# PHENOTYPIC CHARACTERIZATION AND MOLECULAR IDENTIFICATION OF A LUMINESCENT MARINE BACTERUM ISOLATED FROM THE NW SHELF OF CUBA

## CARACTERIZACIÓN FENOTÍPICA E IDENTIFICACIÓN MOLECULAR DE UNA BACTERIA LUMINISCENTE AISLADA DE LA PLATAFORMA NW DE CUBA

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## ABSTRACT

Light emitted by luminescent bacteria is sensitive to several toxic compounds; therefore, some luminous species have been used to evaluate the quality of water environments. In the present study we carried out the phenotypic characterization and molecular identification of a marine luminescent isolate (CBM-784) from the NW Cuban coast. The identification of the CBM-784 luminous isolate was based on phenotypic and genotypic characteristics. Phenotypically, the CBM-784 strain revealed the following characteristics: Gram negative, positive to oxidase and catalase reactions, bioluminescent, and facultative anaerobic respiration. The bacterial strain produces enzymes with gelatinase, lysine carboxylase and amylase activity. Taken together, these assays indicated that CBM-784 showed a high phenotypic similarity to the *Vibrio harveyi* ATCC 14126 strain. On the basis of 16S rRNA gene sequencing, CBM-784 was closely related to *Vibrio harveyi* and *Vibrio rotiferianus* (94% similarity). Sequence analysis of *gyrB* gene, has shown that CBM-784 shares taxonomic position with *Vibrio campbellii* and *Vibrio harveyi* isolates, with 95% of bootstrap value. In addition, sequence analysis of *pyrH* gene, grouped this isolate to the *Vibrio harveyi* cluster with a strong bootstrap support (99%). The multilocus sequence analysis and phenotypic characterization of CBM-784 indicated that this strain have a strong relation to *Vibrio harveyi*.

**Keywords:** luminescent bacteria; Vibrionaceae; *Vibrio harveyi;* Mutilocus sequence analysis.

## RESUMEN

La luz emitida por las bacterias luminiscentes es sensible a varios compuestos tóxicos; por lo tanto, algunas especies luminiscentes se han utilizado para evaluar la calidad de los ambientes acuáticos. En el presente estudio se realizó la caracterización fenotípica y la identificación molecular de un aislado marino luminiscente (CBM-784) de la costa cubana del NW. La identificación del aislado luminiscente CBM-784 se basó en características fenotípicas y genotípicas. Fenotípicamente, la cepa CBM-784 reveló las siguientes características: Gram negativa, oxidasa positiva, reacción catalasa positiva, bioluminiscencia y respiración anaeróbica facultativa. La cepa bacteriana produce enzimas con actividad gelatinasa, lisina carboxilasa y amilasa. Tomados en su conjunto, estos ensayos indicaron que CBM-784 mostró una alta similitud fenotípica con la cepa *Vibrio harveyi* ATCC 14126. Sobre la base de la secuenciación del gen 16S rRNA, CBM-784 mostró una estrecha relación con *Vibrio harveyi* y *Vibrio rotiferianus* (94% de similitud). El análisis secuencial del gen *gyrB*, ha demostrado que CBM-784 comparte la posición taxonómica con *Vibrio campbellii* y *Vibrio harveyi*, con un 95% de valor de *bootstrap*. Además, el análisis de secuencias del gen *pyrH*, agrupó este aislamiento en el *cluster* de *Vibrio harveyi* con un fuerte *bootstrap* (99%). El análisis de la secuencia multilocus y la caracterización fenotípica de CBM-784 indicaron que esta cepa tiene una fuerte relación con *Vibrio harveyi*.

**Palabras claves:** Análisis de secuencias multilocus; bacteria luminiscente; Vibrionaceae; *Vibrio harveyi*

## INTRODUCTION

Early bacteriologists observed phenotypic similarities between some bacterial isolates, and started classifying bacteria into species based on their phenotypic characters (Drews, 2000). Microbial taxonomists later supplemented the phenotypic character analyses with chemotaxonomy, DNA–DNA hybridization, 16S rRNA gene sequence analysis and multilocus sequence analysis (MLSA). Eventually, microbial taxonomy developed polyphasic approaches, which take into account all available information to classify strains into species (Vandamme et al., 1996; Urbanczyk et al., 2014).

Bacterial luminescence occurs mainly, though not exclusively, in species living in marine environments (Nealson and Hastings, 1979; Gómez-Gil et al., 2012). Light-emitting bacteria are the most abundant and widespread of luminescent organisms (Dunlap and Urbanczyk, 2013).

According to Dunlap and Urbanczyk (2013), 25 species of luminous bacteria have been described and located taxonomically into six genera belonging to the families:

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*Vibrionaceae*, *Enterobacteriaceae*, and *Shewanellaceae*. Thereby, *Aliivibrio* (*Aliivibrio fischeri*), *Vibrio* (*Vibrio harveyi*) and *Photobacterium* (*P. leiognathi* and *P. phospohoreum*) constitute the majority of luminous species identified so far (Chiu et al., 2007).

Bioluminescent proteins are invaluable biochemical tools with a wide variety of applications in some fields including: gene expression analysis, drug discovery, study of protein dynamics and mapping of signal transduction pathway (Sáenz and Nevárez, 2010). Currently, the bioluminescent bacterial gene (*lux*) has been employed in the molecular biology and environmental biotechnology fields as a genetic reporter and contaminant biosensor, respectively (Podgórska and Węgrzyn, 2007; Shanware et al., 2013).

Podgórska et al. (2007) have developed a novel microbiological assay for a rapid assessment of mutagenicity of samples from the natural environment. This assay, based on bioluminescence of *Vibrio harveyi* mutant strain, proved to be useful in testing samples of marine water and plant tissues.

Bacteria-based bioassays are gaining popularity as useful method as early warning systems for environmental monitoring and pollution management due to its sensitivity to toxic / xenobiotic substances and the fast results that can be obtained from such tests (Girotti et al., 2008).

In the present study we identified a marine luminescent bacterium isolated from NW Cuban coastal as *Vibrio harveyi* by the phenotypic characterization and molecular identification.

## MATERIALS AND METHODS

### Phenotypic characterization

The selected CBM-784 strain (based on its biotechnological application, e.g. bioindicator) belongs to the Marine Microbial Cultures collection from the Center of Marine Bioproducts (CEBIMAR), Cuba, and was isolated from Cuban NW shell waters.

A total of 40 phenotypic characteristics were examined for the CBM-784 isolate through morphological, physiological, and biochemical tests. The cell shape and its arrangements were determined by standard Gram and endospore stain procedures. Biochemical characterization was performed using the standardized API 20E, API 20NE identification system (BioMeriux, SA, France) in microtitre plates with incubation at 28 °C, according to the manufacturer´s instructions. The inoculated API stripes were incubated for 4 h and the results were determined by color intensity (Alsina and Blanch, 1994).

Growth pattern was determined by culturing the cells in LM broth (Baumann and Baumann, 1981). Bacterial growth was followed by recording variations of apparent absorbance at λ=620 nm with a Shimadzu spectrophotometer while luminiscence was measured with a spectrofluorimeter (Shimadzu RF-5301pc, Japan).

In order to assess the effect of temperature, the strain was grown in an orbital Ecotron shaker at 160 rpm for 24 h at 4, 15, 30, 35, and 40 °C. The ability of CBM-784 to grow in absence or presence of 1%, 3% and 6% NaCl was also determined. Indole production was assessed by the Kovac´s method (Harrigan and McCance, 1968) after incubating tryptone broth cultures for 2-5 days.

The results of the phenotypic characterization were coded as 1 or 0 for positive and negative results, respectively, and 9 for non-comparable data. The data matrix was analyzed with the Statistic v7.0 software, excluding parameters for which no variation was recorded. The similarities were calculated using the simple matching coefficient ($S_{SM}$). Clustering was achieved by unweighted pair group mathematical averaging (UPGMA) (Bagordo et al., 2012). The correlation between the respective values in the similarity matrix and the corresponding dendrogram (cophenetic correlation) were calculated for each coefficient using the Pearson correlation coefficient (*r*).

### Molecular characterization

*Total DNA extraction and PCR reaction.* Total genomic DNA extraction of bacterial CBM-784 isolate was performed using phenol / chloroform method, according to Thompson et al. (2005). For bacterial taxonomy, specific primers were employed (Table 1) by PCR-mediated amplification of the 16S rRNA, *gyrB* and *pyrH* genes. PCR reactions were prepared in a final volume of 20 µL using 10X buffer (200 mM TrisHCl, 500 mM KCl, pH 8.4), Taq DNA polymerase (1 U/µL) (Invitrogen™), 0.2 mM desoxynucleoside triphosphates, 0.4 µM forward and reverse primers, 2.5 mM $MgCl_2$ and 100 ng of template DNA. Thermocycler parameters for the three genes consisted of an initial denaturalization at 95˚C for 5 min, followed by 36 cycles of denaturalization (95 ˚C, 45 s), alignment (58-62 ˚C, 1 min, 30 s), extension (72 ˚C, 1 min, 15 s) and a final extension at 72 ˚C for 7 min. PCR reactions were performed using a thermal cycler (Proflex PCR System; Applied Biosystems, Life Technologies, USA). PCR products were visualized by agarose gel electrophoresis (1.5%) TBE 1X (Tris base, boric acid, EDTA, pH 8), stained with GelRed® (Biotium). GeneRuler 100 bp DNA Ladder Plus (Fermentas®) was used as ladder. Molecular biology grade water (Fermentas®) was used as negative control.

*Sequencing and sequence analysis.* PCR products were purified using the PureLink Purification kit™ (Invitrogen), quantified with a NanoDrop spectrophotometer (Thermo Scientific™) and used for direct DNA sequencing. Partial gene fragments of 16S rRNA, gyrase beta subunit and uridylate kinase (isolate CBM-784) were sequenced in both directions, using the same forward and reverse amplification primers (BigDye Terminator™ V3.1, Applied Biosystems), according to manufacturer´s instructions. The resulting products were purified with the Xterminator™ Kit (Applied Biosystems), and then run on a DNA multicapillary sequencer (Model 3130, Applied Biosystems) at the Laboratorio de Análisis Genómico, Escuela de Ciencias Biológicas, Universidad Nacional de Costa Rica. Recovered sequences were edited using Geneious (R8 version, Biomatters Limited). The 16S rRNA local sequence of CBM-784 and all sequences of the same gene were processed and nucleotides from positions 300 to 400

**Table 1.** Specific primers for the amplification of bacterial taxonomy genes (multilocus sequence analysis).
**Tabla 1.** Cebadores específicos para la amplificación de los genes taxonómicos bacterianos (análisis de secuencias multilocus).

| Primer name | Sequence (5'-3') | Annealing temp. (°C) | Priming site | Target gene | Reference |
|---|---|---|---|---|---|
| 27f | AGAGTTTGATCMTGGCTCAG | 58 | 9 - 27 | 16S rRNA | Lane (1991) |
| 1492r | TACGGYTACCTTGTTACGACTT | 58 | 1513-1492 | 16S rRNA | Lane (1991) |
| gyrBf | GAAGTTATCATGACGGTACTTC | 62 | 11359-11380 | gyrase beta subunit (*gyrB*) | Ast and Dunlap (2005) |
| gyrBr | AGCGTACGAATGTGAGAACC | 62 | 12582-12602 | gyrase beta subunit (*gyrB*) | Ast and Dunlap (2005) |
| pyrHf | ATGASNACBAAYCCWAAACC | 58 | 1-20 | Uridylate kinase (*pyrH*) | Thompson et al. (2005) |
| pyrHr | GTRAABGCNGMYARRTCCA | 58 | 599-618 | Uridylate kinase (*pyrH*) | Thompson et al. (2005) |

were removed from the partial sequences, because of low quality. Sequences were analyzed using the BLAST algorithm (Altschul et al., 1990) with default parameters, and compared with other available 16S rRNA, *gyrB* and *pyrH* sequences from the GenBank database. Our nucleotide sequence data was deposited in the GenBank data library under accession numbers KU761562 and KU761563 for 16S rRNA partial gene sequences. Sequences obtained from this study and those obtained from the GenBank were replicated (clustering of duplicate sequences) by USEARCH v7.0 software (Edgar, 2010) through cluster fast command application (under a threshold identity of 0.99000). Then, non-duplicates clusters sequences were aligned using MAFFT 7.0 on-line program with iterative refinement method (FFT-NS-i) and 1PAM / k=2 parameters. Phylogenetic tree was performed using maximum likelihood (ML) by randomized accelerated maximum likelihood graphical user interface (raxMLGUI v.7.4.2) (Stamatakis et al., 2005; Silvestro and Michalak, 2012) software by general time reversible model (GTRGAMMA substitution model and 1000 rapid bootstrap inferences). The consensus trees were visualized and edited in FigTree 1.4 (Rambaut, 2009).

## RESULTS

Phenotypically, CBM-784 strain revealed the following characteristics: light yellow and translucent colonies with entire margin, Gram negative, oxidase and catalase positive, polar flagellar motility, absence of poly-p-hydroxybutyrate (PHB) accumulation, bioluminescence, and facultative anaerobic respiration. The bacterial strain produces enzymes with gelatinolytic, lysine carboxylase and amylase activity. However no arginine dehydrolase or ornithine carboxylase activities were present; Tween 80 hydrolysis (lipase activity), ortho-nitrophenyl-β D-galactopyranose (ONPG), indol production and casein hydrolysis were detected. CBM-784 strain fermented glucose but was unable to produce gas according to the method of Baumann and Baumann (1981). CBM-784 reduced nitrates, grew in TCBS medium, and was able to grow at 15, 30, 35 and 40 °C, but not at 4 °C (Table 2).

Strain CBM-784 did not grow in the absence of NaCl, which shows its halophile character. Although the bacteria grew from 15 to 37 °C, the growth was inhibited at 5 °C. Nevertheless the strain could grow and emit luminescence in a range between 1-5% NaCl, the highest light intensity was achieved at 3.5%.

The simplified dendrogram obtained by $S_{SM}$/UPGMA of the CBM-784 phenotypic characteristics is shown in Figure 1. Ten tests were deleted from the data matrix because these results were as the positive (i.e., Kovacs' cytochrome oxidase, O/F, motility) or negative (i.e., Gram, $H_2S$ production) responses.

The dendrogram showed that CBM-784 strain showed a high phenotypic similarity (84%) to the *Vibrio harveyi* ATCC 14126 strain. However, CBM-784 presents three different characteristics, for instance, CBM-784 did not exhibit ODC activity or positive response to ONPG and grew at 40 °C, differently from *V. harveyi* ATCC 14126.

Sequence analysis confirmed the identity of CBM-784 strain. According to the phylogenetic positioning from 16S ribosomal RNA gene topology, this isolate was related to the Vibrionaceae cluster, *Vibrio* group (associated specifically to *Vibrio harveyi* and *Vibrio rotiferianus*), showing a strong bootstrap support (94%) (Figure 2). Besides, CBM-784 isolate was located into *gyrB* taxonomic tree in the *Vibrio* cluster with maximum bootstrap support (100%), sharing taxonomic position with isolates of *Vibrio campbellii* and *Vibrio harveyi* with 95% of bootstrap value (Figure 2). In addition, CBM-784 strain was also located in the Vibrionaceae cluster (82% bootstrap support) within uridylate kinase (pyrH) gene topology, sharing specific position into *V. harveyi* cluster with a strong bootstrap support (99%) (Figure 2). On the other hand, *Aliivibrio* group, Photobacteriaceae, Enterovibrionaceae, and

**Table 2.** Main phenotypic characteristics differentiating the luminescent bacteria species.
**Tabla 2.** Principales características fenotípicas de las especies de bacterias luminiscentes.

| Characteristics | CBM-784 | *P.l* | *P.p* | *V.h* | *V.f* | *V.l* | *V.s* | S.h | S.w |
|---|---|---|---|---|---|---|---|---|---|
| Oxidase | + | + | + | + | + | + | + | + | + |
| Catalase | + | - | + | + | + | + | + | + | + |
| Gram | - | - | - | - | - | - | - | - | - |
| Motility | + | + | + | + | + | + | + | + | + |
| Luminescence | + | + | + | + | + | + | + | + | + |
| Nitrate reduction | + | + | + | + | + | + | + | + | + |
| Indole production | - | - | - | + | - | - | + | - | - |
| O/F test | F | F | F | F | F | F | F | O | O |
| ADH | - | + | + | - | - | - | + | - | - |
| LDC | + | - | - | + | + | + | - | - | - |
| ODC | - | - | - | + | - | - | - | - | - |
| ONPG | - | + | + | + | + | - | + | + | + |
| Citrate utlization | - | - | - | - | - | - | + | - | - |
| Urease | - | - | - | - | + | - | - | - | - |
| Gelatinase | + | - | - | + | - | - | + | + | + |
| Aesculin | + | - | - | + | + | + | + | - | - |
| Amylase | + | - | - | + | + | + | + | - | + |
| Lipase | + | - | - | + | + | + | + | + | - |
| $H_2S$ | - | - | - | - | - | - | - | - | - |
| VP | - | + | + | - | - | - | - | - | - |
| Growth at temperature | | | | | | | | | |
| 4°C | - | - | + | - | - | + | + | + | + |
| 15°C | + | + | + | + | + | + | + | + | + |
| 30°C | + | + | + | + | + | - | + | + | + |
| 35°C | + | + | - | + | - | - | - | - | - |
| 40°C | + | + | - | - | - | - | - | - | - |
| Growth in salinity | | | | | | | | | |
| 0% | - | - | - | - | - | - | - | - | - |
| 1% | + | + | + | + | + | + | + | + | + |
| 3% | + | + | + | + | + | + | + | + | + |
| 6% | + | + | + | + | + | + | + | - | - |

+ = positive, – = negative, V = uncertain, F = fermentative, and O = oxidative, ADH = Arginine dihydrolase, LDC =Lysine decarboxilase, ODC = Ornithine decarboxylase, ONPG = ortho-nitrophenyl-β D-galactopyranose, VP = Vogues- Proskauer. Data on standard strains taken from previous studies*. *P.l.*, *Photobacterium leiognathi* ATCC 25521; *P.p.*, *Photobacterium phosphoreum* ATCC 11040; *V.h.*, *Vibrio harveyi* ATCC 14126; *V.f.*, *Vibrio fischeri* ATCC 7744; *V.l.*, *Vibrio logei* ATCC 29985; *V.s.*, *Vibrio splendidus* biovar I ATCC 33125; *S.h.*, *Shewanella hanedai* ATCC 33224; *S.w.*, *Shewanella woodyi* ATCC 51908.
+ = positivo, - = negativo, V = dudoso, F = fermentativo, O = oxidativo, ADH = arginina dihidrolasa, LDC = Lisina descarboxilasa, ODC = Ornitina descarboxilasa, ONPG = orto nitrofenil-β D- galactopiranosa, VP = Vogues- Proskauer.
Datos de cepas estándares tomados de estudios previos *. *P.l.*, *Photobacterium leiognathi* ATCC 25521; *P.p.*, *Photobacterium phosphoreum* ATCC 11040; *V.h.*, *Vibrio harveyi* ATCC 14126; *V.f.*, *Vibrio fischeri* ATCC 7744; *V.l.*, *Vibrio logei* ATCC 29985; *V.s.*, *Vibrio splendidus* biovar I ATCC 33125; *S.h.*, *Shewanella hanedai* ATCC 33224; *S.w.*, *Shewanella woodyi* ATCC 51908.
*(Makemson et al., 1997; Farto et al., 1999; Venkateswaran et al., 1999; Lunder et al., 2000; López-Caballero et al., 2002; Satomi et al., 2003; Bagordo et al., 2012)

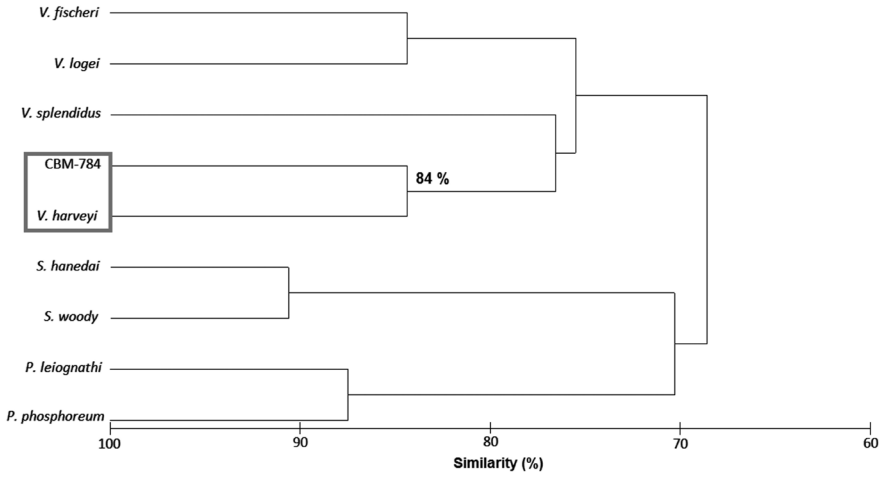


**Figure 1.** Simplified dendrogram based on the $S_{SM}$ coefficient and UPGMA clustering, showing the relationship between CBM-784 strain and standard strains at phenotypical level.
**Figura 1.** Dendograma simplificado basado en el coeficiente $S_{SM}$ y el agrupamiento UPGMA donde se muestra la relación entre la cepa CBM-784 y otras cepas estándares a nivel fenotípico.

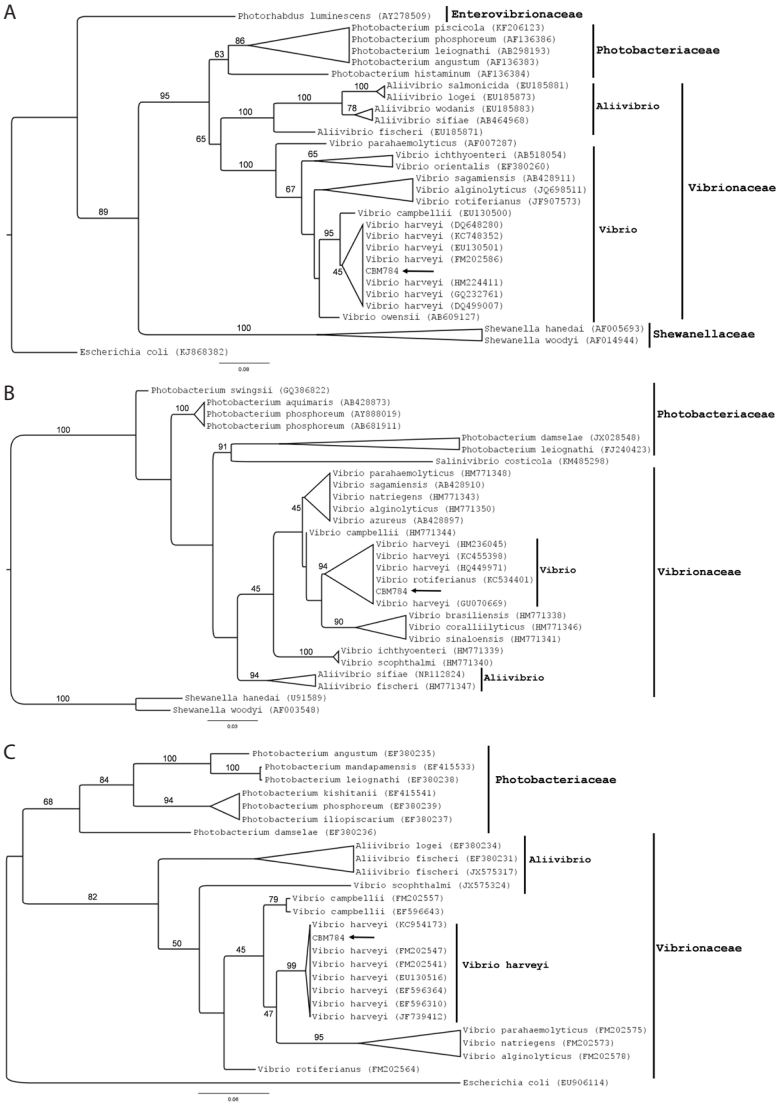


**Figure 2.** Maximum-likelihood phylogenetic tree based on partial sequences of the A) 16S ribosomal RNA gene, B) gyrase beta subunit (*gyrB*) gene and C) uridylate kinase (*pyrH*) gene obtained from GenBank and the sequence from this study (CBM-784 with bold arrow). Numbers at the branches indicates bootstrap support (percentages of 1000 permutations). Branching with a bootstrap value lower than 45% over 1000 repetitions were collapsed or not showed. *Shewanella sp.* and Escherichia coli was set as outgroup. GenBank accession numbers are given in brackets.
Figura 2. Árbol filogenético construido mediante el método de máxima verosimilitud basado en las secuencias parciales de A) gen ribosomal 16S RNA, B) gen de la subunidad beta girasa (gyrB) y C) gen de la urilidato quinasa (pyrH) obtenida del GenBank y la secuencia de este estudio (CBM-784 con la flecha en negrita). Los números en las ramas indican el Bootstrap (porcentajes de 1000 permutaciones). Las ramificaciones con un valor bootstrap inferior a 45% sobre 1000 repeticiones fueron colapsadas o no mostradas. Shewanella spp. y Escherichia coli se establecieron como grupos externos. Los números de acceso de GenBank se indican entre paréntesis.

Shewanellaceae cluster revealed apparent separation between CBM-784 isolate located into *Vibrio* group. Therefore, multilocus sequence analysis and phenotypic approach of CBM-784 indicate that this strain is positioned into *Vibrio* group with a strong relation to *V. harveyi*.

## DISCUSSION

Traditional methods of bacteriology can help to detect common and easily cultured pathogens but can be time-consuming (Balcázar et al., 2007), less sensitive and subject to misinterpretation (Ransangan and Mustafa, 2009) in comparison with molecular methods. In the present study, both methods were used to identify a bioluminescent strain isolated from the Cuban marine shelf, the CBM-784 isolate with potential applications for devising an ecotoxicological test to assess the environmental quality. The biochemical characterization and multilocus sequence analysis converged in a similar identification of the bacterial strain as *V. harveyi*.

Previous studies based on phenotypic characterization have shown that *Vibrio harveyi*, *Vibrio fischeri*, and *Photobacterium leiognathi* are generally the most frequently encountered species of luminous bacteria in temperate and tropical near shore seawaters (Chiu et al., 2007). The species distribution patterns of these bacteria, however, may vary greatly due to geographical and seasonal differences.

The results of the phenotypic analysis showed that *Vibrio harveyi* CBM-784 strain has several metabolic features differing from the ATCC strain. The variability in the biochemical characteristics of *Vibrio harveyi*, could have resulted from gene transfer that occurs both within the same species and among different bacterial genera (Mohammad et al., 2013). Urbanczyk et al. (2014) demonstrated horizontal transference of lux genes in nature from luminous to nonluminous vibrios (from *V. harveyi* to *V. vulnificus* and *V. chagasii,* respectively). The lux genes of VVL1 (*V. vulnificus*) and 21N-12 (*V. chagasii*) instead were evolutionarily very closely related to those of *V. harveyi*, a species that, based on the divergence in sequences of *gyrB*, *recA*, and *pyrH*, is phylogenetically distinct from *V. vulnificus* and *V. chagasii* (Urbanczyk et al., 2014). The discordance between housekeeping and lux genes phylogenies supports the hypothesis that VVL1 and 21N-12 acquired its lux genes by horizontal transfer of *V. harveyi*.

For instance, the bacterial strain isolated in this study differed from the reference strain (*V. harveyi* ATCC 14126) in response to the ONPG test, similarly to the results recently found by Lal and Ransangan (2013) for a strain isolated from Malaysia. The horizontal gene transference in bacterial system was reported for citrate utilization (Ishiguro and Sato, 1979) and urease production (Dupy et al., 1997).

The different results in the identification of *V. harveyi* achieved from biochemical and molecular tools are not surprising and have been reported in the past. *Vibrio harveyi* has been shown to be phenotypically heterogeneous (Alsina and Blanch, 1994). Moreover, *V. harveyi* has also been reported to contain mobile genetic elements, such as bacteriophages, which contribute to new phenotypic characteristics of the bacterium (Cano-Gómez et al., 2011). These make it extremely difficult to identify *V. harveyi* with conventional bacteriological tests. Although more complete biochemical characterizations have been proposed to differentiate closely related *Vibrio* spp (Dunlap and Urbanczyk, 2013), these may be unreliable given variability in biochemical traits and the possibility of errors in interpreting the results.

Molecular tools, such as 16S rDNA sequencing, have proven to be useful for classification and identification of bacterial species (Ransangan and Mustafa, 2009; Cano-Gómez et al., 2015). However, 16S rDNA sequences may not be sufficient for a definite identification, particularly for those bacteria having extremely high rates of horizontal gene transfer and recombination between related species, such as it happens in the *Vibrio* genus. Because of this, Thompson et al. (2005) proposed analysis of other genes, such as *recA*, *pyrH*, *rpoA*, *atpA*, and *obg* or *gyrB* (Ast and Dunlap, 2005) for greater confidence in *Vibrio* species identification.

Regarding the phylogenetic positioning of CBM-784 *V. harveyi* strain, the strong bootstrap (94% and 95% in 16S rRNA and *gyrB* gene topology, respectively) associated to *V. harveyi* cluster including *V. rotiferianus and V. campbellii* showed that the strains are closely related. The Harveyi group consists of five closely related species: *V. harveyi*, *V. rotiferianus*, *V. campbellii*, *V. owensii* and *V. jasicida* according to Urbanczyk et al. (2013) and Cano-Gómez et al. (2015). They found a close relationship between the number of interspecies recombination events and the overall genomic identity, as inferred from the average nucleotide identity (ANI). The relationship between the ANI and the number of detected interspecies recombination events was comparable when they analyzed strains isolated over 80 years apart, from different hemispheres, or from different ecosystems, as well as in strains isolated from the same geographic location within a short time frame (Urbanczyk et al., 2014). On the other hand, topology based on *pyrH* protein-coding gene revealed a strong supported cluster (99% bootstrap) identified as *V. harveyi,* in which CBM-784 strain was positioned. However, the single use of uridylate kinase gene is not adequate for species identification within the Harveyi clade (Pascual et al., 2010). Recombination events in bacteria are possible, generating errors in the clustering, for this reason, at least five housekeeping genes are recommended in multilocus sequence analysis (MLSA) (Cano-Gómez et al., 2011).

Finally, through clustering of phenotypic characteristics and molecular approach (MLSA), we determine that there is sufficient evidence to support that CBM-784 strain corresponds to *Vibrio harveyi.*

## CONCLUSIONS

Combining conventional and molecular techniques allowed determining that the bioluminescent strain CBM-784 isolated from Cuban marine waters, intended to be used as a key component of a water quality bioassay, corresponds to *Vibrio harveyi*.

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