Analysis of Antigens Present in the Extracellular Products and Cell Surface of *Vibrio anguillarum* Serotypes O1, O2, and O3

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Antigens present in the extracellular products (ECP) and cell walls of strains of *Vibrio anguillarum* of serotypes O1, O2, and O3 isolated from different fish species in distinct geographic areas were characterized. The usefulness of slide agglutination, dot blot assay, and quantitative agglutination for subtyping *V. anguillarum* serovars was also evaluated. The three serological assays used to establish the serogroups within *V. anguillarum* isolates demonstrated that serotype O1 constitutes a homogeneous group, whereas within serotypes O2 and O3, two different patterns of serological reactions were detected. Among the three serological methods used, only dot blot and quantitative agglutination assays differentiated subgroups within serotypes O2 and O3 with unabsorbed sera. Electrophoretic analysis and immunoblot assays of cell envelope and ECP components showed that strains belonging to serotype O1 possessed immunologically related lipopolysaccharide (LPS) and proteins, while *V. anguillarum* isolates grouped in serotypes O2 and O3 exhibited internal heterogeneity in their LPS and protein banding patterns. On the other hand, although the LPS present in the ECP and those obtained from cell envelopes of *V. anguillarum* strains showed apparently different gel patterns, a strong relationship between both types of LPS was seen by immunoblot assay. From these results, it can be concluded that *V. anguillarum* strains representative of each of the antigenic groups (O1, O2 α , O2 β , O3A, and O3B) and their ECPs should be included in the formulation of vaccines against vibriosis in areas where the three serotypes coexist.

Vibriosis caused by *Vibrio anguillarum* is one of the major diseases in aquaculture affecting not only fish but bivalve mollusks and crustaceans as well (3, 4, 6, 16, 32). Although epidemiological studies focused on the taxonomic characterization of *V. anguillarum* strains isolated from diseased fish and from the environment have shown biochemical variations in a number of phenotypic traits (8, 13), this information is not sufficient to support the establishment of specific biotypes. Alternatively, serotyping has become one of the most reliable tools for strain separation. Among the different serotyping systems proposed for *V. anguillarum* (21, 32), the typing scheme developed by Sørensen and Larsen (30), in which 10 distinct O serotypes were recognized, has gained general acceptance. Following this system, only serotypes O1, O2, and O3 appear to be important fish pathogens (21, 32). Within serotype O2, two antigenic entities (designated subgroups O2 α and O2 β [24] or subgroups O2 α and O2 β [5]) are distinguished by immunoelectrophoresis, dot blot, and enzyme-linked immunosorbent assays. Serotype O3 has also been subdivided into groups A and B (23). Some of the remaining serotypes (O4 to O10), considered to include only environmental strains, have recently been described to be virulent for fish (serotype O4) (24) or associated with mortalities of artificially reared cod (serotypes O4, O6, and O8) (21).

Besides being important from an epizootiological point of view, serological studies allow identification of the dominant antigens. In this sense, the lipopolysaccharide (LPS) and some proteins of the cell wall have previously been reported to be antigenic determinants of *V. anguillarum* (9, 11, 19, 22, 29). However, in most of these studies, only serotype O2 of *V. anguillarum* was analyzed (19, 22), or information about the

serotype of the strains used was not provided (9, 11, 29). Moreover, no studies exist in which the antigens present in the extracellular products (ECP) of *V. anguillarum* were analyzed. This work describes the characterization of antigens present in the ECP and cell wall of strains of *V. anguillarum* of serotypes O1, O2, and O3 isolated from different fish species in distinct geographic areas.

MATERIALS AND METHODS

Bacterial strains. Forty *V. anguillarum* strains isolated during vibriosis outbreaks were used in the present study. The source of isolation, geographic origin, and serogroup of the strains are shown in Table 1. Stock cultures were kept frozen at -70°C in tryptic soy broth with 1% NaCl (TSB-1) and 15% (vol/vol) glycerol until passage on tryptic soy agar containing 1% NaCl (TSA-1) immediately before use. All culture media were purchased from Difco Laboratories (Detroit, Mich.).

Extraction of ECP. The ECP from a group of *V. anguillarum* strains representative of each serogroup were obtained by the cellophane plate technique (27). Briefly, sterilized cellophane sheets were placed on the surface of TSA-1 plates and inoculated by spreading 0.5 ml of a 24-h-old broth culture of each strain with a sterile swab. After 24 h of incubation at 25°C , cells were washed off the cellophane with phosphate-buffered saline (PBS, pH 7.4). The cell suspensions were centrifuged at $10,000 \times g$ for 30 min at 4°C , and the resulting supernatants were filtered (0.45- μm Millipore membranes) and stored at -30°C until use. The protein concentration of ECP samples was determined by the method of Bradford (7) with bovine serum albumin (Sigma) as the standard. The lipopolysaccharide (LPS) content of ECP was evaluated by the method of Keler and Novotny (18) with LPS from *Vibrio cholerae* INABA 569B (Sigma) as the standard.

Preparation of rabbit antisera. Antisera against *V. anguillarum* strains R-82 (serotype O1), ATCC 19264 (serotype O2, subgroup O2 α), RV-22 (serotype O2, subgroup O2 β) (5), ATCC 43307 (serotype O3, subgroup O3A), and B1.1-2/4 (serotype O3, subgroup O3B) (23) were obtained as described by Sørensen and Larsen (30) with formalin-killed cells as the antigen. For subgrouping serovars O2 and O3, rabbit antisera against strains ATCC 19264 (O2 α), RV-22 (O2 β), ATCC 43307 (O3A), and B1.1-2/4 (O3B) were prepared basically as described by Larsen et al. (21). Briefly, bacterial suspensions of each strain, prepared in PBS, were heated at 100°C for 1 h to obtain the heat-stable O antigen, and the supernatant was removed by centrifugation at 4,000 rpm for 20 min. For absorption, the cell pellets of each strain (washed once with PBS) were mixed with 4 ml of heterologous antiserum, incubated at 37°C for 1 h, stored at 4°C overnight,

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TABLE 1. Origin of the *V. anguillarum* strains used in this study^a

Strain	Serotype	Origin
NCIMB2129	O1	Rainbow trout (Norway)
6018/1	O1	Rainbow trout (Denmark)
TM-10.1	O1	Rainbow trout (Spain)
TM 14	O1	Rainbow trout (Spain)
SO104.2	O1	Pacific salmon (Spain)
SO86-3.	O1	Pacific salmon (Spain)
775	O1	Pacific salmon (United States)
SP-1.1	O1	Atlantic salmon (Spain)
DH11	O1	Striped bass (France)
103F	O1	Striped bass (United States)
R-82	O1	Turbot (Spain)
RT-32	O1	Turbot (Spain)
840817-2/6	O2	Rainbow trout (Denmark)
6087-D/2	O2	Rainbow trout (Denmark)
2887	O2	Rainbow trout (Italy)
820723-2/8	O2	Rainbow trout (Denmark)
860619-3/2	O2	Rainbow trout (Denmark)
N1801	O2	Atlantic salmon (Norway)
43F	O2	Striped bass (United States)
100F	O2	Striped bass (United States)
NCIMB6	O2	Cod (Denmark)
1466/1	O2	Cod (Denmark)
1049/2	O2	Cod (Denmark)
1474/1	O2	Cod (Denmark)
RC71	O2	Turbot (Spain)
RG111	O2	Turbot (Spain)
91079	O2	Turbot (Scotland)
RV22	O2	Turbot (Spain)
ATCC 43307	O3	Rainbow trout (Denmark)
11008	O3	Sea bass (France)
11044	O3	Sea bass (France)
1321/1	O3	Cod (Denmark)
ET208	O3	Japanese eel (Japan)
PT493	O3	Ayu (Japan)
V123	O3	Ayu (Japan)
13A5	O3	Seawater (Spain)
2.2.2.21-P/2	O3	Seawater (Denmark)
B1.1-2/4	O3	Seawater (Denmark)
1.1.2-13/2	O3	Seawater (Denmark)
1.1.2-20/1	O3	Seawater (Denmark)

^a Serotypes follow the European typing scheme of Sørensen and Larsen (30). NCIMB, National Collection of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, Scotland; ATCC, American Type Culture Collection, Rockville, Md.

and centrifuged at 4,000 rpm for 20 min. All sera were tested for specificity with the homologous and heterologous strains. When necessary, the procedure was repeated.

Rabbit antisera against formalin-inactivated ECP from strains of serotypes O1 (R-82) and O2 (RV-22) were prepared as described by Hastings and Ellis (14).

Serological assays. The serological relationships between strains were determined by a slide agglutination test, quantitative agglutination test, and dot blot assay with the heat-stable O antigen (10⁹ cells/ml, McFarland standard no. 3) and unabsorbed and absorbed rabbit whole-cell antisera.

Slide agglutination tests were conducted by mixing a drop of each O antigen suspension with a drop of 1:10-diluted rabbit antiserum on a multiwell glass slide. Strong and rapid agglutination was registered as a positive result.

Quantitative agglutination tests were performed in microtiter plates with serial twofold dilutions of 25-μl aliquots of the antisera. The agglutination titer was considered the reciprocal of the highest dilution of the antiserum giving a positive reaction after incubation with the antigen overnight at 15°C.

The dot blot assay was performed basically as described by Cipriano et al. (10). For this assay, O antigens obtained from each strain were dotted onto nitrocellulose membranes (0.45-μm HA filter; Millipore). Filters were blocked for 1 h with 3% gelatin in Tris-buffered saline (TBS-3, pH 7.5), washed twice with TBS plus 0.05% Tween 20 (TTBS), and exposed to the antisera diluted 1:1,000 in TBS containing 1% gelatin (TBS-1). Goat anti-rabbit immunoglobulin G (IgG) with alkaline phosphatase (Bio-Rad) was used as the conjugate, and 0.1 M carbonate buffer (pH 9.8) containing tetrazolium blue (0.3 mg/ml) and 5-bromo-4-chloro-3-indolylphosphate toluidine salt (0.15 mg/ml) was used to develop the reaction. Only a reaction similar to that exhibited by the homologous strain was scored as positive.

Membrane protein extraction. The cell envelope proteins from *V. anguillarum* were prepared from bacterial cultures grown on TSB-1. The cultures were centrifuged at 7,000 × g for 10 min at 4°C, and the cell pellets were resuspended in 3 ml of 10 mM Tris buffer (pH 8.0) containing 0.3% NaCl and sonicated with a Branson sonifier 250 (60 W, 30 s, three times). After centrifugation at 10,000 × g for 2 min, to eliminate possible whole cells, the supernatants were transferred to new tubes and centrifuged again for 60 min at 20,000 × g at 4°C. The resultant precipitates were kept at -30°C until use. The outer membrane fractions were obtained by the method of Filip et al. (12) with 3% (wt/vol) sodium lauryl sarcosinate (Sarkosyl) in 10 mM Tris buffer.

LPS extraction from whole-cell lysates and ECP. The LPS from the cell envelope was obtained basically by the method of Hitchcock and Brown (15). Briefly, bacterial cultures on TSA-1 were removed, washed once, and suspended to an optical density (OD) of 0.8 at 650 nm in PBS. Bacterial suspensions (1.5 ml) were centrifuged (10,000 × g for 5 min), and the pellets were resuspended in 0.05 ml of sample buffer (12.5 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% [vol/vol] glycerol, 0.002% bromophenol blue, 5% 2-mercaptoethanol). After boiling for 10 min, 0.01 ml of proteinase K (2.5 mg/ml) in sample buffer was added to the bacterial suspensions, and the mixture was incubated at 60°C for 1 h. LPS from ECP were extracted by mixing (5:1) aliquots of 10-min-boiled ECP and the above-described proteinase K solution. The mixture was incubated as for extraction of LPS from bacteria.

Electrophoresis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (20). Cell envelope components (membrane proteins and LPS) and ECP samples (LPS and proteins) solubilized in sample buffer were stacked in 3% (wt/vol) acrylamide gels (10 mA constant current) and separated on 12.5% (wt/vol) acrylamide (20 mA). Standard protein markers were obtained from Bio-Rad. Proteins were stained with Coomassie brilliant blue R-250 (Sigma), and LPS were silver stained by the procedure of Tsai and Frasch (33).

TABLE 2. Slide agglutination test and dot blot assay with O antigen and unabsorbed and absorbed rabbit whole-cell antisera

Antigen	Slide agglutination test ^a										Dot blot assay ^b							
	1	2α	2β	2α (β) ^c	2β (α)	3A	3B	3A (B)	3B (A)	1	2α	2β	2α (β)	2β (α)	3A	3B	3A (B)	3B (A)
Serotype O1 (12 strains)	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Serotype O2																		
O2α (10 strains) ^d	-	+	+	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-
O2β (6 strains) ^e	-	+	+	-	+	-	-	-	-	-	-	+	-	+	-	-	-	-
Serotype O3																		
O3A (7 strains) ^f	-	-	-	-	-	+	+	+	-	-	-	-	-	-	+	-	+	-
O3B (5 strains) ^g	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	+	-	+

^a Strong and rapid agglutination was registered as a positive result. Asterisks indicate different results detected with absorbed and unabsorbed antisera.

^b A reaction was considered positive if it was similar to the reaction exhibited by the strain used to obtain the rabbit sera.

^c The antigen used to absorb the serum is shown in parentheses.

^d Strains grouped into subgroup O2α were ATCC 19264, 6087-D/2, 2887, N1801, 840817-2/6, 1466/1, 1049/2, RC-71, RG-111, and 91079.

^e Strains grouped into subgroup O2β were RV-22, 820723-2/8, 860619-3/2, 43-F, 100-F, and 1474/1.

^f Strains grouped into subgroup O3A were ATCC 43307, 1321/1, 11008, 11044, PT493, V123, and 13A5.

^g Strains grouped into subgroup O3B were B1.1-2/4, ET208, 2.2.2.21-P/2, 1.1.2-13/2, and 1.1.2-20/1.

TABLE 3. Microagglutination test with O antigen and unabsorbed or absorbed rabbit whole-cell antisera^a

Antigen	Range of agglutination titers with serum (absorbing antigen):								
	1	2 α	2 β	2 α (β)	2 β (α)	3A	3B	3A (B)	3B (A)
Serotype O1 (12 strains)	640–1,280	<20	<20	<20	<20	<20	<20	<20	<20
Serotype O2									
O2 α (10 strains)	<20	320–1,280	<20–40	160–640	<20–40	<20	<20	<20	<20
O2 β (6 strains)	<20	<20	320–640	<20	160–320	<20	<20	<20	<20
Serotype O3									
O3A (7 strains)	<20	<20	<20	<20	<20	640–1,280	40–80	320–640	20–40
O3B (5 strains)	<20	<20	<20	<20	<20	<20–40	320–640	<20	160–320

^a See Table 2, footnotes d, e, f, and g, for strains in each subgroup.

Immunoblotting analysis (Western blotting). After SDS-PAGE, fractionated antigens (LPS, outer membrane proteins, and ECP) were electroblotted (at 60 V for 4 h) from the gel onto 0.45- μ m nitrocellulose paper (Bio-Rad) with a transfer buffer composed of 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 20% methanol. After transfer, the nitrocellulose membranes were blocked for 1 h with TBS-3, washed twice with TTBS, and incubated for 1 h in control (nonimmune rabbit serum) or immune rabbit serum diluted 1:1,000 in TBS-1. The membranes were again washed with TTBS and then incubated for 1 h with goat anti-rabbit IgG-alkaline phosphatase conjugate diluted 1:3,000 in TBS-1. The reaction was developed as for the dot blot assay.

RESULTS

Serological testing. Slide agglutination tests with unabsorbed sera and O antigens showed that *V. anguillarum* strains reacted only with antisera of their own serotype, with no cross-reaction being detected (Table 2). However, subtyping of O2 and O3 strains into subgroups (O2 α , O2 β , O3A, and O3B) was only possible with absorbed sera. In contrast, the dot blot assay (Table 2) and quantitative agglutination test (Table 3) detected the existence of subgroups within serotypes O2 and O3 even when unabsorbed sera were used.

Analysis of cell envelope components. The SDS-PAGE analysis of cell envelope proteins showed that, regardless of the serogroups and source of isolation, all the strains possessed protein components with molecular masses ranging between 97 and 14 kDa. All the strains of serotype O1 showed the same protein profile, with a prominent band with an apparent molecular mass of 40 kDa and two minor proteins with molecular masses of 45 and 55 kDa (Fig. 1). The isolates of serotypes O2 showed major outer membrane proteins with slightly different mobilities (molecular masses ranging between 35 and 38 kDa) (Fig. 2). The major proteins of the strains of serotype O3 had molecular masses between 37 and 48 kDa (Fig. 3a).

The immunoblot assay revealed that unabsorbed antisera against whole cells of serotypes O1, O2, and O3 of *V. anguillarum* reacted strongly with the membrane proteins of the strains of their own serotype. No to moderate reactions were observed with sera against *V. anguillarum* of heterologous serotypes (data not shown). Three antigenic proteins with apparent molecular masses of 40, 45, and 55 kDa were detected in all the outer membrane preparations from strains of serotype O1 (data not shown). The results of immunoblot analysis of the outer membrane proteins of *V. anguillarum* strains of serotype O3 with antisera against strain ATCC 43307 (serotype O3, subgroup O3A) are shown in Fig. 3b. Two antigenic proteins of 45 and 65 kDa were detected in the membrane preparation of all strains of serotype O2. However, the major outer membrane protein of strains belonging to subgroups O2 α and O2 β reacted only with the homologous sera. In contrast, *V. anguillarum* O3 antiserum raised against strain ATCC 43307 (subgroup O3A) bound to the major proteins from strains of both O3 subgroups (Fig. 3b).

Silver-stained SDS-PAGE gels showed that the LPS preparation from *V. anguillarum* strains of three serotypes presented the typical ladder-like structure described as smooth LPS (9). All the strains belonging to serotype O1 showed an identical banding pattern (data not shown). However, on the basis of the different mobilities of the LPS components, two different profiles were detected within serotype O2 as well as within serotype O3, which corresponded with the serological subgroups (O2 α , O2 β , O3A, and O3B) established by slide agglutination, dot blot, and quantitative agglutination assays (Tables 2 and 3, Fig. 4a and 5a). The LPS preparations from nine strains of serotype O2 gave a gel pattern resembling the profile displayed by the *V. anguillarum* reference strain ATCC 19264 (subgroup O2 α) (Fig. 4a). The other five isolates of serotype O2 showed the same LPS pattern as strain RV-22 (subgroup O2 β) (Fig. 4a). Similarly, six strains grouped within serotype O3 presented LPS gel patterns analogous to that shown by the reference strain ATCC 43307 (subgroup O3A), while the remaining isolates gave an LPS profile similar to that observed in strain B.1.1-2/4 (subgroup O3B) (Fig. 5a).

Immunoblot profiles obtained with unabsorbed sera against whole cells of the three serotypes corroborated the dissimilarities in LPS structure observed among the different serotypes as well as between strains belonging to the distinct subgroups of serotypes O2 and O3. Figures 4b and 5b show the immunoblot analysis of LPS from *V. anguillarum* strains of serotypes O2 and O3 with antisera against strains RV-22 (serotype O2, subgroup O2 β) and ATCC 43307 (serotype O3, subgroup O3A), respectively.

Analysis of ECP. The SDS-PAGE analysis of ECP from representative strains of serotypes O1, O2, and O3 showed the existence of different protein (Fig. 6a and b) and LPS (Fig. 7a)

FIG. 1. SDS-PAGE of outer membrane proteins of strains of *V. anguillarum* of serotype O1. Lanes: A, NCIMB2129; B, 6018/1; C, TM14; D, SO104.2; E, SO86-3; F, 775; G, SP-1.1; H, DH11; I, 103F; J, R-82; K, RT-32. Numbers on the left indicate molecular sizes (in kilodaltons).

FIG. 2. SDS-PAGE of outer membrane proteins of strains of *V. anguillarum* of serotype O2. Lanes: A, 840817-2/6; B, 6087-D/2; C, 2887; D, 820723-2/8; E, N1801; F, 860619-3/2; G, 1466/1; H, 1049/2; I, 1474/1; J, NCIMB6; K, 43F; L, 100F; M, RV22; N, RG111; O, 91079; P, RC71. Numbers on the left indicate molecular sizes (in kilodaltons).

profiles not only among distinct serotypes but also within the same serotype. The molecular masses of the protein components ranged from 14 to 60 kDa (Fig. 6a and b), although in the ECP from some strains of serotype O2, proteins with molecular masses of 97 kDa were also observed (Fig. 6a). The LPS present in the ECP from *V. anguillarum* strains showed gel patterns (Fig. 7a) apparently different from those obtained with the LPS of the cell envelopes (Fig. 4a and 5a).

An immunoblot assay with rabbit serum against the ECP from strains R-82 (serotype O1) and RV-22 (serotype O2, subgroup O2 β) demonstrated that strains of *V. anguillarum* of serotypes O1, O2, and O3 produced ECP components (LPS and proteins) that were immunologically different (Fig. 7b). Moreover, the serum against the ECP of strain R-82 strongly bound the ECP components of all strains of serotype O1, while

FIG. 3. (a) SDS-PAGE of outer membrane proteins of strains of *V. anguillarum* of serotype O3 and (b) corresponding immunoblot with unabsorbed antisera against strain ATCC 43307. Lanes: A, ATCC 43307; B, 11008; C, 1321/1; D, ET208; E, PT493; F, V123; G, 13A5; H, 2.2.2.21-P/2; I, B1.1-2/4. Numbers on the left indicate molecular sizes (in kilodaltons).

FIG. 4. (a) SDS-PAGE of LPS components of strains of *V. anguillarum* of serotype O2 and (b) corresponding immunoblot with unabsorbed antisera against strain RV22. Lanes: A, 840817-2/6; B, 6087-D/2; C, 2887; D, 820723-2/8; E, N1801; F, 860619-3/2; G, 1466/1; H, 1049/2; I, 1474/1; J, NCIMB6; K, 43F; L, 100F; M, RV22; N, RG111; O, 91079; P, RC71.

the LPS (Fig. 7b) and proteins present in the ECP of the strains of the two O2 subgroups were not immunologically related. Figure 7b shows the immunoblot analysis of LPS present in the ECP from *V. anguillarum* of serotypes O1, O2, and O3 with the antisera against the ECP from strain RV-22 (serotype O2, subgroup O2 β). On the other hand, serum raised against ECP displayed a strong reaction with the LPS obtained from the cell envelopes of strains of the same serotype (Fig. 7b).

DISCUSSION

Vibriosis caused by *V. anguillarum* of serotypes O1, O2, and O3 is one of the most important bacterial infections of fish, threatening marine aquaculture throughout the world. Because of the economic importance of the disease, the aquaculture industry is increasingly relying on vaccines for disease control. Available commercial vaccines, composed of killed bacterial preparations of *V. anguillarum* O1 with *V. ordalii* (formerly *V. anguillarum* biotype II) as the representative for the O2 group, gave unsatisfactory results in areas where serotypes of *V. anguillarum* other than O1 are responsible for disease (1). In addition, it has been demonstrated that many antigenic determinants of *V. anguillarum* of serotype O2 do not occur in strains of *V. ordalii* and *V. anguillarum* of serotype O1 (22, 31). All these findings increased the need to identify dominant disease antigens in a particular area before formulating a vaccine. On the other hand, although it is well known that ECP

FIG. 5. (a) SDS-PAGE of LPS components of strains of *V. anguillarum* of serotype O3 and (b) corresponding immunoblot with unabsorbed antiserum against strain ATCC 43307. Lanes: A, ATCC 43307; B, 11008; C, 11044; D, ET208; E, PT493; F, V123; G, 13A5; H, 2.2.2.21-P/2; I, B1.1-2/4; J, 1.1.2-13/2; K, 1.1.2-20/1.

are highly toxic for fish, few efforts have been made to determine their role as protective antigens (17, 27). To our knowledge, this is the first work in which the dominant antigens present in the ECP and cell envelopes of strains of *V. anguillarum* of serotypes O1, O2, and O3 have been analyzed simultaneously.

The results of serological characterization by slide agglutination, dot blot, and quantitative agglutination tests showed

FIG. 6. SDS-PAGE of protein components of ECP from *V. anguillarum* of serotypes (a) O1 and O2 and (b) O3. (a) Lanes: A, 775; B, 103F; C, R-82; D, TM14; E, NCIMB6; F, RG111; G, 91079; H, 43F; I, RV22. (b) Lanes: A, R-82; B, RV22; C, NCIMB6; D, ATCC 43307; E, 11008; F, B1.1-2/4; G, 1.1.2-13/2; H, 1.1.2-20/1. Numbers on the left indicate molecular sizes (in kilodaltons).

FIG. 7. (a) SDS-PAGE of LPS present in the ECP from *V. anguillarum* of serotypes O1, O2, and O3 and (b) corresponding immunoblot with antiserum against the ECP from strain RV22. Lanes: A, TM14; B, 775; C, 103F; D, R-82; E, RV22; F, NCIMB6; G, RG111; H, ATCC 43307; I, B1.1-2/4; J, 1.1.2-13/2; K, LPS from cell envelope of strain R-82; L, LPS from cell envelope of strain RV22.

that *V. anguillarum* isolates of serotype O1 constitute a homogeneous group, while within serotypes O2 and O3, two different patterns of serological reactions were detected. These results are in agreement with those described by other authors (5, 23, 25). Among the three serological methods used, only dot blot and quantitative agglutination assays were able to differentiate subgroups within serotypes O2 and O3 with unabsorbed sera. Moreover, the dot blot assay allowed the processing of a high number of samples with very low quantities of antisera and was a fast and reproducible method.

SDS-PAGE analysis of cell envelope components showed that all the strains belonging to serotype O1 showed identical LPS and membrane protein banding patterns. In contrast to reports by other authors (19, 22), who detected differences in LPS between subgroups O2 α and O2 β only by immunological techniques but not in silver-stained polyacrylamide gels, we have observed that *V. anguillarum* isolates grouped into serotypes O2 and O3 exhibited internal heterogeneity in their LPS and membrane protein banding patterns. The immunoblot assays confirmed these results, because a close immunological relation was detected among the cell envelope components of *V. anguillarum* strains of serotype O1, while both the LPS and peptides of strains of the two O2 subgroups as well as the LPS of the strains of the two subgroups of serotype O3 were shown to be immunologically unrelated. The present results point out the need to include *V. anguillarum* strains representative of each of the antigenic groups (O1, O2 α , O2 β , O3A, and O3B) in the formulation of vaccines in areas where the three serotypes are responsible for vibriosis.

The analysis by SDS-PAGE of ECP components from

strains of the three serotypes showed the existence of heterogeneity not only among different serotypes but also within the same serotype. The results of the immunoblot assay corroborated the lack of immunological relationship among the ECP components from strains of distinct serotypes and indicated that all strains of serotype O1 produce antigenically similar ECP. On the other hand, contradictory reports exist over the nature of the LPS present in the ECP of bacterial fish pathogens. While Evelyn (11) described the existence of similarities between the LPS present in the cell envelope and ECP of *V. anguillarum*, in other fish pathogens such as *Yersinia ruckeri* (26) and *Vibrio vulnificus* (2), both types of LPS seem to be different. In the present study, LPS present in the ECP from *V. anguillarum* strains showed gel patterns apparently different from those obtained with cell envelopes. However, a strong relationship between both types of LPS was seen by immunoblot assay, which indicates that differences in LPS structure are not always important with regard to antigenic similarity.

In conclusion, the present findings indicate that in fish vaccination programs against vibriosis caused by *V. anguillarum*, (i) the antigenic composition of strains causing disease in a particular area must be considered and (ii) the ECP antigens must be included in vaccine formulation.

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