Photobacterium lipolyticum sp. nov., a bacterium with lipolytic activity isolated from the Yellow Sea in Korea

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A Gram-negative, motile, non-spore-forming, pleomorphic and lipolytic bacterial strain, M37^T, was isolated from an intertidal sediment of the Yellow Sea in Korea. This organism grew optimally at 25–28 °C and in the presence of 1–2 % NaCl. It did not grow without NaCl or in the presence of more than 6 % NaCl. Strain M37^T was characterized chemotaxonomically by having Q-8 as the predominant respiratory lipoquinone and C_{16:1}ω7c and/or iso-C_{15:0} 2-OH and C_{16:0} as the major fatty acids. The DNA G+C content was 47 mol%. Phylogenetic analyses based on 16S rRNA gene sequences placed strain M37^T within the clade comprising *Photobacterium* species, forming a coherent cluster with the type strains of *Photobacterium profundum* and *Photobacterium indicum* (16S rRNA gene similarity levels of 97.5–98.0 %). The mean DNA–DNA relatedness levels between strain M37^T and *P. profundum* JCM 10084^T and *P. indicum* DSM 5151^T were in the range 12–15 %. Similarities between 16S rRNA gene sequences of strain M37^T and those of the type strains of the other *Photobacterium* species ranged from 93.9 % (with *Photobacterium fischeri*) to 96.2 % (with *Photobacterium phosphoreum*). On the basis of phenotypic properties and phylogenetic and genomic distinctiveness, strain M37^T (=KCTC 10562BP^T = DSM 16190^T) should be placed in the genus *Photobacterium* as a novel species, for which the name *Photobacterium lipolyticum* sp. nov. is proposed.

The genus *Photobacterium* was one of the earliest known bacterial taxa and was first proposed by Beijerinck (1889); at present, the genus comprises eight species with validly published names: *Photobacterium phosphoreum* (Reichelt & Baumann, 1973), *Photobacterium leiognathi* (Boisvert *et al.*, 1967), *Photobacterium angustum* (Reichelt *et al.*, 1976), *Photobacterium damsela* (Smith *et al.*, 1991), *Photobacterium iliopiscarium* (Onarheim *et al.*, 1994; Urakawa *et al.*, 1999) and *Photobacterium profundum* (Nogi *et al.*, 1998); *Photobacterium fischeri* (Beijerinck, 1889; Reichelt & Baumann, 1973) and *Vibrio fischeri* (Beijerinck, 1889; Lehmann & Neumann, 1896) have the same type strain and are therefore homotypic synonyms. Recently, a further species, *Photobacterium indicum*, was described through reclassification of *Hyphomicrobium indicum* (Xie & Yokota, 2004). Phylogenetic analyses based on 16S rRNA gene sequences have shown that the genus *Photobacterium* falls within the γ -subclass of the *Proteobacteria* and, in particular,

is closely related to the genus *Vibrio* (Nogi *et al.*, 1998; Anzai *et al.*, 2000). The genus *Photobacterium* is characterized chemotaxonomically by having Q-8 as the predominant respiratory lipoquinone and C_{16:1} and C_{16:0} as the major fatty acids (Nogi *et al.*, 1998).

In this study, we describe a slightly halophilic bacterial strain, M37^T, with lipolytic activity, isolated from an intertidal sediment collected from the Yellow Sea of Korea. This organism was considered to be phylogenetically related to the *Photobacterium* and *Vibrio* group on the basis of 16S rRNA gene sequence comparison. Accordingly, the aim of the present work was to establish the exact taxonomic position of strain M37^T by means of a polyphasic approach that combined phenotypic characterization, detailed phylogenetic analysis and genetic analysis.

Strain M37^T was isolated from intertidal sediment collected in Kaehwa-do of the Yellow Sea on TCN-LB agar medium at 25 °C. TCN-LB medium for screening of lipase-producing bacterial strains was prepared as follows: tricaprilyn was emulsified in 20 mM NaCl, 1 mM CaCl₂ and 0.5 % (w/v) gum arabic, using a Waring blender. The tricaprilyn emulsion (50 ml) was mixed with 450 ml LB agar [1 % (w/v)

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tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl and 1.5 % (w/v) agar containing 3 % (w/v) sea salts (Sigma)]. Strain M37^T, which showed an excellent ability to form a halo on the TCN-LB agar medium, indicating lipase activity, was selected and used for further study. The characterization of the lipase produced by strain M37^T is described elsewhere. Cell morphology was examined by using light microscopy (Nikon E600) and transmission electron microscopy. The flagellum type was determined by using transmission electron microscopy with cells from exponentially growing cultures. The Gram reaction was determined using the bioMérieux Gram Stain kit according to the manufacturer's instructions. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber, using marine agar 2216 (MA; Difco) and MA, supplemented with nitrate, that had been prepared anaerobically. Growth in the absence of NaCl was investigated in trypticase soy broth without NaCl. Growth at various NaCl concentrations (0.5–15 %, w/v) was investigated in marine broth 2216 (MB; Difco) or trypticase soy broth. Growth at various temperatures (4–40 °C) was measured on MA. Catalase activity was determined by bubble production in a 3 % (v/v) hydrogen peroxide solution. Oxidase activity was determined by oxidation of 1 % *p*-aminodimethylaniline oxalate as described by Cowan & Steel (1965). Hydrolysis of Tweens 20, 40, 60 and 80 was determined as described by Cowan & Steel (1965). Hydrolysis of casein, starch, hypoxanthine, tyrosine and xanthine was examined on MA plates with the substrate concentrations described previously (Cowan & Steel, 1965). Hydrolysis of aesculin and gelatin and nitrate reduction were determined as described by Lanyi (1987), with the modification that artificial sea water was used. The artificial sea water contained the following (l⁻¹ distilled water): 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O and 1.3 g CaCl₂·2H₂O (Bruns *et al.*, 2001). H₂S production was tested as described previously (Bruns *et al.*, 2001). The API ZYM system (bioMérieux) was used to determine enzyme activity. Acid production from carbohydrates was determined as described by Leifson (1963). Utilization of substrates as sole carbon and energy sources was tested as described by Yurkov *et al.* (1994). The API 20E and API 20NE systems (bioMérieux) were used to examine other physiological and biochemical properties.

Cell biomass of strain M37^T for respiratory lipoquinone analysis and for DNA extraction was obtained from cultivation in MB at 25 °C. Isoprenoid quinones were extracted and analysed as described by Komagata & Suzuki (1987) using reverse-phase HPLC. Chromosomal DNA was isolated and purified according to the method described previously (Yoon *et al.*, 1996), with the exception that ribonuclease T1 was used together with ribonuclease A. For fatty acid methyl ester analysis, a loop of cell mass of strain M37^T was obtained from agar plates after cultivation for 2 days at 25 °C on MA. The fatty acid methyl esters were extracted and prepared according to the standard

protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984), with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reverse-phase HPLC.

The 16S rRNA gene was amplified by PCR using two universal primers as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analyses were performed as described by Yoon *et al.* (2003). DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained in each sample were excluded and the means of the remaining three values are quoted as DNA relatedness values.

Morphological, cultural, physiological and biochemical characteristics of strain M37^T are shown in Table 1 or are given in the species description (see below). Strain M37^T had an unsaturated ubiquinone with eight isoprene units (Q-8), at a peak area ratio of approx. 86 %, as the predominant respiratory lipoquinone, which is in agreement with previous findings that *Photobacterium* species contain Q-8 as the predominant respiratory lipoquinone (Nogi *et al.*, 1998). Strain M37^T had a cellular fatty acid profile containing large amounts of straight-chain and unsaturated fatty acids: the major components were C_{16:1}ω7c and/or iso-C_{15:0} 2-OH (51.3 %), C_{16:0} (25.9 %) and C_{18:1}ω7c (5.9 %), C_{12:0} 3-OH (4.0 %) and C_{14:0} (3.8 %). This fatty acid profile was similar to those of *Photobacterium* species (Nogi *et al.*, 1998). The DNA G + C content of strain M37^T was 47 mol%, which is higher than those of other *Photobacterium* species (Table 1).

An almost complete 16S rRNA gene sequence of strain M37^T comprising 1498 nt (equivalent to approx. 96 % of the *Escherichia coli* 16S rRNA sequence) was determined directly after PCR amplification. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain M37^T fell within the radiation of the cluster comprising *Photobacterium* species. In the phylogenetic tree based on the neighbour-joining algorithm, strain M37^T formed a coherent cluster with *P. profundum* and *P. indicum* at a high bootstrap confidence level (Fig. 1). Strain M37^T exhibited 16S rRNA gene similarity levels of 97.5–98.0 % with respect to *P. indicum* NBRC 14233^T and *P. profundum* JCM 10084^T, and 93.9–96.2 % with respect to the type strains of the other *Photobacterium* species (Fig. 1). Sequence similarities to all other species included in the phylogenetic analysis were lower than 94.9 % (Fig. 1). Mean DNA–DNA relatedness levels between strain M37^T and *P. profundum* JCM 10084^T and between strain M37^T and *P. indicum* DSM 5151^T were 12 and 15 %, respectively, when their DNAs were used individually as labelled DNA probes for cross-hybridization. These DNA relatedness values indicate that strain M37^T is a member of a genomic species that is distinct from *P. profundum* and *P. indicum* (Wayne *et al.*, 1987).

Table 1. Differential phenotypic characteristics of *Photobacterium* species

Species: 1, *P. angustum* (data from Baumann & Baumann, 1984; Nogi *et al.*, 1998); 2, *P. damsela* (Smith *et al.*, 1991; Gauthier *et al.*, 1995; Nogi *et al.*, 1998); 3, *P. iliopiscarium* (Onarheim *et al.*, 1994); 4, *P. leiognathi* (Baumann & Baumann, 1984; Nogi *et al.*, 1998); 5, *P. phosphoreum* (Reichelt & Baumann, 1973; Baumann & Baumann, 1984; Nogi *et al.*, 1998); 6, *P. profundum* (Nogi *et al.*, 1998); 7, *P. indicum* (Xie & Yokota, 2004); 8, *P. lipolyticum* sp. nov. +, Positive; –, negative; v, variable; ND, not determined. Data in parentheses are for type strains. All taxa are positive for utilization of D-glucose. All taxa are negative for Gram stain, spore formation, reduction of nitrite to nitrogen gas, ornithine decarboxylase and utilization of L-arabinose and lactose.

Characteristic	1	2*	3	4	5	6	7	8
Optimum temperature for growth (°C)	(25)	22–26	20	(26)	(18)	10–15	25	25–28
Growth at:								
4 °C	v	–	+	–	+	+	+	+
35 °C	+	+	–	+	–	–	–	–
Motility	+	v	+	+	v (+)	+	+	+
Catalase	(–)	+	+	(–)	(+)	+	–	+
Oxidase	(–)	+	ND	(+)	(+)	+	–	+
Indole production	(–)	(–)	–	(–)	(–)	+	+	+
H ₂ S production	(–)	(–)	–	(–)	(–)	–	+	–
Reduction of nitrate to nitrite	v (–)	v	+	+	+	+	+	+
Arginine dihydrolase	(+)	+	+	(+)	(+)	+	ND	–
Lysine decarboxylase	(–)	(–)	+	(–)	(–)	–	–	–
Hydrolysis of gelatin	v	–	–	–	–	ND	–	–
Utilization of:								
D-Fructose	+	(+)	+	+	+	v (–)	ND	+
D-Galactose	+	+	+	+	v (–)	+	ND	–
D-Cellobiose	–	+	–	–	–	v (–)	ND	–
D-Mannose	+	+	+	+	+	+	ND	–
D-Trehalose	v (–)	(–)	v	–	–	+	ND	–
Sucrose	v (+)	(–)	–	–	–	–	+	+
Maltose	v (+)	(+)	ND	v (+)	+	+	+	+
Acetate	+	ND	ND	+	–	ND	ND	+
Pyruvate	+	+	ND	+	–	ND	ND	+
DNA G+C content (mol%)	40–42	(42)	38–40	40–44	39–42	42	40	47

*Data in parentheses are for the type strain of *P. damsela* subsp. *damsela*.

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of *P. lipolyticum* sp. nov. M37^T and representatives of some other related taxa on the basis of 16S rRNA gene sequences. Scale bar represents 0.01 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branch points.

Chemotaxonomic analysis supports the monothetic phylogenetic classification of strain M37^T as a member of the genus *Photobacterium*. The predominant respiratory lipoquinone and the cellular fatty acid profile of strain M37^T were most similar to those of *Photobacterium* species (Nogi *et al.*, 1998). The phylogenetic distinctiveness and DNA–DNA relatedness data show that strain M37^T is distinct from previously described *Photobacterium* species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Physiological and biochemical characteristics that differentiate strain M37^T from existing *Photobacterium* species are given in Table 1. Therefore, on the basis of the data presented, there is sufficient evidence to classify strain M37^T in the genus *Photobacterium* as a novel species, for which the name *Photobacterium lipolyticum* sp. nov. is proposed.

Description of *Photobacterium lipolyticum* sp. nov.

Photobacterium lipolyticum (li.po.ly'ti.cum. Gr. n. *lipos* fat; Gr. adj. *lytikos* dissolving; N.L. neut. adj. *lipolyticum* dissolving fat or lipid).

Cells are pleomorphic. Motile by means of a single polar flagellum. Colonies on MA are circular, smooth, glistening, low-convex, cream-coloured and 1.5–2.0 mm in diameter after 2 days incubation at 25 °C. Optimal growth occurs between 25 and 28 °C; growth occurs at 4 and 34 °C but not at 35 °C. Optimal growth pH is between 7.0 and 8.0; growth is observed at pH 5.0 but not at pH 4.5. Optimal growth occurs in the presence of 1–2 % NaCl; no growth occurs without NaCl or in the presence of more than 6 % NaCl. Growth occurs under anaerobic conditions on MA and MA with nitrate. Aesculin, starch and Tweens 20, 40, 60 and 80 are hydrolysed. Casein is weakly hydrolysed. Hypoxanthine, tyrosine, urea and xanthine are not hydrolysed. When assayed with the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase are present, but lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase are absent. Malate and succinate are utilized for growth. D-Xylose, benzoate, butyrate, citrate, glutamate, lactate, formate, ethanol and methanol are not utilized for growth. Acid is produced from D-cellobiose, D-glucose, D-fructose, maltose, D-mannose, D-ribose, sucrose and D-trehalose. Acid is not produced from adonitol, myo-inositol, D-mannitol, D-sorbitol, L-arabinose, D-galactose, lactose, D-melezitose, melibiose, D-raffinose or L-rhamnose. The predominant respiratory lipoquinone is Q-8. The major fatty acids are C_{16:1 ω 7c} and/or iso-C_{15:0} 2-OH and C_{16:0}. The DNA G+C content is 47 mol% (HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, M37^T (= KCTC 10562BP^T = DSM 16190^T), was isolated from an intertidal sediment from the Yellow Sea of Korea.

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