Photobacterium kishitanii sp. nov., a luminous marine bacterium symbiotic with deep-sea fishes

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Six representatives of a luminous bacterium commonly found in association with deep, cold-dwelling marine fishes were isolated from the light organs and skin of different fish species. These bacteria were Gram-negative, catalase-positive, and weakly oxidase-positive or oxidase-negative. Morphologically, cells of these strains were coccoid or coccoid-rods, occurring singly or in pairs, and motile by means of polar flagellation. After growth on seawater-based agar medium at 22 °C for 18 h, colonies were small, round and white, with an intense cerulean blue luminescence. Analysis of 16S rRNA gene sequence similarity placed these bacteria in the genus *Photobacterium*. Phylogenetic analysis based on seven housekeeping gene sequences (16S rRNA gene, *gapA*, *gyrB*, *pyrH*, *recA*, *rpoA* and *rpoD*), seven gene sequences of the *lux* operon (*luxC*, *luxD*, *luxA*, *luxB*, *luxF*, *luxE* and *luxG*) and four gene sequences of the *rib* operon (*ribE*, *ribB*, *ribH* and *ribA*), resolved the six strains as members of the genus *Photobacterium* and as a clade distinct from other species of *Photobacterium*. These strains were most closely related to *Photobacterium phosphoreum* and *Photobacterium iliopiscarium*. DNA–DNA hybridization values between the designated type strain, *Photobacterium kishitanii pjapo*.1.1^T, and *P. phosphoreum* LMG 4233^T, *P. iliopiscarium* LMG 19543^T and *Photobacterium indicum* LMG 22857^T were 51, 43 and 19 %, respectively. In AFLP analysis, the six strains clustered together, forming a group distinct from other analysed species. The fatty acid $C_{17:0}$ cyclo was present in these bacteria, but not in *P. phosphoreum*, *P. iliopiscarium* or *P. indicum*. A combination of biochemical tests (arginine dihydrolase and lysine decarboxylase) differentiates these strains from *P. phosphoreum* and *P. indicum*. The DNA G+C content of *P. kishitanii pjapo*.1.1^T is 40.2 %, and the genome size is approximately 4.2 Mbp, in the form of two circular chromosomes. These strains represent a novel species, for which the name *Photobacterium kishitanii* sp. nov. is proposed. The type strain, *pjapo*.1.1^T (=ATCC BAA-1194^T=LMG 23890^T), is a luminous symbiont isolated from the light organ of the deep-water fish *Physiculus japonicus*.

Photobacterium (Gammaproteobacteria: *Vibrionaceae*) comprises at present 14 species with validly published names, many members of which are luminous and some of which enter into bioluminescent symbioses with marine animals (Dunlap & Kita-Tsukamoto, 2006; Dunlap *et al.*, 2007). *Photobacterium phosphoreum* (Beijerinck, 1889), the type species of the genus *Photobacterium*, was thought to be the light-organ symbiont of deep-water fishes (Hastings & Nealson, 1981), although the type strain of this species was isolated from the surface of a non-luminous fish (Ast & Dunlap, 2005). Recent analyses showed that *P. phosphoreum* was not recovered from light organ symbiosis; instead, the light-organ symbionts of deep-sea fishes formed a clade distinct from *P. phosphoreum*, designated previously as *Photobacterium kishitanii* (Dunlap & Ast, 2005; Dunlap *et al.*, 2007). *P. kishitanii* is closely related to *P. phosphoreum* and *Photobacterium iliopiscarium*, although

Abbreviations: AFLP, amplified length polymorphisms; Mbp, mega base pairs.

The GenBank/EMBL/DDBJ accession number for gene sequences reported in this paper are EF415487–EF415631 and EF441349.

Supplementary material is available with the online version of this paper.

based on phylogenetic analysis, the latter two species are more closely related to each other than either is to *P. kishitanii* (Ast & Dunlap, 2005). Here, we present comprehensive phylogenetic, genomic and phenotypic evidence that strains of *P. kishitanii* represent a novel species, within the genus *Photobacterium*, for which the name *Photobacterium kishitanii* sp. nov. is proposed. The type strain is *pjapo*.1.1^T (=ATCC BAA-1194^T=LMG 23890^T).

Six strains representing *P. kishitanii* sp. nov. were examined here (Table 1), three isolated from fish light-organs (Ast & Dunlap, 2005; Dunlap & Ast, 2005; Haygood *et al.*, 1992) and three from enrichments of fish skin (Ast & Dunlap, 2005; Hendrie *et al.*, 1970; Georgala, 1958; Reichelt & Baumann, 1973). To obtain DNA sequences for phylogenetic analysis, genomic DNA was purified using a DNA extraction kit (Qiagen) following the manufacturer's protocol for Gram-negative bacteria. Seven housekeeping genes (16S rRNA, *gapA*, *gyrB*, *pyrH*, *recA*, *rpoA* and *rpoD*) were amplified from the six strains and from eleven representative strains of the genus *Photobacterium*. In addition, the genes of two contiguous operons, the *lux* operon (*luxC*, *luxD*, *luxA*, *luxB*, *luxF*, *luxE* and *luxG*, the products of which are responsible for the luminescent phenotype) and the *rib* operon (*ribE*, *ribB*, *ribH* and *ribA*, the products of which are involved in riboflavin synthesis) were amplified from luminous strains, for a total of more than 17 kbp of sequence. See Supplementary Tables S1–S4 for PCR amplification profiles and primer sequences (available in IJSEM Online). Amplified products were purified by using a PCR clean-up kit (Millipore) and directly sequenced by using the University of Michigan Sequencing Core. Sequences for housekeeping, *lux* and *rib* genes were obtained for two strains of *Vibrio fischeri* to serve as outgroups. GenBank accession numbers for all DNA sequences, including those obtained previously, are listed in Supplementary Table S5.

To test the evolutionary relationships of these six strains to other species of the genus *Photobacterium*, phylogenetic analysis was performed on the concatenated dataset with the program PAUP* (Swofford, 2002) using the 18 genes listed above (see Supplementary Material for details of phylogenetic analysis). Support values were calculated using 5000 jackknife resampling replicates. The resulting most parsimonious phylogenetic hypothesis clearly demonstrates that the novel strains identified as *P. kishitanii* represent a lineage separate from other species of *Photobacterium* (Fig. 1), with robust resampling support. Within the genus *Photobacterium*, the representatives of *P. kishitanii* form a clade with the species *P. phosphoreum* and *P. iliopiscarium*.

To test further the hypothesis that *P. kishitanii* is a separate species, several additional analyses were performed. For per cent identities among 16S rRNA gene sequences, sequences were aligned using direct optimization analysis (Wheeler, 1996; Wheeler *et al.*, 2006), as implemented by the program POY (Wheeler *et al.*, 2003). Direct optimization iteratively evaluates alignment in the context of phylogeny; it therefore produces a rigorously tested alignment, unlike single-pass multiple sequence alignment algorithms used by other alignment programs. Details of the POY analysis can be found in Supplementary Material. Identities of *P. kishitanii pjapo*.1.1^T to other species were 97.7 % to *P. indicum* LMG 22857^T, 99.6 % to *P. iliopiscarium* LMG 19543^T and 99.7 % to *P. phosphoreum* LMG 4233^T. These values are within the range of per cent identities between other recognized species of *Photobacterium*; for example, the identity between *P. indicum* LMG 22857^T and *P. phosphoreum* LMG 4233^T is 97.9 %.

To characterize genomic features that distinguish the strains of *P. kishitanii* from other species of *Photobacterium*, we performed two tests of genomic similarity, DNA–DNA hybridization and amplified length fragment polymorphism (AFLP) analysis. For DNA–DNA hybridization, high molecular mass DNA was prepared by the method of Wilson (1987) with minor modifications (Cleenwerck *et al.*, 2002). DNA quality and quantity were determined by measuring absorptions at 260, 280 and 234 nm. Only high quality DNA with A_{260}/A_{280} and A_{234}/A_{260} ratios of 1.8–2.0 and 0.40–0.60 was used. Hybridizations were performed using a modification (Goris *et al.*, 1998; Cleenwerck *et al.*, 2002) of the microplate method described by Ezaki *et al.* (1989) with a hybridization temperature of 37 °C. Reported values are

Table 1. Source of the novel strains investigated in this study

LO, Light organ; skin, enrichment from fish skin.

Strain	Previous designation; origin	Reference
LMG 23890^T	ATCC BAA-1194^T, *pjapo*.1.1^T; LO, *Physiculus japonicus*, Japan, 1982	Dunlap & Ast (2005), Ast & Dunlap (2005)
LMG 23891	*ckamo*.1.1; LO, *Caelorinchus kamoharai*, Japan, 2004	Ast & Dunlap (2005)
LMG 23892	Og61; LO, *Opisthoproctis grimaldii*, Cape Verde, 1990	Haygood *et al.* (1992)
LMG 23893	B-421; skin, *Trachurops crumenophthalmus*, Hawaii, 1973	Reichelt & Baumann (1973)
LMG 23894	FS-8.1; skin, 'bluenosed grouper', Florida, 2003	Ast & Dunlap (2005)
LMG 23895	NCIMB 844; skin, *Merluccius capensis*, South Africa, 1958	Georgala (1958); D. L. Georgala, personal communication

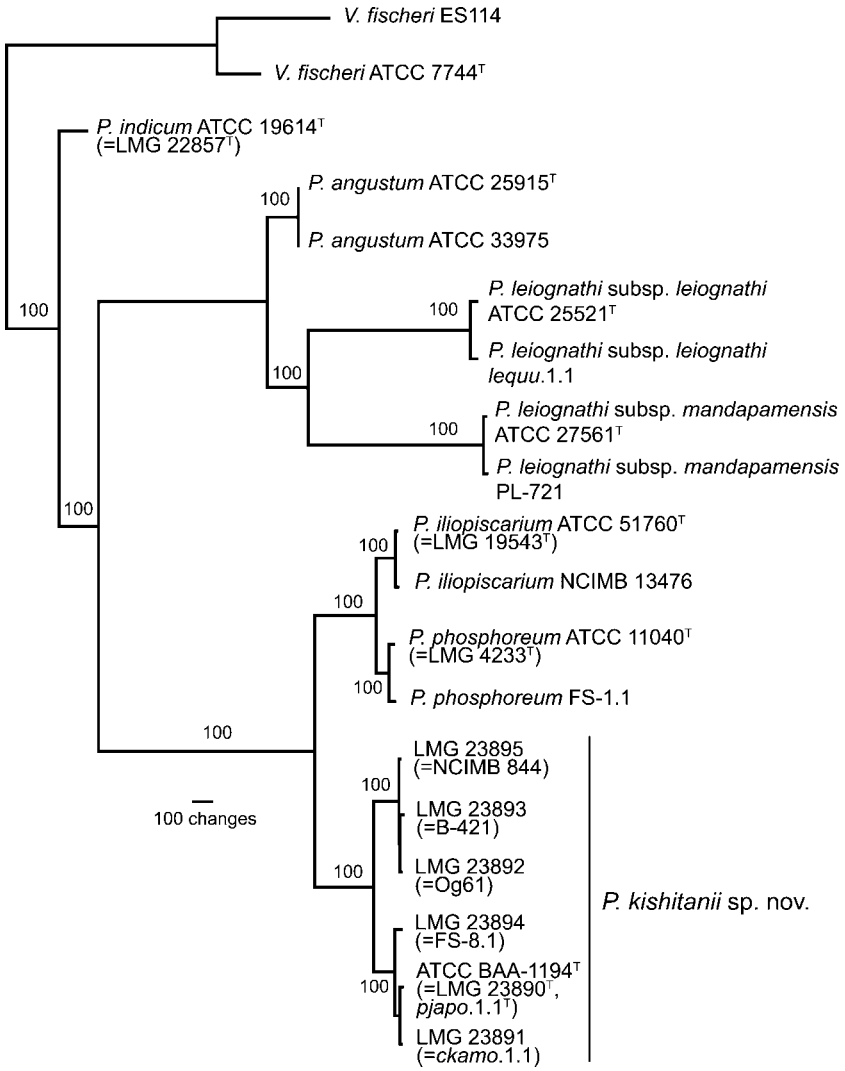

Fig. 1. Phylogenetic tree of *Photobacterium* including the six strains of *P. kishitanii* sp. nov. Parsimony analysis of the concatenated dataset comprising seven housekeeping gene sequences (16S rRNA, *gapA*, *gyrB*, *pyrH*, *recA*, *rpoA* and *rpoD*), seven gene sequences of the *lux* operon (*luxC*, *luxD*, *luxA*, *luxB*, *luxF*, *luxE* and *luxG*) and four gene sequences of the *rib* operon (*ribE*, *ribB*, *ribH* and *ribA*) resulted in the single most parsimonious hypothesis shown here (5018 informative characters, tree length=8540, ensemble consistency index=0.808, ensemble retention index=0.877). Inferred gaps were treated as informative data, and for taxa lacking *lux* and *rib* genes or operons, the missing genes were treated as missing data. Numbers at nodes are jackknife support values based on 5000 resampling replicates.

the mean of a minimum of four hybridizations. The values for hybridization between strain *P. kishitanii pjapo*.1.1^T and other species of *Photobacterium* were 51 % to *P. phosphoreum* LMG 4233^T, 43 % to *P. iliopiscarium* LMG 19543^T and 19 % to *P. indicum* LMG 22857^T. These values, which are below the current level that delimits separate species, demonstrates that strains of *P. kishitanii* are distinct from other species of *Photobacterium*.

For AFLP analysis, DNA was prepared as above, and template preparation, PCR reactions and PAGE were performed as described by Thompson *et al.* (2001). Electrophoretic patterns were tracked and normalized using the GENESCAN 3.1 software (Applera). Normalized tables of peaks, containing fragments of 50–539 bp were transferred to the BioNumerics 4.5 software (Applied Maths) for numerical analysis. Patterns were clustered using the Dice coefficient and the UPGMA algorithm. A band position tolerance value of 0.3 % was allowed to compensate for misalignment of similarly sized bands due to technical imperfections. The profiles were compared with the profiles of other species of *Vibrionaceae* using the database at the BCCM/LMG Bacteria Collection. The dendrogram of the AFLP profiles demonstrates that the strains of *P. kishitanii* cluster together and are clearly distinct from other analysed species of *Photobacterium* and *Vibrio* (Fig. 2).

For determination of whole-cell fatty acid content of strains of *P. kishitanii* compared with strains of *P. phosphoreum*, *P. iliopiscarium* and *P. indicum*, cells were grown for 24 h at 20 °C on plates of M12 (Marine agar; Difco) medium. Harvesting of cells conformed to the recommendations of the manufacturer of the MIDI identification system (Microbial Identification System) for *P. indicum* LMG 22857^T. In the case of the strains of *P. kishitanii*, cells of all strains were harvested from two plates, and for *P. phosphoreum* LMG 4233^T and *P. iliopiscarium* LMG 19543^T, the complete growth on one plate was used to acquire a sufficient concentration of fatty acids for the analysis. Extraction and analysis were performed according the manufacturer's instructions. The

Fig. 2. Dendrogram of AFLP patterns of novel strains of *P. kishitanii* compared with other species of *Photobacterium* and *Vibrio*. The six strains of *P. kishitanii* (numbers 10–15) are in bold. Numbers represent the following strains: 1, *Photobacterium damselae* subsp. *damselae* LMG 7892^T; 2, *V. fischeri* LMG 4414^T; 3, *Vibrio harveyi* LMG 4044^T; 4, *P. iliopiscarium* LMG 19543^T; 5, *Photobacterium angustum* LMG 8455^T; 6, *Photobacterium leiognathi* LMG 4228^T; 7, *P. phosphoreum* LMG 4233^T; 8, *Photobacterium profundum* LMG 19446^T; 9, *Photobacterium rosenbergii* LMG 22223^T; 10, *P. kishitanii* sp. nov. Og61 (=LMG 23892); 11, *P. kishitanii* sp. nov. B-421 (=LMG 23893); 12, *P. kishitanii* sp. nov. NCIMB 844 (=LMG 23895); 13, *P. kishitanii* sp. nov. *ckamo*.1.1 (=LMG 23891); 14, *P. kishitanii* sp. nov. FS-8.1 (=LMG 23894); 15, *P. kishitanii* sp. nov. *pjapo*.1.1^T (=LMG 23890^T); 16, *P. lipolyticum* LMG 23071^T.

overall profiles of the four tested species are similar (Supplementary Table S6), but strains of *P. kishitanii* differ by the presence of the fatty acid $C_{17:0}$ cyclo, which is not present in strains of *P. phosphoreum*, *P. iliopiscarium* or *P. indicum*.

Phenotypic characterizations, e.g. cell morphology, response to Gram staining, motility, oxidase and catalase tests were performed using standard methods. Additional biochemical tests were performed using API 20E and API 20NE tests (bioMérieux). Cells for inoculation of the strips were grown for 24 h at 20 °C on M12 medium and results were visually interpreted according to the manufacturer's instructions. On the basis of the arginine dihydrolase test, novel strains of *P. kishitanii* can be differentiated from strains of *P. phosphoreum*, and the lysine decarboxylase test differentiates novel strains of *P. kishitanii* from strains of *P. phosphoreum* and *P. indicum*. However, no single biochemical trait or combination of traits distinguishes strains of *P. kishitanii* from strains of *P. iliopiscarium*. Complete phenotype data may be found in Supplementary Table S7.

To characterize further *P. kishitanii pjapo*.1.1^T, DNA base composition was determined by HPLC according to the method of Mesbah *et al.* (1989). Non-methylated phage λ was used as a reference. The DNA G+C content of *P. kishitanii pjapo*.1.1^T is 40.2 mol%, which is consistent with other species of *Photobacterium* (Baumann & Baumann, 1984).

To estimate genome size and chromosome composition, genomic DNA inserts were prepared according to Lucangeli *et al.* (2000) with modifications. DNA fragments of undigested inserts and inserts digested with *Not*I or *I-Ceu*I enzyme (New England Biolabs) were separated by PFGE using standard conditions (see Supplementary Material). Based on the electrophoretic banding patterns, the *P. kishitanii pjapo*.1.1^T genome is approximately 4.2 Mbp, configured in two circular chromosomes of sizes about 2.8 and 1.4 Mbp (Fig. 3). Strains B-421, *ckamo*.1.1, FS-8.1 and NCIMB 844 have genome sizes ranging from 4.0 to 4.7 Mbp (data not shown). Similar analyses of the genomes of 27 additional strains of *P. kishitanii* (data not shown) revealed that genomes can range in size from 3.9 to 4.9 Mbp with an average of 4.2 Mbp; all analysed strains were found to have two circular chromosomes, as in other species of *Vibrionaceae* (Okada *et al.*, 2005). The *I-Ceu*I enzyme digestion resulted in eight fragments, suggesting that *P. kishitanii pjapo*.1.1^T has at least eight *rrn* loci (*I-Ceu*I cuts most bacterial genomes only within *rrn* loci), a result consistent with the high *rrn* copy number in other species of *Vibrionaceae* (Klappenbach *et al.*, 2001).

Culture collections currently hold many bacterial strains identified as *P. phosphoreum*. To test if any of these strains are in fact of *P. kishitanii*, we analysed them phylogenetically by using gene sequences of *gyrB*, *recA* and *luxA*. Gene sequences were aligned by inferred amino acid sequence and analysed with a heuristic parsimony search using the program TNT (Goloboff *et al.*, 2005; analysis details can be found in Supplementary Material). These results indicate that the following strains should be reclassified as *P. kishitanii* sp. nov.: ATCC 35080, ATCC 35081, ATCC 35082, NCIMB 61, NCIMB 62, NCIMB 64, NCIMB 65, NCIMB 66, NCIMB 68, NCIMB 69, NCIMB 70, NCIMB 71, NCIMB 844, NCIMB 1276, NCIMB 1277, NCIMB 12838 and NCIMB 12839. The following strains were

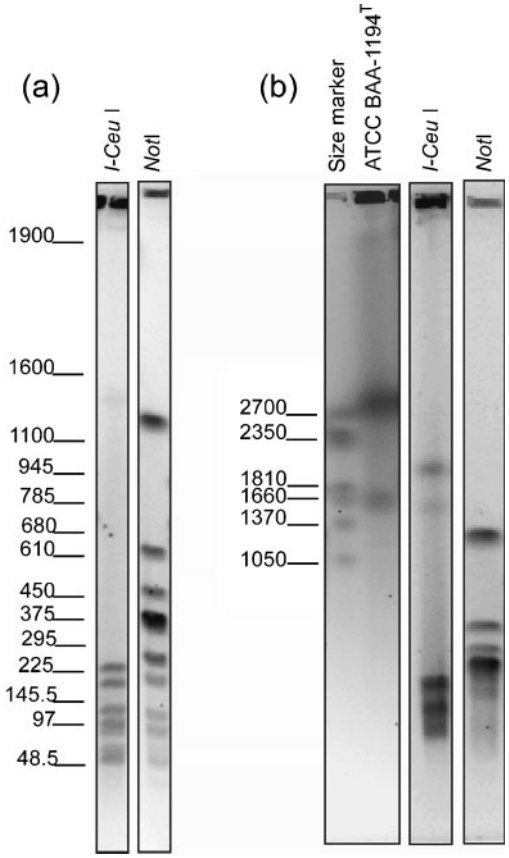

Fig. 3. PFGE of *P. kishitanii pjapo*.1.1^T genomic DNA. Sizes in kb are shown on the left of each picture. (a) Resolution of mid-sized, restriction enzyme digested genomic DNA. (b) Resolution of undigested genomic DNA and large-size, restriction enzyme digested fragments.

confirmed as belonging to *P. phosphoreum*: NCIMB 7, NCIMB 188, NCIMB 193, NCIMB 395, NCIMB 1275 and NCIMB 1279. In previous work (Ast & Dunlap, 2005), three other strains from NCIMB identified as belonging to the species *P. phosphoreum* were resolved as belonging to the species *P. iliopiscarium* (NCIMB 13476, NCIMB 14378 and NCIMB 13481). A phylogenetic tree showing the results from the analysis including all NCIMB and ATCC strains mentioned above is available as Supplementary Fig. S1. All of these strains that originated from fish light-organs are *P. kishitanii* sp. nov., and to date, no strain identified by these criteria as *P. phosphoreum* has been isolated from the light organ of a fish or a squid (Ast & Dunlap, 2005; Dunlap & Ast, 2005; Dunlap *et al.*, 2007; this study). Therefore, in contrast to *P. kishitanii* sp. nov., which can be isolated from light organs of several deep, cold-dwelling fishes, strains of *P. phosphoreum* apparently do not occur as a bioluminescent symbiont of marine animals.

On the basis of these phylogenetic, genomic and taxonomic analyses, strains identified as *P. kishitanii* clearly represent a separate species of *Photobacterium*, for which the name *Photobacterium kishitanii* sp. nov. is proposed.

Description of *Photobacterium kishitanii* sp. nov.

Photobacterium kishitanii (ki.shi.tan′i.i. N.L. gen. n. *kishitanii* of Kishitani, to honour the deceased Japanese scientist Teijiro Kishitani, who first isolated luminous bacteria from the light organ of *Physiculus japonicus*). The following description is based on analyses of six strains (Table 1).

Cells are Gram-negative, coccoid or coccoid-rods, motile, occurring singly or in pairs, 0.9 μm in width by 1.2–3.0 μm in length. After 18 h, colonies grown on LSW-70 at 22 °C are small, round, white and strongly luminous. Catalase-positive. Oxidase-negative or weakly positive. Genome size of the type strain is approximately 4.2 Mbp (ranging within the six strains from 4.0 to 4.7 Mbp), consisting of two circular chromosomes. Cells produce the fatty acid $C_{17:0}$ cyclo. Light-organ symbiont of many fishes, may also be found on the surfaces of fishes and in seawater. The DNA G+C content of the type strain is 40.2 mol%.

The type strain, *pjapo*.1.1^T (=ATCC BAA-1194^T=LMG 23890^T), was isolated in 1982 from the light organ of the deep-sea fish *Physiculus japonicus*.

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