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Toxic and Enzymatic Activities of *Vibrio vulnificus* Biotype 2 with Respect to Host Specificity

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In this work, the enzymatic activities of selected strains of biotypes 1 and 2 of *Vibrio vulnificus* were analyzed by using conventional methods and the API ZYM system. The toxic activities of extracellular products (ECPs) were further evaluated by in vitro and in vivo experiments. The ECPs of both biotypes (i) showed high-level hydrolytic activities, (ii) displayed cytotoxicity for fish cell lines, and (iii) were lethal for eels. Exotoxins seem to be proteinaceous since heat treatment of ECP samples destroyed their toxicity. Only biotype 2 strains were virulent for eels, suggesting that host specificity must be related to differences in cell surface properties. Infectivity trials with other fish species also revealed that only biotype 2 strains were virulent.

Vibrio vulnificus is a pathogenic bacterium whose two biotypes are mainly defined by host range (36). Biotype 1 inhabits marine and estuarine environments and may cause primary septicemia or a wound infection in humans after consumption of seafood or contact with seawater (30). In contrast, the recovery of biotype 2 has been reported only from diseased eels (8, 29, 36). This biotype seemed to be restricted to the Asiatic continent (29, 35, 36) until it was recently recovered from diseased eels in Europe (8). Biotype 2 has been the etiological agent of several epizootic outbreaks at a Spanish eel farm between 1989 and 1992 (7, 8, 14). Biotype 1 has also been recovered from eels and tank water at that farm but never from diseased eels (7, 10, 14). Even though the two biotypes present high levels of phenotypic homology, they are biochemically and serologically distinguishable. (i) Indole production seems to be the only biochemical test to differentiate biotypes (2, 8, 10, 14), and (ii) biotype 1 is serologically heterogeneous while strains of biotype 2 seem to constitute a homogeneous serogroup (4, 12, 14, 28).

Because of fatalities caused by biotype 1 infections in humans, as well as its ubiquity in the aquatic environment, this biotype has been recognized as a potential public health hazard for a long time. This explains why investigations of this species have mainly focused on biotype 1. There are many studies concerning the virulence mechanisms of biotype 1 responsible for its human pathogenicity. From these studies, it seems that the infection caused by this biotype is multifaceted, probably involving the following virulence determinants: (i) a polysaccharidic capsule that confers resistance to phagocytosis and serum complement (22, 25, 34, 41, 43); (ii) various iron uptake systems, including siderophore production and the ability to use hemoglobin and hemin as iron sources (20, 33, 42, 44); (iii) dependence between iron availability in host fluids and the degree of virulence (20, 40); and (iv) lesional factors such as exoenzymes and exotoxins, including a cytotoxin with hemolytic activity that is responsible for extensive tissue damage (18, 26, 31).

On the contrary, biotype 2 has been considered an obligate eel pathogen until recently. From our studies, we know that this biotype is a primary eel pathogen that may also act as an opportunistic pathogen for humans (1, 2, 13, 14, 39). Moreover, we have recently confirmed that biotype 2, like biotype 1, is able to survive in the aquatic environment away from its natural host and to use water as a route of infection (3, 9). However, few studies have been carried out to clarify the virulence factors implicated in the septicemic infection produced by *V. vulnificus* biotype 2 in eels. From these studies, we know that some virulence mechanisms responsible for human or eel infection, such as iron uptake and capsule expression, do not differ substantially between the two biotypes (5, 11, 13). We have also reported that biotype 2 produces a variety of exoenzymes, hemolysins, and cytotoxins, with its exotoxins being implicated in mouse virulence (2, 5, 13). However, these studies have been based on a limited number of strains. Further, none of these studies have investigated the toxicity in vivo for eels of the culture supernatant filtrates from biotype 2 strains, nor have they examined the susceptibilities of other fish species to the vibriosis caused by *V. vulnificus* biotype 2. To address these issues, we have analyzed the exoenzymatic and toxic activities of the extracellular products (ECPs) isolated from selected *V. vulnificus* biotype 2 strains and compared them with those of live cells, both in vitro and in vivo, with biotype 1 strains used for comparative purposes. For the host range study, we used eels, trout, turbot, sea bass, and sea bream as experimental animals.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Of the 11 biotype 2 strains used in this study, 6 strains (E22, E39, E58, E86, E105, and E116) were from diseased European eels and representative of isolates recovered during different outbreaks of vibriosis in Spain (7, 8, 10, 14) and 5 were reference strains (ATCC 33149, NCIMB 2136, NCIMB 2137, NCIMB 2138, and UE516) from diseased Japanese eels (Table 1). Of the eight biotype 1 strains used for comparative purposes, two were environmental strains (TW1 and E109) isolated from the same Spanish eel farm where epizootics were registered (2, 7, 10, 14) and six were clinical strains, including the type strain (T) of the species (ATCC 27562^T, CDC7184, L-180, VvL1, 374, and UMH1) (Table 1). All *V. vulnificus* strains used in this study are of opaque morphotype, with the exceptions of biotype 2 strain NCIMB 2137 and biotype 1 strain TW1, which are of translucent morphotype.

Strains were routinely grown in Trypticase soy broth or Trypticase soy agar (Difco) supplemented with 0.5% NaCl (wt/vol). Unless otherwise indicated, isolates were incubated at 25°C.

Collection of ECPs. ECPs were obtained by the cellophane plate technique as previously described (2, 27). Briefly, Trypticase soy agar plates supplemented with 0.5% NaCl were overlaid with sterile cellophane sheets and inoculated with 1 ml of an overnight culture of isolated translucent and opaque colonies from each strain. After incubation for 24 h at 25°C, cells were recovered with phosphate-buffered saline (PBS)–1% NaCl (pH 7.0) (PBS-1) and centrifuged at 12,000 × *g* for 30 min at 4°C. The resulting supernatants were filter sterilized by

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TABLE 1. Biotypes, sources, and origins of the *V. vulnificus* strains used in this study

Strain(s)[a]	Origin
Biotype 2	
European isolates	
E22[b]	Diseased European eel, Spain (1989)
E39, E58, E86, E105[b]	Diseased European eel, Spain (1990)
E116[b]	Diseased European eel, Spain (1992)
Asiatic isolates	
ATCC 33149	Diseased Japanese eel, Japan
NCIMB 2136	Diseased Japanese eel, Japan
NCIMB 2137	Diseased Japanese eel, Japan
NCIMB 2138	Diseased Japanese eel, Japan
UE516[c]	Diseased Japanese eel, Taiwan
Biotype 1	
Clinical isolates	
ATCC 27562^T	Human blood, United States
CDC7184[d]	Human blood, United States
L-180[d]	Septicemia case, United States
VvL1[d]	Fatal wound infection, United States
374[d]	Septicemia case, United States
UMH1[d]	Fatal wound infection, United States
Environmental isolates	
TW1[b]	Tank water from an eel farm, Spain (1990)
E109[b]	Surface of European eels, Spain (1990)

[a] ATCC, American Type Culture Collection, Rockville, Md.; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland; CDC, Centers for Disease Control and Prevention, Atlanta, Ga.; T, type strain.
[b] See references 2, 8, 10, and 14.
[c] Supplied by Y.-L. Song, National Taiwan University, Taipei, Taiwan.
[d] Supplied by J. D. Oliver, University of North Carolina, Charlotte.

using nitrocellulose filters of 0.45-μm pore size (Millipore). Aliquots (1.5 ml) of each ECP sample were stored at −20°C until used. ECP protein concentrations were determined by the Bradford method (15), using bovine serum albumin as the standard. The lipopolysaccharide (LPS) contents of ECP samples were evaluated by the procedure of Keler and Novotny (23), using purified LPS from *Vibrio cholerae* INABA 569 B (Sigma) as the standard.

Exoenzymatic and hemolytic activities of ECPs. The total proteolytic activity present in ECP samples was evaluated by using the multiprotein substrate Azocoll (Sigma) according to the manufacturer's instructions. One unit of protease activity was defined as an A_{520} of 1.0 after a 30-min assay at 37°C.

The hydrolytic activities of ECP samples were evaluated on agarose plates by the method of Amaro et al. (2). Briefly, agarose plates (0.8% [wt/vol] agarose) (Oxoid) were diluted with PBS-1 supplemented with one of the following substrates: 2% (wt/vol) skim milk, 0.1% (wt/vol) elastin, 1% (wt/vol) mucin, 0.28% (wt/vol) fibrinogen, 1% (wt/vol) Tween 80, 5% (wt/vol) egg yolk emulsion, or 5% (wt/vol) human or eel erythrocytes. DNase production was assayed on DNase agar (Oxoid). Five microliters of each ECP sample was inoculated in 2- to 3-mm-diameter wells made on these plates. The diameter of the hydrolysis halo around the inoculated well in duplicate experiments served as a relative quantification of the lytic activities.

API ZYM system. The API ZYM system (BioMerieux) was used for further characterization of the general enzymatic activities of live cells and ECPs of the *V. vulnificus* strains studied. Assays were performed according to the manufacturer's recommendations at 25°C. The heat stabilities of the enzymatic activities of ECPs were tested after heating at 80°C for 30 min.

Cytotoxic activities of ECPs. Cytotoxicity assays were performed as previously described (2) on the following fish cell lines: CHSE-214 (chinook salmon embryo), RTG-2 (rainbow trout gonad), and EPC (epithelioma papillosum of carp). Cell monolayers were grown in 24-well plates, inoculated with 0.1 ml of serial dilutions of each ECP sample, and incubated at 20°C. Toxicity titers were expressed as the reciprocal of the highest dilution of crude ECP able to produce total or partial destruction of the monolayer within 24 h. Trypan blue staining was used to evaluate cell viability after ECP effects.

To investigate the heat stabilities of the cytopathic effects of ECPs, samples were also assayed after heat treatment at 80°C for 30 min by using the most sensitive cell line (RTG-2).

Virulence and lethality assays. Assays for pathogenicity were made on juvenile fish (average, 10 g) of different species (eels, sea bass, rainbow-trout, turbot, and sea bream) by intraperitoneal (i.p.) inoculation (2, 13). The toxicity of ECPs for eels was evaluated by i.p. injection of 0.1 ml of serial twofold dilutions of each ECP sample per fish.

To determine the thermal stabilities of the toxic activities of live cells and ECPs for eels, bacterial-cell suspensions and ECP samples were also tested after heat treatment at 100°C for 60 min and at 80°C for 30 min, respectively. Dead cells were washed in PBS-1 and adjusted to 10^9 cells per ml, and 0.1 ml of this bacterial suspension per fish was administered i.p.

Groups of six fish were used for virulence and lethality assays. Sets of animals inoculated with sterile PBS-1 were also included in these experiments as negative controls. Fish were anesthetized by adding a 10% (wt/vol) benzocain solution to the tank water. Mortalities were recorded daily for 1 week, and the 50% lethal doses (LD_{50}) of bacterial cells and toxins were calculated by the method of Reed and Münch (32).

RESULTS AND DISCUSSION

Hydrolytic activities of ECPs in vitro. We have previously reported the proteolytic activity exhibited by biotype 2 ECPs (2). The results obtained in the present study, using a larger number of strains and substrata, have confirmed that the ECPs of the two biotypes of *V. vulnificus* are highly proteolytic. ECP samples showed high levels of protein content (0.7 to 3.9 mg/ml) and exhibited similar proteolytic capabilities against gelatin, casein, elastin, and fibrinogen (Table 2). In general, the fibrinolysin activities of Spanish biotype 2 isolates were stronger, while the hydrolysis of elastin was variable in both biotypes. In contrast to our previous report (2), the concentrated culture supernatants of both biotypes displayed phospholipasic activities and were negative for lipase production (Table 2). Mucinase and DNase activities were also negative (Table 2). All ECP samples of biotype 1 and 2 strains were hemolytic for human and eel erythrocytes, exhibiting similar activities, which were higher for human blood (Table 2). In general, the results show high levels of hydrolytic potential among ECPs regardless of the biotype, source, or geographical origin of isolates. The overall results are consistent with the high levels of proteolytic activity detected by the Azocoll assay (Table 2), as well as with our previous studies using live cells of both biotypes (13, 14). This indicates that most of the enzymatic activities displayed by live cells are extracellular. In fact, the caseinase, elastase, fibrinolysin, phospholipasic, and hemolytic activities of supernatant fluids were similar to the ones previously found for live cells (14). Elastase production was variable, as it was in previous reports using live cells, being absent in the ECPs of some strains that are positive for this activity (14). However, we detected slight differences with respect to those of live cells in mucinase, DNase, and lipase activities, which have been reported as positive for live cells but negative for ECPs (13, 14). The high levels of proteolytic activity, as well as hemolytic and phospholipasic activities, displayed by culture supernatants correlate with our previous studies showing that biotype 2 strains possess high levels of exoenzymatic potential. This suggests that these hydrolytic properties contribute to the colonization and invasion of the host. Finally, all hydrolytic activities were lost after heat treatment, suggesting that these heat-labile factors are proteins.

Cytotoxic activities of ECPs. We previously found the ECPs of biotype 2 cells to be cytotoxic for homeothermic and poikilothermic cell lines (2). The toxicity study performed in this work included more strains and confirmed our previous observations. All ECPs of both biotypes displayed a strong toxicity, with their toxins producing total cell detachment of fish cell lines. The minimal protein concentration causing a cytopathic effect on EPC cells was around 1 μg/ml in both biotypes (Table 2). Degenerative changes became apparent in a period ranging from 3 to 4 h and generally consisted of cell elongation and rounding, with subsequent cell detachment from the mono-

TABLE 2. Enzymatic, cytotoxic, and lethal activities displayed by ECPs obtained from selected *V. vulnificus* strains

Strain (colony type)[a]	[Protein] (mg/ml)[b]	Proteolytic activity (U/ml)[c]	[LPS] (mg/ml)[d]	Production of[e,f]:								Hemolysis of erythrocytes from[f]:		Cytopathic effect[g]			Lethality for eels[h]
				Gel	Cas	Elas	Muc	Fib	DNase	Lipase	Phos	Humans	Eels	CHSE-214	RTG-2	EPC	
Biotype 2																	
European isolates																	
E22 (O)	1.6	6.2	1.2	++	++	−	−	++	−	−	+	+	+	+	+	+ (1.5)	+ (10)
E39 (O)	3.0	5.8	1.3	++	+++	++	−	+++	−	−	+	++	+	+	+	+ (1.0)	+ (1.5)
E86 (O)	3.75	6.3	1.3	+++	+++	++	−	+++	−	−	+	+++	+	+	+	+ (0.8)	+ (1.0)
E58 (O)	1.8	5.7	0.7	+	+	−	−	+	−	−	+	+	+	+	+	+ (1.3)	+ (3.5)
E105 (O)	1.9	5.9	1.25	++	++	−	−	+	−	−	+	++	+	+	+	+ (1.1)	+ (3.8)
E116 (O)	2.2	6.1	1.1	++	++	−	−	+	−	−	+	+	+	+	+	+ (1.2)	+ (>5)
Asiatic isolates														+	+	+	
ATCC 33149 (O)	1.52	4.4	1.25	++	++	+	−	+	−	−	++	+	+	+	+	+ (1.0)	+ (7.5)
NCIMB 2136 (O)	2.5	ND[i]	1.0	++	+	−	−	+	−	−	ND	+	+	ND	ND	ND	ND
NCIMB 2137 (T)	0.8	5.8	1.5	++	+	−	−	+	−	−	+	+	+	ND	ND	ND	+ (5.5)
NCIMB 2138 (O)	1.6	ND	1.1	++	++	−	−	+	−	−	ND	+	+	ND	ND	ND	ND
UE516 (O)	0.7	4.6	1.1	++	+	−	−	+	−	−	−	+	+	ND	ND	ND	+ (>5)
Biotype 1																	
Clinical isolates																	
ATCC 27562 (O)	2.1	7.4	1.1	+	++	−	−	+	−	−	+	+	+	+	+	+ (1.4)	+ (4.0)
CDC7184 (O)	3.05	8.4	1.4	++	++	−	−	+	−	−	+	+	+	+	+	+	+ (3.7)
UMH1 (O)	1.9	ND	1.2	++	+	ND	−	+	−	ND	+	+	ND	ND	ND	ND	+
L-180 (O)	3.9	ND	1.0	++	++	ND	−	+	−	ND	+	++	ND	ND	ND	ND	ND
374 (O)	2.5	ND	1.0	++	+	ND	−	+	−	ND	+	+	ND	ND	ND	ND	+
VvL1 (O)	1.6	ND	1.4	+	+	ND	−	−	−	ND	+	+	ND	ND	ND	ND	ND
Environmental isolates																	
E109 (O)	1.70	6.2	1.0	+++	+++	++	−	+	−	−	+	+++	+	+	+	+ (1.2)	+ (3.5)
TW1 (T)	2.3	6.7	1.25	+++	+++	++	−	++	−	−	+	+++	+	+	+	+ (1.1)	+ (3.3)

[a] O, opaque; T, translucent.
[b] Protein content of undiluted ECP samples.
[c] Proteolytic activity was determined on Azocoll substrate. One unit of proteolytic activity produced an A_{520} reading of 1.0.
[d] LPS content of undiluted ECP samples.
[e] Gel, gelatinase; Cas, caseinase; Elas, elastase; Muc, mucinase; Fib, fibrinogenase; Phos, phospholipase.
[f] Ratio of hydrolysis halo to well diameter (+++, ≥3.1; ++, 2.1 to 3.0; +, 1.1 to 2.0; −, 0).
[g] +, rounding and total cell detachment within 3 to 4 h. The minimal protein doses noted parenthetically (in micrograms per milliter) that produced cytotoxic effects on the ECP cell line were around 1 μg/ml for both biotypes.
[h] +, lethal effect of undiluted ECP; −, no lethal effect of undiluted ECP. The data in parentheses are the LD_{50} expressed in micrograms of ECP protein per gram of fish.
[i] ND, not done.

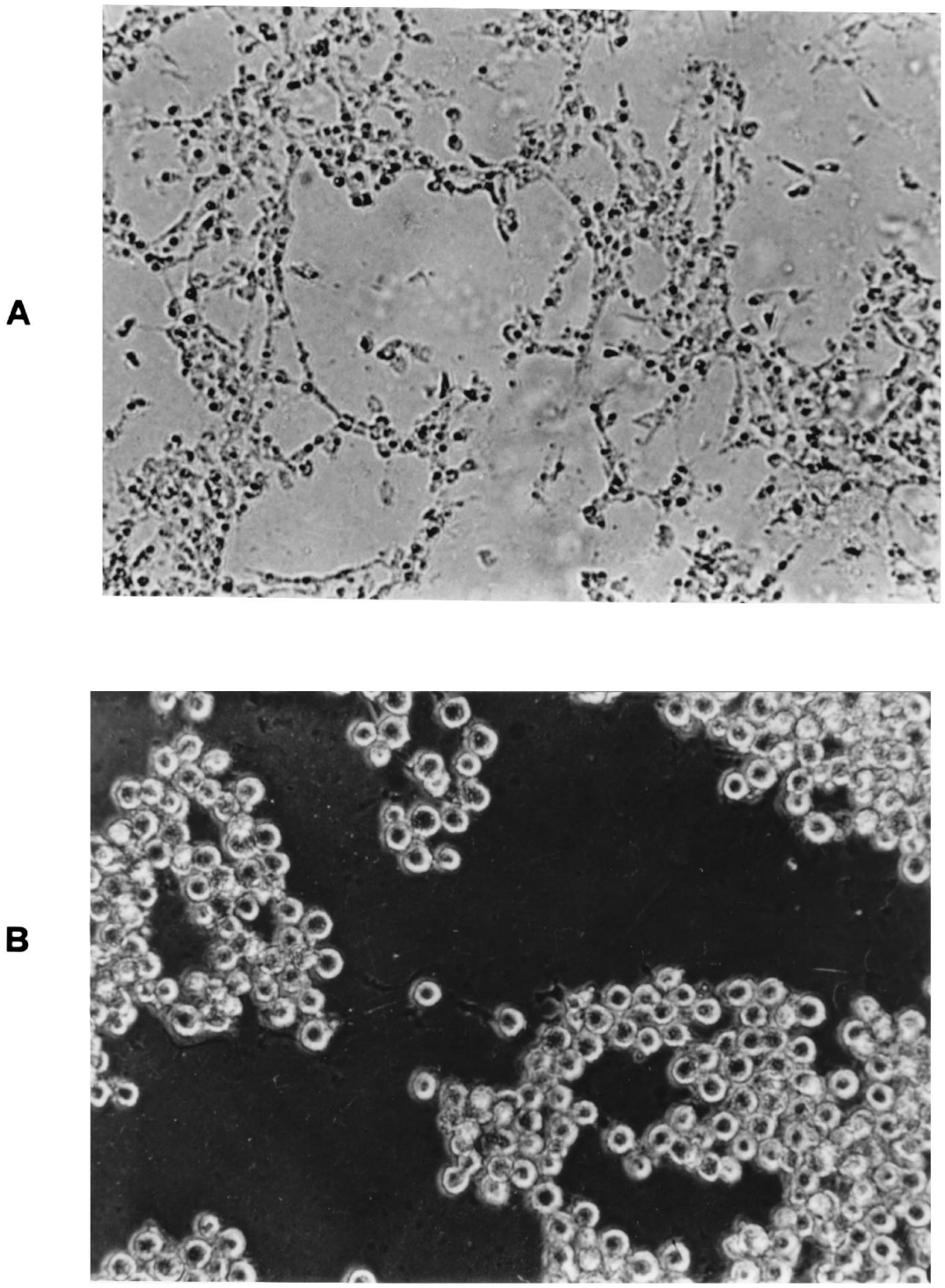

FIG. 1. Cytotoxic effects of *V. vulnificus* ECPs on homeothermic cells. (A) Shrinking and dendritic elongation; (B) rounding and cell detachment.

layer (Fig. 1). Trypan blue staining revealed that more than 80% of cells remained viable, indicating that the ECP samples from *V. vulnificus* mainly displayed proteolytic activity, which was responsible for the detachment of cells from the monolayer. Interestingly, the strongest cytotoxic response was observed for Spanish biotype 2 isolates, followed by those belonging to biotype 1. Apart from that, no apparent qualitative differences in cytotoxic activities related to the biotype, source, or geographical origin of strains were found. Cytotoxicity disappeared after heat treatment of supernatant fluids, suggesting the thermolabile nature of exotoxins.

Lethal activities of ECPs and heat-killed cells for eels. We have recently demonstrated that *V. vulnificus* biotype 2 is virulent for eels and mice (2, 5, 13, 14), with its exotoxins being

TABLE 3. General enzymatic reactions displayed by live cells and ECPs from selected *V. vulnificus* strains in the API ZYM system

Strain	Exoenzymatic activity[a]												
	Ak-phos	E	E-lip	Lipase	L-aryl	V-aryl	Trypsin	α-Chemo	Ac-phos	Naf-phos	β-Gal	α-Gluco	Acet-gluco
Biotype 2													
European isolates													
E86 (LC)[b]	1	3	3	1	4	0	0	0	1	1	1	0	1
E86 (ECP)	3	3	4	1	1	0	3	3	0	1	0	0	0
Asiatic isolates													
ATCC 33149 (LC)	1	2	4	1	4	0	0	0	1	1	2	0	1
ATCC 33149 (ECP)	2	2	4	3	3	0	1	1	1	1	0	0	1
NCIMB 2137 (LC)	4	1	2	1	4	0	0	0	3	3	1	0	2
NCIMB 2137 (ECP)	5	3	3	3	1	0	0	0	1	1	0	0	0
Biotype 1													
Clinical isolates													
ATCC 27562 (LC)	4	2	4	0	5	1	0	0	1	1	2	0	1
ATCC 27562 (ECP)	1	1	2	0	1	0	0	0	0	1	0	0	0
CDC7184 (LC)	2	1	2	0	1	1	0	0	1	1	4	2	2
CDC7184 (ECP)	3	4	4	1	5	0	0	0	0	1	0	0	2
Environmental isolates													
E109 (LC)	2	3	3	0	2	1	0	0	1	1	1	0	1
E109 (ECP)	2	3	4	0	0	0	2	2	0	1	0	0	0
TW1 (LC)	4	5	4	1	2	1	0	0	1	1	2	0	2
TW1 (ECP)	1	1	1	0	0	0	2	2	0	1	0	0	0

[a] Ak-phos, alkaline phosphatase; E, esterase; E-lip, esterase lipase; L-aryl, leucine arylamidase; V-aryl, valine arylamidase; α-Chemo, α-chemotrypsin; Ac-phos, acid phosphatase; Naf-phos, naphthol-A-S-BI-phosphohydrolase; β-Gal, β-galactosidase; α-Gluco, α-glucosidase, Acet-gluco, *N*-acetyl-β-glucosaminidase. Activity grades are indicated (in nanomoles of hydrolized substrate) as follows: 5, ≥40; 4, $30 < x < 40$; 3, $20 < x < 30$; 2, $10 < x < 20$; 1, $5 < x < 10$; 0, negative reaction.

[b] LC, live cells.

implicated in mouse virulence (5). To determine whether the susceptibility of eels to infection is related to their sensitivity to biotype 2 ECPs, we investigated the lethal activities of ECPs in vivo by i.p. administration of serial dilutions of ECPs. The toxicity of ECPs in vivo revealed the strong lethality of each biotype for eels, regardless of the strain's origin. Eel mortalities were recorded between 4 and 48 h postinoculation. No significant differences in the mean lethal dose, expressed as protein content, were detected between biotypes (LD_{50} range, 1 to 10 μg of protein per g of fish) (Table 2). Thus, toxins produced by biotype 1 isolates were equally as lethal for eels as those produced by biotype 2 strains. The mean lethal dose ranges observed were comparable to those reported for other fish pathogens of the genera *Vibrio* and *Aeromonas* (16, 21, 24). Interestingly, ECPs of both biotypes produced in eels the disease signs observed in eels infected naturally with *V. vulnificus* biotype 2. Extensive areas of skin hemorrhage on the body surface were the main pathological sign in eels. The results of these experiments have revealed the relationship between the high levels of hydrolytic and cytotoxic potential in vitro and the lesional capability in vivo displayed by the biological activities present in the ECPs of *V. vulnificus.*

Lethality for eels was lost after heat treatment of supernatant fluids, suggesting the involvement of proteolytic exoenzymes in the production of lethal effects in both biotypes, as well as the lack of toxicity of LPS contained in ECPs (around 1 μg/μl in the two biotypes) (Table 2). To account for the role of the *V. vulnificus* endotoxin in the pathogenesis of this disease, we also inoculated eels with heat-killed cells. No mortalities were recorded for 1 week after i.p. inoculations of approximately 10^8 killed cells per g of fish, indicating the lack of toxicity of the cell-associated LPS of this species for eels. Since we did not test purified LPS, we cannot preclude the possibility that this molecule plays some role in the virulence of this microorganism. In this regard, further studies are in progress to clarify the biological activities of this endotoxin.

API ZYM. The API ZYM system is a semiquantitative micromethod used for rapid detection of general enzymatic activities, such as proteases, esterases, aminopeptidases, phosphatases, and glycosidases. Thus, the enzymatic patterns of both live cells and ECP samples were characterized by using this system. In general, the two biotypes of *V. vulnificus* exhibited similar enzymatic patterns, with the following nine activities in common: alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, acid phosphatase, naphthol-A-S-BI-phosphohydrolase, β-galactosidase, and *N*-acetyl-β-glucosaminidase (Table 3). Although lipase activity was detected in both biotypes, it was 100% positive only in biotype 2 strains; among biotype 1 strains, only the tank water isolate displayed this activity. These results are in contrast to those for lipase activity evaluated on Tween 80 plates, suggesting that this substrate is not suitable for the detection of any type of lipase activity. It is interesting that all biotype 1 strains tested were positive for valine arylamidase production, which was not detected among biotype 2 strains. In general, ECP samples shared the above-mentioned activities for live cells. The main differences were detected in trypsin, α-chemotrypsin, and β-galactosidase activities. The two protease activities were detected only in ECPs, confirming their exoenzymatic nature, whereas β-galactosidase seems to be a cell-associated enzyme. No significant differences were detected among the enzymatic patterns of ECPs from both biotypes. In addition, the API ZYM results correlate well with the specific hydrolytic properties exhibited by ECPs, as well as with previous studies using live cells (13, 14). Finally, all enzymatic activities were practically lost after heat treatment, suggesting the heat-labile nature of exoenzymes.

The API ZYM system has been proposed as a rapid and simply performed tool for the discrimination of related microorganisms which are difficult to separate by conventional biochemical tests (19). We have recently demonstrated a high level of phenotypic homology between the two biotypes of *V. vulnificus*, with indole production as the only biochemical trait that seems to distinguish them. In fact, the negative results for

TABLE 4. Susceptibilities of different fish species to *V. vulnificus* infection by i.p. inoculation

Strain	Colony type	LD_{50} (CFU/fish) for:				
		Warm-water fish			Cold-water fish	
		Eel	Sea bream	Sea bass	Trout	Turbot
Biotype 2						
E22	Opaque	7.2×10^2	ND[a]	ND	$>10^8$	1.7×10^6
E86	Opaque	3.8×10^1	$>10^8$	2.1×10^5	$>10^8$	3.0×10^5
ATCC 33149	Opaque	4.2×10^4	ND	ND	$>10^8$	ND
NCIMB 2137	Translucent	1.2×10^4	ND	ND	$>10^8$	$>10^8$
Biotype 1						
ATCC 27562	Opaque	$>10^8$	ND	ND	$>10^8$	$>10^8$
CDC7184	Opaque	$>10^8$	$>10^8$	$>10^8$	$>10^8$	$>10^8$
E109	Opaque	$>10^8$	ND	ND	$>10^8$	$>10^8$
TW1	Translucent	$>10^8$	ND	ND	$>10^8$	$>10^8$

[a] ND, not determined.

biotype 2 strains with this test are mainly responsible for their typical profiles in the API 20E system being different from those profiles presented by biotype 1 strains (10). To determine the utility of the API ZYM system for discrimination between biotypes, we examined and compared the API ZYM profiles of selected *V. vulnificus* strains (Table 3). Although both biotypes had similar enzymatic patterns, a difference in valine arylamidase activity was detected. Since few strains were studied, this difference could not be generalized at the biotype level. Thus, because of the similarities found in the enzymatic profiles of both biotypes in this system, we could not associate a typical pattern with either biotype. In fact, the general enzymes released by *V. vulnificus* cells were also similar to those previously reported for different species of the *Vibrionaceae* family (19). Therefore, the API ZYM system may not be suitable for biotype discrimination or for species identification.

Host range. Previous pathogenicity studies have shown that the two biotypes of *V. vulnificus* are virulent for mice and that only biotype 2 strains are pathogenic for eels (2, 5, 13, 14). In this work, our virulence study has been extended to other fish species in an attempt to determine other putative hosts for *V. vulnificus*. To this end, we carried out pathogenity assays using both warm- and cold-water fish species as well as selected strains of both biotypes. Eel susceptibilities to biotypes 1 and 2 were reassessed as a control for the virulence of strains. According to our previous reports, the LD_{50} of Spanish biotype 2 isolates are lower than those of Japanese eel isolates, with strain E86 being the most virulent one (LD_{50}, 3.8×10^1 CFU per fish) (Table 4). This isolate's strong lethality for eels correlates with its high-level production of factors related to the invasive potential detected among all biotype 2 ECPs. Thus, we chose strain E86 for selected experiments. The results in Table 4 show that *V. vulnificus* biotype 2 is able to infect other fish species of economic importance in marine aquaculture, such as turbot and sea bass, showing lethal doses higher (LD_{50}, 10^5 to 10^6 CFU per fish) than those observed for eels. These data are in accordance with those previously reported for other fish bacterial pathogens with the same fish species (17). The pathological signs observed in freshly dead fish were those corresponding to hemorrhagic septicemia. Pure cultures of the inoculated strains were recovered from the kidneys and livers of moribund fish. In contrast, biotype 2 cells failed to produce infection in sea bream and trout in assays using 10^8 CFU per fish (Table 4). This lack of virulence of biotype 2 cells for trout correlates with the previously reported sensitivity of these cells to the bactericidal action of fresh trout serum (13). For biotype 1 strains, pathogenicity assays resulted in 0% mortalities for all fish species assayed (Table 4). These results are consistent with our previous observations of adhesion studies to cell lines, showing that while both biotypes were able to adhere to mammalian cells, only biotype 2 strains were adherent to fish cells, suggesting the production of specific adhesins (2).

The overall data presented here demonstrate that *V. vulnificus* biotype 2 is able to infect both warm- and cold-water fish species. We have recently demonstrated its ability to cause infections in mice as well. This broad host range correlates with the wide temperature range for growth (20 to 40°C) reported for biotype 2 strains (7, 14). The recent implications of biotype 2 strains in septicemic human infections (37–39), together with our recent discovery of a clinical biotype 2 strain from the American Type Culture Collection (1), show that this biotype may also act as an opportunistic human pathogen. In contrast, biotype 1 strains are avirulent for fish, despite the fact that the ECPs of both biotypes exhibit high levels of both hydrolytic and cytotoxic potential, as well as a strong lethality for eels. Thus, the extracellular activities displayed by biotype 2 cells are not the only required determinant for the development of vibriosis in eels, although they may be valuable for the spread of this bacterium in the body of the host. These results confirm the previously observed similarities in virulence mechanisms exhibited by the two biotypes (5, 11, 13). Biotype 2 also shares many biochemical traits with biotype 1, but they differ in serology (2, 4, 12, 36). In fact, we have already proposed that biotype 2 constitutes a homogeneous serogroup, with the molecule responsible for this being LPS (14). This serogroup includes only the strains of this species which are pathogenic for eels. Since differences in cellular surface properties could be related to host specificity, further characterization of purified LPS is in progress.

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