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Morphological, Cultural, Biochemical, and Serological Comparison of Japanese Strains of *Vibrio parahemolyticus* with Related Cultures Isolated in the United States

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Morphological, cultural, biochemical, and serological characteristics of 79 strains of *Vibrio parahemolyticus* isolated from patients suffering from gastroenteric disease in Japan were compared with 17 suspected *V. parahemolyticus* cultures isolated from wound infections and 14 nonpathogenic vibrios isolated from an estuarine environment in the United States. These groups were differentiated on the basis of several key reactions which included: the range of growth temperature and salt tolerance; the production of catalase and acetoin; the hydrolysis of starch; the fermentation and utilization as single carbon source of sucrose, cellobiose, and arabinose; and the ability to swarm on 1% agar. The separation of the groups on the basis of cultural and biochemical analyses was confirmed by means of slide agglutinations with specific anti-K antisera. The results of this study strongly suggest that the wound infection isolates are *V. parahemolyticus* species which are easily distinguished from the nonpathogenic estuarine vibrios.

The halophilic microorganism *Vibrio parahemolyticus* has been implicated as the causative organism responsible for 70% of the outbreaks of gastroenteritis in Japan (19). Since the first report of Fujino et al. (4), Japanese workers have made numerous investigations of *V. parahemolyticus*. The organism has been isolated from stools of patients and from sea fish and sea fish products, as well as from sea water (20). In addition to its seasonal occurrence in the coastal waters of Japan (16), the halophile has been found in sea water in the continental United States, Germany, the Far East and Hawaii (1, 18, 19, 24, 25). Although the enteropathogenicity of *V. parahemolyticus* isolated from patients with enteritis has been established (26), the pathogenic significance of related species, such as *V. alginolyticus* and *V. anguillarum*, which are primarily isolated from sea water and sea fish, is doubtful.

Recent isolations of suspected *V. parahemolyticus* from localized tissue infections acquired by individuals living in coastal regions of the United States pose new problems. These organisms may be enteropathogens with an altered route of entry, or "nonpathogenic" marine vibrios with a previously unsuspected virulence.

The present study was undertaken to clarify the relationship of these isolates from marine bathing beach infections with enteropathogenic and nonpathogenic vibrios. Therefore, a morphological, cultural, biochemical, and serological comparison was made among a group of tissue-infection *Vibrios*, a group of known *V. parahemolyticus* strains of gastroenteric origin from Japan, and a group of nonpathogenic vibrios from the estuarine environment of Chesapeake Bay.

MATERIALS AND METHODS

Cultures examined. A total of 110 cultures (Table 1) were used for the present studies. The first group of 79 *V. parahemolyticus* included 78 strains isolated in Japan from feces of patients suffering from gastroenteritis or from food implicated in food poisoning outbreaks. These strains represented all known K antigens with the exception of K-22 antigen. One strain, OY-G_1-3, isolated from Puget Sound, was identified as *V. parahemolyticus* by specific bacteriophage susceptibility (1). The *V. parahemolyticus* group was obtained from the following donors: K. Aiso, Institute of Food Microbiology, Chiba University, Chiba, Japan; Riichi Sakazaki, National Institute of Health, Tokyo; Sanko Junyoku Co., Tokyo; Y. Miyamoto, Kanagawa Prefectural Public Health Laboratory, Yokohama; R. Weaver, Communicable Disease Center, Atlanta, Ga.; and J. Liston, Univer-

TABLE 1. *Culture origin and designation of vibrios*

Strain	Strain designation	Source	Donor
Group I			
KA	K1, K4, K8, K18, K19, T2194 (K6)	Seawater, sea fish, patients' stools; Japan	K. Aiso
RS	K1, K3, K4, K6, K8, K12, K13, K15, K18, K19, K20, K23, K25, K28	Seawater, sea fish patients' stools; Japan	R. Sakazaki
SJ	K1, K18, K20, K21, K23, K32, T2450 (K5), HP838 (K7), HP792 (K29), T1965 (K30), T2081 (K30)	Seawater, sea fish, patients' stools; Japan	Sanko Junyoku Co.
YM	K33-K46	Seawater, sea fish, patients' stools; Japan	K. Miyamoto
CDC	9234–9235	Seawater, sea fish, patients' stools; Japan	R. Weaver
SAK	1–4, 13	Seawater and sediment; Japan	J. Liston
JL	4, 15	Patients stools; Japan	J. Liston
JL	OY-G_1-3	Puget Sound oyster; U.S.	J. Liston
Group II			
CDC	A8633, A5704, A6202, A8198, A6670, A4871, A5002, A7606, A3637, A3454, A1889, A8694, A6614, A1334	Patient isolates; U.S.	R. Weaver
CDC	A6540	Fish isolate; U.S.	R. Weaver
	A4280	Bovine isolate; U.S.	R. Weaver
	A4281	Rat isolate; U.S.	R. Weaver
Group III			
CB	34, 62, 64	Chesapeake Bay water; U.S.	R. Colwell
	25, 137, 139, 166	Chesapeake Bay mud; U.S.	R. Colwell
	1, 4, 15, 47, 116, 153	Chesapeake Bay oysters; U.S.	R. Colwell
MB	22	Chesapeake Bay water; U.S.	R. Colwell

sity of Washington, Seattle. A second group of 17 suspected *V. parahemolyticus* included 14 cultures isolated from localized tissue infections of individuals living in the United States. These infections were apparently acquired by swimmers and others in recreational contact with the marine environment. These strains were cultivated from blood, sputum, discharge from eye and ear, and lesions of wrist, leg, and foot. Two additional animal isolates and a fish isolate resembled the previous 14. This group of cultures was obtained from R. Weaver. A third group included 14 nonpathogenic *Vibrio* cultures isolated from Chesapeake Bay by R. Colwell, Georgetown University, Washington, D.C.

Culture maintenance. Unless otherwise stated, the following conditions applied throughout this study. Stock cultures of all strains except CB and MB were grown at 37 C on Trypticase Soy Agar (BBL) plus 2.5% NaCl. The CB and MB strains were somewhat more nutritionally demanding and did not grow well on this medium. They were therefore grown at 30 C on maintenance medium (CM) with the following composition: 2.4% NaCl, 0.07% KCl, 0.53% $MgCl_2$, 0.7% $MgSO_4 \cdot 7H_2O$, 0.3% Yeast Extract (Difco), 1.0% Proteose Peptone (Difco), and 1.5% agar (Difco), at *p*H 7.2 to 7.4 (R. Colwell, *personal communication*). Incubation was for 18 to 24 hr in screw-cap tubes. Stock cultures were maintained on agar slopes at room temperature.

Agar and broth media for biochemical tests were prepared by making appropriate additions to the maintenance media. Individual carbohydrates and organic and amino acids for fermentation and utilization tests were obtained from BBL, Difco, Nutritional Biochemicals Corp., Cleveland, Ohio, and Calbiochem, Los Angeles, Calif.

Determination of morphological characteristics. Cell form and gram-reaction were studied from growth on maintenance media. Flagella were observed in stained preparations as follows. Each culture was point-inoculated onto agar plates of semisolid motility medium (7). Growth from the leading edge of an 18–hr culture was transferred to maintenance broth and incubated for 5 hr. A loopful of broth culture was placed on a slide, and a smear was prepared (14). Each preparation was stained by a modification of the Bailey method (22). Motility was observed in SIM Medium (Difco). Swarming was detected on maintenance agar plates containing 1.0 and 1.5% agar.

Determination of cultural characteristics. Growth

in a medium subjected to a variable range of *p*H, temperature, or salt concentration was observed after incubating the cultures for 2 days in 1.0% Trypticase (BBL), 0.2% Yeast Extract. The CB and MB strains were tested in CM. The NaCl concentration in both media was 3.0% except in the salt-range experiment, in which concentrations of 0, 3, 5, 7, 10, and 13% were tested. The growth was observed at temperatures of 2, 22, 30, 37, and 42 C, and at initial *p*H 4, 5, 6, 7, 8, 9, 10, and 11.

Determination of oxygen metabolism. Oxidation and fermentation of glucose were observed with Hugh-Leifson medium (8), catalase production on maintenance agar was tested with 10% H_2O_2, and cytochrome oxidase production in maintenance broth was detected by the method of Gaby and Hadley (6).

Determination of carbohydrate metabolism. Starch hydrolysis on maintenance agar plates containing 0.2% Soluble Starch (Difco) was tested by flooding with Gram's iodine solution. Acetoin production from glucose was tested in MR-VP Medium (Difco) or in CM broth plus 0.5% dextrose and 0.5% K_2HPO_4 with 48-hr cultures. The strains were tested for acid production from ethyl alcohol during a 14-day incubation period in the medium of Shimwell, Carr, and Rhodes (21) modified to the following composition: 0.1% $Na(NH_4)HPO_4 \cdot 4H_2O$, 0.1% K_2HPO_4, 2.8% NaCl, 0.02% $MgSO_4$, 2.0% $CaCO_3$, 2.0% ethyl alcohol, 1.0% Yeast Extract, and 1.5% agar, at *p*H 7.0. Carbohydrate fermentation was observed during a 14-day incubation period using modified marine oxidation-fermentation medium (MOF) of Leifson (15); tris-(hydroxymethyl)aminomethane (Tris) concentration was 0.6%, initial *p*H 8.0. Concentrations of 1% of each of the following were tested: glucose, fructose, sorbitol, mannitol, mannose, galactose, dulcitol, arabinose, adonitol, xylose, glycerol, maltose, lactose, sucrose, cellobiose, melibiose, raffinose, salicin, inositol, trehalose, and melizitose. The utilization of carbohydrates as sole carbon sources was observed on a modified Koser (11) inorganic base of 0.15% $Na(NH_4)HPO_4 \cdot 4H_2O$, 0.1% KH_2PO_4, 0.02% $MgSO_4$, 2.86% NaCl, and 0.85% Ionagar 2 (Oxoid). The CB and MB strains were tested on an inorganic base prepared by omitting Yeast Extract and Proteose Peptone from the CM. The inoculum was 0.1 ml of a twice-washed 18-hr culture of each strain diluted 1:100. Sterile, uniform, filter paper discs (Schleicher and Schuell Co., Keene, N.H.; 740-E) were saturated with sterile carbohydrate solutions, dried in vacuum, and then applied, four to a plate, to the surface of the seeded minimal agar. Zones of growth surrounding the discs within 2 days of incubation were interpreted as utilization of the carbohydrate. Solutions (10%) of the 21 carbohydrates listed above and also solutions of the following were sterilized by passage through 0.45 μm membrane filters (Millipore Corp., Bedford, Mass.) and tested: 10% sorbose, 10% α-methyl-D-glucoside, 10% rhamnose, 10% erythritol, 3% salicin, 6% trehalose, and 4% *i*-inositol.

Determination of nitrogenous metabolism. Indole production was tested with Kovac's reagent (12) using 48-hr cultures grown in a medium of 1.0% Trypticase (BBL), 0.2% Yeast Extract, and 3.0% NaCl. For the CB and MB strains, the CM was modified by substituting Trypticase for Proteose Peptone. Urease production was detected by a rapid micro method (23). Nitrate reduction was tested with 48-hr cultures grown in maintenance broth with 0.1% KNO_3 added; gelatin hydrolysis after 5 days of incubation with 12% added gelatin (Difco). Casein hydrolysis was observed during a 3-day incubation by using maintenance agar containing 10% skim milk; H_2S production during a 4-day incubation in SIM Medium with 0.02% cysteine added. Amino acid oxidase production in phenylalanine agar was tested by the method of Ewing, Davis, and Reavis (3). For the CB and MB strains, CM was modified by eliminating Proteose Peptone and adding 0.2% DL-phenylalanine and 0.1% Na_2HPO_4. Amino acid decarboxylation was observed over a 14-day incubation period in the medium of Møller (17) modified to the following composition: 0.5% peptone, 0.3% Yeast Extract, 0.1% dextrose, 3.0% NaCl, and 0.002% Brom Cresol Purple (Difco), at *p*H 7.0. For the CB and MB strains, CM was modified by substituting peptone for 50% of the Proteose Peptone concentration and adding 0.1% dextrose. We added L-lysine and L-arginine·HCl in 0.5% concentration each to the above media. For the cholera-red reaction, the medium of Fujino et al. (5) was used. For the CB and MB strains, CM was appropriately modified. Hemolysis was observed on sheep blood (5%) added to Brain Heart Infusion Agar (Difco).

Determination of organic acid metabolism. The utilization and hydrolysis of alginate was observed during a 10-day incubation (2) and the utilization of D-tartrate, citrate, and mucate during a 14-day incubation in the medium of Kauffmann and Petersen (10) modified by adding 0.2% Yeast Extract. Neutral 1% salt solutions of tartrate, citrate, or mucate were added to the base medium, initial *p*H 7.2. The utilization of sodium malonate was observed during a 14-day incubation period (13). The utilization of organic acids as sole carbon source was observed after 2-day incubation on the modified Koser inorganic media (see above). Neutral salt solutions in the following concentrations were prepared, filter-sterilized, and tested for utilization: 10% lactate or gluconate; 2.5% pyruvate, acetate, formate, citrate, succinate, malonate, malate, or oxalate; 1% DL-phenylalanine, L-tryptophan, or benzoate; and 0.5% phenol.

Serology. Cultures were examined serologically by the use of adsorbed, specific, high-titer antisera prepared by Y. Miyamoto. The titers of these antisera ranged from 1/40 to 1/2560; the mode titer was 1/320. Slide agglutinations were first carried out with polyvalent Anti-K antisera and then by reaction with individual sera.

RESULTS

Morphological characteristics. The morphological examination of the cultures (Table 2) showed the three groups to be composed of short, gram-negative rods exhibiting pleomorphism. Slightly curved, straight, coccoid, and swollen forms were observed. All cultures demonstrated a single polar flagellum.

On maintenance agar plates, most of the cultures were smooth, moist, circular, and opaque

TABLE 2. *Morphological characteristics of vibrios*

Characteristic	Strains in group[a]		
	I (79)	II (17)	III (14)
Gram stain	Negative	Negative	Negative
Form	Short rods	Short rods	Short rods
Flagella	Monotrichous	Monotrichous	Monotrichous
Motility	+79	+17	+14
Swarming			
1.0% Agar	±39	+12	−1
1.5% Agar	−20	−4	−1

[a] Number of strains in each group tested is expressed parenthetically. Values represent the numbers of strains in each group which were positive for the characteristic indicated. Groups were considered positive (+) when 75% or more strains were positive, negative (−) when 25% or less were positive, and variable (±) when the number of positive strains was between 25 and 75%.

with entire edges and a dark center. Four *V. parahemolyticus* cultures were mixtures of smooth colonies and rough-textured variants having serrated edges, a "raspberry" center, and a dull appearance. Repeated subculture of these strains, RS4, SJ12, RS18, and CDC9235, yielded apparently pure cultures of both forms. Sakazaki et al. (20) have described a more complex series of variations of *Vibrio* cultures.

All cultures were motile in hanging drop preparation and SIM agar. The degree to which these organisms swarmed varied with the concentration of agar in the maintenance media and also showed some variation among the three groups. On 1.0% agar, 50% of the *V. parahemolyticus*, most of the group II cultures, but only one of the group III cultures demonstrated the swarming phenomenon. When the agar concentration was elevated, swarming was reduced in strains of groups I and II. The distribution of swarming among the cultures studied here was almost the reverse of that reported by Sakazaki (19). He found none of 1,500 cultures of *V. parahemolyticus* capable of swarming on a 1% agar, whereas 643 of 671 cultures of nonpathogenic *V. alginolyticus*, most of which were isolated from sea fish and seawater, exhibited definite swarming ability.

Cultural characteristics. Examination of the cultures revealed that all strains of the three groups failed to grow at 2 C but grew readily at the other temperatures tested with one exception (Table 3). As expected, considering the estuarine environment from which they were isolated, most of the vibrios of group III did not grow at 42 C.

The *p*H range over which growth occurred was similar in all three groups. All cultures grew well at the initial *p*H values tested except at *p*H 4.0 and 5.0. *V. parahemolyticus* strains showed a higher degree of resistance to *p*H 4.0 than either of the other groups. Growth of group III was inhibited at *p*H 5.

The three groups differed in salt tolerance. The 79 *V. parahemolyticus* strains were obligate halophiles; only a few grew without NaCl. This condition was also true for the pathogenic *Vibrio* cultures of group II. The latter were tolerant to 10% salt; 16 of 17 cultures grew at this concentration. Tolerance of *V. parahemolyticus* to 10% salt was variable; only 50% of these strains grew at that concentration. With three exceptions, the nonpathogens of group III failed to grow at 10% NaCl. Growth at 13% salt was infrequent in all three groups of cultures.

Oxygen metabolism. The slight differences in cultural characteristics manifested by the nonpathogens of group II were enhanced by the results of oxygen metabolism tests (Table 4). All

TABLE 3. *Cultural characteristics of vibrios*

Characteristic	Strains in group[a]		
	I (79)	II (17)	III (14)
Temp			
2 C	−0	−0	−0
22 C	+79	+17	+14
30 C	+79	+17	+14
37 C	+79	+17	+14
42 C	+79	+16	−3
*p*H			
4	−20	−1	−1
5	+76	+15	±8
6	+79	+17	+14
7	+79	+17	+14
8	+79	+17	+14
9	+79	+17	+14
10	+79	+17	+14
11	+79	+17	+14
Salt concn			
0%	−7	−3	ND[b]
1%	+79	+17	+14
3%	+79	+17	+14
5%	+79	+17	+14
7%	+79	+17	+14
10%	±45	+15	−3
13%	−8	±5	−3

[a] Number of strains in each group tested is expressed parenthetically. Values represent the numbers of strains exhibiting positive growth characteristics. Again, groups were considered positive (+) when 75% or more strains were positive, negative (−) when 25% or less were positive, and variable (±) when the number of positive strains was between 25 and 75%.

[b] Not done.

cultures were facultatively anaerobic by the Hugh-Leifson test and the cytochrome–oxidase test. Group III vibrios differed from the other groups, however, in that they did not produce a strong catalase reaction.

Carbohydrate metabolism. Group II strains (tissue infection isolates) exhibited several variations in carbohydrate metabolism which distinguished them from the 79 strains of *V. parahemolyticus* (Table 5). Most of the group I strains fermented arabinose but failed to produce acetoin, ferment sucrose, or utilize sucrose or cellobiose as a single source of carbon. The pathogenic vibrios of group II exhibited variable ability in each of these characteristics.

The nonpathogenic third group was more sharply distinguished from either of the other groups. None of these cultures fermented arabinose, nor did they utilize any of the carbohydrates tested as a single source of carbon. Like the strains of group II, they possessed a variable ability to ferment sucrose, and they were further distinguished by variable ability to ferment dulcitol and galactose and hydrolyze starch.

Although, in most cases, utilization of carbohydrates as single sources of carbon for cultures of groups I and II paralleled fermentation of the carbohydrates by these cultures, there were two notable exceptions. Most of the cultures utilized sorbitol as a single carbon source without demonstrating a fermention reaction (13). Furthermore, *V. parahemolyticus* cultures did not utilize cellobiose as a single carbon source even though 22 strains fermented this sugar in a complex medium.

Nitrogenous metabolism. The biochemical reactions dependent on the metabolism of nitrogenous material failed to differentiate clearly among the three groups of organisms. The results of these examinations (Table 6) demonstrate a close relationship between the *V. parahemolyticus* strains, the tissue infection vibrios, and the estuarine nonpathogens. Slight differences between the nonpathogens of group III and the other two groups existed in variable indole production, nitrate reduction, and gelatin hydrolysis.

The cholera-red reaction was found to be characteristic of a majority of the group I *V. parahemolyticus* strains (Table 6). The failure of Sakazaki et al. (20) to demonstrate this reaction may be explained by the different test media used. Fujino et al. (5) showed that the optimal relationship between essential constituents of the medium and a positive cholera–red reaction could be achieved in a medium containing 10^{-2} M tryptophan and 10^{-3} M $NaNO_3$ (5).

TABLE 4. *Oxygen metabolism of vibrios*

Characteristic	Strains in group[a]		
	I (79)	II (17)	III (14)
Oxidation and fermentation of glucose	+79	+17	+14
Catalase production	+79	+17	−1
Cytochrome oxidase production	+78	+14	+13

[a] Values are expressed as in Table 2.

Organic acid metabolsm. The utilization of organic acids in complex nutrient media failed to separate clearly the three groups (Table 7). Group III exhibited only slight differences compared with the other two groups. The nonpathogenic vibrios from an estuarine environment failed to utilize alginate. Group III cultures were sharply defined by their failure to utilize any of the organic-C compounds with these compounds as a sole source of carbon.

The organic acid reactions exhibited by group I were markedly different from the results of Sakazaki et al. (20), who reported the utilization of lactate, pyruvate, acetate, succinate, and malate to be a characteristic of $\geq 95\%$ of the strains tested. Furthermore, utilization of tartrate by all strains, but utilization of alginate, citrate, and mucate by $\leq 1\%$ of the strains, was reported for strains tested in a complex nutrient medium.

Serological examination. Serological analysis for these groups of cultures (Table 8) reveals that the nonpathogenic *Vibrio* cultures isolated from Chesapeake Bay samples lacked serological relationship with the strains *V. parahemolyticus*. However, 11 of the group II strains isolated from tissue infections were agglutinated by anti-K antiserum. Four of the cultures exhibited multiple agglutination reactions.

DISCUSSION

Close morphological, cultural, and physiological similarity exists between the 79 strains of *V. parahemolyticus* of group I, the tissue infection vibrios of group II, and the nonpathogenic vibrios of group III. All proved to be short, gram-negative, polar-flagellated motile rods. They were rapidly growing, facultative aerobes of which the range of cultural parameters included tolerance of: 22 to 37 C temperature, *p*H 5 to 11, and NaCl concentration 1 to 7%. Most of the strains fermented glucose, fructose, mannitol, mannose, glycerol, galactose, maltose, and trehalose anaerogenically; produced indole, H_2S, and lysine decarboxylase; hydrolyzed starch, gelatin, and casein; reduced nitrate; were positive for cholera-red; were hemolytic; and failed to produce urease, amino acid oxidase, or arginine decarboxylase. Most of the strains failed to utilize malonate, tartrate, citrate, mucate, or alginate in a complete medium.

Despite these similarities, key differences among the three groups were encountered. For example, no carbohydrate or organic acid was utilized as a single carbon source by the 14 non-

TABLE 5. *Carbohydrate metabolism of vibrios*

Characteristic	Strains in group[a]		
	I (79)	II (17)	III (14)
Starch hydrolysis	+78	+16	±8
Acetoin production	−3	±5	−1
Ethyl alcohol oxidation	−0	−0	−0
Fermentation of:			
Glucose	+78	+17	+13
Fructose	+75	+17	+12
Sorbitol	−0	−0	−0
Mannitol	+78	+16	±8
Mannose	+77	+15	+13
Galactose	+78	+14	±10
Dulcitol	−2	−0	±5
Arabinose	+63	±8	−0
Adonitol	−1	−1	−2
Xylose	−0	−0	−2
Glycerol	+62	+16	+12
Maltose	+76	+16	+12
Lactose	−2	−0	−2
Sucrose	−1	±10	±10
Cellobiose	±22	±4	+11
Melibiose	−6	−0	−1
Raffinose	−1	−0	−0
Salicin	−3	−1	−2
Inositol	−0	−0	−1
Trehalose	+78	+17	+12
Melizitose	−0	−0	−0
Utilization as a single carbon source:			
Dextrose	+57	+16	−0
Fructose	+59	+17	−0
Sorbitol	±54	+13(13/16)[b]	−0
Mannitol	+66	+16	−0
Sorbose	−0	−0(0/16)	−0
Mannose	+57	±11	−0
Galactose	+58	+14	−0
Dulcitol	−0	−0(0/16)	−0
α-Methyl-D-glucoside	−2	−0(0/16)	−0
Potassium gluconate	+60	+11(11/16)	−0
Arabinose	±44	±6(6/16)	−0
Adonitol	−3	−0(0/16)	−0
Xylose	−4 (4/77)	−1(1/7)	−0
Rhamnose	−5	−0(0/16)	−0
Erythritol	−0	−0(0/16)	−0
Glycerol	+60	+9(9/16)	−0
Maltose	+58	+15	−0
Lactose	−1 (1/77)	−0	−0
Sucrose	−1	±5(5/16)	−0

TABLE 5—*Continued*

Characteristic	Strains in group[a]		
	I (79)	II (17)	III (14)
Utilization as a single carbon source:			
Cellobiose	−0	±6	−0
Melibiose	−3	−0(0/16)	−0
Raffinose	−1	−0(0/16)	−0
Salicin	−0	−0(0/16)	−0
Inositol	−1	−0(0/16)	−0
Trehalose	+61	+17	−0
Melizitose	−1	−0(0/16)	−0

[a] Values are expressed as in Tables 2 and 4.

[b] Fractions in parentheses represent numbers of strains which were positive for the reaction over total numbers tested, where less than the usual numbers per group were tested.

TABLE 6. *Nitrogenous metabolism of vibrios*

Characteristic	Strains in group[a]		
	I (79)	II (17)	III (14)
Indole production	+78	+13	±8
Urease production	−6	−0	−0
Nitrate reduction	+79	+15	±9
Gelatin hydrolysis	+79	+16	±7
Casein hydrolysis	+64	+16	+8 (8/11)
H_2S production	+79	+17	+12
Amino acid oxidase production	−10	−2	−0
Amino acid decarboxylase production			
L-Lysine	+78	+14	+14
L-Arginine	−0	−3	−3
Cholera-red reaction	+62	+12	+11
Hemolysis	+71	+17	+12

[a] Values are expressed as in Table 5.

pathogenic estuarine *Vibrio* cultures of group III. Very likely these strains require either a growth factor or an organic-N source. A useful set of differential reactions for the three groups is summarized in Table 9.

Group III nonpathogens are easily identified, but distinguishing between the *V. parahemolyticus* strains (group I) and the tissue infection pathogens (group II) would be most difficult even with these "key" reactions. Sakazaki et al. (19, 20) proposed six differential parameters for separating pathogenic and nonpathogenic species of halophilic *Vibrio*: salt tolerance; acetoin production; sucrose, cellobiose, and arabinose fermentation;

TABLE 7. *Organic acid metabolism of vibrios*

Characteristic	Strains in group[a]		
	I (79)	II (17)	III (14)
Utilization in complex medium:			
Alginate	−0	−2	−0
Malonate	±24	±8	−2
Tartrate	±19	±6	−3
Citrate	±18	±7	±6
Mucate	±25	−3	±6
Utilization as single carbon:			
Lactate	+60	+15 (15/16)	−0
Gluconate	+60	+11 (11/16)	−0
Pyruvate	−13	−2 (2/16)	−0
Acetate	±52	±7 (7/16)	−0
Formate	−3	−0 (0/16)	−0
Citrate	+58	+11 (11/16)	−0
Succinate	+62	+13 (13/16)	−0
Malonate	−1	−0 (0/12)	−0
Malate	+59	+15	−0
Oxalate	−1	−0 (0/16)	−0
DL-Phenylalanine	−1	−0 (0/16)	−0
L-Tryptophane	−3	−0 (0/7)	−0
Benzoate	−3	−0 (0/13)	−0
Phenol	−3	−0 (0/16)	−0

[a] Values are expressed as in Tables 5 and 6.

and swarming on 1% agar. These characteristics, indeed, appear to be useful in differentiation. However, to this list, we have added growth temperature, catalase production, starch hydrolysis, and the utilization of the above carbohydrates as single carbon sources.

Results of slide agglutinations confirm the evidence for separating the groups on the basis of the cultural and biochemical analyses. The nonpathogenic vibrios bear no serological relationship to *V. parahemolyticus*. Yet, the tissue infection isolates of group II are clearly identified with the 79 Japanese strains.

The results of our study differ from those of Sakazaki et al. (20) in three respects. They found that *V. parahemolyticus* grew well in peptone water with 7% NaCl added but not with 10% NaCl; *V. alginolyticus* grew in both; *V. anguillarum* grew in neither. Our evidence demonstrates that group I *V. parahemolyticus* and group III estuarine vibrios do not exhibit such a clear-cut pattern of salt tolerance. In a more limited examination of carbohydrate metabolism, they reported that 15% of *V. parahemolyticus* strains, but none of *V. alginolyticus* or *V. anguillarum*, fermented arabinose, whereas only 4% of *V. parahemolyticus* strains fermented cellobiose. Almost 80% of the *V. parahemolyticus* cultures tested in our study fermented arabinose and 28% fermented cellobiose. Finally, they showed that H_2S was not produced in Triple Sugar Iron Agar (TSI; Difco) or SIM Medium. In a brief preliminary study of 57 selected *V. parahemolyticus* strains, we compared the effectiveness of three media for demonstrating H_2S production. Incubation of the test cultures for 4 days at 37 C on these media revealed the following results: no cultures were positive in TSI, 48 were positive in Lead Acetate Agar (Difco), and 56 were positive in SIM Medium. Thus, the medium employed will have a profound effect on the test results.

Kato et al. (9) report that the possible relationship between hemolytic activity and pathogenicity for man supports differentiating *V. parahemolyticus* into two subgroups on the basis of hemol-

TABLE 8. *Serological analysis of groups II and III vibrios with V. parahemolyticus sera*

Strain	K reactions[a]	O group
Group II		
A 8633	2 +++, 4 ++, 37 ++++, 33 ++, 43 +++, 9 ++++, 8 +++, 11 +++, 42 ++, 34 ++, 18 ++	II, III, IV, VI
A 5704	25 ++++	I
A 6202	3 ++, 17 +++	II, V
A 8198	33 +++	III
A 6670	None	
A 4871	None	
A 5002	29 + (slight), 9 ++	III, IV
A 7606	17 +++	V
A 3637	None	
A 3454	None	
A 1889	28 ++++	II
A 8694	3 ++++	II
A 6614	17 +++	V
A 1334	17 +++	V
A 6540	None	
A 4280	41 +++, 45 ++++	I, III
A 4281	None	
Group III		
All CB + MB	None	

[a] Slide agglutination titer, indicated by relative number of plus (+) signs, was determined by the size of clumps and the rapidity of their formation. Each K antigen and the O group to which it belongs were determined by the schema of Y. Myamoto.

TABLE 9. *Differential reactions of three groups of vibrios*

Characteristic	Strains in group[a]		
	I	II	III
Swarming on 1% agar	±	+	−
Growth at 42 C	+	+	−
Growth in 10% NaCl	±	+	−
Catalase production	+	+	−
Starch hydrolysis	+	+	±
Acetoin production	−	±	−
Fermentation:			
Arabinose	+	±	−
Sucrose	−	±	±
Cellobiose	±	±	+
Single carbon utilization:			
Arabinose	±	±	−
Sucrose	−	±	−
Cellobiose	−	±	−

[a] Symbols are the same as in Tables 2–7.

ysis on human blood agar. None of the *V. parahemolyticus* isolated from North or Baltic Sea fish by Nakanishi et al. (18) hemolyzed human blood, although many demonstrated strong serological reactions with anti-K antigen sera. The three groups of cultures in our study could not be differentiated by hemolysis on sheep blood agar. It is possible that an analysis of hemolytic activity on human blood and blood from several animal species might provide a useful separation of these strains.

In summary, our results strongly suggest that the pathogenic vibrios isolated from tissue infections are *V. parahemolyticus* species involved in a hitherto unsuspected mode of infection, i.e. via a wound or tissue injury.

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