacteria clone of *Osedax japonicus* and to members of the genus *Neptunomonas*. In this paper, we characterize and describe four strains isolated from the sediment adjacent to the sperm whale carcasses that are members of the class *Gammaproteobacteria* and are related to the symbiont-like bacteria clone of *Osedax japonicus* and the genus *Neptunomonas*.

The four strains, JAMM 0745^T, JAMM 1380, JAMM 1475 and JAMM 1610, were isolated from the sediment adjacent to the whale carcasses, especially adjacent to the cephalic soft tissues, by the unmanned ROV *Hyper Dolphin* off Kagoshima, Japan (dive no. 331, 31° 20.991' N 129°

Abbreviation: ASW, artificial seawater.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences acquired during this study are AB288092, AB291226–AB291228 and AB293969–AB293970.

59.160' E; dive no. 452, 31° 20.997' N 129° 59.162' E; dive no. 456, 31° 20.997' N 129° 59.158' E and dive no. 458, 31° 18.842' N 129° 59.521' E) at a depth of 226–246 m during cruises NT04-08 and NT05-12. The sediment samples were collected by the ROV's manipulator 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 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. *Neptunomonas naphthovorans* ATCC 700637^T was used as a reference strain. Unless otherwise indicated, physiological tests were performed with a slight modification of the general procedures described by Barrow & Feltham (1993) and Baumann *et al.* (1972) that used artificial seawater (ASW; 1 × ASW consisted of 2.75 % NaCl, 0.07 % KCl, 0.54 % MgCl₂ · 6H₂O, 0.68 % MgSO₄ · 7H₂O, 0.14 % CaCl₂ · 2H₂O and 0.02 % NaHCO₃).

Transmission electron microscopy of negatively stained cells was conducted as described by Nogi *et al.* (1998). Cells of strain JAMM 0745^T grown on MA at optimal temperature and in the mid-exponential phase of growth were used for electron microscopic observations (JEM-1210; JEOL).

Growth at various temperatures (0–30 °C) was measured in MB. The test strains retained viability for about 7 days at optimal temperature. 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). The optimal pH and pH range for growth were 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, 8.5 and 9.0. Acid production from sugars was assessed using modified O/F (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 activities were detected on MA plates using substrate concentrations of 1 %. Urease activity was detected using the method described by Maslen (1952) and DNase activity was assessed using DNase test agar (Difco). Hydrogen sulfide production from thiosulfate and the production of indole were assessed using sulfide indole motility agar (Nissui Pharmaceutical) stabs prepared with 0.5 × ASW instead of water. Susceptibility to antimicrobial substances was examined on MA using Sensi-Disks (Becton Dickinson). Any sign of growth inhibition after 48 h incubation at 20 °C was recorded as sensitivity to the respective antimicrobial agent. The following antibiotics (Becton Dickinson) were examined: 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 U), streptomycin (10 µg), and tetracycline (30 µg).

Cellular fatty acids were extracted and analysed as described by Komagata & Suzuki (1987). Isolated strains and *Neptunomonas naphthovorans* ATCC 700637^T were cultured in MB at the optimal temperatures. Fatty acids were extracted using the method of 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 high-performance liquid chromatography (HPLC) (Komagata & Suzuki, 1987).

Chromosomal DNA was purified using the standard 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 42 °C for 4 h and measured fluorometrically according to the method of Ezaki *et al.* (1989).

The 16S rRNA gene was amplified using the PCR method with primers 27F and 1492R (Lane, 1991). The PCR product was sequenced with the dideoxynucleotide chain-termination method, using a DYEnamic ET terminator (MegaBACE) reagent premix (GE Healthcare UK Ltd) and a MegaBACE 1000 DNA sequencer (GE Healthcare UK Ltd). Primers 27F, 350F, 520R, 780F, 907R, 1100F and 1492R were used in the gene sequencing reaction. Nucleotide substitution rates (K_{nuc}) (Kimura, 1980) were determined and a distance matrix tree was constructed with the neighbour-joining method (Saitou & Nei, 1987) using the CLUSTAL_X program (Thompson *et al.*, 1997). The topology of the phylogenetic tree was evaluated by performing bootstrap analysis with 1000 replicates. The GenBank/DDBJ/EMBL accession numbers for the 16S rRNA gene sequences of the isolates are shown in Fig. 1. Other reference sequences were obtained from the GenBank database.

Colonies on MA were circular with entire edges, smooth, convex and cream coloured and were 0.5 to 1.0 mm in diameter after 1–2 days of incubation at 20 °C. Cells of the four novel strains were found to be Gram-negative rods, 1.6–1.8 µm long and 0.8–1.0 µm wide and were motile by means of a single, unsheathed, subterminal flagellum (Fig. 2).

Culture, physiological and biochemical characteristics of the isolates are shown in Table 1 or given in the species descriptions below. The novel strains were facultative anaerobes and were capable of respiratory metabolism. Growth occurred at temperatures from 5 to 25 °C, with an optimum of 20 °C. No growth occurred at temperatures higher than 27 °C. Growth occurred at NaCl concentrations of 1–5 %, but not in the absence of NaCl, and the optimal concentration for growth was 2 %. Growth

Fig. 1. Phylogenetic tree constructed using the neighbour-joining method and based on 16S rRNA gene sequences showing the relationships of strain JAMM 0745^T within the genus *Neptunomonas* and related genera. Bootstrap values were calculated from multiple resamplings of the sequence dataset, which are the basis for multiple tree topologies. Bar, 0.01 nucleotide substitution per site.

occurred at pH values ranging from 7.0 to 8.5. No growth occurred at pH values greater than 9.0. Catalase, cytochrome oxidase, gelatinase, DNase and lipase (hydrolysis of tri-*n*-butyrin) tests were positive. Protease, amylase, agarase and urease tests were negative. Aesculin was not hydrolysed. H₂S and indole were not produced. Reduction of nitrate to nitrite occurred. Acid was not formed oxidatively from sugar. When the four isolated strains were analysed for their susceptibility to 11 antimicrobial compounds, all displayed susceptibility to the same compounds, varying only in the diameters of the zones of inhibition. They were susceptible to ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), neomycin (30 µg), penicillin (10 IU) and streptomycin (10 µg). No inhibition zones were detected around the discs containing novobiocin (30 µg) or tetracycline (30 µg). The isoprenoid quinones of both the newly isolated strains and *Neptunomonas naphthovorans* ATCC 700637^T were the respiratory quinones detected, with Q-8 (99%) predominating and Q-9 (1%) present in minor amounts.

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

the conclusions described below and further clarify the taxonomic and phylogenetic positions of the four isolated strains. The four novel strains showed high sequence similarities to the following species or genera: *Neptunomonas naphthovorans* ATCC 700637^T (95.6–96.0%), the genus *Oceanospirillum* (92.3–93.5%) and *Neptuniibacter caesariensis* MED92^T (93.4–93.6%). However, they were more closely related to the 16S rRNA gene sequences of the symbiotic bacterial clone R46 of *Osedax japonicus* (99.6–99.9%) and to the sequence of the uncharacterized strain UMB3A (96.0–96.4%) isolated from Boston Harbor surface water. 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 (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002). Phylogenetic analysis revealed that the 16S rRNA gene sequence of strain JAMM 0745^T was grouped with the symbiotic bacterial clone sequence of *Osedax japonicus*, the related strain *Neptunomonas naphthovorans* ATCC 700637^T and the uncharacterized strain UMB3A. These strains were included in the sequences of species representing the genus *Neptunomonas*. The results of DNA–DNA hybridization analysis showed that the four isolated strains showed more than 72% DNA–DNA relatedness with each other, but DNA–DNA relatedness values between these strains and *Neptunomonas naphthovorans* ATCC 700637^T were less than 12% and each group was clearly separate, representing distinct species according to the recommendations of Wayne *et al.* (1987). The G + C content of the DNA of the four novel strains was 43.6–43.8 mol%.

The whole-cell fatty acid contents of the novel strain JAMM 0745^T, *Neptunomonas naphthovorans* ATCC 700637^T and reference strains are shown in Table 2. The major fatty acids of strain JAMM 0745^T were C_{16:1} (hexadecenoic acid) and C_{18:1} (octadecenoic acid) and the major 3-hydroxy fatty acid was C_{10:0}. For *Neptunomonas naphthovorans* ATCC 700637^T, C_{16:0} (hexadecanoic acid), C_{16:1} and C_{18:1} were the major fatty acids with C_{10:0} as the major 3-hydroxy fatty acid. The fatty acid profile of strain JAMM 0745^T showed low levels of similarity to those of the reference strains. For example, strain JAMM 0745^T

Fig. 2. Electron micrograph of a negatively stained cell of strain JAMM 0745^T. Bar, 1.0 µm.

Table 1. Differential characteristics of strain JAMM 0745^T and related taxa

Taxa: 1, *Neptunomonas japonica* sp. nov. JAMM 0745^T; 2, *Neptunomonas naphthovorans* ATCC 700637^T; 3, *Oceanospirillum linum* ATCC 11336^T; 4, *Neptuniibacter caesariensis* MED92^T. All taxa are motile, amylase-negative and catalase- and oxidase-positive. +, Positive; –, negative; ND, no data. Data are from this study, Arahall *et al.* (2007), Garrity *et al.* (2005), Hedlund *et al.* (1999), Holt *et al.* (1994), Krieg (1984), Labrenz *et al.* (2003), Sakane & Yokota (1994) and Satomi *et al.* (2002).

Characteristic	1	2	3	4
Cell shape	Rods	Rods	Spirillum	Rods
Length × width	1.6–1.8 × 0.8–1.0	2.0–3.0 × 0.7–0.9	1.8–4.0 × 0.8–1.4	1.5–2.3 × 0.5–0.8
Flagella	Polar/subterminal	Polar	Bipolar	ND
Coccoid body formation	–	ND	+	+
Growth at/in:				
30 °C	–	–	+	+
7 % NaCl	–	+	+	–
Gelatinase	+	–	+	–
Protease	–	ND	–	–
Urease	–	ND	–	ND
DNase	+	–	–	–
Lipase	+	–	ND	–
Hydrolysis of aesculin	–	ND	–	ND
Production of:				
H ₂ S	–	–	ND	ND
Indole	–	+	–	–
Nitrate reduced	+	–	–	–
Acid production from sugars	–	+	–	ND
DNA G + C content (mol%)	43.6	46.3	48–50	46.6

Table 2. Fatty acid contents of the novel isolate JAMM 0745^T and reference strains

1, *Neptunomonas japonica* sp. nov. JAMM 0745^T; 2, *Neptunomonas naphthovorans* ATCC 700637^T; 3, *Oceanospirillum linum* NBRC 15448^T; 4, *Neptuniibacter caesariensis* MED92^T. tr, Trace (<1 %). Data are from this study, Arahall *et al.* (2007), Labrenz *et al.* (2003) and Sakane & Yokota (1994).

Fatty acid	1	2	3	4
Nonpolar*				
C _{10:0}				tr
C _{12:0}		7	3	
C _{12:1}	3	4	2	
C _{14:0}			1	tr
C _{16:0}	11	21	16	17
C _{16:1}	51	41	48	44
C _{18:0}	tr	tr		1
C _{18:1}	27	28	30	38
C _{18:2}	5			
C _{20:2}	2			
3-Hydroxy†				
C _{10:0}	100	100	100	100

*Numbers refer to the percentage of an acid relative to the total nonpolar fatty acids.

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

contained relatively large amounts of C_{16:1} and small amounts of C_{16:0}, C_{18:2} (octadecadienoic acid) and C_{20:2} (eicosadienoic acid) were produced, but C_{12:0} (dodecanoic acid) was not produced.

The similarity of the four novel strains to the *Osedax* symbiont-like bacteria was estimated based on the basis of the phenotypic, genotypic and phylogenetic data and it is evident that they represent a novel species within the genus *Neptunomonas*. We suggest that strains JAMM 0745^T, JAMM 1380, JAMM 1475 and JAMM 1610 represent a novel species, for which the name *Neptunomonas japonica* sp. nov. is proposed.

Description of *Neptunomonas japonica* sp. nov.

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

Cell are rod-shaped; cell width ranges from 0.8 to 1.0 µm and cell length ranges from 1.6 to 1.8 µm. Cells are Gram-negative and motile by means of a single polar or subterminal flagellum. Colonies on MA are circular with entire edges, smooth, convex, and cream coloured, 0.5 to 1.0 mm in diameter after 1 to 2 days of incubation at 20 °C. The bacteria are psychrotrophic. The optimal growth temperature is 20 °C. Growth occurs at 5 and 25 °C, but not at 0 °C or above 27 °C. Optimal growth occurs in the presence of 2 % NaCl. Growth occurs in the presence of 1 % and 5 % NaCl, but not without NaCl or in the presence of >6 % NaCl. The optimal

pH value for growth is 7.5. Growth occurs at pH 7.0 and pH 8.5, but not at pH 6.5 or greater than pH 9.0. Facultatively anaerobic and capable of respiratory metabolism. The catalase and cytochrome oxidase tests are positive. Does not produce H₂S or indole. Nitrate is reduced to nitrite, but nitrite is not reduced. Gelatinase, DNase and lipase activities are positive. Protease, amylase, urease and agarase activities are negative. Acid is not formed oxidatively from sugars. Susceptible to ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), neomycin (30 µg), penicillin (10 U) and streptomycin (10 µg), but resistant to novobiocin (30 µg) and tetracycline (30 µg). The G+C content of the DNA is about 43.6–43.8 mol% (determined by HPLC). The major isoprenoid quinone is Q-8. The dominant cellular fatty acids are C_{16:0}, C_{16:1} and C_{18:1}.

The type strain, JAMM 0745^T (=JCM 14595^T=DSM 18939^T), was isolated from the sediment adjacent to a sperm whale carcass off Kagoshima, Japan.

Acknowledgements

We would like to thank Mr Katsuyuki Uematsu for assistance in preparing electron micrographs. We are very grateful to the ROV *Hyper Dolphin* operation team and the captain and crew of the R/V *Natushima* for helping to collect the deep-sea samples.

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