Full Paper

Amplification and sequence analysis of the full length *toxR* gene in *Vibrio harveyi*

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(Received May 19, 2006; Accepted September 8, 2006)

This study was focused on obtaining the complete gene sequence of the *toxR* gene in *V. harveyi* by using *toxR*-targeted PCR to amplify 5' and 3' regions flanking the 576-bp *Vibrio harveyi* (NBRC 15634) *toxR* gene fragment previously amplified using degenerate PCR. To obtain the 5' flanking sequences, a forward PCR primer (*VhtoxRpv*) was designed based on known sequences upstream of *toxR* in *V. parahaemolyticus* and *V. vulnificus*. The reverse primer (*VctoxR2R*) was based on the sequence of the 576-bp *Vibrio harveyi toxR* fragment. The resulting 750-bp amplicon was sequenced, providing the 5' sequences of the *V. harveyi* (NBRC 15634) *toxR* gene. The 3' flanking region was amplified using a primer pair *toxRS1* and *toxRS2* based on *V. parahaemolyticus* and *V. vulnificus toxR* and *toxS*, resulting in a 900-bp amplicon that contained the remaining 3' sequences of the *V. harveyi* NBRC 15634 *toxR*. This paper reports, for the first time, a complete 882-bp nucleotide sequence for *toxR* in *Vibrio harveyi*. Sequence analysis and alignment revealed that the complete *toxR* gene in *V. harveyi* shares 87% sequence similarity with *toxR* of *V. parahaemolyticus*, 84% similarity with *V. fluvialis*, 83% with *V. vulnificus* and partial sequence of *V. campbellii*. The phylogenetic trees revealed wider divergence in *toxR* compared to 16S rRNA genes, so that *V. harveyi* could easily be distinguished from *V. campbellii* and *V. parahaemolyticus*.

Key Words—luminous vibriosis; PCR; sequence analysis; *toxR*; *Vibrio harveyi*

Introduction

Vibrio harveyi, the bacterium implicated in shrimp and fish disease is a Gram-negative curved luminous bacterium ubiquitous in marine ecosystems (Baumann et al., 1980) and is the primary causative agent of lumi-

nous vibriosis. In order to gain more insights into the disease-causing mechanisms in this species, it is necessary to study genes and proteins implicated in virulence. To date, there are a few studies on *V. harveyi* genes implicated in virulence such as the *hemolysin* and *toxR* genes. Complete sequence of the *V. harveyi* hemolysin gene (Zhang et al., 2001) whose gene product is an exotoxin responsible for lysis of erythrocytes and host cell damage (Zhang and Austin, 2005), has been reported. ToxR, the gene product of *toxR*, is a transmembrane transcription regulator, which controls the coordinate expression of virulence genes in *Vibrio cholerae* (Miller et al., 1987) and has been well studied

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in *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (DiRita et al., 1996), but not in *V. harveyi*.

The ancestral role of ToxR appears to be in the direct regulation of the transcription of genes encoding for the outer membrane proteins in *Vibrio cholerae* and some related species (Chakrabarti et al., 1996). Ancestral genes reflect the phylogenetic relationship of bacterial species. To be able to do this, sequence data for each species should be generated. Gene sequences for *toxR* have been amplified from *V. alginolyticus*, *V. mimicus*, *V. fluvialis* and *V. hollisae* (Gutacker et al., 2003; Osorio and Klose, 2000; Vudhakul et al., 2000). Conejero and Hedreyda (2003) were able to isolate and sequence a 576-bp *toxR* gene homologue from *V. harveyi* (NBRC 15634). This paper reports the design and selection of primers to amplify regions of the *V. harveyi toxR* gene flanking the 576-bp internal fragment. Amplification and sequence analysis of target *toxR* gene fragments resulted in the complete *toxR* gene sequence of *V. harveyi* NBRC 15634 and *V. harveyi* CAIM 512, type strains that exhibit 100% *toxR* gene sequence similarity. The complete *toxR* gene sequence is expected to provide new information needed to gain insights into the possible

role of the protein product, ToxR, in the virulence of *V. harveyi*. Moreover, availability of the complete gene sequence of *toxR* will allow studies on pathogenicity and phylogeny of the *Vibrios*.

Materials and Methods

Bacterial strains and media. Type strain *Vibrio harveyi* NBRC 15634 from the NITE Biological Resource Center, Japan, and *Vibrio harveyi* CAIM 512 from Collection of Aquatic Infectious Microorganisms, Mexico, were used in this study. The *Vibrio* isolates were cultured at 28°C and maintained in Nutrient broth (NB) or Nutrient Agar (N.A) with 1.5% NaCl.

DNA extraction. Genomic DNA of the *Vibrio harveyi* NBRC 15634 and *Vibrio harveyi* CAIM 512 were isolated using the Nucleospin® Tissue Kit (Clontech Laboratories, Inc., USA) following the protocol prescribed by the manufacturer.

Selection and use of primers to amplify target *toxR* gene fragments from type strain *Vibrio harveyi*. To obtain the 5' sequence flanking the 576-bp internal *Vibrio harveyi* (NBRC 15634) *toxR* gene fragment, a forward primer was designed based on 3' sequences

Table 1. Primers used in this study.

Primer name	Sequence 5'–3'	Expected product size	Primer annealing site	Reference
<i>Vh toxRpv</i> Forward	AGT ACT AAT ATC GAC AC	750 bp	Base pair 1–17 of <i>V. harveyi</i> NBRC 15634 <i>toxR</i>	This study
<i>Vc toxR2R</i> Reverse	ATG ACT TCA ACT GGT G		Base pair 557–537 of <i>V. harveyi</i> NBRC 15634 <i>toxR</i>	San Luis and Hedreyda, 2005
<i>toxRS1</i> Forward	CGA TTA GGN AGC AAC GAA AGC CG	900 bp	Base pair 100–122 of <i>V. vulnificus toxR</i>	Lee et al., 2000
<i>toxRS2</i> Reverse	GTC ACT NCC CCA NTA NAA CC		A region of <i>toxS</i> downstream of <i>toxR</i>	
<i>toxRPV</i> Forward	5' ATG ACT AAT ATC GGC AC 3'	900 bp	Base pair 1–17 of <i>V. harveyi</i> NBRC 15634	This study
<i>toxR3R</i> Reverse	5' TCA GCC CCT AAT GCA AAT GTC 3'		Base pair 862–882 of <i>V. harveyi</i> NBRC 15634	

of the *toxR* gene in *V. parahaemolyticus* and *V. vulnificus* that are available in the database. This forward primer was paired with the reverse primer, *Vc toxR2R* (Table 1), which was designed to anneal within the 576-bp internal *V. harveyi toxR* sequence (San Luis and Hedreyda, 2005). PCR was conducted using a *V. harveyi* DNA template, and 30 cycles of the following PCR conditions: initial denaturation at 94°C (5 min), denaturation at 94°C (1 min), annealing at 42°C (1 min), elongation at 72°C (1 min) and final elongation at 72°C (5 min). The 3' sequence flanking the 576-bp *toxR* was amplified using primers designed on the basis of the *toxS* sequence downstream of *toxR* or close to the 3' end of *V. parahaemolyticus* and *V. vulnificus* (DiRita and Mekalanos, 1991; Lee et al., 2000), a DNA template from *V. harveyi* and PCR parameters described above except for a different annealing temperature of 57°C. Purified amplicons were submitted for sequencing at Molecular Biotechnology and Genome Research Laboratory, National Institute of Molecular Biology and Biotechnology, UP-Diliman, Philippines or Macrogen, Korea.

Nucleotide sequence analysis of the complete *toxR* gene of *Vibrio harveyi*. The nucleotide sequence of the complete *V. harveyi* (NBRC 15634) *toxR* was obtained after sequence alignment and assembly of the internal 576-bp *toxR* fragment and the 5' and 3' flanking regions generated in this study using Clustal W (<http://www.ebi.ac.uk/clustalw/>). Homology search was done using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>).

The complete *toxR* nucleotide sequence of another type strain *V. harveyi* (CAIM 512) was obtained after amplification of the entire gene using *toxR*-targeted primers designed on the basis of sequences obtained from *V. harveyi* NBRC 15634.

Amino acid sequence analysis of *ToxR* protein. The amino acid sequence of the *Vibrio harveyi* *ToxR* protein was decoded using the DNASIS version 2.1 software (Molecular Biology Insights, Inc., Cascade, CO, USA) with the MULTALIN 5.4.1 program (<http://www.toulouse.inra.fr/multalin.html>), and was aligned with the *ToxR* protein sequences of other *Vibrio* species in the database.

Nucleotide sequence accession number. The complete *toxR* sequences of *V. harveyi* NBRC 15634 and CAIM 512 were deposited in the GenBank database.

Results

Amplification of 5' and 3' target toxR gene fragments from Vibrio harveyi

Preliminary amplification experiments using a forward primer that was designed on the basis of sequences upstream of the *toxR* gene in *Vibrio parahaemolyticus* and *Vibrio vulnificus* did not amplify the 5' flanking region of the 576-bp *toxR* fragment of *V. harveyi* (NBRC 15634), suggesting significant sequence variation in sequences upstream of *toxR* between *V. harveyi* and the two related *Vibrio* species. A

Fig. 1. Diagrammatic representation of PCR-generated *toxR* gene (gray) fragments relative to the complete *toxR* gene in *Vibrio vulnificus*.

A, complete *toxR* gene in *V. vulnificus* including gene for heatshock protein *htpG* (upstream) and *toxS* (downstream); B, internal 576-bp *toxR* fragment of *V. harveyi* NBRC 15634 obtained using degenerate PCR; C, *V. harveyi toxR* fragment produced in PCR using primer pair *Vh toxRpv/Vc toxR2R*; D, *toxR* fragment from PCR using the primer pair *toxRS1/toxRS2*.

forward primer (*Vh toxRpv*, Table 1) based on 5' *toxR* sequences of *V. parahaemolyticus* and *V. vulnificus* was used with a reverse primer (*Vc toxR2R*, Table 1) that was expected to anneal to a region of the 576-bp *toxR* fragment, producing the expected size amplicon of approximately 750 bp (Fig. 1) which generated 710 nucleotides completing the 5' *V. harveyi toxR*. For the 3' flanking sequence, a forward primer based on the sequence of the 576-bp *V. harveyi* fragment paired with a reverse primer based on the *toxS* sequence (downstream of *toxR*) in *V. parahaemolyticus* and *V. vulnificus*, did not amplify 3' flanking regions of *V. harveyi toxR*, suggesting that significant variation exists in the *toxS* sequence between *V. harveyi* and the two species. Surprisingly, the 3' flanking region in *V. harveyi* (NBRC 15634) was amplified by a primer pair, *toxRS1* and *toxRS2* (Table 1), previously reported to amplify in *V. parahaemolyticus* and *Vibrio vulnificus*, indicating significant similarity of sequences within the primer annealing regions in *V. harveyi* and the two

species. An approximately 900-bp amplicon (Fig. 1) was produced generating 776 3' nucleotides of *V. harveyi toxR*.

Sequence analysis of the complete *Vibrio harveyi* (NBRC 15634) *toxR* gene

After sequence alignment and further analysis, the entire 882-bp *toxR* gene sequence in type strain *Vibrio harveyi* NBRC 15634 was completed (Fig. 2). Based on this complete *toxR* sequence, a set of primers (*toxRPV/toxR3R*) that amplified the entire *toxR* gene in *V. harveyi*, was designed and used to amplify *toxR* from other strains of *Vibrio harveyi*, including CAIM 512. The resulting 900-bp size amplicon generated the complete 882-bp *toxR* gene for type strain *V. harveyi* CAIM 512, which exhibited 100% sequence similarity with *toxR* of *Vibrio harveyi* NBRC 15634. The complete *toxR* sequences of *V. harveyi* NBRC 15634 and CAIM 512 were deposited in the GenBank database under the accession numbers DQ403146 and

Fig. 2. Multiple alignment using Multalin of complete *toxR* gene sequences from *V. harveyi* NBRC 15634, *V. parahaemolyticus* and *V. vulnificus* (GenBank Accession No. Q403146, AB063113 and AF166120, respectively).

Fig. 3. Alignment of ToxR amino acid sequences from *V. harveyi* NBRC 15634 (*Vha*), *V. parahaemolyticus* (*Vpa*) and *V. vulnificus* (*Vvu*).

The consensus (Cons.) sequence is shown at the bottom in boldface type. Residues identical in at all 3 sequences are shown in uppercase letters while residues identical in 2 of the 3 sequences are shown in lowercase letters.

DQ503438, respectively.

The 882-bp *toxR* gene of type strain *Vibrio harveyi* (NBRC 15634 and CAIM 512) shared 86% sequence similarity with *toxR* of *V. parahaemolyticus*, 85% with partial sequence of *V. campbellii*, 84% similarity with *V. fluvialis*, and 83% with *V. vulnificus* (Fig. 2). The full-length *toxR* sequence from *V. harveyi* was also predicted to generate 294 deduced amino acids and was observed to display a divergent membrane "tether" region flanked by well-conserved transcriptional activation and transmembrane domains, similar to *V. parahaemolyticus* and *V. vulnificus* (Fig. 3, Osorio and Klose, 2000). A phylogenetic tree based on available *toxR* gene sequences revealed wider divergence in *toxR* compared to 16S rRNA. (Fig. 4), suggesting the possible use of *toxR* as a gene marker to differentiate closely related *Vibrios*.

Discussion

The use of *toxR*-targeted PCR using primers based on available *toxR* sequences from related *Vibrio* species (*V. parahaemolyticus* and *V. vulnificus*) resulted in the amplification of missing 5' flanking sequences of an internal *toxR* fragment from type strain *Vibrio harveyi* NBRC 15634. Primers previously reported and used to amplify *Vibrio vulnificus* *toxRS* genes were also used (Lee et al., 2000) to complete the 3' end of the *V. harveyi* *toxR* gene. This was possible because although significant sequence variation exists between the complete *toxR* gene in *V. harveyi*

and any of two *Vibrio* species, the terminal 5' and 3' sequences appeared to be relatively conserved, allowing amplification of *V. harveyi* genes using primers previously designed and used for *V. parahaemolyticus* and *V. vulnificus*. As a result, this paper reports for the first time complete sequences for the *toxR* gene in type strain *Vibrio harveyi* NBRC 15634 and CAIM 512 and for the species in general.

The complete *toxR* gene sequence in *V. harveyi* was observed to be significantly different from other *Vibrios*, 86% sequence similarity with *V. parahaemolyticus*, 85% with partial sequence of *V. campbellii*, 84% with *V. fluvialis*, and 83% with *V. vulnificus*, and is expected to exhibit greater variation from other phenotypically distant *Vibrios*. The *toxR* gene, therefore, may be an important molecular marker for distinguishing different species under *Vibrionaceae*. Moreover, gene sequence data could be used to distinguish *V. harveyi* from close relatives like *V. campbellii*, *V. parahaemolyticus*, and *V. alginolyticus* (Fig. 4) as shown by the phylogenetic tree.

Sequence analysis revealed that the *V. harveyi* *toxR* gene and the predicted ToxR protein have a divergent tether region, flanked by two more conserved regions (Fig. 3) also present in *toxR* of previously studied *Vibrios* (Osorio and Klose, 2000). This observation supports the hypothesis that *toxR* may be an ancestral gene of the *Vibrios* and is therefore present and relatively conserved in all members of *Vibrionaceae*. Complete sequences of the gene from different *Vibrios* would allow sequence comparison among known *Vib-*

16s rRNA ***toxR***

Fig. 4. Unrooted trees constructed by the neighbor-joining method showing the phylogenetic interrelationships of *Vibrio* species based on 16S rRNA and *toxR* gene nucleotide sequences.

Bootstrap values are given at the branching points and bars show sequence divergence.

rios in order to generate baseline information that could be crucial in the identification of unknown and variant environmental strains. In addition to identification, and as described in an earlier study (Conejero and Hedreyda, 2003), the hypervariable region of *toxR* could be targeted in primer design for species detection. The availability of the complete *toxR* sequence of *V. harveyi* is necessary in order to evaluate the useful-

ness of *toxR* as a gene marker for species detection and classification.

Based on the phylogenetic trees constructed (Fig. 4), there is wider degree of divergence in *toxR* gene between *V. harveyi* and closely related *Vibrio* species (particularly between *V. harveyi* vs *V. campbellii* and *V. harveyi* vs *V. parahaemolyticus*) compared to divergence in the 16S rRNA gene. Whereas, 16S rRNA gene sequence analysis is not successful in distinguishing *V. harveyi* from closely related species, particularly, *V. campbellii* (Rañoa and Hedreyda, 2005), the *toxR* divergent regions as well as the total gene sequence deviation between *V. harveyi* and *V. campbellii* could easily help distinguish one from the other.

Lastly, *toxR* codes for a transcriptional regulator of several virulence factors in well studied *Vibrios* such as *Vibrio cholerae* and *Vibrio parahaemolyticus* (DiRita et al., 1996; Kim et al., 1999). It could be hypothesized that the ToxR protein also performs the same function in *V. harveyi* which is implicated in fish and shrimp disease. It would be interesting to know the role of this gene and its gene product in pathogenicity. One approach to take is to correlate gene sequences and gene sequence variation and protein expression, with pathogenicity. This approach could now be taken, not only because complete *toxR* sequences are available for type strains of *V. harveyi*, but also because primers that could amplify the entire gene in pathogenic and non-pathogenic strains are available or could easily be designed based on known sequences. Complete sequence for *toxR* in different species of *Vibrio* would be valuable for future studies on phylogeny, virulence, and genes regulated by the *toxR* gene product.

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