Numerical Taxonomy of *Vibrios* Isolated from Estuarine Environments

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We used numerical taxonomy procedures to analyze data obtained from 227 strains belonging to either the genus *Vibrio* or related genera; included were pathogenic and nonpathogenic vibrios isolated from a variety of samples and geographic locations. Each strain was tested for 150 unit characters. At a similarity coefficient of 70 to 75% or more, the 227 strains clustered into 33 phena, representing *Vibrio anguillarum*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio metschnikovii*, *Aeromonas hydrophila*, and new species of *Vibrio*. Non-O1 and O1 serovars of *V. cholerae* clustered at the species level of relationship (i.e., at a similarity coefficient of $\geq 75\%$). Furthermore, subdivision of *V. cholerae* into classical, El Tor, *proteus*, and *albensis* biovars was not observed. In fact, the biovar *proteus* was found to warrant separate species status as *Vibrio proteus*. Only the sucrose-negative members of Heiberg group V were distinguishable as a separate cluster and were recognized as a separate biovar. Sucrose-positive strains and urease-positive strains of *V. parahaemolyticus* were identified. The differences among the four groups of *V. anguillarum* examined did not warrant recognition of biovars.

Species of the genus *Vibrio* have become increasingly important, especially in medical and environmental microbiology. The type species of *Vibrio*, *Vibrio cholerae*, is the causative agent of Asiatic cholera, and *Vibrio parahaemolyticus* is associated with food poisoning, especially from ingestion of contaminated seafood. Vibrios have been shown to be important in the aquatic environment, for they constitute a significant portion of marine and estuarine bacterial populations in water and are associated with aquatic animals and plants. Species of *Vibrio* found in the environment include pathogens for fish (20), snails (24), and other invertebrates.

The taxonomy of the genus *Vibrio* has undergone major revision in recent years. In *Bergey's Manual of Determinative Bacteriology*, 7th ed (10), Breed et al. listed 34 species for the genus and included aerobes, facultative anaerobes, and obligate anaerobes, strains of which had little in common except for being curved, motile rods. In the 8th edition of *Bergey's Manual* (82), Shewan and Veron took into account more recent knowledge and provided a much improved classification, but they still left many unanswered questions.

Recently several new species have been pro-

posed for inclusion in the genus *Vibrio*. Lee et al. (60, 62) redescribed *Vibrio metschnikovii* and also named a new species, *Vibrio fluvialis* (the group F vibrios; i.e., group EF6). The latter is considered to be intermediate between the genera *Vibrio* and *Aeromonas* (62). An organism capable of extensive tissue damage or death or both in humans was demonstrated by Clark and Steigerwalt (12) to be closely related to species of the genus *Vibrio* and was subsequently named *Vibrio vulnificus* by Farmer (28). These organisms were formerly referred to as "lactose-positive vibrios." *Vibrio damsela* (65) and *Vibrio hollisae* (42) have also been described. There is no doubt that other new species will be proposed as current research continues.

The objective of this study was to examine a large set of vibrios representing as many species as could be obtained from a variety of sources, including environmental and medical, throughout the world. Numerical taxonomy was used to measure phenotypic similarities and differences and to analyze the relationships, if any, among phenotypes associated with geographic sources or types of sample. Our working hypothesis was that answers to environmental and medical questions concerning vibrios can be best answered by using an integrated (i.e., polyphasic) approach to the systematics of the genus *Vibrio* (13). Thus, the results obtained in this study are discussed in light of other criteria, as well as numerical taxonomy.

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TABLE 1. Sources of strains included in the numerical taxonomy analysis

Strain(s)	Source
F687/75, F500/75, F702/75, F704/75, F706/75, F406/75, F407/75, F580/75, F678/75, F71/75, F543/75, F647/75, F673/75, F685/75, F708/75, 269F/75, 279F/75, F565/75, F633/75, F559/75, F643/75, F581/75	Fish wound, Denmark
1280/75, 1298/75, 1279/75, 1289/75a, 1289/75b, 1291/75, 1302/75	Sugar mill ponds, Denmark
DAM85/75, DAM80/75, DAM84/75, DAM83/75, DAM81/75, DAM86/75, DAM114/75, DAM12/75, DAM27, DAM82/75, DAM108, DAM114	Sewage water, Denmark
715/75, 1208/75, 1210/75, 1213/75, 1218/75, 1223/75, 311/75, 416/75, 562/75, 639/75, 905/75, 1104/75, 1105/75, 1106/75, 1194/75a, 1206/75, 1211/75, 1214/75, 1219/75, 1220/75, 1239/75, 1241/75, 1242/75, 1243/75, 1244/75, 1245/75, 1248/75, 1249/75, 1255/75, 1258/75, 1259/75, 1266/75, 1267/75, 1268/75, 1269/75, 1292/75, 1283/75, 1290/75, 1296/75, 1297/75, 1301/75, 939/75, COD 3, 1194/75b, 110/75, 116/75 ^a	Seawater, Denmark
1225/75, 1238/75, 1222/75, 1221/75a, 1221/75b, 1237/75, 309/75, 1157/75, 1111/75, 1114/75, 1160/75, 819/75, 824/75, 958/75, 264/75, K19, K22B, K23B, K204V, K221V, KALV 18, NCMB, 274/75, 931/75, 952/75, 1109/75, NA10B, 307/75, 1118/75, 613/75, 1275, 1202/75, 943/75, AEJERB, 934B/75, 1274, F651/75, F640/75	Miscellaneous environmental samples
V2, V3, V4, V5, V10, V11, V15, V19, V20, V24, V25, V26, V29, V38, V31, V33, V35, V36, V37	Presumptive <i>V. cholerae</i> from Chesapeake Bay
D1, D2, D3, D4, D5, D6, D7, D8, D9, D11, D12, D15, D16, D17, D19, D22, D23, D24, D25, D26, D10, D13, D14	Shrimp mortalities, Mexico
VPDG	Stool, Maryland
GP243G	Rainbow trout, Italy
V16, V22, V27, V28, V30, V34	Chesapeake Bay

^a The culture received as strain 1290/75 yielded two distinct colonial types, both of which were included in the analyses.

MATERIALS AND METHODS

Bacterial strains. A total of 227 strains was examined by numerical taxonomy methods. These strains included pathogenic and nonpathogenic vibrios and vibrio-like bacteria isolated from the Chesapeake Bay, Denmark, and Mexico and represented strains isolated from a variety of sources, including fish, shrimp, seawater, and sewage. The clinical isolates included strains of *V. cholerae* isolated from various sources throughout the world. Reference cultures, which represented a diverse range of facultative anaerobes, were obtained from the American Type Culture Collection. The strains used in this study and their sources are shown in Tables 1 and 2.

Upon isolation or receipt from a collection, each culture was examined for purity by serial streaking three times on tryptic soy agar (TSA; Difco Laboratories, Detroit, Mich.). Stock cultures were maintained on TSA slants that were overlaid with sterile mineral oil and stored at 25°C.

Characterization of strains. Each strain was examined for 150 unit characters. All inocula were taken from cultures that were less than 48 h old. Whenever possible, TSA was used as the basal medium for tests involving dyes, salt, etc. Unless otherwise stated, all inoculated media were incubated at 25°C, and all

media contained NaCl at a final concentration of 0.5%. Tests were repeated in the event of inconclusive results.

Morphology. Colonial morphologies were determined on TSA after incubation for 2 days by using the criteria of Colwell and Weibe (17). The following characteristics were scored for each strain: the presence of creamy white, yellow, orange or red, or diffusible pigments; translucent, opaque, shiny or matte, round, flat, raised, domed, wrinkled, or spreading colonies; and undulate, irregular, or rhizoid colony edges. The presence of fluorescein and the presence of pyocyanin were detected by examining with a fluorescent light the growth obtained after 7 or more days of incubation on the media described by King et al. (56).

Micromorphology and Gram-staining reactions were recorded from heat-fixed smears of fresh cultures after staining with the Hucker modification of the Gram stain (45). We also recorded the presence of pleomorphism, curved and straight rods, chains, filaments, and granular cytoplasm. The approximate ratio of rod length to rod width was measured, and motility was determined both microscopically (by wet mount) and macroscopically (SIM medium [Difco]).

Physiology and resistance to physical and chemical factors. The ability to grow at 5, 10, 15, 37, 42, and 44°C was determined by using TSA and air incubators.

TABLE 2. Reference strains included in the numerical taxonomy analyses

Culture name as received	Strain	Source	Culture name as received	Strain	Source
<i>V. cholerae</i>	ATCC 14035 (O-1) ^a	ATCC ^b	<i>V. proteus</i>	10157-74 (SG 1043) ^c	Sakazaki
	ATCC 14033 (O-1) ^a	ATCC		10041-74 (NCTC 8563) ^c	Sakazaki
	4202-64 (O-5) ^a	Sakazaki	<i>V. metschnikovii</i>	10035-74 (CIP A267) ^c	Sakazaki
	7007-64 (O-6) ^a	Sakazaki		10036-74 (CIP 6914) ^c	Sakazaki
	8394-62 (O-7) ^a	Sakazaki		10038-74 (239) ^c	Sakazaki
	10317-62 (O-8) ^a	Sakazaki		10040-74 (7) ^c	Sakazaki
	112-68 (O-9) ^a	Sakazaki		10042-74 (NCTC 8443) ^c	Sakazaki
	218-68 (O-10) ^a	Sakazaki		10121-74 (211) ^c	Sakazaki
	10843-62 (O-11) ^a	Sakazaki		10122-74 (ATCC 7708) ^c	Sakazaki
	211-72 (O-12) ^a	Sakazaki	<i>Vibrio natriegens</i>	VNATR	Zachary
	11416-62 (O-13) ^a	Sakazaki	<i>V. anguillarum</i>	ATCC 14181	ATCC
	8645-64 (O-14) ^a	Sakazaki	<i>V. marinus</i>	ATCC 15382	ATCC
	1944 (O-1) ^a	Smith		PS207	ATCC
	5040R	Smith	<i>B. pelagia</i>	ATCC 25916	ATCC
	5040S	Smith	<i>B. harveyi</i>	B384	Sakazaki
	8357	Smith	<i>Plesiomonas shigelloides</i>	302-73	Sakazaki
	5049	Smith		306-73	Sakazaki
	7583	Smith		319-73	Sakazaki
	5114	Smith		314-73	Sakazaki
	8338	Smith		318-73	Sakazaki
	8336	Smith		355-73	Sakazaki
	5071	Smith	<i>Shigella flexneri</i>	138-59	Sakazaki
	5695	Smith	<i>Shigella sonnei</i>	269-60	Sakazaki
<i>V. parahaemolyticus</i>	ATCC 17802	ATCC	<i>Shigella dysenteriae</i>	1984-56	Sakazaki
	ATCC 27969 (fc1011) ^c	ATCC		42-67	Sakazaki
	SAK 4	Sakazaki			
<i>V. alginolyticus</i>	ATCC 17749	ATCC			
<i>V. proteus</i>	10037-74 (NCTC 8563) ^c	Sakazaki			

^a The information in parentheses is the serovar of the strain according to the scheme of R. Sakazaki (personal communication).

^b ATCC, American Type Culture Collection, Rockville, Md.

^c The designations in parentheses are other strain designations.

The ability to grow at pH 4.5, 8.0, 9.0, and 9.5 was determined by using TSA adjusted to the appropriate pH with HCl or NaOH. The ability to grow in final NaCl concentrations of 0, 3, 7, and 10% was determined by using 1% peptone broth supplemented with appropriate amounts of NaCl.

The ability to tolerate brilliant green (0.00125%), pyronin G (0.001%), neutral red (0.001%), and crystal violet (0.001%) was tested on TSA appropriately modified. Cultures were also tested for the ability to grow on thiosulfate-citrate-bile salts (TCBS) agar (Difco) and salmonella-shigella (SS) agar (Difco). Susceptibility to the following antibiotics was assessed by the disk diffusion method (BBL Microbiology Systems, Cockeysville, Md.): gentamicin (10 µg), penicillin (10 U), streptomycin (10 µg), chloramphenicol (30 µg),

erythromycin (15 µg), kanamycin (30 µg), chlortetracycline (30 µg), novobiocin (30 µg), polymyxin B (50 U), oxytetracycline (30 µg), nalidixic acid (30 µg), tetracycline (30 µg), and ampicillin (10 µg). Susceptibility to vibriostatic agent 0/129 (2,4-diamino-6,7-diisopropylpteridine) was determined by dropping a crystal of 2,4-diamino-6,7-diisopropylpteridine phosphate (Sigma Chemical Co., St. Louis, Mo.) onto a TSA plate immediately after seeding, as for antibiotic testing. Susceptibility to vibriostatic agent 0/129 was recorded as positive when a zone of clearing was observed. Strains which gave doubtful or unusual reactions were further tested for minimal inhibitory concentrations of vibriostatic agent 0/129 by using plates of heart infusion agar (Difco) containing 0, 5, 10, 50, 100, 150, or 300 µg of 2,4-diamino-6,7-diisopro-

FIG. 1. Similarity matrix for phena 1 through 16, determined by using the Jaccard similarity coefficient and unweighted average linkage.

FIG. 2. Similarity matrix for phena 17 through 33, determined by using the Jaccard similarity coefficient and unweighted average linkage.

FIG. 3. Simplified dendrogram of strains examined in this study, based on the Jaccard similarity coefficient and unweighted average linkage.

pylpteridine phosphate (Sigma) per ml (J. V. Lee, P. A. Court, T. J. Donovan, and A. L. Furniss, J. Appl. Bacteriol. 45:viii, 1978).

Biochemical tests. The following tests were read after incubation for 5 days unless otherwise noted: arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, and histidine decarboxylase (Moeller medium [Difco]) (67); nitrate and nitrite reduction, methyl red, Voges-Proskauer, and phenylalanine deaminase (prepared culture medium [Difco]); oxidase (58); catalase (17); hydrogen sulfide production from sodium thiosulfate (triple sugar iron agar [TSI; Difco]) after 7 days; urease production (broth medium of Stuart et al. [95]); indole production (1% peptone broth and Kovacs reagent [57]); growth and acid production in iodoacetate after incubation for 7 days (method of Colwell and Wiebe [17]); levan production after incubation for 7 days on TSA supplemented with 5% (wt/vol) sucrose; and ammonia production (17).

The test for fermentative or oxidative metabolism of glucose was performed by the method of Hugh and Leifson (47); the test media were examined after incubation for 1, 2, and 7 days. Acid production from each of the following carbohydrates was determined by using the medium of Hugh and Leifson (Difco) supplemented with the carbohydrate at a final concentration of 1% (wt/vol): adonitol, amygdalin, arabinose, butanol, cellobiose, dextrin, dulcitol, erythritol, etha-

nol, fructose, galactose, glycerol, inositol, lactose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, salicin, sorbitol, sorbose, sucrose, trehalose, and xylose. Carbohydrate solutions were filter sterilized and aseptically added to basal medium which had been autoclaved. The resulting media were dispensed in 24-well cluster dishes (Bellco Glass, Inc., Vineland, N.J.) (90) and inoculated by using a multi-point inoculator (66). Because of the high ratio of surface area to volume of the cluster dish wells, initial acid reactions often became alkaline due to oxidation. To guard against this possibility, reactions were read and recorded after incubation for 1, 2, 5, and 7 days. Production of gas from glucose and lactose was tested for in the oxidative-fermentative (OF) basal medium of Hugh and Leifson without agar. Inverted Durham tubes were inserted into the broth to capture any gas produced by the culture. Formation of gas was recorded after incubation for 7 days.

Degradation of organic compounds. Unless specified otherwise, all of the tests were read after incubation for 7 and 14 days. Gelatin hydrolysis was tested by the method of Smith and Goodner (84). Hemolysis was tested on TSA basal medium supplemented with sheep erythrocytes at a final concentration of 5% (vol/vol). Plates were examined after 48 h.

Deoxyribonuclease was detected on DNase test agar (Difco) supplemented with toluidine blue at a final

concentration of 0.1% (wt/vol) (9). Plates were read after 3, 5, and 7 days and then discarded.

The production of lipase was determined by the method of Sierra (83), using four different substrates (viz. Tween 20, Tween 40, Tween 60, and Tween 80) at final concentrations of 1% (wt/vol). Esculin hydrolysis was determined by the method of Sneath (87). The following two reactions were recorded: (i) browning of agar resulting from precipitation of ferric ion in the medium; and (ii) disappearance of the characteristic fluorescence under ultraviolet (UV) light. Degradation of allantoin and tyrosine was detected by the method of Gordon (35). Lecithinase was detected by the method of Baird-Parker (3); the medium used consisted of 100 ml of egg yolk emulsion (Oxoid Ltd., London, England) and 900 ml of TSA. Another method of Baird-Parker (3) was used to detect phosphatase activity; in this test, phenolphthalein diphosphate was employed as the substrate. Cellulose hydrolysis was determined on Whatman filter paper partially immersed in the salt solution recommended by DuBos (23). Casein hydrolysis was determined on TSA containing 5% skim milk.

Chitin digestion was detected by the method of Hsu and Lockwood (44), and chondroitinase activity was detected by the method of Smith and Willett (86).

Utilization of sole carbon sources. Sole carbon source utilization tests were performed by the method of Stevenson (94). The carbon sources used included β -alanine, DL-alanine, arginine, aspartate, glycine, inulin, lysine, phenylalanine, proline, propanol, serine, sodium acetate, sodium alginate, sodium citrate, sodium formate, sodium glutamate, sodium lactate, sodium malonate, sodium mucate, sodium pyruvate, sodium succinate, sodium tartrate, tryptophan, and uracil. The substrates serving as carbon sources were sterilized as 10% (wt/vol) solutions by tyndallization for 1 h at 100°C on 3 consecutive days. To obtain 500 ml (final volume) of medium containing a sole carbon source, 440 ml of water agar base containing 5 g of purified agar (Difco) and 2.5 g of NaCl was autoclaved at 121°C for 15 min. A supporting nutrient base was made up of 80.4 g of yeast nitrogen base (Difco) and 0.12 g of Casamino Acids (Difco) in 1,200 ml of glass-distilled water. The solution was filter sterilized and then neutralized by adding 300 ml of K_2HPO_4 (10%, wt/vol) that had been autoclaved at 121°C for 15 min. A 50-ml portion of the buffered base was added aseptically to the water agar, which had been cooled to 30°C, and then 10 ml of a 10% solution of a carbon source was added. Each lot of medium was thoroughly mixed and dispensed via a peristaltic pump into polystyrene cluster dishes, which were subsequently inoculated by using a multipoint inoculator. The inoculated plates were examined after incubation for 14 days, and the presence or absence of growth was recorded after comparisons with positive controls (glucose as the carbon source) and negative controls (medium lacking the carbon source).

Computer analysis. The test results were coded 0 for negative results, 1 for positive results, and 9 for noncomparable or missing data. The combined data were entered into a Univac model 1108 computer and analyzed to determine whether any of the tests were uniformly positive or negative and, therefore, non-discriminatory. Such tests and other tests believed to be highly subjective (viz., pleomorphism of cells) were

deleted from the data matrix, leaving a total of 129 tests for the final analysis.

The data matrix was prepared by using the simple matching coefficient (92), which includes both positive and negative matches, and the Jaccard coefficient (88), which excludes negative matches. Clustering was by unweighted average linkage and by single linkage (89). The programs used included the Taxan6 and IGPS3 program packages, both of which were available on the Univac model 1108 computer at the University of Maryland.

RESULTS

Vibrio spp. The 227 strains examined clustered into 33 phena at a similarity coefficient of 70 to 75% or more when the Jaccard coefficient was used and clustering was achieved by unweighted average linkage. The similarity matrix derived in this way is shown in Fig. 1 and 2, and the related dendrogram is shown in Fig. 3.

The first four clusters contained strains of *Vibrio anguillarum*. Phenon 1 consisted of eight environmental isolates from Denmark, including four strains isolated from diseased fish (Fig. 3). Phenon 2 was composed of two strains from Denmark and one strain, strain V27, isolated from the Chesapeake Bay. Cluster 3 consisted of 13 strains, 11 from fish and waters of Denmark, one (strain V34) from the Chesapeake Bay, and the reference (type) strain of the species, *V. anguillarum* ATCC 14181. Cluster 4 consisted of a pair of Danish isolates, strains NA10B and 307/75.

The strains of all four of these clusters were very similar to *V. anguillarum* (25, 82). These strains were gram-negative, motile, facultatively anaerobic, rod-shaped organisms which are oxidase positive and susceptible to vibriostatic agent 0/129 (Table 3). They grew in media containing 3% NaCl but not in media lacking NaCl and produced acid, but no gas, from glucose, fructose, mannitol, sucrose, and trehalose. These strains were negative for acid production from adonitol, inositol, lactose, sorbose, and xylose and produced a variety of extracellular enzymes which degraded starch, gelatin, deoxyribonucleic acid (DNA), and Tween 20, Tween 40, Tween 60, and Tween 80. They were able to utilize DL-alanine, citrate, inulin, proline, pyruvate, and serine as sole carbon sources, but not β -alanine, alginate, formate, lysine, malonate, tryptophan, or uracil. Most of the strains were positive for arginine dihydrolase but negative for lysine and ornithine decarboxylases.

Several phenotypic differences were observed among the clusters, as shown in Table 3. For example, strains in clusters 1 and 4 were able to grow at 42°C; this characteristic was variable in the case of clusters 2 and 3. Most of the *V. anguillarum* strains did not grow at this tempera-

TABLE 3. Characteristics of phena 1 through 11^a

Determination	% of strains positive in phenon:										
	1 (n = 9)	2 (n = 3)	3 (n = 13)	4 (n = 2)	5 (n = 5)	6 (n = 3)	7 (n = 2)	8 (n = 33)	9 (n = 4)	10 (n = 2)	11 (n = 3)
Colonial mor- phology											
White or cream	13(8) ^b	33	15	50	80	0	0	19(32)	0	100	100
Yellow	38(8)	0	8	0	20	0	0	0(32)	0	0	0
Colorless	11	67	77	50	0	100	100	81(32)	100	0	0
Diffusible pig- ment	0	0	0	0	0	0	0	3	0	0	0
Mucoid	0	0	0	0	0	0	0	0(32)	0	0	0
Matt	0	0	0	0	0	0	0	0	0	0	0
Translucent	67	100	85	100(1)	100(4)	100	100	82	100	50	33
Round	100	100	100	100	100	100	100	100	100	50	100
Irregular mar- gin	0	0	0	0	0	0	0	0	0	50	0
Rhizoid margin	0	0	0	0	0	0	0	0	0	0	0
Flat	0	33	0	0	0	33	0	3	0	0	0(2)
Spreading	0	0	0	0	0	0	0	0	0	0	0
Micromorpholo- gy											
Gram variable	0	0(2)	0(12)	0	200	0	0	0	0	0	0
Length/width ratio of ≤6:1											
Filaments	0	0(2)	0(12)	0	20	0	0	0	0	0	0
Motility	100	100	89(8)	ND ^c	100	100	100	100(30)	100(3)	50	100(1)
Fluorescence	0	0	0(12)	0	0	0	0	0(27)	0	0	0
Growth at:											
5°C	100(8)	100(2)	82(11)	100	20	0	0	9	25	0	100
10°C	100	100	100	100	100	67	100	84(32)	100	50	100
15°C	100	100	100	100	100	100	100	100(29)	100	100(1)	100
42°C	100	50(2)	69	100	100	100	100	100	100(3)	100	50(2)
44°C	11	0	25(12)	ND	100	100	100	77(31)	100	50	0
pH 4.5	63(8)	0	33(8)	ND	60	0(1)	0	7(14)	33(3)	0(1)	100(2)
pH 9.0	100	100	92	100	100	100	100	100	100	100	100
pH 9.5	ND	100	100	50	ND	100	ND	97	100	100	100
Growth on:											
0% NaCl	0(8)	0(2)	0	0	100	100	100	100(32)	100(3)	100	100(2)
7% NaCl	0	100(2)	92	100	0	33	0	9	25	50	33
10% NaCl	0	0	8	50	0	0	0	0	0	0	0
Sodium lauryl sulfate	89	100	100	100	100	100	100	97	100	100	100
Crystal violet	50(6)	100	100	100	100	100	100(1)	97	100	100	100
Brilliant green	89	100	100	100	80	100	50	93(30)	100	100(1)	100
TCBS agar	100	100	100	100	100	100	100	100	100	100	100
SS agar	56	100	46	100	100	100	50	100	100	100	100
Resistant to:											
Gentamicin	11	0	0(12)	0(1)	20	0(2)	0	0	0	0	0
Penicillin	89	0	67(12)	100(1)	100	100(2)	0	9	0	100	0
Chlorampheni- col	0	0	0(12)	0(1)	0	0(2)	0	3	0	0	0
Erythromycin	11	0	10(10)	0(1)	0	0(2)	0	0	0	0(1)	0
Kanamycin	0	0	8	0(1)	0	0(2)	0	3	0	0(1)	0
Chlortetracy- cline	ND	0	0(12)	0(1)	ND	100(2)	ND	3(31)	0	0	0
Novobiocin	0	0	8(12)	0(1)	0	0(2)	0	0	0	0	0
Polymyxin B	67	100	83(12)	100(1)	100	100(2)	100	73	100	100	33
Oxytetracy- cline	0(8)	0	0	0(1)	20	100(2)	0	0	0	0(1)	0
Tetracycline	11	0	8	0(1)	0	100(2)	100	0	0	0(1)	0
Ampicillin	56	0	42(12)	100(1)	100	100(2)	0	3	25	100(1)	0
Streptomycin	ND	33	0(12)	0(1)	ND	0(2)	ND	6	0	0	0
Susceptible to vi- brio static agent 0/129	100(8)	100	100	100	80	100	100	100(32)	25	100	67

TABLE 3—Continued

Determination	% of strains positive in phenon:										
	1 (n = 9)	2 (n = 3)	3 (n = 13)	4 (n = 2)	5 (n = 5)	6 (n = 3)	7 (n = 2)	8 (n = 33)	9 (n = 4)	10 (n = 2)	11 (n = 3)
Biochemical tests											
Catalase	78	100	100	100	100	100	50	100	100	100	100(2)
Oxidase	100	67	100	100	100	100	100	100	100	100	100(2)
Arginine dihydro- lase	22	100	92	50	0	0	0	0	100	0	33
Histidine de- carboxylase	0	0	0	0	0	0	50	0	0	0	0
Lysine decar- boxylase	0	33	23	0	100	100	100	100	100	100	67
Ornithine de- carboxylase	0	0	15	0	100	100(2)	100	100	50	100	33
H ₂ S	0(8)	0	0	0	0	0	0	0	0	0	0
Methyl red	17(6)	0	15	0	0	0	0	24	100	0	33
Voges-Proskauer	43(7)	33	58(12)	0	100	33	100	73	50	100	0
Phenylalanine deaminase	0(8)	0	0	0	0	0	0	0	0(2)	0	0
Nitrate reduction	100(8)	67	100	0	100	100	100	100	100	100	100
Nitrite reduction	ND	67	100	0	ND	100	ND	100	100	100	100
Phosphatase	100(8)	100	100	100	100	100	100	100(32)	100	100	100
Indole	50(8)	0	39	0	100	100	100	91	100	50	67
Levan	0	0	33(12)	0	0	0	0	0(18)	0	0	0
Melanin	25(8)	0	0	0	0	0	0	0(32)	0	0	0
Iodoacetate, growth	0	33	92	100	0	100(1)	0	70(30)	100(3)	0	100(2)
Iodoacetate, acid	0	0	0	0	0	0(1)	0	0(30)	0(3)	0	0(2)
Alpha-hemolysis	0	0	0(12)	ND	0	0	0	0(32)	25	0	0
Beta-hemolysis	11	0	75(12)	ND	40	100	100	100(32)	50	100	100
Simmons citrate	67(6)	67	100(9)	ND	80	100(1)	100	100(13)	100(2)	100	100(2)
Ammonia production	100	ND	ND	ND	100	ND	100	ND	ND	ND	ND
Fermentative metabolism	100	100	100	100	100	100	100	100	100	100	100(1)
Acid from:											
Arabinose	22	0	85	0	0	0	0	0	50	0	33
Butanol	0	0	8	0	0	0	0	0	0	0	0
Cellobiose	71(7)	0	77	100	75	33(4)	100	18	100	50	0
Dextrin	89	67	100	100	100	100	100	55	100	100	100
Erythritol	0	0	0	0	0	0	0	0	0	0	0
Ethanol	0	0	0	0	40	0	0	3	0	0	0
Fructose	100(7)	100	100	100	80	100	50	100	100	100	100
Galactose	100(8)	67	92	100	100	33	50	84(32)	100	100	100
Glycerol	89	100	62	100	100	100	100	42	100	50	100
Inositol	0	0	0	0	0	0	0	0	0	0	33
Lactose	0(8)	0	15	0	0	0	100	0	0	0	0
Mannitol	100(8)	100	100	100	100	100	100	100	100	100	100
Mannose	89	100	100	100	0	33	0	67	100	100	100
Melibiose	0	0	0	0	0	0	0	0	0	0	0
Raffinose	0	0	0	0	0	0	0	0	0	0	0
Rhamnose	0(8)	0	8	0	0	0	0	0	0	0	0
Salicin	0	0	0	50	0	0	0	0	0	0	33
Sorbitol	44	100	77	0	0	0	0	6	25	0	0
Sorbose	0	0	0	0	0	0	0	0	0	0	0
Sucrose	100(6)	100(2)	100	50	100	100	100	73	50	100	100
Trehalose	100	100	100	100	40	100	0(1)	91	100	100	100
Xylose	0	0	8	0	0	0	0	0	0	0	0

TABLE 3—Continued

Determination	% of strains positive in phenon:										
	1 (n = 9)	2 (n = 3)	3 (n = 13)	4 (n = 2)	5 (n = 5)	6 (n = 3)	7 (n = 2)	8 (n = 33)	9 (n = 4)	10 (n = 2)	11 (n = 3)
Gas from:											
Glucose	0	33	0	0	0	0	0	3(32)	100	0	33
Lactose	0	0	0	0	0	0	0	0(32)	0	0	0
Degradation of:											
Urea	0	0	8	0	0	0	0	0	0	0	0
Starch	86(6)	100	100	100(1)	100(4)	100	100	72(29)	75	100	100
Chitin	100(1)	100(2)	60(10)	0(1)	100	100	100	94(32)	100	100(1)	100
Gelatin	100(1)	ND	100(5)	ND	100	100(1)	100	100	100	100	100(2)
Casein	88(7)	100	100	0(1)	100	100	100	100	100	100	100
DNA	50(4)	100	100	100	100	100	0	91(32)	100	100	100
Tween 20	100	100	100	100	100	100	100	100	100	100	100
Tween 40	100	100	100	100	100	100	100	100(31)	100	100	100
Tween 60	100	100	100	100	100	100	100	100	100	100	100
Tween 80	100(8)	100	100	100	100	100	100	100	100	100	100
Lecithin	50(8)	100	100(10)	100	80	67	50	94	75	100	100
Tyrosine	33	0	8	0	0	0	0	3(32)	0	0	0(2)
Allantoin	0	ND	ND	ND	0	ND	0	ND	ND	ND	ND
Chondroitin	ND	0(1)	60(5)	100(1)	ND	67	ND	21(19)	50(2)	0	100(1)
Esculin (by color)	0(6)	0	8	50	0(2)	0	0	0	0	0	67
Esculin (by UV light)	0(6)	0	0	0	0(1)	0	0	0	0	0	50(2)
Utilization as sole carbon source:											
Acetate	89	100(1)	18(11)	0	100	0	50	50(30)	50	0	0
Beta-alanine	0	ND	0(8)	0	0	0(1)	0	0(2)	ND	ND	0(1)
DL-Alanine	100	100	100	100	100	67	100	88	100	100	100
Alginate	ND	0	0	50	ND	0	ND	0	0	0	0
Arginine	89	0	92	100	100	33	100	97(30)	100	100(1)	100
Citrate	100	100	100	100	100	100	100	94	100	100	100
Formate	0	0	0	0	0	0	0	0	0	0	0
Glycine	0	0	17(12)	100	100	33	100	18	75	0	33
Inulin	100	100	92	100	100	67	50	97	100	100	100
Lysine	0	0	0	0	0	0	0	3	0	0	0
Malonate	0	0	0	0	0	0	0	0	0	0	0
Phenylalanine	0	0	8	0	0	0	0	12	0(3)	0	0
Proline	67	100	100	100	100	100	100	91	100	100	100
Propanol	0	0	8	0	0	0	0	6	75	0	0
Pyruvate	100	100(2)	100	100	100	67	100	97(32)	100	0	100
Serine	100	100	69	100	100	100	100	78(32)	100	50	100
Succinate	100	33	54	100	80	100	100	23(31)	75	0	33
Tartrate	0	0	23	50	0	0	0	3	0	0	0
Tryptophan	0	0	15	0	0(3)	33	0	0	0	0	0
Uracil	0	0	0	0	0	0	0	0	0	0	0

^a All phena were positive for the following tests: growth at 37°C and pH 8, growth on 3% NaCl, neutral red, and pyronin G, and rod-shaped cells. All phena were negative for production of acid from adonitol, dulcitol, amygdalin, and melizitose, resistance to nalidixic acid, cellulose hydrolysis, and formation of chains.

^b The numbers in parentheses indicate the numbers of strains tested for different traits.

^c ND, Not done.

ture. Cluster 1 strains did not grow in media containing 7% NaCl, whereas strains in clusters 2 through 4 did grow at this salinity. We also observed differences in the production of acid from arabinose, cellobiose, and sorbitol and in the utilization of acetate and arginine. The clus-

ter 4 strains differed from the strains of the other three clusters in that they were negative for chitin hydrolysis, casein hydrolysis, and nitrate reduction.

The strains in clusters 5 through 8 were all identified as members of *V. cholerae*. Cluster 5

consisted of five strains, all of which were isolated from Copenhagen sewage. Cluster 6 contained three strains, strain DAM12/75 from Copenhagen sewage and two strains of *V. cholerae* received from R. Sakazaki (strain 10317-74 [serovar 8] and strain 11416-62 [serovar 13]). Two additional reference strains of *V. cholerae* were recovered in cluster 7; these were strain 7007-64 (serovar 6) and strain 4202 (serovar 5). Cluster 8 was the largest cluster recovered in the analysis. This cluster contained 33 strains, including 19 strains of *V. cholerae* isolated from the Chesapeake Bay (strains V2, V3, V35, V33, V31, V37, V4, V5, V10, V36, V38, V11, V15, V25, V26, V20, V24, V19, and V29), 3 strains of *V. cholerae* received from R. Sakazaki (strains 211-72, 218-68, and 8645-64 [serovars 12, 10, and 14, respectively]), 9 strains of *V. cholerae* non-O1 received from H. Smith and isolated in Thailand, in Czechoslovakia, from the Chesapeake Bay, and in the Philippines, 1 strain of *V. cholerae* O1 (strain Inaba, isolated in Bangladesh), and 2 reference strains of *V. cholerae* O1 obtained from the American Type Culture Collection (ATCC 14033 and ATCC 14035, representing the El Tor and classical biovars, respectively).

The strains of clusters 5 through 8 exhibited characteristics ascribed to *V. cholerae*; i.e., they were gram-negative, facultatively anaerobic, rod-shaped organisms that were oxidase positive, susceptible to vibriostatic agent 0/129, and motile and produced acid but not gas from glucose and mannitol, but not from arabinose, inositol, or xylose. These strains were positive for nitrate reduction, lysine decarboxylase, ornithine decarboxylase, growth in 0 and 3% NaCl, hydrolysis of Tween, gelatin, starch, and casein, and indole production, but negative for arginine dihydrolase and growth in 7% NaCl. The guanine-plus-cytosine (G+C) contents of the DNAs of selected strains were 47 to 48 mol% (16), values typical of *V. cholerae* (82).

Certain differences were observed among clusters 5 through 8. For example, antibiotic resistance patterns revealed that strains of clusters 5 and 6 were ampicillin resistant, whereas strains of clusters 7 and 8 were ampicillin susceptible; strains of clusters 6 and 7 were tetracycline resistant, and strains of cluster 7 were also resistant to oxytetracycline.

We also observed differences in the utilization of sole carbon sources; strains of clusters 5 and 7 utilized glycine, whereas strains of clusters 6 and 8 were not able to utilize this compound. Strains of cluster 5 were uniformly positive for acetate utilization, whereas strains of cluster 6 were negative and strains of the remaining two clusters were variable for utilization of this carbon source. Other notable exceptions in

characteristics include the lack of deoxyribonuclease activity in strains of cluster 7 and the production of colorless colonies by strains of cluster 5. Acid production from certain carbohydrates also revealed differences; strains of cluster 7 were lactose positive, whereas the other strains were negative for this characteristic.

Strains of cluster 9 were identified as being members of *Aeromonas hydrophila*; these were strains V16 and V22 from the Chesapeake Bay, strain K204V from seawater in Denmark, and strain 112-68, which was received from R. Sakazaki as *V. cholerae* serovar 9. In general, these strains adhered to the description of *A. hydrophila* provided by various investigators (26, 71, 81). The cluster 9 strains were gram-negative, facultatively anaerobic, rod-shaped organisms which were oxidase positive and motile. They were resistant to vibriostatic agent 0/129, produced acid and gas from a variety of sugars, and gave a positive reaction for arginine dihydrolase. However, strains K204V and 112-68 were ornithine positive, a characteristic which has not been reported previously for *A. hydrophila*. These two strains were unusual in that they were susceptible to vibriostatic agent 0/129, which, together with the ornithine decarboxylase reaction, suggests that they are vibrios. However, these strains produced gas from glucose and acid from arabinose, characteristics which are usually negative for *V. cholerae* but positive for aeromonads.

Cluster 10 contained two strains, strain 1282-75 from Denmark and strain 5049, which was received from H. Smith and was originally isolated from freshwater in Thailand. The strains in this cluster were very similar to strains in the main cluster of *V. cholerae* and possessed the major characteristics of this species (see above).

Three strains were recovered in cluster 11; two of these were from Denmark, and one, strain 10037-74, was received as *Vibrio proteus* from R. Sakazaki. These strains appear to be intermediate strains, belonging to neither *Vibrio* nor *Aeromonas*, as Table 4 shows. On the basis of the data gathered in this study, no definitive species assignment of the strains of phenon 11 can be made at the present time.

A total of 14 vibrio strains isolated from diseased shrimp were recovered in cluster 12, along with the reference strain of *V. parahaemolyticus* (strain ATCC 17802). Also recovered in cluster 12 was a reference culture of *Beneckea harveyi*. This phenon was identified as *V. parahaemolyticus* on the basis of biochemical characteristics (Table 4) and the inclusion of the type strain of this species in the cluster. The base composition of selected strains was 45 mol% G+C. The biochemical characteristics of these strains agree well with the accepted definition of

TABLE 4. Characteristics of *Vibrio phena* 12 through 22

Determination	% of strains positive in phenon:										
	12 (n = 16)	13 (n = 2)	14 (n = 5)	15 (n = 2)	16 (n = 2)	17 (n = 6)	18 (n = 16)	19 (n = 3)	20 (n = 7)	21 (n = 3)	22 (n = 2)
Colonial morphology											
White or cream	25	50	0(4) ^a	0	100	0	53(15)	0	0	0	0
Yellow	0	0	0(4)	0	0	67	7(15)	0	0	0	100
Colorless	75	50	100(4)	100	0	33	40(15)	100	100	100	0
Diffusible pigment	0	0	0(4)	0	0	0	0	0	0	0	0
Mucoid	0	0	0	0	0	0	13(15)	0	0	0	0
Matt	0	0	0	0	0	0	0	0	0	33	0
Translucent	94	100	100	100	100	100	63	100	100	67	50
Round	100	100	100	100	100	100	100	100	100	100	100
Irregular margin	0	0	0	0	0	0	0	0	0	0	0
Rhizoid margin	0	0	0	0	0	0	0	0	0	0	0
Flat	6	50	20	100	0	100	44	100	0	0	0
Spreading	13	0	0	0	0	0	0	0	0	0	0
Micromorphology											
Gram variable	0(15)	0	0(4)	0	0(1)	0	0	0	14	0	0
Length/width ratio of $\geq 6:1$	0(15)	0	0(4)	0	0(1)	17	6	0	100	0	0
Filaments	0(15)	0	0(4)	0	0(1)	33	0	0	43	0	50
Motility	92(13)	100	100(2)	100(1)	100(1)	100	100(15)	100	100(4)	100(2)	100
Fluorescence	0(9)	0	0	0	0	0	0	0	0	0	0
Growth at:											
5°C	0(15)	0	0	0	0	100	100	100	86	100	50
10°C	94	100	100	100	100(1)	100	100	100	100	100	100
15°C	94	100	100	100	100	100	100	100	100	100	100
42°C	100(15)	100	100	100	100	100	92(13)	100(2)	71	0	100
44°C	88	0	40	0	100	100	19	0	14	0	50
pH 4.5	0(14)	ND ^b	0	100(1)	100(1)	75(4)	6	0	0(4)	0	100(1)
pH 9.0	100	100	100	100	100(1)	100	100	100	86	100	100
pH 9.5	100(14)	100	100	ND	100	ND	100(12)	100	100(3)	100(2)	ND
Growth on:											
0% NaCl	0	0	0	0	100(1)	17	93(15)	100	100	0(2)	50
7% NaCl	100	100	60	0	100	100	100	100	100	50(2)	100(1)
10% NaCl	0	0	0	0	50	0	0	0	0	0	0
Sodium lauryl sulfate	88	100	100	50	50	100	100	100	33(6)	100(2)	0
Crystal violet	100	100	100	0(1)	100	100	100(15)	100	100(5)	100	0
Brilliant green	100	100	75(4)	100	50	100	100	100	14	100	100
TCBS agar	100	100	100	100(1)	100	100	100	100	14	100	100
SS agar	81	100	0	50	0	100(5)	94	100	0(6)	33	100
Resistant to:											
Gentamicin	0(13)	0	0(1)	0	0	0	0	0	0	0	50
Penicillin	100(13)	100	100(1)	100	0	100	6	33	0	0	100
Chloramphenicol	0(13)	0	0(1)	0	100	0	0	0	0	0	0
Erythromycin	0(12)	0	0(1)	0	0	0	0	0	0	33	0
Kanamycin	0(13)	0	0(1)	0	0	0	0	0	0	0	0
Chlortetracycline	0(9)	0	ND	ND	0	ND	0(12)	0	0(4)	0	ND
Novobiocin	0(13)	0	0(1)	0	0	0	0	0	0	0	0
Polymyxin B	15(13)	50	0(1)	50	100	20(5)	75	33	43	100	100
Oxytetracycline	0(13)	0(1)	0(1)	0(1)	0	0	0	0	0	0	0
Tetracycline	0(13)	0	0(1)	0(1)	0	0	0	0	0	0	0
Ampicillin	92(13)	50	100(1)	50	0	50	6	67	0	0	100
Streptomycin	0(12)	0	0(1)	ND	0	ND	8(12)	0	0(4)	0	ND
Susceptible to vi- briostatic agent 0/ 129	15(13)	0	100	100	50	100	100(13)	100	100	100	50
Biochemical tests											
Catalase	100	100	100(4)	100	100	100	100	100	100	100	100
Oxidase	100	100	100(4)	100	100	0	0	0	0	100	100
Arginine dihydro- lase	0(15)	0	0(2)	100	100	0	31	0	0	100	0
Histidine decar- boxylase	7(15)	0	0	0	0	33	0	0	43	0	50

TABLE 4—Continued

Determination	% of strains positive in phenon:										
	12 (n = 16)	13 (n = 2)	14 (n = 5)	15 (n = 2)	16 (n = 2)	17 (n = 6)	18 (n = 16)	19 (n = 3)	20 (n = 7)	21 (n = 3)	22 (n = 2)
Lysine decarboxylase	100(15)	100	40	50	50	80(5)	75	67	71	0	50
Ornithine decarboxylase	93(15)	100	100(1)	0	50	0	0	0	14	0	100
H ₂ S	0	50	0	0(1)	0	0	0	0	0	0	0
Methyl red	100	100	40	ND	0	83	20(15)	0	0(6)	0	0
Voges-Proskauer	0	0	0	50	100	67	73(15)	67	86	100	100(1)
Phenylalanine deaminase	13(15)	0	0	0(1)	0	0	0	0	0	0	0
Nitrate reduction	100(15)	100	67(3)	100(1)	100	0	0	0	14	100(2)	100
Nitrite reduction	100(15)	100	67(3)	ND	100	0	0(15)	0	0(6)	100(2)	ND
Phosphatase	100	100	100(4)	ND	100	100	87(15)	100	67(6)	100	100
Indole	83(12)	100	100(4)	0	0	17	19	0	57	0	100
Levan	0(2)	ND	ND	0	0	0	0	0	0	0	0
Melanin	0(2)	0	0(2)	0	0	17	0	0	0	0	0
Iodoacetate, growth	100	100	100	0	100	0	69	67	0(3)	67	0(1)
Iodoacetate, acid	0	0	0	0	0	0	0	0	0(3)	0	0(1)
Alpha-hemolysis	64(14)	50	0(4)	0	0(1)	0	6	0	0	0	0
Beta-hemolysis	0(14)	0	50(4)	50	0(1)	33	81	100	57	67	50
Simmons citrate	ND	ND	ND	0(1)	0(1)	100	81	100	75(4)	100(2)	100(1)
Ammonia production	ND	ND	ND	100	ND	100	100(14)	ND	100(4)	ND	100
Fermentative metabolism	100(15)	100	40	100	50	100	100	100	100(5)	100	100
Acid from:											
Arabinose	31	100	0	0(1)	0	0(5)	0(15)	0	0	33	0
Butanol	0	0	0	0	0	0	0	0	0	0	0
Cellobiose	100	50	60	0(1)	100	33	38	0	0	100	100
Dextrin	50	100	0	0(1)	50	100	100	100	100	100	50
Erythritol	0	0	0	0	0	0	0	0	0	0	0
Ethanol	0	0	0	0	0	0	0	0	0	0	0
Fructose	100	100	100	100(1)	100	100	100	100	100(6)	100	100
Galactose	94	100	100	100(1)	50	67	94	0	100	100	100
Glycerol	75	50	60	100(1)	0	100	100	100	86	100	100
Inositol	0	0	0	0	0	33	69	0	100	0	0
Lactose	0	0	0	0	0	67	88	0	43	0	0
Mannitol	94	100	80	0(1)	100(1)	100	100	100	100(6)	100	100
Mannose	93(14)	100	40	100	100	100(5)	88	100	100	100	50
Melibiose	0	0	0	0(1)	0	67	94	0	0	0	0
Raffinose	0	0	0	0	0	17	0	0	0	0	0
Rhamnose	0	0	0	0	0	0	0	0	0	0	0
Salicin	0	0	20	0	0	0	13	0	0	0	0
Sorbitol	0(14)	0	33(3)	0	0	33	38	0	0	0	50
Sorbose	0	0	0	0	0	0	0	0	0	0	0
Sucrose	94	0	100	0(1)	100	100	100(14)	100	100	100	100(1)
Trehalose	100	100	100(4)	100	100	100	100	100	100	100	50
Xylose	0	0	ND	0	0	0	6	0	0	0	0
Gas from:											
Glucose	13(8)	0	0(2)	0	0	0	7(15)	0	0	0(2)	0
Lactose	0	0	0	0	0	0	7(15)	0	0	0(2)	0
Degradation of:											
Urea	81	0	0	50	0	0	0	0	0	0	0
Starch	100	100	100(4)	100(1)	100	100(3)	100	100	100(5)	100	100
Chitin	73(15)	100	100(4)	ND	0(1)	100(4)	100(15)	100	100(6)	100	100(1)
Gelatin	82(11)	100	100	ND	0(1)	100(3)	91(11)	100	83(6)	100(1)	100(1)
Casein	40(15)	100	75(45)	100	50	100	69	ND	100	100	100
DNA	100(15)	100	100(4)	100(1)	100	80(5)	100(14)	100(2)	100(4)	100	100(1)
Tween 20	100	100	100	100	100	100	100	100(2)	100(6)	100	0
Tween 40	100	100	100	100	0(1)	100	100	100	100	100	0
Tween 60	100	100	100	100	100	100	100	0	100	100	50
Tween 80	100	100	100	100	100	100(5)	93(15)	100(2)	100	100	0
Lecithin	80(15)	50	100(4)	0(1)	100	100(5)	100(13)	100	67(6)	50(2)	100

TABLE 4—Continued

Determination	% of strains positive in phenon:										
	12 (n = 16)	13 (n = 2)	14 (n = 5)	15 (n = 2)	16 (n = 2)	17 (n = 6)	18 (n = 16)	19 (n = 3)	20 (n = 7)	21 (n = 3)	22 (n = 2)
Tyrosine	0(14)	0	0(2)	0	0	17	0	0	0	33	0
Allantoin	ND	ND	ND	0	ND	0	0(14)	ND	0(4)	ND	0
Chondroitin	69	0	25(4)	ND	50	ND	0(1)	ND	0(3)	50(2)	ND
Esculin (by color)	7(15)	0	100	0(1)	100(1)	75(4)	87(15)	67	100(4)	0	0
Esculin (by UV light)	0(15)	0	100	0(1)	100(1)	100(1)	75(12)	0	0(2)	0	0
Utilization as sole carbon source:											
Acetate	40(15)	0	0	0	0	67	75	0	14	0	0
Beta-alanine	0	ND	0	0	0	0	0(4)	ND	0(4)	ND	0
DL-Alanine	100	100	100	100	50	100	100	100	100	67	100
Alginate	94	0	100	ND	0	ND	17(12)	0	0(3)	0	ND
Arginine	94	100	100	100	100	83	94	100	86	0	100
Citrate	94	50	100(4)	100	100	100	94	100	100	33	100
Formate	0	0	0	0	0	0	0	0	0	0	0
Glycine	40(15)	50	0	0	0	17	0	0	0	0	0
Inulin	88	100	100(4)	50	100	83	93(15)	67	86	0	100
Lysine	50(14)	0	20	0	0	0	0	0	0	0	0
Malonate	0	0	0	0	0	0	0	0	0	0	0
Phenylalanine	13	100	20	0	0	17	0	0	0	0	0
Proline	100	50	100	50	100	83	94	100	43	33	100
Propanol	88	0	0	0	0	0	0	0	0	0	0
Pyruvate	100(15)	100	100	100	100	100	88	33	57	0	100
Serine	81	100	25(4)	100	0(1)	100	100	0	57	0	100
Succinate	93(15)	0(1)	60	100	100	100	63	0	71	0	100
Tartrate	15(13)	0	0(4)	0	0	0	0	0	0	0	0
Tryptophan	70(10)	0	ND	0	0	0	0	0	14	0	0(1)
Uracil	0	0	0	0	0	0	0	0	0	0	0

^a The numbers in parentheses indicate the numbers of strains tested for different traits.

^b ND, Not done.

V. parahaemolyticus, with the exception of sucrose fermentation, which was positive for these strains. Other characteristics that are useful for distinguishing *V. parahaemolyticus* from *Vibrio alginolyticus* (viz., acetoin production, growth in 10% NaCl, swarming on 1.5% agar plates, and methyl red reaction) were found to be typical of *V. parahaemolyticus* (75). Sucrose fermentation was tested with several media, including Hugh-Leifson medium (47), OF basal medium, Leifson MOF medium (63), TSI agar, and TCBS agar. All methods gave the same results (i.e., positive sucrose fermentation).

Two other noteworthy characteristics demonstrated by strains of cluster 12 were that 81% of the strains were urease positive and 17% were indole negative.

Cluster 13 consisted of two reference strains of *V. parahaemolyticus* which clustered at a slightly lower value (72%). These strains, strains SAK 4 and ATCC 27969 (= FC 1101), gave the biochemical reaction patterns expected for *V. parahaemolyticus* and differed from cluster 12 in being sucrose negative, unable to grow at 44°C,

and unable to utilize alginate, propanol, or succinate as a sole carbon source.

Five shrimp isolates comprised cluster 14. This cluster was very similar to clusters 12 and 13 and was also identified as *V. parahaemolyticus*. The strains of cluster 14 also were sucrose positive, but possessed none of the other key characteristics of *V. alginolyticus*. These strains differed from the strains of cluster 12 by being urease negative, unable to grow on SS agar, variable for the lysine decarboxylase reaction, and esculin positive.

Phenon 15 comprised only two strains, strains F580/75 and 715. Both of these strains were from Denmark; strain F580/75 was isolated from a fish wound, and strain 715 was isolated from salt water. The strains of cluster 15 were closely related to the strains of cluster 1 (*V. anguillarum*) but were distinguished by the flat, colorless colonies produced, in addition to the lack of acid production from mannitol or dextrin and the lack of growth at 5°C. Additional characteristics of this cluster are given in Table 4.

Two more strains from Denmark, strain

F647/75 from a fish infection and strain 274/75 from seawater, comprised cluster 16, which was rather heterogeneous. Strain F647/75 is a *Pseudomonas* sp. belonging to group A of Lee et al. (Lee et al., J. Appl. Bacteriol. 45:viii, 1978) and was identified on the basis of its oxidative metabolism, motility, oxidase reaction, arginine dihydrolase, resistance to chloramphenicol, lack of ornithine decarboxylase, and growth on succinate and proline as sole carbon sources. Strain 274/75 satisfied the minimum requirements for assignment to the genus *Vibrio*, but could not be unequivocally assigned to any species.

Strains of phenon 17 through 20 resembled the species described by Lee et al. (60) and named *V. metschnikovii*. These strains were gram-negative, curved or straight, motile, rod-shaped organisms that were facultatively anaerobic and produced acid, but no gas, from glucose, mannitol, and sucrose. They were susceptible to vibriostatic agent 0/129 and negative for ornithine and lysine decarboxylases, arginine dihydrolase, nitrate reduction, and cytochrome oxidase.

The cytochrome oxidase reaction is the major characteristic that distinguishes *V. metschnikovii* from other *Vibrio* species. The other characteristics given by Lee et al. (60) are in agreement with our results (i.e., production of acid from mannose and trehalose and no acid produced from arabinose, raffinose, rhamnose, salicin, or xylose). Our results and those of Lee et al. also agreed with respect to utilization of sole carbon sources; nearly all strains were unable to utilize formate, glycine, lysine, malonate, phenylalanine, propanol, or tartrate, whereas they were able to utilize alanine and proline. Variable reactions were observed in the arginine, pyruvate, serine, and succinate tests. In addition, almost all strains hydrolyzed starch, chitin, gelatin, and casein and produced deoxyribonuclease and lipase (Tween 80).

The largest of the *V. metschnikovii* clusters was cluster 18; this cluster consisted of 16 strains, all of which were environmental isolates from Denmark. Three more isolates from Denmark were recovered in cluster 19, which joined cluster 18 at the 71% level. These strains were distinguished from the cluster 18 strains and other *V. metschnikovii* strains by not producing acid from galactose, inositol, lactose, and melibiose and by not being able to utilize succinate as a sole carbon source or hydrolyze Tween 60, although all strains could hydrolyze Tween 80.

Phenon 20 is a very interesting group, consisting entirely of reference strains of *V. metschnikovii* and *Vibrio proteus*. *V. proteus* is an illegitimate name and is a synonym for *V. metschnikovii* (60). The strains in this group included strain 10041-74 (*V. proteus* NCTC 8563), strain 10040-74 (*V. metschnikovii* 7), strain 10122-74 (*V.*

metschnikovii ATCC 7708), strain 10121-74 (*V. metschnikovii* 211), strain 10042-74 (*V. metschnikovii* NCTC 8443), strain 10036-74 (*V. metschnikovii* CIP 6914), and strain 8394-62 (*V. cholerae* O-7). These reference strains clustered with the wild strains at similarity values of only $\geq 66\%$; this finding was similar to the finding of Lee et al. (60), who reported that all of the reference strains of both *V. proteus* and *V. metschnikovii* clustered separately from the wild strains. The strains which we used behaved exactly like the strains in the previous study in that all strains except one were unable to grow on TCBS agar, the primary isolation medium used for *Vibrio* species, and none could grow on SS agar. All of the wild strains grew well on these media. Table 4 shows that, in addition to not growing on TCBS agar, most of the phenon 20 strains did not grow on agar containing sodium lauryl sulfate or brilliant green. All strains produced unusually long cells, with ratios of length to width of $>6:1$. One notable biochemical reaction was the production of acid from inositol by all of these strains, a characteristic negative or variable for the other clusters of *V. metschnikovii* strains. The phenon 20 strains were not as nutritionally diverse as the wild strains; the percent carbon source utilization was generally lower for these strains as a cluster than for the strains in the other clusters of *V. metschnikovii*.

Cluster 21 consisted of three strains (strains 639/75, 1106/75, and 1211/75), all from Denmark. These strains were closely related to the strains of phenon 2; in addition, their characteristics were very similar to those of *V. anguillarum* and, therefore, they were identified as *V. anguillarum*. These strains differed from those of phenon 2 by not being able to utilize citrate or proline (Table 5).

Two more of the Danish strains were grouped in phenon 22; these were strain F702/75, which was originally isolated from a fish infection, and strain DAM81/75, which was isolated from Copenhagen sewage waters. This cluster had an intergroup similarity value of 70% compared with phenon 5, which included strains of *V. cholerae* from Copenhagen sewage water. We concluded that strain DAM81/75 is a strain of *V. cholerae*; this strain had a mean similarity value of 76% compared with group 5. When clustering was performed by single linkage rather than average linkage, strain DAM81/75 clustered with phenon 5, having a similarity value of 81% compared with strain DAM86/75 in phenon 5 (Kaper and Colwell, unpublished data). However, because of lower similarity values in comparisons with other strains of phenon 5, the average level of similarity was higher with strain F702/75 than with all strains of phenon 5. The same

TABLE 5. Characteristics of *Vibrio phena* 23 through 33

Determination	% of strains positive in phenon:										
	23 (n = 3)	24 (n = 2)	25 (n = 2)	26 (n = 2)	27 (n = 2)	28 (n = 2)	29 (n = 3)	30 (n = 6)	31 (n = 2)	32 (n = 13)	33 (n = 2)
Colonial morphology											
White or cream	100	0(1) ^a	0	0	0	0	33	60(5)	0	100	100
Yellow	0	0(1)	0	0	0	0	0	20(5)	0	0	0
Colorless	0	100(1)	50	0	0	100	0	20(5)	100	0	0
Diffusible pigment	0	0	0	0	0	0	0	33	0	0	0(1)
Mucoid	0	0(1)	0	0	0	0	0	0(5)	0	0	0
Matt	0	0	0	0	50	0	0	17	0	31	100(1)
Translucent	0	50	100	100	100	100	67	83	50	15	100(1)
Round	100	50	100	100	50	100	100	100	100	69	100(1)
Irregular margin	0	0	0	0	50	0	0	0	0	15	0(1)
Rhizoid margin	0	50	0	0	0	0	0	0	0	15	0(1)
Flat	0	0	50	50	100	0	0	0	0	92	100(1)
Spreading	0	0	0	0	0	0	0	0	0	0	0(1)
Micromorphology											
Gram variable	0	0	ND ^b	0(1)	ND	50	0	0(5)	0(1)	50(12)	100(1)
Length/width ratio of ≥6:1	33	0	ND	0	0(1)	0	0	0(5)	100(1)	0(12)	0(1)
Filaments	67	0	ND	0	0	0	0	20(5)	0(1)	0(12)	0(1)
Motility	100(1)	100(1)	100	100	0	100(1)	ND	100(4)	ND	100(3)	100(1)
Fluorescence	0	0	0	0	0	0	0	50	0	0	0
Growth at:											
5°C	100(2)	0	0	0	0	0	100	100	0	0(11)	0
10°C	100	100	ND	100(1)	100(1)	100	100	100	50	85	100
15°C	100	100	100	100	100	100	100	100	50	100	100
42°C	67	100	100	100	100	100	100	67	100	100	100
44°C	33	100	100	100	100	0	33	50	100	100(12)	100
pH 4.5	100(2)	100(1)	50	100	100	50	100	100(5)	ND	67(3)	ND
pH 9.0	100	100(1)	100	100	100	0	100	83	0	100	100
pH 9.5	ND	100	ND	ND	ND	ND	ND	83	0	100	100
Growth on:											
0% NaCl	0(2)	0	100	100	100	0	100	100(5)	100(1)	100(11)	100
7% NaCl	0	100	0	0	0	0	50(2)	33	100	100	100
10% NaCl	0	100	0	0	0	0	0	33	100	92	100
Sodium lauryl sulfate	100	100	100	100	100	50	100	100	100	39	100
Crystal violet	0(2)	100	50	50	100	0(1)	100	100	100	100(12)	100
Brilliant green	33	100	0	100	0	0	100	100	100	92	100(1)
TCBS agar	100	100	ND	50	50	100	100(1)	100	0	100	100
SS agar	33	100	100	100	100	0	100	100	0	0	0
Resistant to:											
Gentamicin	0	0	0	0	0	0	0	0(4)	ND	0	0(1)
Penicillin	100	50	100	100	100	100	100	25(4)	ND	0	0(1)
Chloramphenicol	0	0	50	0	0	0	100	0(4)	ND	8	0(1)
Erythromycin	0	0	0	50	50	0	100	33(3)	ND	0(12)	0(1)
Kanamycin	0	0	0	0	0	0	0	0(4)	ND	0	0(1)
Chloramphenicol	ND	0	ND	ND	ND	ND	ND	0(4)	ND	0	0(1)
Novobiocin	0	0	0	0	100	0	100	0(4)	ND	0	0(1)
Polymyxin B	33	100	0	100	100	50	100	75(4)	ND	69	0(1)
Oxytetracycline	ND	0	0	0	50	0	33	0(4)	ND	0	0(1)
Tetracycline	0	50	0	0	0	0	0	0(4)	ND	0	0(1)
Ampicillin	0	50	50	0	0	50	100	50(4)	ND	0	0(1)
Streptomycin	ND	0	ND	ND	ND	ND	ND	0(4)	ND	0	0(1)
Susceptible to vibriostatic agent O/129	100	0(1)	100	0	0	100(1)	0	33	ND	100	0
Biochemical tests											
Catalase	100	100	100	100	100	50	100	100	100	100	100(1)
Oxidase	100	100	100	50	0	100	100	100	100	0(12)	0(1)
Arginine dihydro-lase	0	100	100	100	100	0	100	67	ND	0	0(1)
Histidine decarboxylase	0	0	100	50	50	0	0	0	0(1)	0	0(1)

TABLE 5—Continued

Determination	% of strains positive in phenon:										
	23 (n = 3)	24 (n = 2)	25 (n = 2)	26 (n = 2)	27 (n = 2)	28 (n = 2)	29 (n = 3)	30 (n = 6)	31 (n = 2)	32 (n = 13)	33 (n = 2)
Lysine decarboxylase	0	100	100	50	0	50	0	0	0(1)	0(11)	0
Ornithine decarboxylase	0	100	100	50	50	0	0	0	ND	8	0
H ₂ S	0	0	0	0	0	0	0	0	0	0	0
Methyl red	0	50	100	100	100	100	0	0	50	23	0
Voges-Proskauer	100(1)	0	0	0	50	0(1)	ND	67	0	92	50
Phenylalanine deaminase	0	0	0	0	0	0	0	0	0(1)	0	0
Nitrate reduction	100	100	100	100	100	100	ND	0	100	0	0
Nitrite reduction	ND	100	ND	ND	ND	ND	ND	0	50	0	0
Phosphatase	0(1)	100	100(1)	100	100	100	100	100	100	31	100
Indole	67	0	100	100	0	0	0	0	ND	0	0
Levan	0	0	0	0	0	0	0	0(5)	0	0	0
Melanin	0	0	0(1)	0	0(1)	0	0	0	0	0	0
Iodacetate, growth	0	100	0	0	0	0	100	83	0(1)	75(8)	0(1)
Iodacetate, acid	0	0	0	0	0	0	0	67	0(1)	0(8)	0(1)
Alpha-hemolysis	0	0	0	0	0	0	0	0	0	0(11)	50
Beta-hemolysis	100	0	0	0	0	0	67	67	0	73(11)	50
Simmons citrate	ND	100(1)	ND	0	0	0	ND	80(5)	ND	33(3)	ND
Ammonia production	100	ND	0	50	100	100	100	ND	ND	ND	ND
Fermentative metabolism	100	100(1)	100	100	100	100(1)	0	0	50	8(12)	0
Acid from:											
Arabinose	67	100	0	0	0	0	100	100	0	0	0
Butanol	0	50	0	0	0	0	0	50	0	0	0
Cellobiose	100	50	0	0	0	100(1)	33	100	0	67(12)	50
Dextrin	0	100	0	0	0	0	0	0	50	0	0
Erythritol	0	0	0	0	0	0	100	0	0	0	0
Ethanol	0	100	0	0	0	0	67	50	0	0	0
Fructose	100	100	0	50	100(1)	100	100	100	100	23	0
Galactose	100	100	100	0	50	0	100	100	0	0	0
Glycerol	100	100	100	0	0	0	100	33	50	61	50
Inositol	0	0	100	50	0	0	0	40(5)	0	0	0
Lactose	0	0	100	0	0	0	0(2)	0	0	0(12)	0
Mannitol	100	100	0	0	0	100(1)	100	100	50	67(12)	100
Mannose	67	100	0	50	0	50	100	100	0	100	50
Melibiose	0	0	100	0	0	0	100	100	0	0	0
Raffinose	0	0	0	0	0	0	0	0	0	0	0
Rhamnose	0	0	0	0	0	50	100	100	0	8	0
Salicin	0	0	0(1)	0(1)	0	50	0	0	0	75(12)	50
Sorbitol	100	0	0	0	0	0	100	83	0	0	0
Sorbose	0	0	0	0	0	0	0	0	0	0	0
Sucrose	ND	0	ND	0	50	100(1)	ND	17	100	8	0
Trehalose	100	100	100	100(1)	0	0	0	17	0	0	0
Xylose	0	0	0	0	0	0	100	100	0	0	0
Gas from:											
Glucose	0	0	0	0	0	0	0	0	0(1)	0(12)	0(1)
Lactose	0	0	0	0	0	0	0	0(5)	0	0	0
Degradation of:											
Urea	0	0	0	0	0	0	0(2)	0	0(1)	0	0
Starch	50(2)	100	0	0	0(1)	100	50(2)	0(5)	100	0	0
Chitin	ND	100	ND	0	0(1)	ND	ND	0(5)	ND	0(12)	0(1)
Gelatin	ND	100(1)	ND	0	0	ND	ND	83	50	100	100
Casein	100(2)	0	100	100	100	0	67	100	0	100	50
DNA	ND	100	ND	0	0	100(1)	ND	0(5)	0(1)	46	0
Tween 20	100	100	100	100	100	100	100	100	100	33(12)	100
Tween 40	100	50	100	100	100	100	100	100(5)	100	22(9)	100(1)
Tween 60	100	50	100	100	100	100	100	100	100	15	50
Tween 80	100	100	100	100	100	100	100	100	0	100	100
Lecithin	100(2)	50	0(1)	0	0	ND	0(2)	0(5)	0	100(11)	100

TABLE 5—Continued

Determination	% of strains positive in phenon:										
	23 (n = 3)	24 (n = 2)	25 (n = 2)	26 (n = 2)	27 (n = 2)	28 (n = 2)	29 (n = 3)	30 (n = 6)	31 (n = 2)	32 (n = 13)	33 (n = 2)
Tyrosine	0	0	0(1)	0	0	0	100	83	0	0	0
Allantoin	0	ND	50	100	100	0	0	ND	ND	ND	ND
Chondroitin	ND	0(1)	ND	ND	ND	ND	ND	20(5)	0	10(10)	0
Esculin (by color)	ND	100	0	0	0	100	0	33	0	77	100(1)
Esculin (by UV light)	ND	0(1)	0	0	0	ND	0	0(5)	0	77	100(1)
Utilization as sole carbon source:											
Acetate	0	50	0	100	0	0	67	33	100	25(12)	100
Beta-alanine	0	0	0	0	0	0	0	100(5)	100	0(9)	50
DL-Alanine	67	100	100	100	100	100	100	100	100	100	100
Alginate	ND	50	ND	ND	ND	ND	ND	100	100	15	0
Arginine	33	100	100	100	100	50	67	100	100	100	50
Citrate	100	100	100	100	50	100	100	100(5)	100	100(12)	100
Formate	0	0	0	0	0	0	67	67	0	0	0
Glycine	0	100	0	0	0	0	0	17	0	8	100
Inulin	67	100	100	100	100	100	67	100	100	100	100
Lysine	0	0	0	0	0	0	0	100	100	0(12)	0
Malonate	0	0	0	0	0	0	0	50	100	0	100
Phenylalanine	0	100	0	0	0	0	0	100	100	0	100
Proline	67	100	50	0	100	0	33	100	100	100	100
Propanol	0	100	0	0	0	0	0	100	100	0	100
Pyruvate	100	100	100	100	100	100	100	100	100	100	100
Serine	67	100	100	100	100	100	100	100	50	42(12)	100
Succinate	67	100	100	100	100	100	67	83	100	100	100
Tartrate	0	50	0	0	0	0	0	100	100	0(12)	100
Tryptophan	0	100	0	0	0	0	0	100	100	0	50
Uracil	0	0	0	0	0	0	0	17	0	0	0

^a The numbers in parentheses indicate the numbers of strains tested for different traits.

^b ND, Not done.

situation exists for strain F702/75, which had a similarity value of 80% compared with *V. anguillarum* strain F706/75, but lower similarity values compared with other strains of phenon 1. Strain F702/75 was assigned to phenon 1 by single linkage but not on the basis of average linkage. Thus, cluster 22 consists of strains of two species, *V. cholerae* (strain DAM81/75) and *V. anguillarum* (strain F702/75); this is apparently an aberrancy arising from the limitations of two-dimensional cluster analysis, when in fact multidimensional relationships are involved.

Three strains isolated from fish wounds were recovered in cluster 23. We concluded that strains F406/75, F407/75, and F678/75 are *Vibrio* sp.; these organisms were gram negative, facultatively anaerobic, motile, rod-shaped, oxidase positive, and susceptible to vibriostatic agent 0/129. They did not grow in 0 or 7% NaCl, produced acid but no gas from glucose, cellobiose, galactose, glycerol, mannitol, and trehalose, and reduced nitrate to nitrites, in addition to being lipolytic and proteolytic (Table 5). Decarboxylase reactions were negative for all amino acids tested. Assignment to a described spe-

cies of the genus *Vibrio* was difficult. We concluded that these strains comprise a new species.

Phenon 24 consists of two strains (strains K19 and KALV 18), both of which were isolated from seawater in Denmark. These strains were motile, facultatively anaerobic, rod-shaped organisms which did not grow in 0% NaCl or at 5°C, but did grow at all other salinities and temperatures tested. They were resistant to vibriostatic agent 0/129 at concentrations of 100 and 150 µg/ml but not at higher concentrations (e.g., 300 µg/ml), characteristic of *Aeromonas* spp. They were extremely versatile, both physiologically and biochemically, and utilized all sole carbon sources tested except β-alanine, formate, lysine, malonate, and uracil (Table 5). These strains could not be classified as *Vibrio*, *Beneckeia*, or *Aeromonas* and did not fall into any other genus comprised of facultatively anaerobic rod-shaped organisms. Hence, identification must await further characterization.

Phena 25 through 27 contained reference strains of *Plesiomonas* and *Shigella* spp. which were provided by R. Sakazaki. None of the

environmental isolates of vibrios clustered with these reference strains.

Cluster 28 consisted of two strains (strains 1157/75 and 1114/75); both of these strains were from Denmark, and they clustered at the 71% level of similarity. They were gram-negative, oxidase-positive, motile, facultatively anaerobic, rod-shaped organisms that were halophilic, growing at 3% NaCl but not at 0 or 7% NaCl; they were susceptible to vibriostatic agent 0/129 and produced acid from cellobiose, fructose, mannitol, and sucrose. These strains digested starch but not casein and were lipolytic. Strains 1157/75 and 1114/75 are considered to be representatives of a *Vibrio* sp., but were intermediate to, rather than belonging in, previously described species of this genus.

Pseudomonas spp. were recovered in phenon 29 and 30, which contained three and six strains, respectively, all of which were isolated in Denmark. These strains possessed the minimal characteristics of the genus *Pseudomonas* (46). They were gram-negative, aerobic, motile, rod-shaped organisms that did not produce acid from glucose in closed tubes. They were oxidase and catalase positive and negative in the indole and methyl red tests. These strains were identified as *Pseudomonas aeruginosa* on the basis of a positive arginine dihydrolase test, growth at 42°C, and production of acid from xylose, mannitol, arabinose, and rhamnose but not from lactose or salicin (Table 5).

Two reference strains of *Vibrio marinus* grouped together in cluster 31. No other strains examined in this study clustered with *V. marinus*.

Another *Pseudomonas* species was found in phenon 32. The 13 strains of phenon 32 were all isolated in Denmark and exhibited key characteristics of the genus *Pseudomonas* (see above), except that they were oxidase negative, suggesting that these strains are *Pseudomonas maltophilia*. The other characteristics of these strains (Table 5) support this conclusion (viz., motility, production of lipase (Tween 80), no starch hydrolysis, gelatin hydrolysis, and production of acid from mannose).

The final cluster in the analysis consisted of two strains, strain 613/75 from Denmark and strain ATCC 25916, which was originally deposited as *Beneckea pelagia*. This cluster varied in many characteristics from the description of *B. pelagia* by Baumann et al. (6), such as being nonfermentative and oxidase negative (both should be positive). The reason for the discrepancies is not known.

DISCUSSION

The strains of *Vibrio* and related organisms examined in this study clustered into 33 phenon,

the formation of which crossed geographic and specimen boundaries. Conclusions relating to the ecology and taxonomy of these strains are discussed below by species.

V. cholerae. A total of 43 strains of *V. cholerae* was examined by numerical taxonomy. The results of this analysis support the conclusions of Colwell (13), Sakazaki et al. (76), Shewan and Veron (82), and other workers in that no taxonomic separation could be made in this species on the basis of serology (specifically, the possession of the O1 antigen by these strains). Although this classification is not accepted by many medical microbiologists and epidemiologists (18, 30), it is consistent with all principles of taxonomy and has been confirmed by DNA-DNA hybridization (11, 97). Recent genetic evidence has demonstrated that some strains of *V. cholerae* non-O1 possess genes encoding cholera toxin, whereas many strains of *V. cholerae* O1 do not possess such genes (54). Thus, the results of a molecular genetic analysis of the cholera toxin genes refute the argument for a species defined by possession of a single antigen. Similarly, in the present study strains of *V. cholerae* possessing the O1 antigen clustered with *V. cholerae* non-O1 strains.

The biotyping scheme of *Bergey's Manual* (82) is not supported by our results (i.e., the scheme separating *V. cholerae* into the following four biotypes: *cholerae* [the so-called classical biotype], *El Tor*, *proteus*, and *albensis*). Classical and *El Tor* strains were grouped together in the present study and were not separated into biotypes or subclusters. On the other hand, the *proteus* biotype was grouped relatively distantly from *V. cholerae* (clusters 17 through 20). Unfortunately, strains of biotype *albensis* were not included in this study; therefore, we can make no comment on this biotype. The conclusions described above were reached by the Subcommittee on the Taxonomy of Vibrios (48), which recommended that the *proteus* and *albensis* groups should not be designated biotypes of *V. cholerae*. We hope that the next edition of *Bergey's Manual* will reflect these recommendations.

The strains of *V. cholerae* used in this study formed a fairly homogeneous group, but slight differences among the strains allowed separation into subclusters 5 through 8. The most notable feature of this separation was the antibiotic resistance demonstrated by strains of clusters 5 through 7, particularly in the case of the antibiotics ampicillin and tetracycline (tetracycline is the antibiotic of choice in the treatment of cholera). Since the majority of the resistant strains were isolated from Copenhagen sewage water (the DAM strains), it is possible that resistance was transferred from coliform bacteria in the sew-

age. Tetracycline- and ampicillin-resistant *V. cholerae* strains have recently been isolated in Bangladesh (96), and in vitro transfer of R plasmids from *Escherichia coli* to *V. cholerae* has been reported by several investigators (21, 69, 72). Interestingly, strains that acquired a plasmid conferring resistance to chloramphenicol, streptomycin, and sulfonamide grew poorly on TCBS agar at temperatures above 37°C (100). Since TCBS agar is the standard recovery medium used for *V. cholerae*, caution must be exercised in the choice of the medium used when one is looking for plasmids in *V. cholerae*.

Another characteristic that distinguished *V. cholerae* strains of cluster 7 was fermentation of lactose. *V. cholerae* is considered to be lactose negative. However, this species is sometimes lactose positive after delayed incubation. Sanyal et al. (78) reported that they isolated strains of *V. cholerae* which were capable of rapid fermentation of lactose. Such strains could be misidentified easily. Lactose-positive *Salmonella* strains have been reported (27, 52), which no doubt would have been discarded as coliforms if salmonellae were sought. The ability to ferment lactose has been shown to be plasmid mediated in *Salmonella* (52) but not in *V. cholerae*. Incidentally, these strains should not be confused with so-called lactose-positive vibrios originally described by Hollis et al. (43) and redescribed as *V. vulnificus* (28).

Historically, *V. cholerae* has been divided into biotypes on the basis of sucrose, mannose, and arabinose fermentation reactions. These biotypes have been recognized as Heiberg groups (39). Strains classified as Heiberg groups I, II, and V clustered together by numerical taxonomy in this study. Thus, there seems to be poor reproducibility of groupings of strains based on the Heiberg groups, with the possible exception of the sucrose-negative strains or Heiberg group V. This group was recently reported to be a separate species on the basis of DNA-DNA hybridization values (G. R. Fanning, B. R. Davis, A. G. Steigerwalt, I. K. Wachsmuth, F. W. Hickman, J. J. Farmer III, and D. J. Brenner, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, D57, p. 47).

However, our data suggest the sucrose-negative *V. cholerae*-like strains are a diverse group and that the strains that share the O-1 antigen are most probably a biovar of *V. cholerae*. Studies in progress (P. A. West and R. R. Colwell, unpublished data) clearly indicate heterogeneity within the taxon called *Vibrio mimicus*. The Heiberg scheme is further weakened by the fact that the arabinose-positive strains (Heiberg groups III and IV) are now recognized to comprise largely aeromonads (76). Clearly, the Heiberg groups, although useful in the early days of

bacteriology, no longer can be relied upon for identification of *V. cholerae*.

The largest cluster, cluster 8, contained strains of various serotypes isolated from around the world. Interestingly, a subgroup of strains was identified within cluster 8. Strains V11, V15, V25, V20, V26, V24, V19, and V29 clustered tightly and differed from the main body of group 8 *V. cholerae* strains by their failure to ferment dextrin and sucrose, their negative reaction in the Voges-Proskauer test, and their inability to hydrolyze starch. These strains (all Heiberg group V strains) were isolated from a single sampling of Jones Falls water in October 1976. The sample was taken from a single carboy of water analyzed within 45 min of collection. The fact that these strains are nearly identical (simple matching coefficients as high as 98% were found) indicates that these strains may be merely clones of an organism found in Baltimore Harbor of Chesapeake Bay, a situation entirely consistent with the hypothesis that *V. cholerae* is an autochthonous bacterial species in Chesapeake Bay and, therefore, can multiply in the bay.

V. parahaemolyticus and *V. alginolyticus*. The taxonomy of *V. parahaemolyticus* has been reviewed by Colwell (14), who traced the succession of names given to this organism, including *Pasteurella parahaemolytica*, *Pseudomonas enteritis*, *Oceanomonas enteritidis*, *Oceanomonas parahaemolytica*, and *V. parahaemolyticus* (so named by Sakazaki et al. [79] after examination of 1,072 strains of this species). Two biotypes were identified, which Zen-Yoji et al. (101) concluded should be separated into two species. In 1968 this was proposed by Sakazaki, who designated biotype 2 as *V. alginolyticus* (75). The main differences between the biotypes are acetoin production, sucrose fermentation, growth in 10% NaCl, and swarming on agar plates containing 2 to 7% NaCl. *V. parahaemolyticus* is negative for these characteristics, whereas *V. alginolyticus* is positive. Later, investigators reported specific DNA homology values of 56 to 70%; these values were higher than the other interspecies homology values for the genus *Vibrio* (1, 92). In the 8th edition of *Bergey's Manual* these two taxa were combined as *V. parahaemolyticus*, but the Subcommittee on the Taxonomy of Vibrios of the International Committee on Systematic Bacteriology recognized separation of these two taxa as distinct species (48). The widespread acceptance of *V. alginolyticus* by environmental and medical microbiologists portends the acceptance of this species in microbial systematics.

In the present study, 21 strains were identified as *V. parahaemolyticus*. The variation observed in certain characteristics merits discussion. Su-

crose fermentation is an important feature and is useful for distinguishing *V. parahaemolyticus* from *V. alginolyticus*, but it is not the only feature which can be used for identification. Most of the shrimp isolates in this study were sucrose-positive *V. parahaemolyticus* strains, which is unusual but not unheard of. Colwell (13) found that 6% of the strains of this species that she examined were sucrose positive, and Gilmour (33) also found several such strains. Kampelmacher (53) isolated sucrose-positive *V. parahaemolyticus* strains from mussels, and Fujino et al. (31) found that 2% of their marine isolates were sucrose positive. Of 1,484 strains of this species isolated from British coastal waters, 6.9% were sucrose positive (2). Detection of acid from sucrose fermentation by *V. parahaemolyticus* can vary, according to the composition of the medium and the concentration of sucrose used. Baross (Ph.D. thesis, University of Washington, Seattle, 1972) studied this problem and recommended use of the Hugh-Leifson OF base supplemented with 0.5% (final concentration) sucrose. Several methods were used in the present study (see above), and all yielded the same results. Many of the strains were examined for the presence of extrachromosomal DNA, and the results which we obtained suggest that sucrose fermentation in these strains is not plasmid mediated (Kaper, unpublished data).

Conversely, Stephen et al. (93) found strains of *V. alginolyticus* which did not ferment sucrose but were otherwise identical to the type strains. Golten and Scheffers (34) described strains intermediate between *V. parahaemolyticus* and *V. alginolyticus* which were methyl red and arabinose positive (*V. alginolyticus* is negative for both tests), sucrose positive, capable of swarming, and able to grow in 10% NaCl. We observed other unusual characteristics for several strains of *V. parahaemolyticus*. For example, many strains were positive in the urease test, a test which can vary greatly according to the composition of the medium used and how strongly the medium is buffered. Results of two large studies have been contradictory. Sakazaki et al. (77) reported that 4% of the strains which they tested were urease positive, whereas Colwell (13) reported that 97% of her strains were urease positive. Recently, a number of clinical isolates of urease-positive *V. parahaemolyticus* have been reported (50, 59). The esculin hydrolysis test, which distinguishes the third subgroup of *V. parahaemolyticus* (cluster 14) from the other two, is another test which may provide different results depending on the method used. *V. parahaemolyticus* is usually thought of as being esculin negative (29), but Fujino and co-workers (32) reported that 99% of their strains

were esculin positive. A common test for this characteristic uses a ferric salt indicator, with a brown to black color indicating esculin hydrolysis. Another method (9) calls for the disappearance of UV fluorescence. In the present study we used both methods and found that several strains were positive if the color reaction was the key feature but negative if the tests were read by the UV light reaction. The UV test tended to be too sensitive; even a small amount of residual esculin fluoresced under UV light and gave a false-negative reaction.

V. parahaemolyticus is consistently reported as being negative for production of hydrogen sulfide (28). Sakazaki et al. (77) reported that none of the 1,702 strains which they examined were positive for H₂S when TSI or SIM agar was used. Using more sensitive methods, Colwell (13) found that nearly all of the *V. cholerae* and *V. parahaemolyticus* strains which she examined produced H₂S. Twedt and co-workers (98) found that nearly all of their cultures were positive when they used SIM agar and lead acetate agar (Difco). Indeed, hydrogen sulfide production on Russell triple sugar agar but not on TSI has been suggested as an aid in the identification of this species (51). In the present study we used TSI for determinations of H₂S production and, not surprisingly, found no strains of *V. parahaemolyticus* that were positive for H₂S in this medium.

V. anguillarum and other marine vibrios. *V. anguillarum* has long been implicated as a causative agent of fish disease. Originally described in 1909, the phenon *V. anguillarum* has been repeatedly subdivided and recombined in the literature since that time. This species is readily separated from *V. cholerae*, *V. parahaemolyticus*, and *V. alginolyticus* by a number of characteristics, mainly the decarboxylase reactions. Differentiation from *Aeromonas* species can be problematic, unless the proper tests are run (i.e., salt requirement, susceptibility to vibriostatic agent 0/129, and, especially, DNA base composition). Differentiation from other marine vibrios is still difficult, largely because few marine species have been well characterized.

In 1971, Hendrie et al. (41) proposed combining three *Vibrio* species (*V. anguillarum*, *Vibrio piscium*, and *Vibrio ichthyoderms*) and retaining the name *V. anguillarum* for the phenon. This recommendation was followed in the 8th edition of *Bergey's Manual* (82). The description provided in *Bergey's Manual* is very restrictive, however. Many workers have shown that there are significant biochemical variations in a number of features, including the Voges-Proskauer test, the methyl red test, citrate utilization, and fermentation of lactose, arabinose, and sucrose (25, 64). The description of Evelyn (25) accounts

for these variations and appears to be a more reliable description for the species.

A number of biotyping schemes have been proposed for *V. anguillarum*; the first of these was proposed by Nybelin (68), who described two biotypes (type A, which produced indole and acid from mannitol and sucrose, and type B, which was negative for these characteristics). Smith (85) described a third biotype, biotype C, which produced acid from mannitol and sucrose but did not produce indole. Hastein and Smith (38) examined 163 strains of *V. anguillarum* by using principle component analysis and reported two groups which did not correspond to any of the previously described subgroups. Group 1 of Hastein and Smith was arabinose positive, cellobiose positive, and variable for citrate, lactose, and trehalose, whereas group II was arabinose negative, variable for cellobiose, negative for citrate and lactose, and trehalose positive. Pacha and Kiehn (70) studied marine vibrio strains isolated from fish in the Pacific Northwest and identified several of these strains as *V. anguillarum* on the basis of morphological and biochemical characteristics. Variations were observed in indole production, gelatin hydrolysis, citrate utilization, and fermentation of arabinose, sucrose, lactose, and glycerol. No consistency was found in these variations, but most strains belonged to group A (68). A biotyping scheme for *V. anguillarum* was recently proposed by Baumann et al. (4), who defined two biotypes on the basis of DNA-DNA hybridization and noted a number of phenotypic reactions that distinguished the strains, including arginine dihydrolase, Voges-Proskauer reactions, growth at 30°C, reduction of nitrate, and utilization of several compounds as sole carbon sources, including mannose, trehalose, gluconate, succinate, fumarate, and alpha-ketoglutarate. Biotype I strains were positive for all of these tests, whereas biotype II strains were negative for these tests.

Because of the difficulty in distinguishing these strains phenotypically, DNA homology was used to clarify the situation. Kiehn and Pacha (55) hybridized the same strains which they examined phenotypically and found strong correlations between phenotypic and genotypic analyses in a group of eight strains of marine vibrios that were pathogenic for fish. Three groups were discerned by serology, and these were also distinguished by DNA homology. The first group formed on the basis of DNA homology consisted of strains NCMB 6 (= ATCC 19264), V-2911, V-2916, 30, and 2.1, which were isolated from cod, finnock, Steelhead trout, and Chinook salmon. Strains isolated from Pacific herring (strains 56 and 60) comprised the second group. These strains were not *V. anguillarum* on the basis of the DNA homology results, but they

differed only slightly in biochemical characteristics; viz., they were Voges-Proskauer negative, gelatinase negative, and resistant to dihydrostreptomycin. In addition, strain 60 (which had a G+C content of 41 mol%) fermented lactose and was indole negative and sucrose negative; this strain was designated a third homology group.

Anderson and Ordal (1) distinguished five DNA homology groups among the marine vibrios, as follows: (i) *V. parahaemolyticus*; (ii) *V. alginolyticus*; (iii) *V. anguillarum*, containing strains NCMB 6, V-2911, and V-2916; (iv) group 4, strains of which were isolated from diseased herring, including strain 56 of Pacha and Kiehn (67); (v) group 5, which also consisted of strains that were herring isolates (including strain 60) but were genetically distinct from groups 3 and 4. In contrast to the results of Pacha and Kiehn, the results of Anderson and Ordal showed that serology of these strains had little taxonomic value. Subsequent studies have identified two other homology groups. Ordal (unpublished data) defined a homology group of vibrio strains isolated from shellfish, seawater, and sediments. The members of this group were not pathogenic for salmonid fishes, and the group was designated Ki 72-7. Schiewe et al. (80) examined a group of vibrios described by Harrell et al. (36), which exhibited 83% within-group homology but only about 60% homology with *V. anguillarum* V-2911. A notable phenotypic feature of these strains is the consistently negative arginine dihydrolase reaction. This group was designated simply by a strain number (V-1669) by Schiewe et al. (80), but was described as *V. ('Ben-eckea')* *anguillarum* biotype II by Baumann et al. (4).

The results of the present study cannot answer unequivocally any of the questions raised above because strains NCMB 6, V-2911, V-1669, and Ki 72-7 and other representatives of the DNA homology groups were not included in this analysis. However, cluster 4 did resemble group V-1669 of Harrell et al. (36). Nevertheless, our results do demonstrate that the biotyping schemes proposed by Nybelin, Smith, and others are not robust. The strain variability in this study was no greater than that observed by Evelyn (25) and other workers for this species. Thus, the differences among the four groups defined in this study were not sufficiently great to warrant separate biotype status. More recently, Schiewe and Crosa (79) reported results of polynucleotide sequence relationship determinations, showing the discrete nature of *V. anguillarum* biotypes 1 and 2. Furthermore, biotype 2 strains were found to contain a multicopy plasmid that was either identical or highly conserved and unrelated to the virulence plasmid (pJM1) of *V. anguillarum* biotype 1.

In general, the source of the strains used in

this study was wound infections of fish. Even though fish pathogenicity tests were not done, we assumed that strains isolated from wounds, which often were in almost pure culture in the wounds, were pathogenic. There was no obvious correlation between pathogenicity and any of the biochemical characteristics. Indeed, characteristics associated with pathogenicity in fish often appear to be plasmid mediated (19, 79). It would be interesting to examine selected Danish strains for DNA-DNA hybridization with representatives of the DNA homology groups cited above (all of which were isolated in North America) to assess geographic variation.

V. marinus-Vibrio fischeri. *V. marinus* was reisolated by Colwell and Morita in 1964 (15), who also gave an emended description of the species. In 1970, Hendrie and co-workers (40) noted similarities between *V. marinus* and *V. fischeri* (formerly *Photobacterium fischeri*) and proposed that these organisms be amalgamated under the name *V. fischeri*. This approach was followed in the 8th edition of *Bergey's Manual* (82). Reichelt and Baumann (73) disagreed with the generic assignment of Hendrie and colleagues (40) and argued that *V. fischeri* should remain in the genus *Photobacterium*. A later paper by these investigators (74) presented data showing essentially no genetic similarity between "*P. fischeri*" and other *Photobacterium* spp. Recently, Baumann et al. (5) proposed that these strains, which are distinguished from the rest of the genus *Vibrio* by low G+C contents (39 to 42 mol%), should be separated into three species, *V. fischeri*, *Vibrio logei*, and *V. marinus*, reaffirming the results of Colwell and Morita (15) for *V. marinus*. All of these species except *V. marinus* contain luminescent strains.

Unfortunately, in the present study the relationship between *V. marinus* and *V. fischeri* could not be clarified further. No other strains grouped with *V. marinus*, which is not surprising since the majority of strains used in this study were isolated from estuarine rather than marine environments and the isolation procedures used included methods which would not enhance recovery of psychrophiles. The reference strains of *V. marinus* examined in this study were distantly related to other *Vibrio* spp., indicating that *V. marinus* is indeed a separate species. In the future, it may be shown that this organism is a member of a new genus.

V. metschnikovii. Anyone with extensive experience in the use of TCBS agar has undoubtedly encountered bacteria which, in pure culture, yield sucrose-positive colonies. Upon further testing, these are found to be oxidase negative and, most often, are quickly discarded as not being a *Vibrio* sp. In fact, many of these strains may belong to the species *V. metschnikovii*, which was recently redescribed by Lee and

colleagues (60). Several of the strains included in the present study were identified as *V. metschnikovii*. These clustered together in groups 17 through 20. Interestingly, the analysis of Lee et al. (60) also yielded four clusters of *V. metschnikovii* strains. These authors concluded that the clusters should be put together as a single species and that this species should also include strains heretofore known as *V. proteus*, the original strain of which was isolated in 1884 from feces of patients suffering from cholera (60). This species was listed as a biotype of *V. cholerae* in the 8th edition of *Bergey's Manual* (82), even though it differs from other biotypes of *V. cholerae* in a number of important features, such as decarboxylase reactions, salt requirements, oxidase reaction, nitrate reduction, and fermentation of lactose and inositol. DNA-DNA hybridization data revealed only 12 to 22% homology between this species and *V. cholerae* ATCC 14035 (74).

Despite the fact that this species is oxidase negative and not able to reduce nitrates, we concluded that the strains should be classified in the genus *Vibrio*, based on overall phenotypic similarity and DNA base composition (45.6 mol% G+C). West et al. (99) examined the cytochrome distribution in *V. metschnikovii* and other *Vibrio* spp. and found that *V. metschnikovii* lacks cytochrome *c* but shares cytochromes *b*, *o*, *d*, and *a* with other *Vibrio* spp. These workers hypothesized that the different respiratory system compositions may reflect different evolutionary pathways to suit habitats which are dissimilar in degree of anaerobiosis. The answers to this question and questions concerning the taxonomic relationships of *V. metschnikovii* to the other vibrios will require nucleic acid reassociation studies.

One interesting sidelight of our study concerns two strains of *V. proteus*, strains 10037-74 and 10041-74, which are reported to be subcultures of the same strain (strain NCTC 8563). These strains were received from Sakazaki, who had received them from different investigators. These two cultures of the same strain clustered with different phenotypes; the former clustered with group 11, and the latter clustered with group 20. A parallel situation is found in the work of Lee and co-workers (60), who examined duplicate cultures of three type strains (the duplicate strains were received from different sources). None of the duplicate strains clustered closely together, as might be expected. One of these strains was strain NCTC 8563, as in our study. Probable explanations include the possibility that the strains were mixed in a laboratory and the possibility that the duplicate cultures simply changed (i.e., lost or gained genetic material upon repeated subculturing).

Another curious result was the presence in

cluster 20 of all of the reference strains which have been in culture collections for several years. These strains utilized fewer organic compounds and were more susceptible to agents such as dyes and detergents than recently isolated environmental isolates. In fact, these strains were not able to grow on TCBS agar, the primary *Vibrio* isolation medium, whereas fresh isolates readily grew on this medium. This phenomenon was also reported by Lee et al. (60), who observed that all of their reference strains clustered separately from fresh isolates. A possible explanation for this observation is that strains which are held in a culture collection and, particularly in this case, transferred many times change (viz., they grow less rapidly, are less nutritionally versatile, and are highly adapted) to the point of being unable to grow on selective media, such as TCBS agar. Such a situation has been reported for strains of *V. cholerae* (60).

There are a number of species of *Vibrio*-like organisms which have been described that were not examined in this study. Some species, such as *V. vulnificus* (formerly lactose-positive vibrios) (8, 28) and *V. fluvialis* (synonym, group F vibrios, group EF6) (49, 62; J. V. Lee, P. Shread, and A. L. Furniss, J. Appl. Bacteriol. 45:ix, 1978) have recently been recognized as human enteric pathogens. Other species, such as *Vibrio nigrapulchritudo* (7) and *Vibrio gazogenes* (37), are distinguished chiefly by production of pigment (black and red pigments, respectively). Together, these and similar strains represent a rather diverse group of organisms covering a wide range of salt requirements (5 to 700 mM Na⁺), DNA base compositions (39 to 51 mol% G+C), DNA homology groups, and ecological niches.

Several genera (*Beneckea*, *Lucibacterium*, *Oceanomonas*, and others) have been proposed to accommodate some of these species. Recently, Baumann et al. (5) proposed that 20 such species should be combined in the genus *Vibrio*, since no consistent internal subdivisions suitable for generic separation are evident in this complex of strains. We support this concept, having doubted the validity of the above-listed three genera, and offer evidence in the present study, in which strains from a variety of geographic areas, ecological niches, and specimen types were grouped together on the basis of numerical taxonomy. Nevertheless, some species of the genus *Vibrio* which we examined were quite distantly related to other species in this genus, but reproducible subdivisions could not be discerned. Thus, the internal diversity of this genus is far greater than that of a typical genus of the *Enterobacteriaceae*. Few other bacterial phyla are subdivided and split to the extent of the

Enterobacteriaceae. There is no compelling set of arguments to apply such a degree of division to the genus *Vibrio*. With our present state of knowledge of the genus *Vibrio*, subdivisions would be arbitrary, confusing, and, in our opinion, of little value to microbiologists who must deal with these species in clinical and environmental laboratories. For these reasons, we will await the results of experiments in progress in our laboratory before proposing new genera for organisms presently classified as *Vibrio* spp., regardless of the extent of internal diversity in the genus *Vibrio* observed in this study.

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