Photobacterium aplysiae sp. nov., a lipolytic marine bacterium isolated from eggs of the sea hare *Aplysia kurodai*

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A bacterium, named GMD509^T, showing lipolytic activity was isolated from the eggs of the sea hare *Aplysia kurodai* collected at Mogiyeo (depth, 12 m), an uninhabited small island in the South Sea of Korea. The strain is Gram-negative, motile, facultatively anaerobic, mesophilic and weakly halophilic. Optimal growth of strain GMD509^T occurs in the presence of 3·0 % (w/v) NaCl and at pH 8 and 25 °C. The whole-cell fatty acid profile of the isolate includes C16 : 1, C16 : 0 and C18 : 1 as major fatty acids and its DNA G+C content is 45 mol%. Phylogenetic analyses of 16S rRNA gene sequences place this bacterium in the γ-*Proteobacteria*, within the genus *Photobacterium*. The 16S rRNA gene sequence of strain GMD509^T is most similar to those of *Photobacterium frigidiphilum* (97·8 %), *Photobacterium profundum* (97·5 %) and *Photobacterium indicum* (97·4 %). DNA–DNA relatedness levels between the isolate and its closest known phylogenetic relatives, *P. frigidiphilum* and *P. indicum*, are 25·3 and 13·7 %, respectively. Strain GMD509^T therefore represents a novel species, for which the name *Photobacterium aplysiae* sp. nov. is proposed, with the type strain GMD509^T (=KCTC 12383^T=JCM 12948^T).

The genus *Photobacterium*, with *Photobacterium phosphoreum* as the type species, belongs to the γ-subclass of the *Proteobacteria* and was first proposed by Beijerinck (1889). Members of this genus are Gram-negative, straight, plump rods with one to three polar flagella (although some are not motile), and they are facultatively anaerobic and isolated from the marine environment. They cannot utilize the exogenous monomer β-hydroxybutyrate. Presently, the genus includes 11 species with validly published names: *Photobacterium leiognathi* (Boisvert *et al.*, 1967), *P. phosphoreum* (Reichelt & Baumann, 1973), *Photobacterium fischeri* (Beijerinck, 1889; Reichelt & Baumann, 1973), *Photobacterium angustum* (Reichelt *et al.*, 1976), *Photobacterium damselae* subsp. *damselae* (Smith *et al.*, 1991), *Photobacterium damselae* subsp. *piscicida* (Gauthier *et al.*, 1995), *Photobacterium iliopiscarium* (Onarheim *et al.*, 1994), *Photobacterium profundum* (Nogi *et al.*, 1998), *Photobacterium indicum* (Xie & Yokota, 2004), *Photobacterium rosenbergii* (Thompson *et al.*, 2005), *Photobacterium lipolyticum* (Yoon *et al.*, 2005) and *Photobacterium frigidiphilum* (Seo *et al.*, 2005). Among them, *P. profundum* (Nogi *et al.*, 1998) and *P. frigidiphilum* were isolated from deep-sea sediments and *P. damselae* subsp. *damselae*, *P. iliopiscarium* and *P. leiognathi* were isolated from marine animals, i.e. skin ulcers of damselfish (Smith *et al.*, 1991), intestine of herring (Onarheim *et al.*, 1994) and the light organ of teleostean fish (Boisvert *et al.*, 1967), respectively.

In this article, we describe the morphological, phenotypic, phylogenetic and genomic characteristics of strain GMD509^T, isolated from eggs of the sea hare *Aplysia kurodai*. Based on this polyphasic evidence, it is proposed that GMD509^T be assigned as the type strain of the novel species *Photobacterium aplysiae* sp. nov.

Eggs of the sea hare *Aplysia kurodai* were collected in August 2003 by diving at Mogiyeo (depth, 12 m; 34° 04′ 35″ N, 127° 15′ 18″ E), an uninhabited small island in the South Sea of Korea. Immediately after sampling, a 1 g sample of egg was ground in a mortar containing 3 ml aged sea water that was autoclaved at 121 °C for 20 min. An aliquot (100 µl) was diluted serially (10^{-1}, 10^{-2} and 10^{-3}) in sterile-filtered (0·2 µm) or autoclaved aged sea water. Diluted sample (100 µl) was spread onto plates of marine 2216E agar (MA; Difco) supplemented with 1 % (v/v) tributyrin (TBN). The

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GMD509^T is AY781193.

A transmission electron micrograph of a negatively stained cell of strain GMD509^T is available as supplementary material in IJSEM Online.

plates were then incubated at 10 °C for 7 days. Individual colonies with a halo were isolated from the TBN plates. The process was repeated on MA until pure cultures were obtained. Strain GMD509^T was selected as a lipase-producing bacterium and studied further.

Gram staining was performed and growth characteristics were determined as described previously (Bae *et al.*, 2005) and growth potential under anaerobic conditions was tested as described by Sohn *et al.* (2004). Cell morphology and flagellum type were observed under a transmission electron microscope at ×12 000, with cells grown for 2 days at 25 °C on MA (Seo *et al.*, 2005). Susceptibility to vibriostatic agent 2,4-diamino-6,7-diisopropylpteridine (O/129; Sigma) was determined by the disc-diffusion method (Seo *et al.*, 2005). The isolate is Gram-negative, rod-shaped, motile by means of a single polar flagellum, facultatively anaerobic, approximately 0·5–0·8 μm in width by 1·0–4·0 μm in length (see Supplementary Figure, available in IJSEM Online) and susceptible to vibriostatic agent O/129. Colonies grown for 2 days at 25 °C on MA are cream-coloured and opaque with a smooth surface, circular form and convex elevation, with entire margins, and 3·0–4·0 mm in diameter. Strain GMD509^T can grow between 10 and 31 °C, at pH 4–9 and between 1 and 5 % NaCl (w/v). Optimal growth occurs at 25 °C, pH 8 and in the presence of 3 % (w/v) NaCl.

Results of the physiological characterization and chemotaxonomic analyses are given in the species description with methods as described previously (Seo *et al.*, 2005), except that the concentration of NaCl was 3 % (w/v) instead of 2 % in the modified saline solution used as the bacterial suspension solution and cells were incubated in marine 2216E broth (MB; Difco) at 25 °C. The major fatty acids of strain GMD509^T are similar to those reported for other *Photobacterium* species (Nogi *et al.*, 1998). The phenotype of strain GMD509^T, including characters for differentiating it from all other all *Photobacterium* species, is indicated in Table 1. The most clear differentiating characters between strain GMD509^T and its closest phylogenetic neighbours, *P. frigidiphilum*, *P. indicum* and *P. profundum*, are indole production and cellobiose utilization, as well as optimal temperature for growth, catalase and oxidase activities and the utilization of *N*-acetyl-D-glucosamine, D-fructose, D-galactose, sucrose and D-mannose.

Table 1. Differential phenotypic characteristics of strain GMD509^T and related *Photobacterium* species

Taxa: 1, GMD509^T; 2, *P. frigidiphilum* KCTC 12384^T [data from Seo *et al.* (2005)]; 3, *P. indicum* NBRC 14233^T [data from the present study and Xie & Yokota (2004)]; 4, *P. profundum* JCM 10084^T [data from Nogi *et al.* (1998) and Seo *et al.* (2005)]; 5, *P. lipolyticum* KCTC 10562BP^T [data from Yoon *et al.* (2005)]; 6, *P. angustum* ATCC 25915^T [data from Nogi *et al.* (1998) and Seo *et al.* (2005)]; 7, *P. phosphoreum* ATCC 11040^T [data from Nogi *et al.* (1998) and Seo *et al.* (2005)]; 8, *P. iliopiscarium* ATCC 51760^T [data from Onarheim *et al.* (1994)]; 9, *P. damselae* subsp. *damselae* JCM 8968 [data from Nogi *et al.* (1998)]; 10, *P. damselae* subsp. *piscicida* NCIMB 25918 [data from Gauthier *et al.* (1995)]; 11. *P. leiognathi* ATCC 25521^T [data from Nogi *et al.* (1998)]; 12, *P. rosenbergii* LMG 22223^T [data from Thompson *et al.* (2005)]. +, Positive; −, negative; V, variable; W, weak; ND, not determined. All species were positive for utilization of α-D-glucose. All species 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	9	10	11	12
Optimal temperature for growth (°C)	25	14	25	10	25–28	25	18	20	26	25–35	26	20–30
Catalase	+	+	−	+	+	−	+	+	+	+	−	ND
Oxidase	+	+	−	+	+	−	+	ND	+	+	+	+
Lipase	+	+	ND	+	+	−	−	ND	ND	−	ND	+
Nitrate reduction	+	+	+	+	+	−	+	+	+	−	+	+
Indole production	−	+	+	+	+	−	−	−	−	−	−	−
Arginine dihydrolase	+	+	+	+	−	+	+	+	+	ND	+	+
Hydrolysis of gelatin	+	+	−	ND	−	V	−	−	ND	−	−	−
Utilization of:												
N-Acetyl-D-glucosamine	+	+	+	−	ND	+	+	ND	+	+	+	+
D-Fructose	+	+	−	−	+	+	+	+	+	+	+	+
D-Galactose	W	+	−	+	−	+	−	+	+	W	+	+
D-Mannose	+	+	−	+	−	+	+	+	+	−	+	+
D-Trehalose	+	+	+	+	−	−	−	V	+	−	+	+
Cellobiose	+	−	−	−	−	−	−	−	+	−	+	+
Sucrose	+	+	+	−	+	+	−	−	−	W	+	+
Maltose	+	+	+	+	+	+	+	ND	+	−	+	+
DNA G+C content (mol%)	45	44	40	42	47	40	39	38–40	42	ND	40	48

The genomic DNA G+C content was determined by using the HPLC method as described by Mesbah *et al.* (1989). Unmethylated lambda phage DNA (Sigma) was used as the calibration reference. The DNA G+C content of strain GMD509^T was 45 mol%, which is within the accepted range for the genus *Photobacterium* (38–48 mol%).

The 16S rRNA gene was analysed as described previously (Seo *et al.*, 2005). The 16S rRNA gene sequence (1504 bp) of strain GMD509^T was aligned manually with representative sequences of the genus *Photobacterium* and related taxa by using known 16S rRNA secondary-structure information. Phylogenetic trees were inferred by the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1993) and maximum-parsimony (Fitch, 1971) methods. Evolutionary-distance matrices (for the neighbour-joining method) were generated according to the model of Jukes & Cantor (1969). The trees were rooted by using *Escherichia coli* (GenBank accession no. X80725) as an outgroup. The PHYLIP package (Felsenstein, 1993) was used for all analyses. The resultant unrooted tree topology was evaluated by bootstrap analyses (1000 replicates; Felsenstein, 1985) using the neighbour-joining method.

Sequence-similarity analysis indicated that the closest relatives of strain GMD509^T were *P. frigidiphilum* (97·8%), *P. profundum* (97·5%) and *P. indicum* (97·4%) (Fig. 1). Sequence similarities to all other species included in the phylogenetic analyses were $<97·0$%.

DNA–DNA hybridization was performed according to the method of Ezaki *et al.* (1989). The resultant estimates of DNA relatedness between the isolate and the type strains of the closely related species *P. frigidiphilum* and *P. indicum* were 25·3 and 13·7%, respectively, which is significantly lower than that accepted as the genotypic definition of a species (Wayne *et al.*, 1987).

Description of *Photobacterium aplysiae* sp. nov.

Photobacterium aplysiae (ap.ly′si.ae. N.L. gen. fem. n. *aplysiae* of *Aplysia*, a zoological genus, referring to the isolation of the type strain from a sea hare, genus *Aplysia*).

Cells are rod-shaped, 0·5–0·8 μm in width and 1·0–4·0 μm in length, motile by means of a polar flagellum and Gram-negative. Cream-coloured, opaque, smooth, circular, convex colonies with entire margins are formed after 2 days on MA at 25 °C. Facultatively anaerobic. Susceptible to vibriostatic agent O/129. Mesophilic. Growth occurs between 10 and 31 °C, with an optimum at 25 °C. The pH range for growth is 4–9, with an optimum at pH 8. Obligate requirement for NaCl for growth; growth occurs at concentrations of 1–5% (w/v) and is optimal at 3% (w/v). Catalase, oxidase, arginine dihydrolase, β-galactosidase, α-glucosidase, alkaline phosphatase, esterase/lipase, lipase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities and hydrolysis of gelatin are positive. Reduction of nitrate to nitrite occurs and acid is produced from glucose. Does not utilize β-hydroxybutyrate. The following carbon sources are utilized: dextrin, glycogen, *N*-acetyl-D-glucosamine, cellobiose, D-fructose, maltose, D-mannitol, D-mannose, sucrose, D-trehalose, DL-lactic acid, L-alanine, methyl pyruvate, glycyl L-aspartic acid, L-serine, L-threonine, L-alanyl glycine, glutamic acid, inosine, uridine, glycerol and glucose 6-phosphate. Tweens 40 and 80, D-galactose, D-psicose, D-gluconic acid, α-ketoglutaric acid, succinic acid, glycyl L-glutamic acid and asparagine are utilized weakly. The major fatty acids are palmitoleic acid (C16 : 1), palmitic acid (C16 : 0) and oleic acid (C18 : 1). The DNA G+C content is 45 mol%.

The type strain, GMD509^T (=KCTC 12383^T=JCM 12948^T), was isolated from the eggs of a sea hare (*Aplysia kurodai*) collected in August 2003 at Mogiyeo (depth, 12 m), an uninhabited small island in the South Sea of Korea.

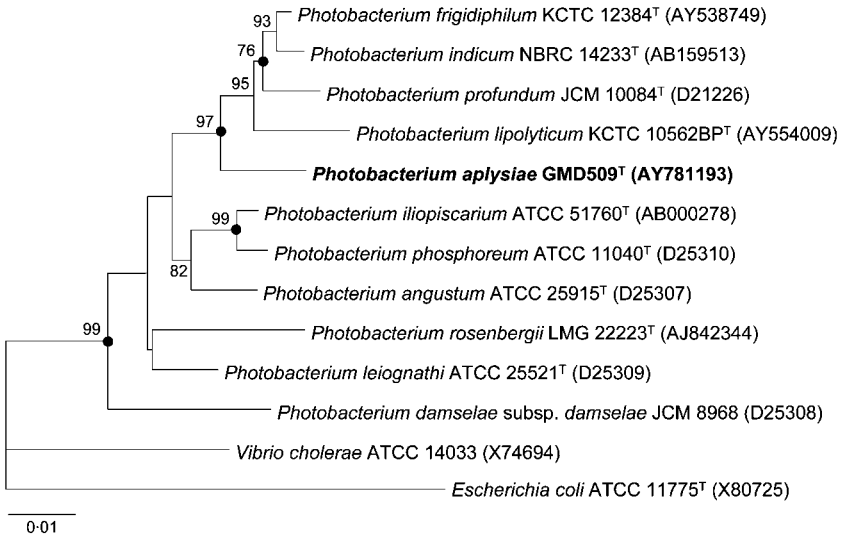

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strain GMD509^T and related taxa, based on 16S rRNA gene sequences. Numbers at nodes represent the levels of bootstrap support based on neighbour-joining analyses of 1000 resampled datasets; only values >70% are given. Solid circles represent clades that were also recovered in the maximum-parsimony and maximum-likelihood trees. Bar, 0·01 substitutions per site.

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