Psychromonas japonica sp. nov., *Psychromonas aquimarina* sp. nov., *Psychromonas macrocephali* sp. nov. and *Psychromonas ossibalaenae* sp. nov., psychrotrophic bacteria isolated from sediment adjacent to sperm whale carcasses off Kagoshima, Japan

Masayuki Miyazaki, Yuichi Nogi, Yoshihiro Fujiwara and Koki Horikoshi

Correspondence
Miyazaki Masayuki
miyazakim@jamstec.go.jp

The XBR Group, Japan Agency for Marine-Earth Science and Technology, 2-15 Natsushima-cho, Yokosuka 237-0061, Japan

Four novel species of psychrotrophic bacteria were isolated from sediment adjacent to sperm whale carcasses off Kagoshima, Japan, at a depth of 228–250 m. Cells of the five isolated strains, JAMM 0394^T, JAMM 0404^T, JAMM 0415^T, JAMM 0700 and JAMM 0738^T, were Gram-negative, rod-shaped, non-sporulating and motile by means of a single polar flagellum. The novel strains were able to produce isoprenoid quinone Q-8 as the major component. The predominant fatty acids were C_{16:0} and C_{16:1}. Phylogenetic analysis based on 16S rRNA gene sequences showed that the novel strains represent a separate lineage within the genus *Psychromonas*. The G + C contents of the novel strains were 38.8–42.8 mol%. DNA–DNA hybridization values between these strains and reference strains of the genus *Psychromonas* were significantly lower than that generally accepted as the threshold for the phylogenetic definition of species. The names *Psychromonas japonica* sp. nov. (type strain JAMM 0394^T=JCM 14783^T=ATCC BAA-1525^T), *Psychromonas aquimarina* sp. nov. (type strain JAMM 0404^T=JCM 14784^T=ATCC BAA-1526^T), *Psychromonas macrocephali* sp. nov. (two strains, type strain JAMM 0415^T=JCM 14785^T=ATCC BAA-1527^T) and *Psychromonas ossibalaenae* sp. nov. (type strain JAMM 0738^T=JCM 14786^T=ATCC BAA-1528^T) are proposed.

The genus *Psychromonas* was proposed by Mountfort *et al.* (1998) for a novel psychrophilic and aerotolerant anaerobic bacterium that belonged to the class *Gammaproteobacteria*. The genus includes piezophilic and halophilic species that are widely distributed in nature, especially in aquatic environments such as polar sea ice, high-salinity ponds and the ocean (Kawasaki *et al.*, 2002; Nogi *et al.*, 2002; Groudieva *et al.*, 2003; Xu *et al.*, 2003; Auman *et al.*, 2006; Nogi, 2007). Seven species of the genus *Psychromonas* have been described to date. We investigated a sperm whale carcass ecosystem off Kagoshima, Japan, for three years between 2003 and 2005 (Fujiwara *et al.*, 2007).

Abbreviation: ASW, artificial seawater.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the five strains described in this paper are AB304804–AB304808.

Transmission electron micrographs of negatively stained cells of the four novel species and a table detailing the results of DNA–DNA hybridization experiments between the novel strains and closely related species of the genus *Psychromonas* are available as supplementary material with the online version of this paper.

About 800 aerobic bacteria were isolated from the sediment adjacent to the sperm whale carcasses, of which five strains were related to the genus *Psychromonas*. In this paper, we describe the results of taxonomic studies on these isolates. Several lines of evidence indicate that these isolates represent four novel species within the genus *Psychromonas*.

The five strains, JAMM 0394^T, JAMM 0404^T, JAMM 0415^T, JAMM 0700 and JAMM 0738^T, were collected from marine sediment adjacent to sperm whale carcasses by the unmanned ROV *Hyper Dolphin* off Kagoshima, Japan (dive no. 189, 31° 20.720' N 129° 59.285' E; dive no. 192, 31° 20.730' N 129° 59.314' E; dive no. 193, 31° 20.716' N 129° 59.284' E; dive no. 328, 31° 20.722' N 129° 59.283' E; dive no. 330, 31° 18.518' N 129° 59.372' E) at a depth of 228–250 m during cruises NT03-08 and NT04-08. 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 in marine agar 2216 (MA; Difco) at 15 °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. *Psychromonas antarctica* DSM 10704^T and *Psychromonas ingrahamii* CIP 108865^T were used as reference strains. Unless otherwise indicated, physiological tests (Baumann *et al.* 1972; Barrow & Feltham 1993) were performed with a slight modification 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). Growth at various temperatures (0–30 °C) was measured in MB. The novel strains retained viability for about 7 days at the optimal temperature (20 °C). 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 0–7 % (w/v). The optimal pH and pH range for growth were determined in media containing 0.5 % peptone, 0.5 % yeast extract, 0.32 % MgSO₄·7H₂O and 3.0 % NaCl (w/v) with pH adjusted to 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0 and 10.5. Acid production from sugars was assessed using modified O/F 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 % (w/v). DNase activity was assessed using DNase test agar (Difco). Hydrogen sulfide production from thiosulfate and the production of indole were assessed using SIM medium (Nissui Pharmaceutical) stabs prepared with 0.5×ASW instead of water.

Transmission electron microscopy of negatively stained cells was performed as described by Nogi *et al.* (1998). Cells of strains JAMM 0394^T, JAMM 0404^T, JAMM 0415^T and JAMM 0738^T grown in MA at the optimal temperature and in the mid-exponential phase of growth were used for electron microscopic observations (JEM-1210; JEOL).

Cellular fatty acids were extracted and analysed as described by Komagata & Suzuki (1987). The novel strains were cultured in MB at the optimal temperatures and 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 HPLC (Komagata & Suzuki, 1987).

Chromosomal DNA was purified according to a 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 41 °C for 4 h and measured fluorometrically using the method of Ezaki *et*

al. (1989). 16S rRNA and gene sequences were obtained by direct sequencing of PCR-amplified DNA as described previously (Miyazaki *et al.*, 2008). 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). Alignment gaps and unidentified base positions were not taken into consideration in the calculations. The topology of the phylogenetic tree was evaluated by performing bootstrap analysis with 1000 replicates. The GenBank/DBJ/EMBL accession numbers for the 16S rRNA gene sequences of the novel isolates are shown in Fig. 1. Other reference sequences were obtained from the GenBank database.

Cultural, physiological and biochemical characteristics of the novel isolates are shown in Table 1 or given in the species descriptions below. Cells of the novel strains were Gram-negative, non-sporulating rods that were motile by means of a single polar unsheathed or sheathed flagellum (see Supplementary Fig. S1 in IJSEM Online). Colonies on MA were circular with entire edges, smooth and convex. The novel strains were facultatively anaerobic chemo-organotrophs and the major isoprenoid quinone type was Q-8. Detailed physiological and biochemical characteristics of the novel strains are shown in Table 1 and in the species descriptions.

The whole-cell fatty acid contents of the novel and reference strains are shown in Table 2. The cultivation temperatures of the strains differed and thus the fatty acids of these strains were analysed after growth at the optimal temperatures for the strains. The major fatty acids detected for the novel strains were C_{16:0} and C_{16:1}. The fatty acid profiles of the novel strains showed only low levels of similarity with those of the reference strains. For example, strain JAMM 0394^T contained only small amounts of C_{14:0} and C_{15:0}, but did not contain C_{22:6}, strain JAMM 0404^T contained small amounts of C_{14:0} and C_{15:0}, strain JAMM 0415^T contained small amounts of C_{14:0} and strain JAMM 0738^T contained small amounts of C_{14:0} and C_{22:6}.

The G+C contents of the novel strains and those of other species of the genus *Psychromonas* are shown in Table 1. 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 novel strains in the genus *Psychromonas*. Strains JAMM 0394^T, JAMM 0415^T, JAMM 0700 and JAMM 0738^T were most closely related to *P. antarctica* DSM 10704^T (97.2–98.0 % gene sequence similarity). Strain JAMM 0404^T was most closely related to *P. ingrahamii* CIP 108865^T (97.3 % similarity). 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 (see Supplementary Table S1 in IJSEM Online) of the novel strains and reference strains

Fig. 1. Phylogenetic tree, constructed using the neighbour-joining method and based on 16S rRNA gene sequences, showing the relationships of the five novel strains within the genus *Psychromonas* and related genera. The percentage of 1000 bootstrap resamplings that support branching points above 50 % confidence is indicated. Bar, 0.01 nucleotide substitution per site.

showed that the DNA–DNA relatedness was less than 47 %, which is significantly lower than that generally accepted as the phylogenetic definition of a species. This suggests that the isolated strains represent novel species of the genus *Psychromonas*.

The five isolated strains are psychrotrophic and, on the basis of the phenotypic, genotypic and phylogenetic data, evidently represent novel species within the genus *Psychromonas*. We therefore propose the names *Psychromonas japonica* sp. nov., *Psychromonas aquimarina* sp. nov., *Psychromonas macrocephali* sp. nov. and *Psychromonas ossibalaenae* sp. nov. for these new strains.

Description of *Psychromonas japonica* sp. nov.

Psychromonas japonica (ja.pon'i.ca. N.L. adj. *japonica* pertaining to Japan, where the isolate originated).

Cells are rod-shaped; cell width ranges from 0.5 to 0.8 µm and cell length ranges from 1.6 to 2.2 µm. Cells are Gram-negative and are motile by means of a single polar flagellum. Colonies on MA are circular with entire edges, smooth, convex and translucent cream-coloured. Colonies on MA are 0.5–1.0 mm in diameter after 1–2 days of incubation at 20 °C. The bacteria are psychrotrophic. The optimal growth temperature is 20–22 °C. Growth occurs at 5 and 25 °C, but not at 0 °C or above 27 °C. Optimal growth occurs in the presence of 3 % NaCl (w/v). Growth occurs in the presence of 2 % and 5 % NaCl, but not without NaCl or in the presence of >6 % NaCl. The optimal pH value for growth is pH 9.0. Growth occurs at pH 6.5 and pH 9.5, but not at pH 6.0 or at greater than pH 10.0. 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. DNase is positive. Gelatin is weakly hydrolysed. Protease, amylase and lipase tests are negative. Hydrolysis of Tween 20 is positive, but hydrolysis of Tween 40, Tween 80 and ONPG is negative. Acid is formed oxidatively from cellobiose, D-fructose, D-glucose and D-mannose. No acid is produced from L-arabinose, D-galactose, glycerol, *myo*-inositol, D-lactose, maltose, D-mannitol, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose or xylose. The G + C content of the DNA is about 38.8 mol% (determined by HPLC). The major isoprenoid quinone is Q-8. The dominant cellular fatty acids are C_{16:0} and C_{16:1}.

The type strain, JAMM 0394^T (=JCM 14783^T=ATCC BAA-1525^T), was isolated from the sediment adjacent to a sperm whale carcass off Kagoshima, Japan.

Description of *Psychromonas aquimarina* sp. nov.

Psychromonas aquimarina (a.qui.ma.ri'na. L. n. *aqua* water; L. adj. *marinus* of the sea; N.L. fem. adj. *aquimarina* of/from seawater).

Cells are rod-shaped; cell width ranges from 0.9 to 1.1 µm and cell length ranges from 1.6 to 3.2 µm. Cells are Gram-negative and are motile by means of a single polar sheathed flagellum. Colonies on MA are circular with entire edges, smooth, convex and cream-coloured. Colonies on MA are 1.0–2.0 mm in diameter after 1–2 days of incubation at 20 °C. The bacteria are psychrotrophic. The optimal growth temperature is 20–25 °C. Growth occurs at 5 and 27 °C, but not at 0 °C or above 30 °C. Optimal growth occurs in the presence of 3 % NaCl (w/v). Growth occurs in the presence of 2 % and 5 % NaCl, but not without NaCl or in the presence of >6 % NaCl. The optimal pH value for growth is pH 8.5. Growth occurs at pH 6.0 and pH 9.0,

Table 1. Differential characteristics of species of the genus *Psychromonas*

Taxa: 1, *Psychromonas japonica* sp. nov. JAMM 0394^T; 2, *Psychromonas aquimarina* sp. nov. JAMM 0404^T; 3, *Psychromonas macrocephali* sp. nov. JAMM 0415^T; 4, *Psychromonas ossibalaenae* sp. nov. JAMM 0738^T; 5, *P. antarctica* DSM 10704^T; 6, *P. ingrahamii* CIP 108865^T. Cells of all species are Gram-negative and the major isoprenoid quinone type is Q-8. The following tests were positive for all taxa: oxidase activity and utilization of D-fructose and D-glucose. The following tests were negative for all taxa: production of indole and utilization of L-arabinose, myo-inositol, D-lactose and D-sorbitol. +, Positive; –, negative; w, weakly positive; ND, no data; TC, translucent cream; C, cream; W, white. Data are from this study, Mountfort *et al.* (1998), Nogi *et al.* (2002) and Auman *et al.* (2006).

Characteristic	1	2	3	4	5	6
Cell						
Shape	Rods	Rods	Rods	Rods	Ovoid rods	Large rods
Length (µm)	1.6–2.2	1.6–3.2	2.2–2.9	1.9–3.7	2.5–6.0	6–14
Colony colour	TC	C	C	C	W	W
Motility	+	+	+	+	+	–
Growth at/in:						
25 °C	+	+	+	+	–	–
10 % NaCl (w/v)	–	–	–	–	–	+
pH 9.0	+	+	+	+	–	–
Catalase	+	–	–	–	+	+
Gelatinase	w	+	+	–	+	–
Protease	–	w	+	–	ND	ND
Amylase	–	+	–	–	+	–
DNase	+	+	+	+	ND	ND
Lipase (tri- <i>n</i> -butyrin)	–	–	–	+	ND	ND
Hydrolysis:						
Tween 20	+	+	+	–	ND	ND
Tween 40	–	+	+	–	ND	ND
Tween 80	–	+	+	–	ND	ND
ONPG test	–	+	+	+	ND	ND
Nitrate reduction to nitrite	+	+	+	+	–	+
Utilization as carbon source:						
Cellobiose	+	–	–	+	+	+
D-Galactose	–	+	+	+	+	+
Glycerol	–	+	+	+	–	+
Maltose	–	+	+	+	+	ND
D-Mannitol	–	+	–	–	+	+
D-Mannose	+	+	+	+	w	–
Raffinose	–	–	–	w	–	ND
L-Rhamnose	–	–	–	–	–	ND
Sucrose	–	+	–	+	+	+
Trehalose	–	+	+	+	+	+
Xylose	–	–	–	+	–	–
DNA G + C content (mol%)	38.8	42.2	41.5	41.3	42.8	40

but not at pH 5.5 or at greater than pH 9.5. Facultatively anaerobic and capable of respiratory metabolism. The cytochrome oxidase test is positive, but catalase is negative. Does not produce H₂S or indole. Nitrate is reduced to nitrite, but nitrite is not reduced. Gelatinase, amylase and DNase are positive. Protease is weakly positive. Lipase is negative. Hydrolysis of Tween 20, Tween 40, Tween 80 and ONPG is positive. Acid is formed oxidatively from D-fructose, D-galactose, D-glucose, glycerol, maltose, D-mannitol, D-mannose, sucrose and trehalose. No acid is produced from L-arabinose, cellobiose, myo-inositol, D-lactose, raffinose, L-rhamnose, D-sorbitol or xylose. The G + C content of the DNA is about 42.2 mol%

(determined by HPLC). The major isoprenoid quinone is Q-8. The dominant cellular fatty acids are C_{16:0} and C_{16:1}.

The type strain, JAMM 0404^T (=JCM 14784^T=ATCC BAA-1526^T), was isolated from the sediment on the seabed adjacent to a sperm whale carcass off Kagoshima, Japan.

Description of *Psychromonas macrocephali* sp. nov.

Psychromonas macrocephali (mac.ro.ceph'a.li. N.L. gen. n. *macrocephali* of *Physeter macrocephalus*, the Latin name for the sperm whale).

Table 2. Fatty acid contents of the novel strains and other members of the genus *Psychromonas*

Taxa: 1, *P. japonica* sp. nov. JAMM 0394^T; 2, *P. aquimarina* sp. nov. JAMM 0404^T; 3, *P. macrocephali* sp. nov. JAMM 0415^T; 4, *P. ossibalaenae* sp. nov. JAMM 0738^T; 5, *P. antarctica* DSM 10704^T; 6, *P. ingrahamii* CIP 108865^T. tr, Trace (<1 %). Data are from this study, Nogi *et al.* (2002) and Auman *et al.* (2006).

Fatty acid	1	2	3	4	5	6
C _{12:0}	tr	3.6	4.0	tr	1	2.5
C _{14:0}	2.0	1.5	1.3	1.9	—	—
C _{15:0}	5.0	2.8	tr	tr	—	—
C _{16:0}	21.7	28.9	26.7	25.4	24	18.7
C _{14:1}	7.3	4.3	4.5	5.1	8	—
C _{16:1}	52.5	49.0	51.0	55.5	58	67
C _{18:1}	2.7	1.8	4.3	1.4	3	3.6
C _{22:6}	—	tr	tr	1.2	—	—
C _{14:0} 3-OH	6.2	7.2	7.5	8.4	6	4.5*

*C_{12:0} alde, C_{16:1} iso or C_{14:0} 3-OH.

Cells are rod-shaped; cell width ranges from 0.6 to 0.8 µm and cell length ranges from 2.2 to 2.9 µm. Cells are Gram-negative and are motile by means of a single polar sheathed flagellum. Colonies on MA are circular with entire edges, smooth, convex and cream-coloured. Colonies on MA are 1.0–2.0 mm in diameter after 1–2 days of incubation at 20 °C. The bacteria are psychrotrophic. The optimal growth temperature is 20 °C. Growth occurs at 0 °C and 25 °C, but not above 27 °C. Optimal growth occurs in the presence of 3 % NaCl (w/v). Growth occurs in the presence of 2 % and 6 % NaCl, but not without NaCl or in the presence of >7 % NaCl. The optimal pH value for growth is pH 8.5. Growth occurs at pH 6.0 and pH 9.0, but not at pH 5.5 or at greater than pH 9.5. Facultatively anaerobic and capable of respiratory metabolism. The cytochrome oxidase test is positive, but catalase is negative. Does not produce H₂S or indole. Nitrate is reduced to nitrite, but nitrite is not reduced. Gelatinase, protease and DNase are positive, but amylase and lipase are negative. Hydrolysis of Tween 20, Tween 40, Tween 80 and ONPG is positive. Acid is formed oxidatively from D-fructose, D-galactose, D-glucose, glycerol, maltose, D-mannose and trehalose. No acid is produced from L-arabinose, cellobiose, *myo*-inositol, D-lactose, D-mannitol, raffinose, L-rhamnose, D-sorbitol, sucrose or xylose. The G + C content of the DNA is about 41.5 mol% (determined by HPLC). The major isoprenoid quinone is Q-8. The dominant cellular fatty acids are C_{16:0} and C_{16:1}.

The type strain, JAMM 0415^T (=JCM 14785^T=ATCC BAA-1527^T), was isolated from the sediment adjacent to a sperm whale carcass off Kagoshima, Japan. Strain JAMM 0700 is a reference strain.

Description of *Psychromonas ossibalaenae* sp. nov.

Psychromonas ossibalaenae (os.si.ba.la.e.na'e. L. n. *os* ossis bone; L. n. *balaena* -ae whale; N.L. gen. n. *ossibalaenae* of a bone of a whale).

Cells are rod-shaped; cell width ranges from 0.7 to 1.1 µm, and cell length ranges from 1.9 to 3.7 µm. Cells are Gram-negative and are motile by means of a single polar flagellum. Colonies on MA are circular with entire edges, smooth, convex and cream coloured. Colonies on MA are 1.0–2.0 mm in diameter after 1–2 days of incubation at 20 °C. The bacteria are psychrotrophic. The optimal growth temperature is 20 °C. Growth occurs at 0 and 25 °C, but not above 27 °C. Optimal growth occurs in the presence of 3 % NaCl (w/v). Growth occurs in the presence of 2 % and 5 % NaCl, but not without NaCl or in the presence of >6 % NaCl. The optimal pH value for growth is pH 9.0. Growth occurs at pH 6.5 and at pH 9.5, but not at pH 6.0 or at greater than pH 10.0. Facultatively anaerobic and capable of respiratory metabolism. The cytochrome oxidase test is positive, but catalase is negative. Does not produce H₂S or indole. Nitrate is reduced to nitrite, but nitrite is not reduced. DNase and lipase are positive, but gelatinase, protease and amylase are negative. Hydrolysis of ONPG is positive, but that of Tween 20, Tween 40 and Tween 80 is negative. Acid is formed oxidatively from cellobiose, D-fructose, D-galactose, D-glucose, glycerol, maltose, D-mannose, sucrose, trehalose and xylose. Acid is weakly formed oxidatively from raffinose. No acid is produced from L-arabinose, *myo*-inositol, D-lactose, D-mannitol, L-rhamnose or D-sorbitol. The G + C content of the DNA is about 41.3 mol% (determined by HPLC). The major isoprenoid quinone is Q-8. The dominant cellular fatty acids are C_{16:0} and C_{16:1}.

The type strain, JAMM 0738^T (=JCM 14786^T=ATCC BAA-1528^T), was isolated from the sediment adjacent to a sperm whale carcass off Kagoshima, Japan.

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