Psychromonas boydii sp. nov., a gas-vacuolate, psychrophilic bacterium isolated from an Arctic sea-ice core

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A gas-vacuolate bacterium, strain 174^T, was isolated from a sea-ice core collected from Point Barrow, Alaska, USA. Comparative analysis of 16S rRNA gene sequences showed that this bacterium was most closely related to *Psychromonas ingrahamii* 37^T, with a similarity of >99 %. However, strain 174^T could be clearly distinguished from closely related species by DNA–DNA hybridization; relatedness values determined by two different methods between strain 174^T and *P. ingrahamii* 37^T were 58.4 and 55.7 % and those between strain 174^T and *Psychromonas antarctica* DSM 10704^T were 46.1 and 33.1 %, which are well below the 70 % level used to define a distinct species. Phenotypic analysis, including cell size (strain 174^T is the largest member of the genus *Psychromonas*, with rod-shaped cells, 8–18 μm long), further differentiated strain 174^T from other members of the genus *Psychromonas*. Strain 174^T could be distinguished from its closest relative, *P. ingrahamii*, by its utilization of D-mannose and D-xylose as sole carbon sources, its ability to ferment *myo*-inositol and its inability to use fumarate and glycerol as sole carbon sources. In addition, strain 174^T contained gas vacuoles of two distinct morphologies and grew at temperatures ranging from below 0 to 10 °C and its optimal NaCl concentration for growth was 3.5 %. The DNA G+C content was 40 mol%. Whole-cell fatty acid analysis showed that 16 : 1ω7*c* and 16 : 0 comprised 44.9 and 26.4 % of the total fatty acid content, respectively. The name *Psychromonas boydii* sp. nov. is proposed for this novel species, with strain 174^T (=DSM 17665^T =CCM 7498^T) as the type strain.

Since the description of *Psychromonas antarctica* (Mountfort *et al.*, 1998), additional members of the genus *Psychromonas* have been isolated and described. All members are Gram-negative rods exhibiting cold-temperature aerobic growth, an optimum salt concentration of greater than 0 %, a DNA G+C content around 40±3 mol% and membranes that are enriched in 16-carbon saturated and unsaturated fatty acids (Auman *et al.*, 2006; Breezee *et al.*, 2004; Brenner *et al.*, 2005; Groudieva *et al.*, 2003; Hosoya *et al.*, 2008; Kawasaki *et al.*, 2002; Miyazaki *et al.*, 2008; Mountfort *et al.*, 1998; Nogi *et al.*, 2002, 2007; Xu *et al.*, 2003). Beyond these similarities, members of the genus *Psychromonas* described to date have a great deal of variety in their other physiological characteristics including cell size, temperature range for growth, piezophily, presence of gas vacuoles and carbon source utilization. Here, strain 174^T, a novel psychrophilic member of this genus is described. Strain 174^T, isolated from a polar sea-ice core collected near Point Barrow, Alaska, USA, has the largest cell size of the genus *Psychromonas*, contains two distinct types of gas vacuole and can be distinguished from its closest phylogenetic relatives, *Psychromonas ingrahamii* 37^T and *P. antarctica*

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 174^T is FJ822615.

DSM 10704^T, based upon DNA–DNA hybridization results as well as several physiological characteristics.

Strain 174^T was isolated from an ice core obtained in May 1991. Specifically, strain 174^T was isolated 40–60 cm from the ice/water interface from a 1.8 m ice core sampled from an open water site (Gosink *et al.*, 1993) and grown on Ordal's seawater cytophaga medium (SWC_m) using full-strength artificial seawater (Irgens *et al.*, 1989). Colonies of 174^T grown on SWC_m plates were white, circular and convex with an entire margin and a smooth appearance. Phenotypic properties of strain 174^T and other members of the genus *Psychromonas* are shown in Table 1.

Cell morphology was determined by phase-contrast microscopy with a Zeiss Photomicroscope I using cells grown in Difco marine broth 2216 (Becton Dickinson) and by electron microscopy with a JEOL-100B transmission electron microscope at 60 kV using unstained whole cells grown in SWC_m broth. Cells of 174^T were very long rods, ranging in length from 8 to 18 μm, with a mean length of 14 μm; these are the largest cells found to date in the *Psychromonas* species (Fig. 1, Table 1). Cells were arranged singly or in chains. Motility was assessed by regular examination over the course of 12 days by phase-contrast microscopy of cells grown in SWC_m broth; cells were found to be non-motile. In addition, no flagella were observed by electron microscopy. This lack of motility is shared by only one other member of the genus *Psychromonas*, *P. ingrahamii* 37^T, which was shown experimentally to be non-motile (Auman *et al.*, 2006) but has been shown more recently to contain an operon for flagellar synthesis the functionality of which has yet to be verified (Riley *et al.*, 2008).

Gas vacuoles were visible, however, as bright, refractile intracellular structures in both phase-contrast and electron micrographs (Fig. 1). Two distinct morphologies of gas vacuole, plentiful short and wide cylinders and less numerous long and narrow cylinders, both with conical ends, were visible by electron microscopy. The presence of multiple gas vacuole types has been observed previously in *Halobacterium halobium* (Walsby, 1994) and, more recently, in *P. ingrahamii* 37^T (Auman *et al.*, 2006); recent genome sequencing of *P. ingrahamii* 37^T revealed the presence of two *gvp* gene clusters encoding the two gas vacuole types (Riley *et al.*, 2008). Strain 174^T represents only the second of the 13 currently described species of the genus *Psychromonas* to contain gas vacuoles. The presence of gas vacuoles, however, appears to be widespread in various taxonomic groups isolated from sea-ice microbial communities (Irgens *et al.*, 1989; Gosink *et al.*, 1993, 1998; Staley *et al.*, 1989; Gosink & Staley, 1995; Staley & Gosink, 1999; Bowman *et al.*, 1998), the source of both strain 174^T and *P. ingrahamii* 37^T (Auman *et al.*, 2006), but their function remains unknown.

The temperature and pH ranges for growth of strain 174^T were determined on SWC_m medium. To determine the temperature range, SWC_m plates were incubated at 0 to 20 °C at intervals of 5 °C. Growth was observed at 0, 5 and 10 °C, but not at temperatures above 10 °C, indicating that strain 174^T is a true psychrophile according to the definition of Morita (1975). Growth at temperatures below 0 °C was not assessed due to the technical difficulties inherent in examining growth at subfreezing temperatures. To determine the pH range, SWC_m broth was buffered to various pH with 25 mM solutions of MES (pH 5.7), ACES (pH 6.6), TAPSO (pH 7.4), TAPS (pH 8.3) and CHES (pH 9.0) (Dyksterhouse *et al.*, 1995) and growth at each pH was monitored spectrophotometrically at 600 nm using a Bausch and Lomb 20-D spectrophotometer. Growth was observed within a neutral pH range of 6.5 to 7.4, but not under more acidic (pH 5.0) or basic (pH 8.3 or 9.0) conditions.

Growth in the presence of various NaCl concentrations (0–20.0 %) was monitored on CLED agar (Difco). Optimum growth occurred at 3.5 % NaCl; growth was observed at 2.0–18.0 % NaCl, although it was weak at the extremes of this range. No growth was observed at NaCl concentrations at or below 1.0 % or at 20.0 %. This range of growth is more restrictive than that observed for *P. ingrahamii* 37^T, the strain most closely related to strain 174^T (Auman *et al.*, 2006).

Carbon source utilization was tested by inoculating strain 174^T into microtitre plate wells of SWC_m broth each containing 0.2 % substrate (with each substrate represented in triplicate); plates were incubated at 5 °C for 21 days and growth was regularly monitored spectrophotometrically at 600 nm using a DeltaSoft II microplate reader. Unlike *P. ingrahamii* 37^T, strain 174^T was able to use D-mannose and D-xylose as sole carbon sources, but was unable to use fumarate or glycerol. Genome sequencing data of *P. ingrahamii* 37^T supports previously reported results indicating that 37^T lacks the ability to utilize xylose, but can use fumarate (Riley *et al.*, 2008; Auman *et al.*, 2006). In addition, the genome sequence of *P. ingrahamii* 37^T confirmed the presence of the glycerol utilization enzymes needed for the experimentally determined ability of this strain to use glycerol as a sole carbon source (Riley *et al.*, 2008; Auman *et al.*, 2006). Interestingly, glycerol was the carbon source provided to *P. ingrahamii* 37^T in experiments to determine its generation time at subzero temperatures because glycerol is known to lower the freezing point of water (Breezee *et al.*, 2004). In those experiments, glycerol was provided in excess of that used for growth by 37^T, but showed no growth inhibition (Breezee *et al.*, 2004). Thus, the lack of glycerol utilization reported here for strain 174^T is a substantial physiological difference between this isolate and *P. ingrahamii* 37^T, but the presence of glycerol in the growth medium may not be inhibitory to the subzero growth of strain 174^T. In fact, a number of other members of the genus *Psychromonas*, including the closely related *P. antarctica* star-1^T, also appear to lack the ability to use glycerol as a sole carbon source for growth (Table 1) (Miyazaki *et al.*, 2008; Mountfort *et al.* 1998; Nogi *et al.*, 2002).

Table 1. Characteristics of strain 174^T (*Psychromonas boydii* sp. nov.) and other members of the genus *Psychromonas*

Strains: 1, *P. boydii* 174^T; 2, *P. ingrahamii* 37^T; 3, *P. antarctica* DSM 10704^T; 4, *P. arctica* Pull 5.3^T; 5, *P. aquimarina* JAMM 0404^T; 6, *P. ossibalaenae* JAMM 0738^T; 7, *P. hadalis* K41G^T; 8, *P. heitensis* AK15-027^T; 9, *P. japonica* JAMM 0394^T; 10, *P. kaikoae* JT7304^T; 11, *P. macrocephali* JAMM 0415^T; 12, *P. marina* 4-22^T; 13, *P. profunda* 2825^T. All strains are Gram-negative, oxidase-positive, catalase-positive and able to use both D-glucose and D-fructose as sole carbon sources. +, Positive; −, negative; W, weakly positive after 3 weeks; (W), weakly positive after 6 weeks; NR, not reported; ND, not determined. Data are from this study, Auman *et al.* (2006), Breezee *et al.* (2004), Brenner *et al.* (2005), Groudieva *et al.* (2003), Hosoya *et al.* (2008), Kawasaki *et al.* (2002), Miyazaki *et al.* (2008), Mountfort *et al.* (1998), Nogi *et al.* (2002, 2007), Riley *et al.* (2008) and Xu *et al.* (2003).

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Cell morphology and arrangement*	LR, S, C	LR, S, P	OR, S, P	R, S, P	R	R	R	R	R	OR	R	R	R
Cell length (μm)	8–18	6–14	2.5–6.0	1.3–2.6	1.6–3.2	1.9–3.7	1.5–2.0	1.0–1.5	1.6–2.2	2–4	2.2–2.9	1.5–2.0	2.0–5.5
Production of gas vesicles	+	+	−	−	−	−	−	−	−	−	−	−	−
Colony colour†	WH	WH	WH	WH	CR	CR	NR	NR	TCR	NR	CR	NC	NC
Motility	−	−‡	+	+	+	+	+	+	+	+	+	+	+
Utilization of:													
D-Galactose	+	+	+	NR	+	+	+	−	NR	+	+	+	+
D-Mannose	+	−	−	+	+	+	+	−	+	+	+	−	−
D-Mannitol	+	+	+	+	+	−	NR	−	−	+	−	+	W
D-Sorbitol	−	−	−	NR	NR	NR	NR	NR	NR	−	NR	−	−
N-Acetylglucosamine	+	+	+	NR	NR	NR	NR	−	NR	NR	NR	+	NR
Arabinose	−	−	NR	NR	NR	NR	−	−	NR	−	NR	−	−
D-Xylose	+	−	−	−	−	+	NR	−	−	−	−	+	+
Cellobiose	+	+	−	NR	−	+	NR	+	+	+	−	+	+
Lactose§	−	−	−	+	ONPG +	ONPG +	NR	ONPG +	ONPG −	−	ONPG +	+	+
Maltose	+	+	+	+	+	+	NR	+	NR	+	+	+	+
Sucrose	+	+	+	+	+	+	NR	+	−	+	−	+	W
Fumarate	−	+	−	+	NR	NR	NR	NR	NR	NR	NR	NR	+
DL-Lactate	+	+	−	−	NR	NR	NR	NR	NR	NR	NR	NR	(W)
DL-Malate	−	−	−	−	NR	NR	NR	+	NR	NR	NR	NR	NR
Glycerol	−	+	−	+	+	+	+	NR	−	−	+	+	(W)
Fermentation of:													
D-Glucose‖	+	+	+ (G)	+ (G)	+	+	−	+	+	+	+	+	+
myo-Inositol	+	−	−	NR	−	−	−	−	−	−	−	−	+
Lactose	+	+	−	NR	−	−	−	−	−	−	−	+	+
Trehalose	+	+	+	NR	+	+	+	+	−	+	+	−	+
D-Xylose	−	−	−	−	−	+	−	−	−	−	−	+	+
Hydrolysis of:													
Starch	−	−	+	+	+	−	−	+	−	−	−	+	W
Gelatin	−	−	+	−	+	−	−	+	W	+	+	−	−
NaCl concentration for growth (%)													
Range	2–18¶	1–12	0–4	1–7	2–5	2–5	>0	>0	2–5	>0	2–6	>0–7	>0
Optimum	3.5	ND	3	2	3	3	3	3–4	3	NR	3	3–5	NR
Growth temperature range (°C)	⩽0 to 10#	−12 to 10	2–17	0–25	5–27	0–25	2–12	2–30	5–25	4–15	0–25	0–25	2–14
pH for growth													
Range	6.5–7.4	6.5–7.4	NR	6.5–9.8	6.0–9.0	6.5–9.5	NR	6.0–9.0	6.5–9.5	NR	6.0–9.0	NR	NR
Optimum	ND	ND	6.5	8.8	8.5	9.0	NR	NR	9.0	NR	8.5	NR	NR
O_2 requirement for growth**	FAN	FAN	ATAN	A	FAN	FAN	FAN	FAN	FAN	FAN	FAN	FAN	FAN
Growth at atmospheric pressure	+	+	+	+	+	+	−	+	+	−	+	+	+
Indole production	−	−	−	NR	−	−	−	−	−	−	−	−	+
Nitrate reduction	+	+	−	−	+	+	+	−	+	+	+	+	+
DNA G+C content (mol%)	40	40	42.8	40.1	42.2	41.3	39.1	38	38.8	43.8	41.5	43.5	38.1

*LR, Large rods; OR, ovoid rods; R, rods; C, chains; P, pairs; S, single cells.

†CR, Cream; TCR, translucent cream; WH, white; NC, colourless.

‡Recent genomic analysis by Riley *et al.* (2008) revealed that the genome of *P. ingrahamii* 37^T contains a cluster of flagellar genes, but motility was not observed experimentally.

§Results prefixed with ONPG indicate that strains were not tested for lactose utilization directly but, rather, for ONPG hydrolysis/β-galactosidase activity.

‖(G) indicates gas production.

¶Growth was seen at concentrations of 2–18 %, but was weak at the extremes of this range.

#Growth below 0 °C was not determined.

**A, Aerobe; ATAN, aerotolerant anaerobe; FAN, facultative anaerobe.

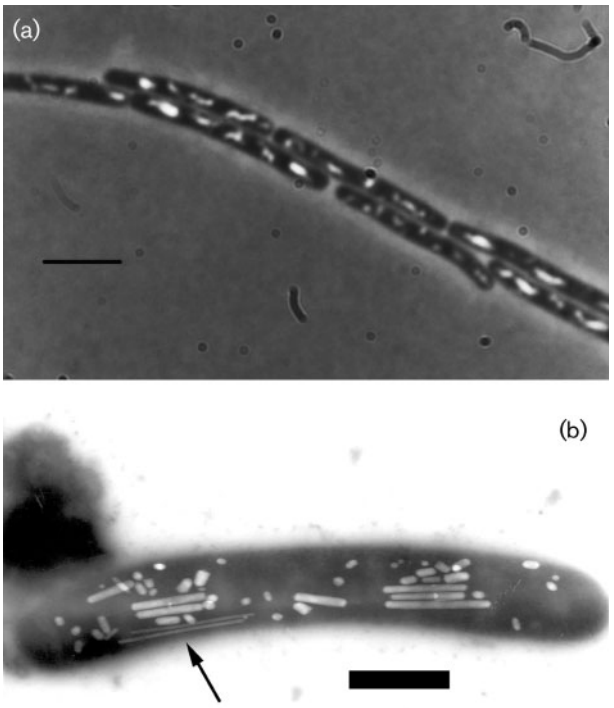

Fig. 1. Phase-contrast micrograph (a) and transmission electron micrograph (b) of cells of strain 174^T. Bars, 5 μm (a) and 1 μm (b). Bright areas within the cells are gas vacuoles. The characteristic morphology of the subunit gas vesicles, i.e. their cylindrical shape with the conical polar caps, is shown in the transmission electron micrograph preparation; note that two different sizes of gas vesicle are present, the more common wide type and the thin type (arrow).

Sugar fermentation by strain 174^T was tested using the Hugh–Leifson method as described previously (Gerhardt *et al.*, 1981) using OF basal medium (BBL) dissolved in ONR-7a salt solution (Dyksterhouse *et al.*, 1995), with each carbon source tested diluted to a final concentration of 1 %. Inoculation with *Vibrio splendidus* ATCC 33125^T was used as a positive control, whereas inoculation of medium lacking an added carbon source was used as a negative control. Strain 174^T was grown in SWC_m broth with added glucose and an inverted Durham tube for the detection of gas production from glucose utilization. Strain 174^T was facultatively anaerobic and was able to ferment several carbon sources including lactose, sucrose, D-mannitol, salicin, *myo*-inositol, maltose, trehalose, cellobiose, D-galactose, melibiose and glucose (with no production of gas), but not dulcitol, D-sorbitol, L-arabinose or D-xylose. The carbon sources fermented by strain 174^T are similar to those fermented by *P. ingrahamii* 37^T, with the exception that strain 174^T could ferment *myo*-inositol (Auman *et al.*, 2006). The ability to ferment *myo*-inositol is shared by only one other member of the genus, *Psychromonas profunda* 2825^T (Xu *et al.*, 2003).

Several biochemical tests were also carried out as described previously (Gerhardt *et al.*, 1981), inoculating strain 174^T into SWC_m medium supplemented with the appropriate substrate. A nitrate reduction assay was performed in the presence of 0.1 or 0.01 % $NaNO_3$ and 0.17 % agar. Strain 174^T was found to be Gram-negative, oxidase-positive, weakly catalase-positive and positive for nitrate reduction, which is consistent with data for other members of the genus *Psychromonas*. Like *P. ingrahamii* 37^T, *Psychromonas hadalis* K41G^T and *Psychromonas ossibalaenae* JAMM 0738^T, strain 174^T was unable to hydrolyse starch or gelatin. Monitoring of the three stages of growth in SWC_m in the absence of succinate indicated that strain 174^T was negative for indole production, a result that is consistent with all other members of the genus *Psychromonas* with the exception of *P. profunda* 2825^T (Xu *et al.*, 2003). Strain 174^T and *P. ingrahamii* 37^T were also tested for H_2S production as described by Gerhardt *et al.* (1981) using Kligler iron agar slants (Becton Dickinson) made up in 2216 broth (Difco). Growth at 5 °C was monitored periodically over 3 weeks. Both strains were negative for H_2S production.

Whole-cell fatty acid analysis was carried out using the MIDI protocol as described previously (MIDI, 1993). Briefly, cells of 174^T grown at 4 °C on SWC_m plates were collected into 13 × 100 mm Teflon-lined tubes, frozen at −80 °C and lysed. The resulting fatty acids were saponified with methanolic base and converted to fatty acid methyl esters with HCl prior to identification and quantification by GC (Hewlett Packard model 5890 Series II). Like other members of the genus *Psychromonas* (Auman *et al.*, 2006; Breezee *et al.*, 2004; Brenner *et al.*, 2005; Groudieva *et al.*, 2003; Hosoya *et al.*, 2008; Kawasaki *et al.*, 2002; Miyazaki *et al.*, 2008; Mountfort *et al.*, 1998; Nogi *et al.*, 2002, 2007; Xu *et al.*, 2003), strain 174^T possessed a high concentration of unsaturated and saturated 16-carbon fatty acids, comprising 46.2 and 26.4 %, respectively, of the whole-cell fatty acid content (Table 2). Other fatty acids produced by strain 174^T in measurable quantities included 12 : 0 (7.1 %), 14 : 0 (4.5 %), one or more of 18 : 1ω7*c*, ω9*t* and ω12*t* (2.3 %) and 18 : 1ω9*c* (1.6 %). Our methodology was unable to distinguish between the fatty acids 12 : 0 alde, iso-16 : 1 and 14 : 0 3-OH; a significant proportion (9.4 %) of the fatty acids from strain 174^T belonged to this group. In addition, 1 % of the fatty acids produced by strain 174^T could not be identified. Compared with *P. ingrahamii* 37^T, strain 174^T produced a greater variety of fatty acid types and 16 : 1ω7*c* made up less than half of the total fatty acid content of strain 174^T, whereas it accounted for 67 % in *P. ingrahamii* 37^T (Auman *et al.*, 2006). Like *P. ingrahamii* 37^T, strain 174^T lacked the 14 : 1 fatty acids found in all other members of the genus *Psychromonas*, as well as the larger (20 or more carbons) fatty acids found in some other members of the genus (Auman *et al.*, 2006).

For determination of the G+C content of strain 174^T, genomic DNA was isolated using a standard hexadecyltrimethylammonium bromide protocol (Ausubel *et al.*,

Table 2. Fatty acid compositions of strain 174^T (*P. boydii* sp. nov.) and other members of the genus *Psychromonas*

Strains: 1, *P. boydii* 174^T; 2, *P. ingrahamii* 37^T; 3, *P. antarctica* DSM 10704^T; 4, *P. arctica* Pull 5.3^T; 5, *P. aquimarina* JAMM 0404^T; 6, *P. ossibalaenae* JAMM 0738^T; 7, *P. hadalis* K41G^T; 8, *P. heitensis* AK15-027^T; 9, *P. japonica* JAMM 0394^T; 10, *P. kaikoae* JT7304^T; 11, *P. macrocephali* JAMM 0415^T; 12, *P. marina* 4-22^T; 13, *P. profunda* 2825^T. Values are percentages of total fatty acids. Isomers are shown in parentheses if known. Results below 1 % are not shown. Data for other *Psychromonas* species were taken from Auman *et al.* (2006), Breezee *et al.* (2004), Brenner *et al.* (2005), Groudieva *et al.* (2003), Hosoya *et al.* (2008), Kawasaki *et al.* (2002), Miyazaki *et al.* (2008), Mountfort *et al.* (1998), Nogi *et al.* (2002, 2007) and Xu *et al.* (2003).

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12	13
12 : 0	7.1	2.5	1	2.7–5.2	3.6		1			1	4.0		
14 : 0	4.5				1.5	1.9	1	0.5–7.9	2.0	6	1.3		
15 : 0					2.8				5.0	1			
16 : 0	26.4	18.7	24	7.0–16.2	28.9	25.4	31	16.8–20.6	21.7	15	26.7	43.6	31
14 : 1			8 (ω7*t*)	2.7–5.2 (ω5*t*)	4.3	5.1	17	6.1–9.9 (ω7*c*)	7.3	10 (ω7*t*)	4.5	3.2	15
16 : 1	44.9 (ω7*c*), 1.3 (2-OH)	67 (ω7*c*)	58 (ω7*c*)	~50 (ω7*c*), 7.0–16.2 (ω7*t*)	49.0	55.5	37	57.7–66.6 (ω7*c*)	52.5	52 (ω7*c*), 2 (ω9*c*)	51.0	39.4	44
18 : 1	2.3 (ω7*c*, ω9*t*, ω12*t*), 1.6 (ω9*c*)	3.6	3 (ω7*c*)	7.0–16.2 (ω7)	1.8	1.4		3.5–5.1 (ω7*c*)	2.7	2 (ω7*c*)	4.3	3.1	
20 : 5ω3										2			
22 : 6						1.2	8			2		1.6	
12 : 0 3-OH										2			
12 : 0 alde, iso-16 : 1 or 14 : 0 3-OH	9.4	4.5	6 (14 : 0 3-OH)		7.2 (14 : 0 3-OH)	8.4 (14 : 0 3-OH)	3 (14 : 0 3-OH)	1.5–2.9 (14 : 0 3-OH)	6.2 (14 : 0 3-OH)	4 (14 : 0 3-OH)	7.5 (14 : 0 3-OH)	2.7 (iso-16 : 1)	
Unknown	1.0												

1989) and subjected to HPLC analysis according to the protocol of Mesbah *et al.* (1989). The G+C content of strain 174^T was 40 mol%, which is similar to values determined for other members of the genus *Psychromonas* (Auman *et al.*, 2006; Breezee *et al.*, 2004; Brenner *et al.*, 2005; Groudieva *et al.*, 2003; Hosoya *et al.*, 2008; Kawasaki *et al.*, 2002; Miyazaki *et al.*, 2008; Mountfort *et al.*, 1998; Nogi *et al.*, 2002, 2007; Xu *et al.*, 2003).

For determination of the 16S rRNA gene sequence, genomic DNA was isolated using standard methods (Sambrook *et al.*, 1989). A 16S rRNA gene fragment of about 1450 bp was amplified from a 100-fold dilution of a chromosomal DNA sample from strain 174^T using primers F27 and 1492R (Lane, 1991). The reactions were carried out in a Perkin Elmer Gene Amp 2400 thermocycler with an initial denaturation step of 5 min at 94 °C, followed by 40 cycles of 92 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, with a final extension of 72 °C for 5 min. The PCR mixture contained final concentrations of 1 × PCR buffer with $(NH_4)_2SO_4$ (Fermentas Life Sciences), 2 mM $MgCl_2$ (Fermentas Life Sciences), 0.33 μM F27 (Operon Biotechnologies), 0.33 μM 1492R (Operon Biotechnologies), 0.166 nM dNTPs (Fermentas Life Sciences) and 2.5 U *Taq* polymerase (Fermentas Life Sciences) in a total volume of 30 μl. Following purification of the PCR product with the QIAquick PCR Purification kit (Qiagen), sequencing of the 16S rRNA PCR-generated gene fragment was carried out on both strands using the ABI Prism Big Dye terminator sequencing kit (PE Applied Biosystems). Sequencing reactions and analysis were performed using the University of Washington Department of Biochemistry Sequencing Facility using an Applied Biosystems automated sequencer. Sequences obtained using various primers [F27, 1492R, 530F (5′-AGCAGCCGCGGTAATAC-3′) and 530rc (5′-GTATTACCGCGGCTGCT-3′)] were checked for base determination accuracy, assembled and aligned using the computer program SEQUENCHER v. 4.8 (Gene Codes) into a 1415 bp double-stranded fragment. Sequencing analysis of the PCR product revealed degeneracy in two nucleotide positions, probably due to slight differences in the multiple copies of the 16S rRNA genes found in strain 174^T; *P. ingrahamii* 37^T has been shown to contain 10 rRNA operons (Riley *et al.*, 2008).

Phylogenetic analysis was performed on the 16S rRNA gene fragment from strain 174^T and a number of closely related strains whose 16S rRNA gene sequences were obtained from the Ribosomal Database Project II (Cole *et al.*, 2007). The GenBank/EMBL accession numbers for the additional 16S rRNA gene sequences used are given in parentheses in Fig. 2. These sequences were aligned using CLUSTAL W v. 2.0 (Larkin *et al.*, 2007) and GENEDOC (Nicholas *et al.*, 1997). Dendrograms were constructed using the programs DNADIST (distance options according to Kimura's two-parameter model), NEIGHBOR, SEQBOOT and CONSENSE in the PHYLIP software package v. 3.5c (Felsenstein, 1989). The resulting tree was visualized using TREEVIEW (Page, 1996). Bootstrap values were determined based upon 100 replications. Phylogenetic analysis revealed that strain 174^T was a member of the class *Gammaproteobacteria*, order

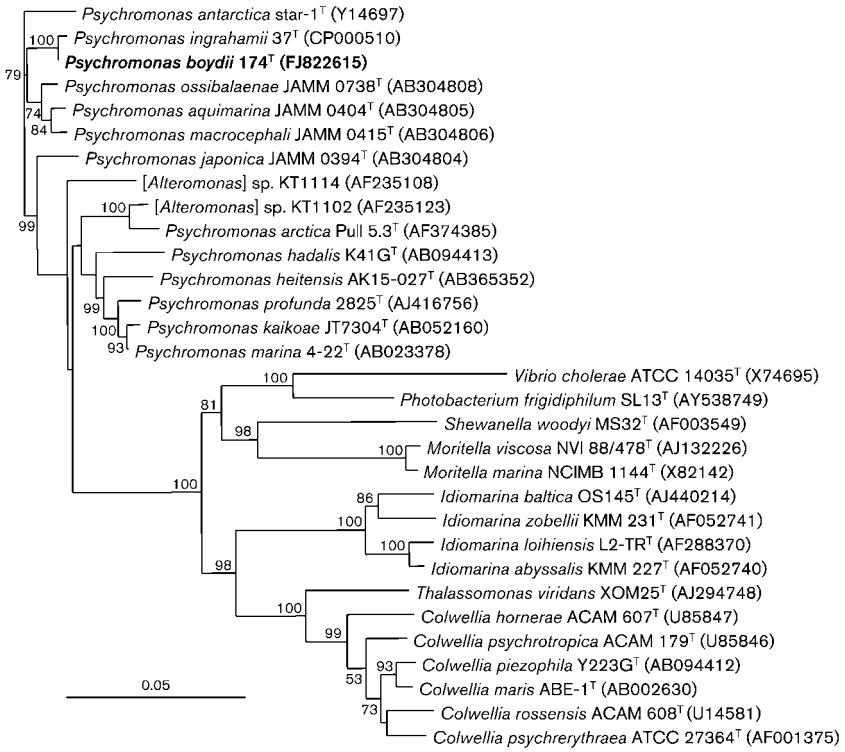

Fig. 2. Phylogenetic analysis based on 16S rRNA gene sequences (accession numbers in parentheses) available from the Ribosomal Database Project II (Cole *et al.*, 2007) constructed after multiple alignment of data by using CLUSTAL W v. 2.0 (Larkin *et al.*, 2007) and GENEDOC (Nicholas *et al.*, 1997). Distances (distance options according to Kimura's two-parameter model) and clustering with the neighbour-joining method were determined by using the programs DNADIST, NEIGHBOR, SEQBOOT and CONSENSE in the PHYLIP software package version 3.5c (Felsenstein, 1989). The resulting tree was visualized using TREEVIEW (Page, 1996). Bootstrap values (%) based on 100 replications are given at branch points. Bar, 0.05 substitutions per nucleotide position.

Alteromonadales, family *Psychromonadaceae*, according to the classification proposed by Ivanova *et al.* (2004), was most closely related to *P. ingrahamii* 37^T (having over 99 % similarity at the nucleotide level) and clustered with other members of the genus *Psychromonas* (Fig. 2). This analysis also revealed distinct clustering of all members of the genus *Psychromonas*, clearly separating *Psychromonas* species from members of closely related genera. Included within this *Psychromonas* cluster are sequences from North Sea pelagic bacterial strains that, prior to efforts to better distinguish between families within the order *Alteromonadales* (Ivanova *et al.*, 2004), have been identified as [*Alteromonas*] strains (Eilers *et al.*, 2000), but are probably members of the genus *Psychromonas*.

Although phenotypic analysis revealed differences between strain 174^T and *P. ingrahamii* 37^T, phylogenetic analysis suggested that strain 174^T might be a second strain of *P. ingrahamii*. To clarify the relationship between these two strains further, DNA–DNA hybridization experiments were performed using genomic DNA isolated from strain 174^T with that from either *P. ingrahamii* 37^T or *P. antarctica* DSM 10704^T according to two methods. DNA was isolated using a French pressure cell (ThermoSpectronic) and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). For the first method, DNA–DNA hybridization experiments were carried out in 2 × SSC buffer at 65 °C as described by De Ley *et al.* (1970) using the modifications described by Huß *et al.* (1983). Reassociation curves were recorded by using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). The reassociation value between strain 174^T and *P. ingrahamii* 37^T was 58.4 % (63.1 % in a repeat experiment) and that between strain 174^T and *P. antarctica* DSM 10704^T was 46.1 % (41.5 % in a repeat experiment). For the second method, DNA–DNA hybridization experiments were performed with genomic DNA isolated from strain 174^T with either *P. ingrahamii* 37^T or *P. antarctica* DSM 10704^T using the method described by Ziemke *et al.* (1998) except that, for nick translation, 2 μg DNA was labelled during a 3 h incubation at 15 °C. With this method, the reassociation value between strain 174^T and *P. ingrahamii* 37^T was 55.7 % (67.9 % in a repeat experiment) and that between strain 174^T and *P. antarctica* DSM 10704^T was 33.1 % (46.1 % in a repeat experiment). All these reassociation values are well below the widely accepted value of 70 % that is used to delineate a distinct species (Wayne *et al.* 1987), thus confirming that strain 174^T represents a novel species.

Members of the genus *Psychromonas* share several characteristics that probably result from commonalities in the cool marine environments from which they have been isolated most frequently: growth at low temperatures and optimal growth in the presence of NaCl. However, members of this genus also exhibit a great variety of other phenotypic characteristics that may allow the various members to adapt to the specific characteristics of the microenvironments that they inhabit. Within this genus, there is variability in, for example, cell size, growth temperature range, the presence of gas vacuoles, apparent flagellar motility, piezophilic growth and carbon source utilization capabilities. This range of phenotypes is not unique to the genus *Psychromonas*, but is also seen in the closely related genus *Colwellia*, another genus containing a wide variety of members isolated from cool environments (Bowman *et al.*, 1998; Deming *et al.*, 1988; Nogi *et al.*, 2004).

Here, we add to the growing collection of members of the genus *Psychromonas* through the description of *Psychromonas boydii* sp. nov., with strain 174^T as the type strain. Strain 174^T can be distinguished from closely related strains by its unusually large cell size, the presence of two types of gas vacuole within its cells and its carbon source utilization characteristics. Within this genus, which, at the time of writing, contained 12 previously described species, it represents only the second gas-vacuolate species isolated and characterized to date; it can be distinguished from its closest relative *P. ingrahamii* 37^T based upon both its low DNA–DNA reassociation value with this organism as well as several differences in carbon source usage.

Description of *Psychromonas boydii* sp. nov.

Psychromonas boydii (boy′di.i. N.L. masc. gen. n. *boydii* of Boyd, in honour of William L. Boyd, whose career was dedicated to researching psychrophilic bacteria. This epithet was also chosen to honour Josephine W. Boyd).

Cells are Gram-negative, non-motile, large rods, 8–18 μm long (mean 14 μm), found either singly or in chains and containing two gas vesicle morphologies. On SWC_m, colonies are white, circular and convex, with a smooth appearance and an entire margin. Growth occurs at NaCl concentrations between 2 and 18 %, with optimum growth occurring at 3.5 % NaCl. Psychrophilic, with growth occurring between 0 and 10 °C (not tested below 0 °C; not tested between 10 and 15 °C, but no growth is observed at 15 °C). Growth occurs at pH 6.5–7.4, but not at pH 5.0, 8.3 or 9.0. Grows at atmospheric pressure. Facultative anaerobe, capable of both respiratory and fermentative metabolism. Positive for catalase and cytochrome oxidase. Negative for H_2S production. Reduces inorganic nitrate, but does not produce indole. The major membrane fatty acids are 16 : 1ω7*c* and 16 : 0. Uses the following as sole carbon sources: D-glucose, D-ribose, D-fructose, sucrose, L-glutamate, L-cysteine, DL-aspartate, citrate, pyruvate, acetate, *N*-acetylglucosamine, cellobiose, DL-lactate, D-mannitol, D-mannose, salicin, trehalose and D-xylose. Cannot use the following as sole carbon sources: lactose, L-leucine, L-proline, α-ketoglutarate, succinate, propionate, benzoate, glycolate, glycerol, methanol, glucosamine, arabinose, caproate, D-gluconate, DL-malate, D-sorbitol, D-glucuronate and Tweens 20, 40 and 80. Ferments lactose, sucrose, D-mannitol, salicin, *myo*-inositol, maltose, trehalose, cello-

biose, D-galactose, melibiose and D-glucose (with no gas production), but not dulcitol, D-sorbitol, L-arabinose or D-xylose. Does not hydrolyse starch or gelatin.

The type and only known strain is 174^T (=DSM 17665^T =CCM 7498^T), isolated from an open water site at Point Barrow, Alaska, USA, about 40–60 cm from the ice/water interface from a 1.8 m ice core. The DNA G+C content of the type strain is 40 mol%.

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