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Cell-surface properties of *Vibrio ordalii* strains isolated from Atlantic salmon *Salmo salar* in Chilean farms

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ABSTRACT: *Vibrio ordalii* is the causative agent of atypical vibriosis and has the potential to cause severe losses in salmonid aquaculture, but the factors determining its virulence have not yet been elucidated. In this work, cell-surface-related properties of the isolates responsible for outbreaks in Atlantic salmon were investigated. We also briefly examined whether pathogenicity against fish varied for *V. ordalii* strains with differing cell-surface properties. Hydrocarbon adhesions indicated the hydrophobic character of *V. ordalii*, although only 4 of 18 isolates induced haemagglutination in Atlantic salmon erythrocytes. A minority of the studied isolates (6 of 18) and the type strain ATCC 33509^T produced low-grade biofilm formation on polyethylene surface after 2 h post-inoculation (hpi), but no strains were slime producers. Interestingly, *V. ordalii* isolates showed wide differences in hydrophobicity. Therefore, we chose 3 *V. ordalii* isolates (Vo-LM-03, Vo-LM-18 and Vo-LM-16) as representative of each hydrophobicity group (strongly hydrophobic, relatively hydrophobic and quasi-hydrophilic, respectively) and ATCC 33509^T was used in the pathogenicity studies. All tested *V. ordalii* strains except the type strain resisted the killing activity of Atlantic salmon mucus and serum, and could proliferate in these components. Moreover, all *V. ordalii* isolates adhered to SHK-1 cells, causing damage to fish cell membrane permeability after 16 hpi. Virulence testing using rainbow trout revealed that isolate Vo-LM-18 was more virulent than isolates Vo-LM-03 and Vo-LM-16, indicating some relationship between haemagglutination and virulence, but not with hydrophobicity.

KEY WORDS: *Vibrio ordalii* · Cell-surface properties · Vibriosis · Atlantic salmon · Chile

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INTRODUCTION

Vibrio ordalii is a Gram-negative, motile, rod-shaped bacterium that is fermentative, catalase and oxidase positive, and sensitive to the vibriostatic agent O/129 (Farmer et al. 2005). It causes septicaemia and death of fish with gross pathological symptoms similar to those of *Vibrio anguillarum* (Toranzo & Barja 1993). Indeed, *V. ordalii* has been established to accommodate strains formerly classified as *V. anguillarum* biotype 2 (Schiewe et al. 1981), which were isolated for the first time from diseased salmon cultured in coastal waters of the Pacific Northwest of the USA (Harrel et al. 1976). Since its first isolation, *V. ordalii* has been also reported in Japan, Australia and New Zealand, affecting mainly the culture of several salmonid species (Ransom et al. 1984, Toranzo et al. 1997). In Chile, the first report

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was described from a population of Atlantic salmon *Salmo salar* (Colquhoun et al. 2004) and, a few years ago, from Pacific salmon *Oncorhynchus kisutch* and rainbow trout *Oncorhynchus mykiss* (Silva-Rubio et al. 2008).

Fish affected by *V. ordalii* show a generalized haemorrhagic septicaemia based on the fins, exophthalmos and especially injury to the area surrounding the anus, including the anal pore (Ransom et al. 1984). However, the mortalities in cultured salmonids in Chile are characterized by the presence of skin lesions and haemorrhaging ulcers, pericarditis, peritonitis, multiple necrotic foci in the liver and signs of systemic septicaemia (Bohle et al. 2007). It is important to note that *V. ordalii* develops the bacteremia only at late stages of the disease and the concentration of bacterial cells in blood of the infected fish is normally less abundant than in skeletal and cardiac muscles, anterior and posterior gastrointestinal tract, and gills. In addition, the bacteria are not evenly dispersed but are present within tissues as colonies or aggregates of cells (Ransom et al. 1984).

Numerous studies describe *V. ordalii* isolated from salmon as a biochemically, antigenic and genetically 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 that the Chilean strains may constitute a new serological subgroup within this bacterial species. Nevertheless, despite these studies there is a limited amount of information about the nature of the virulence factors involved in the pathogenesis of the infection that *V. ordalii* causes in fish.

Evidence suggests that the production of a leucocytolytic factor may play an important role in pathogenesis (Ransom et al. 1984). Other factors are the ability of this pathogen to resist the bactericidal action of fish serum (Trust et al. 1981) and to agglutinate different eukaryotic cell types, suggesting that *V. ordalii* attaches to and interacts with the host cells (Larsen & Mellergaard 1984). More recently, in the strain ATCC 33509^T genome, Naka et al. (2011) identified biosynthesis and transport genes for a yersiniabactin-like siderophore, which could play a role in virulence. In addition, in the same strain, all hemolysin genes were conserved except for *vah4*, and an entire biofilm formation cluster was identified: the Syp system, except for SypE, a negative regulator. Yet, these genes have been identified only in the type strain so far and the exact role and contribution of many of these virulence factors in full *V. ordalii* virulence remain largely unknown.

For the development of microbial pathogenesis, the infectious agent must be able to penetrate the protective barriers that shelter the host from its environment. A first prerequisite for successful colonization is the ability to adhere to the host. Then, the agent must be able to survive the many defence mechanisms, cellular and humoral, which attack it as soon as it reaches the tissues. The purpose of this study was to evaluate some virulence determinants of *V. ordalii* isolated from outbreaks in cultured Atlantic salmon, such as its hydrophobic and haemagglutination properties, adhesion to mucus and salmon head kidney (SHK-1) cells, survival and biofilm formation in skin mucus and serum resistance. Furthermore, we attempted to determine whether *V. ordalii* isolates with different cell-surface-associated properties of this bacterium are more or less pathogenic for fish.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Eighteen Chilean *Vibrio ordalii* isolates recovered from Atlantic salmon from enzootics on farms in Southern Chile during 2003–2011 were examined in this study. The Atlantic salmon isolates were obtained from internal organs (kidney or spleen) of diseased cage-cultured Atlantic salmon. The identity of each isolate was initially confirmed as *V. ordalii* by standard phenotypical procedures (Silva-Rubio et al. 2008, Poblete-Morales et al. 2013) and using the PCR-based analysis described by Avendaño-Herrera et al. (2014). In addition, profiles of our isolates were compared with those of the *V. ordalii* type strain ATCC 33509^T from the American Type Culture Collection (ATCC).

For all tests, the isolates were routinely grown on trypticase soy agar or in broth supplemented with 1% (w/v) NaCl (TSA-1 and TSB-1, respectively) and incubated at 18°C for 48 to 72 h. Stock cultures were maintained frozen at −80°C in Criobilles tubes (AES Laboratory) or in TSB-1 containing 15% (v/v) glycerol. The reference strains *Yersinia ruckeri* CECT 955 (equivalent to strain 11.4) and *Vibrio anguillarum* ATCC 43307 were included in the analysis of cell-surface characteristics and biofilm formation ability as positive and negative controls, respectively (Magariños et al. 1996a, González-Contreras et al. 2011). In addition, *V. anguillarum* ATCC 43307 was also included in adherence and invasion assays.

Analysis of cell-surface characteristics

The cell-surface hydrophobicity of the 18 *V. ordalii* isolates, the type strain ATCC 33509^T and each control was evaluated using 2 standard methods: (1) microbial adhesion to hydrocarbons (MATH) and (2) the salt aggregation test (SAT) according to the procedure described by Rosenberg et al. (1980), with some modifications (Lindahl et al. 1981, Abdelsalam et al. 2009, González-Contreras et al. 2011).

In the MATH assay, bacterial cells were harvested and washed twice with 0.1 M potassium phosphate buffer (pH 7.0), and resuspended in the same buffer; the absorbance (initial optical density, OD) was measured at 400 nm (approximately 10^9 *V. ordalii* cells ml^{-1}). A small amount of n-octane (0.4 ml, Winkler) was mixed with the cell suspension (2.4 ml) for 2 min using a vortex mixer. The mixture was allowed to stand for 15 min, and the aqueous phase was carefully transferred to a 1-ml cuvette and the absorbance was measured at 400 nm. The result was expressed as the decrease of OD recorded for the aqueous phase relative to the initial OD (as a percentage).

For the SAT, bacteria were salted out with ammonium sulphate, $(NH_4)_2SO_4$ (Merck). Volumes (25 µl) containing 10^9 *V. ordalii* cells ml^{-1} suspended in phosphate-buffered saline (PBS, pH 7.0) were mixed with 25 µl of a series of $(NH_4)_2SO_4$ concentrations (0–4.0 M) on a microscope slide. After 2 min of mixing on a rocking shaker at room temperature, the bacteria were examined with a stereomicroscope (120×). The lowest final concentration of $(NH_4)_2SO_4$ that caused aggregation was recorded as the SAT value.

The ability of the 18 *V. ordalii* isolates and the type strain ATCC 33509^T to agglutinate erythrocyte suspensions from Atlantic salmon was determined according to Toranzo et al. (1983). Isolates were considered negative if visible haemagglutination did not occur within 10 min. As controls we included the same reference strains that were used in the SAT assays.

Phenotypic characterization of slime-producing bacteria

Biofilm formation was studied by culturing the 18 *V. ordalii* isolates and the *V. ordalii* type strain on Congo red agar (CRA) plates made by mixing 50 g of saccharose with 0.8 g Congo red in 1 litre of TSA-1 and incubing at 18°C for 96 h under aerobic conditions (Freeman et al. 1989). The CRA plates were observed every day and the results were interpreted as follows: very black, black and almost black or reddish-black colonies with a rough, dry and crystalline consistency were considered to be normal slime-producing strains; while very red, red, bordeaux and smooth colonies were classified as non-slime-producing strains, as described previously (Arciola et al. 2002). The reference strain *Staphylococcus aureus* ATCC 25923, known to possess the ability to form biofilms (Jain & Agarwal 2009), was included as positive control.

Biofilm formation ability of *V. ordalii*

Biofilm production by the 18 *V. ordalii* isolates and ATCC 33509^T grown in TSB-1 was determined using a semi-quantitative adherence assay on 96-well tissue culture plates (NUNC^{TM} Surface) as described previously (Retamales et al. 2012). One hundred microlitres of each overnight bacterial culture grown in TSB-1 was added into each well containing 100 µl of PBS (pH 7.2). Each strain was tested in triplicate, and to confirm the absence of a bacterial contamination, an additional 3 wells with sterile TSB-1 inoculated alone with PBS were included as negative control.

Following 2 h of incubation at 18°C, the medium was removed and the microtiter wells were washed vigorously 3 times with PBS to remove the non-adherent bacteria and were then dried in an inverted position. Ten microlitres of 1% crystal violet was added to each well and incubated for 20 min; stained wells were then washed 3 times with sterile distilled water to remove excess stain and air-dried. The remaining crystal violet was quantified by adding 200 µl of 99.8% ethanol to each well, shaken gently for 30 min and the OD of the extracted stain was measured at 595 nm. Based on the OD produced by bacterial films and considering the average OD_{595} for the 3 replicates as the value for the strain, each strain was classified as highly positive ($OD_{595} \geq 1$), low-grade positive ($0.1 \leq OD_{595} < 1$) or negative ($OD_{595} < 0.1$) (Kouidhi et al. 2010).

Survival of *V. ordalii* in skin mucus

Based on the analysis of cell-surface characteristics, we decided to use *V. ordalii* Vo-LM-03, Vo-LM-16 and Vo-LM-18 for the rest of our study. These 3 isolates were chosen as representative of *V. ordalii* because they were classified as strongly hydropho-

bic, relatively hydrophobic and quasi-hydrophilic, respectively.

To obtain the mucus, all fish were anaesthetized by immersion in 30 µg ml^{-1} BZ-20 (Veterquímica) according to the Ethics Committee for Animal Experiments of the Universidad Andrés Bello.

Mucus was scraped from the skin of 5 *V. ordalii*-free Atlantic salmon (weighing 100–120 g) with a glass slide passed from the operculum to the caudal peduncle. These fish were analysed with plating and PCR techniques to confirm the absence of *V. ordalii* as well as other bacterial fish pathogens according the protocols described by Avendaño-Herrera et al. (2014) and Tapia-Cammas et al. (2011), respectively. The pool of mucus samples was centrifuged for 5 min at 2655 × *g* to remove particulate material, and the supernatant (3 ml) was dissolved in sterile seawater (1:1), mixed thoroughly, filter-sterilized onto 0.22 µm and stored at −20°C until use. The protein concentration of the mucus samples was determined using a Micro BCA^{TM} Protein Assay (Pierce). Ten microlitres of the samples was streaked for isolation of bacteria onto TSA-1 and incubated at 20°C for 72 h to check for bacterial contamination.

The antibacterial activity of the skin mucus was performed with the 3 selected *V. ordalii* isolates according to the procedure described by González-Contreras et al. (2011), except that *Streptococcus phocae* was replaced by *V. ordalii*. The antibacterial activity was tested using a sterile 6-well multidish containing the mucus samples diluted 1:10 (v/v) in sterile saline solution (0.85% NaCl) and then mixed by repeated pipetting. Each well was seeded with 100 µl of each isolate, re-suspended in sterile saline solution to a final concentration of 2.2 ± 0.3 × 10^7 colony-forming units (CFU) ml^{-1}. For *V. anguillarum* ATCC 43307 and *V. ordalii* ATCC 33509^T, the bacterial suspension contained approximately 7.5 ± 0.05 × 10^7 CFU ml^{-1}. Samples were incubated at 18°C, and surviving bacteria were enumerated by viable counts of 10-fold serial dilutions of each supernatant in sterile saline solution of 100 µl onto TSA-1 plates at 1, 6, 12 and 24 hpi. Positive controls consisted of the same treatments, with the exception of the mucus, which was replaced by TSB-1, while other wells were maintained with TSB-1 without bacteria (negative control). For reference purposes, viable counts were also made on each control. The survival of mucus-treated bacteria was expressed as CFU recovered compared with that of untreated samples. The reproducibility of results was assessed by repetition in triplicate of at least 2 independent antibacterial activity assays.

To determine the possible involvement of complement in the bacterial activity, the mucus preparations were heated at 56°C for 30 min. Following this, the mucus preparations were assayed for antibacterial activity under the same conditions as those described above.

Biofilm formation ability of *V. ordalii* in skin mucus

The detection of biofilm formation in this study was based on the ability of the *V. ordalii* cells to form biofilms on mucus. This assay was carried out indirectly by determining the extent of crystal-violet-stained *V. ordalii* cells' adherence to the wells of 96-well flat-bottom polystyrene plates (NUNC^{TM} Surface) (O'Toole & Kolter 1998) with 10 µg of mucus protein per well with some modifications (González-Contreras et al. 2011). One hundred microlitres of mucus was inoculated onto 96-well polystyrene plates in triplicate and left to air-dry. Following this, 100 µl of each *V. ordalii* isolate obtained from an overnight culture was transferred to each mucus-coated well, in which the final concentration was 10^6 cells ml^{-1}. Bacteria were allowed to adhere to the mucus surface at 18°C for 24 h. Thereafter, 10 µl of 1% crystal violet was added to each well and incubated for 20 min; stained wells were then washed 3 times with sterile distilled water to remove excess stain and air-dried. The remaining crystal violet was quantified by adding 200 µl of 100% ethanol to each well, shaking gently for 30 min, and absorbance of the extracted stain was measured at 595 nm.

To rule out the possible influence of the mucus on adherence, we also repeated the adherence experiment using only air-dried mucus (without bacteria). Control tests using uncoated polystyrene surfaces were performed. ODs of growth treatments and controls were measured at the beginning of and after the adhesion incubation.

Survival in fish serum

Serum was aseptically obtained from the same population of healthy Atlantic salmon described above. Fish were bled by caudal puncture, and approximately 500 µl of blood per fish was obtained. These bloods were placed in the same 1.5 ml Eppendorf tube and allowed to clot overnight at 4°C. The serum was collected following centrifugation at 15 000 × *g* for 3 min and separated serum was stored at −20°C until use. The same 3 *V. ordalii* isolates and

the type strain ATCC 33509^T used in the mucus assay were inoculated in the serum diluted 1:1 in PBS; the isolates were incubated at 18°C with gentle shaking for 1 h, and surviving bacteria were enumerated by viable counts of 100 µl of 10-fold serial diluted bacterial suspension on TSA-1 at 1, 6, 12 and 24 hpi, and bacterial growth was monitored by the plating method. Controls with the addition of bacteria alone and assays with inactivated sera (56°C for 30 min) were run simultaneously under exactly the same conditions as described earlier.

Adherence to fish cell lines

The adherence assays to fish cell lines were performed with the same 3 *V. ordalii* isolates and the type strain ATCC 33509^T according to the procedure described by Magariños et al. (1996a) using SHK-1 cells. SHK-1 cells were grown in duplicate using semiconfluent monolayers in 6-well culture plates (10^5 cells per well) with Leibovitz's L-15 medium (Hyclone Laboratories) supplemented with 10% foetal bovine serum (Hyclone Laboratories), 6 mM L-glutamine (Hyclone Laboratories), 40 µM 2-mercaptoethanol (Gibco, Invitrogen Laboratories) and 10 µg ml^{-1} gentamicin (Gibco, Invitrogen Laboratories). All fish cell cultures were maintained at 16°C and 2 h before performing the adhesion assays, the medium was removed and replaced by fresh medium without antibiotics.

Bacteria were harvested, washed twice with PBS and resuspended in PBS to obtain a concentration of $2.1 \pm 0.7 \times 10^6$ CFU ml^{-1}. One hundred microlitres of the bacterial suspension was added into each well containing the cell monolayers, equivalent to a multiplicity of infection of ~20 bacteria per cell. Following 2 h of incubation at 18°C, the medium was removed and monolayers were washed vigorously 3 times with PBS to remove the non-adherent bacteria. The ability of *V. ordalii* to adhere to the cells was determined by spreading 10-fold serial dilutions of each supernatant onto TSA-1 plates. Results were expressed as the percentage of the bacteria that were recovered in comparison with the number of each bacterium that was initially inoculated.

Invasion assays

The bacterial invasion assay was performed with the same 3 *V. ordalii* isolates and the type strain ATCC 33509^T using 2 standard *in vitro* methods: (1) incubation of the *V. ordalii* isolates with SHK-1 cells followed by selective killing of extracellular bacteria using gentamicin and the release of internalized bacteria by lysis of SHK-1 cells with detergent (Magariños et al. 1996a, González-Contreras et al. 2011) and (2) evaluation of the liberation level of the cytosolic enzyme lactate dehydrogenase (LDH) in the cell supernatant using a commercial LDH cytotoxicity detection kit (Takara).

In the first method, the SHK-1 cells cultured in 24-well culture plates (10^5 cells per well) were co-cultured with each *V. ordalii* isolate using the procedure and strains described previously for the adherence assay. After 1 h of incubation at 18°C, the monolayers were washed with PBS and incubated for a further 2 or 24 h in L-15 containing 50 µg ml^{-1} of gentamicin to kill extracellular bacteria. Then, monolayers were washed 3 times with PBS and lysed with a 1% Triton X-100 (Invitrogen) solution to release bacteria that penetrated the cells. Intracellular bacteria were quantified by spreading 10-fold serial dilutions of each lysate onto TSA-1 plates. According to the criteria described by Janda et al. (1991), an isolate was considered invasive when the number of intracellular bacteria was equal to or exceeded 10^3 CFU per well. Controls were run in parallel to ensure viability of the bacteria in exactly the same conditions as described previously.

In the second assay, *V. ordalii* suspensions prepared in sterile saline solution (10^6 cells per well) were inoculated in 24-well tissue culture plates containing the SHK-1 cell monolayers and incubated at 18°C for 120 hpi. Possible differences in the permeability of the membrane in SHK-1 cells exposed to *V. ordalii* were determined by evaluating the liberation level of LDH in the cell supernatant using a commercial LDH cytotoxicity detection kit (Takara). To calculate the percentage of cytotoxicity, the control cell cultures were run simultaneously without the addition of bacteria as well as the 3 controls required in the Takara kit itself (i.e. background, low and high controls). In brief, samples of 100 µl of supernatant cell culture, taken 2, 16, 24, 48, 72 and 120 hpi, were analyzed in accordance with the supplier's instructions. The results of the reaction were measured using absorbance at 490 nm and expressed as percentage cytotoxicity by calculating the average absorbance values of 2 replicates and subtracting from each of these the absorbance value obtained in the background control. The result was obtained using the equation: cytotoxicity (%) = [(experimental value − cells cultured in L-15 / cells cultured in L-15 with 2% Triton X-100)] × 100.

In addition, the morphology of the fish cell line was examined using a phase-contrast inverted microscope at 400× or 600× magnification.

Virulence for rainbow trout

To determine the virulent capacities of the 3 Chilean isolates and the *V. ordalii* type strain, infectivity trials were conducted. The isolate Vo-LM-13 was also included as a haemagglutination-negative representative of Group 2. Healthy *V. ordalii*-free rainbow trout (weighing 28–30 g) obtained from a fish farm with no history of diseases (located in central Chile, Los Andes) were used. This farm is the oldest in Chile, has produced its own rainbow trout eggs since 1903 and has a certificate of being free of pathogens listed in Chilean Lists 1, 2 and 3 (www.sernapesca.cl). Also, before moving the fish, according to sanitary regulations, fish were certified as being free of any pathogen described in Chile (e.g. infectious salmon anemia virus, infectious pancreatic necrosis virus [IPNV], *Piscirickettsia salmonis* and *Renibacterium salmoninarum*; Avendaño-Herrera 2011).

In addition, to ensure that fish were uninfected with *V. ordalii*, samples were subjected to standard microscopical and bacteriological examination, as well as analysis by PCR (Avendaño-Herrera et al. 2014). Fish were randomly allocated in groups of 5 per 4-l plastic tank and acclimatized for 48 h prior to bacterial challenge.

Groups of 5 rainbow trout were inoculated by intraperitoneal injection with a high and low dose of infection from each bacterial strain (Table 1).

Control fish received 0.1 ml of TSB-1. All fish were maintained in tanks at 18 ± 1°C with aeration during the course of the experiments (21 d), with 50% of the tank water refreshed daily. Dead fish were removed from each tank daily and examined to confirm whether the bacterium inoculated was the cause of mortality by the following methods: (1) kidney, liver, spleen and external lesions were directly streaked onto TSA-1 plates and incubated at 18°C for 1 wk and (2) pure or mixed cultures as well as fish tissues (kidney, liver and spleen) were also tested using the specific PCR (Avendaño-Herrera et al. 2014). In addition, the identity of each isolate was confirmed as *V. ordalii* by standard phenotyping procedures (Silva-Rubio et al. 2008, Poblete-Morales et al. 2013).

Table 1. Dosages of *Vibrio ordalii* isolates for virulence assays inoculated by intraperitoneal injection into groups of rainbow trout (n = 5 per group). Control fish received 0.1 ml of TSB-1

Strain	Dose per fish (CFU × ml^{-1})	
	Low	High
Vo-LM-03	1.38×10^5	1.38×10^7
Vo-LM-13	6.69×10^5	6.69×10^7
Vo-LM-16	2.01×10^5	2.01×10^7
Vo-LM-18	1.29×10^5	1.29×10^7
V. ordalii ATCC 33509^T	3.31×10^5	3.31×10^7

Statistical analysis

The results were subjected to an ANOVA (Sokal & Rohlf 1980) to compare adherence, invasion and survival among treatments and each control. An alpha value of 0.05 was used for all analysis.

RESULTS

Analysis of cell-surface characteristics

Table 2 summarizes the results of the hydrophobic properties of the *Vibrio ordalii* isolates and the reference strains included as controls. In the MATH assay, the 18 *V. ordalii* strains were clearly separated into 3 main groups with percentage of hydrophobicity values ranging from 9.6% to 32.7%. In contrast, the hydrophobic value of the negative control was significantly lower than that of all *V. ordalii* isolates (1.2%), while that of the positive control was 16.8%. By SAT, most of the *V. ordalii* isolates were classified as hydrophilic as they aggregated in more than 3.0 M ammonium sulphate, while 3 strains were relatively hydrophobic with a 2.0 M concentration of ammonium sulphate (Table 2).

With respect to the haemagglutination assays, this cell-surface characteristic was detected in only 4 of the 18 Chilean *V. ordalii* isolates tested (Vo-LM-14, Vo-LM-18, Vo-LM-19 and Vo-LM-20), while the remaining isolates did not agglutinate red blood cells obtained from Atlantic salmon (Table 2).

Phenotypic characterization of slime-producing bacteria

The 18 *V. ordalii* strains studied were non-producers of slime, developing red or bordeaux colonies. As expected, the reference strain *Staphylococcus aureus* ATCC 25923 included as positive control showed very black colonies on the CRA plate, which are acceptable results for the slime-producing tests (Table 2).

Table 2. *Vibrio ordalii* strains included in this study and the results from cell-surface characteristic tests in comparison with reference strains from other bacterial species included as control. ATCC: American Type Culture Collection (Rockville, MD, USA); CECT: Colección Española de Cultivos Tipos (Valencia, Spain); NT: not tested. MATH data: percentage of bacterial hydrophobicity to n-octane. SAT: salt aggregation tests; values represent the lowest molarity of ammonium sulphate yielding aggregation of bacteria. Data are means ± SD of all replicates of each strain. Classification of groups follows MATH results: Group 1, >29.6%; Group 2, 19.6–29.5%; Group 3, 9.6–19.5%

Strain	Hydrophobicity test MATH	SAT	Haemagglutination assay	Congo red assay	Biofilm assay
Group 1					
Vo-LM-03	32.7 ± 1.6	2 ± 0	–	–	Negative
Vo-LM-05	32.3 ± 3.6	2 ± 0	–	–	Negative
Vo-LM-11	31.6 ± 2.4	2 ± 0	–	–	Low-grade positive
Group 2					
Vo-LM-01	20.3 ± 2.6	3 ± 0	–	–	Negative
Vo-LM-02	24.1 ± 2.8	3 ± 0.7	–	–	Negative
Vo-LM-04	21.0 ± 3.6	3 ± 0	–	–	Negative
Vo-LM-09	21.1 ± 2.7	3 ± 0	–	–	Negative
Vo-LM-10	23.0 ± 3.5	3 ± 0	–	–	Negative
Vo-LM-13	19.8 ± 0.8	3 ± 0	–	–	Negative
Vo-LM-14	20.8 ± 2.9	3 ± 0	+	–	Negative
Vo-LM-18	21.2 ± 3.2	3 ± 0	+	–	Negative
Vo-LM-19	25.6 ± 1.5	3 ± 0.7	+	–	Low-grade positive
Group 3				–	
Vo-LM-06	14.3 ± 3.4	3 ± 0	–	–	Negative
Vo-LM-07	16.0 ± 2.8	3 ± 0	–	–	Low-grade positive
Vo-LM-12	15.3 ± 1.9	3 ± 0	–	–	Low-grade positive
Vo-LM-15	14.3 ± 2.6	3 ± 0.6	–	–	Low-grade positive
Vo-LM-16	9.6 ± 1.7	3 ± 0	–	–	Negative
Vo-LM-20	13.0 ± 1.3	3 ± 0	+	–	Low-grade positive
Reference strain					
Vibrio ordalii ATCC 33509^T	12.0 ± 2.2	3 ± 0	–	–	Low-grade positive
Yersenia ruckeri CECT 955	1.2 ± 1.2	4 ± 0	NT	NT	Negative
Vibrio anguillarum ATCC 43307	16.8 ± 2.3	2 ± 0	–	–	Low-grade positive
Staphylococcus aureus ATCC 25923	NT	NT	NT	+	Low-grade positive

Biofilm formation ability of the *V. ordalii* isolates

The semi-quantitative adherence assay on 96-well tissue culture plates showed that the *V. ordalii* strains were separated into 2 main groups. One profile comprised 6 Chilean isolates and the *V. ordalii* type strain classified as low-grade positive biofilm producers, showing an OD between 0.10 ± 0.003 and 0.17 ± 0.03. The second group (12 isolates) showed values between 0.06 ± 0.0007 and 0.09 ± 0.004, with isolates considered negative biofilm producers (Table 2). As expected, the reference strains *Yersinia ruckeri* CECT 955 and *Vibrio anguillarum* ATCC 43307 included as controls in the analysis of biofilm production showed acceptable results, with values of 0.07 ± 0.03 and 0.23 ± 0.014, respectively. It is important to point out that *S. aureus* ATCC 25923 showed a higher OD at 0.33 ± 0.0035, also considered to be a low-grade positive biofilm.

Survival and biofilm formation ability of *V. ordalii* isolates in skin mucus

This assay was applied using final mucus suspensions containing 100 µg protein ml^{-1}, as estimated by the Micro BCA^{TM} Protein Assay, and all samples were culture-negative following plating onto TSA-1.

The survival capacity of all *V. ordalii* isolates studied after 24 h of incubation in mucus is shown in Fig. 1. In general, the standard plate counts showed a slight increase of culturable cells in all Chilean *V. ordalii* isolates at 12 hpi followed by a stabilization around the initial concentration value inoculated (2.2 ± 0.3 × 10^7 CFU ml^{-1}). In contrast, culturability onto TSB-1 seeded with the same initial inoculum of each *V. ordalii* isolate (positive control) was lower than that tested on Atlantic salmon mucus. Interestingly, when the mucus was heated at 56°C for 30 min to destroy complement, a similar growth pattern was scored for the isolates Vo-LM-03 and Vo-LM-16 after 24 hpi, showing a decrease

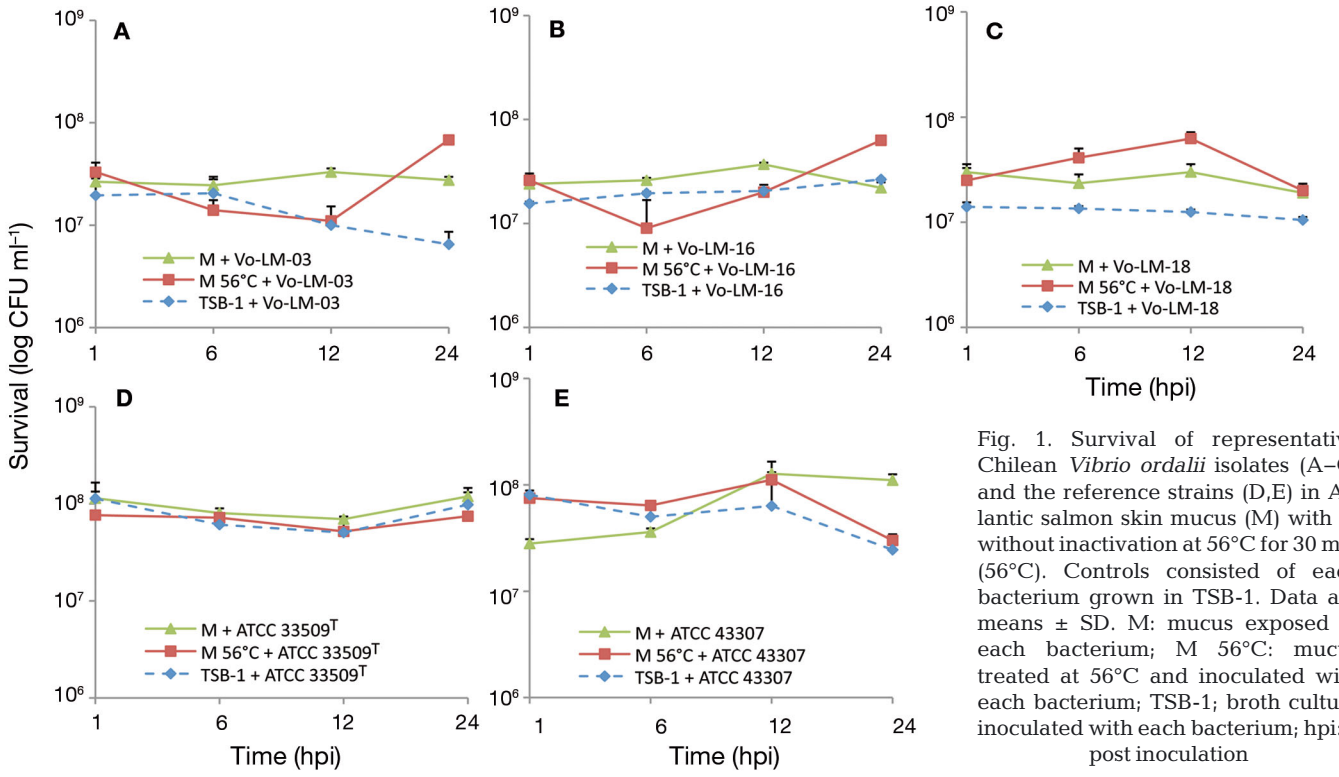

Fig. 1. Survival of representative Chilean *Vibrio ordalii* isolates (A–C) and the reference strains (D,E) in Atlantic salmon skin mucus (M) with or without inactivation at 56°C for 30 min (56°C). Controls consisted of each bacterium grown in TSB-1. Data are means ± SD. M: mucus exposed to each bacterium; M 56°C: mucus treated at 56°C and inoculated with each bacterium; TSB-1; broth culture inoculated with each bacterium; hpi: h post inoculation

in culturable *V. ordalii* during the first 12 hpi and then an increase of approximately 1 log-unit in CFU ml^{-1} (Fig. 1A,B). In the case of isolate Vo-LM-18, differences were indistinguishable with or without treated mucus after 24 hpi. An identical pattern was obtained with the type strain ATCC33509^T inoculated in wells with or without heated Atlantic mucus, as well as in TSB-1 (positive control) (Fig. 1D). The major differences were detected with *V. anguillarum* ATCC 43307, showing an increase of approximately 1 log-unit of the number of culturable bacteria after 24 hpi in the mucus compared with the positive control as well as the heated mucus (Fig. 1E).

Furthermore, the *V. ordalii* isolates and the type strain showed biofilm formation ability to the bottom of each well coated with Atlantic salmon mucus, reaching an average at an OD_{595} of 0.15 ± 0.05 at 24 hpi (considered low-grade positive biofilm), while the adherence using only air-dried mucus (without bacteria) had a value of 0.047 ± 0.001 (data not shown).

Survival in fish serum

Results of the survival experiments showed that in normal Atlantic salmon serum, all Chilean *V. ordalii* tested were resistant to the serum's antibacterial activity and the number of culturable cells increased by at least 1.32 × 10^7 CFU ml^{-1} (equivalent to 41% higher than the positive control) during the incubation period ($p < 0.05$), while the number of *V. ordalii* ATCC 33509^T grown in TSB-1, regardless of the period tested, was always higher than that of the mucus samples ($p > 0.05$). In contrast, culturability of *V. anguillarum* ATCC 43307 in mucus remained relatively constant, varying by approximately 1 log-unit when compared with positive control at 24 h ($p < 0.05$; Fig. 2E).

When the serum was heated, the plate count method showed a similar growth pattern for the isolates Vo-LM-03 and Vo-LM-16 and the 2 reference strains, showing a slight decrease of culturable bacteria at 6 or 12 hpi, followed by a increase of around 1 log-unit at 24 hpi. Interestingly, at 24 hpi, the number of Chilean *V. ordalii* and *V. anguillarum* culturable bacteria grown with or without heated mucus was higher than that of the positive control (bacteria alone with liquid medium).

Adherence to and invasion of fish cell lines

At 2 hpi, all Chilean *V. ordalii* isolates adhered to SHK-1 cells, and showed a percent adhesion from 5.2 to 10.7, while that of *V. anguillarum* ATCC 43307 was lower (4.9%) than that of all Chilean isolates, and that of the type strain ATCC 33509^T was 5.8% (Table 3).

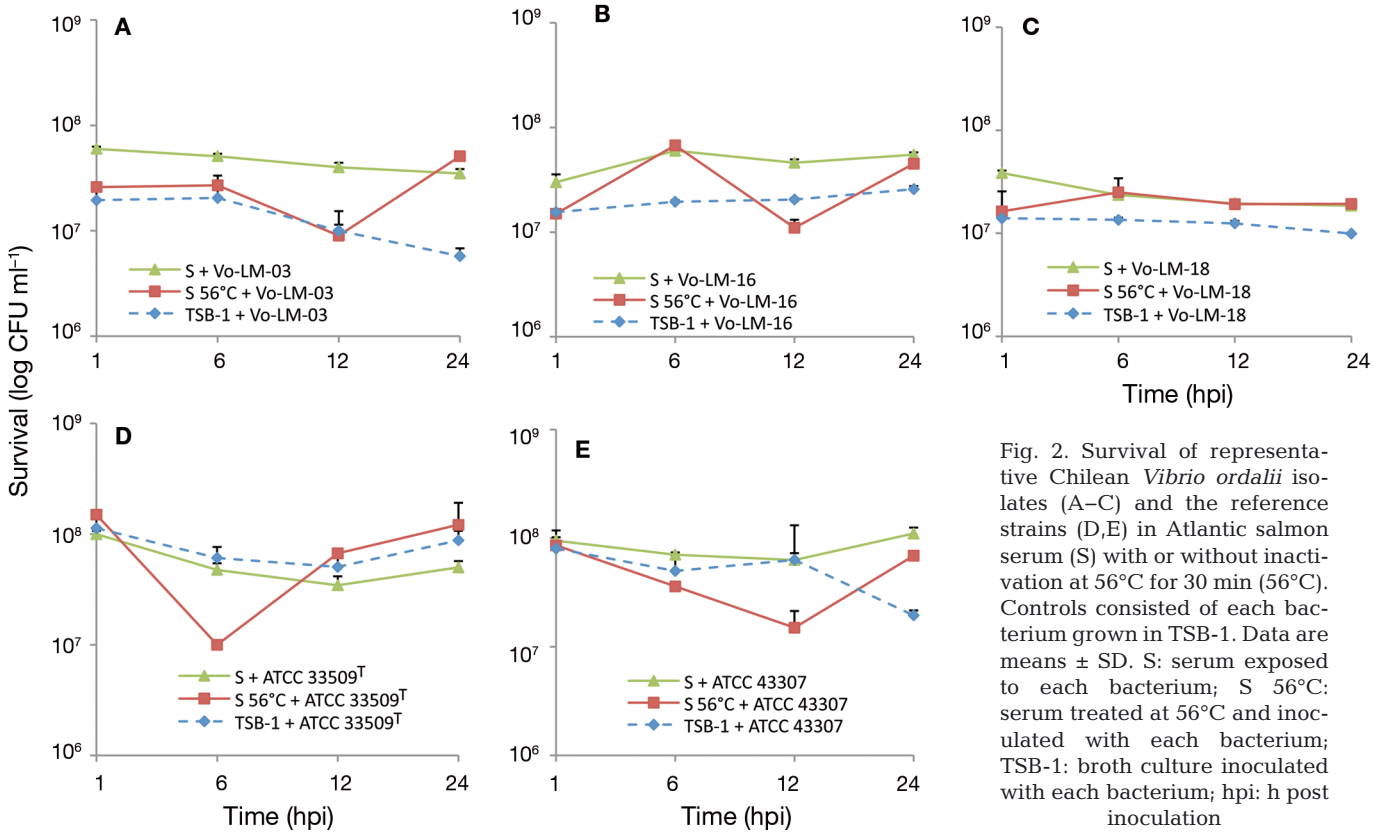

Fig. 2. Survival of representative Chilean *Vibrio ordalii* isolates (A–C) and the reference strains (D,E) in Atlantic salmon serum (S) with or without inactivation at 56°C for 30 min (56°C). Controls consisted of each bacterium grown in TSB-1. Data are means ± SD. S: serum exposed to each bacterium; S 56°C: serum treated at 56°C and inoculated with each bacterium; TSB-1: broth culture inoculated with each bacterium; hpi: h post inoculation

Table 3. Adhesion and invasion of *Vibrio ordalii* isolates and reference strains to salmon head kidney (SHK-1) cells. Adhesion results are expressed as the percentage of the original inoculum used for each strain recovered after 2 hpi. Invasion results are expressed as the percentage of the original inoculum used for each strain recovered after 2 and 24 hpi. Values in parentheses are numbers of culturable cells (CFU ml^{-1})

Strain	Adhesion	Invasion	
		2 hpi	24 hpi
Vo-LM-03	10.7 ± 0.26 ($1.18 \times 10^5 \pm 2.83 \times 10^3$)	0.0009 ± 0.00019 (10 ± 2.07)	0.16 ± 0.08 ($2.21 \times 10^2 \pm 1.11 \times 10^2$)
Vo-LM-16	5.2 ± 0.58 ($1.21 \times 10^5 \pm 1.34 \times 10^4$)	0.0003 ± 0.00007 (7 ± 1.55)	0.01 ± 0.01 ($1.40 \times 10^2 \pm 5.66 \times 10^1$)
Vo-LM-18	5.9 ± 1.58 ($1.26 \times 10^5 \pm 3.39 \times 10^4$)	0.0002 ± 0.00005 (5 ± 1.04)	0.11 ± 0.02 ($2.25 \times 10^2 \pm 3.54 \times 10^1$)
V. anguillarum ATCC 43307	4.9 ± 0.11 ($1.53 \times 10^5 \pm 3.54 \times 10^3$)	0.0031 ± 0.00065 (98 ± 20.2)	0.03 ± 0.01 ($3.25 \times 10^2 \pm 1.06 \times 10^2$)
V. ordalii ATCC 33509T	5.8 ± 0.04 ($1.10 \times 10^5 \pm 7.07 \times 10^2$)	0.0001 ± 0.00003 (3 ± 0.52)	0.08 ± 0.02 ($1.19 \times 10^2 \pm 2.65 \times 10^1$)

However, the invasion results regardless of the time analysed (2 or 24 hpi) showed that the 3 *V. ordalii* isolates tested and the type strain were not able to enter SHK-1 cells, with invasion values ranging from 0.01 to 0.16% of the initial CFU numbers. A similar result was only scored at 24 hpi with *V. anguillarum* ATCC 43307, which was used as negative control (0.03%).

In contrast, microscopic analysis of the SHK-1 cell line revealed cytopathic effect after 16 hpi, with a significant increase in the level of LDH liberation in SHK-1 cells inoculated with all 3 selected Chilean *V. ordalii* isolates on Day 2 of the study, giving percentages of LDH activity between 179.1 and 253.5%, whereas that of the type strain ATCC 33509T was 74.4%. When *V. anguillarum* ATCC 43307 was tested, we observed that the LDH values increased rapidly during the first 16 h, reaching their highest level around 48 h, but then decreasing

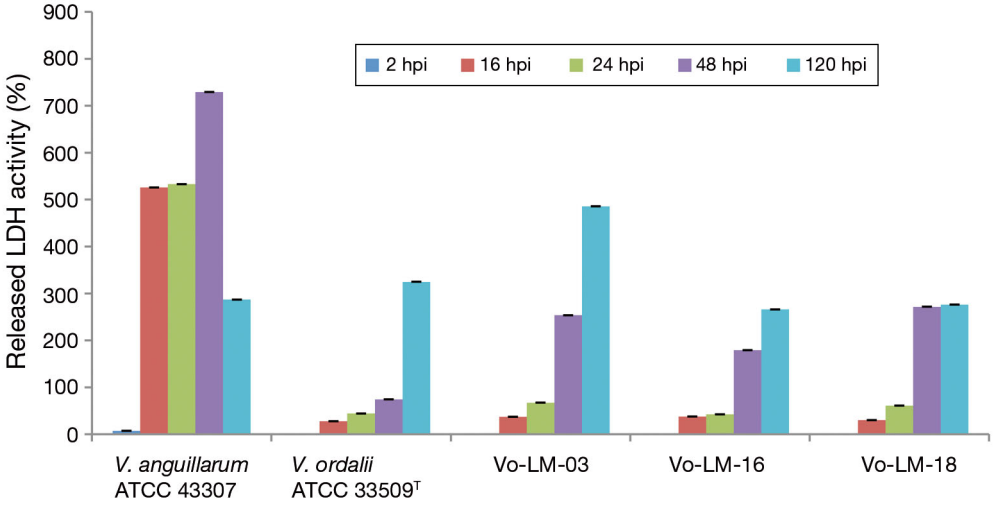

Fig. 3. Percentage of the released lactate dehydrogenase (LDH) activity (mean ± SD) in salmon head kidney (SHK-1) cells infected with representative isolates of *Vibrio ordalii* and over 5 d. 0% corresponds to cells incubated without bacteria, while 100% corresponds to cells incubated without bacteria with 1% Triton X-100

by more than half (729.2% to 286.7%) on Day 5 (Fig. 3). As expected in the monolayers that were not inoculated with the *V. ordalii* isolates, negative results were found for the cytopathic effect test and LDH analysis.

Virulence for rainbow trout

The virulence assays showed that the 4 *V. ordalii* isolates and the type strain were able to cause 100% mortality when fish were intraperitoneally injected with the high doses. In the case of the isolate Vo-LM-18 (Group 2 and haemagglutination positive), total mortality was recorded during the first 3 d post-inoculation (dpi), while with the remaining isolates this occurred after 13 dpi. Interestingly, with the type strain, mortalities began at 5 dpi, and were 100% at Day 18. In contrast, no mortalities during 21 d were observed for the low dose of the type strain, while the mortalities reached 40, 80 and 100% for fish challenged with low doses of Vo-LM-03, Vo-LM-16 and Vo-LM-18, respectively. Also, 100% mortality was obtained with the isolate Vo-LM-13. No mortalities were observed in the control groups. *Vibrio ordalii* was recovered from all dead fish, which showed in all cases the typical internal haemorrhage observed in the natural outbreaks. When the PCR protocol was applied to different tissue samples from all clinically diseased fish tested, *V. ordalii* was detected in all samples, yielding the expected 112 bp fragment.

DISCUSSION

The present study aimed to determine some cell-surface properties that have been associated with bacterial virulence in a variety of fish pathogens (Grayson et al. 1995, Abdelsalam et al. 2009, González-Contreras et al. 2011), but using Chilean *Vibrio ordalii* isolates responsible for disease outbreaks in cultured Atlantic salmon.

In this context, an essential initial step in bacterial pathogenicity to host cells is bacterial adhesion to the external surfaces and tissues, because it promotes the delivery of bacterial toxins and precedes penetration into target cells by the microorganism (Montgomery & Kirchman 1994). Hydrophobicity and biofilm production are major factors in the adhesion process and the survival of pathogens in cells (Daly & Stevenson 1987). Specific adherence is mediated through compounds on the surface of the bacteria, which bind via rigid stereochemical bonds to particular molecules on the support to which they are adhering. In contrast, non-specific adherence depends on hydrophobic or ionic interactions between certain structures on the surface of the bacterium and on the support (Ofek & Doyle 1994). A variety of different techniques have been used to assess the ability of bacteria to adhere specifically or non-specifically, including the use of hydrocarbons, the aggregation of particles with different substances, and binding to tissues or cells.

Our data revealed that *V. ordalii* isolates showed wide differences in their hydrophobic properties.

Microbial adhesion to n-octane (a polar solvent) clearly revealed 3 main groups, which we classified as strongly hydrophobic (Group 1), relatively hydrophobic (Group 2) and quasi-hydrophilic (Group 3). However, a discrepant result was obtained when the SAT assay was performed, where the 3 *V. ordalii* isolates (Vo-LM-03, Vo-LM-05 and Vo-LM-11) belonging to Group 1 by MATH were categorized as relatively hydrophobic, and the remaining isolates were categorized as hydrophilic. Interestingly, analogous SAT results were also reported in a previous study with 2 *V. ordalii* strains (Balebona et al. 1995).

Methods for measuring bacterial hydrophobicity differ somewhat in the precise properties they measure; and different types of interactions are considered when different methods are used to estimate hydrophobicity (Kadam et al. 2009). Lee & Yii (1996) suggested that the MATH method using n-octane as solvent is suitable for assaying the hydrophobicity of pathogenic vibrios isolated from diseased aquatic animals.

In contrast, specific carbohydrate-binding proteins or lectins can act as adhesins or haemagglutinins, which adhere to erythrocytes and cause haemagglutination (Ofek & Doyle, 1994). Although *V. ordalii* can agglutinate trout and human erythrocytes (Trust et al. 1981, Larsen & Mellergaard 1984), our study showed that the majority of Chilean *V. ordalii* isolates (14 of 18) lack haemagglutinins to Atlantic salmon red blood cells. The pathogenic bacteria concerned might have different target cells when an infection is initiated, similar to what was reported by Mellergaard & Larsen (1981) for *A. salmonicida* and *V. anguillarum* strains isolated from rainbow trout. However, in the present study, 3 of 4 isolates belonging to Group 2 showed haemagglutination to Atlantic salmon erythrocytes. The potential role of these agglutinins during the onset and progress of infection in fish was unknown. Therefore, we chose 3 *V. ordalii* isolates as representative of each hydrophobic group to be used in the subsequent analyses, including Vo-LM-03 (strongly hydrophobic), Vo-LM-18 (relatively hydrophobic) and Vo-LM-16 (quasi-hydrophilic).

Surface hydrophobicity is not always correlated with adherence ability (Balebona et al. 1995, López-Cortés et al. 1999). In aquatic environments, bacteria rarely exist in a free-floating planktonic state. Bacteria are predominantly found in complex surface-associated microbial communities known as biofilms (Huq et al. 2008). A biofilm consists of surface-attached bacteria enclosed in a self-produced extracellular polymeric substance matrix (Costerton et al. 1999), which provides the bacteria with protection against host defences and antimicrobial drugs (Sundell & Wiklund 2011). In this context, slime-producing strains are more virulent and are responsible for severe infections (Kouidhi et al. 2010). In our work, we found that 6 *V. ordalii* isolates (Vo-LM-07, Vo-LM-11, Vo-ML-12, Vo-LM-15, Vo-LM-19 and Vo-LM-20) and the strain ATCC 33509^T produced low-grade biofilm formation on polyethylene surface after 2 hpi, but none were slime producers on CRA plates. It is important to note that an additional incubation period greater than 2 h could be necessary to detect more noticeable changes with the other 12 *V. ordalii* isolates, especially on inert surfaces such as a polystyrene plate. In fact, when the bottom of each well was coated with Atlantic salmon mucus, 3 isolates previously classified as negative for the formation of biofilm (Vo-LM-03, Vo-LM-18 and Vo-LM-16) showed low-grade positive biofilm after 24 h of incubation.

In this sense, with the exception of the type strain ATCC 33509^T, our results demonstrated that Atlantic salmon mucus favours the growth of *V. ordalii* and *V. anguillarum* ATCC 43307 because they were resistant to the antibacterial activity, showing a higher number of CFU on Atlantic salmon mucus than broth inoculated with each bacterium after 24 h. Garcia et al. (1997) showed that salmon intestinal mucus is an excellent nutrient source for the growth of *V. anguillarum* and that during growth in mucus, a number of proteins are induced in the outer membrane. Based on our data, *V. ordalii* isolates could persist within aquatic environments utilizing the mucus of reservoir fish hosts, similar to other fish pathogens (Bordas et al. 1996, Garcia et al. 1997, Balebona et al. 1998, Avendaño-Herrera et al. 2006, González-Contreras et al. 2011). In addition, those authors have suggested that this ability to exploit the fish surface for growth could facilitate the colonization and subsequent invasion of the host. Ransom (1978) postulated that *V. ordalii* cells present on skin may invade directly through the integument.

Another putative virulence factor examined in *V. ordalii* was its capacity to survive in fish serum. Our data clearly indicate that *V. ordalii*, except again the type strain, can resist the killing activity of Atlantic salmon serum and proliferate in it, suggesting the presence of a cell structure (i.e. capsular material) that confers this property. Sadovskaya et al. (1998) demonstrated that *V. ordalii* produce capsular polysaccharides, but the significance of these findings for pathogenesis is unknown. Trust et al. (1981) found the same ability to resist the bactericidal activity of a

non-immune rainbow trout serum using 7 of 8 *V. ordalii* strains isolated from different *Oncorhynchus* species.

To our surprise, when the levels of antibacterial activity produced by *V. ordalii* cells grown in mucus and heated and non-heated serum were compared, the majority of isolates (with the exceptions of Vo-LM-18 [relatively hydrophobic and haemagglutinative] and the strain ATCC 33509^T) showed a decrease in the number of CFU within 12 h of exposure, and then increased quickly by around one order of magnitude in 24 h. In the present study, we demonstrated that the Atlantic salmon serum is not effective in killing the Chilean *V. ordalii* isolates, regardless of their hydrophobicity properties. Our observations also support the idea that a nutrient source in the mucus or serum is likely affected at 56°C, this being necessary to support the growth of *V. ordalii* for at least the first 12 h after incubation. Denkin & Nelson (1999) noted that there is a specific inducing agent or agents within the mucus that is required for growth of *V. anguillarum*.

In recent years, cell lines have been increasingly used in toxicity assays, gene expression studies and other *in vitro* applications (Pei et al. 2008, Jos et al. 2009, Ruiz et al. 2009, Smith et al. 2010). To our knowledge, studies concerning the adherence and invasive properties of *V. ordalii* have not been conducted. In the present study, we chose a cell line derived from leucocytes that have some of the properties of macrophages (Dannevig et al. 1997). Our findings on SHK-1 cells showed that regardless of the strain studied, the Atlantic salmon *V. ordalii* isolates adhered strongly to the cultured fish cells after 2 hpi, while the bacterial count in SHK-1 cells showed a negligible invasion according to the inoculum size at 24 hpi. We also evaluated the cytopathic effect of each *V. ordalii* isolate into the SHK-1 cell line, but for a longer period of time than 2 hpi. Interestingly, after 16 hpi, and due to the growth conditions, the infection spread from the monolayers to neighbouring cells, with total degenerative changes occurring in the SHK-1 cell line after 5 d of incubation. In addition, the cytopathic effect was accompanied by a significant increase in the level of LDH liberation in SHK-1 cells inoculated with *V. ordalii* from Day 2 to Day 5 of the study, when *V. ordalii* showed the greatest level of liberation owing to greater damage of the cell membrane, which indicates the relative inefficacy of this cell line in killing *V. ordalii*.

The discrepancy in the invasion capacity displayed by *V. ordalii* could be due to differences in incubation time (2 and 24 hpi) as well as the technique used. For example, Schiewe (1983) reported that the number of *V. ordalii* in the liver of salmon declined after 1 h and then increased at 22 hpi, bacterial numbers were high in all organs, and 100% mortality occurred 6 d after infection. In contrast, previous findings for *Streptococcus phocae* demonstrated that the resolution of the invasion process with confocal microscopy (Cortez-San Martín et al. 2012) was clearly higher than when a plate culture procedure was used (González-Contreras et al. 2011).

Finally, when the virulence of each strain assessed by challenge experiments was examined, the isolate Vo-LM-18 was more virulent than the strongly hydrophobic (Vo-LM-03) and quasi-hydrophilic (Vo-LM-16) isolates (considering time to death and trial with low doses). Our findings could be explained because the relatively hydrophobic isolate Vo-LM-18 was also haemagglutination positive; however, similar mortality values were observed with the other isolate included within Group 3 (Vo-LM-13), but this isolate was negative for agglutination of Atlantic salmon erythrocytes. Remarkably, all Chilean *V. ordalii* isolates were originally isolated from Atlantic salmon, but they also caused mortality in rainbow trout, indicating that there is no stringent host specificity for vibriosis. Similar findings were also found in sea bass *Dicentrarchus labrax* by Frans et al. (2013) for *V. anguillarum* isolated from rainbow trout.

Although *V. ordalii* ATCC 33509^T killed all the fish at concentrations from 10^7 CFU per fish, the development of mortality was delayed for several days post-infection. In addition, no mortalities were recorded for challenged fish with the low dose, despite the fact that strain ATCC 33509^T was originally isolated from a diseased Pacific salmon. These results were in line with those of other cell-surface properties of the type strain evaluated here, such as survival and biofilm formation in skin mucus and serum resistance (all of them with results different than Chilean *V. ordalii* isolates). Therefore, the type strain ATCC 33509^T could be classified as having low virulence.

CONCLUSIONS

The evidence presented here suggests for the first time the existence of a widely hydrophobic character of *Vibrio ordalii*, although only 4 of 18 isolates produced haemagglutination activity against Atlantic salmon erythrocytes. Only a minority of all the studied isolates produced low-grade biofilm formation on polyethylene surface after 2 hpi, but when the bottom of each well was coated with At-

lantic salmon mucus, *V. ordalii* can form biofilm after 24 h. Our study also confirmed the survival of *V. ordalii* in Atlantic salmon mucus, which could be relevant *in vivo* to facilitate the colonization and subsequent invasion of the host, for which the pathogen needs to possess the ability to overcome the antibacterial activity of fish serum. *V. ordalii* (except the type strain) was also resistant to the killing activity of Atlantic salmon serum. Finally, virulence experiments using rainbow trout as a model demonstrated mortality or pathological signs in fish injected only with the Chilean *V. ordalii* isolates, regardless of the dose used (high or low). A relationship could be established between virulence and hydrophobicity, but no relationship was found among haemagglutination and biofilm characteristics.

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