Vibrio ichthyenteri sp. nov., a Pathogen of Japanese Flounder (*Paralichthys olivaceus*) Larvae

KATSUYA ISHIMARU,¹ MASAYO AKAGAWA-MATSUSHITA,² AND KIYOKUNI MUROGA^{1*}

Faculty of Applied Biological Science, Hiroshima University, Higashihiroshima-shi 739,¹ and Department of Chemistry, University of Occupational and Environmental Health, Yahatanishi-ku, Kitakyushu-shi 807,² Japan

Seven similar strains which were pathogens of Japanese flounder (*Paralichthys olivaceus*) larvae with opaque intestines had characteristics of the genus *Vibrio*. These strains were divided into two genomic species (species 1 containing six strains, and species 2 containing one strain) on the basis of the results of DNA-DNA hybridization experiments in which the membrane filter method was used, and these two species could be differentiated from each other by the following characteristics: acid production from D-galactose and utilization of D-glucuronate and β -hydroxybutyrate. Strain F-2, the type strain of species 1, exhibited levels of DNA relatedness with 29 previously described *Vibrio* species of 5 to 18%. The flounder isolates belonging to species 1 were also differentiated from the previously described *Vibrio* species phenotypically by the following characteristics: they were nitrate reduction positive; each cell had a single polar flagellum; they did not produce arginine dihydrolase, chitinase, gelatinase, and lipase; they did not utilize D-cellobiose and citrate; and they did not grow at 35°C. The G+C contents of the DNAs of four species 1 strains were 43 to 44 mol%. The name *Vibrio ichthyenteri* sp. nov. is proposed for genomic species 1. The type strain of *V. ichthyenteri* is strain F-2 (= IFO 15847). Species 2 was also considered a new genomic species, but a species name is not proposed in this paper because only one strain is available and the phenotypic variability of the species is not known.

Since 1971, a bacterial disease characterized by opaque intestines or intestinal necrosis and high rates of mortality has occurred often in larval Japanese flounder (*Paralichthys olivaceus*) reared at marine hatcheries in Japan (13). O. Murata (12) isolated the causative agent of the disease, a vibrio, from affected flounder larvae between 1971 and 1973 in Wakayama Prefecture, but the strains isolated by Murata have been lost. Between 1986 and 1988 we isolated the causative agent of the disease mainly in Hiroshima Prefecture. On the basis of its morphological, biochemical, physiological, pathological, and serological characteristics, this flounder pathogen, which was tentatively designated *Vibrio* species INFL (intestinal necrosis of flounder larvae), was thought to be a new species (11). The disease caused by *Vibrio* species INFL is a very unusual fish disease because a local (intestinal) infection develops only in larval fish (14). In this study we determined the taxonomic position of this flounder pathogen and propose the name *Vibrio ichthyenteri* sp. nov. for it.

MATERIALS AND METHODS

Bacterial strains. The strains which we used are listed in Table 1. The seven strains belonging to the INFL group were isolated from the guts of diseased Japanese flounder larvae reared in prefectures in western Japan. These strains were isolated and subcultured on ZoBell 2216e agar plates (1) at 25°C and were maintained in semisolid ZoBell 2216e medium containing 0.5% agar at 20°C; they were subcultured every 6 months. Aged and filtered seawater obtained from the Inland Sea of Japan was used to prepare ZoBell 2216e medium. The pathogenicity of three strains (strains F-1, F-2^T [T = type strain], and FH-8804) for flounder larvae was confirmed experimentally (11).

In the DNA hybridization tests performed in this study we used the type strains of all species except *Vibrio cholerae*. It has been shown previously by DNA-DNA hybridization that the strain of *V. cholerae* used in this study (strain HUPF 5004 [= NIH 35A3]) is closely related to the type strain of this species (strain ATCC 14035) (level of similarity, 98%) (3).

Phenotypic characterization. Arginine dihydrolase activity (Thornley method) was determined as described by West and Colwell (19). Other phenotypic char-

acteristics of the seven strains were determined as described previously (9). The NaCl concentration was adjusted to 1.5% (wt/vol) in all of the test media except those containing seawater or those that were used to determine the range of NaCl concentrations for growth.

Genetic characterization. The DNAs of strains listed in Table 1 were prepared as described previously (9). G+C contents of DNAs were determined by the high-performance liquid chromatography method described by Kumagai et al. (10). DNA-DNA hybridization experiments were performed by using the membrane filter method described previously (2). Briefly, reference DNA was labeled with tritium by nick translation by using a nick translation kit (Takara Shuzo Co., Ltd., Kyoto, Japan). About 10 μ g (the exact amount used was recorded) of unlabeled single-stranded DNA immobilized on a nitrocellulose membrane and 0.1 μ g of labeled reference DNA were reassociated in a solution containing 0.1% sodium dodecyl sulfate and 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After incubation for 40 h at 62°C, the filters were washed and dried. Radioactivity was measured with an Aloca model LSC-3500E liquid scintillation counter. Each hybridization experiment was performed in triplicate, and the same experiment was repeated two or three times for some strains, as indicated in Table 1.

RESULTS AND DISCUSSION

The seven strains isolated from affected flounder larvae were gram-negative, facultatively anaerobic bacilli that were motile by means of a single polar flagellum (Fig. 1). The strains were oxidase positive, susceptible to vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine), required NaCl to grow, and did not accumulate poly- β -hydroxybutyrate. The G+C contents of five strains ranged from 43 to 45 mol%. The seven strains which we studied belonged to two genomic species as shown below. The G+C contents of the species 1 strains were 43.4 to 44.3 mol%, and the G+C content of the single species 2 strain (strain P-8706) was 45.1 mol%. The strains belonging to the INFL group were identified as members of the genus *Vibrio* on the basis of the characteristics described above.

As determined by DNA-DNA hybridization experiments, five strains belonging to the INFL group exhibited 89 to 100% relatedness to strain F-2^T. However, the levels of DNA homology between strain P-8706 and the other INFL strains were only 36 to 42% despite the phenotypic similarities of these organisms. The levels of DNA relatedness between reference strains of 29 previously described *Vibrio* species and strain F-2^T were less than 18%. Strains belonging to 18 *Vibrio* species also

* Corresponding author. Mailing address: Faculty of Applied Biological Science, Hiroshima University, 1-4-4 Kagamiyama, Higashihiroshima-shi, Hiroshima Prefecture, Japan 739. Phone: 0824-24-7977. Fax: 0824-22-7059.

TABLE 1. Levels of DNA relatedness of strains F-2^T and P-8706 with strains of other *Vibrio* species and related genera

Strain ^a	Source ^a	% DNA reassociation with:	
		Strain F-2 ^T	Strain P-8706
<i>Vibrio ichthyenteri</i> F-2 ^T (= IFO 15847 ^T) ^b	Hiroshima Prefecture	100	36 (2) ^c
<i>Vibrio ichthyenteri</i> F-1 (= IFO 15846) ^b	Hiroshima Prefecture	89 (3)	39 (2)
<i>Vibrio ichthyenteri</i> FH-8804 (= IFO 15848) ^b	Hiroshima Prefecture	100 (3)	40 (2)
<i>Vibrio ichthyenteri</i> F-9202	Hiroshima Prefecture	94 (2)	40 (1)
<i>Vibrio ichthyenteri</i> MF-1	Mie Prefecture	92 (3)	39 (2)
<i>Vibrio ichthyenteri</i> P-8603 ^b	Wakayama Prefecture	90 (3)	42 (2)
<i>Vibrio</i> species INFL-2 strain P-8706 ^b	Wakayama Prefecture	37 (3)	100
<i>Vibrio aestuarianus</i> ATCC 35048 ^T	ATCC	11 (2)	10 (1)
<i>Vibrio alginolyticus</i> HUF 9107 ^T	U. Simidu	11 (2)	8 (1)
<i>Vibrio anguillarum</i> HUF 5001 ^T	M. Nishibuchi	14 (2)	11 (1)
<i>Vibrio campbellii</i> HUF 9109 ^T	U. Simidu	11 (2)	14 (1)
<i>Vibrio carchariae</i> HUF 9110 ^T	U. Simidu	11 (2)	12 (1)
<i>Vibrio cholerae</i> HUF 5004	M. Nishibuchi	11 (2)	5 (1)
<i>Vibrio cincinnatiensis</i> ATCC 35912 ^T	ATCC	9 (2)	
<i>Vibrio damsela</i> ATCC 33539 ^T	ATCC	5 (2)	4 (1)
<i>Vibrio diazotrophicus</i> HUF 9301 ^T	H. Kawasaki	11 (2)	
<i>Vibrio fischeri</i> ATCC 7744 ^T	ATCC	9 (1)	
<i>Vibrio fluvialis</i> HUF 9302 ^T	H. Kawasaki	11 (2)	
<i>Vibrio gazogenes</i> HUF 9303 ^T	H. Kawasaki	6 (2)	
<i>Vibrio harveyi</i> HUF 9111 ^T	U. Simidu	12 (2)	13 (1)
<i>Vibrio logei</i> HUF 5007 ^T	M. Nishibuchi	8 (2)	
<i>Vibrio mediterranei</i> ATCC 43341 ^T	ATCC	7 (2)	4 (1)
<i>Vibrio natriegens</i> ATCC 14048 ^T	ATCC	7 (2)	7 (1)
<i>Vibrio navarrensis</i> ATCC 51183 ^T	ATCC	10 (2)	
<i>Vibrio nereis</i> HUF 9112 ^T	U. Simidu	15 (2)	13 (1)
<i>Vibrio nigripulchritudo</i> ATCC 27043 ^T	ATCC	6 (2)	
<i>Vibrio ordalii</i> ATCC 33509 ^T	ATCC	8 (2)	6 (1)
<i>Vibrio orientalis</i> ATCC 33934 ^T	ATCC	18 (2)	18 (1)
<i>Vibrio parahaemolyticus</i> HUF 9114 ^T	U. Simidu	16 (2)	16 (1)
<i>Vibrio pelagius</i> HUF 9115 ^T	U. Simidu	12 (2)	11 (1)
<i>Vibrio penaeicida</i> KH-1 ^T	Kagoshima Prefecture	8 (1)	
<i>Vibrio proteolyticus</i> HUF 9307 ^T	H. Kawasaki	8 (2)	
<i>Vibrio salmonicida</i> ATCC 43839 ^T	ATCC	8 (1)	
<i>Vibrio splendidus</i> HUF 9117 ^T	U. Simidu	10 (2)	13 (1)
<i>Vibrio tubiashii</i> HUF 9118 ^T	U. Simidu	11 (2)	12 (1)
<i>Vibrio vulnificus</i> HUF 5002 ^T	M. Nishibuchi	15 (2)	15 (1)
<i>Photobacterium phosphoreum</i> HUF 9311 ^T	H. Kawasaki	7 (1)	
<i>Escherichia coli</i> K-12	IAM	3 (3)	2 (2)

^a ATCC, American Type Culture Collection, Rockville, Md.; HUF, Hiroshima University Fish Pathology Laboratory Culture Collection, Higashihiroshima-shi, Hiroshima, Japan; IAM, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan; IFO, Institute for Fermentation Osaka, Osaka, Japan.

^b Strains whose G+C content was determined.

^c The numbers in parentheses are the numbers of times that the experiments were performed.

exhibited low levels of DNA relatedness with strain P-8706 (less than 18%). These results indicate that the INFL group consists of two genomic species. Species 1 contains six strains and is represented by strain F-2^T, and species 2 contains one

FIG. 1. Electron micrograph of a negatively stained cell of *V. ichthyenteri* F-2^T showing the single polar flagellum. Bar = 1 µm.

strain (strain P-8706). Both of these species are distinct genetically from the previously described *Vibrio* species examined. The following eight species were not used in the DNA-DNA hybridization experiments performed with strain F-2^T: *Vibrio costicola*, *Vibrio furnissii*, *Vibrio hollisae*, *Vibrio iliopiscarius*, *Vibrio marinus*, *Vibrio metschnikovii*, *Vibrio mimicus*, and *Vibrio mytili*. Three of these species, *V. costicola*, *V. furnissii*, and *V. hollisae*, differ from the flounder pathogens in their DNA G+C contents (50 mol%). These species can also be differentiated from the two species of flounder pathogens by the following phenotypic characteristics, as well as the differential characteristics shown in Table 2: *V. costicola* is positive in the Voges-Proskauer test and grows in the presence of 8% NaCl (19); *V. furnissii* produces gas from D-glucose, grows when no NaCl is present, and utilizes γ-aminobutyrate and β-hydroxybutyrate (8); and *V. hollisae* produces indole and acid from L-arabinose and sucrose (7). *V. iliopiscarius* can be differentiated from flounder isolates by its DNA G+C content (40 mol%) and the following characteristics: it produces arginine dihydrolase and lysine decarboxylase; it produces gas from D-glucose; it is pos-

TABLE 2. Characteristics that distinguish *V. ichthyoenteri* from previously described *Vibrio* species^a

Taxon	Thornley arginine dihydrolase activity ^b	Nitrate reduction	Chitinase activity	Gelatinase activity	Lipase activity	Utilization of:		Growth at 35°C	3-12 polar flagella
						D-Cellobiose	Citrate		
<i>Vibrio ichthyoenteri</i>	— ^c	+	—	—	—	—	—	—	—
<i>Vibrio</i> species INFL-2	—	+	—	—	—	—	—	—	—
<i>Vibrio aestuarianus</i>	+	ND	ND	—	ND	ND	+	+	—
<i>Vibrio alginolyticus</i>	—	+	+	+	+	—	+	+	—
<i>Vibrio anguillarum</i>	+	+	+	+	+	+	+	+	—
<i>Vibrio campbellii</i>	—	+	+	+	+	V	V	+	—
<i>Vibrio carchariae</i> ^d	—	+	+	+	+	ND	—	+	—
<i>Vibrio cholerae</i>	—	+	+	+	+	—	V	+	—
<i>Vibrio cincinnatiensis</i>	—	+	+	—	—	+	+	+	—
<i>Vibrio costicola</i>	V	V	—	—	—	—	—	+	—
<i>Vibrio damsela</i>	+	+	+	—	—	—	—	+	ND
<i>Vibrio diazotrophicus</i>	+	+	—	—	—	+	+	+	—
<i>Vibrio fischeri</i>	—	V	V	—	+	+	V	V	+
<i>Vibrio fluvialis</i>	+	+	+	+	+	V	+	+	—
<i>Vibrio furnissii</i>	+	+	+	+	V	—	+	+	ND
<i>Vibrio gazogenes</i>	—	—	—	+	+	+	+	+	—
<i>Vibrio harveyi</i>	—	+	+	+	+	+	+	+	—
<i>Vibrio hollisae</i>	—	+	ND	—	—	ND	—	+	—
<i>Vibrio iliopiscarius</i> ^e	+	+	ND	—	ND	—	—	—	ND
<i>Vibrio logei</i>	—	+	+	—	V	+	—	—	+
<i>Vibrio marinus</i>	—	+	+	+	+	—	—	—	—
<i>Vibrio mediterranei</i>	+	+	—	—	+	+	ND	ND	—
<i>Vibrio metschnikovii</i>	+	—	+	+	+	—	V	+	—
<i>Vibrio mimicus</i>	—	+	ND	ND	—	ND	+	+	—
<i>Vibrio mytili</i> ^f	+	+	ND	—	+	+	V	+	—
<i>Vibrio natriegens</i>	—	+	—	V	+	V	+	+	—
<i>Vibrio navarrensis</i> ^g	—	+	ND	+	ND	V	V	+	—
<i>Vibrio nereis</i>	+	+	V	V	—	—	+	+	—
<i>Vibrio nigripulchritudo</i>	—	+	+	+	+	+	+	—	—
<i>Vibrio ordalii</i>	—	—	V	+	—	—	+	—	—
<i>Vibrio orientalis</i>	V	+	+	+	+	+	+	+	—
<i>Vibrio parahaemolyticus</i>	—	+	+	+	+	—	+	+	—
<i>Vibrio pelagius</i> biovar 1	—	+	V	—	+	—	+	+	—
<i>Vibrio pelagius</i> biovar 2	—	+	+	+	+	—	+	+	—
<i>Vibrio penaeicida</i> ^h	—	+	+	+	+	V	V	—	—
<i>Vibrio proteolyticus</i>	+	+	+	+	+	—	+	+	—
<i>Vibrio salmonicida</i>	—	—	—	—	—	—	ND	—	+
<i>Vibrio splendidus</i> biovar 1	V	+	+	+	+	+	+	V	—
<i>Vibrio splendidus</i> biovar 2	V	+	+	+	+	V	+	—	—
<i>Vibrio tubiashii</i>	+	+	+	+	+	+	+	V	—
<i>Vibrio vulnificus</i> biovar 1	—	+	+	+	+	+	+	+	—
<i>Vibrio vulnificus</i> biovar 2 ⁱ	—	+	ND	+	+	ND	+	+	—

^a Data from reference 8 except as noted below.^b Data from reference 5.^c +, positive in at least 90% of the strains; —, negative in at least 90% of the strains; V, variable among strains; ND, no data available.^d Data from reference 6.^e Data from reference 15.^f Data from reference 16.^g Data from reference 18.^h Data from reference 9.ⁱ Data from reference 17.

itive in the Voges-Proskauer test; and it grows at 4°C (15). It has been reported previously that the type strain of *V. cholerae* and the type strain of *V. mimicus* (strain ATCC 33653) are closely related as determined by DNA-DNA relatedness experiments (level of relatedness, 67%) (4). However, strains F-2^T and P-8706, the representative strains of species 1 and 2, respectively, exhibited only 11 and 5% DNA relatedness to *V. cholerae* HUF 5004, respectively. Therefore, the flounder pathogens (both species 1 and species 2) are genetically distinct from *V. mimicus*. The remaining three species (*V. marinus*, *V. metschnikovii*, and *V. mytili*) can be clearly distinguished from the flounder isolates by at least three characteristics, as shown in Table 2. In addition to the differences shown in Table

2, the following characteristics differentiate the flounder isolates from these three species: *V. marinus* is lysine decarboxylase positive and grows at 4°C but not 30°C (8, 19); *V. metschnikovii* is Voges-Proskauer positive and produces amylase but not oxidase (8); and *V. mytili* produces gas from D-glucose and acid from L-arabinose but not D-mannose, utilizes D-xylose, and grows in the presence of 8% NaCl (16).

The two genomic species that make up the INFL group, species 1 and 2, can be differentiated phenotypically from each other by three characteristics, acid production from D-galactose and utilization of D-glucuronate and β-hydroxybutyrate (Table 3), and are distinct from the previously described *Vibrio* species (Table 2). On the basis of the results described above,

TABLE 3. Biochemical and physiological characteristics of six *V. ichthyenteri* strains and *Vibrio* species INFL-2 strain P-8706^a

Characteristic	<i>V. ichthyenteri</i>		Reaction of <i>Vibrio</i> species INFL-2 strain P-8706
	% of strains positive	Reaction of strain F-2 ^T	
Fermentation of glucose (4 days)	100	+ ^b	+
Gas produced from glucose (4 days)	0	—	—
Oxidase activity	100	+	+
Catalase activity	100	+	+
Indole production (5 days)	0	—	—
Nitrate reduction (5 days)	100	+	+
Voges-Proskauer test (5 days)	0	—	—
Hydrogen sulfide production (7 days)	0	—	—
Arginine dihydrolase activity (Thornley) (7 days)	0	—	—
Arginine dihydrolase activity (Møller) (7 days)	0	—	—
Lysine decarboxylase activity (Møller) (7 days)	0	—	—
Ornithine decarboxylase activity (Møller) (7 days)	0	—	—
ONPG hydrolysis ^c	0	—	—
Alginase activity (7 days)	0	—	—
Amylase activity (7 days)	0	—	—
Chitinase activity (7 days)	0	—	—
Gelatinase activity (7 days)	0	—	—
Lipase activity (7 days)	0	—	—
PHB accumulation (4 days) ^d	0	—	—
Growth at:			
4°C	0	—	—
15°C	100	+	+
30°C	100	+	+
35°C	0	—	—
Growth in the presence of:			
0% NaCl	0	—	—
1% NaCl	100	+	+
3% NaCl	100	+	+
6% NaCl	100	+	+
8% NaCl	0	—	—
Acid production (7 days) from:			
Adonitol	0	—	—
L-Arabinose	0	—	—
D-Cellobiose	0	—	—
Dextrin	0	—	—
Dulcitol	0	—	—
Erythritol	0	—	—
Fructose	100	+	+
D-Galactose	0	—	+
D-Glucose	100	+	+
Glycerol	0	—	—
Glycogen	0	—	—
myo-Inositol	0	—	—
Inulin	0	—	—
Lactose	0	—	—
Maltose	100	+	+
D-Mannitol	0	—	—
D-Mannose	100	+	+
Melibiose	0	—	—
Raffinose	0	—	—
L-Rhamnose	0	—	—
Salicin	0	—	—
D-Sorbitol	0	—	—
Sucrose	100	+	+
Trehalose	100	+	+
D-Xylose	0	—	—
Utilization of the following compounds as sole carbon sources (14 days):			
γ-Aminobutyrate	0	—	—
D-Cellobiose	0	—	—
Citrate (Simmons)	0	—	—
L-Citrulline	0	—	—
Ethanol	0	—	—
D-Gluconate	50	—	—
D-Glucuronate	0	—	+
β-Hydroxybutyrate	0	—	+

Continued on following page

TABLE 3—Continued

Characteristic	<i>V. ichthyoenteri</i>		Reaction of <i>Vibrio</i> species INFL-2 strain P-8706
	% of strains positive	Reaction of strain F-2 ^T	
L-Leucine	0	—	—
D-Mannitol	33	—	—
Putrescine	0	—	—
Sucrose	17	—	—
D-Xylose	0	—	—

^a All tests except the growth temperature tests were performed at 25°C.

^b +, positive; —, negative.

^c ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

^d PHB, poly-β-hydroxybutyrate.

we propose the name *Vibrio ichthyoenteri* sp. nov. for the species 1 strains, including strain F-2^T. Species 2, which consists of one strain, strain P-8706, is also thought to be a new species of the genus *Vibrio*, but we do not propose a name for this species at this time because only one strain is available and the phenotypic variability of the species is not known. We designated this second genomic species *Vibrio* species INFL-2.

Description of *Vibrio ichthyoenteri* sp. nov. *Vibrio ichthyoenteri* (ich.thy.o.en'te.ri. Gr. n. *ichthys*, fish; Gr. n. *enteron*, gut; Gr. gen. n. *ichthyoenteri*, of fish gut) cells are gram-negative short rods (1.6 to 2.5 by 0.6 to 0.8 μm) that are straight or slightly curved and motile by means of a single polar flagellum when the cells are grown on solid media. No lateral flagella are observed on solid media, and there is no swarming. Spores are not formed. Facultatively anaerobic. Susceptible to 2,4-diamino-6,7-diisopropylpteridine (vibriostatic agent O/129) at a concentration of 10 μg per disc. Neither pigmentation nor luminescence is observed. Colonies of most strains on thiosulfate-citrate-bile-sucrose agar are yellow; strain F-2^T colonies are green because of weak acid production from sucrose. Biochemical and physiological characteristics are shown in Table 3.

The G+C contents of the DNAs of the four strains examined range from 43.4 to 44.3 mol%.

V. ichthyoenteri is the causative agent of intestinal necrosis or bacterial enteritis of Japanese flounder larvae in Japan (14). Isolated from affected fish in hatcheries, but the distribution of this organism in the environment is not known.

The type strain is strain F-2 (= IFO 15847).

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