Photobacterium frigidiphilum sp. nov., a psychrophilic, lipolytic bacterium isolated from deep-sea sediments of Edison Seamount

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A Gram-negative, motile, rod-shaped, psychrophilic and weakly halophilic bacterial strain, SL13^T, was isolated from deep-sea sediments (1450 m depth) of Edison Seamount in the western Pacific Ocean. Optimal growth of SL13^T requires the presence of 1.5 % (w/v) NaCl, a pH of 6.0 and a temperature of 14 °C. The whole-cell fatty acid profile of the isolate includes C16 : 1 and C16 : 0 as major fatty acids and contains C20 : 5 ω 3. This is consistent with corresponding data for *Photobacterium profundum*. The DNA G + C content of strain SL13^T is 43.8 mol%. Phylogenetic analyses of 16S rRNA gene sequences place this bacterium in the 'Gammaproteobacteria', within the genus *Photobacterium*. Sequence similarity analysis indicates that the closest relatives of strain SL13^T are *Photobacterium indicum* (99.3 %), *P. profundum* (98.5 %) and *Photobacterium lipolyticum* (98.2 %). The DNA–DNA hybridization levels between the isolate and its closest known phylogenetic relatives, *P. indicum*, *P. profundum* and *P. lipolyticum*, are 27.1, 52.4 and 20.2 %, respectively. Thus strain SL13^T represents a novel species of the genus *Photobacterium*, for which the name *Photobacterium frigidiphilum* sp. nov. is proposed. The type strain is SL13^T (=KCTC 12384^T=JCM 12947^T).

In terms of taxonomic identification, historically there has been some confusion between the bacterial genera *Photobacterium* and *Vibrio*. The genus *Photobacterium* comprises a group of Gram-negative, facultatively anaerobic, plump and straight rod-shaped bacteria that require sodium ions for growth. These species can accumulate poly- β -hydroxybutyrate under certain culture conditions, and do not utilize exogenous monomers of β -hydroxybutyrate. Unlike the flagella of *Vibrio* species, which are also common in marine environments, the flagella of all *Photobacterium* species lack an enclosing sheath (Baumann & Baumann, 1984). *Vibrio iliopiscarius* was proposed as a species of the genus *Vibrio* because it has sheathed flagella and because poly- β -hydroxybutyrate was not found in this strain (Onarheim *et al.*, 1994). However, this strain was later reclassified as *Photobacterium iliopiscarium* comb. nov. on the basis of phylogenetic analysis of 16S rRNA gene sequences and restriction fragment length polymorphism

patterns of 16S rRNA gene sequences digested with *Hha*I, which differ from those observed in other species of the genus *Vibrio* (Urakawa *et al.*, 1998, 1999).

Species of the genus *Photobacterium*, which belongs to the family *Vibrionaceae* together with the genera *Vibrio* and *Listonella*, are widespread in marine environments (Baumann & Baumann, 1984; Okuzumi *et al.*, 1994; Shieh *et al.*, 2003). *Photobacterium phosphoreum* was first reported in 1889; at present, there are nine known species. *P. phosphoreum*, *Photobacterium leiognathi* and *Photobacterium profundum* were isolated from sea water, the intestinal contents of marine animals, and deep-sea sediment, respectively (Baumann & Baumann, 1984; Nogi *et al.*, 1998). Typically, marine environments have low temperatures; consequently, psychrophiles, whose optimum growth temperature is lower than 20 °C, are abundant in marine environments. *P. iliopiscarium*, *P. phosphoreum*, *P. profundum* and several other species of the genus *Photobacterium* are psychrophilic. Of these, *P. profundum* was the first to be reported from the deep sea, after being isolated from the sediments of the Ryukyu Trench (5110 m deep; Nogi *et al.*, 1998).

Here, we describe the morphological, phenotypic, phylogenetic and genomic characteristics of strain SL13^T isolated from deep-sea sediment samples. On the basis of this polyphasic evidence, we propose that SL13^T be assigned as a

Published online ahead of print on 14 March 2005 as DOI 10.1099/ijs.0.63338-0.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SL13^T is AY538749.

A table of physiological properties of strain SL13^T and a graph showing growth of the strain under varying barometric pressure are available as supplementary material in IJSEM Online.

novel species of the genus *Photobacterium*, for which the name *Photobacterium frigidiphilum* sp. nov. is proposed.

Sediment samples were collected at Edison Seamount (cold seep area at 1500 m depth) using a video-guided grab sampler (TV-Grab; Preuss). Edison Seamount is positioned south of Lihir Island, Papua New Guinea, in the Western Pacific Ocean. Immediately after sampling, ~0.3 g chilled deep-sea sediment was ground in a mortar and inoculated into 30 ml of autoclaved marine broth 2216 (MB; Difco) containing 1% (v/v) hexadecane in a 100 ml Erlenmeyer flask. After incubation at 10 °C for 7 days on an orbital shaker (JEIO TECH; 120 r.p.m.), 150 µl culture broth was spread onto plates of marine agar 2216 (MA; Difco) supplemented with 1% (v/v) tributyrin. The plates were then incubated at 10 °C for 7 days. Individual colonies with halo zones were isolated from the tributyrin plates. This process was repeated on MA until pure cultures were obtained.

Air-dried smears from MA cultures were stained using the Difco Gram-staining kit, according to the manufacturer's instructions. Cell morphology and flagellum type were examined by transmission electron microscopy (model FDL5000; JEOL). Cells grown for 3 days at 10 °C on MA were negatively stained with 1% (w/v) phosphotungstic acid. After being air-dried, the grids were examined by transmission electron microscopy at 12 000× magnification.

Biochemical tests were performed using API 20NE (bioMérieux) according to the manufacturer's instructions, but with a modified saline solution (containing, l⁻¹ distilled water, 8.8 g MgCl₂·6H₂O, 1.8 g CaCl₂·2H₂O and 20 g NaCl) instead of the API 20NE saline solution for bacterial suspension (Sohn *et al.*, 2004a). The cells' ability to utilize different carbon sources were examined using a Biolog GN2 MicroPlate, for which the bacterial suspensions were prepared as described above. Catalase activity was determined by means of bubble production in a 3% (v/v) hydrogen peroxide solution, and lipase activity was assessed by observing the formation of clear zones around colonies on MA containing 1% (v/v) tributyrin. Growth potential under anaerobic conditions was tested as described by Sohn *et al.* (2004b), and the presence of oxygen was monitored using resazurin as an indicator of redox potential. Luminescence was checked, in darkness, after 2 days incubation at 10 °C in MB. Susceptibility to the vibriostatic agent 2,4-diamino-6,7-diisopropylpteridine (O/129; Sigma) was determined by the disc-diffusion method using 10 and 150 µg O/129 (Donovan & Furniss, 1984).

Growth characteristics were examined using MB as a basal medium. To determine the optimal temperature for growth, the growth rates were calculated from optical density values measured in a temperature-gradient incubator (TVS126MA; Advantec). To determine the optimal pH for growth, MB was dissolved in the following buffers (Sigma) at a concentration of 20 mM: for pH 4, 5 and 5.5, MES buffer; for pH 6 and 6.5, PIPES buffer; for pH 7 and

7.5, HEPES buffer; and for pH 8, 9 and 10, AMPSO buffer (Bae *et al.*, 2005). The requirement for NaCl was tested using modified MB, containing (per litre distilled water) 5 g bactopectone, 1 g yeast extract and 0.01 g FePO₄·4H₂O, supplemented with different NaCl concentrations. The requirements for MgCl₂ and/or CaCl₂ were determined using modified MB containing 2% (w/v) NaCl along with 8.8 g MgCl₂ l⁻¹ and/or 1.8 g CaCl₂ l⁻¹.

Strain SL13^T was Gram-negative, rod-shaped, motile by means of a single polar flagellum and approximately 0.8–1.5 µm in width by 2.3–3.8 µm in length (Fig. 1). Colonies on MA (after incubation for 3 days at 10 °C) were cream-coloured, opaque, smooth, circular, convex with entire margins and 2.0–3.0 mm in diameter. Luminescence was not observed. The isolate was susceptible to O/129. Strain SL13^T grew optimally at 14 °C and growth was possible between 6 and 20 °C (Fig. 2). SL13^T did not grow at temperatures higher than 20 °C. The strain grew at rates greater than half-optimal at 6 °C. It grew well within the pH range 5.0–8.5 and optimally at pH 6.0. Optimal growth for SL13^T occurred in the presence of 1.5% (w/v) NaCl; no growth occurred at NaCl concentrations above 4.0% or in the absence of NaCl. In addition, SL13^T required either MgCl₂ or CaCl₂ for growth. The isolate showed catalase, oxidase, arginine dihydrolase, β-glucosidase, protease and lipase activities, but no urease activity. Nitrate was reduced to nitrite, and indole was produced. As the strain was unable to grow in AUX medium (from the API 20NE test kit), no data were obtained with respect to 12C assimilation. On the basis of Biolog tests, strain SL13^T could oxidize the

Fig. 1. Transmission electron micrograph of a negatively stained cell of strain SL13^T. Bar, 1 µm.

Fig. 2. Temperature-dependent growth of SL13^T.

various carbon compounds listed in the species description. The physiological characteristics of strain SL13^T are listed in a supplementary table available in IJSEM Online. On the basis of those phenotypes, SL13^T can be differentiated from related *Photobacterium* type strains (Table 1).

To determine the response of cell growth under pressure, the OD₆₆₀ was measured every 2 or 3 h during incubation at

14 °C in MB under pressure (from 0.1 to 50 MPa). Strain SL13^T was found to be slightly piezophilic: the optimum pressure for growth was 10 MPa (see the supplementary figure available in IJSEM Online). However, this strain could be piezosensitive, as there was no growth under high hydrostatic pressure (50 MPa).

The total lipid component of cells incubated at 10 °C for 3 days was extracted using the method of Folch *et al.* (1957). For the analysis of fatty acid composition, total lipids were converted to fatty acid methyl esters by serial addition of 1.5 % (w/v) NaOH and 5 % (v/v) HCl in methanol; both reactions were repeated at 65 °C for 20 min. Fatty acid methyl esters were analysed with a gas chromatograph (5890II; Hewlett Packard) equipped with a flame-ionization detector and a capillary column (non-polar SPB-1; 25 m × 0.25 mm). Bacterial fatty acid methyl ester standards (Supelco) and equivalent chain-length values (Stransky *et al.*, 1992) were used for fatty acid identification. The DNA G+C content (mol%) of the isolate was determined by the melting temperature method, as described by Sohn *et al.* (2004b). Purified chromosomal DNA extracted from *Escherichia coli* K-12 (=KCTC 2443) served as a control.

Table 1. Physiological characteristics that differentiate strain SL13^T from related *Photobacterium* type species

Strains: 1, SL13^T; 2, *P. indicum* NBRC 14233^T (data from Xie & Yokota, 2004); 3, *P. profundum* JCM 10084^T (Nogi *et al.*, 1998); 4, *P. lipolyticum* KCTC 10562^T (Yoon *et al.*, 2005); 5, *Photobacterium angustum* ATCC 25915^T (Nogi *et al.*, 1998); 6, *P. phosphoreum* ATCC 11040^T (Nogi *et al.*, 1998); 7, *Photobacterium damsela* subsp. *damsela* JCM 8968^T (Okuzumi *et al.*, 1994); 8, *P. leiognathi* ATCC 25521^T (Nogi *et al.*, 1998). Symbols: +, positive; –, negative; ND, not determined. All strains were positive for utilization of α-D-glucose. All strains were negative for Gram stain, nitrite reduction to nitrogen and utilization of L-arabinose and α-D-lactose.

Characteristic	1	2	3	4	5	6	7	8
Optimal growth temp. (°C)	14	25	10	25–28	25	18	26	26
Catalase	+	–	+	+	–	+	–	–
Lipase	+	ND	+	–	–*	–*	ND	ND
API 20NE:								
Nitrate reduction	+	+	+	+	–	+	+	+
Indole production	–	+	+	+	–	–	–	–
Acid from glucose	+	ND	–	+	+	+	ND	ND
β-Glucosidase	+	ND	–*	–	+	+	ND	ND
β-Galactosidase	+	ND	+	–	+	+	ND	ND
Protease	+	–	+	–	+	–	–	–
Oxidase	+	–	+	+	–	+	+	+
Biolog GN2 MicroPlate:								
N-Acetyl-D-glucosamine	+	ND	–	ND	+	+	+	+
D-Fructose	+	ND	–	+	+	+	+	+
D-Galactose	+	ND	+	–	+	–	+	+
D-Mannitol	+	ND	+	–	–	–	–	–
Sucrose	+	+	–	+	+	–	–	–
D-Trehalose	+	ND	+	–	–	–	+	–
Turanose	–	ND	–	ND	+	–	+	+
Glycerol	+	ND	+	ND	+	–	+	+
DNA G+C content (mol%)	43.8	40.0	42.0	47.0	39.6	39.1	41.8	39.7

*Determined in the present study.

Table 2. Cellular fatty acid compositions of strain SL13^T and related *Photobacterium* species

Strains: 1, SL13^T; 2, *P. indicum* NBRC 14233^T (data from Nogi *et al.*, 1998; Xie & Yokota, 2004); 3, *P. profundum* JCM 10084^T (Nogi *et al.*, 1998; Xie & Yokota, 2004); 4, *P. lipolyticum* KCTC 10562^T (Yoon *et al.*, 2005); 5, *P. phosphoreum* ATCC 11040^T (Nogi *et al.*, 1998; Xie & Yokota, 2004). Values are percentages of total fatty acids.

Fatty acid	1	2	3	4	5
12:0	5	4	2	—	6
13:0	2	—	—	—	—
3-OH 12:0	—	2	5	4	9
iso-13:0	—	—	2	—	—
iso-14:0	—	—	4	—	—
14:0	5	7	3	3.8	11
14:1	—	1	3	—	1
3-OH 14:0	—	—	—	—	3
iso-15:0	—	1	2	—	—
15:1	—	—	—	—	—
15:0	—	2	1	—	—
iso-16:0	1	1	15	—	—
16:1	37	41	32	—	40
16:1 ω 7*	—	—	—	51.3	—
16:0	23	26	9	25.9	25
17:1	—	—	—	—	—
iso-17:0	1	1	—	—	—
cyc-17:0	1	—	—	—	—
17:0	—	1	—	—	—
18:1	12	3	9	—	3
18:1 ω 7c	—	—	—	5.9	—
18:0	3	1	1	—	1
20:5 ω 3	6	—	13	—	—

*And/or 2-OH iso-15:0.

The cellular fatty acids ranged in size from C12 to C20 and included saturated, monoenoic and iso-branched components (Table 2). The major fatty acids in the strain were C12:0, C14:0, C16:1, C16:0, C18:1 and C20:5 ω 3, a range similar to that reported for other *Photobacterium* species (Nogi *et al.*, 1998). Of particular note among the fatty acids of SL13^T was 20:5 ω 3, which is usually found in both psychrophilic and piezophilic marine bacteria (DeLong *et al.*, 1997; Nogi *et al.*, 1998). The fatty acid composition of SL13^T is similar to that reported for *P. profundum* JCM 10084^T isolated from the deep sea (Nogi *et al.*, 1998). The DNA G+C content of strain SL13^T was 43.8 mol%, which is within the range (40–44 mol%) for the genus *Photobacterium* (Baumann & Baumann, 1984).

Genomic DNA was extracted from MB-cultured SL13^T using the Wizard genomic DNA purification kit (Promega). The 16S rRNA gene sequence was amplified from genomic DNA by using a PCR with the universal primers 27F and 1522R (Weisburg *et al.*, 1991). The PCR product was

sequenced using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) and the resultant 16S rRNA gene sequences were manually aligned with those from other *Photobacterium* species and related taxa, using known 16S rRNA secondary-structure information. Phylogenetic trees were constructed using neighbour-joining (Saitou & Nei, 1987), Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993) and maximum-parsimony (Fitch, 1972) methods. Evolutionary distance matrices (for the neighbour-joining and Fitch–Margoliash methods) were generated according to the Jukes–Cantor model (Jukes & Cantor, 1969). The trees were rooted using *E. coli* (GenBank accession no. X80725) as an outgroup. The PHYLIP software package (Felsenstein, 1993) was used for all phylogenetic analyses. The resultant unrooted tree topology was evaluated by bootstrap analysis (1000 replicates; Felsenstein, 1985) of the neighbour-joining tree.

The 16S rRNA gene sequence of SL13^T obtained in this study consisted of 1536 nt. In terms of 16S rRNA gene sequence similarity, the closest relatives of strain SL13^T were *Photobacterium indicum* (99.3%), *P. profundum* (98.5%), *Photobacterium lipolyticum* (98.2%), *P. iliopiscarium* (97.4%) and *P. phosphoreum* (97.2%). Lower sequence similarities (<97.0%) were found with all other *Photobacterium* species with validly published names (Fig. 3).

DNA–DNA hybridization was performed according to the method of De Ley *et al.* (1970). The resultant estimates of DNA relatedness between the isolate and the three type strains of the most closely related *Photobacterium* species (*P. indicum*, *P. profundum* and *P. lipolyticum*) were less than 53% (Table 3), which is significantly lower than that accepted as the phylogenetic definition of a species (Wayne *et al.*, 1987).

Description of *Photobacterium frigidiphilum* sp. nov.

Photobacterium frigidiphilum (fri.gi.di.phi'lum. L. adj. *frigidus* cold; N.L. adj. *philus* -a -um from Gr. adj. *philos* friendly to, loving; N.L. neut. adj. *frigidiphilum* cold-loving).

Cells are rod-shaped, 0.8–1.5 μ m in width and 2.3–3.8 μ m in length, motile by means of a polar flagellum and Gram-negative. Colonies are cream-coloured, opaque, smooth, circular, convex, with entire margins, and form on MA at 10 °C after 3 days. Non-luminescent. Susceptible to O/129. Facultatively anaerobic. Psychrophilic. Growth occurs between 6 and 20 °C, with an optimum at 14 °C. The pH range for growth is 5.0–8.5, with an optimum at pH 6.0. Has an absolute requirement for NaCl for growth; growth occurs at NaCl concentrations of 1.0–3.5% (w/v) and is optimal at 1.5% (w/v). Catalase, oxidase, arginine dihydrolase, β -glucosidase, protease, β -galactosidase and lipase are present. Reduction of nitrate to nitrite occurs, and indoles are produced. Does not utilize β -hydroxybutyrate. The following carbon sources are utilized: dextrin, glycogen, Tweens 40 and 80, D-fructose, N-acetyl-D-glucosamine,

Fig. 3. Neighbour-joining tree showing the phylogenetic positions of strain SL13^T and related taxa based on 16S rRNA gene sequences. Numbers at nodes are levels of bootstrap support (%) based on neighbour-joining analyses of 1000 resampled data-sets; only values above 70% are given. Filled circles indicate clades that were also supported by the Fitch–Margoliash, maximum-parsimony and maximum-likelihood trees. Bar, 0.01 substitutions per nucleotide position.

N-acetyl-D-galactosamine, D-fructose, D-galactose, α -D-glucose, *myo*-inositol, maltose, D-mannitol, D-mannose, sucrose, D-trehalose, D-glucuronic acid, DL-lactic acid, propionic acid, succinic acid, D-alanine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-serine, inosine, glycerol and D-glucose-6-phosphate. The major fatty acids are oleic acid (C16:1) and palmitic acid (C16:0); the fatty acid profiles contain eicosapentaenoic acid (C20:5 ω 3). The DNA G + C content is 43.8 mol%.

The type strain, SL13^T (=KCTC 12384^T=JCM 12947^T), was isolated from sediments at Edison Seamount (1500 m depth) in the western Pacific Ocean.

Acknowledgements

We thank the Chief Scientist of the German cruise SONNE-166 (2002), Dr P. M. Herzig, Dr S.-M. Lee and the Captain and crew of the RV Sonne. We also thank Professor H. G. Trüper, Dr C. Kato and Dr Y. H. Lee for nomenclatural assistance, the pressure experiment and manuscript correction, respectively. This work was supported by the Marine and Extreme Genome Research Center Program and the Marine Novel Bioactive Development Program, Ministry of Maritime Affairs and Fisheries, the 21C Frontier Microbial Genomics and Applications Center Program, Ministry of Science and Technology

(grant MG 02-0101-001-1-0-0) and in-house project PE91900 of the Korea Ocean Research and Development Institute, Republic of Korea.

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Table 3. Levels of DNA reassociation between SL13^T and related *Photobacterium* type strains

Source of unlabelled DNA	Reassociation (%) with labelled DNA from SL13 ^T
<i>P. indicum</i> NBRC 14233 ^T	27.1
<i>P. profundum</i> JCM 10084 ^T	52.4
<i>P. lipolyticum</i> KCTC 10562 ^T	20.2

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