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COMPLEMENTARY EXAMINATION OF DNA'S AMONG VIBRIO SPECIES[1]

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SUMMARY The DNA-DNA hybridization test was performed by the DNA-agar method on strains of *Vibrio cholerae El Tor*, *V. parahaemolyticus* and other vibrios. On the basis of the results the tested strains were separated into at least three groups, namely, a *V. cholerae El Tor* group, a *V. parahaemolyticus* group and a *V. costicolus* group.

INTRODUCTION

Chemical analyses of DNA, analysis of purine and pyrimidine bases (GC content) (Lee et al., 1956, Vendrely, 1958) and DNA-DNA hybridization (McCarthy et al., 1963) have recently developed as useful tools for bacterial taxonomy.

Sebald et al. (1963) reported that the GC content of vibrios ranges from 44 to 50%, and Colwell et al. (1968) differentiated *V. cholerae*, a marine *Vibrio* species from *V. parahaemolyticus* by their GC contents: *V. cholerae* had the highest GC content (47–48%), *V. parahaemolyticus* an intermediate content (46%) and *Vibrio* species the lowest (44–45%). This difference in the GC contents of these bacteria was too small considering the reproducibility of the estimation procedure, so we carried out DNA-DNA hybridization following the method of Bolton et al. (1962) to obtain further information on the extent of DNA homology among various vibrios.

MATERIALS AND METHODS

1. *Organisms*

Twenty-nine strains were used. *Vibrio costicolus* NCMB 701, *V. anguillarum* NCMB 829, *V. ichthyodermis* NCMB 1291, *V. piscium* NCMB 571, *V. metchnikovii* IAM 1039, *V. tyrogenus* Deneke, *Vibrio* sp. NCTC 4711 (Gardner & Venkatraman group III), *Vibrio* sp. NCTC 4716 (Gardner & Venkatraman group VI), *Pseudomonas aeruginosa* Iishima and *Aeromonas hydrophila* AB 364 were obtained from Mr. K. KOTERA, Research Institute for Microbial Diseases, Osaka University. *Vibrio parahaemolyticus* (Fujino et al., 1951, Sakazaki et al., 1963), strain Takikawa A55 (O5, K 15) and strain 8–3 (O1, K32), the 10 pilot strains of O antigen

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group 1–10 of *V. parahaemolyticus*, *V. alginolyticus*, *V. cholerae El Tor* and *Escherichia coli* B were given by Dr. G. OMORI, Osaka City Institute of Hygiene. Four strains, 11–1, 15–2, 21–1 and 73–2 were picked up in this study from stocks of strains isolated during examination of cases of food poisoning following the standard method of the Ministry of Welfare, Japan, for *Vibrio parahaemolyticus* (1963)[2]. These strains were suspected to be the causative agents of food poisoning. Strain 73–2 differs from other strains of *V. parahaemolyticus* only in that it gives positive Voges-Proskauer test. As these strains could not be typed serologically using antisera against 42 K antigens of *V. parahaemolyticus*, they are termed serologically untypable *V. parahaemolyticus*.

2. *Culture*

The growth medium employed throughout consisted of tris-HCl 0.01 M, $MgCl_2$ 0.001 M, KH_2PO_4 0.0005 M, KCl 0.04 M, NaCl 0.25 M and Difco Bacto-peptone 0.2%, pH 8.0. For isolation of ^{32}P labeled DNA, 1 mc of carrier free ^{32}P was added to 100 ml of each culture and cultures were incubated for more than three generations. Most organisms were cultured at 30 C with shaking, but *V. parahaemolyticus* and *V. alginolyticus* were grown at 37 C.

3. *Preparation of DNA*

Highly polymerized DNA was prepared by Marmur's method (Marmur, 1961).

4. *GC content*

Purified DNA was digested in 88% formic acid at 175 C for 30 min. (Wyatte et al., 1953). The four bases were separated by column chromatography by the method of Cohn (1949).

5. *DNA-agar method*

The procedures used were those of Bolton et al. (1962) and McCarthy et al. (1963). We usually mixed 0.3 ml of solution containing 10 μg of ^{32}P labeled DNA fragments dissolved in double strength SSC (SSC consists of 0.15 M NaCl and 0.015 M Na-citrate) with 0.3 g of DNA-agar containing 100 μg of DNA and incubated the mixture at 60 C for 20 hr. After incubation, unbound DNA fragments were eluted with double strength SSC at 60 C and bound DNA was eluted with 1 : 100 SSC at 70 C. Each fraction was collected and counted in a Nuclear Chicago liquid scintillation spectrometer.

2 The standard method of the Ministry of Welfare, Japan, for *Vibrio parahaemolyticus*. published by the Ministry of Welfare, Japan (1963).

RESULTS

The extent of homology of the DNA's of various vibrios is shown in Table 1.

Assuming that strains showing more than 50% cross-reactivity can be regarded as belonging to the same group, most of the strains tested can be distinguished into three groups. Group 1 includes *Vibrio cholerae El Tor*, *V.* sp. NCTC 4711 and *V.* sp. NCTC 4716. Group 2 includes *V. parahaemolyticus*, *V. alginolyticus* and the three serologically untypable strains of *V. parahaemolyticus*, 11–1, 15–2 and 21–1. Group 3 includes *V. costicolus*, *V. anguillarum*, *V. ichthyodermis*, *V. piscium* and *V. metchnikovii*. Two strains, *V. tyrogenus* and strain 73–2 of serologically untypable *V. parahaemolyticus* cannot be included in any of these groups.

In contrast to the DNA-DNA hybridization values, the GC contents of one or two strain(s) of Groups 1, 2 and 3 were quite similar to each other, as shown in Table 1.

V. sp. NCTC 4711 and *V.* sp. NCTC 4716 were both more like *V. cholerae El Tor* than *V. parahaemolyticus* A55 and *V. costicolus*.

So, it seems that these strains belong to a group distinguishable from other vibrios.

As shown in Table 2, respective pilot strains of O antigen groups 1–10 of *V. parahaemolyticus* showed 100% cross-reactivity to DNA of A55 (O 5). *V. alginolyticus* showed 56% cross reaction with *V. parahaemolyticus* and less with *V. cholerae El Tor* and *V. costicolus*, so we include it in the same groups as *V. parahaemolyticus*. Three of the four serologically untypable strains of *V. parahaemolyticus*, 11–1, 15–2 and 21–1, gave more than 50% cross reaction with DNA of A55, and less cross reaction with *V. cholerae El Tor- or V. costicolus*-DNA, so we include them in Group 2.

The DNA's of *V. anguillarum*, *V. ichthyodermis*, *V. piscium* and *V. metchnikovii* cross

TABLE 1 *Binding of DNA fragments of various strains to three DNA-agars*

Source of ^{32}P-DNA \ Source of DNA-agar	% DNA bound relative to homologous DNA			Group	GC (%)
	V. parahaemolyticus A55	*V. cholerae El Tor*	*V. costicolus*		
Vibrio cholerae El Tor	25	100	31	1	NT[a]
Vibrio sp. NCTC 4711	24	100	31	1	46.8
Vibrio sp. NCTC 4716	24	100	33	1	NT
Vibrio parahaemolyticus A55 (O 5, K 15)	100	25	30	2	46.0
8–3 (O 1, K 32)	100	23	23	2	NT
11–1 (untypable)	83	23	26	2	NT
15–2 (untypable)	90	27	21	2	NT
21–1 (untypable)	55	30	23	2	NT
73–2 (untypable)	21	21	19	?	NT
Vibrio alginolyticus	56	20	20	2	44.5
Vibrio costicolus NCMB 701	22	31	100	3	NT
Vibrio anguillarum NCMB 829	25	26	80	3	46.0
Vibrio ichthyodermis NCMB 1291	24	28	82	3	NT
Vibrio piscium NCMB 571	30	38	90	3	45.7
Vibrio metchnikovii IAM 1039	21	31	95	3	NT
Vibrio tyrogenus Deneke	25	34	24	?	NT
Pseudomonas aeruginosa Iishima	6	8	8		NT
Aeromonas hydrophila AB 364	7	9	13		NT
Escherichia coli B	4	1	1		NT

a NT; not tested.

TABLE 2 *Binding of DNA fragments of the pilot strains of O group of V. parahaemolyticus to A55 DNA-agar*

O group	K antigen	% DNA bound relative to A55[a] DNA
1	1	100
2	2	100
3	4	100
4	8	100
5	14	100
6	18	100
7	19	100
8	20	100
9	23	100
10	24	100

a A55 (O 5, K 15).

reacted more or less equally well with *V. costicolus*-DNA but less with *V. parahaemolyticus*- or *V. cholerae El Tor*-DNA. Accordingly these four strains were included in Group 3.

The DNA of *V. tyrogenus* cross-reacted less with the three DNA-agars, so, this strain may not fit into any of these three groups. To test this, further studies must be made with its DNA-agar. The serologically untypable *V. parahaemolyticus* strain 73–2 showed a similar cross reaction pattern to *V. tyrogenus*, but the relationship between the two is not yet clear.

Pseudomonas aeruginosa, *Aeromonas hydrophila* and *Escherichia coli* scarcely cross-reacted with vibrios. This can be understood by the difference of the GC contents of their DNA's.

DISCUSSION

Our DNA-DNA hybridization data show that various strains of the genus *Vibrio* can be genetically classified into three groups. These groups are very different from each other, giving only 20–30% cross reaction, although the difference in the GC contents of some of these bacteria is very small relative to the accuracy of the experimental procedure. These data suggest that a similarity in GC contents of bacteria does not necessarily mean that the bacteria have a close genetic relationship.

Basden et al. (1968) also pointed out that the similar GC contents of *Vibrio* species was not necessarily associated with a close genetic relationship in DNA-RNA hybridization experiments on two *Vibrio* species of avian origin with 32% GC content. The RNA of one strain showed only 48% of the cross-reactivity of that of the second strain to the DNA of the second strain. However, they did not report DNA-DNA hybridization data. Hoyer et al. (1968) observed a similar phenomenon in *Brucella* in the competition test of DNA-DNA hybridization.

It is also noteworthy that the cross-reactivity between the three groups of vibrios is much less than that between related genera of *Enterobacteriaceae*: *i.e.*, 70–71% between *E. coli* and *Shigella dysenteriae* or *Salmonella typhimurium*, and 45–60% between *Aerobacter aerogenes* and *E. coli*, *Sh. dysenteriae* or *S. typhimurium* (McCarthy et al., 1963).

The data on *V. parahaemolyticus* are not clear-cut. The extents of cross reaction of its DNA with those of *V. cholerae El Tor* and *V. costicolus* were quite similar, ranging from 19 to 30%, in all the strains tested. However, the extents of cross reaction of DNA of strains within the same species to DNA of strain A55 varied widely from 21 to 100%. The serologically typable strains, respective pilot strains of the O antigen groups and strain 8–3 (O1, K32) showed 100% cross-reactivity. On the other hand, the serologically untypable strains, 11–1, 15–2, 21–1 and 73–2, showed varying degrees of cross-reactivity (83, 90, 55 and 21%, respectively). Strain 73–2 differs from other strains only in its positive Voges-Proskauer test, but it can hardly be regarded as a distinct species from its other biological characters. The other three strains are indistinguishable from serologically typable strains by their biological characters.

DNA of *V. alginolyticus* showed 56% cross-reaction to that of strain A55, and hence it is tentatively included in the same group as *V. parahaemolyticus*.

Although strain 73–2 should be regarded as a member of the *V. parahaemolyticus* group from its biological characters, it shows a distinct difference in DNA homology from serologically typable strains and the difference is much larger than that between *V. alginolyticus* and serologically typable *V. parahaemolyticus*. Although strain 73–2 and *V. tyrogenus* gave similar extents of cross-reaction with the three DNA's, they may differ if tested against the DNA-agar of one of them.

Since the extent of DNA homology of serologically untypable *V. parahaemolyticus* varies widely, further studies on more strains should be performed.

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