ARTICLE

Amplification and sequence analysis of the *luxA* gene in the non-luminescent type strain *Vibrio campbellii* NBRC 15631

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Type strains of *V. campbellii* can be distinguished from its close relative *V. harveyi* by luminescence activity where *V. campbellii* tests negative and *V. harveyi* tests positive. The luminescent phenotype depends on the ability to produce the enzyme luciferase that has two subunits, alpha (α) and beta (β), encoded by adjacent genes *luxA* and *luxB*, respectively. The *luxA* and *luxB* genes have been isolated and sequenced from type strain *V. harveyi*, but only the *luxB* gene has been amplified and sequenced from type strain *V. campbellii*. In this study, the *luxA* gene from *V. campbellii* NBRC 15631 was amplified and sequenced in order to gain insight into the molecular basis of the non-luminescent phenotype in this *Vibrio*. Inverse PCR using circularized *Hind*III-digested genomic DNA of type strain *V. campbellii* as template and primers that amplify genes flanking the *luxB* gene produced a 1,100 bp amplicon containing the 225 bp *luxA* gene. This much shorter *luxA* of *V. campbellii* NBRC 15631 and the full length 1,068 bp type strain *V. harveyi* NBRC 15634 *luxA* showed only 88% sequence similarity. The 225 bp *luxA* of *V. campbellii* corresponds to a 74-amino acid LuxA polypeptide exhibiting only 74% sequence similarity with LuxA of type strain *V. harveyi*. The much shorter *V. campbellii luxA* is expected to give rise to a variant LuxA polypeptide that may have produced a luciferase with an altered and non-functional active site. This study, therefore, provides a molecular basis for the difference in luminescence phenotype between the type strains of the two closely related species, *V. harveyi* and *V. campbellii*. Moreover, the significant difference in the *luxA* gene sequence between type strains of *V. harveyi* and *V. campbellii* can be utilized in the detection and identification of these two closely related species.

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INTRODUCTION

The type strain *Vibrio campbellii* is a Gram-negative, non-luminescent marine bacterium. Like *V. harveyi* that has long been associated with shrimp disease (Lavilla-Pitogo et al. 1990, Karunasagar et al. 1994, Prayitno and Latchford 1995, Leaño et al. 1998, Abraham et al. 1999, Sung et al. 1999, de la Peña et al. 2001), *V. campbellii* has been implicated as a significant pathogen of marine organisms including black tiger shrimp (*Penaeus monodon*). *V. campbellii* and *V. harveyi* are very closely related and members of these species are difficult to discriminate by more than 100 phenotypic features (Gomez-Gil et al. 2004). Biochemical characterization of type strains from both species shows that they only differ in two tests, the ornithine decarboxylase assay and the test for luminescence, where *V. harveyi* is positive and *V. campbellii* is negative (Alsina

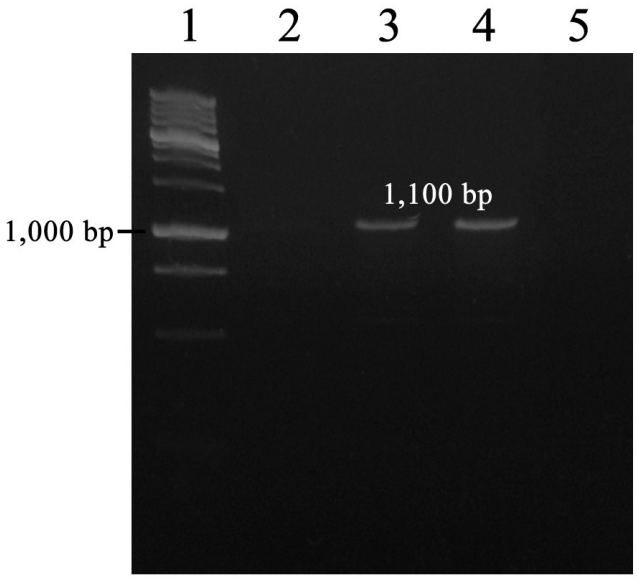

Figure 1. Amplification by inverse PCR of the 1,100 *lux* operon fragment from the circularized *Hind*III-digested type strain *V. campbellii* NBRC 15631 genomic DNA. Lanes 2-4, PCR amplicons from *V. campbellii* NBRC 15631 (10, 50, 100 ng, respectively) using the *VhluxmidR* and *Vc_invluxB* primer pair; lane 5, no-template negative control; lane 1, 1 kb ladder (iNtRON Biotechnology, South Korea).

and Blanch 1994a, Alsina and Blanch 1994b, de la Peña et al. 2001) for both tests. The isolation and complete gene sequencing of the ornithine decarboxylase gene (*odc*) in type strain *V. harveyi* and the absence of an *odc* gene homologue in type strain *V.campbellii* has been reported, providing a basis for the phenotypic difference (Hedreyda and Rañoa 2007, Cua et al. 2011). A molecular basis for the difference in the luminescence phenotype between the two species is yet to be determined.

Bioluminescence is the ability to produce the enzyme luciferase (Lux), catalyzing oxidation of luciferin, with the simultaneous release of blue-green light (Johnston et al. 1986, Fisher et al. 1996). The enzyme is a heterodimer constituting two subunits, alpha (α) and beta (β), encoded by adjacent genes *luxA* and *luxB* (respectively) in the *lux* operon (Swartzman et al. 1990,Fisher et al. 1996). The *lux* operon is very well studied in *V. harveyi* and a total of seven *lux* genes have been discovered from the species, *luxCDABEGH* (in that order, Swartzman et al. 1990). It has been established that the disruption on any of the five essential *lux* genes, *luxCDABE*, will abolish luminescence (Engebrecht et al. 1983, Engebrecht and Silverman 1984, Boylan et al. 1985). Both α and β subunits play the crucial role in luminescence. The α subunit contains the active site of the enzyme (Cline and Hastings 1972), whereas the β subunit stabilizes the conformation of the α subunit (Campbell et al. 2009).

The *luxA* and *luxB* genes have been isolated and studied from a number of bioluminescent vibrios and other related species: *V. fischeri*, *V. harveyi*, *V. logei*, *V. orientalis*, *V. splendidus*, *Photobacterium phosphoreum*, *Photobacterium leiognathi*, and *Photorhabdus luminescens* (Hastings and Nealson 1977, Baumann et al. 1980, Meighen 1991, Bassler et al. 1997). PCR primers which were designed to target the *luxA* and *luxB* genes in *V. harveyi* were able to amplify the *luxB* gene (with 96% sequence similarity with the gene of *V. harveyi*) from type strain *V. campbellii* 15631 (Rañoa and Hedreyda 2006), but the *luxA*-targeted primers did not produce a *luxA* gene homologue in *V. campbellii*. In this study, inverse PCR was used to amplify the type strain *V. campbellii* NBRC 15631 *luxA* gene for sequence analysis and comparison with the *luxA* of type strain *V. harveyi* in order to gain more insight into the non-luminescent phenotype of type strain *V. campbellii*.

MATERIALS AND METHODS

Bacterial isolate or strain used in this study

Type strain *Vibrio campbellii* NBRC 15631 was kindly provided by Dr. Ken Suzuki from the National Institute of Technology and Evaluation (NITE) – Biological Resource Center (NBRC, Japan). The *V. campbellii* cultures were routinely grown in nutrient broth (CONDA, Spain) with 1.5% NaCl (Fisher Scientific, United Kingdom), incubated overnight at 30°C at 222 rpm, and in nutrient agar media (CONDA, Spain) with 1.5% NaCl (Fisher Scientific, United Kingdom), incubated overnight at room temperature (25-27°C).

Genomic DNA extraction

Genomic DNA from type strain of *V. campbellii* NBRC 15631 was extracted from an overnight bacterial culture using the DNeasy® Blood and Tissue Kit (QIAGEN GmbH, Germany) following the manufacturer's protocol. The genomic DNA extracts were stored at 4°C until further use.

PCR amplification of the *luxA* gene of *V. campbellii* NBRC 15631 by inverse PCR

Amplification of the *luxA* gene from *V. campbellii* 15631 was performed using inverse PCR. Genomic DNA of type strain *V. campbellii* (10, 50, 100 ng) was subjected to restriction enzyme digestion with *Hind*III (Roche Diagnostics GmbH, Germany), followed by ligation to circularize the *Hind*III fragments using T4 DNA ligase (Roche Diagnostics GmbH, Germany), following the manufacturer's protocol. The circularized DNA fragments were purified via phenol:chloroform:isoamyl alcohol and ethanol precipitation and were used as template for inverse PCR using the primers *VhluxmidR* (5'-ACTCACTGAACAACTGAAAC-3', Rañoa 2006) and *Vc_invluxB* (5'-CGATTGAAGATAAGC-3', designed in this study) that anneal to regions on the adjacent *luxB* gene (Figure 2).

The PCR mix [*V. campbellii* genomic DNA template, 1X PCR buffer, 0.2 mM dNTP, 0.5 µM of each primer, and 0.025 U/µL Titanium *Taq* DNA polymerase (Clontech Laboratories,

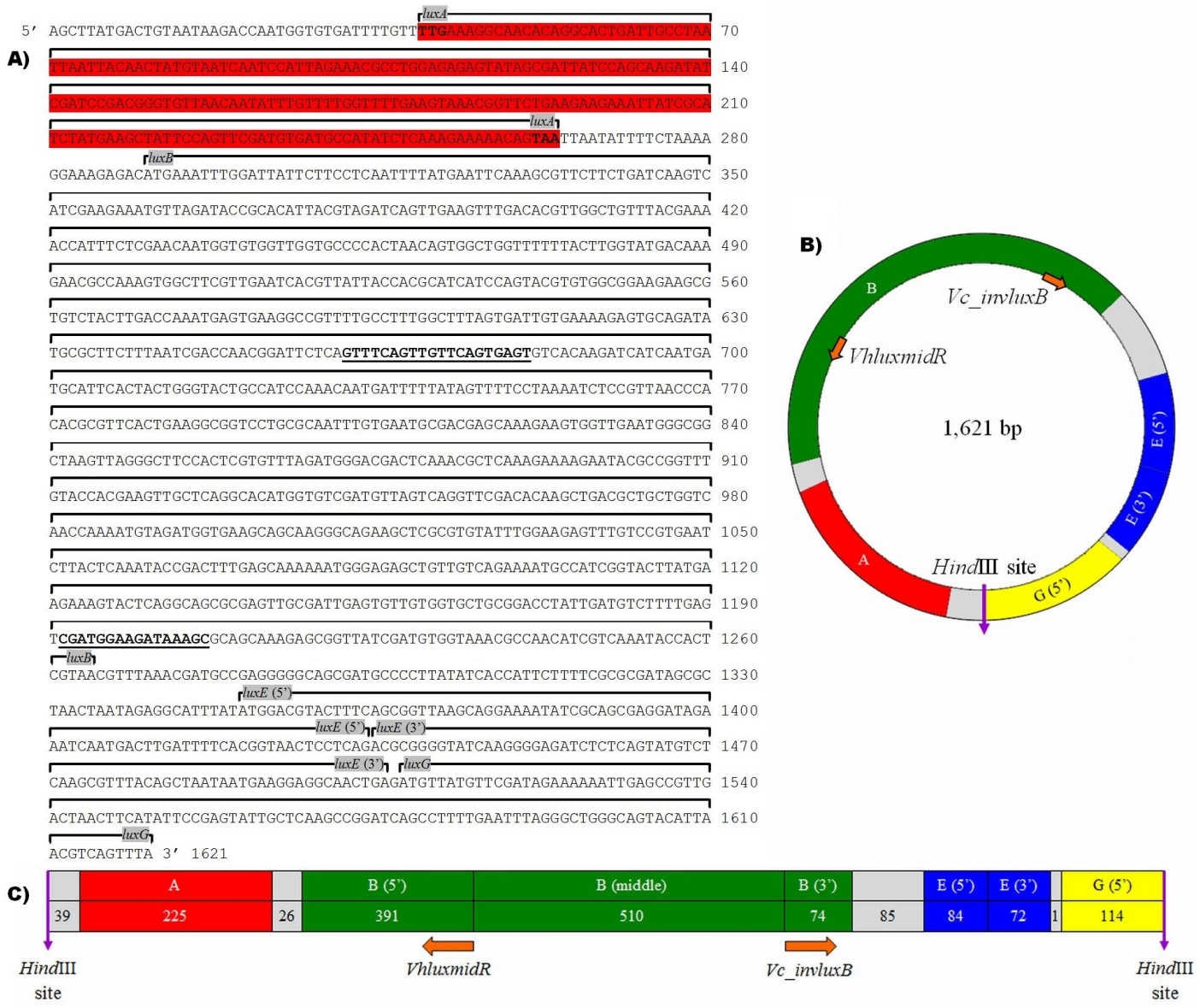

Figure 2. Nucleotide sequence (A) and schematic diagram of the circular (B) and linearized (C) *Hind*III-digested DNA fragment (1,621 bp) containing part of the *lux* operon of type strain *V. campbellii* NBRC 15631. (A) The *luxA* gene is highlighted in red, with the start and stop codons in bold letters, respectively. The underlined and bold regions in *luxB* indicate the respective annealing sites for the *VhluxmidR* and *Vc_invluxB* primers. The different genes are labelled at the start and the end of the gene and were based on BLAST homology search of each region. Unlabelled regions are spacer and undetermined regions. (B and C) The different genes are indicated in different colors: red – *luxA* (full), green – *luxB* (full), blue – 5' and 3' regions of *luxE* (incomplete), yellow – 5' region of *luxG* (partial), gray – spacer and undetermined regions. The sizes (bp) of the different regions are also indicated. The purple arrows indicate ends of the fragment due to the occurrence of *Hind*III restriction sites. The orange arrows indicate regions where primers anneal to the DNA fragment in inverse PCR.

Inc., USA)] was subjected to 30 cycles of the following PCR conditions: denaturation at 94°C for 20 sec, annealing at 50.0°C for 50 sec, and extension at 72°C for 2 min using the Multigene™ Gradient Thermal Cycler (Labnet International, Inc., USA). Initial denaturation and final extension were set at 94°C for 2 min and 72°C for 7 min, respectively. The resulting amplicons were cloned in pCR®4-TOPO® sequencing vector using the TOPO TA Cloning® Kit for Sequencing (Invitrogen Corporation, USA), and the plasmids with inserts were used to transform DH5α™-T1^R chemically competent *E. coli* cells (Invitrogen Corporation, USA), following the manufacturer's protocol. Plasmids containing the amplicons were isolated from transformants using the NucleoSpin® Plasmid Kit (Clontech Laboratories, Inc., USA; Appendix H) and submitted to 1st BASE Pte. Ltd. (Singapore) for sequencing.

Sequence comparison of *luxA* gene and LuxA amino acid sequences

The *luxA* gene homology search was performed using the Basic Local Alignment Search Tool or BLAST (http://www.ncbi.nlm.nih.gov/blast, Altschul et al. 1990). The full-length *luxA* gene sequences of type strains *V. campbellii* NBRC 15631 and *V. harveyi* NBRC 15634 were aligned using MultAlin (http://multalin.toulouse.inra.fr/multalin, Corpet 1988). The amino acid sequence of the *V. campbellii* NBRC 15631 *luxA* gene was deduced using the Expert Protein Analysis System (ExPASY) Translate tool (http://www.expasy.ch/tools/dna.html; Swiss Institute of Bioinformatics, Switzerland), aligned with the LuxA amino acid sequence of *V. harveyi* NBRC 15634 using MultAlin (Corpet 1988), and percent sequence similarity was determined by BLAST (Altschul et al. 1990).

Nucleotide sequence accession number

The nucleotide sequence of the full-length *luxA* gene of type strain *V. campbellii* NBRC 15631 has been assigned the GenBank accession number JF912496 and is available in the database.

RESULTS

Isolation of the putative *luxA* gene homologue of *V. campbellii* NBRC 15631

Inverse PCR produced amplicons of approximately 1,100 bp (Figure 1) which were successfully cloned into pCR®4-TOPO® vector (Invitrogen Corporation, USA). Sequence analysis (Figure 2) revealed that the fragment contained a 225 bp *luxA* sequence, followed by the 5' region of *luxB* of 391 bp (Figure 2). A short stretch of 39 bp was present upstream of the putative *luxA* in *V. campbellii* that did not produce any match in the GenBank database (Benson et al. 2008) by BLAST (Altschul et al. 1990). The 225 bp *luxA* gene from type strain *V. campbellii* NBRC 15631 corresponds to a 74-amino acid (deduced by the ExPASY Translate tool) polypeptide (Figure 5) with a predicted molecular mass of 8,272.37 Da and a pI of 4.09.

Initial homology search by BLAST (Altschul et al.1990) showed highest sequence similarity of 89% with a gene in the *lux* operon of a species of *Vibrio harveyi* BCB494 (Figure 3). Top searches also showed 88% sequence similarity with the *luxA* gene of type strain *V. harveyi* NBRC 15634 (GenBank accession no. DQ436496), *luxA* from some environmental strains of *V. harveyi* (Rañoa and Hedreyda 2006) from the Philippines, and a luminescent *Vibrio* isolate from Southern Thailand identified as *V. campbelli* based on *16S rRNA* gene analysis (Suadee et al. 2007).

Pairwise alignment of the *luxA* gene and LuxA amino acid sequences of *V. campbellii* NBRC 15631 and *V. harveyi* NBRC 15634

The *luxA* gene of *V. campbellii* NBRC 15631 is 225 bp, only about one fifth in length compared to the *luxA* of *V. harveyi*

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU192084.1	Vibrio sp. BCB494 lux operon, complete sequence	289	289	100%	6e-75	89%
CP000790.1	Vibrio harveyi ATCC BAA-1116 chromosome II, complete sequence	289	289	100%	6e-75	89%
X58791.1	V.harveyi luxA and luxB genes for luciferase alpha and beta subunits	283	283	100%	3e-73	89%
EU122288.1	Vibrio harveyi strain ATCC 14126 lux operon, partial sequence	278	278	100%	1e-71	88%
EF394780.1	Vibrio campbellii luciferase subunit A (luxA) and luciferase subunit B (l	278	278	100%	1e-71	88%
DQ436496.1	Vibrio harveyi luciferase alpha subunit (luxA) and luciferase beta subu	278	278	100%	1e-71	88%
HQ259112.1	Synthetic construct Streptomyces coelicolor transposon Tn5068 seq	272	272	100%	6e-70	88%
HM002749.1	Mycobacterial tetracycline-inducible vector pMEND-Lx, complete seq	272	272	100%	6e-70	88%
HM002748.1	Mycobacterial tetracycline-inducible vector pKW08-Lx, complete seq	272	272	100%	6e-70	88%
EU122293.1	Vibrio chagasii strain 21N-12 lux operon, partial sequence	272	272	100%	6e-70	88%
EU122289.1	Vibrio vulnificus strain ATCC 43382 lux operon, partial sequence	272	272	100%	6e-70	88%
EU556495.1	Vibrio orientalis strain ATCC 33934 2-amino-3-ketobutyrate coenzym	272	272	100%	6e-70	88%
AJ297913.2	Plasmid pIPO2T, complete sequence	272	272	100%	6e-70	88%
M10961.1	V.harveyi luciferase alpha and beta subunit (luxA and luxB) genes, co	272	272	100%	6e-70	88%
EU192082.1	Vibrio harveyi BCB440 lux operon, complete sequence	267	267	100%	3e-68	88%
EU192083.1	Vibrio harveyi BCB451 lux operon, complete sequence	261	261	100%	1e-66	87%

Figure 3. Representative result of the BLAST homology search for the 225-bp putative *luxA* gene of type strain *V. campbellii* NBRC 15631.

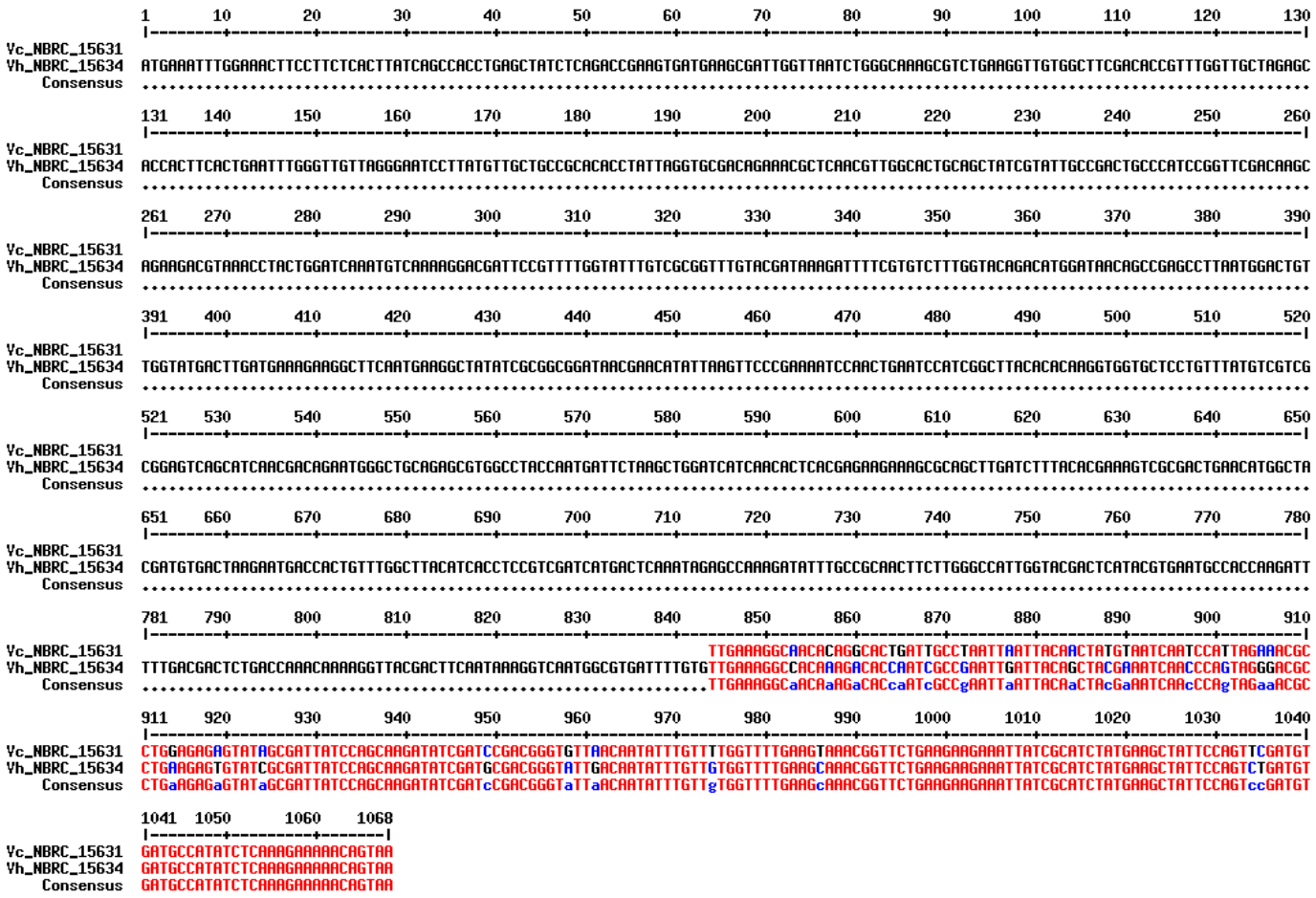

Figure 4. Pairwise sequence alignment using MultAlin of the *luxA* gene sequences of *V. campbellii* NBRC 15631 (from this study) with *V. harveyi* NBRC 15634 *luxA* (Rañoa and Hedreyda 2006). The last row shows the consensus sequence (indicated as "Consensus") where highly conserved regions (≥ 90%) are shown in (red) capital letters, low consensus regions (≥ 50%) in (blue) small letters, and neutral regions in (black) dots (.).

NBRC 15634 which is 1,068 bp (Rañoa and Hedreyda 2006). Pairwise sequence alignment of type strain *V. campbellii* and *V. harveyi luxA* genes (Figure 4) using MultAlin (Corpet 1988) showed that the *luxA* gene of *V. campbellii* is very similar to the 225-bp, 3' end region of the *luxA* gene of *V. harveyi*. This is supported by alignment by BLAST (Altschul et al. 1990), indicating that this region exhibits 88% sequence similarity between the *luxA* of the two species (Figure 3).

The LuxA amino acid sequences of *V. campbellii* NBRC 15631 in this study (deduced by the ExPASY Translate tool) and *V. harveyi* NBRC 15634 (Rañoa and Hedreyda 2006) were aligned using MultAlin (Corpet 1988; Figure 5). As expected, the 74-amino acid LuxA of type strain *V. campbellii* showed similarity with the last 74 amino acids of the 355-amino acid LuxA of *V. harveyi*. Alignment of amino acid sequences by BLAST (Altschul et al. 1990) showed a sequence similarity of 74% in this region between the LuxA of two species (data not shown).

DISCUSSION

Bioluminescence is the ability of organisms to emit light, such as in the luminescent *V. harveyi*, caused by the enzyme luciferase (Lux; Johnston et al. 1986, Fisher et al. 1996). The luciferase enzyme is a heterodimeric protein composed of the alpha (α) and beta (β) subunits, encoded by the adjacent *luxA* and *luxB* genes, respectively, found in the *luxCDABEGH* operon (Swartzman et al. 1990). The ability to produce its own light is one of the major distinctions between *V. harveyi* and its close relative, the non-luminescent type strain *V. campbellii* (Alsina and Blanch 1994a; Alsina and Blanch 1994b).

A study to amplify the *luxA* and *luxB* genes from type strain *V. campbellii* NBRC 15631 (Rañoa and Hedreyda 2006) amplified a *luxB* gene homologue that exhibited 96% sequence

similarity with the *luxB* of type strain *V. harveyi* NBRC 15634, using *luxB* gene-targeted primers based on the *V. harveyi* sequence. PCR using *V. harveyi luxA* gene-targeted primers, however, did not amplify a *luxA* gene homologue in *V. campbellii*. Based on the type strain *V. campbellii luxA* gene sequence obtained in this study, it is now evident that a *luxA* gene homologue was not amplified and isolated in earlier studies because one of the primers used in PCR was based on a sequence not present in the interrupted and much shorter type strain *V. campbellii* 15631 *luxA*. The results of this study revealed that the *luxA* gene could not be amplified from type strain *V. campbellii* in a previous study because a big portion of the 5' region of the *luxA* gene (approximately 850 bp) is absent and the *luxB* gene was amplified because the entire *luxB* gene (Rañoa and Hedreyda 2006) is present.

Sequence determination and analysis revealed that the type strain *V. campbellii* NBRC 15631 *luxA* gene is only 225 bp with the start and stop codons at positions 1-3 and 223-225, respectively (Figure 2). The corresponding polypeptide is 74 amino acids with a calculated molecular weight of approximately 8,200 Da. The initiation codon of the gene is TTG which, based on the standard genetic code, codes for a leucine amino acid (L). In addition to the typical ATG (methionine) start codon, prokaryotes may use alternative start codons GTG (valine) or TTG (leucine) for initiation of translation (Blattner et al. 1997). However, these alternate codons will still be translated as methionine anyway, even if the codon might normally encode a different amino acid, because a separate tRNA is used for initiation of translation (Malys and McCarthy 2010).

BLAST (Altschul et al. 1990) homology search (Figure 3) showed highest sequence similarity of type strain *V. campbellii* with a gene in the *lux* operon of a *Vibrio* species, strain BCB494 (89%). Strain BCB494 is an environmental isolate from Boca Ciega Bay, Florida, USA and is closely related to *V. harveyi* based on the *16S rRNA* and *luxCDABEGH* genes but identified as *V. cincinnatiensis* based on *recA* (O'Grady and Wimpee 2008). BLAST homology search also showed 88% *luxA* gene sequence similarity with an isolate identified as *V. campbellii* (Suadee et al. 2007). The bacterium was isolated from a shore in Southern Thailand and the identity was determined by *16S rRNA* gene analysis (Suadee et al. 2007). This *Vibrio* isolate possess the full length *luxA* like the type strain *V. harveyi* and is therefore not a type strain *V. campbellii*. Further analysis of additional genes from this isolate could be performed to determine if this luminescent bacterium is indeed a luminescent strain of *V. campbellii* and not just a variant of *V. harveyi* that contains both the ornithine decarboxylase and *luxAB* genes. Use of *16s rRNA* gene sequence analysis in identifying *V. harveyi* and *V. campbellii* has been reported to be unreliable at times in distinguishing these two closely related species (Orata and Hedreyda 2011, Kita-Tsukamoto et al. 1993, Cortado et al. 2005) because the gene is at least 97% similar for these two species.

The *V. harveyi luxA* is 1,068 bp, whereas the *V. campbellii luxA* is about 225 bp, only about one-fifth in length as the former. Pairwise alignment of sequences from these two species by MultAlin (Corpet 1988) revealed that the *V. campbellii luxA* is similar to the 3' end region (last 225 bp) of *V. harveyi luxA* (Figure 4). Consequently, alignment of the 74-amino acid LuxA protein sequence of *V. campbellii* with the 355-amino acid LuxA of *V. harveyi* revealed similar results (Figure 5) with 74% sequence similarity. This findings indicate that *luxA* in type strain *V. campbellii* may not be able to produce a complete α subunit of the luciferase enzyme needed to provide the functional active site of the luciferase enzyme, resulting in the inability of the type strain *V. campbellii* to exhibit luminescence.

Because of the significant difference in the *luxA* gene, molecular detection and identification of type strains *V. harveyi* and *V. campbellii* could target these divergent regions. Another reliable molecular detection method could be developed based

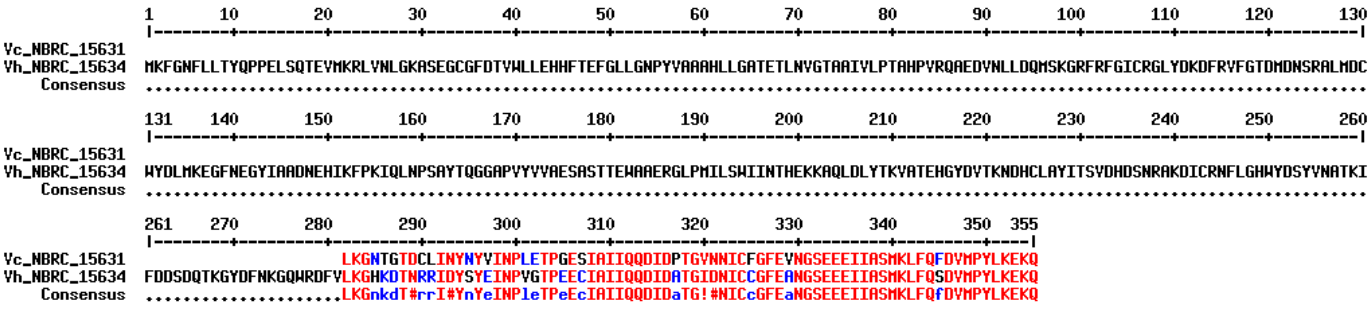

Figure 5. Pairwise sequence alignment using MultAlin of the LuxA amino acid sequences of *V. campbellii* NBRC 15631 (from this study) and *V. harveyi* NBRC 15634 (Rañoa and Hedreyda, 2006). The last row shows the consensus sequence (indicated as "Consensus") where highly conserved regions (≥ 90%) are shown in (red) capital letters or other symbols (#, !), low consensus regions (≥ 50%) in (blue) small letters, and neutral regions in (black) dots (.). The exclamation mark (!) denotes any one of IV amino acids and hash (#) denotes any one of NDQE amino acids.

on the genes in the *lux* operon. The 225 bp region of *luxA* present in both type strains *V. campbellii* and *V. harveyi* exhibit only 88% sequence similarity and could also serve as a distinguishing gene marker.

There is a case where some *Vibrio* isolates are referred to as "natural dark mutants." These natural mutants contain the full set of *lux* genes but contain lesions in them, either an insertion sequence, point mutations, and deletions (O'Grady and Wimpee 2008), causing them to become non-luminescent. Visibly non-luminescent dark mutants of *V. cholerae* and *V. fischeri*, two of the known luminescent vibrios, have been examined to have such mutations (O'Grady and Wimpee 2008). The type strain of *V. campbellii* that is non-luminescent contains an incomplete *luxA* gene. It is possible, however, that non-luminescent strains of *V. campbellii* and *V. harveyi* exist, containing the full set of *luxAB* genes but with other mutations within the *lux* operon. The *luxA* gene could be used to differentiate type strains of the two species but if absence of luminescence is a result of other mutations, multi locus gene sequence analysis should be used to identify variants of both species.

CONCLUSION

The full-length sequence of the *luxA* gene of type strain *V. campbellii* NBRC 15631 was amplified and isolated by inverse PCR using circularized *Hind*III restriction fragments of type strain *V. campbellii* genomic DNA as template and *luxB*-targeted primers. The primers were designed to amplify regions flanking the *luxB* gene which include *luxA* that is upstream of *luxB*. The *luxA* gene sequence is 225 bp, corresponding to a 74-amino acid polypeptide. The presence of a much shorter *luxA* gene explains why the gene could not be amplified in previous studies using primers designed based on the full length 1,068 bp *luxA* of type strain *V. harveyi* 15634. The much shorter gene and the 1,068 bp *luxA* of type strain *V. harveyi* 15634 exhibit only 88% sequence similarity in the 3' end region of *V. harveyi luxA*. Pairwise alignment of the corresponding amino acid sequences revealed a 74% sequence similarity. The presence of a much shorter *luxA* gene and, consequently, an incomplete α subunit of the luciferase enzyme in type strain *V. campbellii,* plus the significant sequence variation of the gene from the *luxA* of luminescent *V. harveyi,* could have resulted in a non-functional enzyme. The α subunit contains the active site of the enzyme and a much smaller polypeptide is expected to be unable to attain the proper structure to retain the activity of the enzyme. The availability of the *luxA* gene sequence provides an additional gene marker to distinguish the type strains of the closely related *V. harveyi* and *V. campbellii*. It would be interesting to determine the *luxAB* gene sequences of environmental strains of both species to relate the gene sequences with their bioluminescence because it is possible that non-type strains of both species may or may not exhibit bioluminescence depending on the mutation in genes within the *lux* operon. Results of this study provide the molecular basis for the inability of the type strain *V. campbellii* to exhibit luminescence, a characteristic that has been widely used to describe the type strain of the species.

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NO CONFLICT OF INTEREST STATEMENT

There is no conflict of interest among authors, institutions, and individuals mentioned above in the conduct of this study and the preparation and submission of this manuscript.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Mr. Fabini D. Orata conducted the experiments under the guidance of Dr. Cynthia T. Hedreyda. Both worked together to write and revise the manuscript.

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