Virulence properties of three new *Photobacterium* species affecting cultured fish

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Abstract

Aims: Several virulence factors of three new *Photobacterium* species: *Photobacterium toruni*, *Photobacterium malacitanum* and *Photobacterium andalusiense* associated with diseases of cultured redbanded seabream (*Pagrus auriga*) were studied. The exoenzymatic activities, adherence and cytotoxic capabilities, and iron-uptake mechanisms were determined both in bacterial extracellular products (ECP) and whole bacterial cells. The histopathology damages provoked on redbanded seabream by the ECP was also studied.

Methods and Results: The highest exoenzymatic activities of the ECP were alkaline- and acid-phosphatase, phosphohydrolase and lipase. The ECP were strongly lethal for fish at 4–96 h post-inoculation (p.i). Histological changes were evident at 96 hpi of ECP, affecting head kidney, splenic parenchyma and heart. Cytotoxicity assays, on three fish lines and one human cell line, were conducted using whole bacterial cells and their ECP. The new species tested were cytotoxic only for fish cell lines using whole bacterial cells. Bacterial adherence showed an adherence index moderate on CHSE-214 cell line. All strains showed variable haemolytic activity, and were able to grow under iron- limiting conditions, although the CAS reactivitiy was very low. However, all strains produced high amounts of extracelullar citrate that could be used as iron carrier, and use haem as iron source, except the *P. toruni* strains because a deletion in the genomic region encoding this ability in all Vibrionaceae members.

Conclusions: The toxic activity of the bacterial ECPs was thermolabile, and not associated with their thermoresistant lipopolysaccharide content. The virulence of the strains tested could not be related to the haemolytic activity. Iron uptake could be based on the use of endogenous citrate as iron carrier and *P. toruni* lacks the ability to use haem as iron source.

Significance and Impact of the study: The study analyses for the first time the virulence properties of three new species of *Photobacterium* pathogenic for fish.

Introduction

The ability of bacteria to cause disease depends mainly on the expression of virulence factors, which help them to invade the host, produce pathological effects and evade host defences (Austin and Austin 2016). Bacterial diseases are the most important causes of economic losses among fish and shellfish aquaculture industry,

and thus the knowledge of the bacterial virulence factors is essential for the development of new immunoprophylactic and chemotherapeutic treatments against the bacterial infections (Cole *et al.* 2009). The genus *Photobacterium* comprises more than 35 species detected worldwide in aquatic ecosystems and shows substantial ecophysiological diversity including free-living, symbiotic, piezophilic and parasitic life styles (Labella *et al.* 2017a). Some species of this genus, including *Photobacterium rosenbergii*, *Photobacterium jea nii* and *Photobacterium sanctipauli*, have been reported to produce several pathologies on animal hosts, such as corals, sponges and zoanthids (Thompson *et al.* 2005; Chimetto *et al.* 2010; Moreira *et al.* 2014). Only *Photobacterium damselae* subspecies have received a great attention as emerging pathogens for many aquatic organisms, including fish, molluscs, crustaceans and even for humans (Labella *et al.* 2011; Rivas *et al.* 2013a; Moi *et al.* 2017). *Photobacterium damselae* subsp. *damselae* is considered a primary pathogen of several species of wild- and cultured-fish causing wound infections and haemorrhagic septicaemia. It is also an opportunistic human pathogen, causing necrotizing fasciitis (Yamane *et al.* 2004; Rivas *et al.* 2013a). The other subspecies, *P. damselae* subsp. *piscicida*, is the causal agent of fish photobacteriosis, a serious bacterial disease affecting different economically important cultured marine fish species (Lopez-Doriga *et al.* 2000; Romalde 2002).

Marine fish farming is a very important activity of European aquaculture industry. The main marine fish species intensively cultured are gilthead seabream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*) and turbot (*Scophthalmus maximus*). In last 15 years, several new marine fish species are being evaluated as potential candidates for aquaculture production. In Southern Spain, studies on the reproductive cycles, nutrition, growth and immune system of species such as Senegelese sole (*Solea senegalensis*), redbanded seabream (*Pagrus auriga*), common seabream (*Pagrus pagrus*), white seabream (*Diplodus sargus*) and meagre (*Argyrosomus regius*) are ongoing (Cardenas and Manchado 2008). However, the intensive culture of these fish species has favoured the appearance of bacterial outbreaks with high and moderate mortalities (Labella *et al.* 2006; Garcia-Rosado *et al.* 2007). Recently, three new species of *Photobacterium* have been described, *Photobacterium toruni*, *Photobacterium malacitanum* and *Photobacterium andalusiense*, isolated from disease outbreaks affecting cultured redbanded seabream in South of Spain (Labella *et al.* 2017b, 2018), which constitute, together with *P. damselae*, the unique species of this genus pathogenic for fish. However, the virulence properties of these newly described species until now remain unknown.

The aim of this work was to study the main virulence factors present in strains of *P. toruni*, *P. malacitanum* and *P. andalusiense* determining their exoenzymatic activities, adherence and cytotoxic capabilities, as well as their iron- uptake mechanisms. In addition, we have analysed the histopathological damages on fish provoked by raw ECP of the strains tested.

Materials and methods

Bacterial strains and culture media

Five *Photobacterium* strains, strains 410B^T, 403B and 405 H belonging to *P. toruni* CECT 9189^T, *P. malacitanum* CECT 9190^T strain 402H and *P. andalusiense* CECT 9192^T strain 409B, associated with epizootic outbreaks affecting cultured redbanded seabream (*P. auriga*) fish species were used. All the strains were obtained from internal organ lesions and were phenotypically and genotypically characterized as described previously (Labella *et al.* 2017b, 2018). *Photobacterium damselae* subsp. *damselae* strain ATCC 33539^T and *P. damselae* subsp. *piscicida* strain ATCC 51736^T were used for comparative purposes. Bacterial strains were routinely cultured on tryptic soy agar or broth (TSA or TSB) (Difco Laboratories, Detroit, MI) supplemented with 1·5% (w/v) NaCl (TSAs or TSBs respectively), and incubated at 20–22°C for 2–5 days. Stock cultures were stored frozen at —80°C in TSBs with 15% (v/v) glycerol. Growth ability in iron deficient conditions was examined in CM9 minimal medium and incubated at 25°C (Lemos *et al.* 1988). Differential iron availability conditions were achieved by adding (final concentrations) $FeCl_3$ 10 lmol (iron excess conditions), or increasing concentrations of the iron chelator 2,2'- dipyridyl (TCI) (iron deficient conditions which mimic the low iron conditions found within the host).

Extracellular products (raw ECPs) extraction

Raw bacterial ECPs were obtained by the cellophane plate technique (Liu 1957) by spreading 3-ml of a 48 h broth culture of each strain over sterilized cellophane sheets placed on TSAs plates. After incubation for 48 h at 20– 22°C, bacterial cells were washed off the cellophane sheets with phosphate-buffered saline (PBS) at pH 7·0. The bacterial suspensions were centrifuged at 13 000 g for 20 min at 4°C, and the respective supernatants were filtered through 0·45 lm pore-size membrane filters. All the ECP samples were inoculated in TSAs plates to verify their sterility and then stored at —20°C with glycerol (J.T. Baker, Deventer, Holland) until used. The protein concentration of the ECPs was spectrophotometrically determined using bicinchoninic acid (Sigma-Aldrich, St. Louis, MO) and bovine serum albumin (Sigma-Aldrich) as standard. To evaluate the total proteolytic activity present in the ECP samples, a multiprotein substrate (Azocoll; Sigma-

Aldrich) was used following manufacturer's instructions. An absorbance reading of 1·0 at 520 nm, after a 30-min assay at 37°C, was defined as one unit of protease activity.

In vivo toxicity of bacterial raw ECPs

The toxicity of the bacterial ECP of each strain was evaluated by intra-peritoneal inoculation of 0·1 ml of each ECP sample in groups of 20 redbanded seabream specimens (5–10 g in weight). Fish were acclimated for a week before starting the experiment. The water temperature was 22 1°C and salinity 35–37 g l^{-1}. The fish were maintained under natural photoperiod conditions, and fed a commercial pellet diet (Skretting, Burgos, Spain) administrated once per day at a rate of 1% fish biomass. Each inoculated fish group was monitored at 4, 24, and 96 hpid periods, and the samples were used for histopathological purposes. Sets of animals (n = 20) inoculated with sterile PBS (pH 7·4) were included in the trials as negative controls. ECPs heated at 100°C for 10 min were also assayed in parallel. For histological examinations, a total of 9 dead or euthanized fish by a MS-222 (Sigma-Aldrich) overdose (100 mg ml^{-1}), were fixed in 10% neutral buffer formalin solution, embedded in paraffin, sectioned at 5 lm. Selected tissues analysed were kidney, liver, spleen and heart, which were stained routinely with haematoxylin and eosin (HE) and examined using light microscope.

All procedures were carried out under the Spanish directive (RD 1201/2005) for the protection of animals used in scientific experiments, and given the registration number 10-06-2016-102 by the Spanish authorities for the regulation of animal care and experimentation.

Determination of enzymatic activities

The enzymatic activities presented in the raw ECPs and in bacterial cultures were evaluated using the API ZYM system (bioMerieux, Marcy l'Etoile, France). Other enzymatic activities, such as caseinase, gelatinase, amylase, phospholipase and lipase, were determined in plates by the diffusion method. Plate dishes of saline basal nutrient agar (Difco) (1·5% NaCl, w/v) were supplemented with 1% (w/v) sodium caseinate (Difco), 1% (w/v) gelatin (Oxoid, Basingstoke, Hampshire), 0·4% (w/v) starch (Difco), 2% (v/v) egg yolk emulsion (Sigma-Aldrich) and 1% (v/v) Tween-20/Tween-80 (Sigma-Aldrich) respectively. In addition, commercial deoxyribonuclease test medium (Biolife, Milano, Italy) was used for the DNase test. To determine the heat stability, all the enzymatic activities above mentioned were also tested after treatment of ECP samples at 100°C for 10 min.

Cytotoxic activity of whole cells and raw ECPs

Cytotoxicity assays were basically carried out as described by Wang *et al.* (1998) on mammalian and fish cell lines. The human epithelial cell line HT-1080 was cultured and maintained using Dulbecco's modified Eagle's medium (HyClone Laboratories, Logan, UT) supplemented with 4 mmol L-glutamine (Sigma-Aldrich), bovine calf serum (Gibco, Carlsbad, CA) to a final concentration of 10% (v/v) and 1% (v/v) penicillin–streptomycin solution (Gibco). Cell cultures were incubated in 5% CO_2 at 37°C and 95% humidity. In addition, three different fish cell lines were tested: SAF-1, a fibroblastic cell line from gilthead seabream *S. aurata* (Bejar *et al.* 1997); BF-2, a fibroblastic cell line from bluegill fry *Lepomis macrochirus* (Wolf *et al.* 1966); and CHSE-214, an epithelial cell line from Chinook salmon *Oncorhynchus tshawytscha* (Fryer *et al.* 1965). Fish cells were cultured and maintained using Leibovitz medium (L-15; Gibco), supplemented with 2% (v/v) L-glutamine solution, 1% (v/v) penicillin– streptomycin solution and 10% (v/v) foetal bovine serum (Cambrex). Two different incubation temperatures in 5% CO_2 atmosphere were used for the maintenance and growth of the cell cultures: 25°C for SAF-1 and BF-2, and 20°C for the CHSE-214 cell line.

For the cytotoxicity assay, four 96-well plates were pre- pared for each cell line and bacterial strain. Cell cultures were inoculated with 25 ll of whole cells (10^5 CFU per ml), their ECP or ECP heated (100°C, 10 min) samples (1 mg ml^{-1}). PBS (pH 7·4) was used as negative control. Each experimental condition was assayed in triplicate. The development of cytotoxic effects was observed at 6, 24 and 48 hpi using a phase-contrast inverted microscope (Nikon) at 2009 magnification.

Adherence tests

For these assays the fish cell lines SAF-1, BF-2 and CHSE-214 were used following the methodology described by Pan *et al.* (2008). The culture and incubation conditions of cell lines were the above described pre viously until the semi-confluent monolayer formation on the well-plates (about 24–36 h). Two hours previous to the assay, the culture medium was removed and washed with PBS, and the cells were cultured using L-15 without antibiotic supplement. Then, each well was inoculated with 10^8 colony-forming units (CFU per ml) of each bac terial strain and incubated at 20°C for 1 h. Then, the culture medium was removed from wells and three washed steps with PBS were carried out to remove the non-adhered bacterial cells and bacterial debris. The cell line

monolayers were lysated with a 1% aqueous solution Tri- ton X-100 for 5 min, and the lysate supernatant was serially diluted and plated on TSAs plates to determine the adherence index (AI) of each strain on each fish cell line. The bacterial adherence was recorded as high (AI > 10), intermediate (1 < AI < 10) or non-adherent (AI < 1).

Haemolytic activity

The production of haemolysins was assayed on Mueller- Hinton agar (Oxoid) supplemented with 5% (v/v) sheep, and defibrinated blood from gilthead seabream, common seabream and redbanded seabream. In all the tests, ali quots of 5 ll of each ECP (raw and heated) or exponential growth bacterial cultures in TSBs were placed on the plates and incubated at 22°C for 24–48 h, following the methodology described by Balebona *et al.* (1998a). Growth under iron limiting conditions, haem utilization, siderophore production and citrate accumulation. To test the ability of each *Photobacterium* strain to grow under iron-limiting conditions, overnight cultures in TSBs were adjusted to an OD_{600} of 0·5 and diluted 1 : 100 in CM9 minimal medium containing increasing concentrations (25, 50, 100 and 150 μmol) of the iron chelator 2,2'-dipyridyl. When required, ferric chloride, ferric-ammonium citrate (Panreac, Barcelona, Spain) or Hemin (Sigma) was added to CM9 medium at a final concentration of 10 lmol. The growth achieved (OD_{600}) by each culture was measured after 12 h of incubation at 25°C with shaking at 150 rev min^{-1}. To analyse either siderophore production or citrate accumulation in cell-free supernatants, bacterial cultures under low-iron conditions in early exponential phase (OD_{600} = 0·6) were centrifuged (5 min at 8000 g) and filtered through 0·22 lm pore-size membrane filters. Siderophore production was measured using the colorimetric liquid assay of Chrome-Azurol-S (CAS) dye, as described previously (Balado *et al.* 2017b). Citrate quantification in the supernatants was achieved using the Citrate Assay Kit (Sigma-Aldrich) according to manufacturer's protocols. The citrate concentration standard curve was obtained with increasing concentrations (ranging from 0·1 to 12·5 mmol) of sodium citrate (Merck, Vienna, Austria) in CM9 medium. All assays were carried out in triplicate, and results shown are the means of three independent experiments.

DNA purification and PCR

Total genomic DNA from *Photobacterium* strains was isolated with the Easy–DNA kit (Invitrogen, Eugene, OR) according to manufacturer's protocols. PCR reactions were routinely carried out in a T-Gradient Thermal Cycler (Biometra, Jena, Germany), with Taq polymerase KAPA Taq (Kapa Biosystems, Wilmington, MA). Primers used to test the presence of genes encoding haemolysins Dly and HlyA, described in *P. damselae* subsp *damselae* (Rivas *et al.* 2011), and the deletion affecting haem utilization gene cluster are listed in Table S1. PCR conditions used were those previously described (Osorio *et al.* 2000; Rivas *et al.* 2011). Amplification products were analysed on 1% (w/v) agarose gels with TAE (0·04 mol l^{-1} Tris-acetate, 1 mmol l^{-1} EDTA) electrophoresis buffer, and were visualized on a UV transilluminator after staining with ethidium bromide. A high molecular weight DNA marker (Roche Diagnostics, Rotkreuz, Switzerland) was included in all the gels. When required, DNA from *P. damselae* subsp. *piscicida* ATCC 51736^T and P. *damselae* subsp. *damselae* ATCC 33539^T were used as PCR controls.

Bioinformatic analysis of genomes

The genomes of *P. toruni* CECT9189^T (accession no. GCF_900166975.1), *P. malacitanum* CECT9190^T (acces sion no. GCF_900185615.1) and *P. andalusiense* CECT9192^T (accession no. GCF_900185625.1) strains available through NCBI were analysed using the bioinformatics software tool antiSMASH (Blin *et al.* 2017), which identify putative nonribosomal peptide synthetases (NRPS) genes that would encode siderophore synthesis- related functions. BlastN searchs were also used to anal- yse the presence of *P. damselae* subsp. *damselae* haem utilization system homologue (accession no. AJ830893·1) or presence of *dly* and $hlyA_{cr}$ genes homologues (Rivas *et al.* 2013b).

Results

In vivo toxicity of bacterial raw ECP and histopathological examinations

The ECP of the *Photobacterium* strains tested were strongly lethal for redbanded seabream, resulting in death between 4 and 96 h post-inoculation (hpi). The main clinical signs and gross changes in the ECP-inoculated fish were lethargy, increase in the respiratory frequency and mucus production. The inoculated fish became excited and disorientated, bumping into the aquarium walls. Internally, presence of ascitic liquid, haemorrhagic and enlarged liver and haemorrhages in the abdominal cavity were the main signs. In contrast, heated ECP samples (100°C for 10 min) of all the strains tested did not produce toxic effects in fish after 7 dpi, although fish

sur- vivors exhibited abnormal enlarged spleen and kidney.

Gross alterations of tissue and/or cellular histopatologies were not detected in organs and tissues analysed of redbanded seabream after 4 h of all bacterial ECP hpi (Fig. 1a). However, important histopathological findings were detected at 24 h of *P. toruni* and *P. malacitanum* ECP pi affecting mainly to kidney, spleen and heart. At 24 hpi, epithelial cells from renal tubules showed cytoplasmic vacuolisations and nuclear pyknosis. Vessels of kidney showed hyperaemic and haemorrhagic areas within interstitial haematopoietic tissue (Fig. 1b). Spleen presented hyaline material filling vessels and capillary hyperaemia related to signs of bacterial septicaemia response (Fig. 1b). In the heart, lumen of ventricle and atrium were filled with abundant erythrocytes in the heart of post-inoculated redbanded seabream specimens (Fig. 1b). In *P. andalusiense* these histopathological changes were weak at 24 hpi. However the liver of affected specimens showed normal structures without signs of histopathological damages (Fig. 1b). Histological changes were more evident at 96 hpi post- ECP inoculation of the three new bacterial species tested (Fig. 1c). Head kidney showed an increase of melanoma- crophaghic centres within interstitial tissue (Fig. 1c), and the excretory caudal kidney showed hyaline droplet degeneration of epithelial tubular cells (Fig. 1c). In spleen, the splenic parenchyma displayed from focal to diffuse haemosiderosis (Fig. 1c). In the case of heart, there is a degeneration of muscle bundles of myocardium and focus of necrosis. Endocardial oedema was also fre- quent as well as haemorrhagic focus with erythrocytes and necrotic material filling the endocardial spaces (Fig. 1c). Similar to 24 h of ECP post-inoculation, the liver from inoculated redbanded seabream specimens showed normal hepatic organization (Fig. 1c).

Enzymatic activities of bacterial raw ECP and whole cells

The characterization of ECP from seven *Photobacterium* strains (five belonging to new species and two reference strains) was performed studying their enzymatic activities. All ECP samples showed remarkable content of protein (1570–5930 lg ml^{-1}) and were adjusted at 1 mg ml^{-1} for all analysis performed, although the total proteolytic activity, as determined using azocoll, was very low (<10 U ml^{-1}). Furthermore, specific proteolytic enzymes, such as amylase or gelatinase, were not detected.

Nevertheless, ECP samples exhibited other enzymatic activities such as lipase (Tween-20 and Tween-80). None of the ECP samples analysed was able to hydrolyse DNA (Table 1). Similar to the results obtained for ECP, the whole cells of seven strains tested did not present gelatinase and DNase, activities. Amylase activity was recorded in *P. malacitanum*, while lipase (Tween-20) activity was obtained for *P. damselae* subsp. *damselae*, *P. malacitanum*, and two strains of *P. toruni* (410B and 405H). In the case of lipase (Tween-80) activity, the strains *P. malacitanum* and *P. toruni* 410B were positive (Table 1). The enzymatic activities of the ECP samples were also evaluated using the API ZYM system. ECP of *Photobacterium* strains tested were positive for acid phosphatase and phosphohydrolase activities, being negative for the presence of valine arylamidase, trypsin, b-glucuronidase, a- and b-galactosidase, a- and b-glucosidase, a-mannosidase and a-fucosidase. Variable results were obtained with the remaining enzymatic activities (Table 2). All enzymatic activities of bacterial ECP were lost by heating of the samples, except the phospholipase activity of *P. damselae* subsp. *damselae* ATCC 33539^T. Haemolytic activity and detection of the phospholipase- D *dly* and pore-forming toxin *hlyA* genes. The whole cells from strains of *Photobacterium* strains tested showed an evident haemolytic capacity on Mueller- Hinton plates supplemented with gilthead seabream, red- banded seabream, common seabream and sheep blood. *Photobacterium andalusiense* and *P. malacitanum* whole cells did not show haemolytic activity except for gilthead seabream blood (Table 1). The ECP samples produced no haemolysis for any of fish blood tested in the case of *P. andalusiense* and *P. malacitanum* (Table 1). The ECP of two strains of *P. toruni* (403B and 405H) showed haemolytic activity against the blood of the three fish spe- cies tested. The haemolytic activity of sheep blood was only recorded in ECP of *P. damselae* subsp. *damselae* ATCC 33539^T, this activity being lost by heating of ECP at 100°C for 10 min, which indicates the production of thermolabile haemolysins. The amplification of the two *Photobaterium* virulence genes encoding phospholipase-D Dly and the pore-forming toxin HlyA failed for all *P. toruni*, *P. malacitanum* and *P. andalusiense* strains tested. Only the strongly hae- molytic *P. damselae* subsp. *damselae* ATCC 33539^T strain, used as positive control, showed the specific PCR prod- ucts (Fig. S1). *In silico* analysis of the three genomes of these species also showed the absence of homologues to *dly* and $hlyA_{ch}$ genes from *P. damselae* subsp. *damselae* (Rivas *et al.* 2013b). However, the genome of *P. toruni* shows a filamentous haemagglutinin N-terminal domain- containing protein (accession number WP_080176293.1) that could explain the higher haemolytic activity of this species. This protein is not present in the other two *Photobacterium* species studied.

Cytotoxicity of raw ECP and whole cells using mammalian and fish cell lines

The ECP of *Photobacterium* strains isolated from fish were assayed to evaluate their *in vitro* toxicity using both mammalian and fish cell lines. The ECP samples tested displayed variable cytotoxic effects at 48 hpi depending on the cell line type considered (Table 3). Similar results for cytotoxicity test were recorded on SAF-1 and CHSE-214, being only cytotoxic for these cell lines the strain of *P. damselae* subsp. *damselae* ATCC 33539^T. On the

contrary, a total destruction of the cell monolayer at 48 hpi was observed on BF-2 cell line for all the *Photobacterium* strains tested, except for *P. andalusiense*, which did not produce cytotoxic effects. In the case of the mammalian cell line HT-1080, only positive cytotoxicity was obtained for *P. damselae* subsp. *damselae* ATCC 33539^T ECP. The degenerative changes mainly consisted of the appearance of clusters of round cells and dendritic elongations (Fig. 2a,b), and finally cell detachment (Fig. 2c). How- ever, the ECP heated at 100°C had no effects on the different cell lines used (data not shown). Cytotoxicity of the whole cells was very different of their ECP. Only *P. damselae* subsp. *piscicida* strain ATCC 51736^T showed cytotoxicity capability on the three fish cell lines tested, and variable results for the other *Photobacterium* strains were obtained depending the fish cell line considered (Table 3). Interestingly, *P. damselae* subsp. *damselae* ATCC 33539^T was cytotoxic for the unique sparid cell line used (SAF-1) (Fig. 2d), being negative for the other fish cell lines (Fig. 2e). For the mammalian HT-1080 cell line the whole cells of all strains tested were negative for cytotoxicity activity (Fig. 2f and Table 3).

Adherence capacity

Adherence capacity of the *Photobacterium* strains tested was strongly dependent on the origin of the cell line. Thus, all the bacterial strains showed an AI lower than 1 (non-adherent) for fibroblastic cell line (SAF-1 and BF- 2); whereas for epithelial cell line (CHSE-214) all the strains tested, except *P. toruni* 403B and 405H, showed an intermediate AI ($1 < AI < 10$).

Production of siderophores and use of haem as iron source

To study the production of siderophores by the *Photobacterium* strains tested, growth in iron deficient conditions was firstly assayed. All strains could grow in presence of 2,2'-dipyridyl at concentrations of 25 or 50 μmol, but none of them could grow at a concentration above 100 μmol (Fig. 3). To test siderophore levels, a CAS assay was performed on cell-free supernatants after growing each strain under iron deficiency conditions that did not limit growth (2,2'-dipyridyl 25 μmol). All strains showed a weak positive CAS phenotype (Fig. 4a), which does not allow us to ensure the production of siderophores. In addition, an *in silico* analysis of *P. toruni* CECT9189^T, *P. malacitanum* CECT9190^T and *P. andalusiense* CECT9192^T genomes did not show any suitable candidate genes that could encode a putative siderophore system. Thus, all strains tested would produce low levels of siderophores or produce a siderophore with a weak iron chelating activity.

All strains could grow using ferric citrate as an iron source (Fig. 3). To verify if the strains tested could secrete and use endogenous citrate as siderophore for iron scavenging from the cell environment, we performed a citrate quantification in cell-free supernatants of *P. toruni*, *P. malacitanum* and *P. andalusiense* strains grown in the presence of 2,2'-dipyridyl (25 μmol). All strains showed a significant endogenous citrate accumulation (Fig. 4b). Moreover citrate accumulation in *P. toruni* and *P. malacitanum* supernatants was between 5 and 12 mmol, equivalent to the levels found in *P. damselae* strains lacking vibrioferrin production (Balado *et al.* 2017a). Haem utilization is a widespread trait in Vibrionaceae (Lemos and Osorio 2007). In the present study *P. andalusiense* and *P. malacitanun* strains were able to use haem as an iron source, whereas the three *P. toruni* isolates did not grow when haem was added to a low-iron medium (Fig. 4). We performed a further analysis of *P. toruni* genomes and found a large deletion that includes both the *tonB1* system and the haem utilization genes that are very conserved in Vibrionaceae family (Fig. 5a). PCR analysis confirmed that all *P. toruni* strains assayed harbour the same gene deletion (Fig. 5b).

Discussion

Until now, the only *Photobacterium* members involved in fish pathologies belong to the two subspecies of *P. damselae*. Recently, we described three new *Photobacterium* species (*P. toruni*, *P. malacitanum* and *P. andalusiense*) isolated from epizootic outbreaks in captived redbanded seabream, causing a cumulative mortality of 28% (Labella *et al.* 2017b, 2018). The diseased fish showed clinical symptoms of lethargy, distended abdomen and haemorrhages at the base of the pectoral fins and caudal fins. However, the virulence factors of these new bacterial species remain unknown. We considered that these pathogenic *Photobacterium* species might possess some virulence factors that enable them to evade the host defense system, spread within the host and eventually causing the dead of the host. Some of the factors that may play a role in pathogenesis are cell surface components, toxins, enzymes, iron acquisition systems, secretion systems and others (Pablos *et al.* 2010). In other fish pathogenic *Photobacterium*, some of these virulence determinants are secreted bacterial factors contained in the ECPs (Wu *et al.* 2008; Labella *et al.* 2010). The analysis of the ECPs secreted by bacteria has been a common strategy for studying the virulence factors of fish-pathogenic bacteria and the host-pathogen interactions (Ellis *et al.* 1981). ECPs contain diverse enzymatic activities such as haemolytic, cytolytic, proteolytic or lipolytic activities (Balebona *et al.* 1998b; Rodkum *et al.* 2005), which can provoke tissue destruction and haemorrhages, playing an important role in colonization, invasiveness and dissemination within the host (Balebona *et al.* 1998a; Hamed *et al.* 2018).

Although the proteolytic activity of the ECPs of the *Photobacterium* strains tested *in vitro* in the present study was weak, the intraperitoneal inoculation of these ECPs was lethal for redbanded seabream after 4–96 hpi.

Ellis *et al.* (1988) indicated that the presence of protease and haemolysin activities in the raw ECP of *Aeromonas salmonicida* was correlated with the development of lesions but not with the lethal toxicity of the ECP in rainbow trout, concluding that, an unidentified component of ECP was responsible for killing fish. Similar conclusions have been pointed out for *Vibrio anguillarum* and *P. damselae* subsp. *damselae* in other fish species (Lamas *et al.* 1994; Fouz *et al.* 1995; Labella *et al.* 2010). In our case, heating the ECPs eliminates their capacity to kill the fish, which suggests that the active fraction of the toxins present in the ECPs is thermolabile, and it is not associated with their thermoresistant lipopolysaccharide content. Fish inoculated with heat-inactivated ECP samples showed enlarged lymphohaematopoietic organs suggesting a stimulation of immune response with cellular accumulation, similarly to what as described for the ECP of *Aeromonas hydrophila* by Rey *et al.* (2009).

Histopathological effects caused by ECP of the newly described *Photobacterium* species involved necrotic lesions in the spleen and kidney with accompanying granulomatous inflammation (Fig. 1). In addition, the increase of melanomacrophage centres and haemosiderosis in kidney and spleen suggests an increase in the rate of destruction of erythrocytes within melanomacrophage centres. Haemosiderin has been observed to increase considerably after prolonged starvation (Agius 1981), and it invariably reflects a pathological process (Wolke *et al.* 1985). Functions ascribed to melano-macrophage centres included the deposition of resistant pathogens such as intracellular bacterial and parasitic spores (Roberts 2001), and antigen processing in immune responses (Agius and Roberts 2003). Although iron capture and storage in haemolytic diseases appears to be a primary function, several studies indicate that the general function of these centres is the focalization of destruction, detoxification or recycling of endogenous and exogenous materials, including infectious agents (Herraez and Zapata 1986; Agius and Roberts, 2003; Suresh 2009). The presence of endocardial oedemas in affected redbanded seabream suggests that it may be a response to the presence of microbial toxins and/or exoenzymatic activities present in the bacterial ECP (Stephens *et al.* 2006; Labella *et al.* 2010).

Cell lines possess an important role in investigating bacterial–host interactions because the easy manipulation of cells and their maintenance under controlled conditions. In this study, all lethal ECP samples presented cytotoxic effects for some fish cell lines (Fig. 2). The cytotoxicity was totally lost on heated ECP samples, indicating the presence of a thermolabile extracellular cytolysin in *P. malacitanum*, *P. toruni* and both subspecies of *P. damselae* (Table 3). It has been proposed that bacterial peptidases provoke degradation of host tissues, playing an important role in bacterial pathogenesis as they allow the pathogen invasion (Miyoshi and Shinoda 2000). In addition, peptidases enable the evasion of the bacteria from several fish defense mechanisms (Vivas *et al.* 2004). Bacterial peptidases are well-known proteins that bacterial pathogens use to invade host tissues, and to evade innate fish defense mechanisms (Miyoshi and Shinoda 2000). However, the proteolytic activities of the *P. toruni*, *P. malacitanum* and *P. andalusiense* ECPs tested were very weak, whereas other enzymatic activities such as acid- and alkaline-phosphatase, esterase, phosphohydrolase and leucine-arylamidase were identified (Table 2). Moreover the cytotoxicity on four cell lines was totally lost on heated ECP samples (data not shown), indicating the presence of a thermolabile extracellular cytolysin. In previous works, phospholipase D (Dly, damselysin) and cytotoxins demonstrated to play an important role in the pathogenesis of the disease (Labella *et al.* 2010; Rivas *et al.* 2013b). However, the new *Photobacterium* species tested here did not present this cytolysin (Fig. S1). Thus, the histopathological damages observed in kidney, spleen and heart in the present study should be explained by other factors different to damselysin.

In this regard, the identification of a haemaglutinin in the genome of *P. toruni* that is absent in the other two species could explain in part the higher haemolytic activ- ity of this species. Haemolysis of erythrocytes in several fish organs has previously been reported during infections caused by fish pathogenic bacteria (Grizzle and Kiryu 1993). Futhermore, several authors have demonstrated that the pathogenicity of bacterial fish pathogens was related to the ability to haemolyse the host erythrocytes (Borrego *et al.* 1991; Fouz *et al.* 1993; Pedersen *et al.* 2009). The *Photobacterium* strains analysed exhibit differential haemolytic activity against erythrocytes from different fish species (Table 1). These result are in agreement with previous studies of Cutter and Kreger (1990) and Thyssen *et al.* (1998), who described variable responses dependent on both the source of the erythrocytes and the strain tested.

One of the first lines of defense against bacterial infections is the sequestration of iron. Thus, bacterial pathogens must circumvent iron withholding to cause disease (Skaar 2010). Two of the most important systems to obtain iron from host sources are siderophore production and utilization of haem (Wandersman and Deleoelaire 2004). These systems are present in most bacterial pathogens and their inactivation significantly reduces the ability of most pathogenic bacteria to cause disease (Henderson and Payne 1994). In this study, the three *Photobacterium* species studied were evaluated for both siderophore pro- duction and haem utilization. Unexpectedly, all *Photobacterium* strains evaluated showed a low MIC for the iron chelator 2,2'-dipyridyl, failed in siderophore detection in CAS assays and the *in silico* analysis of genomes did not identify suitable candidate genes to encode siderophore- related functions. Notably, although the utilization of haem as iron source is an ability widespread in Vibrionaceae and the genes that code these functions are highly conserved (Lemos and Osorio 2007), all *P. toruni* strains failed to use haem as iron source since they harbor a large deletion that affects not only the haem utilization system but also the TonB1 energy transduction system. TonB1 is one of the energy transducer systems that proportionate the energy necessary for siderophore and haem uptake (Kuehl and Crosa 2010). These findings greatly suggest that the iron necessary to support growth during the disease caused by the three *Photobacterium* species studied must be supplied by an unidentified system. Interestingly, previous works found that *P. damselae* subsp. *damselae* strains showing a strong positive CAS reaction are likely to produce the siderophore vibrioferrin

(Puentes *et al.* 2017) while other virulent strains showing weak CAS phenotype secrete endogenous citrate and use it as iron scavenger (Balado *et al.* 2017b). The present study shows not only that the three *Photobacterium* spp studied use citrate as iron source, but also that they accumulate a significant amount of extracellular citrate that could be used as siderophore. While most bacteria can use externally supplied ferric citrate to fulfill their nutritional requirement for iron (Mazoy *et al.* 1997; Marshall *et al.* 2009), there are few examples of bacteria that secrete citrate in order to get iron. *Pseudomonas syringae* (Jones and Wildermuth 2011), *Bradyrhizobium japonicum* (Guerinot *et al.* 1990) and *P. damselae* subsp. *damselae* (Balado *et al.* 2017b) are the unique examples to date. Our findings greatly suggest that *P. toruni*, *P. malacitanum* and *P. andalusiense* secret endogenous citrate and use it as iron carrier. Further work must be carried out to identify the specific genes and proteins that mediate the utilization of endogenous citrate as siderophore in *Photobacterium.*

In conclusion, *P. toruni*, *P. malacitanum* and *P. andalusiense* produce some virulence factors, many of them are extracellular, and that could explain the pathogenicity of the disease observed in fish infected by these bacteria. Although, most enzymatic activities detected were low, they could be enough to cause disease in immunocompromised individuals. Furthermore, ECPs from the three species were highly toxic for redbanded seabream due to an unidentified factor. Further studies will contribute to increase our knowledge about the virulence properties of these novel pathogens.

Acknowledgements

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Conflicts of Interest

The authors declare there is not conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. PCR results of *Photobacteriun hlyA* and *dly* detection. M denotes 1 kb DNA ladder with fragments ranging from 250 bp to 10 kb (250 bp, 500 bp, 750 bp, 1 kb, 1·5 kb, 2 kb, 2·5 kb, 3 kb, 4 kb, 5 kb, 6 kb, 8 kb and 10 kb).

Table S1. Primers used in this study.

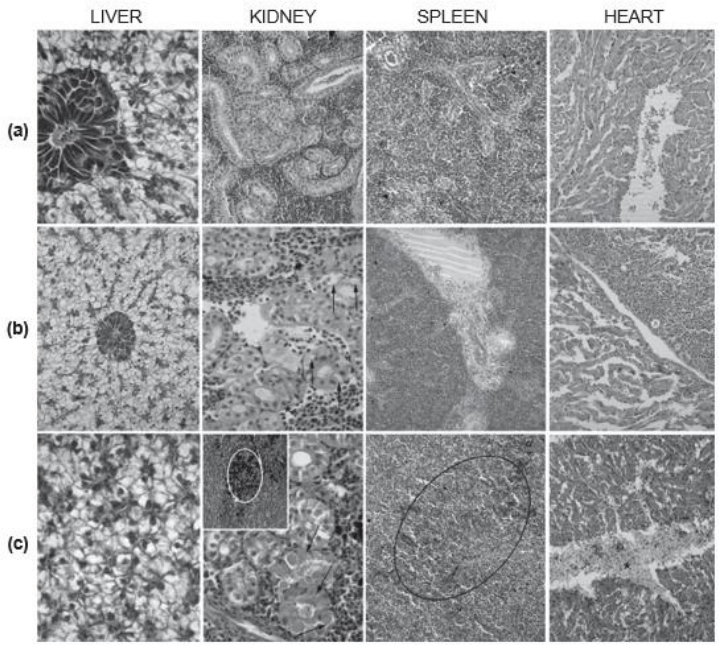

Figure 1 Histological sections of liver, kidney, spleen and heart of redbanded seabream stained with HE after inoculation of *Photobacterium tor- uni*, *Photobacterium malacitanum* and *Photobacterium andalusiense* ECPs. (a) 4 hpi showing normal structures of different organ systems and tis- sues (hepatocytes, sinusoids, bile canaliculus; renal tubules and ducts and haematopoietic tissue, as well as atrium, ventricle and bulbus arterious, muscle fibres). (b) 24 hpi showing the liver with no signs of histopathological damages, epithelial cells from renal tubules with cytoplasmic vac- uolizations (arrows) and nuclear pyknosis (asterisk), spleen showing hyaline material filling vessels and lumen of ventricle and atrium (asterisk) of the heart filled with abundant erythrocytes. Weak or non-histopathological damages of *P. andalusiense* ECP were recorded. (c) 96 hpi showing an increase of melanomacrophage centres within interstitial tissue (white circle), hyaline droplet degeneration of epithelial tubular cells (arrows), haemosiderosis (black circle) in the splenic parenchyma and endocardial oedema as well as haemorrhagic focus with erythrocytes and necrotic material filling the endocardial spaces (asterisk) in the heart.

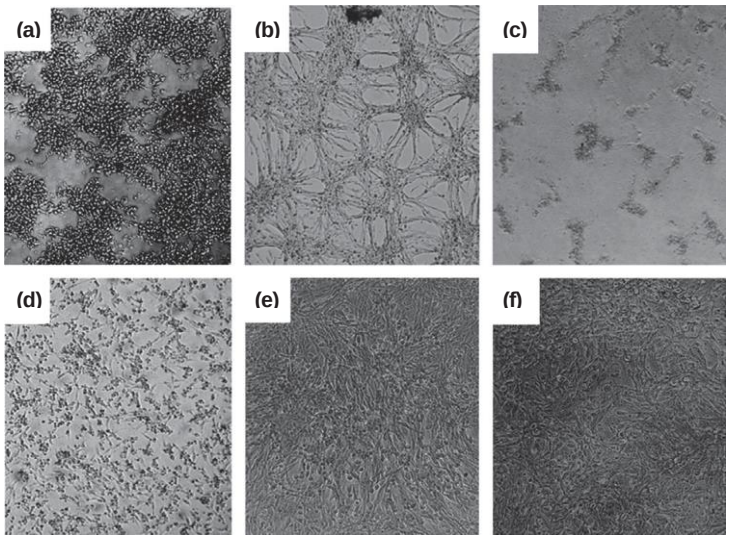

Figure 2 Cytotoxicity of ECP and whole cells (10^5 cells per ml) of different *Photobacterium* species tested using mammalian and fish cell lines (see "Materials and Methods': Cytotoxicity activity). (a) Clusters of round cells on SAF-1 cell line produced by inoculation of *Photobacterium damselae* subsp. *damselae* ECP samples. (b) Dendritic elongations of BF-2 cells produced by *Photobacterium malacitanum* ECP. (c) Detachment of BF-2 cell line provoked by inculation of *Photobacterium toruni* 410B ECP samples. (d) Cytotoxic activity of *P. damselae* subsp. *damselae* whole cell on SAF-1 cell line. (e) Negative effects of *Photobacterium andalusiense* whole cell on CHSE-214. (f) Negative effects of *P. damselae* subsp. *piscicida* whole cell on HT-1080.

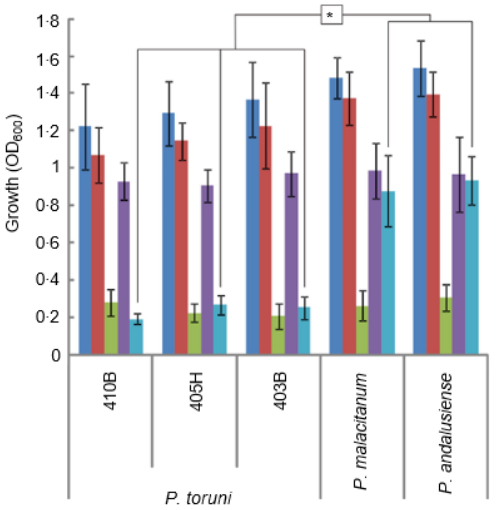

Figure 3 Growth under iron limiting conditions and use of ferric citrate and haem as iron sources by the strains tested. Significant dif- ferences (at $P < 0{\cdot}05$) are marked with an asterisk. (■) 10 μmol l^{-1} $FeCl_3$; (■) 25 μmol l^{-1} 2,2'-dipyridyl; (■) 100 μmol l^{-1} 2,2'-dipyridyl; (■) 100 μmol l^{-1} 2,2'-dipyridyl+Citrate; (■) 100 μmol l^{-1} 2,2'-dipyri- dyl+Haem.

(

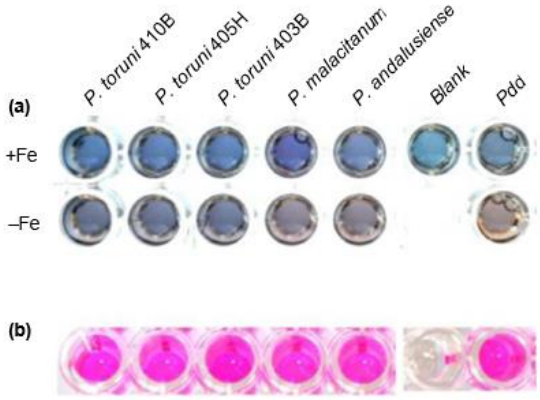

Figure 4 Quantification of siderophore production by CAS assay (a) and endogenous citrate accumulation (b) in cell-free supernatants.

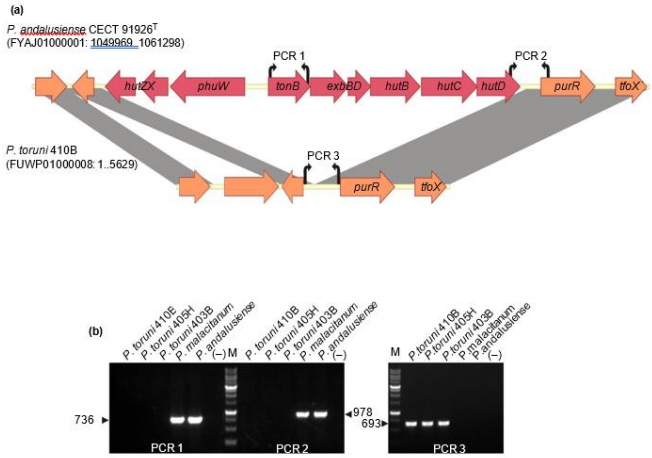

Figure 5 Gene map of the genomic region of *Photobacterium andalusiense* encoding haem uptake/utilization and TonB1 systems and its counter- part in *Photobacterium toruni* (a) (■) Haem/hemin utilization protein; ((■) Other function. PCR results to detect the presence of *tonB1* and haem uptake genes (PCR 1 and PCR 2) or the large deletion (PCR 3). M denotes 1 kb DNA ladder with fragments ranging from 250 bp to 10 kb (250 bp, 500 bp, 750 bp, 1 kb, 1·5 kb, 2 kb, 2·5 kb, 3 kb, 4 kb, 5 kb, 6 kb, 8 kb and 10 kb).

Table 1 Enzymatic activities of extracellular products (ECP) and bacterial cells of *Photobacterium* species using plate tests

	Activity in ECP/bacterial cells								
						Haemolysis activity			
Species	Amylase	Gelatinase	DNase	Lipase (T20)	Lipase (T80)	GSB	RSB	CSB	S
Photobacterium andalusiense	−/−	−/−	−/−	−/−	−/−	−/+	−/+	−/−	−/−
P. malacitanum	−/+	−/−	−/−	+/+	−/+	−/+	−/+	−/−	−/−
P. toruni 410B	−/−	−/−	−/−	+/+	+/+	−/+	+/+	−/+	−/−
P. toruni 403B	−/−	−/−	−/−	−/−	−/−	+/+	+/+	+/+	−/−
P. toruni 405H	−/−	−/−	−/−	−/+	−/−	+/+	+/+	+/+	−/−
P.d.d.	−/−	−/−	−/−	+/+	+/−	+/+	+/+	+/+	+/+
P.d.p.	−/−	−/−	−/−	+/−	+/−	+/−	+/−	+/−	−/−

+, positive result; −, negative result.
T20, Twen 20; T80, Tween 80; GSB, gilthead seabream; RSB, redbanded seabream; CSB, common seabream; S, sheep.
P.d.d., *P. damselae* subsp. *damselae*; *P.d.p.*, *P. damselae* subsp. *piscicida*.

Table 2 Enzymatic activity profiles of extracellular products (ECP) of *Photobacterium* species using the API ZYM system

	Activity (nmol)																		
Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Photobacterium andalusiense	30–40	5–10	5–10	<5	20–30	<5	<5	<5	<5	>40	20–30	<5	<5	<5	<5	<5	10–20	<5	<5
P. malacitanum	20–30	10–20	<5	<5	<5	<5	10–20	<5	<5	>40	20–30	<5	<5	<5	<5	<5	10–20	<5	<5
P. toruni 410B	<5	<5	<5	<5	<5	<5	5–10	<5	<5	20–30	10–20	<5	<5	<5	<5	<5	5–10	<5	<5
P. toruni 403B	>40	<5	<5	<5	10–20	<5	10–20	<5	<5	>40	10–20	<5	<5	<5	<5	<5	<5	<5	<5
P. toruni 405H	>40	10–20	<5	5–10	20–30	<5	10–20	<5	<5	>40	>40	<5	<5	<5	<5	<5	10–20	<5	<5
P.d.d.	>40	10–20	10–20	<5	10–20	<5	5–10	<5	<5	>40	20–30	<5	5–10	<5	<5	<5	<5	<5	<5
P.d.p.	5–10	<5	<5	<5	5–10	<5	<5	<5	<5	>40	>40	<5	<5	<5	<5	<5	<5	<5	<5

1, alkaline phosphatase; 2, esterase; 3, esterase lipase; 4, lipase; 5, leucine arylamidase; 6, valine arylamidase; 7, cystine arylamidase; 8, trypsin; 9, a-chymotrypsin; 10, acid phosphatase; 11, naftol-AS-BI-phosphohydrolase; 12, a-galactosidase; 13, ß-galactosidase; 14, ß-glucuronidase; 15, a-glucosidase; 16, ß-glucosidase; 17, N-acetyl-ß-glucosaminidase; 18, a-mannosidase; 19, a-fucosidase.
P.d.d., *P. damselae* subsp. *damselae*; *P.d.p.*, *P. damselae* subsp. *piscicida*.

Table 3 Cytotoxicity of *Photobacterium* species on *in vitro* cell line cultures

	ECP		Cytotoxicity on		Whole cells		Cytotoxicity on	
Species	SAF-1	BF-2	CHSE-214	HT-1080	SAF-1	BF-2	CHSE-214	HT-1080
P. andalusiense	−	−	−	−	+	+	−	−
P. malacitanum	−	+	−	−	+	+	−	−
P. toruni 410B	−	+	−	−	+	−	−	−
P. toruni 403B	−	+	−	−	+	+	−	−
P. toruni 405H	−	+	−	−	+	+	−	−
P.d.d.	+	+	+	+	+	−	−	−
P.d.p.	−	+	−	−	+	+	+	−

+, positive result; −, negative result.
P.d.d., *P. damselae* subsp. *damselae*; *P.d.p.*: *P. damselae* subsp. *piscicida*.