Vibrio mytili sp. nov., from Mussels

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Five strains isolated from mussels (harvested off the Atlantic Spanish coast in 1985 and 1986) were found to be phenotypically distinct from previously described *Vibrio* species. They showed 94 to 100% intragroup relatedness as determined by DNA-DNA hybridization (S1 nuclease method) but were found to be only 1 to 25% related to other *Vibrio* species. These strains have all of the properties that define the genus *Vibrio* and can be clearly differentiated from other species by their positive responses in tests for Thornley's arginine dihydrolase, gas production from glucose, growth in media containing 10% NaCl, and acid production from sucrose, L-arabinose, D-xylose, and D-cellobiose and their negative responses in tests for lysine decarboxylase, the Voges-Proskauer reaction, growth without NaCl and at 40°C, hydrolysis of gelatin, casein, starch, DNA, and alginate, and acid production from D-mannose. The G+C ratio of the DNA is 45 to 46 mol%. The name *Vibrio mytili* is proposed for the new species; strain 165 (= CECT 632) is the type strain.

The genus *Vibrio* currently contains more than 35 species, most of which are of marine origin. In addition to the 22 *Vibrio* species described in *Bergey's Manual of Systematic Bacteriology* (2), 14 new species have been recognized (3, 4, 7, 9, 15, 17, 18, 20, 24, 31, 33, 36, 37, 41). Phenotypic characterization data for many fresh isolates indicate that it is often difficult to correctly identify these organisms to species level and suggest that a number of isolates might correspond to undescribed species (6, 21, 28, 39, 40).

It is generally agreed that a bacterial species is a group of strains with high levels of intragroup DNA relatedness which can also be recognized by phenotypic traits (38). There is no such operational definition of a genus at the present time. Although phylogenetic criteria are of tremendous importance in delineating a genus (and higher taxa), it has been agreed that genera should contain species with common phenotypic traits to allow phenotypic identification at the genus level (26). Although splitting of the genus *Vibrio* into *Vibrio* sensu stricto and the genus *Listonella* on the basis of molecular data has been proposed (25), there is not general agreement about the constituent species of the genus *Listonella* (10, 35) or the phylogenetic justification of this proposed genus (8, 27), and most workers still refer to *Listonella* spp. as *Vibrio* spp. for phenotypic reasons.

Five strains isolated from mussels could not be identified as any known *Vibrio* species (28). The purpose of this work was to examine the taxonomic position of these strains relative to known species by DNA-DNA hybridization and extensive phenotypic tests. The outcome is the description of a new species named *Vibrio mytili*.

MATERIALS AND METHODS

Bacterial strains. *V. mytili* 163, 165^T (T = type strain), 167, 178, and 185, the strains that constituted phenon 3 (28), were isolated from *Mytilus edulis* and were maintained as working stocks at room temperature in semisolid seawater agar containing 1% (wt/vol) peptone, 0.3% meat extract, and 0.3% purified agar dissolved in 75% (vol/vol) aged, filtered seawater in distilled water; the pH was adjusted to 7.3 after

boiling and filtering of the medium. For DNA hybridization experiments, a collection of *Vibrio* DNAs was used (37) (Table 1).

Physiological and conventional biochemical characterization. Unless otherwise indicated, incubations were done at 28°C and commercial media were supplemented with 1% (wt/vol) NaCl. The oxidation-fermentation test and the test for gas production from glucose, both performed in F1 medium (1), tests for motility, swarming, and a specific requirement for sodium, and nutritional screening on multi-inoculated basal medium plates were performed by using previously described methods (1). The oxidase test, the Voges-Proskauer test (24 and 48 h), tests for Moeller's decarboxylases for lysine and ornithine, the salt tolerance test, and tests for acid production from carbohydrates and esculin hydrolysis were performed by using the methods of Lee and Donovan (22). Arginine dihydrolase (ADH) activity was tested by two methods: Thornley's method, as modified by Baumann and Baumann (1), and Moeller's method in decarboxylase medium base (Difco) containing 0.5% L-arginine (Merck) and 1% NaCl (i.e., as lysine decarboxylase and ornithine decarboxylase). Although these methods are generally considered to be equivalent, only the first method gives reliable differential results in some species (29, 32). Alginate hydrolysis, amylase, Tween 80 hydrolysis, and nitrate reduction tests were done as described by Furniss et al. (13). Indole production and H₂S production were tested on SIM medium (Difco) after 24 h. Growth at different temperatures was tested on tryptone soya broth after 24 h for 37, 40, and 42°C, after 7 days for 10°C, and after 2 weeks for 4°C. Catalase activity was detected by flooding a slant of nutrient agar with 10 volumes of H₂O₂. Luminescence was visualized in the dark after 10 min of adaptation with 24-h cultures grown on seawater agar. Susceptibility to vibriostatic agent O/129 (150 µg) was tested as described by Baumann et al. (2) by using Oxoid discs and nutrient agar containing 0.9% NaCl. Urease activity was tested on urea broth (Difco) incubated for 6 days. Citrate utilization was tested on Simmons citrate agar (Oxoid). DNase activity was read after 48 h of incubation by flooding DNase agar (Oxoid) plates with a 0.1% (wt/vol) aqueous solution of toluidine blue. A pink coloration around the growth was recorded as

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positive. The gelatin liquefaction test was performed by using method 1 of Smibert and Krieg (34). Tyrosine, xanthine, and casein media were prepared as double-layered plates containing marine agar 2216 (Difco) in the bottom layer and marine agar supplemented with the substrate (1%, wt/vol) in the upper layer. Plates were spot inoculated and incubated for 14 days. Flagella were visualized after staining by the method of Heimbrook et al. (19).

Assimilation tests. Carbon source utilization tests were performed with Biotype strips (BioMérieux, La Balme-les-Grottes, France), which previously have been referred to as specially manufactured API strips (37). A 60-ml portion of Biotype medium no. 1 (BioMérieux) was supplemented with 2.5% NaCl and 0.02% MgCl₂ and was inoculated with 2 ml of a 100-Klett unit suspension of bacterial cells in 2.5% NaCl in water. The strips were completely filled with the seeded medium so as to obtain a convex meniscus and incubated at 30°C for 4 days. The strips were examined visually for growth after 2 and 4 days. The results were entered in an Apple Macintosh computer by using the Recognizer program (Institut Pasteur Taxolab, Paris, France).

Determination of G+C content. The melting temperatures of DNA samples in 0.1× SSC buffer were measured with a Gilford spectrophotometer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). DNA from *Escherichia coli* B was used as a standard (guanine-plus-cytosine [G+C] content, 51.2 mol%). G+C contents were calculated as described by Owen and Hill (30).

DNA-DNA hybridizations. We used previously described procedures to extract, purify, and shear unlabeled DNA (5). In vitro labeling of DNA with tritiated nucleotides and hybridization by the S1 nuclease method have been described in detail previously (16). Hybridization experiments were conducted at 60°C.

RESULTS AND DISCUSSION

The phenotypic properties common to the strains from mussels which we studied are given in the species description below.

Identification to the genus level. The five strains from mussels (163, 165^T, 167, 176, and 185) had all of the properties of the genus *Vibrio*. The cells were gram negative and polarly flagellated. The strains were facultative anaerobes that were able to produce acid from glucose under anaerobic conditions. All required sodium ions for growth. The strains were oxidase positive, reduced nitrates to nitrites, used D-mannitol as a sole carbon and energy source, and were susceptible to vibriostatic compound O/129 (2,4-diamino-6,7-diisopropylpteridine). The G+C contents of the DNAs from strains 165^T and 163 were 45.6 and 45.1 mol%, respectively.

DNA relatedness. Three other strains isolated from mussels were 94 to 100% related to strain 165^T (Table 1). Known *Vibrio* species included in the study were 1 to 25% related to strain 165^T. The species most closely related to the mussel isolates was *Vibrio parahaemolyticus*. Clearly, the strains from mussels constitute a genomic species distinct from known *Vibrio* species.

Phenotypic differentiation of the new genomic species. The major characteristics that differentiate the new genomic species from other halophilic, ADH-positive *Vibrio* species are given in Table 2.

The strains from mussels were first thought to be gas-producing *Vibrio diazotrophicus* strains. It should be noted that these strains are only 11% related to *V. diazotrophicus*

TABLE 1. Levels of reassociation between DNA from *V. mytili* 165^T and DNAs from members of other *Vibrio* species and related genera

Source of unlabeled DNA ^a	% Reassociation with labeled DNA from strain 165 ^T
<i>Vibrio mytili</i> 165 ^T	100 (0.0) ^b
<i>V. mytili</i> 163	94 (0.5)
<i>V. mytili</i> 167	95 (0.0)
<i>V. mytili</i> 185	100
<i>V. parahaemolyticus</i> CECT 512 ^T	25
<i>V. pelagius</i> (<i>Listonella pelagia</i>) CIP 102762 ^T	19
<i>V. harveyi</i> NCIMB 1280 ^T	18
<i>V. alginolyticus</i> NCIMB 1903 ^T	17
<i>V. campbellii</i> NCIMB 1894 ^T	17
<i>V. natriegens</i> ATCC 14048 ^T	15
<i>V. diazotrophicus</i> ATCC 33466 ^T	11
<i>V. proteolyticus</i> ATCC 15338 ^T	10
<i>V. tubiashii</i> CIP 102760 ^T	9
<i>V. orientalis</i> CIP 102891 ^T	9
<i>V. fluvialis</i> ATCC 33809 ^T	8
<i>V. navarrensis</i> 1397-6 ^T	8
<i>V. nereis</i> NCIMB 1897 ^T	8
<i>V. vulnificus</i> ATCC 27562 ^T	7
<i>V. ordalii</i> NCIMB 2167 ^T	7
<i>V. (Listonella) anguillarum</i> NCIMB 6 ^T	6
<i>V. (Listonella, Photobacterium) damsela</i> NCIMB 2184 ^T	6
<i>V. mimicus</i> ATCC 33653 ^T	6
<i>V. cholerae</i> CECT 514 ^T	6
<i>V. cincinnatiensis</i> ATCC 35912 ^T	5
<i>V. mediterranei</i> CECT 514 ^T	5
<i>V. metschnikovii</i> ATCC 7708 ^T	4
<i>V. (Photobacterium) fisheri</i> NCIMB 1281 ^T	3
<i>V. costicola</i> NCIMB 701 ^T	1
<i>Aeromonas hydrophila</i> ATCC 7966 ^T	2
<i>A. caviae</i> ATCC 15468 ^T	1
<i>Escherichia coli</i> K-12	1

^a ATCC, American Type Culture Collection, Rockville, Md.; CECT, Colección Española de Cultivos Tipo, Universitat de València, Valencia, Spain; CIP, Collection de l'Institut Pasteur, Paris, France; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom.

^b The values in parentheses are ΔT_m values (in degrees Celsius) (ΔT_m is the difference between the melting temperature of the homologous hybrid and the melting temperature of the heterologous hybrid).

as determined by DNA-DNA hybridization and that phenotypic tests are available to distinguish these taxa (Table 2). Moreover, a numerical taxonomic study which included 20 type strains of *Vibrio* species showed no relationship at the species level between the strains in phenon 3 (163, 165^T, 167, 178, and 185) and *V. diazotrophicus* NCIMB 2169^T (28).

Some of the unidentified *Vibrio* strains characterized by Lee and coworkers as phenon 5 (23) appear to be very similar to the mussel strains. These strains remained an unidentified phenon in the study of Bryant et al. (6) and showed no genetic relationship at the species level to *V. diazotrophicus* (17). Their phenotype suggests that they may be similar to the mussel strains.

The following *Vibrio* species were not included in the DNA-DNA reassociation experiments: *Vibrio aestuarianus*, *Vibrio hollisae*, *Vibrio nigrapulchritudo*, *Vibrio splendidus*, *Vibrio marinus*, *Vibrio (Photobacterium) logei*, *Vibrio gazogenes*, *Vibrio furnissii*, *Vibrio carchariae*, and *Vibrio salmonicida*. Each of these species differs by at least eight traits from the mussel strains. *V. furnissii* is the only species that presents a phenotypic profile that might be confused with the

TABLE 2. Characteristics useful for differentiating *V. mytili* from other Thornley's ADH-positive and/or gas-producing *Vibrio* species

Taxon	Voges-Proskauer reaction	Gas production	Growth in the presence of 10% NaCl	Growth at 40°C	Hydrolysis of:		Acid produced from:				Use of GABA ^a
					Starch	Tween 80	Arabinose	Xylose	Mannose	Cellobiose	
<i>V. mytili</i>	— ^b	+	+	—	—	+	+	+	—	+	—
<i>V. furnissii</i>	—	+	d	+	d	+	+	—	+	—	+
<i>V. fluvialis</i>	—	—	d	+	+	+	+	—	+	d	+
<i>V. diazotrophicus</i>	—	—	—	d	+	—	+	+	d	+	+ ^c
<i>V. splendidus</i> biogroup I	—	—	—	—	+	+	—	—	+	+	—
<i>V. nereis</i>	—	—	—	d	—	—	—	d	+	d	+
<i>V. anguillarum</i>	+	—	—	—	+	+	d	—	+	d	—
<i>V. tubiashii</i>	—	—	—	— ^c	+	+	—	—	+	d	—
<i>V. aestuarianus</i>	—	—	—	—	+ ^c	+ ^c	—	—	+	+	— ^c
<i>V. orientalis</i>	—	—	— ^c	—	+	+	—	— ^c	+	+	— ^c
<i>V. mediterranei</i>	—	—	—	—	d	+	—	d	+	+	—
<i>V. costicola</i> ^d	+	—	+	+	—	d	—	—	d	—	—
<i>V. metschnikovii</i> ^e	+	—	—	+	+	+	—	—	d	—	—
<i>V. gazogenes</i> ^e	—	+	—	+	+	+	+	+	+	ND	—
<i>V. (Listonella, Photobacterium) damsela</i> ^f	+	+	—	—	d	+	—	—	+	—	—
<i>V. proteolyticus</i> ^f	+	—	+	+	+	+	—	—	+	+	—

^a GABA, 4-aminobutyrate.^b +, 90% or more of the strains are positive; —, 90% or more of the strains are negative; d, 11 to 89% of the strains are positive; ND, no data available.^c Results obtained with the type strain of the species.^d *V. costicola* is a moderately halophilic organism that grows in the presence of NaCl concentrations up to 20%. Most strains are nitrate negative (14).^e Species is negative for oxidase activity and nitrate. *V. gazogenes* is ADH negative and red pigmented.^f Species is sucrose negative.

mussel strain profile in short, routine identification, but is easily distinguished by its inability to ferment D-xylose, cellobiose, and arbutin, its positive response in the DNase test, and its growth at 40°C. The assimilation properties are also clearly different; *V. furnissii* uses D-galacturonate, 4-aminobutyrate, putrescine, and L-arginine, while the mussel strains do not use these compounds but do utilize D-xylose. Since *V. furnissii* and *Vibrio fluvialis* are closely related (40 to 64% [5]) and *V. fluvialis* is only 8% related to mussel isolate 165^T, one can assume that *V. furnissii* is also genetically distinct from the mussel isolates. Another species that uses xylose and produces gas, *V. gazogenes*, differs from the mussel strains by its negative oxidase, nitrate, and ADH reactions and by fermenting D-mannose (2, 11, 12). *V. hollisae*, *V. nigripulchritudo*, *V. splendidus* biogroup II, *V. marinus*, *V. (Photobacterium) logei*, and *V. salmonicida* are differentiated from the mussel strains by their negative reactions for gas production, Thornley's ADH, growth in the presence of 10% NaCl, and acid production from mannose (11, 15). The other three species not tested in hybridization experiments (*V. furnissii*, *V. aestuarianus*, and *V. splendidus* biogroup I) are Thornley's ADH positive and could be clearly separated from the new species on the basis of their phenotypes, as shown in Table 2.

It must be emphasized that arginine alkalization was negative on Moeller's medium (it was positive only as determined by Thornley's method, a behavior reported previously for *Vibrio mediterranei* [32] and observed in several strains of *V. splendidus* biogroup I, *Vibrio orientalis*, and *Vibrio tubiashii* [29]).

When the Recognizer program was used, the nutritional properties of *V. mytili* did not fit with those of any known species (even when the numbers of allowed differences with known species were 5 positive tests and 10 negative tests).

On the basis of the criteria recommended by a group of experts (38), these strains constitute a new species, for which the name of *Vibrio mytili* is proposed.

Description of *Vibrio mytili* sp. nov. *Vibrio mytili* (my.ti'.li. L. gen. n. *mytili*, of mussels [the genus *Mytilus*]). Gram-negative coccobacilli motile by means of one polar flagellum. Grows readily on marine agar 2216 (Difco), yeast extract agar (1, 2), and tryptone soya agar (Oxoid) containing 1% NaCl and in semisolid seawater agar (stab cultures). Colonies develop within 24 h at 28°C and are regular in shape and unpigmented. Grows on thiosulfate-citrate-bile-sucrose agar (yellow colonies) but not on MacConkey agar. Swarming has not been detected in any of the media used. Glucose metabolism is fermentative, with production of gas. Tests for catalase, oxidase, and nitrate reduction to nitrite are positive. No growth occurs without addition of NaCl to the culture medium. The salt requirement is specific for sodium ions. Grows in the presence of 1 to 10% (wt/vol) NaCl but not in the presence of 20% (wt/vol) NaCl. Susceptible to vibriostatic agent O/129 (150 µg per disc). Growth occurs at temperatures between 10 and 37°C, but not at 4 or 40°C. Not luminescent. Voges-Proskauer, indole, H₂S, lysine and ornithine decarboxylase, and β-glucuronidase negative. Thornley's ADH positive. The strains are not able to hydrolyze urea, DNA, gelatin, casein, starch, alginate, and xanthine but degrade Tween 80, esculin, and tyrosine. Acid is produced from sucrose, L-arabinose, arbutin, D-cellobiose, D-galactose, maltose, D-trehalose, salicin, and D-xylose, but not from D-mannose, D-sorbitol, myo-inositol, D-melibiose, L-rhamnose, and adonitol.

All *V. mytili* strains utilize the following substrates as sole carbon and energy sources: *N*-acetyl-D-glucosamine, *cis*-aconitate, *trans*-aconitate, D-alanine, L-alanine, L-aspartate, D-cellobiose, esculin, D-fructose, fumarate, D-galactose, gentibiose, D-gluconate, D-glucosamine, D-glucose, L-glutamate, DL-glycerate, glycerol, 2-ketoglutarate, DL-lactate, L-malate, maltose, maltotriose, D-mannitol, 1-*O*-methyl-β-D-glucoside, palatinose, L-proline, D-raffinose, D-ribose,

L-serine, succinate, sucrose, D-trehalose, D-turanose, L-tyrosine, and D-xylose. Four of the five strains use L-arabinose, betaine, citrate, lactose, D-malate, and D-melezitose.

None of the strains utilizes the following substrates as sole carbon and energy sources: adonitol, 4-aminobutyrate, 5-aminovaleate, D-arabitol, L-arabitol, benzoate, caprate, caprylate, *m*-coumarate, dulcitol, *i*-erythritol, ethanolamine, L-fucose, D-galacturonate, gentisate, D-glucuronate, glutarate, histamine, L-histidine, 3-hydroxybenzoate, 4-hydroxybenzoate, 3-hydroxybutyrate, *myo*-inositol, itaconate, 2-ketogluconate, 5-ketogluconate, D-lyxose, malonate, D-mannose, D-melibiose, 1-*O*-methyl- α -galactoside, 3-*O*-methyl-D-glucose, 1-*O*-methyl- α -D-glucoside, mucate, phenylacetate, 3-phenylpropionate, protocatechuate, putrescine, quinate, L-rhamnose, D-saccharate, D-sorbitol, L-sorbose, D-tagatose, D-tartrate, L-tartrate, *meso*-tartrate, tricarballoylate, trigonelline, tryptamine, tryptophan, and xylitol.

The G+C content is 45 to 46 mol% (as determined by the thermal method).

Isolated from mussels (*Mytilus edulis*) commercialized in Valencia but harvested off the Atlantic Spanish coast (Galicia). Not known to be pathogenic for humans or mussels.

The type strain is strain 165.

Description of the type strain. Strain 165^T has all of the properties given above for the species. In addition, L-arabinose, betaine, citrate, lactose, lactulose, D-malate, D-melezitose, and 1-methyl- β -galactoside are utilized in 2 or 4 days and maltitol is not utilized. The G+C content of the DNA is 45.6 mol%. This strain has been deposited at the Colección Española de Cultivos Tipo (Valencia, Spain) as strain CECT 632.

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