Psychromonas heitensis sp. nov., a psychrotolerant bacterium isolated from seawater in Japan

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Four bacterial strains, 04HE-4-40, A4I-9, A4I-21 and AK15-027^T, were isolated from Heita Bay off Kamaishi in Japan. Based on 16S rRNA gene sequence analysis, the isolates were placed as a new lineage within the genus *Psychromonas*. The 16S rRNA gene sequence similarity values between the isolates and type strains of recognized *Psychromonas* species were less than 97 %. The DNA G + C contents of the isolates were about 38 mol%. The major respiratory quinone was Q-8, and the predominant cellular fatty acids were 16 : 1 ω 7c and 16 : 0. The isolates were able to grow at 30 °C, unlike other *Psychromonas* species. Based on the results of the phylogenetic analysis and phenotypic characteristics and DNA–DNA hybridization data, the isolates represent a novel species, for which the name *Psychromonas heitensis* sp. nov. is proposed. The type strain is AK15-027^T (=MBIC 06898^T=NCIMB 14416^T).

The genus *Psychromonas*, the only member of the family *Psychromonadaceae* (Ivanova *et al.*, 2004), belongs to the order *Alteromonadales* (Bowman & McMeekin, 2005) within the class *Gammaproteobacteria*. This genus includes motile, Gram-negative, rod-shaped or oval-shaped, facultatively anaerobic bacteria. Members of this genus are psychrophilic species, which are defined by an optimal temperature for growth of about 15 °C or below, a maximal growth temperature of about 20 °C and the ability to grow at 0 °C (Morita, 1975), or psychrotolerant species, which are capable of growing at below 5 °C with optimum and maximum growth temperatures of 20 °C and higher (Ingraham & Stokes, 1959; Morita, 1975). Some piezophilic bacteria are also included in this genus (Brenner *et al.*, 2005). Members of the genus *Psychromonas* have been isolated from cold environments, including polar regions [*Psychromonas antarctica* (Mountfort *et al.*, 1998), *P. arctica* (Groudieva *et al.*, 2003) and *P. ingrahamii* (Auman *et al.*, 2006)], deep-sea sediments [*P. kaikoe* (Nogi *et al.*, 2002), *P. profunda* (Xu *et al.*, 2003) and *P. hadalis* (Nogi *et al.*, 2007) and drifting ice [*P. marina* (Kawasaki *et al.*, 2002)]. In this study, four psychrotolerant strains isolated from Heita Bay off Kamaishi in Japan were analysed.

Strains 04HE-4-40, A4I-9 and A4I-21 were isolated using a polyurethane foam (PUF) block supplemented with sterile

seawater containing 1.5 % (w/v) agar (Yasumoto-Hirose *et al.*, 2006). The PUF block was immersed in the sea (Heita Bay, Iwate, Japan) for 3 days during May 2004. The block was recovered and cut into small pieces (1 cm³), each being homogenized in 5 ml sterile seawater by using a glass rod. The homogenate was diluted to 1/1000 with seawater. Strain 04HE-4-40 was isolated from the diluted homogenate using NSW medium (1.0 g NH₄NO₃, 0.02 g FeC₆H₅O₇·nH₂O, 0.2 g K₂HPO₄, 10 g sodium pyruvate, 800 ml filtered seawater, 200 ml distilled water and 15.0 g agar at pH 7.8). Strains A4I-9 and A4I-21 were isolated from the diluted homogenate using natural seawater agar containing 90 % (v/v) seawater and 1.5 % (w/v) agar, supplemented with 2 g tryptophan l⁻¹. Strain AK15-027^T was isolated from seawater taken from Heita Bay in July 2003, using full-strength seawater agar that contained 100 % (v/v) filtered seawater and 1.5 % (w/v) agar, supplemented with 5 % (v/v) vitamin solution of the DSM141 medium (DSMZ, 1993). The isolates were cultured and maintained on 1/5-strength marine agar [800 ml filtered seawater, 200 ml distilled water, 7.48 g marine broth (Difco) and 15 g agar].

The 16S rRNA genes were amplified for the phylogenetic analysis by using primers 27F and 1492R (Weisburg *et al.*, 1991). Sequences similar to those of the isolates were obtained by using BLAST (Altschul *et al.*, 1990). According to the BLAST search, the isolates were closely affiliated with the genus *Psychromonas*. A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) with the CLUSTAL_X program (version 1.83; Thompson *et al.*, 1997) after multiple alignments [the sequences used corresponded to positions 95–175,

Abbreviation: PUF, polyurethane foam.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 04HE-4-40 (=MBIC 08331), A4I-9 (=MBIC 08332), A4I-21 (=MBIC 08330) and AK15-027^T (=MBIC 06898^T=NCIMB 14416^T) are AB365349, AB365350, AB365351 and AB365352, respectively.

193–442, 454–819 and 845–1393 of the sequence of *Aeromonas allosaccharophila* CECT 4199^T (S39232), using the method of Ivanova *et al.* (2004)]. A bootstrap analysis was conducted from 1000 trials. The 16S rRNA gene sequence similarity was calculated according to the method of Ivanova *et al.* (2004). The results of the phylogenetic analysis based on 16S rRNA gene sequences are shown in Fig. 1. The four isolates fell within the genus *Psychromonas* and demonstrated a high level of 16S rRNA gene sequence similarity (>99%). Based on the sequence similarity values, the closest relatives of the isolates were *P. profunda* 2825^T, *P. kaikoae* JT7304^T, *P. marina* 4-22^T and *P. hadalis* K41G^T (each 96%); the 16S rRNA gene similarities with *P. antarctica* DSM 10704^T, *P. arctica* Pull 5.3^T and *P. ingrahamii* 37^T were 94, 95 and 94%, respectively. The 16S rRNA gene sequence similarity values between the isolates and recognized species of the genus *Psychromonas* were <97%, indicating that the isolates represent a novel genomic species (Stackebrandt & Goebel, 1994; Xu *et al.*, 2003). Genus affiliation was investigated by comparing the isolates with recognized species of the genus *Psychromonas* by using signature nucleotide sites and 16S rRNA gene sequence similarities, according to the method of Ivanova *et al.* (2004). The signature nucleotide sites of members of the genus *Psychromonas* were defined in the family *Psychromonadaceae* at positions 385(T), 811(A), 842(A), 845(T) and 1336(T). The signature nucleotide at position 385 deviated from T to C in the isolates and the nucleotides at the other positions were conserved. Similarity values with recognized species of the genus *Psychromonas* were >93% and the 16S rRNA signature nucleotide sites were almost identical, indicating that the isolates represent a member of the genus *Psychromonas*.

DNA–DNA hybridization was carried out at 40 °C by using genomic DNA prepared by the method of Saito & Miura

(1963). The formation of a hetero-duplex was measured fluorometrically by using the method of Ezaki *et al.* (1989). The high level of DNA–DNA relatedness (86–107%) among the four isolates indicated that they represented a single species (Wayne *et al.*, 1987).

The following phenotypic characteristics were determined. Cells were grown for 24 h at 20 °C on marine agar and observed by TEM after negative staining with uranyl acetate. Gram staining was performed using the Hucker staining method described by Smibert & Krieg (1994). The DNA G + C content was determined by HPLC according to the method of Tamaoka & Komagata (1984). Analysis of the respiratory quinone and cellular fatty acid compositions was performed using previously described methods (Katsuta *et al.*, 2005). For analysis of the cellular fatty acids, cells were grown for 24 h at 20 °C in marine broth. Growth at different temperatures (2, 8, 15, 20, 25, 30 and 37 °C) was tested on marine agar. The pH range for growth was tested on half-strength (1/2) SP5-Tween 20 agar as 1/2 SP5 agar plus 0.1% (v/v) Tween 20 (SP5 agar; Hosoya *et al.*, 2006), using 10 mM MES buffer to adjust the pH to 5.5–7.0; 10 mM TAPS buffer was used to adjust the pH to 8.0–9.0 and 100 mM NaHCO₃/Na₂CO₃ buffer to adjust the pH to 10.0. Salt tolerance was tested on saline-free 1/2 SP5-Tween 20 agar, supplemented with 0–10% NaCl (w/v). Oxidase activity was tested by spreading cell pellets on oxidase test paper (Eiken), and catalase activity was tested by using a 3% H₂O₂ solution. Growth under anaerobic conditions was tested on marine agar for 1 week using the AnaeroPack system (Mitsubishi Gas Chemical). Degradation of starch was tested on 1/2 SP5-Tween 20 agar containing 0.2% (w/v) soluble starch using the method of Smibert & Krieg (1994). Hydrolysis of Tweens 20, 40, 60 and 80 was determined on 1/2 SP5 agar supplemented with 0.1% (v/v) Tweens by the observation

Fig. 1. Neighbour-joining phylogenetic tree of *Psychromonas heitensis* sp. nov. and other members of the genus *Psychromonas* based on 16S rRNA gene sequence analysis. Bootstrap values greater than 500 are given at branching points. Bar, 0.01 K_{nu}.

of opaque halo formation. Degradation of L-tyrosine was tested on 1/2 SP5-Tween 20 agar containing 0.5 % L-tyrosine according to the method of Barrow & Feltham (1993). Hydrolysis of DNA was determined on DNA agar (Nissui) supplemented with half-strength artificial seawater (ASW; Hosoya *et al.*, 2006). Degradation of casein was tested on 1/2 SP5-Tween 20 agar supplemented with 4 % (w/v) litmus milk (Difco). Acid production from sugars was assessed by using API 50 CH and API 50 CHB/CHE medium supplemented with half-strength ASW. Tests with API ZYM and API 20NE (bioMérieux) were generally performed according to the manufacturer's instructions, with the test strip inoculum prepared as suspensions in half-strength ASW. Assimilation of carbon sources (API 20NE) was tested with a suspension medium consisting of 0.05 % $\text{NH}_4\text{H}_2\text{PO}_4$, 0.005 % yeast extract, 0.05 % Tris, 0.15 % agar (all w/v) and half-strength ASW. API 50 CH tests were read after 24 h incubation at 30 °C, the API ZYM tests were read after 4 h incubation at 37 °C and the API 20NE tests were read after 48 h incubation at 30 °C.

The phenotypic characteristics are given in the species description and in Table 1. Strains 04HE-4-40, A4I-9, A4I-21 and AK15-027^T did not reduce nitrate to nitrite, unlike *P. hadalis*, *P. ingrahamii*, *P. profunda*, *P. marina* and *P. kaikoeae*. The isolates were unable to produce acid from D-galactose. The major fatty acids of the isolates and recognized *Psychromonas* species were 16:1 and 16:0 (Table 2). In addition, the maximum temperature for growth of the isolates was 30 °C, although recognized species of the genus *Psychromonas* could not grow at 30 °C. The results of the phylogenetic analysis based on the 16S rRNA gene sequence suggested that the isolates belonged to the genus *Psychromonas*. The sequence similarity values and signature nucleotides of the 16S rRNA gene, together with the DNA–DNA hybridization data, indicated that the isolates represent a novel species of the genus *Psychromonas*. Based on the results described above, we propose that the novel isolates represent a novel species of the genus *Psychromonas*, for which the name *Psychromonas heitensis* sp. nov. is proposed.

Table 1. Differential characteristics of strains AK15-027^T, 04HE-4-40, A4I-9 and A4I-21, and recognized species of the genus *Psychromonas*

Taxa: 1, strains AK15-027^T, 04HE-4-40, A4I-9 and A4I-21 (*Psychromonas heitensis* sp. nov.); 2, *P. antarctica* DSM 10704^T; 3, *P. arctica* Pull 5.3^T; 4, *P. hadalis* K41G^T; 5, *P. ingrahamii* 37^T; 6, *P. kaikoeae* JT7304^T; 7, *P. marina* 4-22^T; 8, *P. profunda* 2825^T. Data are from this study, Kawasaki *et al.* (2002), Groudieva *et al.* (2003), Xu *et al.* (2003), Brenner *et al.* (2005), Auman *et al.* (2006) and Nogi *et al.* (2007). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8
Temperature range (optimum) (°C)	2–30 (20–25)	2–17 (12)	0–25 (20)	2–12 (6)	–12 to 10 (5)	4–15 (10)	0–25 (14–16)	2–14 (10)
Growth at atmospheric pressure	+	+	+	–	+	–	+	+
Motility	+	+	+	+	–	+	+	+
DNA G + C content (mol%)	38	42.8	40.1	39.1	40	43.8	43.5	38.1
Hydrolysis of:								
Gelatin	+	+	–	–	–	+	–	–
Starch	+	–	+	–	–	–	+	+
Production of:								
H ₂ S	–	–	ND	–	ND	–	+	+
Indole	–	–	ND	–	–	–	–	+
Reduction of nitrate	–	–	–	+	+	+	+	+
Production of acid from:								
Cellobiose	+	+	ND	–	+	+	+	+
D-Galactose	–	+	ND	+	+	+	+	+
Glycerol	–	–	ND	+	ND	+	+	+
myo-Inositol	–	–	ND	–	–	–	–	+
D-Lactose	–	–	ND	–	+	–	+	+
D-Mannose	–	–	ND	+	ND	+	–	+
L-Rhamnose	–	–	ND	–	ND	–	–	+
Sucrose	+	+	ND	–	+	+	+	+
Trehalose	+	+	ND	+	+	+	–	+
DL-Xylose	–	–	–	–	–	–	+	+

Table 2. Fatty acid profiles of strains AK15-027^T, 04HE-4-40, A4I-9 and A4I-21, and recognized species of the genus *Psychromonas*

Taxa: 1, strains AK15-027^T, 04HE-4-40, A4I-9 and A4I-21 (*Psychromonas heitensis* sp. nov.); 2, *P. antarctica* DSM 10704^T; 3, *P. arctica* Pull 5.3^T; 4, *P. hadalis* K41G^T; 5, *P. ingrahamii* 37^T; 6, *P. kaikoe* JT7304^T; 7, *P. marina* 4-22^T; 8, *P. profunda* 2825^T. Data are from this study, Kawasaki *et al.* (2002), Groudieva *et al.* (2003), Auman *et al.* (2006) and Nogi *et al.* (2007). Each value is expressed as a percentage of the total fatty acids. Values below 1 % are not shown.

Fatty acid	1	2	3	4	5	6	7	8
12:0		1	2.7–5.2	1	2.5	1		
14:0	0.5–7.9			1		6		
15:0						1		
16:0	16.8–20.6	24	7.0–16.2	31	18.7	15	43.6	31
i-16:0							2.7	
14:1	6.1–9.9 (ω 7c)	8 (ω 7t)	2.7–5.2 (ω 5t)	17		10 (ω 7t)	3.2	15
16:1	57.7–66.6 (ω 7c)	58 (ω 7c)	~50 (ω 7c), 7.0–16.2 (ω 7t)	37	67	52 (ω 7c), 2 (ω 9c)	39.4	44
18:1	3.5–5.1 (ω 7c)	3 (ω 7c)	7.0–16.2 (ω 7)		3.6	2 (ω 7c)	3.1	
20:5 ω 3						2		
22:6				8		2	1.6	
3-OH-12:0						2		
3-OH-14:0	1.5–2.9	6		3		4		10
12:0 alde, i-16:1 or 3-OH-14:0					4.5			

Description of *Psychromonas heitensis* sp. nov.

Psychromonas heitensis (hei.ten'sis. N.L. fem. n. *heitensis*, pertaining to Heita bay, where the organisms were isolated).

Cells are Gram-negative, facultatively anaerobic, rod-shaped, approximately 1.0–1.5 μ m long by 0.5–1.0 μ m wide. Cells are motile by means of single subpolar flagella. Cytochrome oxidase- and catalase-positive. Optimal growth temperature is 20–25 °C; growth occurs at 2 or 30 °C, but not at 37 °C. pH range for growth is 6.0–9.0. Growth occurs at NaCl concentrations of 3–4 %; no growth occurs in the absence of NaCl. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α -glucosidase. Positive for degradation of DNA, aesculin, starch and Tweens 20, 40, 60 and 80. Does not reduce nitrate (API 20E). Acid is produced by fermentation from glucose (API 20NE), and also by oxidation from glucose, fructose, mannitol, cellobiose, maltose, sucrose, trehalose and starch (API 50 CH). Acid production from glycogen is variable. Does not decompose tyrosine or urea. Indole is not produced. Negative for arginine dihydrolase, lipase (C4), cystine arylamidase, valine arylamidase, trypsin, chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, *N*-acetylglucosamidase, α -mannosidase and α -fucosidase. Does not produce acid from glycerol, erythritol, DL-arabinose, ribose, DL-xylose, adonitol, methyl β -D-xylopyranoside, D-galactose, D-mannose, sorbose, L-rhamnose, dulcitol, myo-inositol, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, D-lactose, melibiose, inulin, melezitose, raffinose,

xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, DL-fucose, DL-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate (API 50 CH). Assimilation of the following carbon sources is positive for maltose, DL-malate and citrate, and negative for glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, gluconate, *n*-capric acid, adipic acid and phenylacetate (API 20NE). Predominant fatty acids are 16:1 ω 7c and 16:0. Major respiratory quinone is Q-8. The DNA G + C content is 38 mol%.

The type strain, AK15-027^T (=MBIC 06898^T=NCIMB 14416^T), was isolated from seawater taken from Heita Bay off Kamaishi in Japan. Strains 04HE-4-40 (=MBIC 08331), A4I-9 (=MBIC 08332) and A4I-21 (=MBIC 08330) are additional reference strains.

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