Vibrio scopthalmi sp. nov., a New Species from Turbot (*Scophthalmus maximus*)

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Six strains isolated from the intestines of juvenile turbot in a fish hatchery in the north of Spain were found to be phenotypically members of the genus *Vibrio*. However, the phenotypic traits of these organisms did not place them in any of the currently known *Vibrio* species. These isolates formed an homogeneous group in which the DNA-DNA similarity values (the differences between the thermal denaturation midpoints of the homologous and heterologous duplexes) with reference strain A089^T (T = type strain) ranged from 0 to 1.7°C. The results of a 16S rRNA sequence analysis of A089^T placed this strain in the genus *Vibrio* in the gamma subclass of the *Proteobacteria*. The closest relative is *Vibrio aestuarianus*, with a sequence similarity of 97.8%. This group of strains can be easily differentiated from the other *Vibrio* species by their clear phenotype. We propose the name *Vibrio scopthalmi* sp. nov. for these strains; the type strain is strain A089 (= CECT 4638).

The genus *Vibrio* comprises more than 35 species, most of which are widely distributed in marine environments. Some of the members of the genus are pathogenic for humans and marine animals; the pathogenicity is particularly severe if the hosts are under stressed conditions (11). However, members of the genus *Vibrio* are the most common bacteria in the gastrointestinal flora of many fish (7, 21, 22). Turbot (*Scophthalmus maximus*) is a flatfish that is important in aquaculture. High rates of mortality of turbot larvae have been reported during the first days of feeding (36), and these high rates coincide with high levels of a heterogeneous microflora in the gastrointestinal tracts of the larvae. However, when a homogeneous microflora is established in the gut, the fish show enhanced resistance to colonization by bacterial pathogens. The gut microflora may play an important role in preventing colonization by bacterial pathogens (8, 20).

A number of phenotypically similar strains were isolated as the dominant bacteria in the gastrointestinal tracts of juvenile turbot (*S. maximus*) which were reared in a fish hatchery in the north of Spain. These bacteria belong to genus *Vibrio* on the basis of their phenotypic characteristics. However, these strains could not be identified as members of any of the previously described *Vibrio* species (6, 11, 23, 24, 34). In this study we demonstrate that this group of bacteria constitutes a genotypically well-defined new *Vibrio* species with a phenotype that is clearly distinct. The name *Vibrio scopthalmi* is proposed for the new species.

MATERIALS AND METHODS

Bacterial strains. The six *V. scopthalmi* strains studied (strains A089^T [T = type strain], A102, A105, A107, A110, and SL/G2) were isolated from the gastrointestinal tracts of 35- to 40-day-old turbot. The fish were anesthetized with 10 mg of MS222 (tricaine methane sulfonate) per liter, and after surface disinfection with a 0.1% benzalkonium chloride saline solution and thorough rinsing with sterile marine phosphate-buffered saline (PBS) (20 g of NaCl per liter, 0.2 g of KCl per liter, 1.15 g of PO₄HNa₂ per liter, 0.2 g of PO₄H₂K per liter; pH 7.1 to 7.2), the intestine was completely removed from each fish and both the intestine and its contents were homogenized in 10 ml of sterile marine PBS.

Serial 10-fold dilutions of the homogenate were plated onto tryptone soy agar supplemented with 1.5% NaCl. The plates were incubated at 20°C for 48 h. For reference purposes, the following three strains of different *Vibrio* species were used in the genotypic characterization: *Vibrio aestuarianus* LMG 7909^T, *Vibrio mediterranei* LMG 11258^T, and *Vibrio splendidus* ATCC 33125^T.

Biochemical characterization. Biochemical characterization was performed by using standard methods. The oxidation-fermentation test and the motility test were performed in Hugh and Leifson's oxidation-fermentation basal medium. The oxidase test, decarboxylase tests, and starch hydrolysis tests were performed as described by Furniss et al. (13). Catalase activity was tested by flooding a nutrient agar plate with H₂O₂ (10%). Luminiscence was visualized in the dark by using an overnight culture grown on marine agar (Pronadisa) after 10 min of adaptation to the dark. Sensitivity to vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine) (10 and 150 µg) was determined by using Oxoid discs as recommended by the manufacturer. DNase activity was detected by flooding an 18-h culture grown on DNase agar with 1 N HCl. Citrate utilization was determined on Simmons citrate agar (Oxoid) as described by Smibert and Krieg (29). Urease activity was tested on Christensen urea agar (Oxoid) as described by Smibert and Krieg (29). The *o*-nitrophenyl-β-D-galactopyranoside test was performed with Difco discs and marine PBS. Salt tolerance tests were performed on Bacto Peptone (Difco) medium supplemented with NaCl (0, 0.5, 1, 2, 3, 6, 8, 10%). Growth in peptone water at different temperatures was tested at 4, 22, 30, 37, and 40°C. Growth on thiosulfate-citrate-bile salts-sucrose agar (9, 15) and VAM (3) was also tested. The test preparations were incubated at 23°C and were examined after 24 h. Some test preparations were examined again after 48 h. All of the test media were supplemented with 1.5% NaCl. In addition, API 20E strips (BioMérieux SA, Marcy, l'Étoile, France) and Biolog microplate tests (Biolog, Inc., Hayward, Calif.) were used. Both test kits were inoculated and incubated by using the modifications described by Austin et al. (4). A similarity matrix for previously described vibrios (1, 2) and the software Bacterial Identifier (T. N. Bryant, Blackwell Scientific Publications, Oxford, United Kingdom) were used to identify the isolates.

DNA isolation and 16S rRNA sequencing. The bacterial strains were grown in 500 ml of marine broth (Difco) for 24 h at 23°C. Bacteria were harvested by centrifugation and washed twice in marine PBS. Cells were lysed and DNAs were isolated by the method of Marmur (18). In vitro amplification of 16S rRNA genes and direct sequencing of the amplified DNA fragments were performed as previously described (30).

Sequence data analysis. The sequence determined in this study was added to an alignment of about 3,000 homologous bacterial 16S rRNA primary structures by using the aligning tool of the ARB program package (30a). Similarity and distance matrices were calculated with the program ARB-PHYL of the same package. Phylogenetic trees were constructed by using subsets of data that included representative sequences of *Vibrio* spp. strains (17). We used distance matrix, bootstrapped distance matrix, and maximum-likelihood methods as implemented in the programs of the PHYLIP (12), ARB, and fastDNAm1 (17) program packages, respectively.

Determination of G+C content. The guanine-plus-cytosine (G+C) content was determined by hydrolyzing the DNA enzymatically and quantifying the nucleosides by high-performance liquid chromatography (31) with the modifications described previously (26). DNA from *Escherichia coli* B (G+C content, 52

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TABLE 1. Phenotypic properties of *V. scophtalmi* strains^a

Test	Reaction of strain A089 ^T	Strain(s) that give different results
L-Alanine	—	SL/G2
Glycogen	—	A107, A110, SL/G2
D-Trehalose	—	A102, SL/G2
Turanose	—	A107, SL/G2
D-Glucuronic acid	—	A102, A110, SL/G2
Succinamic acid	—	A110, SL/G2
Glucuronamide	—	A110, SL/G2
L-Alanylglycine	—	A110, SL/G2
L-Aspartic acid	—	A110, SL/G2
Glycyl-L-aspartic acid	—	A110, SL/G2
Glycyl-L-glutamic acid	—	A110, SL/G2

^a Tests that were positive for all of the strains were the tests for motility, oxidase, catalase, NO₂ production, arginine dihydrolase, and utilization of α -D-glucose, sucrose, D-fructose, N-acetyl-D-glucosamine, and inosine. Tests that were negative for all of the strains were the tests for luminescence, lysine decarboxylase, ornithine decarboxylase, H₂S production, urease, gelatinase, amylase, DNase, β -galactosidase, tryptophan deaminase, indole production, the Voges-Proskauer reaction, and utilization of α -cyclodextrin, Tween 80, citrate, adonitol, D-arabitol, i-erythritol, m-inositol, xylitol, D-raffinose, L-rhamnose, D-galactose, gentiobiose, α -lactose, α -D-lactose, lactulose, D-melibiose, D-sorbitol, D-mannitol, β -methyl-D-glucoside, formic acid, D-galactonic acid lactone, D-glucosaminic acid, α -hydroxybutyric acid, γ -hydroxybutyric acid, p-hydroxyphenylacetic acid, itaconic acid, α -ketovaleric acid, malonic acid, quinic acid, D-saccharic acid, sebacic acid, urocanic acid, D-alanine, L-histidine, hydroxy-L-proline, L-phenylalanine, L-pyrogutamic acid, D-serine, DL-carnitine, phenylethylamine, 2-amino-ethanol, glycerol, putrescine, and glucose 1-phosphate.

mol%) and lambda DNA (G+C content, 49.9 mol%; Sigma Chemical Co., St. Louis, Mo.) were used as standards.

DNA-DNA hybridization. Purified DNAs of *V. scophtalmi* A089^T and *V. aestuarianus* LMG 7909^T were labeled with ¹²⁵I by the method described by Selin et al. (28). The quantitative bacterial dot method (33) was used in the DNA-DNA hybridization experiment. The hybridization temperature was 25°C below the thermal denaturation midpoint (*T_m*) of the reference strain. DNA-DNA similarity was determined as the difference between the *T_m* of the homologous duplex and the *T_m* of the heterologous duplex (ΔT_m). The pooled standard deviation in this experiment was 0.43°C.

Nucleotide sequence accession number. The 16S rRNA gene sequence determined in this study has been deposited in the GenBank database under accession number U46579.

RESULTS AND DISCUSSION

Phenotypic characterization. The six strains isolated from turbot have all of the properties of the genus *Vibrio*; they are facultatively anaerobic short rods that are Gram stain negative and oxidase positive, are susceptible to vibriostatic compound O/129, and are able to reduce nitrates to nitrites. All of these strains require NaCl, and the optimal range of NaCl concentrations is 1 to 3% (wt/vol). None of the six strains grew in alkaline peptone water containing no NaCl, and none grew in the presence of an NaCl concentration of 6% (wt/vol) or more. All of the strains grow well at 22 and 30°C.

A total of 97 phenotypic features were investigated for the six isolates. This group of organisms was phenotypically homogeneous, and there were only 11 variable traits (Table 1). The biochemical properties of the six strains studied did not correspond to those of any previously described *Vibrio* species (6, 11, 23, 24, 25, 34), as determined by the methods used for identification (1, 2). The whole group could be easily differentiated from *V. aestuarianus* and other close relatives by its clearly distinct phenotype (Table 2).

Phylogenetic affiliation. The phylogenetic position of strain A089^T was investigated by analyzing the 16S rRNA gene sequence. The results of this analysis showed that the closest relative of A089^T is *V. aestuarianus* (27, 32) (sequence similarity, 97.8%) (Table 3). A089^T is a member of the gamma subclass of the *Proteobacteria*, and as shown in Fig. 1, A089^T appeared to be affiliated with the true vibrios (10, 16) together with the marine vibrios (5). In all cluster analyses a common root with *V. aestuarianus* was observed, and the new organisms formed an isolated branch within the vibrio cluster. In any case, both A089^T and *V. aestuarianus* appeared to be on the same phylogenetic branch as *Vibrio metschnikovii*, as was observed when partial sequences were used (14). The sequence signatures for the genus described by Dorsch et al. (10) were also conserved in A089^T. The 16S rRNA similarity to the closest relative, *V. aestuarianus*, is the same as the mean of the similarities among the 13 species included on branch B1 (Fig. 1). Some of the species on this branch have nearly identical se-

TABLE 2. Characteristics that are useful for distinguishing *V. scophtalmi* from its closest relatives^a

Test	<i>V. scophtalmi</i>	<i>V. aestuarianus</i>	<i>V. splendidus</i> I	<i>V. splendidus</i> II	<i>V. vulnificus</i>	<i>V. mediterranei</i>
Arginine dihydrolase	+ ^b	+	(+)	v	—	—
Lysine decarboxylase	—	v	—	—	+	+
Ornithine decarboxylase	—	—	—	—	+	—
Indole	—	+	+	d	+	+
ONPG ^c	—	+ ^d	(+)	v	+	+ ^d
Citrate	—	+	+	+	+	—
D-Glucosamine	+	— ^d	—	—	+	+ ^d
Utilization of:						
Sucrose	+	+	(+)	—	(—)	+
D-Mannitol	—	+	+	+	(+)	+
Production of:						
Gelatinase	—	+	+	+	+	—
Amylase	—	+ ^d	+	+	+	v
Growth in the presence of 6% NaCl	—	+	+	v	d	+
Growth at:						
4°C	—	+	d	—	—	—
35°C	+	+	v	—	+	(+)
40°C	—	—	—	—	+	—

^a The data for taxa other than *V. scophtalmi* were obtained from references 1, 2, 6, 11, and 25.

^b +, more than 90% of the strains are positive; (+), 75 to 89% of the strains are positive; v, 26 to 74% of the strains are positive; (—), 11 to 25% of the strains are positive; —, less than 10% of the strains are positive; d, values determined in previous studies differ.

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

^d Result obtained for the type strain.

TABLE 3. Genotypic characteristics of strains of *V. scopthalmi* and closely related *Vibrio* species

Species	Strain ^a	ΔT_m (°C) with:		% 16S rRNA sequence similarity with:		G+C content (mol%)
		A089 ^T	<i>V. aestuarianus</i>	A089 ^T	<i>V. aestuarianus</i>	
<i>V. aestuarianus</i>	LMG 7909 ^T	7.1	0	97.8		43–44 ^b
<i>V. mediterranei</i>	LMG 11258 ^T	8.5	6	96.3	96.9	42–43 ^c
<i>V. splendidus</i>	ATCC 33125 ^T	10.2	7.4	96.2	97.0	45–46 ^d
<i>V. scopthalmi</i>	A089 ^T	0	6.8		97.8	45.3
	A102	0	7.7			44.7
	A105	1.7				43.8
	A107	1.1				44.4
	A110	0.8				44.0
	SL/G2	0.7				45.5

^a ATCC, American Type Culture Collection, Rockville, Md.; LMG, Laboratorium voor Microbiologie, Rijksuniversiteit, Ghent, Belgium.

^b Data from reference 32.

^c Data from reference 25.

^d Data from reference 6.

quences, although genomically they are clearly separate species (19).

G+C content and DNA-DNA hybridization. The G+C contents of the isolates ranged from 44 to 45.5 mol% (Table 3), which was consistent with the overall values for the genus (16). These G+C contents are similar to the value observed for *V. aestuarianus* (32). DNA-DNA hybridization studies were performed with all of the isolates and representative *Vibrio* species. All of the new isolates were closely related to each other, and the ΔT_m with reference strain A089^T was never higher than 1.7°C. However, this group of strains was genomically distant (ΔT_m , 6.8 to 7.1°C) from the phylogenetically closest known species, *V. aestuarianus* (Table 3).

The six new isolates constitute a new species within the genus *Vibrio*, as shown by the overall results. The differences in ΔT_m between the new isolates and the phylogenetically closest species, together with the distinct phenotypic profile of the new isolates, indicate that the new strains are members of a new *Vibrio* species (35). We propose the name *Vibrio scopthalmi* sp. nov. for this group of strains.

Description of *Vibrio scopthalmi* sp. nov. *Vibrio scopthalmi* (scoph. thal' mi. L. gen. n. *scophthalmi*, of turbot [*Scophthalmus maximus*]). Gram-negative, motile, short rods. Colonies

are regular and unpigmented. Does not swarm. Grows on thio-sulfate-citrate-bile salts-sucrose agar, producing yellow colonies, and does not grow on VAM. Both oxidative metabolism and fermentative metabolism occur. Oxidase and catalase tests are positive. Reduces nitrate to nitrite. Sensitive to vibriostatic agent O/129 (10 or 150 µg per disc) and does not produce gas from glucose. *V. scopthalmi* cannot grow at 4, 37, or 40°C, but grows well at 22 and 30°C. It grows weakly in culture media supplemented with 0.5% (wt/vol) NaCl and does not grow when NaCl is absent. The optimal NaCl concentration for growth is 1 to 3% (wt/vol), and it does not grow at higher NaCl concentrations (6, 8, 10%). Additional properties are showed in Tables 1 and 2. *V. scopthalmi* has a G+C content of 44 to 45.5 mol%.

Description of the type strain. Strain A089 is the type strain of *V. scopthalmi*. It has biochemical and molecular characteristics described above. The G+C content of its DNA is 45.3 mol%, and it has a T_m of 69.7°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Strain A089^T has been deposited in the Colección Española de Cultivos Tipo, València, Spain, as strain CECT 4638^T.

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FIG. 1. 16S rRNA-based tree reflecting the phylogenetic relationships of *V. scopthalmi* A089^T and a selection of available type strains of members of the genus *Vibrio*. The tree is based on the results of a distance matrix analysis of complete or almost complete 16S rRNA sequences. The common root of A089^T and *V. aestuarianus* and their isolated position within the genus were not different in trees calculated with other methods. Branch 1 (B1) includes *V. carchariae*, *V. campbellii*, *V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, *V. natriegens*, *V. pelagius*, *V. proteolyticus*, *V. nereis*, *V. tubiashi*, *V. orientalis*, *V. mediterranei*, and *V. fluvialis* (mean similarity level, 97.8%); branch 2 (B2) comprises *V. cincinnatiensis*, *V. metschnikovii*, and *V. gazogenes* (mean similarity level, 95.7%); and branch 3 (B3) includes *V. damsela*, *V. costicola*, and *V. hollisae* (mean similarity level, 92.4%).

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