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Polyphasic Taxonomy of the Genus *Vibrio*: Polynucleotide Sequence Relationships Among Selected *Vibrio* Species

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Polynucleotide relationships among selected *Vibrio* species were examined by means of deoxyribonucleic acid (DNA) reassociation reactions and chromatography on hydroxyapatite. Relative levels of intraspecific DNA duplex formation (*V. cholerae-V. cholerae* and *V. parahaemolyticus-V. parahaemolyticus*) were found to be high at 60 C (>80%), and only minimally reduced at 75 C. Interspecific DNA duplexes between *V. cholerae* DNA and that of the non-cholera vibrios also exhibited high relative levels of formation at 60 C (>80%) and, with one exception, were only slightly reduced at 75 C. The thermal stability of these duplexes formed at 60 or 75 C was virtually identical to that of homologous *V. cholerae* DNA duplexes. The degree of reassociation and the thermal stability of *V. cholerae*-non-cholera vibrio DNA duplexes suggests relatively little evolutionary divergence in these organisms. In all other interspecific DNA reassociation reactions, only low levels of DNA duplex formation were noted at 60 C (<25%), and these were drastically reduced (>50%) at 75 C. The degree of nucleotide sequence divergence indicated by these reactions suggests that these *Vibrio* species are not significantly related to *V. cholerae* or *V. parahaemolyticus*. Reassociation reactions between *V. cholerae* DNA and the DNA of *V. parahaemolyticus* indicated these species were not significantly related to each other.

Recent advances in the biochemistry of nucleic acids have led to the accumulation of data which attempt to assess genetic and phylogenetic relationships amongst the bacteria by the study of polynucleotide similarities (29, 32). If the nucleic acids of two organisms are unable to reassociate under ideal conditions, it is clear that these organisms are not related. Conversely, it is considered that the extent and perfection of polynucleotide reassociations do validly reflect genetic and existent evolutionary relationships. However, data of this type, considered in the absence of carefully assessed and correlated phenetic data, lack practical usefulness. Estimates of relationships among bacteria which are based on phenetic data alone have certain limitations. In this regard, one can consider the implications of a degenerate genetic code in terms of protein structure and function (47). The concept of a polyphasic taxonomy was developed to overcome these shortcomings. One assimilates and correlates data derived from many informational levels and by all available methodologies (12).

The aim of the investigation reported in this paper is to provide an assessment of genetic relatedness for members of selected *Vibrio* species from measurements of the extent of intra- and interspecific nucleic acid reassociations.

MATERIALS AND METHODS

Organisms and media. Strains used in this investigation are listed in Table 1. The strains of *V. cholerae*, *V. cholerae* var. El Tor, and non-cholera vibrios were selected with consideration for historial interest and for diversity in the time and place of their isolation.

Brain Heart Infusion (Difco) was used for routine culture of all strains except MP-1, MB-22, PS-207, and MB-1. These marine strains were grown in a medium (*p*H 8.0) containing (g/liter): NaCl, 24.0; KCl, 0.7; $MgCl_2 \cdot 6H_2O$, 5.3; $MgSO_4 \cdot 7H_2O$, 7.0; Trypticase (BBL), 10.0; Yeast Extract (Difco), 5.0.

To prepare radiolabeled deoxyribonucleic acid (DNA) of *V. cholerae* strain NIH 35A3 cells were grown overnight at 37 C in a medium containing (g/liter): NaCl, 10.0; $MgSO_4$, 0.05; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 0.013; tris(hydroxymethyl)aminomethane(Tris), 12.1; glucose, 5.0; and casein hydrolysate, salt-free and vitamin-free (Nutritional Bio-

chemicals Co.), 0.5; *p*H was adjusted to 8.0 with HCl. These cells were diluted 1:150 with fresh media, 10 μCi per ml of carrier-free $H_3{}^{32}PO_4$ (Tracerlab) was added, and the culture was incubated at 37 C for 20 hr. Similarly, radiolabeled DNA was prepared from cells of *V. parahaemolyticus* strain 4, after growth for 20 hr at 37 C in a medium containing (g/liter): NaCl, 24.0; KCl, 0.7; $MgCl_2 \cdot 6H_2O$, 5.3: Tris, 12.1; casein hydrolysate, salt-free and vitamin-free (Nutritional Biochemicals Co.), 0.5; glucose, 5.0; 10 μCi per ml of carrier-free $H_3{}^{32}PO_4$ (Tracerlab); *p*H was adjusted to 8.0 with HCl.

Preparation of DNA. DNA was extracted essentially by the method of Marmur (31), but modified to include the use of phenol and predigested Pronase (Calbiochem) to effect more complete removal of protein. Bacterial cells in the stationary phase of growth were harvested by centrifugation and resuspended in saline-EDTA (0.15 M NaCl + 0.15 M ethylenediaminetetraacetate, *p*H 9.0) to a volume one-tenth that of the original broth culture. Sodium lauryl sulfate was added to a final concentration of 1%, and an equal volume of cold (6 to 8 C) redistilled, water-saturated phenol was added. The phenol and aqueous phases were well mixed by shaking for several minutes, and the two phases were separated by centrifugation. The aqueous phase was removed and mixed well with an equal volume of chloroform-isoamyl alcohol (24:1, v/v); the two phases were separated by centrifugation. This step was repeated three times. Two volumes of 95% ethanol were gently layered on the separated aqueous phase, and the DNA fibers precipitated during mixing were collected on a glass rod. After washing the fibers with 95% ethanol, the DNA precipitate was air-dried and dissolved in 0.1 × SSC (0.15 M NaCl + 0.015 M trisodium citrate, *p*H 7.0). Bovine pancreatic ribonuclease (Calbiochem), previously heated to 80 C for 10 min to inactivate any contaminating deoxyribonuclease, was added to a final concentration of 50 μg per ml, and the solution was incubated for 1 hr at 37 C and then for 30 min at 60 C. Pronase, predigested for 2 hr at 37 C, was added to a final concentration of 100 μg per ml, and the DNA solution was incubated at 37 C for 4 to 6 hr. After cooling the solution in an ice-water bath, an equal volume of phenol was added; the two phases were mixed well and then separated by centrifugation. The recovered aqueous phase was then repeatedly extracted with chloroform-isoamyl alcohol until no visible interfacial material remained. To the final recovered aqueous phase, 0.1 volume of acetate-EDTA (3.0 M sodium acetate + 0.001 M EDTA, *p*H 7.0) was added. The DNA was precipitated and recovered from solution by the dropwise addition of 0.54 volume of isopropyl alcohol while stirring with a glass rod. After washing with 95% ethanol, the DNA was dissolved in 0.1 × SSC and precipitated with two volumes of 95% ethanol; this step was repeated four to six times. The final DNA precipitate was dissolved in 0.014 M PB (phosphate buffer, and equimolar mixture of Na_2HPO_4 and NaH_2PO_4, *p*H 6.8), and fragmented by mechanical shear at 50,000 psi to a molecular weight of approximately 2×10^5 (5, 6). The solution was filtered through a Metricel filter disc (0.45-μm pore size, Gelman Instrument Co.), dialyzed against 0.14 M PB and stored at −20 C for further use.

TABLE 1. *Vibrio strains employed*[a]

V. cholerae (classical)	*V. parahaemolyticus*
NIH 35A3	Strain 1
NIH 41	Strain 4
C-401	Strain 5
VC-9	Strain 13
ATCC[b] 14035	Strain 15
P 33/58	Strain 17
V. cholerae (El Tor)	Strain 21
ATCC 14033	Strain 23
NCTC[c] 2890	*V. alginolyticus*
NCTC 6563	5144[d]
HK-1	5146[d]
2A/62	5162[d]
SLH 29803	"Marine" vibrios
Non-cholera vibrios	*V. marinus* MP-1
NCTC 4711	*V. marinus* PS-207
NCTC 4715	*P. piscicida* MB-1
NCTC 4716	"Marine" vibrio MB-22
NCTC 8042	
NCTC 30	
S-860	

[a] See Colwell (13) for details of isolation, description, etc.
[b] American Type Culture Collection, Rockville, Md.
[c] National Collection Type Cultures, London, England.
[d] Received as *V. parahaemolyticus* biotype 2.

Further purification of labeled DNA preparations. Before shearing, labeled preparations of DNA were chromatographed on methylated albumin kieselguhr (MAK) columns (30) to remove non-DNA labeled contaminants. DNA dissolved in 0.5 M NaCl + 0.05 M PB (*p*H 6.8) was loaded onto columns equilibrated with the same buffer. Contaminants were removed by washing the columns with 10 bed volumes of 0.5 M NaCl + 0.05 M NaCl, and the DNA was recovered by eluting with 10-ml volumes of 2.0 M NaCl + 0.05 M PB. Fractions containing the DNA were pooled, concentrated, dialyzed against 0.014 M PB, and then sheared at 50,000 psi. After filtration through Metricel filters, labeled preparations were dialyzed against 0.14 M PB, denatured by boiling at 100 C for 10 min, and passed through an hydroxyapatite (HA) column equilibrated with 0.14 M PB held at 60 C. Material bound to the column under these conditions was discarded (4). Recovered DNA fragments were dialyzed against 0.14 M PB and stored at 4 C over chloroform until used.

DNA-DNA reassociation reactions. Reaction mixtures contained 2.0 optical density units at 260 nm (OD_{260}) of unlabeled DNA fragments plus 0.004 OD_{260} units of labeled DNA fragments in 0.5 ml of 0.14 M PB. These were placed in sealed vials (4-ml capacity), heated to 100 C for 5 min, and immediately transferred to a sealed water bath held at 60 or 75 C for 20 hr of incubation. This period of incubation was of sufficient duration to assure at least 80% reassociation of unlabeled fragments, whereas reassociation of labeled fragments with one another would be less than 4%. At the conclusion of the incubation period the vials were rapidly chilled in an ice-water bath and

stored at −20 C until chromatographed on hydroxyapatite.

Separation of single- and double-stranded DNA on hydroxyapatite (HA). HA, prepared by the method of Miyazawa and Thomas (37), was boiled for 20 min in 0.14 M PB before use to reduce nonspecific adsorption of single-stranded DNA (D. Kohne, *personal communication*). To process the large number of reaction mixtures at hand, the "batch" procedure of Brenner et al. was used (3). This permits the simultaneous chromatography of 8 to 12 samples. An amount of HA, two- to threefold in excess of that required to bind all the DNA contained in the reaction mixture, was equilibrated with 0.14 M PB and placed in a polycarbonate centrifuge tube (50-ml capacity). The tubes are placed in a water bath held at 60 or 75 C, the temperature at which the reaction mixtures were previously incubated for 20 hr. The frozen reaction mixtures were thawed, diluted to 18 ml with prewarmed 0.14 M PB, added to the tubes, and mixed thoroughly with the HA. After allowing 20 min for temperature equilibration, the tubes were rapidly transferred to a Sorvall centrifuge (type SS-1) maintained at 60 or 75 C in an incubator and centrifuged at 3,000 rev/min for 2 min. The centrifuge rotor was rapidly stopped, and the supernatants were poured directly into counting vials. The tubes were replaced in the water bath and given four additional washes with 18-ml volumes of 0.14 M PB. Reassociated DNA fragments were then eluted by raising the temperature of the water bath to 100 C and washing the HA with three 18-ml volumes of 0.4 M PB. To insure complete recovery of all material, the HA was dissolved in 5.0 M HCl (18 ml, final volume) and placed in a counting vial. The elution procedure described above was used only for those experiments in which the percentage of reassociated fragments was determined. When it was desired to determine the thermal stability of reassociated DNA fragments, the following elution procedure was used. Polycarbonate tubes, containing HA and the reaction mixtures, were placed in a circulating water bath (Haake, type FT) and washed as described above with five 18-ml volumes of 0.14 M PB at 60 or 75 C, the temperature at which the reaction mixtures were previously incubated for 20 hr. This effectively removed nonreassociated fragments. The temperature of the water bath was then increased in increments of 3 or 5 C to 100 C. At each temperature increment, the HA was washed with one 18-ml volume of 0.14 M PB (two at 100 C). Finally, the HA was dissolved in 5.0 M HCl (18 ml, final volume) and placed in a counting vial. As the temperature of the water bath exceeded the thermal stability of the reassociated DNA fragments, the resultant single strands were eluted from the HA. In this manner, temperature elution profiles were obtained for selected reassociation reactions.

Radioactivity assay. All supernatant fractions and dissolved HA were placed directly into counting vials and assayed for radioactivity by Cerenkov counting (8) in a Packard liquid scintillation spectrometer (model 3310).

Analytical CsCl density gradient analysis. Highly purified DNA extracted from each strain used in this study was examined as described previously (48). A "marker" DNA of predetermined density, *M. lysodeikticus* (ρ = 1.731 g/cm^3), was included in each analysis. Calculations of buoyant density and guanine plus cytosine (GC) content were made by using the relationships of Schildkraut et al. (43).

RESULTS

CsCl density gradient analysis. A knowledge of the overall DNA base composition provides useful preliminary data of an exclusionary nature, for assessing relatedness (29, 32). Comparisons between species possessing different overall DNA base compositions has revealed that little, if any, relatedness exists (10). Although highly related organisms do possess nearly identical DNA base compositions, no prediction of relatedness should be made solely on this basis (29, 32). From the data presented in Table 2, one may reasonably expect that relatedness between species with different DNA base compositions would not be very great. This does not preclude the possible existence of small conserved regions of polynucleotide similarity within the genomes of different species (4).

Quantitative nucleic acid relationships. Factors affecting the extent and specificity of nucleic acid reassociations have been discussed previously (4). The concentrations of labeled and unlabeled DNA fragments contained in the reassociation reaction mixtures were chosen to insure low levels of labeled-labeled fragment reassociation while permitting labeled-unlabeled fragment reassociation to proceed to high levels of completion. The lower of the incubation temperatures (60 C) used in this study was sufficiently high to preclude the formation of spuriously reassociated fragments.

Table 3 presents a compilation of reassociation (binding) studies designed to examine possible polynucleotide sequence similarities between *V. cholerae* strain NIH 35A3 and members of the other *Vibrio* species, and between *V. parahaemolyticus* strain 4 and the other *Vibrio* species. Relative binding values are percentage values normalized to the homologous intraspecific value [(source of radioactive fragments), e.g., NIH 35A3/NIH 35A3] arbitrarily designated as 100%. The thermal binding index is the ratio of relative reassociation (binding) at 75 C and 60 C (4, 33). It is especially useful in detecting the presence or absence of highly related genetic material in interspecific DNA reassociation reactions. A low thermal binding index indicates significant polynucleotide sequence disparity as evidenced by the inability of the majority of those reassociated fragments formed at 60 C to

TABLE 2. *Base composition [moles per cent guanine plus cytosine (GC)] of DNA of Vibrio species*

Organism	Strain no.	Buoyant density[a]	GC[a]
		g/cm³	%
V. cholerae (classical)	NIH 35A3	1.707 ± 0.001	48 ± 1
	NIH 41	1.707 ± 0.001	48 ± 1
	C-401	1.707 ± 0.001	48 ± 1
	VC-9	1.707 ± 0.001	48 ± 1
	ATCC[b] 14035	1.707 ± 0.001	48 ± 1
	P 33/58	1.707 ± 0.001	48 ± 1
V. cholerae (El Tor)	ATCC 14033	1.707 ± 0.001	48 ± 1
	NCTC[c] 2890	1.707 ± 0.001	48 ± 1
	NCTC 6563	1.707 ± 0.001	48 ± 1
	HK-1	1.707 ± 0.001	48 ± 1
	SLH 29803	1.707 ± 0.001	48 ± 1
	2A/62	1.707 ± 0.001	48 ± 1
Non-cholera vibrios	NCTC 4711	1.707 ± 0.001	48 ± 1
	NCTC 4715	1.707 ± 0.001	48 ± 1
	NCTC 4716	1.707 ± 0.001	48 ± 1
	NCTC 8042	1.707 ± 0.001	48 ± 1
	NCTC 30	1.707 ± 0.001	48 ± 1
	S-860	1.707 ± 0.001	48 ± 1
V. parahaemolyticus	1	1.705 ± 0.001	46 ± 1
	4	1.705 ± 0.001	46 ± 1
	5	1.705 ± 0.001	46 ± 1
	13	1.705 ± 0.001	46 ± 1
	15	1.705 ± 0.001	46 ± 1
	17	1.705 ± 0.001	46 ± 1
	21	1.705 ± 0.001	46 ± 1
	23	1.705 ± 0.001	46 ± 1
V. alginolyticus	5144[d]	1.698 ± 0.001	39 ± 1
	5146[d]	1.698 ± 0.001	39 ± 1
	5162[d]	1.698 ± 0.001	39 ± 1
"Marine" vibrios	PS-207	1.702 ± 0.001	43 ± 1
	MP-1	1.700 ± 0.001	41 ± 1
	MB-22	1.707	48 ± 1
	MB-1	1.704 ± 0.001	45 ± 1

[a] Schildkraut, Marmur, and Doty (43).
[b] American Type Culture Collection, Rockville, Md.
[c] National Collection Type Cultures, London, England.
[d] Received as *V. parahaemolyticus* biotype 2.

survive incubation at the more stringent incubation temperature, 75 C.

Several general features of the data in Table 3 should be noted. The "classical" cholera strains were virtually indistinguishable in their relative relatedness to the reference strain, NIH 35A3. Although the El Tor cholera vibrios and the non-cholera vibrios also exhibited a high degree of relatedness to NIH 35A3, the levels of relatedness were more diverse. With the exception of strain NCTC 30, the levels of relative binding were minimally affected by increasing the incubation temperature from 60 to 75 C, indicating a high degree of base sequence complementarity in the reassociated DNA fragments. Similarly, levels of relative binding observed for the *V. parahaemolyticus* intraspecific reassociations were not greatly depressed by incubation at the high temperature.

Only low levels of relative binding were obtained in all interspecific reactions, <25%. At 75 C of incubation, these values were reduced by more than half, indicating a substantial portion of unpaired bases, within the DNA duplexes.

Thermal stability of intra- and interspecific V. cholerae DNA duplexes. The thermal stability of reassociated DNA fragments formed at

TABLE 3. *Nucleic acid reassociation (binding)[a] relationships among Vibrio species*

Nucleic acid reaction	Actual binding (60 C)	Relative binding (60 C)[b]	Actual binding (75 C)	Relative binding (75 C)	Thermal binding index[c]
	%	%	%	%	
V. cholerae NIH35A3[d]/35A3	94 ± 0.4 (4)	100	86 ± 1.0 (5)	100	1.00
V. cholerae NIH35A3[d]/NIH 41	93 ± 0.7 (3)	99	86 ± 0.9 (3)	100	1.01
V. cholerae NIH35A3[d]/P 33-58	93 ± 1.1 (3)	99	90 ± 1.4 (3)	105	1.06
V. cholerae NIH35A3[d]/ATCC 14035	92 ± 1.8 (3)	98	86 ± 1.3 (3)	100	1.02
V. cholerae NIH35A3[d]/VC-9	93 ± 0.6 (3)	99	85 ± 0.9 (3)	99	1.00
V. cholerae NIH35A3[d]/C-401	94 ± 0.5 (3)	100	87 ± 1.2 (3)	101	1.01
V. cholerae NIH35A3[d]/ATCC 14033	82 ± 0.6 (3)	87	71 ± 1.1 (3)	83	0.95
V. cholerae NIH35A3[d]/NCTC 2890	86 ± 0.3 (3)	91	76 ± 1.4 (3)	88	0.97
V. cholerae NIH35A3[d]/NCTC 6563	83 ± 0.8 (3)	88	75 ± 1.3 (3)	87	0.99
V. cholerae NIH35A3[d]/HK-1	91 ± 0.7 (3)	97	82 ± 0.7 (3)	95	0.98
V. cholerae NIH35A3[d]/SLH 29803	90 ± 0.6 (3)	96	82 ± 1.1 (3)	95	0.99
V. cholerae NIH35A3[d]/2A-62	91 ± 0.8 (3)	97	81 ± 2.5 (3)	94	0.97
V. cholerae NIH35A3[d]/NCTC 4711	80 ± 0.4 (3)	85	70 ± 0.9 (3)	81	0.95
V. cholerae NIH35A3[d]/NCTC 4715	81 ± 0.3 (3)	86	73 ± 0.6 (3)	85	0.99
V. cholerae NIH35A3[d]/NCTC 4716	80 ± 0.7 (3)	85	71 ± 0.5 (3)	83	0.98
V. cholerae NIH35A3[d]/NCTC 8042	81 ± 0.9 (3)	86	71 ± 0.1 (3)	83	0.97
V. cholerae NIH35A3[d]/NCTC 30	77 ± 0.8 (3)	82	64 ± 1.1 (3)	74	0.90
V. cholerae NIH35A3[d]/S 860	81 ± 0.7 (3)	86	72 ± 1.0 (3)	84	0.98
V. cholerae NIH35A3[d]/*V. parahaemolyticus* 4	19 ± 0.9 (3)	20	1 ± 0.2 (3)	1	0.05
V. cholerae NIH35A3[d]/*V. parahaemolyticus* 15	16 ± 0.4 (3)	17	0 (3)	0	0.00
V. cholerae NIH35A3[d]/*V. parahaemolyticus* 21	22 ± 0.7 (3)	23	5 ± 0.5 (3)	5	0.22
V. cholerae NIH35A3[d]/5144	4 ± 0.2 (3)	4	0 (3)	0	0.00
V. cholerae NIH35A3[d]/5146	4 ± 0.2 (3)	4	1 ± 0.6 (3)	1	0.25
V. cholerae NIH35A3[d]/5162	12 ± 0.6 (3)	13	5 ± 0.9 (3)	6	0.46
V. cholerae NIH35A3[d]/PS-207	12 ± 0.9 (3)	13	0 (3)	0	0.00
V. cholerae NIH35A3[d]/MP-1	7 ± 0.2 (3)	7	0 (3)	0	0
V. cholerae NIH35A3[d]/MP-22	14 ± 0.5 (3)	15	0 (3)	0	0
V. cholerae NIH35A3[d]/MB-1	5 ± 0.2 (3)	5	0 (3)	0	0
V. parahaemolyticus 4[d]/*V. parahaemolyticus* 4	91 ± 0.8 (3)	100	83 ± 0.5 (4)	100	1.00
V. parahaemolyticus 4[d]/*V. parahaemolyticus* 1	78 ± 0.4 (3)	86	70 ± 0.7 (4)	84	0.98
V. parahaemolyticus 4[d]/*V. parahaemolyticus* 5	80 ± 0.4 (3)	88	71 ± 0.9 (4)	86	0.98
V. parahaemolyticus 4[d]/*V. parahaemolyticus* 13	76 ± 1.0 (3)	84	70 ± 1.2 (4)	84	1.00
V. parahaemolyticus 4[d]/*V. parahaemolyticus* 15	80 ± 1.5 (3)	88	70 ± 0.9 (4)	84	0.95
V. parahaemolyticus 4[d]/*V. parahaemolyticus* 17	74 ± 0.6 (3)	81	68 ± 1.2 (4)	82	1.01
V. parahaemolyticus 4[d]/*V. parahaemolyticus* 21	82 ± 0.3 (3)	90	74 ± 0.6 (4)	89	0.99
V. parahaemolyticus 4[d]/*V. parahaemolyticus* 23	79 ± 0.5 (3)	87	70 ± 1.0 (4)	84	0.97
V. parahaemolyticus 4[d]/NIH 14035	14 ± 1.1 (3)	15	1 ± 0.3 (3)	1	0.07
V. parahaemolyticus 4[d]/ATCC 14033	15 ± 0.6 (3)	16	3 ± 0.1 (3)	4	0.25
V. parahaemolyticus 4[d]/NCTC 4715	13 ± 0.9 (3)	14	1 ± 0.3 (3)	1	0.07
V. parahaemolyticus 4[d]/5144	3 ± 0.3 (3)	3	0 (3)	0	0
V. parahaemolyticus 4[d]/5146	4 ± 0.2 (3)	4	0 (3)	0	0
V. parahaemolyticus 4[d]/5162	7 ± 0.4 (3)	7	2 ± 0.6 (3)	2	0.28
V. parahaemolyticus 4[d]/MB-22	22 ± 0.7 (3)	24	9 ± 0.6 (3)	11	0.46
V. parahaemolyticus 4[d]/PS-207	8 ± 0.5 (3)	9	1 ± 0.1 (3)	1	0.11
V. parahaemolyticus 4[d]/MP-1	4 ± 0.8 (3)	4	0 (3)	0	0
V. parahaemolyticus 4[d]/MB-1	5 ± 0.3 (3)	5	1 ± 0.4 (3)	1	0.20

[a] Reassociation = binding.

[b] Per cent values normalized to the intraspecific reassociation value (e.g., *V. cholerae* NIH 35A3[d]/35A3), arbitrarily designated as 100%. Numbers in parentheses show the number of experiments.

[c] Ratio of relative binding at 75 C to relative binding at 60 C.

[d] Source of radioactive fragments.

various incubation temperatures may be used as an index of the extent and specificity of base pairing. Comparative information may be obtained as to the relative amounts of closely and partially related base sequences between the DNA of one organism and a related one. Previously reassociated DNA fragments are dissociated and eluted by increasing temperature stepwise, and assaying the eluted fractions by measurement of radioactivity. The thermal midpoint of elution $T_{m(e)}$ is defined as that temperature at which 50% of the reassociated fragments have become dissociated by strand separation and eluted. If one determines the thermal denaturation of DNA optically (determination of T_m = 50% hyperchromicity increase), one finds that the

T_m and $T_{m(e)}$ agree closely (4). Studies with synthetic polynucleotides demonstrated that the thermal stabilities of double-stranded nucleic acid complexes are highly sensitive to small proportions of unpaired bases (2, 5). It was estimated that the presence of approximately 1% unpaired bases within a reassociated polynucleotide fragment lowers its thermal stability by 1 C (2).

Table 4 presents the results of thermal stability studies of intraspecific *V. cholerae-V. cholerae* and interspecific *V. cholerae*-non-cholera vibrio DNA duplexes. DNA fragments previously reassociated at 60 or 75 C were dissociated, eluted, and assayed by the methods above. All of the reassociated DNA reaction mixtures had nearly identical $T_{m(e)}$ values with the possible exception of strain NCTC 30, which shows a slightly lower $T_{m(e)}$. Those polynucleotide sequences shared in common between *V. cholerae* NIH 35A3 and each of the other strains are highly similar with respect to nucleotide sequence.

DISCUSSION

It is presumed that the interactions observed at the molecular level, i.e., DNA reassociations, reasonably reflect genetic and phylogenetic relationships among the species examined. Because there are no formal guidelines available within which to correlate genetic and molecular information to taxonomic groupings, caution and the assistance of all available comparative data are required in the interpretation of these data. Since the degree of interspecific DNA reassociation and the stability of these DNA duplexes can vary greatly as the experimental conditions are varied, it is imperative that reaction conditions of sufficient stringency be employed to rule out nonspecific reassociations. In addition, the levels of relative relatedness reported in this study are meaningful only when the assumption is made that all of the organisms tested possess genomes of equal size. Present limitations of the methodology involved in determining genome size, based on the kinetics of DNA fragment reassociation, prevent the detection of differences of at least 10% (D. E. Kohne, *personal communication*). The reasonably good agreement of the levels of relative relatedness obtained in reciprocal reactions between *V. cholerae* and *V. parahaemolyticus* support the assumption of equivalent genome size.

Member species of the genus *Vibrio* were difficult to identify and classify, and several proposed revisions of the genus were made in recent years (14, 44). The long-standing controversy regarding the relationship of the "classical" cholera vibrios to the El Tor vibrios has only recently been resolved with the inclusion of the El Tor strains within the species *V. cholerae* (17, 35). The reluctance of some investigators to accept the fact of the pathogenicity of El Tor vibrios, on the one hand, and the relatively few differential characteristics separating El Tor strains from the classical *V. cholerae* strains, on the other, prolonged the dispute. The extensive phenetic analysis provided by Colwell (13) and the molecular data reported here strongly support the combining of these two groups within a single species.

Recent work with cholera vibrios revealed a more serious problem. The non-cholerae vibrios [NAG (not agglutinable) vibrios, not agglutinable in O group I cholera antisera] include many poorly characterized isolates and even some isolates more appropriately assigned to the genera *Pseudomonas* and *Aeromonas* (1, 7, 21,

TABLE 4. *Thermal elution midpoints ($T_{m(e)}$)[a] of selected reassociation reactions performed at incubation at 60 or 75 C*

Nucleic acid reaction	$T_{m(e)}$ from 60 C incubation	Relative binding	$T_{m(e)}$ from 75 C	Relative binding	Thermal binding index[c]
	C	%	*C*	%	
NIH35A3[b]/NIH35A3	87.8 ± 0.3	100	88.1 ± 0.3	100	1.00
NIH35A3[b]/ATCC14035	88.0 ± 0.4	100	88.2 ± 0.7	98	0.98
NIH35A3[b]/ATCC14033	86.4 ± 0.9	86	87.7 ± 0.6	81	0.94
NIH35A3[b]/NCTC4711	87.1 ± 0.1	85	87.3 ± 0.4	82	0.97
NIH35A3[b]/NCTC4715	87.3 ± 0.4	86	87.7 ± 0.9	83	0.97
NIH35A3[b]/NCTC4716	87.3 ± 0.2	86	87.5 ± 0.2	82	0.97
NIH35A3[b]/NCTC8042	87.2 ± 0.7	86	87.2 ± 0.3	82	0.97
NIH35A3[b]/S860	87.2 ± 0.2	87	87.4 ± 0.2	83	0.95
NIH35A3[b]/NCTC30	85.7 ± 0.7	83	85.4 ± 0.3	71	0.86

[a] Thermal midpoint of elution of DNA duplexes from hydroxyapatite.
[b] Source of radioactive fragments.
[c] Ratio of relative binding at 75 C to relative binding at 60 C.

41); however, the true *Vibrio* cultures of this group pose a real dilemma to the epidemiologists and the public health workers in the field. NAG vibrios are frequently isolated from patients suffering a cholera-like diarrhea, from ground waters in endemic cholera areas, and from asymptomatic carriers (15, 16, 20, 21, 38). An increasing number of reports implicate these organisms as the probable causative organism of diarrheal disease states in man (34, 35). Because of the hemolytic activity of many of these isolates, they have occasionally been classified as El Tor vibrios. Six NCV strains included in this study were isolated from patients suffering diarrheal disease. Phenetic comparisons with *V. cholerae* strains showed high levels of similarity (13). Polynucleotide sequence relationships between *V. cholerae* and these NCV strains were found to be high, 74 to 85% at 75 C. When examined for nucleotide sequence complementarity, by the criterion of thermal stability, these were found to be nearly identical to the *V. cholerae* intraspecific reassociations ($T_{m(e)}$, Table 4). Thus, the proposal that at least some of the NCV strains should be included in the species *V. cholerae* requires some consideration (13). Among the *Enterobacteriaceae*, high levels of genetic relatedness (>80%) are rarely observed in interspecific nucleic acid reassociation studies; the exception being *Escherichia coli-Shigella flexneri* and *Salmonella typhi-S. typhimurium* reactions (4). A recent study of polynucleotide sequence relatedness among different serotypes of *E. coli* reveals levels of DNA relatedness as low as 63 to 76% between some serotypes and as high as 96% for others (D. Brenner, *personal communication*). Therefore, after observing the high phenetic similarity and the high level of genetic relatedness measurable between *V. cholerae* and some of the NCV isolates, serious attention should be given to the inclusion of some of the NCV strains into the species *V. cholerae*.

The isolation of *V. parahaemolyticus* and its wide spread occurrence in the marine environment have generated considerable interest in recent years because of the association of this microorganism with massive outbreaks of gastroenteritis in Japan (18, 19, 39, 40). *V. parahaemolyticus* appears to be readily isolated from coastal waters of Japan and the United States (26, 45). Three strains of *V. alginolyticus*, originally classified as "biotype" 2 of *V. parahaemolyticus* by Sakazaki (42), were found to be minimally related to *V. parahaemolyticus*. Hanaoka et al. (22) reported the base composition of *V. alginolyticus* DNA to be 44.5 moles % GC. Further, these investigators reported 56% relative binding between the DNA of *V. alginolyticus* and *V. parahaemolyticus*, and 20% relative binding between *V. alginolyticus* and *V. cholerae* DNA. In view of this information, it appears most probable that strains of *V. alginolyticus*, including the *V. parahaemolyticus* biotype 2, are not a homogeneous group and require further study. Only low levels of relatedness were observed between *V. parahaemolyticus* and *V. cholerae*, and between *V. parahaemolyticus* and each of the marine vibrios included in this study. Unfortunately, these data could be interpreted to signify the separate generic status of *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus*, and *V. marinus*. Additional work is required to determine whether these microorganisms represent separate genera. *Vibrio* strain MB-22, originally isolated and reported by Tyler et al. (46), revealed somewhat higher polynucleotide relatedness to *V. cholerae* and *V. parahaemolyticus* DNA than that shown by the other marine vibrio species; the higher relationship was observed with *V. parahaemolyticus* DNA. A comparison of the reassociation data obtained from incubation reactions at 60 and 75 C reveals that almost 50% of the DNA duplexes persist at 75 C, whereas the values obtained for the other marine vibrio species with *V. parahaemolyticus* DNA were more drastically reduced (Table 3).

Finally, strain MB-1 is a representative of the species *Pseudomonas piscicida*. Although phenetic relationships measured by numerical taxonomy methods showed a possible generic level of relationship of this microorganism with *Pseudomonas*, subsequent analysis of overall DNA base composition eliminated this possibility, at least for the terrestrial and freshwater forms of *Pseudomonas* since the GC composition of *P. piscicida* strains were found to be approximately 44%. The GC range for *Pseudomonas* DNA is generally accepted to be about 58 to 65% (10, 24). Because of the GC content of *P. piscicida* DNA, it was suggested that these strains could more reasonably be placed in the genus *Vibrio* (23). However, marine strains which possess DNA base composition in the range of 44 ± 2 moles % GC and demonstrate a number of characteristics in common with *Pseudomonas* strains have been isolated (Colwell, *unpublished data*). Miyamoto et al. (36) proposed separate genus status for strains subsequently designated as *V. parahaemolyticus*. Their proposal to create a new genus *Oceanomonas*, however, seems to be based on very tenuous grounds, i.e., salt requirement. It is well known among marine microbiologists that salt requirement and salt dependence (27, 40) are rather variable characteristics of marine strains. Also, taking into account the rather wide range of DNA base compositions displayed

by species presently considered members of a single genus (24), it appears the more pragmatic decision to make is to retain these microorganisms within the genus *Vibrio* but to note their markedly different species status.

V. marinus strains MP-1 and PS-207 were originally described as separate biotypes (9, 11, 13). These strains have little significant relationship either to *V. cholerae* or *V. parahaemolyticus*. The difference in DNA base compositions of these two strains suggests that they are not identical strains (*see* Table 2).

The results of this study provide some valuable insights in the relationships extant among those microorganisms presently classified within the genus *Vibrio*. The precision of the measurement of nucleotide sequence relatedness permits much less arbitrary conclusions to be drawn regarding the classification of these bacteria. Furthermore, by a complementary analysis of phenetic and genetic relatedness, a certain degree of "synergism" is effected, with greater reliability provided to the taxonomic conclusions than if only one approach, i.e., numerical taxonomy or measurement of nucleotide sequence relatedness, was undertaken.

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