Vibrio sinaloensis sp. nov., isolated from the spotted rose snapper, *Lutjanus guttatus* Steindachner, 1869

Bruno Gomez-Gil,[1] Emma Fajer-Avila,[1] Javier Pascual,[2,3] M. Carmen Macián,[3,4] María J. Pujalte,[2,3] Esperanza Garay[2,3,4] and Ana Roque[5]

Correspondence
Bruno Gomez-Gil
bruno@ciad.mx

[1]CIAD, A.C., Mazatlán Unit for Aquaculture, AP. 711 Mazatlán, Sinaloa 82000, Mexico

[2]Instituto Cavanilles de Biodiversidad y Biología Evolutiva, Universitat de València, Spain

[3]Departamento de Microbiología y Ecología, Universitat de València, Spain

[4]Colección Española de Cultivos Tipo (CECT), Universitat de València, Spain

[5]Institut de Reçerca i Tecnologia Agroalimentaries, Centre d'Aquicultura, AP 200 Sant Carles de la Rapita 43540, Spain

Nine bacterial strains were studied by means of rep-PCR, 16S rRNA gene sequence analysis, DNA–DNA hybridization and physiological characterization. Typing analysis by means of rep-PCR showed that all nine strains were highly homogeneous, with similarities above 94 %. The strains were isolated from the same geographical area (Mazatlán, Sinaloa state, Mexico) and the same type of host (cultured rose snapper, *Lutjanus guttatus*), although from different individuals and organs. Comparison of the almost-complete 16S rRNA gene sequences of five strains showed that they belonged to the genus *Vibrio* and are closely related to the type strains of *Vibrio brasiliensis* and *Vibrio hepatarius*, with similarity values ranging from 97.9 to 98.1 % and from 97.4 to 97.8 %, respectively. The DNA–DNA hybridization value of strain CAIM 797^T with the type strain of *V. brasiliensis* (CAIM 495^T) was 47.5 %, with a reciprocal value of 44.7 %. The main phenotypic features of the strains were in agreement with the phylogenetic and genomic data. The results presented here support the description of a novel species, for which the name *Vibrio sinaloensis* sp. nov. is proposed, with strain CAIM 797^T (=CECT 7298^T) as the type strain.

Many species of the genus *Vibrio* have been described in recent years, especially since molecular fingerprinting methods that interrogate the bacterial genome have been available. There are now over 70 species in the genus (Euzéby, 1997; last full update 4 October 2007), many isolated from cultured marine organisms. Vibrios can cause severe infections in humans and in many marine organisms, but also are considered to be part of the normal microbiota of marine invertebrates and fish (Thompson *et al.*, 2004; Gomez-Gil *et al.*, 2007). Most of the pathogenic vibrios studied are of an opportunistic nature, causing disease only when the health status of the host is compromised (Lightner & Redman, 1998). Variations in virulence between strains of the same *Vibrio* species are a common phenomenon (Soto-Rodriguez *et al.*, 2003) but, perhaps, pathogenic strains are the exception rather than the rule. Therefore, it is important to know the normal *Vibrio* microbiota of a cultured marine organism in order to understand better the involvement of a certain bacterial strain or species in a pathogenic process.

Nine bacterial strains were isolated on TCBS agar during November 2003 from cultured rose snappers (*Lutjanus guttatus*) kept at the Research Centre for Nutrition and Development (CIAD) in Mazatlán, Sinaloa state, Mexico, and they were tentatively called '*Vibrio* sp. nov. 3' (cluster number 2; Gomez-Gil *et al.*, 2007). Two strains were obtained from the liver (CAIM 648 and CAIM 694), three from the spleen (CAIM 695, CAIM 792 and CAIM 797^T), one from the kidney (CAIM 752) and three from external lesions (CAIM 750, CAIM 793 and CAIM 798), each from a different fish. The strains were deposited at the Collection of Aquatic Important Microorganisms (http://www.ciad.mx/caim).

As recommended by Figueras *et al.* (2006), these nine strains and strains from the closely related species *Vibrio*

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CAIM 695, CAIM 797^T, CAIM 798, CAIM 752, CAIM 648 and CAIM 636 are respectively DQ451210, DQ451211 and EU043379–EU043382.

brasiliensis (five strains; Thompson *et al.*, 2003a) and *Vibrio hepatarius* (one strain; Thompson *et al.*, 2003b) were fingerprinted by rep-PCR as described previously (Cabanillas-Beltran *et al.*, 2006). Briefly, DNA was extracted with a commercial kit (Wizard Genomic DNA purification; Promega) according to the manufacturer's instructions following growth on TSA supplemented with 2.0 % (w/v) NaCl. The DNA was adjusted to a concentration of 50 ng μl^{-1} and amplified with the primer GTG_5 (GTG_5-PCR), the amplification products were resolved electrophoretically (1.2 % 20 × 20 cm agarose gel for 140 min at 90 V in 1 × TAE buffer at room temperature) and the resulting bands were analysed with the GelCompar II software (version 4.5; Applied Maths). A similarity matrix was calculated with the Jaccard coefficient (Kosman & Leonard, 2005) with a band position tolerance of 0.59 %, and a dendrogram was constructed with the Ward algorithm.

The dendrogram showed clear separation between species (Fig. 1) and was also able to identify strains that had identical or very similar (>94 %) GTG_5-PCR patterns and thus could be considered clones. Because this rep-PCR analysis has been applied to all strains in the CAIM collection (>1800 strains), it allowed us to identify another strain already deposited at the CAIM (CAIM 636 =LMG 21563) as genomically related to these snapper strains. This strain was previously identified as *Vibrio fortis* by FAFLP (Thompson *et al.*, 2003b), although it was not sequenced or compared by DNA hybridization with the type strain of that species. However, the rep-PCR analysis showed

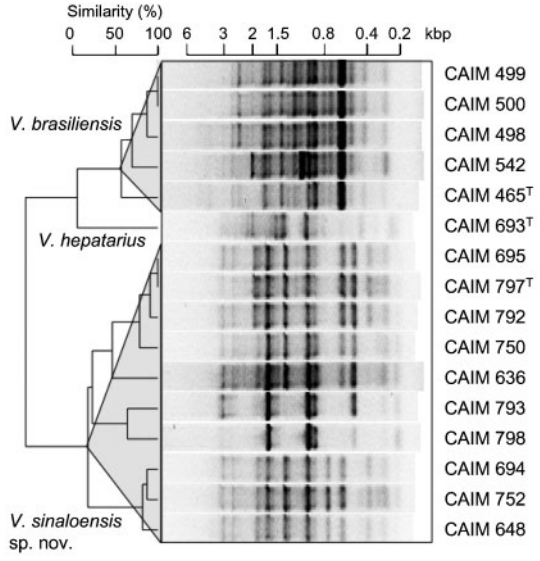

Fig. 1. rep-PCR of the novel snapper strains (*Vibrio sinaloensis* sp. nov.) compared with strains of *V. brasiliensis* and *V. hepatarius*. The similarity matrix was calculated with the Jaccard coefficient with a position tolerance of 0.59 % and the dendrogram was constructed with the Ward algorithm.

similarity values above 44 % (Jaccard, 0.84 % optimization, 0.7 % tolerance) for strain CAIM 636 with the other members of the proposed species represented by the snapper strains and below 40 % with strains belonging to *V. fortis* or *V. brasiliensis*. rep-PCR analysis of the whole database with members of almost all species of the *Vibrionaceae* (type, reference and environmental strains) using the Jaccard algorithm (0.59 % tolerance, 0.35 % optimization) has consistently shown a similarity value above 40–42 % to delineate a species, supported by a group separation statistical analysis (jackknife maximum similarities with tie handling assigned to its own group).

The 16S rRNA genes of five strains were sequenced as described previously (Gomez-Gil *et al.*, 2007) and analysed with the MEGA software version 3.1 (Kumar *et al.*, 2001). The 16S rRNA gene sequence of strain CAIM 797^T was compared with sequences of the type strains of previously described species (NCBI/Megablast) and the closest species were *V. brasiliensis* (98.17 %) and *V. hepatarius* (97.72 %) (Fig. 2). The sequences of the other four strains showed sequence similarity between 97.9 and 98.1 % with *V. brasiliensis* LMG 20546^T and between 97.4 and 97.9 % with *V. hepatarius* LMG 20362^T. Strain CAIM 636 was also sequenced, showing 99.4 % similarity with CAIM 797^T, 98.1 and 97.8 % similarity, respectively, with the type strains of *V. brasiliensis* and *V. hepatarius*, and only 96.7 % similarity with *V. fortis* LMG 21557^T.

DNA–DNA hybridization experiments were done with the hydroxyapatite/microtitre plate method (Ziemke *et al.*, 1998) with a hybridization temperature (T_m) of 50 °C. Results of individual DNA–DNA hybridization determinations of strain CAIM 797^T against the type strains of the two closest species ranged between 36.8 % with CAIM 495^T and 47.7 % with CAIM 693^T (Table 1), well below the 70 % threshold established to delineate a species (Wayne *et al.*, 1987). Hybridization values between three strains (CAIM 797^T, CAIM 695 and CAIM 648) of the proposed novel species showed values above 70 %. Reciprocal values with the labelled strain CAIM 495^T were in agreement with previous results (Table 1).

Phenotypic characterization was performed on the nine novel strains plus the type strains of *V. brasiliensis*, CAIM 495^T, and *V. hepatarius*, CAIM 693^T, and included the determination of temperature and salinity growth ranges, the ability to hydrolyse several macromolecules extracellularly, the determination of different enzyme activities and the use of 54 substrates as sole carbon and energy sources, as described previously (Macián *et al.*, 2001). In addition, profiles in API 20E strips were also determined as recommended by the manufacturer with the exception that the bacterial suspension was prepared in 2.5 % sterile saline solution. The strains of the novel species showed the basic traits of *Vibrio* species: they have Gram-negative, oxidase-positive cells that ferment glucose without gas production and require sodium ions for growth. Phenotypic tests were observed that could differentiate

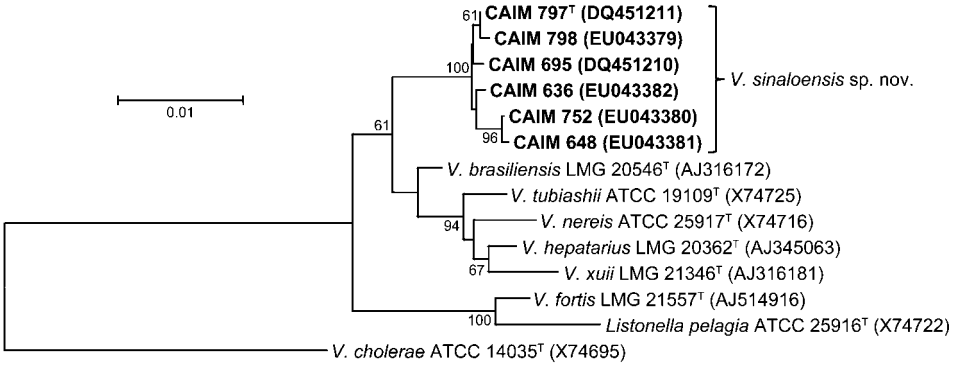

Fig. 2. Phylogenetic tree of six strains of *Vibrio sinaloensis* sp. nov. and the closest *Vibrio* species, derived from almost-complete 16S rRNA gene sequences. The tree topology was obtained by the neighbour-joining method (pairwise deletions, Jukes–Cantor correction). Numbers at nodes indicate percentages of bootstrap support (1000 replicates). Bar, 1 % sequence divergence.

the novel species from phylogenetically related and arginine dihydrolase-positive and lysine and ornithine decarboxylase-negative *Vibrio* species, as shown in Table 2.

The genotypic and phenotypic data presented here support the proposal of a novel species, for which the name *Vibrio sinaloensis* sp. nov. is proposed.

Description of *Vibrio sinaloensis* sp. nov.

Vibrio sinaloensis (si.na.lo.en′sis. N.L. masc. adj. *sinaloensis* from the Mexican state of Sinaloa, the source of the first strains).

Gram-negative, curved bacilli that grow as bright-yellow colonies on TCBS agar, are not luminescent and do not swarm on marine agar or on TSA with 2.0 % (w/v) NaCl. Growth occurs with 0.35–9 % (w/v) NaCl; no growth without NaCl or with more than 10 % (w/v) NaCl. Grows at 15 and 37 °C but not at 4 °C. Growth at 40 °C is variable, being negative for strains CAIM 695, CAIM 793 and CAIM 798. Strains are sensitive to the vibriostatic

Table 1. DNA–DNA hybridization results

All analyses were done in duplicate.

Source of unlabelled DNA	Hybridization (%) with labelled DNA from:	
	V. sinaloensis CAIM 797^T	*V. brasiliensis* CAIM 495^T
V. sinaloensis CAIM 797^T	100	44.7
V. sinaloensis CAIM 695	78.6	46.1
V. sinaloensis CAIM 648	91.3	66.4
V. brasiliensis CAIM 495^T	47.5*	100
V. hepatarius CAIM 693^T	49.7*	61.7

*Mean of two hybridization experiments.

Table 2. Phenotypic characteristics that differentiate *Vibrio sinaloensis* sp. nov. from phylogenetically related and arginine dihydrolase-positive, lysine and ornithine decarboxylase-negative *Vibrio* species

Taxa: 1, *V. sinaloensis* sp. nov. (nine strains); 2, *V. brasiliensis*; 3, *V. furnissii*; 4, *V. hepatarius*; 5, *V. nereis*; 6, *V. pacinii*; 7, *V. splendidus* I; 8, *V. tubiashii*; 9, *V. xuii*. +, Positive; −, negative; (+), 75–89 % of strains positive; d, doubtful; V, variable; ND, no data available. Data for reference species were taken from Alsina & Blanch (1994) and Macián *et al.* (2004) (*V. furnissii*, *V. nereis*, *V. splendidus* I, *V. tubiashii*), Gomez-Gil *et al.* (2003) (*V. pacinii*), Thompson *et al.* (2003a) (*V. brasiliensis*, *V. xuii*) and Thompson *et al.* (2003b) (*V. hepatarius*). Some additional data were obtained in this study for all reference species with the exception of *V. pacinii*.

Test	1	2	3	4	5	6	7	8	9
Growth at/in:									
0.35 % NaCl	+	+	+	−	−	−	−	−	−
9 % NaCl	+	−	+	−	+	+	+	+	+
4 °C	−	−	−	−	d	+	+	−	−
Production of indole	−	+	−	+	+	−	−	+	+
Utilization of:									
α-Ketoglutaric acid	−	−	−	−	ND	−	+	−	+
Acetic acid	(+)	+	d	+	d	−	d	−	+
Cellobiose	+	+	−	+	−	+	+	+	+
Citrate	+	+	+	+	+	−	+	+	−
D-Galacturonic acid	−	−	+	−	−	−	−	−	−
D-Gluconate	+	+	+	+	+	−	V	+	+
D-Mannose	+	+	+	+	−	−	+	+	+
L-Aspartic acid	−	+	ND	−	d	ND	+	−	+
Putrescine	−	+	+	−	+	−	−	d	ND
L-Serine	+	+	ND	−	d	+	+	−	+
L-Alanine	+	+	ND	−	+	ND	+	−	+
4-Amino-L-butyric acid	+	+	+	−	+	−	ND	ND	ND
L-Ornithine	V	+	−	−	+	−	−	−	+

agent O/129 at 10 and 150 μg ml^{-1}. Positive for arginine dihydrolase (Thornley) and tryptophan deaminase (TDA) and negative for lysine and ornithine decarboxylases (Moeller). Ferments glucose without gas formation; positive for ONPG, nitrate reduction, methyl red and gelatinase. Hydrolyses casein, starch, Tween 80, DNA and blood (except CAIM 797^T). Negative for production of indole and H_2S; negative for citrate utilization, the Voges–Proskauer reaction and urease. Negative for hydrolysis of alginate, agar and lecithin (except CAIM 793). Utilizes the following substrates as sole sources of carbon: 3-hydroxybutyrate, acetate (except CAIM 797^T), cellobiose (weak reaction for most strains), citrate, D-fructose, D-galactose (except CAIM 797^T), D-glycerate (weak reaction), D-gluconate, D-glucose, D-mannitol (except CAIM 797^T and CAIM 792), D-mannose, D-ribose, trehalose, fumarate, glycerol (except CAIM 797^T), glycine, L-glutamic acid, 4-amino-L-butyric acid, lactate, L-alanine, L-arginine (weak reaction), L-citrulline (weak reaction), L-histidine (except CAIM 797^T; weak reaction for CAIM 792), L-serine, L-tyrosine, L-threonine, malate (weak reaction; negative for CAIM 752), maltose, *N*-acetyl-D-glucosamine, pyruvate, propionate, sucrose, succinate and *trans*-aconitate (weak reaction). Negative for utilization of 2-ketoglutarate, L-aspartic acid, amygdalin, betaine, butyrate, D-galacturonate, D-glucuronate, D-sorbitol, D-xylose, lactose, L-arabinose, L-leucine (except CAIM 648, which presents a weak reaction), L-lysine, L-rhamnose, L-sarcosine, *myo*-inositol and putrescine. Variable results are obtained for melibiose (weak reaction; negative for CAIM 797^T, CAIM 695 and CAIM 792), salicin (positive for CAIM 750, CAIM 793 and CAIM 798) and L-ornithine (positive for CAIM 695, CAIM 750 and CAIM 792).

The type strain is CAIM 797^T (=CECT 7298^T). The type strain and the reference strains CAIM 648, CAIM 694, CAIM 695, CAIM 750, CAIM 752, CAIM 792, CAIM 793 and CAIM 798 were isolated from cultured spotted rose snapper, *Lutjanus guttatus* Steindachner, 1869, in Mazatlán, Sinaloa state, Mexico.

Acknowledgements

We are grateful to Carmen Bolán-Mejía, Roxana Atondo-Mexía and Beatríz Lacuesta for technical work. This study was financed by FOSEMARNAT project 2004-01-33 (Mexico), the CGL2005-02299/BOS project (Spanish Ministry of Education and Science) and JACUMAR-Programa Nacional de Cultivo de Lenguado (Spanish Ministry of Agriculture, Fisheries and Food).

References

Alsina, M. & Blanch, A. R. (1994). A set of keys for biochemical identification of environmental *Vibrio* species. *J Appl Bacteriol* **76**, 79–85.

Cabanillas-Beltran, H., Llausas-Magana, E., Romero, R., Espinoza, A., Garcia-Gasca, A., Nishibuchi, M., Ishibashi, M. & Gomez-Gil, B. (2006). Outbreak of gastroenteritis caused by the pandemic *Vibrio parahaemolyticus* O3 : K6 in Mexico. *FEMS Microbiol Lett* **265**, 76–80.

Euzéby, J. P. (1997). List of bacterial names with standing in nomenclature: a folder available on the internet. *Int J Syst Bacteriol* **47**, 590–592.

Figueras, M. J., Alperi, A., Guarro, J. & Martinez-Murcia, A. J. (2006). Genotyping of isolates included in the description of a novel species should be mandatory. *Int J Syst Evol Microbiol* **56**, 1183–1184.

Gomez-Gil, B., Thompson, F. L., Thompson, C. C. & Swings, J. (2003). *Vibrio pacinii* sp. nov., from cultured aquatic organisms. *Int J Syst Evol Microbiol* **53**, 1569–1573.

Gomez-Gil, B., Fajer-Avila, E. & Garcia-Vargas, F. (2007). Vibrios of the spotted rose snapper *Lutjanus guttatus* Steindachner, 1869 from northwestern Mexico. *J Appl Microbiol* **102**, 1518–1526.

Kosman, E. & Leonard, K. J. (2005). Similarity coefficients for molecular markers in studies of genetic relationships between individuals for haploid, diploid, and polyploid species. *Mol Ecol* **14**, 415–424.

Kumar, S., Tamura, K., Jakobsen, I.-B. & Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245.

Lightner, D. V. & Redman, R. M. (1998). Shrimp diseases and current diagnostic methods. *Aquaculture* **164**, 201–220.

Macián, M. C., Ludwig, W., Aznar, R., Grimont, P. A. D., Schleifer, K. H., Garay, E. & Pujalte, M. J. (2001). *Vibrio lentus* sp. nov., isolated from Mediterranean oysters. *Int J Syst Evol Microbiol* **51**, 1449–1456.

Macián, M. C., Garay, E., Grimont, P. A. D. & Pujalte, M. J. (2004). *Vibrio ponticus* sp. nov., a neighbour of *V. fluvialis-V. furnissii* clade, isolated from gilthead sea bream, mussels and seawater. *Syst Appl Microbiol* **27**, 535–540.

Soto-Rodriguez, S. A., Roque, A., Lizarraga-Partida, M. L., Guerra-Flores, A. L. & Gomez-Gil, B. (2003). Virulence of luminous vibrios to *Artemia franciscana* nauplii. *Dis Aquat Organ* **53**, 231–240.

Thompson, F. L., Li, Y., Gomez-Gil, B., Thompson, C. C., Hoste, B., Vandemeulebroecke, K., Rupp, G. S., Pereira, A., De Bem, M. M. & other authors (2003a). *Vibrio neptunius* sp. nov., *Vibrio brasiliensis* sp. nov. and *Vibrio xuii* sp. nov., isolated from the marine aquaculture environment (bivalves, fish, rotifers and shrimps). *Int J Syst Evol Microbiol* **53**, 245–252.

Thompson, F. L., Thompson, C. C., Hoste, B., Vandemeulebroecke, K., Gullian, M. & Swings, J. (2003b). *Vibrio fortis* sp. nov. and *Vibrio hepatarius* sp. nov., isolated from aquatic animals and the marine environment. *Int J Syst Evol Microbiol* **53**, 1495–1501.

Thompson, F. L., Iida, T. & Swings, J. (2004). Biodiversity of vibrios. *Microbiol Mol Biol Rev* **68**, 403–431.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Ziemke, F., Höfle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998). Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.