

Brief Original Article

Draft genome sequence of first *Vibrio diabolicus* in Mexico strain InDRE-D1-M1, an emergent threat

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Abstract

Introduction: The complete genome of the marine environmental bacterium *Vibrio diabolicus* isolated from raw shrimp in the city of Guadalajara in the state of Jalisco in Mexico is reported here.

Methodology: *Vibrio spp.* it was isolated and identified using standard microbiological and molecular techniques. Whole genome sequencing was performed using the Miseq system (Illumina, USA).

Results: The Multi Locus Sequence Typing profile of the isolated *Vibrio* bacteria coincided only with 4 specific loci (*atpA*, *gyrB*, *pyrH* and *recA*) and with a total coverage of the species belonging to *Vibrio spp.* Analysis of the complete genome of the *Vibrio* isolate and other closely related species, using the genomic fingerprints of the Virtual Analysis Method for PHylogenomic fingerprint estimation (VAMPHyRe) software, revealed the clustering of this species among the clade *Vibrio diabolicus*. The antibiogram revealed that this strain of *Vibrio diabolicus* is resistant to ampicillin, which is consistent with the bioinformatic finding of the β -lactamase enzyme that hydrolyzes carbenicillin class A.

Conclusions: This study demonstrated that the environmental marine bacterium *Vibrio diabolicus* contains carrier genes associated with pathogenicity and ecological function, which could represent a threat to public health.

Key words: *Vibrio diabolicus*; Mexico; environmental; pathogenicity.

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Introduction

Vibrio is a diverse genus of Gram negative bacteria, comprising about 139 species in various groups where at least 12 are human pathogens. The best known are *V. cholerae* and *V. parahaemolyticus*, which have been associated with pandemic diseases. The *Vibrio harveyi* group includes some shellfish pathogens, such as *V. diabolicus* and *V. antiquarius* that exhibit a cosmopolitan distribution in oceans, estuaries, and marine environments. This genus has developed specific genes that promote survival and persistence in

hydrothermal vent ecosystems [1]. This genus produces several proteins such as ToxR, Tlh, Tdh, hemolysins and type III secretion systems (T3SS) that are necessary to form biofilms and interact with other microbial communities, but are also considered factors associated with virulence in pathogens of the species *Vibrio* [2]. Maintaining these characteristics in the deep sea has been mentioned as evidence that secreted toxins are ancient evolutionary characteristics that may play a greater role in environmental adaptation [1,3]. Climate change, as well as many human activities, could affect

the redistribution of this type of microorganism [4], so that strict monitoring of seafood in the coming years could become a fundamental issue for public health.

Methodology

Vibrio bacterial cultures were cultivated in TCBS media, these cultures were sucrose positive and tolerant to NaCl up to 10%, which is not typical in this microorganism. Subsequently, antimicrobial resistance was tested through diffusion of the disk in culture (Table 1) [5]. Amplification of *ompW* was carried out by PCR assay using 5'-CACCAAGAAGGTGACTTTATTGTG-3' (forward) and 5'-GAACTTATAACCACCCGCG-3' (reverse) primers [6] to amplify a 588bp fragment. The reaction mixture contained 5 µL of template DNA, 1 µL of each primer (20 pmol/µL), 2.5 µL of 10 mM deoxynucleoside triphosphates, 0.25 µL (5 U/µL) of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA), 2.5 µL of 10× reaction buffer, 2 µL of 25 mM MgCl₂, and 10.75 µL of distilled water. A 40 cycle PCR program consisting of the following steps: denaturation at 95 °C for 30 seconds, annealing at 50 °C for 30 seconds, and extension at 72 °C for 40 seconds. Before initiation of the first cycle, the reaction mixture was heated at 95 °C for 10 minutes to allow complete denaturation of the template. PCR products, thus obtained, were electrophoresed through 2.5% (wt/vol) agarose gel to resolve the amplified products that were visualized under UV light. The *ompW* sequencing was performed using the ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA) with both primers previously mentioned and the Big Dye Terminator v3.1 Cycle Sequencing kit according to the manufacturer's instructions. *ompW* sequence identification was established according to sequence similarity in BLAST.

Genomic DNA extraction was performed manually using ion exchange columns with a DNA kit according

to the recommended manufacture (Qiagen, GE). Quality (A260/280 nm ratio) and DNA concentration were determined using the Qubit 2.0 fluorometer (Thermo Fisher Scientific, USA). Fragmentation and library preparation was performed by the Nextera XT kit. The DNA sequence was performed according to the instructions of the Miseq system guide (Illumina, USA). A final torque chemistry was used in a 300 cycle flow cell (2X300 v3), a pool density of 765 k/mm² was obtained with a yield of 6.2 Gb. 90.9% of the clusters passed quality filters and 76.4% of the readings scored higher than Q30. The quality of fastq files was verified with Fast QC software version 0.11.5. Data were deposited in Genbank under the numbers: BioSample: SAMN14845524 and BioProject PRJNA630816.

Results

As part of the epidemiological surveillance system program for food control, the Public Health Laboratory of the State of Jalisco (LESPJ) isolated a biochemically unclassifiable *Vibrio* from commercial shrimp and sent it for identification to the Enterobacteria Laboratory of the Diagnostic Institute. Epidemiological and Reference (InDRE). The first attempt to identify the organism was PCR amplification and the nucleotide sequence of the 16S ribosomal RNA gene. However, the PCR amplification product could not be detected (data not shown), therefore a *Vibrio*-specific PCR-Sanger sequence was performed for the *ompW* gene. The *ad hoc* database was constructed and aligned with the sample sequence using MEGA X software. The sample was grouped into phylogenetic trees belonging to the clade *Vibrio harveyi*, closely related to *V. alginolyticus* and *V. parahaemolyticus*. However, no differences were found between *Vibrio antiquarius* and *Vibrio diabolicus* (Figure 1). So we decided to biochemically analyze the microorganisms through a disk diffusion antibiogram. As shown in Table 1, this new *Vibrio* exhibited phenotypic antimicrobial

Table 1. Antimicrobial disc diffusion tests.

Antimicrobial	Disc diffusion test for <i>Vibrio</i> strain					
	<i>Vibrio</i> ALI-210		** <i>E. coli</i> 25922		** <i>P. aeruginosa</i> 27853	
	Diameter (mm)	S/R*	Diameter (mm)	S/R	Diameter (mm)	S/R*
Sulfametaxol 1.25 µg	24	S	26	S	ND	ND
Ampicillin 10 µg	6	R	20	S	ND	ND
Colicin 30 µg	30	S	27	S	ND	ND
Ciprofloxacin 5 µg	25	S	37	S	32	S
Cefotaxime 30 µg	32	S	33	S	22	S
Meropenem 10 µg	31	S	29	S	25	S
Nalidixic Acid 30 µg	24	S	22	S	24	S
Gentamycin M 10 µg	22	S	25	S	20	S
Chloramphenicol 10 µg	13	S	15	S	14	S

*S/R: Sensitive or Resistant; **Strains of *Escherichia coli* ATCC® 25922 and *Pseudomonas aeruginosa* ATCC® 27853 were used as quality control of the Muller Hinton agar plates and the antibiotic discs used; ND: Not determined.

resistance to ampicillin, consistent with inhibition in growth diameter (approximately one third over the sensitive control strain). In light of our results, we decided to use Next Generation Sequencing (NGS) to carry out a correct identification of this microorganism.

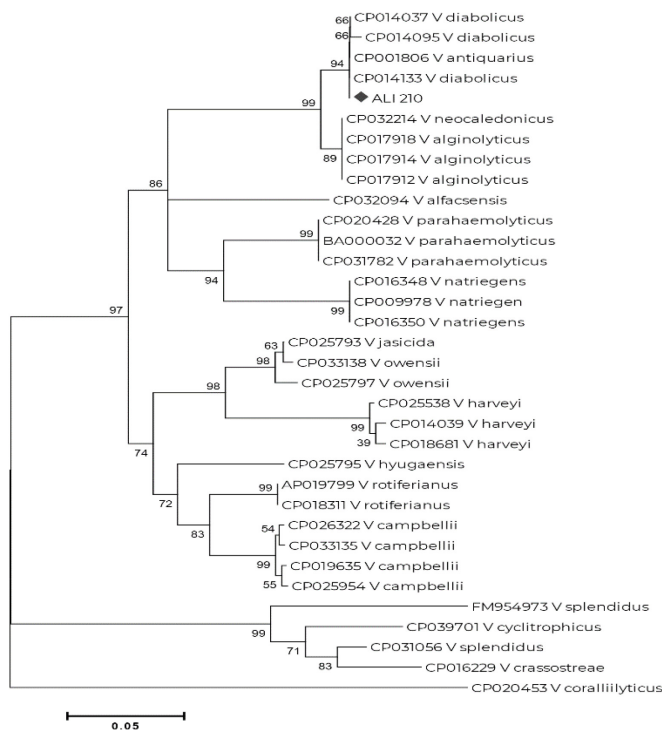
One hundred and seventy-seven contigs (large contig = 243933 bp) with a total size of 5094644 bp were assembled using the Newbler software in the 454 suite package, providing 40X deep coverage. The median contig length is 28783 bp and N50 = 75245, with an overall G + C content of the *Vibrio diabolicus* genome sets of 44.8%. Raw data (fastq files) were obtained using whole genome multilocus sequence typing (MLST) from the Center for Genomic Epidemiology website [7]. The analysis of the whole genome of the isolate of *Vibrio* and other closely related species (*V. antiquarius*, *V. alginolyticus* and *V. diabolicus*) was examined, using the genomic fingerprints of the Virtual Analysis Method for Phylogenomic fingerPrint Estimation (VAMPHYRE) software. The 13AM VAMPHYRe probe was used, which allowed for a mismatch. Five nucleotide extension left and right, 22 nucleotide threshold (data not shown). The sequence was deposited in Genbank (BioSample: SAMN14845524, BioProject PRJNA630816).

The MLST profile of the isolated *Vibrio* bacteria coincided only with 4 specific *loci* and with a total coverage (*atpA*, *gyrB*, *pyrH* and *recA*) of the species belonging to *Vibrio spp.* not with another available profile of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* or *V. tapetis*.

Antimicrobial resistance elements were sought on two different platforms: The Comprehensive Antibiotic Resistance Database (CARD) [8] and Resfinder 2.0 from the Genomic Epidemiology Center website, both highlighting a *blaCARB* gene for the hydrolyzing of carbenicillin class A beta-lactamase called CARB -42, with 100% coverage and 37X deep coverage (data not shown). To verify this finding, a reference mapping was performed using the NCAR_048745.1 *Vibrio parahaemolyticus* TUMSAT_H03_S5 *blaCARB* gene, in Roche's GS Mapper Ver 1.0 software, confirming the last result.

To establish similarities and differences of this organism with previously reported virulence factors, an *ad hoc* database was developed for these genes found in *V. diabolicus*, including *ompA*, *tdh*, *tdl*, *tlh*, *toxR*, *toxS* and secretion systems T3SS1 and 2 [2, 8]. This database was used to map genes in GS Mapper Software ver. 1.0 (Roche, SW). After mapping, we found that 3 genes match T3SS1, thermolabile *tlh*, and *toxR* (data not shown).

Figure 1. Phylogenetic Tree constructed using the *ompW* sequence from 33 representative *Vibrio* genes plus the sample 210ALI studied in this manuscript.



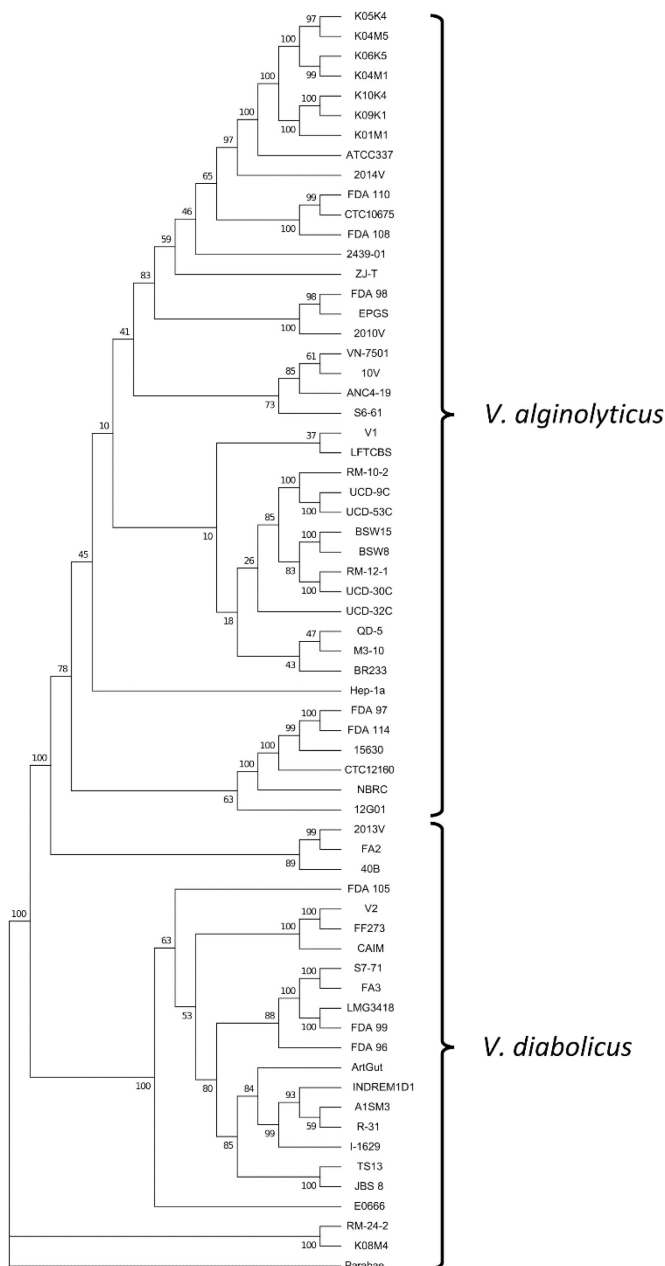
Discussion

The taxonomy of the genus *Vibrio* is a history in constant revision, especially at the boundaries of particular species among the members of the *Harveyi* group (*V. alginolyticus*, *V. parahaemolyticus*, *V. campbellii*, *V. harveyi*, *V. rotiferianus*, *V. natriegens*, to name a few) are closely related species and especially difficult to distinguish from *V. diabolicus* and *V. antiquarius*. The sample examined at InDRE turned out to be one of those cases since the results of its identification by microbiological and biochemical techniques were not conclusive. In fact, we concluded that the strain was an unclassifiable *Vibrio*. Therefore, according to the epidemiological surveillance system, the InDRE carried out the initial identification of this new strain. First, attempts to amplify the *16S* rRNA gene were unsuccessful, then the *ompW* gene was amplified by PCR and the DNA sequence determined by the Sanger method revealed that the *Vibrio* isolate is part of the *Harveyi* group members and that it is closely related to *V. alginolyticus* and *V. parahaemolyticus*, but is indistinguishable from *Vibrio antiquarius* and *Vibrio diabolicus*. It is presumed that the relationship between the two species is due to evolutionary processes such as

genetic recombination [1]. So we decided to determine the sequencing of the entire genome for the correct identification of the culture of this *Vibrio sp.* using NGS (Illumina Sequencing Platform). The sequence was then analyzed using VAMPHyRe software to identify the specific genomic fingerprints of the *Vibrio* species. The genomic fingerprints of closely related species were used to construct a higher resolution phylogenetic tree to determine the identity of the *Vibrio* species isolate. The phylogenetic tree obtained was in agreement with previous results where *V.*

parahaemolyticus and *V. alginolyticus* were the closest species, while *V. antiquarius* and *V. diabolicus* remained undefined (Figure 2). This is explained by the recent discovery that the strains of *V. diabolicus* (Art-Gu C1 and CNCM I-1629), *V. antiquarius* (939 and EX25) and *V. alginolyticus* (E0666, FF273, TS13 and V2) share a genome with similarity greater than 97%, which shows that they constitute the same species and that they are members of the subgroup of *V. diabolicus* [1]. Raw sequencing data was used for MLST analysis, resulting in the presence of 4 loci (*atpA*, *gyrB*, *pyrH*, and *recA*) corresponding to *Vibrio* species other than *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. tapetis*. These results are consistent with a previous study [1]. It is important to mention that genes for ToxR proteins, thermolabile hemolysin (TLH) and type III secretion system (T3SS) were found in the *V. diabolicus* strain examined. Taking into account the generalized and progressive trend of resistance to antibiotics in *V. cholerae*, it is important to note that the genes associated with resistance to fluoroquinolone, fosfomicin, tetracycline, penicillin and cephalosporin, in addition to the genes for resistance to carbenicillin (CARB-42) were identified in the *Vibrio diabolicus* genome that is reported here. Finally, the presence of a specific subsystem of efflux pumps that confer resistance to cobalt, zinc and cadmium was found [1]. We must not forget that pathogenic bacteria with these resistance properties can represent a threat to public health as well as a bacterial survival mechanism in the aquatic environment, so the isolation and molecular identification of the *V. diabolicus* strain described here becomes relevant to serve as a reference for study, epidemiological surveillance and resulting in schemes to prevent diseases [1,8].

Figure 2. Phylogenetic Tree constructed using the full genome of the sample InDRE-D1-M1 studied in this manuscript.



Conclusions

The sequence of the first Mexican environmental genome of *V. diabolicus* is reported; pathogenicity genes were found, associated with resistance and survival in the aquatic environment.

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