Isolation and sequence analysis of the full-length *toxR* gene of type strain *Vibrio campbellii* and use of the *toxR* gene sequence to evaluate variation and relatedness with other *Vibrio* species

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Availability of complete sequences of the *toxR* gene (which are universally present yet exhibit significant variations) from type strains of different vibrios will be valuable in evaluating phylogenetic relatedness as well as developing a rapid species-specific identification and differential detection protocols for unknown vibrios. This study focused on the isolation and determination of the complete sequence of the *toxR* gene from type strain *Vibrio campbellii* NBRC 15631. Also, the effectiveness of *toxR* in the evaluation of variation and relatedness among *Vibrio* and *Photobacterium* species is compared with the *16S rRNA* gene. Using a combination of *toxR*- and *toxS*-targeted PCR primers, the full-length *toxR* gene homologue from type strain *V. campbellii* NBRC 15631 was amplified and amplicons were subjected to sequencing. Nucleotide sequence analysis of the full-length 873-bp *toxR* revealed highest sequence similarity with the previously sequenced partial *toxR* of *V. campbellii* CAIM 519 (100%) and exhibited lower sequence similarity with *toxR* from *V. harveyi* (79%), *V. parahaemolyticus* (75%), *V. anguillarum* (64%), *V. vulnificus* (63%), *V. cholerae* (59%), *V. fischeri* (53%), *V. hollisae* (50%), and *Photobacterium profundum* (26%). The gene encodes for a 290-amino acid polypeptide, and multiple alignment of the *V. campbellii* ToxR with reported full-length sequences from other vibrios in the database revealed sequence similarity with *V. harveyi* (85%) and other vibrios (38-76%), but a fairly conserved transcription activation (62-98% between *V. campbellii* and other *Vibrio* and *Photobacterium* species) and transmembrane and periplasmic domains (30-85% between *V. campbellii* and other species) flanking a highly divergent membrane "tether" region (6-62% between *V. campbellii* and other species) is observed. The use of the *toxR* and *16S rRNA* genes to evaluate the relatedness among *Vibrio* and *Photobacterium* species were compared. The phylogenetic tree based on *toxR* sequence variation showed that the different vibrios formed separate clusters, including the closely related *V. campbellii* and *V. harveyi*, illustrating that *V. campbellii* and *V. harveyi* are indeed distinct species. In contrast, the phylogenetic

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tree based on the *16S rRNA* gene is less specific as indicated by lower bootstrap values. Because of greater sequence variation among species, the use of the *toxR* gene becomes more effective than the *16S rRNA* gene in distinguishing different species of *Vibrio*. The divergent region in *toxR*, the membrane "tether" region, could be targeted in designing *V. campbellii*-specific and other species-specific *toxR*-targeted PCR primer pairs for the rapid differential detection of closely related vibrios.

KEYWORDS

Vibrio, *Vibrio campbellii*, *Vibrio harveyi*, *toxR*

INTRODUCTION

Vibrio campbellii is a Gram-negative, non-luminescent marine bacterium that has recently been identified as an important species involved in shrimp disease through experimental infection of black tiger shrimp, *Penaeus monodon* (de la Peña et al. 2001), the main penaeid species cultured in the Philippines. *V. campbellii* is very closely related to its sister species, *V. harveyi* (Pedersen et al. 1998), the species 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), that identification between the two became an issue. Both *V. campbellii* and *V. harveyi* cannot be distinguished in more than 100 phenotypic characters (Gomez-Gil et al. 2004). Also, using genomic fingerprinting and DNA-DNA hybridization techniques, 39 previously identified strains of *V. harveyi* were in fact *V. campbellii* (Gomez-Gil et al. 2004). Eventually, *V. campbellii* emerged as an equally important pathogen of reared aquatic organisms which is the reason why interest in studying and detecting *V. campbellii* was initiated a few years back (San Luis and Hedreyda 2005; San Luis and Hedreyda 2006).

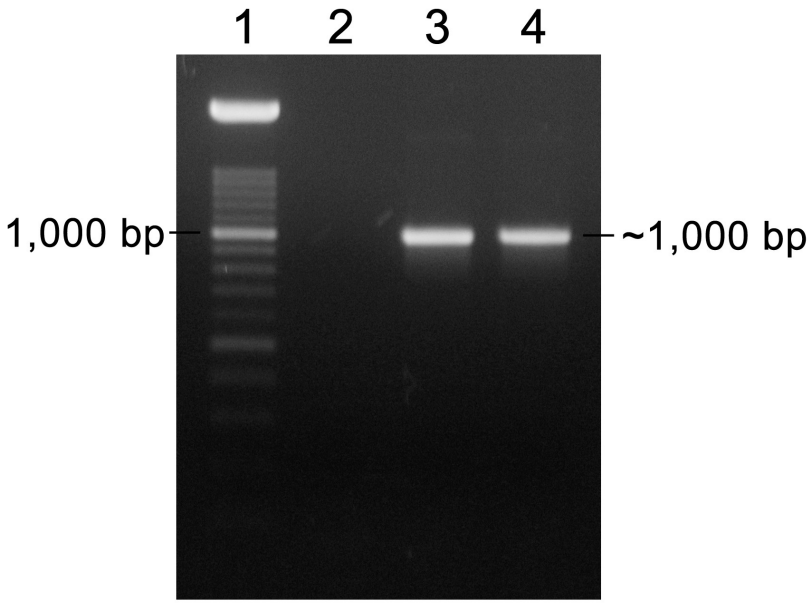

Figure 1. PCR amplification of the putative *toxR* gene in type strain *V. campbellii* NBRC 15631. Lane 2, no-template negative control; lanes 3 and 4, amplicons of PCR using the *toxRPV* and *Vib_toxS-R* primer pair; lane 1, 100 bp ladder (Roche Diagnostics GmbH, Germany).

The gold standard in the identification and taxonomic differentiation of closely related members of the *Vibrionaceae* family is with the use of the highly conserved *16S rRNA* gene (Dorsch et al. 1992; Cortado et al. 2005). However, because of high sequence similarity among taxa, comparison using the *16S rRNA* gene could not distinguish the closely related vibrios (Kita-Tsukamoto et al. 1993; Cortado et al. 2005). Therefore, other gene markers present in *V. campbellii* as well as other vibrios were exploited for a more accurate molecular detection and identification. One of the earlier studies to detect *V. campbellii* at the molecular level involves isolating the full-length sequence of the *V. campbellii* hemolysin gene (*vch*; San Luis and Hedreyda 2006), which codes for the virulence factor of the same name that causes the lysis of erythrocytes and other cells and competes with the iron acquisition system with the host (Zhang and Austin 2005). The use of hemolysin gene-targeted primers for species detection was also performed in the same study (San Luis and Hedreyda 2006).

This study was focused on the isolation and sequencing of the *V. campbellii toxR* gene, another gene implicated in virulence, that codes for the transmembrane protein ToxR that was first identified to function in the coordinated regulation of virulence gene expression in *V. cholerae* (Miller et al. 1987; Miller and Mekalanos 1988; DiRita 1992). The gene product of *toxR* regulates the transcription of toxin, porin, and pilus genes in *V. cholerae* (Miller and Mekalanos 1988; DiRita 1992; Higgins and DiRita 1996). Aside from *V. cholerae*, the *toxR* gene has been isolated in *V. parahaemolyticus* (Lin et al. 1993), *V. fischeri* (Reich and Schoolnik 1994), *V. vulnificus* (Lee et al. 2000), *V. alginolyticus*, *V. fluvialis*, *V. mimicus* (Osorio and Klose 2000), *V. hollisae* (Vuddhakul et al. 2000), *V. anguillarum* (Okuda et al. 2001), and *V. harveyi* (Franco and Hedreyda 2006).

Only a partial sequence of the *V. campbellii toxR* gene has been obtained in earlier studies (San Luis and Hedreyda 2005) and *toxR*-targeted PCR primers for *V. campbellii* detection was designed based on the partial *toxR* sequence (Castroverde et al. 2006). The availability of the complete *toxR* gene sequence for type strains of vibrios, like *V. campbellii,* will allow the use of the *toxR* gene sequence analysis for a more accurate species identification of unknown *Vibrio* isolates. The availability of complete *toxR* sequences for type strains of different *Vibrio* species is required

to be able to identify variable regions and species-specific sequences for designing species-specific PCR detection primers. Moreover, additional complete *toxR* sequences of vibrios contribute data that could be used for phylogenetic analysis of known and unknown *Vibrio* isolates. In this study, therefore, the complete sequence of the *V. campbellii toxR* gene was obtained and used to evaluate variation and relatedness of *V. campbellii* with other vibrios.

MATERIALS AND METHODS

Bacterial strain used in this study

Type strain *Vibrio campbellii* NBRC 15631 was kindly provided by Dr. Ken Suzuki from the National Institute of

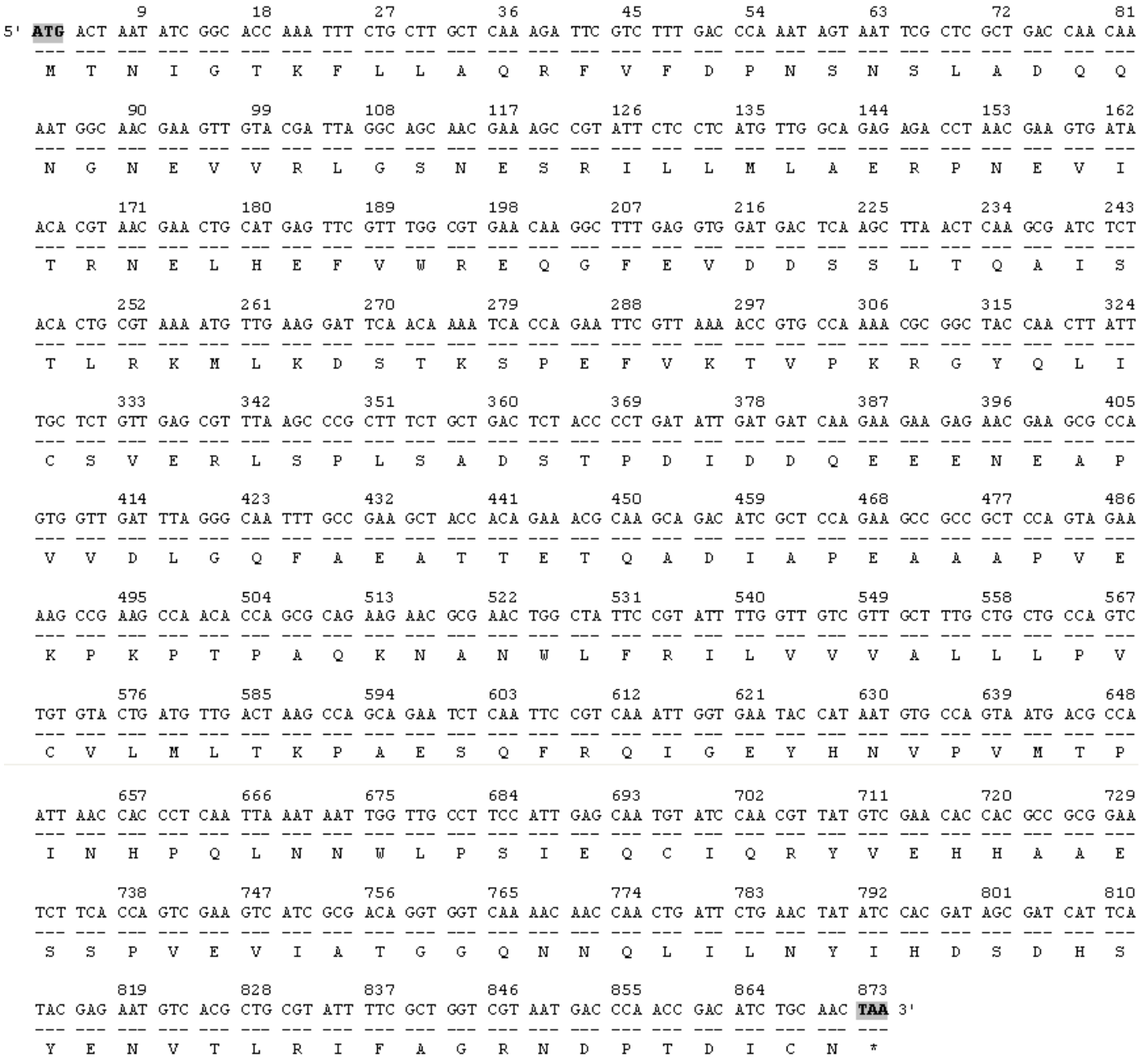

Figure 2. Nucleotide and deduced amino acid sequences of the full-length *toxR* gene of type strain *V. campbellii* NBRC 15631. The codons in bold text and shaded indicate the start and stop codons, respectively.

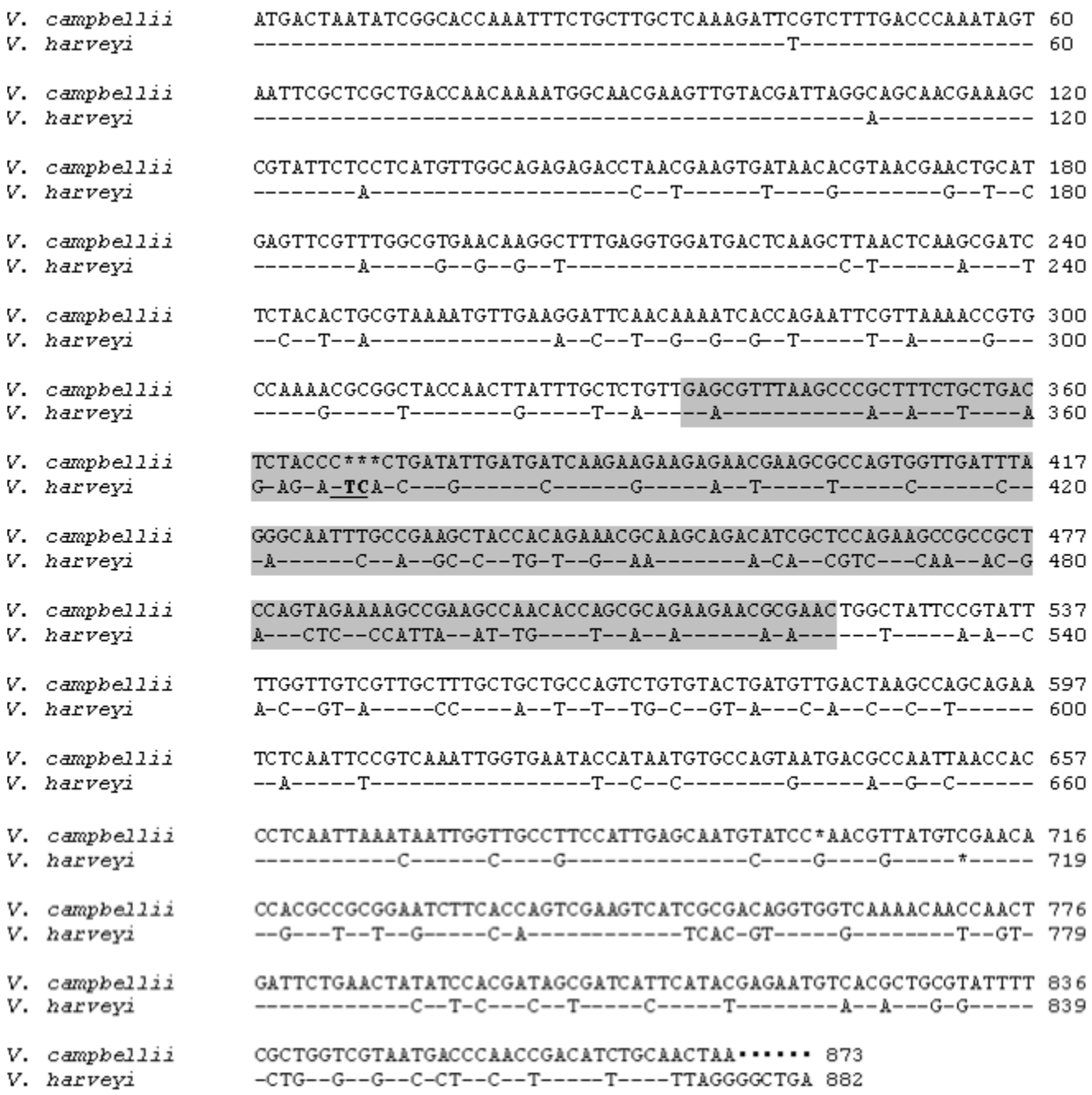

Figure 3. Pairwise alignment of *V. campbellii* NBRC 15631 and *V. harveyi* NBRC 15634 *toxR* gene using ClustalW2. Identical nucleotides are indicated by gaps (–). The shaded nucleotides code for the membrane "tether" region. The codon in bold text and underlined corresponds to a leucine amino acid (CTC). Astersisks (*) refer to deleted nucleotides in *V. campbellii toxR*. Black dots (•) indicate the end of the sequence of the *V. harveyi toxR* gene. Numbers on the right refer to the position of the last nucleotide on each line. GenBank accession numbers for the full-length *toxR* sequences are given in the text.

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 at room temperature (25-27°C).

Genomic DNA extraction

Type strain *V. campbellii* NBRC 15631 genomic DNA 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 extract was stored at 4°C until further use.

PCR amplification of the full-length *toxR* gene of type strain *V. campbellii*

The forward primer *toxRPV* (5'-ATGACTAATA-TCGGCAC-3'), that was previously designed to target the *toxR* gene in type strain *V. harveyi* NBRC 15634 (Franco and Hedreyda 2006), was paired with a degenerate reverse primer *Vib_toxS-R* (5'-GCCATTCTTTAGAGGTCARNAVYTGYT-C-3'; where R = A or G; N = A, T, C or G; V = A, C or G; Y = C or T). The degenerate reverse primer was designed in this study to target a region in *toxS*, which lies downstream of *toxR* in the *toxRS* operon (DiRita and Mekalanos 1991), based on *toxS* of other *Vibrio* species: *V. parahaemolyticus* (GenBank no. AAA27577), *V. fischeri* (GenBank no. AAA20503), *V. harveyi* (GenBank no. ABW96215.1), *V. cholerae* (GenBank no. AAA63559), and *V. vulnificus* (GenBank no. AAD53267) available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank; Benson et al. 2008). The Consensus-Degenerate Hybrid Oligonucleotide Primer (CODEHOP) program (http://bioinformatics.weizmann.ac.il/blocks/codehop.html; Rose et al. 1998) was used to design the degenerate reverse primer following the designer's protocol. The primer pair was synthesized by AITBiotech Pte. Ltd. (Singapore). The expected product in PCR is approximately 1,000 bp.

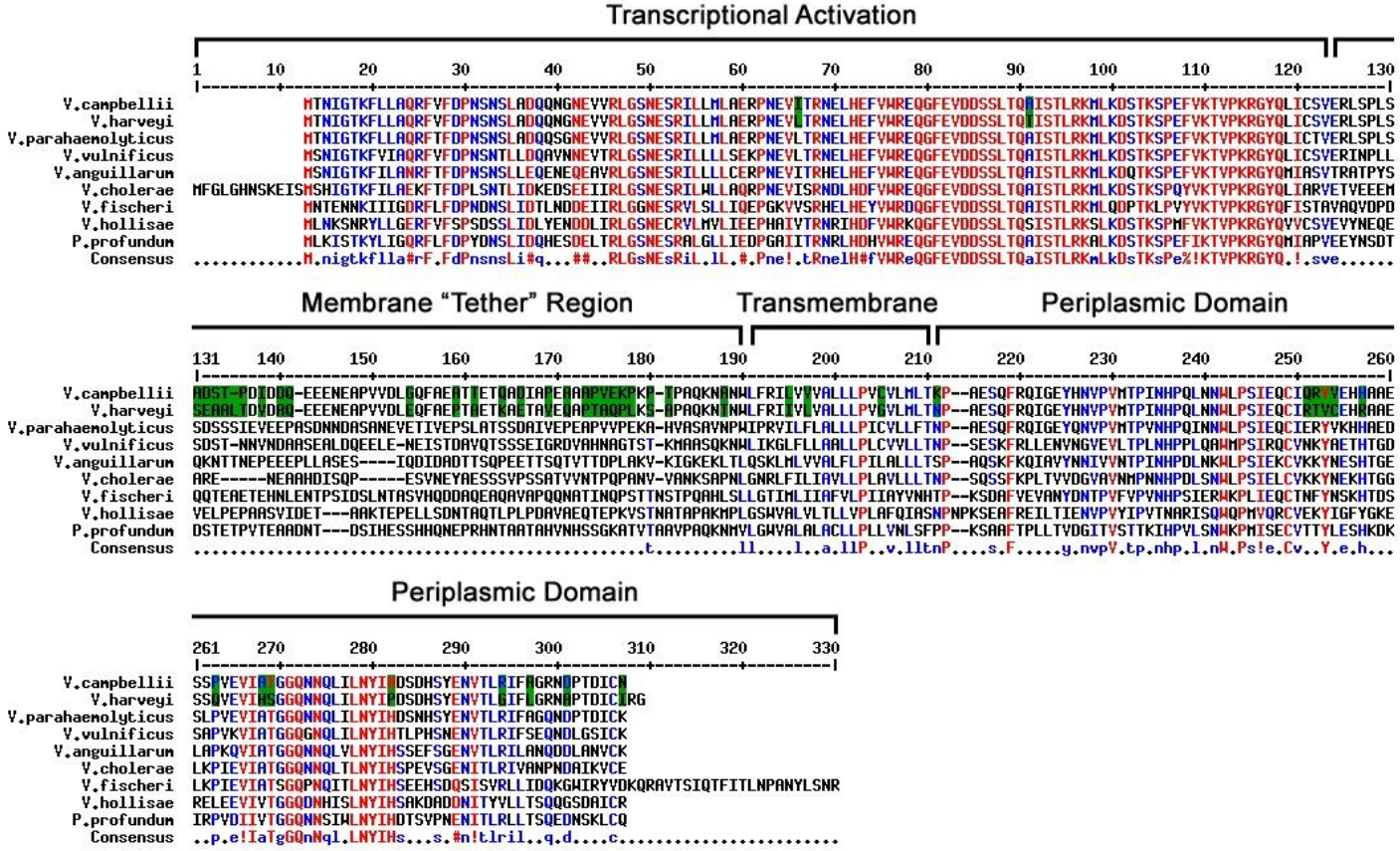

Figure 4. Multiple sequence alignment using MultAlin of the deduced *V. campbellii* NBRC 15631 ToxR amino acid sequence with sequences from other *Vibrio* and *Photobacterium* species. The last row shows the consensus sequence (indicated as "Consensus") where highly conserved regions (≥90%) are shown in red, low consensus regions (≥50%) in blue, and neutral regions in black. The exclamation mark (!) denotes any one of IV, percent symbol (%) denotes any one of FY, and hash (#) denotes any one of NDQEBZ. Amino acid residue differences between *V. campbellii* and *V. harveyi* are highlighted in green. GenBank accession numbers for the full-length *toxR* sequences are given in the text.

The PCR mix [*V. campbellii* genomic DNA template, 1X PCR buffer, 0.8 mmol l^{-1} dNTP, 0.5 µmol l^{-1} of each primer, and 0.025 U μl^{-1} *Taq* DNA polymerase (Roche Diagnostics GmbH, Germany)] was subjected to 30 cycles of the following PCR conditions: denaturation at 94°C for 1 min, annealing at 51.4°C for 1 min, and extension at 72°C for 1 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 PCR products, together with the 100 bp ladder (Roche Diagnostics GmbH, Germany), were run at 100 V in 1.2% agarose gel in 0.5X TAE and stained with ethidium bromide. The gel was viewed with an AlphaDigiDoc Pro UV transilluminator (Alpha Innotech, USA).

Purification and direct sequencing of PCR products

The PCR product containing the putative *toxR* gene from type strain *V. campbellii* was purified using the Nucleospin® Nucleic Acid Purification Kit (Clontech Laboratories, Inc., USA) following the manufacturer's protocol. DNA sequencing was performed by 1st BASE Pte. Ltd. (Singapore) using the 3730XL Genetic Analyzer together with the BigDye® Terminator v3.1 Cycle Sequencing Kit (both from Applied Biosystems, USA) and *toxRPV* and *Vib_toxS-R* as sequencing primers (from this study) following the manufacturers' protocols.

Sequence comparison and phylogenetic analysis

The *toxR* 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 *toxR* and *16S rRNA* sequences of *V. campbellii* and other *Vibrio* and *Photobacterium* species were aligned using ClustalW2, version 2.0 (http://www.ebi.ac.uk/Tools/clustalw2; Larkin et al. 2007). The amino acid sequence of the *V. campbellii toxR* was deduced using the Expert Protein Analysis System (ExPASY) Translate tool (http://www.expasy.ch/ tools/dna.html; Swiss Institute of Bioinformatics, Switzerland) and aligned with ToxR amino acid sequences from other *Vibrio* and *Photobacterium* species using MultAlin (Corpet 1988) and ClustalW2 (Larkin et al. 2007). Phylogenetic trees were constructed based on *toxR* and *16S rRNA* gene sequences of *V. campbellii* and other *Vibrio* and *Photobacterium* species using the Molecular Evolutionary Genetics Analysis (MEGA), version 4.0 software (Tamura et al. 2007). The construction of phylogenetic trees employed the neighbour-joining (NJ), p-distance method with the reliability of topologies assessed by the bootstrap method with 10,000 replicates.

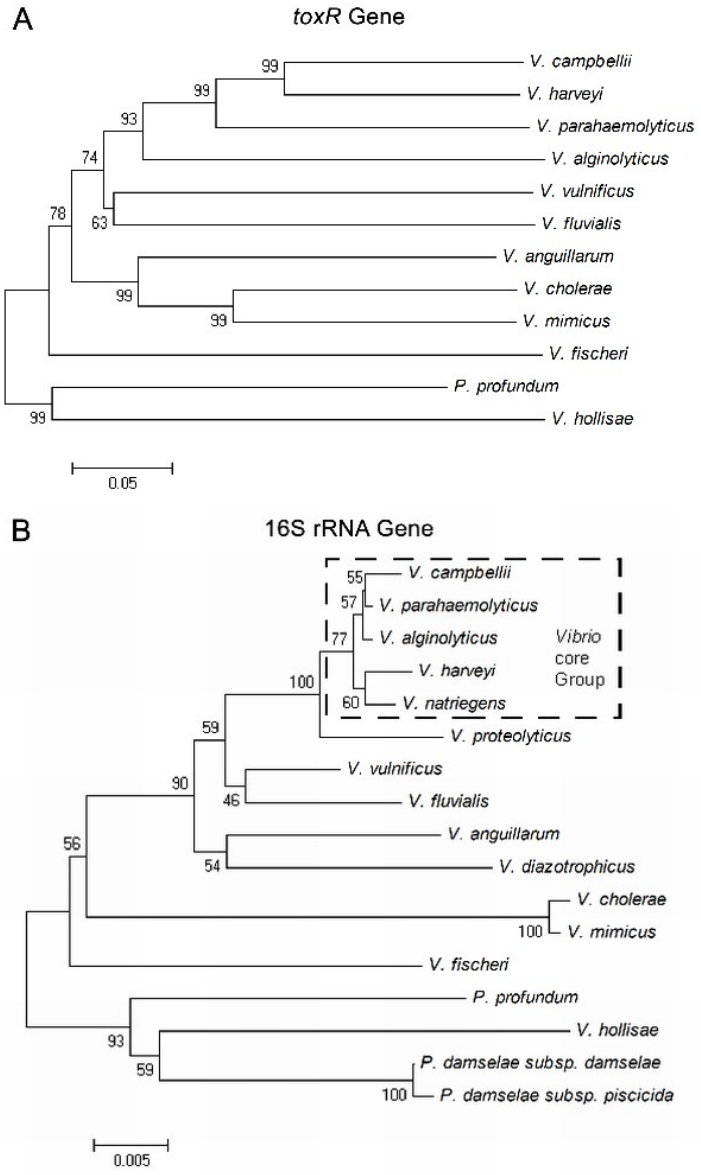

Figure 5 Unrooted neighbor-joining tree constructed by MEGA 4.0 showing the phylogenetic interrelationship of different species of *Vibrio* and *Photobacterium* based on their *toxR* (A) and *16S rRNA* (B) gene sequences. Percent bootstrap values (from 10,000 replicates generated) are given at the branching points. The bars (⊢⊣) indicate genetic distance. GenBank accession numbers for the additional *toxR* genes: *V. alginolyticus*, AF170882; *V. fluvialis*, AF170885; *V. mimicus*, AF170881. GenBank accession numbers for the *16S rRNA* genes: *V. campbellii*, X56575; *V. parahaemolyticus*, X74720; *V. alginolyticus*, X74690; *V. harveyi*, X56578; *V. natriegens*, X56581; *V. proteolyticus*, X56579; *V. vulnifcus*, X76334; *V. fluvialis*, X7 6335; *V. anguillarum*, X16895; *V. diazotrophicus*, X56577; *V. cholerae*, X76337; *V. mimicus*, X74713; *V. fischeri*, X74702; *P. profundum*, D21226; *V. hollisae*, X74707; *P. damselae* subsp. *damselae*, X74700; *P. damselae* subsp. *piscicida*, Y18496.

Table 1. Representative result of the BLAST homology search for the 873-bp putative *toxR* gene of type strain *V. campbellii* NBRC 15631.

Species Name	Strain	GenBank Accession Number	Percent Identity with reported *V. campbellii toxR*
V. campbellii	CAIM 519	AY946038	100%
V. parahaemolyticus	ATCC 17802	AY527396	88%
V. parahaemolyticus	OYVP2	DQ092577	87%
V. alginolyticus	a10(071022)	AB372532	87%
V. alginolyticus	a20-6-23	AB372526	86%
V. harveyi	CAIM 520	DQ517446	81%
V. harveyi	CAIM 512	DQ503438	80%
V. harveyi	NBRC 15634	DQ403146	80%
V. anguillarum	PT087050	AB042547	78%

Nucleotide sequence accession number

The nucleotide sequence of the full-length *toxR* gene of type strain *V. campbellii* NBRC 15631 has been assigned the GenBank accession number HQ318823 and is available in the database.

RESULTS

Isolation of the full-length putative *toxR* gene of type strain *V. campbellii* by PCR

PCR amplification of the putative *toxR* gene of type strain *V. campbellii* using the forward primer (*toxRPV*) and a degenerate reverse primer (*Vib_toxS-R*) generated the expected amplicon of approximately 1,000 bp (Figure 1). The forward primer, *toxRPV*, was designed to target base pairs 1-17 of the *toxR* gene in type strain *V. harveyi* NBRC 15631 (Franco and Hedreyda 2006), while the reverse primer, *Vib_toxS-R*, targeted a region of *toxS*, about 100 bp downstream of *toxR*, in the *toxRS* operon (DiRita and Mekalanos 1991).

Sequencing the putative *toxR* gene amplicon from type strain *V. campbellii* NBRC 15631 revealed the 873-bp gene which corresponds to a 290-amino acid polypeptide (Figure 2) with a predicted molecular mass of 32.53 kDa and a pI of 4.83. Initial analysis using BLAST (Altschul et al. 1990) homology search (Table 1) showed that the putative *V. campbellii toxR* gene displayed 100% sequence similarity with the previously reported partial *toxR* gene of another *V. campbellii* type strain, CAIM 519 (GenBank no. AY946038). In addition, the *V. campbellii* NBRC 15631 *toxR* gene showed lower sequence similarity (Table 1) with the *toxR* gene of multiple strains of *V. parahaemolyticus* (87-88%), multiple strains of *V. alginolyticus* (86-87%), multiple strains of *V. harveyi* (80-81%) and a *V. anguillarum* strain (78%).

Multiple sequence alignment of the *toxR* gene and ToxR amino acid sequences

Multiple nucleic acid sequence alignment using ClustalW2 (Larkin et al. 2007; Table 2) of the *V. campbellii toxR* gene from this study (GenBank no. HQ318823) with other *toxR* gene sequences from other *Vibrio* and *Photobacterium* species available in the GenBank database, revealed highest percent sequence similarity with *V. harveyi* NBRC 15634 (GenBank no. DQ403146) with 79%, followed by *V. parahaemolyticus* (GenBank no. L11929), *V. anguillarum* (GenBank no. AB042547), *V. vulnificus* (GenBank no. AF166120), *V. cholerae* (GenBank no. M21249), *V. fischeri* (GenBank no. L29053), *V. hollisae* (GenBank no. AB027503), and *P. profundum* (GenBank no. U77060) with 75%, 64%, 63%, 59%, 53%, 50%, and 26%, respectively (Table 2). Close comparison of the *toxR* gene of *V. campbellii* NBRC 15631 with its close relative, *V. harveyi* NBRC 15634, showed that they share only 79% sequence similarity with 191 nucleotide substitutions out of 882 bases, including a deletion of three bases at position 368 in *V. campbellii toxR* corresponding to a leucine amino acid (CTC) residue in *V. harveyi* ToxR (Figure 3).

Multiple ToxR amino acid sequence alignment using MultAlin (Corpet 1988; Figure 4) and ClustalW2 (Larkin et al. 2007; data not shown) of type strain *V. campbellii* (deduced by ExPASY Translate tool in this study) with ToxR of other *Vibrio* species obtained from the GenBank database revealed highest percent sequence similarity with *V. harveyi* (85%), followed by *V. parahaemolyticus* (76%), *V. vulnificus* (61%), *V. anguillarum* (59%), *V. cholerae* (52%), *P. profundum* (43%), *V. fischeri* (40%), and *V. hollisae* (38%). In addition, alignment of individual regions of ToxR (alignment data not shown) revealed relatively conserved transcriptional activation (62-98% between *V. campbellii* and other *Vibrio* and *Photobacterium* species), transmembrane and periplasmic (30-85% between *V. campbellii* and other species) flanking a highly divergent membrane "tether" region (6-62% between *V. campbellii* and other species), consistent with the findings of Osorio and Klose (2000). The divergence of ToxR in the membrane "tether" region is also present between *V. campbellii* and the closest relative *V. harveyi* (Figure 4). It is in this region where most of the amino acid residue differences in ToxR between the two species are found (Figures 3 and 4). These results indicate that *toxR* gene sequence analysis could easily differentiate *V. campbellii* from other related vibrios.

Phylogenetic analysis based on the *toxR* and *16S rRNA* gene sequences

Two phylogenetic trees were constructed with the MEGA 4.0 software (Tamura et al. 2007) using the neighbor-joining (NJ), p-distance method. The first one is based on the *toxR* gene sequences (Figure 5A), while the second one is based on the *16S rRNA* gene sequences (Figure 5B) of different *Vibrio* and *Photobacterium* species, including the *toxR* gene sequence of *V. campbellii* from this study. The additional *toxR* and all of the *16S rRNA* gene sequences used were obtained from the GenBank database. The phylogenetic tree based on the *toxR*

gene sequences (Fig. 5A) showed *V. harveyi* and *V. campbellii* in a separate cluster, supported by a bootstrap value of 99%. The same goes with most of the other *Vibrio* species forming more distinct, separate clusters represented by relatively higher boostrap values (63-99%) than in the *16S rRNA* phylogenetic tree. The phylogenetic tree based on the *16S rRNA* gene sequences (Fig. 5B) clustered all *Vibrio* species belonging to the *Vibrio* core group (Thompson and Swings, 2006) in one group (boxed in Fig. 5B) and showed relatively lower bootstrap values (55-77%) as compared to the *toxR* phylogenetic tree.

DISCUSSION

Partial to complete *toxR* gene sequences from different *Vibrio* species, including *V. cholerae* (Miller et al. 1987; Miller and Mekalanos 1988; DiRita 1992), *V. parahaemolyticus* (Lin et al. 1993), *V. fischeri* (Reich and Schoolnik 1994), *V. vulnificus* (Lee et al. 2000), *V. alginolyticus*, *V. fluvialis*, *V. mimicus* (Osorio and Klose 2000), *V. hollisae* (Vuddhakul et al. 2000), *V. anguillarum* (Okuda et al. 2001), and *V. harveyi* (Franco and Hedreyda 2006), have been reported. This study reports another complete *toxR* gene sequence for an additional *Vibrio* species, type strain *Vibrio campbellii* NBRC 15631. The presence of the *toxR* gene from all these *Vibrio* species is consistent with the observation that *toxR* appears to be an ancestral gene of the family *Vibrionaceae*.

Table 2. Comparison of the *toxR* gene sequences from different *Vibrio* and *Photobacterium* species, including the *V. campbellii* NBRC 15631 *toxR* gene obtained in this study, by multiple sequence alignment using ClustalW2. GenBank accession numbers for the full-length *toxR* sequences are given in the text.

	Percent Sequence Similarity								
***Vibrio* Species**	*V. campbellii*	*V. harveyi*	*V. parahaemolyticus*	*V. anguillarum*	*V. vulnificus*	*V. cholerae*	*V. fischeri*	*V. hollisae*	*P. profundum*
V. campbellii		79%	75%	64%	63%	59%	53%	50%	26%
V. harveyi	79%		73%	60%	62%	57%	52%	22%	25%
V. parahaemolyticus	75%	73%		64%	63%	55%	23%	18%	24%
V. anguillarum	64%	60%	64%		62%	63%	58%	22%	26%
V. vulnificus	63%	62%	63%	62%		59%	24%	22%	26%
V. cholerae	59%	57%	55%	63%	59%		23%	22%	57%
V. fischeri	53%	52%	23%	58%	24%	23%		24%	24%
V. hollisae	50%	22%	18%	22%	22%	22%	24%		26%
P. profundum	26%	25%	24%	26%	26%	57%	24%	26%	

In this study, isolation and amplification of the complete *toxR* gene of *V. campbellii* was achieved using a pair of PCR primers targeting the *toxR* gene observed in other *Vibrio* species. The design and selection of primers were based on the hypothesis that certain regions of the *toxR* gene may be conserved among species. The forward primer, *toxRPV*, was designed to target the 5' region of the *toxR* gene in *V. harveyi* (Franco and Hedreyda 2006). Since *V. harveyi* is known to be a very close relative of *V. campbelii* based on various biochemical and molecular identification methods (Alsina and Blanch 1994a; Alsina and Blanch 1994b; de la Peña et al. 2001; Gomez-Gil et al. 2004), the *toxRPV* forward primer was considered for the isolation of the *toxR* gene in *V. campbellii*. The reverse primer is a degenerate primer, *Vib_toxS-R*, which targets the *toxS* gene, the gene downstream of *toxR*, reported in other *Vibrio* species: *V. parahaemolyticus*, *V. fischeri*, *V. harveyi*, *V. cholerae*, and *V. vulnificus*. Lee and colleagues (2000) designed a similar degenerate reverse primer targeting *toxS* for the amplification of the *toxRS* gene fragment in *V. vulnificus*. As hypothesized, the sequence targeted by the primers was conserved enough, allowing the

amplification of the expected 1,000 bp fragment containing the *V. campbellii toxR* gene homologue (Figure 1).

Sequence analysis revealed that the type strain *V. campbellii* NBRC 15631 *toxR* gene is 873 bp with start and stop codons at positions 1-3 and 871-873, respectively (Figure 2). BLAST (Altschul et al. 1990) homology search showed 100% sequence identity with the partial *toxR* sequence of the type strain *V. campbellii* CAIM 519 (GenBank no. AY946038; Table 1).

Multiple nucleotide sequence alignment (Table 2) showed that the *toxR* gene from *V. campbellii* is significantly different from the *toxR* gene of other *Vibrio* and *Photobacterium* species with the highest sequence similarity of only 79% with *V. harveyi* NBRC 15634 *toxR* (Franco and Hedreyda 2006), which is said to be a very close relative of *V. campbellii* (Alsina and Blanch 1994a; Alsina and Blanch 1994b; Pedersen et al. 1998; de la Peña et al. 2001; Gomez-Gil et al. 2004). Sequence similarity of the *V. campbellii toxR* with the gene from *V. parahaemolyticus*, *V. anguillarum, V. vulnificus, V. cholera, V. fischeri*, *V. hollisae* and *P. profundum* is 75%, 64%, 63%, 59%, 53%, 50%, and 26%, respectively (Table 2). This is significantly lower compared to multiple sequence alignment of the *16S rRNA* gene of various vibrios, showing a generally higher sequence similarity of about 89-99% among species (alignment data not shown). Analysis of the *V. campbellii* sequence data plus available sequences on other vibrios in the database revealed that although the *toxR* gene seems to be universally present in vibrios, significant gene sequence variation exists in *toxR* among the different *Vibrio* species. The significant variation in the *toxR* sequences observed among the different *Vibrio* species could be useful in distinguishing one species from the other and in designing PCR primers for species detection and identification.

Multiple alignment of ToxR proteins from different *Vibrio* and *Photobacterium* species, including the 290-amino acid polypeptide of *V. campbellii* from this study (Figure 4), revealed relatively conserved transcription activation (62-98% between *V. campbellii* and other *Vibrio* and *Photobacterium* species; alignment data not shown) and transmembrane and periplasmic domains (30-85% between *V. campbellii* and other species; alignment data not shown) flanking a highly divergent membrane "tether" region (6-62% between *V. campbellii* and other species; alignment data not shown), consistent with the findings of Osorio and Klose (2000). Even the closely related *V. campbellii* and *V. harveyi* that are almost indistinguishable phenotypically (Gomez-Gil et al. 2004), exhibited amino acid differences that are mostly within the divergent membrane "tether" region (Figures 3 and 4). A deletion of three nucleotides from *V. campbellii toxR* at position 368 corresponding to a leucine amino acid (CTC) residue in *V. harveyi,* also appears in the membrane "tether" region (Figure 3). This region is hypothesized to function as an anchor between the transcriptional activation and transmembrane domains and can therefore tolerate several insertions and/or deletions (Osorio and Klose 2000). These results indicate that *toxR* gene sequence analysis could easily differentiate *V. campbellii* from other related vibrios. The divergent region of *toxR* has been utilized in the designing of PCR primers for species-specific detection and identification of *V. harveyi* (Conejero and Hedreyda 2003; Castroverde et al. 2006) and could be used for designing species-specific primers for other vibrios.

The phylogenetic tree that was constructed using available full-length *Vibrio toxR* gene sequences from the database and from this study (Figure 5A) revealed significant divergence comparable to the phylogenetic tree based on the *16S rRNA* gene (Figure 5B), which could not clearly distinguish the closely related species of vibrios (Kita-Tsukamoto et al. 1993; Osorio and Klose 2000; Cortado et al. 2005). This is indicated by higher percent bootstrap values seen in the phylogenetic tree based on *toxR* than the one based on the *16S rRNA* gene. The phylogenetic tree based on the *16S rRNA* gene grouped the closely related members of the *Vibrio* core group – *V. alginolyticus*, *V. parahaemolyticus*, *V. harveyi*, *V. campbellii*, and *V. natriegens* (Thompson and Swings 2006). On the other hand, the phylogenetic tree based on *toxR* clearly separated the different *Vibrio* species into different clusters, including *V. campbellii* and *V. harveyi* which are not only phenotypically indistinguishable but also produce almost identical results in several biochemical tests (Alsina and Blanch 1994a; Alsina and Blanch 1994b; de la Peña et al. 2001; Gomez-Gil et al. 2004). This supports the claim by Thompson and colleagues (2007) that *V. campbellii* and *V. harveyi* are clearly genetically distinct from one another and should not be classified under one group.

The results suggest that using the *toxR* gene rather than the *16S rRNA* gene, considered as the gold standard for identification and has been used extensively in taxonomic differentiation of vibrios (Dorsch et al. 1992; Cortado et al. 2005), the very closely related vibrios could be distinguished better when it comes to identification at the species level. In addition, the observed divergence in the *toxR* gene, mainly found in the membrane "tether" region, may serve as a useful tool as the basis for the distinction of the closely related *V. campbellii* and *V. harveyi* as well as other *Vibrio* and *Photobacterium* species. For example, *V. campbellii* and *V. harveyi*, which have *16S rRNA* gene sequence similarity of 97% (alignment data not shown), only have 79% similarity with *toxR* (Table 2). PCR primers could be designed to target the divergent membrane "tether" region for species-specific identification.

Based on previous studies, comparison of DNA sequences of vibrios appears to be a reliable method in species detection and identification (Gomez-Gil et al. 2004; Thompson et al. 2004; Thompson et al. 2007) than conventional methods (Alsina and Blanch 1994a; Alsina and Blanch 1994b; Vandenberghe et al. 2003; Thompson et al. 2004). The study by Thompson and colleagues (2007) used multilocus sequence typing, which involved the study of the sequences of several genetic loci, to type different *Vibrio* isolates. Other genes such as *rpoA*, *recA*, and *pyrH* have been studied in multiple *Vibrio* species and have

been used extensively in the taxonomic identification vibrios with 98%, 94%, and 94% sequence similarity, respectively (Thompson et al. 2005), with the idea of replacing the *16S rRNA* gene for a more reliable identification method. The *toxR* gene shows even more divergence among species (i.e., lower sequence similarity, highest of which is only 79% between *V. campbellii* and *V. harveyi*), suggesting that the gene is a better distinguishing marker in differentiating different *Vibrio* species. The availability of the full-length *toxR* gene sequence from type strain *V. campbellii* contributes to the list of genes, aside from the *16S rRNA*, *rpoA*, *recA*, and *pyrH* genes, that could be analyzed and compared in order to generate baseline information important in distinguishing and identifying known, unknown, and variant environmental *Vibrio* strains.

CONCLUSION

This study reports, for the first time, the complete 873-bp *toxR* sequence of type strain *V. campbellii* NBRC 15631 which is translated to produce a polypeptide of 290 amino acids. Although the *toxR* gene seems to be universal and ancestral in the *Vibrionaceae* family, multiple sequence alignment of *toxR* from different *Vibrio* species revealed a significant sequence difference between *V. campbellii* NBRC 15631 and its close relative *V. harveyi* NBRC 15634 (79%), and even lower similarity with *toxR* of other vibrios (ranging from 26-75%). Alignment of different ToxR amino acid sequences showed a highly divergent membrane "tether" region flanked by relatively conserved transcriptional activation, transmembrane, and periplasmic regions. This high divergence could be used for species-specific identification of *V. campbellii* and other vibrios. Moreover, a phylogenetic tree constructed based on the *toxR* gene of different vibrios showed a greater divergence among *Vibrio* species compared to a phylogenetic tree that was constructed based on the *16S rRNA* gene. The *toxR* gene may be used as a gene marker for a more accurate identification and classification of known and unknown *Vibrio* isolates.

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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, who mainly conceptualized the study. Both worked together to write and revise the manuscript.

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