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

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Two luminous marine bacterial strains LC2-065T 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 LC2-065T and Photobacterium kishitanii ATCC BAA-1194T was 42.1%. The major fatty acids were C\textsubscript{12}:0, C\textsubscript{14}:0, C\textsubscript{16}:0, C\textsubscript{18}:0 and C\textsubscript{18}:0 iso 2-OH and/or C\textsubscript{16}:1\textsubscript{ω}7c (summed feature 3). The DNA G+C contents of strains LC2-065T 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 LC2-065T (=NBRC 104633\textsuperscript{T} = KCTC 22356\textsuperscript{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 luminous strains were isolated from seawater and analysed using 16S rRNA, the alpha-subunit of luciferase (luxA), uridylate 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 LC2-065T 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 LC2-065T 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 mm; 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 LC2-065T 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.

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

Based on the \textit{luxA} gene sequence analysis (611 bp), the novel strains are distinct from other luminous strains of the genus \textit{Photobacterium} (Fig. 3). The \textit{luxA} gene sequence similarities between strain LC2-065\textsuperscript{T} and related species were 84.9\% to \textit{P. leiognathi} ATCC 27561, 70.6\% to \textit{P. phosphoreum} ATCC 11040\textsuperscript{T}, 70.4\% to \textit{P. kishitanii} pjapo.1.1\textsuperscript{T} and 63.7\% \textit{P. leiognathi} ATCC 25521\textsuperscript{T}. The phylogenetic placement of strains LC2-065\textsuperscript{T} and LC2-086 based on the \textit{luxA} gene sequences did not match that determined on the basis of the housekeeping genes. The discordance between the housekeeping and \textit{luxA} gene phylogenies supports the hypothesis of horizontal transfer of the \textit{lux} genes (Urbanczyk \textit{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 \textit{Photobacterium}. In contrast to most of their phylogenetic neighbours, these novel strains can produce \textit{z}-galactosidase and \textit{\beta}-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\textsuperscript{T} were C\textsubscript{15}:0 iso 2-OH and/or C\textsubscript{16}:1 \textit{v7c} (summed feature 3; 36.5\%), C\textsubscript{16}:0 (18.7\%), C\textsubscript{18}:0 (10.8\%), C\textsubscript{14}:0 (8.6\%) and C\textsubscript{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\textsuperscript{T} was distinctive due to the presence of the fatty acids C\textsubscript{8}:0 3-OH and C\textsubscript{10}:0 which were not present in strains of \textit{P. kishitanii}, \textit{P. phosphoreum}, \textit{P. leiognathi} or \textit{P. angustum}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{phylogenetic_tree.png}
\caption{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. \textit{Aliivibrio fischeri} was used as an outgroup. Bar, 2\% estimated sequence divergence.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{luxA_tree.png}
\caption{Phylogenetic tree based on \textit{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. \textit{Aliivibrio fischeri} was used as an outgroup. Bar, 5\% estimated sequence divergence.}
\end{figure}
The morphological, cultural, physiological and biochemical characteristics of strain LC2-065T are given in the species description or are shown in Table 1. The DNA G+C contents of strains LC2-065T and LC2-086 were 42.2 and 42.9 mol%, respectively. The results of this study revealed that strain LC2-065T 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 LC2-065T represents a novel species in the genus *Photobacterium*, for which the name *Photobacterium aquimaris* sp. nov. is proposed.

### Description of *Photobacterium aquimaris* sp. nov.


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 C15 : 0iso 2-OH and/or C16 : 1v7c, C16 : 0, C18 : 0, C14 : 0, C12 : 0, C16 : 1iso I and/or C14 : 03-OH, C14 : 1v5c, C12 : 03-OH, C15 : 0 and C18 : 1v7c.

The type strain, LC2-065T (=NBRC 104633T=KCTC 22356T), 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.

### Acknowledgements

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

<table>
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<td>Luminescence</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Production of:</td>
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<tr>
<td>Alkaline phosphatase</td>
<td>w</td>
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<td>w</td>
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<td>Esterase (C4)</td>
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<td>w</td>
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<td>w</td>
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<td>−</td>
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<td>42.9</td>
<td>40.2*</td>
<td>40.8*</td>
<td>41.6*</td>
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*Data from Thyssen & Ollevier (2005).
Japan Science Society and a research grant from Institute for Fermentation, Osaka, Japan.

References


