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Characterization and pathogenicity of *Vibrio splendidus* strains associated with massive mortalities of commercial hatchery-reared larvae of scallop *Argopecten purpuratus* (Lamarck, 1819)

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ABSTRACT

Three strains (VPAP16, VPAP18 and VPAP23 strains) were isolated as the most predominant organisms from 3 different episodes of massive mortalities of larval cultures of the Chilean scallop *Argopecten purpuratus* occurred in different commercial hatcheries located in northern Chile. The main aims of this study were to identify the pathogenic strains and investigate their pathogenic activity. Based on selected phenotypic features and sequence identity of the 16S rRNA gene and the housekeeping gene, RNA polymerase α-chain *rpoA*, all pathogenic strains were identified as *Vibrio splendidus*. Healthy 10–day–old scallop larvae cultures exhibited mortality percentages of 69.61 ± 3.35%, 79.78 ± 6.11% and 61.73 ± 3.71% after 48 h when were inoculated with 1×10^6 CFU (colony forming units) mL^{-1} of VPAP16, VPAP18 and VPAP23 strains, respectively, and evidenced that concentrations $\geqslant 10^4$ CFU mL^{-1} would probably be detrimental for the larval culture. The main clinical signs observed in challenged larvae for 24 h were bacterial swarms on the margins of the larvae, extension and disruption of the velum, detachment of velum cilia cells and digestive tissue necrosis. Otherwise, challenge assays using pathogenic strains stained with 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein hydrochloride (5-DTAF) evidenced that after 1 h stained bacteria were detected in high density in the digestive gland and the margin of the shell. When larval cultures were inoculated with cell-free extracellular products (ECP) of *V. splendidus* strains, exhibited larval mortalities higher than 70% (VPAP16), 80% (VPAP18) and 50% (VPAP23) after 24 h, even when ECP were treated with proteinase K or heat, indicating that extracellular pathogenic activity is mainly mediated by non-proteic thermostable compounds. In this study all Koch's postulates were fulfilled and it was demonstrated for the first time the pathogenic activity of *V. splendidus* strains on reared-larvae of scallop *A. purpuratus* and prompt the necessity to maintain this species at concentrations lower than 10^4 CFU mL^{-1} to avoid episodes of mass mortalities in scallop hatcheries.

1. Introduction

The culture of Chilean scallop *Argopecten purpuratus* (Lamarck, 1819) is one of the most commercially important industries of Chilean mariculture and is mainly concentrated in the North region of the country (von Brand et al., 2006). Although efficient culture techniques for scallop larvae production have been developed, Chilean hatcheries occasionally suffer episodes of massive mortalities of reared-larvae, mainly caused by bacterial pathogens. Some of these pathogens have been previously identified as *Halomonas* sp. (Rojas et al., 2009), *Vibrio anguillarum*-related (Riquelme et al., 1995a), and the association of *Aeromonas hydrophila* and *Vibrio alginolyticus* (Riquelme et al., 1996), but the information of larval bacterial pathogens in the culture of *A. purpuratus* is still scarce.

Many *Vibrio* species have been reported to produce high mortalities in larvae and juveniles stages of reared molluscs (Jeffries, 1982; Nicolas et al., 1992; Freites et al., 1993; Sainz et al., 1998; Lacoste et al., 2001; Estes et al., 2004; Gómez-León et al., 2005; Kesarcodi-Watson et al., 2009b; Romalde et al., 2014). Clinical signs frequently observed in mollusc larvae suffering vibriosis

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outbreaks include bacterial swarms on the margins of the larvae, detachment of velum cells, colonization of the mantle, invasion of the internal cavity and subsequently a necrosis of soft tissues (Tubiash et al., 1965; Tubiash and Otto, 1986; Beaz-Hidalgo et al., 2010) and these clinical signs have been described in different molluscan species including clam *Ruditapes decussatus* (Gómez-León et al., 2005), oysters *Crassostrea virginica* (Gómez-León et al., 2008) and *Crassostrea gigas* (Estes et al., 2004; Gay et al., 2004b; Garnier et al., 2007), and scallops *Pecten maximus* (Nicolas et al., 1996; Torkildsen et al., 2005) and *Argopecten ventricosus* (Luna-González et al., 2002). In addition, virulence of pathogenic *Vibrio* strains isolated from molluscs has been shown to be related to their ability to produce extracellular products (ECPs) (Elston and Leibovitz, 1980; Nottage and Birkbeck, 1987a, 1987b; Labreuche et al., 2006; Hasegawa et al., 2008).

Previous reports show an important incidence of *Vibrio* spp. in Chilean commercial hatcheries of *A. purpuratus* (Riquelme et al., 1995b; Avendaño et al., 2001; Jorquera et al., 2004), suggesting that *Vibrio* could be a pathogen of major concern for the pectinid industry. The proper identification and the understanding of pathogenic activity of bacterial strains causing epizootics in larval cultures are essential for developing efficient management strategies to prevent and control outbreaks in intensive scallop larvae husbandry. Considering the occurrence of episodes of massive mortalities of reared larvae occurring in Chilean scallop culture that are mainly caused by bacterial pathogens as well as the absence of knowledge on the pathogenic mechanisms involved in these outbreaks, the aims of this study were to identify pathogenic *Vibrio* strains recovered from massive losses in the pectinid culture industry and to characterize their pathogenic activity on scallop larvae.

2. Materials and Methods

2.1. Sampling and bacterial isolation

Larval samples were taken from three different events of massive mortalities of reared-larvae of scallop *A. purpuratus* occurred in 3 commercial hatcheries located in the north of Chile. In each larval outbreak, samples of settled moribund larvae were collected from the rearing tanks in sterile glass flasks during the water exchange process. To reduce bacterial contamination, scallop larvae were mesh concentrated and washed with 0.45 μm-filtered, autoclaved seawater and resuspended in 500 mL of sterile seawater. Larval samples were centrifuged at 3000 rpm for 5 min and ground by hand using a sterile glass digester added with 2 mL of sterile physiological saline (0.85%) (PS) to obtain a homogenate according to Nicolas et al. (1996). The homogenate and tenfold dilutions prepared in PS were seeded (0.1 mL) in triplicate onto Tryptic Soy Agar (Difco) supplemented with 2% of NaCl (Oxoid) (TSA2) and TCBS agar (Difco) and plates were incubated at 20 °C for 48 h. In each case a dense bacterial culture was grown and a predominant colony grew almost as a pure culture on TSA2 and TCBS media, suggesting its role as the etiological agent of each larval disease. Predominant colonies observed in each event were purified in TSA2 and stored at −85 °C in CryoBank (Mast Diagnostic) vials until use.

2.2. Bacterial characterization

2.2.1. Phenotypic analysis

The phenotypic tests Gram stain, cell morphology, motility, oxidase and catalase production, oxidation/fermentation of glucose, gas from glucose, growth on thiosulfate-citrate-bile salts sucrose (TCBS) agar, swarming on solid media and susceptibility to the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine) (10 and 150 μg per disc) were determined according to the procedures described in Barrow and Feltham (1993) in media supplemented with NaCl (2%). Luminiscence was observed in the dark on Marine agar and β-hemolysis of sheep erythrocytes was determined using Columbia Blood agar (Oxoid). Møller's L-lysine and L-ornithine decarboxylase, and Thornley's arginine dihydrolase were performed according to Hansen and Sörheim (1991). Growth at different temperatures (4, 20, 30, 35, 40 °C) was tested on Tryptic soy broth (Difco labs.) supplemented with 2% NaCl, whereas growth at different salinities (0%, 3%, 6%, 8% and 10% of NaCl) was tested using peptone broth (Difco). The results were recorded after incubation at 20 °C for 48 h. Additional phenotypic characteristics were determined by using the API 20E system (bioMérieux, Marcy-l'Etoile, France), and strains were additionally screened for their ability to utilize 95 organic substrates inoculating the GN2 microplates of the Biolog system (Biolog Inc., Hayward, CA) assay. For the API system *Vibrio* strains were inoculated according to the manufacturer's instructions with the modifications suggested by MacDonnell et al. (1982) and API strips were incubated at 20 °C for 48 h. For the Biolog system strains were inoculated by using a solution containing 2.5% NaCl, 0.8% $MgCl_2$ and 0.05% KCl according to the instructions of the manufacturer and microplates were incubated aerobically in the dark at 20 °C, and duplicate readings were made after 48 h and 72 h of incubation, but only results after 72 h of incubation were considered. Furthermore, enzymatic activities of strains were determined utilizing the API ZYM system according to the manufacturer's guidelines (bioMérieux, France). Briefly, isolated colonies were cultured overnight in Tryptic Soy broth (Difco) supplemented with 2% NaCl (Oxoid) (TSB2), centrifuged at 5000 rpm at 4 °C and resuspended in sterile 3% (w/v) NaCl solution to obtain a turbidity of 5–6 McFarland (1.5–1.8 × 10^9 CFU mL^{-1}). This suspension (65 μL) was added to each cupule and the test strips were incubated for 4 h at 22 °C and read as indicated by the manufacturer. All assays were performed twice.

2.2.2. Molecular analysis

Bacterial strains were resuspended in TE buffer (Tris 0.01 M, EDTA 0.001 M, NaCl 0.15 M, pH 7.8), and cell lysis was performed using sodium dodecyl sulfate and incubation at 70 °C. The DNA were extracted with phenol/chloroform and subsequently precipitated with ethanol as previously described (Romero et al., 2002). A final purification was carried out using Wizard DNA Clean Up (Promega, Madison, WI, USA). PCR reactions were performed as described in Romero and Navarrete (2006) with a reaction mixture (30 μL) containing 0.2 mM of each deoxynucleoside triphosphate, 0.05 U μL^{-1} Platinum Taq DNA polymerase (Invitrogen, San Diego, CA, USA), 1 × polymerase reaction buffer, 2 mM $MgCl_2$, and 0.25 pmol μL^{-1} of each primer. To identify the bacterial strains, amplification of 16S rRNA from positions 28 to 1492 was performed using primers 27F and 1492R as previously described (Navarrete et al., 2010). For *rpoA* gene analysis, PCR mixtures were identical as described before for 16S rDNA, but the primers used were rpoAF: ATGCAGGGTTCTGTDACAG and rpoAR: GHGGCCARTT TTCHARRCGC (Thompson et al., 2005). The thermal program consisted of (i) 5 min at 95 °C; (ii) 25 cycles of 30 s at 95 °C, 30 s min at 55 °C, and 30 s at 72 °C; and (iv) a final 5 min at 72 °C. All PCR products were analyzed by polyacrylamide electrophoresis and silver nitrate staining according to Romero et al. (2002) and were purified using Wizard PCR Preps (Promega) and then sequenced by the Macrogen USA sequencing service. The 16S rRNA gene sequences were compared with those available in the public Ribosomal Database Project II (Cole et al., 2007) (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp), whereas *rpoA* gene sequences were compared with those available in the National Center for Biotechnology Information (NCBI) Reference Sequence

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database by using a BLAST search in order to ascertain their closest relatives.

2.3. Bacterial pathogenicity assay

Larval challenges were performed using healthy 10 day-old scallop larvae of *A. purpuratus* obtained from the Marine Culture Laboratory of the Universidad Católica del Norte. Health status of larval cultures were confirmed when larvae showed active swimming, intact velum exhibiting intense ciliar movement and with digestive glands with full microalgal content. Bacterial isolates were tested for pathogenicity using 12-well microplates (Orange Scientific) in triplicate and different microplates for each isolate were used to avoid cross-contamination. Scallop larvae were added to each well containing microfiltered (0.22 μm) seawater (salinity 36‰) at a final concentration of 20 larvae mL^{-1}. Pathogenic strains were grown on TSA2 agar and top portion of colony material was collected using a loop without touching the agar surface and resuspended in microfiltered sterile seawater, adjusted by absorbance determination at 600 nm and added to the challenged larval groups at the final concentration of 1×10^6 colony forming units (CFU) mL^{-1}. Larval control groups were inoculated with an equal volume of sterile seawater. Bacterial concentration was confirmed by enumeration on TSA2 agar plates using a standard dilution plating technique with incubation at 20 °C for 48 h. Microplates were incubated for 48 h in the dark at 18 °C and clinical symptoms of disease as well as the proportion of dead larvae was recorded at 0, 6, 12, 18, 24, 30, 36 and 48 h by using the inverted microscope Olympus CKX41 (Tokyo, Japan). Larvae were considered dead when no valvar movement was observed. To fulfill with all Koch's postulates an analysis of pathogenic *V. splendidus* recovery was performed using culture independent techniques based on the examination of the DNA extracted from samples of larval cultures not challenged (control) and challenged with the pathogenic strains for a period of 24 h. The larvae homogenates were weighed and an equal weight of cold sterile TE buffer was added. DNA from homogenates was extracted as described previously (Trabal Fernández et al., 2014) and were PCR-amplified using primers targeting 16SrRNA; 27F and 1492R and primers targeting *rpoA* (described above). Amplicons were sequenced using Macrogen USA sequencing service. In the larvae samples, levels of pathogenic vibrios were determined by using qPCR, using primers targeting *rpoA* (described above), respectively. qPCR reactions were performed as described (Abid et al., 2013). Melting curve of the products was checked in each reaction.

2.4. Pathogenic activity of different concentrations of V. splendidus strains

Healthy nine-day old scallop larval cultures at a density of 20 larvae mL^{-1} in 12-well microplates (Orange Scientific) were inoculated with the appropriate bacterial suspensions of overnight cultures of each pathogenic strain to obtain a final concentrations of 1×10^1, 1×10^2, 1×10^3, 1×10^4, 1×10^5 and 1×10^6 CFU mL^{-1}. Bacterial concentrations were confirmed by a standard dilution plating technique as previously described. Larval cultures inoculated with sterile seawater were used as controls. All assays were performed in triplicate and larval cultures were maintained at 18 °C in the dark for 36 h and observed at 0, 12, 24 and 36 h to determine larval survival using the inverted microscope Olympus CKX41 (Olympus, Japan).

2.5. Invasive pathogenic activity

The methodology of Sherr et al. (1987) to label bacteria with 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein hydrochloride (5-DTAF, Sigma–Aldrich, D-0531, St. Louis) was modified to obtain the best conditions for *Vibrio* strains (VPAP16, VPAP18 and VPAP23). Pathogenic strains were recovered in TSB2 (Difco) at 20 °C and agitated at 100 rpm in orbital shaker (WiseShake SHO-2D) for 24 h. The broth was centrifuged at 8000 rpm for 8 min at 20 °C, the bacterial pellet was resuspended in 10 mL of sterile seawater and optical density was adjusted to 0.8–1.3 at 610 nm in a spectrophotometer (PG Instruments T70) under sterile conditions. 5-DTAF was dissolved in sterile 0.22 μm filtered PBS (pH 7.4) at 0.5 mg mL^{-1}. A 0.5 mL aliquot of 5-DTAF solution was added to 9.5 mL of the bacterial suspension, and the mixture was incubated for 1 h in total darkness with shaking at 90 rpm at 20 °C. After incubation, cultures were pelletized by centrifugation (8000 rpm at 20 °C for 6 min) and resuspended in 0.22 μm filtered seawater. This procedure was repeated until an unstained suspension was observed and healthy 10 day-old scallop larvae of *A. purpuratus* maintained in 12-well microplates (Orange Scientific) at a density of 20 mL^{-1} larvae were inoculated in triplicate with the 5-DTAF-stained bacterial strains to obtain a final concentration of 1×10^5 - CFU mL^{-1} and observed at 6, 12, 18, 24 and 30 h using the Nikon's fluorescence microscope Eclipse 50i. Bacterial concentrations were confirmed by a standard dilution plating technique as previously described. Larval cultures inoculated with unstained pathogenic strains as well as larval cultures not inoculated with the assayed bacterial strains were included as controls. Experiments were repeated 2 times to confirm reproducibility.

2.6. Pathogenic activity of extracellular products (ECP)

The ECP were obtained by the cellophane overlay plate method as described by Munro et al. (1980). Briefly, a volume of 2 mL of 36 h cultures of *Vibrio* strains grown in TSB2 were spread onto sterile cellophane films placed on the surface of TSA2 plates. After incubation at 20 °C for 24 h, the cellophane overlay was transferred to an empty Petri dish and bacterial cells were washed off from the cellophane sheet using phosphate buffered saline (PBS, pH 7.4) and removed by centrifugation at 13,000 rpm at 4 °C for 20 min. Supernatants were sterilized by filtration through a 0.22 μm filter (Sartorious Stedim Biotech, Germany) and stored at −80 °C until use. Total protein concentrations of the supernatans were measured using the commercial kit cupric BCA (Novagene) and then were read at 562 nm using the T70 UV/VIS spectrophotometer (PG Instruments Ltd, Leicestershire, United Kingdom). In addition, cell-free supernatant samples were treated with proteinase K and heat to evaluate the proteinaceous composition of the virulence factors included in the bacterial supernatants. Cell-free supernatants of *Vibrio* strains were treated with proteinase K (Sigma) at a final concentration of 1 mg mL^{-1}. The enzyme was initially dissolved in PBS and added to a subsample of the supernatant at a 1:10 dilution (one part enzyme, nine parts supernatant). The reaction was incubated at room temperature for 2 h and subsequently heated for 5 min at 100 °C to inactivate the enzyme. The heat treatments of the cell-free supernatants were performed by heating a subsample at 125 °C for 15 min. The sample was diluted with PBS in the same ratio as for the proteinase K. Ten day-old scallop larvae were added to each well of 12-well microplates (Orange Scientific) added with 3.8 mL of microfiltered seawater containing a concentration of 20 larvae mL^{-1} and then inoculated in triplicate with 200 μL of the non-treated and treated cell-free supernatant to obtain a final concentration of 4 μg protein mL^{-1}. Larval cultures inoculated with 200 μL of PBS only were used as controls. Microplates were incubated for 24 h in the dark at 18 °C and the proportion of dead larvae was determined at 12 and 24 h by using the Olympus's inverted microscope CKX41 (Tokyo, Japan). Larvae were considered dead when no movement was observed with the valves.

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2.7. Detection of siderophores

To determine if pathogenic strains were able to produce siderophores, the chrome azurol S (CAS) agar assay was performed according to Schwyn and Neilands (1987). Bacterial cultures in stationary phase (3×10^8 CFU mL^{-1}) grown for 24 h at 22 °C in CM9 medium (Gómez-León et al., 2005) were spotted on CAS agar plates, which were incubated for 72 h at 28 °C and appearance of yellow orange halos around bacterial spots grown on the CAS agar indicated the production of siderophores. The size of the halo was used as a way to estimate the siderophore level produced. *Salmonella* Typhimurium wild type (Luo et al., 2011) and *S.* Typhimurium *Δfur-44* (Santander et al., 2012) were used as controls for siderophore synthesis.

2.8. Statistical analyses

For larval assays, mortality percentages were transformed to arcsin (square root [mortality rate ration]) and were analyzed using one-way analysis of variance (ANOVA). Normality of the variables was determined by using the Kolmogorov–Smirnov test, whereas homogeneity of the variances was determined by using the Levene's test (Zar, 1999). When overall differences were significant ($P < 0.05$), a Tukey's multiple range test was used to determine significant differences ($P < 0.05$) among the proportions of mortality of challenged and control scallop larvae. All statistical analyses were performed by using the Sigma version 3.1 computer program (Systat Software Inc.).

3. Results

3.1. Bacterial characterization

Pathogenic strains exhibited phenotypic characteristics of bacteria belonging to the genus *Vibrio* (Farmer and Janda, 2004). All were Gram-negative, short, motile rods, oxidase and catalase producers, susceptible to O/129 and required NaCl for growth. All strains were consistently positive for β-galactosidase and arginine dihydrolase, indole production, acid from glucose, mannitol and amygdalin, and consistently negative for tryptophane deaminase, lysine decarboxylase and ornithine decarboxylase, H_2S production, acetoin production, and acid from mannose, inositol, sorbitol, rhamnose, sucrose, melibiose and arabinose, matching with the description of *Vibrio splendidus* in Bergey's Manual of Systematic Bacteriology (Farmer and Janda, 2004). Additionally, pathogenic strains exhibited phenotypic characteristics that distinguish *V. splendidus* from other lysine and ornithine decarboxylase-negative *Vibrio* species, including hydrolysis of gelatin, growth on TCBS medium, no acid from sucrose and no growth at 4 °C (Macián et al., 2001).

Further phenotypical characterization of all *V. splendidus* strains indicated a high phenotypical homogeneity showing utilization of the following Biolog substrates as sole carbon sources: α-cyclodextrin, dextrin, glycogen, tween 40, tween 80, D-cellobiose, D-fructose, D-galactose, α-D-glucose, α-D-lactose, maltose, D-mannitol, D-mannose, D-trehalose, methyl-pyruvate, acetic acid, cis-aconitic acid, citric acid, D-gluconic acid, α-keto glutaric acid, D,L-lactic acid, succinic acid, bromo succinic acid, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, L-proline, D-serine, L-threonine, inosine, uridine, thymidine, glycerol, D,L-α-glycerol phosphate, glucose-1-phosphate and glucose-6-phosphate. It must be noted that the sole difference among strains was the use of α-D-lactose by the VPAP16 and VPAP18 strains, but not by the VPAP23 strain.

No differences in the enzymatic profiles obtained by the API ZYM tests were observed among the *V. splendidus* – related strains, being able to produce the alkaline phosphatase, esterase (C_4), esterase lipase (C_8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase enzymes. It must be noted that enzymatic patterns of pathogenic strains were identical to the exhibited by the *V. splendidus* biotype 2 NCMB2251 (Lunder et al., 2000).

V. splendidus 16S rDNA sequences were compared with homologous published sequences. The 16S rDNA closest sequences of pathogenic *Vibrio* strains belonged to *V. splendidus* strains. VPAP16 strain (GenBank accession number KF880674) gave 99.77% sequence similarity with *V. splendidus* (GenBank accession number FR750951) and 99.6% sequence similarity with *V. splendidus* EU091329. VPAP18 strain (GenBank accession number KF880675) gave 99.7% sequence similarity with *V. splendidus* (GenBank accession number EU091325) and 99.6% sequence similarity with *Vibrio* sp. (GenBank accession number HM012747), whereas VPAP23 strain (GenBank accession number KF880676) gave 99.8% sequence identity to *V. splendidus* (GenBank accession numbers KF009762 and KF009757).

The 16S rRNA gene sequence differences to each other *V. splendidus* strains were very low, observing differences of 0.075% between the VPAP16 and VPAP18 strains, 0.15% between the VPAP16 and VPAP18 strains and 0.30% between VPAP16 and VPAP18 strains. Only 1 nucleotide base was different between strain VPAP16 and VPAP18 (C × T in position 420), 2 nucleotide bases were different between strain VPAP18 and VPAP23 (T × G in position 400 and G × A in position 900), and 3 nucleotide bases were different between strain VPAP16 and VPAP23 (T × G in position 400, C × T in position 420 and G × A in position 900).

Otherwise, the *rpoA* gene sequences of pathogenic *Vibrio* strains VPAP16, VPAP18 and VPAP23 gave 100% sequence similarity with *V. splendidus* (protein id = YP_002418345.1 of NCBI Reference Sequence NC_011753.2), exhibiting a 100.0% of *rpoA* gene sequence similarity to each other.

3.2. Bacterial pathogenicity assays

All strains showed high levels of pathogenicity on the assayed scallop larvae but VPAP23 strain was less pathogenic than the other strains. After 24 h of exposure, VPAP16 and VPAP18 strains caused larval mortalities of 42.25 ± 2.70 and 38.50 ± 4.11%, respectively, being significantly higher than the VPAP23 strain (20.41 ± 2.10%, $P < 0.001$). Larval mortality at 48 h post inoculation was 69.61 ± 3.35%, 79.78 ± 6.11% and 61.73 ± 3.71% for strains VPAP16, VPAP18 and VPAP23, respectively observing no significant differences between strains VPAP16 and VPAP18 ($P = 0.056$) and between VPAP16 and VPAP23 strains ($P = 0.245$), but VPAP18 strain was significantly ($P < 0.05$) more pathogenic than VPAP23 strain (Fig. 1). No larval mortality was observed in the control groups, until 48 h of incubation (2.31 ± 1.16%).

All *V. splendidus* strains produced the classical symptoms of bacillary necrosis and were similar to those of the reared-larvae during the vibriosis outbreaks in the sampled commercial hatcheries. The main clinical signs exhibited by infected larvae were swarms of bacteria on the margins of the larvae, disruption and/or extension of the velum, detachment of velum cilia cells and necrosis of digestive tissue (Fig. 2), after 24 h of bacterial exposure.

When different concentrations of the *V. splendidus* strains were inoculated in the scallop larvae cultures no larval mortality or very low levels of larval mortality was observed after 24 and 36 h of exposure to concentrations equal or less than 10^3 CFU mL^{-1} (Fig. 3). At 36 h, when bacterial concentrations of 10^4 CFU mL^{-1} were inoculated only VPAP16 and VPAP18 strains exhibited important pathogenic activity (27.25 ± 2.70 and 35.30 ± 3.90% of larval mortality, respectively) and were significantly higher than the

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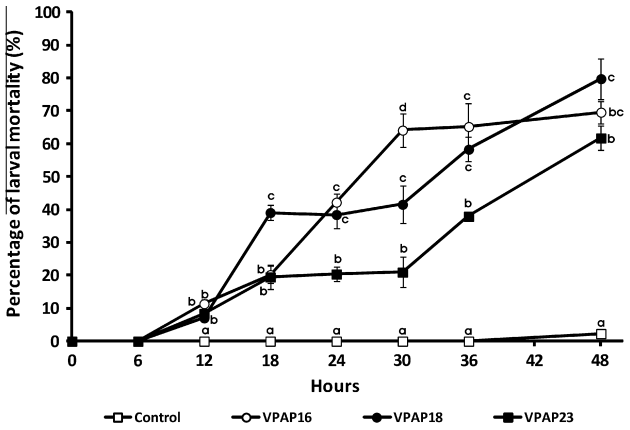

Fig. 1. Mortality of 10-day-old scallop larvae unchallenged (control) and challenged with 1×10^6 CFU mL^{-1} of *Vibrio* strains VPAP16, VPAP18 and VPAP23), over a 48 h period. Values are means of three replicates. Letters indicate significant differences between treatments (mean ± SD, ANOVA $P < 0.05$).

produced by the VPAP23 strain (11.40 ± 5.81% of larval mortality) (Fig. 3B). Significative differences ($P < 0.05$) in larval mortalities produced by the *V. splendidus* strains inoculated at concentrations of 10^6 CFU mL^{-1} after 36 h were observed, observing that VPAP16 and VPAP18 strains produced mortalities of 79.22 ± 2.84 and 88.47 ± 3.63%, respectively, whereas VPAP23 strain only produced a larval mortality of 44.50 ± 4.78% (Fig. 3B).

Molecular tools assessed the presence of pathogenic strains in moribund and dead larvae after 24 h of bacterial challenge with the 3 *V. splendidus* strains. In challenged cultures the levels of each pathogenic strain were $9.59 \times 10^4 \pm 1.07 \times 10^4$ of *V. splendidus* VPAP16 per gram of larvae, $2.08 \times 10^5 \pm 8.13 \times 10^3$ of *V. splendidus* VPAP18 per gram of larvae and $2.83 \times 10^4 \pm 8.89 \times 10^2$ of *V. splendidus* VPAP23 per gram of larvae. Sequencing *rpoA* amplicons obtained directly from larvae homogenates allowed the validation of quantification and the identification of each pathogenic *V. splendidus* strain. The presence of each pathogenic *V. splendidus* strain in moribund and dead larvae was also corroborated after PCR amplification and sequencing of 16S rRNA fragment; the sequence analysis revealed 100% identity between of 16S rRNA from larvae and from *V. splendidus* strain samples.

3.3. Invasive pathogenic activity

All *V. splendidus* strains were efficiently stained using 5-DTAF and the fluorescence was maintained for at least 36 h. Stained-bacterial cells of the pathogenic strains were detected in a high concentration surrounding the larval shells as well as inside the digestive gland of the scallop larvae, as soon as 1 h after the bacterial inoculation (Fig. 4A), and after 12 and 24 h bacterial cells still remained inside the infected larvae (Fig. 4B and C), whereas only after 30 h a decrease in the intensity of fluorescence was detected in the digestive gland (Fig. 4D).

3.4. Pathogenicity of bacterial extracellular products (ECP)

In general, when scallop larval cultures were exposed to ECP of pathogenic strains identical symptoms produced during bacterial challenges were observed. After 12 h of inoculated, ECP of VPAP18 strain produced the highest larval mortality (72.67 ± 5.03%), followed by VPAP16 (46.67 ± 4.16%) and VPAP23 (40.67 ± 5.03%) strains. Larvae inoculated with the cell-free supernatants of the pathogenic strains exhibited high levels of larval mortality over a period of 24 h, and the pathogenic activity of ECP from VPAP16 and VPAP18 strains was significantly ($P < 0.05$) higher than the produced by the ECP from the VPAP23 strain (Table 1). It must be noted the high levels of virulence of ECP produced by the VPAP16 and VPAP18 strains, considering that after 24 h of exposure, larval mortalities of 77.00 ± 3.00% and 92.00 ± 2.00% were produced by the ECP from the VPAP16 and VPAP18 strains, respectively (Table 1). After 12 h of exposure no larval mortality was observed in the control groups and only a 2.50 ± 1.00% of larval mortality was observed after 24 h. Otherwise, results from Table 1 evidenced that toxic activity of bacterial supernatants was not affected by treatment with proteinase K or heat (Table 1). At 12

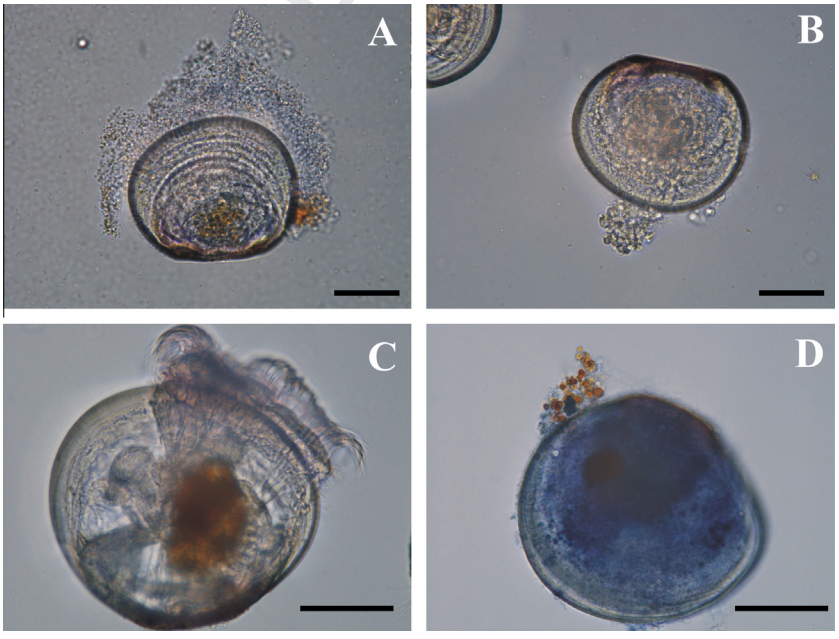

Fig. 2. Main symptoms of pathogenic activity of *Vibrio splendidus* strains on experimentally infected *Argopecten purpuratus* larvae after 24 h exposure. (A) Swarms of bacteria on the margins of the larvae (bacterial swarming), (B) velum disruption, (C) extension of the velum and (D) detachment of the cilia cells of the velum and necrosis of digestive tissue stained with trypan blue. Scale bars: 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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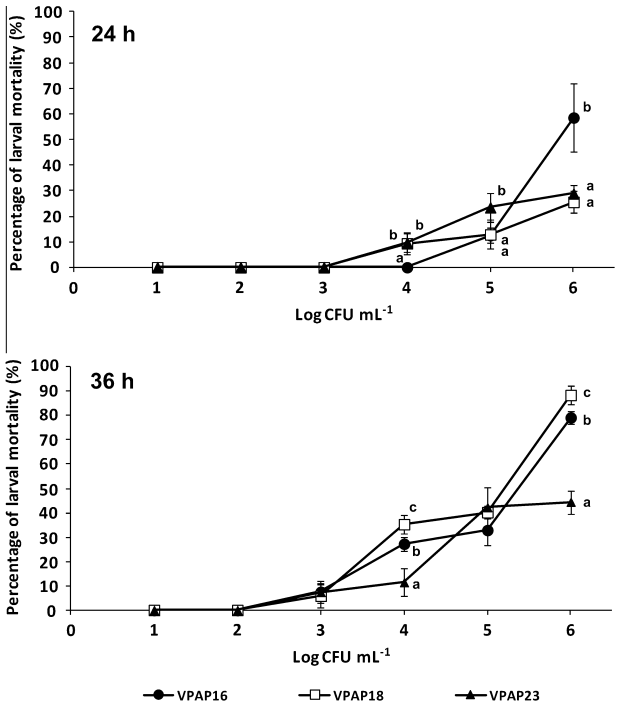

Q7 **Fig. 3.** Mortality of 10-day-old scallop larvae challenged with various concentrations of *Vibrio splendidus* strains over a 24 h (A) and 36 h (B) period. Values are means of three replicates. Letters indicate significant differences between treatments (mean ± SD, ANOVA $P < 0.05$).

and 24 h, larval mortality of cultures inoculated with non-treated cell-free supernatants from the 3 strains was not significantly different than those of cultures inoculated with cell-free supernatants treated with proteinase K or heat. After 24 h of exposure only a small reduction in the larval mortality of cultures inoculated with supernatans treated by heat when compared with untreated and treated with proteinase K supernatants was observed, but these differences were not significantly ($P < 0.05$) different.

3.5. Detection of siderophores

All pathogenic strains were able to produce siderophores, but the size of orange halos around bacterial spots grown on the CAS agar was small, indicating that a low level of siderophores was produced by the pathogenic strains.

4. Discussion

The present study demonstrated that mortality events occurred in different locations and in various scallop hatcheries in Chile are associated with highly pathogenic strains belonging to the *V. splendidus* group. Currently, more than 70 *Vibrio* species are recognized, grouped in 14 clades (Sawabe et al., 2007) and the Splendidus clade contains the highest number of species, which have been found to be the dominant *Vibrio* species in marine environments and bivalves (Lambert et al., 1998; Sobecky et al., 1998; Romalde et al., 2014). Phenotypic characteristics that distinguished *V. splendidus* strains with phenotypically and phylogenetically related species of the genus *Vibrio* included β-galactosidase and indole production, growth on TCBS agar, hydrolysis of gelatin, acid production from mannitol, no growth at 4 and 35 °C, no acetoin production and no acid from melibiose, arabinose and sucrose (Lambert et al., 1998; Macián et al., 2001; Diéguez et al., 2011). In this study, pathogenic *Vibrio* strains were identified as *V. splendidus* by using both traditional biochemical and molecular techniques, observing that classification of VPAP16, VPAP18 and VPAP23 strains by traditional phenotypical techniques were in agreement with those obtained by using the sequence of their 16S rRNA and *rpoA* genes. The three pathogenic strains showed 99.70–99.93% 16S rRNA gene sequence similarity to each other, being above the limit of intraspecies variability (98.7%) proposed by Stackebrandt and Ebbers, 2006. It must be noted that use of 16S rDNA-sequencing is considered not useful for *Vibrio* species diferentiation, mainly because of divergence between cistrons and usually is not correlated with DNA-DNA hybridisation results (Fox et al., 1992; Le Roux et al., 2004; Thompson et al., 2004; Montes et al., 2006). As was noted by Le Roux et al. (2004) 16S rDNA sequence analysis is not effective in identifying closely related species such as belonging to the Splendidus clade (LeRoux Q4

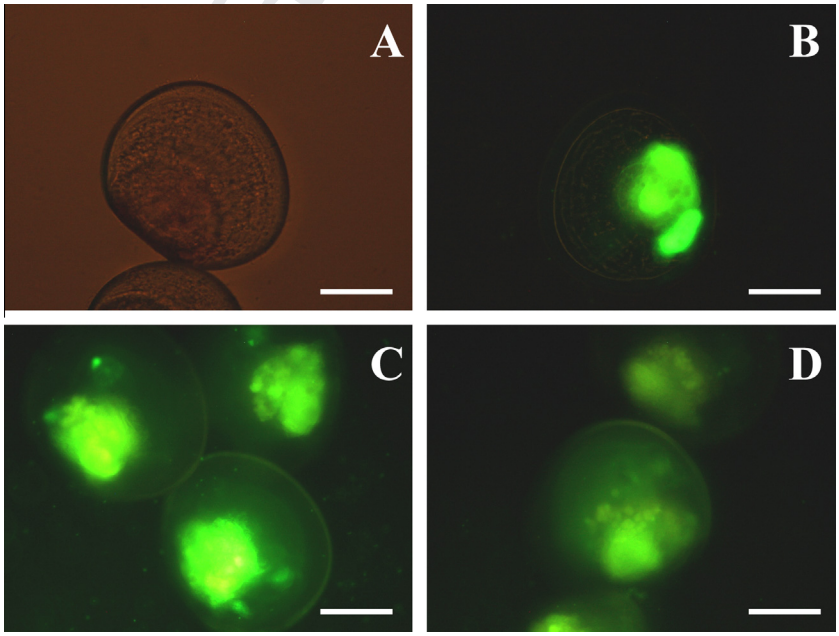

Fig. 4. Scallop larvae inoculated with the VPAP16 strain not stained (A), and stained with 5-DTAF after 1 h (B), 24 h (C) and 30 h (D) of exposure. Scale bars: 50 μm.

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Table 1
Mortality of 10-day-old scallop larvae challenged with non-treated and treated (proteinase K and heat) supernatants of *Vibrio splendidus* strains (VPAP16, VPAP18 and VPAP23) after of 12 h and 24 h of exposure.

Hour	Strain	Percentage of mortality (mean of 3 replicates ± SD)				
		Untreated	Treated			
			Proteinase K	*P*-value	Heat	*P*-value
12	None	0.00 ± 0.00				
	VPAP16	46.67 ± 4.16	48.00 ± 3.46	0.727	42.00 ± 4.00	0.380
	VPAP18	72.67 ± 5.03	70.33 ± 4.51	0.393	68.00 ± 4.00	0.008*
	VPAP23	40.67 ± 5.03	42.00 ± 5.29	0.006*	38.00 ± 4.00	0.420
24	None	2.50 ± 1.00				
	VPAP16	77.00 ± 3.00	78.00 ± 5.29	0.645	74.00 ± 5.29	0.758
	VPAP18	92.00 ± 2.00	89.33 ± 3.06	0.394	86.00 ± 4.00	0.373
	VPAP23	55.33 ± 4.62	53.33 ± 5.03	0.320	50.67 ± 1.15	0.287

* Significant differences ($P < 0.05$) between larval mortalities produced by non-treated and treated supernatants.

et al., 2002; Thompson et al., 2003). Recently, the multilocus sequence analysis (MLSA) of housekeeping genes, such as *rpoA*, *rpoD*, *recA*, *gyrB*, and *atpA* has been considered more appropriate for studying the biodiversity of vibrios (Thompson et al., 2005, 2007; Sawabe et al., 2007). Otherwise, other molecular approaches using real-time PCR have been proposed to identify environmental *Vibrio* species (Tall et al., 2012).

In this study, in addition to sequencing of the 16S rRNA, one housekeeping gene *rpoA* were used to identify the pathogenic *Vibrio* strains, because the *rpoA* gene, encoding for the α-chain of RNA polymerase has been previously demonstrated to be useful to differentiate *Vibrio* species belonging to the Splendidus clade (Beaz-Hidalgo et al., 2009; Diéguez et al., 2011; Lasa et al., 2013). For gene *rpoA* sequence analyses, considered specific for *Vibrio* species differentiation (Thompson et al., 2005; Dalmasso et al., 2009), all strains exhibited 100% similarities with *V. splendidus* LGP32 (Le Roux et al., 2009), confirming the species identity of pathogenic strains.

Although the *V. splendidus* strains displayed very similar phenotypic and genotypic characteristics, their virulence was markedly different. All strains were highly virulent to scallop larvae but VPAP23 strain produced remarkable lower levels of larval mortality than the other strains. The different virulence for scallop larvae exhibited by the *V. splendidus* strains could be explained by a carriage of a variable set of virulence genes, as was previously reported for *V. cholerae* (Rahman et al., 2008), as well as for *V. splendidus* (LeRoux et al., 2009). LeRoux et al. (2009) found a different distribution of putative virulence determinants among the 3 *V. splendidus* strains analyzed, suggesting that high versatility and efficient horizontal gene transfer of *V. splendidus* strains can produce a large number of phenotypes exhibiting diverse virulence traits.

V. splendidus has been recognized as a dominant *Vibrio* species in seawater and bivalves (Farto et al., 1999; Le Roux and Austin, 2006; Le Roux et al., 2009) and has been associated with mortality events of diverse bivalve species, including the scallop species *Pecten maximus*, *Ruditapes decussatus*, *Perna canaliculus, Crassostrea virginica* and *Crassostrea gigas* (Nicolas et al., 1996; Sugumar et al., 1998; Lambert et al., 1999; Lacoste et al., 2001; Le Roux et al., 2002; Waechter et al., 2002; Gay et al., 2004a; Garnier et al., 2007; Kesarcodi-Watson et al., 2009a), and more recently was identified as a pathogen to Yesso scallop, *Patinopecten yessoensis* (Liu et al., 2013), but to our knowledge, this is the first worldwide report of the pathogenic activity of strains belonging to the *V. splendidus* group on the reared-larvae of the scallop, *A. purpuratus*.

The clinical signs caused by the three pathogenic strains resembled those previously described for larval vibriosis and bacillary necrosis occurred in larval stages of oysters (Freites et al., 1993; Sugumar et al., 1998; Lacoste et al., 2001; Elston et al., 2008) and scallops (Nicolas et al., 1996; Riquelme et al., 1996; Lambert et al., 1998; Torkildsen et al., 2005), with the only difference that in this study pathogenic strains produced loss of cilia of larval velum, not previously described as a symptom of bacillary necrosis.

From this study it is not clear that pathogenic activity of *V. splendidus* strains include invasive-mediated activities. Challenge tests using 5-DTAF-stained bacterial cells clearly showed that *V. splendidus* strains can easily reach the digestive gland after 30 min, and then concentrating in the digestive gland and surrounding organs.

In this study, the *V. splendidus* strains exhibited the production of β-haemolysins and as was noted by Nottage and Birkbeck (1986), a major feature of vibriosis is an extensive necrosis followed by rapid death, that is clearly consistent with the involvement of proteinases and haemolysins. Furthermore, all *V. splendidus* strains exhibited the valine aminopeptidase activity, stated by Labreuche et al. (2006) as a marker of virulent bacteria.

Our results demonstrate that ECPs of *V. splendidus* strains are involved in the pathogenesis of scallop larval vibriosis, producing high levels of mortality after 24 h of exposure. The results evidence that extracellular toxigenic activity of the *V. splendidus* strains during the larval stages, is mainly mediated by the production of heat-stable non-proteic compounds, causing larval necrosis, in agreement with several reports which demonstrated that many *Vibrio* species are able to produce heat stable ciliostatic toxins and proteinases that degrade larval tissue (DiSalvo et al., 1978; Nottage et al., 1989).

The production of siderophores by the *V. splendidus* strains could explain in part the virulence of all assayed *V. splendidus* strains, in accordance with other studies which reported the relationship of siderophore production and virulence in various *Vibrio* species (Okujo and Yamamoto, 1994; Biosca et al., 1996; Colquhoun and Sørum, 2001), including *V. splendidus* (Gómez-León et al., 2005), but further studies are needed to elucidate if other bacterial structures such as lipopolysaccharide or fragments of peptidoglican are involved in the virulence of the *V. splendidus* strains. When LeRoux et al. (2009), studied the complete genome of some *V. splendidus* strains, they found homologues of genes associated to a type VI secretion system and siderophore transport and utilization, suggesting that these systems could be involved in the virulence of studied pathogenic vibrios, as was previously demonstrated for *V. parahaemolyticus* (Salomon et al., 2013). Furthermore, recent findings have demonstrated various classes of regulators sensitive to environmental cues which specifically modulate the activity of the VI secretion system, such as iron, bacterial enhancer binding proteins, surface association, quorum sensing and other bacteria-derived signals (Silverman et al., 2012).

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Clearly, concentrations of pathogenic *V. splendidus* strains of 10^3 CFU mL^{-1} or lower are not sufficient to produce important levels of scallop larval mortality after 36 h of bacterial exposure. This fact prompts the urgent necessity that shellfish hatcheries could implement routine programmes of detection and identification of pathogenic vibrios especially in the larval rearing system. Such programmes must be efficient to detect levels of pathogenic vibrios that could represent a potential risk to the larval culture.

In Chilean commercial scallop hatcheries routine bacteriologic monitoring of reared-larvae mainly consider the control of levels of vibrios by using TCBS medium, but the results of the study suggest that a preliminary detection of virulent *Vibrio* strains in the culture is an urgent need in order to predict and consequently prevent the collapse of larval culture. It has extensively been noted that there is no phenotypic or genotypic markers available to distinguish pathogenic from non-pathogenic *V. splendidus* strains (Le Roux et al., 2002), so currently the only way to determine the virulence of a given strain remains experimental infection, as was noted by Kesarcodi-Watson et al. (2009a).

In conclusion, we demonstrated for the first time in experimentally challenged scallop larvae a toxic effect of *V. splendidus* strains and extracts against *A. purpuratus*, confirming the role of *V. splendidus* strains as the causative agents of the observed disease outbreaks in reared scallop larvae in Chilean hatcheries. Results from this study demonstrate that *V. splendidus* strains penetrate the scallop larvae accumulating in the digestive gland, and developing their toxic activity by means of extracellular heat-stable non proteic toxins producing some clinical signs of bacillary necrosis. It is concluded that the high pathogenic activity of the *V. splendidus* strains are enough to provoke mass mortalities even when proper rearing and sanitation measures are developed, representing a high health risk for reared-larvae in commercial scallop hatcheries in Chile.

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