Photobacterium aquimaris sp. nov., a luminous marine bacterium isolated from seawater

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Two luminous marine bacteria, strains LC2-065^T and LC2-102, were isolated from seawater at Sagami Bay in Japan. These bacteria were Gram-negative, oxidase-negative, catalase-positive, motile and coccoid-rods. 16S rRNA gene sequence analysis and multilocus sequence analysis (MLSA) using six loci (*ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH* and *topA*) and sequence analysis of the alpha subunit of luciferase (*luxA*) gene revealed that these bacteria were distinct from other species of the genus *Photobacterium*. These novel strains were most closely related to *Photobacterium kishitanii*. The DNA–DNA hybridization value between strain LC2-065^T and *Photobacterium kishitanii* ATCC BAA-1194^T was 42.1 %. The major fatty acids were C_{12:0}, C_{14:0}, C_{16:0}, C_{18:0} and C_{15:0} iso 2-OH and/or C_{16:1}ω7c (summed feature 3). The DNA G+C contents of strains LC2-065^T and LC2-086 were 42.2 and 42.9 mol%, respectively. The phenotypic features of the novel strains were similar to those of *P. kishitanii* and *P. phosphoreum*, but there were sufficient physiological differences for the novel strains to be easily differentiated. On the basis of these results, these new strains represent a novel species, for which the name *Photobacterium aquimaris* sp. nov. is proposed. The type strain is LC2-065^T (=NBRC 104633^T=KCTC 22356^T).

The genus *Photobacterium* was first described by Beijerinck (1889). At present, the genus comprises 19 species and five species in this genus contain luminous strains: *Photobacterium phosphoreum* (Reichelt & Baumann, 1973), *Photobacterium leiognathi* (Boisvert *et al.*, 1967), *Photobacterium angustum* (Reichelt *et al.*, 1976) (Dunlap & Kita-Tsukamoto, 2001), *Photobacterium ganghwense* (Park *et al.*, 2006) and *Photobacterium kishitanii* (Ast *et al.*, 2007). Recent molecular techniques based on multilocus sequence analysis (MLSA) have revealed that some luminous bacteria are the light-organ symbionts of certain fishes (Ast & Dunlap, 2005; Dunlap *et al.*, 2007; Thompson *et al.*, 2007a; Wada *et al.*, 2006).

In this study, two luminescent strains were isolated from seawater and analysed using 16S rRNA, the alpha-subunit

of luciferase (*luxA*), uridylylate kinase (*pyrH*), a cell division protein (*ftsZ*), a rod shaping protein (*mreB*), the DNA gyrase B-subunit (*gyrB*), topoisomerase I (*topA*) and glyceraldehyde 3-phosphate dehydrogenase (*gapA*) gene sequences. Based on the phylogenetic analyses, the novel strains formed a tight cluster in which no recognized type species of the genus *Photobacterium* was grouped. This suggests that the new isolates represent a novel species of the genus *Photobacterium*. Therefore, we conducted a series of analyses to fully identify these isolates. Based on the results of phylogenetic and physiological and analyses, these strains are proposed to represent a novel species.

Strains LC2-065^T and LC2-086 were isolated from seawater samples taken from Sagami Bay (35° 00' N, 139° 20' E) during the KT-05-16 cruise of RV 'Tansei Maru' [Ocean Research Institute, The University of Tokyo, and Japan Agency for Marine-Earth Science and Technology (JAMSTEC)] from 13–21 July 2005. Strains LC2-065^T and LC2-086 were isolated from samples collected at a depth of 50 m, 15.6 °C, and at 1000 m, 3.2 °C, respectively. The seawater samples were filtered through a polycarbonate filter (pore size 0.2 µm; Whatman International Ltd) and then the filter was placed on half strength marine agar 2216E (MA; Difco) and maintained at 20 °C. Luminous colonies that grew on the agar plates were isolated with

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH* and *topA* gene sequences used and determined in this study are given in Supplementary Table S1, available with the online version of this paper.

Supplementary tables detailing the GenBank accession numbers for the 16S rRNA, *ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH* and *topA* gene sequences used in this study and the fatty acid contents of strain LC2-065^T are available with the online version of this paper. Seven additional phylogenetic trees based on analysis of the 16S rRNA (maximum-likelihood), *ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH* and *topA* (neighbour-joining) gene sequences are also available.

sterile toothpicks utilizing a CCD camera and were transferred to fresh MA plates for reisolation. Cell morphology and flagella were observed using atomic force microscopy (SPM-9500 J2, Shimadzu) as previously described by Nishino *et al.*, (2004). The temperature range for growth was determined by incubating the isolates on the half strength MA plates. Growth at different NaCl concentrations, 0.5–10 % (w/v), was determined on tryptone soy agar (Difco) plates. Catalase activity was determined by bubble formation in a 3 % H₂O₂ solution. Oxidase activity was determined by cytochrome oxidase paper (Nissui Pharmaceutical). API 20E and API ZYM strips (bioMérieux) were used to determine physiological and biochemical characteristics. All suspension media for the API test strips were supplemented with 2 % (w/v) NaCl solution (final concentration). API 20E and API ZYM were read after 48 h incubation at 20 °C and 6 h incubation at 20 °C, respectively.

Cellular fatty acid composition (MIDI system) was determined as described previously (Xie & Yokota, 2003). The cells were grown for 48 h at 20 °C on plates of half-strength MA. DNA was prepared according to the method of Marmur (1961) from cells grown on half-strength MA and the DNA base composition was determined by using the HPLC method (Mesbah *et al.*, 1989). DNA–DNA hybridizations were performed with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989). DNA quality and quantity were determined by the measuring absorbance at 260 and 280 nm. Only high quality DNA with an A₂₆₀:A₂₈₀ ratio of 1.8–2.0 was used. The hybridization temperature was set at 41 °C. Hybridization was performed using five replications for each strain. The highest and lowest values obtained were excluded and the mean of the remaining three values was quoted as the final DNA–DNA relatedness value.

A fragment of approximately 1500 bp of the 16S rRNA gene was amplified from the extracted DNA by using bacterial universal primers specific to the 16S rRNA gene (27F and 1492 R) (Lane, 1991). The genes encoding *ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH* and *topA* were used for multilocus sequence analysis (MLSA) (Ast *et al.*, 2007; Thompson *et*

al., 2007b). PCR primers for the six genetic loci and the reaction conditions were according to Sawabe *et al.* (2007). The *luxA* gene was used for phylogenetic analysis of luminous bacteria. PCR primers described by Wimpee *et al.* (1991) were used. To test the evolutionary relationships of the members of the genus *Photobacterium*, phylogenetic analysis was performed with the MEGA 3.1 program (Kumar *et al.*, 2004) and PHYLIP 3.67 software (developed by Dr Joe Felsenstein; (<http://evolution.genetics.washington.edu/phylip.html>)). Multiple alignments of the sequences were performed using CLUSTAL W (version 1.6) (Thompson *et al.*, 1994). Distances were calculated using the Kimura two-parameter model (Kimura, 1980). Clustering based on the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony and maximum-likelihood methods was determined using bootstrap values based on 1000 replications (Felsenstein, 1985). Sequence data for other species of the genus *Photobacterium* were obtained from GenBank.

The 16S rRNA gene sequence identified strains LC2-065^T and LC2-086 as members of the genus *Photobacterium* using the neighbour-joining, maximum-parsimony and maximum-likelihood methods (Fig. 1 and also see Supplementary Fig. S1 in IJSEM Online). The closest phylogenetic neighbours were *P. kishitanii* (99.8 % 16S rRNA gene sequence similarity), *Photobacterium iliopiscarium* (99.8 %) and *Photobacterium phosphoreum* (99.7 %). All phylogenetic trees based on MLSA (*ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH* and *topA*, 3981 bp) using the neighbour-joining, maximum-parsimony and maximum-likelihood methods confirmed the clustering of strains LC2-065^T and LC2-086, with a bootstrap value of 100 %, and their separate position from their closest phylogenetic neighbours *P. kishitanii* (93.2 % similarity), *P. phosphoreum* (91.8 %) and *P. iliopiscarium* (91.2 %) (Fig. 2). The phylogenetic trees constructed using each gene separately are presented in Supplementary Figs S2–S7 in IJSEM Online.

The DNA–DNA hybridization value between strains LC2-065^T and LC2-086 was 78.3 %. The two new strains should therefore be considered to be members of a single species. The DNA–DNA relatedness values between strain

Fig. 1. Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing the relationships between strains LC2-065^T and LC2-086 and members of the genus *Photobacterium*. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resamplings; only values >50 % are shown. *Aliivibrio fischeri* was used as an outgroup. Bar, 0.5 % estimated sequence divergence.

Fig. 2. Phylogenetic tree based on the concatenated gene sequences of the six loci (3981 bp). This tree combines the results of the neighbour-joining, maximum-parsimony and maximum-likelihood treeing methods. Numbers at the nodes denote the level of bootstrap support based on 1000 replicates, NJ/MP/ML. The topology shown was obtained using the neighbour-joining method. *Aliivibrio fischeri* was used as an outgroup. Bar, 2% estimated sequence divergence.

LC2-065^T and related species of the genus *Photobacterium* were 42.1% to *P. kishitanii* ATCC BAA-1194^T, 38.7% to *P. phosphoreum* IAM 14401^T and 38.8% to *P. iliopiscarium* DSM 9896^T. These results demonstrate that strain LC2-065^T is distinct from other species of the genus *Photobacterium*.

Based on the *luxA* gene sequence analysis (611 bp), the novel strains are distinct from other luminous strains of the genus *Photobacterium* (Fig. 3). The *luxA* gene sequence similarities between strain LC2-065^T and related species were 84.9% to *P. leiognathi* ATCC 27561, 70.6% to *P. phosphoreum* ATCC 11040^T, 70.4% to *P. kishitanii* pjapo.1.1^T and 63.7% to *P. leiognathi* ATCC 25521^T. The phylogenetic placement of strains LC2-065^T and LC2-086 based on the *luxA* gene sequences did not match that determined on the basis of the housekeeping genes. The discordance between the housekeeping and *luxA* gene phylogenies supports the hypothesis of horizontal transfer of the *lux* genes (Urbanczyk *et al.*, 2008).

The two new isolates form luminous and non-pigmented translucent colonies on MA. Strain LC2-086 forms green colonies on thiosulfate citrate bile sucrose (TCBS) agar. On the basis of the API 20E and API ZYM tests, the two strains can be discriminated from other species of the genus *Photobacterium*. In contrast to most of their phylogenetic neighbours, these novel strains can produce α -galactosidase and β -galactosidase. The novel strains cannot produce arginine dihydrolase, esterase (C4) or esterase lipase (C8) and cannot utilize glucose or sucrose (Table 1).

The predominant cellular fatty acids of strain LC2-065^T were C_{15:0} iso 2-OH and/or C_{16:1} ω 7c (summed feature 3; 36.5%), C_{16:0} (18.7%), C_{18:0} (10.8%), C_{14:0} (8.6%) and C_{12:0} (5.9%). The fatty acid profiles of the five strains tested in this study were similar (see Supplementary Table S2 in IJSEM Online), but strain LC2-065^T was distinctive due to the presence of the fatty acids C_{8:0} 3-OH and C_{10:0} which were not present in strains of *P. kishitanii*, *P. phosphoreum*, *P. leiognathi* or *P. angustum*.

Fig. 3. Phylogenetic tree based on *luxA* gene sequences (611 bp). This tree combines the results of the neighbour-joining, maximum-parsimony and maximum-likelihood methods. The topology shown was obtained using the neighbour-joining method. Numbers at nodes denote the level of bootstrap support based on 1000 replicates, NJ/MP/ML. *Aliivibrio fischeri* was used as an outgroup. Bar, 5% estimated sequence divergence.

Table 1. Differential phenotypic characteristics of strains LC2-065^T and LC2-086 and related species of the genus *Photobacterium*

Taxa: 1, strain LC2-065^T; 2, strain LC2-086; 3, *P. kishitanii* ATCC BAA-1194^T; 4, *P. phosphoreum* IAM 14401^T; 5, *P. leiognathi* NCIMB 2193^T; 6, *P. angustum* CIP 75.10^T. +, Positive; w, weakly positive; –, negative; G, green colony; Y, yellow colony.

Characteristic	1	2	3	4	5	6
Luminescence	+	+	+	+	+	–
Production of:						
Alkaline phosphatase	w	–	w	w	w	w
Arginine dihydrolase	–	–	+	–	+	–
Esterase (C4)	–	–	w	w	w	w
Esterase lipase (C8)	–	–	w	–	–	w
α -Galactosidase	w	w	–	–	–	–
β -Galactosidase	w	w	–	w	–	–
Lysine decarboxylase	–	–	+	w	+	+
Valine arylamidase	–	–	–	w	–	–
Citrate utilization	–	–	–	–	–	+
Nitrate reduced to nitrite	+	+	+	+	+	–
Fermentation of:						
Glucose	–	–	+	–	+	–
Sucrose	–	–	–	–	–	+
Growth at (°C):						
4	–	+	+	+	–	–
30	–	+	+	–	+	+
37	–	–	–	–	+	–
Growth in NaCl (%; w/v):						
0.5	–	+	+	+	+	+
6	–	–	–	–	+	+
TCBS agar	–	G	–	G	G	Y
DNA G + C content (mol%)	42.2	42.9	40.2*	40.8*	41.6*	41*

*Data from Thyssen & Ollevier (2005).

The morphological, cultural, physiological and biochemical characteristics of strain LC2-065^T are given in the species description or are shown in Table 1. The DNA G + C contents of strains LC2-065^T and LC2-086 were 42.2 and 42.9 mol%, respectively. The results of this study revealed that strain LC2-065^T is closely related to *P. kishitanii* and *P. phosphoreum*, but that it can be differentiated from these species based on phylogenetic analyses, DNA–DNA relatedness and several phenotypic traits. Therefore, strain LC2-065^T represents a novel species in the genus *Photobacterium*, for which the name *Photobacterium aquimaris* sp. nov. is proposed.

Description of *Photobacterium aquimaris* sp. nov.

Photobacterium aquimaris (a.qui.ma'ris. L. n. *aqua* water; L. gen. n. *maris* of the sea; N.L. gen. n. *aquimaris* of water of the sea).

Gram-negative, oxidase-negative, catalase-positive and motile by means of more than one polar flagellum. Non-pigmented, translucent, luminescent colonies on MA with no swarming. Round, 2–3 mm green colonies with very poor growth on TCBS agar. Growth occurs in the presence of 0.9–3.5 % NaCl (w/v), but not at 0.5, 6, 8 or 10 % NaCl. Grows at 10–25 °C, but not at 4, 30 or 37 °C. Using the

API 20E and API ZYM systems (bioMérieux), alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase and *N*-acetyl- β -glucosaminidase are present, but arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, tryptophan deaminase, gelatinase, esterase (C4), esterase lipase (C8), lipase (C4), valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, β -glucuronidase, α -glucosidase, β -glucosidase, α -mannosidase and α -fucosidase are absent. Reduces nitrate to nitrite, but not further to N₂. The predominant cellular fatty acids of the type strain are C_{15:0} iso 2-OH and/or C_{16:1} ω 7c, C_{16:0}, C_{18:0}, C_{14:0}, C_{12:0}, C_{16:1} iso I and/or C_{14:0} 3-OH, C_{14:1} ω 5c, C_{12:0} 3-OH, C_{15:0} and C_{18:1} ω 7c.

The type strain, LC2-065^T (=NBRC 104633^T=KCTC 22356^T), was isolated from seawater of Sagami bay in Japan. The DNA G + C content of the type strain is 42.2 %. Strain LC2-086 (=NBRC 104591=KCTC 22357) is a reference strain.

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References

- Ast, J. C. & Dunlap, P. V. (2005). Phylogenetic resolution and habitat specificity of members of the *Photobacterium phosphoreum* species group. *Environ Microbiol* **7**, 1641–1654.
- Ast, J. C., Cleenwerck, I., Engelbeen, K., Urbanczyk, H., Thompson, F. L., De Vos, P. & Dunlap, P. V. (2007). *Photobacterium kishitanii* sp. nov., a luminous marine bacterium symbiotic with deep-sea fishes. *Int J Syst Evol Microbiol* **57**, 2073–2078.
- Beijerinck, M. W. (1889). Le *Photobacterium lamosum*, bacterie lumineuse de la Mer du Nord. *Arch Neerl Sci Exactes Nat* **23**, 401–427.
- Boisvert, H., Chatelain, R. & Bassot, J. M. (1967). Étude d'un *Photobacterium* isolé de l'organe lumineux de poissons Leionathidae. *Ann Inst Pasteur (Paris)* **112**, 520–524.
- Dunlap, P. V. & Kita-Tsukamoto, K. (2001). Luminous bacteria. In *The Prokaryotes*, pp. 863–892. Edited by M. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt. New York: Academic Press.
- Dunlap, P. V., Ast, J. C., Kimura, S., Fukui, A., Yoshino, T. & Endo, H. (2007). Phylogenetic analysis of host-symbiont specificity and codivergence in bioluminescent symbioses. *Cladistics* **23**, 507–532.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide-sequences. *J Mol Evol* **16**, 111–120.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Nishino, T., Ikemoto, E. & Kogure, K. (2004). Application of atomic force microscopy to observation of marine bacteria. *J Oceanogr* **60**, 219–225.
- Park, Y. D., Baik, K. S., Seong, C. N., Bae, K. S., Kim, S. & Chun, J. (2006). *Photobacterium ganghwense* sp. nov., a halophilic bacterium isolated from sea water. *Int J Syst Evol Microbiol* **56**, 745–749.
- Reichelt, J. L. & Baumann, P. (1973). Taxonomy of marine, luminous bacteria. *Arch Mikrobiol* **94**, 283–330.
- Reichelt, J. L., Baumann, P. & Baumann, L. (1976). Study of genetic relationships among marine species of genera *Beneckea* and *Photobacterium* by means of in vitro DNA-DNA hybridization. *Arch Microbiol* **110**, 101–120.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sawabe, T., Kita-Tsukamoto, K. & Thompson, F. L. (2007). Inferring the evolutionary history of vibrios by means of multilocus sequence analysis. *J Bacteriol* **189**, 7932–7936.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Thompson, C. C., Thompson, F. L., Vicente, A. C. & Swings, J. (2007a). Phylogenetic analysis of vibrios and related species by means of *atpA* gene sequences. *Int J Syst Evol Microbiol* **57**, 2480–2484.
- Thompson, F. L., Gomez-Gil, B., Vasconcelos, A. T. R. & Sawabe, T. (2007b). Multilocus sequence analysis reveals that *Vibrio harveyi* and *V. campbellii* are distinct species. *Appl Environ Microbiol* **73**, 4279–4285.
- Thyssen, A. & Ollevier, F. (2005). Genus *Photobacterium*. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2, part B, *The Proteobacteria*, pp. 546–552. Edited by D. J. Brenner, N. R. Krieg & J. T. Staley. New York: Springer.
- Urbanczyk, H., Ast, J. C., Kaeding, A. J., Oliver, J. D. & Dunlap, P. V. (2008). Phylogenetic analysis of the incidence of *lux* gene horizontal transfer in *Vibrionaceae*. *J Bacteriol* **190**, 3494–3504.
- Wada, M., Kamiya, A., Uchiyama, N., Yoshizawa, S., Kita-Tsukamoto, K., Ikejima, K., Yu, R., Imada, C., Karatani, H. & other authors (2006). *LuxA* gene of light organ symbionts of the bioluminescent fish *Acropoma japonicum* (Acropomatidae) and *Siphamia versicolor* (Apogonidae) forms a lineage closely related to that of *Photobacterium leiognathi* ssp. *mandapamensis*. *FEMS Microbiol Lett* **260**, 186–192.
- Wimpee, C. F., Nadeau, T. L. & Nealson, K. H. (1991). Development of species-specific hybridization probes for marine luminous bacteria by using in vitro DNA amplification. *Appl Environ Microbiol* **57**, 1319–1324.
- Xie, C. H. & Yokota, A. (2003). Phylogenetic analyses of *Lamproedia hyalina* based on the 16S rRNA gene sequence. *J Gen Appl Microbiol* **49**, 345–349.