Photobacterium swingsii sp. nov., isolated from marine organisms

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Six Gram-negative coccobacilli were isolated from Pacific oysters (*Crassostrea gigas*) from Mexico and haemolymph of spider crabs (*Maja brachydactyla*) from Spain. All of the isolates grew as small green colonies on thiosulphate-citrate-bile salts-sucrose (TCBS) agar and were facultatively anaerobic, oxidase-positive and sensitive to the vibriostatic agent O/129. Repetitive palindromic PCR analysis revealed a high degree of genomic homogeneity among the isolates. Several phenotypic traits differentiated the isolates from the type strains of species of the genus *Photobacterium*. DNA–DNA relatedness between two representative isolates and their closest phylogenetic neighbours by 16S rRNA gene sequence similarity, *Photobacterium aplysiae* CAIM 14^T and *Photobacterium frigidiphilum* CAIM 20^T, was 44.01–53.85%. We propose a novel species of the genus *Photobacterium* to accommodate the six isolates, with the name *Photobacterium swingsii* sp. nov. The type strain is CAIM 1393^T (=CECT 7576^T).

Photobacterium is one of the oldest established genera in the family *Vibrionaceae* of the class *Gammaproteobacteria*. The type species is *Photobacterium phosphoreum* Beijerinck 1889, which had been described by Ferdinand Cohn in 1878 as '*Micrococcus phosphoreus*'. At the time of writing, the genus *Photobacterium* contained 17 species (<http://www.vibriobiology.net>). The genus has a worldwide distribution and most species include strains that exhibit luminescence and that are able to grow not only with general media but also with selective media, such as thiosulphate-citrate-bile salts-sucrose (TCBS), which is widely used for vibrio isolation. Some strains (*Photobacterium damsela* subsp. *piscicida*) are pathogenic for aquatic animals, but most strains have no described pathogenic traits and are common inhabitants of marine ecosystems and marine animals.

During a survey of vibrios in cultured oysters in Mexico and haemolymph of wild spider crabs in Spain, genomic characterization by repetitive palindromic PCR (rep-PCR) revealed that six isolates were closely related and formed a cluster that was separate from all of the other isolates analysed. These six isolates were cultured from homogenates of the Pacific oyster (*Crassostrea gigas*), collected from three different localities in Mexico and the haemolymph of spider crabs (*Maja brachydactyla*), collected from the wild in the Canary Islands (Table 1). Another two strains were isolated from corals in Brazil but died before they could be characterized and only the 16S rRNA gene sequence of one (R13) was obtained. The isolates grow on TCBS agar as small, green, round colonies with smooth borders, 2–3 mm in diameter, and were non-luminescent. On marine agar after 24 h incubation, colonies were white and smooth, 2–3 mm in diameter, and were non-luminescent. Strains CAIM 1119 and CAIM 1206 showed weak agarolytic activity on marine agar.

Phenotypic characterization of the isolates used standard methods as described elsewhere (Macián *et al.*, 2001) and the API 20E and API ZYM systems (bioMérieux), using

Abbreviation: rep-PCR, repetitive palindromic PCR.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CAIM 1439, CAIM 1206, CAIM 1119, CAIM 1394, CAIM 1395 and CAIM 1393^T are GQ386816–GQ386818 and GQ386820–GQ386822, respectively.

Table 1. Origins of strains of *Photobacterium swingsii* sp. nov.

Strain	Source	Locality	Isolation date	Coordinates
CAIM 1439	Pacific oyster homogenate	Manuela lagoon, Mulegé, Baja California, Mexico	7 November 2003	28° 01' 23" N 114° 06' 22" W
CAIM 1119	Pacific oyster homogenate	El Cardón estuary, Baja California Sur, Mexico	7 November 2003	26° 47' 36" N 108° 50' 53" W
CAIM 1206	Pacific oyster homogenate	La Cruz, Sinaloa, Mexico	13 February 2004	24° 05' 44" N 108° 50' 46" W
CAIM 1393 ^T , CAIM 1394, CAIM 1395	Spider crab haemolymph	Taliarte Bay, Gran Canaria, Canary Islands, Spain	30 November 2006	27° 59' N 15° 22' W

cultures prepared on TSA plus 2.0% NaCl and cell suspensions prepared in 2.5% sterile saline solution to a density that equalled 0.5 MacFarland standard. Tests included temperature and NaCl required for growth, several biochemical characteristics and the use of 49 substrates as sole carbon and energy sources. The results of the phenotypic characterization are presented in the species description and Tables 2 and 3. Several phenotypic characteristics differentiated the isolates from the type strains of closely related species of the genus *Photobacterium* and some differed among the isolates.

The 16S rRNA gene was amplified using universal primers (V16S-9F, 5'-AGAGTTTGATCATGGCTCAG-3'; V16S-1491R, 5'-AGCGCTACCTTGTTACGACTT-3') and a thermal cycling program of 94 °C for 2 min, 35 cycles at 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min and finally 72 °C for 5 min. Purification and sequencing of the amplification products was done at Macrogen (Korea). The sequences of the isolates were analysed with those of type strains of the genus *Photobacterium* available in public databases, using the consensus when more than one sequence of a strain was available. Phylogenetic analysis was conducted in MEGA4 (Kumar *et al.*, 2001) using evolutionary distances computed using the maximum-composite-likelihood method (Tamura *et al.*, 2004) and the neighbour-joining method (Saitou & Nei, 1987) to infer evolutionary history. Alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). Bootstrap calculations (Felsenstein, 1985) were based on 1000 replicates. The Jukes-Cantor correction was used in the calculation of 16S rRNA gene sequence similarity. An almost-complete sequence (>1450 bp) of the 16S rRNA gene was obtained for each of the isolates. 16S rRNA gene sequence similarity among the isolates was ≥98.6%. Phylogenetic analysis showed that the isolates belonged to the genus *Photobacterium*. The optimal tree with the sum of branch length=0.1766 is shown in Fig. 1. The isolates exhibited 96.8–95.6% 16S rRNA gene sequence similarity to *Photobacterium frigidiphilum* SL13^T and 96.7–95.5% similarity to *Photobacterium aplysiae* GMD509^T.

Genomic characterization of the isolates and the reference strains *P. frigidiphilum* CAIM 20^T, *P. aplysiae* CAIM 14^T and *P. phosphoreum* CAIM 328^T was performed using

rep-PCR fingerprinting, using methods described previously (Cabanillas-Beltran *et al.*, 2006). Briefly, DNA was extracted using a Wizard genomic DNA purification kit (Promega) according to the manufacturer's instructions. The DNA was adjusted to a concentration of 50 ng µl⁻¹.

Table 2. Phenotypic differences between *Photobacterium swingsii* sp. nov. and some of its closest phylogenetic neighbours

Strains: 1, *Photobacterium swingsii* sp. nov. (n=6); 2, *P. frigidiphilum* CAIM 20^T; 3, *P. aplysiae* CAIM 14^T (data from this study and Seo *et al.*, 2005); 4, *P. profundum* CAIM 1796^T (this study and Nogi *et al.*, 1998); 5, *P. lipolyticum* CAIM 15^T (this study and Yoon *et al.*, 2005); 6, *P. phosphoreum* CAIM 328^T; 7, *P. iliopiscarium* CAIM 909^T. Data were obtained in this study unless indicated. +, Positive; (+), most strains positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7
Arginine dihydrolase	+	–	+	+	–	+	+
Lysine decarboxylase	–	+	–	–	–	–	–
Citrate utilization	–	+	–	ND	ND	–	+
Indole production	–	–	–	+	+	–	–
Gelatin hydrolysis	(+)	+	+	ND	–	–	–
Growth with/at:							
6% NaCl	+	+	–	+	–	–	+
4 °C	(+)	–	–	+	+	+	+
30 °C	+	+	+	–	+	+	+
Utilization of:							
L-Arabinose	+	+	–	–	–	+	+
Cellobiose	+	+	+	–	–	+	+
D-Fructose	+	+	+	–	+	–	+
D-Galactose	+	–	+	+	–	–	–
Maltose	+	+	+	+	+	–	+
D-Mannose	+	+	+	+	–	–	+
Sucrose	–	+	+	–	+	+	+
Trehalose	+	+	+	+	–	–	+
L-Alanine	+	+	+	+	–	–	+
L-Aspartate	+	+	+	+	–	–	+
L-Glutamate	+	+	+	+	–	–	+
L-Threonine	+	+	+	+	–	–	–
D-Glucuronate	–	+	+	ND	+	–	+
α-Ketoglutarate	+	+	+	+	+	–	+
Propionate	+	+	+	+	–	–	+

Table 3. Phenotypic variability of strains of *Photobacterium swingsii* sp. nov.

Strains: 1, CAIM 1393^T; 2, CAIM 1394; 3, CAIM 1395; 4, CAIM 1119; 5, CAIM 1206; 6, CAIM 1439. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5	6
Utilization of:						
DL-Lactate	+	–	–	+	+	–
D-Mannitol	–	–	+	–	–	+
Putrescine	–	–	+	–	–	+
Production of:						
N-Acetyl- β -glucosaminidase	+	+	+	–	+	–
Cystine arylamidase	w	w	w	–	–	w
Valine arylamidase	+	+	+	–	w	+
Growth at 35 °C	+	–	+	–	–	–
ONPG (β -galactosidase) test	+	+	+	–	–	–

and amplified using the GTG₅ primer (5'-GTGGTGG-TGGTGGT-3'). The amplification products were electrophoretically resolved on a 20 × 20 cm 1.2 % agarose gel at 90 V in 1 × TAE buffer at room temperature for 140 min. The resulting bands were analysed using GelCompar II version 4.5 (Applied Maths). A similarity matrix was calculated with the Jaccard coefficient (Kosman & Leonard, 2005) with a band-position tolerance of 0.59 % (0.34 % optimization) and a dendrogram was constructed with the Ward algorithm. The six isolates showed genomic homogeneity and were clearly differentiated from the reference strains (Fig. 2).

The DNA G+C content of selected isolates, strains CAIM 1393^T, CAIM 1439 and CAIM 1394, was determined by quantitative PCR as described by Gonzalez & Saiz-Jimenez (2002) with some modifications. Cells were prepared in

50 ml TSB plus 2.0 % NaCl under constant agitation, pelleted by centrifugation at 4000 r.p.m. at 4 °C for 30 min, washed with 1 × TAE buffer, resuspended gently in 467 μ l 1 × TAE and lysed with 50 μ l 10 % SDS and 4 μ l 20 mg proteinase K ml⁻¹ at 37 °C for 45 min and 65 °C for 15 min. DNA was extracted with 550 μ l phenol/chloroform/isoamyl alcohol (24:24:1; Sigma). After centrifugation (14 000 r.p.m. at room temperature for 5 min), the upper aqueous phase was transferred to a Phase Lock Gel tube (5Prime), washed with 1 vol. phenol/chloroform/isoamyl alcohol, incubated with 4 μ l RNase (Promega) at 37 °C for 30 min and washed with 1 vol. chloroform/isoamyl alcohol. Finally, the DNA was precipitated with 0.1 vol. cold sodium acetate and 0.6 vol. cold 2-propanol and collected by spooling onto a heat-sealed Pasteur pipette. The DNA concentration was measured spectrophotometrically and adjusted to 1 μ g μ l⁻¹. For the quantitative PCR, 6 μ l DNA solution was mixed with 7.5 μ l of 0.1 μ l SYBR Green I (Molecular Probes) ml⁻¹, 7.5 μ l 1 × SSC and 54.0 μ l water (total 75 μ l) and 25 μ l was dispensed in triplicate in wells of a 96-well microtitre plate. An iCycler real-time thermocycler (3.0; Bio-Rad) was used for thermal denaturation with the following protocol: 25 °C for 15 min, 0.2 °C increments for 12 s until 100 °C was reached and a final step of 60 °C for 30 min. Fluorimetric measurements were taken during the temperature increase every 0.2 °C. The melting temperature (T_m) was calculated using the negative first derivative of the temperature versus fluorescence ($-dF/dT$). The percentage of guanine and cytosine was calculated using a formula obtained from the thermal denaturation analysis of six strains with published whole-genome sequences and, therefore, known DNA G+C contents: *Bacillus subtilis* CECT 461 (43.5 mol%), *Lactobacillus lactis* CECT 4433 (35.3 mol%), *Escherichia coli* CECT 433 (50.8 mol%), *Pseudomonas aeruginosa* CECT 4122 (66.6 mol%), *Corynebacterium glutamicum* CECT 77 (53.8 mol%) and *Vibrio*

Fig. 1. Neighbour-joining dendrogram, based on almost-complete (1336 bp) 16S rRNA gene sequences, showing the position of strains of *Photobacterium swingsii* sp. nov. among their closest phylogenetic neighbours. When more than one sequence from a strain was available, the consensus was used. Bootstrap values based on 1000 replicates are shown at branch nodes. A consensus of two sequences from *Vibrio cholerae* ATCC 14035^T was used as an outgroup. Bar, 0.005 substitutions per nucleotide position.

Fig. 2. Genomic comparison of strains of *Photobacterium swingsii* sp. nov., based on rep-PCR analysis.

campbellii ATCC BAA-1116 (45.4 mol%) [this last strain had been originally assigned to *Vibrio harveyi*, but was recently reassigned to *V. campbellii* by Lin *et al.* (2010)]. The formula obtained was $G + C \text{ content (mol\%)} = 2.002 \times T_m - 111.5$ ($R^2 = 0.97$). The DNA G + C contents determined were 46.7 mol% (T_m 79.0 °C) for strain CAIM 1393^T, 47.5 mol% (T_m 79.4 °C) for strain CAIM 1439 and 48.7 mol% (T_m 80.0 °C) for strain CAIM 1394.

For DNA–DNA hybridization, genomic DNA was extracted using a DNeasy Blood & Tissue kit (Qiagen). The hydroxyapatite/microtitre plate method (Ziemke *et al.*, 1998) with a hybridization temperature of 60 °C was used. DNA–DNA relatedness was determined between strains CAIM 1393^T and CAIM 1439 and *P. aplysiae* CAIM 14^T and *P. frigidophilum* CAIM 20^T. Reciprocal reactions were performed and DNA–DNA relatedness was calculated as the mean of at least three replications. DNA–DNA relatedness between strain CAIM 1393^T and *P. aplysiae* CAIM 14^T was 51.4 % (reciprocal value 53.8 %), between strain CAIM 1393^T and *P. frigidophilum* CAIM 20^T was 44.0 % (50.0 %) and between strains CAIM 1393^T and CAIM 1439 was 86.5 % (89.6 %).

On the basis of phenotypic, genotypic and chemotaxonomic characterization, the six isolates belong to a novel *Photobacterium* species, for which the name *Photobacterium swingsii* sp. nov. is proposed.

Description of *Photobacterium swingsii* sp. nov.

Photobacterium swingsii (swing'si.i. N.L. gen. n. *swingsii* of Swings, in honour of the Belgian microbiologist Jean Swings).

The species description is based on data for six isolates. Cells are Gram-negative, small coccobacilli. Facultatively anaerobic. Positive for oxidase, catalase (except strain CAIM 1395), nitrate reduction, gelatin hydrolysis (except strain CAIM 1119), arginine dihydrolase and Voges–Proskauer reaction. Negative for urease, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase (except strain CAIM 1395), citrate and production of

H₂S and indole. Sensitive to the vibriostatic agent O/129 (10 and 150 µg). Grows with 3 and 6 % NaCl, but not with 0 or 10 % NaCl; only strain CAIM 1395 grows with 8 % NaCl. Grows at 4 °C (except strain CAIM 1395), 20 °C and 30 °C, but not at 35 °C (except strains CAIM 1393^T and CAIM 1395) or 40 °C. As sole sources of carbon, utilizes amygdalin (except strain CAIM 1119), L-arabinose, cellobiose, D-fructose, D-galactose, D-glucosamine, D-glucose, maltose, D-mannose, melibiose (except strain CAIM 1395), D-ribose, D-salicin, trehalose, methanol, glycerol, L-alanine, glycine, L-aspartate, L-glutamate, L-glutamine, L-ornithine, L-threonine, acetate, citrate, fumarate, ketoglutarate, pyruvate, propionate and succinate, but not lactose (except strain CAIM 1395), sucrose, D-xylose, *myo*-inositol, D-sorbitol, L-histidine (except strain CAIM 1395), L-leucine (except strain CAIM 1395), L-lysine, L-rhamnose, D-galacturonate, D-gluconate (except strain CAIM 1395), D-glucuronate, malate, *p*-hydroxybenzoate or γ -aminobutyrate (except strain CAIM 1395). Produces acid phosphatase, alkaline phosphatase, esterase lipase (C8), lipase (C14), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and esterase (C4) (weak; except strain CAIM 1119), but not α -chymotrypsin, α -fucosidase, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, α -glucosidase (except strain CAIM 1119; weak) or α -mannosidase. Variable for utilization of DL-lactate, D-mannitol and putrescine, production of *N*-acetyl- β -glucosaminidase, cystine arylamidase and valine arylamidase and ONPG (β -galactosidase) test. The DNA G + C content is 46.7–48.7 mol% (type strain 46.7 mol%).

The type strain, CAIM 1393^T (=CECT 7576^T), was isolated from the haemolymph of wild spider crabs (*Maja brachydactyla*) from the Canary Islands, Spain. Five other strains were isolated from the same source and from homogenates of cultured Pacific oysters (*Crassostrea gigas*) from north-western Mexico (Table 1).

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