Phenotypic Characteristics and Virulence of *Vibrio anguillarum*-Related Organisms

F. PAZOS,* Y. SANTOS, B. MAGARIÑOS, I. BANDÍN, S. NÚÑEZ, AND A. E. TORANZO

Departamento de Microbiología y Parasitología, Facultad de Biología, Universidad de Santiago, Santiago de Compostela 15706, Spain

Received 23 February 1993/Accepted 15 June 1993

The phenotypic, molecular, and virulence properties of 46 *Vibrio anguillarum*-related (VAR) strains isolated from diseased fish and shellfish and from the environment were investigated. Twelve reference strains belonging to the 10 serotypes of *V. anguillarum* and the *Vibrio splendidus* type strain were included for comparison. Numerical taxonomy studies allowed us to group the isolates into four phena. The main phenotypic traits to differentiate VAR strains from *V. anguillarum* were fermentation of arabinose and mannitol, indole and Voges-Proskauer reactions, gelatin and casein hydrolysis, hemolytic activity, growth at 37 and 4°C, and resistance to ampicillin. Serological analysis confirmed that phena I and II were composed mainly of strains of *V. anguillarum*, while phena III and IV included VAR strains. Excluding the reference strains, the typeable isolates belonged to serotypes O3 (15 strains), O4 (3 strains), and O5 (2 strains) of *V. anguillarum*. The infectivity trials showed that only 9 of a total of 24 strains tested displayed virulence for rainbow trout. Virulent strains (50% lethal dose ranging from 10^2 to 10^6 cells) included *V. anguillarum* strains belonging to serotypes O1 (one strain), O2 (one strain), O3 (three isolates), and O4 (one isolate) and only three strains of the VAR group. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of lipopolysaccharide and outer membrane proteins showed heterogeneity not only among the 10 *V. anguillarum* serotypes but also within the VAR group. Immunoblot assays demonstrated a close relationship among *V. anguillarum* strains from the same serotype, while strains from different serotypes were not antigenically related. The VAR strains did not share antigenic components with the serotypes of *V. anguillarum* tested (serotypes O1 to O5). Plasmids were detected in only 19 of the total of 59 strains. The majority of the strains carrying plasmids were grouped within phenon IV, in which plasmid bands of 27 and 36 MDa were found in all the isolates. No correlation between the plasmid content of VAR microorganisms and their phenotypic or virulence characteristics was observed. From these results it can be concluded that VAR strains associated with disease should be included together with *V. anguillarum* in the formulation of vaccines against vibriosis.

Vibriosis, caused mainly by *Vibrio anguillarum*, is a scourge of marine aquaculture throughout the world (1, 26, 34), affecting a great diversity of fish, bivalve molluscs, and crustaceans (6, 7, 17).

Although 10 serotypes are defined for this species (O1 to O10, European serotype designation [32]), only serotypes O1, O2, and, to a lesser extent, O3 have been associated with mortality in cultured or feral fish. The remaining serotypes have been considered to be composed of environmental strains without pathogenic importance (22, 25, 32, 34), and they are very similar to other *Vibrio* species closely related to *V. anguillarum*. These related bacteria correspond to different biotypes of *Vibrio splendidus* and *Vibrio pelagius*, and they can be grouped under the designation *V. anguillarum*-like or *V. anguillarum*-related (VAR) (15, 23, 26, 34).

In Galicia (northwestern Spain), vibriosis has been a significant disease problem for the last 10 years. Although *V. anguillarum* serotypes O1 and O2 have repeatedly been isolated from diseased fish, since 1989 mortality due to VAR strains has gradually increased to become a threatening problem in marine cultures (37). Vibriosis caused by VAR microorganisms has also been reported in other geographical areas (3, 10, 26, 27).

In the present study, the biochemical, serological, and molecular characteristics of a group of VAR organisms with

different sources of isolation and geographical origins were compared with those of *V. anguillarum*. The relationships among *V. anguillarum* and VAR strains were evaluated by numerical taxonomy, lipopolysaccharide (LPS) and protein profiles, immunoblot assays, and plasmid analysis. In addition, the degree of virulence of VAR organisms for fish was also evaluated.

MATERIALS AND METHODS

Bacterial strains. In the present study we used 46 strains isolated from diseased fish (37 isolates) and shellfish (2 isolates) and from the environment (7 isolates) in our area and other countries (United States, Chile, Denmark, France, and Japan) (see Table 2). Bacterial strains were assigned to the *Vibrio* genus on the basis of Gram stain (negative), colony and cell morphologies, motility, fermentation of glucose, growth on thiosulfate citrate bile sucrose agar (Oxoid Ltd., Basingstoke, England), and sensitivity to the vibriostatic compound 2,4-diamino-6,7-diisopropylpteridine (O/129) (Oxoid). Reference strains of *V. anguillarum* from the American Type Culture Collection (ATCC 43305 [serotype O1], ATCC 43306 [serotype O2], ATCC 43307 [serotype O3], ATCC 43308 [serotype O4], ATCC 43309 [serotype O5], ATCC 43310 [serotype O6], ATCC 43311 [serotype O7], ATCC 43312 [serotype O8], ATCC 43313 [serotype O9], and ATCC 43314 [serotype O10]) and from our own collection (strain R-82 of serotype O1 and strain RV-22 of serotype O2), as well as the type strain of *V.*

* Corresponding author.

splendidus, ATCC 33125 (biotype I), were also included for comparative purposes. Bacterial strains were cultured on tryptic soy agar or tryptic soy broth containing 1% NaCl (Difco Laboratories, Detroit, Mich.) and kept frozen at -70°C in tryptic soy broth–1% NaCl with 15% (vol/vol) glycerol.

Phenotypic characterization and numerical analysis. All of the strains were examined by a total of 42 physiological and biochemical tests (Table 1). The assays were performed mainly according to the criteria of Bryant et al. (8, 9), Fouz et al. (15), Myhr et al. (26), and West and Colwell (42). The results of the phenotypic tests of the 59 strains were subjected to numerical taxonomy analysis. Similarity among strains was calculated by using the simple matching coefficient. Clustering was carried out by the unweighted pair group method with arithmetic averages (30). The numerical analysis was performed with a UNIVAC 1100 computer after the appropriate programs were developed (5).

In addition, the sensitivities of the isolates to chemotherapeutic agents were determined by the disc diffusion method (2) on Mueller-Hinton agar (Oxoid) with 1% NaCl added, using the following antimicrobial substances (micrograms per disc): ampicillin (10), streptomycin (10), tetracycline (30), oxytetracycline (30), oxolinic acid (2), chloramphenicol (30), trimethoprim-sulfamethoxazole (25), and nitrofurantoin (300). All chemotherapeutic agents were supplied by Oxoid.

Serotyping. Slide agglutination tests were conducted according to the procedures of Sørensen and Larsen (32) and Toranzo et al. (36). The tests were carried out by using the thermostable O antigen and rabbit whole-cell antisera against reference strains of *V. anguillarum* (serotypes O1 to O10), kindly provided by J. L. Larsen (Department of Veterinary Microbiology, Royal Veterinary and Agricultural University, Fredriksberg, Denmark). Bacterial strains that could not be grouped by the *V. anguillarum* O1 to O10 antisera were retested by using serum against *V. splendidus* ATCC 33125 (biotype I). This antiserum was produced in rabbits as described by Sørensen and Larsen (32).

LPS and membrane protein preparations. The LPS was obtained by the method of Hitchcock and Brown (16).

The total membrane proteins from *Vibrio* strains were prepared from overnight bacterial cultures grown on tryptic soy broth–1% NaCl. The cultures were centrifuged at $7,000 \times g$ 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). After centrifugation at $10,000 \times g$ for 2 min, the supernatants were centrifuged again at $20,000 \times g$ for 60 min 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. (14), using 3% (wt/vol) sodium lauryl sarcosinate (Sarkosyl) in 10 mM Tris buffer.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (19). Membrane proteins or LPS solubilized in sample buffer were stacked in a 3% (wt/vol) acrylamide gel (10-mA constant current) and separated on 12.5% (wt/vol) acrylamide (20 mA). Standard protein markers were from Bio-Rad Laboratories, Richmond, Calif. Proteins were stained with Coomassie brilliant blue R-250 (Sigma Chemical Co., St. Louis, Mo.), and LPS was silver stained by the procedure of Tsai and Frasch (40).

Immunoblot analysis. Immunoblot assays were performed basically as described by Towbin et al. (39). Briefly, after SDS-PAGE, fractionated antigens (LPS and outer membrane proteins) were electroblotted (at 60 V for 4 h) from the

gel onto 0.45- μm nitrocellulose paper (Bio-Rad) by using 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 3% gelatin in Tris-buffered saline (TBS) (pH 7.5), washed twice with TBS plus 0.05% Tween 20, and incubated for 1 h in control or diluted immune rabbit serum in TBS containing 1% gelatin. The membranes were again washed with TBS–0.05% Tween 20 and then incubated for 1 h with goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Bio-Rad) diluted in TBS–1% gelatin. Bands were visualized by incubating membranes in 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).

Plasmid screening and restriction endonuclease cleavage analysis. Plasmid DNA was isolated as described by Birnboim and Doly (4). Electrophoresis was done on a horizontal apparatus with 0.7% agarose gels and Tris-borate-EDTA buffer at a current of 3 V/cm. The gels were stained in 0.5 μg of ethidium bromide (Sigma) per ml and photographed under 254-nm light. Plasmids from *Escherichia coli* V-517 were used as size markers.

In order to determine the genetic homology of the plasmids shared by some *Vibrio* isolates, purified plasmid bands extracted from the gels were subjected to cleavage with the restriction enzyme *EcoRI* (obtained from Boehringer, Mannheim, Germany) under the conditions recommended by the manufacturer. Reactions were stopped by the addition of 5 μl of a mixture containing 7% SDS, 33% glycerol, and 0.07% bromophenol blue. The samples were electrophoresed in 1.0% agarose gels at 70 V, stained with ethidium bromide, and destained in water before visualization. Digests of λ DNA were used as controls.

Virulence test. A total of 24 strains representative of the different phenotypes were tested for virulence in fingerling rainbow trout (mean weight, 7 g) kept in freshwater tanks at a temperature ranging from 17.2 to 19°C with aeration. Fishes were injected intraperitoneally with bacterial suspensions in phosphate-buffered saline (pH 7.4) (PBS) containing 10^2 to 10^8 cells (six fish per dose), following the procedures of Toranzo et al. (35). Control fish were injected with PBS. Mortalities were recorded over a 14-day period. The degree of virulence (expressed as 50% lethal dose [LD_{50}]) was calculated by the method of Reed and Muench (28). Bacteria displaying an LD_{50} of $\geq 10^7$ were considered not virulent.

RESULTS

Biochemical and physiological characterization. The results of the phenotypic characterization (Table 1) indicated that all of the strains were rod shaped, motile, gram negative, oxidase and catalase positive, halophilic, positive for L-arginine dihydrolase reaction, and negative for ornithine decarboxylase. All fermented D-glucose without gas production, reduced nitrate, and were sensitive to the vibriostatic agent O/129. None of the isolates produced acid from inositol or rhamnose, and none grew at 44°C .

Although the reference strains produced hemolysins against human and turbot erythrocytes, possessed the enzymes gelatinase and caseinase, and fermented mannitol, variable results were found for these activities among the remaining isolates. For the other biochemical tests, variation among the strains was also detected (Table 1).

The results of the assays for susceptibility to chemotherapeutic agents indicated that *V. anguillarum* and *V. splendidus* reference strains were susceptible to the majority of

TABLE 1. Phenotypic characteristics of the phenon of the *Vibrio* strains evaluated in this study

Test	% of strains positive in phenon:			
	I (n = 24) ^a	II (n = 13)	III (n = 10)	IV (n = 12)
Motility	100	100	100	100
Gram-negative stain	100	100	100	100
Oxidase	100	100	100	100
Catalase	100	100	100	100
β-Galactosidase	95.8	100	60	8.3
Arginine dihydrolase	100	100	100	100
Lysine decarboxylase	0	23.1	0	0
Ornithine decarboxylase	0	0	0	0
Yellow colonies on TCBS ^b	100	92.3	10	100
Citrate utilization	83.3	15.4	80	75
Urease	0	0	0	0
Indole	95.8	100	10	8.3
Nitrate reduction	100	100	100	100
Gas from glucose	0	0	0	0
Voges-Proskauer	95.8	53.8	20	25
Salt tolerance (% NaCl)				
0	0	0	0	0
3	100	100	100	100
5	100	100	100	100
8	0	0	0	0
10	0	0	0	0
Growth at (°C):				
4	100	84.6	30	16.7
15	100	100	100	100
25	100	100	100	100
37	100	84.6	50	16.7
44	0	0	0	0
Acid from:				
Glucose	100	100	100	100
Mannitol	95.8	92.3	40	25
Inositol	0	0	0	0
Sorbitol	87.5	7.7	0	0
Rhamnose	0	0	0	0
Sucrose	100	92.3	10	91.7
Melibiose	0	0	0	0
Amygdalin	58.3	92.3	10	8.3
Arabinose	83.3	15.4	10	8.3
Hemolysis of erythrocytes				
Human	95.8	92.3	90	0
Turbot	95.8	100	100	0
Production of:				
Amylase	95.8	76.9	100	8.3
Gelatinase	95.8	100	100	8.3
Caseinase	95.8	84.6	100	25
Elastase	75	23.1	0	0
Lipase	58.3	84.6	60	0
Lecithinase	45.8	100	90	0
Sensitivity to:				
Vibriostatic agent (O/129)	100	100	100	100
Chloramphenicol	100	100	90	100
Ampicillin	0	7.7	10	8.3
Oxytetracycline	100	92.3	80	100
Tetracycline	95.8	100	90	91.7
Oxolinic acid	100	100	60	91.7
Streptomycin	4.2	0	10	0
Trimethoprim-sulfamethoxazole	87.5	69.2	70	75
Nitrofurantoin	100	84.6	80	83.3

^a Number of strains in phenon.^b TCBS, thiosulfate citrate bile sucrose.

FIG. 1. Simplified dendrogram showing relationships among *Vibrio* strains determined by the simple matching coefficients and the unweighted pair group method with arithmetic averages.

compounds utilized; they were resistant to ampicillin and streptomycin. The remaining *Vibrio* isolates were sensitive to chloramphenicol, tetracycline, and oxolinic acid, resistant to ampicillin and streptomycin, and variable in their susceptibilities to the other agents assayed.

Grouping of the strains by numerical taxonomy. The 59 *Vibrio* strains subjected to numerical analysis were grouped in four phenons (Fig. 1), defined at similarity levels of 88% (phenon I), 87.5% (phenon II), 83% (phenon III), and 84.5% (phenon IV).

Phenon I contained 24 strains. Almost all of the *V. anguillarum* reference strains were included in this group. The majority of the strains grew at 4°C, were Voges-Proskauer and indole positive, produced acid from arabinose, mannitol, and sorbitol, and showed elastase activity. Phenon II is composed of 13 strains which were lecithinase positive, and a high percentage produced acid from amygdalin. Three of the strains were positive for lysine decarboxylase, an enzyme not detected in the other phenons. In contrast to phenon I, most of the strains of phenon II did not ferment arabinose and sorbitol. Phenon III comprised 10 strains, which lacked elastase activity. Most of them possessed amylase and caseinase activities, and only 10% produced acid from amygdalin and arabinose. Phenon IV included 12 isolates and is the most distinct phenon, showing a relatively low similarity (76%) to the other phenons. All strains of this phenon lacked lecithinase, lipase, elastase, and hemolytic activities. Only one strain exhibited gelatinase and amylase activities.

Serological testing. Only 33 of 59 strains agglutinated with *V. anguillarum* and *V. splendidus* antisera (Table 2). Excluding the reference strains (13 isolates), the remaining typeable isolates belonged to serotypes O3 (15 of 33), O4 (3 of 33), and O5 (2 of 33) of *V. anguillarum*. None of the strains agglutinated with the *V. splendidus* biotype I antiserum.

Phenon I grouped all the strains of serotypes O1 (2 of 24), O2 (2 of 24), O6 (ATCC 43310), O7 (ATCC 43311), and O10 (ATCC 43314) of *V. anguillarum* and the majority of serotype O3 (12 of 24) and O4 (3 of 24) isolates. Phenon II included the rest of strains belonging to serotypes O3 (4 of 13), O4 (1 of 13), O5 (ATCC 43309), O8 (ATCC 43312), and O9 (ATCC 43313). Only three strains of phenon III were typeable, showing a positive reaction with the antisera against *V. anguillarum* serotype O5 (two strains) and *V. splendidus* (ATCC 33125) (Table 2). None of the phenon IV strains could be characterized serologically as *V. anguillarum* or *V. splendidus*.

LPS analysis. The LPS electrophoretic analysis of a representative group of strains of each phenon revealed the

existence of several banding patterns (Fig. 2a). As expected, heterogeneity among the 10 *V. anguillarum* serotypes was observed. However, minor differences between strains of the same serotype were observed. Among VAR isolates, different LPS patterns were also observed. The differences involved the core and lipid A positions and the O chain structure. Interestingly, the strains of VAR groups which displayed more similarities at the phenotypic level also showed similar LPS profiles.

Immunoblot assays using sera against whole cells of different serotypes (O1 to O5) of *V. anguillarum* confirmed the dissimilarities in LPS structure observed among the different serotypes of *V. anguillarum*. Moreover, these assays demonstrated that *V. anguillarum* isolates of the same serotype shared antigenic components. The LPS from the VAR strains analyzed did not react with the antisera raised against serotypes O1 to O5 of *V. anguillarum*. Figure 2b shows the immunoblot assay of LPS from *V. anguillarum* and VAR strains with the whole-cell antiserum against the reference strain ATCC 43306 of serotype O2.

Protein analysis and Western blot (immunoblot). SDS-PAGE analysis of total and outer membrane proteins showed heterogeneity among the isolates. The VAR strains analyzed showed major proteins distributed from 33 to 42 kDa (Fig. 3). The *V. anguillarum* strains possessed different patterns not only among distinct serotypes but also within the same serotype. The major proteins of this group of strains had molecular masses ranging from 27 to 69 kDa (Fig. 4a).

The Western blot studies revealed that antisera against whole cells of serotypes O1 to O5 of *V. anguillarum* reacted strongly with the membrane proteins of the strains of these serotypes. However, only a few immunoreactive bands were observed when the outer membrane proteins from VAR strains and from *V. anguillarum* isolates of serotypes O6 to O10 were analyzed. Figure 4b shows the immunoblot analysis of the outer membrane proteins of *V. anguillarum* strains (serotypes O1 to O10) with the antiserum against the reference strain ATCC 43305 of serotype O1.

Plasmid composition and restriction analysis. All of the strains were examined for plasmid content. Only 19 isolates (32.2%) harbored one or more plasmids, ranging from 1.7 to 60 MDa (Table 2).

The collection strains of *V. anguillarum* serotype O1 harbored a 47-MDa plasmid that was similar to plasmid pJM1 of type strain 775. The *V. anguillarum* strains belonging to serotypes O2, O4, O6, O7, O8, O9, and O10 did not carry any plasmids. In a *V. anguillarum* serotype O3 strain isolated from seawater and one serotype O5 strain from rainbow trout, several low-molecular-weight plasmids were detected. Fifteen VAR strains contained plasmids. The majority of them (11 strains) belonged to phenon IV and shared two plasmid bands of 27 and 36 MDa which are usually harbored in combination with some other plasmids. In addition, the restriction endonuclease analysis allowed us to confirm the genetic similarity of these plasmids among the different VAR strains (data not shown). In the remaining phenons the presence of plasmids was unusual. No correlation between the presence of plasmids and resistance to chemotherapeutic agents or phenotypic traits was observed.

Virulence test. The virulence assays demonstrated that of 24 strains tested, only 9 (37.5%) were virulent for fingerling rainbow trout, showing an LD₅₀ ranging from 10² to 10⁶ cells (Table 2). Most of the VAR strains were avirulent for rainbow trout. Among *V. anguillarum* isolates, the strains of serotype O3 showed a variable degree of virulence, ranging

TABLE 2. Plasmid profiles of the *Vibrio* strains analyzed by the method of Birnboim and Doly (4)

Phenon and strain	Species (serotype) ^a	Origin	Virulence (LD ₅₀) ^b	Plasmid content ^c
I				
ATCC 43305	<i>V. anguillarum</i> (O1)	Rainbow trout (Denmark)	NT	47
R-82	<i>V. anguillarum</i> (O1)	Turbot (Spain)	+9.0 × 10 ²	47
RV 22	<i>V. anguillarum</i> (O2)	Turbot (Spain)	+2.0 × 10 ²	—
ATCC 43306	<i>V. anguillarum</i> (O2)	Cod (Denmark)	NT	—
ATCC 43307	<i>V. anguillarum</i> (O3)	Seawater (Spain)	+1.1 × 10 ⁵	—
13A5	<i>V. anguillarum</i> (O3)	Seawater (Spain)	—>1.0 × 10 ⁷	36, 2.6
ET-208	<i>V. anguillarum</i> (O3)	Japanese eel (Japan)	—>8.0 × 10 ⁷	—
PT-493	<i>V. anguillarum</i> (O3)	Ayu (Japan)	NT	—
PT-82018	<i>V. anguillarum</i> (O3)	Ayu (Japan)	NT	—
V 123	<i>V. anguillarum</i> (O3)	Ayu (Japan)	NT	—
11008	<i>V. anguillarum</i> (O3)	Sea bass (France)	+8.6 × 10 ⁵	—
11044	<i>V. anguillarum</i> (O3)	Sea bass (France)	+8.0 × 10 ⁴	—
11081	<i>V. anguillarum</i> (O3)	Sea bass (France)	NT	—
1321/1	<i>V. anguillarum</i> (O3)	Cod (Denmark)	NT	—
820806-1/2	<i>V. anguillarum</i> (O3)	Rainbow trout (Denmark)	NT	—
820806-1/3	<i>V. anguillarum</i> (O3)	Rainbow trout (Denmark)	NT	—
ATCC 43308	<i>V. anguillarum</i> (O4)	Cod (Denmark)	—>3.1 × 10 ⁷	—
RPM 41.11	<i>V. anguillarum</i> (O4)	Turbot (Spain)	+7.8 × 10 ³	—
RM 40.1	<i>V. anguillarum</i> (O4)	Turbot (Spain)	NT	—
ATCC 43310	<i>V. anguillarum</i> (O6)	Cod (Denmark)	NT	—
ATCC 43311	<i>V. anguillarum</i> (O7)	European eel (Denmark)	NT	—
ATCC 43314	<i>V. anguillarum</i> (O10)	Cod (Denmark)	NT	—
RPM 232.1	VAR	Turbot (Spain)	NT	—
LV 29	VAR	Seawater tank (Norway)	NT	60
II				
1.1.2-13/2	<i>V. anguillarum</i> (O3)	Seawater (Denmark)	NT	—
B.1.1.2/4	<i>V. anguillarum</i> (O3)	Seawater (Denmark)	NT	—
2.2.2-21-P/2	<i>V. anguillarum</i> (O3)	Seawater (Denmark)	NT	—
1.1.2-20/1	<i>V. anguillarum</i> (O3)	Seawater (Denmark)	NT	—
AZ 8.2	<i>V. anguillarum</i> (O4)	Turbot (Spain)	—>7.3 × 10 ⁷	—
ATCC 43309	<i>V. anguillarum</i> (O5)	Cod (Denmark)	—>5.3 × 10 ⁷	—
ATCC 43312	<i>V. anguillarum</i> (O8)	Cod (Denmark)	NT	—
ATCC 43313	<i>V. anguillarum</i> (O9)	Cod (Denmark)	NT	—
OG	VAR	Scallop (Chile)	+8.0 × 10 ³	17
TKI	VAR	Seawater tank (Chile)	+1.0 × 10 ⁶	—
LL	VAR	“Loco” (Chile)	+7.5 × 10 ³	—
NF 25.1	VAR	Salmon (Spain)	NT	—
SB	VAR	Striped bass (United States)	NT	—
III				
TE 5.2	<i>V. anguillarum</i> (O5)	Rainbow trout (Spain)	—>9.0 × 10 ⁷	3.6, 2.6, 2.2, 1.7
PC 22.2	<i>V. anguillarum</i> (O5)	Sea bream (Spain)	—>1.5 × 10 ⁷	—
ATCC 33125	<i>V. splendidus</i> (biotype I)	Marine fish (United States)	—>5.2 × 10 ⁷	—
88589-1	VAR	Tropical fish (United States)	NT	—
88589-2	VAR	Tropical fish (United States)	NT	—
SE 2.1	VAR	Salmon (Spain)	NT	27
RA 60.2	VAR	Turbot (Spain)	—>1.0 × 10 ⁷	4.2, 2
RI 41	VAR	Turbot (Spain)	—>1.2 × 10 ⁸	—
RC 25.1	VAR	Turbot (Spain)	NT	—
RPM 167.2	VAR	Turbot (Spain)	NT	—
IV				
AZ 1.1	VAR	Turbot (Spain)	NT	36, 27, 2
PC 23.1	VAR	Turbot (Spain)	NT	27
RG 22.2	VAR	Turbot (Spain)	—>1.6 × 10 ⁸	43, 36, 27, 6.1
RQ 1.2	VAR	Turbot (Spain)	NT	43, 36, 27, 6.1, 4
RM 36.1	VAR	Turbot (Spain)	NT	36, 27, 6.1
RM 51	VAR	Turbot (Spain)	NT	43, 36, 27
RPM 30.2	VAR	Turbot (Spain)	—>2.2 × 10 ⁷	43, 36, 27
RPM 36.1	VAR	Turbot (Spain)	NT	36, 27, 6.1, 4
RPM 40.1	VAR	Turbot (Spain)	—>2.2 × 10 ⁷	43, 36, 27, 6.1
RPM 41.1	VAR	Turbot (Spain)	—>4.2 × 10 ⁷	43, 36, 27, 6.1
SE 8.1	VAR	Salmon (Spain)	—>2.8 × 10 ⁷	36, 27
RB 798	VAR	Striped bass (United States)	NT	—

^a VAR organisms were not typeable with the sera raised against *V. anguillarum* of serotypes O1 to O10 or with serum against *V. splendidus* (biotype I).^b Number of viable cells needed to kill 50% of inoculated fish in a 14-days period. NT, not tested.^c Numbers indicate plasmid molecular masses in megadaltons. —, no plasmids detected.

FIG. 2. Silver-stained polyacrylamide gel (a) and the corresponding Western blot (immunoblot) analysis (b) of the LPS extracted from *Vibrio* strains. Lanes A to M, *V. anguillarum* strains; lanes N to P, VAR strains. Lanes: A, ATCC 43305 (serotype O1); B, R-82 (serotype O1); C, ATCC 43306 (serotype O2); D, RV-22 (serotype O2); E, ATCC 43307 (serotype O3); F, PT-493 (serotype O3); G, ATCC 43308 (serotype O4); H, ATCC 43309 (serotype O5); I, ATCC 43310 (serotype O6); J, ATCC 43311 (serotype O7); K, ATCC 43312 (serotype O8); L, ATCC 43313 (serotype O9); M, ATCC 43314 (serotype O10); N, RPM 30.2; O, RPM 40.1; P, AZ 1.1. The antiserum used in the immunoblot was prepared against reference strain ATCC 43306 of *V. anguillarum* serotype O2.

from 8×10^4 to $>1 \times 10^7$ cells, and only one of our isolates of serotype O4 was highly virulent ($LD_{50} = 7.8 \times 10^3$). No deaths occurred among the control fish. Interestingly, virulent strains were in phena I and II, while the avirulent ones ($LD_{50} \geq 10^7$) were in phena III and IV.

DISCUSSION

In the estuarine environment can be found a number of vibrios, taxonomically related to *V. anguillarum*, which have recently been associated with diseased fish and shell-

FIG. 3. SDS-PAGE of the outer membrane proteins from *Vibrio* strains. Lanes A to F, *V. anguillarum* strains; lanes G to N, VAR strains. Lanes: A, ATCC 43305 (serotype O1); B, RPM 41.11 (serotype O4); C, AZ 8.2 (serotype O4); D, ATCC 43309 (serotype O5); E, TE 5.2 (serotype O5); F, PC 22.2 (serotype O5); G, *V. splendidus* ATCC 33125; H, RI 41; I, RA 60.2; J, RQ 1.2; K, RPM 30.2; L, RPM 40.1; M, AZ 1.1; N, SE 8.1. Numbers indicate molecular size markers in kilodaltons.

fish. These microorganisms belong to the species *V. splendidus* and *V. pelagius*, and they can be designated VAR organisms. A clear distinction between VAR strains and *V. anguillarum* is not easy because of the great biochemical similarity and the existence of variable reactions within each species. At present, only a few studies on the taxonomic relationships among these groups of vibrios have been conducted. This is the first work in which the biochemical, serological, molecular, and pathogenic characteristics of VAR organisms have simultaneously been analyzed.

Our taxonomic study has confirmed the previously reported biochemical similarity between the VAR group and *V. anguillarum* (8, 10, 15, 34). In agreement with these authors, we believe that the following phenotypic traits can be helpful to differentiate VAR strains from *V. anguillarum*: fermentation of arabinose and mannitol, indole and Voges-Proskauer reactions, gelatin and casein hydrolysis, hemolytic activity, growth at 4 and 37°C, and resistance to ampicillin (Table 1).

In the numerical analysis the *Vibrio* strains were distributed in four phena, with similarity values among them ranging from 76 to 87% and levels of internal similarity ranging from 84.5 to 88%. Similar results have previously been obtained by other authors (8, 15, 20, 21).

On the basis of serological characterization, antigenic differences were detected among the strains, regardless of their biochemical similarities. Serology allowed us to confirm that the majority of strains in phena I and II belonged to the different serotypes established for *V. anguillarum* species (32).

The analysis of LPS and cell envelope proteins showed the existence of heterogeneity within VAR isolates as well as among *V. anguillarum* strains belonging to distinct O serotypes. Moreover, the strains of *V. anguillarum* grouped in the same serotype exhibited slight differences in their LPS and membrane protein banding patterns. The Western blot

FIG. 4. SDS-PAGE of outer membrane proteins from *V. anguillarum* strains (a) and the corresponding Western blot (immunoblot) (b) with antiserum against *V. anguillarum* reference strain ATCC 43305 (serotype O1). Lanes: A, ATCC 43305 (serotype O1); B, R-82 (serotype O1); C, ATCC 43306 (serotype O2); D, RV-22 (serotype O2); E, ATCC 43307 (serotype O3); F, 820806-1/2 (serotype O3); G, 1321/1 (serotype O3); H, 11008 (serotype O3); I, ET-208 (serotype O3); J, PT-493 (serotype O3); K, ATCC 43308 (serotype O4); L, ATCC 43309 (serotype O5); M, ATCC 43310 (serotype O6); N, ATCC 43311 (serotype O7); O, ATCC 43312 (serotype O8); P, ATCC 43313 (serotype O9); Q, ATCC 43314 (serotype O10). Numbers on the left indicate molecular sizes in kilodaltons.

assays confirmed these results because a close relationship was detected among isolates of *V. anguillarum* from the same serotype (although we detected differences in the intensity of the reaction), while strains from different serotypes were not immunologically related. Immunoblot assays also demonstrated that LPS and membrane proteins from *V. anguillarum* (serotypes O1 to O5) and from VAR organisms are immunologically different. From these results we have concluded that VAR strains must be included together with

virulent *V. anguillarum* serotypes in the formulation of vaccines against vibriosis in areas where these microorganisms are repeatedly associated with disease.

Several studies have evaluated in vivo pathogenicity of *V. anguillarum* strains (24, 25, 29, 41, 43). However, to our knowledge, this is the first work in which the possible virulence of VAR strains for fish has been determined. In this study, only 33.3% of the strains tested were virulent for rainbow trout. None of the virulent strains belonged to phenon III or IV. The three virulent VAR strains were included in phenon II and had been isolated from seawater and diseased shellfish. Although serotype O3 of *V. anguillarum* has been reported as pathogenic only in Japan and Taiwan (18, 25, 31), we have demonstrated that some European isolates were virulent for fish. Interestingly, one of our isolates from turbot identified as *V. anguillarum* serotype O4 was highly virulent for rainbow trout. Until now, this serotype has not been considered to be pathogenic. The virulence of VAR strains for marine species should be tested in order to determine the real risk that these microorganisms represent for marine farming.

It has been demonstrated that the virulence of serotype O1 strains of *V. anguillarum* is related to the presence of a 47-MDa plasmid (13, 38) that enables these bacteria to grow under conditions of iron limitation (11–13, 33). Our study indicated that, as expected, the *V. anguillarum* strains belonging to serotype O1 carried a plasmid which had a mobility close to that of the described 47-MDa plasmid. None of the remaining isolates harbored a similar plasmid. It is interesting that in the VAR strains of phenon IV we have found a characteristic plasmid profile consisting of 27- and 36-MDa plasmid bands, which proved to be genetically similar among the isolates by restriction endonuclease analysis. Although so far the role of these plasmids is not known, they are in the same molecular weight range as those detected in *V. splendidus* isolated from healthy Atlantic salmon and sea bass (26) and in VAR strains from diseased clams (10). In the present study no correspondence between biochemical profiles, resistance patterns, or virulence and the plasmid content of the VAR organisms was observed.

ACKNOWLEDGMENTS

This investigation was supported by grant MAR 91-1133-C02-01 from the Comisión Interministerial de Ciencia y Tecnología (C.I.C.I.T.) and grant XUGA 20009A92 from the Xunta de Galicia, Spain. I. Bandín thanks the Ministerio de Educación y Ciencia, and F. Pazos and B. Magariños thank the Xunta de Galicia (Spain), for research fellowships.

We thank the donors who kindly supplied *Vibrio* strains, and we thank Javier Barja for his contribution to the presentation of the data.

REFERENCES

1. Austin, B., and D. A. Austin. 1987. Vibrios, p. 263–296. In L. M. Laird (ed.), Bacterial fish pathogens. Disease in farmed and wild fish. Ellis Horwood Publishers, Chichester, United Kingdom.
2. Barry, A. L., and C. Thornsberry. 1991. Susceptibility tests: diffusion test procedures, p. 1117–1125. In A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
3. Baticados, M. C. L., C. R. Lavilla-Pitogo, E. R. Cruz-Lacierda, L. D. de la Peña, and N. A. Suñaz. 1990. Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *Vibrio splendidus* isolated from diseased *Penaeus monodon* larvae and rearing

- water. *Dis. Aquat. Org.* **9**:133-139.
4. Birnboim, H., and J. Doly. 1979. A rapid extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**:1513-1525.
 5. Bolinches, J., M. L. Lemos, and J. L. Barja. 1988. Population dynamics of heterotrophic bacterial communities associated with *Fucus vesiculosus* and *Ulva rigida* in a estuary. *Microb. Ecol.* **15**:345-357.
 6. Bolinches, J., A. E. Toranzo, A. Silva, and J. L. Barja. 1986. Vibriosis as the main causative factor of heavy mortalities in the oyster culture industry in northwestern Spain. *Bull. Eur. Assoc. Fish Pathol.* **6**:1-4.
 7. Bowser, P. R., R. Rosemark, and C. R. Reiner. 1981. A preliminary report of vibriosis in cultured American lobsters, *Homarus americanus*. *J. Invertebr. Pathol.* **37**:80-85.
 8. Bryant, T. N., J. V. Lee, P. A. West, and R. R. Colwell. 1986. Numerical classification of species of *Vibrio* and related genera. *J. Appl. Bacteriol.* **61**:437-467.
 9. Bryant, T. N., J. V. Lee, P. A. West, and R. R. Colwell. 1986. A probability matrix for the identification of species of *Vibrio* and related genera. *J. Appl. Bacteriol.* **61**:469-480.
 10. Castro, D., E. Martínez-Manzanares, A. Luque, B. Fouz, M. A. Morínigo, J. J. Borrego, and A. E. Toranzo. 1992. Characterization of strains related to brown ring disease outbreaks in southwestern Spain. *Dis. Aquat. Org.* **14**:229-236.
 11. Conchas, R. F., M. L. Lemos, J. L. Barja, and A. E. Toranzo. 1991. Distribution of plasmid- and chromosome-mediated iron uptake systems in *Vibrio anguillarum* strains of different origins. *Appl. Environ. Microbiol.* **57**:2956-2962.
 12. Crosa, J. H., L. A. Actis, Y. Mitoma, J. Pérez-Casal, M. E. Tolmasky, and M. A. Malvano. 1985. Plasmid mediated iron sequestering systems in pathogenic strains of *Vibrio anguillarum* and *Escherichia coli*, p. 759-774. In D. R. Helinski, S. N. Cohen, D. Clewell, D. Jackson, and A. Hollander (ed.), *Plasmids in bacteria*. Plenum Publishing Corp., New York.
 13. Crosa, J. H., L. L. Hodges, and M. H. Schiewe. 1980. Curing of a plasmid is correlated with an attenuation of virulence in the marine fish pathogen *Vibrio anguillarum*. *Infect. Immun.* **27**:897-902.
 14. Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart. 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium lauryl sarcosinate. *J. Bacteriol.* **115**:717-722.
 15. Fouz, B., R. F. Conchas, J. Bolinches, J. L. Romalde, J. L. Barja, and A. E. Toranzo. 1990. Relationship among pathogenic *Vibrio anguillarum* and *Vibrio tubiashii* with environmental vibrios, p. 77-89. In F. O. Perkins and T. C. Cheng (ed.), *Pathology in marine science*. Academic Press, Inc., New York.
 16. Hitchcock, P. J., and T. M. Brown. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J. Bacteriol.* **154**:269-272.
 17. Jeffries, V. E. 1982. Three *Vibrio* strains pathogenic to larvae of *Crassostrea gigas* and *Ostrea edulis*. *Aquaculture* **29**:201-226.
 18. Kitao, T., T. Aoki, M. Fukudome, K. Kawano, Y. Wada, and Y. Mizuno. 1983. Serotyping of *Vibrio anguillarum* from diseased freshwater fish in Japan. *J. Fish Dis.* **6**:175-181.
 19. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
 20. Larsen, J. L. 1982. *Vibrio anguillarum*: prevalence in three carbohydrate loaded marine recipients and a control. *Zentralbl. Bakteriell. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe C* **3**:519-530.
 21. Larsen, J. L. 1983. *Vibrio anguillarum*: a comparative study of fish pathogenic, environmental and reference strains. *Acta Vet. Scand.* **24**:456-476.
 22. Larsen, J. L., H. B. Rasmussen, and I. Dalsgaard. 1988. Study of *Vibrio anguillarum* strains from different sources with emphasis on ecological and pathobiological properties. *Appl. Environ. Microbiol.* **54**:2264-2267.
 23. Lupiani, B., C. P. Dopazo, A. Ledo, B. Fouz, and J. L. Barja. 1989. New syndrome of mixed bacterial and viral etiology in cultured turbot *Scophthalmus maximus*. *J. Aquat. Anim. Health* **1**:197-204.
 24. Muroga, K. 1975. Studies on *Vibrio anguillarum* and *Vibrio anguillarum* infection. *J. Fac. Fish. Anim. Husb. Hiroshima Univ.* **14**:101-215.
 25. Muroga, K., H. Yamanoi, Y. Hironaka, S. Yamamoto, M. Tatani, Y. Jo, S. Takahashi, and H. Hanada. 1984. Detection of *Vibrio anguillarum* from wild fingerlings of ayu *Plecoglossus altivelis*. *Bull. Jpn. Soc. Sci. Fish.* **50**:591-596.
 26. Myhr, E., J. L. Larsen, A. Lillehaug, R. Gudding, M. Heum, and T. Håstein. 1991. Characterization of *Vibrio anguillarum* and closely related species isolated from farmed fish in Norway. *Appl. Environ. Microbiol.* **57**:2750-2757.
 27. Paillard, C., and P. Maes. 1990. Etiologie de la maladie de l'anneau brun chez *Tapes philippinarum*: pathogénicité d'un *Vibrio* sp. *C.R. Acad. Sci.* **310**:15-20.
 28. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27**:493-497.
 29. Santos, Y., R. Lallier, I. Bandín, J. Lamas, and A. E. Toranzo. 1991. Susceptibility of turbot (*Scophthalmus maximus*), coho salmon (*Oncorhynchus kisutch*), and rainbow trout (*O. mykiss*) to strains of *Vibrio anguillarum* and their exotoxins. *J. Appl. Ichthyol.* **7**:160-167.
 30. Sneath, P. H. A. 1972. Computer taxonomy, p. 29-98. In J. R. Norris and D. W. Ribbons (ed.), *Methods in microbiology*, vol. 7A. Academic Press, Inc. (London) Ltd., London.
 31. Song, Y. L., S. N. Chen, and G. H. Kou. 1988. Serotyping of *Vibrio anguillarum* strains isolated from fish in Taiwan. *Fish Pathol.* **23**:185-189.
 32. Sørensen, U. B. S., and J. L. Larsen. 1986. Serotyping of *Vibrio anguillarum*. *Appl. Environ. Microbiol.* **51**:593-597.
 33. Tolmasky, M. E., L. A. Actis, A. E. Toranzo, J. L. Barja, and J. H. Crosa. 1985. Plasmids mediating iron uptake in *Vibrio anguillarum* strains isolated from turbot in Spain. *J. Gen. Microbiol.* **131**:1989-1997.
 34. Toranzo, A. E., and J. L. Barja. 1990. A review of the taxonomy and seroepizootiology of *Vibrio anguillarum*, with special reference to aquaculture in the northwest of Spain. *Dis. Aquat. Org.* **9**:73-82.
 35. Toranzo, A. E., J. L. Barja, S. A. Potter, R. R. Colwell, F. M. Hetrick, and J. H. Crosa. 1983. Molecular factors associated with virulence of marine vibrios isolated from striped bass in Chesapeake Bay. *Infect. Immun.* **39**:1220-1227.
 36. Toranzo, A. E., A. M. Baya, B. S. Roberson, J. L. Barja, D. J. Grimes, and F. M. Hetrick. 1987. Specificity of slide agglutination test for detecting bacterial fish pathogens. *Aquaculture* **61**:81-97.
 37. Toranzo, A. E., Y. Santos, I. Bandín, J. L. Romalde, A. Ledo, B. Fouz, and J. L. Barja. 1990. A five years survey of bacterial fish infections in continental and marine aquaculture in northwest of Spain. *World Aquacult.* **21**:91-93.
 38. Toranzo, A. E., Y. Santos, M. L. Lemos, A. Ledo, and J. Bolinches. 1987. Homology of *Vibrio anguillarum* strains causing epizootics in turbot, salmon and trout reared on the Atlantic coast of Spain. *Aquaculture* **67**:41-52.
 39. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350-4354.
 40. Tsai, C. M., and C. E. Frasch. 1982. Staining of lipopolysaccharide in SDS polyacrylamide gels using silver-staining method. *Anal. Biochem.* **119**:115-119.
 41. Uno, M. 1976. Experimental pathogenicity of *Aeromonas salmonicida*, *Vibrio anguillarum* and *Pseudomonas anguilliseptica* for Iwana and other trout. *Fish Pathol.* **11**:5-9.
 42. West, P. A., and R. R. Colwell. 1984. Identification and classification of *Vibrionaceae*. An overview, p. 258-363. In R. R. Colwell (ed.), *Vibrios in the environment*. John Wiley & Sons, Inc., New York.
 43. Wiik, R., K. Andersen, F. L. Daae, and K. A. Hoff. 1989. Virulence studies based on plasmid profiles of the fish pathogen *Vibrio salmonicida*. *Appl. Environ. Microbiol.* **55**:819-825.