Numerical Taxonomy and Deoxyribonucleic Acid Relatedness of Environmental and Clinical *Vibrio* Species Isolated in Indonesia

E. MOLITORIS,^{1,2} M. A. MARIJ,^{1,3} S. W. JOSEPH,^{1*} M. I. KRICHEVSKY,² G. R. FANNING,⁴ G. LAST,¹
A. M. EL-MISHAD,³ Y. A. EL BATAWI,³ AND R. R. COLWELL¹

Department of Microbiology, University of Maryland, College Park, Maryland 20742¹; Microbial Systematics Section, National Institute of Dental Research, Bethesda, Maryland 20205²; Department of Microbiology, Faculty of Medicine, Cairo University, Cairo, Egypt³; and Biochemistry Division, Walter Reed Army Institute of Research, Washington, D.C. 20307⁴

Received 4 January 1989/Accepted 31 July 1989

Numerical taxonomic methods were used to analyze taxonomic data gathered for 229 *Vibrio* strains isolated from fecal, seawater, and seafood specimens collected in Jakarta, Indonesia. Simple matching and Jaccard coefficients yielded similar results. Most of the clinical isolates were identified as *Vibrio parahaemolyticus*, *V. cholerae*, or *V. mimicus*. Environmental isolates included *V. alginolyticus* and one strain of *V. vulnificus*. Clusters of *V. parahaemolyticus* included both clinical and environmental isolates. Biotypes of *V. parahaemolyticus* were not associated with a specific source. Reference strains of other *Vibrio* species did not cluster with any of the isolates examined in this study. Four groups remained initially unidentified and were further subjected to deoxyribonucleic acid hybridization analysis, which enabled their identification as *V. parahaemolyticus*, *V. alginolyticus*, or possible new *Vibrio* spp. Descriptions of *Vibrio* species already published proved useful in identifying and classifying most of the isolates from a tropical climate.

The taxonomy of the genus *Vibrio* has undergone significant revision during the past 15 years. There are currently some 20 species, and at least 14 more have been proposed (2). Recently, separation of the genus *Vibrio* into three genera, *Vibrio*, *Shewanella*, and *Listonella*, has been recommended (20).

At least 11 *Vibrio* species have been found to be associated with human gastroenteritis, wound infections, or septicemia. *Vibrio cholerae* and *V. parahaemolyticus* cause gastroenteritis and have been isolated worldwide (13). *V. vulnificus*, *V. alginolyticus*, and *V. damsela* have been isolated from wounds of patients after exposure to seawater and also have a worldwide distribution (13, 19, 26). *V. fluvialis*, *V. furnissii*, *V. metschnikovii*, *V. mimicus*, *V. cincinnatiensis*, and *V. hollisae* have been associated with gastroenteritis or wound infections (3, 6, 16, 18). Generally, these pathogens share estuarine, neritic, or brackish marine environments and can be isolated from a variety of environmental samples. Other species, including *V. fischeri*, *V. splendidus*, *V. natriegens*, *V. nereis*, and *V. pelagius*, inhabit pelagic regions and may occur only transiently in estuarine environments (1, 2).

Nonpathogenic *Vibrio* species, including *V. aestuarianus*, *V. anguillarum*, *V. campbelli*, *V. carchariae*, *V. costicola*, *V. diazotrophicus*, *V. gazogenes*, *V. harveyi*, *V. logei*, *V. marinus*, *V. mediterranei*, *V. nigripulchritudo*, *V. ordalii*, *V. orientalis*, *V. proteolyticus*, *V. psychroerythrus*, *V. salmonicida*, and *V. tubiashii*, are currently recognized as members of the genus (10).

Development of biochemical and physiological methods for classification of members of the family Vibrionaceae has changed rapidly in recent years (2, 20, 33). Kaper et al. (14) stated that the best approach to the enigma of environmental versus clinical origin of *Vibrio* species requires integration of systematics and ecology. In this study, both environmental and clinical *Vibrio* isolates from a tropical country, Indone-

sia, were subjected to numerical taxonomy. The strains were initially characterized by methods developed for enteric organisms, except for the use of increased NaCl concentrations in the media. The description of *Vibrio* species has recently been expanded by incorporation of nutritional data derived from sole carbon source utilization tests (2). In this study, nutritional screening of 229 strains was done and the characteristics of both temperate and tropical *Vibrio* spp. were recorded. Selected unidentified strains were further characterized by using deoxyribonucleic acid (DNA) hybridization analysis.

MATERIALS AND METHODS

Bacterial strains. Presumptive *Vibrio* strains ($n = 229$) were isolated from seafood, seawater, and human fecal specimens collected in Jakarta, Indonesia (12), and in the original selection, emphasis was placed on the isolation of species other than *V. cholerae*. Isolates were lyophilized for long-term storage.

Upon initiation of the study reported here, lyophilized cultures were revived by suspending the pellets in 1% tryptone broth (Oxoid Ltd., Basingstoke, England) supplemented with 1% (wt/vol) NaCl (T_1N_1) and incubated at 25°C for 48 h. Each isolate was examined for purity by being subcultured twice onto nutrient agar (NA) (Lab-Lemco agar; Oxoid) supplemented with 1% NaCl. Working cultures were maintained at ambient temperature on NA slants under mineral oil.

Reference strains, including pathogenic and nonpathogenic *Vibrio* species, were treated similarly. The following strains were obtained from the American Type Culture Collection (ATCC), Rockville, Md.: *V. splendidus* ATCC 33125^T (type strain), *V. natriegens* ATCC 14048^T, *V. pelagius* biovar I ATCC 25916^T, *V. pelagius* biovar II ATCC 33504, *V. anguillarum* ATCC 19264^T, *V. harveyi* ATCC 14126^T, and *V. nereis* ATCC 25917^T. The following strains were supplied by R.R.C. (equivalent ATCC or National Collection of Type Cultures [NCTC; London, United King-

* Corresponding author.

dom] designations are also given): *V. alginolyticus* 4228 (= ATCC 17749^T), 186, 11-10, 11-20, and 11-36; *V. parahaemolyticus* 196 (= ATCC 17802^T), 192, and 191; *V. vulnificus* 4435 (= ATCC 27567^T), 425, and 181; *V. cholerae* 123 (= ATCC 14035^T); *V. fluvialis* 11327 (= NCTC 11327^T); *V. furnissii* 2386 (= NCTC 11328); *V. metschnikovii* 7708 (= ATCC 7708); *V. damsela* 4440; *V. hollisae* 4047; *V. splendidus* biovar II 4236 (= ATCC 25914); and *Aeromonas hydrophila* 4160.

Biochemical and physiological tests. Unless otherwise stated, all media were amended with 1% (wt/vol) NaCl, inoculated with 1 or 2 drops from a Pasteur pipette of an overnight T₁N₁ broth culture, and incubated at 25°C. All media were purchased from Difco Laboratories, Detroit, Mich., unless otherwise noted.

Strains were tested for production of amylase (33), arginine dihydrolase (28), lysine and ornithine decarboxylases (28), chitinase (33), oxidase (22), and acid from L-arabinose, D-cellobiose, salicin, and sucrose in O/F basal medium (33). Methyl red and Voges-Proskauer reactions were determined in methyl red Voges-Proskauer broth after 7 days. Reactions in triple-sugar iron (TSI) agar (Oxoid) were read after 18 to 24 h at 35°C and recorded as alkaline or acid. The presence of oxidative or fermentative metabolism was determined by using O/F basal medium containing 1% (wt/vol) glucose.

A requirement for NaCl was determined visually as absence of growth after 48 h in 1% tryptone broth without added NaCl. Growth in the presence of NaCl was tested in 1% tryptone broth supplemented with the appropriate concentration of NaCl (34). Growth at 40°C was determined visually after 48 h in T₁N₁ broth.

Hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (22) was tested by using a suspension of cells from a 48-h NA plate supplemented with 1% (wt/vol) lactose (33). Sensitivity to O/129 (2,4-diamino-6,7-diisopropylpteridine) was determined by spot inoculation of NA containing 10, 50, and 150 μg/ml (33). Production of urease was determined by spot inoculation of urea agar plates incubated for 20 to 24 h (28).

Sole carbon and energy source. The ability to utilize a variety of compounds as sole carbon and energy sources was tested by adding the following compounds to a basal mineral salts medium (33) at a concentration of 0.1% (wt/vol; vol/vol for liquids). All strains were tested for utilization of 4-aminobutyrate, L-arabinose, butyrate, D-cellobiose, ethanol, galacturonate, glucuronate, L-leucine, propanol, putrescine, and sucrose. Supplemental tests were conducted on 43 isolates for utilization of L-rhamnose, D-galactose, 5-aminovaleate, L-arginine, glycine, hippurate, L-ornithine, mannose, glutarate, glycerate, gluconate, α-ketoglutarate, valerate, and L-citrulline. Sole carbon source plates were spot inoculated with an A400 multipoint inoculator (Denley, Sussex, England). The inoculum was prepared by diluting 1 or 2 drops of a 3- to 5-h T₁N₁ broth culture from a Pasteur pipette into 3 ml of fresh T₁N₁ broth. The biochemical and nutritional tests chosen were selected as a means of distinguishing among *Vibrio* species specifically from estuarine environments and not from pelagic regions of the oceans (2).

Morphology. Gram stains were prepared from overnight NA cultures. Flagellar staining was performed by the method of Mayfield and Inniss (23) on air-dried smears from overnight T₁N₁ broth cultures stained for 30 to 60 s.

Test errors. Sixteen isolates were inoculated in duplicate to estimate the average probability of test errors. Errors were scattered among the tests, and the average probability of error was calculated to be approximately 5%.

Moles percent guanine-plus-cytosine determinations. Prep-

aration of DNA samples was performed as described by Johnson (11), with the following modifications. The cells were suspended and lysed in Tris-saline buffer (pH 8), containing 0.05 M Tris (Bethesda Research Laboratories, Inc., Rockville, Md.) and 0.2 M NaCl; the Tris-saline buffer was supplemented with sodium EDTA (0.02 M final concentration) at the time of lysis, and the cell suspension was frozen at -70°C until required for further preparation.

The DNA was precipitated and purified (11), and after 1 h of incubation in RNase, pronase (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 100 μg/ml and incubation was continued for 1 h at 35°C. The DNA was examined for purity by determining the A₂₃₀, A₂₆₀, and A₂₈₀ in a model 2600 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The same instrument was used to melt the DNA at temperatures increasing by 1°C/min to determine the moles percent guanine plus cytosine by the method of Mandel et al. (21). *V. cholerae* ATCC 14035 (47 mol% guanine plus cytosine) and *V. parahaemolyticus* ATCC 17802 (46 mol% guanine plus cytosine) were used as standards for moles percent guanine-plus-cytosine calculations.

DNA relatedness analysis. DNA-DNA hybridization was conducted with 14 of the isolates (see Table 4) by using the batch hydroxyapatite thermal elution procedure (4, 5). DNAs from the type strains of *V. parahaemolyticus* (ATCC 27969), *V. alginolyticus* (ATCC 17749), and *V. natriegens* (ATCC 14048) were nick translated with ³²P (NEK-004; Dupont, NEN Bioproducts, Boston, Mass.), and reacted with unlabeled DNA from representative *Vibrio* isolates at both the optimal reassociation temperature of 60°C and the stringent incubation temperature of 75°C. Relatedness was expressed as the relative binding ratio (RBR) and as the divergence in the melting temperature (dT_m or ΔT_m) (see Table 4). RBR is the amount of double-stranded DNA formed between labeled and unlabeled DNAs from different strains divided by the amount of double-stranded DNA formed between labeled and unlabeled DNAs from the same strain. RBR was calculated as a percentage to enable determination of similarity between strains. RBRs of <70% for reactions at 60°C and <55% for reactions at 75°C were considered the cutoff values for DNA relatedness at the species level.

The dT_m expressed in degrees Celsius, is the difference in melting temperature between the homologous and heterologous DNA duplexes. A dT_m of 5°C was considered the cutoff value for DNA relatedness at the species level.

Control reactions in which labeled DNA was incubated without unlabeled DNA were included, and the label-only control values were subtracted from all reactions before the RBR was calculated.

DNA from *V. parahaemolyticus* ATCC 17802, *V. alginolyticus* ATCC 17749, and *Neisseria meningitidis* Norway H355 were used as control reactions for the DNA-DNA hybridizations (see Table 4).

Computer analysis. Computer analysis was performed by using programs available from the Microbial Systematics Section, National Institute of Dental Research, Bethesda, Md. Biochemical and physiological data were submitted to the program TAXON (31) for cluster analysis using simple matching (*S*) and Jaccard (*S_j*) coefficients and unweighted average linkage.

RESULTS

A total of 240 strains (229 isolates and 11 reference strains) clustered into 22 groups when the *S* coefficient and un-

FIG. 1. Simplified dendrogram of the strains examined in this study by the *S* (A) and *S_j* (B) coefficients, both with unweighted average linkage. The groups in panel B were numbered for consistency with those in panel A. Uniformly positive and negative features were omitted to increase the sensitivity of the analysis.

weighted average linkage were used (Fig. 1). Analysis using the *S_j* coefficient produced similar clusters. The two largest groups were identified as *V. parahaemolyticus* and *V. alginolyticus*. Three other groups also found as results of the analysis were identified as *V. cholerae*, *V. mimicus*, and *V. vulnificus* (Fig. 1).

Groups 1, 10–13, and 19 bordered on and were biochemically similar to *V. parahaemolyticus* and *V. alginolyticus*. Forty-three isolates within these groups and 22 reference strains were analyzed by using additional sole carbon source utilization tests and growth at 40°C to clarify relationships with *V. parahaemolyticus* and *V. alginolyticus*. Results of the second analysis are presented in the dendrogram shown in Fig. 2. In general, the groupings were not altered. A new group (group 23) was composed of strains H6922 and N186 (Fig. 2).

V. harveyi, *V. furnissii*, *V. natriegens*, *V. nereis*, *V. anguillarum*, *V. fluvialis*, *V. metschnikovii*, *V. hollisae*, *V. damsela*, *V. splendidus*, *V. pelagius*, and *A. hydrophila* did not cluster with any isolates in either analysis.

The DNA base composition of strain N219 was 44 ± 1 mol% guanine plus cytosine. Group 1 was the only group in which all strains were capable of utilizing galacturonate and aminobutyrate (Table 1).

Groups 2 to 9 (Fig. 1) clustered at $S \geq 85\%$ ($S_j \geq 70\%$) with the type strain of *V. parahaemolyticus* 196 in group 3. Strains within these clusters demonstrated characteristics of *V. parahaemolyticus* (Table 1). Distinguishing features of

the larger subgroups of *V. parahaemolyticus* are shown in Table 2. *V. parahaemolyticus* reference strain 191 did not cluster with other strains when either the *S* or *S_j* coefficient was used.

Groups 10 to 12 occupied a position between the *V. parahaemolyticus* and *V. alginolyticus* groups and clustered at $S \geq 78\%$ ($S_j \geq 63\%$) (Fig. 1). A consistent feature for all strains within these three groups was the inability to utilize ethanol or propanol (Tables 1 and 2).

Groups 10 and 11 were identified as *V. parahaemolyticus*. Group 10 contained two environmental isolates that clustered at $S \geq 88\%$ ($S_j \geq 81\%$) after the primary analysis (Fig. 1). After further testing and analysis, these strains joined *V. parahaemolyticus* 196^T and 192 at $S \geq 82\%$ ($S_j \geq 73\%$) (Fig. 2). Group 11 included two environmental and seven clinical isolates that clustered at $S \geq 84\%$ ($S_j \geq 73\%$) (Fig. 1). After further testing and analysis, these strains joined *V. parahaemolyticus* 196^T and 192 at $S \geq 82\%$ ($S_j \geq 73\%$) (Fig. 2).

Group 12 contained two environmental and three clinical isolates that clustered at $S \geq 90\%$ ($S_j \geq 81\%$) and joined *V. parahaemolyticus* at $S \geq 77\%$ ($S_j \geq 70\%$) (Fig. 1). After further analysis, these strains clustered at $S \geq 86\%$ ($S_j \geq 75\%$) and joined *V. parahaemolyticus* 196 and 192 at $S \geq 80\%$ ($S_j \geq 69\%$) (Fig. 2). The DNA base composition of strain H4309 was 45 ± 1 mol% guanine plus cytosine.

Group 13 consisted of nine environmental isolates that clustered at $S \geq 82\%$ ($S_j \geq 73\%$) and joined *V. alginolyticus* groups at $S \geq 79\%$ ($S_j \geq 68\%$) (Fig. 1). Some features shared

FIG. 2. Dendrogram resulting from taxonomic analysis of strains subjected to further testing with the *S* coefficient and unweighted average linkage. The data included both the original and supplemental results. Uniformly positive and negative features were omitted to increase the sensitivity of the analysis. The groups are numbered for consistency with those in Fig. 1. H, Human clinical isolates; N, environmental isolates.

by this group with *V. alginolyticus* were the ability to produce acid from sucrose, production of acetoin, growth in the presence of 10% NaCl, growth at 40°C, and inability to produce acid from arabinose (Table 1). All strains were gram-negative rods that produced polar flagella in T₁N₁ broth. The DNA base composition of two strains, N339 and N57, was 43 ± 1 mol% guanine plus cytosine. An interesting difference between strains in group 13 and *V. alginolyticus* was the TSI reaction (Table 1).

Groups 14 to 18 contained 48 environmental, 1 clinical, and 5 *V. alginolyticus* reference strains that clustered at *S* ≥ 85% (*Sj* ≥ 77%) (Fig. 1). Distinguishing features of the larger subgroups of *V. alginolyticus* are shown in Table 3.

Group 19 contained one clinical and nine environmental isolates that clustered at *S* ≥ 84% (*Sj* ≥ 79%) and joined the *V. alginolyticus* strains at *S* ≥ 78% (*Sj* ≥ 70%) (Fig. 1). After further analysis, the nine environmental strains clustered at

S ≥ 86% (*Sj* ≥ 75%) and joined the *V. alginolyticus* reference strains at *S* ≥ 83% (*Sj* ≥ 72%) (Fig. 2), while the clinical strain (H6922) separated into a new group (group 23). The DNA base composition of two strains, N122 and N104, was 44 ± 1 mol% guanine plus cytosine. Although these traits initially identified this group as *V. alginolyticus*, the DNA relatedness data (Table 4) confirmed that group 19 is a species separate from *V. alginolyticus*.

Group 20 was identified as *V. cholerae* and contained one environmental isolate (from a milkfish specimen), four clinical isolates, and the *V. cholerae* type strain. These strains clustered at *S* ≥ 85% (*Sj* ≥ 68%) and joined the *V. alginolyticus* and *V. parahaemolyticus* strains at *S* ≥ 60% (*Sj* ≥ 44%) (Fig. 1). Distinctive features of this group are provided in Table 1.

Group 21 contained two clinical isolates that clustered at *S* ≥ 86% (*Sj* ≥ 68%) (Fig. 1) and was identified as *V. mimicus*. The identification of these isolates was based on the ability to grow in tryptone broth without supplemental NaCl and the utilization of glucuronate (Table 1).

Group 22 was composed of one environmental isolate (from a barracuda specimen) and three *V. vulnificus* reference strains that clustered at *S* ≥ 84% (*Sj* ≥ 69%) (Fig. 1). These strains required NaCl for growth in tryptone broth but did not grow in 8% NaCl (Table 1).

The second analysis created a new group, 23, that clustered at *S* ≥ 87% (*Sj* ≥ 81%) and included one environmental isolate (from a spinyfoot specimen) and one clinical isolate (Fig. 2). This was the only group in which strains produced acid from both L-arabinose and sucrose (Table 1). Both strains required added NaCl for growth in tryptone broth but did not grow in 10% NaCl and produced amylase, lysine, and ornithine decarboxylases; and acid slant-acid butt reactions in TSI. They differed from *V. parahaemolyticus* in the utilization of sucrose and galactose and differed from *V. alginolyticus* in the utilization of L-arabinose, ethanol, and propanol (Table 1).

DNA relatedness analysis was conducted with 14 isolates belonging to groups 1, 2, 9 to 14, 18, 19, and 23. The isolates were selected to represent the unidentified groups (groups 1, 12, 13, and 23), groups that bordered on these unidentified groups in either of the previous two analyses (groups 10, 11, and 19), *V. parahaemolyticus* (groups 2 to 9), and *V. alginolyticus* (groups 14 to 18), together with one of the nongroupable strains (N287). It was possible to confirm the identification of groups 1, 2, 9 to 12, and 23 as *V. parahaemolyticus* and groups 13, 14, and 18 as *V. alginolyticus*, but group 19 remained unidentified and could be described only as a *Vibrio* species.

Except for groups 10 and 23, all isolates belonging to *V. parahaemolyticus* (groups 1, 2, 9, 11, and 12) were 78 to 93% related to the type strain of *V. parahaemolyticus* at both 60 and 75°C, with dTms of -0.2 to 1.1°C. Groups 10 and 23 were 70 and 72% related to *V. parahaemolyticus*, respectively. Although these values are very close to the cutoff for DNA relatedness at 60°C (<70%), identification of group 10 as *V. parahaemolyticus* was based on its dTm (0.4°C) and relatedness at 75°C (72%) (Table 4). Group 23 (strain H6922) was 72% related to *V. parahaemolyticus* at 60°C, with a dTm of 0.2°C and relatedness of 67% at 75°C.

Groups 1, 2, 9 to 12, and 23 were 23 to 53% related to the type strain of *V. alginolyticus* (ATCC 17749) at 60°C, with dTms of 10.1 and 8.8°C for groups 1 and 2, respectively. Groups 1 and 2 were 26 and 23% related to *V. alginolyticus* at 75°C, respectively (Table 4).

V. alginolyticus (groups 13, 14, and 18) were 85 to 96%

TABLE 1. Feature frequencies for the major groups obtained in this analysis

Feature ^a	% Frequency in:								
	<i>V. para-haemolyticus</i> (n = 141)	<i>V. alginolyticus</i> (n = 64)	<i>Vibrio</i> sp.				<i>V. cholerae</i> (n = 6)	<i>V. mimicus</i> ^b (n = 2)	<i>V. vulnificus</i> (n = 4)
			Group 1 (n = 4)	Group 12 (n = 5)	Group 13 (n = 9)	Group 23 (n = 2)			
Methyl red	95	98	100	100	78	100	50	50	75
Voges-Proskauer	0	83	0	0	100	0	100	50	0
Acid from:									
L-Arabinose	77	0	100	100	0	100	0	0	0
Salicin	53	33	100	40	11	0	17	0	100
D-Cellobiose	99	86	100	80	78	100	50	100	75
Sucrose	1	100	0	0	100	100	100	0	0
Production of:									
Amylase	99	67	100	100	44	100	100	0	75
Chitinase	74	46	100	100	20	0	NT ^c	100	50
L-Arginine dihydrolase	5	0	25	0	0	0	0	0	0
Decarboxylation of:									
L-Lysine	100	100	100	100	100	100	100	100	50
L-Ornithine	100	53	100	100	33	100	100	50	50
ONPG hydrolysis	21	2	50	20	0	0	100	100	100
TSI									
K/A	100	0	100	100	100	0	83	100	100
A/A	0	100	0	0	0	100	17	0	0
Gas	36	33	75	80	44	0	50	50	25
NaCl requirement	100	100	100	100	100	100	0	0	100
NaCl tolerance (%):									
8	92	98	100	100	100	100	0	0	0
10	0	80	25	0	89	0	0	0	0
O/129 (µg/ml):									
10	99	97	100	100	100	100	83	100	25
50	56	79	67	0	56	50	83	100	0
150	7	3	0	0	11	0	0	50	0
Growth at 40°C	100	93	100	100	100	100	100	NT	33
Utilization of:									
L-Arabinose	73	0	100	100	0	100	0	0	0
D-Cellobiose	60	20	100	20	22	50	0	0	100
Sucrose	4	98	50	0	100	100	100	0	0
Ethanol	83	20	75	0	44	100	0	0	0
Propanol	82	33	100	0	78	100	0	0	0
Galacturonate	3	0	100	0	0	0	0	0	0
Glucuronate	40	0	50	20	0	50	0	100	50
4-Aminobutyrate	13	3	100	20	11	50	0	0	0
L-Leucine	87	84	75	0	89	50	0	0	0
Putrescine	94	78	100	100	100	100	0	0	0
D-Galactose	77	33	50	80	11	0	0	NT	33
Mannose	100	73	100	100	33	100	0	NT	0
Glutarate	0	0	0	20	0	0	0	NT	0
Glycerate	100	87	100	60	100	100	0	NT	0
5-Aminovalerate	0	0	0	20	0	0	0	NT	0
L-Arginine	100	100	100	100	100	100	0	NT	100
Glycine	100	100	100	60	100	100	0	NT	0
L-Ornithine	31	80	0	0	78	50	0	NT	0
L-Citrulline	31	67	0	0	100	0	0	NT	0

^a All strains possessed fermentative metabolism, produced oxidase, and utilized α-ketoglutarate and gluconate. None produced H₂S in TSI after 24 h or urease or utilized butyrate, L-rhamnose, valerate, or hippurate. ONPG, o-nitrophenyl-β-D-galactopyranoside. TSI reactions: K/A, alkaline slant-acid butt; A/A, acid slant-acid butt.

^b *V. mimicus* was not tested for utilization of α-ketoglutarate, gluconate, L-rhamnose, valerate, or hippurate.

^c NT, Not tested.

TABLE 2. Features distinguishing the larger subgroups of *V. parahaemolyticus*

Feature	% Frequency in:			
	Group 2 (n = 49)	Group 3 (n = 24)	Group 7 (n = 12)	Group 11 (n = 9)
Acid from:				
L-Arabinose	100	100	0	0
Salicin	59	38	0	82
O/129 (50 µg/ml)	95	13	36	30
Utilization of:				
L-Arabinose	98	92	0	0
Ethanol	98	100	100	0
Propanol	100	87	100	0
Glucuronate	10	83	0	73

related to the type strain of *V. alginolyticus* at 60°C and 83 to 93% related to the same type strain at 75°C, and the dTm was 0.3 to 0.6°C.

These groups were 37 to 50% related to the type strain of *V. parahaemolyticus* (ATCC 27969) at 60°C. The dTm for group 18 was 11°C. Group 18 was also 28% related to *V. parahaemolyticus* in reactions at 75°C (Table 4).

Group 19 (strain N329) was 68% related to *V. alginolyticus* (ATCC 17749) at 60°C. Although this value is very close to the cutoff for DNA relatedness at 60°C (<70%), separation of group 19 from *V. alginolyticus* was based on the dTm (5.2°C for group 19 versus a cutoff value of ≤5.0°C) and on relatedness at 75°C (53% for group 19 versus a cutoff value of <55%). This group was 48% related to *V. parahaemolyticus* ATCC 27969 at 60°C, with a dTm of 9.1°C and relatedness of 22% at 75°C (Table 4).

Nongroupable strain N287 remained unidentified, as revealed by its low relatedness to *V. parahaemolyticus* (41% at 60°C; 18% at 75°C; 8.5°C dTm) and *V. alginolyticus* (66% at 60°C; 47% at 75°C; 4.7°C dTm) (Table 4).

All isolates in this analysis were only 15 to 28% related to the type strain of *V. natriegens* (ATCC 14048) (Table 4). These values were sufficient to exclude the possibility of identification for any of the isolates examined as *V. natri-*

iegens without determining the dTms or relatedness at 75°C. *V. natriegens* was included in the study because previously (32) this species was observed to cluster very closely with *V. parahaemolyticus*.

DISCUSSION

The environmental and clinical isolates examined in this study clustered into 23 groups. Five species were identified by use of reference strains and/or characteristic features. It has been noted that the use of a range of tests to identify a particular species from one environmental habitat may not be applicable in another habitat (34). Nevertheless, results of this study indicate that taxonomic criteria for identification of *V. parahaemolyticus*, *V. alginolyticus*, *V. cholerae*, *V. mimicus*, and *V. vulnificus* are useful for identification of strains from seawater, seafood, and clinical specimens collected in Indonesia and included in this study.

The clusters of *V. parahaemolyticus* were mixtures of environmental and clinical isolates with no separation of the groupings ascribable to the source. Nutritional characteristics were in general agreement with those for *V. parahaemolyticus* (2). Results in some cases, e.g., fermentation of L-arabinose and salicin, production of chitinase, and sensitivity to vibriostat O/129 (50 µg/ml), were useful in distinguishing biotypes of *V. parahaemolyticus* but, in general, did not separate clinical and environmental isolates.

Identification of *V. alginolyticus* isolated from tropical waters and seafood was achieved by using standard tests (32, 33). The *V. alginolyticus* strains examined in this study were environmental isolates, except for a single clinical isolate. Interestingly, *V. alginolyticus* is rarely isolated from fecal specimens (13, 25).

Two diagnostic features of *V. alginolyticus* (groups 14 to 18), growth in 10% NaCl and acetoin production, were positive for only about 80% of the strains (Table 1). The strains that contributed most of the negative results for these features were in group 18. Acetoin production has been reported to be relatively low and transient for some *Vibrio* strains (2) and is influenced by incubation time and temperature (33). Other studies have reported negative acetoin reactions (15). Inspection of specimens and dates of isolation for strains in group 18 revealed no consistent pattern to explain the negative results for growth in 10% NaCl. Larsen et al. (15) found that only 86% of human *V. alginolyticus* strains could grow in 10% NaCl, and others have also isolated strains negative for this characteristic (17, 27).

Two clinical isolates were identified as *V. mimicus* on the basis of their feature frequencies (6, 7). However, these strains produced acid from cellobiose (Table 1). Isolates from tropical areas in other studies were predominantly from clinical specimens, rarely from environmental sources (6, 7). However, many strains have been isolated from water or shellfish specimens in temperate climates. The possibility that a reservoir of *V. mimicus* exists in tropical environments requires further investigation.

Clinical and environmental strains of *V. vulnificus* have been isolated from many temperate areas (13, 24) and have been shown to be indistinguishable phenotypically (29). It should be pointed out that *V. vulnificus* has been isolated from fish in Senegalese coastal waters (26). In this study, *V. vulnificus* was observed to be distinct from other *Vibrio* species on the basis of taxonomic criteria (2).

The taxonomic traits of any group of organisms should be applicable to isolates from the environment. In this study, the taxonomic criteria used to describe *Vibrio* sp. were

TABLE 3. Features distinguishing the larger subgroups of *V. alginolyticus*

Feature	% Frequency in:			
	Group 15 (n = 15)	Group 16 (n = 10)	Group 17 (n = 10)	Group 19 (n = 10)
Acid from:				
Salicin	53	10	0	70
D-Cellobiose	93	60	80	100
Gas in TSI	0	90	30	50
Decarboxylation of L-ornithine	73	30	40	90
Susceptibility to O/129 (50 µg/ml)	100	100	0	89
Utilization of:				
D-Cellobiose	7	0	10	80
Ethanol	0	0	0	80
Propanol	20	10	0	100

TABLE 4. DNA relatedness among representative *Vibrio* isolates and reference strains (*V. parahaemolyticus*, *V. alginolyticus*, and *V. natriegens*)

Source of unlabeled DNA			Data for labeled DNA from ^a :							Confirmed identification
Strain ^b	Group	Preliminary identification	<i>V. parahaemolyticus</i> ATCC 27969			<i>V. alginolyticus</i> ATCC 17749			<i>V. natriegens</i> ATCC 14048 RBR (60°C)	
			RBR (60°C)	dTm (°C)	RBR (75°C)	RBR (60°C)	dTm (°C)	RBR (75°C)		
N248	1	<i>Vibrio</i> sp.	93	1.1	89	53	10.1	26	15	<i>V. parahaemolyticus</i>
H7417	2	<i>V. parahaemolyticus</i>	80	−0.2	78	44	8.8	23		<i>V. parahaemolyticus</i>
H5247	9	<i>V. parahaemolyticus</i>	89	0.7	95	36			28	<i>V. parahaemolyticus</i>
N73	10	<i>V. parahaemolyticus</i>	70	0.4	72	23			18	<i>V. parahaemolyticus</i>
H4517	11	<i>V. parahaemolyticus</i>	86	0.7	96	34			23	<i>V. parahaemolyticus</i>
N265	11	<i>V. parahaemolyticus</i>	89	0.3	82	33			19	<i>V. parahaemolyticus</i>
H4309	12	<i>Vibrio</i> sp.	89	0.3	90	25			19	<i>V. parahaemolyticus</i>
N364	12	<i>Vibrio</i> sp.	84	−0.1	78	27			18	<i>V. parahaemolyticus</i>
H6922	23	<i>Vibrio</i> sp.	72	0.2	67	32			23	<i>V. parahaemolyticus</i>
N533	13	<i>Vibrio</i> sp.	37			93	0.4	89	25	<i>V. alginolyticus</i>
H7180	14	<i>V. alginolyticus</i>	38			85	0.3	83	23	<i>V. alginolyticus</i>
N72	18	<i>V. alginolyticus</i>	50	11.0	28	96	0.6	93	23	
N329	19	<i>V. alginolyticus</i>	48	9.1	22	68	5.2	53	15	<i>Vibrio</i> sp.
N287	Nongroupable		41	8.5	18	66	4.7	47	18	<i>Vibrio</i> sp.
11-36VA	Reference	<i>V. alginolyticus</i>	38			91	0.4	87	22	
ATCC 17749	Reference	<i>V. alginolyticus</i>	42	9.3	23	100	0.0	100		
ATCC 17802	Reference	<i>V. parahaemolyticus</i>	87	0.6	88					

^a Blank space indicates not done. The results presented are averages of two separate analyses; the deviations were within acceptable limits relative to the cutoff values.

^b N, Environmental isolate; H, human clinical isolate.

found to be useful in identifying environmental and clinical strains. Biotypes of *V. parahaemolyticus* and *V. alginolyticus* in this study could not be correlated with sources. The variability of selected taxonomic features in the unidentified groups emphasizes the need to use DNA hybridization for resolving their species designations.

DNA relatedness analysis proved useful in identifying the representative isolates of groups 1, 12, and 23 as *V. parahaemolyticus* and those of group 13 as *V. alginolyticus*. It also confirmed identification of groups 2 and 9 to 11, 14 and 18, and 19 as *V. parahaemolyticus*, *V. alginolyticus*, and *Vibrio* sp., respectively.

Another interesting finding was that group 19 appeared to be most similar to *V. alginolyticus* on the basis of numerical taxonomic and sole carbon source utilization data. The isolate (N32a) selected to represent this group was similar to *V. alginolyticus* by DNA relatedness analysis; e.g., it was 68% related to the type strain of *V. alginolyticus* (ATCC 17749) in reactions at 60°C. However, the dTm was very high (5.2°C) and relatedness dropped to 53% in the reaction at 75°C. Thus, this group is concluded to be a separate species, distinct from *V. alginolyticus*. Group 19 and strain N287, which did not cluster with any of the groups, remained unidentified. Further research with additional reference strains should resolve the species designation of these organisms within the genus *Vibrio*.

The recently described species *V. aestuarianus*, *V. orientalis*, *V. diazotrophicus*, and *V. tubiashii* differ from the isolates examined in this study by at least two of the following features: arginine dihydrolase; lysine and ornithine decarboxylases; acid from L-arabinose, sucrose, or salicin; and growth at 40°C (8, 9, 30, 35).

The possible existence of new *Vibrio* spp. previously unrecognized, as seen in this study, suggests that further studies of vibrios in tropical climates will prove both informative and useful.

ACKNOWLEDGMENTS

This study was conducted in part by S.W.J. at Naval Medical Research Unit-2, Jakarta Detachment, Jakarta, Indonesia, and the Naval Medical Research Institute, Bethesda, Md. This work was supported in part by National Science Foundation systematics grant BSR8401397, World Health Organization grant C6-181-70, and Naval Medical Research and Development Command work unit MF51.524.009.0040B.

LITERATURE CITED

- Baumann, P., L. Baumann, and M. Mandel. 1971. Taxonomy of marine bacteria: the genus *Beneckea*. J. Bacteriol. 107:268-294.
- Baumann, P., and R. H. W. Schubert. 1984. Vibrionaceae, p. 516-538. In N. R. Krieg (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
- Brayton, P. R., R. B. Bode, R. R. Colwell, M. T. MacDonell, H. L. Hall, D. J. Grimes, P. A. West, and T. N. Bryant. 1986. *Vibrio cincinnatiensis* sp. nov., a new human pathogen. J. Clin. Microbiol. 23:104-108.
- Brenner, D. J., G. R. Fanning, A. V. Rake, and K. E. Johnson. 1969. Batch procedure for thermal elution of DNA from hydroxyapatite. Anal. Biochem. 28:447-459.
- Brenner, D. J., A. C. McWhorter, J. K. Leete-Knutson, and A. G. Steigerwalt. 1982. *Escherichia vulneris*: a new species of *Enterobacteriaceae* associated with human wounds. J. Clin. Microbiol. 15:1133-1190.
- Davis, B. R., G. R. Fanning, J. M. Madden, A. G. Steigerwalt, H. B. Bradford, Jr., H. L. Smith, Jr., and D. J. Brenner. 1981. Characterization of atypical *Vibrio cholerae* strains and description of a new pathogenic species, *Vibrio mimicus*. J. Clin. Microbiol. 14:631-639.
- Desmarchelier, P. M., and J. L. Reichelt. 1984. A phenotypic and genetic study of sucrose nonfermenting strains of *Vibrio mimicus* and *Vibrio cholerae*. Curr. Microbiol. 10:41-48.
- Guerinot, M. L., P. A. West, J. V. Lee, and R. R. Colwell. 1982. *Vibrio diazotrophicus* sp. nov., a marine nitrogen-fixing bacterium. Int. J. Syst. Bacteriol. 32:350-357.
- Hada, H. S., P. A. West, J. V. Lee, J. Stemmler, and R. R. Colwell. 1984. *Vibrio tubiashii* sp. nov., a pathogen of bivalve

- mollusks. *Int. J. Syst. Bacteriol.* **34**:1-4.
10. Janda, J. M., C. Powers, R. G. Bryant, and S. L. Abbott. 1988. Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clin. Microbiol. Rev.* **1**:245-267.
 11. Johnson, J. L. 1981. Genetic characterization, p. 450-472. In P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), *Manual of methods for general bacteriology*. American Society for Microbiology, Washington, D.C.
 12. Joseph, S. W. 1974. Observations on *Vibrio parahaemolyticus* in Indonesia, p. 35-40. In T. Fujino, G. Sakaguchi, R. Sakazaki, and Y. Takeda (ed.), *International Symposium on Vibrio parahaemolyticus*. Saikon Publishing Co., Tokyo.
 13. Joseph, S. W., R. R. Colwell, and J. B. Kaper. 1983. *Vibrio parahaemolyticus* and related halophilic vibrios. *Crit. Rev. Microbiol.* **10**:77-124.
 14. Kaper, F. B., H. Lockman, E. F. Remmers, K. Kristensen, and R. R. Colwell. 1983. Numerical taxonomy of vibrios isolated from estuarine environments. *Int. J. Syst. Bacteriol.* **33**:229-255.
 15. Larsen, J. L., A. F. Farid, and I. Dalsgaard. 1981. A comprehensive study of environmental and human pathogenic *Vibrio alginolyticus* strains. *Zentralbl. Bakteriol. Mikrobiol. Hyg. 1 Abt. Orig. A* **251**:213-222.
 16. Lee, J. V., T. J. Donovan, and A. L. Furniss. 1978. Characterization, taxonomy, and emended description of *Vibrio metschnikovii*. *Int. J. Syst. Bacteriol.* **28**:99-111.
 17. Lee, J. V., M. S. Hendrie, and J. M. Shewan. 1979. Identification of *Aeromonas*, *Vibrio*, and related organisms, p. 151-166. In F. A. Skinner and D. W. Lovelock (ed.), *Identification methods for microbiologists*, 2nd ed. Academic Press, London.
 18. Lee, J. V., P. Shread, and A. L. Furniss. 1981. Taxonomy and description of *Vibrio fluvialis* sp. nov. (synonym group F vibrios, group EF6). *J. Appl. Bacteriol.* **50**:73-94.
 19. Love, M. D., Teebken-Fisher, J. H. Hose, J. J. Farmer III, F. W. Hickman, and G. R. Fanning. 1981. *Vibrio damsela*, a marine bacterium, causes skin ulcers on the damselfish *Chromis punctipinnis*. *Science* **214**:1139-1140.
 20. MacDonell, M. T., and R. R. Colwell. 1985. Phylogeny of the Vibrionaceae, and recommendation for two new genera, *Listonella* and *Shewanella*. *Syst. Appl. Microbiol.* **6**:171-182.
 21. Mandel, M., L. Iganbi, J. Bergendahl, M. L. Dodson, Jr., and E. Scheltgen. 1970. Correlation of melting temperature and cesium chloride buoyant density of bacterial deoxyribonucleic acid. *J. Bacteriol.* **101**:333-338.
 22. MacFaddin, J. F. 1980. Biochemical tests for identification of medical bacteria. The Williams & Wilkins Co., Baltimore.
 23. Mayfield, C. I., and W. E. Inniss. 1977. A rapid, simple method for staining bacterial flagella. *Can. J. Microbiol.* **23**:1311-1313.
 24. Oliver, J. D., R. A. Warner, and D. R. Cleland. 1982. Distribution and ecology of *Vibrio vulnificus* and other lactose-fermenting marine vibrios in coastal waters of the southeastern United States. *Appl. Environ. Microbiol.* **44**:1404-1414.
 25. Sakazaki, R., S. Iwanami, and H. Fukumi. 1963. Studies on the enteropathogenic, facultatively halophilic bacteria, *Vibrio parahaemolyticus*. I. Morphological, cultural and biochemical properties and its taxonomical position. *Jpn. Med. Sci. Biol.* **16**:161-188.
 26. Schandevyl, P., E. Van Dyck, and P. Piot. 1984. Halophilic *Vibrio* species from seafish in Senegal. *Appl. Environ. Microbiol.* **48**:236-238.
 27. Schmidt, U., H. Chmel, and C. Cobbs. 1979. *Vibrio alginolyticus* infections in humans. *J. Clin. Microbiol.* **10**:666-668.
 28. Smibert, R. M., and N. R. Krieg. 1981. General characterization, p. 409-443. In P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), *Manual of methods for general bacteriology*. American Society for Microbiology, Washington, D.C.
 29. Tison, D. L., and R. J. Seidler. 1981. Genetic relatedness of clinical and environmental isolates of lactose-positive *Vibrio vulnificus*. *Curr. Microbiol.* **6**:181-184.
 30. Tison, D. L., and R. J. Seidler. 1983. *Vibrio aestuarianus*: a new species from estuarine waters and shellfish. *Int. J. Syst. Bacteriol.* **33**:699-702.
 31. Walczak, C. A., and M. I. Krichevsky. 1980. Computer methods for describing groups from binary phenetic data: modification of numerical taxonomy programs to increase flexibility. *Int. J. Syst. Bacteriol.* **30**:622-626.
 32. West, P. A., P. R. Brayton, T. N. Bryant, and R. R. Colwell. 1986. Numerical taxonomy of *Vibrios* isolated from aquatic environments. *Int. J. Syst. Bacteriol.* **36**:531-543.
 33. West, P. A., and R. R. Colwell. 1984. Identification and classification of Vibrionaceae—an overview, p. 285-363. In R. R. Colwell (ed.), *Vibrios in the environment*. John Wiley & Sons, Inc., New York.
 34. West, P. A., J. V. Lee, and T. N. Bryant. 1983. A numerical taxonomic study of species of *Vibrio* isolated from the aquatic environment and birds in Kent, England. *J. Appl. Bacteriol.* **55**:263-282.
 35. Yang, Y., L. pen Yeh, Y. Cao, L. Baumann, P. Baumann, J. Sung-en Tang, and B. Beaman. 1983. Characterization of marine luminous bacteria isolated off the coast of China and description of *Vibrio orientalis* sp. nov. *Curr. Microbiol.* **8**:95-100.