

Toxicity of *Photobacterium damsela* subsp. *damsela* strains isolated from new cultured marine fish

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ABSTRACT: The *in vivo* and *in vitro* toxicity of bacterial cells and their extracellular products (ECPs) from 16 strains of *Photobacterium damsela* subsp. *damsela* isolated from 7 epizootic outbreaks were evaluated. On the basis of their 50% lethal dose (LD₅₀) values (about 1×10^5 CFU), these strains may be considered as moderately virulent. However, their ECPs were strongly lethal for redbanded seabream *Pagrus auriga* causing fish death within 2 h post-inoculation (protein concentration ranged between 2.1 and 6.41 $\mu\text{g g}^{-1}$ fish). The bacterial ECPs tested exhibited several enzymatic activities, such as amylase, lipase, phospholipase, alkaline phosphatase, esterase-lipase, acid phosphatase, and β -glucosaminidase. These ECPs displayed a strong cytotoxic effect on 4 fish and 2 mammalian cell lines, although this activity disappeared when ECPs were heated at 100°C. The virulence of the strains tested could not be related to the hemolytic activity or to the production of the toxin damselysin. Therefore, another unknown type of toxin could play an important role in the virulence mechanisms of this bacterial pathogen.

KEY WORDS: Toxicity · ECP · *Photobacterium damsela* subsp. *damsela* · Cultured marine fish

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INTRODUCTION

Photobacterium damsela subsp. *damsela* is a halophilic bacterium associated with several diseases affecting both wild and cultured fish species (Fouz et al. 1991, 1992, Vera et al. 1991) and mammals, including humans (Clarridge & Zigelboim-Daum 1985, Coffey et al. 1986, Buck et al. 1991, Yamane et al. 2004). Epizootic outbreaks of this bacterial pathogen have been associated with immunocompromised or stressed fish by overcrowding (Thune et al. 1993, Garcia-Rosado et al. 2007). Symptoms of these bacterial pathologies are not specific, including hemorrhagic septicemia with extensive skin lesions and focal necrosis of the liver, spleen, kidney, and other tissues (Hjeltnes & Roberts 1993).

Several new marine species are being evaluated as potential candidates for aquaculture production. In southern Europe, studies on reproductive cycles, nutri-

tion, growth, and immune system of species such as redbanded seabream *Pagrus auriga*, white seabream *Diplodus sargus*, and meagre *Argyrosomus regius* are ongoing (Cardenas & Manchado 2008, Martin-Antonio et al. 2009). However, the intensive culture of these fish species and others has favored the appearance of several outbreaks with moderate mortality involving *Photobacterium damsela* subsp. *damsela* (Labella et al. 2006, Garcia-Rosado et al. 2007, Kanchanopas-Barnette et al. 2009).

Several virulence factors are involved in the pathogenesis of *Vibrio* and *Photobacterium* genera (Norqvist et al. 1990, David et al. 1992, Toranzo & Barja 1993, Magariños et al. 1994, Bakopoulos et al. 1997, Perez et al. 1998, Farto et al. 2002, 2006, Ishihara et al. 2002, Wang et al. 2007). In the case of *P. damsela* subsp. *damsela* only an extracellular cytolytic toxin, named damselysin, which is a phospholipase-D active against the sphingomyelin of the sheep erythrocyte mem-

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brane, has been described (Kreger 1984, Kothary & Kreger 1985, Kreger et al. 1987). In addition, a relationship between the degree of virulence and the hemolytic activity has been demonstrated in *P. damsela* subsp. *damsela* strains isolated from fish (Fouz et al. 1993). However, Cutter & Kreger (1990) found that not all the *P. damsela* subsp. *damsela* strains presented the damselysin gene (*dly*), but only those strains showing intense hemolytic activity. A further study demonstrated that the presence of this gene was not correlated to the virulence in mice and fish of 17 *P. damsela* subsp. *damsela* strains isolated from different sources (Osorio et al. 2000).

The extracellular products (ECPs) are produced by bacterial pathogens to facilitate the uptake of nutrients from the surrounding environment, and/or for the successful penetration and survival of pathogens inside the host (Bakopoulos et al. 2003). However, the role of ECPs in the pathogenesis of *Photobacterium damsela* subsp. *damsela* in fish is poorly known, which is a considerable disadvantage for the development of vaccines and vaccine strategies, since it has been suggested that the ECP components are major antigenic compounds of several vaccine formulations (Collado et al. 2000, Bakopoulos et al. 2004).

The aim of this study was to determine the toxicity of different *Photobacterium damsela* subsp. *damsela* strains isolated from cultured diseased fish. For this purpose, we performed *in vivo* and *in vitro* assays using bacterial cultures and their ECPs. In addition, the enzymatic activities of the ECPs and their cytotoxicity in fish and mammalian cell lines were compared.

MATERIALS AND METHODS

Bacterial strains. In this study, 16 *Photobacterium damsela* subsp. *damsela* strains associated with epizootic outbreaks affecting various cultured marine fish species (Table 1) were used. All the isolates were phenotypically and genotypically characterized as previously described (Labella et al. 2006, 2009, Kanchanopas-Barnette et al. 2009). *P. damsela* subsp. *damsela* strain ATCC 33539^T, isolated from damselfish, was used for comparative purposes.

Strains were routinely cultured on tryptic soy agar or broth (TSA or TSB) (Difco Lab) supplemented with 1.5% (w/v) NaCl (TSAs or TSBs, respectively), and incubated at 22°C for 2 to 5 d. Stock cultures were stored frozen at –80°C in TSBs with 15% (v/v) glycerol.

ECPs extraction. Bacterial ECPs were obtained by the cellophane plate technique (Liu 1957) by spreading 3 ml of a 24 h broth culture of each strain over sterilized cellophane sheets placed on TSAs plates. After incubation for 48 h at 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 µm pore-size membrane filters. All the ECP samples were stored at –20°C until used. The protein concentration of the ECPs was spectrophotometrically determined by using bicinchoninic acid (Sigma-Aldrich) 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 the

Table 1. Source of the *Photobacterium damsela* subsp. *damsela* strains and protein content and total proteolytic activity of their extracellular products. ATCC: American type culture collection

Strain	Source	Isolation organ	Outbreak date	Proteins (µg ml ⁻¹)	Proteolytic activity (U ml ⁻¹)
203H	Redbanded seabream <i>Pagrus auriga</i>	Liver	Winter 2003	910.3	0.23
401H	Redbanded seabream <i>P. auriga</i>	Liver	Spring 2004	594.16	0.04
402O	Redbanded seabream <i>P. auriga</i>	Eye	Autumn 2004	412.50	0.59
403D1	Redbanded seabream <i>P. auriga</i>	Intestine	Autumn 2004	533.05	0.16
412B	Redbanded seabream <i>P. auriga</i>	Spleen	Autumn 2004	473.05	<0.01
9401H	Redbanded seabream <i>P. auriga</i>	Liver	Summer 2004	877.08	<0.01
501H	Redbanded seabream <i>P. auriga</i>	Liver	Summer 2005	613.61	<0.01
501R	Redbanded seabream <i>P. auriga</i>	Kidney	Summer 2005	346.38	<0.01
501B	Redbanded seabream <i>P. auriga</i>	Spleen	Summer 2005	218.05	<0.01
601R	Redbanded seabream <i>P. auriga</i>	Kidney	Summer 2006	414.72	0.05
602R	Redbanded seabream <i>P. auriga</i>	Kidney	Summer 2006	347.50	<0.01
D408U	Gilt-head seabream <i>Sparus aurata</i>	Ulcers	Autumn 2004	835.27	0.93
Lb501R	European seabass <i>Dicentrarchus labrax</i>	Kidney	Summer 2005	564.72	0.12
S503C	White seabream <i>Diplodus sargus</i>	Brain	Summer 2005	554.72	<0.01
C601R1	Meagre <i>Argyrosomus regius</i>	Kidney	Summer 2006	774.16	<0.01
LCA240907	Barramundi <i>Lates calcarifer</i>	Liver	Autumn 2007	1282.39	0.38
ATCC 33539 ^T	Damselfish <i>Chromis punctipinnis</i>	–	–	420.00	0.02

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.

Determination of enzymatic activities. The enzymatic activities presented in the ECPs and in bacterial cultures were evaluated using the API ZYM system (bioMérieux). Other enzymatic activities, such as caseinase, gelatinase, amylase, phospholipase, and lipase, were determined in plates by a 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), 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) was used for the DNase test. The production of hemolysins was assayed on Mueller-Hinton agar (Oxoid) supplemented with 5% (v/v) sheep, calf, or redbanded seabream blood. In all the tests, aliquots of 5 µl of each ECP or exponential growth bacterial cultures in TSBs were placed on the plates and incubated at 22°C for 24 to 48 h, following the methodology described by Balebona et al. (1998a). To determine the heat stability, all the enzymatic activities were also tested after treatment of ECP samples at 100°C for 10 min.

Assessment of *in vivo* toxicity of *Photobacterium damsela* subsp. *damsela* and their ECPs. The determination of the 50% lethal dose (LD₅₀) was conducted by intraperitoneal (IP) inoculation of redbanded seabream specimens (5 to 10 g in weight). Groups of 20 fish were inoculated with washed bacterial cells in PBS at doses ranging from 10² to 10⁸ CFUs per fish, held at 22°C for 15 d, and observed daily for pathological signs. Groups of control fish were inoculated with 0.1 ml of sterile PBS (pH 7.4). Mortalities were considered to be due to the inoculated bacterial strain only when it was recovered from internal organs as a pure culture. The LD₅₀ was determined by the Reed & Muench (1938) method.

The toxicity of the ECPs of the *Photobacterium damsela* subsp. *damsela* strains was evaluated by IP inoculation of 0.1 ml of each ECP sample in groups of 20 redbanded seabream specimens (5 to 10 g in weight). The mortality rate of each group was monitored over a 7 d period. Sets of animals 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.

Cytotoxic activity of the ECPs. Cytotoxicity assays were basically carried out as described by Wang et al. (1998) on mammalian and fish cell lines. Two different mammalian cell lines were employed: NIH/3T3, a murine fibroblastic cell line, and HT-1080, a human

epithelial cell line. Cells were cultured and maintained using Dulbecco's modified Eagle's medium (HyClone Laboratories) supplemented with 4 mM L-glutamine (Sigma-Aldrich), bovine calf serum (Gibco) to a final concentration of 10% (v/v) and 1% (v/v) penicillin-streptomycin solution (Gibco). Cell cultures were incubated in 5% CO₂ at 37°C and 95% humidity. Four different fish cell lines were tested: SAF-1, a fibroblastic cell line from gilt-head seabream *Sparus aurata* (Bejar et al. 1997); BF-2, a fibroblastic cell line from bluegill fry *Lepomis macrochirus*; CHSE-214, an epithelial cell line from Chinook salmon *Oncorhynchus tshawytscha*; and SSN-1, an epithelial cell line derived from striped-snakehead fish *Ophicephalus striatus*. 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 were used for the maintenance and growth of the cell cultures: 25°C for SAF-1, BF-2, and SSN-1 cell lines, and 20°C for the CHSE-214 cell line.

For the cytotoxicity assay, 12-well plates were prepared for each cell line and bacterial strain. Cell cultures were inoculated with 0.1 ml of the ECP or ECP heated (100°C, 10 min) samples, PBS (pH 7.4) being used as negative control. Each experimental condition was assayed in triplicate. The development of cytotoxic effects was observed at 6, 24 and 48 h post-inoculation using a phase-contrast inverted microscope (Nikon) at 200× magnification.

Detection of phospholipase-D (*dly*) gene. Bacterial cells from a 24 h culture in TSBs were resuspended in 500 µl of TES buffer (Sigma-Aldrich) supplemented with 5 µl of lysozyme (10 mg ml⁻¹ stock solution) (Sigma-Aldrich) and incubated at 37°C for 30 min. Then, 5 µl each of Proteinase K (10 mg ml⁻¹ stock solution; Sigma-Aldrich) and RNase (10 mg ml⁻¹ stock solution; Sigma-Aldrich) were added, and the solution was incubated for 1 h at 65°C. After addition of 50 µl of 20% (w/v) SDS (Sigma-Aldrich), tubes were returned immediately to the 65°C water bath for a further 10 min. After cell lysis, a phenol/chloroform extraction was performed. DNA was precipitated with cold ethanol (-20°C), and resuspended in 50 µl of sterile distilled water. DNA concentration was spectrophotometrically determined at 260 nm.

Two primer sets designed for the amplification of the complete *dly* gene (*dly*-1f/*dly*-2r) and a fragment of 567 bp (*dly*-5'f/*dly*-3'r) were used, according to Osorio et al. (2000). PCR amplifications were performed in a DNA thermocycler (Eppendorf Iberica). A typical reaction mixture (100 µl) consisted of 0.5 µg of each primer, 2 U of *Taq* polymerase (Perkin Elmer Tech. Center), *Taq* polymerase buffer (Perkin Elmer), 2 mM MgCl₂,

200 μM of each deoxynucleoside triphosphate, and 100 ng of DNA. The amplification conditions for the complete *dly* gene were 95°C for 4 min followed by 30 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 3 min. A final extension step of 10 min at 72°C was carried out. For the 567 bp fragment, the amplification conditions were 95°C for 4 min, 30 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 40 s, and 72°C for 5 min. Amplification products were analysed on 1% (w/v) agarose gels with TAE (0.04 M Tris-acetate, 1 mM EDTA) electrophoresis buffer, and were visualized on a UV transilluminator after staining with ethidium bromide. A high molecular weight DNA marker (Roche Diagnostics) was included in all the gels. DNA from *Photobacterium damsela* subsp. *piscicida* ATCC 17911^T was used as negative control.

RESULTS

In vivo toxicity and enzymatic activities of ECPs

The extracellular products of the *Photobacterium damsela* subsp. *damsela* strains tested were strongly lethal for redbanded seabream, resulting in death between 2 and 4 h after inoculation. The main clinical signs and gross changes 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, hemorrhagic and enlarged liver, and

hemorrhages in the abdominal cavity were the main signs. In contrast, ECP from the strain 9401H and heated ECP samples (100°C for 10 min) of all the strains tested did not produce toxic effects in fish after a 7 d post-inoculation period, although fish survivors exhibited abnormal enlarged spleen and kidney.

The characterization of ECPs from *Photobacterium damsela* subsp. *damsela* was performed studying their enzymatic activities. All ECP samples showed remarkable content of protein (218 to 1282 $\mu\text{g ml}^{-1}$), although the total proteolytic activity, as determined by using azocoll, was very low (<10 U ml^{-1}) (Table 1). Furthermore, specific proteolytic enzymes, such as caseinase or gelatinase, were not detected, except for the strain 412B, which exhibited gelatinase activity. Nevertheless, ECP samples exhibited other enzymatic activities such as amylase, lipase (Tween-20), and phospholipase. None of the ECP samples analyzed was able to hydrolyze DNA or Tween-80 (Table 2).

The enzymatic activities of the ECP samples were also evaluated by using the API ZYM system. ECPs of *Photobacterium damsela* subsp. *damsela* strains tested were positive for alkaline phosphatase, esterase-lipase, acid phosphatase, and N-acetyl- β -glucosaminidase activities, being negative for the presence of β -glucuronidase. Variable results were obtained with the remaining enzymatic activities (Table 3).

All enzymatic activities of *Photobacterium damsela* subsp. *damsela* ECPs were lost by heating of the samples, except the phospholipase activity of strains 402O and ATCC 33539^T.

Table 2. Enzymatic activities of extracellular products (ECPs) and bacterial cells of *Photobacterium damsela* subsp. *damsela* strains using plate tests. +: positive result; -: negative result. T20: Tween-20; T80: Tween-80

Strain	Activity in ECP/bacterial cells								
	Caseinase	Gelatinase	Amylase	DNase	Lipase (T20)	Lipase (T80)	Phospholipase	Hemolysis (sheep and calf)	Hemolysis (redbanded seabream)
203H	-/-	-/-	+/+	-/-	+/+	-/-	-/+	-/+	-/-
401H	-/-	-/-	-/-	-/-	+/+	-/-	+/+	-/-	-/+
402O	-/-	-/-	-/-	-/-	+/+	-/-	+/+	+/+	+/+
403D1	-/-	-/-	-/-	-/-	+/+	-/-	+/+	-/+	-/+
412B	-/-	+/+	-/-	-/-	+/+	-/-	-/-	-/-	-/+
9401H	-/-	-/-	+/+	-/-	-/-	-/-	-/+	-/+	-/+
501H	-/-	-/-	-/-	-/-	-/-	-/-	-/+	-/+	-/+
501R	-/-	-/-	-/-	-/-	+/+	-/-	+/+	-/+	-/+
501B	-/-	-/-	-/-	-/-	-/-	-/-	+/+	-/+	-/+
601R	-/-	-/-	+/+	-/-	-/-	-/-	+/+	-/+	-/+
602R	-/-	-/-	+/+	-/-	-/-	-/-	+/+	-/+	-/+
D408U	-/-	-/-	-/-	-/-	+/+	-/-	+/+	-/+	-/+
Lb501R	-/-	-/-	-/-	-/-	+/+	-/-	+/+	-/+	-/-
S503C	-/-	-/-	-/-	-/-	+/+	-/-	+/+	-/+	-/-
C601R1	-/-	-/-	-/-	-/-	+/+	-/-	+/+	-/+	-/+
LCA240907	-/-	-/-	+/+	-/-	+/+	-/-	-/+	-/+	-/+
ATCC 33539 ^T	-/-	-/-	+/+	-/-	+/+	-/-	+/+	-/+	-/+

Lethal dose and enzymatic activities of bacterial cultures

The LD₅₀ obtained by IP inoculation of *Photobacterium damsela* subsp. *damsela* strains were very similar, with a mean value of about 1 × 10⁵ CFU, except for the strain 9401H with a value higher than 10⁸ CFU. According to Santos et al. (1988) criteria, these strains may be considered as moderately virulent, and non-virulent in the case of the strain 9401H.

Similar to the results obtained for ECPs, the 17 strains tested did not present caseinase, gelatinase, DNase, and lipase (Tween-80) activities, except the strain 412B, which was gelatinase positive. Amylase and lipase (Tween-20) activities were recorded in 35.3 and 70.6%, respectively of the *Photobacterium damsela* subsp. *damsela* strains tested. All *P. damsela* subsp. *damsela* strains displayed phospholipase activity, except the strain 412B (Table 2).

However, several differences were determined in the enzymatic activities presented in ECPs versus bacterial cells using the API ZYM system (Tables 3 & 4). Thus, the leucine arylaminidase activity was present in all bacterial cells tested whilst this enzymatic activity was absent or in low levels in their corresponding ECPs. Similarly, β-glucosidase activity was recorded in the bacterial cultures of 9 strains tested, but was absent in their ECPs, except for the strain LCA240907. For N-acetyl-β-glucosaminidase, higher levels of activity were obtained for the cells compared to their ECPs. In contrast, α-chymotrypsin activity was recorded in ECPs of 11 strains tested, but was absent in their bacterial cells, except for the strain 412B.

Cytotoxicity of ECPs using mammalian and fish cell lines

The ECPs of *Photobacterium damsela* subsp. *damsela* strains isolated from different fish species were assayed to evaluate their *in vitro* toxicity using both mammalian and fish cell lines. The ECP samples tested displayed cytotoxic effects on all the cell lines used at 6 h post-inoculation, with a total destruction of the cell monolayers at 48 h post-inoculation, except for the strain 9401H, which did not produce cytotoxic effects. The degenerative changes mainly consisted of the appearance of clusters of round cells and dendritic elongations (Fig. 1), and finally cell detachment. However, the ECPs heated at 100°C had no effects on the different cell lines used.

Table 3. Enzymatic activity profiles of extracellular products of *Photobacterium damsela* subsp. *damsela* strains using the API ZYM system. 1, alkaline phosphatase; 2, esterase; 3, esterase lipase; 4, lipase; 5, leucine arylamidase; 6, valine arylamidase; 7, cystine arylamidase; 8, trypsin; 9, α-chymotrypsin; 10, acid phosphatase; 11, naphthol-AS-BI-phosphohydrolase; 12, α-galactosidase; 13, β-galactosidase; 14, β-glucuronidase; 15, α-glucosidase; 16, β-glucosidase; 17, N-acetyl-β-glucosaminidase; 18, α-mannosidase; 19, α-fucosidase. Activity expressed as nmol of hydrolyzed substrate

Strain	Activity (nmol)																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
203H	30-40	10-20	20-30	<5	5-10	<5	<5	<5	5-10	>40	5-10	<5	<5	<5	<5	<5	20-30	<5	5-10
401H	>40	5-10	5-10	<5	<5	<5	<5	<5	<5	>40	5-10	<5	<5	<5	<5	<5	5-10	<5	<5
402O	20-30	5-10	10-20	<5	<5	<5	<5	<5	<5	>40	20-30	<5	<5	<5	<5	<5	5-10	<5	<5
403D1	>40	<5	5-10	<5	5-10	<5	<5	<5	30-40	<5	<5	<5	<5	<5	<5	<5	5-10	<5	<5
412B	>40	10-20	20-30	<5	10-20	<5	<5	<5	5-10	5-10	5-10	<5	<5	<5	<5	<5	20-30	<5	<5
9401H	30-40	5-10	5-10	<5	<5	<5	<5	<5	5-10	30-40	10-20	<5	<5	<5	<5	<5	5-10	<5	<5
501H	30-40	5-10	5-10	<5	5-10	<5	<5	<5	5-10	>40	10-20	5-10	5-10	<5	<5	<5	5-10	5-10	5-10
501R	5-10	<5	10-20	<5	<5	<5	<5	<5	5-10	>40	10-20	<5	<5	<5	<5	<5	30-40	<5	<5
501B	30-40	5-10	5-10	<5	5-10	<5	<5	<5	5-10	>40	10-20	<5	<5	<5	<5	<5	5-10	<5	<5
601R	20-30	<5	10-20	5-10	10-20	<5	<5	<5	5-10	>40	10-20	<5	<5	<5	<5	<5	10-20	<5	<5
602R	>40	<5	5-10	<5	5-10	5-10	<5	<5	5-10	>40	10-20	5-10	5-10	<5	<5	5-10	10-20	5-10	5-10
D408U	30-40	5-10	10-20	<5	<5	<5	<5	<5	5-10	>40	10-20	<5	<5	<5	<5	<5	5-10	<5	<5
Lb501R	20-30	5-10	10-20	<5	<5	<5	<5	<5	5-10	>40	5-10	<5	<5	<5	<5	<5	5-10	<5	<5
S503C	20-30	5-10	10-20	<5	<5	<5	<5	<5	5-10	>40	5-10	<5	<5	<5	<5	<5	5-10	<5	<5
C601R1	20-30	5-10	10-20	<5	<5	<5	<5	<5	5-10	>40	10-20	<5	<5	<5	<5	<5	5-10	<5	<5
LCA240907	30-40	5-10	10-20	<5	5-10	<5	<5	<5	<5	>40	5-10	<5	<5	<5	<5	5-10	5-10	<5	<5
ATCC 33539 ^T	30-40	10-20	10-20	<5	5-10	<5	<5	<5	5-10	30-40	20-30	<5	5-10	<5	<5	<5	10-20	5-10	<5

Table 4. Enzymatic activity profiles of bacterial cells of *Photobacterium damsela* subsp. *damsela* strains using the API ZYM system. Activity expressed as nmol of hydrolyzed substrate. For abbreviations see Table 3

Strain	Activity (nmol)																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
203H	>40	5–10	20–30	<5	20–30	<5	<5	<5	<5	>40	10–20	<5	<5	<5	30–40	10–20	>40	5–10	<5
401H	30–40	10–20	10–20	<5	10–20	<5	<5	<5	30–40	30–40	5–10	<5	<5	<5	5–10	<5	30–40	<5	<5
402O	30–40	5–10	10–20	<5	10–20	<5	<5	<5	30–40	30–40	5–10	<5	<5	<5	5–10	<5	30–40	<5	<5
403D1	30–40	5–10	10–20	<5	10–20	<5	<5	<5	30–40	30–40	5–10	<5	<5	<5	5–10	<5	30–40	<5	<5
412B	20–30	5–10	10–20	<5	20–30	5–10	<5	5–10	30–40	30–40	5–10	<5	<5	<5	<5	<5	30–40	<5	<5
9401H	30–40	20–30	10–20	<5	20–30	5–10	10–20	<5	30–40	30–40	10–20	<5	<5	<5	30–40	<5	30–40	<5	<5
501H	30–40	10–20	10–20	<5	20–30	5–10	<5	<5	30–40	30–40	5–10	<5	<5	<5	5–10	<5	30–40	<5	<5
501R	20–30	5–10	10–20	<5	20–30	<5	<5	<5	30–40	30–40	5–10	<5	<5	<5	5–10	<5	30–40	<5	<5
501B	30–40	10–20	10–20	<5	20–30	<5	<5	<5	30–40	30–40	5–10	<5	<5	<5	<5	<5	30–40	<5	<5
601R	30–40	10–20	10–20	<5	20–30	<5	<5	<5	30–40	30–40	5–10	<5	<5	<5	<5	<5	30–40	<5	<5
602R	30–40	5–10	5–10	<5	10–20	<5	<5	<5	30–40	30–40	5–10	<5	<5	<5	<5	<5	30–40	<5	<5
D408U	30–40	5–10	10–20	<5	10–20	<5	<5	<5	30–40	30–40	5–10	<5	<5	<5	<5	<5	30–40	<5	<5
Lb501R	30–40	20–30	20–30	10–20	20–30	<5	<5	<5	30–40	30–40	10–20	<5	<5	<5	5–10	<5	30–40	<5	<5
S503C	30–40	10–20	20–30	<5	20–30	<5	<5	<5	30–40	30–40	5–10	<5	<5	<5	5–10	<5	30–40	<5	<5
C601R1	30–40	5–10	10–20	<5	20–30	<5	<5	<5	30–40	30–40	5–10	<5	<5	<5	<5	<5	30–40	<5	<5
LCA240907	30–40	10–20	20–30	<5	20–30	<5	<5	<5	30–40	30–40	10–20	<5	<5	<5	30–40	5–10	30–40	5–10	<5
ATCC 33539 ^T	30–40	10–20	20–30	<5	20–30	<5	<5	<5	30–40	30–40	5–10	<5	<5	<5	<5	5–10	30–40	5–10	<5

Hemolysis and detection of the phospholipase-D gene

The different strains of *Photobacterium damsela* subsp. *damsela* tested showed an evident hemolytic capacity on Mueller-Hinton plates supplemented with sheep, calf, and redbanded seabream blood, excepting for the strains 401H and 412B for sheep and calf blood, and 203H, Lb501R and S503C for fish blood (Table 2). The ECP samples produced no hemolysis for any of the strains tested, except in the case of the strain 402O, which produced hemolysis using all the erythrocyte types tested. The hemolytic activity of ECP of 402O strain was lost by heating at 100°C for 10 min, which indicates the production of thermolabile hemolysins by this strain.

Only the strongly hemolytic strains (402O and ATCC 33539^T) showed the specific 567 bp PCR product corresponding to the phospholipase-D gene using the primers dly-5'f and dly-3'r (Fig. 2). The amplification of the complete gene using dly-1f and dly-2r primers failed for all the strains tested.

DISCUSSION

Photobacterium damsela subsp. *damsela* is recognized as an opportunistic pathogen for a wide variety of hosts, mainly fish, dolphins, and humans (Fujioka et al. 1988, Pedersen et al. 1997, Yamane et al. 2004), being the causative agent of hemorrhagic septicemia in several cultured fish species (Labella et al. 2006, Kanchanopas-Barnette et al. 2009). The continuous isolation of this bacterial pathogen from diseased specimens of newly cultured fish species (Table 1) reveals that the syndrome may well be an emerging disease in cultured marine fish in several geographical areas.

Photobacterium damsela subsp. *damsela* possesses a variety of virulence factors that affect both mammals and fish, provoking symptoms such as septicemia, hemorrhages, and massive ulcers and necrosis on the skin surface or in other tissues. These data suggest that several secreted ECPs of the pathogen may contribute to the bacterial growth inside the hosts (Sakai 1985) and induce an infection and disease. Main ECP components related to virulence include proteases, hemolysins, and siderophore-mediated iron sequestering systems (Balebona et al. 1998b, Rodkum et al. 2005, Wang et al. 2007). These mechanisms can provoke tissue destruction and hemorrhages, playing an important role in colonization, invasiveness, and dissemination within the host (Finkelstein et al. 1992, Silva et al. 2003).

In the present study, the pathogenicity of *Photobacterium damsela* subsp. *damsela* strains isolated from different outbreaks affecting newly cultured marine

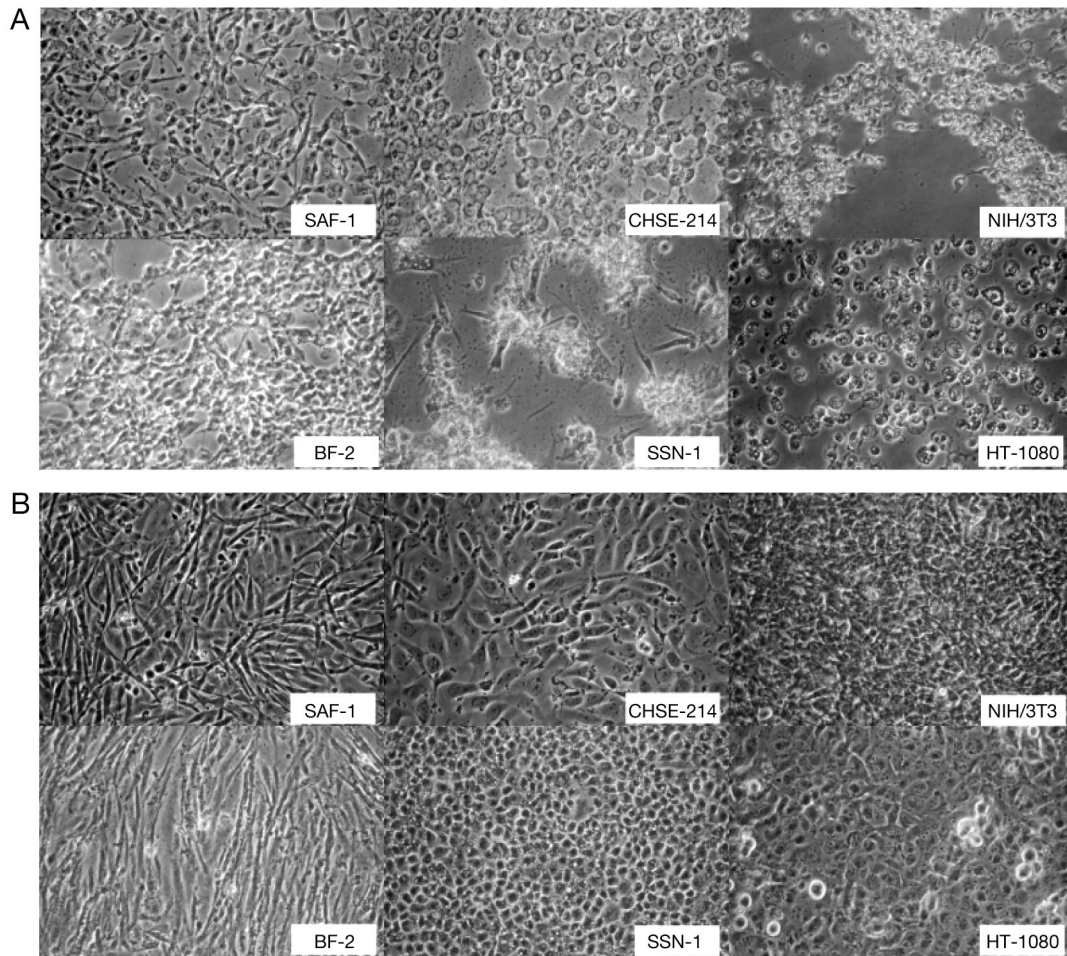


Fig. 1. *Photobacterium damsela* subsp. *damsela* extracellular product (ECP) cytotoxicity assay using mammalian and fish cell lines (see 'Materials and methods: Cytotoxic activity of the ECPs' for details). (A) Clusters of round cells and dendritic elongations produced by inoculation of ECP samples. (B) Inoculation of heated ECP samples produced no cytotoxic effects

fish have been evaluated. All the strains may be considered as moderately virulent according to Santos et al. (1988) criteria, with LD₅₀ values about 1 × 10⁵ CFU, with the exception of one non-virulent strain (LD₅₀ > 10⁸ CFU). The intraperitoneal inoculation of ECPs from the virulent strains was lethal for redbanded seabream at 2 to 4 h post-inoculation. Tissue damage was more

severe than those observed after bacterial inoculation (data not shown). Similar results have been reported for *Listonella* (= *Vibrio*) *anguillarum* and *P. damsela* subsp. *damsela* in other fish species (Lamas et al. 1994, Fouz et al. 1995). The inoculation of heated ECPs to fish did not produce deaths, which suggests that the active fraction of the exotoxin present in the ECPs is

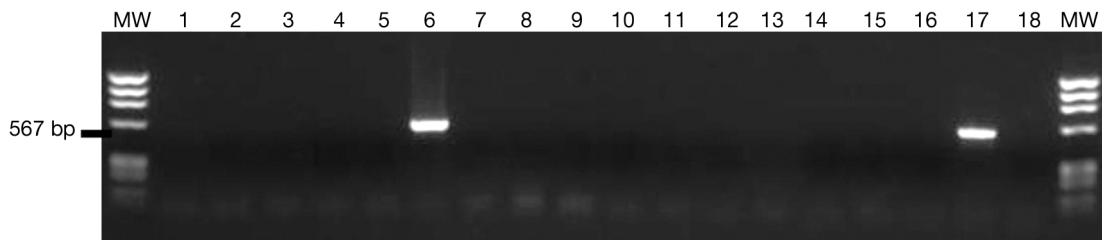


Fig. 2. PCR amplification of the 567 bp *dly* gene fragment of *Photobacterium damsela* subsp. *damsela* strains using *dly*-5' and *dly*-3' primers. Lanes: 1: 203H; 2: 401H; 3: 9401H; 4: 412B; 5: 403D1; 6: 402O; 7: 501H; 8: 501R; 9: 501B; 10: 601R; 11: 602R; 12: D408U; 13: C501R; 14: S503C; 15: LB501R; 16: LCA907; 17: ATCC 33539^T; 18: Negative control (DNA from *P. damsela* subsp. *piscicida* ATCC 17911^T); MW: 72 to 1353 bp ladder

thermolabile, and it is not associated with their thermoresistant lipopolysaccharide content. Fish inoculated with heat-inactivated ECP samples showed enlarged lymphohematopoietic organs suggesting a stimulation of immune response with cellular accumulation. Rey et al. (2009) reported that ECPs of *Aeromonas hydrophila* induced larger accumulations of hyaline structures resembling necrotic erythrocytes, into the lymphoid organs compared to the signs induced by the live bacterial cells alone. Hemolysis of erythrocytes in several fish organs has previously been reported during infections with fish pathogenic bacteria (Grizzle & Kiryu 1993).

Tissue cultures have played a crucial role in investigating bacterial–host interactions due to the easy manipulation of cells and their maintenance under controlled conditions. In the present study, all lethal ECP samples were also cytotoxic both for mammalian and fish cell lines (Fig. 1). The cytotoxicity was totally lost on heated ECP samples, indicating the presence of a thermolabile extracellular cytolysin.

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 & Shinoda 2000). In addition, peptidases enable the evasion of the bacteria from several fish defense mechanisms (Vivas et al. 2004). In the present study, the proteolytic activities of the *Photobacterium damsela* subsp. *damsela* ECPs tested were very low (Table 1), without production of caseinase or gelatinase (Table 2), but they exhibited other activities such as phospholipase, lipase, amylase, phosphatases, or glycosidases (Tables 2 & 3). Nevertheless, none of these activities could be related with the high degree of toxicity both *in vivo* and *in vitro* presented by the ECPs, in contrast to results reported for other fish pathogens (Esteve et al. 1995).

On the other hand, several authors have demonstrated that the pathogenicity of bacterial fish pathogens was related to the capability to hemolyse the host erythrocytes (Borrego et al. 1991, Fouz et al. 1993, Pedersen et al. 2009). The differences found in the hemolytic activity of the *Photobacterium damsela* subsp. *damsela* strains (Table 2) are in agreement with the results obtained by Cutter & Kreger (1990) and Thyssen et al. (1998), who reported variable responses dependent on both the source of the erythrocytes and the strain source. Surprisingly, the hemolytic activity disappeared in the ECPs, except for the strain 402O (Table 2). A correlation between the hemolytic activity of *P. damsela* subsp. *damsela* strains and their virulence properties could not also be established.

Fouz et al. (1993) reported that *Photobacterium damsela* subsp. *damsela* (formerly *Vibrio damsela*) strains isolated from diseased fish secreted a cytolysin

with phospholipase activity named damselysin, which plays an important role in the virulence mechanism of this pathogen for both fish and mammals. However, in the present study, although 75 % of the virulent strains showed phospholipase activity in their ECPs, only 2 of them (strains 402O and ATCC 33539^T) showed the specific amplicon corresponding to the phospholipase-D (*dly*) gene (Fig. 2). Similarly, other authors have concluded that not all *P. damsela* subsp. *damsela* strains carry the *dly* gene (Clarridge & Zighelboim-Daum 1985, Osorio et al. 2000). Therefore, a correlation between the presence of *dly* gene and the pathogenicity of the *P. damsela* subsp. *damsela* might not be established. Consequently, we can hypothesize that another unknown type of toxin, different to the *dly* gene, could be involved on the toxicity of the *P. damsela* subsp. *damsela* ECPs. A neurotoxin possessing an acetylcholine-esterase activity has been described in strains of *Aeromonas hydrophila* and several species of *Vibrio*, including *V. damsela* (*P. damsela* subsp. *damsela*), which could be responsible for several clinical signs observed in the present study (Balebona et al. 1998b, Perez et al. 1998).

In conclusion, the cytopathology induced by ECPs in cell lines would be an adequate *in vitro* model for the determination of *Photobacterium damsela* subsp. *damsela* virulence. On the other hand, more efforts would be necessary to demonstrate the neurotoxic effect of *P. damsela* subsp. *damsela* ECPs by determination of the acetylcholine-esterase activity. In addition, the presence of phospholipases (including damselysin) is not directly related to the pathogenic properties of *P. damsela* subsp. *damsela* strains.

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