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ORIGINAL ARTICLE

Characterization of *Vibrio harveyi* strains recovered from diseased farmed Senegalese sole (*Solea senegalensis*)

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Abstract

Aim: To characterize 16 *Vibrio harveyi* strains isolated from different epizootic outbreaks affecting farmed Senegalese sole.

Materials and Results: The *Vibrio harveyi* strains tested have broad phenotypic diversity based on their biochemical and exoenzymatic patterns, outer membrane proteins (OMP), extracellular product (ECP) patterns and presence of prophages. Lethal dose 50 (LD_{50}) of the strains and *in vitro* antagonism tests with two probiotic strains were also determined. The OMP analysis revealed three different patterns (A, M and V). The electrophoretic analysis of the ECP showed two different groups. All strains considered virulent based on their LD_{50} exhibited the same protein pattern in their ECP (pattern I), while all nonvirulent strains showed a different profile (pattern II). About 32% of the tested strains were positive for prophages, although a clear relationship between virulence and the presence of prophages has not been established.

Conclusions: The results obtained have shown differences between virulent and avirulent strains isolated from diseased farmed Senegalese sole based on the protein patterns of their ECP. However, a clear relationship between virulence and presence of prophages has not been established.

Significance and Impact of the Study: The differences observed between virulent and nonvirulent strains could be used to design prophylactic strategies against diseases caused by *V. harveyi* in farmed Senegalese sole.

Introduction

Marine aquaculture in southern Europe is an expanding industry requiring diversification of the production in culture. A new species, being cultured in Portugal and Spain, is the Senegalese sole (*Solea senegalensis*, Kaup). The most significant factor affecting fish culture is the high incidence of diseases of bacterial origin. Several pathogenic micro-organisms have been involved in epizootic outbreaks in sole culture, *Photobacterium damselae* ssp. *piscicida* (Zorrilla *et al.* 1999; Magariños *et al.* 2003) and *Vibrio harveyi* (Zorrilla *et al.* 2003a; Arijo *et al.* 2005) being frequently isolated.

Vibrio harveyi is one of the most frequently isolated marine *Vibrio* species (Arias *et al.* 1999; Pujalte *et al.* 1999, 2003), and it has been associated with large-scale losses of larval and juvenile penaeids (Liu *et al.* 1996; Robertson *et al.* 1998; Diggles *et al.* 2000), and several opportunistic diseases of marine fish (Hispano *et al.* 1997; Company *et al.* 1999; Alcaide *et al.* 2001; Liu *et al.* 2003, 2004; Zorrilla *et al.* 2003b; Arijo *et al.* 2005). *Vibrio harveyi* has also been associated with outbreaks affecting farmed Senegalese sole with moderate mortalities (Zorrilla *et al.* 2003a). The role of the extracellular products (ECP) in the disease caused by this pathogen in Senegalese sole has been demonstrated (Zorrilla *et al.* 2003a). Liu *et al.* (1996), Montero and Austin (1999) and Zhang and Austin (2000) also demonstrated that the pathogenicity of different isolates of *V. harveyi* isolated from prawns and salmonids was associated with their ECP, and Harries and Owens (1999) isolated two exotoxins, which were the probable virulence factors for *V. harveyi.*

The development of aquaculture requires the typing of the microbial strains isolated from diseased fish to exert

an adequate control, applying strategies, such as vaccination. For this reason, it is necessary to use techniques that enable a distinction to be made between virulent and nonvirulent strains of the micro-organism. Different typing methods have been used to dilucidate the epidemiology and pathobiology of several *Vibrio* species, including *Vibrio vulnificus* (Biosca *et al.* 1997; Arias *et al.* 1998), *Vibrio anguillarum* (Austin *et al.* 1995) and *Vibrio ordalii* (Williams *et al.* 1988). There are several studies concerning the molecular typing of *V. harveyi* strains isolated from farmed marine fish, such as gilthead seabream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*) and common dentex (*Dentex dentex*) (Pujalte *et al.* 2003), and shrimp (Hernández and Olmos 2004; Alavandi *et al.* 2006), but they are null with regard to Senegalese sole.

The aim of the present work is to characterize virulent and nonvirulent strains of *V. harveyi* according to their ECP and outer membrane protein (OMP) patterns and prophage presence, in order to establish a possible relationship with virulence for Senegalese sole.

Materials and methods

Bacterial strains

The study included 16 isolates obtained from five epizootic outbreaks affecting Senegalese sole specimens cultured from 2000 to 2004 in fish farms in Spain (Table 1). Diseased specimens showed the following symptoms: dark skin, haemorrhagic fins, septicemia, and occasionally, ulcers on the skin surface. Internally, diseased fish accumulated fluids in the peritoneal cavity and haemorrhagic livers were observed in some specimens. The isolates were identified as *V. harveyi* by biochemical characterization following the criteria proposed by Alsina and Blanch (1994) and Ortigosa *et al.* (1994). The reference strain *V. harveyi* CECT 525 was also included in this study. Oakey *et al.* (2003) developed a PCR to confirm *V. harveyi* strains, and although it does not produce bands exclusively for *V. harveyi* and cross-reactions with some *Vibrio alginolyticus* could occur, production of acetoin (VP, Voges–Proskauer test) by the latter species enables differentiation between *V. alginolyticus* and *V. harveyi.*

The PCR reaction described by Oakey *et al.* (2003) was employed to confirm the strains as *V. harveyi*. This reaction was carried out using the puReTAq Ready-To-Go PCR Beads (Amersham Biosciences, UK), according to the manufacturer's instructions. *Vibrio harveyi* DNA was extracted following the methodology proposed by Martínez *et al.* (1998). DNA was quantified using a spectrophotometer. The primers used in the PCR reaction described by Oakey *et al.* (2003) were VH-1 (5′-AAC GAG TTA TCT GAA CCT TC-3′) and VH-2 (5′-GCA GCT ATT AAC TAC ACT ACC-3′). A positive PCR for *V. harveyi* genome created a 413-bp long fragment (Oakey *et al.* 2003). Reaction mixtures contained 2·5 units of pureTaq DNA polymerase, 3 mmol l^{-1} magnesium

Table 1 Characteristics of the *Vibrio harveyi* strains used in this study

Strains	Swarming	LD_{50}	OMP profile	ECPs profile	Prophage presence	Haemolytic activity	Inhibition		Phenotype
							Pdp9	51M6	
Lg 6/00	–	$>10^8$	A	II	–	–	–	–	1
Lg 14/00	+	2.1×10^5	V	I	+	+	–	–	2
Lg 15/00	VR	$>10^8$	A	II	–	+	–	–	3
Lg 16/00	+	7.4×10^4	V	I	+	+	–	–	4
Lg 7/01	–	$>10^8$	A	II	+	–	–	+	5
Lg 12/01	+	$>2 \times 10^7$	M	II	–	+	–	+	6
Lg 13/01	+	4.8×10^6	M	I	–	+	+	+	7
Lg 14/01	+	2.3×10^6	V	I	–	+	–	–	8
Lg 25/01	+	6.5×10^6	M	I	–	+	+	+	8
Lg 26/01	–	$>10^8$	M	II	–	+	–	–	8
Lg 48/01	+	3.2×10^5	V	I	–	+	–	–	8
Lg 32/03	VR	4×10^6	V	I	+	+	–	–	4
Lg 34/03	VR	3.6×10^5	V	I	+	+	–	–	10
Lg 35/03	+	9.1×10^4	V	I	+	+	–	–	11
Lg 41/03	+	5.6×10^6	M	I	+	+	+	+	12
Lg 13/04	+	2.4×10^5	V	I	–	+	–	–	12
CECT 525	–	$>10^8$	A	II	–	–	–	–	9

OMP, outer membrane proteins; ECP, extracellular product; VR, variable result.
Swarming on tryptic soya agar; lethal dose 50 (LD_{50}) values expressed as CFU g^{-1} fish.

chloride, 200 μmol l^{-1} each dNTP in 10 mmol l^{-1} Tris-HCl (pH 9·0), 50 mmol l^{-1} KCl, 10 pmol each primer and 50 ng template DNA per 25 μl. PCR cycle parameters were: initial denaturation at 94°C for 2 min, 40 cycles at 94°C for 1 min and 65°C for 1 min, and a final extension at 72°C for 2 min. The first thermal cycler used was the Eppendorf Mastercycler. Amplicons were visualized by running 10 μl through 2% agarose electrophoresis and staining with ethidium bromide.

Phenotypic characterization

The phenotypes of the 16 strains were compared using the following tests: Gram stain, cell morphology, cytochrome-oxidase, motility, O-F test, susceptibility to 150 μg of the vibriostatic agent O/129, growth on thiosulfate-citrate-bile salt-sucrose agar (TCBS), swarming, growth in 0%, 6% and 8% NaCl, growth at 4, 22 and 37°C, gas production from glucose, indole and Voges–Proskauer tests, arginine dihydrolase, decarboxylation of lysine and ornithine, acid production from arabinose, sucrose, lactose, mannitol and mannose, and the extracellular enzymatic activities, namely caseinase, gelatinase, lipase, phospholypase, amylase, and hydrolysis of aesculin. Strains were grown on tryptone soya agar (TSA; Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with 1·5% NaCl and in tryptone soya broth (TSB; Oxoid) with 1·5% NaCl and incubated at 22°C for 24–48 h. The tests were performed following the methodology described by Smibert and Krieg (1981). Haemolytic activity was assayed on Columbia agar base (Oxoid) supplemented with 1·5% (w/v) NaCl and 5% (v/v) sheep blood (BioMerieux, Marcy l'Etoile, France), following incubation at 22°C for 24–48 h.

After 24 h incubation in TSB, *V. harveyi* strains were subcultured, streaked on luminescent agar (10 g l^{-1} peptone, 30 g l^{-1} NaCl, 2 g l^{-1} K_2HPO_4, 0·25 g l^{-1} $MgSO_4$, 2 g l^{-1} glycerol, 1 l distilled water) incubated at 22°C and observed after 24 and 48 h incubation.

Virulence testing

The Lethal dose 50 (LD_{50}) of strains was determined following the protocols described by Nieto *et al.* (1984). LD_{50} represents the number of bacteria needed to kill 50% of the inoculated fish. Bacterial isolates were intraperitoneally injected to juvenile Senegalese sole specimens (5–10 g body weight), using 0·1 ml of serial bacterial dilutions in phosphate-buffered saline (PBS). Groups of five fish were inoculated with bacterial doses ranging from 10^3 to 10^8 CFU. The same number of fish specimens inoculated with 0·1 ml PBS (pH 7·4) was used as a control. Inoculated fish were kept at 22°C and observed daily for 14 days after inoculation and all mortalities recorded, considering only the bacterial origin when the bacterial strain was reisolated in pure culture from the internal organs of dead fish. Surviving fish were killed and reisolation of the inoculated strain was attempted to test for the possible carrier state.

In all cases, the fish specimens were kept and manipulated according to European and Spanish legislation concerning the use of experimental animals.

Determination of OMP profiles

The OMP profiles were determined using the method previously described by Crosa and Hodges (1981). Outer membranes (OM) were obtained by treatment of total cell envelopes with Sarcosyl [1·5% (w/v)] in 0·01 mol l^{-1} Tris-HCl (pH 8·0) for 20 min at room temperature. After centrifugation (100 000 ***g***, 1 h at 4°C), the OM pellets were washed twice in 10 $mmol^{-1}$ Tris-HCl (pH 8·0) and stored at −20°C. The OMP were examined by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970) with 12·5% acrylamide in the separating gel and 4·5% acrylamide in the stacking gel. The proteins were stained with Coomassie brilliant blue R-250 (Sigma Chemical Co., St Louis, MO, USA) and the molecular weights were determined by comparison with a mixture of commercial markers (SDS-6H) (Sigma).

ECP and Western blotting

The ECP of the bacterial isolates were prepared according to Amaro *et al.* (1992). The total protein content of the ECP samples was determined using a commercially available protein determination reagent (Bio-Rad Laboratories), and bovine serum albumin (Sigma) as standard. The enzymatic activities of ECP were evaluated using the API ZYM system (BioMérieux, Charbonnières-les-Bains, France).

The ECP were obtained as previously described and separated by SDS-PAGE with 12·5% and 3% acrylamide in the running and stacking gels, respectively. The protein bands were visualized by staining with Coomassie brilliant blue R250. According to Towbin *et al.* (1979), ECP separated by SDS-PAGE were transferred onto nitrocellulose membranes (Bio-Rad) with a transfer buffer comprising 25 mmol l^{-1} Tris-HCl (pH 8·3), 192 mmol l^{-1} glycine and 20% methanol, by electroblotting at 350 mA for 1 h in a semi-dry transblot apparatus (Mini Trans-Blot Electrophoretic Transfer Cell, Bio-Rad). After transfer, immobilized ECP were detected using mouse antisera raised against ECP obtained from the virulent strain Lg 16.00.

Identification of bacterial host strains for bacteriophage

Vibrio harveyi strains were examined for the presence of prophages using the methods described by Oakey and Owens (2000). *Vibrio* strains were cultured in 20 ml aliquots of TSB. All cultures were grown at optimal temperatures until OD_{600} was *c.* 0·2. The cultures were aseptically divided into two equal aliquots and one of each pair was treated with 30 ng ml^{-1} mitomycin C. Cultures were returned to optimal incubation temperatures and the OD_{600} recorded periodically for 24 h. Cell lysis, as indicated by a relative decrease in OD_{600} compared with the untreated control, was considered to indicate the presence of prophages.

Confirmation of *V. harveyi* myovirus-like (VHML) infection of *V. harveyi* strains was determined by PCR specific for VHML following the methodology proposed by Vidgen *et al.* (2006). The extraction of DNA from *V. harveyi* strains was carried out according to Martínez *et al.* (1998). The confirmatory VHML PCR was performed using primers VMF1 (5′-TTCCGGCGAAG CGTTTGA-3′) and VMR1 (5′-CGTGCTCGGCCTTAATC-CATA-3′). The reaction mixture consisted of 2·5 mmol l^{-1} magnesium chloride, 5 μl of 10× polymerase buffer, 200 μmol l^{-1} each dNTP, 1 U *Taq* polymerase (Bioline, London, UK), 10 pmol of each primer and 50–250 ng template DNA per 50 μl. The parameters for cycling consisted of initial denaturation at 94°C for 7 min, 36 cycles at 94°C for 50 s, 59°C for 50 s and 72°C for 1 min, and final extension for 7 min at 72°C. Amplicons were visualized by gel electrophoresis at 200 V using 1·5% agarose containing ethidium bromide and 10 μl of PCR mixture. Samples were VHML positive if a 250 bp band was present.

In vitro antagonism tests

Probiotic strains Pdp 9 and 51M6 were selected based on their ability to inhibit several pathogenic strains of *V. harveyi* and *P. damselae* ssp. *piscicida* demonstrated in previous studies (Chabrillón *et al.* 2005a,b). The antagonistic activity was detected by using the double layer method described by Dopazo *et al.* (1988). Briefly, 10 μl of overnight cultures of the probiotics in TSB were grown on TSA. After incubation for 48 h at 22°C, bacteria were killed by exposure to chloroform vapours for 30 min. Subsequently, 100 μl of *V. harveyi* overnight cultures in TSB were mixed with 5 ml of soft agar (TSB supplemented with 0·7% agar) and poured over the dish surface. The double-layer dishes were incubated at 22°C and the inhibition of pathogen growth around and/or over the macrocolony was recorded after 24 and 48 h. Control plates to test the potential effect of the chloroform on the growth of the target bacteria were also included. In addition, antagonism tests among the isolates and the pathogenic strains were carried out to detect auto and cross-inhibition.

Results

Identification and phenotyping characterization

All strains tested were biochemically identified as *V. harveyi*, producing fragments corresponding to the expected size of 413 bp as confirmed by PCR (data not shown). All the strains were fermentative and produced acids from glucose, sucrose and mannose, but no acids were produced from arabinose, rhamnose and inositol. They were Voges–Proskauer and arginine dihydrolase negative, but positive for lysine and ornithine decarboxylase testing. All strains grew in 6% NaCl and at 22°C, but none in 0% NaCl or at 4°C. Ten out of 17 strains tested showed swarming on TSA, while three showed a variable result. The 16 strains isolated from Senegalese sole were distributed in 12 phenotypes. Variable responses to biochemical tests were detected for growth with 8% NaCl; use of citrate; production of acids from mannitol, sorbitol, melobiose and amygdalin; and hydrolysis of aesculin. Phenotypes 1, 3, 5 and 7 did not produce acids from mannitol, while phenotypes 5, 9 and 11 were negative for acid production from sorbitol. Only phenotype 9 produced acids from melobiose, and phenotypes 1–4, 7–9 and 12 from amygdalin. Hydrolysis of aesculin was positive for phenotypes 5–9 and 12. The reference strain was included in phenotype 9. The LD_{50} values determined for *V. harveyi* strains assayed ranged from $7{\cdot}4 \times 10^4$ to $>10^8$ CFU g^{-1} fish (Table 1).

Vibrio harveyi strains hydrolysed casein and produced gelatinase, lecithinase and amylase. Most of them (14 out of 17) exhibited haemolytic activity, including three strains with $LD_{50} > 2 \times 10^7$ CFU g^{-1}, and all strains showing LD_{50} values ranging from $7{\cdot}4 \times 10^4$ to $6{\cdot}5 \times 10^6$ CFU g^{-1}. The growth of three out of 17 strains assayed was inhibited by the action of both the probiotic micro-organisms assayed. This group included strains with LD_{50} values ranging from $4{\cdot}8 \times 10^6$ to $6{\cdot}5 \times 10^6$ CFU g^{-1} (or $>2 \times 10^7$ g^{-1}). Only the reference strain showed luminescence.

OMP analysis

The analysis of OMP revealed three different patterns (A, M and V) for the *V. harveyi* strains tested (Fig. 1). The A pattern included four strains which showed in all cases LD_{50} values $>10^8$ CFU g^{-1} fish, and included 12 protein bands ranging from 17·18 to 70·30 kDa (Table 1). The

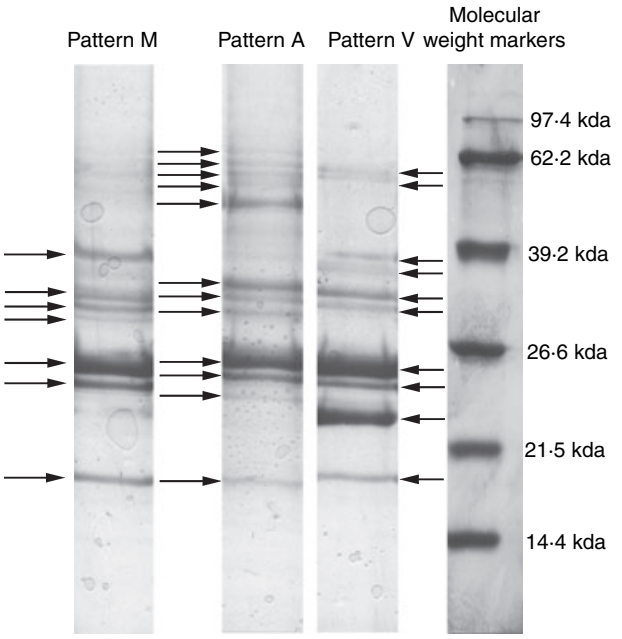

Figure 1 SDS-PAGE of outer membrane proteins from *Vibrio harveyi* strains.

V pattern included eight strains showing values of LD_{50} from 4×10^4 to 4×10^6 CFU g^{-1} fish. This profile included 10 protein bands with molecular weights ranging from 17·52 to 64·93 kDa. Five strains showed the M profile, which exhibited seven protein bands with molecular weights from 17·18 to 44·53 kDa, and corresponded to strains with LD_{50} ranging from $4{\cdot}8 \times 10^6$ to $>10^8$ CFU g^{-1} fish.

ECP analysis

The results obtained using API ZYM tests showed that strain Lg 15/00 exhibited a positive activity only for α-galactosidase, while all other strains showed the following common enzymatic activities: alkaline phosphatase, esterase (C1), esterase lipase, leucine arylamidase, valine arylamidase, cistine arylamidase, trypsin, chemotrypsin, acid phosphatase, phosphohydrolase. The strains showed variability in the case of lipase (C14) (negative for phenotypes 1, 3, 9 and 10), α-galactosidase (negative for phenotypes 1, 2, 9 and 10), β-glucuronidase (positive for phenotypes 1–2 and 7–11); and *n*-acetyl-β-glucosaminidase (negative only for phenotype 9). The ECP of all strains showed gelatinase, amylase, phospholipase and caseinase activities.

Considering the electrophoretic analysis of the protein contents of ECP from *V. harveyi*, the assayed strains were included in two different groups (Fig. 2). All strains showing LD_{50} ranging from $7{\cdot}4 \times 10^4$ to $6{\cdot}5 \times 10^6$ CFU g^{-1} fish

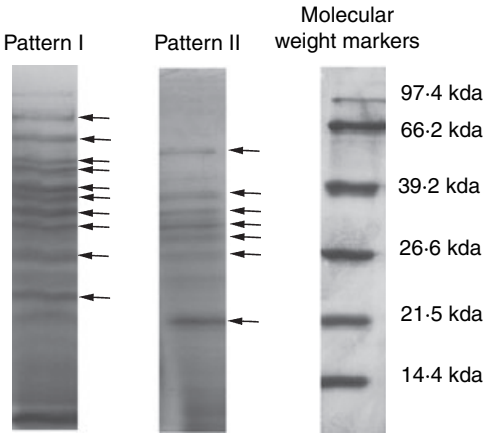

Figure 2 SDS-PAGE of extracellular products from *Vibrio harveyi* strains.

exhibited the same protein profile (pattern I), including 10 bands with molecular weights from 22·1 to 65 kDa. All strains with values of $LD_{50} > 10^7$ CFU g^{-1} fish showed a common profile (pattern II) with seven bands with molecular weights ranging from 21·3 to 50·1 kDa. Pattern I showed bands with molecular weights of 65, 56·2, 44·6, 38·6, 35·2 and 22·1 kDa, which are not present in pattern II. In contrast, pattern II exhibited bands of 50·1, 27·3 and 21·3 kDa, which did not appear in the other pattern.

Due to the fact that all virulent strains exhibited the same protein pattern in their ECP, polyvalent antiserum against ECP of *V. harveyi* Lg 16/00 was employed for Western blotting. All the virulent strains again showed the same pattern, including three bands which corresponded to protein bands detected in pattern I with molecular weights of 27·5, 31·6 and 39·8 kDa (Fig. 3). The highest intensity corresponded to the 39·8 kDa band. All Western blots carried out by applying the serum against strain Lg 16/00 to all avirulent strains showed a unique intense band of 35·5 kDa, which was also present in the Western blot obtained with strain Lg 16/00, although less intense.

Bacteriophage testing

More than 41% of the strains tested were positive for prophages. The confirmation of infection of *V. harveyi* strains by VHML bacteriophage was determined by PCR specific for this phage. The absence of VHML bacteriophage in these strains was assumed by the absence of PCR product of the expected size (data not shown).

Discussion

All the isolates were identified as *V. harveyi* based on their biochemical activities and their identification was

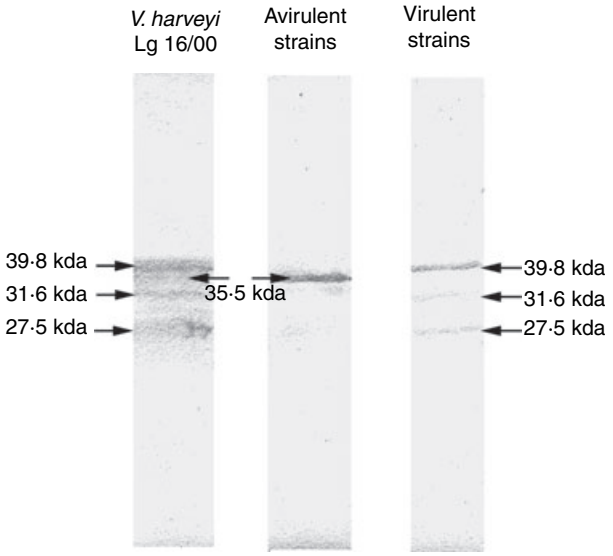

Figure 3 Western blots derived from SDS-PAGE of extracellular products from avirulent and virulent *Vibrio harveyi* strains.

confirmed by PCR. In this study, a high phenotypic diversity (12 different phenotypes) was detected. This result is in agreement with those obtained by other authors (Zorrilla *et al.* 2003b; Gómez-Chiarri *et al.* 2004). In this study, 11 strains showing LD_{50} values ranging from $7{\cdot}4 \times 10^4$ to $6{\cdot}5 \times 10^6$ CFU g^{-1} were considered as virulent micro-organisms according to the criteria proposed by Santos *et al.* (1988). All these strains exhibited swarming on TSA. However, the relationship between virulence and phenotypic characteristics has not been established. Eight out of 11 strains showed OMP profile V, while three out of 11 exhibited the OMP profile M. Six strains showed LD50 $> \times 10^7$ CFU g^{-1} and were considered as nonvirulent micro-organisms (Santos *et al.* 1988). Four showed OMP profile A and the other two, pattern M. Similar results were obtained by Pizzutto and Hirst (1995), who studied the protein profiling of *V. harveyi* strains affecting shrimp larvae, and concluded that there is no defined grouping that differentiates virulent strains from nonvirulent ones. A previous study carried out with *V. harveyi* and *Vibrio parahaemolyticus* strains isolated from diseased Senegalese sole, Zorrilla *et al.* (2003a) suggested that the ability to develop swarming could be a marker to differentiate between virulent and avirulent strains of both *Vibrio* species. However, in this study, one avirulent strain Lg 12/01 showed swarming growing on TSA, while two virulent strains Lg 32/03 and 34/03 and one avirulent strain Lg 15/00, exhibited a variable result for swarming.

Vibriosis, especially luminous disease, has caused serious losses in prawn hatcheries, and there are data reporting a correlation between the capability of *V. harveyi* to show luminescence and to produce toxicity in shrimp (*Penaeus japonicus*) (Nakayama *et al.* 2005). In this study, a correlation between the production of luminescence and virulence has not been established because only the reference strain, CECT 525, which was not virulent for sole showed luminescence, while virulent strains for Senegalese sole, did not show luminescence. Similarly, Nakayama *et al.* (2006) did not observe a clear correlation between the ability to produce luminescence and cytotoxicity, and Alavandi *et al.* (2006) concluded that nonluminescent *V. harveyi* also appear to be important aetiological agents of vibriosis in shrimp larvae.

Only three out of 17 strains assayed were inhibited by the action of the two probiotics assayed. The results of our study showed that the strains having the lowest LD_{50} values were not affected by the probiotics. This data could be in agreement with those obtained by Nakayama *et al.* (2006), who, carrying out a comparison of the cytotoxicity and antibiotic resistance of *V. harveyi* strains, observed that the strains resistant to higher concentrations of oxytetracycline were also more toxic, suggesting that the development of antibiotic resistance and higher toxicity are related to environmental adaptation.

The ECP are considered to be important determinants of the virulence in *V. harveyi* (Liu *et al.* 1996; Soto-Rodriguez *et al.* 2003). In this study, proteolytic and phospholipase activities were exhibited by all the strains assayed. This result is in agreement with that obtained by Liu *et al.* (1996), who demonstrated a significant correlation between the mortality of *Artemia franciscana* nauplii inoculated with *V. harveyi* isolates, and the ability of these isolates to produce proteases and phospholipases.

Zhang and Austin (2000) concluded that haemolytic activity was involved in the pathogenesis of *V. harveyi* in

salmonids. In another study, Zhang *et al.* (2001) demonstrated that the most pathogenic strain tested contained two closely related haemolysin genes, while less-virulent strains lacked haemolysin genes or contained a single gene. On the contrary, in this study, several strains with haemolytic activity showed high values of LD_{50}, compared with the virulent strains without this ability. However, Nakayama *et al.* (2006) demonstrated that one nonhaemolytic strain of *V. harveyi* isolated from prawns, showed higher cytotoxic ability than other strains with this activity, suggesting that the cytotoxic ability was the result of a toxin which was not a haemolysin.

All virulent strains exhibited the same protein pattern in their ECP (profile I), while those considered as nonvirulent shared another common protein pattern (profile II). In the case of the virulent strains, the number of bands detected was higher than the number observed in the case of avirulent strains. The results derived from the Western blotting demonstrated, in the case of virulent strains, three protein bands that were the most immunogenic for mouse. However, results obtained from Western blottings corresponding to nonvirulent strains showed only one protein band, which was not in the virulent strain, except in the case of strain Lg 16/00, where this band is less intense than in avirulent strains. This result could suggest that this protein is expressed in higher intensity in nonvirulent strains than in virulent strains, in which its expression is very scarce or even null. The results obtained in this study could be useful to design a vaccine against *V. harveyi* enriched with proteins shown to be more immunogenic, thus avoiding the use of a highly polyvalent vaccine, which could be less efficient.

More than 41% of the strains tested were positive for prophages. This percentage is similar to that obtained by Jiang and Paul (1998), who reported frequencies of lisogenic prophages in marine bacteria of 25–62% depending upon the geographical area. It is also close to the percentage reported by Oakey and Owens (2000), who detected that 46% of *V. harveyi* strains tested positive for prophages. Several authors have concluded that bacteriophages have been known to confer virulence to bacteria upon infection (lysogenic conversion) (Waldor and Mekalanos 1996; Faruque *et al.* 1998). In the case of *V. harveyi*, several studies have reported that a bacteriophage may sometimes mediate the toxicity of this micro-organism in *Penaeus monodon* by transfer of toxin gene (Ruangpan *et al.* 1999; Oakey and Owens 2000). Munro *et al.* (2003) demonstrated that infecting naïve avirulent *V. harveyi* strains with the bacteriophage VHML, described by Oakey and Owens (2000), converted them into virulent strains. These authors concluded that bacteriophage VHML either fully or partly confers virulence to *V. harveyi* resulting in up-regulation of haemolysin and up-regulation of protein synthesis with some proteins being recognized as the same toxic subunits found in the virulent strain. Vidgen *et al.* (2006) proposed a confirmation of VHML bacteriophage infection of *V. harveyi* strains by PCR specific for VHML, with primers designed from the genome of this phage (Vidgen *et al.* 2006). These authors considered that the strains were VHML positive if a 250 bp band was present. In this study, applying the PCR protocol proposed by Vidgen *et al.* (2006), none of the strains, which previously had exhibited the presence of prophages with the treatment of mitomycin C, showed the 250 bp band obtained by PCR specific amplification for VHML (Vidgen *et al.* 2006) (data not shown). Although in this study, the frequency of bacteriophagic presence was higher in the virulent strains, it is not clear that the presence of lysogenic bacteriophages is directly linked to the virulence, as only five out of 11 virulent strains showed the presence of bacteriophages, and although the most virulent strain (Lg 16/00) was positive for prophages, other strains, such as Lg 35/03 with similar LD_{50} values were negative for prophage presence. Even, the nonvirulent strain Lg 7/01 exhibited bacteriophagic presence. This result is in agreement with authors, such as Pasharawipas *et al.* (2005) and Khemayan *et al.* (2006), who did not conclude a clear relationship between temperate phage presence and strain virulence. However, in this study, haemolytic activity, prophage presence and virulence are not found to be correlated. Furthermore, the protein patterns of bacteriophage-positive strains were not influenced by the presence of the prophage.

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