JOURNAL OF BACTERIOLOGY, Feb. 1972, p. 696-706

Vol. 109, No. 2
Printed in U.S.A.

Deoxyribonucleic Acid Relationships Among Marine Vibrios

ROBERT S. ANDERSON AND ERLING J. ORDAL
Department of Microbiology, University of Washington, Seattle, Washington 98195

Received for publication 27 October 1971

The deoxyribonucleic acid (DNA) relationships of 80 strains identified either as *Vibrio parahaemolyticus*, *V. alginolyticus*, and *V. anguillarum*, or as allied marine vibrios were delineated by DNA-DNA competition experiments as well as by measuring the thermal stabilities of the DNA-DNA duplexes formed in direct binding studies. The tested strains included isolates from Japan, Europe, and the United States. The *V. parahaemolyticus* and *V. alginolyticus* groups showed an average of 67% homology to one another and 30% to strains of *V. anguillarum*. Significantly, a number of the isolates from the Pacific Northwest which had been previously identified as *V. parahaemolyticus* based on morphological, biochemical, and serological evidence were shown either to be strains of *V. anguillarum* or to belong to as yet unnamed groups. Most strains isolated from diseased salmon in the Pacific Northwest proved to be virtually identical with *V. anguillarum* type C by DNA homology experiments, thereby differentiating them from similar strains isolated from diseased herring and occasionally from salmon. The latter Pacific Northwest isolates fell into two distinct genotypic groups. A plot of the per cent homology by competition versus the difference in the thermal stabilities of heterologous and homologous duplexes ($\Delta T_{m,e}$) between the same DNA species shows a linear decline in homology of 4.25% per degree of $\Delta T_{m,e}$. The use of this relationship for estimating the percentage of the mispaired bases distinguishing DNA preparations directly from competition experiments is discussed.

Vibrios isolated from marine environments have been the subject of considerable recent study (2, 5, 11, 17). One species, *Vibrio parahaemolyticus*, has been of particular interest because of its apparent diverse host range of pathogenicity that includes humans as well as marine invertebrates. In Japan, *V. parahaemolyticus* has been established as the principal agent of gastroenteritis associated with the consumption of raw or partially cooked seafoods (20, 26). In the United States, *V. parahaemolyticus* has been identified as the causative agent involved in mass mortalities of shellfish (12, 24), as well as the cause of severe wound infections in humans (18, 24). In addition to *V. parahaemolyticus*, the related biotype *V. alginolyticus*, a dubious, but possible, cause of gastroenteritis (20), and the fish pathogen *V. anguillarum* (19, 20) have been found in inshore marine environments.

Numerous epizootics due to marine vibrios have occurred in the Pacific Northwest in stocks of young salmon reared in seawater and in natural populations of Pacific herring (19). A number of isolates from different epizootics have been identified as *V. anguillarum*, but others have remained unidentified (11, 17).

Since the recent isolations of *V. parahaemolyticus* from United States waters by several independent investigators (1, 2, 12, 25), certain problems have arisen concerning a practical phenotype-based scheme for the identification and differentiation of *V. parahaemolyticus* from related marine vibrios. Sakazaki's original scheme (21) for the differentiation of *V. parahaemolyticus* from the related biotypes *V. alginolyticus* and *V. anguillarum* was based on the differential ability of these organisms to grow at various salt concentrations, to ferment various sugars, and to produce acetylmethylcarbinol. Subsequent investigations have shown that these tests are not adequate, as there are numerous marine vibrios whose identity remains ambiguous because they do not wholly resemble any one of the three biotypes (23).

Recent attempts to clarify the taxonomic position and relationships within the marine vibrio group have relied on determinative characterization, including Adansonian analysis and deoxyribonucleic acid (DNA)-DNA hybridization experiments as well as an amalgamation of these procedures (4, 5, 8, 11, 17). Hanaoka et al., using the DNA agar hybridization technique, determined the inter- and intraspecific relationships between selected strains of *V. parahaemolyticus* and *V. cholerae* (8). Also included in their study was a cursory survey of a variety of other marine vibrios, including a single strain of *V. alginolyticus* which showed 56% homology to *V. parahaemolyticus*.

Colwell (5) and Citarella and Colwell (4) on the basis of "complete" information, i.e., genetic, biochemical, ecological, etc., also reported on the inter- and intraspecific relationships between strains of *V. parahaemolyticus* and *V. cholerae*. However, the inter- and intraspecific relationships between *V. parahaemolyticus* and the biotype *V. alginolyticus* received only cursory attention, and *V. anguillarum* was not included at all. Further, confusion concerning the actual DNA homology between *V. parahaemolyticus* and *V. alginolyticus* arose as a consequence of their study because they reported less than 10% homology between these strains (4) whereas, as noted above, Hanaoka et al. (8) reported 56%. In acknowledging the discrepancy between their findings and that of Hanaoka et al., Citarella and Colwell suggested that *V. alginolyticus* was not a homogeneous group and that additional study was required.

Kiehn and Pacha, using the DNA agar hybridization technique, determined the DNA homology of a number of strains of pathogenic vibrios isolated from fish epizootics in the Pacific Northwest to the type strain of *V. anguillarum* type A, NCMB 6 (11). They showed that several Pacific Northwest isolates and *V. anguillarum* type C (V-2911) were 80% or more homologous to *V. anguillarum* type A (NCMB 6). However, they did not include type strains of *V. parahaemolyticus* and *V. alginolyticus* in their study.

This study was undertaken to compare the vibrios from epizootics in fish in the Pacific Northwest with known species of vibrios, including strains of *V. anguillarum* from Europe and Japan and Japanese strains of *V. parahaemolyticus* and *V. alginolyticus*, by making inter- and intraspecific comparisons by use of DNA-DNA competition experiments. Subsequently, a number of strains of vibrios isolated in the Pacific Northwest and identified as *V. parahaemolyticus* and *V. alginolyticus* were included in the study (1, 2). The differences in the thermal stabilities ($\Delta T_{m,e}$) were determined between the homo- and the hetero-DNA-DNA duplexes to characterize further these phenotypically similar organisms at the nucleic acid level. The relationship between the $\Delta T_{m,e}$ and the per cent homology as determined by DNA-DNA competition experiments is noted, and a model is proposed calculating the per cent mispaired bases distinguishing DNA species directly from their homology results.

MATERIALS AND METHODS

Bacteria. The strains used in this investigation and their sources are listed in Table 1.

Cultivation. For the isolation of unlabeled DNA, the organisms were grown in 2% Brain Heart Infusion broth (Difco) with 1.5% sodium chloride at *p*H 7.3. A medium containing 0.9% Brain Heart Infusion broth and 1.5% NaCl at *p*H 7.3 was employed for the incorporation of ^{3}H-adenine (New England Nuclear Corp., Boston, Mass.) into the DNA. The specific activity of the six reference DNA preparations was between 2,000 and 3,500 counts per min per μg.

DNA preparation. The cells were suspended in 0.15 M NaCl and 0.015 M sodium citrate (1 × SSC) and lysed by the addition of sodium lauryl sulfate to 1%. After a preliminary extraction with chromatography-grade liquid phenol, the DNA was purified according to the method of Marmur (14).

The competitor DNA preparations, dissolved in 0.1 × SSC, were sheared by passage through a French pressure cell at 0.8 to 1 kilobar, and their concentrations were adjusted to 1.5 mg/ml. The competitor DNA preparations then were denatured by immersion in a boiling-water bath for 5 min, dialyzed at 5 C to 2.2 × SSC. The completed DNA preparations were stored frozen until used.

The labeled DNA preparations were treated as above except that their concentrations were adjusted to 100 μg/ml and their salt concentration remained at 0.1 × SSC.

DNA base compositions. The base compositions of the DNA preparations were determined by their thermal melting points (T_m) by use of an automatic recording spectrophotometer (Gilford Instrument Laboratories). The DNA samples were dialyzed to 1 × SSC and heated at a rate of 30 C/hr. The per cent guanine plus cytosine (GC) was calculated from the equation of Marmur and Doty (16). *Escherichia coli* B DNA was used as the standard.

DNA immobilization. Native DNA (50 μg/ml) was denatured by boiling for 5 min in 0.1 × SSC and was diluted with cold 6 × SSC to 5 μg/ml. Denatured DNA was immobilized either on 15-cm membrane filters (B6, Schleicher & Schuell Co., Keene, N.H.) or 45-mm membrane filters by slow filtration (7). The filters were then dried at room temperature, followed by 2 hr of drying at 80 C in a

TABLE 1. *Organisms employed*

Strain	Source or reference
Vibrio parahaemolyticus	
ATCC 17802	American Type Culture Collection
K-1 pilot (0, 1; K, 1)[a]	Japan, H. Zen-Yoji
K-25 pilot (0, 1; K, 1)	Same as above
K-3 pilot (0, 2; K, 3)	Same as above
K-4 pilot (0, 3; K, 4)	Same as above
K-5 pilot (0, 3; K, 5)	Same as above
T3991-1 (0, 3; K, 7)	Same as above
T3960-1 (0, 4; K, 4)	Same as above
K-10 pilot (0, 4; K, 10)	Same as above
T3937-1 (0, 4; K, 12)	Same as above
T3880-1 (0, 4; K, 13)	Same as above
K-28 pilot (0, 4; K, 28)	Same as above
K-15 pilot (0, 5; K, 15)	Same as above
T3979-1 (0, 5; K, 15)	Same as above
K-17 pilot (0, 5; K, 17)	Same as above
K-18 pilot (0, 6; K, 18)	Same as above
K-19 pilot (0, 7; K, 19)	Same as above
K-20 pilot (0, 8; K, 20)	Same as above
K-21 pilot (0, 8; K, 21)	Same as above
K-22 pilot (0, 8; K, 22)	Same as above
K-23 pilot (0, 9; K, 23)	Same as above
K-24 pilot (0, 10; K, 24)	Same as above
8 1BOH4	Pacific Northwest, J. Baross and J. Liston
7 1BOH4	Same as above
SOY-1 (0, 2; K, 3)	Same as above
SOY-2A (0, 2; K, 3)	Same as above
SOY-11 (0, 2; K, 3)	Same as above
1 POY3A	Same as above
SWA-50 (0, 2; K, 3)	Same as above
AEOY1(7) (0, 4)	Same as above
POYAJ2	Same as above
PCL-5	Same as above
PCL-10	Same as above
BBc115	Same as above
V. alginolyticus	
ATCC 17749	American Type Culture Collection
V-373	Japan, H. Zen-Yoji
V-374	Same as above
V-375	Same as above
V-385	Same as above
V-386	Same as above
V-388	Same as above
V-389	Same as above
V-390	Same as above
V-393	Same as above
5 1BOH	Pacific Northwest, J. Baross and J. Liston
9 28 POB2	Same as above
13 2BhC	Same as above
12 28 POB5	Same as above
20 1BSpC11	Same as above
24 28P1A	Same as above
V. anguillarum	
V-2911	Scotland, I. W. Smith
V-2916	Same as above
NCMB 6	Denmark, J. M. Shewan
2.1	Bowman's Bay, Washington; *Oncorhynchus tshawytscha*
4.3	Same as above
30	Vancouver, Wash.; *Salmo gairdinerii*
73	Same as above
83	Same as above
BB61-b	Bowman's Bay, Wash.; *O. tshawytscha*
PB-1	Japan, S. Egusa
PB-15	Same as above
PB-30	Same as above
PB-34	Same as above
V. piscium NCMB 571	Scotland, J. M. Shewan
V. ichthyodermis NCMB 407	Same as above
V. cholerae	
OGAWA 5596	E. S. Boatman[b]
ElTOR 8457	Same as above[b]
NIH 35A3	R. R. Colwell[b]
E. coli B	Univ. of Wash. Culture Collection
Unnamed strains	
GH-20	Gig Harbor, Wash.; *Clupea pallasi*
GH-10	Same as above
GH-15	Same as above
GH-41	Same as above
BB-3	Bowman Bay, Wash.; *O. kisutch*
BB-6	Same as above
BB-10	Same as above
56	Same as above
62	Gig Harbor, Wash.; *C. pallasi*
63	Same as above
90	Same as above
92	Same as above
94	Same as above
b60	Same as above
b61	Same as above
$b60_2$	Same as above

[a] Serotypes.
[b] DNA provided by J. Baross.

vacuum oven. The dried filters were stored in envelopes in a desiccator at room temperature. Prior to use, 3 by 9 mm filters were cut from the large filters with a Keysort card punch (no. 52-3, McBee Systems). Approximately 8 μg of DNA was immobilized on the 3 by 9 mm filters.

DNA duplex formation. Single-point competition experiments were performed by use of a variation of the Denhardt procedure (6), as previously described by Johnson and Ordal (10). The 3 by 9 mm filters were incubated overnight at 63 C (10, 14) in 2.5-cm vials made from 5-mm glass tubing containing 0.11 ml of reaction mixture topped with several drops of mineral oil.

For the competition experiments, the reaction mixture consisted of 0.01 ml of 0.1 × SSC containing 1.0 μg of denatured labeled DNA and 0.10 ml of 2.2 × SSC containing 150 μg of denatured competitor DNA. The 1:150 ratio of labeled to competitor DNA was more than sufficient to insure that the plateau of maximal competition was reached. The reaction mixture for the thermal denaturation experiments was the same as above, except for the omission of the 150 μg of competitor DNA in the 2.2 × SSC. After incubation, the filters were rinsed and soaked for 5 min in 2 × SSC at 63 C, dried, skewered on pins, and counted in toluene scintillation fluid in a liquid scintillation counter.

The homology values were calculated by dividing the depression in binding caused by 150 μg of the heterologous competitor DNA by the depression in binding caused by the 150 μg of unlabeled homologous DNA. These values, multiplied by 100, are listed as per cent homology.

The thermal denaturation profiles of the duplexes were determined as follows. The filters were washed as above, skewered on pins, and transferred successively through culture tubes (12 by 75 mm) containing 1.0 ml of 0.5 × SSC; they were held for 10 min at each increment of 5 C from 50 through 95 C by use of a Haake water bath. The amount of dissociated labeled DNA for each increment of 5 C was then determined by adding 30 μg of bovine serum albumin and 0.5 ml of 50% trichloroacetic acid. The resulting precipitate was collected on membrane filters (B-6, 27 mm; Schleicher & Schuell Co.), which were dried and counted as above.

The thermal denaturation profiles were computed by plotting the percentage of the summation of counts eluted from the filters at each increment of 5 C. The thermal elution points ($T_{m,e}$) were defined by the temperature at which 50% of the labeled DNA was eluted from the filter. Optical thermal denaturation profiles of the reference DNA preparations were determined in 0.5 × SSC for comparison.

RESULTS

The morphological, nutritional, serological, and ecological similarities of *V. parahaemolyticus*, *V. alginolyticus*, and *V. anguillarum* suggest them to be of a common phylogenetic source. The present study supports this view in that all of the strains tested showed DNA homologies of 30% or more.

Tables 2 and 3 include the names and strain numbers of the organisms, the per cent GC of the reference strains, the per cent DNA-DNA homology to the reference strains as determined by DNA-DNA competition experiments, and, in many instances, the $\Delta T_{m,e}$ values. The organisms were assigned to groups 1 to 5 on the basis of showing 50% or more homology to one of the reference strains, *V. parahaemolyticus* (ATCC 17802), *V. alginolyticus* (ATCC 17749), *V. anguillarum* (V-2911), GH-20, $b60_2$. They were then assigned to the group to which they showed the highest degree of homology.

Group 1 contains 25 of the 34 strains received as *V. parahaemolyticus*. As can be seen in Table 2, all of the strains in group 1 showed 91% homology or more to the reference strain *V. parahaemolyticus* (ATCC 17802). Their close similarity to the reference strain, ATCC 17802, is further emphasized by noting that of the strains tested there was a difference of less than 1 degree C between the $T_{m,e}$ values of the reference strain homoduplexes and those of the heteroduplexes.

Table 1 includes the serotypes of 21 of the Japanese strains of *V. parahaemolyticus* studied. There were one or more K serotype strains from each of the O groups 1 to 10 included in the study, and the results are consonant with those of Hanaoka et al., who reported 100% homology among a similar representative sample using the DNA agar method (8).

Significantly, only 3 of the 12 strains received from Baross as having been isolated from seawater or shellfish from the Puget Sound region and identified as *V. parahaemolyticus* on the basis of morphological, biochemical, and serological evidence showed sufficient DNA homology with ATCC 17802 to be included in group 1. Although these data confirm the presence of *V. parahaemolyticus* in Pacific Northwest waters, they suggest that this strain is not found with the frequency previously reported (1, 2). The remaining nine phenotypically similar strains, as will be discussed below, were either identifiable as strains of *V. anguillarum* or as strains belonging to as yet unnamed or undelineated groups.

The 16 strains of group 2 (Table 2), regarded as *V. alginolyticus*, averaged 67% homology to *V. parahaemolyticus* (ATCC 17802). These data are comparable with the result of Hanaoka et al., who reported 56% homology between these strains employing but a single strain of *V. alginolyticus* in a direct binding study (8).

TABLE 2. *Homology values of DNA preparations to reference strains ATCC 17802, ATCC 17749, and V-374*

Strain	Per cent GC	Reference strains						Group
		ATCC 17802		ATCC 17749		V-374		
		Homology	$\Delta T_{m,e}$[a]	Homology	$\Delta T_{m,e}$[a]	Homology	$\Delta T_{m,e}$[a]	
Vibrio parahaemolyticus								
ATCC 17802	47	100	0	66	9	33	16.25	1
K-1 pilot		100		63				
T3937-1		100		67				
K-22 pilot		98		68				
K-10 pilot		97	0	67	8			
K-18 pilot		97	0	72	8			
K-21 pilot		97		63				
K-24 pilot		97	0	66	9			
K-25 pilot		97		65				
T3880-1		97		64				
T3960-1		97		64				
T3979-1		97		65				
1 POY 3A[b]		97						
K-23 pilot		96		61				
K-19 pilot		95	0.3	61	8			
K-4 pilot		94	0.5	58				
K-15 pilot		94	0.3	67	8			
K-17 pilot		94	0.2	67	8			
K-20 pilot		94	0.3	66	8.7			
K-28 pilot		94	0.5	65	8			
K-3 pilot		92	0.8	60	9			
K-5 pilot		92		62	7.6			
T3991-1		91		65				
8 1BOH4[b]			0					
7 1BOH4[b]			0					
V. alginolyticus								
ATCC 17749	47	67	9	100	0	82	4.6	2
V-373		63		93	1.5	80	5.4	
V-390		67	9	96	0.7	86		
V-385		63		90		83		
V-375		66		89	3.2	81	4.5	
V-393		70		82	5	92	1.0	
V-386		69		84				
V-389		67		84		92		
V-388		68	7	84	4.5	84	4	
V-374		70	7	80	5	100	0	
5 1BOH					3.3		1.7	
9 28POB2					3.2		0.5	
13 2B hC					4.2		0.5	
12 28 POB5					4.3		0.5	
20 1BSpC11					4.4		1.8	
24 28P1A					4.5		1.8	
V-2911	44	31		31				3
GH-20	45	28		29				4
$b60_2$	41	27		32				5
PCL-5[b]	43		17.5		18.2			6
PCL-10[b]	43		17		18			
BBc115[b]	47		17		17			
V. cholerae								
El TOR 8457		24		29	17.5	33	15	
OGAWA 5596		31	16.2	32	17	29	15	
NIH 35A3		33	16.5	30	17	32	15	
E. coli B		5		8				

[a] The $\Delta T_{m,e}$ values were derived from thermal elution profiles run in parallel with the reference strains.
[b] Strains received from J. Baross as *V. parahaemolyticus*.

TABLE 3. *Homology values of DNA preparations to reference strains V-2911, GH-20, and b60₂*

Strain	Per cent GC	Reference strains						Group
		V-2911		GH-20		b60₂		
		Homology	$\Delta T_{m,e}$[a]	Homology	$\Delta T_{m,e}$[a]	Homology	$\Delta T_{m,e}$[a]	
Vibrio anguillarum								
V-2911[b]	44	100	0	26		30		3
V-2916[b]		100						
AEO1 (7)[c]		100						
PB-1		100						
BB61-b		96	0					
SOY-11[c]		96						
2.1[b]		95	0					
4.3[b]		95						
30[b]		95						
73		94						
83[b]		93						
SOY-2A[b]		93						
SOY-1[b]		92						
PB-15		92						
V. piscium								
NCMB 571		92						
SWA50[b]		90						
V. ichthyodermis NCMB 407		90	0.7					
V. anguillarum								
NCMB 6[b]		90	1.0					
PB-39		87						
GH-20	45	30		100		22		4
GH-10				100				
BB-3				100				
56[b]				100				
90				100				
63				98				
GH-15				91				
GH-41				86				
BB-6				86				
b61				85				
92				84				
94				80				
POYAJ2[c]				79				
b60₂	45	29		22		100		5
60[b]						100		
62						99		
BB-10						81		
PC1-5[c]	45		17		16.5		18	6
PC1-10[c]	45		17		17.5		18	
BBc115	43		17		17		18.2	
E. coli B		0		4				

[a] The $\Delta T_{m,e}$ values were determined from thermal elution profiles run in parallel with the reference strains.

[b] Strains common with Pacha and Kiehn (17) and Kiehn and Pacha (11) studies.

[c] Strains received from J. Baross as *V. parahaemolyticus*.

The *V. anguillarum* reference strain, V-2911, and the *V. cholerae* strains were approximately 30% homologous to *V. parahaemolyticus* (ATCC 17802), a result comparable to those reported by Hanaoka et al.

Group 2 encompasses the 16 strains received as *V. alginolyticus*, 6 of which were Puget Sound isolates. These organisms exhibited a broader spectrum of homologies than did the *V. parahaemolyticus* strains of group 1. All of

the tested strains showed 80% or more homology to the reference strain *V. alginolyticus* ATCC 17749, and their $\Delta T_{m,e}$ values ranged from less than 1 to 5 C. Strain V-374, which showed 80% homology and a $\Delta T_{m,e}$ of 5 C to ATCC 17749, was used as a second reference strain (Table 2) in hopes of discriminating the subgroupings of *V. alginolyticus*. The results suggest that this group consists of strains showing a continuum of differentiable homologies between 80 and 100%.

The 18 strains comprising group 3 (Table 3) showed 87% or more homology with the reference strain *V. anguillarum* V-2911. Included in this group are representative strains received as *V. anguillarum* from Japanese and Pacific Northwest sources. Also included in group 3 are *V. piscium* (NCMB 571), *V. ichthyodermis* (NCMB 407), and five strains isolated from Puget Sound which had been considered to be *V. parahaemolyticus* because of their ability to grow in peptone broth plus 7% NaCl, their variable utilization of sucrose, their hemolysis of blood, and their sharing of O and K antigens with *V. parahaemolyticus*.

Group 4 (Table 3) is composed of 12 strains, 10 of which had been isolated from diseased herring during fish epizootics in Pacific Northwest waters. Also included in this group is strain POYAJ2, a Puget Sound isolate which had been considered *V. parahaemolyticus* for the aforementioned reasons.

Group 5 (Table 3) consists of four strains, three of which had also been isolated from herring during epizootics in Pacific Northwest waters but which proved genotypically distinct from groups 3 and 4.

The three strains comprising group 6 are strains of those Puget Sound isolates which had previously been considered to be *V. parahaemolyticus* and which did not show significant homology to any one of the five reference strains employed in this study.

The quantitative relationship of the expressed "homology" between DNA preparations that results from DNA direct binding or DNA-DNA competition experiments and the number of mispaired bases that actually distinguish those DNA preparations has remained unclear. However, Bautz and Bautz (3) and Laird et al. (13) have shown that the five mispaired bases in heteroduplexes can be quantitated to the $\Delta T_{m,e}$ of the heteroduplexes. The latter estimated a 0.7 C $\Delta T_{m,e}$ per 1% mispaired bases. Ullman and McCarthy, in a more recent study (1970), revised this estimate to 1.6 C $\Delta T_{m,e}$ per 1% mispaired bases (*personal communication*). Given Ullman and McCarthy's estimate of 1.6 C $\Delta T_{m,e}$ per 1% mispaired bases, and if the quantitative relationship between $\Delta T_{m,e}$ and DNA direct binding of DNA-DNA competition experiments were known, it would then be possible to estimate the per cent mispaired bases separating two DNA preparations directly from DNA direct binding or DNA-DNA competition studies.

Figure 1 is a plot of the per cent homology as determined in competition experiments with respect to the $\Delta T_{m,e}$ between the same DNA preparations (Table 2), and shows a linear decline of 4.25% homology per degree C of $\Delta T_{m,e}$. Extrapolating to 0% homology shows the maximum $\Delta T_{m,e}$ to be 23.5 C, which is the maximum $\Delta T_{m,e}$ expected given the temperature of incubation employed in this study. The hybridization reaction mixtures employed in this study were incubated at a temperature of 25 C below the T_m of the native reference DNA preparations as determined spectrophotometrically. Figure 2 shows that the T_m of the native reference DNA (*V. parahaemolyticus*

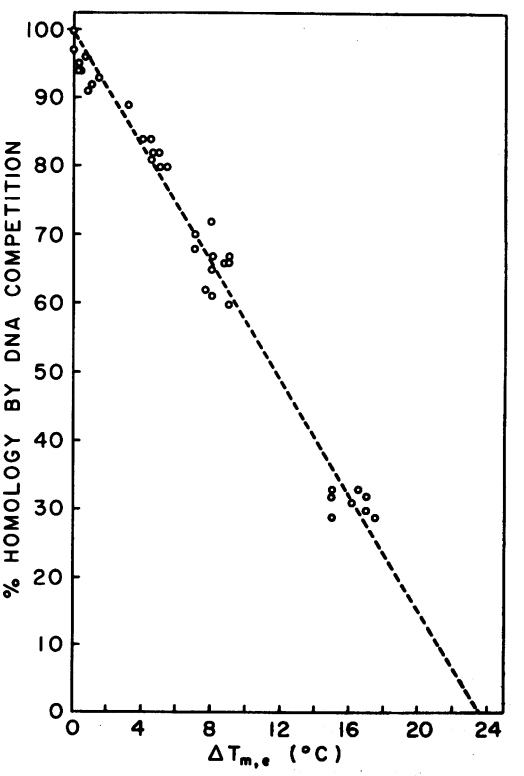

FIG. 1. *Plot of the per cent homology by competition versus the difference in the thermal stabilities of the heterologous and homologous filter-bound duplexes ($\Delta T_{m,e}$) between the same DNA preparations. Conditions of incubation, 2 × SSC and 63 C, were identical in each instance. $T_{m,e}$ of the filter bound DNA duplexes were determined in 0.5 × SSC.*

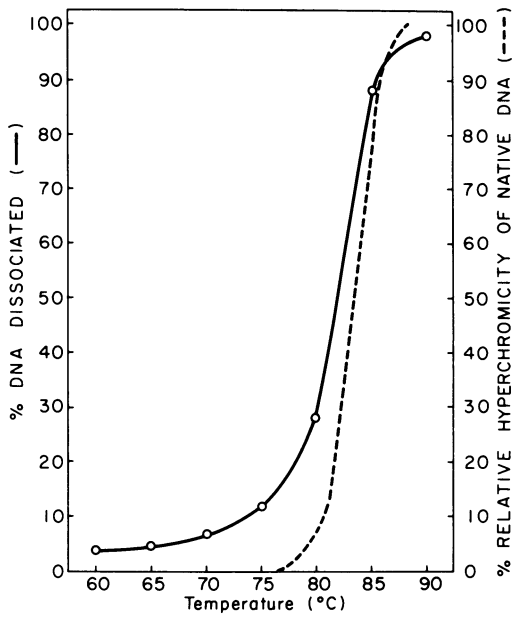

FIG. 2. *Thermal stability of Vibrio parahaemolyticus ATCC 17802 filter-bound DNA duplexes in 0.5 × SSC. The DNA duplexes were formed at 63 C in 2 × SSC. ^{3}H-labeled fragments (3,100 counts per min per μg), 1 μg. Immobilized DNA, 8 μg per 3 by 9 mm filter. Symbols: O, V. parahaemolyticus fragments; dashed line, optical melting profile of native V. parahaemolyticus DNA in 0.5 × SSC.*

ATCC 17802) is 1.5 C higher than the $T_{m,e}$ of its respective homoduplexes. Theoretically, the $T_{m,e}$ should be higher than the T_m for the same DNA, because the $T_{m,e}$ is the measurement of complete strand separation whereas the T_m is the measurement of the hypochromic shift occurring incident to strand separation. However, experimentally the $T_{m,e}$, when membrane filters are used, is generally slightly lower than the T_m of the same DNA. Presumably, this is mainly due to the release of the total DNA from the filters with increasing temperature. Therefore, one would expect the maximal range of $\Delta T_{m,e}$ values for DNA heteroduplexes to be the difference between the temperature of incubation and the $T_{m,e}$ of the reference DNA homoduplexes, in this instance an interval of 23.5 C.

With the use of Ullman and McCarthy's estimate of 1.6 C $\Delta T_{m,e}$ per 1% mispaired bases and our determination of a 4.25% decline in homology per degree of $\Delta T_{m,e}$, it may be computed that there is a 6.8% decline in homology per per cent mispaired bases, or:

$$\% \text{ mispaired bases} = \frac{100 - \% \text{ homology}}{6.8}$$

The reassociation data of Tables 2 and 3 and the above formula are used in Table 4 to summarize the nucleotide divergence among the strains of *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, and the unnamed herring vibrios employed in this study by noting the per cent DNA that did not react with the reference strains, i.e., the per cent DNA differing from the reference DNA by at least 14.7% base pairs, as well as the per cent unpaired bases of that conserved DNA which did cross-react.

DISCUSSION

In summary, all of the Japanese strains received as *V. parahaemolyticus* were isolated from patients afflicted with acute gastroenteritis, and all proved to be virtually indistinguishable from the type strain of *V. parahaemolyticus* (ATCC 17802) by the DNA-DNA comparison procedures employed in this study. The singularity of these strains' homology to the type strain of *V. parahaemolyticus* (ATCC 17802) suggests that Sakazaki's scheme (Table 5) is clinically valuable for accurately identifying *V. parahaemolyticus* when used for discriminating isolates from human sources. However, when classifying isolates from marine environments, the accepted cultural criteria for discriminating *V. parahaemolyticus* from *V. anguillarum*, and from other marine vibrios, are not wholly reliable. The difficulty in accurately classifying these phenotypically similar oganisms is made explicitly evident by noting that only 3 of the 12 strains isolated from marine environments and identified as *V. parahaemolyticus* because of their ability to grow on peptone broth plus 7% NaCl, their variable utilization of sucrose, their hemolysis of blood, and their sharing of O and K antigens with *V. parahaemolyticus* did prove to be *V. parahaemolyticus* when tested by the DNA homology procedures employed in this study. Therefore, in view of this apparent unreliability in the diagnostic key for discriminating *V. parahaemolyticus* from *V. anguillarum* and from other similar marine vibrios, particular caution should be exercised in its use for identifying with finality suspected strains of *V. parahaemolyticus* isolated from marine environments.

Recently, several studies have reported the isolation of *V. parahaemolyticus* from marine environments (1, 2, 12, 25), as well as characterizing *V. parahaemolyticus* as the etiological agent responsible for mortalities in some shellfish (12, 24). The present study suggests that the identification of these strains as *V. parahaemolyticus* should be considered tentative

TABLE 4. *Reciprocal nucleotide sequence divergence among V. parahaemolyticus, V. alginolyticus, V. anguillarum type C, and allied marine vibrios*

Organism	Reference strains									
	V. parahaemolyticus ATCC 17802		*V. alginolyticus* ATCC 17749		*V. anguillarum* V-2911		GH-20		$b60_2$	
	Unrelated DNA	Unpaired bases	Unrelated DNA	Unpaired bases	Unrelated DNA	Unpaired bases	Unrelated DNA	Unpaired bases	Unrelated DNA	Unpaired bases
V. parahaemolyticus strains	0–9	0–1.3	28–42	4.1–6.2						
V. alginolyticus strains	30–37	4.4–5.4	0–20	0–2.9						
V. anguillarum strains	69	10.1	69	10.1	1–13	0–1.9	74	10.8	70	10.3
GH-20 strains	72	10.5	71	10.4	70	10.3	0–21	0–3.1	78	11.5
$b60_2$ strains	73	10.6	68	10	71	10.4	78	11.5	0–19	0–2.8

[a] Results show the per cent unrelated DNA and the per cent unpaired bases of conserved DNA.

TABLE 5. *Differentiation of V. parahaemolyticus from V. alginolyticus and V. anguillarum*[a]

Characteristic	*V. parahaemolyticus*	*V. alginolyticus*	*V. anguillarum*
Growth in 1% Trypticase broth			
with 3% NaCl	+	+	+
with 7% NaCl	+	+	−
with 10% NaCl	−	+	−
Voges-Proskauer reaction	−	+	+ or −
Sucrose fermentation	−	+	+ or −
Cellobiose fermentation within 24 hr	−	−	+
Colony morphology on starch-agar	Smooth	Swarming	Smooth

[a] The differentiation scheme is based on data of Sakazaki et al. (21).

until their speciation is confirmed by DNA homology procedures.

The reciprocal DNA homology experiments between isolates of *V. parahaemolyticus* and *V. alginolyticus* showed them to be 67% homologous to one another. These results sharply contrast with those of Citarella and Colwell, who in a similar study reported less than 10% homology between *V. parahaemolyticus* and *V. alginolyticus* (4). This discrepancy between Citarella and Colwell's findings and those of our study is attributable to their use of distinctly different organisms as representative strains of *V. alginolyticus*. The Citarella and Colwell strains have a GC content of 39% and are clearly distinguishable from the primary reference strain of *V. alginolyticus*, ATCC 17749, employed in this present study which was deposited by Sakazaki with the ATCC as the type strain of *V. alginolyticus* and which has a GC content of 47%. This difference in GC content—8%—as well as the differences in their relative relatedness to *V. parahaemolyticus*—10 and 67%, respectively—indicates that the strains used by Citarella and Colwell as *V. alginolyticus* were misnamed and their homology to *V. parahaemolyticus* and *V. alginolyticus* should consequently be disregarded.

Pacha and Kiehn (17) and Kiehn and Pacha (11) compared the cultural, serological, and DNA homology relationships among a limited sample of pathogenic marine vibrios isolated from fish in the Pacific Northwest with strains of *V. anguillarum*. They concluded that, on the basis of morphological and cultural characteristics, the Pacific Northwest isolates were a homogenous group that were closely related to *V. anguillarum* and divisible by serological analysis into three serotypes. Serotype 1 consisted of organisms isolated from Pacific Northwest salmon; serotype 2, of the European strains of *V. anguillarum;* and serotype 3, of the strains isolated from Pacific Northwest herring. Additionally, Kiehn and Pacha (11) supported the above serological groupings by data derived from DNA competition experiments. The present study confirms and amplifies their DNA homology results, with but few differences. However, the interpretation of their serological groups 1 to 3 as distinguishable genotypic groups is questionable.

Table 3 includes the majority of the strains included in both of the Pacha and Kiehn studies. By the DNA homology techniques employed in this study, those vibrios isolated from salmon (Table 3, group 3) and *V. anguillarum*, type 3 (V-2911), proved to be virtually indistinguishable. This demonstrable synonomy conflicts with the serological findings of Pacha and Kiehn in which they placed these strains into two distinct though similar serological groups. Pacha and Kiehn included the

herring vibrios in a single and distinct serological group. But, as shown (Table 3, groups 4 and 5), the herring vibrios fall into two clearly distinguishable and distantly related genotypic groups. These discrepancies between the serological groupings and the genotypic groupings clearly illustrate the limitations of serological analysis as a taxonomic tool for delimiting genotypic groups and quantitating the relatedness of those groups.

As noted in Table 3, group 3 includes *V. piscium* var. *japonicus* (NCMB 571) and *V. ichthyodermis* (NCMB 407) which show 92 and 90% homology, respectively, to *V. anguillarum* type C (V-2911). Their similarity and relatedness morphologically and nutritionally to *V. anguillarum* was previously reported by Smith (22) and, as the above DNA homology results confirm, these strain names should be changed to *V. anguillarum*. Also included in group 3 is *V. anguillarum* type A (NCMB 6) which shows 90% homology to *V. anguillarum* type C (V-2911) and is comparable to the reciprocal 88% result of Kiehn and Pacha (11).

Studies employing DNA-DNA direct binding and DNA-DNA competition procedures for determining genetic relationships have generally expressed relatedness between DNA preparations as per cent relative binding or per cent homology. Though these procedures have been most useful for determining and expressing the relative relatedness between DNA preparations, the quantitative relationship between such expressions of DNA relatedness and the actual number of base substitutions distinguishing those DNA preparations has remained obscure. However, the formula of

$$\% \text{ mispaired bases} = \frac{100 - \% \text{ homology}}{6.8}$$

developed above allows one to make such an estimate. Of course this formula has only been shown to be useful for homology experiments run under the temperatures and salt concentrations employed in this study. Also, it assumes that the lengths of the genomes being compared are the same, that the distribution of bases along the DNA molecule is random, and that distribution of the bases distinguishing the DNA molecules is also random. This formula is further qualified by the accuracy with which the relationship between the per cent mispaired bases and the $\Delta T_{m,e}$ values is known. The current best estimate, by Ullman and McCarthy, is 1.6 $\Delta T_{m,e}$ per 1% mispaired bases, based on extensive deamination and depurination studies of *E. coli* DNA. However, they qualified their estimate by noting that it was unlikely that such studies on chemically altered base pairs could provide the precise solution to the problem of delineating the relationship between $\Delta T_{m,e}$ and per cent mispaired bases. This is because the mispaired bases found in the heteroduplexes formed between deaminated or depurinated and unaltered DNA are not the same as, nor are they wholly analogous to, those mispaired bases found in the heteroduplexes of unaltered DNA. Furthermore, since their estimate is based on studies in which *E. coli* DNA (50% GC) was used, the answer to the question of whether it is accurate for DNA of other GC contents is not known. However, they did show that alkali deamination of DNA of low GC, 33% (*Chondrococcus columnaris*), middle GC, 43% (*Bacillus globigii*), and high GC, 64% (*Pseudomonas aeruginosa*), had about the same effect on the thermal stability of the DNA per 1% altered base pairs as that found for *E. coli* DNA. This suggests that their estimate may be reasonable for bacterial DNA preparations regardless of GC content.

ACKNOWLEDGMENTS

We thank John Baross for his generosity in providing the DNA from some of his isolates included in this study. Our thanks also to Robert Bigford, Dudley Eirich, and Joyce Durnford for technical assistance.

This investigation was supported by the Atomic Energy Commission under contract AT(45-1)-2225, Task agreement no.10.

LITERATURE CITED

1. Baross, J., and J. Liston. 1968. Isolation of *Vibrio parahaemolyticus* from the Northwest Pacific. Nature (London) **217**:1263-1264.
2. Baross, J., and J. Liston. 1970. Occurrence of *Vibrio parahaemolyticus* and related hemolytic vibrios in marine environments of Washington State. Appl. Microbiol. **20**:179-186.
3. Bautz, E. K. F., and F. A. Bautz. 1964. The influence of noncomplementary bases on the stability of ordered polynucleotides. Proc. Nat. Acad. Sci. U.S.A. **52**:1476-1481.
4. Citarella, R. V., and R. R. Colwell. 1970. Polyphasic taxonomy of the genus *Vibrio:* polynucleotide sequence relationships among selected *Vibrio* species. J. Bacteriol. **104**:434-442.
5. Colwell, R. R. 1970. Polyphasic taxonomy of the genus *Vibrio:* numerical taxonomy of *Vibrio cholerae*, *Vibrio parahaemolyticus*, and related *Vibrio* species. J. Bacteriol. **104**:410-433.
6. Denhardt, D. T. 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641-646.
7. Gillespie, D., and S. Speigelman. 1965. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J. Mol. Biol. **12**:829-842.
8. Hanaoka, M., V. Kato, and T. Amano. 1969. Complementary examinations of DNA's among *Vibrio* species. Biken's J. **12**:181-185.
9. Horie, S., K. Seheki, and M. Okuzuma. 1967. Quantitative enumeration of *Vibrio parahaemolyticus* in sea

and estuarine waters. Bull. Jap. Soc. Sci. Fish. **33**:126–130.
10. Johnson, J. L., and E. J. Ordal. 1968. Deoxyribonucleic acid homology in bacterial taxonomy: effect on incubation temperature on reaction specificity. J. Bacteriol. **95**:893–900.
11. Kiehn, E. D., and R. E. Pacha. 1969. Characterization and relatedness of marine vibrios pathogenic to fish: deoxyribonucleic acid homology and base composition. J. Bacteriol. **100**:1248–1255.
12. Krantz, G. E., R. R. Colwell, and E. Loveliace. 1969. *Vibrio parahaemolyticus* from the blue crab *Callinectes sapictus* in Chesapeake Bay. Science **164**:1286–1287.
13. Laird, C. D., B. L. McConaughy, B. J. McCarthy. 1969. Rate of fixation of nucleotide substitution in evolution. Nature (London) **224**:149–154.
14. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3**: 208–218.
15. Marmur, J., and P. Doty. 1961. Thermal renaturation of deoxyribonucleic acids. J. Mol. Biol. **3**:585–594.
16. Marmur, J., and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. **5**:109–118.
17. Pacha, R. E., and E. D. Kiehn. 1969. Characterization and relatedness of marine vibrios pathogenic to fish: physiology, serology, and epidemiology. J. Bacteriol. **100**:1242–1247.
18. Roland, F. P. 1970. Leg gangrene and endotoxin shock due to *Vibrio parahaemolyticus* an infection acquired in New England coastal waters. N. Engl. J. Med. **282**: 1306.
19. Rucker, R. R., B. J. Earp, and E. J. Ordal. 1953. Infectious diseases of Pacific salmon. Trans. Amer. Fish. Soc. **83**:297–312.
20. Sakazaki, R. 1969. Halophilic vibrio infections. Foodborne infections and intoxications. Academic Press Inc., New York.
21. Sakazaki, R., S. Iwanami, and H. Fukumi. 1963. Studies on the enteropathogenic, facultatively halophilic bacteria, *Vibrio parahaemolyticus*. I. Morphological, cultural and biochemical properties and its taxonomical position. Jap. J. Med. Sci. Biol. **16**:161–188.
22. Smith, I. W. 1961. A disease in finnock due to *Vibrio anguillarum*. J. Gen. Microbiol. **24**:247–252.
23. Twedt, R. M., P. L. Spaulding, and H. E. Hall. 1969. Morphological, cultural, biochemical, and serological comparison of Japanese strains of *Vibrio parahaemolyticus* with related cultures isolated in the United States. J. Bacteriol. **98**:511–518.
24. Vanderzant, C., R. Nickelson, and J. C. Parker. 1970. Isolation of *Vibrio parahaemolyticus* from Gulf Coast shrimp. J. Milk Food Technol. **33**:161–162.
25. Ward, B. Q. 1968. Isolations of organisms related to *Vibrio parahaemolyticus* from American estuarine sediments. Appl. Microbiol. **16**:543–546.
26. Zen-Yoji, H., S. Sakai, T. Ferayama, Y. Kudo, T. Ito, M. Benoki, and M. Nagasaki. 1965. Epidemiology, enteropathogenicity and classification of *Vibrio parahaemolyticus*. J. Infec. Dis. **115**:436–444.