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Survival behaviour and virulence of the fish pathogen *Vibrio ordalii* in seawater microcosms

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ABSTRACT: *Vibrio ordalii*, the causative agent of atypical vibriosis, is a Gram-negative, motile, rod-shaped bacterium that severely affects the salmonid aquaculture industry. *V. ordalii* has been biochemically, antigenically and genetically characterized. However, studies on the survival behaviour of this bacterium in aquatic environments are scarce, and there is no information regarding its disease transmission and infectious abilities outside of the fish host or regarding water as a possible reservoir. The present study investigated the survival behaviour of *V. ordalii* Vo-LM-06 and Vo-LM-18 in sterile and non-sterile seawater microcosms. After a year in sterile seawater without nutrients, 1% of both *V. ordalii* strains survived (~10^3 colony-forming units ml^{-1}), and long-term maintenance did not affect bacterial biochemical or genetic properties. Additionally, *V. ordalii* maintained for 60 d in sterile seawater remained infective in rainbow trout *Oncorhynchus mykiss*. However, after 2 d of natural seawater exposure, this bacterium became non-culturable, indicating that autochthonous microbiota may play an important role in survival. Recuperation assays that added fresh medium to non-sterile microcosms did not favour *V. ordalii* recovery on solid media. Our results contribute towards a better understanding of *V. ordalii* survival behaviour in seawater ecosystems.

KEY WORDS: *Vibrio ordalii* · Vibriosis · Survival · Microcosm

INTRODUCTION

Vibrio spp. are among the most frequently occurring and harmful bacteria for global fish and shellfish aquaculture industries (see review by Thompson et al. 2004). In fact, these bacteria form a significant part of the microbiota present in marine environments (Chatterjee & Haldar 2012). The Gram-negative, motile, rod-shaped bacterium *Vibrio ordalii* is the causative agent of septicemia, which can result in fish death (Toranzo & Barja 1993). This microorganism was isolated for the first time from diseased salmon cultured in coastal waters of the American Pacific Northwest (Harrell et al. 1976). Since its first isolation, *V. ordalii* has also been reported in Japan, Australia, New Zealand and Chile, mainly affecting salmonid farming (Wards et al. 1991, Colquhoun et al. 2004). This bacterium has also been reported in other fish species, such as the ayu *Plecoglossus altivelis* and rockfish *Sebastes schlegeli* in Japan (Muroga et al. 1986) and, more recently, in gilthead sea bream *Sparus aurata* in Turkey (Akayli et al. 2010).

Numerous studies describe *V. ordalii* isolated from salmon as a biochemically, serologically and geneti-

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cally homogeneous taxon (Mutharia et al. 1993, Larsen et al. 1994, Grisez & Ollevier 1995, Tiainen et al. 1995, Toranzo et al. 1997), although Silva-Rubio et al. (2008) suggested the possibility that the Chilean strains constitute a new serological subgroup of this bacterial species. In addition to the fact that its taxonomic classification is still evolving, our understanding of the pathogenic mechanisms of *V. ordalii* is likewise incomplete. While a previous study found that *V. ordalii* pathogenicity is not correlated to the haemagglutination of Atlantic salmon *Salmo salar* red blood cells, the pathogenicity of this bacterium could be related to hydrophobic properties (Ruiz et al. 2015). Furthermore, *V. ordalii* can survive in the mucus of Atlantic salmon. This trait probably plays a role in *in vivo* host colonization and in evading the immune response of the host, even including bactericidal activities (Ruiz et al. 2015). However, while the ability of *V. ordalii* to survive and remain infective in the aquatic environment may act as a major determinant to its pathogenicity, this has not yet been assessed.

Related to environmental adaptations, several microorganisms have developed coping strategies in response to adverse conditions, such as changes in temperature, salinity, or nutrient deprivation (Roszak & Colwell 1987). For example, temperature is known to affect the viability and virulence expression of several *Vibrio* spp. that are fish pathogens (Biosca et al. 1996, Colquhoun & Sørum 2001, Armada et al. 2003). In fact, early studies on *V. ordalii* growth showed that 18°C might be more optimal for growth than 22°C (Poblete-Morales et al. 2013). Another study in *Vibrio* spp. and *Pseudomonas* spp. found that one of the initial responses to starvation is cell-rounding, together with a reduction in cell size (Kjelleberg et al. 1983).

Currently, there is limited knowledge regarding the behaviour of *V. ordalii* in aquatic environments, particularly since this bacterium has not been isolated directly from marine ecosystems. Therefore, the possible role of water as a natural reservoir and route of transmission for the disease is still unknown. Clarifying this subject is especially important for the Chilean aquaculture industry as, following a widespread outbreak between 2004 and 2009, sporadic *V. ordalii* infections occur yearly for reasons yet unknown. Therefore, the aims of this study were to determine the possible effects of biotic factors on *V. ordalii* survival; monitor the survival behaviour of *V. ordalii* in seawater microcosms; analyse changes in morphological, biochemical and molecular characteristics under survival conditions; and evaluate *V. ordalii* virulence after a period in sterile water.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Two *Vibrio ordalii* strains were used in this study, Vo-LM-06 and Vo-LM-18. These strains were originally isolated in Atlantic salmon from 2 enzootics detected at marine farms in southern Chile during 2008. Each strain was obtained from the kidney of different cage-cultured Atlantic salmon. Both strains belonged to the O2 serotype of this bacterial species, the members of which show similar DNA patterns, as supported by RAPD, REP-PCR and ERIC-PCR techniques (Silva-Rubio et al. 2008). These strains were chosen based on the analysis of cell-surface characteristics reported by Ruiz et al. (2015). *V. ordalii* Vo-LM-18 was classified as relatively hydrophobic, while Vo-LM-06 was classified as quasi-hydrophilic. Standard phenotyping initially identified each strain as *V. ordalii* (Silva-Rubio et al. 2008, Poblete-Morales et al. 2013), a classification later confirmed using PCR analysis based on the *vohB* (haemolysin) gene described by Avendaño-Herrera et al. (2014).

Vo-LM-06 and Vo-LM-18 were routinely grown on trypticase soy broth or agar supplemented with 1% (w/v) NaCl (TSB-1 and TSA-1, respectively) and incubated at 18 ± 1°C for 48–72 h. Following collection in 2008, stock cultures were maintained frozen at –80°C in Criobilles tubes (AES Laboratory) or in TSB-1 containing 15% (v/v) glycerol.

Experimental design for microcosms

Survival assays were carried out in seawater (36‰ salinity, 18°C and pH 7.83) collected from the Centro de Investigación Marina Quintay (CIMARQ), located 50 km south of Viña del Mar (V Región, Chile). The collected seawater was pre-filtered through a 50 µm quartz filtration system and then filtered through 1 µm membranes. Water samples were transported to the laboratory in cold-storage containers and kept at 18°C until use (within 24 h). For the sterile microcosm, water was filtered through 0.22 µm pore-size membranes (Millipore), and the filtrate was autoclaved at 121°C for 15 min (pH 8.0) following the protocol reported by Avendaño-Herrera et al. (2006). To assess the possible effects of biotic factors on *V. ordalii* survival, a non-sterile seawater microcosm was prepared using seawater that was not filtered through pore-sized membranes or autoclaved.

The initial experimental assays were conducted in triplicate in 500-Schott Duran® flasks containing

300 ml of sterile or non-sterile seawater inoculated with each bacterial suspension. Inocula were prepared from cells scraped off TSA-1 plates after 52–56 h of growth, washed in sterile saline solution (0.85% NaCl) and resuspended in the sterile seawater to achieve an initial bacterial concentration of approximately 10^6 cells ml^{-1}, as determined by the direct microscopy count methodology (see subsection 'Total bacterial count using epifluorescence microscopy'). Parallel to this, each inoculum was serial-diluted in a sterile saline solution, and 0.1 ml of each 10-fold dilution was plated on TSA-1 plates to count the total amount of culturable heterotrophic cells (colony-forming units, CFU). Flasks were incubated at 18°C, the average summer temperature during vibriosis outbreaks, and agitated at 120 rpm for 365 d in the case of the sterile microcosm and 67 d in the case of the non-sterile microcosm.

Moreover, 3 additional non-sterile seawater microcosms were separately prepared for each *V. ordalii* strain to evaluate the recuperation and possible growth of Vo-LM-06 and Vo-LM-18. These microcosms were prepared 10 d after the initial experiments, and recuperation assays were performed in triplicate (see subsection 'Recuperation of non-culturable cells'). The CFU counts of *V. ordalii* were based on the characteristic morphotype of this bacterium and were biochemically confirmed using the miniaturized API 20E system (bioMérieux). The *V. ordalii* strains were detected by the quantitative PCR (qPCR) protocol described in the subsection 'Quantitative real-time PCR analysis'.

Finally, sterile and non-sterile microcosm controls without the addition of *V. ordalii* strains were simultaneously maintained and incubated under the same conditions described above.

Bacterial count

To determine culturable cell numbers, 0.5 ml samples were aseptically collected from each microcosm immediately after inoculation at Days 1, 2, 3, 4, 5, 6, 7, 10, 36, 60 and 67 in the case of the 67 d non-sterile microcosm and at Days 1, 2, 3, 7, 14, 35, 60, 90, 168, 203 and 365 in the case of the 365 d sterile microcosm. Samples were serial-diluted in a 0.85% sterile saline solution, and 0.1 ml of each 10-fold dilution was plated on TSA-1 to count the total amount of culturable heterotrophic marine bacteria. Culturable cells of *V. ordalii* were determined after 1 wk of incubation at 18°C. Plating was performed in duplicate for each experimental and control sterile and non-sterile microcosm, and the number of culturable bacterial cells in each microcosm was calculated from dilutions that showed between 30 and 300 colonies on the agar plates.

In the case of the non-sterile microcosms, CFU counts were performed at the times previously mentioned using as initial criteria the characteristic morphotype of *V. ordalii* (i.e. for 24–48 h of growth, a 3–5 mm circular, raised, yellow-brown, opaque colony; for 4–6 d of growth, a 1–2 mm, circular, convex, white, translucent colony) (Schiewe et al. 1981). Furthermore, biochemical and PCR assays were carried out to verify the presence of the inoculated Vo-LM-06 and Vo-LM-18 strains. Various phenotypical and biochemical traits were examined, including colony morphology and pigmentation, cell morphology and motility, Gram-staining, cytochrome oxidase, oxidation/fermentation reactions, catalase reaction (3% H_2O_2), susceptibility to the vibriostatic agent O/129 and the growth or lack thereof on thiosulfate–citrate–bile salts–sucrose (TCBS) agar. Additionally, a total of 30 colonies representative of the predominant morphotype from each plate were selected, and the DNA of each colony was extracted and analysed by PCR to confirm the presence of *V. ordalii* (Avendaño-Herrera et al. 2014). All results, based on 3 replicates per microcosm seeded in duplicate, represent the average bacterial count in each microcosm ± standard deviation.

Total bacteria count using epifluorescence microscopy

The total numbers of bacteria in sterile and non-sterile microcosms were determined by epifluorescence microscopy using 4′,6-diamidino-2-phenylindone (DAPI) staining (Porter & Feig 1980). Samples (1 ml) were taken after inoculation during the first week and then periodically, as previously noted, until 67 and 365 d of incubation in the non-sterile and sterile microcosms, respectively. Samples were fixed in a final volume of 3 ml with 1% formalin (30 µl), diluted and stained with DAPI (final concentration: 0.1 mg ml^{-1}). After a 10 min incubation period, samples were filtered through 0.22 µm black Nuclepore filters previously washed twice with 5 ml of sterile saline solution to prevent bacteria lysis. Filters were observed using epifluorescence microscopy (Olympus BX41) on at least 10 randomly selected fields. This provided a total count of >300 cells, from which the number of bacteria was calculated.

Quantitative real-time PCR analysis

Samples (1 ml) were collected from each microcosm, and DNA was extracted using the commercial InstaGene Matrix Kit (Bio-Rad) according to the manufacturer's instructions. After extraction, each DNA sample was maintained at −20°C until use in PCR analysis.

All qPCR analyses used for *V. ordalii* detection in the microcosms used the primer set VohB_Fw + VohB_Rv, which was designed to amplify a 112 bp fragment flanking the *vohB* gene (Avendaño-Herrera et al. 2014). The reactions and amplification cycles used for denaturation, primer annealing and extension were carried out according to the published qPCR protocol. Samples were amplified using a Stratagene Mx3000P qPCR System, and the amplicons were confirmed by electrophoresis on 2% Seakem LE agarose gel and with ethidium bromide staining.

To determine the number of *V. ordalii* present in each microcosm sample, the threshold value (Ct) was extrapolated using the values from a quantification curve prepared with known concentrations and constructed using the vohB-112 plasmid as indicated by Avendaño-Herrera et al. (2014). The Ct values were converted to genomic equivalents based on the assumption that single copies of the *vohB* target sequence are present in the *V. ordalii* genome.

Analysis of phenotypical and genotypical changes

Changes in bacteria morphology and size were monitored periodically by epifluorescence microscopy observations of filters with DAPI dye. Moreover, to determine if *V. ordalii* exhibited long-term phenotypical or genetic changes, one representative *V. ordalii* Vo-LM-06 colony was evaluated using culturable cells grown 35, 60, 90 and 168 d after inoculation in sterile microcosms. Biochemical alterations during the maintenance period were examined using the miniaturized API 20E system (bioMérieux) according to the manufacturer's instructions, with the exception of the incubation temperature which was fixed at 18°C. To determine if there were alterations in the bacterial cell wall component, the lipopolysaccharide and protein profiles were analysed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970) and posterior silver staining following the procedure of Tsai & Frasch (1982). The lipopolysaccharides were obtained from the cell envelope using the Proteinase K method described by Hitchcock & Brown (1983), while protein components were obtained as described by Avendaño-Herrera et al. (2004).

Additionally, ERIC-PCR and REP-PCR amplifications were used to assess the genetic pattern of starved *V. ordalii* Vo-LM-06 cultures. The obtained pattern was then compared against the original *V. ordalii* strain using the method described by Silva-Rubio et al. (2008).

Recuperation of non-culturable cells

Two days after the start of the experiments, the culture plates seeded with 0.1 ml of the non-sterile microcosm were confirmed negative for *V. ordalii*. These plates did present other bacteria, but these differed from the typical morphology of *V. ordalii* and were confirmed *V. ordalii*-negative through PCR analysis. To increase the possibility of *V. ordalii* recuperation, the experiments were repeated by seeding 1 ml of the non-sterile microcosm directly onto plates.

The recuperation experiments were conducted at Days 5, 10 and 60 by extracting 1 ml of each of the 3 non-sterile seawater microcosms and separately seeding these samples into a sterile solution of TSB-1 (25%) in an attempt to reverse any non-culturable *V. ordalii* cells back into a culturable state. For recuperation, flasks were incubated at 18°C for 24–48 h. Samples (1 ml) were taken after 24 and 48 h from the non-sterile microcosms and plated on TSA-1. The CFUs were quantified after 72 h of incubation at 18°C to confirm the absence or presence of *V. ordalii*.

Simultaneously, 1 and 100 ml samples were also collected from each non-sterile microcosm and centrifuged at 7000 × *g* for 10 min. Then the pellet was resuspended in 1 ml. Finally, DNA was extracted using the InstaGene Matrix (Bio-Rad) to confirm the presence or absence of *V. ordalii* in the microcosms (Avendaño-Herrera et al. 2006) via qPCR. After qPCR confirmation that there was no *V. ordalii* DNA present, the cells were considered absent in the microcosm.

Sequencing of 16S rRNA from autochthonous aquatic microbiota

The dominant colonies that grew in the non-sterile microcosms during the first week of study were subjected to taxonomical analysis using 16S rRNA sequencing. Total DNA was extracted from a colony of each strain using the InstaGene DNA Purification

Matrix (Bio-Rad) following the manufacturer's instructions. The 16S rRNA gene was amplified using the universal primer pair pA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and pH (5′-AAG GAG GTG ATC CAG CCG CA-3′), as described by Edwards et al. (1989). The expected 1500 bp amplicon was obtained and then sequenced by Macrogen (Seoul, Korea). The resulting 16S rRNA sequences were analysed using the on-line EzTaxon tool available at www.ezbiocloud.net/eztaxon (Chun et al. 2007). Then, the type strain sequences of other species from the same genus were aligned against the obtained sequences using MEGA software v.5 (Tamura et al. 2011). Genetic distances were obtained using Kimura's 2-parameter model (Kimura 1980) and clustered with the neighbour-joining algorithm.

Screening for bacteriophages

In addition to being passed through a 1 µm filter, the natural seawater employed in this study was also examined for the presence or absence of bacteriophages and protozoans. Furthermore, samples from each of the 3 non-sterile microcosms were also taken on Days 5, 10 and 60 (before exposure to TSB-1) to assess the presence of bacteriophages. Bacteriophage presence was evaluated by the double-agar-layer technique using an enrichment procedure. For this procedure, seawater was treated with 100 µl of chloroform to remove cellular components. Then, the seawater was treated with TSB-1 containing a 1:10 (v/v) dilution of an overnight culture of *V. ordalii* Vo-LM-06 or Vo-LM-18. The enriched mixture was incubated overnight at 18°C, treated again with chloroform 1:10 to eliminate all viable bacterial cells and centrifuged at 7000 × *g* for 5 min to precipitate the cellular components. Bacteriophage detection in the supernatant was carried out by a plaque assay with lawns of each *V. ordalii* strain. In the case of protozoans, 100 ml samples were centrifuged (7000 × *g*), resuspended in 1 ml of the same seawater and filtered for microscopy observation.

Screening autochthonous aquatic microbiota for inhibitory activities

Representatives of the numerically most abundant morphotypes grown on TSA-1 plates from samples of non-sterile microcosms seeded with *V. ordalii* were tested to determine the possible role of native bacterial populations in the survival of *V. ordalii*. The bactericidal effect of these isolates against *V. ordalii* Vo-LM-06 or Vo-LM-18 was examined using the double-layer method of Dopazo et al. (1988). Briefly, a 10 µl suspension of each isolate was inoculated overnight on agar plates with TSA-1. The obtained cells were washed twice in a sterile saline solution at 10 000 × *g* for 15 min. The macrocolonies that subsequently grew on the agar plates were exposed to chloroform vapour for 45 min and were then covered by a second layer of soft agar (TSB-1 and 0.9% agar) that contained 100 µl of a 1:10 (v/v) dilution of either *V. ordalii* Vo-LM-06 or Vo-LM-18 (10^8 cells ml^{-1}). The plates were observed after a 72 h incubation period at 18°C, where a clear zone of *V. ordalii* inhibition around the cell paste indicated antibacterial activity.

Fish infection

The Vo-LM-06 strain was selected to investigate virulence capacity. For this, infectivity trials were conducted with the strain recovered after 60 d from the sterile microcosm, as described by Ruiz et al. (2015). Briefly, *V. ordalii*-free rainbow trout (*Oncorhynchus mykiss*, weighing 28–30 g) were obtained from the 'Río Blanco Federico Alber Taupp' fish farm, which is located in Los Andes, Chile, and belongs to the Pontificia Universidad Católica de Valparaíso. These specimens were healthy and had no history of diseases. To ensure that fish were uninfected with *V. ordalii*, 5 specimens were subjected to standard microscopic and bacteriological examinations, as well as to qPCR analysis (Avendaño-Herrera et al. 2014). Fish were randomly allocated into 3 different groups containing 15 specimens per 10 l plastic tank with seawater and acclimatized for 48 h prior to the bacterial challenge. This work was carried out in accordance with the Ethics Committee for Animal Experiments of the Universidad de Andrés Bello.

Bacterial inocula were prepared from a single colony scraped off of TSA-1 plates, suspended in 10 ml of TSB-1 and incubated overnight at 18 ± 1°C with shaking at 120 rpm. After this, an initial bacterial concentration of 1×10^7 cells ml^{-1}, as determined by direct microscopy count, was adjusted in the same broth.

Two groups of 15 rainbow trout were moved to a separate 5 l plastic tank, anesthetized using 1.5 ml benzocaine (BZ-20®, Veterquímica S.A.) and inoculated by intraperitoneal injection with a total volume of 0.1 ml containing either 1.12×10^6 CFU of the *V. ordalii* Vo-LM-06 strain recovered from the sterile microcosm after 60 d or 9.04×10^5 CFU of the original

Vo-LM-06 strain. Another group of 15 fish were anesthetized as described above and then injected with 0.1 ml of TSB-1 as a control. Each group of fish (treatment and control) was maintained in a separate 20 l plastic tank with seawater at 18 ± 1°C with aeration during the course of the experiments (21 d) and with 50% of the tank water refreshed daily. Fish were fed commercial food pellets daily at a ratio corresponding to 1% body weight. Dead fish were removed from each tank daily, and the following methods were used to confirm whether the inoculated bacterium was the cause of mortality. Kidney, liver, spleen and external lesions, if existent, were directly streaked onto TSA-1 plates and incubated at 18°C for 1 wk. Then, suspected *V. ordalii* colonies were identified using phenotypic and biochemical traits as described above. Additionally, pure or mixed cultures were also tested using a specific qPCR (Avendaño-Herrera et al. 2014).

RESULTS

The survival of the *Vibrio ordalii* strains inoculated in sterile seawater microcosms is shown in Fig. 1. In general, the CFU per milliliter of LM-Vo-06 and Vo-LM-18 declined by 1 log-unit in the first 7 d of the experiment as compared to the original inoculum. After this, standard plate counts showed a slight decline of culturable *V. ordalii*, and from Day 60 onwards, the bacterial concentration of both strains stabilized at 10^3 CFU ml^{-1} until Day 365 (Fig. 1). Regardless of the *V. ordalii* strain, variations lower than a single log-unit were observed through epifluorescence microscopy during the experimental period, with values reaching $2.1 \times 10^5 \pm 0.5 \times 10^5$ bacteria ml^{-1}. Moreover, the applied qPCR protocol positively amplified the *V. ordalii vohB* hemolysin gene from all microcosms during the study period. The Ct value obtained between Days 140 and 365 was 29.3 ± 1.8, values corresponding to the standard vohB-112 plasmid curve produced by 2858 quantified copies of *vohB*.

Microscopic observations demonstrated that the morphology and size of the *V. ordalii* cells in the sterile microcosm changed after the first 3 d of incubation from rods to a cocci form, a form maintained for 365 d regardless of the strain studied (Fig. 2). Additionally, after 35, 60, 90 and 168 d in the sterile microcosm, analysis through the miniaturized API 20E kit rendered the 0004024 profile for all Vo-LM-06 strains, the same profile as that for the original strain (data not shown). Additionally, the protein and LPS patterns were the same as those of the original Vo-

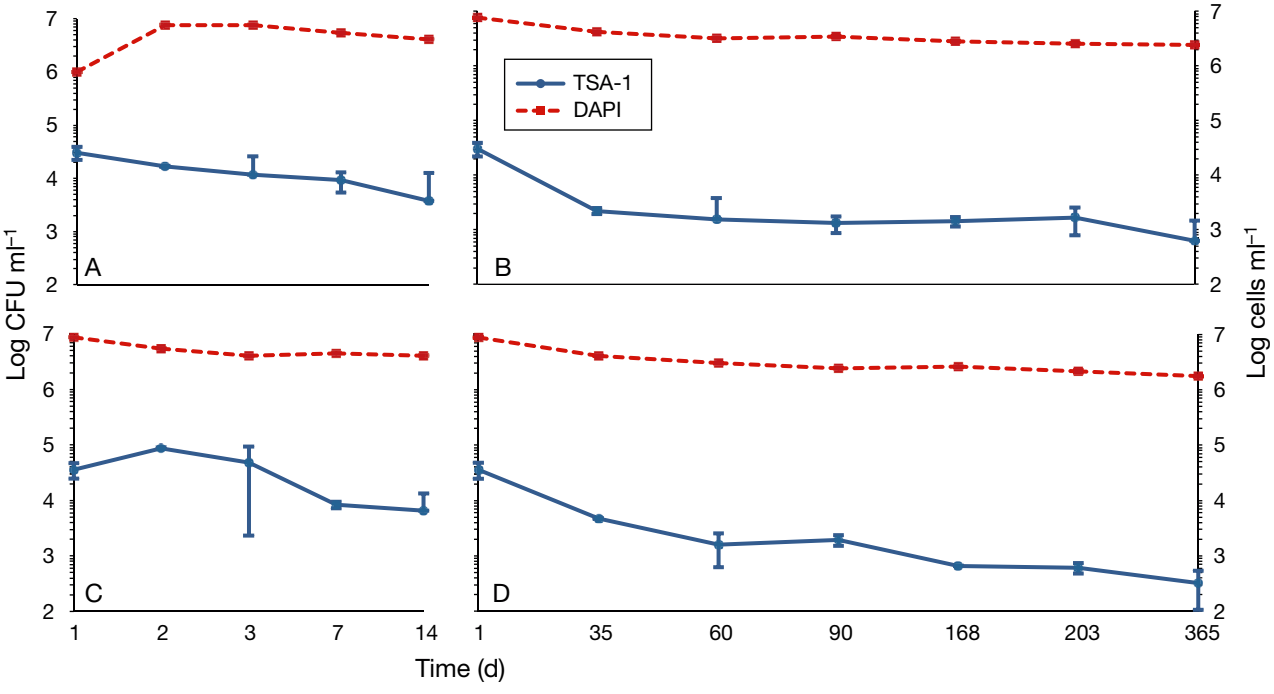

Fig. 1. Survival of *Vibrio ordalii* (A,B) LM-Vo-06 and (C,D) LM-Vo-18 in sterilized seawater microcosms: (A,C) 14 d and (B,D) the experimental period obtained for *V. ordalii*. Culturable cells (colony-forming units [CFU] ml^{-1}) were counted on TSA-1 medium and cell number was determined by DAPI direct counts (cells ml^{-1}). Error bars: standard deviation

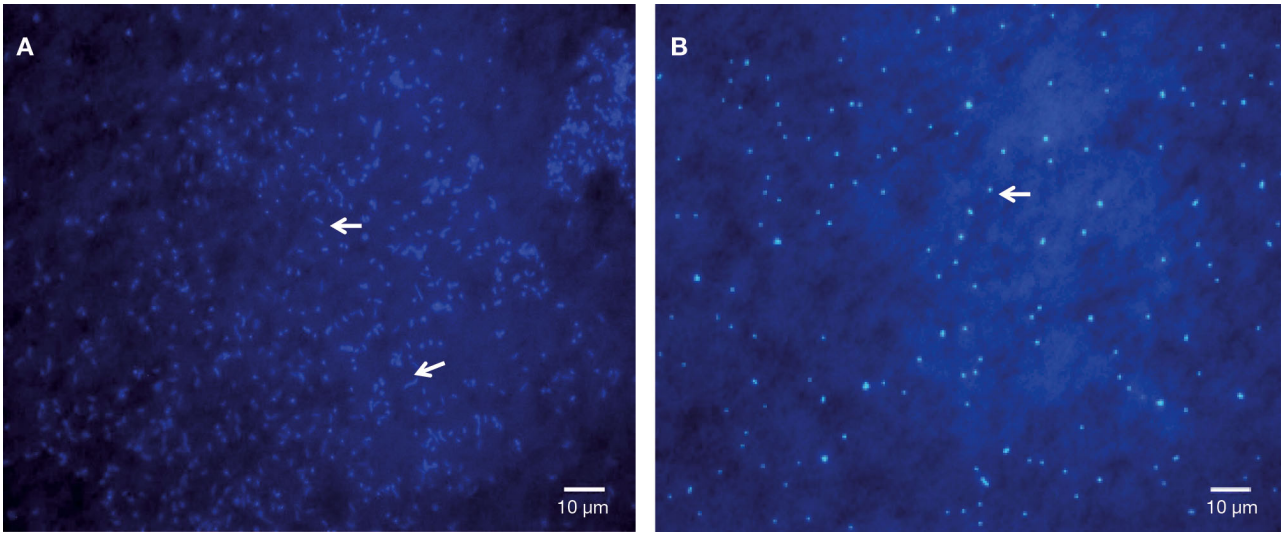

Fig. 2. Morphological characteristics of *Vibrio ordalii* under a fluorescence microscope using the population collected from a sterile microcosm after (A) 3 and (B) 168 d of incubation. Arrows: cells that change their morphology from rod to cocci. Scale bars = 10 µm

LM-06 strain (Fig. 3A), and, when ERIC-PCR and REP-PCR techniques were applied, cluster analysis of the patterns clearly showed the same profile as that of the original strain (Fig. 3B).

In contrast, under non-sterile conditions the number of culturable *V. ordalii* Vo-LM-06 and Vo-LM-18 declined very rapidly (Fig. 4), with no *V. ordalii* colonies detectable by biochemical tests just 2 d after the start of the experiment. This observation was supported by results obtained from qPCR analysis for *V. ordalii* DNA in the microcosms, where qPCR gave positive reactions only during the first 2 and 3 d of the

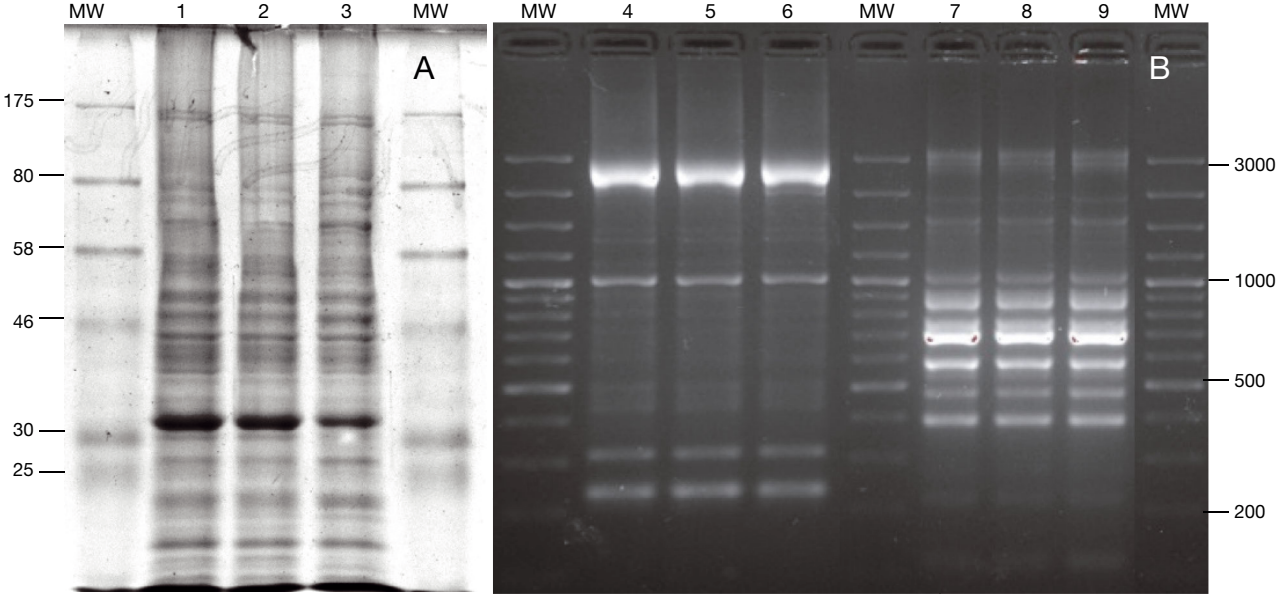

Fig. 3. (A) Electrophoresis protein profiles and (B) amplification fingerprints obtained for *Vibrio ordalii* LM-Vo-06 using ERIC-PCR (Lanes 4–6) and REP-PCR (Lanes 7–9). Lanes — MW: molecular size markers; 1, 4 and 7: whole-cell protein and DNA obtained from original strain; 2, 5 and 8: whole-cell protein and DNA obtained from colonies after 60 d of incubation in a sterile microcosm; 3, 6 and 9: whole-cell protein and DNA obtained from colonies after 168 d of incubation in a sterile microcosm. Numbers on the left indicate positions in the molecular size marker (kDa). Numbers on the right indicate the molecular size in base pairs (bp)

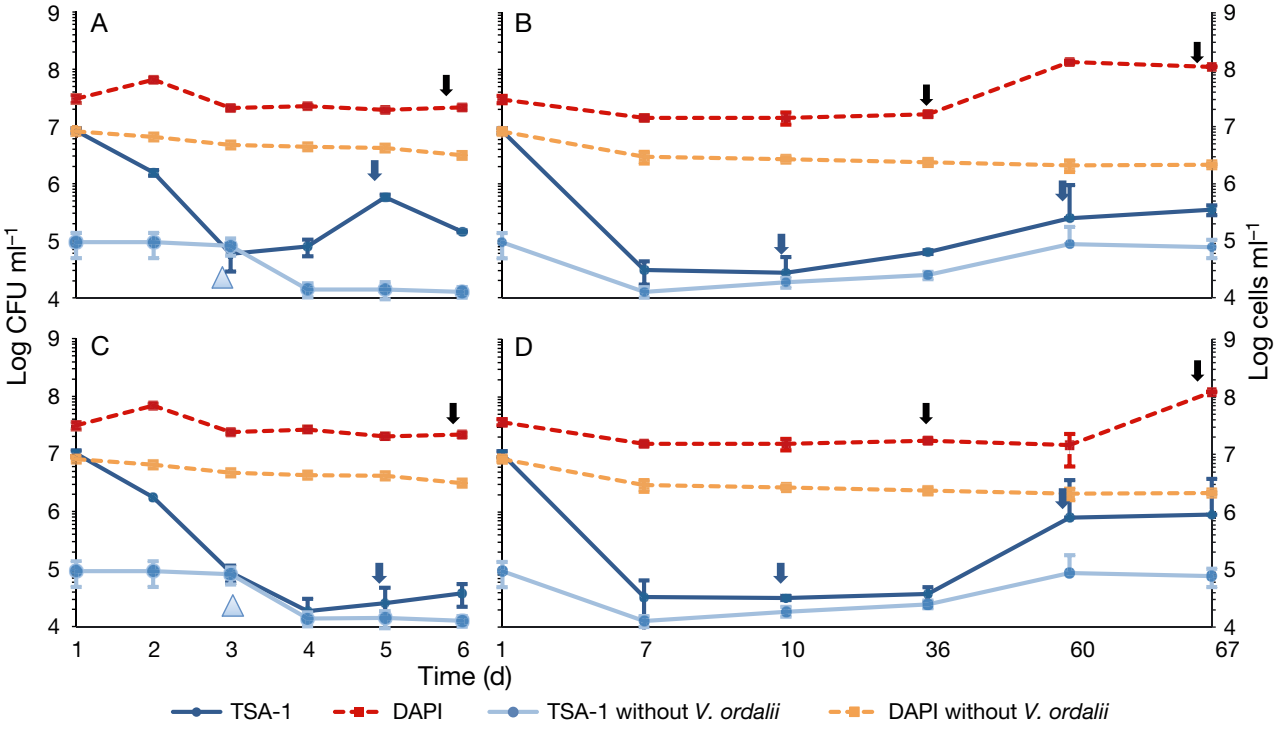

Fig. 4. Survival of *Vibrio ordalii* (A,B) LM-Vo-06 and (C,D) LM-Vo-18 in non-sterilized seawater microcosms: (A,C) 6 d and (B,D) the experimental period. Culturable cells (CFU ml^{-1}) were counted on TSA-1 medium and cell number was determined by DAPI direct counts (cells ml^{-1}). Arrowheads (Day 2): failure to isolate and identify *V. ordalii* strains; arrows: revival of non-culturable cells. Error bars: standard deviation

experiment, with Ct values of 31.4 ± 0.9 and 32.6 ± 0.2 for the Vo-LM-06 and Vo-LM-18 microcosms, respectively. As expected, no fluorescent signal was detected in the nuclease-free water blank used as a negative technical control or in the microcosms without *V. ordalii* Vo-LM-06 and Vo-LM-18. It is important to note that the culturability of autochthonous microbial communities in the non-sterile microcosms without *V. ordalii* remained relatively constant until Day 67, with approximately 10^5 CFU ml^{-1} (Fig. 4). Interestingly, a similar behaviour was observed for all non-sterile microcosms with *V. ordalii* Vo-LM-06 and Vo-LM-18.

To test whether native microbiota decreased the survival of *V. ordalii* in non-sterile microcosms, different bacterial phenotypes isolated from this microcosm were analysed during the first week of the study. On TSA-1 plates, 3–4 different bacterial colony types were consistently observed. Based on 16S rRNA gene analysis, these colonies were *Halomonas saccharevitans* (98.2%), *Ornithinimicrobium kibberense* (98.7%) and *Pseudoalteromonas hadiensis* (100%). Specific *V. ordalii* bacteriophages or protozoans were not detected in non-sterile microcosms. Moreover, the antagonism assays showed that *P. hadiensis* inhibited growth of the *V. ordalii* strains, displaying inhibition areas ranging from 25 to 34 mm in diameter.

To induce the recuperation and possible growth of the *V. ordalii* strains, fresh nutrients were added to the non-sterile microcosms at 3 different times (5, 10 and 60 d). At 24 and 48 h after the addition of fresh media, no *V. ordalii* colonies were observed on the agar plates, due to an overgrowth of bacteria naturally present in the water, and at least 3 distinct colony types or morphotypes were abundant on TSA-1 plates. When the qPCR for *V. ordalii* DNA was employed, amplification products were not obtained from these water samples, regardless of the volume used to extract DNA (1 or 100 ml). An identical finding was obtained from the DNA extracted from each colony examined. According to these predictions, no colonies were recovered from non-inoculated sterile microcosms. Moreover, qPCR amplification products were not obtained for non-inoculated sterile or non-sterile microcosms (data not shown).

To determine if *V. ordalii* Vo-LM-06 remained infective in rainbow trout after 60 d in sterile seawater, a challenge experiment was performed using intraperitoneal inoculation. Mortalities began 5 d after

inoculation, and there was a dramatic increase by Day 7 and 100% mortality 13 d post-infection. Likewise, the original Vo-LM-06 strain caused 100% mortality 14 d post-inoculation. In contrast, the control groups registered no mortalities. Samples from all dead fish showed the presence of *V. ordalii* Vo-LM-06, and, as in naturally occurring cases, the internal haemorrhaging caused by this bacterium was found. Some infected fish presented haemorrhagic lesions in tissues surrounding the ventral fin and anal pore, and, internally, infected fish presented splenomegaly and, in some cases, a pale liver with ecchymosis. The recovered strains were confirmed to be *V. ordalii* by PCR amplification using the VohB primers.

DISCUSSION

Although vibriosis caused by *Vibrio ordalii* is a disease that has affected important farmed marine fish species since 1981 (Schiewe et al. 1981), the niches and behaviour of this microorganism outside its fish hosts are unknown. In fact, the route of infection and the possible role of the marine environment as a reservoir of this pathogen are not yet clear. Indeed, a large body of the knowledge available on the physiology of pathogenic bacteria has been obtained in laboratory-based microcosm experiments using pure cultures (McKay 1992).

Even today, the majority of studies are still performed under sterile conditions, despite increasing interest in applying water microcosms to determine the period of survival for fish pathogens in the aquatic environment. These studies have included species such as *Vibrio vulnificus* (Oliver et al. 1991), *Photobacterium damselae* ssp. *piscicida* (Magariños et al. 1994), *Yersinia ruckeri* (Romalde et al. 1994), *Streptococcus parauberis* (Currás et al. 2002), *Flavobacterium psychrophilum* (Madetoja et al. 2003), *Tenacibaculum maritimum* (Avendaño-Herrera et al. 2006), *Francisella noatunensis* (Duodu & Colquhoun 2010) and *Flavobacterium columnare* (Arias et al. 2012).

In the present evaluation of *V. ordalii* survival in a sterile microcosm, the concentration of bacteria was higher in DAPI staining as compared to the CFUs on Day 1. This suggests that a high number of non-culturable or dead cells may be introduced into sterile microcosms. Despite this, *V. ordalii* survived in a culturable state for 365 d. Over the entire experimental period, DAPI staining indicated that total cell counts remained very high (10^5 cells ml^{-1} average value), and the bacterial count remained at 10^3 CFU ml^{-1} until Day 365. Therefore, seawater could be a significant infection route for this bacterium. However, since culturability in a non-sterile microcosm was limited to 2 d, careful consideration should be given when interpreting laboratory results. It is important to note that this behaviour may not be related to the strain employed, but rather to *V. ordalii* in general. Both Vo-LM-06 and Vo-LM-18 declined identically in terms of culturability in non-sterile microcosms, regardless of the times that the assay was repeated.

Since DNA can persist in metabolically inactive or dead cells, and in the environment after cell lysis (Deere et al. 1996, Keer & Birch 2003), a specific qPCR was performed to detect *V. ordalii* DNA, the detection limits of which ranged between 5.3×10^2 and 4.13×10^3 CFU ml^{-1} (equivalent to Ct values between 31.66 and 33.96) (Avendaño-Herrera et al. 2014). The survival dynamic was corroborated by data from the PCR quantification analysis, where no amplification product was observed for DNA extracted from non-sterile microcosms after 3 d, regardless of the microcosms tested (old or new set). A similar decline in culturability has been reported for other fish pathogens under natural fresh- and seawater conditions, such as *Aeromonas hydrophila*, *V. vulnificus*, *T. maritimum* and *F. noatunensis* (Maalej et al. 2004, Marco-Noales et al. 2004, Vivas et al. 2004, Avendaño-Herrera et al. 2006, Duodu & Colquhoun 2010).

To clarify this point, recuperation assays were performed for the *V. ordalii* strains Vo-LM-06 and Vo-LM-18 after 5, 10 and 60 d of the experiment in the non-sterile microcosm. Neither strain showed the ability to recuperate growth capacities under the tested experimental conditions, as confirmed by plate seeding and qPCR analysis for *V. ordalii* DNA in samples obtained from water in which this bacterium should have been present. Similar results have been described for other fish pathogens (Avendaño-Herrera et al. 2006). Although these findings could indicate that surviving *V. ordalii* cells may exist at levels below the PCR detection limit, it was not possible to reactivate these cells into a culturable state.

Competition, predation, temperature and pH fluctuations all have significant influences on the ability of a bacterium to survive in its environment (Panicker et al. 1994). In this study, temperature was kept the same for all microcosms, while water pH was 7.8 before sterilization and 8.0 after autoclaving. However, the change in pH did not affect the results, since all microcosms were stored for a week and

because water pH was 7.7 ± 0.3 before bacteria were inoculated.

Biological factors, such as bacteriophages and protozoans, also play an important role in the survival of a variety of bacteria in aquatic systems; however, the presence of these bacteriovorous microorganisms was not detected in the seawater employed in this study. One explanation is that all *V. ordalii* cells were lysed by the autochthonous microbiota, followed by rapid degradation of the DNA. Data obtained from the autochthonous population were analysed using standard protocols (Dopazo et al. 1988) to determine the existence of antagonistic properties against *V. ordalii*. It was revealed that the extracellular products of *P. hadiensis*, an agarolytic bacterial isolate, inhibited Vo-LM-06 and Vo-LM-18 cell growth (Chi et al. 2014). Interestingly, *P. hadiensis* is a bacterial component consistently found in seawater at the CIMARQ facility, and its further use as a biotechnological tool against *V. ordalii* is open for discussion. The present results indicate that bacterial antagonism is linked to the survival of *V. ordalii* in seawater microcosms.

However, other alternative strategies may be employed by *V. ordalii* in the aquatic environment, such as biofilm formation. According to Wai et al. (1998) and Lipp et al. (2002), biofilm formation by *Vibrio* spp. on exoskeletons of crustaceans and other marine organisms may constitute a survival strategy during starvation and/or other adverse environmental conditions, including the presence of autochthonous aquatic bacteria with inhibitive properties. Therefore, attachment to a natural surface is an integral part of the aquatic lifestyle of many *Vibrio* spp., representing a successful survival mechanism (Vezzulli et al. 2010). Additionally, starved cells exhibit enhanced resistance to heat and to oxidative and osmotic shock (Barcina et al. 1997); in some cases increased surface hydrophobicity occurs, as in *P. damselae* spp. *piscicida* (Magariños et al. 1994). *V. ordalii* is able to form biofilm, adhere to glass surfaces coated with fish mucus and possesses a hydrophobic character, which could be relevant in the marine aquatic environment to facilitate survival, colonization and host invasion (Ruiz et al. 2015). This result is in accordance with that described by Naka et al. (2011), whereby the *V. ordalii* type strain carries the *syp* genes involved in biofilm formation.

Starved *V. cholerae* cells tend to show a reduction in size and, consequently, an increase in the surface-to-volume ratio, which may facilitate the uptake of substrates from a nutrient-poor environment (Wai et al. 1998). In the present study, a visual light microscopy analysis of *V. ordalii* cells indicated that these were reduced in size after the third day of incubation in the sterile microcosm; however, future analysis through scanning electronic microscopy is needed to confirm this observation. Such a drastic reduction in cell size may be a survival strategy to confer resistance to environmental stress, as has been observed during the starvation survival process for several bacterial species in the aquatic environment (Amy & Morita 1983, Kjelleberg & Hermansson 1984, Jiang & Chai 1996, Vatsos et al. 2003, Avendaño-Herrera et al. 2006, Du et al. 2007, Arias et al. 2012). Also in the sterile microcosm, *V. ordalii* cells changed from a rod to a cocci shape. Although the virulence of *V. ordalii* colonies recovered after 365 d in the microcosm was not evaluated, it is probable that the rod to cocci change in morphology did not affect infective capacity. This assumption is based on virulence tests performed after 60 d, or post-change in morphology, which provide evidence that *V. ordalii* Vo-LM-06 retained the ability to cause fish mortalities.

Analysis of cell surface components and the phenotypic characteristics of *V. ordalii* Vo-LM-06 recovered at Days 35, 60, 90 and 168 from the sterile microcosm revealed that cells maintained the same biochemical and genetic characteristics as the original strain. These phenotypic and genetic characterization results are in line with findings for other Chilean isolates (Silva-Rubio et al. 2008).

Finally, when the virulence of *V. ordalii* Vo-LM-06 (after 60 d of incubation) was assessed by challenge experiments, Vo-LM-06 was able to initiate infection in rainbow trout after prolonged incubation in a sterile microcosm. Similar infectious potential is maintained by other fish pathogens during prolonged periods under survival conditions (Magariños et al. 1994, Romalde et al. 1994, Huq et al. 2000), suggesting that long-term maintenance may not affect the ability of *V. ordalii* to maintain virulence.

CONCLUSIONS

In conclusion, Duodu & Colquhoun (2010) denoted that microcosm studies are a relevant initial phase towards gaining knowledge on the ecology of bacterial pathogens, with subsequent studies taking place under natural farming conditions. The present data suggest that *V. ordalii* can survive in sterile seawater without nutrients for extended periods of time and that long-term maintenance does not affect biochemical and genetic properties or virulence. However, this bacterium does not appear

to remain culturable after exposure to natural seawater, indicating that autochthonous microbiota may play an important role in the survival of this fish pathogen. These findings contribute towards a better understanding of *V. ordalii* survival behaviour in seawater ecosystems.

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