Numerical Taxonomy of Nitrogen-Fixing “Decarboxylase-Negative” *Vibrio* Species Isolated from Aquatic Environments

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A total of 95 strains of the genus *Vibrio* were studied by using numerical taxonomic techniques to determine phenotypic relationships among “decarboxylase-negative” strains, nitrogen-fixing (¹⁵N method) strains, and other members of the *Vibrionaceae*. Taxonomic resemblance was estimated on the basis of 129 tests by using Euclidean distance. Most strains clustered in phena, enabling identification with existing type strains. Nitrogen-fixing strains of *Vibrio natriegens* were identified, but other nitrogen-fixing isolates could not be assigned to any specific species or biovar.

Within the last decade, extensive taxonomic characterization of strains belonging to the genus *Vibrio* has revealed the existence of three broad phenotypic groups based on the ability to decarboxylate either L-arginine, L-lysine, or L-ornithine. The so-called “arginine decarboxylase-positive” group contains species that are pathogenic for humans and fish, such as *Vibrio fluvialis* and *Vibrio anguillarum*, respectively, as well as organisms which are probably saprophytic in aquatic environments. Several important pathogens, including *Vibrio cholerae* and *Vibrio vulnificus*, belong to the “arginine decarboxylase-negative” group, which contains organisms that do not decarboxylate L-arginine and vary in their ability to utilize other amino acids (21).

Strains assigned to the decarboxylase-negative phenotypic group within the genus *Vibrio* do not produce alkaline end products during growth in Møller medium containing L-arginine, L-lysine, or L-ornithine (15). Data from previous taxonomic studies on this group have suggested that most strains are saprophytic in marine environments and are associated with such metabolic functions as mineralization of organic compounds (1–4). Recent impetus has been given to further taxonomic investigation following reports that a variety of decarboxylase-negative *Vibrio* species are associated with nitrogen fixation (8), fish disease (17), and diarrheal disease in humans (12).

We describe here a taxonomic investigation of the nitrogen-fixing ability of decarboxylase-negative *Vibrio* species that were isolated from a diverse range of aquatic environments in different global regions.

MATERIALS AND METHODS

Bacterial strains. The sources of the 79 strains of decarboxylase-negative *Vibrio* species studied are listed in Table 1. Most strains were isolated from water, but some were associated with sediments or bivalve shellfish. All strains provisionally identified as *Vibrio* sp. were originally grown on thiosulfate-citrate-bile salts-sucrose agar, and most had been isolated after enrichment in alkaline peptone water before characterization (20). A total of 16 type and reference

strains representing species in the family *Vibrionaceae* were also used (Table 1).

Phenotypic characterization. The tests listed in Table 2 were performed without modification by using the methods described by West et al. (22). All decarboxylase-negative *Vibrio* strains were screened for the presence of nitrogenase activity by using the acetylene reduction assay (11) in semisolid N-deficient glucose medium, as described previously (8, 9). A total of 12 strains exhibited nitrogenase activity (see below), and the capacity to fix nitrogen was tested with 8 of these strains by using ¹⁵N-labeled nitrogen gas. Each isolate was grown in 10 ml of N-deficient or N-amended (2.5 g of NH₄Cl per liter) medium in 25-ml test tubes to a density of approximately 10⁶ cells per ml. The headspace of each test tube was purged with either helium (to create anaerobic conditions) or a helium-oxygen gas mixture and spiked with 99% ¹⁵N₂ (Monsanto Research Corp., Mound-Stable Isotope Sales, Miamisburg, Ohio) at a pressure of 0.2 atmosphere (20 kPa). Cultures were incubated for 48 h in the dark at 25°C before the assay was halted by adding 2 ml of 5 N H₂SO₄. Cells were harvested by centrifugation at 7,000 rpm for 20 min and transferred to 5-ml ampoules. The bacterial mass in each ampoule was freeze-dried and prepared for the ¹⁵N assay by the Dumas technique (6). Total nitrogen contents and atom percentages of ¹⁵N were measured by using a model MS10S mass spectrometer (AEI).

Analysis of results. Each isolate was characterized by using 5 quantitative multistate characters and 134 qualitative characters. The multistate characters, the minimum and maximum salt concentrations for growth, the minimum and maximum growth temperatures, and susceptibility to vibriostatic agent 0/129 were scored in a range from 0 to 1. Binary characters were scored as 0 (negative) or 1 (positive). A resemblance matrix was calculated by using Euclidean distance, and a phenogram was constructed by unweighted pair group arithmetic average sorting (18). The cophenetic correlation coefficient associated with construction of the phenogram was calculated by the method of Sokal and Rohlf (19). Cluster analysis was performed by using the CLUSTAN package (version 2, release 1) (23) available on the University of Surrey Prime 750 computer system. The GPROPS and GBEST programs (22) were used to determine the properties of each phenon, the phenon centrotyle, and the best diagnostic characters for each phenon.

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TABLE 1. Designations and sources of strains assigned to phenon derived from analysis by Euclidean distance unweighted pair group arithmetic average sorting

Phenon	Taxon	Strain(s) ^a	Source and/or site of isolation
1	<i>V. natriegens</i>	ATCC 14048 ^T W66 W70, W344 W361 W362 W285 W387 W396	Salt marsh mud Unknown Water, Chesapeake Bay, Maryland Sediment, Chesapeake Bay, Maryland Oysters, Chesapeake Bay, Maryland Sediment, Yaquina Bay, Oregon Water, Yaquina Bay, Oregon Oysters, England
2	Unidentified marine <i>Vibrio</i> spp.	W433 W353, W384, W388 W383, W385 W432 W389 W390, W392 W381	Water, Chesapeake Bay, Maryland Water, Tillamook River, Oregon Clams, Oregon Sediment, Garibaldi River, Oregon Freshwater stream, England Brackish water ditch, England Sediment, Louisiana
3	<i>V. alginolyticus</i>	ATCC 17749 ^T W335	Spoiled fish Water, Bangladesh
4	<i>V. pelagius</i>	ATCC 25916 ^T W411, 487	Seawater Seawater, Puerto Rico
5	Unidentified marine <i>Vibrio</i> spp.	W349 W391 W398 W386 W417	Water, Mud Lake, Louisiana Brackish water ditch, England Water, Chesapeake Bay, Maryland Water, Coos Bay, Oregon Seawater, Puerto Rico
6	Unidentified marine <i>Vibrio</i> spp.	W294, W298, W302, W304, W307, W310	Seawater, Florida-Puerto Rico, transect
7	<i>V. campbellii</i>	ATCC 25920 ^T W291, W293, W296, W301, W312, W315, W399, W403, W404, W405, W407, W408, W409, W410, W412, W414, W415, W416, W418, W420, W422, W423, W434 W482, W484, W485, W486, W488, W489, W491	Seawater Seawater, Florida-Puerto Rico transect Seawater, Puerto Rico
8	<i>V. splendidus</i>	ATCC 33125 ^T W413, W424	Marine fish Seawater, Florida-Puerto Rico transect
9	<i>V. diazotrophicus</i>	ATCC 33466 ^T W380	Sea urchin Water, Louisiana
10	Unidentified marine <i>Aeromonas</i> -like spp.	W345, W346, W348	Water, Chesapeake Bay, Maryland
Unclustered strains	<i>V. fluvialis</i>	NCTC 11327 ^T	Human feces
	Unidentified <i>Vibrio</i> sp.	W352	Water, Chesapeake Bay, Maryland
	<i>Aeromonas hydrophila</i>	ATCC 7966	Milk
	<i>Aeromonas hydrophila</i>	ATCC 15467	Oil emulsions
	<i>V. cholerae</i>	ATCC 14035 ^T	Human feces
	<i>V. anguillarum</i>	ATCC 19264 ^T	Diseased fish
	Unidentified <i>Vibrio</i> sp.	W364	Water, Chesapeake Bay, Maryland
	<i>Vibrio parahaemolyticus</i>	ATCC 17082 ^T	Human feces
	Unidentified <i>Vibrio</i> sp.	W490	Seawater, Puerto Rico
	Unidentified <i>Vibrio</i> sp.	W205	Estuary water, Oregon
	<i>Vibrio nigripulchritudo</i>	ATCC 27043 ^T	Seawater
	Unidentified <i>Vibrio</i> sp.	W290	Seawater, Florida-Puerto Rico, transect
	Unidentified <i>Vibrio</i> sp.	W316	Water, Chesapeake Bay, Maryland
	Unidentified <i>Vibrio</i> sp.	W397(Bar)	Sea urchin
	<i>Vibrio fischeri</i>	ATCC 7744 ^T	Seawater
	<i>Vibrio nereis</i>	ATCC 25917 ^T	Seawater
	<i>V. splendidus</i>	ATCC 25914	Seawater
	Unidentified <i>Vibrio</i> sp.	W421	Seawater, Florida-Puerto Rico, transect
	Unidentified <i>Vibro</i> sp.	W179	Unknown
	Unidentified <i>Vibrio</i> sp.	W372	Water, Bangladesh

^a ATCC, American Type Culture Collection, Rockville, Md; NCTC, National Collection of Type Cultures, London, England; W, culture collections of P.A.W. held at the University of Maryland, College Park. The following strains were phenon centrotypes: W384 (phenon 2), W411 (phenon 4), W349 (phenon 5), W307 (phenon 6), W482 (phenon 7), W413 (phenon 8), and W346 (phenon 10).

TABLE 2. Nitrogen fixation in *Vibrio* species as determined by ^{15}N enrichment

Strain	Presence of N fixation	Incubation conditions	Amt of N (μmol)	^{15}N enrichment (atom %) ^a
W362	+	N-deficient anaerobic	1.1	0.022
W362	+	N-amended anaerobic	5.3	0.018
W432	+	N-deficient anaerobic	0.6	0.042
W432	+	N-amended anaerobic	3.5	0.016
W383	+	N-deficient anaerobic	0.8	0.068
ATCC 33466 ^T	+	N-deficient anaerobic	0.8	0.056
ATCC 33466 ^T	—	N-deficient aerobic	2.3	0.0
W344	—	N-deficient anaerobic	1.9	0.0
W344	—	N-amended anaerobic	3.2	0.0
W344	—	N-amended anaerobic	6.4	0.0
W410	—	N-deficient anaerobic	2.1	0.0
W410	—	N-amended anaerobic	3.4	0.0
W380	—	N-amended anaerobic	5.2	0.0
W396	—	N-amended anaerobic	7.7	0.0

^a ^{15}N enrichment was calculated as follows: ^{15}N enrichment (atom%) = atom of ^{15}N — 0.366%.

RESULTS AND DISCUSSION

Each strain was examined for 139 characters. All strains were gram negative, oxidase positive, and motile, grew at pH 10.0, and fermented glucose. All strains failed to produce hydrogen sulfide, as well as grow on butyrate, dulcitol, *p*-hydroxybenzoate, and valerate as sole carbon sources.

Exclusion of these 10 characters resulted in basing the taxonomic resemblance among the 95 strains tested on 129 characters. Nitrogen-fixing data are shown in Table 2.

The relationships among phena based on the Euclidean distance coefficient and unweighted pair group arithmetic average sorting are illustrated in Fig. 1. The Euclidean distance values were transformed into percentages of similarity. The cophenetic correlation coefficient associated with the phenogram was 0.875, indicating the suitability of the data for hierarchical clustering (13). All but 20 strains fell into 10 phena.

The characteristics of the phena are shown in Table 3. The characters which distinguish phena are shown in Table 4.

Phenon 1 was identified as *Vibrio natriegens*. The strains in this phenon were isolated from a geographically diverse range of habitats and formed a cluster at the 86.5% level of similarity. Of the nine strains in this phenon, seven exhibited various degrees of nitrogenase activity in the acetylene reduction assay. The nitrogen-fixing capacities of selected strains were tested by using the ^{15}N assay. Strain W362, which was isolated from a Chesapeake Bay water sample, was able to fix nitrogen when it was grown anaerobically in both N-deficient and N-amended media (Table 2). The phenon centrotypic, strain W344, failed to fix nitrogen under similar growth conditions. Strain W396 failed to show nitrogen fixation but was tested only in N-amended medium incubated anaerobically.

The strains in phenon 2 formed a cluster at the 89.5% level of similarity. Characteristically, all of the phenon 2 strains degraded tyrosine and xanthine and, more unusually, grew on adenine and ethanolamine. These strains could not be assigned to a known species or biovar in the family *Vibrion-*

FIG. 1. Phenogram showing relationships among phena based on the Euclidean distance coefficient and unweighted pair group arithmetic average sorting, with distances transformed into percentages of similarity.

TABLE 3. Characteristics of major and minor phena

Characteristic ^a	Phenon 1 (<i>V. natrigens</i>) (n = 9)	Phenon 2 (unidentified marine <i>Vibrio</i> spp.) (n = 11)	Phenon 3 (<i>V. alginolyticus</i>) (n = 2)	Phenon 4 (<i>V. pelagius</i>) (n = 3)	Phenon 5 (unidentified marine <i>Vibrio</i> spp.) (n = 5)	Phenon 6 (unidentified marine <i>Vibrio</i> spp.) (n = 6)	Phenon 7 (<i>V. campbellii</i>) (n = 31)	Phenon 8 (<i>V. splendidus</i>) (n = 3)	Phenon 9 (<i>V. diazotrophicus</i>) (n = 2)	Phenon 10 (unidentified marine <i>Aeromonas</i> -like spp.) (n = 3)
Growth in:										
0% NaCl	— ^b	—	—	—	—	—	—	—	—	+
3% NaCl	+	+	+	+	+	+	+	+	+	+
6% NaCl	+	+	+	2	+	+	+	+	+	+
8% NaCl	—	7	+	1	—	—	2	—	1	—
10% NaCl	—	—	+	—	—	—	—	—	—	—
Growth at:										
4°C	—	—	—	—	—	—	—	1	1	—
10°C	1	+	+	—	4	—	11	1	+	+
35°C	+	+	+	+	+	1	28	—	+	+
42°C	—	—	—	1	2	—	—	—	—	+
Susceptibility to:										
0/129 (10 µg/ml)	—	3	1	—	3	3	10	2	1	—
0/129 (50 µg/ml)	1	10	1	+	+	5	25	+	+	—
0/129 (150 µg/ml)	+	+	1	+	+	+	+	+	+	—
Nitrate reduction	+	+	+	+	+	1	25	+	+	+
ONPG test ^c	6	+	—	+	+	—	—	1	+	—
Voges-Proskauer test	—	—	+	—	—	—	—	—	—	—
Indole	—	3	+	2	+	—	29	+	+	+
Glucuronate oxidation	—	—	—	—	—	—	—	—	—	—
Arginine decarboxylase	—	—	—	—	—	—	—	—	1	—
Lysine decarboxylase	—	—	1	—	—	—	1	—	—	—
Ornithine decarboxylase	—	—	—	—	—	—	—	—	—	—
Thornley arginine	—	—	—	—	—	—	—	—	1	—
Growth at pH 4.5	—	—	—	—	—	—	—	—	—	2
Chick cell agglutination	4	2	—	—	1	—	3	—	—	—
Hemolysis	—	1	—	1	2	1	—	1	—	—
Luminescence	—	—	—	—	—	—	—	—	—	—
Catalase	+	+	+	+	+	+	30	+	+	+
Swarming	—	8	1	—	—	—	—	—	—	—
Glucose gas	—	—	—	—	—	—	—	—	—	—
Acid production from:										
L-Arabinose	+	10	—	—	2	—	—	—	+	—
Arbutin	+	1	—	—	2	—	—	—	+	+
Cellobiose	7	1	+	+	4	—	+	+	+	—
m-Inositol	5	—	—	—	—	—	1	—	—	+
D-Mannitol	+	10	+	+	+	1	12	1	+	+
D-Mannose	4	1	+	+	4	1	14	+	—	+
Salicin	+	1	—	—	3	—	25	2	1	+
Sorbitol	2	—	—	—	3	—	—	—	—	—
Sucrose	+	+	+	+	+	—	1	—	+	—
Trehalose	+	+	+	+	+	4	+	+	+	—
Growth on:										
D-Arabitol	+	—	—	—	—	—	—	—	—	+
Ethanol	6	3	—	1	—	—	1	—	—	—
D-Glycerol	+	+	+	3	4	5	30	—	—	+
D-Mannitol	+	+	+	3	+	—	10	—	+	+
1-Propanol	6	10	—	—	—	—	—	—	—	—
Sorbitol	1	—	—	—	1	—	—	—	—	—
D-Alanine	+	+	+	+	4	5	+	—	1	+
L-Alanine	+	+	+	2	+	+	28	—	1	+
L-Arginine	+	+	+	+	+	4	13	1	+	—
L-Asparagine	+	9	—	+	4	4	14	—	1	—
L-Aspartate	7	+	+	2	4	2	9	—	—	—

Continued

TABLE 3—Continued

Characteristic ^a	Phenon 1 (<i>V. natriegens</i>) (n = 9)	Phenon 2 (unidentified marine <i>Vibrio</i> spp.) (n = 11)	Phenon 3 (<i>V. alginolyticus</i>) (n = 2)	Phenon 4 (<i>V. pelagius</i>) (n = 3)	Phenon 5 (unidentified marine <i>Vibrio</i> spp.) (n = 5)	Phenon 6 (unidentified marine <i>Vibrio</i> spp.) (n = 6)	Phenon 7 (<i>V. campbellii</i>) (n = 31)	Phenon 8 (<i>V. splendidus</i>) (n = 3)	Phenon 9 (<i>V. diazotrophicus</i>) (n = 2)	Phenon 10 (unidentified marine <i>Aeromonas</i> -like spp.) (n = 3)
γ-Aminobutyrate	+	—	—	2	—	—	—	—	—	—
Citrulline	+	—	—	+	—	+	6	—	+	—
L-Glutamate	+	+	+	+	+	5	+	—	+	+
Glycine	+	+	+	+	—	5	7	—	—	—
L-Histidine	+	+	+	+	+	4	15	2	+	+
L-Leucine	6	2	+	—	—	—	2	—	—	—
L-Ornithine	+	+	+	+	+	5	4	—	+	—
L-Proline	+	8	+	+	+	+	28	—	+	+
L-Hydroxyproline	1	—	+	—	—	—	—	—	—	+
L-Serine	+	+	—	1	+	5	14	—	—	+
Taurine	4	—	—	—	—	—	—	—	—	—
L-Threonine	+	+	+	+	+	+	29	—	1	—
δ-Aminovalerate	+	2	—	—	—	—	—	—	—	—
D-Amygdalin	3	—	—	—	—	—	—	—	—	—
L-Arabinose	+	+	1	—	1	—	1	—	+	+
Arbutin	+	1	—	—	—	—	—	—	—	+
Cellobiose	+	—	—	+	4	—	29	—	+	—
D-Fructose	+	+	+	+	+	4	8	—	+	+
D-Galactose	+	+	—	+	+	—	4	—	+	+
D-Gluconate	8	+	+	+	+	—	1	—	+	+
D-Glucose	+	+	+	+	+	+	+	—	+	+
D-Glucosamine	+	+	1	2	4	—	1	—	+	+
N-Acetyl-D-glucosamine	+	+	+	+	4	5	29	—	+	+
Glycogen	+	+	+	+	4	—	30	—	+	—
Lactose	1	—	—	—	—	—	—	—	—	—
Maltose	+	+	+	+	+	—	28	—	+	—
D-Mannose	4	—	—	+	3	—	11	—	—	+
Melibiose	6	—	—	1	—	—	—	—	—	—
D-Ribose	8	+	+	+	+	1	29	—	+	+
Salicin	+	5	—	—	3	—	2	—	—	+
Sucrose	+	+	+	+	4	—	18	—	+	+
Trehalose	+	+	+	+	+	+	16	—	+	1
D-Xylose	—	1	—	—	—	—	—	—	1	—
Acetate	+	+	+	2	+	—	1	—	—	+
Phenylacetate	—	2	—	1	—	—	—	—	—	—
DL-3-Hydroxybutyrate	+	+	—	—	—	—	—	—	—	—
Citrate	+	+	+	+	4	—	18	—	+	+
Fumarate	+	+	+	+	+	4	30	—	+	+
Glutarate	3	—	—	—	—	—	—	—	—	—
α-Ketoglutarate	3	+	+	+	3	—	30	—	1	—
DL-Glycerate	8	—	+	1	—	—	10	—	—	—
DL-Lactate	7	2	+	—	2	—	2	—	—	—
DL-Malate	1	10	—	—	2	—	1	—	—	+
Malonate	+	—	—	—	—	—	—	—	—	—
Propionate	8	+	+	—	1	—	—	—	—	—
Succinate	8	+	+	+	4	2	26	1	—	+
Adenine	—	+	—	—	—	—	—	—	—	—
Ethanolamine	—	+	—	—	—	—	—	—	—	—
D-Galacturonate	1	—	—	—	—	—	—	—	—	—
D-Glucuronate	2	—	—	—	—	—	—	—	—	—
m-Inositol	5	—	—	—	—	—	—	—	—	+
Putrescine	+	+	—	+	—	—	—	—	—	—
Sarcosine	6	—	—	—	—	—	—	—	—	—
Tyrosine	4	+	—	—	3	—	—	—	—	+
Xanthine	1	—	—	—	—	—	—	—	—	—
Degradation of:										
Albumin	—	+	1	—	+	1	15	1	—	—
Casein	7	+	+	+	+	3	+	2	—	—

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TABLE 3—Continued

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Tween 20	+	+	+	+	+	+	+	+	+	—
Tween 40	+	+	+	+	+	+	+	+	+	—
Tween 60	+	+	+	+	+	+	+	+	+	—
Tween 80	+	+	+	+	+	5	+	+	—	—
Tyrosine	+	+	+	—	4	—	18	1	—	+
Xanthine	—	+	—	—	—	—	—	—	—	—
Elaboration of:										
Alginase	—	—	—	2	—	—	—	—	1	—
Amylase	+	+	1	+	—	—	+	+	+	—
Chitinase	—	10	+	+	3	3	+	+	—	—
Chondroitinase	—	4	—	—	1	1	—	—	—	—
Deoxyribonuclease	5	10	+	+	+	+	+	+	—	—
Elastase	—	6	—	—	4	1	2	—	—	—
Gelatinase	+	+	+	+	+	+	+	+	—	—
Lecithinase	+	+	+	+	4	+	+	+	—	—
Phosphatase	+	+	+	+	+	+	+	+	+	+
Sulfatase	1	—	—	2	—	—	—	—	—	—
Urease	6	3	—	1	—	—	—	—	—	+
Susceptibility to:										
Ampicillin (10 µg)	+	+	—	1	4	2	—	1	+	+
Novobiocin (5 µg)	1	7	—	—	3	+	+	+	—	—
Polymyxin (50 IU)	+	+	+	1	—	4	6	+	+	—
Streptomycin (10 µg)	+	+	+	2	+	+	29	+	+	+
Resistance to:										
Bile salts (0.5%)	+	+	+	+	+	+	30	+	+	+
Brilliant green (0.002%)	—	6	1	—	—	—	—	—	—	—
Crystal violet (0.002%)	—	+	—	—	—	—	—	—	—	—
Methylene blue (0.01%)	5	1	—	—	—	2	1	—	—	+
Methyl violet (0.002%)	7	3	1	+	2	3	+	+	—	+
Pyronin Y (0.002%)	—	8	—	—	—	—	—	—	—	+
Teepol 610 (0.4%)	+	+	+	+	+	+	+	2	+	+
Tellurite (0.0005%)	4	+	—	—	4	—	—	—	+	—
Neutral red (0.002%)	+	+	+	+	+	+	+	+	+	+

^a All of the strains studied were motile, oxidase positive, and gram negative, fermented glucose, and grew at pH 10.0. All strains were negative for the following traits: production of hydrogen sulfide and growth on butyrate, dulcitol, *p*-hydroxybenzoate, and valerate.

^b +, All strains positive; —, all strains negative. The numbers indicate the number of positive strains.

^c ONPG, *o*-Nitrophenyl-β-D-galactopyranoside.

aceae, although they most closely resembled a recently described pathogen of bivalve molluscs, *Vibrio tubiashii* (10). Additional key characteristics of strains in phenon 2 include susceptibility to vibriostatic agent 0/129 (150 µg), anaerogenic fermentation of glucose, positive *o*-nitrophenyl-β-D-galactopyranoside reaction, negative Voges-Proskauer reaction, no growth in the absence of NaCl, growth on DL-3-hydroxybutyrate, propionate, D-gluconate, putrescine, and 1-propanol as sole carbon sources, and failure to utilize DL-glycerate, malonate, γ-aminobutyrate, citrulline, cellobiose, D-mannose, and D-glucuronate. Two phenon 2 strains

(strains W383 and W432) which exhibited nitrogenase activity in the acetylene reduction assay fixed nitrogen when they were tested by the ¹⁵N technique (Table 2). The centrotpe of phenon 2 is strain W384, which was isolated from Oregon waters and did not exhibit nitrogenase activity in the acetylene reduction assay.

Phenon 3 comprised two strains, *Vibrio alginolyticus* ATCC 17749^T (T = type strain) and an unidentified decarboxylase-negative *Vibrio* sp. strain isolated at Teknaf, Bangladesh, from water; these strains clustered at a similarity level of 93.4%.

Three strains were linked at a similarity level of 89.7% in phenon 4 and were identified as *Vibrio pelagius*. These strains produced amylase, gelatinase, and alginate and grew on D-gluconate and putrescine.

The strains in phenon 5 clustered at the 84.7% level of similarity. These strains were isolated from a diverse range of locations and could not be identified from their characteristics when they were compared with the data available in the literature. In general, the phenon 5 strains resembled *Vibrio splendidus*, but the failure of the type strain of this species to cluster in phenon 5 means that this cluster remains unidentified. None of the isolates in this group exhibited nitrogenase activity in the acetylene reduction assay. Similarly, the six strains which clustered at the 89.0% level of similarity to form phenon 6 could not be identified. All of the phenon 6 strains were isolated from water near Puerto Rico and were in general unreactive in key characterization tests, including nitrate reduction tests. These strains resembled *Vibrio ordalii*, but the failure of any type or reference strain to cluster in phenon 5 or 6 suggests that further study will be required. No strain in phenon 6 exhibited nitrogenase activity.

Phenon 7 formed at the 86.7% level of similarity and contained 31 strains, including strain ATCC 25920^T; this phenon was identified as *Vibrio campbellii*. All wild strains were isolated from the top 1 m of ocean water near Puerto Rico. Only one strain (strain W410) exhibited trace amounts of nitrogenase activity in the acetylene reduction assay; subsequently this strain was shown not to fix nitrogen by the ¹⁵N method. Phenon 7 strains characteristically produced amylase but failed to utilize γ -aminobutyrate, sucrose, L-arabinose, D-galactose, and putrescine as sole carbon sources. Curiously, only strain ATCC 25920^T demonstrated lysine decarboxylase activity in Møller medium.

Phenon 8 was identified as *V. splendidus*; the strains in this phenon formed a cluster at the 91.3% level of similarity. *V. splendidus* type strain ATCC 33125 was a member of this cluster.

Phenon 9 comprised strain ATCC 33466^T, which is the type strain of *Vibrio diazotrophicus*, and a wild isolate from an oyster sample collected in Louisiana. Phenon 9 formed at the 92.3% level of similarity. The wild isolate, which exhibited trace amounts of nitrogenase activity, failed to fix ¹⁵N anaerobically in N-amended medium. *V. diazotrophicus* was the first *Vibrio* described which fixed nitrogen, as tested by the acetylene reduction assay (9). We report, for the first time, the ability of strain ATCC 33466^T to fix nitrogen when it is tested by the definitive ¹⁵N method.

A very closely related cluster formed at the 98.7% level of similarity; this was phenon 10. All phenon 10 strains had been isolated from Chesapeake Bay shellfish and phenotypically were very different from the other strains in this study. These strains were urease positive, produced acid from arbutin and inositol but not trehalose, and failed to grow on L-arginine and asparagine. The strains in phenon 10 were not identified, and further genotypic characterization is warranted. No strain exhibited nitrogenase activity in the acetylene reduction assay.

The ability of one or two species or strains within a genus to fix nitrogen is not uncommon (5). We report here the ability of strains belonging to the genus *Vibrio* to fix nitrogen. Current taxonomic criteria for the genus *Vibrio* (1, 4) require expansion of the description to include the ability to fix molecular nitrogen. The failure to detect nitrogen-fixing *Vibrio* strains in earlier taxonomic studies was most likely due to the use of insensitive assay methods (2).

TABLE 4. Characteristics useful for identifying phenon

Characteristic	Phenon 1 (<i>V. natriegens</i>)	Phenon 2 (unidentified marine <i>Vibrio</i> spp.)	Phenon 3 (<i>V. alginolyticus</i>)	Phenon 4 (<i>V. pelagius</i>)	Phenon 5 (unidentified marine <i>Vibrio</i> spp.)	Phenon 6 (unidentified marine <i>Vibrio</i> spp.)	Phenon 7 (<i>V. campbellii</i>)	Phenon 8 (<i>V. splendidus</i>)	Phenon 9 (<i>V. diazotrophicus</i>)	Phenon 10 (unidentified marine <i>Aeromonas</i> -like spp.)
Tyrosine decomposition	+	+	+	-	+	-	d	d	-	+
Albumin hydrolysis	-	+	d	-	+	-	d	d	-	-
Acid production from:										
Cellobiose	+	-	+	+	+	-	+	+	+	-
D-Mannose	d	-	+	+	+	-	d	+	-	+
Sucrose	+	+	+	+	+	-	-	-	+	-
Growth on:										
L-Arabinose	+	+	d	-	d	-	-	-	+	+
D-Cellobiose	+	-	-	+	+	-	+	-	+	+
D-Galactose	+	+	-	+	+	-	-	-	+	+
D-Mannose	d	-	-	+	d	-	d	-	-	+
Trehalose	+	+	+	+	+	+	d	-	+	d
Sucrose	+	+	+	+	+	-	d	-	+	+
L-Arginine	+	+	+	+	+	d	d	d	+	-
L-Asparagine	+	+	-	+	+	d	d	-	d	-
L-Aspartate	+	+	+	d	+	d	d	-	-	-
Citrate	+	+	+	+	+	-	d	-	+	+
Citrulline	+	-	+	+	+	+	-	-	+	-
DL-Glycerate	+	-	+	d	-	-	d	-	-	-
D-Glycerol	+	+	+	d	+	+	+	-	-	+
Glycine	+	+	+	-	+	+	d	-	-	-
L-Histidine	+	+	+	+	+	+	d	d	+	+
L-Ornithine	+	+	+	+	+	+	-	-	+	-
Succinate	+	+	+	+	+	d	d	d	-	+

^a +, Character present in at least 80% of the strains; -, character absent in at least 80% of the strains; d, character present in 21 to 79% of the strains.

Many of the strains used in the present study were not able to decarboxylate amino acids in Møller medium. It is not clear whether these strains do not possess constitutive decarboxylase pathways or whether the medium used lacks the specificity and sensitivity to detect end products of decarboxylation. The limitations of Møller medium in detecting decarboxylase activity by coliform bacteria have been noted previously (16). Similar deficiencies in this test when it is adapted for use with marine isolates may explain some unusual strain groupings within phenon 3, 7, and 9. In addition, phenon 10 was not identified, although possession of arginine decarboxylase would have provided presumptive identification as an anaerogenic *Aeromonas* sp. Unfortunately, possession of amino acid decarboxylases is a heavily weighted characteristic during primary identification of strains belonging to the genus *Vibrio* (21). Failure of Møller medium to reflect decarboxylase activity may lead to problems in identifying strains according to some existing classification schemes.

Recently, a new species, *Vibrio hollisae*, was described

(12) which has been associated with human disease. The ecology of this pathogen is not yet fully understood, but interestingly, many strains have been isolated from cases of disease involving consumption of raw seafood. This suggests an aquatic environmental reservoir although in the present study we did not detect these organisms in coastal regions of the United States.

The contribution of nitrogen-fixing microorganisms to the transfer of nitrogen to animals from their environmental milieu has been recognized recently. Studies have shown that microbially fixed nitrogen is incorporated into sea urchin tissue (7), and it is likely that similar commensal relationships exist elsewhere (14). In this study we demonstrated, for the first time, the presence of nitrogen-fixing ability in the genus *Vibrio*, which often is the predominant bacterial group in estuarine and marine environments. The extent to which these organisms contribute to the nitrogen cycle and protein nutrition of estuarine and marine animals requires evaluation (14).

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