Systematic and Applied Microbiology 41 (2018) 444–451

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Photobacterium malacitanum sp. nov., and *Photobacterium andalusiense* sp. nov., two new bacteria isolated from diseased farmed fish in Southern Spain

Alejandro M. Labella[a], M. Dolores Castro[a], Manuel Manchado[b], Teresa Lucena[c], David R. Arahal[c], Juan J. Borrego[a,*]

[a] Universidad de Málaga, Departamento de Microbiología, Facultad de Ciencias, 29071 Málaga, Spain
[b] IFAPA Centro El Toruño, Junta de Andalucía, Puerto de Santa María, 11500 Cádiz, Spain
[c] Departamento de Microbiología y Ecología, and Colección Española de Cultivos Tipo (CECT), Universidad de Valencia, Valencia, Spain

ARTICLE INFO

Article history:
Received 29 January 2018
Received in revised form 12 April 2018
Accepted 20 April 2018

Keywords:
Photobacterium malacitanum
Photobacterium andalusiense
ANI
DDH
Genome
Fish pathogen

ABSTRACT

Three strains, H01100409B^T, H01100413B, and H27100402H^T, were isolated from several internal organs of diseased redbanded seabream (*Pagrus auriga*) reared in Andalusia (Southern Spain). All strains were studied by phenotypic, including chemotaxonomy, and genomic characteristics. Phylogenetic analysis based on concatenated sequences of six housekeeping genes (*gyrB*, *ftsZ*, *topA*, *mreB*, *gapA*, and 16S rRNA) supported the inclusion of the strains within the clade Phosphoreum of the genus *Photobacterium*, and two of the strains (H27100402H^T and H01100409B^T) formed a tight group separated from the closest species *P. aquimaris*. Genomic analyses, including average nucleotide identity (ANIb and ANIm) and DNA–DNA hybridization (DDH), clearly separated strains H27100402H^T and H01100409B^T from the other species within the clade Phosphoreum with values below the thresholds for species delineation. The chemotaxonomic features (including FAME analysis and MALDI-TOF-MS) of H27100402H^T and H01100409B^T strains confirmed their differentiation from the related taxa. The results demonstrated that strain H01100413B was classified as *P. aquimaris* and the strains H27100402H^T and H01100409B^T represented a new species each in the genus *Photobacterium*, for which we propose the names *Photobacterium malacitanum* sp. nov., type strain H27100402H^T (=CECT 9190^T = LMG 29992^T), and *Photobacterium andalusiense* sp. nov., type strain H01100409B^T (=CECT 9192^T = LMG 29994^T).

The GenBank/EMBL/DDBJ accession numbers for the housekeeping gene sequences used in this study are listed in Fig. 2 and Supplementary Table S2. The draft genome sequences of strains, H27100402H^T, H01100409B^T and H01100413B are under accession numbers, FYAK01, FYAJ01 and FYAH01 respectively.

Currently the genus *Photobacterium* comprises about 30 species inhabitants of saline environments and of a variety of marine organisms, some photobacteria are symbiotic or pathogenic for their hosts, including fish, molluscs, crustaceans, zoanthids, corals, and mammals [6,25]. Two subspecies of *P. damselae* (*P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida*) and the novel species *P. toruni* are involved in diseases of wild and cultured fish [7,16], the former species are included in a well-defined clade (Damsela), whereas *P. toruni* has been included into the clade Phosphoreum.

Three strains, H0110409B^T, H0110413B, and H2710402H^T, collected from an outbreak affecting diseased redbanded seabream (*Pagrus auriga*, Valenciennes) reared in an experimental research fish centre, have been taxonomic and genomic characterized. The results demonstrated that two of them (strains H27100402H^T and H01100409B^T) constituted two new species within the genus *Photobacterium*, while strain H0110413B could be classified as *P. aquimaris* a species that previously was isolated and described to be exclusively from seawater [28].

Fish samples were obtained from one outbreak affecting captived redbanded seabream (*P. auriga*), ranging from 485 to 555 g with a cumulative mortality of 28%, reared in the experimental research fish centre IFAPA, Centro El Toruño (Puerto de Santa Maria, Cadiz, SW, Spain) during autumn 2014. Diseased fish specimens were anaesthetised by immersion in seawater supplemented with MS-222 (Sigma Chemical Co.) at a final concentration of

* Corresponding author at: Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, Campus Universitario Teatinos, 29071 Malaga, Spain.
E-mail address: jjborrego@uma.es (J.J. Borrego).

https://doi.org/10.1016/j.syapm.2018.04.005
0723-2020/

0.065 g mL^{-1} prior to dissection and sampling to pathological examination. For bacterial isolation, samples were collected from affected organs and tissues, such as eyes, spleen, liver, and head-kidney, and seeded on Tryptic Soy agar (Difco) supplemented with 1.5% NaCl (TSAS), Tryptic Soy broth (Difco) supplemented with 1.5% NaCl (TSBS), and Marine agar (MA, Difco). The inoculated media were incubated at 22 °C for 2–5 days.

Pure cultures were carried out onto TSAS Petri dishes, and the isolates were subjected to 16S rRNA gene sequence and phenotypic analysis. A phylogenetic tree was constructed using the 16S rRNA gene sequences of strains H0110409B^T, H0110413B, and H2710402H^T, together with the types of all species of the genus *Photobacterium*, using the Maximum-Likelihood (ML), Neighbour-Joining (NJ), and Maximum-Parsimony (MP) methods. Phenotypic features were studied using the classical procedures, as well as the miniaturized systems API 20NE, ID 32E, and API ZYM (BioMerieux). The reference strains, *P. aquimaris* LC2-065^T, *P. iliospicarium* ATCC 51760^T, *P. kishitanii* ATCC BAA-1194^T, *P. phosphoreum* ATCC 11040^T, *P. piscicola* NCCB 100098^T, and *P. toruni* CECT 9189^T, were tested alongside for comparative purposes. Biolog GN microplate and API 20NE were used for the determination of the utilization of substrates as sole carbon and energy source by the tested strains.

The strains tested shared the main properties of the genus *Photobacterium* [24]. They are motile rods, facultative anaerobic, Gram-negative and catalase positive, capable of reducing nitrates to nitrites, ornithine decarboxylase and gelatinase negatives, and unable to grow in media with lower than 100 mM Na^+. The isolates showed high phenotypical homogeneity, although variable reactions were observed for several traits among other species tested (Table 1).

The phenotypic differential characters of the strains tested are given in the species description and Table 1. Colonies growing either on TSAS agar were beige with entire edges and not produced a diffusible pigment. Strains H01100409B^T, H01100413B and H27100402H^T grew on TCBS as green colonies, thus being unable to ferment sucrose. All strains were psycrophilic-mesophilic and slightly halophilic, and their extracellular hydrolytic abilities were limited to urease, amylase, lipase (API 20NE), alkaline and acid phosphatases, phosphohydrolase, esterase (C4), esterase–lipase (C8), leucine arylamidase and glucosaminidase activities. The complete results of these strains using API 20NE, ID 32E, API-ZYM and Biolog GN microplates are given in the species description. Strains tested were able to metabolize a large number of substrate contained in the different test galleries.

On the basis of the phenotypic homogenity obtained for the problem strains, we performed three PCR-based techniques (RAPD, ERIC-PCR and REP-PCR) for the analysis of genetic variability of the tested strains. Chromosomal DNA was extracted using the InstaGene Matrix (Bio-Rad) as previously described [5]. The RAPD reactions were performed using Ready-togo RAPD analysis beads (Amersham Pharmacia Biotech), containing buffer, nucleotides and Taq DNA polymerase. Reagents that must be added to the reaction were template DNA (1 μL) and 25 pmol of respective RAPD primers (provided in the kit) to complete a 25-μL total volume. The oligonucleotide sequences of the primers used were the following: P4 (5′-AAGAGCCCCT-3′) and P5 (5′-AACGCGCAAC-3′). ERIC-PCR and REP-PCR were carried out with Readyto-go-PCR beads (Amersham Pharmacia Biotech). These commercial beads have been optimized for PCR and contain buffer, nucleotides and *Taq* DNA polymerase. For this reason, the only reagents added to the reaction were template DNA (1 μL), primers (100 pmol each) and water to complete a volume of 25 μL. The ERIC and REP primers used in this study were ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) [melting temperature (Tm) 65.1 °C], ERIC 2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) (Tm 66.4 °C), REP1D (5′-NNHRCGYCGNCATCMGGC-3′) (Tm 62.5 °C) and REP2D (5′-RCGYCTTATCMGGCCTAC-3′) (Tm 55.1 °C), respectively [26]. All PCR amplifications were performed in a T-gradient thermocycler (Biometra). The amplification protocol for ERIC-PCR was one step of 95 °C for 5 min, 35 cycles of 92 °C for 45 s, 52 °C for 1 min and 70 °C for 10 min, and a final extension step of 70 °C for 10 min. In the case of REP-PCR, one step of 95 °C for 7 min, 35 cycles of 92 °C for 45 s, 40 °C for 1 min, 72 °C for 8 min and a final

Table 1
Phenotypic characteristics distinguishing *Photobacterium malacitanum* sp. nov., and *P. andalusiense* sp. nov. from phylogenetically related species. (1) *P. malacitanum* sp. nov. (strain H27100402H^T), (2) *P. andalusiense* sp. nov. (strain H01100409B^T), (3) *P. aquimaris* H01100413B, (4) *P. aquimaris* LC2-065^T, (5) *P. iliopiscarium* ATCC 51760^T, (6) *P. kishitanii* ATCC BAA-1194^T, (7) *P. phosphoreum* ATCC 11040^T, (8) *P. piscicola* NCCB 100098^T, (9) *P. toruni* CECT 9189^T. All results from this study.

Characteristics	1	2	3	4	5	6	7	8	9
Oxidase	+	+	+	–	–	–	–	–	+
Growth at:									
4 °C	+	+	+	–	+	+	+	+	+
35 °C	+	w	+	–	–	–	+	w	–
Acetoine production	–	–	–	–	–	+	+	–	+
Arginine dehydrolase	+	+	+	–	+	+	+	+	+
Lysine decarboxylase	+	+	+	–	+	–	+	+	+
ONPG	+	w	+	w	–	–	+	–	–
Urease	+	+	+	–	–	–	–	–	+
Lipase	+	+	+	–	–	–	–	–	+
β-Galactosidase	+	–	+	+	–	+	+	–	–
Amylase	+	+	+	–	–	–	–	–	+
Trypsin	–	–	+	–	–	–	–	–	–
α-Chymotrypsin	+	+	+	–	w	–	w	–	–
α-Galactosidase	–	–	–	+	–	–	–	–	–
Esterase (C4)	+	+	+	–	+	+	+	w	+
Esterase–lipase (C8)	+	+	+	–	w	+	–	w	+
Acids from:									
D-Glucose	+	+	+	–	+	w	–	w	+
D-Trehalose	–	–	–	–	w	–	–	+	–
D-Cellobiose	–	–	–	w	–	–	–	w	–
Assimilation of:									
D-Glucose	+	+	v	+	w	w	–	+	+
D-Mannose	+	+	+	+	–	–	–	+	+
N-Acetylglucosamine	v	v	–	+	+	–	–	+	+
D-Maltose	v	v	–	–	+	–	+	+	–
Malonate	w	w	–	–	–	–	–	+	+

+, positive; –, negative; w, weak; v, variable result depending on the method used.

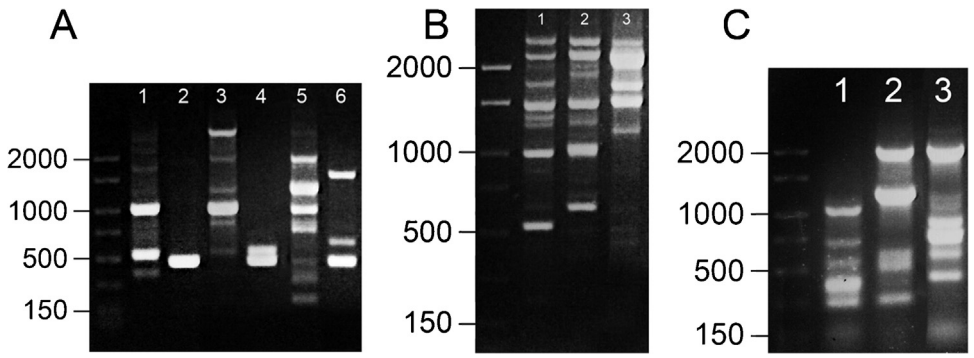

Fig. 1. Genomic differentiation of *Photobacterium* sp. strains H01100409B^T, H0110413B, and H27100402H^T using RAPD (A), REP-PCR (B), and ERIC-PCR (C). A: Lanes 1 and 2; strain H01100409B^T bands profiles using random primers 5 and 4, respectively. Lanes 3 and 4; strain H01100413B bands profiles using random primers 5 and 4, respectively. Lanes 5 and 6; strain H27100402H^T bands profiles using random primers 5 and 4, respectively. B: Lane 1; strain H01100409B^T bands profile. Lane 2; strain H01100413B bands profile. Lane 3; strain H27100402H^T bands profile. C: Lane 1; strain H01100409B^T bands profile. Lane 2; strain H01100413B bands profile. Lane 3; strain H27100402H^T bands profile. Molecular weight marker ranged from 50 to 2000 bp ladder (Sigma-Aldrich).

extension of 72 °C for 15 min. Finally, for RAPD amplifications, one step of 95 °C for 1 min, 30 cycles of 95 °C for 1 min, 35 °C for 1 min, 72 °C for 2 min and a final step of 72 °C for 5 min. DNA extracted from *Escherichia coli* BL21 was used for annealing temperature gradient assays of ERIC-PCR and REP-PCR. The annealing temperatures ranged from 52 to 66 °C and from 40 to 64 °C for ERIC-PCR and REP-PCR, respectively. In all cases, amplification products were analysed by 10 cm horizontal 1% (w/v) agarose gels with TAE (0.04 mol L^{-1} Tris–acetate, 1 mmol L^{-1} EDTA) as electrophoresis buffer. After staining with ethidium bromide, gels were photographed under UV light. A 50- to 2000-bp ladder (Sigma Chemical Co.) was included as molecular weight marker. To determine significant differences in the patterns, reproducibility of results was assessed by repetition in at least three independent RAPD, ERIC-PCR and REP-PCR assays. All the gels were scanned, and the images captured by a GelDoc-2000 gel documentation system (Bio-Rad). The data analysis was performed using the Diversity Database software (Bio-Rad), and a band position tolerance value of 0.5% was allowed to compensate for misalignment of homologous bands because of technical imperfections.

These three fingerprinting methods generated reproducible band patterns, differing in number and molecular mass of amplicons (Fig. 1), which were suitable for an accurate discrimination among the problem strains. According to their RAPD genetic profiles using primer P5, each strain rendered a unique profile, which differed in the number of bands (from 6 to 13) and in their molecular size (between 150 and >2000 bp) (Fig. 1A). In the case of primer P4, again almost each strain rendered a unique profile, presenting 2–4 bands with sizes between 400 and 2000 bp. A common band of about 400 bp was observed in all the profiles (Fig. 1A). REP-PCR amplification rendered unique profiles for each tested strain with the higher number of bands (from 11 to 12) with molecular weights between 250 and >2000 bp (Fig. 1B). With ERIC-PCR procedure, profiles obtained were unique for each strain, showing a pattern formed between 6 and 9 bands whose molecular weights ranged between 150 and >2000 bp (Fig. 1C).

Table 2
Fatty acid composition (%) of *Photobacterium malacitanum* sp. nov. and *P. andalusiense* sp. nov. and related *Photobacterium* species. (1) *P. malacitanum* sp. nov. (strain H27100402H^T), (2) *P. andalusiense* sp. nov. (strain H01100409B^T), (3) *P. aquimaris* H01100413B, (4) *P. aquimaris* LC2-065^T, (5) *P. iliopiscarium* ATCC 51760^T, (6) *P. kishitanii* ATCC BAA-1194^T, (7) *P. phosphoreum* ATCC 11040^T, (8) *P. piscicola* NCCB 100098^T, (9) *P. toruni* CECT 9189^T.

Major fatty acids	1	2	3	4	5	6	7	8	9
C10:0	–	0.2	–	–	–	–	–	–	–
$C_{11:0}$ 3-OH	0.6	–	–	–	–	–	–	–	–
C12:484	–	–	1.1	1.4	0.6	0.7	0.8	0.7	–
$C_{12:0}$ 3-OH	3.8	4.0	8.0	8.1	3.8	4.6	7.1	3.5	4.6
C12:0	5.3	5.4	7.0	6.9	4.4	5.9	6.0	4.7	5.1
C13:0	0.4	0.3	–	–	–	–	–	–	–
C14:0	3.2	3.5	4.4	4.1	2.5	7.3	6.5	2.5	3.2
$C_{15:1}$ *w8c*	0.3	0.2	–	–	–	–	–	–	–
iso-$C_{16:0}$	0.2	–		–	–	–	–	–	–
C16:0	17.6	15.8	12.7	12.3	19.4	20.4	20.9	16.5	20.8
$C_{16:1}$ *w5c*	–	–	–	–	–	–	0.5	–	0.3
$C_{17:1}$ *w8c*	1.1	0.7	–	–	–	–	–	–	0.3
C17:1 *w6c*	0.5	0.6	–	–	–	–	–	–	–
C17:0	2.3	1.4	–	–	3.5	5.2	–	0.9	0.6
$C_{18:1}$ *w7c*	0.4	0.3	9.2	9.6	9.3	5.4	2.5	11.2	0.7
$C_{18:1}$ *w9c*	–	–		–	0.4	1.5	–	–	–
$C_{18:1}$ *w6c*	8.4	9.0		–	–	–	7.1	–	7.1
C18:0	0.6	0.5	–	–	0.8	1.1	0.7	0.7	0.5
Summed features[a]									
1	0.8	0.5	0.3	–	–	–	–	–	0.3
2	5.3	5.3	10.1	10.6	4.2	5.3	8.5	5.7	5.8
3	48.8	52.0	46.8	47.0	50.7	42.6	42.4	52.3	50.4
7	0.4	0.3	0.4	–	0.4	–	–	1.3	0.3

–, trace quantities (<0.1%);

[a] Summed features represent two or three fatty acids that cannot be separated by the MIDI. Summed feature 1 consisted of $C_{13:0}$ 3-OH and iso-$C_{15:1}$ H, summed feature 2 consisted of $C_{14:0}$ 3-OH and iso-$C_{16:1}$ I, summed feature 3 consisted of $C_{16:1}$ *w7c* and $C_{16:1}$ *w6c*, and summed feature 7 consisted of $C_{19:1}$ *w6c*/$C_{19:1}$ *w7c*/and $C_{19:0}$ cyclo *w10c*.

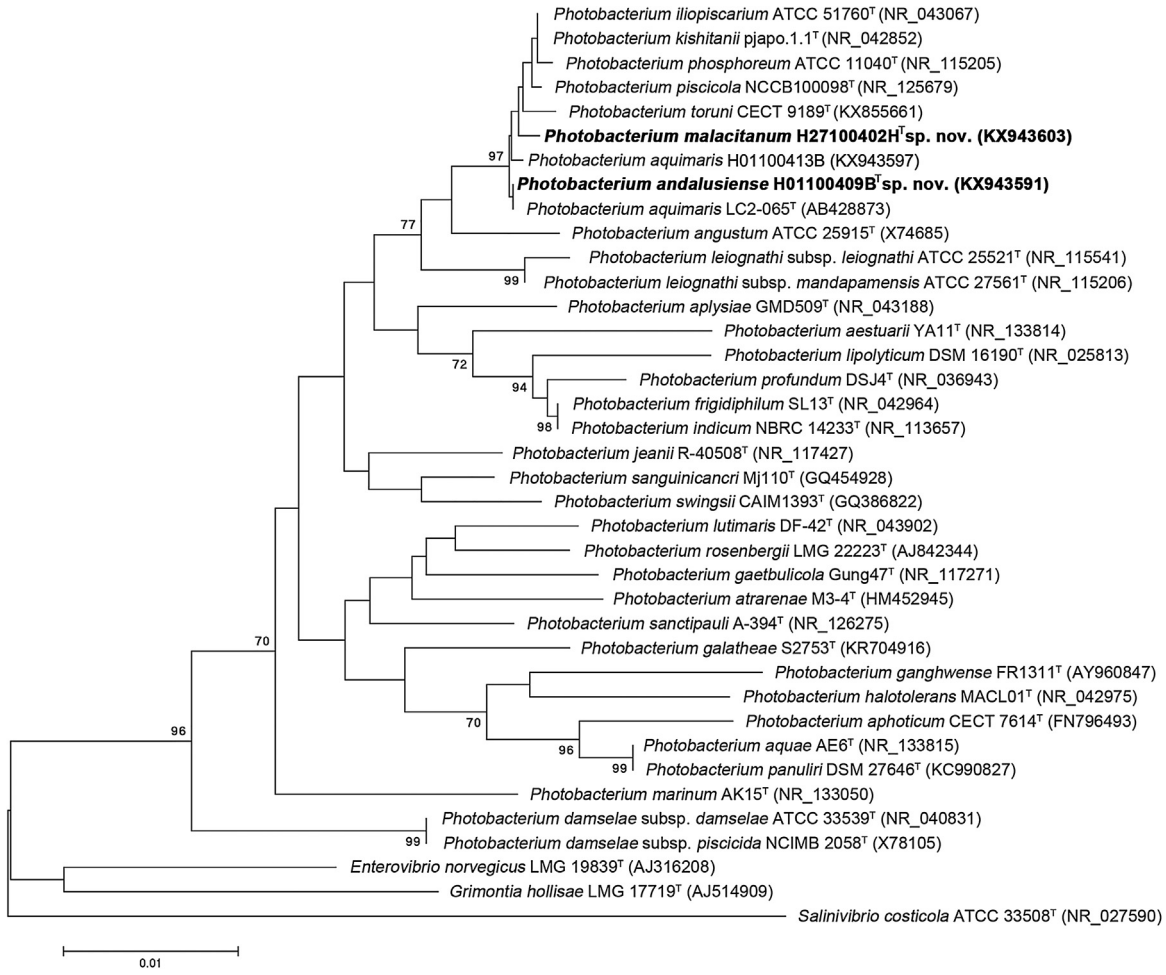

Fig. 2. Neighbor-joining phylogenetic tree based on nearly complete sequences of the 16S rRNA gene of *Photobacterium andalusiense* H01100409B^T, *Photobacterium aquimaris* H01100413B, and *Photobacterium malacitanum* H27100402H^T, plus the type strains of other *Photobacterium* species. *Grimontia hollisae*, *Enterovibrio norvegicus* and *Salinivibrio costicola* type strain sequences have been added as outgroup. Sequence accession numbers are given in parentheses. Bootstrap values greater than 70% confidences are shown at branching points (percentage of 1000 resamplings). Bar indicates number of substitutions per position.

Cellular fatty acids analysis was performed on strains H01100409B^T, H01100413B, and H27100402H^T, and on the reference strains of *P. aquimaris*, *P. iliospicarium*, *P. kishitanii*, *P. phosphoreum*, *P. piscicola*, and *P. toruni*. Strains were grown on TSAS and incubated at 22 °C for 24–48 h. Fatty acid methyl esters were extracted and prepared according to standard protocols as described for the MIDI Microbial Identification System [18]. Cellular fatty acid content was analysed by gas chromatography using an Agilent 6850 chromatographic unit, with the MIDI Microbial Identification System using the TSBA6 method [11], and identified using the Microbial Identification Sherlock software package. The majority of the fatty acids were similar for the tested strains, although differences in their percentages were observed (Table 2). Among others differences it can be pointed that $C_{11:0}$ 3-OH and $C_{16:0}$ iso were present exclusively, as minor component, in strain H27100402H^T, and absent in all other strains tested. Furthermore, $C_{10:0}$ is detected exclusively, as minor component, in strain H01100409B^T (Table 2). Other variations in the FAME profiles were the exclusive presence of $C_{13:0}$, $C_{15:1}$ *w8c* and $C_{17:1}$ *w6c* in strains H01100409B^T and H27100402H^T. The major fatty acids were $C_{16:0}$, $C_{18:1}$ *w6c*, and summed feature 3 ($C_{16:1}$ *w7c*/$C_{16:1}$ *w6c*) (Table 2). Strain H01100413B shared similar FAME profile with *P. aquimaris* LC2-065^T, although it possessed two peaks (summed feature 1 consisting of $C_{13:0}$ 3-OH and iso-$C_{15:1}$ H, and summed feature 7 consisting of $C_{19:1}$ *w6c*/$C_{19:1}$ *w7c*/and $C_{19:0}$ cyclo *w10c*), not found in *P. aquimaris* LC2-065^T (Table 2).

To obtain the MALDI-TOF MS protein profiles of the strains tested, single colonies on the MA dishes were removed with a disposable tip micropippete and resuspended in 50 μL of acetonitrile-trifluoroacetic acid-Milli-Q water solvent (50:2:48), and then mixed 1:1 with matrix solution (saturated a-cyano-4-hydroxy cinnamic acid in the same solvent). 1-μL of sample was spotted in quadruplicate onto a MTP 384-spot AnchorChip target plate BC (Bruker Daltonics) and allowed to air dry. A peptide mixture "Protein calibration Standar I" (Bruker Daltonics) was used as an external standard for mass calibration. The samples were analysed with an Ultraflextreme MALDI-TOF/TOF instrument (Bruker Daltonics). The instrument was operated in positive acquisition mode and controlled by the FlexControl 3.4 software package. All spectra were obtained using the linear mode and covered a mass range of 2–20 kDa, with an acceleration voltage of 25 kV, a linear detector voltage of 2719 kV, and a pulsed ion extraction delay of 250 ns in the positive ion mode. The data was collected from average 5000 laser shots, with the lowest laser energy necessary to obtain sufficient signal to noise ratios. Peak lists were generated from the MS spectra using Bruker FlexAnalysis software (Version 3.4). The results obtained were further processed with MassUp statistical program (www.sing.ei.uvigo.es/mass-up/index.php). Raw data (mzXML) were analysed using MALDIquant, integrated in

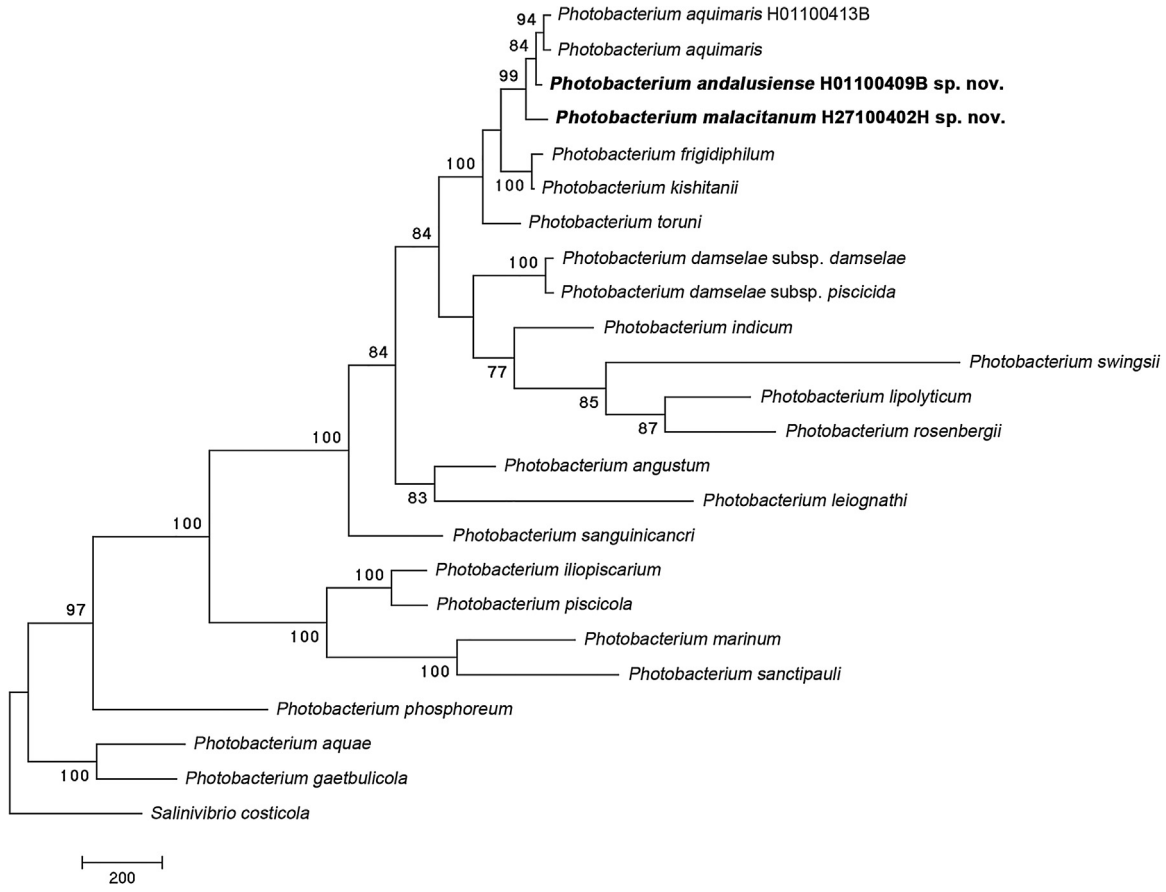

Fig. 3. Maximum Parsimony phylogenetic tree based on 6 concatenated genes (16S rRNA, *gyrB*, *gapA*, *topA*, *ftsZ*, and *mreB*) of strains H01100409B^T, H01100413B and H27100402H^T plus the type strains of other *Photobacterium* species. *Salinivibrio costicola* was included as outgroup. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values greater than 70% confidences are shown at branching points (percentage of 1000 resamplings).

MassUp software. After processing, the two replicate spectra for each bacterial isolate were aligned using the integrated package MALDIquant method. In addition, principal component analysis (PCA) was applied to measure similarity between strains as the result of distances between objects plotted in a three-dimensional space. The PCA was configured with a variance of 0.95, normalized data and discretized [17].

MALDI-TOF MS protein profiles distinguished the tested strains (H27100402H^T, H01100409B^T, and H01100413B) from *P. aquimaris* LC2-065^T, *P. iliopiscarium* ATCC 51760^T, *P. kishitanii* ATCC BAA-1194^T, *P. phosphoreum* ATCC 11040^T, *P. piscicola* NCCB 100098^T, and *P. toruni* CECT 9189^T. MALDI-TOF MS PCA plots indicate that strains H27100402H^T and H01100409B^T, and H01100413B are not clonal (Supplementary Fig. S1).

Strains H01100409B^T, H01100413B, and H27100402H^T were subjected to 16S rRNA gene sequence analysis. The obtained sequences were compared with corresponding sequences of type strains within the family *Vibrionaceae* using alignments retrieved from the latest updates of SILVA and LTP as references [12] and through EzBioCloud identification tool [27]. Alignments were corrected manually based on secondary structure information. Sequence similarities were calculated in ARB without the use of an evolutionary substitution model. Phylogenetic analysis indicates that all strains collected from diseased fish may be considered as members of the genus *Photobacterium*, clustering within the clade Phosphoreum (at a 77% of bootstrap value) (Fig. 2). Data subsets were performed using the appropriate ARB tools [8]. All the novel strains shared a similarity higher than 99% with *P. aquimaris, P. kishitanii, P. piscicola, P. phosphoreum*, *P. iliopiscarium* and *P. toruni.* GenBank accession numbers of 16S rRNA are shown in Fig. 2.

Since the results of the 16S rRNA analysis were inconclusive about the identification of the isolates at the species level, an MLSA scheme was carried out [19]. Genomic DNA extraction, PCR amplification and sequencing of six housekeeping genes were performed according to the methodology previously described [4]: *gapA* (glyceraldehyde-3-phosphate dehydrogenase A), *topA* (DNA topoisomerase I), *mreB* (cell wall structural complex MreBCD), *ftsZ* (GTP-binding tubulin-like cell division protein), *gyrB* (DNA gyrase B subunit), and 16S rRNA. PCR primers used for amplification and sequencing of these genes are listed in Supplementary Table S1. PCR amplification was carried out in a 25-μL reaction mixture containing 5 pmol of each primer, 200 μM of each dNTP, 1× PCR buffer (Promega), 2 mM $MgCl_2$, 1.5 U Bio*Taq* polymerase (Bioline), and 1 μL of bacterial template DNA. PCR amplifications were performed using a Mastercycle thermocyler (Eppendorf). The conditions for 16S rRNA gene amplification were 1 min at 95 °C, 30 cycles of 1 min at 95 °C, 1 min at 50 °C, and 30 s at 72 °C, and a final step of 5 min at 72 °C. For *gapA*, *ftsZ*, and *gyrB* gene amplifications, the thermal program consisted of 2 min at 95 °C, 30 cycles of 1 min at 95 °C, 1 min at 50 °C, and 10 min at 72 °C, and a final step of 5 min at 72 °C. For *mreB* gene amplification, the thermal program consisted of 2 min at 95 °C, 30 cycles of 1 min at 95 °C, 1 min at 55 °C, and 5 min at 72 °C, and a final step of 5 min at 72 °C. Finally, for *topA* gene amplification, the thermal program consisted of 2 min at 95 °C, 30 cycles of 1 min at 95 °C, 1 min at 60 °C, and 5 min at 72 °C, and a final step of 5 min at 72 °C. Amplified products were examined by agarose gel electrophoresis (1.2%) and ethidium bromide staining. Purified

Table 3
Basic features of the genome sequences determined in this study.

Photobacterium species	Assembly accession number	Total size (bp)	Number of contigs	N50 contig length	No. of CDSs	No. of rRNA genes	No. of tRNA genes
P. malacitanum H27100402H^T (CECT 9190^T)	GCA_900185615.1	4,380,538	56	433,037	3785	38	187
P. andalusiense H01100409B^T (CECT 9192^T)	GCA_900185625.1	4,455,676	68	442,834	3878	47	180
P. aquimaris H01100413B (CECT 9191)	GCA_900185565.1	4,306,099	53	550,839	3753	36	189

Table 4
Estimated DNA–DNA hybridization of strains H27100402H^T, H01100409B^T, H01100413B and type strains of other species of the genus *Photobacterium* (clade Phosphoreum). 1, *P. andalusiense* strain H01100409B^T; 2, *P. aquimaris* strain H01100413B; 3, *P. aquimaris* NCCB 100386^T; 4, *P. malacitanum* strain H27100402H^T; 5, *P. piscicola* NCCB 100098^T; 6, *P. toruni* CECT 9189^T; 7, *P. kishitanii* ATCC BAA-1194^T; 8, *P. phosphoreum* ATCC 11040^T; 9, *P. angustum* ATCC 25915^T; 10, *P. leiognathi* ATCC 25521^T and 11, *P. iliopiscarium* ATCC 51760^T.

Strains	1	2	3	4	5	6	7	8	9	10
1										
2	62.1									
3	63.0	76.9								
4	50.3	49.6	49.8							
5	27.2	27.1	27.0	26.4						
6	28.2	28.4	28.2	27.5	32.6					
7	29.4	29.7	29.7	28.9	27.8	28.6				
8	29.2	29.2	29.0	28.3	29.2	29.3	30.6			
9	21.1	21.3	21.3	21.3	21.3	21.0	21.0	21.0		
10	20.9	20.9	20.9	21.0	20.8	20.9	20.8	30.2	20.9	
11	27.1	27.0	26.8	26.4	38.6	31.5	28.1	20.6	24.9	20.5

PCR amplicons were sequenced using a Bigdye Terminator v.3.1 kit (Applied Biosystems) in a 377 DNA sequencer (Applied Biosystems) and Seqman v5.53 (DNASTAR). Phylogenetic analysis based on the individual 16S rRNA gene and concatenated gene sequences was carried out using MEGA 6.0 software [20]. Distance and clustering with Maximum Likelihood (ML), Neighbour-Joining (NJ), and Maximum Parsimony (MP) methods were determined using bootstrap values based on 1000 replications. ML and NJ analyses were carried out based on the Jukes–Cantor model [3]. For MP analyses, the tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm [13].

GenBank accession numbers of gene sequences used in this study are listed in Supplementary Table S2. The phylogenetic tree obtained (3700 nt concatenated sequence lenght) of six housekeeping genes (16S rRNA, *gapA*, *topA*, *mreB*, *ftsZ*, and *gyrB*) using 1000 replications support the tightness of the strains H01100409B^T, H01100413B, and H27100402H^T and clustered together in a branch with *P. aquimaris* with a bootstrap value of 99% (Fig. 3).

The whole genome of the novel strains H01100409B^T, H01100413B, and H27100402H^T, were sequenced at Central Service of Support to Experimental Research (SCSIE) of the University of Valencia (Valencia, Spain) using an Illumina MiSeq technology with 2 × 250 paired-end reads with above 100× sequencing coverage. Genome assembly was performed using SPAdes 3.6 [14]. The resulting draft genome sequence was annotated with Rapid Annotations using Subsystems Technology (RAST Server) [1]. For the quality assessment and applicability of the genome sequences, the recently proposed minimal standards [2] dealing with their use for the taxonomy of prokaryotes have been taken into consideration. The basic features of the genome sequences determined in this study are shown in Table 3. The DNA–DNA hybridization (DDH) was calculated *in silico* by the Genome-to-Genome Distance Calculator (GGDC 2.0) using the BLAST method [10]. Results were based on recommended formula 2 (identities/HSP length), which is independent of genome length and is thus robust against the use of incomplete draft genomes. DNA GC mol % was obtained from the genomic sequences. Calculation of the average identity (ANI) according to MUMmer (ANIm) and BLAST (ANIb) was performed in JSpecies WS [15].

DNA–DNA hybridations (DDH) comparison with the draft genome of the type strain H27100402H^T (*P. malacitanum* sp. nov. proposed) yielded low percentages (<30%) with all the species tested, excepting with the strain of *P. aquimaris* NCCB 100386^T (49.8%) (Table 4). The ANIb and ANIm between the type strain H27100402H^T and the other species tested were lower than 90%, excepting in the case of *P. aquimaris* NCCB 100386^T (92.6 and 93.0%, respectively) (Table 5).

In the case of the strain H01100409B^T (*P. andalusiense* sp. nov. proposed), DNA-DNA hybridations (DDH) comparison with the draft genome of the type strain H01100409B^T yielded low percentages (<30%) with all the species tested, excepting with *P. aquimaris* NCCB 100386^T (63.0%) (Table 4). The ANIb and ANIm between the type strain H01100409B^T and the other species tested were lower than 90%, excepting in the case of *P. aquimaris* NCCB 100386^T (94.7 and 95.5%, respectively) (Table 5). Finally, the DNA–DNA hybridations (DDH) comparison with the draft genome of the strain H01100413B yielded percentages of 76.9% with *P. aquimaris* NCCB 100386^T (considering as the same species), and percentages lower than 30% for the other species tested (Table 4). The ANIb and ANIm between the strain H01100413B and the other species tested were lower than 90%, excepting in the case of *P. aquimaris* NCCB 100386^T (96.8 and 97.4%, respectively) (Table 5).

Currently it is recommended to rely on overall genomic indexes to delineate species (strains from the same species share at least 70% *in silico* DDH or above 95–96% ANI) [2,10,15,21,22], since they are more accurate than traditional wet lab approaches. The DDH between the problem strains was: H27100402H^T (*P. malacitanum* sp. nov. proposed) vs H01100409B^T (*P. andalusiense* sp. nov. proposed) = 50.3% (Table 4). H27100402H^T (*P. malacitanum* sp. nov. proposed) vs H01100413B (*P. aquimaris*) = 49.6%, and H01100409B^T (*P. andalusiense* sp. nov. proposed) vs H01100413B (*P. aquimaris*) = 62.1% (Table 4). The ANIb and ANIm between strains H27100402H^T (*P. malacitanum* sp. nov. proposed) vs H01100409B^T (*P. andalusiense* sp. nov. proposed) were 92.5 and 93.1%, respec-

Table 5
Average nucleotide identity percentages based on BLAST (ANIb) and on MUMmer (ANIm) of strains H27100402H^T, H01100409B^T, H01100413B and type strains of other species of the genus *Photobacterium* (clade Phosphoreum). Taxa indicated as: 1, *P. andalusiense* strain H01100409B^T; 2, *P. aquimaris* strain H01100413B; 3, *P. aquimaris* NCCB 100386^T; 4, *P. malacitanum* strain H27100402H^T; 5, *P. piscicola* NCCB 100098^T; 6, *P. toruni* CECT 9189^T; 7, *P. kishitanii* ATCC BAA-1194^T; 8, *P. phosphoreum* ATCC 11040^T; 9, *P. angustum* ATCC 25915^T; 10, *P. leiognathi* ATCC 25521^T and 11, *P. iliopiscarium* ATCC 51760^T.

		Values of ANIb										
		1	2	3	4	5	6	7	8	9	10	11
ANIm values	1		95.1	94.7	92.5	83.5	84.0	85.2	85.0	75.9	75.5	83.5
	2	95.4		96.8	92.3	83.4	84.0	85.3	85.1	75.8	75.5	83.3
	3	95.5	97.4		92.6	83.5	84.2	85.2	85.1	75.8	75.5	83.4
	4	93.1	93.0	93.0		83.0	83.6	84.6	84.6	75.9	75.4	83.6
	5	86.5	86.5	86.5	86.2		86.7	83.9	84.8	75.7	75.1	89.4
	6	87.0	87.1	87.1	86.7	88.3		84.4	85.0	75.8	75.6	86.3
	7	87.1	87.2	87.2	86.9	86.4	86.8		85.5	75.5	75.0	83.8
	8	86.9	87.0	86.9	86.6	87.2	87.1	87.3		75.4	74.9	85.5
	9	85.3	85.4	85.4	85.4	85.1	85.0	85.1	84.7		81.6	75.4
	10	84.8	84.7	84.8	85.2	84.7	84.6	84.6	84.6	86.2		75.0
	11	86.4	86.4	86.3	86.1	90.3	87.8	86.6	87.6	84.9	84.6	

tively; the ANIb and ANIm between strains H27100402H^T (*P. malacitanum* sp. nov. proposed) vs H01100413B (*P. aquimaris*) were 92.3 and 93.0%, respectively; values clearly below the threshold. On the contrary, the ANIb and ANIm between strains H01100409B^T (*P. andalusiense* sp. nov. proposed) vs H01100413B (*P. aquimaris*) were 95.1 and 95.4%, values closer to the 95–96% threshold. Based on the polyphasic analysis including DDH, ANIb and ANIm, MLSA, MALDI-TOF MS fingerprint profiles, chemotaxonomic and other phenotypic tests presented in this study, we propose to classify the tested isolates as two new species, *Photobacterium malacitanum* sp. nov. (type strain H27100402H^T) and *Photobacterium andalusiense* sp. nov. (type strain H01100409B^T).

Description of *Photobacterium malacitanum* sp. nov.

Photobacterium malacitanum (ma.la.ci.ta'num. L. neut. adj. malacitanum, pertaining to Malaga).

Cells are Gram-negative rods, motile, chemoorganotrophic and facultative anaerobe. They grow on Tryptic Soy agar supplemented with 1.5% NaCl (TSAS) producing regular colonies after 48–72 h at 22 °C without diffusible pigment production. Good growth occurs at 4 °C and 35 °C and weakly at 45 °C. It shows optimal growth on 0.5–8% total salts. Glucose is fermented with weak production of gas. Oxidase and catalase are positive. Reduces nitrate to nitrite, but not to N_2. It is positive for arginine dihydrolase (ADH), lysine decarboxylase (LCD), urease, amylase, acid- and alkaline phosphatases, α-chimotrypsin, phosphohydrolase, esterase, esterase–lipase, leucine-arylamidase, α- and β-galactosidase, and N-acetyl-β-glucosaminidase; but negative for indole production from tryptophan, ornithine decarboxylase (ODC), trypsin, hydrolysis of gelatine and esculin, α- and β-glucosidase, β-glucuronidase, α-maltosidase, α-mannosidase, α-fucosidase, L-aspartic acid arylamidase, valine aryalminase and cystine arylaminase. Produces acids from the following carbohydrates: D-glucose and D-maltose; in contrast, the reactions of fermentation of L- and D-arabitol, D-mannitol, adonitol, palatinose, sucrose, melibiose, L-arabinose, D-trehalose, L-rhamnose, inositol, D-cellobiose, D-sorbitol, phenol red, galacturonate and 5-ketoglutarate are negative. Variable results were obtained for lipase test according to the method used.

The assimilation of the substrates included on API 20NE and Biolog GN galleries show a variable pattern, it is able to grow on dextrin, N-acetyl D-galactosamine, N-acetyl D-glucosamine, D-cellobiose, D-fructose, D-galactose, α-D-lactose, lactulose, maltose, D-mannose, D-melibiose, D-psicose, methyl piruvate, cis-aconitic acid, formic acid, α-ketobutyric acid, α-ketoglutaric acid, D,L-lactic acid, succinic acid, bromo succinic acid, L-alaninamide, L-asparagine, L-aspartic acid, glycil-L-aspartic acid, L-serine, inosine, uridine, thymidine, glycerol, D,L-α-glycerol phosphate, α-D-glucose-1-phosphate and D-glucose-6-phosphate. On the contrary, it is negative for the assimilation of L-arabinose, i-erythritol, β-methyl-D-glucoside, xylitol, D-galactonic acid lactone, γ-hydroxybutiric acid, p-hydroxyphenylacetic acid, itaconic acid, α-ketovaleric acid, sebacic acid, and urocanic acid. Other substrates give weak positive results.

The most abundant cellular fatty acids are $C_{16:0}$, summed in feature 3 ($C_{16:1\ w}7c/C_{16:1\ w}6c$), and $C_{18:1\ w}6c$.

The DNA GC content of the type strain obtained from its draft genome sequence is 39.8%.

The type strain was isolated during one episode of mortality affecting captived redbanded seabream specimens in Southatlantic coast of Spain. The type strain is H27100402H^T (= CECT 9190^T = LMG 29992^T) isolated from liver samples of diseased fish in an outbreak of autumn 2014.

Description of *Photobacterium andalusiense* sp. nov.

Photobacterium andalusiense (an.da.lu.si.en'se.N.L. neut. adj. andalusiense, pertaining to Andalusia).

Cells are Gram-negative rods, motile, chemoorganotrophic and facultative anaerobe. They grow on Tryptic Soy agar supplemented with 1.5% NaCl (TSAS) producing regular colonies after 48–72 h at 22 °C without diffusible pigment production. Good growth occurs at 4 °C, 20 °C and 30 °C, weak at 35 °C, but not at 45 °C. It shows good growth on 0.5–8% total salts. Glucose is fermented with production of gas. Oxidase and catalase are positive. Reduces nitrate to nitrite, but not to N_2. It is positive for arginine dihydrolase (ADH), lysine decarboxylase (LCD), urease, amylase, acid- and alkaline phosphatases, α-chimotrypsin, phosphohydrolase, esterase, esterase-lipase, leucine-arylamidase, α-galactosidase, and N-acetyl-β-glucosaminidase; but negative for indole production from tryptophan, ornithine decarboxylase (ODC), trypsin, hydrolysis of gelatine and esculin, β-galactosidase, α- and β-glucosidase, β-glucuronidase, α-maltosidase, α-mannosidase, α-fucosidase, L-aspartic acid arylamidase, valine arylaminase and cystine arylaminase. Produces acids from the following carbohydrates: D-glucose and D-maltose; however, the reactions of fermentation of L- and D-arabitol, D-mannitol, adonitol, palatinose, sucrose, melibiose, L-arabinose, D-trehalose, L-rhamnose, inositol, D-cellobiose, D-sorbitol, phenol red, galacturonate and 5-ketoglutarate are negative. Variable results are obtained for lipase test according to the method used.

The assimilation of the substrates included on API 20NE and Biolog GN galleries show a variable pattern, it is able to grow on dextrin, N-acetyl D-galactosamine, N-acetyl D-glucosamine, D-cellobiose, D-fructose, D-galactose, α-D-lactose, lactulose, maltose, D-mannose, D-melibiose, D-psicose, methyl piruvate, cis-aconitic

acid, formic acid, α-hydroxybutiric acid, α-ketobutyric acid, α-ketoglutaric acid, D,L-lactic acid, succinic acid, bromo succinic acid, L-alaninamide, L-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycil-L-aspartic acid, L-serine, L-threonine, inosine, uridine, thymidine, glycerol, D,L-α-glycerol phosphate, α-D-glucose-1-phosphate and D-glucose-6-phosphate. On the contrary, it is negative for the assimilation of β-methyl-D-glucoside, itaconic acid, and sebacic acid. The other substrates tested give weak positive results.

The most abundant cellular fatty acids are $C_{16:0}$, summed in feature 3 ($C_{16:1\ w}7c/C_{16:1\ w}6c$), and $C_{18:1\ w}6c$.

The DNA GC content of the type strain obtained from its draft genome sequence is 39.5%.

The type strain was isolated during one episode of mortality affecting captived redbanded seabream specimens in Southatlantic coast of Spain. The type strain is H01100409B^T (=CECT 9192^T = LMG 29994^T) isolated from spleen samples of diseased fish in an outbreak of autumn 2014.

Acknowledgements

The authors thank Carolina Lobo from Unidad de Proteómica (SCI, University of Malaga) for her technical assistance in the MALDI-TOF-MS analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2018.04.005.

References

[1] Aziz, R.K., Bartels, D., Best, A.A., Dejonh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., McNeil, L.K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G.D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., Zagnitko, O. (2008) The RAST Server: rapid annotations using subsystems technology. BCM Genomic 9, 75.

[2] Chun, J., Oren, A., Ventosa, A., Christensen, H., Arahal, D.R., da Costa, M.S., Rooney, A.P., Yi, H., Xu, X.-W., De Meyer, S., Trujillo, M.E. (2018) Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. Int. J. Syst. Evol. Microbiol. 68 (1), 461–466, art. no. 002516.

[3] Jukes, T.H., Cantor, C.R. (1969) Evolution of protein molecules. In: Munro, H.N. (Ed.), Mammalian Protein Metabolism, Academic Press, New York, pp. 21–132.

[4] Labella, A., Vida, M., Alonso, M.C., Infante, C., Cardenas, S., Lopez-Romalde, S., Manchado, M., Borrego, J.J. (2006) First isolation of *Photobacterium damselae* ssp. *damselae* from cultured redbanded seabream, *Pagrus auriga* Valenciennes, in Spain. J. Fish Dis. 29, 175–179.

[5] Labella, A., Manchado, M., Alonso, M.C., Castro, D., Romalde, J.L., Borrego, J.J. (2010) Molecular intraspecific characterization of *Photobacterium damselae* ssp. *damselae* strains affecting cultured marine fish. J. Appl. Microbiol. 108, 2122–2132.

[6] Labella, A.M., Arahal, D.R., Castro, D., Lemos, M.L., Borrego, J.J. (2017) Revisiting the genus *Photobacterium*: taxonomy, ecology and pathogenesis. Int. Microbiol. 20, 1–10.

[7] Labella, A.M., Arahal, D.R., Lucena, T., Manchado, M., Castro, D., Borrego, J.J. (2017) *Photobacterium toruni* sp nov., a bacterium isolated from diseased farmed fish. Int. J. Syst. Evol. Microbiol. 67, 4518–4525.

[8] Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., et al. (2004) ARB: a software environment for sequence data. Nucleic Acids Res. 32, 1363–1371.

[10] Meier-Kolthoff, J.P., Auch, A.F., Klenk, H.P., Göker, M. (2013) Genome sequence-based species delimitation with confidence intervals and improved distance functions. BCM Bioinform. 14, 60.

[11] MIDI, 2008 Sherlock Microbial Identification System Operating Manual, Version 6.1, MIDI Inc., Newark, DE, USA.

[12] Munoz, R., Yarza, P., Ludwig, W., Euzéby, J., Amann, R., Schleifer, K.H., Glöckner, F.O., Roselló-Móra, R. (2011) Release LTPs104 of the all-species living tree. Syst. Appl. Microbiol. 34, 169–170.

[13] Nei, M., Kumar, S. 2000 Molecular Evolution and Phylogenetics, Oxford University Press, New York, NY, USA.

[14] Nurk, S., Bankevich, A., Antipov, D., Gurevich, A., Korobeynikov, A., Lapidus, A., Prjibelsky, A., Pyshkin, A., Sirotkin, A., Sirotkin, Y., Stepanauskas, R., McLean, J., Lasken, R., Clingenpeel, S.R., Woyke, T., Tesler, G., Alekseyev, M.A., Pevzner, P.A. (2013) Assembling genomes and mini-metagenomes from highly chimeric reads, in: Deng, M., Jiang, R., Sun, F., Zhang, X. (Eds.), Lecture Notes in Computer Science, vol. 7821, Springer-Verlag, Berlin, pp. 158–170.

[15] Richter, M., Roselló-Mora, R. (2009) Shifting the genomic gold standard for the prokaryotic species definition. Proc. Natl. Acad. Sci. U. S. A. 106, 19126–19131.

[16] Rivas, A.J., Lemos, M.L., Osorio, C.R. (2013) *Photobacterium damselae* subsp. *damselae*, a bacterium pathogenic for marine animals and humans. Front. Microbiol. 4, 283–289.

[17] Santos, T., Capelo, J.L., Santos, H.M., Oliveira, I., Marinho, C., Gonçalves, A., Araújo, J.E., Poeta, P., Igrejas, G. (2015) Use of MALDI-TOF mass spectrometry fingerprinting to characterize *Enterococcus* spp. and *Escherichia coli* isolates. J. Proteomics 127, 321–331.

[18] Sasser, M. 1990 Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids, MIDI Technical Note 101, MIDI Inc., Newark, DE, USA.

[19] 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.

[20] Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

[21] Thompson, C.C., Vicente, A.C.P., Souza, R.C., Vasconcelos, A.T.R., Vesth, T., Alves, N., Ussery, D.W., Iida, T., Thompson, F.L. (2009) Genomic taxonomy of Vibrios. BCM Evol. Biol. 9, 258.

[22] Thompson, C.C., Chimetto, L., Edwards, R.A., Swings, J., Stackebrandt, E., Thompson, F.L. (2013) Microbial genomic taxonomy. BCM Genomic 14, 913.

[24] Thyssen, A., Ollevier, F. (2005) Genus II. *Photobacterium*. In: Brenner, D.L., Krieg, N.R., Staley, J.T. (Eds.), Bergey's Manual of Systematic Bacteriology, 2nd ed. Vol. 2 The Proteobacteria, Part B The Gammaproteobacteria, Springer, New York, pp. 546–562.

[25] Urbanczyk, H., Ast, J.C., Dunlap, P.V. (2011) Phylogeny, genomics, and simbiosis of *Photobacterium*. FEMS Microbiol. Rev. 35, 324–342.

[26] Versalovic, J., Schneider, M., de Bruijn, F.J., Lupski, J.R. (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol. Cell Biol. 5, 25–40.

[27] Yoon, S.H., Ha, S.M., Kwon, S., Lim, J., Kim, Y., Seo, H., Chun, J. (2017) Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int. J. Syst. Evol. Microbiol. 67, 1613–1617.

[28] Yoshizawa, S., Wada, M., Kita-Tsukamoto, K., Yokota, A., Kogure, K. (2009) *Photobacterium aquimaris* sp nov., a luminous marine bacterium isolated from seawater. Int. J. Syst. Evol. Microbiol. 59, 1438–1442.

Further reading

[9] Marchesi, J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J., Dymock, D., Wade, W.G., (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Environ. Microbiol. 64, 795–799.

[23] Thompson, F.L., Thompson, C.C., Naser, S., Hoste, B., Vandemeulebroecke, K., Munn, C., Bourne, D., Swings, J., (2005) *Photobacterium rosenbergii* sp. nov. and *Enterovibrio coralii* sp. nov., vibrios associated with coral bleaching. Int. J. Syst. Evol. Microbiol. 55, 913–917.