Deoxyribonucleic Acid Reassociation Experiments with a Halophilic, Lactose-Fermenting *Vibrio* Isolated from Blood Cultures

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Hollis et al. (J. Clin. Microbiol. 3:425-431, 1976) recently described an apparently new group of bacteria, with characteristics of the genus *Vibrio*, which was isolated from human blood, spinal fluid, and local infections of the extremities, and which caused fatal fulminant septicemia in at least 5 of 38 cases. The organisms were morphologically and biochemically similar to, but not identical with, both *Vibrio alginolyticus* and *V. parahaemolyticus*; they had different isolation sources from those of the latter two species, and they also fermented lactose and possessed different tolerances for sodium chloride in nutrient broth. This paper reports the results of deoxyribonucleic acid (DNA) reassociation experiments, which showed that DNA fragments from six strains of the newly described organisms, incubated at 60°C with DNA fragments from *V. alginolyticus*, *V. cholerae*, and *V. parahaemolyticus*, resulted in DNA duplex formation showing a level of relatedness consistent with that of different species within a genus. All strains of the newly described vibrio were highly related. Incubation at 75°C produced less than 10% relative duplex formation. When tested for heat stability, these duplexes had low temperatures of dissociation, indicating imperfectly matched DNA molecules. We thus concluded that the newly described vibrio is a species distinct from the other species studied.

Recently Hollis et al. (5) described a halophilic vibrio capable of causing fatal septicemia which was isolated chiefly from human blood, spinal fluid, and localized infections of the extremities. The organism differed from *Vibrio parahaemolyticus* in its ability to ferment lactose and in its lower tolerance for sodium chloride in nutrient broth. In addition, it differed from *V. alginolyticus* in its failure to ferment sucrose and to produce acetylmethylcarbinol. Its isolation sources were also different from those of the latter two vibrios. In their study, they referred to this organism as the lactose-positive (L+) *Vibrio*.

We describe deoxyribonucleic acid (DNA) reassociation experiments with strains of this recently characterized vibrio and of other vibrios. Results indicate the (L+) *Vibrio* is indeed a different species, possessing the minimal morphological and biochemical characteristics of the genus (6), but distinct from other species of *Vibrio*.

MATERIALS AND METHODS

Cultures. We chose the *V. alginolyticus*, *V. parahaemolyticus*, and (L+) *Vibrio* cultures from which the DNAs were prepared from the culture collection of the Special Bacteriology Section, Center for Disease Control, Atlanta, Ga. This culture collection is

maintained frozen in defibrinated rabbit blood at -55°C. Table 1 provides a list of cultures used, and gives their different sources of isolation from various geographic areas.

After the culture was thawed, a droplet was streaked on rabbit blood agar and incubated in a candle jar at 35°C for 18 to 24 h. We prepared working stocks by subculturing from blood agar into motility medium (formula given below), incubating at 25°C until they were well grown, and then maintaining them at room temperature (about 25°C) in corked test tubes. These cultures remained viable for months.

Culture media. Rabbit blood agar, i.e., 5% sterile, defibrinated rabbit blood in heart infusion agar (Difco), was used to propagate thawed bacteria.

Motility medium (16 g of motility test medium [Difco], 4 g of nutrient broth [Difco], 1 g of NaCl, and 1 liter of distilled water) was used for maintaining working stock cultures for the experiments. This medium was dispensed as soft agar deeps, 8 ml per 15-by-125-mm tube.

Brain heart infusion broth (BHI; Difco) was used to propagate the cultures for unlabeled DNA preparation.

We modified Citarella and Colwell's medium (4) by substituting acid-hydrolyzed casein for salt- and vitamin-free casein hydrolysate, and used it to grow the culture A8694 of (L+) *Vibrio* for preparation of radiolabeled DNA. Our medium consisted of the following: NaCl, 24.0 g; KCl, 0.017 g; MgCl₂·6H₂O, 5.3 g; tris(hydroxymethyl)aminomethane (Tris),

TABLE 1. *Bacterial strains*

Species	Strain	Source ^a
<i>Serratia marcescens</i>	SM-6	D. J. Brenner, CDC
<i>Vibrio alginolyticus</i>	ATCC 17749 (type culture)	ATCC
<i>Vibrio alginolyticus</i>	CDC-C6814	Leg wound (Georgia)
<i>Vibrio cholerae</i>	CDC-5540-70	Stool (Dacha)
<i>Vibrio cholerae</i> (El Tor)	CDC-1320-72	Stool (Algeria)
<i>Vibrio cholerae</i> (non-agglutinating)	CDC-1845-73	Stool (Texas)
<i>Vibrio parahaemolyticus</i>	ATCC 17802 (type culture)	ATCC
<i>Vibrio parahaemolyticus</i>	CDC-A8633	Ear (New York)
(L+) <i>Vibrio</i>	CDC-A1402	Corneal ulcer (Virginia)
(L+) <i>Vibrio</i>	CDC-A8694	Blood (Florida)
(L+) <i>Vibrio</i>	CDC-B3547	Leg wound (Rhode Island)
(L+) <i>Vibrio</i>	CDC-C7127	Blood (California)
(L+) <i>Vibrio</i>	CDC-C7684	Thumb drainage (Texas)
(L+) <i>Vibrio</i>	CDC-C8806	Blood (Texas)
<i>Yersinia ruckeri</i> (red mouth bacterium)	4535-69	D. J. Brenner, CDC

^a CDC, Center for Disease Control, Atlanta, Ga.; ATCC, American Type Culture Collection, Rockville, Md.

12.1 g; casein hydrolysate (acid) (Nutritional Biochemicals Corp.), 0.5 g; glucose, 5 g; BHI broth, 4 ml; carrier-free $\text{H}_3^{32}\text{PO}_4$ (New England Nuclear Corp.), 10 $\mu\text{Ci/ml}$; distilled water, 1 liter; pH adjusted to 8.0 with HCl.

Reagents. The reagents used included the following: (i) lysing solution: ethylenediaminetetraacetate, 0.05 M; Tris, 0.05 M; NaCl, 0.1 M; Pronase (Calbiochem), 50 $\mu\text{g/ml}$; (ii) phosphate buffer (PB): an equimolar mixture of monobasic and dibasic potassium phosphate, pH 6.8; (iii) liquefied phenol: Mallinkrodt analytical reagent, 88%; (iv) bovine pancreatic ribonuclease (Calbiochem): heated to 80 to 90°C for 10 min to inactivate any contaminating deoxyribonuclease; (v) Pronase (Calbiochem); (vi) ethoxyethanol (Fisher Cellosolve, purified); (vii) scintillation fluid: Econofluor (New England Nuclear Corp., Boston, Mass.); (viii) calf thymus DNA carrier (Sigma Chemical Co., St. Louis, Mo.); (ix) $^{32}\text{PO}_4$ (New England Nuclear Corp., Boston, Mass.); (x) sodium dodecyl sulfate (SDS) (BDH Chemicals, Ltd., Poole, England); and (xi) hydroxyapatite (HA), DNA grade (BioRad Laboratories, Richmond, Calif.).

Preparation of unlabeled DNA. The following is a brief description of the procedure which Brenner et al. (2) discussed in detail. The stock culture was inoculated into 20 ml of BHI broth and incubated at 37°C on a New Brunswick rotary shaker (model G-25, New Brunswick Scientific Co., New Brunswick, N.J.). The total culture was transferred to 1.5 liters of BHI broth and incubated on the shaker at 37°C for 16 h. The cells were sedimented in a refrigerated centrifuge (model RC-3, Ivan J. Sorvall, Norwalk, Conn.) and suspended into 100 ml of lysing solution. SDS was added to a concentration of 0.5%, and the bacteria were lysed in a 37°C water bath for 10 min. After the cell protein was extracted with 100 ml of phenol, the mixture was shaken for 1 min and then centrifuged at 3,000 rpm in a Sorvall model SS-3 centrifuge to separate the aqueous layer containing DNA. The DNA solution was made 1 M with sodium perchlorate. After residual phenol was extracted

with 100 ml of chloroform, the mixture was centrifuged to separate the aqueous DNA layer. The extraction was repeated. The DNA was precipitated with 200 ml of cold, absolute ethyl alcohol, and the precipitate was spooled. The DNA was dissolved in 25 ml of distilled water and 2 drops of chloroform. After the initial ethanol precipitation of DNA, the procedure was repeated twice, each time insuring that the solution was 0.1 M with NaCl. Lysing solution was added to a total volume of 100 ml; contaminating RNA was removed with ribonuclease (50 $\mu\text{g/ml}$) at 37°C for 1 h. Ribonuclease and contaminating byproducts were removed with Pronase (50 $\mu\text{g/ml}$) and 0.5% SDS at 37°C for 1 h. Residual Pronase was extracted with an equal volume of phenol, and the aqueous DNA layer was separated by centrifugation. Residual phenol was extracted with chloroform twice. The DNA was precipitated three times with ethoxyethanol, each time insuring that the solution was 0.1 M with NaCl. The DNA was made 0.28 M with PB. It was then sonically extracted with a Branson Sonifier (Heat Systems, Ultrasonics, Inc., Plainview, N.Y.) at an energy level of 4 for 1.5 to 3 min. The DNA content of the sample was determined with a Beckman spectrophotometer (model Acta III, Beckman Instruments, Inc., Fullerton, Calif.). The DNA solution was stored with 2 drops of chloroform at 4°C.

D. J. Brenner prepared DNAs from *Vibrio cholerae*, *Serratia marcescens*, and *Yersinia ruckeri* (red mouth bacterium) strains and contributed them for these experiments.

Preparation of ^{32}P -labeled DNA. The stock culture of (L+) *Vibrio* strain A8694 was subcultured into 50 ml of BHI broth and incubated for 8 h on a rotary shaker at 37°C. The resulting culture was transferred into 1.5 liters of the modified Citarella-Colwell medium, without phosphate, and incubated for 1 h at 37°C with shaking. A 10-mCi amount of ^{32}P was added to the culture, which was then reincubated with shaking for an additional 16 h. The culture was centrifuged and the labeled DNA was extracted by using the same procedure described

above, except that only 20 ml of lysing solution was added to the sedimented cells, insuring a high concentration of DNA.

After it was purified and sheared, the labeled DNA was denatured at 100°C for 4 min, chilled at 0°C (quenched), passed through an HA column equilibrated with 0.14 M PB plus 0.4% SDS, and held at 60°C. DNA bound to the column was discarded. This procedure reduced the "zero-time binding" (label bound to the column immediately after DNA has been denatured). Recovered single-stranded DNA fragments were stored at 4°C with chloroform.

DNA reassociation. Thermally denatured, labeled DNA fragments (0.1 µg/ml) were incubated at 60 or 75°C with a 1,500-fold excess of denatured, unlabeled DNA fragments in 0.28 M PB for 16 h. After the incubation procedure was completed, the buffer concentration was diluted to 0.14 M, and the DNA mixture was passed through HA to separate single-stranded DNA by the batch procedure of Brenner et al. (3). HA was equilibrated with 0.14 M PB plus 0.4% SDS and held at 60 or 75°C. Four 15-ml portions of 0.14 M PB plus SDS at the incubation temperature removed unreassociated DNA. HA then was washed with four 15-ml portions of 0.4 M PB, the salt concentration at which the reassociated, double-stranded DNA was removed from the HA. All eluates (except those from reassociation experiments in which tritium-labeled DNA from the non-agglutinating *V. cholerae* strain 1845-73 was used) were collected in counting vials for assay. Tritiated samples were precipitated in 5% trichloroacetic acid in the presence of approximately 100 µg of calf thymus DNA carrier. The precipitates were collected on membrane filters (pore size 0.45 µm) (Millipore Corp., Bedford, Mass.), placed in counting vials, and dried in a hot-air oven at 60°C. A 15-ml volume scintillation fluid was added to each sample prior to assay. Radioactivity was assayed in a Packard liquid scintillation spectrometer (model 2450, Packard Instruments, Downers Grove, Ill.).

Determination of approximate base composition from thermal elution temperatures. Thermal elution experiments were carried out with sufficient (10 ml per tube) HA to adsorb 2 mg of native DNA added to the column for each sample. The temperature of the water bath holding the HA columns was increased in 5°C increments from 60 to 80°C, and thereafter in 2°C increments from 80 to 100°C. At each temperature the HA column was washed with one 10-ml portion of 0.14 M PB. After the 100°C portion had been collected, the sample was given a final wash with 0.4 M PB. To determine the amount of DNA eluted from HA, the optical density of each wash was determined with the spectrophotometer at wavelengths of 258 nm and 300 nm. The temperature at which half of the adsorbed DNA was eluted from HA was taken to be the approximate thermal denaturation temperature ($T_{m(e)}$). The formula of Marmur and Doty, $T_m = 69.3 + 0.41$ (guanine plus cytosine [G+C]) (9), was used to determine the approximate G+C base composition. DNA preparations from *S. marcescens* and *Y. ruckeri* were used as standards.

RESULTS

Table 2 shows the degree of reassociation that occurred when radioactive ³²P-labeled DNA fragments from (L+) *Vibrio* strain A8694 were incubated with unlabeled DNA fragments from the same strain, from other strains of (L+) *Vibrio*, and from *V. alginolyticus*, *V. cholerae*, and *V. parahaemolyticus* at two incubation temperatures (60°C, optimal for reassociation, and 75°C, where only highly related DNA duplexes are stable). The degree of reassociation (relative binding) among labeled DNA from strain A8694 and unlabeled DNA from other strains of the same species was between 85 and 93% at 60°C, and between 82 and 92% at 75°C.

Strain A8694 exhibited 25 to 46% relative binding with *V. alginolyticus*, 15 to 32% with *V. cholerae*, and 41 to 48% with *V. parahaemolyticus* at 60°C.

At the more stringent 75°C incubation, less than 10% reassociation occurred among DNA from (L+) *Vibrio* and DNA from all other species tested.

Table 2 also includes results obtained at 60°C with ³H-labeled DNA (supplied by D. J. Brenner) from *V. cholerae* (non-agglutinating) strain 1845-73. The (L+) *Vibrio* strains were 22 to 37% related to strain 1845-73. *V. alginolyticus* showed 40 to 45% binding, *V. parahaemolyticus* showed 46 to 56% binding, and *V. cholerae* showed 85 to 86% binding.

Thermal stability of reassociated DNA fragments formed at various incubation temperatures is an index of the extent and specificity of base pairing. Bautz (1) estimated that the presence of approximately 1% unpaired bases within a reassociated polynucleotide fragment lowers its thermal stability by 1°C. After radioactive ³²P-labeled DNA fragments of (L+) *Vibrio* A8694 were incubated overnight at 60°C with unlabeled DNA fragments from other strains of (L+) *Vibrio*, *V. alginolyticus*, *V. cholerae*, and *V. parahaemolyticus*, the thermal stability of the reassociated DNA duplexes was tested (Table 3).

The $T_{m(e)}$ of homologous A8694 DNA was about 92.5°C (the range was 90.3 to 93.8°C for six trials). The elution temperatures of reassociated DNA from reactions between DNA fragments of A8694 (labeled) and other (L+) *Vibrio* strains all are in the range of 89.7 to 93.3°C. The decrease in thermal elution temperature for heteroduplexes formed ranges from 0.2 to 3.2°C. Thus it appears that those polynucleotide sequences shared in common among various strains of (L+) *Vibrio* are highly similar.

On the other hand, the elution temperatures

TABLE 2. Reassociation among DNA fragments from *Vibrio* sp. (lactose positive) and from other *Vibrio* strains^a

Source of unlabeled DNA	% Binding		
	(L+) <i>Vibrio</i> A8694 ^b		<i>Vibrio cholerae</i> (non-aggl) 1845-73 ^b ; 60°C
	60°C	75°C	
(L+) <i>Vibrio</i> A8694	100 ^c	100 ^d	37
(L+) <i>Vibrio</i> A1402	90	82	24
(L+) <i>Vibrio</i> B3547	85	83	25
(L+) <i>Vibrio</i> C7127	93	86	22
(L+) <i>Vibrio</i> C7684	87	92	22
(L+) <i>Vibrio</i> C8806	87	83	26
<i>Vibrio alginolyticus</i> ATCC 17749	25	6	40
<i>Vibrio alginolyticus</i> C6814	46	7	45
<i>Vibrio parahaemolyticus</i> ATCC 17802	48	8	56
<i>Vibrio parahaemolyticus</i> A8633	41	1	46
<i>Vibrio cholerae</i> 5540-70	16	0	85
<i>Vibrio cholerae</i> (El Tor) 1320-72	15	0	86
<i>Vibrio cholerae</i> (non-aggl) 1845-73	32	4	100 ^e

^a Each reaction was done three or four times at 60°C and twice at 75°C.

^b Source of labeled DNA.

^{c, d, e} Average binding before normalization was (c) 82%; (d) 87%; (e) 78%.

of reassociated DNA fragments from reactions among (L+) *Vibrio* A8694 (labeled) and *V. alginolyticus*, *V. cholerae*, and *V. parahaemolyticus* are considerably lower (71 to 78°C), indicating a higher percentage of unpaired bases.

Marmur and Doty (7) showed that heating DNA in solution produced an increase in its extinction coefficient at the temperature at which the DNA becomes denatured. The midpoint temperature of the absorbance rise is linearly related to the average DNA base composition; a higher G+C content confers a higher thermal stability.

The $T_{m(e)}$ of native DNA from six strains of (L+) *Vibrio* was determined as described in Materials and Methods in order to find the approximate G+C content in those organisms. Marmur and Doty's formula (8) was used to calculate the base composition of the DNA. The G+C content determined ranged from 49.8 to 53.4 mol%, with an average of 51.0 mol% for the six strains.

DISCUSSION

Hollis and co-workers stated (5) that (L+) *Vibrio* not only showed biochemical characteristics which distinguished it from *V. parahaemolyticus* and *V. alginolyticus*, but also pointed out that the sources of these organisms and illnesses caused by them were different. (L+) *Vibrio* was isolated from blood, local infections, and spinal fluid, and appeared more likely to produce localized infections or systemic illnesses; 2 of 60 strains of *V. alginolyticus* and *V. parahaemolyticus* from extraintes-

tinal sources were isolated from blood. In contrast, *V. alginolyticus* has been implicated in wound infections (10), whereas *V. parahaemolyticus* usually causes gastroenteritis. Hollis et al. suggested that the lactose-positive vibrios should be regarded either as a subspecies of *V. parahaemolyticus* or as a possibly new, distinct species.

The genetic evidence presented here indicates that these organisms belong to a species different from *V. parahaemolyticus*, *V. alginolyticus*, and *V. cholerae*. Brenner et al. (2) showed that, in most cases in the family *Enterobacteriaceae*, interspecies duplex formation is 55% or less, whereas that obtained from intraspecies DNA reassociation is 70% or greater. Extrapolating to other bacterial families, we can say that we were dealing with interspecies relationships among the vibrios we tested, because the degree of DNA homology at 75°C incubation among the newly described group and the three known species tested was always less than 10%.

The homology values of DNA from the various strains of (L+) *Vibrio* reassociated at 60 and 75°C were very close, indicating a precise relationship among the sequences of the reassociated strands (Table 2). With 60°C incubation, relative homology between the new species and any one of the other named species mentioned drops to 50% or lower; at the more stringent 75°C incubation, the homology values drop below 10%. Undoubtedly in (L+) *Vibrio* we are dealing with a distinct species of *Vibrio*.

Although the six strains of (L+) *Vibrio*

TABLE 3. Thermal elution midpoints ($T_{m(e)}$) of reassociated DNA fragments from (L+) *Vibrio* and from other *Vibrio* species bound to hydroxyapatite at 60°C incubation

Nucleic acid reaction	$T_{m(e)}$	$\Delta T_{m(e)}$	Relative binding (%)
(L+) <i>Vibrio</i> A8694	92.5		100
(L+) <i>Vibrio</i> A8694			
(L+) <i>Vibrio</i> A8694	90.2	2.7	89
(L+) <i>Vibrio</i> A1402			
(L+) <i>Vibrio</i> A8694	89.7	3.2	90
(L+) <i>Vibrio</i> B3547			
(L+) <i>Vibrio</i> A8694	93.2	0.2	94
(L+) <i>Vibrio</i> C7127			
(L+) <i>Vibrio</i> A8694	92.2	0.8	86
(L+) <i>Vibrio</i> C7684			
(L+) <i>Vibrio</i> A8694	90.7	2.2	87
(L+) <i>Vibrio</i> C8806			
(L+) <i>Vibrio</i> A8694	78.1	14.0	24
<i>Vibrio alginolyticus</i> ATCC 17749			
(L+) <i>Vibrio</i> A8694	75.8	16.9	43
<i>Vibrio alginolyticus</i> C6814			
(L+) <i>Vibrio</i> A8694	76.9	15.2	46
<i>Vibrio parahaemolyticus</i> ATCC 17802			
(L+) <i>Vibrio</i> A8694	74.9	18.0	36
<i>Vibrio parahaemolyticus</i> A8633			
(L+) <i>Vibrio</i> A8694	72.0	20.9	16
<i>Vibrio cholerae</i> 5540-70			
(L+) <i>Vibrio</i> A8694	70.6	22.3	17
<i>Vibrio cholerae</i> (El Tor) 1320-72			
(L+) <i>Vibrio</i> A8694	76.1	16.6	27
<i>Vibrio cholerae</i> (non-aggl) 1845-73			

tested were selected from widely different sources (Table 1), the group was genetically homogeneous. The strains also were alike morphologically (gram-negative rods, motile with a single polar flagellum) and biochemically (oxidase positive; indole positive; nitrate positive; proteolytic; MacConkey positive; urease negative; VP negative; lysine positive; arginine negative; fermenting glucose, lactose, and maltose; and requiring 0.5 to 6.0% NaCl in growth medium).

The G+C content range of 50 to 53 mol% for this new species is somewhat high for the genus *Vibrio* (limits: 40 to 50 mol% G+C [6]). The fact that the experiment was done with the "batch" HA procedure, in which the binding of DNA to HA could delay elution of single-stranded DNA at the precise temperature of disassociation, could account for the higher-than-expected val-

ues obtained. Had the experiment been performed with DNA strands free in solution during heating, a somewhat lower T_m value (and therefore a lower G+C ratio) might have been obtained. To verify the values we obtained, G+C determinations should be made by other means.

With these experiments, we intended to determine genetically the degree of relationship between the (L+) *Vibrio* and *V. alginolyticus* and *V. parahaemolyticus*, to which species it seemed to be closely related biochemically. We did not attempt to examine the position of (L+) *Vibrio* relative to other recognized species in the genus, i.e., *V. anguillarum*, *V. fischeri*, and *V. costicola* (9). Suffice it to say that the (L+) *Vibrio* phenotypically seems even less closely related to these vibrios than to *V. alginolyticus* and to *V. parahaemolyticus*.

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REPRINT REQUESTS

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