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Polyphasic Taxonomy of the Genus *Vibrio*: Numerical Taxonomy of *Vibrio cholerae*, *Vibrio parahaemolyticus*, and Related *Vibrio* Species

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A set of 86 bacterial cultures, including 30 strains of *Vibrio cholerae*, 35 strains of *V. parahaemolyticus*, and 21 representative strains of *Pseudomonas*, *Spirillum*, *Achromobacter*, *Arthrobacter*, and marine *Vibrio* species were tested for a total of 200 characteristics. Morphological, physiological, and biochemical characteristics were included in the analysis. Overall deoxyribonucleic acid (DNA) base compositions and ultrastructure, under the electron microscope, were also examined. The taxonomic data were analyzed by computer by using numerical taxonomy programs designed to sort and cluster strains related phenetically. The *V. cholerae* strains formed an homogeneous cluster, sharing overall S values of $\geq 75\%$. Two strains, *V. cholerae* NCTC 30 and NCTC 8042, did not fall into the *V. cholerae* species group when tested by the hypothetical median organism calculation. No separation of "classic" *V. cholerae*, El Tor vibrios, and nonagglutinable vibrios was observed. These all fell into a single, relatively homogeneous, *V. cholerae* species cluster. *V. parahaemolyticus* strains, excepting 5144, 5146, and 5162, designated members of the species *V. alginolyticus*, clustered at S $\geq 80\%$. Characteristics uniformly present in all the *Vibrio* species examined are given, as are also characteristics and frequency of occurrence for *V. cholerae* and *V. parahaemolyticus*. The clusters formed in the numerical taxonomy analyses revealed similar overall DNA base compositions, with the range for the *Vibrio* species of 40 to 48% guanine plus cytosine. Generic level of relationship of *V. cholerae* and *V. parahaemolyticus* is considered dubious. Intra- and intergroup relationships obtained from the numerical taxonomy studies showed highly significant correlation with DNA/DNA reassociation data.

Identification and classification have long been activities of primary concern to bacteriologists, particularly for purposes of communication and information storage. The bacteria have been arranged into the classical taxonomists' pigeonholes of orders, classes, families, genera, species, etc. Recent developments in biochemistry, molecular biology, and the computer sciences have intensified the already strong and fundamental interest in identifying, describing, and naming bacterial groups. It has become apparent that the new avenues of research all provide useful and meaningful data. Thus, a taxonomy is required which assembles and assimilates the many levels of information, from the molecular to the ecological, and incorporates the several distinct, and separable, portions of information extractable from a nonhomogeneous system to yield a multidimensional taxonomy. Such a taxonomy has been termed "polyphasic" (8).

The genus *Vibrio* was selected for study with the aim of establishing a polyphasic taxonomy for this genus. It has long been difficult to identify and classify organisms belonging to the genus *Vibrio*. Despite several proposed revisions in recent years (13, 18), the descriptions of the genus and species within the genus, and the intrageneric relationships of these species still need clarification.

Studies have shown the unreliability of a single feature, such as cell curvature, as a sole diagnostic criterion. Before 1953, the genus was identified on the basis of characteristic flagellation, morphology, and curvature of the cells. More recent work has shown that the genus *Vibrio* should be reserved for organisms with a fermentative carbo-

hydrate metabolism since glucose fermentation by *V. comma* (*V. cholerae*, reference 31) yields acid but no gas and proceeds via the Embden-Meyerhof glycolytic scheme (76). Sensitivity to the compound 2,4-diamino 6,4-di-isopropyl pteridine has also been shown to be a useful diagnostic characteristic (68). An overall deoxyribonucleic acid (DNA) base compositional range for the genus *Vibrio* of 44 to 50% guanine plus cytosine (GC), with the *V. cholerae* species range of 46 to 47%, has been proposed (64; Colwell and Yuter, Bacteriol. Proc. p. 18, 1965).

Serious problems are encountered in differentiation of species within the genus *Vibrio* and also in resolution of relationships of *Vibrio* to other genera such as those in the families *Pseudomonadaceae* and *Enterobacteriaceae*. As emphasized by Davis and Park (13), serious difficulties lie in differentiation of *Vibrio* species from anaerogenic *Aeromonas*, with determination of overall DNA base composition being, in some instances, the only definitive means of separation (9, 30, 60).

Aside from the theoretical aspects of classification, some very practical considerations direct the study of the genus *Vibrio*. Outbreaks of cholera occurring recently in the Middle East and in Southeast Asia were attributed to the El Tor variety of *V. cholerae*. Identification and classification of species within the genus *Vibrio*, in particular, *V. cholerae*, the El Tor vibrio ("*Vibrio eltor*"), the nonagglutinable (NAG) vibrios, and the wide variety of strains grouped as "water vibrios" must be accomplished quickly and accurately for epidemiological purposes. The relationship of the El Tor vibrio to *V. cholerae* continues to be disputed. That is, some investigators consider the El Tor vibrio to be a separate species (14, 15, 51) and others a biotype of the species *V. cholerae* (18, 32). In any case, the decision of The International Association of Microbiological Societies, Committee on Taxonomy, Subcommittee on *Vibrio*, was to include the El Tor strains as an infra-subspecific taxon within the species *V. cholerae* (18).

Another important species of the genus, *V. parahaemolyticus*, is currently the subject of intensive research (24). *V. parahaemolyticus* was first isolated in Japan by Fujino et al. (25) from "summer season food poisoning" victims with symptoms of acute gastroenteritis. The organism was subsequently classified by Sakazaki, Iwanami, and Fukumi (61). Recent reports (1, 36, 74, 80) revealed *V. parahaemolyticus*, which requires sodium chloride for optimal growth and is isolated from the marine habitat, to be present in coastal waters of the United States (1, 36, 74). *V. parahaemolyticus* provides an interesting problem in regard to intrageneric relationships of marine and nonmarine species, since *Vibrio* abounds in the estuarine and marine environment (42, 67).

Because the genus *Vibrio* provides a useful and interesting model for the application of a polyphasic taxonomy, the object of the present study is to present the numerical taxonomic (phenetic) analysis of the genus *Vibrio*, with data on molecular genetic relationships and ecological distributions of *Vibrio* species in the natural environment to be published in separate communications (6; Colwell, Krantz, Lovelace, and Wang, *in preparation*).

MATERIALS AND METHODS

Bacterial cultures. A total of 86 strains were included in the study: 30 strains of *V. cholerae*, 35 strains of *V. parahaemolyticus*, and 21 representative strains of *Pseudomonas*, *Spirillum*, *Achromobacter*, *Arthrobacter*, and marine *Vibrio* species. Table 1 lists the strains, source, and details of isolation, etc. The cultures were received as lyophils (*V. cholerae*, *V. parahaemolyticus*, and ATCC strains) or on agar slants. All were subcultured when received and examined for purity. Stock cultures were lyophilized, and reference vials of the *Vibrio* species were retained in storage at room temperature (ca. 25 C).

Nonmarine strains were maintained on a medium (YE, *p*H 7.2) consisting of: Yeast Extract (Difco), 0.3%; Proteose Peptone (Difco), 1.0%; NaCl, 0.5%. The marine strains and *V. parahaemolyticus* were maintained in the same medium but with the following salts solution replacing the NaCl added as diluent: NaCl, 2.4%; KCl, 0.07%; $MgCl_2 \cdot 6H_2O$, 0.53%; $MgSO_4 \cdot 7H_2O$, 0.70% (SWYE). Cultures were routinely incubated at 25 ± 1 C. During testing, cultures were routinely transferred each week, or more frequently as necessary. When cultures were held over time intervals longer than 1 week, the cultures were overlaid with sterile mineral oil to a depth of ca. 1 cm and retained at room temperature (25 C).

Tests and testing methods. The routine tests for scoring morphological and culture characteristics were used. Except where indicated, the testing procedures were as previously published (7, 48, 70). For the marine and *V. parahaemolyticus* strains, testing media were made up with the salts consisting of 2.4% NaCl, 0.07% KCl, 0.53% $MgCl_2 \cdot 6H_2O$, and 0.7% $MgSO_4 \cdot 7H_2O$. Plate inoculations were made by spot-drop (7), replica plate technique (38), or multipoint inoculator (41). Liquid media were inoculated with one drop (ca. 0.05 ml) from a sterile disposable Pasteur pipette of an 18- to 24-hr culture. Tests were carried out at incubation temperatures of 25 ± 1 C, except where otherwise indicated.

Morphological characters were scored from 18- to 24-hr cultures grown in YE or SWYE broth and examined by phase-contrast microscopy.

Motility was scored from wet-mount preparations examined by phase-contrast microscopy. Flagella stains were prepared by the method of Leifson (39).

TABLE 1. *Details of bacterial strains used in this study (total = 86 strains)*

Strain no.	Serotype	Place isolated	Date isolated	Source, details[a]
"Classic" *Vibrio cholerae*[b]				
NIH 41	Ogawa	India	Early 1940's	DBS, NIH, Bethesda, Md., originally obtained from WRAMC, Washington, D.C.
NIH 35A3	Inaba	India	Early 1940's	DBS, originally obtained from WRAMC
C-401	Inaba	Calcutta, India	1953	Originally obtained from W. Burrows
C-441	Ogawa	Calcutta, India	1953	Originally obtained from W. Burrows
VC-9, VC-12	Ogawa	Dacca, E. Pakistan	1960	Isolated by J. Feeley at Mitford Hospital
VC-13	Inaba	Dacca, E. Pakistan	1960	Isolated by J. Feeley at Mitford Hospital
ATCC 14035	Ogawa	*Vibrio cholerae-asiaticae* (*V. comma*)	Type species	ATCC, NCTC 8021
P6/58	Inaba	Calcutta, India	1958	Originally from D. C. Lahiri
P33/58	Ogawa	Calcutta, India	1958	Originally from D. C. Lahiri
El Tor vibrios[b]				
ATCC 14033	Inaba	El Tor Station	1930	ATCC, NCTC 8457; isolated by Doorenbos from a pilgrim at a quarantine camp (suggested neotype)
NCTC 2890	Inaba	El Tor Station	1905	Originally from W. Burrows, Tor A, Gardner and Venkatramen, 1935
NCTC 6560	Ogawa	El Tor Station	1933	Originally from W. Burrows, 34-D 9, Gardner and Venkatramen, 1935
NCTC 6563	Inaba	El Tor Station	1933	Originally from W. Burrows, 34-D 13, Gardner and Venkatramen, 1935
HK-1	Ogawa	Hong Kong	1961	Originally from D. J. M. MacKenzie
HK-25	Ogawa	Hong Kong	1961	Originally from D. J. M. MacKenzie
SLH 29803	Ogawa	Manilla	1961	Isolated by J. Feeley, San Lazaro Hospital
SLH 30810	Ogawa	Manilla	1961	Isolated by J. Feeley, San Lazaro Hospital
2A/62, 2B/62	Ogawa	New Guinea	1962	Originally from S. Mukerjee ("non-hemolytic El Tor vibrio")
Non-cholera vibrio[b]				
NCTC 4711	O group III	Nanking	1932	NCTC, Gardner and Venkatramen, 1935
NCTC 4715	O group V	El Tor Camp	1934	NCTC, Gardner and Venkatramen, 1935
NCTC 4716	O group VI	Kasauli	1932	NCTC, Gardner and Venkatramen, 1935
NCTC 8042	O group II	Nanking	1932	NCTC, Gardner and Venkatramen, 1935
NCTC 30	O group III		1916	NCTC, Gardner and Venkatramen, 1935
S-163, S-165, S170, S-696, S-860		Dacca, E. Pakistan	1962	SEATO Cholera Research Laboratory, isolated by O. R. McIntyre from human diarrhea cases
Vibrio parahaemolyticus[c]				
1	1-1-1[d]		8/12/63	Japanese strain no. 3053-63
2-R, 2-S	1-2-2		8/18/63	Japanese strain no. 3066-63
3	1-2-3		9/2/62	Japanese strain no. 5245-62
4	1-3-4		7/18/61	Japanese strain no. 3866-61
5	1-3-5		8/12/61	Japanese strain no. 5211-61
6-R, 6-S	1-3-6		8/1/62	Japanese strain no. 5007-62
7	1-3-7		9/12/62	Japanese strain no. 5113-62
8	1-4-8		9/20/63	Japanese strain no. 3102-63
9-O, 9-T	1-4-9		6/20/61	Japanese strain no. 5301-61

10	1-4-10		8/2/61	Japanese strain no. 5315-61
11-O, 11-T	1-4-11		8/19/63	Japanese strain no. 3037-63
12	1-4-12		8/11/63	Japanese strain no. 3065-63
13	1-4-13		9/1/62	Japanese strain no. 0028-62
14	1-5-14		8/16/61	Japanese strain no. 5401-61
15-O, 15-T	1-5-15		8/11/61	Japanese strain no. 5299-61
16	1-5-16		8/13/62	Japanese strain no. 5171-62
17	1-5-17		9/14/62	Japanese strain no. 5019-62
18	1-6-18		8/15/62	Japanese strain no. 4826-62
19	1-7-19		9/8/62	Japanese strain no. 5222-62
20	1-8-20		6/1/62	Japanese strain no. 7121-62
21	1-8-21		8/23/61	Japanese strain no. 5000-61
23	1-9-23		9/21/61	Japanese strain no. 3313-61
24	1-10-24		10/1/63	Japanese strain no. 4001-63
25	1-1-25		7/23/63	Japanese strain no. 3010-63
28	1-2-28		8/19/61	Japanese strain no. 3072-61
30	1-3-30		9/12/62	Japanese strain no. 5001-62
5144[e]	2		8/6/62	Japanese strain no. 5144-62, from seawater
5146[e]	2		8/2/62	Japanese strain no. 5146-62, from food
5162[e]	2		9/3/62	Japanese strain no. 5162-62, from food
5403[e]	2		8/23/62	Japanese strain no. 5403-62, from fish
Pseudomonas aureofaciens				
8ab1, 8ab2				8ab1 produces phenazine-α-carboxylic acid, 8ab2 produces chlororaphine (amide of phenazine-α-carboxylic acid); isolated by J. Toohey, Queen's University Biological Field Station, Elgin, Ontario; received April 1963
Arthrobacter sp.				
3C				F. D. Cook, Soil Service, Edmonton, Alberta; received March 1963
Achromobacter georgiopolitanum				
COC21				Chesapeake and Ohio Canal water; Colwell, Smith, and Chapman (12)
Pseudomonas apiseptica				
PA				Honeybee pathogen; received from T. A. Gochnauer, Apiculture Section, Canadian Department of Agriculture, Ottawa, February 1964; Katznelson and Landerkin (37)
Spirillum serpens subsp. *serpens*				
11330				ATCC; A. J. Kluyver strain suggested neotype; Williams and Rittenberg (81)
Pseudomonas cruciviae				
13262				ATCC; Gray and Thornton strain (28)
Spirillum itersonii subsp. *vulgatum*				
11331				ATCC; S. C. Rittenberg strain; type strain (81)
Pseudomonas sp.				
CIM 3, 9A, 9B, C, and D				Cultures isolated from soils enriched with cinnamic acid; isolated by F. J. Simpson, Prairie Regional Laboratory, National Research Council, Saskatoon, Saskatchewan
Ox-Sawyer				Isolated from oyster homogenate, used for cultivation of marine amoeba. Received from T. Sawyer, Biology Laboratory, Bureau of Commercial Fisheries, Oxford, Md., February 1966.

TABLE 1—*Continued*

Strain no.	Serotype	Place isolated	Date isolated	Source, details[a]
Vibrio sp.				Florida coastal waters; Tyler, Bielling, and Pratt (75)
MB-22				
WH-135				W. McElroy strain; received April 1964
Achromobacter fischeri				W. McElroy strain; Department of Biology, The Johns Hopkins University, Baltimore, Md.; received December 1967
4032, 4036, 4037, and 4038				Unidentified laboratory strains

[a] Division of Biologics Standards, DBS; National Institutes of Health, NIH; Walter Reed Army Medical Center, WRAMC; American Type Culture Collection, ATCC; National Collection of Type Cultures, NCTC.

[b] Groupings under which the strains were received. In subsequent tables, test results are reported for *V. cholerae* and include "classic," El Tor, and "non-cholera" vibrios which formed a species cluster.

[c] Except where indicated, strains were isolated from gastro-enteritis victims.

[d] Subgroup-O group-K antigen.

[e] Received as *Vibrio parahaemolyticus*.

Cultures were prepared for flagella stains by aseptically transferring the liquid of syneresis (1 to 2 ml) which contained the bacterial cells (located at the junction of the agar slope and the glass test tube), to 2 ml of sterile distilled water. One drop of 10% Formalin per ml of distilled water was then added as a fixative for the bacteria. The marine and *V. parahaemolyticus* strains were transferred to sterile distilled water pretreated with Formalin to prevent lysis of the cells. Staining time for the strains used in this study was, on average, 15 min. Records of flagella stain results were kept by means of Polaroid photomicrography. The Gram stain used was the Hucker modification (70), and cultures were scored as gram-negative, -positive, or-variable.

Colonial characteristics were determined on YE and SWYE agar at 24 to 48 hr. Pigment production as a diffusible green pigment, diffusible blue pigment, or diffusible brown pigment was scored from examination of a variety of media (7). Pyocyanine and pyorubin were identified by presumptive tests. Sporadic production of oxychlororaphin and chlororaphin crystals was noted for the *Pseudomonas aureofaciens* strains but no single medium served as indicator for consistent production of these compounds. This is in concurrence with observations of other investigators (J. Toohey, *personal communication*).

Growth characteristics in liquid media were scored, with turbidity measured by using Brown's opacity tubes as reference (Burroughs and Wellcome Co., Tuckahoe, N.Y.). Characteristics in liquid media were determined for all isolates in YE and SWYE broth at 48 hr.

Temperature range of growth was measured by incubating inoculated YE and SWYE broth tubes which were preincubated at given test temperatures for 6 to 12 hr before inoculation. The incubation period extended from 5 days to 4 weeks, depending on the temperature of incubation. Salinity range of growth was tested by inoculating YE and SWYE broth tubes with added NaCl.

YE and SWYE broth, adjusted to the desired *p*H with sterile HCl or NaOH, as required, after autoclaving, were inoculated to determine *p*H range of growth. Growth was observed at 1, 2, 7, and 14 days.

Sensitivity to antibiotics and the "vibriostatic" compound (2,4-diamino-6,7-diisopropylpteridine) described by Shewan, Hodgkiss, and Liston (68) were tested. YE or SWYE agar plates were used, with sensitivity discs (BBL) for the following antibiotics: penicillin, 10 units; dihydrostreptomycin, 10 μg; chloramphenicol, 30 μg; erythromycin, 15 μg; kanamycin, 30 μg; aureomycin, 30 μg; novobiocin, 30 μg; polymyxin B, 300 units; terramycin, 30 μg; and tetracycline, 30 μg.

Glucose utilization was determined by the methods of Hugh and Leifson (35) and Board and Holding (4). The Hugh and Leifson medium, without agar and with an inverted inner vial, was also used to detect gas production from carbohydrates. Growth in glucose, with and without 10^{-3} M iodoacetate, was determined. Growth and production of acetic acid in ethanol agar was recorded. Acid production from carbohydrates (1%) and ethanol (5%) was determined in tubes with Hugh and Leifson (35) medium and on agar plates.

Carbohydrates were sterilized by filtration, except for dulcitol, inulin, and dextrin which were steamed for 1 hr on 3 successive days. Twenty-five carbohydrates were tested. Each culture was examined at 1, 7, 14, and 28 days for acid and gas production in all tests. Hydrolysis of aesculin and starch, production of dihydroxyacetone from glycerol, digestion of agar, and examination for levan production were tested by methods previously cited (7, 48).

Methyl red and Voges-Proskauer tests were performed by the techniques described in the Manual of Microbiological Methods (70). The following tests were made by using previously cited methods (7, 48): oxidase, cytochrome oxidase, catalase, production of phosphatase, reduction of nitrate and nitrite, gelatin liquefaction, litmus milk, growth on skim milk agar and casein hydrolysis, production of ammonia from peptone at 14 days and hydrogen sulfide from peptone in lead acetate agar and from cystine and cysteine, urease production, indole production, production of 2-ketogluconic acid, deamidation of acetamide, arginine dihydrolase, lysine and orthinine decarboxylase, production of phenylpyruvic acid from phenylalanine, trimethylamine oxide reduction, lecithinase, lipase, ability to utilize citrate, oxidation of calcium lactate through acetate to carbonate and utilization of 0.3% sodium malonate, 0.1% sodium acetate, 0.1% sodium formate as carbon source and 0.1% $NH_4 \cdot H_2PO_4$ as nitrogen source.

Nutritional tests were carried out by replica plating technique and by using a basal salts medium with added amino acids. A control plate with only the basal salts agar was tested at the same time. The basal salts solution (*p*H 6.8) consisted of: NaCl, 5.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; K_2HPO_4, 1.0 g; distilled water, 1 liter. For the marine and *V. parahaemolyticus* strains, the four-salts solution given above was used. Vitamin-free Casamino Acids, proline, DL-alanine, β-alanine, arginine, lysine, phenylalanine (California Biochemical Corp.) were tested for ability to support growth.

Hemolysis of defibrinated sheep red blood cells and citrated whole human blood was tested by using 5% blood in Blood Agar Base (Difco).

Sensitivity to sodium lauryl sulfate was tested by inoculation of strains into YE or SWYE broth with 0.01% sodium lauryl sulfate added. The tubes were examined for growth at 2, 7, and 14 days and compared with growth in YE or SWYE broth without added sodium lauryl sulfate.

Cellulose digestion was tested by using the cellulose strip-peptone medium of Skerman (69) and by inoculation of a 1.5% purified agar (Difco; *p*H 7.4) with a sterile filter paper disc (Whatman no. 1) over-laid on the agar and inoculation with a cotton-tipped swab from YE or SWYE broth cultures.

Seawater and specific salt requirement were tested by spot-drop inoculation with washed cells of a basal medium with: (i) 2.4% NaCl, (ii) 2.4% NaCl and

0.07% KCl, (iii) 2.4% NaCl and 0.53% $MgCl_2$, (iv) 0.07% KCl, 0.53% $MgCl_2$, and 0.7% $MgSO_4$, (v) 0.6 M NaCl, and (vi) aged seawater. The basal medium (*p*H 7.2) consisted of 0.1% Trypticase (BBL), 2.0% Ionagar (Difco), and distilled water (75).

Base compositions of the highly purified DNA extracted from the strains by the method of Marmur (43) were determined from melting-point temperatures (T_m; reference 44). Methods used were previously cited (10).

Ultrathin sections of bacteria were prepared and examined under the electron microscope. Methods employed in fixing, embedding, sectioning, and staining and the instruments for electron microscopy were described by Felter et al. (22).

A total of 200 characteristics for each strain examined were scored, coded, and transferred to IBM cards for analysis by computer. Similarity and matching values were computed (71), and a highest linkage sorting of strains was done by computer, by using the Georgetown Taxonomy Program (GTP-2) in the Georgetown University Computation Center Program Library. Median organism calculations (40) and intragroup feature frequencies (7) were also computed. The triangular matrices of S values for groups established by the modified linkage program were also prepared by computer (GTP-2-Chart Program, Georgetown University Program Library). An online plotting program was also used for part of the analysis. The programs were written in PL/1 language for use on the IBM Systems 360/40 computer with disc and tape drives and with Cal-Comp (California Computer Products, Inc.) on-line plotter. The data have been transferred to tape and entered in the Georgetown University Computer Center Microbial Taxonomy Library.

RESULTS

The *V. cholerae* strains (including "classic," El Tor, and "non-cholera" vibrios; Table 1) formed an homogeneous cluster and, with the exception of *V. cholerae* NCTC 30, shared *overall* similarity (S) values of $\geq 75\%$ (Fig. 1). The *V. cholerae* strains, grouped by a modified highest linkage sorting, clustered at levels of $\geq 85\%$ S except for *V. cholerae* NCTC 30, which joined the *V. cholerae* cluster at S = 81% through high S values shared with strains 8042, 14033, and 2890 (Fig. 2). When the hypothetical median organism (40) was calculated for the *V. cholerae* and grouping by this method used, two strains of non-cholera vibrios, NCTC 30 and NCTC 8042 (Table 1) did not cluster with the *V. cholerae* at S levels of $\geq 85\%$ as did the other 28 strains. Strain NCTC 8042 was included in the species group at S = 76%, and strain NCTC 30 was not positioned within the group (when membership S level was set at 75%) by the hypothetical median organism calculation. Both strains grouped with other *V. cholerae* strains only at S-value levels which were nonspecific, i.e., $\leq 50\%$. In Fig. 3, a plot of the mean S value and mean number of shared features of each strain of *V. cholerae* with all the other strains of the set is given (56). The essential homogeneity of the species cluster is indicated by this plotting method.

ADANSONIAN ANALYSIS OF VIBRIO CHOLERAE

LEGEND: N = NON-CHOLERA VIBRIO; E = ELTOR VIBRIO; C = CLASSIC VIBRIO CHOLERAE; * = RECOMMENDED NEOTYPE

FIG. 1. *Grouping of Vibrio cholerae, non-cholera vibrios, and El Tor vibrios obtained by highest linkage relationships of strains, by the method of Sokal and Sneath (71).*

S-VALUE GRAPH

FIG. 2. *Total similarity value output for Vibrio cholerae and related strains. Strains arranged by modified highest-linkage sorting by computer by using the GTP-2 program. All S values computed for the set of strains are included in the diagram.*

The feature frequencies for the *V. cholerae* strains are listed in Tables 2 to 5. The *V. cholerae* strains were uniformly gram-negative, polarly flagellated, round-ended, short to medium length rods. Less than two-thirds of the strains examined

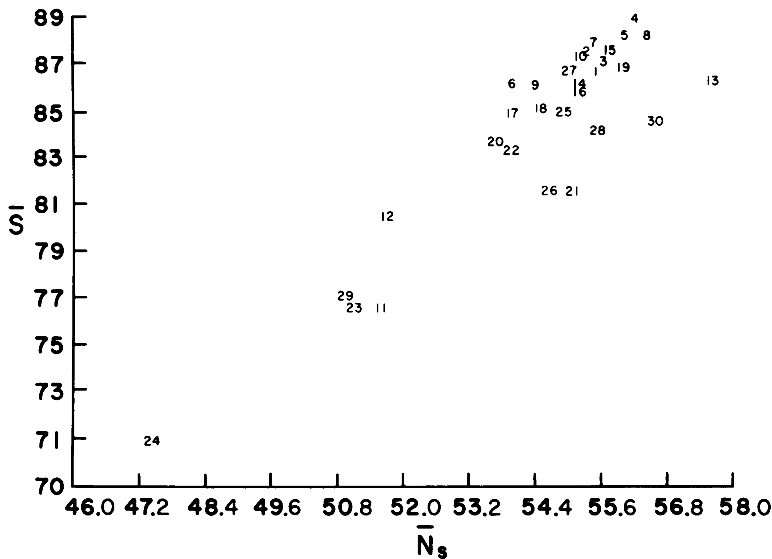

FIG. 3. *Homogeneity of the Vibrio cholerae cluster indicated by plot of mean overall similarity ($\bar{S}$) and mean number of shared features ($\bar{N}s$) of each of the 30 strains of V. cholerae examined in this study with the other 29 strains (56). The order of the strains is as listed in Table 1, except that strain NCTC 6560 is computer identification no. 30. Strain no. 24 in this figure is NCTC30. Strains 11, 23, and 29 are ATCC 14033, 8042, and S 860, respectively.*

demonstrated cell curvature considered typical of vibrios. Colony growth on solid media showed some variation, with entire edge, translucent colonies 2 to 5 mm in diameter dominant in all cultures examined in this study. Growth in liquid media was an even, moderate turbidity with slightly more than half the strains forming a pellicle in liquid media. No pigmentation in liquid or broth culture was noted.

The strains of *V. cholerae* were found to be sensitive to all the antibiotics tested (Table 3), including pencillin and tetracycline but excepting polymyxin B to which half the strains examined were not sensitive. A clear-cut separation of "classical" *V. cholerae* and the El Tor vibrios on the basis of sensitivity to polymyxin B was not found. Sensitivity to the 0/129 vibriostat (68) was observed.

The *V. cholerae* strains were capable of aerobic and anaerobic breakdown of glucose without production of gas. Starch hydrolysis was also noted for the majority of the strains (Table 4). Other biochemical tests, oxidase, catalase, nitrate reductase, tryptophanase, lecithinase, gelatinase, and lysine and ornithine decarboxylase were positive (Table 5). A temperature range of 15 to 42 C, *p*H tolerance of 5.0 to 10.0, and sodium chloride tolerance of 0 to 5.0% was noted. The *V. cholerae* strains were not found to be fastidious, being capable of growth on media with single amino acids (for example, alanine, proline, and arginine) as sole source of carbon and nitrogen (Table 5).

V. parahaemolyticus (Table 1) with the exception of strains 5144, 5146, and 5162, clustered at $S \geq 80\%$ by highest linkage sorting (Fig. 4). *V. parahaemolyticus* strains 5144, 5146, and 5162 clustered together at $S \geq 75\%$, joining the major *V. parahaemolyticus* cluster at $S \leq 72\%$. The *V. parahaemolyticus* and *V. cholerae* clusters joined at $S = 77\%$ (Fig. 5).

The hypothetical median organism calculation resulted in a grouping of the *V. parahaemolyticus* strains at $S \geq 75\%$ with strains 5144, 5146, and 5162 excluded and clustered separately at $S \geq 85\%$. The ranking of strains within the species *V. cholerae* and *V. parahaemolyticus* with respect to S value with the hypothetical median organism of each species, respectively, is given in Table 6.

The total S-value triangular matrix for *V. parahaemolyticus* is given in Fig. 4. The S values for the entire *V. cholerae* and *V. parahaemolyticus*

TABLE 2. *Morphological and cultural characteristics for Vibrio cholerae and Vibrio parahaemolyticus*

Character	Frequency of occurrence[a]	
	V. cholerae	*V. parahaemolyticus*
Cell morphology		
Rods	+(1.00)	+(1.00)
Curved rods	±(0.62)	±(0.63)
Filaments	−(0)	±(0.38)
Spiral (rods)	−(0)	−(0)
Refractile	−(0)	−(0)
Single cells	+(1.00)	+(1.00)
Paired cells	−(0.21)	+(1.00)
Chains of cells	±(0.31)	±(0.38)
Short length (0.2 to 0.6 μm)	+(0.93)	−(0.03)
Medium length (0.6 to 1.2 μm)	±(0.62)	−(0.09)
Long (1.0 to ≥3 μm)	−(0)	+(0.93)
Slender (0.2 to 0.6 μm)	+(1.00)	−(0.21)
Stout (0.6 to 1.0 μm)	−(0)	+(0.78)
Round end	+(1.00)	±(0.41)
Tapered (lanceolate) end	−(0)	±(0.59)
Motile	+(1.00)	+(1.00)
Polar flagella	+(1.00)	+(1.00)
Monotrichous	+(1.00)	+(1.00)
Lophotrichous	−(0)	−(0)
Gram-negative	+(1.00)	+(1.00)
Gram variable	−(0)	−(0)
Colony morphology		
Entire edge	+(1.00)	+(0.91)
Convex	−(0)	+(1.00)
Translucent	+(0.83)	+(0.78)
Opaque	−(0.17)	−(0.25)
Rough	−(0)	−(0)
Small colony (1 to 2 mm)	−(0.21)	+(0.69)
Medium colony (2 to 5 mm)	+(0.79)	−(0.09)
Spreading growth on agar	−(0)	−(0)
Growth in liquid medium		
Even turbidity	+(1.00)	+(0.84)
Granular turbidity	−(0)	−(0.15)
Slight turbidity	−(0.07)	−(0.03)
Moderate turbidity	+(0.93)	±(0.34)
Heavy turbidity	−(0)	±(0.63)
Pellicle	±(0.59)	+(0.72)
Ring	−(0.10)	−(0.28)
Slime formation	−(0)	−(0)
Pigmentation		
Fluorescent under ultraviolet light	−(0)	−(0)
Visible insoluble pigment	−(0)	−(0)
Diffusible pigment	−(0)	−(0)
Pyocyanin produced	−(0)	−(0)
Pyorubin produced	−(0)	−(0)
Fluorescein produced	−(0)	−(0)
Oxychlororaphine produced	−(0)	−(0)
Pigment produced on Sabauraud agar	−(0)	−(0)
White	−(0)	−(0.03)
Off-white	+(1.00)	+(0.66)
Gray	−(0)	−(0.31)

[a] Frequency of occurrence of characteristic in sample set. Total number of *V. cholerae* strains = 29 (NCTC 30 excluded). Total number of *V. parahaemolyticus* strains = 32 (5144, 5146, and 5162 excluded).

TABLE 3. *Antibacterial and antibiotic sensitivity of Vibrio cholerae and Vibrio parahaemolyticus*

Compound tested	Frequency of occurrence[a]	
	V. cholerae reaction	*V. parahaemolyticus* reaction
Chloromycetin (Chloramphenicol), 30 μg	+ (1.00)	+ (0.94)
Dihydrostreptomycin, 10 μg	+ (1.00)	− (0.31)
Erythromycin (Erythrocin), 15 μg	+ (1.00)	+ (0.66)
Kanamycin (Kantrex), 30 μg	+ (1.00)	+ (0.69)
Novobiocin (Albamycin), 30 μg	+ (1.00)	+ (0.91)
Penicillin, 10 units	+ (1.00)	− (0.06)
Polymyxin B (Aerosporin), 300 units	± (0.55)	− (0.19)
Tetracyclin (Achromycin), 30 μg	+ (1.00)	± (0.59)
Oxytetracycline (Terramycin), 30 μg	+ (1.00)	± (0.50)
Chlortetracycline (Aureomycin), 30 μg	+ (1.00)	+ (0.66)
2,4-Diamino-6,7-diisopropylpteridine (Vibriostat 0/129), saturated	+ (1.00)	+ (0.75)
Sodium lauryl sulfate, 0.01%	− (0)	NT

[a] Values in parentheses = frequency of occurrence of characteristic. Total number of *V. cholerae* strains = 29 (NCTC 30 excluded). Total number *V. parahaemolyticus* strains = 32 (5144, 5146, and 5162 excluded). Symbols: + = sensitive, ± = variable, − = insensitive, NT = not tested.

set are presented in Fig. 6. Interspecies S values (Fig. 6) ranged from 60 to 80%, with most of the S values at 66 to 75%.

Characteristics and frequency of occurrence of the characteristics within the *V. parahaemolyticus* cluster are given in Tables 2 to 5. In general, the *V. parahaemolyticus* strains were of larger average cell size (>1.2 μm) than the *V. cholerae*. Cultural characteristics of significant difference were the generally moderate to heavy turbidity in broth compared to the slight to moderate growth of *V. cholerae*. Physiological and biochemical differences between *V. cholerae* and *V. parahaemolyticus* are cited in Table 7.

Characteristics uniformly positive for all *Vibrio* species examined in this study were gram-negative reaction, motility by polar monotrichous flagellum, rod shape, *p*H range of tolerance 5.5 to 10, growth temperature range of 15 to 41 C, salt tolerance of 0.5 to 5.0% sodium chloride, catalase, reduction of nitrate, production of hydrogen sulfide from sodium thiosulfate, growth on vitamin-free Casamino Acids and on skim milk agar, anaerogenic fermentation of glucose, acid production from fructose, growth on L-proline as sole source of carbon and nitrogen, growth on citrate as carbon source, and hemolysis of sheep red blood cells. The strains grouped as *V. alginolyticus* (Table 7) demonstrated negative reactions for oxidase, lipase, and lysine decarboxylase, all of which were positive characteristics in the other *Vibrio* species studied.

The characteristics uniformly negative for the species of the genus *Vibrio* included in this analysis were production of diffusible, insoluble, or fluorescent pigments, growth at *p*H 4.0, production of acid from adonitol, inositol, or melezitose, oxidation of gluconate, reduction of nitrite, growth at 0 to 5 C, production of acetic acid from ethanol, and the Møeller (49) arginine decarboxylase reaction.

The reference strains included in the study were clustered as shown in Fig. 6. *Achromobacter fischeri* strain WH 135 and *Vibrio* species MB-22 grouped at very low levels of similarity with *V. parahaemolyticus*, i.e., S = 65%, S = 60%, respectively. Relationship with other of the *Vibrio* species and reference *Pseudomonas*, *Spirillum*, and *Achromobacter* species was ≤50%. Since the three strains, *A. fischeri*, WH 135, and *Vibrio* MB-22 share characteristics in common with *V. parahaemolyticus*, they are included in the genus *Vibrio*.

A clustering of strains, including *Pseudomonas aureofaciens* 8 ab1 and 8 ab2, *P. fluorescens* Oxford-Sawyer strain, *Spirillum itersonii* ATCC 11331 and CIM3, 9a, 9b, C and D (Table 1) was noted. From Fig. 6, *S. serpens* ATCC 11330, *P. cruciviae* 13262, *A. georgiopolitanum*, *P. apiseptica*, and strains 4032, 4036, 4037, 4038, and 3C were not grouped with any of the three clusters determined from the computed data.

The median organism calculations, from computer output of GTP-3 and GTP-4 programs, showed the following cluster of reference strains when the S level was set at 75%: Cim 9a, 9b, C, D, and *S. itersonii* 11331. Aside from the *V. cholerae* and *V. parahaemolyticus* clusters de-

TABLE 4. *Carbohydrate reactions of Vibrio cholerae and Vibrio parahaemolyticus strains*

Character	Frequency of occurrence[a]	
	V. cholerae	*V. parahaemolyticus*
Acid production		
Glucose aerobic (35)	+(1.00)	+(1.00)
Glucose anaerobic (35)	+(1.00)	+(1.00)
Glucose (4)	+(1.00)	+(1.00)
Adonitol	−(0)	−(0)
Arabinose	−(0)	+(0.84)
Cellobiose	±(0.62)	+(0.97)
Dextrin	+(1.00)	+(0.97)
Dulcitol	−(0)	−(0.03)
Fructose	+(1.00)	+(1.00)
Galactose	+(1.00)	+(0.97)
Glycerol	+(0.97)	+(1.00)
Inositol	−(0)	−(0)
Inulin	−(0.24)	−(0)
Lactose	−(0.14)	−(0.03)
Maltose	+(1.00)	+(0.97)
Mannitol	+(1.00)	+(1.00)
Mannose	+(0.90)	+(1.00)
Melezitose	−(0)	−(0)
Melibiose	−(0)	−(0.13)
Raffinose	−(0)	−(0)
Rhamnose	−(0)	−(0.13)
Salicin	−(0)	−(0.03)
Sucrose	+(0.97)	−(0.06)
Sorbitol	−(0.07)	−(0)
Trehalose	+(1.00)	+(0.97)
Xylose	−(0)	−(0)
Gas production from carbohydrates	−(0)	−(0)
Glucose + iodoacetate → growth	+(1.00)	±(0.47)
Glucose + iodoacetate → acid production	−(0)	−(0.22)
Starch hydrolyzed	+(0.97)	+(1.00)
Levan produced	+(0.97)	+(0.94)
Ethanol → growth	+(0.90)	+(1.00)
Ethanol → acetic acid	−(0)	−(0)
Glycerol → dihydroxyacetone	−(0)	+(0.97)
Aesculin hydrolyzed	−(0)	+(0.88)
Gluconate → 2-keto-gluconate (7)	±(0.38)	−(0.03)
Gluconate → 2-keto-gluconate (48)	−(0)	−(0)
Gluconate oxidized	−(0)	−(0.03)
Cellulose digested	−(0)	NT
Agar digested	−(0)	−(0)

[a] Values in parentheses = frequency of occurrence of characteristics. Total number of *V. cholerae* strains = 29 (NCTC 30 excluded). Total number of *V. parahaemolyticus* strains = 32 (5144, 5146, and 5162 excluded). NT = not tested.

scribed above, the remaining strains did not group with each other or with any of the reference strains by this method of clustering.

Electron microscopy. Thin sections of the *V. cholerae* and *V. parahaemolyticus* strains revealed the typical gram-negative structure, with cell wall, plasma membrane, nuclear material, and ribonucleoprotein particles. The cytoplasmic material was closely approximated by the plasma membrane, with the electron-dense layers of the cell wall clearly separated from the plasma membrane and appearing in wavy or undulating configuration (Fig. 7). Round bodies were frequently observed in sections of pure cultures of the *Vibrio* species, as reported previously for *V. marinus* (22). A round body of *V. cholerae* ATCC 14033, typical of round bodies observed in *Vibrio* species, is shown in Fig. 8.

DNA base composition. The overall DNA base composition range observed for the *Vibrio* species was 40 to 48% GC (Table 8). Three strains, *V. adaptus* ATCC 19263, *V. neocistes* RH 1810, and *V. alcaligenes* ATCC 14736, included in the DNA base composition studies, revealed 63 to 64 moles % GC, which would exclude these strains from the genus. The GC range for the *Pseudomonas* species examined was 61 to 64%, with DNA base compositions of the two *Spirillum* species also falling into this range (Table 8). Thus, the clusters of strains indicated in Fig. 5 showed similar overall DNA base composition, within a deviation ±2%.

DISCUSSION

The first question which might be dealt with is that of the relationship of "classic" *V. cholerae*, El Tor vibrios, and non-cholera, or NAG vibrios. Ten strains of each of these groups were examined in this study (Table 1). The results of the numerical taxonomy analysis clearly showed that all strains formed an homogeneous cluster, with the exception of two NAG vibrios, NCTC 30 and NCTC 8042 (Fig. 2 and 3). No subgrouping of El Tor or NAG strains was observed. Other investigators have concluded that the El Tor strains do not warrant separate species status (18, 31). However, the data reported here do not support the hypothesis of the El Tor vibrios as being a distinguishable infra-subspecific taxon well separated from "classical" cholera and related NAG strains (17). From the numerical taxonomy results, the El Tor vibrios are members of the species *V. cholerae*. On the basis of overall similarity, no significant subgroup or intracluster formation was noted for either the El Tor or NAG strains.

As pointed out by Feeley (17), a functional separation of El Tor vibrios and "classic" cholera vibrios was originally made by the demonstration of hemolytic properties of the El Tor vibrios. Resistance of El Tor vibrios to group IV phage (51), ability to agglutinate chicken erythrocytes (23), and positive Voges-Proskauer reactivity (20, 55, 77) have been added to the differential criteria for *V. eltor* and "classic" *V. cholorae*. Nevertheless, most workers recognize that the "classic" cholera and El Tor vibrios share a very large majority of biochemical and serological

TABLE 5. *General physiological, biochemical, and nutritional characteristics of Vibrio cholerae and Vibrio parahaemolyticus*

Character	Frequency of occurrence[a]	
	V. cholerae	*V. parahaemolyticus*
Temp (C) of growth		
0–5	−(0)	−(0)
15–41	+(1.00)	+(1.00)
42	+(0.90)	+(1.00)
Sodium chloride tolerance (%)		
0	+(0.90)	−(0.25)
0.5–5.0	+(1.00)	+(1.00)
7	±(0.31)	+(1.00)
10	−(0)	+(1.00)
Seawater required for growth	−(0)	+(0.88)
*p*H tolerance		
*p*H 4.0	−(0)	−(0)
*p*H 4.5	±(0.45)	−(0)
*p*H 5.0	+(1.00)	±(0.63)
*p*H 5.5–10.0	+(1.00)	+(1.00)
Oxidase (Kovacs)	+(1.00)	+(1.00)
Catalase	+(1.00)	+(1.00)
Urease	+(0.79)	+(0.97)
Nitrate reduced	+(1.00)	+(1.00)
Nitrite reduced	−(0)	−(0)
Methyl red reaction	+(1.00)	+(0.88)
Voges-Proskauer reaction	±(0.59)	−(0.03)
Indole produced	+(1.00)	+(0.94)
Trimethylamine oxide → trimethylamine	+(1.00)	+(0.88)
Hemolysis (sheep RBC)	+(1.00)	+(1.00)
Lipase (Tween 20, 40, 60, 80)	+(1.00)	+(1.00)
Lipase (Spirit Blue Agar + olive oil)	−(0)	NT
Calcium lactate–acetate–carbonate	−(0.14)	−(0)
Citrate utilization		
Koser's method	+(0.83)	+(1.00)
Simmons' method	+(0.90)	+(0.72)
Christensen's method	+(1.00)	+(1.00)
Lecithinase	+(1.00)	+(0.97)
Skim milk agar-growth	+(1.00)	+(1.00)
Skim milk agar-casein hydrolysis	+(0.90)	+(0.91)
Litmus milk peptonized	+(0.76)	+(0.97)
Litmus milk surface peptonized	−(0.10)	−(0)
Litmus milk acid	+(0.83)	±(0.34)
Litmus milk alkaline	−(0.14)	−(0.22)
Litmus milk reduced	±(0.69)	+(0.97)
Gelatin liquefied	+(1.00)	+(1.00)
Lysine decarboxylase	+(1.00)	+(1.00)
Ornithine decarboxylase	+(1.00)	+(0.97)
Phenylalanine deaminase	−(0)	NT
L-Phenylalanine → phenylpyruvic acid	−(0)	−(0.13)
Arginine dihydrolase (Thornley)	−(0)	−(0.06)
Arginine decarboxylase (Moeller)	−(0)	−(0)
L-Tyrosine → melanin	−(0)	+(0.94)
Peptone → H_2S (lead acetate agar)	−(0.10)	+(0.75)
Cysteine → H_2S	+(1.00)	+(0.97)
Cystine → H_2S	+(1.00)	NT
Sodium thiosulfate → H_2S	+(1.00)	+(1.00)
Peptone → NH_3	+(1.00)	+(1.00)
Acetamide reaction	+(0.90)	−(0)
Growth in vitamin-free Casamino Acids	+(1.00)	+(1.00)
Growth with the following as C and N source		
0.1% L-proline	+(1.00)	+(1.00)
0.1% L-arginine	+(1.00)	+(0.97)
0.1% DL-alanine	+(0.97)	+(0.97)
0.1% L-lysine	+(0.97)	(1.00)
0.1% L-phenylalanine	+(0.79)	+(0.97)
0.1% β-alanine	±(0.66)	−(0)
Utilization of $NH_4H_2PO_4$ as source of N	+(1.00)	+(0.78)
Growth with sodium acetate as C source	+(1.00)	+(0.97)
Growth with sodium formate as C source	+(0.97)	+(1.00)
Growth in 0.3% sodium malonate broth	+(0.86)	+(0.97)

[a] Total number of *V. cholerae* strains = 29 (NCTC 30 excluded). Total number of *V. parahaemolyticus* strains = 32. NT = not tested.

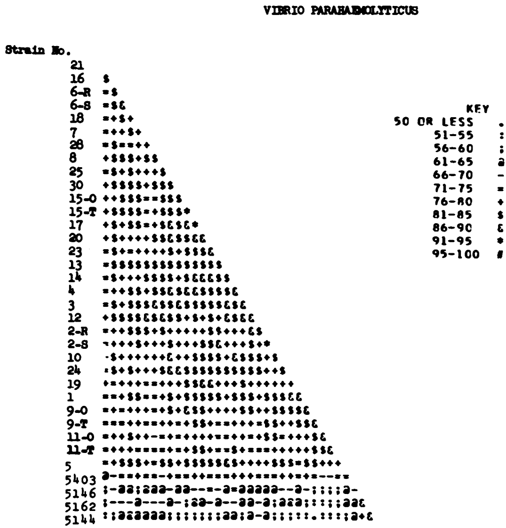

FIG. 4. *Total similarity value triangle output for Vibrio parahaemolyticus. Arrangement of strains was by computer, by using the GTP-2 program.*

properties, and may cause identical disease in man. Furthermore, cholera vaccines show equal cross-protection in animals against "classic" cholera and El Tor vibrios (54). Feeley (17, 19) found that the O group I serotype vibrios from various geographical regions could be divided into five types based on phage IV sensitivity, Voges-Proskauer reaction, chicken-cell agglutination, and hemolysis. Not all of the types appeared to be stable, however, with variations in hemolysis and colony type. Furthermore, each of these subspecific types could be subdivided into either Ogawa or Inaba serotypes by agglutination tests with adsorbed sera. Unfortunately, the five types described by Feeley (17) were not found in the present study to be distinguishable groupings on the basis of overall similarity. The conclusion drawn by Feeley (17) that a single species, *V. cholerae*, consisting of several types, including *V. eltor*, is, on the other hand, fully confirmed by this analysis.

Hugh (31, 33) in a comparison of 120 strains of *V. eltor* with 258 of *V. cholerae* for 52 attributes

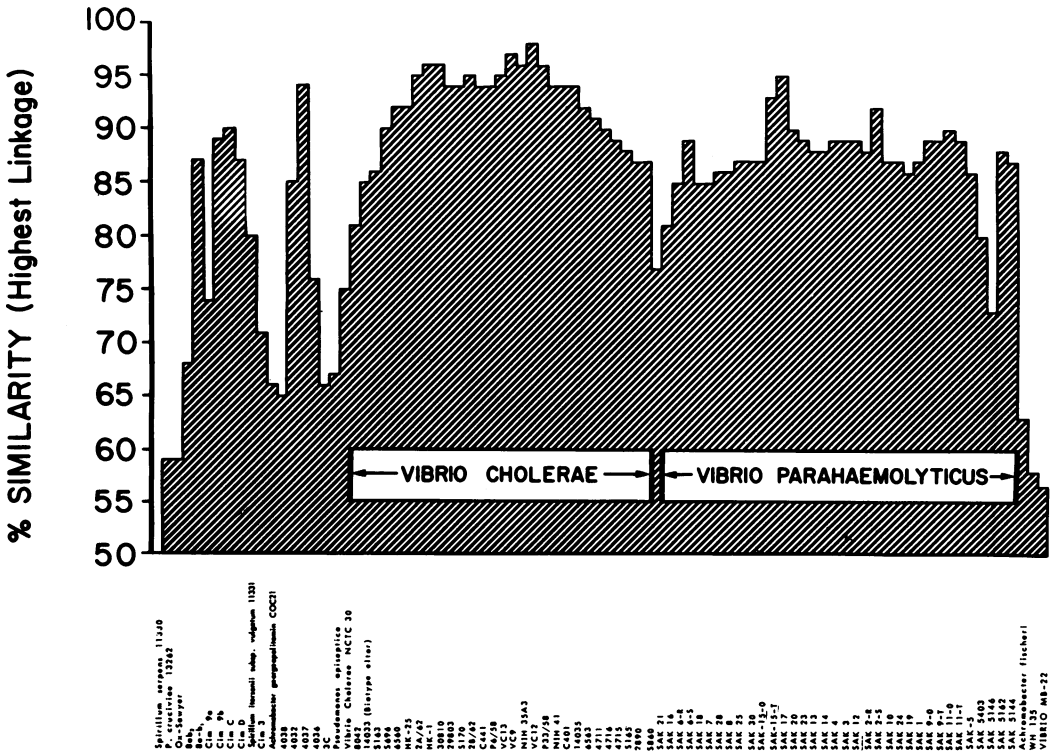

FIG. 5. *Highest pair-linkage sorting of the V. cholerae, V. parahaemolyticus, and reference strains included in the study.*

found the boundary between these two species to be overlapping. He concluded that the differences between *V. cholerae* and *V. eltor* are insufficient for the recognition of the two as distinct species. Hugh (33) made no attempt in his study to classify the strains into subspecies or infrasubspecific forms on the basis of phage sensitivity, hemolysis, O forms (Ogawa and Inaba serotypes), hemagglutinative ability, polymyxin sensitivity, or Voges-Proskauer reaction.

The IAMS Subcommittee on Taxonomy of *Vibrios* (18) recognized that the species *V. cholerae* and *V. eltor* could both be divided into Ogawa and Inaba serotypes, and the majority view was that the described differences between the two organisms are insufficient for their recognition as distinct species.

Felsenfeld (21) has reviewed the literature on cholera, bringing up to date the survey done by Pollitzer (55). As pointed out by Felsenfeld (21), generally speaking, cholera vibrios are considered to be only those agglutinated by Gardner and Venkatraman's (55) vibrio group O–I sera; other vibrios were called nonagglutinating or NAG vibrios. Within this category, further restricted to group I of Heiberg (55), i.e., sucrose-positive, arabinose-negative, maltose-positive strains, there is no dependable means of differentiating El Tor and other vibrios. Felsenfeld (21) states unequivocally that "the differences between 'classical' cholera and El Tor vibrios are insignificant. The main marks of El Tor vibrios are lower susceptibility to chemical and physical factors, a greater readiness to produce hemolysin(s), a tendency to form acetylmethylcarbinol (especially in fresh cultures), resistance to Mukerjee's phage type IV and, often, hemagglutinability. These differences do not justify the designation of a new bacterial species."

Variability in all of the so-called *V. cholerae-V. eltor* differential tests has been reported. Mukerjee and Guha Roy (52) described variability in the hemolytic activity of El Tor vibrios; strains nonhemolytic at the time of isolation became hemolytic after culturing in the laboratory. Transformation from one serological type to another, particularly from Ogawa to Inaba, has been observed during epidemics in endemic areas and also in the laboratory (2). In an accidental laboratory infection in which only the Inaba serotype was extant, the victim, a laboratory technician, shed Inaba type during the first 2 days after infection, but only Ogawa type thereafter (66).

An infection with an El Tor strain of *V. cholerae* belonging to Heiberg group III was reported by Sen, Vaishnav, and Majumder (65). The strain was a sucrose-, mannose-, and arabinose-fermenting, Ogawa type, El Tor vibrio belonging to phage type 6. El-Shawi and Thewaini (16) reported isolation of Inaba type *V. cholerae* during an epidemic caused by organisms of the Ogawa serotype. They also isolated an atypical strain, intermediate between the "classical" and El Tor vibrios. The strain was Heiberg group III of Ogawa type and was isolated from a cholera case during the 1966 epidemic in Iraq. It gave reactions typical of El Tor vibrios but it was susceptible to Mukerjee's type IV phage. Group IV phage, in sufficient titer, has been shown by other

TABLE 6. *S value of Vibrio cholerae and Vibrio parahaemolyticus with the hypothetical median organism of each species*[a]

V. cholerae			*V. parahaemolyticus*		
Strain	S-value	No. of tests[b]	Strain	S-value	No. of tests[b]
VC-9	96.5	115	4	92.8	111
35A3	96.4	114	17	92.1	114
VC-12	96.4	113	25	92.0	113
P33/58	96.4	114	15-T	91.2	114
VC-13	95.7	116	3	91.1	112
P6/58	95.6	113	20	90.3	114
NIH 41	93.9	116	24	89.6	116
2B/62	93.9	116	1	89.4	114
C 441	93.9	115	14	89.4	113
S 170	93.1	117	15-O	88.6	114
C 401	93.1	116	23	88.6	114
14035	93.0	115	9-O	87.5	112
6563	92.3	118	30	87.1	116
6560	92.3	118	2-S	86.8	114
2A/62	92.1	115	5	86.8	114
29803	91.4	117	10	86.1	115
HK-25	91.2	116	12	86.1	115
HK-1	90.6	118	8	85.8	113
30810	90.6	118	13	85.4	117
4716	89.9	119	19	85.3	116
4715	89.6	115	2-R	85.2	115
S-163	89.1	119	16	84.9	113
S-696	89.1	119	18	84.8	119
4711	87.9	116	6-S	84.0	119
S-860	87.5	120	9-T	83.3	114
S-165	86.2	116	28	83.3	114
2890	85.8	113	11-T	82.3	113
14033	85.0	120	6-R	82.2	118
8042	77.3	119	5403	82.2	118
			11-O	80.3	117
			7	79.8	119
			21	75.4	118

[a] The GTP-3 and GTP-4 computer programs were used to calculate the median organism and feature frequencies (40).

[b] Number of tests ($n_s + n_d$) on which the S value ($S = n_s/n_s + n_d$) is based. Total number of coded tests was 210.

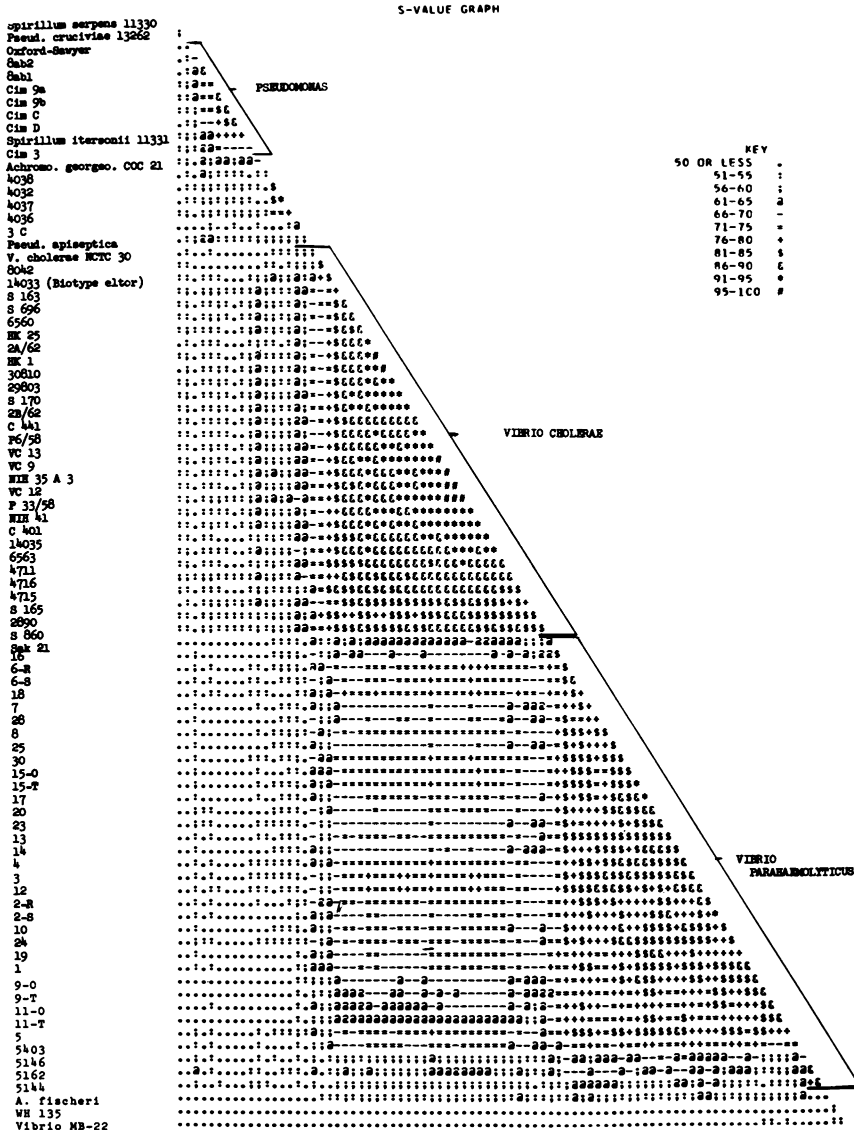

FIG. 6. *Total triangular similarity-value matrix for the entire test set, including V. cholerae, V. parahaemolyticus, and reference strains of the study.*

investigators to produce clearing, resembling phage lysis, on lawns of El Tor vibrios (50). Also, phage-resistant mutants of *V. cholerae* which are exactly like the parent strains in all characteristics except phage reactions can easily be obtained (57). Thus, phage reactions are only a marker and not the final arbiter in identification of the El Tor vibrio. Vibriocine production appears to be a common characteristic of both *V. cholerae* and *V. eltor*, an additional indication of a close relationship between them (79).

The polymyxin B disc test, used in many laboratories to differentiate classical from El Tor cholera strains, was shown by Gangarosa, Bennett, and Boring (27) to be dependent on the medium used. Furthermore, their data showed that 85% of the El Tor strains on Monsur's medium and 100% on thiosulfate citrate bile salts sucrose medium were erroneously classified as "classical" cholera.

During routine characterization of about 1,000 strains of agglutinable vibrios from different parts of the Philippines, Pesigan et al. (53) isolated

TABLE 7. *Differences between Vibrio species examined in this study*

Character	Frequency of occurrence[a]		
	V. cholerae	*V. parahaemolyticus*	*V. alginolyticus*[b]
Dihydrostreptomycin	+ (1.00)	− (0.31)	NT
Penicillin	+ (1.00)	− (0.06)	− (0)
0% NaCl	+ (0.90)	− (0.25)	+ (1.00)
7% NaCl	± (0.31)	+ (1.00)	+ (1.00)
10% NaCl	− (0)	+ (1.00)	+ (1.00)
Seawater required for growth	− (0)	+ (1.00)	− (0)
Voges-Proskauer	± (0.59)	− (0.03)	− (0)
L-Tyrosine → melanin	− (0)	+ (0.94)	+ (1.00)
Peptone → H_2S (lead acetate agar)	− (0.10)	+ (0.75)	+ (1.00)
Acetamide reaction	+ (0.90)	− (0)	− (0)
0/129	+ (1.00)	+ (0.75)	− (0)
4 to 7 C	− (0)	− (0)	+ (1.00)
Gluconate → 2 ketogluconate	± (0.38)	− (0.03)	+ (1.00)
Xylose	− (0)	− (0)	+ (1.00)
Salicin	− (0)	− (0.03)	+ (1.00)
L-Phenylalanine → phenylpyruvic acid	− (0)	− (0)	+ (1.00)
Spreading on agar	− (0)	− (0)	± (0.33)
Starch hydrolyzed	+ (0.97)	+ (1.00)	− (0)
Erythromycin	+ (1.00)	+ (0.66)	− (0)
Tetracyclin	+ (1.00)	± (0.59)	− (0)
Aureomycin	+ (1.00)	+ (0.66)	− (0)
Terramycin	+ (1.00)	± (0.50)	− (0)
Arabinose	− (0)	+ (0.84)	− (0)
Sucrose	+ (0.97)	− (0.06)	+ (1.00)
Glycerol → dihydroxyacetone	− (0)	+ (0.97)	± (0.33)
Aesculin hydrolyzed	− (0)	+ (0.88)	+ (1.00)
Mannitol	+ (1.00)	+ (1.00)	− (0)
Mannose	+ (0.90)	+ (1.00)	− (0)
Oxidase	+ (1.00)	+ (1.00)	− (0)
Lecithinase	+ (1.00)	+ (0.97)	− (0)
Lipase (Tween 40, 60, 80)	+ (1.00)	+ (1.00)	− (0)
Lysine decarboxylase	+ (1.00)	+ (1.00)	− (0)
Ornithine decarboxylase	+ (1.00)	+ (0.97)	− (0)
β Alanine → growth	+ (0.66)	− (0)	− (0)
Dextrin	+ (1.00)	+ (0.97)	− (0)

[a] Values in parentheses denote frequency of occurrence. NT = not tested.
[b] Strains 5144, 5146, and 5162 were received as *V. parahaemolyticus*.

four strains which resembled El Tor vibrios in some respects and "classical" *V. cholerae* in others. That is, hemolysis was variable but present to some degree in all strains, Voges-Proskauer reaction was variable, all were resistant to cholera phage group IV, sensitive to polymyxin B, and consistently nonhemagglutinating for chicken cells. These intermediates, thus, suggested that hemagglutination and resistance to polymyxin B and cholera phage group IV as found in El Tor vibrios are not necessarily linked characters.

Thus, it can clearly be concluded from the data obtained in this study and that appearing in the literature that *V. cholerae* and *V. eltor* are indeed a single species without distinct and unequivocal subspecies grouping. The differences between *V. cholerae* and *V. eltor* are not of much apparent value for the clinician or epidemiologist because of the great variability of the vibrios.

A more serious problem is that of the NAG vibrios. *V. cholerae* and El Tor vibrios are grouped serologically on the basis of their O antigen and are subtyped as Ogawa, Inaba, or Hikojima (21). A change in O agglutination may be observed on occasion. Although the pathogenicity of NAG vibrios has not been accepted by some workers, there are increasing reports of cholera-like disease in which *V. cholerae* has not been isolated but NAG vibrios have been observed (45, 46). These vibrios do not agglutinate with cholera O group I antiserum. McIntyre and

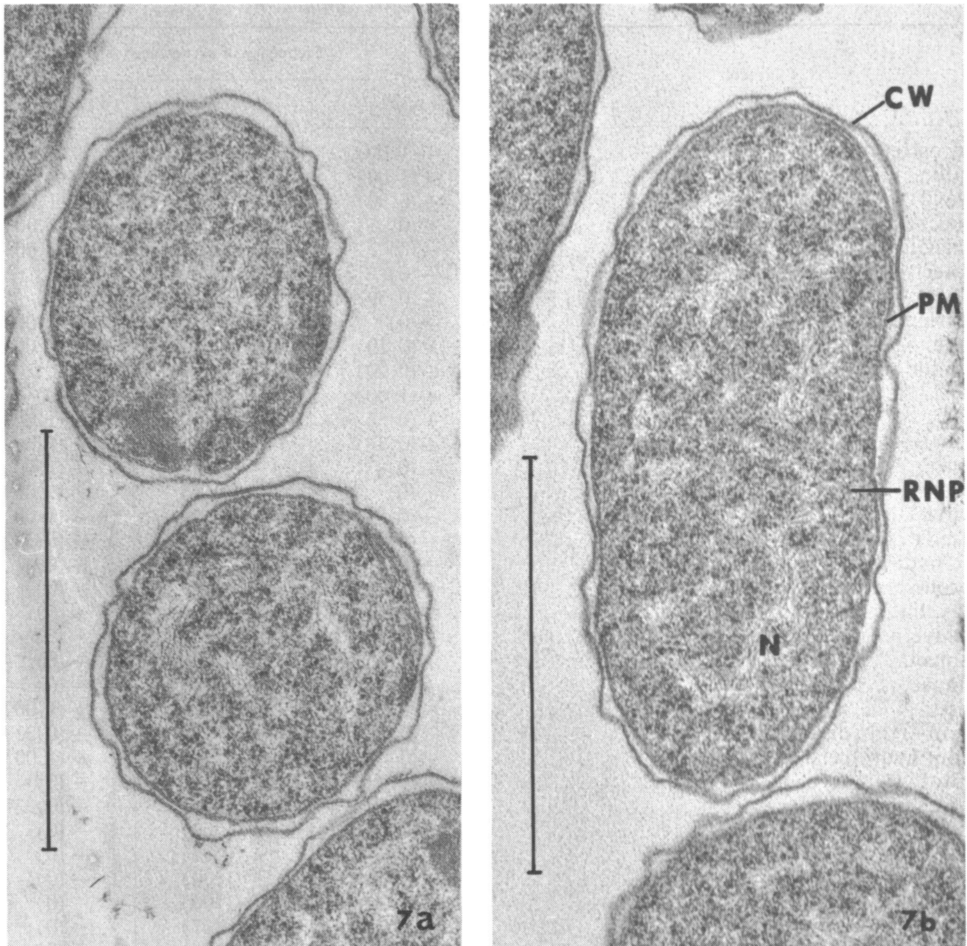

FIG. 7. *Cross sections (a) and longitudinal sections (b) of V. parahaemolyticus strain 23. In the electronmicrographs, the magnification bar represents 1 μm. The tripartite nature of the cell wall (CW) and plasma membrane (PM) may be seen. Nuclear material (N) is axially disposed and densely packed ribosomes (RNP) can be seen.* $\times$ *55,400.*

Feeley (45) were able to show a significant rise in agglutinating antibody titer against the non-cholera vibrios (NAG) isolated when acute and convalescent phase sera were examined. Bhattacharji and Bose (3) claimed to have been able to transform *V. cholerae* strains into NAG vibrios and vice versa. El-Shawi and Thewaini (16) attempted to repeat the experiments of Bhattacharji and Bose (3), but were not able to isolate NAG organisms from water samples inoculated with agglutinable organisms. However, they used Ogawa El Tor vibrios, whereas Bhattacharji and Bose (3) used "classical" *V. cholerae* of Inaba serotype. Nevertheless, El-Shawi and Thewaini (16) did find that three of their El Tor vibrios lost agglutinability after being maintained in the laboratory for longer than 1 year, and one changed from the serological type Ogawa to Hikojima.

Sakazaki, Gomez, and Sebald (60) examined 164 strains of NAG vibrios and found 142 of these to be vibrios, i.e., strains which were monotrichously flagellated, oxidase-positive rods which fermented glucose without gas. The DNA of the NAG *Vibrio* species clustered by numerical taxonomy analysis was found to be 45% GC. These authors concluded that the group of NAG vibrios of biotype 10311 should be included in the species *V. cholerae* because of very high S values (>90%) with reference strains of *V. cholerae*. Hugh (34) also concluded from a study of 52 strains of NAG vibrios which did not possess the O group antigen that they should be classified with *V. cholerae*.

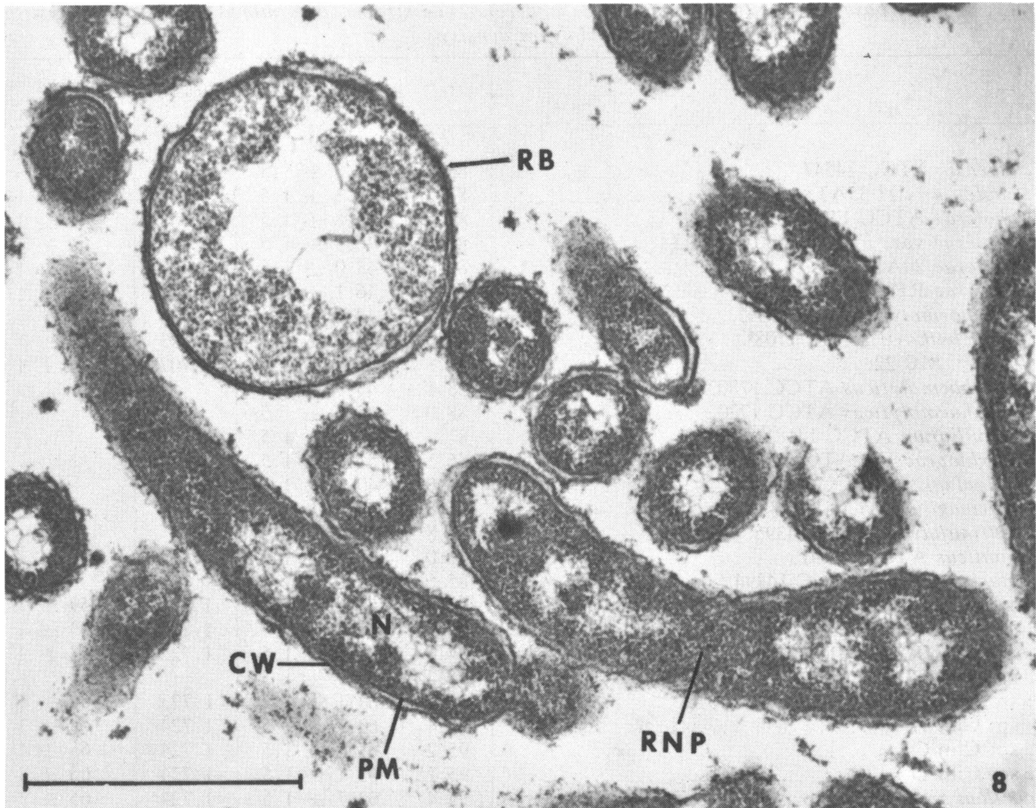

FIG. 8. *Electron micrograph of V. cholerae ATCC 14033. A round body (RB) is shown in cross section. The cell wall (CW), diffuse nuclear material (N), plasma membrane (PM), and ribosomes (RNP) of V. cholerae are shown in longitudinal section.* × *36,000.*

That the so-called NAG vibrios isolated from victims of cholera-like diarrhea belong within the species *V. cholerae* is a conclusion drawn from results reported by Colwell and Yuter (Bacteriol. Proc. p. 18, 1965). The data given by Hugh (34), obtained from studies of 377 strains of *V. cholerae* and 52 NAG, non-cholera, and water vibrios incriminated as a cause of diarrhea in man, show no significant differences between the two groups. The data cited by Hugh (34) and Carpenter et al. (5) and those presented in this study are in excellent agreement. The results of the present study lead to the conclusion that NAG vibrios with a DNA base composition of 46 to 48% GC (within limits of experimental determination, i.e., $\pm 1.5\%$) and S values of $\geq 75\%$ when compared with *V. cholerae* strains, employing the calculated median organism, should be included in the species *V. cholerae*. It is important to note that all the NAG vibrios included in this study were isolated from human diarrhea cases (Table 1).

V. parahaemolyticus strains were originally included in this analysis to determine the relationship of these strains, which are of marine origin, to *V. cholerae*. The unusual characteristics of the strains, i.e., a NaCl requirement for growth, suggested a different line of evolution. On the other hand, the many characteristics of the *V. parahaemolyticus* held in common with other *Vibrio* species resulted in its inclusion in the genus *Vibrio* through a tortured taxonomic path, i.e., via *Pasteurella parahaemolytica* sp. n. (26), *Pseudomonas enteritis* (72), *Oceanomonas enteritidis* sp. n. (47), and finally *V. parahaemolyticus* (61). The level of similarity observed in this study between *V. cholerae* and *V. parahaemolyticus* (S = 66 to 75%), was not high and on the basis of very early studies would be only minimally acceptable for placement of these two species into one single genus.

The original isolation of *V. parahaemolyticus* was by Fujino during examination of clinical specimens of people who had eaten "shirasu"

TABLE 8. *DNA base composition of Vibrio sp. and reference strains, determined by thermal transition and buoyant density*

Organism	T_m	Guanine plus cytosine	Density	Guanine plus cytosine
	C	*%*	*g/cm³*	*%*
V. albensis ATCC 14547	88.8	47.6 ± 1.5		
V. cholerae NIH 35A3	88.7	47.3 ± 1.5	1.707	48 ± 1
V. cholerae ATCC 14035	88.5	46.8 ± 1.5	1.707	48 ± 1
V. cholerae var. *El Tor* ATCC 14033	88.5	46.8 ± 1.5	1.707	48 ± 1
V. cholerae 20A10	89.0	48.0 ± 1.5	1.707	48 ± 1
V. cholerae RH 1094	88.2	46.1 ± 1.5	1.707	48 ± 1
V. parahaemolyticus strain 3[a]	87.7	44.8 ± 1.5		
V. metschnikovii ATCC 7708[b]	88.6	47.0 ± 1.5	1.703	43 ± 1
Vibrio sp. MB-22	87.8	45.1 ± 1.5	1.707	48 ± 1
V. parahaemolyticus ATCC 17803[a]	88.1	45.9 ± 1.5		
V. parahaemolyticus ATCC 17802[a]	88.0	45.6 ± 1.5		
V. anguillarum ATCC 14181	87.4	44.2 ± 1.5		
V. marinopraesens ATCC 19648	86.1	41.0 ± 1.5		
V. haloplanktis ATCC 14393	86.0	40.7 ± 1.5		
V. marinagilis ATCC 14398	85.8	40.2 ± 1.5		
V. marinofulvus ATCC 14395	85.8	40.2 ± 1.5		
V. ponticus ATCC 14391	86.0	40.7 ± 1.5		
V. marinovulgaris ATCC 14394	85.6	40.0 ± 1.5		
Pseudomonas apiseptica PA	94.0	60.2 ± 1.5	1.718	59 ± 1
P. aureofasciens 8aB2	94.7	61.8 ± 1.5	1.722	63 ± 1
P. aureofasciens 8aB1	94.8	62.2 ± 1.5	1.722	63 ± 1
V. adaptus ATCC 19263	95.2	63.1 ± 1.5		
P. sp. Cim 9A	95.2	63.1 ± 1.5	1.723	64 ± 1
P. sp. Cim 9B	95.4	63.7 ± 1.5	1.724	65 ± 1
P. sp. Cim C	95.2	63.2 ± 1.5	1.724	65 ± 1
P. sp. Cim D	95.2	63.1 ± 1.5	1.724	65 ± 1
Spirillum serpens, subsp. *serpens* ATCC 11330	95.4	63.7 ± 1.5	1.724	65 ± 1
V. neocistes RH 1810[b]	95.7	64.4 ± 1.5	1.723	64 ± 1
V. alcaligenes ATCC 14736	95.8	64.6 ± 1.5		
S. itersonii subsp. *vulgatum* ATCC 11331	95.5	64.0 ± 1.5	1.724	65 ± 1
Arthrobacter sp. 3C[c]	96.3	61.0 ± 1.5		
Achromobacter georgiopolitanum COC 21[d]			1.700	41 ± 1
A. fischeri			1.703	44 ± 1
P. sp. Ox-Sawyer	94.3	60.9 ± 1.5		

[a] See Citarella and Colwell (6) for other *V. parahaemolyticus* DNA base composition data.
[b] Colwell, Citarella, and Ryman (11).
[c] Colwell and Mandel, *unpublished data*.
[d] Colwell, Smith, and Chapman (12).

and subsequently suffered severe food poisoning. The bacteria which were isolated were classified by Fujino (25) as *P. parahaemolytica*. Takikawa (73), in investigating an outbreak of food poisoning which occurred among patients and staff at the Yokohama National Hospital, isolated salt-requiring bacteria on media containing 4% NaCl during routine screening for *Staphylococcus*. Comparison of 33 strains isolated from two epidemics and from sporadic cases of food poisoning with a strain from Fujino's study led Takikawa (73) to rename the species *P. enteritis*. Comparison of data reported for the strains examined by Takikawa and those in Tables 2, 4, and 5 show excellent agreement; the only differences were reports of negative H_2S production, urea test, and the methyl red test, which were found to be positive by the methods employed in this study. The remaining 44 tests cited by Takikawa were in perfect agreement with results reported here.

Miyamoto, Nakamura, and Takizawa (47) isolated *P. enteritis* in outbreaks of food poisoning in Kanagawa prefecture, Yokahama, Japan. Seasonal oceanic surveys revealed *P. enteritis* to be isolated directly from seawater in the Sagami and Tokyo Bay areas. These authors decided, on the basis of the NaCl requirement for growth, that the *P. enteritis* strains which they had isolated, along with the Fujino and Takikawa strains

should be placed in a new genus, *Oceanomonas enteritidis*. An additional proposal was made, that on the basis of chitin, alginate, and sucrose utilization, the strains decomposing alginate and fermenting sucrose be placed in the species *O. alginolytica*. It is interesting to note that Miyamoto et al. (47) did find the strains examined by them to be methyl red-positive and hydrogen sulfide-producing when in 3% NaCl-sulfide indole motility medium containing cysteine as was noted in the present study.

An extensive examination of morphological, cultural, biochemical, serological, and enteropathogenic properties of *V. parahaemolyticus* was done by Sakazaki et al. (61–63). A total of 1,679 cultures derived from feces of patients suffering from gastroenteritis, from seafish, food, and seawater were screened. Cultures from the Fujino and Takikawa studies were also included in the testing schedules.

As observed in the present study and by other investigators (61–63), the morphology of *V. parahaemolyticus* is similar to that of *V. cholerae* (Fig. 7 and 8). Colonies of *V. parahaemolyticus* examined from 24- to 48-hr streak plates from log-phase broth cultures are entire, convex, mainly translucent but with some opaque colonies, and 1 to 2 mm in diameter. This is as was found by Sakazaki et al. (61) for organisms freshly plated onto agar. Also to be noted is that stationary-phase cultures show colonial variants of the rough variety. No diffusible or fluorescent pigment has been observed in the *V. parahaemolyticus* cultures examined in this or previous studies.

Since the description of *V. parahaemolyticus* is rather incomplete, some of the characteristics noted for this species are as follows. *V. parahaemolyticus* strains were not sensitive to penicillin or to polymyxin B, and only 31% of the cultures were sensitive to dihydrostreptomycin. Nearly all strains tested, on the other hand, were sensitive to chloramphenicol and novobiocin. Sensitivity to other antibiotics was variable (Table 3). The vibriostatic agent (0/129) was effective in producing bacteriostasis in most of the *V. parahaemolyticus* strains tested (0.75) but was not uniformly reactive for all strains since at least one-fourth of the cultures were not sensitive.

All of the *Vibrio* cultures examined in this study fermented glucose without production of gas. Iodoacetate inhibited acid production from glucose by *V. parahaemolyticus* (Table 4). Carbohydrate reactions for *V. parahaemolyticus* are given in Table 4.

The *V. parahaemolyticus* strains examined in this study did not grow at temperatures below 5 C but did grow within the range 15 to 42 C. Poor or no growth was observed on media without added NaCl, and all strains grew in media with 0.5 to 10.0% added NaCl. Nearly all of the *V. parahaemolyticus* strains required seawater for growth, i.e., Na^+, K^+, and Mg^{2+} in the concentrations found in seawater. All cultures grew in the *p*H range of 5.5 to 10.0, with *p*H 5.0 being the borderline for many strains (Table 7). Hemolysis was noted for all strains.

The *V. parahaemolyticus* strains were oxidase-, catalase-, and urease-positive. Nitrate but not nitrite was reduced; the methyl red test was positive (0.88, Table 5), but the Voges-Proskauer reaction was negative. Other results are given in Table 5. The only test differing from the data reported by Sakazaki et al. (61) was the urea decomposition test, and it appears that different methods were employed from those used in this study. Growth in vitamin-free amino acids and utilization of ammonium phosphate as nitrogen source was noted for the *V. parahaemolyticus* strains tested and several amino acids served as carbon and nitrogen sources (Table 5).

A very distinct subgrouping of strains received as *V. parahaemolyticus* was observed. The subgroup was significantly different from other *V. parahaemolyticus* strains (Fig. 5), joining only at $S \leq 72\%$. The subgroup was similar to that observed by Zen Yoji et al. (82) in a numerical taxonomy study of a set of *V. parahaemolyticus* strains. A subgroup was also recognized by Sakazaki (58), who proposed the name *V. alginolyticus* for the strains originally grouped as "Biotype 2" of *V. parahaemolyticus* by Sakazaki et al. (59). The description cited by Sakazaki (58) for *V. alginolyticus* differs from results of this study, mainly in that growth with 10% added sodium chloride and the Voges-Proskauer tests were not found to differentiate *V. parahaemolyticus* from strains designated in this study as *V. alginolyticus*. That is, in the present study, it was observed that both the *V. parahaemolyticus* and the subgroup strains were capable of growth in 10% NaCl and gave negative Voges-Proskauer reactions. These two tests will give variable results, however, depending on how the tests are done. Species differentiation based on these tests will therefore have a greater probability of error. However, a number of tests (Table 7) were found to be useful in differentiation of *V. parahaemolyticus* and *V. alginolyticus*. The clear separation of the two species groups, *V. parahaemolyticus* and *V. alginolyticus*, which was observed in this study was confirmed on molecular genetic grounds, as discussed in a subsequent paper (6).

Comparison of results cited by Baross and

Liston (1) and Sakazaki (58) for *V. alginolyticus* and the results reported here for the group designated as *V. alginolyticus* reveals a number of discrepancies. For example, in salt tolerance test results, both the results of Sakazaki (58) and the results of this study showed growth of *V. alginolyticus* strains in 10% NaCl, whereas those of Baross and Liston (1) did not. The strains included in the present study were found to be oxidase-negative, as well as lysine- and ornithine-negative. Sakazaki (58) reports these tests as positive for *V. alginolyticus*. Baross and Liston (1) did not publish data on these tests.

Sakazaki (58) cites differences of *V. alginolyticus* and *V. anguillarum* as lack of growth of the latter in 10% NaCl, negative Voges-Proskauer reaction, variable sucrose fermentation and positive cellobiose fermentation within 24 hr. The *V. alginolyticus* strains examined in this study would be considered, on the basis of these criteria, to be intermediate between the *V. alginolyticus* of Sakazaki (58) and *V. anguillarum*. Unfortunately, only one strain of *V. anguillarum*, ATCC 14181, was included in this study. It was examined for overall DNA base composition (Table 8); the GC content of the *V. anguillarum* and *V. parahaemolyticus* strains were similar, i.e., 44 to 45 moles % GC.

The variability among *V. alginolyticus* strains from different sources suggests that either more than one species may be included in *V. alginolyticus* or that the present identification and classification is based on too few characteristics. Hanaoka, Kato, and Amano (29) reported 44.5% GC content of a strain designated as *V. alginolyticus*, whereas the strains of the *V. parahaemolyticus* subgroup, tentatively labeled as *V. alginolyticus* in this study, were found to possess a DNA composition of 39% GC (6). Obviously, the species *V. alginolyticus* requires further study.

Sakazaki (58) separates the three species, *V. alginolyticus* (subgroup 2 of *V. parahaemolyticus*), *V. ichthyodermis* (NCMB 407), and *V. anguillarum* (NCMB 6) on the basis of the indole, Voges-Proskauer, arabinose and sorbitol reactions, and ability to swarm on agar. Each of these characteristics have been found to vary considerably within and between the species groups. To solidify such a diagnostic key prematurely as has been done in some laboratories (namely, Bacteriological Analytical Manual. 1969. Food and Drug Administration, Division of Microbiology, Washington, D.C.) will surely lead to problems in classification and identification of these species. Veron and Sebald (78) measured the overall DNA base composition of *V. ichthyodermis* strain NCMB 407 and found it to be 44.9 ± 0.5% G+C, well in the range of *V. parahaemolyticus* (Table 8). There is good reason with this additional evidence to suspect a high level of similarity to exist among *V. parahaemolyticus*, *V. ichthyodermis*, and *V. anguillarum*.

The reference strains included in this study provided some interesting results. Only the *A. fischeri*, the luminescent vibrio strain WH 135, and *Vibrio* MB-22 showed similarity values of S ≥ 60% with *V. parahaemolyticus*. The *A. fischeri* strain, on the basis of the DNA analysis and phenotypic similarity with the other vibrios studied, should be reclassified as *V. fischeri*.

A clustering of *Pseudomonas* species, including *P. aureofaciens* 8ab1 and 8ab2 and *P. fluorescens* Oxford-Sawyer strain, with *S. itersonii* ATCC 11331 and CIM strains 3, 9a, 9b, C, and D strongly suggests that *S. itersonii* ATCC 11331, a green fluorescent pigment producing organism, and the CIM strains should be placed in the genus *Pseudomonas*. The overall DNA base composition data (Table 8) support this proposal.

The reference strains not clustering with any of the above groupings nor with each other were *Spirillum serpens* ATCC 11330, *P. cruciviae* 13262, *A. georgiopolitanum*, *P. apiseptica* and the strains designated 4032, 4036, 4037, 4038, and 3C. Strain 3C has been identified as an *Arthrobacter* species (F. D. Cook, *personal communication*).

The *P. apiseptica* strain included in this study was received from T. A. Gochnauer (Table 1). The organism was originally isolated as a honeybee pathogen. However, on retesting the pathogenicity of this strain, it was found to be no longer pathogenic for bees. A *P. apiseptica* subculture from a parent strain maintained at the U.S. Dept. of Agriculture, Beltsville, Md., was obtained through the courtesy of H. Shimunaki. This strain of *P. apiseptica* was found to be highly pathogenic for bees (T. A. Gochnauer, *personal communication*). On testing the Beltsville isolate, the DNA base composition was 65 mole % GC, and the culture produced pyocyanine (Colwell, Wang, and Lovelace, *unpublished data*). Thus, the identity of *P. apiseptica* appears to be in doubt and provides an interesting problem for the apiculturalist.

In conclusion, the numerical taxonomy study presented here has provided a measurement of the phenotypic similarity at the intra- and interspecies level within the genus *Vibrio*. Molecular genetic evidence confirming the phenetic groupings is given in the form of DNA compositional measurements. A more significant confirmation of the numerical taxonomy results, however, was observed when the phenotypic similarity was compared directly with DNA/DNA reassociation for several *Vibrio* strains (6; *see* Fig. 9). The

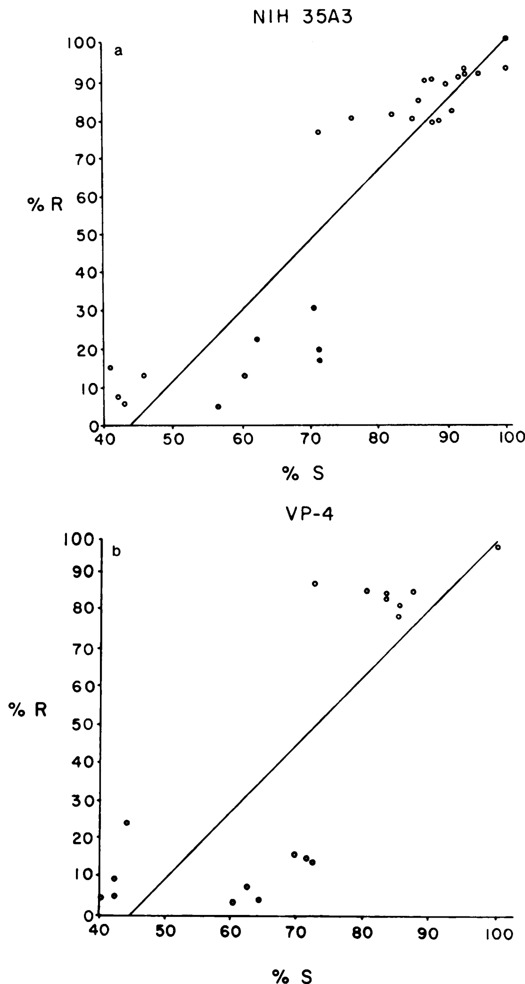

FIG. 9. *Comparison of S values (% S) and nucleic acid reassociation data (% R) for Vibrio species (a) V. cholerae, (b) V. parahaemolyticus. The reference strains selected for comparison are indicated at the top of the graphs. Correlation coefficients = 0.92 and 0.81, respectively.*

significant correlations observed between the two sets of data show that the two analyses, genetic and phenetic, provide complementary information, thereby strengthening taxonomic conclusions drawn from the individual sets of data. Isozyme patterns for *V. cholerae*, *V. parahaemolyticus*, and related vibrios (10), which can be considered an estimate of the similarity of the gene product, i.e., the enzyme, were used to group strains. The *Vibrio* strains with similar isozyme patterns were also grouped together when examined by numerical taxonomy, DNA base composition, and DNA/DNA reassociation methods. Information of an ecological nature, i.e., distribution of *V. parahaemolyticus* and related vibrios in the natural environment has also been obtained and will be published (Colwell, Krantz, Lovelace, and Wang, *in preparation*). Thus, the assimilation of these data, respresenting several levels of informational complexity, represents a major advance towards the achievement of a multiphasic, or polyphasic, taxonomy for the genus *Vibrio*.

Information gathered at all levels, from the molecular to the ecological, when carefully integrated will provide precision and reliability in identification and classification. No one facet of the information is singularly sufficient and all contribute a level of focus, thereby leading to a more complete understanding of the interrelationships within and among the genera of bacteria.

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