Highly Invasive New Bacterium Isolated From US East Coast Waters

National Institutes of Health–supported research scientists warn that physicians in mid-Atlantic coastal areas (and perhaps in other coastal and salt water areas as well) should be aware of an unusually virulent marine bacterium, *Vibrio vulnificus.*

This organism—which is associated with a high incidence of septicemia and high mortality (>40%), especially among persons with chronic liver disease or other dysfunction that increases serum iron levels—has been found to be ubiquitous, both geographically and in a variety of environmental sources.

James D. Oliver, PhD, and colleagues in the Department of Biology, University of North Carolina at Charlotte, working with support from the National Institute of Allergy and Infectious Diseases, recently isolated 3,887 sucrose-negative vibrios from seawater, sediment, plankton, and animal samples taken from 80 sites ranging from Miami to Portland, Me. Of these, 4.2% were able to ferment lactose, a taxonomic trait by which these organisms have been differentiated from other vibrios (*Appl Environ Microbiol* 1983;45:985-998).

In this study, 33 isolates, representing 20% of all lactose-fermenting vibrios, were almost identical phenotypically to clinical strains of *V vulnificus* studied by the Centers for Disease Control (CDC), Atlanta, and by Dr Oliver's laboratory. Their identification was confirmed by DNA-DNA hybridization studies.

Vibrio vulnificus was isolated from water, sediment, plankton, and animals. Comparison of the environmental parameters of the 80 subsites showed no substantial differences. The majority of the isolates were obtained from animals, mainly oysters and clams (84%).

On intraperitoneal injection into mice, 82% of the *V vulnificus* isolates caused death within 2½ to five hours (*Appl Environ Microbiol* 1983;45:985-998). Lethality to animals when injected subcutaneously is unique among vibrios and does not occur even with *Vibrio cholerae* (*Infect Immun* 1978;20:126-129). Another unique sequela was the great amount of fluid accumulation after cutaneous entry of the bacterium into laboratory mice. The hematocrit value of a mouse, normally about 45%, rose rapidly to 65% to 70%, reflecting a loss both of blood fluid and of blood proteins (*Infect Immun* 1981;32:1193-1199).

The investigators confirmed an intestinal route of entry to the blood when they found no contamination of the peritoneum in studies of ligated rat and rabbit ileal loops inoculated with live *V vulnificus* (*Marine Technol Soc J* 1981;15:45-52) (Figs 1 through 3). Injection into ileal loops rapidly produced high-density bacteremia and death, Dr Oliver's team found (*Infect Immun* 1978;20:126-129).

Although *V vulnificus* occurs in relatively low numbers, this halophilic bacterium may be the most invasive of the vibrio species. Infections in humans are almost always seawater associated and generally occur after contamination of a skin lesion or after ingestion of seafood. Incubation is rapid, and symptoms appear, on the average, in about 16 hours.

Two clinical forms of the disease have been recognized. One is fulminating septicemia, in which *V vulnificus* is isolated from blood cultures and, occasionally, from secondary skin lesions. Severe hypotension and shock may result. Indeed, 11 of 24 cases recorded by the CDC in 1979 were fatal, often within two or three days of onset (*N Engl J Med* 1979;300:1-5).

Apparently caused by *V vulnificus* entering through the gastrointestinal tract, this form of the disease occurs primarily in patients with preexisting hepatic disease, commonly characterized by abnormally high levels of serum iron. Iron overload, in turn, might predispose to infection since increasing the availability of this element is known to increase susceptibility to bacterial disease. Withholding iron from bacteria by host proteins, in contrast, produces a type of "nutritional immunity" (*Infect Immun* 1983;41:644-649). Persons with higher than normal iron levels (eg, with chronic cirrhosis, hepatitis, thalassemia major, or hemochromatosis) are especially vulnerable to active bacterial invasion and to potentially fatal bacteremia, the investigators warned (*Infect Immun* 1981;34:503-507).

The other form of *V vulnificus* infection is a rapidly progressive cellulitis resulting from infection of seawater-associated wounds such as those sustained while cleaning

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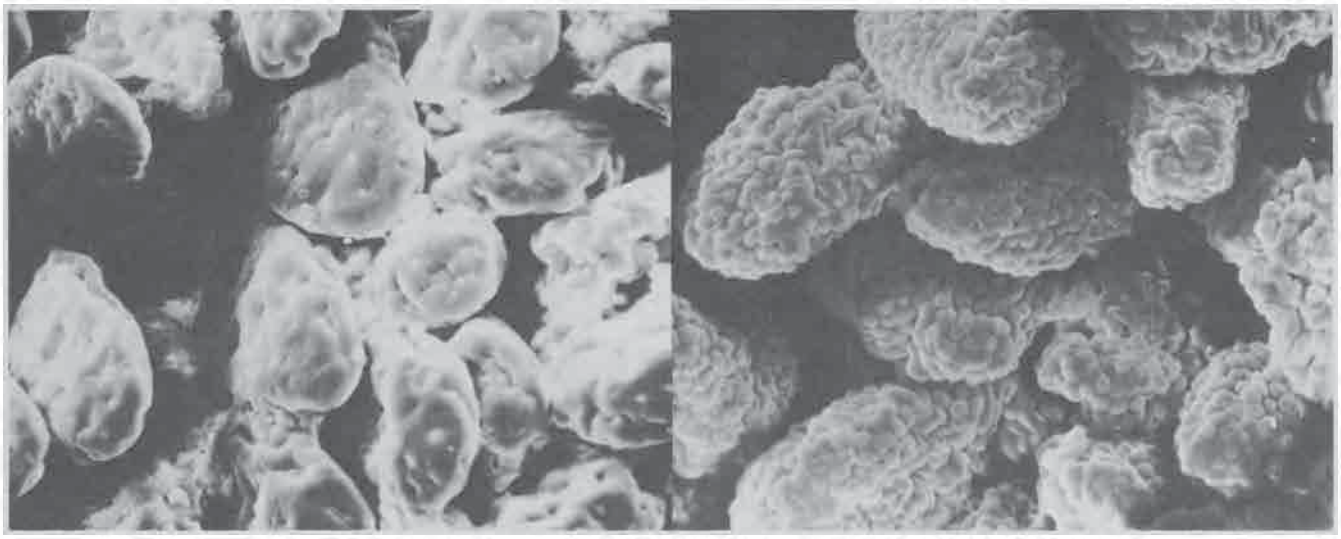
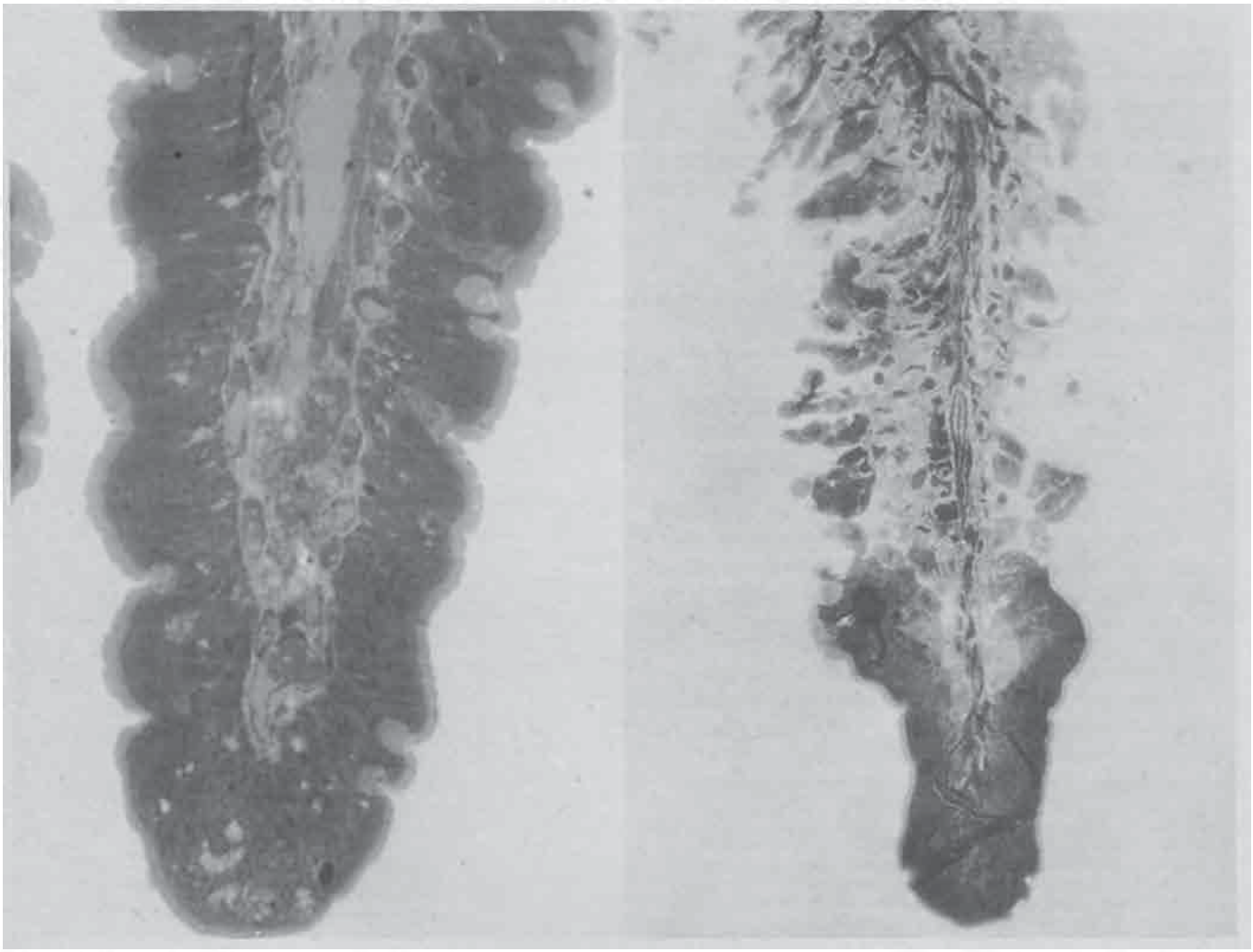


Fig 1.—Scanning electron micrographs of rabbit ileum villi. Left, Control (×320). Right, Experimental, after one hour in presence of *Vibrio vulnificus*. Note development of numerous "blebs" at villi surfaces (×320).

Fig 2.—Light micrographs of rabbit ileum villi. Left, Control (×460). Right, Experimental, after nine hours in presence of *Vibrio vulnificus*. Note extensive degradation of villus (×230).



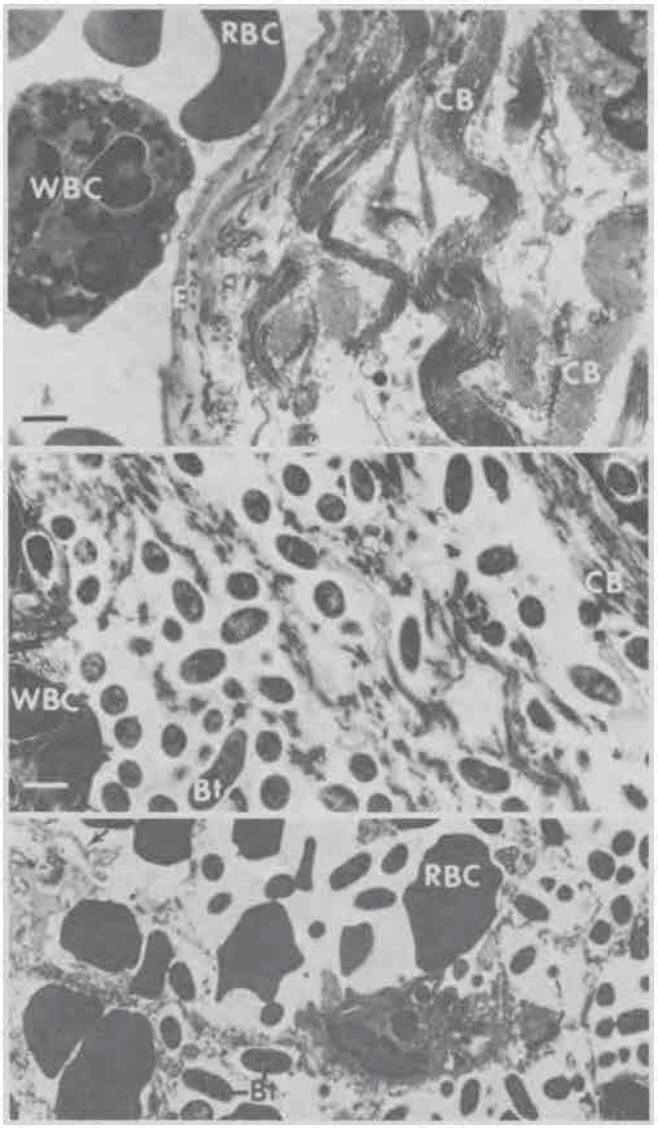


Fig 3.—Transmission electron micrograph of rabbit submucosa. Top, Phosphate-buffered saline-injected control loop tissue shows collagen bundles (CB) and endothelial layer (E) surrounding blood vessel containing WBCs and RBCs. Center, *Vibrio vulnificus*-injected loops show remnants of collagen bundles (CB) and massive invasion by gram-negative bacteria (Bt). Bottom, Numerous RBCs are seen not contained within vessel, suggesting breakdown of endothelial tissue. Vestiges of cytoplasmic components of endothelial cells are seen (arrow). Gram-negative bacteria (Bt) are prevalent within tissue.

shellfish or harvesting oysters and crabs. This form typically occurs in people who seem to be healthy and is characterized by marked edema and necrosis requiring incision, drainage or débridement, and antibiotic therapy. This form of infection is also characterized, on occasion, by septicemia. Cutaneous infections on the extremities frequently result in limb amputations.

Although an association has been established between this infection and the ingestion of raw seafood, especially oysters, properly chilled oysters may not pose a hazard to healthy persons, since chilling greatly reduces the number of bacteria. However, even a small number of surviving *V vulnificus* may pose a serious health hazard to persons with elevated serum iron levels (*Appl Environ Microbiol* 1981;41:710-717).

Clinical studies by other investigators have shown that physicians presented with a case of *V vulnificus* infection should use penicillin or tetracycline, since the bacterium is susceptible to most antibiotics. Time is an essential consideration since the infection spreads so rapidly (*South Med J* 1983;76:296-303).

First reported in 1976, *V vulnificus* was originally known to the CDC as the "lactose-positive" vibrio. Since 1978, the CDC has received more than 100 *V vulnificus* cultures for identification, mostly taken from humans within the United States, although some came from environmental sources and from abroad. As state health departments become more proficient at identifying the bacterium, fewer isolates are referred to the CDC, a spokesman said. As of May 1983, only two *V vulnificus* cultures had been sent to the CDC this year. *Vibrio vulnificus* infections are reported most frequently in the United States during the warm months, May to October.

Current studies are directed at understanding the remarkable invasiveness not only of this pathogen, but of numerous other species of lactose-fermenting vibrios in the marine environment, some of which seem to be potentially pathogenic to man (abstract from the Third International Symposium on Microbial Ecology, August 1983). In addition, Dr Oliver and colleagues are examining the involvement of plasmids in mediating the phenotypic and virulence traits of these bacteria.

For more information on *V vulnificus* research, contact James D. Oliver, PhD, Department of Biology, University of North Carolina at Charlotte, Charlotte, NC 28223; telephone (704) 597-4049.

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Distribution of *Vibrio vulnificus* and Other Lactose-Fermenting Vibrios in the Marine Environment

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During the summer of 1981, 3,887 sucrose-negative vibrios were isolated from seawater, sediment, plankton, and animal samples taken from 80 sites from Miami, Fla., to Portland, Maine. Of these, 4.2% were able to ferment lactose. The lactose-positive strains isolated from the various samples correlated positively with pH and turbidity of the water, vibrios in the sediment and oysters, and total bacterial counts in oysters. Negative correlations were obtained for water salinity. Numerical taxonomy was performed on 95 of the lactose-fermenting environmental isolates and 23 reference strains. Five clusters resulted, with the major cluster containing 33 of the environmental isolates and all of the *Vibrio vulnificus* reference strains. The 33 isolates, which produced an acid reaction in lactose broth within hours of initial inoculation, represented 20% of all lactose-fermenting vibrios studied. These isolates were nearly identical phenotypically to clinical strains of *V. vulnificus* studied by the Centers for Disease Control, Atlanta, Ga., and by our laboratory, and their identification was confirmed by DNA-DNA hybridization studies. *V. vulnificus* was isolated from all sample types and from Miami to Cape Cod, Mass., and comparison of the environmental parameters of the eight subsites yielding this species with those of all 80 subsites revealed no significant differences. The majority of the isolates were obtained from animals, with clams providing most (84%) of these. On injection into mice, 82% of the *V. vulnificus* isolates resulted in death. Members of the remaining four clusters contained strains which differed from *V. vulnificus* in such phenotypic traits as luminescence and in urease or H_2S production. None of the other reference cultures, including nine other *Vibrio* species, were contained in the remaining clusters, and these isolates could not be identified. Most of these were also lethal for mice. Phenotypic differences, potential pathogenicity, and geographic distribution of the five clusters were examined. It is concluded that *V. vulnificus* is a ubiquitous organism, both geographically and in a variety of environmental sources, although it occurs in relatively low numbers. The public health significance of this organism and of the other unidentified lactose-fermenting *Vibrio* species is discussed.

Vibrio vulnificus is a lactose-fermenting, opportunistic human pathogen capable of causing death in mice within 2.5 h (3, 19) and in humans within 2 or 3 days (2, 6). Although infections apparently can occur in otherwise healthy individuals (11), human disease typically results from ingestion of contaminated seafood or from infection of a wound, frequently of crab or oyster origin (2). Studies from our laboratory and from case histories suggest that persons with elevated serum iron levels (owing to, e.g., chronic alcoholism, hepatitis, thalassemia major, or hemochromatosis) are especially vulnerable to infection by this organism (2, 27). The organism is able to cause massive damage to the intestinal wall, which probably allows its penetration to the circulatory system (17; J. B. Dellinger, M.S. thesis, University of North Carolina at Charlotte). A toxin(s) has now been isolated which shows cytolytic, cytotoxic, vascular permeability, and lethal activities (12). Poole et al. (M. D. Poole, J. H. Bowdre, and D. Klapper, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B155, p. 43) have demonstrated an extracellular product with proteolytic (but not hemolytic) activity able to degrade albumin, complement fractions C3 and C4, immunoglobulin G, and elastin. This elastase rapidly produced hemorrhagic necrosis, edema, and muscle tissue disruption when injected into mice at microgram levels. Collagenolytic (23) and hemolytic (7) activities also have been demonstrated.

Infections caused by *V. vulnificus* have been reported from Japan (14), Australia (5), Belgium

TABLE 1. Sites sampled for lactose-fermenting *Vibrio* spp.

Site	No. of subsites
Portland, Maine	3
Boston, Mass.	4
Cape Cod, Mass.	4
New London, Conn.	4
New York City, N.Y.	10
Atlantic City, N.J.	4
Ocean City, Md.	4
Chesapeake Bay, Md.	4
Virginia Beach, Va.	4
Cape Hatteras, N.C.	8
Fort Fisher, N.C.	5
Myrtle Beach, S.C.	2
Charleston, S.C.	4
Savannah, Ga.	4
Jacksonville, Fla.	4
Cocoa Beach, Fla.	4
Miami Beach, Fla.	8

(15), and 20 states, including California, all Gulf Coast states, and all East Coast states except New Jersey and Connecticut (J. Farmer, personal communication). Although isolation of *V. vulnificus* from the marine environment has been reported, only regional studies of its distribution and ecology have been made (10, 17, 18; D. L. Tison, M. Nishibuchi, and R. J. Seidler, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, Q97, p. 216). We have shown (17; J. D. Oliver, D. R. Cleland, and R. A. Warner, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, N16, p. 175) that lactose fermentation is extremely common among vibrios (as evidenced by the ability to hydrolyze *o*-nitrophenyl-β-D-galactopyranoside [ONPG]) and is shared by several species. Along with *V. vulnificus*, one of these appears to be a new group of luminescent vibrios (18).

We have examined 3,887 sucrose-negative marine vibrios isolated from several environments along the entire East Coast of the United States, and we report here on the distribution and ecology of *Vibrio vulnificus* and other lactose-positive, sucrose-negative vibrios from these sites.

MATERIALS AND METHODS

Sampling protocol. A total of 17 major sites (Table 1) along the U.S. East Coast from Miami, Fla., to Portland, Maine were sampled during the summer months (May through August) of 1981. At each site, 2 to 10 subsites, representing distinct sampling locations, were studied for a total of 80 subsites (a detailed description of the subsites is available from J.D.O.). At each subsite, water was tested for the following parameters: temperature, dissolved oxygen, salinity, *pH*, *nitrate*, phosphate, turbidity, fecal coliforms, total bacteria, and total vibrios. In addition to the water samples taken at each subsite, sediment, plankton, and animal samples were taken, homogenized in an artificial seawater solution, and plated onto a marine medium (MSWYE; 19) for total bacteria and onto thiosulfate-citrate-bile salts-sucrose agar (TCBS; BBL Microbiology Systems) for total vibrios. Methods employed for obtaining the various samples and examining the different parameters have been published (18). Through the use of a self-contained mobile microbiology laboratory, bacteriological analysis of all sample types (water, plankton, animal, and sediment) was performed within minutes of collection. All initial isolation plates were incubated at room temperature (ca. 21 to 27°C) for 16 to 18 h. From TCBS plates, all sucrose-negative colonies were picked, inoculated into phenol red lactose broth (Difco Laboratories; made to 1% NaCl [wt/vol]), and incubated at 37°C. Tubes were monitored at frequent intervals, and lactose-fermenting isolates were transferred onto MSWYE slants. On returning to our laboratory, isolates were then restreaked onto TCBS to ensure purity, and the fermentation of lactose was confirmed. Strains not giving K/A reaction (6) on TSI medium were discarded, and 47 phenotypic traits of the remaining 95 isolates, as well as 23 reference strains (see Table 9), were determined. Taxonomic methodology was as previously described (18), except that penicillin and colistin sensitivities were determined by the disk diffusion method with Mueller-Hinton agar (Difco) made up with three-salts (19). Sensitivity to pteridine 0/129 was determined by placing crystals of the antibiotic directly onto the medium surface. Test results were encoded for numerical taxonomic analysis with the Jaccard coefficient and single-linkage clustering and were computed at our laboratory with the program TAXAN 6, developed by R. Colwell, Department of Microbiology, University of Maryland at College Park.

Mouse lethality. All environmental isolates were grown in standing culture for approximately 18 h in brain heart infusion broth (BBL) at 37°C. Intraperitoneal injections (0.5 ml, 10^8 to 10^9 cells per ml) were made in 6- to 8-week-old ICR mice. All reference cultures were similarly tested for lethality.

RESULTS

Occurrence and distribution of lactose-fermenting vibrios. Little difference was observed for the various physical, chemical, or microbiological parameters monitored at the 80 subsites (Table 2). Vibrios comprised a high percentage (26 to 40%) of the total bacterial populations of all sample types. A large percentage (40 to 69%) of the vibrios did not ferment the sucrose present in TCBS agar. These isolates (*n*, 3,887) were picked and tested for their ability to ferment lactose. Of the 3,887 sucrose-negative vibrios, 163, or 4.2%, were able to ferment lactose, as indicated by acid production in phenol red lactose broth. The percentage of the sucrose-negative vibrios which were able to ferment lactose varied from 1.6 to 9.7%, depending on the type of sample (Table 2). Standard (Pearson) correlation coefficient analysis performed between the bacterial populations and the seven chemical

TABLE 2. Means of environmental parameters examined at 80 sites

Parameter	Mean ± SEM	No. of samples[a]
Water temperature (°C)	25.8 ± 1.82	84
Salinity (‰)	25.6 ± 2.81	84
Water pH	7.6 ± 0.56	84
Dissolved oxygen (mg/liter)	6.8 ± 1.48	81
Turbidity (FTU)[b]	14.7 ± 4.34	81
NO_3 (μg/liter)	207 ± 18.8	81
PO_4 (μg/liter)	544 ± 25.5	82
Water		
Fecal coliforms (per 100 ml)	221 ± 28.7	80
Total bacteria[c]	$4.7 \times 10^3 \pm 9.0 \times 10^1$	82
Total vibrios[d]	$7.1 \times 10^2 \pm 3.5 \times 10^1$	81
% Vibrios[e]	31.2 ± 5.73	81
% Sucrose-negative vibrios[f]	51.6 ± 9.34	81
% Lactose-positive vibrios[g]	5.3 ± 3.63	77
Sediment		
Total bacteria	$2.2 \times 10^5 \pm 9.0 \times 10^2$	40
Total vibrios	$6.3 \times 10^4 \pm 4.7 \times 10^2$	42
% Vibrios	35 ± 5.9	40
% Sucrose-negative vibrios	41 ± 5.2	41
% Lactose-positive vibrios	2.5 ± 2.34	43[h]
Plankton		
Total bacteria	$5.3 \times 10^4 \pm 3.2 \times 10^2$	24
Total vibrios	$5.9 \times 10^3 \pm 9.4 \times 10^1$	27
% Vibrios	40.1 ± 5.88	25
% Sucrose-negative vibrios	57.0 ± 5.67	29
% Lactose-positive vibrios	1.6 ± 2.30	28[h]
Oysters		
Total bacteria	$7.6 \times 10^6 \pm 4.4 \times 10^3$	7
Total vibrios	$4.6 \times 10^6 \pm 3.5 \times 10^3$	7
% Vibrios	38 ± 6.2	7
% Sucrose-negative vibrios	40 ± 5.4	7
% Lactose-positive vibrios	9.2 ± 4.49	12[h]
Crab and fish		
Total bacteria	$4.1 \times 10^5 \pm 5.9 \times 10^2$	3
Total vibrios	$7.2 \times 10^4 \pm 2.0 \times 10^2$	2
% Vibrios	26 ± 0.8	2
% Sucrose-negative vibrios	69 ± 3.6	2
% Lactose-positive vibrios	9.7 ± 3.27	11[h]

[a] Number of samples may exceed 80 when a parameter was monitored more than once at a subsite.
[b] FTU, Formazin turbidity units.
[c] Total viable counts (CFU) on MSWYE agar.
[d] Total viable counts on TCBS.
[e] Average of individual [(counts on TCBS)/(counts on MSWYE)] × 100.
[f] [(Sucrose-negative colonies on TCBS)/(Total number of colonies on TCBS)] × 100.
[g] [(Lactose-fermenting sucrose-negative vibrios)/(Total number of sucrose-negative vibrios tested)] × 100.
[h] Exceeds number of total vibrio (TCBS count) samples because of nonquantitative swab samples.

TABLE 3. Correlation studies

Variable pair		No. of cases	P value <[a]:	Correlation
First element	Second element			
Total bacteria in:				
Water	Nitrates	79	0.046	0.1904
	Fecal coliforms	78	0.010	0.2618
Plankton	Salinity	24	0.015	−0.4456
	Dissolved oxygen	22	0.001	0.6098
	Total bacteria in water	24	0.001	0.6620
	Total bacteria in sediment	15	0.001	0.8930
Oysters	Phosphates	6	0.001	0.9914
Crabs	Water temperature	3	0.032	0.9948
	Total bacteria in sediment	3	0.032	0.9948
	Total bacteria in water	3	0.013	−0.9993
Fecal coliforms	Salinity	80	0.004	−0.2988
	Dissolved oxygen	77	0.002	−0.3239
	Turbidity	77	0.001	0.4425
	Nitrates	77	0.001	0.8456
	Phosphates	78	0.001	0.6904
	Total bacteria in water	78	0.010	0.2618
	Total vibrios in water	77	0.001	0.5376
Total vibrios in:				
Water	Turbidity	78	0.036	0.2047
	Nitrates	79	0.001	0.4337
	Phosphates	79	0.001	0.4492
	Fecal coliforms	77	0.001	0.5376
	Total bacteria in water	79	0.001	0.5087
	Total bacteria in oysters	7	0.047	0.6777
Sediment	Turbidity	41	0.001	0.4921
	Total bacteria in crabs	3	0.033	0.9947
Plankton	Water temp	27	0.003	0.5188
	pH	27	0.025	−0.3806
Oysters	Phosphates	6	0.001	0.9921
	Total bacteria in water	7	0.048	0.6738
	Total bacteria in oysters	7	0.001	1.0000
% Vibrios in:				
Water	Total bacteria in oysters	7	0.001	0.9724
	Total vibrios in oysters	7	0.001	0.9713
Sediment	% Vibrios in plankton	15	0.009	0.6014
Plankton	Salinity	24	0.048	0.3481
	% Vibrios in sediment	15	0.009	0.6014
Oysters	Total vibrios in oysters	6	0.037	0.7680

TABLE 3—*Continued*

Variable pair		No. of cases	*P* value <[a]:	Correlation
First element	Second element			
% Sucrose-negative vibrios in:				
Water	Total vibrios in plankton	25	0.050	−0.3373
Sediment	Total bacteria in crabs	3	0.036	−0.9938
Plankton	Nitrates	25	0.017	0.4528
Oysters	Water temp	7	0.017	0.7910
	Salinity	7	0.011	−0.8262
	pH	7	0.024	0.7605
	Total bacteria in water	7	0.023	0.7620
	Total vibrios in oysters	6	0.048	−0.7349
	% Sucrose-negative vibrios in sediment	6	0.028	0.7988
% Lactose-positive vibrios in:				
Water	pH	77	0.049	0.1899
	Total bacteria in oysters	7	0.003	0.9000
	Total vibrios in oysters	7	0.003	0.9002
	% Sucrose-negative vibrios in oysters	7	0.010	0.8320
Sediment	Salinity	43	0.001	−0.4915
	Turbidity	42	0.047	0.2616
	Total vibrios in sediment	41	0.004	0.4074
	% Sucrose-negative vibrios in oysters	7	0.020	0.7776
Plankton	Total bacteria in oysters	4	0.001	1.000
	Total vibrios in oysters	4	0.001	1.000
Oysters	% Vibrios in sediment	9	0.007	0.7731
	% Sucrose-negative vibrios in sediment	9	0.036	−0.6238
	% Sucrose-negative vibrios in oysters	6	0.009	0.8874
Crabs	Salinity	11	0.008	−0.6991

[a] Only *P* values of <0.05 are included.
[b] Standard correlation coefficient (Pearson).

and physical environmental parameters measured at each site indicated that several significant correlations exist with total bacterial counts, total vibrio counts, and fecal coliform populations (Table 3). Lactose-fermenting vibrio isolates correlated positively with pH of the water, total vibrios in the sediments and oysters, and the total viable counts and percentages of sucrose-negative vibrios associated with oysters (Table 3). Negative correlations were observed between salinity of the water column and the incidence of lactose fermenters.

V. vulnificus (identified as described below) was isolated in low numbers (*n*, 33) throughout the eastern seaboard (Table 4, cluster III) and from all sample types examined (19 from animal samples, 4 from sediment samples, 8 from water samples, and 1 from a Miami plankton sample). The majority of the positive isolates were from bivalves (16 from 3 different clams and 1 from an oyster), with the remaining 2 isolates coming from crabs. Of the four sediment samples in which *V. vulnificus* was found, two were brownish ocean beach sand (Jacksonville, Fla., and Flander's Beach, N.Y.), and two were from river beach sand (Fort Fisher, N.C.). None of the isolates was from an estuarine mud sample. Comparison of the environmental parameters of the 8 sites yielding *V. vulnificus* isolates to those of the entire study (80 sites) revealed no significant differences (Table 5).

Taxonomy. All 95 environmental isolates were *Vibrio* spp. which fermented lactose. Lactose fermentation (as indicated by an acid reaction in phenol red lactose broth at 37°C) was typically quite rapid (an average of 2 h) and frequently reverted to a neutral or alkaline pH after overnight incubation (R. Warner, D. Cleland, and J.

TABLE 4. Distribution, geographically and by sample type, of lactose-fermenting vibrio isolates contained in the five taxonomic clusters

Sample sources	No. of samples in cluster[a]:				
	I	II	III	IV	V
Water	4	3	8		2
Sediment	4	3	4	2	
Plankton	3	1	1		
Animals	2	1	19	11	3

[a] Sources for clusters: Cluster I: Savannah, Ga.; North Myrtle Beach, S.C.; Fort Fisher, N.C.; Wrightsville Beach, N.C.; Virginia Beach, Va.; Atlantic City, N.J.; New York City, N.Y. Cluster II: Miami, Fla.; Jacksonville, Fla.; Savannah, Ga.; Charleston, S.C.; New York City, N.Y. Cluster III: Miami, Fla.; Jacksonville, Fla.; Fort Fisher, N.C.; Cape Hatteras, N.C.; Atlantic City, N.J.; New York City, N.Y.; Cape Cod, Mass. (the sample source of one isolate from Cape Cod, Mass., is unknown). Cluster IV: Cape Hatteras, N.C. Cluster V: Cape Hatteras, N.C.

Oliver, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, N106, p. 195). Thus, numerous lactose-fermenting isolates would have been missed if a single observation of the lactose reaction had been made after overnight incubation.

Numerical taxonomy performed on the isolates resulted in five major clusters (defined as groups containing five or more isolates) comprising 70 of the environmental strains (Fig. 1). Clusters IV and V contained isolates from several subsites of a single site (Cape Hatteras, N.C.), whereas members of the remaining three clusters were not regional (Table 4). None of the isolates, except for members of cluster III, grouped with any of the 23 reference strains. (A sixth grouping, identified on Fig. 1 as Vc + Vm, was comprised only of the four reference strains of *Vibrio cholerae* and *Vibrio mimicus*.) (See Table 6 for phenotypic traits of the five clusters.)

Cluster I was comprised of 13 isolates, obtained from Savannah, Ga., to New York City, isolated from all sample types (four water, four sediment, three plankton, and two from animals; Table 4). Phenotypically, this cluster consisted of H_2S-producing strains and was one of two groups containing isolates which were bioluminescent (Table 6). Lethality studies showed only 16.7% to be lethal for mice.

Cluster II contained 8 isolates obtained from Miami, Fla., to New York City and from all sample types (three water, three sediment, one plankton, and one animal). Members of this cluster were similar to cluster I in being H_2S positive but contained no luminescent strains. Along with H_2S production, these strains differed from *V. vulnificus* in their penicillin resistance, general lack of salicin fermentation, and ability to grow at higher NaCl concentrations. Despite these differences, all eight strains of the cluster showed a high similarity (80 to 90%) to a *V. vulnificus* type strain (ATCC 27562) appearing in cluster III and included in this study as one of the reference cultures. Members of cluster II demonstrated one of the highest degrees of lethality to mice observed in this study, with 87.5% causing death on peritoneal injection.

The largest grouping of environmental isolates (*n*, 33) occurred in cluster III, which also contained all of the eight reference *V. vulnificus* strains. Geographically, cluster III contained isolates from eight sites from Miami, Fla., to Cape Code, Mass. (Table 4). The majority (*n*, 19) of the cluster members were from animal samples, with 8 from water, 4 from sediment, and 1 from a plankton sample. Some 10 of the 33 strains of cluster III were selected at random

TABLE 5. Environmental data on sites yielding *V. vulnificus*

Water parameter	Mean values for:	
	Sites positive for *V. vulnificus*[a]	All 80 sites
Temperature (°C)	26.3 (19–32)	25.8
Salinity (‰)	22.5 (9–30)	25.6
pH	7.4 (6.8–7.8)	7.6
Dissolved oxygen (mg/liter)	6.4 (2.5–9.2)	6.8
Turbidity (FTU)[b]	6.4 (<2–22)	14.7
NO_3 (μg/liter)	252 (44–1020)	267
PO_4 (μg/liter)	529 (170–1170)	544
Fecal coliforms (per 100 ml)	155 (<1–880)	221
Total bacteria[c] (per ml)	9.6×10^3 (3.4×10^2–4.2×10^4)	4.7×10^3
Total vibrios[d] (per ml)	6.6×10^2 (4×10^1–2.9×10^3)	7.1×10^2
% Sucrose-negative vibrios[e]	59 (25–76)	51.6

[a] Values in parenthesis are ranges observed for each parameter.
[b] Formazin turbidity units.
[c] Total viable counts on MSWYE.
[d] Total viable counts on TCBS.
[e] [(Sucrose-negative vibrios on TCBS)/(total colonies on TCBS)] × 100.

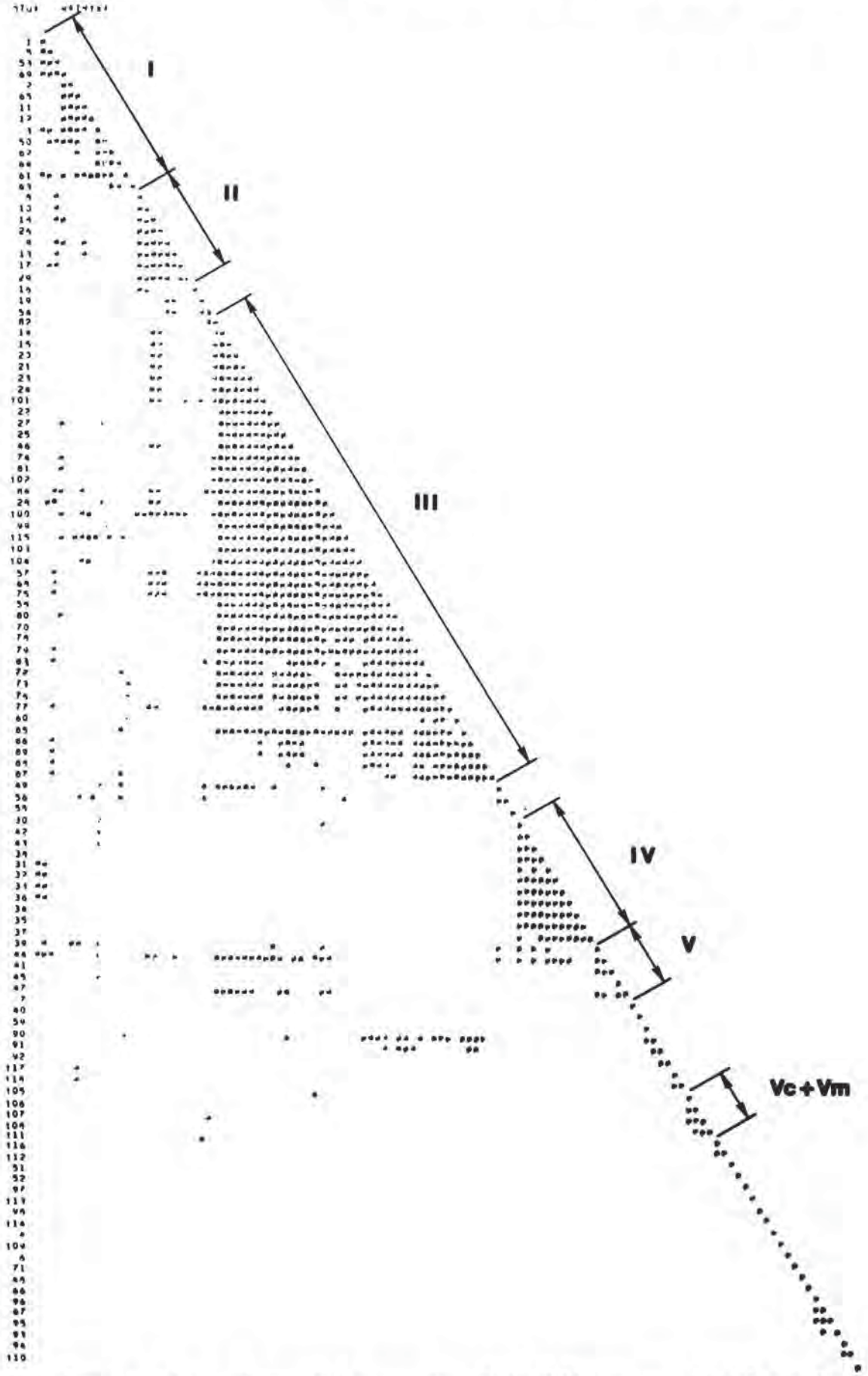

FIG. 1. Computer printout of numerical taxonomic analysis of 95 sucrose-negative, lactose-fermenting marine vibrios. Five clusters of the environmental isolates are indicated. Also shown is the clustering of the four reference strains of *V. cholerae* and *V. mimicus* (Vc + Vm). The printout symbol denotes intrastrain similarities of ≥80%.

TABLE 6. Phenotypic traits[a] of taxonomic clusters

Phenotypic trait	Presence of trait by cluster no:				
	I (n = 13)	II (n = 8)	III (n = 33)	IV (n = 11)	V (n = 5)
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
Fermentation of:					
Lactose (24 h)[b]	V (46)	+	V (33)	+	+
Sucrose	−	−	−	−	−
Salicin	V (69)	V (38)	+	+	+
Gas from dextrose	−	−	−	− (5)	−
Hydrolysis of:					
Starch	+	+ (88)	+	−	+
Gelatin	V (38)	+	+ (76)	− (9)	− (20)
Agar	−	−	−	−	−
TSI = K/A[c]	+ (80)	+	+	+	+
H_2S (on TSI agar)	+	+	− (6)	V (27)	+
Voges-Proskauer	−	−	−	−	−
Citrate	−	− (13)	−	−	−
Lysine decarboxylase	V (69)	+	+	+ (91)	+
Ornithine decarboxylase	V (46)	+	+ (79)	V (36)	+ (80)
Arginine dihydrolase	−	−	−	− (9)	−
Urease	− (20)	− (13)	−	+ (91)	V (60)
Indole	V (38)	V (50)	V (55)	−	−
NO_3 reduction	+	+	+	+	+
Growth in NaCl:					
0%	−	V (25)	−	−	−
3%	+	+	+	+	+
6%	+ (92)	+	V (64)	+ (82)	V (60)
10%	−	V (50)	−	−	−
Growth at:					
5°C.	−	−	−	−	−
42°C.	V (33)	+	+	+	V (40)
Luminescence	V (54)	−	−	−	+ (80)
Sensitivity to:					
Penicillin	− (15)	V (25)	+ (88)	−	−
Pteridine 0/129	+ (75)	+ (75)	+	V (36)	+ (80)
Colistin	V (46)	− (13)	−	+ (91)	−
Pigment	−	−	−	−	−
Spreading growth	−	−	−	−	−

[a] All isolates were gram-negative rods which fermented glucose. +, −: ≥97% of strains tested gave indicated result if no number is shown in parenthesis. Numbers in parentheses indicate percentage of strains positive for the trait. +, ≥70% positive; −, ≤20% positive; V, Variable result (21 to 69% positive).

[b] All strains examined in this study fermented lactose. Lactose reaction indicated here is result observed in phenol red lactose broth after 24 h of incubation at 37°C.

[c] Alkaline (or no reaction) slant, acid butt in triple sugar iron agar.

TABLE 7. Lethality in mice of lactose-fermenting vibrios

Group (no. of strains)	% Lethality[a]
Cluster I (13)	16.7
Cluster II (8)	87.5
Cluster III (33)	82.4
Cluster IV (11)	18.2
Cluster V (5)	0
Nonclustering isolates (25)	64
H_2S-positive isolates (27)	40.7

[a] A 0.5-ml dose of a stationary-phase culture grown at 23°C in brain heart infusion broth was injected intraperitoneally into ICR mice.

and examined by D. Tison, Oregon State University, Corvallis, Oreg., for genetic homology to *V. vulnificus* by DNA-DNA hybridization studies. All 10 of the strains were found to be *V. vulnificus*, with homologies of 77 to 95% at stringent temperatures. Of the 33 members of cluster III, 82.4% were lethal for mice.

Members of cluster IV were all collected from the Cape Hatteras, N.C., area. Of these isolates, 11 were obtained from animals, and the remaining 2 were from sediments (Table 4). Phenotypically, these isolates differed significantly from *V. vulnificus* in their production of urease, in the general lack of extracellular enzymes, and in their pattern of antibiotic sensitivity (Table 6). Several members produced H_2S in TSI medium. Lethality studies showed that only 15.4% were lethal for mice.

Cluster V contained five members which were isolated from waters from Cape Hatteras, N.C. This cluster could be characterized by the production of H_2S by all members and of urease by three of the five. In addition, most of the isolates were observed to be luminescent. Cluster V was, therefore, phenotypically similar to cluster I. None of the strains was lethal for mice.

Of the 25 lactose-fermenting isolates which did not form clusters, 16 (64%) were also lethal for mice (Table 7).

DISCUSSION

Distribution of *V. vulnificus*. In this study of 80 coastal sites from Miami, Fla., to Portland, Maine (Table 1), an average count (on the marine medium MSWYE) of 4.7×10^3 bacteria per ml of seawater was obtained (Table 2). Total bacteria in the water column were seen to correlate with two of the pollution indicators monitored, nitrates and fecal coliforms (Table 3). Whereas the number of bacteria associated with plankton increased as the total population in the water column increased, a negative correlation with salinity was observed (Table 3). This result is in accord with the study reported by Kaneko and Colwell (9) on bacteria-plankton associations in Chesapeake Bay. The validity and effectiveness of the methods used for monitoring the various environmental parameters and studying their correlations is indicated by the correlations observed with fecal coliforms in the water column (Table 3). As would be expected, positive correlations were obtained for the presence of fecal coliforms and four water pollution indicators, namely, turbidity, nitrates, phosphates, and total bacteria. Also as would be expected, negative correlations were seen between numbers of fecal coliforms and the salinity and dissolved oxygen of the water column. A significant portion (26 to 30%) of the total bacterial population were *Vibrio* spp. (defined as cells giving rise to colonies on TCBS medium on overnight incubation). Although our taxonomic studies have indicated relatively few nonvibrios growing on TCBS, we recognize the likelihood of such bacteria appearing on this medium. The actual number of vibrios present in the water column may, therefore, be less than that reported here. Our observations, however, are consistent with previous studies (8, for example) which have shown vibrios to make up a high percentage of the aquatic bacterial population, especially in summer. Several correlations were also observed with total vibrios from the various samples (Table 3). Of note were those of vibrios in the water column and several pollution indicators, and of vibrios associated with oysters and the total number of bacteria in oysters. The correlation between vibrios in the water column and water temperature reported by Kaneko and Colwell (8) was not observed in the present study but can be explained by the lack of variation in water temperature (Table 2). The total number of bacteria and the percentage of vibrios in the non-filter-feeding animals examined (two crabs and one fish) were considerably less (Table 2).

The observation that 4.2% of all sucrose-negative vibrios tested were able to dissimilate lactose is similar to that recently reported by J. T. Graikoski and J. E. Houser (Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, Q69, p. 221), who found 2 to 5% of all bacterial isolates from seawater, sediment, and animals to be able to ferment this sugar. Of the lactose-fermenters we studied, a total of 33 strains (20%) of the sucrose-negative, lactose-positive vibrios were identified as *V. vulnificus* by numerical taxonomic methods. Identification of these strains was confirmed by DNA-DNA homology studies. Thus, the 33 isolates of *V. vulnificus* constituted only 0.85% of the 3,887 sucrose-negative vibrios examined. In a previous study (18), we observed that over 42% of all sucrose-negative

TABLE 8. Comparison of environmental (n = 33) and clinical isolates of *V. vulnificus*

Taxonomic character	% Positive		
	Environmental strains	Clinical strains	
		Hollis et al. (6)	Present study[a]
Gram-negative rods	100	100	100
Motile	100	100	100
Oxidase	100	100	100
Fermentative metabolism	100	100	100
Acid from:			
Lactose	100	81	100[b]
Sucrose	0	3	0
Dextrose	100	100	100
Salicin	100	100	100
Gas from dextrose	0	0	0
Hydrolysis of:			
Starch	97	100	90
Gelatin	76	97	100
Agar	0	NR[c]	0
TSI = K/A[d]	97	100	90
H_2S production (TSI agar)	6	0	0
Voges-Proskauer	0	0	0
Decarboxylation of:			
Lysine	100	97	100
Ornithine	79	66	100
Arginine dihydrolase	0	0	0
NO_3^- reduction	100	100	100
NO_2^- reduction	0	NR	0
Growth in NaCl:			
0%	3	NR	70
3%	100	NR	100
6%	64	100	100
8%	9	8	30
10%	0	0	10
Growth at:			
5°C	0	NR	40
25°C	100	NR	100
37°C	100	NR	100
42°C	100	NR	100
Catalase	100	NR	100
Urease	0	0	0
Indole	55	97	0
Citrate	0	76	0
Luminescence	0	NR	0
Pigment	0	NR	0

TABLE 8—*Continued*

Taxonomic character	% Positive		
	Environmental strains	Clinical strains	
		Hollis et al. (6)	Present study[a]
Spreading growth	0	NR	20
Sensitivity to:			
Penicillin	88	100	100
Pteridine 0/129	100	NR	100
Colistin	0	0	0

[a] Eight clinical strains received from the Centers for Disease Control were studied.
[b] Some strains negative by 24 h; see text.
[c] NR, Not reported.
[d] Alkaline (or no reaction) slant, acid butt on triple sugar iron agar.

marine vibrios tested were positive for ONPG hydrolysis. Of these, only 3 to 4% could be identified as *V. vulnificus*. It is evident, therefore, that as we have previously emphasized (17, 18), numerous lactose-fermenting *Vibrio* species other than *V. vulnificus* exist in the marine environment. A similar conclusion was reached by Tison et al. (25; D. L. Tison, M. Nishibuchi, and R. J. Seidler, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, Q97, p. 216), who described several taxonomically undefined groups of lactose-fermenting marine vibrios and showed them to be genetically distinct from *V. vulnificus*.

In the present study, *V. vulnificus* was isolated from water, sediment, plankton, and animal samples obtained from widespread geographical regions of the East Coast of the United States (Table 4). The isolation of sucrose-negative, lactose-fermenting vibrios correlated with several parameters (Table 3), but as observed by Tamplin et al. (M. L. Tamplin, C. E. McKnight, G. E. Rodrick, and N. E. Blake, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, Q72, p. 212), no correlation with fecal coliform levels was seen. Nor were any statistically significant differences observed between the values recorded for the various environmental parameters of those subsites which yielded *V. vulnificus* and the mean values of all 80 subsites (Table 5).

Numerical taxonomic analysis of the 95 sucrose-negative, lactose-positive isolates resulted in five major clusters (Fig. 1) comprising 70 of the 95 strains.

Cluster I, unusual in containing H_2S-, urease-, and luminescence-positive strains, were $\leq$ 70% similar to all of the 23 reference strains included in the analysis and were unlike any described *Vibrio* spp. However, when several strains from clusters IIa and IIb from our previous study (18) of ONPG-hydrolyzing marine vibrios were included in the taxonomic analysis, a high similarity (80 to 100%) to these strains was observed. Clusters IIa and IIb of that study also consisted of strains that were luminescent and H_2S positive, with some urease producers. Lethality for mice was also similar for the two groupings (16.7% for cluster I of the present study and 22% of the clusters IIa and IIb of the previous study). As previously concluded (18), these isolates appear to represent a new taxonomic group of vibrios.

Like cluster I strains, all members of cluster II were H_2S positive. They differed from cluster I, however, in their gelatin, decarboxylase, salt tolerance, and growth temperature responses (Table 6). None of the cluster II members was luminescent. Also like cluster I, none of the reference strains appeared in this cluster, nor were its members similar to published descriptions of *Vibrio* spp. When taxonomic data from strains described as clusters IIa and IIb in a previous study (18) were added to the present numerical taxonomic analysis, these H_2S-producing strains appeared with this H_2S-producing cluster. When potential pathogenicity is considered, this cluster is quite significant, with over 80% of the member strains causing death in mice within 24 h.

The phenotypic traits of the 33 members of cluster III were typical of *V. vulnificus*, and this identification was confirmed by DNA hybridization studies performed on 10 randomly selected member strains. The cluster contained all eight reference *V. vulnificus* strains and demonstrated a high percentage of similarity to members of cluster Ib (also identified as *V. vulnificus*) of our previous study (18). Cluster III could be easily differentiated from the other four clusters obtained in the present study (Table 6) and was nearly identical phenotypically to the 38 clinical strains of *V. vulnificus* described by Hollis et al. (6), several of which have been characterized in our laboratory (Table 8). Tison and Seidler (25) have also demonstrated that *V. vulnificus* strains isolated from a wide range of estuarine environ-

ments are genetically indistinguishable from clinical isolates. Differences between our environmental isolates and the clinical strains studied by Hollis et al. (6) were observed in the ability to grow on citrate as a sole carbon source, NaCl tolerance, and indole formation. The negative citrate reaction observed for the 33 cluster III isolates, however, agrees with the result we have observed for *V. vulnificus* reference strains using Simmons citrate medium (Table 8) and with our previous observations (18). A negative citrate reaction was also reported by Matsuo et al. (14). The reason for this discrepancy is not known. We also found that strains from the Centers for Disease Control (Atlanta, Ga.) are able to grow over a wider range of NaCl concentrations than that reported by Hollis et al (6), although the determination of this trait is quite subjective. Differences in indole production between the clinical and environmental isolates were also noted, although negative reactions for this test have also been previously reported (15). Two strains in cluster III failed both to produce indole and to decarboxylate ornithine; thus, they were similar to *V. vulnificus* biogroup 2 recently described by Tison et al. (24). These two strains differed from this new biogroup, however, in their ability to grow at 42°C.

Another aspect of the taxonomic identification of *V. vulnificus* which deserves mention concerns the time required for lactose fermentation. Hollis et al. (6) described their 38 clinical isolates as usually fermenting lactose within 24 h, although a few isolates required 3 to 7 days. Baumann et al. (1) have stated that in fact wild-type strains of *V. vulnificus* are unable to utilize lactose (20) and that lactose fermentation is detected only after 1 to 3 days of incubation because of spontaneously arising mutants which are capable of fermenting this sugar. All of our (presumably wild-type) isolates, on the other hand, were capable of producing an acid reaction in phenol red lactose broth within several hours of inoculation (R. Warner, D. Cleland, and J. Oliver, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, N106, p. 195). This finding has not been reconciled with that of Baumann et al. (1), although the discrepancy might conceivably be due to differences in the medium employed by those authors to determine the ability to ferment lactose.

Cluster IV also contained several H_2S-producing strains but could be distinguished from other clusters by the production of urease by its members (Table 6). Urease production is highly unusual among vibrios, having been described only for *Vibrio damsela* (13), occasional strains of *Vibrio parahaemolyticus* (16), and an unnamed vibrio causing infections in flounder (21).

TABLE 9. Reference strains employed in taxonomic and lethality studies

Reference strain and source[a]	Lethality[b]	Strain no. (Fig. 1)
V. vulnificus:		
ATCC 27562	+	103
ATCC 29306; CDC A1402	+	115
ATCC 29307	+	102
CDC B3547	+	99
CDC C2756	+	100
CDC C7184	+	944
CDC C8806	+	104
CDC D9889	+	101
CDC E2272	+	NR[c]
CDC H3308	+	NR
J. Oliver, butcher	+	NR
V. cholerae:		
CDC C4752	+	106
CDC C6487	+	107
Lab strain	+	108
V. mimicus CDC C6713	+	105
V. parahaemolyticus:		
ATCC 27519	+	NR
K. Nealson, B113	+	111
Vibrio alginolyticus K. Nealson, B86	−	116
Vibrio fischeri K. Nealson, B64	−	110
Vibrio harveyi:		
K. Nealson, B332	−	117
K. Nealson, B352	−	NR
K. Nealson, B376	−	118
V. Neptuna, ATCC 25919	−	97
Vibrio marinofulvus[d] ATCC 14395	−	98
Vibrio algosus[d] ATCC 14390	NT[e]	114
Vibrio marinagilis[d] ATCC 14398	−	113
Photobacterium leiognathi K. Nealson, B474	−	109
Photobacterium phosphoreum K. Nealson, B404	−	NR
Aeromonas hydrophila ATCC 9071	+	112

[a] ATCC, American Type Culture Collection, Rockville, Md.; CDC, Centers for Disease Control.
[b] Examined as described in Table 7.
[c] NR, Not a reference strain in the numerical taxonomic study.
[d] Not a recognized scientific name.
[e] NT, Not tested.

However, members of cluster IV differed from *V. damsela* and *V. parahaemolyticus* in numerous phenotypic traits, including lactose fermentation, H_2S production, lack of gas production in carbohydrate broth, and salicin fermentation, and from the flatfish vibrio in the reactions to sucrose, indole, and starch and in penicillin sensitivity. No similarity to the ONPG-hydrolyzing vibrios studied previously (18) could be demonstrated for cluster IV, and the identity of this group was undetermined.

Cluster V also contained H_2S- and urease-producing members, most of which were luminescent (Table 6). Except for our previous study (18), H_2S-producing luminescent bacteria have not been previously described (18, 20). Members of cluster IIb of that study also contained H_2S- and urease-producing strains and were luminescent. In neither study did our isolates cluster with the luminescent reference strains included for comparison. We were not able to identify these isolates as being members of a described *Vibrio* species, and we suggest that they, along with the luminescent strains of cluster I, represent a new taxonomic group of marine vibrios.

The fact that four of the five taxonomic clusters described here could not be identified is not unusual. Graikoski and Houser (Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, Q69, p. 221) recently reported that ca. 60% of all isolates from marine waters, sediments, and animals could not be identified. One aspect of the present study which has made specific identification difficult was the frequent occurrence of H_2S-producing isolates among the 95 lactose-fermenting vibrios. Although strains producing H_2S from thiosulfate have been reported for *V. cholerae* and *V. parahaemolyticus* (4), this trait has not generally been observed among vibrios (22, 26; P. A. West, Ph.D. thesis, University of Kent, Canterbury, England, 1980). The possibility that the H_2S trait may be plasmid mediated is currently being investigated in our laboratory. What is especially relevant to the present study is the extent to which these undescribed species were virulent for mice. When even large inocula of the various reference strains were injected intraperitoneally into mice, more than half of the species failed to cause death (Table 9). Thus, it is evident that the technique employed can differentiate the environmental isolates and reference strains tested in our study regarding virulence for laboratory animals (Tables 7 and 9). Although mouse lethality as determined by the method used in this study cannot be equated to human pathogenicity, the lethality studies reported here indicate that numerous lactose-fermenting marine vibrios, in addition to *V. vulnificus*, are potentially pathogenic for humans. Among these unidentified *Vibrio* species are a large percentage (28.4%) of H_2S producers which were, as a group, quite virulent for mice (Table 7). Tison and Seidler (25) concluded that environmental isolates of *V. vulnificus* may be of public health significance. We agree with that conclusion and suggest that although considerably more work is warranted on all aspects of the biology of *V. vulnificus*, the other taxonomically undefined clusters of lactose-fermenting vibrios described here deserve particular attention as possible public health hazards.

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