Vibrio tubiashii sp. nov., a Pathogen of Bivalve Mollusks

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The genotypic and phenotypic properties of six strains that were isolated during two unrelated incidents of a bacterial disease of bivalve mollusk larvae were compared with phenotypically similar *Vibrio* species. The strains of this bivalve mollusk larval pathogen are distinct from other *Vibrio* spp. phenotypically and as determined by deoxyribonucleic acid-deoxyribonucleic acid hybridization and are described here as *Vibrio tubiashii* sp. nov. The base composition of the overall deoxyribonucleic acid is 43 to 45 mol% guanine plus cytosine. All strains of *V. tubiashii* degrade xanthine and tyrosine extracellularly. Strain ATCC 19109 is designated the type strain of *V. tubiashii*.

Tubiash et al. (7) described strains of *Vibrio* spp. that were pathogenic for the larvae of bivalve mollusks. These organisms were tentatively identified as *Vibrio anguillarum* and were deposited in the American Type Culture Collection as strains ATCC 19105, ATCC 19106, and ATCC 19109^T (T = type strain). In the last decade, knowledge of the taxonomy of the genus *Vibrio* has advanced rapidly, and many bacteria that would have been identified as *V. anguillarum* previously can now be allocated to genotypically and phenotypically distinct species, including *V. anguillarum* sensu stricto, *Vibrio ordalii*, *Vibrio nereis*, *Vibrio fluvialis*, *Vibrio diazotrophicus*, and *Vibrio splendidus* (1, 9).

In a numerical taxonomic study of 237 strains of the *Vibrionaceae*, including type and reference strains of most of the species of *Vibrio* and over 50 wild isolates of *V. anguillarum*, strains ATCC 19105, ATCC 19106, and ATCC 19109^T were shown to be closely related to one another but phenotypically distinct from *V. anguillarum* and all other *Vibrio* spp. (10). Recently, Jeffries (3) isolated strains of *Vibrio* spp. in England which were pathogenic for oyster larvae and were phenotypically similar to the strains which Tubiash et al. isolated from diseased hardshell clams on the east coast of the United States (7).

In view of the potential economic importance of these vibrios in the cultivation of bivalve mollusk larvae, the work described here was undertaken to establish the genetic relationship between the larval bivalve pathogens and phenotypically similar *Vibrio* spp.

MATERIALS AND METHODS

Bacterial strains and phenotypic characterization. The strains which we examined and their sources are listed in Table 1. The methods used for phenotypic characterization of each strain have been described previously (9, 10). All preparations were incubated at 25°C.

DNA extraction. Deoxyribonucleic acid (DNA) was isolated essentially by the methods of Marmur and Doty (5). Bacteria harvested from 2-liter cultures by centrifugation ($6,500 \times g$, 15 min, 5°C) were washed twice in a solution containing 0.1 M NaCl, 0.05 M tris(hydroxymethyl)aminomethane base, and 0.05 M ethylenediaminetetraacetate (pH 8.1) (TES). The cells were resuspended in TES, incubated with 1 mg of lysozyme (Sigma Chemical Co., St. Louis, Mo.)

per ml and 50 µg of pronase (Calbiochem-Behring Corp., La Jolla, Calif.) per ml for 30 min at room temperature, and lysed by adding 6 mg of sodium dodecyl sulfate per ml. A 0.2 volume of TES-saturated, distilled phenol was added, and the mixture was shaken for 30 min; this was followed by centrifugation at $6,000 \times g$ for 15 min. The upper aqueous layer was collected, mixed with 30 ml of a chloroform-isoamyl alcohol mixture (24:1), and centrifuged at $6,000 \times g$ for 15 min at 5°C; 2 volumes of cold (-20°C) 95% ethanol was added to the collected aqueous solution, and the nucleic acid precipitated was spooled onto glass rods. The nucleic acid was dissolved in $0.1 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) and incubated with 250 µg of pancreatic ribonuclease (Sigma Chemical Co.) per ml for 3 h at 37°C. A single phenol extraction was followed by three to five chloroform-isoamyl alcohol extractions. The DNA was precipitated by using 2 volumes of 95% ethanol and then dissolved in $0.1 \times$ SSC. A second precipitation was accomplished by adding 0.1 volume of 2.2 M sodium acetate-0.01 M ethylenediaminetetraacetate (pH 7.2) and 0.6 volume of isopropyl alcohol (-20°C) while spooling with a glass rod. The spooled DNA was dissolved in $0.1 \times$ SSC.

Determination of G+C content. The purity of each DNA preparation was determined spectrophotometrically. Values of 2.0 for the ratio of optical density at 260 nm to optical density at 230 nm and 1.75 to 1.85 for the ratio of optical density at 260 nm to optical density at 280 nm were used to indicate a lack of protein and ribonucleic acid contamination. The mean guanine-plus-cytosine (G+C) content of the DNA was calculated by estimating the midpoint of the optical melting curves (4). The melting temperatures were determined by establishing an absorbance-temperature profile (60 to 90°C) at 260 nm with a Gilford model 2400-S spectrophotometer. Control DNA from *Escherichia coli* ATCC 11775^T was included in all determinations.

DNA-DNA hybridization. Unlabeled DNA was adjusted to a concentration of 10 µg/ml with $0.1 \times$ SSC, denatured by adding 0.1 volume of 1.0 N NaOH for 10 min, and chilled in an ice bath. After neutralization with 1.8 M tris(hydroxymethyl)aminomethane hydrochloride-tris(hydroxymethyl)aminomethane base, the DNA was adjusted to a concentration of 5 µg/ml with $3 \times$ SSC, and 10 ml of this solution was gravity filtered through nitrocellulose filters (diameter, 25 mm; pore size, 0.45 µm; type BA85; Schleicher-Schuell, Dassel, Germany). The amount of filter-bound DNA was estimated by spectrophotometrically measuring the DNA in the filtrate (2). The filters were washed with 10 ml of $3 \times$ SSC, dried overnight at 20°C, and vacuum dried for 2 h at

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TABLE 1. Bacterial strains used and their sources

Species	Strain ^a	Source
<i>Vibrio tubiashii</i>	ATCC 19105	Hard clam larvae
	ATCC 19106	Oyster spat
	ATCC 19109 ^T	Juvenile hard clams
	NCMB 2164 (= B-Z)	Oyster larvae ^b
	NCMB 2165 (= B-SS)	Oyster larvae
	NCMB 2166 (= B-TE)	Oyster larvae
<i>Vibrio anguillarum</i>	ATCC 19264 ^T	Diseased cod
<i>Vibrio diazotrophicus</i>	ATCC 33466 ^T	Sea urchin
<i>Vibrio fluvialis</i>	NCTC 11327 ^T	Human feces
<i>Vibrio metschnikovii</i>	ATCC 7708	
<i>Vibrio nereis</i>	ATCC 25917 ^T	Seawater
<i>Vibrio proteolyticus</i>	ATCC 15338 ^T	Wood borer
<i>Vibrio splendidus</i>	ATCC 33125 ^T	Marine fish
<i>Vibrio alginolyticus</i>	ATCC 17749 ^T	Mackerel
<i>Vibrio cholerae</i>	ATCC 14035 ^T	Human feces
<i>Vibrio fischeri</i>	ATCC 25918	Seawater
<i>Vibrio parahaemolyticus</i>	ATCC 17802 ^T	Seafood
<i>Escherichia coli</i>	ATCC 11775 ^T	Urine

^a ATCC, American Type Culture Collection, Rockville, Md.; NCMB, National Collection of Marine Bacteria, Aberdeen, Scotland; NCTC, National Collection of Type Cultures, Colindale, England.

^b See reference 3.

80°C and a pressure of 15 lb/in².

In vitro radioactive labeling of DNA was performed by using the nick translation method (6), in which 1.5 µg of DNA in 0.2 ml of 0.1× SSC was incubated with 0.2 ml of a nucleotide-buffer solution containing 100 µM deoxyribosyladenosine triphosphate, 100 µM deoxyribosylguanosine triphosphate, and 100 µM deoxyribosylthymine triphosphate in 250 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 7.8), 25 mM MgCl₂, and 50 mM 2-mercaptoethanol and 0.1 ml of 25 µM [α-³²P]deoxycytidine triphosphate (The Radiochemical Center, Amersham, Ill.). The reaction was initiated by adding 0.1 ml of an enzyme solution containing 100 U of DNA polymerase I and 0.02 µg of deoxyribonuclease I. After incubation at 15°C for 2 h, the mixture was eluted through a Pasteur pipette column containing TES-saturated polyacrylamide (Bio-Gel P-60; Bio-Rad Laboratories, Richmond, Calif.). A series of 10 fractions (200 µl each) were collected and monitored with a Geiger counter to determine the labeled double-stranded DNA peak.

Labeled DNA was hybridized with nitrocellulose-fixed unlabeled DNA by alkaline denaturation (2) of the labeled DNA in PM buffer (3× SSC, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.01% sodium dodecyl sulfate). Filters were placed in preheated (65°C) stoppered vials containing PM buffer and denatured labeled DNA and incubated at 65°C for 24 h. The filters were removed, thoroughly washed with 3× SSC, and air dried, and the radioactive counts were determined by using a Beckman model LS-7500 spectrometer and a scintillation fluid containing 4 g of Omnifluor (New England Nuclear Corp., Boston, Mass.) per liter of toluene.

RESULTS AND DISCUSSION

There was a high degree of homology between the DNAs from the strains of Tubiash et al. (7) (strains ATCC 19105, ATCC 19106, and ATCC 19109^T) and the DNAs from the strains of Jeffries (3) (strains NCMB 2164, NCMB 2165, and

NCMB 2166), with the degree of reassociation varying from 68 to 98% (Table 2). No other strain showed more than 26% reassociation with DNA from strain NCMB 2166. Similarly, when DNA from *V. anguillarum* ATCC 19264^T was used as the reference DNA, it had low (≤27%) degrees of reassociation with the DNAs of strains ATCC 19105, ATCC 19106, ATCC 19109^T, NCMB 2164, NCMB 2165, and NCMB 2166. These results suggest that the latter six strains are sufficiently related to one another genotypically and sufficiently distinct from other phenotypically similar vibrios to be considered members of a separate species.

It was only possible to study six strains, but these strains were isolated from estuarine areas at least 2,000 miles (3,218 km) apart and have been shown to be responsible for collapses of cultures of bivalve larvae (3, 8). In view of the potential economic importance of these strains, we believe that a species should be formally described, and we propose that this species be named *Vibrio tubiashii*. A species description is given below.

Vibrio tubiashii sp. nov. (tu.bi.ash'i.i. L. gen. n. *tubiashii* named after H. S. Tubiash, who first isolated the organism [7]) cells are gram-negative short rods (0.5 by 1.5 µm) that are straight or curved and motile by means of a single polar flagellum when they are grown in liquid media. The cells do not swarm on solid media, but lateral short-wavelength flagella may be produced. Colonies on marine agar (Difco Laboratories, Detroit, Mich.) are smooth, circular, and off-white and may be mucoid. Colonies on TCBS agar (Oxoid Ltd., Basingstoke, England) are smooth, circular, and yellow (sucrose fermenting). No pigments are produced, and strains do not luminesce. Sodium chloride is required for growth, the optimum concentration being 1 to 3% (wt/vol); strains are not able to grow in 8% (wt/vol) NaCl. Facultatively anaerobic. Acid but no gas is produced from glucose. Kovacs oxidase positive. Catalase positive. Nitrate reduced to nitrite. Chitin, DNA, gelatin, lecithin, starch, Tween 80, tyrosine, and xanthine are hydrolyzed extracellularly, but sodium alginate and elastin are not. Voges-Proskauer nega-

TABLE 2. DNA base compositions and levels of DNA-DNA relatedness of *V. tubiashii* and *V. anguillarum* strains to other strains of *V. tubiashii* and strains of *Vibrio* spp.

Test strain	G+C content (mol%) ^a	% Homology using ³² P-labeled DNA from strain: ^b		
		NCMB 2166	ATCC 19109 ^T	ATCC 19264 ^T
NCMB 2164	43	98	79	27
NCMB 2165	44	89	85	22
NCMB 2166	43	100	68	27
ATCC 19105	44	97	83	18
ATCC 19106	45	85	96	19
ATCC 19109 ^T	45	71	100	15
ATCC 19264 ^T	45	22	28	100
ATCC 33466 ^T	44	19	ND ^c	ND
NCTC 11327 ^T	48	17	ND	ND
ATCC 7708	46	16	ND	ND
ATCC 25917 ^T	46	19	ND	ND
ATCC 15338 ^T	50	13	ND	ND
ATCC 33125 ^T	45	26	ND	ND
ATCC 17749 ^T	47	24	ND	ND
ATCC 17802 ^T	47	24	ND	ND
ATCC 14035 ^T	48	13	ND	ND
ATCC 25918 ^T	40	19	ND	ND

^a Mean of three determinations. The level of accuracy was ±1.5%.

^b Mean of three determinations. The level of accuracy was ±14%.

^c ND, Not determined.

TABLE 3. Characteristics of *V. tubiashii* which differ from strain to strain

Trait	% of strains positive	Reaction of strain ATCC 19109 ^T	Strains of <i>V. tubiashii</i> that are positive
Acid from cellobiose	33	+ ^a	ATCC 19106, ATCC 19109 ^T
Møllers arginine ^b	83	+	ATCC 19105, ATCC 19109 ^T , NCMB 2164, NCMB 2165, NCMB 2166
Growth on:			
DL-3-Hydroxybutyrate	33	+	ATCC 19106, ATCC 19109 ^T
D-Alanine	67	+	ATCC 19105, ATCC 19106, ATCC 19109 ^T , NCMB 2166
Melibiose	50	+	ATCC 19106, ATCC 19109 ^T , NCMB 2165
Putrescine	17	—	ATCC 19106
Adenine	50	—	ATCC 19105, ATCC 19106, NCMB 2165
Growth at 37°C	33	—	NCMB 2164, NCMB 2165
Growth in:			
4% NaCl	83	+	ATCC 19105, ATCC 19106, ATCC 19109 ^T , NCMB 2165, NCMB 2166
5% NaCl	83	+	ATCC 19105, ATCC 19106, ATCC 19109 ^T , NCMB 2165, NCMB 2166
6% NaCl	17	—	NCMB 2166

^a +, Positive; —, negative.^b Character useful for differentiating *V. tubiashii* from other species of *Vibrio*.

tive. Grows on simple mineral media on a variety of organic carbon sources, including glycine, mannose, and galactose but not D-sorbitol or γ -aminobutyrate. Produces β -galactosidase. Acid is produced from sucrose and D-mannose but not from L-arabinose, arbutin, *m*-inositol, lactose, raffinose, L-rhamnose, salicin, D-sorbitol, or D-xylose. Susceptible to 2,4-diamino-6,7 diisopropylpteridine (agent 0/129) phosphate (10- μ g disk) and polymyxin B (50-Iu disk). Not able to decarboxylate lysine and ornithine. Chicken erythrocytes are not agglutinated. Pathogenic for oyster (*Crassostrea virginica*, *Crassostrea gigas*, and *Ostrea edulis*) and clam (*Mercuraria mercenaria*) larvae. The characteristics which vary among strains are listed in Table 3. The base composition of the DNA ranges from 43 to 45 mol% G+C.

All strains have been isolated from diseased larvae of clams and oysters.

The type strain is ATCC 19109. The description of the type strain is the same as that given above for the species,

and other characteristics are given in Table 3. The base composition of the DNA is 43 to 45 mol% G+C, as determined by thermal denaturation.

Additional characteristics of strains ATCC 19105, ATCC 19106, and ATCC 19109^T have been given elsewhere (9). Characteristically, all strains of the species are able to hydrolyze xanthine and tyrosine extracellularly over a 14-day incubation period. Although the zone of clearing in these tests may only be below the colony, it is a character that is easy to determine and useful for identification. No other species of *Vibrio* described so far can degrade xanthine, and tyrosine hydrolysis is restricted to only a few strains (9). Some strains of *V. tubiashii* are also unusual for vibrios in being able to grow on adenine and melibiose (Table 3). In addition, strains of *V. tubiashii* can grow on glycine, are Voges-Proskauer negative, do not produce acid from L-arabinose or D-sorbitol, do not agglutinate chicken erythrocytes, and are not able to grow on sorbitol. These features

TABLE 4. Characters useful for distinguishing *V. tubiashii* from phenotypically similar species^a

Trait	<i>V. tubiashii</i>	<i>V. anguillarum</i>	<i>V. nereis</i>	<i>V. fluvialis</i>	<i>V. splendidus</i> biovar 1	<i>V. ordalii</i>	<i>V. diazotrophicus</i>	<i>V. metschnikovii</i>
Luminescence	— ^b	—	—	—	+	—	—	—
Gas from glucose	—	—	—	V	—	—	—	—
Oxidase	+	+	+	+	+	+	+	—
Nitrate reduction	+	+	+	+	+	—	+	—
Voges-Proskauer	—	+	—	—	—	—	—	+
Acid from:								
L-Arabinose	—	V	—	+	—	—	+	—
Cellobiose	V	+	—	V	+	+	+	V
Salicin	—	—	—	V	—	V	+	—
D-Sorbitol	—	V	—	—	—	—	—	V
Growth on D-xylose	—	—	—	—	—	—	+	—
Extracellular degradation of:								
Casein	+	+	—	+	V	+	—	+
Chitin	+	+	+	+	+	+	—	+
DNA	+	+	+	+	+	+	—	+
Gelatin	+	+	—	+	+	+	—	+
Lecithin	+	V	—	+	+	—	—	+
Tween 80	+	+	—	+	+	—	—	+
Tyrosine	+	—	+	V	—	—	—	—
Xanthine	+	—	—	—	—	—	—	—

^a Data from references 9 and 10.^b —, Negative; +, positive; V, variable among strains.

readily distinguish *V. tubiashii* from *V. anguillarum*, and *V. tubiashii* may be distinguished from other similar species of *Vibrio* on the basis of the tests shown in Table 4.

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