Psychromonas agarivorans sp. nov., a novel agarolytic bacterium

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Two heterotrophic, agarolytic bacteria were isolated from marine environments. A 16S rRNA gene sequence analysis showed the isolates (designated strains J42-3A^T and 04OZ-AS15-7A) to be positioned in a separate lineage within the genus *Psychromonas*. Members of the genus *Psychromonas* are recognized as being psychrophilic or psychrotolerant, whereas none of the currently established *Psychromonas* species is known to be agarolytic. The DNA G + C contents of the isolates were about 42 mol% and the predominant cellular fatty acids were 16 : 1ω7c and 16 : 0. Based on the results of the phylogenetic and phenotypic analyses and DNA–DNA hybridization data, the isolates represent a novel species, for which the name *Psychromonas agarivorans* sp. nov. is proposed. The type strain is J42-3A^T (=NBRC 104585^T=KCTC 22285^T).

Jean *et al.* (2006) reported that five bacterial species within the class *Alteromonadales* (Bowman & McMeekin, 2005a) were recognized as being agarolytic: *Pseudoalteromonas atlantica* (Akagawa-Matsushita *et al.*, 1992; Bowman & McMeekin, 2005b), *Pseudoalteromonas agarivorans* (Romanenko *et al.*, 2003a), *Glaciecola mesophila* (Romanenko *et al.*, 2003b), *Shewanella olleyana* (Skerratt *et al.*, 2002) and *Thalassomonas agarivorans* (Jean *et al.*, 2006). In this study we characterized two novel agarolytic strains (designated J42-3A^T and 04OZ-AS15-7A) that were isolated from marine sediment and seawater in Japan. Our results suggest that the novel isolates should be classified as representing a novel species of the genus *Psychromonas*.

Strain J42-3A^T was isolated from sediment collected at Kori, off Nomozaki, Nagasaki (32° 36′ 04.7″ N 129° 46′ 34.2″ E) in Japan. The strain was isolated using solidified 1/10 strength marine broth [900 ml filtered seawater and 100 ml marine broth (Difco)] with 1.5 % (w/v) agar and supplemented with 0.5 % Rose bengal. The other strain, 04OZ-AS15-7A, was isolated from a polyurethane foam (PUF) block supplemented with sterile seawater containing 1.5 % (w/v) agar. The PUF block was placed in the sea (Otsuchi Bay, Iwate, Japan) for 3 days during May 2004 (Yasumoto-Hirose *et al.*, 2006). The sampling point was sand close to the beach (39° 20′ 59.6″ N 141° 56′ 09.8″ E). The recovered PUF block was cut into small pieces (1 cm³) and homogenized with 5 ml of sterile seawater by using a

glass rod. The homogenate was diluted to 1/100 with sterile seawater and the bacterium was isolated from this mixture using a medium that comprised sterile seawater with 0.1 % (w/v) 7-hydroxy-flavon and 1.5 % (w/v) agar.

Nucleotide sequences of the 16S rRNA genes amplified by using primers 27F and 1492R (Weisburg *et al.*, 1991) were determined to identify the phylogenetic relationships of the novel isolates. Sequences very similar to those of the isolates were obtained by using BLAST (Altschul *et al.*, 1990). A phylogenetic tree was constructed with the neighbour-joining method (Saitou & Nei, 1987) using CLUSTAL_X (version 1.83; Thompson *et al.*, 1997) after multiple alignment; the sequence used corresponded to positions 95–175, 193–442, 454–819 and 845–1393 of that of *Aeromonas allosaccharophila* CECT 4199^T (GenBank accession no. S39232) obtained using the method of Ivanova *et al.* (2004). A bootstrap analysis was conducted from 1000 bootstrapped trials. 16S rRNA gene sequence similarity was calculated according to the method of Ivanova *et al.* (2004). As shown in Fig. 1, the two novel isolates were positioned within the genus *Psychromonas*, with a high level of 16S rRNA gene sequence similarity (>99%). The highest levels of 16S rRNA gene sequence similarity were found with *Psychromonas macrocephali* JAMM 0415^T, *Psychromonas japonica* JAMM 0394^T, *Psychromonas aquimarina* JAMM 0404^T, *Psychromonas kaikoe* JT7304^T and *Psychromonas profunda* 2825^T (each showing 96 % sequence similarity). Levels of similarity with respect to other *Psychromonas* species were 94–95 %. To determine the genus affiliation, nucleotides at 16S rRNA gene signature sites of the novel isolates and members of the genus *Psychromonas* were compared, according to the method of Ivanova *et al.* (2004). The nucleotide signature

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains J42-3A^T and 04OZ-AS15-7A are AB374544 and AB374545, respectively.

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequence analysis, showing strains J42-3A^T and 04OZ-AS15-7A and other members of the genus *Psychromonas*. Bootstrap values greater than 500 are given at branch points. Bar, 0.01 K_{nuc} .

sites of the genus *Psychromonas* were defined in the family *Psychromonadaceae* at positions 385(T), 811(A), 842(A), 845(T) and 1336(T). The nucleotides at these positions in the novel isolates were conserved, except at position 385, which was C instead of T. The levels of 16S rRNA sequence similarity and the nucleotide signature sites observed indicated that the isolates represent a novel species of the genus *Psychromonas* (Stackebrandt & Goebel, 1994; Ivanova *et al.*, 2004).

Genomic DNA was extracted by using the method of Saitou & Miura (1963). To determine the level of DNA–DNA relatedness, DNA–DNA hybridization was carried out at 41.5 °C and was measured fluorometrically by using the method of Ezaki *et al.* (1989). A high level of DNA–DNA relatedness (89.3–91.9 %) was found between the two novel isolates, indicating that they represent a single species (Wayne *et al.*, 1987).

The DNA G + C contents were determined by using HPLC according to the method of Tamaoka & Komagata (1984). The DNA G + C contents of the novel isolates were 41–42 mol%. The respiratory quinone and cellular fatty acid compositions of cells grown for 48 h at 20 °C in marine broth were determined using previously described methods (Katsuta *et al.*, 2005). The major respiratory quinone of the isolates was Q-8 and the major fatty acids were 16:1 ω 7c and 16:0; the isolates also contained 16:1 ω 7t (Table 1).

Phenotypic characteristics of the novel isolates were determined as follows. Cells were grown for 48 h at

20 °C on marine agar (Difco) and were observed by using transmission electron microscopy after negative staining with uranyl acetate. Gram staining was performed by using the Hucker staining method described by Smibert & Krieg (1994). Growth at various 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 agar (Hosoya *et al.*, 2006), using appropriate buffers: 10 mM MES was used to adjust the pH to 5.5–7.0, 10 mM TAPS for pH 8.0–9.0 and 100 mM NaHCO₃/Na₂CO₃ for pH 10.0. Salt tolerance was tested on salinity-free 1/2 SP5 agar supplemented with 0–10 % NaCl (w/v). Oxidase activity was tested by using oxidase test paper (Eiken), and catalase activity was tested using a 3 % H₂O₂ solution. Growth under anaerobic conditions was tested on marine agar for 2 weeks with the AnaeroPack system (Mitsubishi Gas Chemical). Degradation of starch was tested on 1/2 SP5 agar containing 0.2 % (w/v) soluble starch by using the method of Smibert & Krieg (1994). Hydrolysis of Tweens 20, 40, 60 and 80 was determined on 1/2 SP5 agar adjusted to pH 8.0, supplemented with 0.1 % (v/v) Tweens, by observing the opaque halo formed. Hydrolysis of agar was tested by inoculating stab cultures prepared by using Sap2 medium (Hosoya *et al.*, 2006) containing 1 % agar. Degradation of casein [4 % (w/v) litmus milk (Difco)] and L-tyrosine (0.5 %, w/v) was determined on 1/2 SP5 agar by observing the clear zone formed. Hydrolysis of DNA was determined on DNA agar (Nissui) supplemented with half-strength artificial seawater (ASW). Acid production from sugars was assessed using API 50 CH and API 50

Table 1. Fatty acid profiles of strains J42-3A^T and 04OZ-AS15-7A and other members of the genus *Psychromonas*

Taxa: 1, strains J42-3A^T and 04OZ-AS15-7A (*Psychromonas agarivorans* sp. nov.); 2, *P. antarctica* DSM 10704^T; 3, *P. marina* 4-22^T; 4, *P. kaikoeae* JT7304^T; 5, *P. profunda* 2825^T; 6, *P. arctica* Pull 5.3^T; 7, *P. ingrahamii* 37^T; 8, *P. hadalis* K41G^T; 9, *P. japonica* JAMM 0394^T; 10, *P. aquimarina* JAMM 0404^T; 11, *P. macrocephali* JAMM 0415^T; 12, *P. ossibalaenae* JAMM 0738^T; 13, *P. heitensis*. Data are from this study and Kawasaki *et al.* (2002), Groudieva *et al.* (2003), Xu *et al.* (2003), Brenner *et al.* (2005), Auman *et al.* (2006), Nogi *et al.* (2007), Miyazaki *et al.* (2008) and Hosoya *et al.* (2008). Values are percentages of the total fatty acids. Fatty acids not detected or amounts below 1% are not shown.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12	13
12:0	5.9–7.6	1		1		2.7–5.2	2.5	1		3.6	4		
14:0	2.6–3.2			6				1	2	1.5	1.3	1.9	0.5–7.9
15:0				1					5	2.8			
16:0	37.1–38.2	24	43.6	15	31	7.0–16.2	18.7	31	21.0	28.9	26.7	25.4	16.8–20.6
i-16:0			2.7										
14:1	3.7–4.4(ω 7c)	8(ω 7t)	3.2	10(ω 7t)	15	2.7–5.2(ω 5t)		17	7.3	4.3	4.5	5.1	6.1–9.9(ω 7c)
16:1	35.0–35.3(ω 7c), 6.9–7.0(ω 7t)	58(ω 7c)	39.4	52(ω 7c), 2(ω 9c)	44	~50(ω 7c), 7.0–16.2(ω 7t)	67	37	52.5	49	51	55.5	57.7–66.6(ω 7c)
18:1	3.0–3.4(ω 7c)	3(ω 7c)	3.1	2(ω 7c)		7.0–16.2(ω 7)	3.6		2.7	1.8	4.3	1.4	3.5–5.1(ω 7c)
20:5 ω 3				2									
22:6			1.6	2				8				1.2	
3-OH-12:0				2									
3-OH-14:0	2.0–2.8	6		4	10			3	6.2	7.2	7.5	8.4	1.5–2.9
12:0 alde, i-16:1 or 3-OH 14:0							4.5						

CHB/CHE media (bioMérieux) supplemented with half-strength ASW. The cells used to inoculate the API test strips were suspended in half-strength ASW. Assimilation of carbon sources (API 20NE) was tested using a suspension medium (Hosoya *et al.*, 2008). The API 50 CH tests were read after incubation for 24 h at 30 °C, the API ZYM tests after incubation for 4 h at 37 °C and the API 20NE tests after incubation for 48 h at 30 °C.

The phenotypic characteristics are given in the species description and in Table 2. The novel isolates caused agar plates to be pitted and eventually the agar was liquefied, unlike all other members of the genus *Psychromonas*. The isolates grew at 30 °C, although no recognized *Psychromonas* species, except for *Psychromonas heitensis*, could grow at 30 °C. As shown in Table 1, the novel isolates and *P. arctica* contained 16:1 ω 7t, unlike other recognized *Psychromonas* species. The levels of sequence similarity and signature nucleotides of the 16S rRNA gene suggested that the isolates represent a novel species of the genus *Psychromonas*. On the basis of the data from this study, strains J42-3A^T and 04OZ-AS15-7A represent a novel species of the genus *Psychromonas*, for which the name *Psychromonas agarivorans* sp. nov. is proposed.

Description of *Psychromonas agarivorans* sp. nov.

Psychromonas agarivorans (a.ga.ri.vo'rans. N.L. n. *agarum* agar; L. part. adj. *vorans* devouring, destroying; N.L. part. adj. *agarivorans* agar-devouring).

Cells are Gram-negative, facultatively anaerobic, rod-shaped, approximately 0.8–1.2 µm long and 0.5–0.8 µm

wide. Cells are motile by means of a subpolar flagellum. Cytochrome oxidase-positive and catalase-negative. Optimal growth temperature is 20–25 °C. Growth occurs at 2 and 30 °C; no growth occurs at 37 °C. The pH range for growth is 6.0–9.0. Growth occurs at NaCl concentrations of 1–4 %. Does not grow in the absence of NaCl. Pitting of agar plates occurs and eventually the agar is liquefied. Positive for degradation of DNA and aesculin. Positive for alkaline phosphatase, esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β -galactosidase. Does not reduce nitrate (API 20E; bioMérieux). Acid is produced from glucose by fermentation (API 20NE). Acid is produced by oxidation from galactose, glucose, cellobiose and maltose (API 50 CH). Hydrolysis of and acid production from starch is variable. Does not decompose gelatin, Tweens 20, 40, 60 and 80, tyrosine or urea. Does not produce indole. Negative for arginine dihydrolase, esterase (C4), lipase (C14), cystine arylamidase, valine arylamidase, trypsin, chymotrypsin, α -galactosidase, β -glucuronidase, β -glucosidase, *N*-acetyl- β -glucosamidase, α -mannosidase and α -fucosidase. Does not produce acid from glycerol, erythritol, DL-arabinose, ribose, DL-xylose, adonitol, methyl β -D-xylopyranoside, fructose, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, DL-fucose, DL-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate (API 50 CH). Assimilation of the following

Table 2. Differential characteristics of strains J42-3A^T and 04OZ-AS15-7A and other members of the genus *Psychromonas*

Taxa: 1, strains J42-3A^T and 04OZ-AS15-7A (*Psychromonas agarivorans* sp. nov.); 2, *P. antarctica* DSM 10704^T; 3, *P. marina* 4-22^T; 4, *P. kaikoae* JT7304^T; 5, *P. profunda* 2825^T; 6, *P. arctica* Pull 5.3^T; 7, *P. ingrahamii* 37^T; 8, *P. hadalis* K41G^T; 9, *P. japonica* JAMM 0394^T; 10, *P. aquimarina* JAMM 0404^T; 11, *P. macrocephali* JAMM 0415^T; 12, *P. ossibalaenae* JAMM 0738^T; 13, *P. heitensis* AK15-027^T. Data are from this study and Kawasaki *et al.* (2002), Groudieva *et al.* (2003), Xu *et al.* (2003), Brenner *et al.* (2005), Auman *et al.* (2006), Nogi *et al.* (2007), Miyazaki *et al.* (2008) and Hosoya *et al.* (2008). +, Positive; –, negative; w, weakly positive; ND, no data available; v, variable reaction.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Temperature range for growth (°C)	2–30	2–17	0–25	4–15	2–14	0–25	–12 to 10	2–12	5–25	5–27	0–25	0–25	2–30
Optimum growth temperature (°C)	20–25	12	14–16	10	10	20	20–25	6	20–22	20–25	20	20	20–25
Growth at atmospheric pressure	+	+	+	–	+	+	+	–	+	+	+	+	+
Motility	+	+	+	+	+	+	–	+	+	+	+	+	+
Catalase activity	–	+	+	+	+	ND	+	+	+	–	–	–	+
DNA G + C content (mol%)	41–42	42.8	43.5	43.8	38.1	40.1	40	39.1	38.8	42.2	41.5	41.3	38
Hydrolysis of:													
Agar*	+	–	–	–	–	–	–	–	–	–	–	–	–
Gelatin	–	+	–	+	–	–	–	–	w	+	+	–	+
Starch	v	–	+	–	+	+	–	–	–	+	–	–	+
Production of:													
H ₂ S	–	–	+	–	+	ND	ND	–	–	–	–	–	–
Indole	–	–	–	–	+	ND	–	–	–	–	–	–	–
Reduction of nitrate	–	–	+	+	+	–	+	+	+	+	+	+	–
Acid produced 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	+	+	–	+	+	+	+
Xylose	–	–	+	–	+	–	–	–	–	–	–	+	–

*Data were obtained through personal communication with Dr Yuichi Nogi and Masayuki Miyazaki.

carbon sources is positive for glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, maltose, gluconate, DL-malate and citrate. Assimilation is negative for n-capric acid, adipic acid and phenyl acetate (API 20NE). Predominant fatty acids are 16:1 ω 7c and 16:0. The respiratory quinone is Q-8.

The type strain, J42-3A^T (=NBRC 104585^T=KCTC 22285^T), was isolated from marine sediments collected at Kori, off Nomozaki, Nagasaki in Japan. Strain 04OZ-AS15-7A (=NBRC 104632) is a reference strain. The DNA G + C contents of the type strain and strain 04OZ-AS15-7A are 41–42 mol%.

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