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Vibrio vulnificus Biotype 2, Pathogenic for Eels, Is Also an Opportunistic Pathogen for Humans

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We report that the eel pathogen *Vibrio vulnificus* biotype 2 is also an opportunistic pathogen for humans. Results from a detailed comparative study using reference strains of both biotypes revealed that the clinical strain ATCC 33817, originally isolated from a human leg wound and classified as *V. vulnificus* (no reference on its biotype is noted), belongs to biotype 2 of the species. As a biotype 2 strain, it is negative for indole and pathogenic for eels and mice, harbors two plasmids of high M_rs, and belongs to serogroup E, recently proposed as characteristic of biotype 2 strains. In consequence, appropriate measures must be taken by consumers, particularly by those running a health risk, and by fish farmers, above all when manipulating eels during epizootic outbreaks.

The bacterial species *Vibrio vulnificus* includes two biotypes that have been defined on the basis of differences in biochemical and serological properties as well as in host range (24). Seawater is the normal habitat of biotype 1, where it is widespread but generally not numerous (13, 18, 19). This biotype can use humans as hosts and behave as an opportunistic pathogen capable of producing fatal disease after ingestion of raw shellfish or wound infection (17). Strains of biotype 1 are serologically heterogeneous and phenotypically similar to the type strain of the species (15, 24). The worldwide distribution of biotype 1 and its pathogenicity for humans is the reason that our knowledge of this species is based mainly on studies performed with biotype 1 strains. Thus, there are a considerable number of reports about both its isolation and the virulence mechanisms responsible for its pathogenicity for humans. The following virulence determinants have been characterized: (i) a polysaccharidic capsule that confers resistance to phagocytosis and serum complement, (ii) various iron uptake systems, including siderophore production and the ability to use hemoglobin and hemin as iron sources, and (iii) lesional factors such as exoenzymes and exotoxins, including a potent cytotoxin with hemolytic activity (12, 14, 20, 22, 23, 26).

On the other hand, biotype 2 seems to inhabit only eels under normal circumstances, given that it is reported to have been isolated only from diseased eels. This biotype includes the strains of the species pathogenic for eels and seems to constitute a serologically homogeneous group (10, 16, 24). Biotype 2 strains can also be differentiated from biotype 1 strains by their negative response to the indole test (1, 10, 24). This biotype seemed to be restricted to areas of Japan and Taiwan until we isolated it from diseased eels cultured in Spain in November 1989 (5). From that moment, we focused our investigations on (i) its biochemical and serological characterization and (ii) the determination of virulence factors responsible for its pathogenicity for eels. Surprisingly, this biotype turned out not to be an obligate eel pathogen, since it was able to survive outside its host and to use water as a route of infection (2). Moreover, it was also pathogenic for mice and able to express the same virulence factors as those related to pathogenicity for humans in biotype 1: capsule expression (9), siderophore production (7), ability to use hemin and hemoglobin as iron sources (4), and exotoxin production (1, 10).

All these data lead us to believe that biotype 2, like biotype 1, is an opportunistic pathogen for humans. This biotype had probably not been related to any clinical case previously because it was misidentified as biotype 1 because of the high phenotypic similarity between the two biotypes (1, 6, 10, 24). The consequence of this erroneous classification is most serious, given that biotype 2 is considered to be an obligate eel pathogen and no special measures are taken by fish farmers during epizootic outbreaks. In fact, in 1992, an indole-negative strain of *V. vulnificus* was isolated from a septicemic patient: a man was infected after manipulating contaminated eels (25). This finding seemed to confirm our suppositions.

To probe the hypothesis that biotype 2 could be an opportunistic pathogen for humans, we tested numerous clinical strains, classified as or considered to be biotype 1 strains, together with various strains of biotype 2 with a great number of biochemical and serological tests. The clinical strains serologically and biochemically similar to biotype 2 strains were further characterized by molecular analysis and virulence assays. We report here that *V. vulnificus* ATCC 33817, originally isolated from a patient with wound infection, is a strain belonging to biotype 2, capable of infecting eels. The results of our investigation are described in this article.

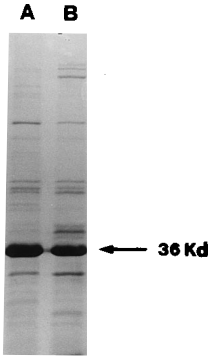

FIG. 1. OMP profiles of strains E22 (lane A) and ATCC 33817 (lane B). OMPs were extracted and stained as described by Biosca et al. (8).

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

Strain(s)[a]	Origin
Biotype 1	
Clinical	
ATCC 27562[T]	Blood, United States
ATCC 29306 (CDC A1402)	Corneal ulcer, United States
ATCC 33814	Blood, United States
ATCC 33815	Leg ulcer, United States
ATCC 33817 (CDC B3547)	Leg wound, United States
CDC C7184[b]	Human blood, United States
L-180[b]	Septicemia case, United States
VVL1[b]	Fatal wound infection, United States
374[b]	Septicemia case, United States
UMH1[b]	Fatal wound infection, United States
MO6-24 (FDA)[b]	Blood, United States
CDC H3308[b]	Clinical, United States
Environmental	
UNCC 890[b]	Environmental, United States
TW1[c]	Tank water from an eel farm, Spain
E109, E110, E112, E113, E114[c]	Surface of European eels, Spain
Biotype 2	
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[d]	Diseased Japanese eel, Taiwan
E4, E12, E22[c]	Diseased European eel, Spain (1989)
E24, E27, E32[c]	Diseased European eel, Spain (1989)
E37, E39, E40[c]	Diseased European eel, Spain (1990)
E52, E56, E58[c]	Diseased European eel, Spain (1990)
E80, E86, E92[c]	Diseased European eel, Spain (1990)
E103, E105, E106[c]	Diseased European eel, Spain (1990)
E112, E113, E116[c]	Diseased European eel, Spain (1992)

[a] ATCC, American Type Culture Collection, Rockville, Md.; CDC, Centers for Diseases Control and Prevention, Atlanta, Ga.; FDA, Food and Drug Administration, Cincinnati, Ohio; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland; T, type strain.
[b] Supplied by J. D. Oliver, University of North Carolina, Charlotte.
[c] See references 1, 5, 6, and 10.
[d] Supplied by Y.-L. Song, National Taiwan University, Taipei.

TABLE 2. API 20E profiles, indole production, and ODC activities of *V. vulnificus* strains

Strain(s)[a]	API 20E profile	Indole	ODC	Serogroup E[b]
Biotype 1				
ATCC 27562[T], VVL1, UMH1, TW1	5146105	+	+	−
ATCC 29306, UNCC 890	5146005	+	+	−
ATCC 33814, CDC C7184, L-180, 374, CDC H3308, E110, E113, E114	5346105	+	+	−
ATCC 33815	5346145	+	+	−
MO6-24 (FDA)	4346105	+	+	−
E109	5346005	+	+	−
E112	5246105	+	−	−
Biotype 2				
ATCC 33149, UE516, E4, E12, E24, E27, E32, E52, E56, E58, E80, E92	5006005	−	−	+
NCIMB 2136	4206005	−	−	+
NCIMB 2137	5006004	−	−	+
NCIMB 2138	5206005	−	−	+
E22, E37, E39, E40, E86	5206005	−	−	+
E103, E105, E106, E112, E113, E116	5306005	−	+	+
ATCC 33817	5306005	−	+	+

[a] E103, E105, E106, E112, E113, and E116 were isolated in epizootics on a Spanish eel farm occurring at low salinities (around 0.3% NaCl).
[b] Determined by slide agglutination using O antigens and antisera against whole cells of the *V. vulnificus* biotype 2 strain E22. +, positive agglutination in less than 5 min; −, negative agglutination.

The strains used in this work are listed in Table 1. The phenotypic traits studied are shown in Tables 2 and 3. Strain ATCC 33817 was the only clinical isolate that agglutinated with the antiserum against whole cells of the biotype 2 strain E22. This clinical isolate was indole negative and ornithine decarboxylase (ODC) positive, as were the strains of biotype 2 from the Spanish farm recovered during the outbreaks registered at low salinities. We recently reported that biotype 2 strains are variable for ODC and homogeneously negative for indole production (10). ATCC 33817 also showed a typical biotype 2 profile in the API 20E system (6). This profile was identical to the one presented by the eel strains mentioned above. In the other phenotypic traits shown in Table 3, the clinical isolate turned out to be very similar to clinical and environmental isolates of both biotypes. This isolate was further analyzed by molecular characterization, and the results were compared with the ones previously published by us (3, 8, 10).

In previous reports we demonstrated that all biotype 2 strains belonged to the same serogroup, serogroup E, and presented the same lipopolysaccharide (LPS) and similar outer membrane protein (OMP) profiles, with the surface molecule LPS being responsible for the serological specificity (3, 8, 10). LPS and OMPs were extracted and electrophoresed as previously described (3, 8). LPS was revealed by immunoblotting with polyclonal antiserum against strain E22, and OMPs were stained with Coomassie brilliant blue R250 (Sigma). As can be seen in Fig. 1, the OMP profile of this clinical isolate was similar to the one presented by the selected eel isolate (Fig. 1). This strain also presented a major OMP of around 36 kDa and minor bands similar to those previously reported by us for biotype 2 isolates (8). Moreover, LPS from this strain was immunostained with antiserum against the eel isolate E22 and presented a similar ladder-like structure (Fig. 2). This result demonstrates that the clinical isolate ATCC 33817 belongs to serogroup E.

We have recently demonstrated that strains of biotype 2 harbored two plasmids of high M_rs whereas biotype 1 strains generally lacked these extrachromosomal elements (10). The absence of plasmids among biotype 1 strains has been con-

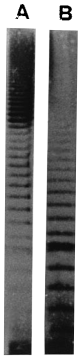

FIG. 2. LPS profiles of strains E22 (lane A) and ATCC 33817 (lane B). LPSs were extracted and immunostained with rabbit antiserum against strain E22 as described by Amaro et al. (3).

TABLE 3. Comparison of phenotypes of strain ATCC 33817 and reference strains of biotypes 1 and 2[a]

Characteristic	ATCC 33817	Biotype 2			Biotype 1		
		Spanish		Japanese, ATCC 33149	Environmental		Clinical, ATCC 27562
		E22	E105		E109	TW1	
Gram stain	−	−	−	−	−	−	−
Cytochrome oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+
ONPG test	+	+	+	+	+	+	+
Indole production	−	−	−	−	+	+	+
Polar flagellation	+	+	+	+	+	+	+
Colonial dimorphism	+	+	+	+	+	−	+
Swarming	−	−	−	−	−	−	−
O/F test	OF	OF	OF	OF	OF	OF	OF
Voges-Proskauer	−	−	−	−	−	−	−
Sensitivity to O/129	+	+	+	+	−	−	+
Production of:							
Gas from glucose	−	−	−	−	−	−	−
H_2S	−	−	−	−	−	−	−
Pigment	−	−	−	−	−	−	−
ADH (Moeller's)	−	−	−	−	−	−	−
LDC (Moeller's)	+	+	+	+	+	+	+
ODC (Moeller's)	+	−	+	−	+	+	+
Growth with NaCl (%)							
0	−	−	−	−	−	−	−
0.5	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+
8	−	−	−	−	−	−	−
Growth at temp (°C):							
37	+	+	+	+	+	+	+
40	+	+	−	+	+	+	+
42	NT	−	−	−	−	−	+
Growth on TCBS agar[b]	+	+	+	+	+	+	+
Acid from:							
D-Amigdaline	+	+	+	+	+	+	+
L-Arabinose	−	−	−	−	−	−	−
Arbutin	+	+	+	+	+	+	+
D-Cellobiose	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+
Lactose[c]	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+
D-Melibiose	−	+	+	+	+	+	−
D-Raffinose	−	−	−	−	−	−	−
L-Rhamnose	−	−	−	−	−	−	−
Sucrose	−	−	−	−	−	−	−
D-Salicin	+	+	+	+	+	+	+
D-Trehalose	+	+	+	+	+	+	+
D-Xylose	−	−	−	−	−	−	−
Adonitol	−	−	−	−	−	−	−
Dulcitol	−	−	−	−	−	−	−
Glycerol	+	+	+	+	+	+	+
meso-Inositol	−	−	−	−	−	−	−
D-Mannitol	−	−	−	−	−	+	+
D-Sorbitol	−	−	−	−	−	−	−
Starch	+	+	+	+	+	+	+
Hydrolysis of:							
Gelatin	+	+	+	+	+	+	+
Mucin	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+
Chitin	+	+	+	+	+	+	+
Phospholipids	+	+	+	+	+	+	+
Human erythrocytes	+	+	+	+	+	+	+

[a] ONPG, β-galactosidase; O/F, oxidative/fermentative metabolism; ADH, arginine dihydrolase; LDC, lysine decarboxylase; TCBS, TCBS cholera medium (Oxoid); NT, not tested.

[b] Green colonies.

[c] Positivity can be delayed for 14 days.

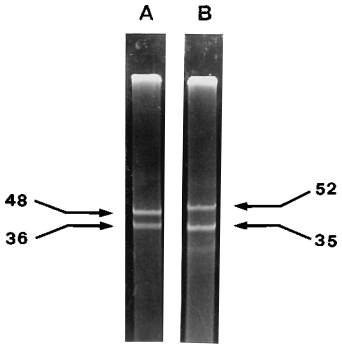

FIG. 3. Plasmid profiles of strains E22 (lane A) and ATCC 33817 (lane B). Plasmids were extracted as previously described (10). Molecular masses (in megadaltons) are indicated.

firmed by other authors (11). To examine whether the clinical isolate carried plasmids, we attempted plasmid extraction by the method of Kado and Liu slightly modified by Biosca et al. (10). As can be seen in Fig. 3, the clinical isolate harbored two plasmids of M_rs similar to those of the plasmids of Spanish isolates belonging to biotype 2.

In previous works we showed that all biotype 2 strains were virulent for mice and eels, with pathogenicity to eels being exclusive to this biotype (10). The 50% lethal doses (21) for strain ATCC 33817 injected into mice and eels by the intraperitoneal route (1) were found to be ca. 10^4 CFU per fish and 10^6 CFU per mouse, values similar to those reported for some slightly virulent biotype 2 strains (7, 10). As has previously been reported for both biotypes, this strain was iron responsive, with 10^3- to 10^4-fold decreases in the lethal doses for eels and mice made hyperferremic (4, 7). Biotype 1 strains ATCC 27562, E109, and TW1 were also tested and proved to be avirulent for eels (Table 4). Thus, as we expected, this clinical strain of serogroup E was virulent for eels.

The overall data demonstrate that the clinical isolate ATCC 33817 belongs to biotype 2. This isolate belongs to serogroup E, is negative for the indole test and virulent for eels and presents an LPS profile typical of biotype 2 strains and similar OMP and plasmid patterns. Therefore, the species *V. vulnificus* comprises phenotypically different strains; some of them can be opportunistic pathogens for humans, but only those belonging to serogroup E can colonize and develop infections in eels. Consequently, the LPS characteristic on a bacterial surface is correlated to pathogenicity for eels. Since biotype 2 can also be virulent for humans and can be transmitted by water (2), special measures must be taken by fish farmers, especially by those running a health risk, during epizootic outbreaks.

TABLE 4. Virulence of *V. vulnificus* strains for elvers and mice

Strain and colony type	50% lethal dose[a]	
	Elver	Mouse
Biotype 2, E22		
Opaque	4.1×10^2	7.2×10^5
Translucent	3.5×10^4	5.8×10^7
Biotype 1		
ATCC 27562, opaque	$>10^8$	NT
ATCC 33817		
Opaque	7.8×10^4	1.8×10^6
Translucent	NT	$>10^8$

[a] CFU per fish or mouse. NT, not tested.

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