Antonie van Leeuwenhoek (2015) 108:355–364
DOI 10.1007/s10482-015-0488-1

ORIGINAL PAPER

Vibrio mexicanus sp. nov., isolated from a cultured oyster *Crassostrea corteziensis*

Adrián González-Castillo · Julissa Enciso-Ibarrra · M. Carmen Bolán-Mejia · Sabela Balboa · Aide Lasa · Jesús L. Romalde · Hector Cabanillas-Beltrán · Bruno Gomez-Gil

Received: 3 March 2015 / Accepted: 19 May 2015 / Published online: 29 May 2015

Abstract A bacterial strain was taxonomically characterised by means of a genomic approach comprising 16S rRNA gene sequence analysis, multilocus sequence analysis (MLSA), the DNA G+C content, whole genome analyses (ANI and GGDC) and phenotypic characterisation. The strain CAIM 1540^T was isolated from a cultured oyster *Crassostrea corteziensis* in La Cruz, Sinaloa state, México. The isolate was found to be catalase and oxidase positive, cells were observed to be motile, O/129-sensitive and facultatively anaerobic. The almost-complete 16S rRNA gene sequence placed this strain within the genus *Vibrio*; the closest related species were found to be *Vibrio aestivus*, *Vibrio marisflavi*, *Vibrio maritimus* and *Vibrio variabilis* with similarity values of 99.02, 97.05, 96.70, and 96.59 % respectively. MLSA of four housekeeping genes (*ftsZ*, *gapA*, *recA,* and *topA*) was performed with the closely related species. A draft genome sequence of strain CAIM 1540^T was obtained. The DNA G+C content of this strain was determined to be 43.7 mol%.The ANI values with *V. aestivus* were 89.6 % (ANIb), 90.6 % (ANIm) and a GGDC value of 39.5 ± 2.5 % was obtained; with *V. marisflavi* the genomic similarities were 71.5 % (ANIb), 85.5 % (ANIm) and 20.2 ± 2.3 % (GGDC); with *V. maritimus* 72.6 % (ANIb), 85.7 % (ANIm) and 22.0 ± 2.0 % (GGDC); and with *V. variabilis* 72.6 % (ANIb), 85.8 % (ANIm) and 21.6 ± 1.6 % (GGDC). These ANI and GGDC values are below the threshold for the delimitation of prokaryotic species, i.e. 95–96 and 70 %, respectively. Phenotypic characters also showed differences with the closest related species analysed. The results presented here support the description of a novel species, for which the name *Vibrio mexicanus* sp. nov. is proposed, with strain CAIM 1540^T (= CECT 8828^T, = DSM 100338^T) as the type strain. In addition, we found that the recently described species *Vibrio thalassae* and *Vibrio madracius* might be a single species because the values of ANIb 95.8 %, ANIm 96.6 % and GGDC 70.2 ± 2.9 % are above the accepted species thresholds.

Keywords *Vibrio mexicanus* · *Vibrio naceae* · *Vibrio aestivus* · *Vibrio marisflavi*

Abbreviations
ANI Average nucleotide identity

Electronic supplementary material The online version of this article (doi:10.1007/s10482-015-0488-1) contains supplementary material, which is available to authorized users.

A. González-Castillo · J. Enciso-Ibarrra · M. C. Bolán-Mejia · B. Gomez-Gil (✉)
CIAD, A.C., Mazatlán Unit for Aquaculture, AP. 711, 82000 Mazatlán, Sinaloa, Mexico
e-mail: bruno@ciad.mx

S. Balboa · A. Lasa · J. L. Romalde
Departamento de Microbiología y Parasitología, CIBUS, Universidad de Santiago de Compostela, Campus Sur s/n, 15782 Santiago de Compostela, Spain

H. Cabanillas-Beltrán
Instituto Tecnológico de Tepic, Av. Tecnológico 2595, 63175 Tepic, Nayarit, Mexico

GGDC Genome-to-genome distance calculator
MLSA Multilocus sequence analysis

Introduction

Members of the family *Vibrionaceae* are Gram-negative Gammaproteobacteria and are found in a wide variety of aquatic biotopes, including extremophile species that can live at great depths such as *Photobacterium profundum* or symbionts such as *Aliivibrio fischeri* (Thompson and Swings 2006).Most members of the family *Vibrionaceae* are free-living marine bacteria but many can also form biofilms on marine invertebrate exoskeletons or other surfaces (Thompson and Swings 2006). The members of this family have also an important impact on aquaculture; *Aliivibrio salmonicida, Vibrio anguillarum* and *Vibrio vulnificus* are major fish pathogens, while *Vibrio harveyi* and *Vibrio parahaemolyticus* are the most important pathogens of shrimp (Austin and Austin 1999; Gomez-Gil et al. 1998, 2003; Tran et al. 2013; Soto-Rodriguez et al. 2015).

Notably, the genus *Vibrio* consists of over 90 species (http://www.bacterio.net/vibrio/html). However, within this genus there are closely related species that are difficult to identify. The genus is divided in many clades according to their phylogenetic relationships established by Multilocus Sequence Analyses (MLSA, Sawabe et al. 2007, 2013). In particular, the so-called Marisflavi clade (Lucena et al. 2012) consists of three species, *Vibrio aestivus*, *Vibrio marisflavi* and *Vibrio stylophorae* based on the 16S rRNA gene sequences. These species were recently isolated from seawater and corals (Wang et al. 2011; Sheu et al. 2011, Lucena et al. 2012). However, the robustness of this clade has not yet been supported by MLSA. Furthermore, the closely related Mediterranei clade consists of five species, *Vibrio maritimus*, *Vibrio variabilis*, *Vibrio mediterranei*, *Vibrio madracius* and *Vibrio thalassae* (Sawabe et al. 2013; Moreira et al. 2014; Tarazona et al. 2014).

DNA–DNA hybridization (DDH) is still considered a gold standard for species delineation, despite that other methods have greater resolving power and are easier to perform. Such methods comprise of genomic taxonomy techniques such as genome-to-genome distance (GGD), average nucleotide identity (ANI), and genotype-to-phenotype on the basis of whole genome sequences (Amaral et al. 2014; Thompson et al. 2015). In this study, a genomic taxonomy study was performed to classify a novel *Vibrio* strain, CAIM 1540^T.

Materials and methods

Bacterial strain and growth conditions

The strain CAIM 1540^T was isolated on Marine Agar from a cultured oyster (*C. corteziensis*) in La Cruz, Sinaloa state, México (23°55′05.9″N 106°53′24.7″W) on February 13th, 2004. The type strains of *V. aestivus* CAIM 1861^T and *V. marisflavi* CAIM 1886^T were obtained from the Collection of Aquatic Important Microorganisms (CAIM). The strains were grown on Tryptone Soy Agar (TSA; Oxoid) + 2 % NaCl (w/v) and incubated at 30 °C for 24 h. Cultures were maintained frozen at −80 °C in Tryptone Soy Broth (TSB) supplemented with 2 % NaCl (w/v) and 15 % (v/v) glycerol as preservative.

Phenotypic analyses

The following phenotypic tests were performed with the strains (MacFaddin 1993; Noguerola and Blanch 2008): Gram staining, catalase and oxidase activities, cell morphology, motility, oxidation/fermentation test, methyl red test, Voges–Proskauer test, utilisation of citrate, arginine dihydrolase, lysine and ornithine decarboxylation, and nitrate reduction. Further characterisation was done using API 20E test strips (bioMérieux) and Biolog GN MicroPlate™. The strains were grown onTSB to determine salt tolerance (0–10 % NaCl), growth at different temperatures (8, 20, 30, 37, 40 °C) and pH (2–14). The sensitivity to the vibriostatic agent O/129 (2,4-diamino-6, 7- diisopropylpteridine, 150 μg per disc) and the use of 44 substrates as sole carbon and energy sources were determined as described previously (Macián et al. 2001).

Analysis of fatty acid methyl esters (FAMEs) was performed according to the Microbial Identifications Systems (MSI) (MIDI, Newark, DE, USA) protocol as described by Sasser (1990). The cells were grown on

TSA supplemented with 2.0 % NaCl (w/v) and incubated at 25 °C for 24 h.

DNA isolation, amplification, sequencing, and sequence analysis

Genomic DNA was extracted from 24 h cultures on TSA supplemented with 2 % NaCl (w/v) using a Promega kit (Wizard® Genomic DNA Purification Kit). The amplification and sequencing of the 16S rRNA gene, and of the genes *ftsZ* (cell division protein), *gapA* (glyceraldehyde 3-phosphate dehydrogenase), *recA* (recombinase A gene) and *topA* (topoisomerase I) were performed as previously described (Pascual et al. 2010; Cano-Gomez et al. 2010; Yoshizawa et al. 2010). Sequence data analysis was carried out with the DNASTAR Lasergene SEQMAN program. Sequence similarity of the 16S rRNA was determined using the EzTaxon-e server (Kim et al. 2012). Phylogenetic trees were reconstructed using the neighbour-joining, maximum-likelihood, and maximum parsimony algorithms with MEGA ver. 5.05 (Tamura et al. 2011). Sequences of phylogenetically closely related species were obtained from GenBank/EMBL/DDBJ (Supplementary Table 1).

The draft genome of CAIM 1540^T was sequenced by means of the Ion Torrent PGM platform as described earlier (Quail et al. 2012; Moreira et al. 2014) with minor modifications as follows. Library preparation was carried out using the Ion Plus Fragment Library Kit, with 1 µg DNA (in Low TE, 50 µL). DNA was fragmented using the BioRuptor®Sonication System as described in the Ion Plus Fragment Library Kit protocol. End repair, adapter ligation, nick repair, and amplification (10 cycles) were also performed as described in the Ion Plus Fragment Library protocol. 300 and 350 bp fragments were selected through agarose gel (2 % m/v) electrophoresis (E-Gel SizeSelect, Life technologies). Quality and concentration of the libraries were determined using an Agilent 2100 Bioanalyzer with the associated High Sensitivity DNA kit (Agilent Technologies), as well as with an Ion library Quantization kit using TaqMan® in a CFX96™ Real-Time PCR System (Bio-Rad). The amount of library required for template preparation was calculated using the Template Dilution Factor calculation described in the manufacturers protocol. Emulsion PCR and enrichment steps were carried out in the Ion OneTouchTM 200 Template Kit v2. Ion Sphere Particle quality assessment was carried out as outlined in this protocol. Sequencing was done using a 318 chip with barcoding. The Ion PGM™ 200 Sequencing Kit was used for sequencing following the recommended protocol and Torrent Suite 1.5 was used for analyses. The reads were assembled de novo with Newbler (RunAssembly ver. 2.3).

The DNA G+C mol% was estimated using a method described previously (Moreira et al. 2011) and from the draft genome sequence.

Accession numbers

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *ftsZ*, *gapA*, *recA,* and *topA* gene sequences of strain CAIM 1540^T are JQ434105, KP698215, KP698212, KP698213, and KP698214 respectively; all other gene sequences used in this study are listed in the supplementary Table 1. The genome accession number of the type strain CAIM 1540^T is JYJP00000000.

Result and discussion

Bacterial characterisation

Strain CAIM 1540^T was isolated from a cultured oyster (*C. corteziensis*) in La Cruz, Sinaloa state, México. The strain showed phenotypic characteristics that place it clearly asa member of the genus *Vibrio*: the cells were observed to be motile small rods, Gram-negative, facultatively anaerobic, oxidase and catalase positive. The strain was found to require sodiumions for growth; the optimal salinity range obtained was between 3 and 5 % (Fig. S1). No growth was observed at NaCl concentrations of 7 % or higher. Optimal pH was established at a range of 6–8 (Fig. S2). The optimal temperature was found to be 30 °C (Fig. S3) and no growth was observed at 40 °C. The strain was found to grow on thiosulfate-citrate-bile-sucrose agar (TCBS) agar (Difco) as green colonies. Strain CAIM 1540^T was also found to be able to reduce nitrates to nitrites, to be positive for methyl red test, urea hydrolysis, L-tryptophan deaminase, indole production, and bovine gelatin hydrolysis. Strain CAIM 1540^T was found to be negative for citrate utilisation, the Voges–Proskauer test, arginine dihydrolase and lysine and ornithine

decarboxylase. Several phenotypic tests could be selected to clearly differentiate CAIM 1540^T from the closest related species *Vibrio* species (Table 1).

The fatty acid profile of strain CAIM 1540^T showed the main features (Table 2) of the members of the genus *Vibrio*.

Phylogenetic analysis

The closest related species identified by 16S rRNA gene sequence were found to be *V. aestivus* and *V. marisflavi*, with similarity values of 99.02–97.05 % respectively (Fig. 1); the third member of the proposed Marisflavi clade (Lucena et al. 2012), *V. stylophorae*, was found to show a more distant relationship (95.04 %) and this species was well separated in the phylogenetic tree (Fig. 1). Similarities with members of the Mediterranei clade were 96.7 % with *V. maritimus* and 96.6 % with *V. variabilis*. The 16S rRNA gene sequence analysis clearly separates CAIM 1540^T from *V. aestivus* forming a separate branch, supported with a high bootstrap value(Fig. 1).

MLSA has been proposed as a valuable technique for the identification and classification of vibrios (Cano-Gomez et al. 2010). In this study, sequences of the following four housekeeping genes were obtained: cell division protein (*ftsZ,* 432 pb), glyceraldehyde-3-phosphate dehydrogenase A (*gapA*,602 bp), protein recombinase A (*recA*, 494 bp), and DNA topoisomerase 1 (*topA*, 402 bp); these sequences were compared with those of related species. The phylogenetic trees based on the housekeeping genes *ftsZ* (Fig. S4), *gapA* (Fig. S5), *recA* (Figs. S6, S10), *topA* (Fig. S7) and the 16S rRNA gene (Fig. 1), and on the concatenated sequences of these five genes (Figs. 2, S8, S9), confirmed the clustering of the proposed novel *Vibrio* species as a independent branch with high bootstrap values, and its distinction from the closest phylogenetic neighbours. In all individual trees the isolate formed a monophyletic group with *V. aestivus*. Notably these analyses suggest that this lineage is well separated from *V. marisflavi*, suggesting that neither *V. aestivus* nor CAIM 1540^T should be considered members of the Marisflavi clade.

Each MLSA gene was analysed for recombination events with the program SplitsTree v4 (Huson and Bryant 2006) (Figs. S11–S18).Recombination analyses are useful to detect recombination events that may affect the topology of phylogenetic trees; a recombination event increases the distance between species. Several recombination events were observed, especially in *recA* as seen for other vibrios (González-Castillo et al. 2014). Nevertheless, the analysis of concatenated gene sequences provides more informative data and minimizes the weight of recombination events, making it a tool that increases the quality of

Table 1 Phenotypic characteristics that distinguish *V. mexicanus sp. nov.* from related *Vibrio* species

Test	1	2	3	4	5	6	7	8
Arginine dihydrolase (Moller)	−	−	+	+	+	−	−	−
Lysine decarboxylase (Moller)	−	−	+	+	−	+	v	+
Growth at 8 % NaCl	−	−	−	+	−	+	−	−
Growth at 40 °C	−	−	−	−	+	+	−	−
Citrate utilisation	−	−	−	+	−	−	+	+
Voges–Proskauer	−	−	−	−	−	−	−	−
Indole production	+	−	−	+	+	+	+	+
ONPG	−	+	+	−	+	ND	ND	ND
Gelatin hydrolysis	+	−	+	+	+	−	−	+
L-Tryptophan deaminase	+	−	−	−	−	+	ND	ND

Taxa are indicated as: 1. *V. mexicanus* sp. nov. CAIM 1540^T, 2. *V. aestivus* CAIM 1861^T, 3. *V. marisflavi*CAIM 1886^T, 4. *V. maritimus* CAIM 1455^T, 5. *V. variabilis*CAIM 1454^T, 6. *V. madracius*A-354^T, 7. *V. thalassae* MD16^T, 8. *V. mediterranei* CAIM 316^T

+, positive for ≥90 %; (+), positive for 75–89 %; −, negative; *ND* no data available, *D* discrepancies exist, *V* variable results, *W* weakly positive, *CS* carbon source. Data in columns 1–5, 8 are from this study; data in columns 6 and 7 are from Moreira et al. (2014) and Tarazona et al. (2014)

Table 2 Total fatty acid content (%) of *V. mexicanus* sp. nov. (CAIM 1540^T) and of related *Vibrio* species

Fatty acids	1	2	3	4	5	6	7	8
12:0	2.7	3.9	3.1	5	2.3	6.5	5.1	5.7
12:0 3 OH	1.4	2.3	1.7	2.5	1.6	2.9	2.5	2.8
14:0	6.6	8	4.7	7.5	5.7	6.1	7.4	5.9
15:0 Iso	3.8	–	1.4	2	5.5	2	ND	2
15:0 Anteiso	1	1.5	–	ND	ND	1.8	–	1.4
16:0	20.9	15.2	13.2	16	13	12.9	14	10
16:0 Iso	–	3	3.4	–	–	6.2	2.9	4.8
17:0 Iso	3.2	–	1.9	–	9	1.6	ND	1.7
18:0	–	ND	ND	1.4	–	ND	ND	ND
18:1 ω7c	–	ND	ND	23.5	19.4	14.1	ND	ND
18:1 ω9c	–	ND	ND	–	–	ND	ND	ND
Summed feature 2	1.8	2.4	2.6	2.9	2.1	4.3	3.8	4.4
Summed feature 3	36.4	42.1	43.4	30.5	31.5	27.8	34.8	38.8
Summed feature 8	15.1	11.7	13.1	ND	ND	ND	18.5	16.9

Taxa are indicated as: 1. *V. mexicanus* sp. nov. CAIM 1540^T, 2. *V. aestivus* CAIM 1861^T, 3. *V. marisflavi*CAIM 1886^T, 4. *V. maritimus* CAIM 1455^T, 5. *V. variabilis*CAIM 1454^T, 6. *V. madracius*A-354^T, 7. *V. thalassae* MD16^T, 8. *V. mediterranei* CAIM 316^T

Values are percentages of the total fatty acids. *ND* no data available; −, values below 1 %. *Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed Feature 2 consisted of 14:0 3OH and/or 16:1 iso I; Summed Feature 3 consisted of 16:1 w6c and/or 16:1 w7c and/or 15:0 iso 2-OH; Summed Feature 8 consisted of 18:1 w7c and/or 18:1 w6c. Data in columns 1, 2 and 3 are from this study; data in columns 4–8 are from Chimetto et al. (2011), Moreira et al. (2014) and Tarazona et al. (2014)

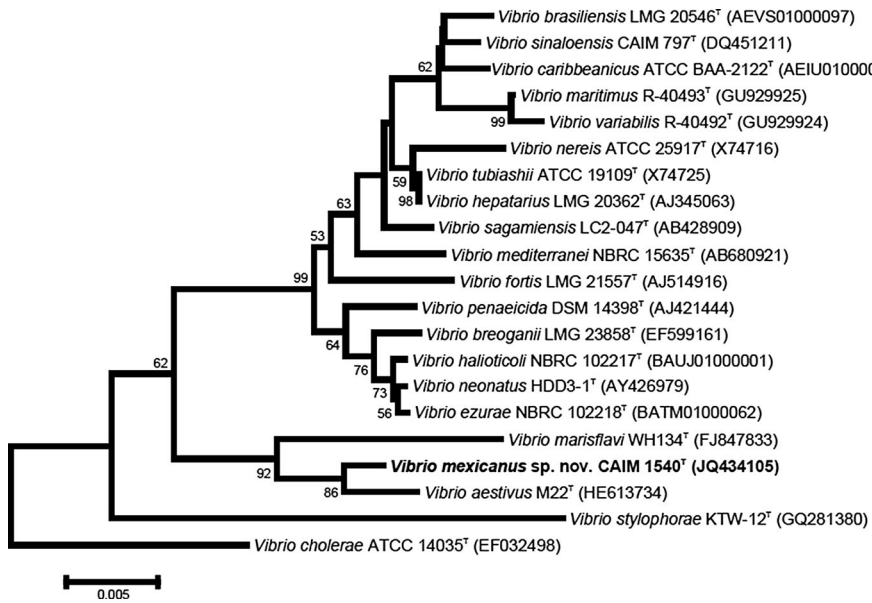

Fig. 1 Phylogenetic tree based on partial 16S rRNA gene sequences obtained by the neighbour joining method based on the Jukes–Cantor model. GenBank sequence accession numbers are given in parentheses. *Numbers* at nodes denote the level of bootstrap based on 1000 replicates; only values greater than 50 % are shown. *Vibrio cholerae* was used as bacterial outgroup. *Bar* 0.5 % estimated sequenced divergence. *Scale bar*, base substitutions per site

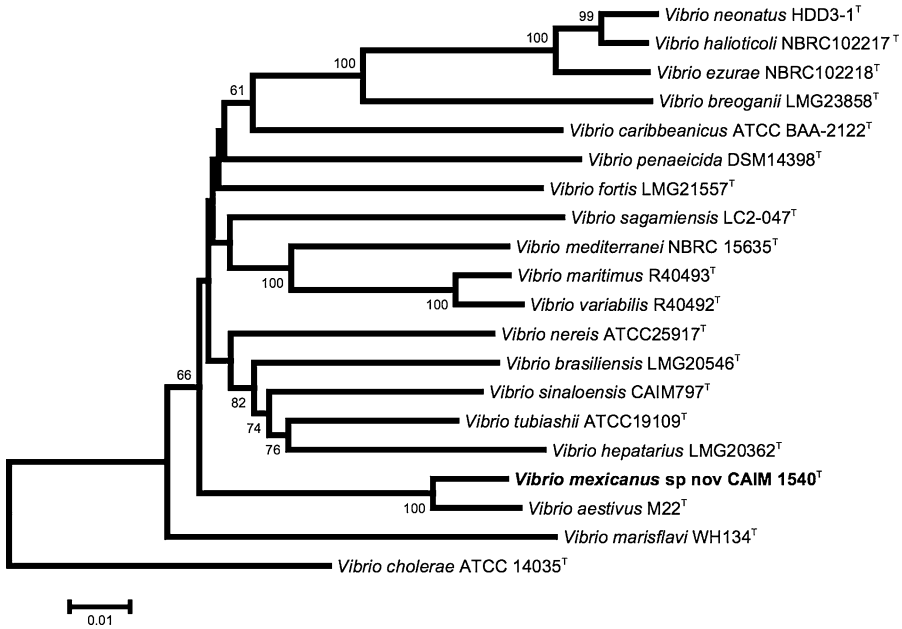

Fig. 2 Phylogenetic tree based on concatenated sequences (3131 bp) of the four housekeeping genes *ftsZ* (432 bp), *gapA* (602 bp), *recA* (494 bp) and *topA* (402 bp), and the 16S rRNA gene (1261 bp) sequences available from the GenBank (accession numbers are listed in Supplementary Table S1) Neighbour Joining. *Numbers* at nodes denote the level of bootstrap based on 1000 replicates; only values greater than 50 % are shown. *V. cholerae* was used as bacterial out-group. *Scale bar*, base substitutions per site

phylogenetic analyses and provides a greater power of taxonomic resolution (Pascual et al. 2010). In this study recombination events were minimized by using concatenated gene sequences and, therefore, phylogenetic tree topology was unaffected.

Genomic analysis

DDH has been the gold standard for prokaryotic classification at the genomic level as it offers a numerical and relatively stable limit, but in the era of genomics this method seems to be out-dated and could be replaced by a comparison between sequenced genomes. ANI with at least 20 % of the genome of the query strains rather than its complete sequence, is enough to clearly differentiate species (Richter and Rosselló-Móra 2009).ANI is calculated with two algorithms, BLAST and MUMmer. The proposed limits for the definition of species are set at 95–96 % of ANI values (Lucena et al. 2012). Therefore, shotgun genome sequencing was performed on strain CAIM 1540^T. The subsequent assembly produced 167 contigs (N 50 = 88,690 bp, G+C 43.7 %) for a 5.4 Mb genome.

Comparison of the draft genome of strain CAIM 1540^T yielded ANI values (Table 3) of 89.6 % (ANIb) and 90.6 % (ANIm) with the closest related species *V. aestivus*, 71.5 % (ANIb) and 85.5 % (ANIm) with *V. marisflavi*, 72.6 % (ANIb) and 85.7 % (ANIm) with *V. maritimus,* and 72.6 % (ANIb) and 85.8 % (ANIm) with *V. variabilis*. These values are clearly below the species-delineating threshold of 96 %, indicating that strain CAIM 1540^T does not belong to these previously described species.

Digital DDH calculations were also performed for these genome sequences. The genomic distance was calculated using the Genome-To-Genome Distance Calculator (GGDC) (Auch et al. 2010; Meier-Kolthoff et al. 2013). In silico DDH is calculated by the Genome Blast Distance Phylogeny (GBDP), which was devised as an approach for the inference of phylogenetic trees or networks from a given set of wholly (or even incompletely) sequenced genomes (Henz et al. 2005) and was subsequently revisited and enhanced (Auch et al. 2010). Strains from the same prokaryotic species share >70 % in silico GGDC (Thompson et al. 2013). Comparisons with the draft genome of strain CAIM 1540^T yielded GGDC values as low as 39.5 ± 2.5 % with *V. aestivus* and 20–23 % with other closely related species (Table 4), confirming that the strain does not belong to any of these species.

Another tool with a high resolving power, which uses genome sequences is vibrio phenotyping, this program is based on the search for those enzymes related to the phenotype of interest. It uses BLAST to

Table 3 Results of ANI calculations (%) using JS pecies software

ANIb	1	2	3	4	5	6	7	8
1. *V. mexicanus* sp. nov. CAIM 1540^T	–	89.67	71.52	72.64	72.65	72.52	72.56	72.25
2. *V. aestivus* CAIM 1861^T	89.62	–	71.46	72.56	72.55	72.48	72.54	72.3
3. *V. marisflavi* CAIM 1886^T	71.42	71.40	–	70.88	70.83	71.18	71.20	70.92
4. *V. maritimus* CAIM 1455^T	72.61	72.46	70.95	–	92.51	76.73	76.77	76.73
5. *V. variabilis* CAIM 1454^T	72.69	72.56	71.01	92.52	–	76.67	76.72	76.73
6. *V. madracius* A-354^T	72.80	72.67	71.37	76.88	76.76	–	95.87	85.6
7. *V. thalassae* MD16^T	72.82	72.77	71.49	76.94	76.82	95.81	–	86.38
8. *V. mediterranei* CAIM 316^T	72.59	72.55	71.19	76.90	76.80	85.64	86.44	–
ANIm	1	2	3	4	5	6	7	8
1. *V. mexicanus* sp. nov. CAIM 1540^T	–	90.68	85.52	85.73	85.82	86.15	86.28	86.08
2. *V. aestivus* CAIM 1861^T	90.68	–	85.08	85.91	85.64	85.75	86.58	86.14
3. *V. marisflavi* CAIM 1886^T	85.55	85.12	–	85.61	85.65	86.52	86.66	86.18
4. *V. maritimus* CAIM 1455^T	85.75	85.92	85.60	–	93.37	85.28	85.61	85.52
5. *V. variabilis* CAIM 1454^T	85.82	85.60	85.62	93.38	–	85.24	85.37	85.27
6. *V. madracius* A-354^T	86.24	85.84	86.47	85.29	85.21	–	96.65	88.46
7. *V. thalassae* MD16^T	86.26	86.57	86.63	85.61	85.36	96.65	–	89.27
8. *V. mediterranei* CAIM 316^T	86.07	86.19	86.10	85.51	85.26	88.46	89.28	–

Comparison between 1. *V. mexicanus* sp. nov. CAIM 1540^T(JYJP00000000), 2. *V. aestivus* CAIM 1861^T (JYJN00000000), 3. *V. marisflavi*CAIM 1886^T (JYJM00000000), 4. *V. maritimus* CAIM 1455^T (JYJJ00000000), 5. *V. variabilis*CAIM 1454^T (JYJK00000000), 6. *V. madracius*A-354^T (ASHK00000000), 7. *V. thalassae* MD16^T (PRJEB5327), 8. *V. mediterranei* CAIM 316^T (JYJL00000000)

Table 4 Results of GGDC calculations using BLAST+

	1	2	3	4	5	6	7
V. mexicanus sp. nov. CAIM 1540^T	39.5 ± 2.51	20.20 ± 2.31	22.00 ± 2.35	21.60 ± 2.34	22.60 ± 2.36	23.20 ± 2.38	22.60 ± 2.36

Comparison between *V. mexicanus* sp. nov. CAIM 1540^T(JYJP00000000), 1. *V. aestivus* CAIM 1861^T(JYJN00000000), 2. *V. marisflavi*CAIM 1886^T(JYJM00000000), 3. *V. maritimus* CAIM 1455^T(JYJJ00000000), 4. *V. variabilis*CAIM 1454^T(JYJK00000000), 5. *V. madracius*A-354^T(ASHK00000000), 6. *V. thalassae* MD16^T(PRJEB5327), 7. *V. mediterranei* CAIM 316^T(JYJL00000000)

assign a positive match if the identity is greater than 40 % with a sequence query length greater than 70 %. It is an alternative for the phenotypic identification of vibrios (Amaral et al et al. 2014; Thompson et al 2015). CAIM 1540^T showed similar results to those obtained with commercial systems (Table 5).

In addition to our genomic studies of strain CAIM 1540^T, we found that the recently described species *V. thalassae*, which was assigned to the Mediterranei clade (Tarazona et al. 2014; Validation List 160, Oren and Garrity 2014) and *V. madracius* (Moreira et al. 2014; Validation List 161, Oren and Garrity 2015) comprise a single species because the values of ANIb 95.8 %, ANIm 96.6 % and GGDC 70.2 ± 2.9 % (Tables 3, 4) are above the accepted thresholds for delineating prokaryotic species. Thus, *V. madracius*can likely be considered a later heterotypic synonym of *V. thalassae*.

The DNA G+C mol% range reported for members of the genus *Vibrio* is between 38 and 51 % (Dieguez et al. 2011).The precise DNA G+C mol% for strain CAIM 1540^T was obtained from the draft genome sequence to be 43.7 %; this value was also estimated by a real time-PCR method (Moreira et al. 2011) to be 44.3 mol%.

Table 5 In silico phenotypic characteristics that distinguish *V. mexicanussp. nov.*from related *Vibrio* species

Test	1	2	3	4	5	6	7
L-Arabinose	−	−	−	−	−	−	−
Sucrose	−	−	−	+	+	−	−
Ornithine	−	−	−	−	−	−	−
Vogues	−	−	−	−	−	−	−
Galactose	−	−	−	−	−	−	+
Cellobiose	−	−	−	+	+	−	−
D-Mannitol	−	−	−	+	+	−	+
Arginine	−	−	−	+	+	−	−
Trehalose	+	+	+	+	+	+	−
D-Sorbitol	−	−	−	−	−	+	−
Indole	+	+	+	+	+	−	+
M-Inositol	−	−	−	+	−	−	−
D-Mannose	+	−	+	+	+	+	+

Genotypic and phenotypic similarities were calculated using the Jaccard coefficient based on the presence or absence of the diagnostic phenotypic features

Taxa are indicated as: 1. *V. mexicanus* sp. nov. CAIM 1540^T, 2. *V. aestivus* CAIM 1861^T, 3. *V. marisflavi*CAIM 1886^T, 4. *V. maritimus* CAIM 1455^T, 5. *V. variabilis*CAIM 1454^T, 6. *V. madraciusA*-354^T, 7. *V. mediterranei* CAIM 316^T

The polyphasic taxonomic study which involved phenotypic, genotypic, genomic and phylogenetic analyses support the proposal of a novel species, for which the name *Vibrio mexicanus sp. nov*. is proposed, with CAIM 1540^T as the type strain.

Description of *Vibrio mexicanus sp. nov*

Vibrio mexicanus (me.xi.cánus N.L. masc. adj. *mexicanus* from México).

Gram-negative, curved bacilli that grow as green colonies on TCBS agar, motile and facultatively anaerobic, not luminescent and do not swarm on marine agar or on TSA with 2.0 % NaCl. Growth occurs at 1–6 % NaCl (optimally in 4 % NaCl), no growth without NaCl or with more than 7 % NaCl. Grows at 20, 30, 37 °C (optimum 30 °C). Grows at 4–11 pH (optimally at pH 6–8). Sensitive to the vibriostatic agent O/129 (150 µl per disc). Oxidase and catalase positive. Negative for arginine dihydrolase, lysine and ornithine decarboxylases, positive for nitrate reduction, methyl red test, urea, L-tryptophan, indole, and bovine gelatin; negative reaction for utilisation of citrate and the Voges–Proskauer test. Ferments D-glucose and amygdalin but not D-mannitol, L- rhamnose, inositol, D-sorbitol, D-melibiose, L-arabinose and D-sucrose (in API 20E tests). Utilises the following substrates as sole sources of carbon: 2-ketoglutarate, L-arabinose, L-aspartate, D-cellobiose, D-glucosamine, D-mannitol, D-fructose, glycerol, D-glucose, L-alanine, D-galactose, L-glutamate, lactose, D,L-lactate, malate, maltose, D-mannose, L-leucine, D-melibiose, *N*-acetyl-D-glucosamine, L-ornithine, L-rhamnose, D-ribose, succinate, sucrose, D-xylose and D-trehalose. Negative for utilisation ofacetate, citrate, D-galacturonate, ϒ-aminobutyrate, D-gluconate, pyruvate, D-glucuronate, salicin, glycine, p-hydroxybenzoate, L-lysine, L-histidine, m-inositol, propionate, putrescine, L-threonine and tyrosine. Using Biolog GN2 MicroPlates, oxidizes the following substrates: D-melibiose, glycerol, D,L, α glycerolphosphate, D-fructose, D-cellobiose, glucose-1-phosphate, glucose-6-phosphate, p-hydroxyphenylacetic acid, bromosuccinic acid, β-methyl-D-glucoside, itaconic acid, succinamic acid, hydroxy-L-proline, L-fucose, glucuronamide, L-leucine, L-rhamnose, glycogen, D-galactose, formic acid, α-ketoglutaric acid, L-alaninamide, L-ornithine, Tween 40, Tween 80, α-D-glucose, D-sorbitol, D-galacturonic acid, D,L-lactic acid, L-alanine, sucrose, D-gluconic acid, malonic acid, *N*-acetyl-D-glucosamine, α-D-lactose, D-trehalose, propionic acid, adonitol, lactulose, turanose, D-glucuronic acid, quinic acid, L-aspartic acid, L-arabinose, maltose, D-saccharic acid, L-glutamic acid, D-mannitol, sebacic acid and D-mannose; weak positive reactions for inosine, thymidine and phenylethylamine. Negative for I-erythritol, D-alanine, m-inositol, putrescine, 2-aminoethanol, acetic acid, L-histidine, α-cyclodextrin, cis-aconitic acid, dextrin, D-psicose, citric acid, α-ketobutyric acid, D-raffinose, gentiobiose, D-galactonic acid lactone, α-ketovaleric acid, L-phenylalanine, L-proline, *N*-acetyl-D-galactosamine, L-alanyl-glycine, L-pyroglutamic acid, D-glucosaminic acid, L-asparagine, D-serine, L-serine, 2,3-butanediol, urocanic acid, uridine, xylitol, α-hydroxybutyric acid, L-threonine, D-arabitol, methyl pyruvate, β-hydroxybutyric acid, glycyl-L-aspartic acid, D,L-carnitine, monomethyl-succinate, γ-hydroxybutyric acid, succinic acid, glycyl-L-glutamic acid, and γ-aminobutyric acid. The major fatty acids are summed feature 3 (comprising C16:1 w7c and/or C16:1 w6c and/or C15:0 iso 2–OH), C16:0, summed feature 8 (C18:1 w6c and/or C18:1 w7c) and C14:0. The following fatty acids are

present in small amounts: C15:0 iso, C17:0 iso, C12:0, summed feature 2 (C14:0 3OH and/orC16:1iso) and C12:0 3OH.

The type strain is CAIM 1540^T. The type strain was isolated from a cultured oyster, *C. corteziensis*, in La Cruz, Sinaloa state, México and deposited as CAIM 1540^T and as CECT 8828^T. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *ftsZ*, *gapA*, *recA,* and *topA* gene sequences of strain CAIM 1540^T are JQ434105, KP698215, KP698212, KP698213, and KP698214 respectively. The genome accession number of the type strain CAIM 1540^T is JYJP00000000.

Acknowledgments This work was partially funded by CONACYT project CB-2009-01 132328 and Grant AGL–2010–18438 from the Ministerio de Ciencia e Innovación (Spain).

References

Amaral GRS, Dias GM, Wellington-Oguri M, Chimetto L, Campeão ME, Thompson FL, Thompson CC (2014) Genotype to phenotype: identification of diagnostic vibrio phenotypes using whole genome sequences. Int J Syst Evol Microbiol 64:357–365

Auch AF, von Jan M, Klenk H-P, Göker M (2010) Digital DNA–DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. Stand Genom Sci 2:117–134

Austin B, Austin DA (1999) Bacterial fish pathogens: disease of farmed and wild fish, 3rd edn. Springer, Berlin

Cano-Gomez A, Goulden EF, Owens L, Høj L (2010) *Vibrio owensii* sp. nov., isolated from cultured crustaceans in Australia. FEMS Microbiol Lett 302:175–181

Chimetto LA, Cleenwerck I, Moreira APB, Brocchi M, Willems A, De Vos P, Thompson FL (2011) *Vibrio variabilis* sp. nov. and *Vibrio maritimus* sp. nov., isolated from *Palythoa caribaeorum*. Int J Syst Evol Microbiol 61:3009–3015

Dieguez AL, Beaz-Hidalgo R, Cleenwerck I, Balboa S, de Vos P, Romalde JL (2011) *Vibrio atlanticus* sp. nov. and *Vibrio artabrorum* sp. nov., isolated from the clams *Ruditapes philippinarum* and *Ruditapes decussates*. Int J Syst Evol Microbiol 61:2406–2411

Gomez-Gil B, RoqueA TurnbullJF, Tron-Mayen L (1998) Species of *Vibrio* isolated from hepatopancreas, hemolymph and digestive tract of a population of healthy juvenile *Penaeus vannamei*. Aquaculture 163:1–9

Gomez-Gil B, Thompson FL, Thompson CC, Swings J (2003) *Vibrio rotiferianus* sp. nov., isolated from cultures of the rotifer *Brachionus plicatilis*. Int J Syst Evol Microbiol 53:239–243

González-Castillo A, Balboa S, Romalde JL, Gomez-Gil B (2014) *Vibrio crosai* sp. nov., isolated from a cultured oyster *Crassostrea gigas*. Antonie Van Leeuwenhoek 106:457–463

Henz S, Huson D, Auch AF, Nieselt-Struwe K, Schuster S (2005) Whole-genomeprokaryotic phylogeny. Bioinformatics 21(10):2329–2335

Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. Mol Biol Evol 23(2):254–267

Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chun J (2012) Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 62:716–721

Lucena T, Ruvira MA, Arahal DR, Macián MC, Pujalte MJ (2012) *Vibrio aestivus* sp. nov. and *Vibrio quintilis* sp. nov., related to Marisflavi and Gazogenes clades, respectively. Syst Appl Microbiol 35:427–431

MacFaddin JF (1993) Pruebas bioquímicas para la identificación de bacterias de importancia clínica (translation by Médica Panamericana SA). Williams & Wilkins, Baltimore

Macián MC, Ludwig W, Aznar R, Grimont P, Schleifer KH, Garay E, Pujalte MJ (2001) *Vibrio lentus* sp. nov., isolated from Mediterranean oysters. Int J Syst Evol Microbiol 51:1449–1456

Meier-Kolthoff JP, Alexander AF, Hans-Peter K, Markus K (2013) Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinform 14:60

Moreira AP, Pereira N Jr, Thompson FL (2011) Usefulness of a real-time PCR platform for G+C content and DNA–DNA hybridization estimations in vibrios. Int J Syst Evol Microbiol 61:2379–2383

Moreira AP, Duytschaever G, Tonon LAC, Dias GM, Mesquita M, Cnockaert M, Francini-Filho RB, DeVos P, Thompson CC, Thompson FL (2014) *Vibrio madracius* sp. nov. isolated from *Madracisdecactis* (*Scleractinia*) in St. Peter & St. Paul Archipelago, Mid-Atlantic Ridge, Brazil. Curr Microbiol 69(4):405–411

Noguerola I, Blanch AR (2008) Identification of *Vibrio* spp. with a set of dichotomous keys. J Appl Microbiol 105:175–185

Oren A, Garrity GM (2014) List of new names and new combinations previously effectively, but not validly, published. Int J Syst Evol Microbiol 64:3603–3606

Oren A, Garrity GM (2015) List of new names and new combinations previously effectively, but not validly, published. Int J Syst Evol Microbiol 65:1–4

Pascual J, Macián MC, Arahal DR, Garay E, Pujalte MJ (2010) Multilocus sequence analysis of the central clade of the genus *Vibrio* by using the 16S rRNA, *recA*, *pyrH*, *rpoD*, *gyrB*, *rctB* and *toxR* genes. Int J Syst Evol Microbiol 60:154–165

Quail MA, Smith M, Coupland P, Otto TD, Harris SR, Connor TR, Bertoni A, Swerdlow HP, Gu Y (2012) A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and IlluminaMiSeq sequencers. BMC Genom 13(1):341

Richter M, Rosselló-Móra R (2009) Shifting the genomic gold standard for the prokaryotic species definition. Proc Natl Acad Sci USA 106:19126–19131

Sasser M (1990) Identification of bacteria by gas chromatography of cellular fatty acids. Microbial ID Inc, Newark

Sawabe T, Kita-Tsukamoto K, Thompson FL (2007) Inferring the evolutionary history of vibrios by means of multilocus sequence analysis. J Bacteriol 189:7932–7936

Sawabe T, Ogura Y, Matsumura Y, Feng G, Rohul Amin AKM, Mino S, Nakagawa S, Sawabe T, Kumar R, Fukui Y, Satomi M, Matsushima R, Thompson FL, Gomez-Gil B, Christen R, Maruyama F, Kurokawa K, Hayashi T (2013) Updating the Vibrio clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *Vibrio tritonius* sp. nov. Front Microbiol 4:(414)1–14

Sheu S-Y, Jiang S-R, Chen CA, Wang JT, Chen WM (2011) *Vibrio stylophorae* sp. nov., isolated from the reef-building coral *Stylophora pistillata*. Int J Syst Evol Microbiol 61:2180–2185

Soto-Rodriguez SA, Gomez-Gil B, Lozano-Olvera R, Betancourt-Lozano M, Morales-Covarrubias MS (2015) Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in Northwestern Mexico. Appl Environ Microbiol 81:1689–1699

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Tarazona E, LucenaT Arahal DR, Macián MC, Ruvira MA, Pujalte MJ (2014) Multilocus sequence analysis of putative *Vibrio mediterranei* strains and description of *Vibrio thalassae* sp. nov. Syst Appl Microbiol 37:320–328

Thompson FL, Swings J (2006) Taxonomy of the vibrios. In: Thompson FL, Austin B, Swings J (eds) The biology of vibrios. American Society for Microbiology, Washington, pp 29–43

Thompson CC, Amaral GR, Campeão M, Edwards RA, Polz MF, Dutilh BE, Ussery DW, Sawabe T, Swings J, Thompson FL (2015) Microbial taxonomy in the post-genomic era: rebuilding from scratch? Arch Microbiol 197:359–370

Thompson CC, Chimetto L, Edwards RA, Swings J, Stackebrandt E, Thompson FL (2013) Microbial genomic taxonomy. BMC Genom 14:913

Tran L, Nunan L, Redman RM, Mohney LL, Pantoja CR, Fitzsimmons K, Lightner DV (2013) Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis Aquat Organ 105:45–55

Wang H, Liu J, Wang Y, Zhang X-H (2011) *Vibrio marisflavi sp. nov*., isolated from sea water. Int J Syst Evol Microbiol 61:568–573

Yoshizawa S, Wada M, Yokota A, Kogure K (2010) *Vibrio sagamiensis* sp. nov., luminous marine bacteria isolated from sea water. J Gen Appl Microbiol 56:499–507