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Vibrio vulnificus Biogroup 2: New Biogroup Pathogenic for Eels†

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Clinical and nonclinical isolates of the lactose-positive *Vibrio vulnificus* were compared with *Vibrio* strains isolated from lesions on eels (*Anguilla japonica*) cultured commercially in Japan. Strains were compared phenotypically and antigenically, for pathogenicity to mice and eels, and for genetic relatedness. The strains isolated from diseased eels differed phenotypically from the original species description of *V. vulnificus* in that they were negative for indole production, ornithine decarboxylase activity, growth at 42°C, and acid production from mannitol and sorbitol. No relationship between the surface antigens of *V. vulnificus* strains from environmental and clinical sources and the strains from diseased eels was observed. Typical *V. vulnificus* strains and the eel isolates were pathogenic to mice; however, only those strains originally isolated from diseased eels were found to be pathogenic to eels. Results of DNA-DNA competition experiments revealed that there was greater than 90% relative reassociation between clinical and nonclinical *V. vulnificus* and strains from diseased eels. Based on the results of the DNA-DNA competition experiments, we conclude that the strains isolated from diseased eels were *V. vulnificus*; however, the differences in phenotypic characteristics and eel pathogenicity indicated that these strains represent a different biogroup. Therefore, we propose that strains phenotypically similar to the type strain of the species (ATCC 27562) be classified as *V. vulnificus* biogroup 1 and the strains phenotypically similar to those isolated from diseased eels be classified as *V. vulnificus* biogroup 2 represented by the reference strain ATCC 33148.

Interest in the clinical and ecological significance of a halophilic, lactose-fermenting *Vibrio* strain has increased as this organism is increasingly recognized as an agent of septicemia resulting from the consumption of contaminated raw shellfish and wound infections after exposure to seawater (2, 13, 14). In the past, this organism has been referred to as lactose-positive *Vibrio* (11) and *Beneckea vulnifica* (28) and is currently classified as *Vibrio vulnificus* (1, 9).

Hollis et al. (11) have shown that *V. vulnificus* can be distinguished from other *Vibrio* spp. phenotypically. *V. vulnificus* has also been shown to be genetically distinct from other *Vibrio* spp. based on the results of DNA homology studies (6, 28). Clinical and environmental isolates of *V. vulnificus* have also been shown to be indistinguishable phenotypically and genetically (31).

A recent series of reports described a *Vibrio* strain pathogenic to eels cultured in Japan (20–25). Strains which were phenotypically and genotypically identical were isolated from diseased eels from six different localities in three different prefectures in Japan during seasonal outbreaks of vibriosis from 1975 to 1977. This organism was classified as *Vibrio anguillarum* type B (*V. anguillarum* f. *anguillicida*) as described by Nybelin (26), but since this name did not have valid taxonomic standing, a revival of the name *V. anguillicida* (4) was suggested (20, 24).

In this report, we compare clinical and environmental isolates of *V. vulnificus* to strains of the *Vibrio* eel pathogen. Based on the results of phenotypic comparisons, eel and mouse pathogenicity, and DNA-DNA hybridization studies, we conclude that these organisms are of the same species in the genus *Vibrio* and propose that the eel pathogen be classified as *V. vulnificus* biogroup 2.

MATERIALS AND METHODS

Bacterial cultures. Nine strains of *V. vulnificus* and four strains of *Vibrio* sp. isolated from diseased eels were used in this study. Bacterial strains and their sources are listed in Table 1. The *Vibrio* strains

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TABLE 1. Source of strains tested

Strain	Source
CDC B9629 (ATCC 27562)[a]	Human blood, Florida
CDC E9315	Clinical, Centers for Disease Control, Atlanta, Ga.
CDC A8694 (ATCC 29307)	Human blood, Florida
CDC B51	Human blood, Hawaii
80-02-125	Human blood, Texas
79-08-9	Seawater, Texas
79-11-114	Seawater, Texas
A38	Crab, Oregon
LSU 11-3M-30	Crab, Louisiana
ES-7601 (ATCC 33147)	Diseased eel, Japan
ET-7617 (ATCC 33148)	Diseased eel, Japan
KV-1 (ATCC 33149)	Diseased eel, Japan
ET-517	Diseased eel, Japan

[a] Type strain of *V. vulnificus*.

isolated from diseased eels are representative of 24 strains which have been extensively characterized (20–25; M. Nishibuchi, M.A. thesis, Hiroshima University, Hiroshima, Japan, 1979). The selected strains were isolated from different localities during two seasonal outbreaks of vibriosis in eel culture ponds in Japan.

Biochemical characteristics and morphological examinations. Test cultures were grown on tryptic soy agar (Difco Laboratories) for approximately 24 h before inoculation into test media. All test media contained 0.5% NaCl except the lecithinase (egg yolk) agar, which was amended to 1.5% NaCl, and the peptone water for the NaCl tolerance test. Since usual incubation temperatures for fish and human pathogens are 20 to 25°C and 35 to 37°C, respectively, all biochemical tests were performed at both 25 and 35°C except where otherwise indicated. Cultures used for examination of motility and flagella morphology were grown in tryptic soy broth without dextrose (Difco) for approximately 8 h at 25°C before examination. Flagellar morphology was determined by microscopic examination of stained specimens (Leifson flagella stain [27]) and confirmed for selected strains by shadow casting with platinum-palladium (80:20) through a Phillips EM-300 transmission electron microscope. Motility was tested by the hanging-drop method after passage of test organisms through tryptic soy broth (Difco) amended with 0.3% agar as described by Craigie (8). Gas from glucose was tested in MR-VP broth (32). Sensitivity to 2,4-diamino-6,7-diisopropyl pteridine phosphate (0/129) (Sigma Chemical Co.) was tested by the method of Seidler et al. (29). Sensitivity to novobiocin was tested on heart infusion agar (Difco) treated with 5-μg disks (Difco). Production of 2,3-butanediol was tested by the method of Bullock (5), Tween 80 hydrolysis was carried out by the method of Sierra as described by Cowan (7), and lecithinase activity on blood agar base was amended to 1.5% NaCl and 10% egg yolk emulsion (Oxoid Ltd.). Luminescence was tested by visual examination of growth on luminescence agar for vibrios, based on the medium described by Furness et al. (10). Casein hydrolysis was tested on a two-layer medium as described by Sizemore and Stevenson (30), with tryptic soy agar used for the upper layer. Carbohydrate fermentations were tested in Hugh-Leifson O/F medium (Difco) amended with 1% of the given carbohydrate. Inoculated media were overlaid with sterile mineral oil, and results were observed for up to 14 days. NaCl tolerance was tested in 1% peptone water amended with given concentrations of NaCl. Growth at 5, 37, and 42°C was tested in 1% peptone water containing 0.5% NaCl. Hemolysis of horse blood was tested on blood agar base (Difco) with 10% fresh erythrocytes. All other traits were tested by conventional methods by procedures outlined by Lennette et al. (16). Selected methods and media are given in parentheses in Table 2.

Mouse LD_{50}s. Test organisms were grown in tryptic soy broth without dextrose (Difco) supplemented with an additional 0.5% sodium chloride (TSB') for 10 h at 25°C. Tenfold dilutions were made in 0.01 M phosphate-buffered physiological saline (pH 7.2), and each diluted cell suspension was injected intraperitoneally into five Swiss-Webster mice weighing 17 to 20 g each. After 72 h, mortality was recorded, and 50% lethal doses (LD_{50}s) were determined by the method of moving averages (19). Common log LD_{50}s were compared by a t test, using a pooled sample estimator of population variance (18).

Suckling mouse assay. Test organisms were grown in TSB' at 37°C for 18 h on a reciprocal shaker. A 0.1-ml amount of the bacterial culture in 0.01% Evans blue dye was orally administered to five 3-day-old mice, strain CF1. Inoculated animals were incubated on a white filter paper at 25°C for 18 h. At the end of the incubation, mortality was recorded and the number of feces stained with Evans blue dye was counted as an indicator of diarrhea.

Pathogenicity for eels. Eel (*Anguilla japonica*) pathogenicity tests were performed as described by Muroga et al. (21).

Serology. Rabbit antisera were prepared against Formalin-killed cells of representative strains (three human, three environmental, and three eel isolates) by the method described earlier (23). The rapid slide agglutination test was carried out as previously reported (25).

DNA studies. Cells were grown in TSB' for 24 h at 37°C on a reciprocal shaker, harvested by centrifugation at 15,000 × g, washed with 0.01 M phosphate-buffered physiological saline (pH 7.2), and stored frozen until DNA extraction was done. DNA was extracted and purified with phenol by conventional techniques previously published (12). Labeled reference DNA was prepared by growing cells in 200 ml of TSB' plus 5 mCi of [^{3}H]thymidine (New England Nuclear Corp.). Cultures were incubated for 18 h at 37°C on a shaker. Cells were harvested and the DNA was purified as described above. Purified ^{3}H-labeled reference DNA had a specific activity of 4×10^4 to 5×10^4 cpm/μg of DNA.

The guanine-plus-cytosine DNA base composition was determined by the thermal melting technique, employing the relationship between midpoint temperature (T_m) and percent guanine plus cytosine described by Mandel et al. (17). DNA competition experiments were done by the membrane filter technique (12). Renaturation buffer consisted of 2× SSC (1× SSC = 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7) plus 40% (vol/vol) dimethyl sulfoxide. Renaturation was carried out at a temperature 15°C below the DNA

TABLE 2. Biochemical and morphological characteristics of *V. vulnificus* and *Vibrio* sp. isolated from diseased eels

Test	*V. vulnificus* biogroup (no.)	
	1 (9)	2 (3)
Shape	Short rod	Short rod
Single polar flagellum (Leifson)	+[a]	+
Motility	+	+
Gram stain (modified Huckers's)	−[b]	−
Acid fastness (Ziehl-Neelsen)	−	−
Fermentation of glucose (Hugh-Leifson)	+	+
Gas from glucose	−	−
Oxidase	+	+
Catalase	+	+
0/129 Sensitivity	+	+
Novobiocin sensitivity	+	+
Litmus milk	+	+
Nitrate reduction	+	+
Gelatin liquefaction	+	+
Indole production (Kovaks)	+	−
Voges-Proskauer	−	−
2,3-Butanediol	−	−
Methyl red	+	+
Hydrogen sulfide production (SIM)	−	−
Arginine dihydrolase (Moeller)	−	−
Lysine decarboxylase (Moeller)	+	+
Ornithine decarboxylase (Moeller)	d[c] (89[d])	−
Phenylalanine deaminase	−	−
Urease	−	−
Citrate (Simmons's)	+	+
Tartrate	+	+
Malonate	−	−
Starch hydrolysis	+	+
Tween 80 hydrolysis	+	+
Lecithinase (egg yolks)	+	+
Luminescence	−	−
Casein hydrolysis	+	+
Acid from		
D-Fructose	+	+
D-Galactose	+	+
D-Glucose	+	+
D-Mannose	+	+
Maltose	+	+
Trehalose	+	+
Dextrin	+	+
Starch	+	+
Glycogen	+	+
Cellobiose	+	+
Melibiose	+	(+)[e]
Salicin	+	+
D-Amygdalin	+	+
L-Arabinose	−	−
Sucrose	−	−
D-Mannitol	d (44)	−
Glycerol	+	(+)
i-Inositol	−	−
D-Sorbitol	d (44)	−
Lactose	+	(+)

TABLE 2—*Continued*

Test	*V. vulnificus* biogroup (no.)	
	1 (9)	2 (3)
Inulin	−	−
L-Rhamnose	−	−
Raffinose	−	−
D-Xylose	−	−
Adonitol	−	−
Dulcitol	−	−
NaCl tolerance (%)		
0	−	−
0.5	+	+
3	+	+
5	+	(+)
7	−	−
10	−	−
Growth at (°C)		
5	−	−
37	+	+
42	d (44)	−
Hemolysis of horse blood	+	+

[a] Total of >90% positive.
[b] Total of <10% positive.
[c] Total of 11 to 89% positive.
[d] Percent positive.
[e] Positive reaction in >3 days.

thermal denaturation temperature as measured in the renaturation buffer.

RESULTS AND DISCUSSION

There were no differences in the results of biochemical tests run at 25 and 37°C, except for the fact that some positive reactions were observed earlier with the cultures incubated at 25°C (Table 2).

The phenotypic characteristics tested were essentially the same as those examined by Muroga, et al. (21) in previous work on the eel isolates. Results obtained with the three eel isolates (ES-7601, ET-7617, and KV-1) shown in Table 2 agreed with those previously reported (21) and with those of a subsequent examination of 24 isolates from diseased eels (Nishibuchi, M.A. thesis), except for acid production from lactose and glycerol, which had been reported as negative. These discrepancies may be explained by the difference in methodologies used. Also, eel isolates are slow, weak lactose fermentors (delayed 5 to 10 days), as are some of the *V. vulnificus* strains (unpublished data).

The major phenotypic difference observed between the strains was that the eel isolates were negative for indole production, whereas the *V. vulnificus* strains were indole positive. Other differences (Table 2) were ornithine decarboxylase activity, growth at 42°C, and acid production from mannitol and sorbitol by *V. vulnificus*, whereas the eel isolates were consistently negative for these traits.

Mouse LD_{50}s. Preliminary experiments showed that three animal passages through mice reduced the LD_{50} of *V. vulnificus* B51 by only 1 log. Therefore, tests were performed with cultures stored at −80°C without animal passage.

TABLE 3. Mouse LD_{50}s of *V. vulnificus* from clinical and environmental sources and from diseased eels

Origin	Strain	LD_{50} (CFU[a])
Clinical	E9315	3.40×10^6
	B51	3.80×10^5
	A8867	8.59×10^6
	80-02-125	3.50×10^7
Environmental	79-08-9	2.77×10^8
	A38	2.69×10^7
	LSU 11-3M-30	1.00×10^8
	CL-0-010	2.45×10^7
Diseased eel	ES-7601	9.27×10^4
	ET-7617	2.47×10^6
	KV-1	4.27×10^6

[a] CFU, Colony-forming units.

TABLE 4. Results of suckling mouse assay for *V. vulnificus* from clinical and environmental sources and from diseased eels

Origin	Strain	Suckling mouse assay	
		% Mortality	Stained feces[a]
Clinical	E9315	40	−
	B51	80	−
	80-02-125	20	++
Environmental	79-08-9	40	−
	A38	100	−
	LSU 11-3M	40	+
Diseased eel	ES-7601	100	+
	ET-7617	100	−
	KV-1	100	−

[a] Stained feces excretion from five animals was recorded as negative (−), positive (+), and strongly positive (++) for zero to one, two to three, and four or more spots, respectively, of stained feces.

Equal numbers of male and female mice were employed, but there was no effect of sex on LD_{50}s when tested by factorial analysis ($P < 0.001$), and hence the data were pooled to calculate LD_{50}s (Table 3). LD_{50}s of clinical strains of *V. vulnificus* were on the order of 10^5 to 10^7 colony-forming units, in agreement with a previous report (15). Statistical analysis revealed that LD_{50}s of the clinical stains of *V. vulnificus* were indistinguishable from those of the strains isolated from diseased eels. The former and latter values, however, were significantly different ($0.025 < P < 0.050$ and $0.005 < P < 0.010$, respectively) from those of the environmental strains of *V. vulnificus*.

Suckling mouse assay. The results of suckling mouse assays are shown in Table 4. All of the tested strains of *V. vulnificus* of clinical and environmental origin and the strains isolated from diseased eels caused mortality in suckling mice. The highest incidence of mortality was achieved by the eel isolates. One of three test strains in each group induced stained feces, indicative of diarrheal symptoms. These results suggest that there may be strain-to-strain differences in the pathogenicity for suckling mice among these three groups.

Pathogenicity for eels. Results of pathogenicity tests for eels are shown in Table 5. Neither clinical nor environmental isolates of *V. vulnificus* showed evidence of pathogenicity for eels (Table 5), whereas eel isolate ET-7617 was pathogenic, as previously reported (21).

Serology. Results of rapid slide agglutination tests to compare surface antigens are shown in Table 6. In most cases, weak reactions occurred, but only a strong reaction was recorded as positive. The results indicate that surface antigens of *V. vulnificus* strains were heterologous. Positive reactions were observed with the strains tested against their homologous antisera and between strains E9315 and 80-02-125. Although strain 79-08-9 agglutinated with heterologous antisera, the reciprocal reactions did not occur. On the other hand, the surface antigens of *Vibrio* isolates from diseased eels were shown to be homologous, as previously reported (25). There appears to be no strong relationship between the surface antigens of *V. vulnificus* strains and eel isolates. (Although *V. vulnificus* strain A38 agglutinated with antisera against eel isolates, the reciprocal reactions were not observed.) However, *V. vulnificus* and the eel isolates shared many soluble intracellular antigens, including an antigen specific to *V. vulnificus* and the eel isolates but not to other *Vibrio* spp. when examined by Ouchterlony immunodiffusion and one- and two-dimensional immunoelectrophoresis (M. Nishibuchi and R. J.

TABLE 5. Pathogenicity for Japanese eel (*A. japonica*) of *V. vulnificus* from clinical and environmental sources and from diseased eels[a]

Origin	Strain	Dose (CFU[b] × 10^8)	Pathological symptom in eels	
			% Mortality	Red swelling or necrosis or both at injection site
Clinical	B51	1.02	0	−
	ATCC 27562	4.45	0	−
Environmental	79-08-9	4.10	0	−
	LSU-11-3M-30	7.55	0	−
Diseased eel	ET-7617	4.85	80	+

[a] Bacterial culture grown on nutrient agar (Eiken Co., Tokyo, Japan) at 25°C for 24 h was suspended in 0.85% saline. One milliliter of appropriately diluted bacterial cell suspension was injected intramuscularly into five healthy eels. Injected fish were kept in aquaria at 25°C and observed for evidence of pathogenicity for 1 week.
[b] CFU, Colony-forming units.

TABLE 6. Comparison of surface antigens of *V. vulnificus* from clinical and environmental sources and from diseased eels by rapid agglutination test

Agglutinin[a]		Agglutination with rabbit antiserum prepared against[b]:								
		V. vulnificus						Eel strains of *V. vulnificus*		
Source	Strain	E9315	B51	A8867	80-02-125	79-08-9	A38	ET-517	KV-1	ET-7617
Clinical	E9315	+[c]	−	−	+	−	−	−	−	−
	B51	−	+	−	−	−	−	−	−	−
	A8867	−	−	+	−	−	−	−	−	−
Environmental	80-02-125	+	−	−	+	−	−	−	−	−
	79-08-9	−	+	−	+	+	−	−	−	−
	A-38	−	−	−	−	−	+	+	+	+
Diseased eel	ET-517	−	−	−	−	−	−	+	+	+
	KV-1	−	−	−	−	−	−	+	+	+
	ET-7617	−	−	−	−	−	−	+	+	+

[a] Bacterial cells grown on tryptic soy agar (Difco) supplemented with 0.5% sodium chloride were suspended in phosphate-buffered saline (pH 7.2).
[b] Antisera were prepared against Formalin-killed cells.
[c] The only strong reaction occurring in 1 min was recorded as positive (+).

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DNA studies. Previously reported DNA base compositions of 45.7 to 47.8 mol% guanine plus cytosine for the *V. vulnificus* strains (31) and the eel isolates (24) were reconfirmed in this study. The results of DNA-DNA competition experiments (Table 7) indicate that there was greater than 90% relative reassociation at stringent temperature (T_m − 15°C) between the eel isolates and *V. vulnificus* strains of clinical and environmental origin. This level of reassociation indicates that these strains are of the same species (3).

Taxonomic considerations. The results of the DNA-DNA competition experiments clearly indicate that the strains isolated from diseased eels are *V. vulnificus*. However, there were phenotypic differences between the eel isolates and typical *V. vulnificus* strains, the major ones being that strains isolated from diseased eels were consistently indole and ornithine decarboxylase negative, whereas clinical and environmental strains of *V. vulnificus*, as described in the original species description (28), were indole positive and generally ornithine decarboxylase positive. There were also differences in the pathogenicity. Although all strains were lethal to mice, only those strains originally isolated from diseased eels were found to be pathogenic to eels. *V. vulnificus* strains of human origin and phenotypically identical strains isolated from estuarine environments produced neither swelling nor necrosis at the site of injection, nor mortality of eels.

Based on the phenotypic differences and the differences in host range, we conclude that these strains represent different biogroups of *V. vulnificus*. Therefore, we propose that those strains classified as *V. vulnificus* in the original description of the species (28) be classified as *V. vulnificus* biogroup 1 (type strain ATCC 27562), whereas those strains phenotypically resembling the eel isolates be classified as *V. vulnificus* biogroup 2 (reference strain ATCC 33148, originally submitted as *Vibrio* sp.).

TABLE 7. Reassociation of *V. vulnificus* DNA with the *V. vulnificus* eel isolate DNA at stringent temperature (T_m − 15°C)

Competitor DNA from eel isolate	% Relative reassociation of *V. vulnificus* strain[a]:		
	B51	CDC B9629 (ATCC 27562)	11-3M-30
ES-7601	106	94	100
ET-7617	103	93	97
KV-1	104	91	99

[a] Source of labeled reference DNA.

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