Psychroflexus tropicus sp. nov., an obligately halophilic *Cytophaga–Flavobacterium–Bacteroides* group bacterium from an Hawaiian hypersaline lake

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A Gram-negative bacterium designated LA1^T was isolated from water collected in hypersaline Lake Laysan on Laysan Island in the Northwestern Hawaiian Islands. Cells occurred singly as fine rods to short filaments. Growth in 50 % strength marine broth occurred optimally when the medium contained 7·5–10 % (w/v) NaCl. The major fatty acids in LA1^T grown at 15 and 30 °C were 12-methyl tetradecanoic acid and 13-methyl tetradecanoic acid, respectively. The nucleotide sequence of the 16S rRNA gene showed that LA1^T belonged in the *Cytophaga–Flavobacterium–Bacteroides* (CFB) group in the domain *Bacteria*. The closest described neighbour in terms of 16S rRNA gene sequence identity was *Psychroflexus torquis* ACAM 623^T (94·4 % over 1423 bases), an obligate psychrophile from Antarctic sea-ice. The G+C content of 35·0 mol% was consistent with this affiliation. Phenotypic and genotypic analyses, including DNA hybridization, indicated that LA1^T could be assigned to the genus *Psychroflexus* but, based on significant differences, including growth at 43 °C, it constitutes a novel species, *Psychroflexus tropicus* sp. nov., for which LA1^T (=ATCC BAA-734^T=DSM 15496^T) is the type strain.

Cytophaga–Flavobacteria species occur in marine sediments, freshwater biofilms and hypersaline lakes (Manz *et al.*, 1999; Ravenschlag *et al.*, 2001; Dobson *et al.*, 1993). We isolated strain LA1^T from subtropical, hypersaline Lake Laysan on a remote atoll in the Northwestern Hawaiian Islands. The closest described neighbour, on the basis of 16S rRNA sequence identity, is *Psychroflexus torquis* ACAM 623^T, an obligate psychrophile from Antarctic sea-ice. With differences in 16S rRNA sequences of 5 % or more within the genera of the *Flavobacteriaceae* (Bernardet *et al.*, 2002), we assumed that we had isolated a mesophilic *Psychroflexus* species. Bowman *et al.* (1998) cited ecophysiological differences between two *Psychroflexus* species as an example of how phylogenetically related bacteria adapt to different habitats. A defining characteristic of these authors' strains was obligate psychrophily which, with phylogenetic evidence and fatty acid data, led to inclusion of psychrotolerant [*Flavobacterium*] *gondwanense* in the new genus *Psychroflexus* (Bowman *et al.*, 1998). Here, we describe the phenotypic and genotypic characterization of strain LA1^T. On the basis of polyphasic evidence, we propose that this strain be included in the genus *Psychroflexus* as *Psychroflexus tropicus* sp. nov.

Abbreviation: CFB group, *Cytophaga–Flexibacter–Bacteroides* group.

The GenBank accession number for the 16S rRNA gene sequence of LA1^T is AF513434.

Scanning electron micrographs of *Psychroflexus tropicus* cells and growth curves in relation to salinity are available as supplementary material in IJSEM Online.

Water from 0·3 m depth at the centre of L. Laysan (25° 46′ N 171° 44′ W) was spread on marine agar (MA) (Difco) and incubated at 25 °C. An orange colony (LA1) that arose after 5 days was transferred to MA for purification and incubated at 30 °C. Strain LA1^T was thence maintained on MA or in marine broth (MB) (Difco). Stock cultures were stored at −80 °C in MB with 30 % glycerol (w/v). Initial identification of LA1^T, based on a fragment of the 16S rRNA gene, showed that the strain's closest neigbours are in the genus *Psychroflexus*, which contains obligate psychrophiles and psychrotolerant [*Flavobacterium*] *gondwanense* (Bowman *et al.*, 1998). LA1^T is the first mesophilic *Psychroflexus* species to be identified.

The tolerance to NaCl of LA1^T was tested on tryptic soy agar (TSA) (BBL) with 0·5–20 % (w/v) NaCl, and on 50 % MA with 1–20 % (w/v) NaCl, both at 30 °C for 10 days. The 50 % MA was half-strength MA diluted with distilled water,

but supplemented with agar to 1·3 % (w/w). The optimum salinity for growth was determined in 50 % MB, in the range 1–20 % (w/v) NaCl, incubated with shaking (100 r.p.m.) at 30 °C. The 50 % MB was half-strength MB. MB (8·5 ml) was inoculated with 50 µl of a 72 h, 30 °C culture in MB containing 2 % (w/v) NaCl. Growth was followed by measuring the turbidity at λ_{605} of 100 µl subsamples, over 85 h, in a Beckman DU650 spectrophotometer. The growth temperature range was determined on MA from 4 to 60 °C. Anaerobic growth was checked on MA in the BBL GasPak Pouch system, with oxygen and carbon dioxide concentrations of <2 % and >4 %, respectively.

Motility was observed by hanging drop under a 1000 × objective with oil immersion after 24 h in MB, or from colonies on MA (7·5 %, w/v, NaCl) in sterile 7·5 % (w/v) saline. Flexirubin-type pigments were checked for by flooding colonies with 20 % KOH (Reichenbach, 1989). Single colonies on MA were tested for catalase and cytochrome oxidase *c* with 3 % hydrogen peroxide (Sigma) and tetramethyl-*p*-phenylenediamine (BBL), respectively. Nitrate reduction was determined in nitrate broth (Difco) containing NaCl to 7·5 % (w/v); standard reagents for reductase were added after 48 h at 30 °C. Amylase was tested on starch medium (Difco) with NaCl concentrations of 0–7·5 % (w/v) by flooding inoculated plates with iodine after 7 days incubation at 30 °C. Hydrolysis of DNA was checked on DNase test agar with methyl green (Difco), and hydrolysis of gelatin in gelatin nutrient medium (Difco), each in the presence of 1 and 7 % (w/v) NaCl.

Growth on and acidification of carbohydrates in API 50 CH (bioMérieux Vitek) were followed over 5 days in CHB/E medium with SL-8 trace elements solution (Atlas, 1997), rather than Cohen-Bazire mineral base, and 7·5 % (w/v) NaCl. Constitutive enzyme activities were assayed in API ZYM. Oxidation of carbohydrates, alcohols, organic acids, amino acids and nucleosides as single carbon sources was checked in Biolog GN. Fatty acids in whole cells grown on MA (15 and 30 °C) were analysed in the MIDI system (Sasser, 1997). Cells grown for 24 h and 5 days in MB were prepared for scanning electron microscopy (Donachie *et al.*, 2002).

Genomic DNA was extracted from 48 h cultures in MB using the G NOME kit (Qbiogene). A fragment of the 16S rRNA gene was amplified from the DNA by PCR with *Pfu* DNA polymerase and primers 27F and 1492R (Lane, 1991). Thermal cycling conditions comprised initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 90 s. Final extension was carried out at 72 °C for 7 min, followed by cooling to 4 °C. The PCR product was purified with a Qiagen PCR purification kit (Qiagen) and sequenced in both directions in a Beckman CEQ2000 DNA analyser using the Beckman sequencing kit with primers 27F, 519R, 533F and 1492R (Lane, 1991). 16S rDNA sequences were assembled in Seqman (DNASTAR). Genomic DNA extracted with phenol/chloroform (Marmur, 1961) from LA1^T grown in MB was hybridized with DNA from the type strains of *P. torquis* and *Psychroflexus gondwanensis* (Huß *et al.*, 1983; Bowman *et al.*, 1998). Hybridizations were carried out in 2 × SSC buffer, with renaturation at 64 °C with *P. torquis* DNA (assuming 32 and 35 mol% G + C, for *P. torquis* and LA1^T, respectively), and at 65 °C with *P. gondwanensis* DNA (assuming 36 and 35 mol% G + C, for *P. gondwanensis* and LA1^T, respectively). The G + C content of the DNA was determined following Sly *et al.* (1986). The relationship of LA1^T with other *Bacteria* was visualized on the basis of their 16S rRNA gene sequences in a phylogenetic tree constructed from a CLUSTAL X alignment (Thompson *et al.*, 1997), using the neighbour-joining method (Saitou & Nei, 1987) corrected for multiple substitutions, and rerooted in NJPlot (Perrière & Gouy, 1996).

Morphological, physiological and biochemical characteristics of LA1^T are given in the species description. In addition, LA1^T did not produce flexirubin-type pigments. Coccoid bodies developed in older cultures (see Supplementary Fig. A in IJSEM Online). Although LA1^T appeared non-motile in a hanging-drop preparation, the eroded aspect of colony margins might indicate gliding motility (Bernardet *et al.*, 2002). LA1^T grew on TSA only in the presence of 7·5 or 10 % (w/v) NaCl, while growth on MA covered the 1–20 % salinity range. Differences in growth on these media with the same NaCl concentrations may reflect a requirement for yeast extract, since this is absent from TSA (Bowman *et al.*, 1998). NaCl-supplemented TSA also lacks other inorganic salts found in MA or MB, a valid consideration because L. Laysan contains evaporated sea water. The salinity optimum for *P. tropicus* LA1^T (see Supplementary Fig. B in IJSEM Online) is greater than that for *P. torquis* ACAM 623^T (optimum 3 %) and *P. gondwanensis* (5 %). LA1^T required 7·5 % (w/v) NaCl in the medium for some activities: nitrate and nitrite reduction took place only in the presence of 7·5 % (w/v) NaCl; there was weak amylase activity on starch plates containing 7·5 % (w/v) NaCl, but not on those with 0, 2 or 4 % (w/v) NaCl; and substrates in Biolog GN were oxidized only when cells were inoculated in 7·5 % (w/v) NaCl. In this respect, it is notable that the salinity in L. Laysan was 7·6 %, as measured by an AGE model 2100 Minisal salinometer calibrated against IAPSO standard (Wormley) sea water. The halophilic and mesophilic nature of LA1^T (growth on MA at 43 °C, but not at 50 °C), compared with its nearest *Psychroflexus* neighbours, confirms the phenotypic diversity, a probable consequence of adaptation, that may occur among phylogenetically close bacteria (Bowman *et al.*, 1998).

LA1^T produced acid from carbohydrates (Table 1) and expressed constitutive lipolytic, proteolytic and saccharolytic enzymes in API ZYM. The dominant fatty acid changed with incubation temperature (Table 2). Monounsaturated fatty acids were a greater fraction of the total fatty acid pool at 15 than 30 °C. Iso-branched acids (i15 : 0, i15 : 1, 3-OH i17 : 0) comprised much of the fatty acids in LA1^T, while

Table 1. Differentiation of *P. tropicus* LA1^T, *P. torquis* ACAM 623^T and *P. gondwanensis* ACAM 48^T on the basis of selected phenotypic characteristics and acid production from carbohydrates

For *P. torquis* ACAM 623^T and *P. gondwanensis* ACAM 48^T, data from Bowman *et al.* (1998). All type strains express alkaline phosphatase, α-glucosidase and β-glucosidase. None express α-galactosidase, β-glucosidase or α-fucosidase, produce acid from cellobiose, glycerol or rhamnose or hydrolyse gelatin. Acid production data for *P. tropicus* determined in API 50 CH. +, Positive reaction; −, negative reaction; V, variable.

Characteristic	*P. tropicus* sp. nov. LA1^T	*P. torquis* ACAM 623^T	*P. gondwanensis* ACAM 48^T
Motility	Gliding	Gliding	Non-motile
NaCl optimum (%, w/v)	7·5–10	3	5
NaCl range (%, w/v)	1–20	1–8	0–15
Temperature range (°C)	4–43	−16–20	−5–30
Hydrolysis of:			
DNA	−	+	+
Gelatin	−	−	−V
Aesculin	−	−	+
Tween 80	−	+	+
Acid from:			
Glucose	−	+	+
Maltose	−	+	+
Arabinose	−	−	+
Xylose	−	−	+
D-Mannose	−	+	+
Fructose	+	−	−
Mannitol	+	−	−
Sorbitol	+	−	−
Arabitol	+	−	−

anteiso-branched fatty acids (e.g. a15:0, a15:1, 3-OH a17:0) dominate in other *Psychroflexus* species (Bowman *et al.*, 1998). The ratio of anteiso- to iso-branched fractions changed with temperature, with a ~3·5-fold increase in this ratio when cells were grown at 15 °C compared to that at 30 °C. The fact that monounsaturated straight-chain fatty acids were essentially lacking is consistent with the description of *Psychroflexus*.

The 16S rRNA gene nucleotide sequence in LA1^T shared 94·4 % identity over 1423 bases with that of *P. torquis* ACAM 623^T. LA1^T fell firmly within the genus *Psychroflexus* in the *Cytophaga–Flexibacter–Bacteroides* (CFB) group of the domain *Bacteria* (Fig. 1). DNA–DNA hybridization revealed only 4 % DNA reassociation between LA1^T and *P. torquis* ACAM 623^T DNA, and 17 % DNA reassociation between LA1^T and *P. gondwanensis* ACAM 48^T DNA. Therefore, LA1^T does not belong to *P. torquis* or to *P. gondwanensis* (Wayne *et al.*, 1987). The G+C content of LA1^T was in the range 32–36 mol% reported for *Psychroflexus* species (Bowman *et al.*, 1998). In light of the phenotypic and genotypic differences between LA1^T and other members of *Psychroflexus*, we propose that LA1^T represents the type strain of a novel species within the genus, *Psychroflexus tropicus* sp. nov.

Description of *Psychroflexus tropicus* sp. nov.

Psychroflexus tropicus [trop′ic.us. L. masc. adj. *tropicus* tropical, of or pertaining to the tropic(s) or solstice, relating to its isolation from a subtropical lake].

Gram-negative, non-motile, straight to slightly curved rods 0·18–0·25 μm wide and 2·0–2·5 μm long. Old cultures in MB produce 'coccoid bodies'. Orange, circular, butyrous, convex, opaque, entire, smooth, glistening colonies of 2–4 mm diameter on MA. Gliding motility. Grows aerobically on MA between 4 and 43 °C, but not at 50 °C, and on 50 % strength MA with 1–20 % (w/v) NaCl. Moderately halophilic, with optimal growth in 50 % strength MB at 30 °C with 7·5–10 % (w/v) NaCl. No growth on MA in a CO_2-enriched atmosphere at 30 °C. Catalase positive, oxidase negative; nitrate reductase expressed in the presence of 7·5 % (w/v) NaCl, but not in the absence of NaCl or with 3·2 % (w/v) NaCl. Other characteristics are listed in Table 1. Alkaline phosphatase, esterase (C_4), esterase lipase (C_8), lipase (C_{14}), leucine, valine and cystine arylamidases, trypsin, chymotrypsin, acid phosphatase and phosphohydrolase activities expressed in API ZYM. L-Alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-leucine, L-ornithine, L-proline, L-serine, L-threonine, mono-methyl succinate and L-alaninamide are oxidized in

Table 2. Fatty acid composition of *P. tropicus* sp. nov. LA1^T, *P. torquis* ACAM 623^T and *P. gondwanensis* ACAM 48^T

Data for *P. torquis* ACAM 623^T and *P. gondwanensis* ACAM 48^T derived from Bowman *et al.* (1998), determined by GC-MS after growth at 15 °C.

Fatty acid	*P. tropicus* sp. nov. LA1^T*	*P. torquis* ACAM 623^T	*P. gondwanensis* ACAM 48^T
Saturated fatty acids:			
15:0	0·6 (3·7)	1·2	1·9
16:0	– (–)	0·6	1·1
Sum saturated fatty acids	0·6 (3·7)	1·8	3·0
Branched-chain fatty acids:			
i13:0	0·5 (–)	0·7	1·4
a13:0	0·6 (–)	–	–
i14:1ω9*c*	– (–)	–	2·8
i14:0	2·7 (4·7)	1·0	4·8
i15:1ω10*c*	11·8† (9·9†)	0·4	2·2
a15:1ω10*c*	12·9† (2·4†)	16·9	18·4
i15:0	16·7 (29·8)	1·1	2·1
a15:0	19·3 (10·2)	35·2	23·0
i16:0	3·4 (3·6)	6·0	10·9
Sum branched-chain fatty acids	66·8 (60·6)	60·3	65·6
Ratio anteiso/iso	0·93 (0·26)	6·4	1·7
Hydroxy fatty acids:			
3-OH i14:0	0·4 (1·0)	–	–
2-OH 15:0	2·8 (1·4)	–	–
3-OH i15:0	2·9 (4·7)	0·3	0·9
3-OH 15:0	– (2·2)	2·5	0·9
3-OH i16:0	10·1 (11·0)	15·4	18·5
3-OH 16:0	0·4 (0·9)	1·2	0·4
3-OH i17:0	10·0 (11·0)	0·2	0·9
3-OH a17:0	– (–)	10·9	6·6
3-OH 17:0	– (1·2)	0·4	0·7
2-OH 17:0	3·9 (–)		
Sum hydroxy fatty acids	30·5 (33·4)	30·9	28·9
Polyunsaturated fatty acids:			
20:4ω6	– (–)	2·1	–
20:5ω3	– (–)	4·9	–
Total	99·0 (97·7)	99·2	97·5

*At 15 °C (30 °C)

†Data for *P. tropicus* are based on MIDI analyses that do not definitively identify these components, but they are common to most *Flavobacteriaceae* and the genus *Psychroflexus*. Only major PLFA components are shown.

Biolog GN in the presence of 7·5 % (w/v) NaCl, but not in the presence of 2 or 4 % (w/v) NaCl. Grows on glycerol, D-glucose, D-fructose, D-mannose, sorbitol, trehalose, starch and D-arabitol in API 50 CH strips. Acid produced from sucrose and 5-ketogluconate. The dominant fatty acids at 15 and at 30 °C are 12-methyl tetradecanoic acid and 13-methyl tetradecanoic acid, respectively. The G+C content is 35±0·8 mol%.

The type strain, LA1^T (=ATCC BAA-734^T=DSM 15496^T), was isolated from water collected at a depth of 0·3 m in hypersaline L. Laysan on Laysan Island in the Northwestern Hawaiian Islands. On the basis of the 16S rDNA sequence, *Psychroflexus torquis* ACAM 623^T from Antarctic sea-ice is the closest described relative (94·4 % sequence identity over 1423 bases). LA1^T and *P. torquis* ACAM 623^T share morphological and nutritional traits, but the fact that LA1^T grows well at temperatures above 20 °C, together with genotypic differences, support the placement of LA1^T as a novel species in *Psychroflexus* as *Psychroflexus tropicus* sp. nov.

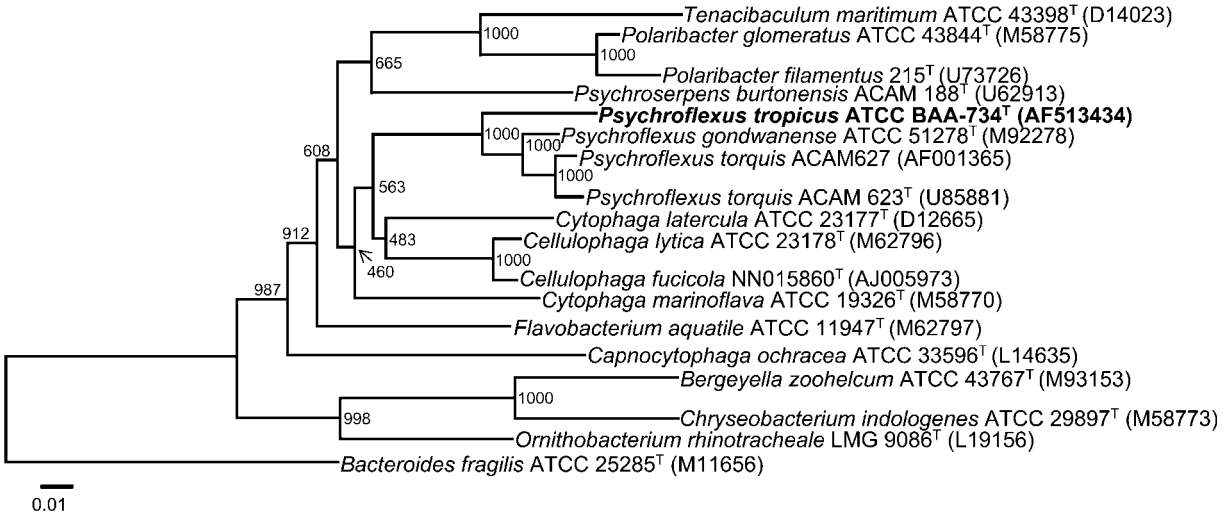

Fig. 1. Phylogenetic tree showing the relationship of *Psychroflexus tropicus* sp. nov. LA1^T (bold) to representatives of the CFB group, on the basis of 1272 nt aligned in CLUSTAL X. Bootstrap values for 1000 replicates are shown. See text for alignment and tree construction details (Page, 1996). *Bacteroides fragilis* (M11656) is the outgroup. Bar, 0·01 nt substitutions per site.

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