Antigenic characterization of *Vibrio anguillarum*-related organisms isolated from turbot and cod

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ABSTRACT: This work reports on the antigenic characterization and virulence for rainbow trout *Oncorhynchus mykiss* of *Vibrio anguillarum*-related (VAR) strains isolated from diseased turbot *Scophthalmus maximus* and cod *Gadus morhua* in Spain and Denmark. These vibrio strains belong to *Vibrio splendidus* biovar I (serogroups B, C, D, F and G) and *V. pelagius* biovar I (serogroup A). The pathogenicity assays demonstrated that 10 of 15 (67%) VAR strains tested were virulent for rainbow trout, showing an LD₅₀ (50% lethal dose) ranging from 8.4×10^3 to 7×10^5 CFU (colony forming units) per fish. Moreover, all the *V. splendidus* biovar I strains of serogroup F, which is the predominant type among strains isolated from cod, were virulent for rainbow trout, while *V. splendidus* biovar I strains isolated from turbot were avirulent. Analysis of lipopolysaccharides (LPS) and membrane proteins showed that VAR strains belonging to different serogroups possessed distinct electrophoretic banding patterns. The immunoblot analysis demonstrated a high immunorelation among VAR strains of the same serogroups, while strains from different serogroups were not immunologically related. Immunoblot assays also confirmed the dissimilarities in LPS structure observed among the distinct subgroups of serogroup F. These findings suggest that present vibriosis vaccines for cod could be improved if *V. splendidus* biovar I representative of serogroup F (subgroups α and β) were tested in trial vaccines.

KEY WORDS: *Vibrio anguillarum*-related organisms · Turbot · Cod · LPS · Proteins · Virulence

INTRODUCTION

Vibrio anguillarum (serotypes O1 and O2) is the main causative agent of vibriosis among cultured marine fish, particularly turbot *Scophthalmus maximus* and cod *Gadus morhua*, in European countries (Toranzo & Barja 1990, Larsen et al. 1994). Immunization of turbot with vaccines which contain antigen preparations of *V. anguillarum* serotypes O1 and O2 and vaccination of cod with monovalent vaccines containing formalin-killed bacteria representative of *V. anguillarum* serotype O2 have proved to be effective for the control of vibriosis caused by these serotypes (Myhr et al. 1991, Santos et al. 1991, Knappskog et al. 1993). However, *V. splendidus* biovars I and II and other *V. anguillarum*-related (VAR) species, commonly considered environmental strains without pathogenic im-

portance, have been isolated from vaccinated farmed turbot (both adults and fry) and cod during disease outbreaks (Toranzo et al. 1990, Myhr et al. 1991, Knappskog et al. 1993, Pazos et al. 1993, Santos et al. 1996). VAR organisms of possible clinical significance have also been isolated from other marine fish species such as sea bass *Dicentrarchus labrax* and sole *Solea solea* as well as from diseased crustaceans and molluscs (Baticados et al. 1990, Myhr et al. 1991, Castro et al. 1992, Riquelme et al. 1995), but these microorganisms appeared to be of no clinical importance among farmed salmonid fish (Myhr et al. 1991). Despite an increasing number of reports that describe the isolation of VAR organisms in association with diseased fish and shellfish, few efforts (Pazos et al. 1993, Riquelme et al. 1995) have been made in order to assess the precise role of these bacteria as primary pathogens.

The VAR organisms can be differentiated from *Vibrio anguillarum* on the basis of biochemical and serological characteristics (Myhr et al. 1991, Pazos et al.

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1993, Santos et al. 1996). Moreover, it has been demonstrated (Santos et al. 1996) that VAR strains can be typed on the basis of heat stable O-antigens and that strains isolated from diseased fish can be grouped into 6 serogroups (A, B, C, D, F, and G), the serotypes C and F being the predominant types among strains isolated from turbot and cod, respectively. This work reports on the antigenic characterization of VAR organisms isolated from diseased turbot and cod in Spain and Denmark. In addition, pathogenicity for rainbow trout *Oncorhynchus mykiss* was examined in order to assess the importance of these microorganisms for fish farming.

MATERIALS AND METHODS

Bacterial strains and growth conditions. In the present work we used 20 strains of *Vibrio anguillarum*-related organisms (VAR) isolated from diseased turbot *Scophthalmus maximus* (9 strains) and cod *Gadus morhua* (11 strains) in northwestern Spain and Denmark (Table 1). As previously reported (Santos et al. 1996), these strains were identified as *V. splendidus* (18 strains) and *V. pelagius* (2 strains) using standard morphological, physiological, and biochemical plate and tube tests (Bryant et al. 1986a, b, Fouz et al. 1990, Myhr et al. 1991).

The bacteria were routinely cultured on tryptic soy agar (TSA, Difco Laboratories, Detroit, MI, USA) supplemented with 1% NaCl (TSA-1). Bacterial strains were stored frozen at -70°C in tryptic soy broth containing 1% NaCl and 15% (v/v) glycerol until tested.

Serological characterization. The serological identity of each strain was confirmed by the slide agglutination test using the heat stable O-antigen (10^9 cells ml^{-1} , McFarland standard No. 3) and rabbit antisera against formalin-killed suspensions of the various strains: RM51 (serogroup A), RC25.1 (serogroup B), RPM30.2 (serogroup C), RM36.1 (serogroup D), 95-3-72 (serogroup F), and 95-3-88 (serogroup G). The O-antigens were prepared by heating bacterial suspensions of each strain in phosphate buffered saline (PBS, pH 7.4) at 100°C for 1 h. The basis for serotyping VAR organisms was previously established by Santos et al. (1996). Sera were obtained as described by Sørensen & Larsen (1986).

Virulence assay. The VAR strains were tested for virulence in fingerling rainbow trout (mean weight, 10 g) kept in freshwater at 20°C with aeration. The assay was performed by intraperitoneal inoculation of bacterial doses ranging from 10^2 to 10^8 CFU (colony forming units) per fish as previously described (Toranzo et al. 1987). Control fish were injected with PBS. Mortalities were recorded over a 14 d period. The degree of virulence, expressed as the 50% lethal dose (LD_{50}), was calculated by the method of Reed & Muench (1938).

Preparation of lipopolysaccharides (LPS) and electrophoresis analysis. LPS from the cell envelope were obtained following basically the method of Hitchcock & Brown (1983). Briefly, bacterial cultures on TSA-1 were harvested, washed once, and suspended to an optical density (OD) of 0.8 at 650 nm in PBS. Aliquots of these suspensions (1.5 ml) were centrifuged ($10\,000 \times g$ for 5 min) and the cell pellets, resuspended in 50 μl of

Table 1. Bacterial strains used in the present study. LD_{50} : number of viable cells needed to kill 50% of inoculated fish in a 14 d period. The virulence assay was performed by intraperitoneal inoculation of rainbow trout. NT: not tested

Strain	Species	Origin	Serogroup	Virulence (LD_{50})
RQ1.2	<i>Vibrio pelagius</i> (bv. I)	<i>Scophthalmus maximus</i> (Spain)	A	5.0×10^4
RM51	<i>V. pelagius</i> (bv. I)	<i>S. maximus</i> (Spain)	A	3.0×10^5
RC25.1	<i>V. splendidus</i> (bv. I)	<i>S. maximus</i> (Spain)	B	$> 9.0 \times 10^8$
RPM167.2	<i>V. splendidus</i> (bv. I)	<i>S. maximus</i> (Spain)	B	NT
RPM30.2	<i>V. splendidus</i> (bv. I)	<i>S. maximus</i> (Spain)	C	$> 2.0 \times 10^8$
RPM136.1	<i>V. splendidus</i> (bv. I)	<i>S. maximus</i> (Spain)	C	$> 1.5 \times 10^8$
RPM40.1	<i>V. splendidus</i> (bv. I)	<i>S. maximus</i> (Spain)	C	$> 3.0 \times 10^8$
RPM41.1	<i>V. splendidus</i> (bv. I)	<i>S. maximus</i> (Spain)	C	$> 2.0 \times 10^8$
RM36.1	<i>V. splendidus</i> (bv. I)	<i>S. maximus</i> (Spain)	D	NT
95-3-71	<i>V. splendidus</i> (bv. I)	<i>Gadus morhua</i> (Denmark)	F	NT
95-3-72	<i>V. splendidus</i> (bv. I)	<i>G. morhua</i> (Denmark)	F	1.0×10^4
95-3-74	<i>V. splendidus</i> (bv. I)	<i>G. morhua</i> (Denmark)	F	6.0×10^5
95-3-89	<i>V. splendidus</i> (bv. I)	<i>G. morhua</i> (Denmark)	F	8.4×10^3
95-3-90	<i>V. splendidus</i> (bv. I)	<i>G. morhua</i> (Denmark)	F	7.0×10^4
95-3-91	<i>V. splendidus</i> (bv. I)	<i>G. morhua</i> (Denmark)	F	1.0×10^5
95-3-92	<i>V. splendidus</i> (bv. I)	<i>G. morhua</i> (Denmark)	F	8.0×10^4
95-3-93	<i>V. splendidus</i> (bv. I)	<i>G. morhua</i> (Denmark)	F	NT
95-3-94	<i>V. splendidus</i> (bv. I)	<i>G. morhua</i> (Denmark)	F	NT
95-3-95	<i>V. splendidus</i> (bv. I)	<i>G. morhua</i> (Denmark)	F	7.0×10^5
95-3-88	<i>V. splendidus</i> (bv. I)	<i>G. morhua</i> (Denmark)	G	1.4×10^5

electrophoresis sample buffer [12.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 0.002% bromophenol blue, and 5% 2-mercapto-ethanol], were boiled for 10 min and treated with 10 μ l of 2.5 mg ml⁻¹ proteinase K in sample buffer at 60°C for 1 h. Samples (15 μ l) were electrophoresed in sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) at constant current (Laemmli 1970). The LPS were silver stained by the procedure of Tsai & Frasch (1982).

Analysis of cell-envelope proteins. The cell-envelope proteins were prepared from overnight *Vibrio* cultures grown on TSB-1. The cells were recovered by centrifugation, resuspended in 3 ml of 10 mM Tris buffer containing 0.3% (w/v) NaCl (pH 8.0), and sonicated with a Branson sonifier 250 (60 W, 30 s, 3 times). After centrifugation at 10 000 $\times g$ for 2 min, the supernatants were transferred to new tubes and centrifuged for 1 h at 17 000 $\times g$ at 4°C. Total membrane proteins were examined by SDS-PAGE as described for the LPS analysis. Proteins were stained with Coomassie brilliant blue R-250 (Sigma Chemical Co., St. Louis, MO, USA) and the molecular masses (MM) were determined by comparison with a mixture of MM protein markers (Bio-Rad, Richmond, CA, USA).

Immunoblotting analysis of LPS and proteins. Cell envelope components separated by SDS-PAGE were

electroblotted from the gels onto nitrocellulose (NC) membranes (Bio-Rad) using the procedures of Towbin et al. (1979). The NC membranes were then separately exposed to the different polyclonal antisera mentioned above. LPS and protein components recognized by the antisera were visualized by reacting the rinsed NC membranes with goat anti-rabbit IgG alkaline phosphatase conjugate for 1 h and the appropriate substrate (0.3 mg ml⁻¹ tetrazolium blue and 0.15 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl phosphate toluidine salt in 0.1 M carbonate buffer, pH 9.8).

RESULTS AND DISCUSSION

The experimental infections carried out in the present study (Table 1) demonstrated that 10 of 15 (67%) VAR strains tested were virulent for rainbow trout, showing an LD₅₀ ranging from 8.4×10^3 to 7×10^5 CFU per fish. These LD₅₀ values are in the same range as those reported for *Vibrio anguillarum* strains of pathogenic serogroups O1, O2, and O3 (Toranzo et al. 1987, Santos et al. 1991, Pazos et al. 1993). Moreover, differences in the degree of virulence were observed between strains of serogroups C and F, which are the predominant types detected among *V. splendidus*

Fig. 1. (A) SDS-PAGE electrophoresis of lipopolysaccharide (LPS) components of *Vibrio anguillarum*-related (VAR) strains isolated from turbot and (B) the corresponding immunoblot using whole cell antiserum against strain RPM30.2 of serogroup C. Lanes: (1), RQ1.2; (2), RM51; (3), RC25.1; (4), RPM167.2; (5), RPM30.2; (6), RPM36.1; (7), RPM40.1; (8), RPM41.1; (9), RM36.1

Fig. 2. (A) SDS-PAGE electrophoresis of LPS components of VAR strains isolated from cod and (B) the corresponding immunoblot using whole cell antiserum against strain 95-3-72 (serogroup F, subgroup F α). Lanes: (1), 95-3-72; (2), 95-3-74; (3), 95-3-89; (4), 95-3-90; (5), 95-3-91; (6), 95-3-92; (7), 95-3-93; (8), 95-3-94; (9), 95-3-95; (10), 95-3-88

strains isolated from turbot and cod, respectively (Table 1). Whereas all the *V. splendidus* biovar I strains belonging to the serotype F tested were virulent for fish, avirulent strains predominated among the *V. splendidus* biovar I strains grouped into the serotype C. The 2 *V. pelagius* biovar I strains (serogroup A) isolated from turbot showed a degree of virulence similar to that found among *V. splendidus* isolated from cod (serogroup F strains). During the course of the experiments, no deaths occurred among the control fish.

The results of the virulence assays indicated that VAR organisms are potentially virulent for salmonid fish. These results differ from those published by Myhr et al. (1991), who reported that VAR strains seem to be of no clinical importance among farmed salmonid fish. However, it must be considered that the experimental challenges used in the present work do not necessarily mimic the natural route of infection.

The analysis of LPS (Figs. 1A & 2A) showed that, except strain 95-3-88 which belongs to serogroup G, all of the VAR isolates tested produced LPS with O-chains of heterogeneous length (i.e. yielded ladder-like patterns). VAR strains belonging to different sero-

groups possessed distinct electrophoretic banding patterns (Figs. 1A & 2A). In general, differences between LPS profiles could be seen in the O-chain pattern as well as in the position of the lipid A and core (the faster migrating bands). Moreover, on the basis of the different mobilities of the LPS components, 2 different profiles (Fig. 2A) were detected within serogroup F, which corresponded with the serological subgroups previously detected using quantitative agglutination assays (Santos et al. 1996). We have designated these serological entities as F α and F β . The existence of variability in profiles of LPS among strains belonging to different serogroups and also between strains of the same serotype has been previously reported in *Vibrio* isolated from salmonid and non-salmonid fish (Nomura & Aoki 1985, Pazos et al. 1993, Santos et al. 1995). The Western Blot analysis confirmed the dissimilarities in LPS structure observed among the different serogroups as well as between strains belonging to the distinct subgroups of serogroup F. Figs. 1B & 2B show the immunoblot analysis of LPS from VAR strains isolated from turbot and cod with the antisera against the strain RPM30.2 (serogroup C) and 95-3-72 (serogroup F, subgroup F α), respectively.

Fig. 3. (A) SDS-PAGE electrophoresis of total membrane proteins of VAR strains isolated from turbot and cod and (B) the corresponding immunoblot using whole cell antiserum against strain RPM30.2 of serogroup C. Lanes: (1), RQ1.2; (2), RM51; (3), RPM30.2; (4), RPM36.1; (5), RPM40.1; (6), RPM41.1; (7), RPM167.2; (8), RC25.1; (9) RM36.1(10), 95-3-72; (11), 95-3-74; (12), 95-3-89; (13), 95-3-90; (14), 95-3-95. St: molecular mass protein markers in kDa

Analysis of cell envelope proteins showed that VAR strains possessed major protein components with molecular weight ranging from approximately 33 to 45 kDa (Fig. 3A). Distinct protein patterns were observed depending on the serotype of the VAR strains. However, minor differences between strains of the same serotype were detected (see Fig. 3, lanes 1 and 2 and lanes 7 and 8). Immunoblot assays revealed that antisera against whole cell of *Vibrio splendidus* strains of serogroups B, C, D, F, and G reacted strongly only with the membrane proteins of the strains of these serogroups (data shown only for reactions with serotype C antiserum). None to moderate reactions were observed using sera against *V. splendidus* of heterologous serotypes (Fig. 3B). Similar results were obtained with the whole cell antiserum against the strain of *V. pelagius* RM51 (serogroup A).

The present results suggest that *Vibrio splendidus* biovar I representative of serogroup F (subgroups α and β) should be included in trial vaccines against vibriosis for use in the cod farming industry. In the case of turbot, additional serological and virulence studies with a larger number including a higher number of VAR organisms are needed to determine if there is a serogroup that should be included in the vibriosis vaccines.

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