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Variable repeat regions in the genome of *Vibrio vulnificus* and polymorphism in one of the loci in strains isolated from oysters

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Abstract

Vibrio vulnificus an estuarine bacterium is associated with severe wound infections and fatal septicemia related to consumption of raw shellfish. In this study we screened the two whole genome sequences available for *V. vulnificus* in GenBank for the presence of variable number of tandem repeat (VNTR) regions. Five potential VNTR loci with unit repeat size ranging from 6–7 nucleotides were identified for *V. vulnificus* genome. One of the loci designated *Vv*1 was selected to detect the repeat number present in *V. vulnificus* strains isolated from oyster samples in India. Twenty six of the thirty samples tested were found to be highly polymorphic for the *Vv*1 locus. Copy numbers for the hexanucleotide motif ranged from 4–55, giving rise to a total of 17 polymorphic groups. Our analysis, shows that different genotypic variants exist in the environment and the VNTR loci studied can be used as a marker for strain discrimination and in epidemiological study of this organism.

Keywords: *Vibrio vulnificus*; Genotyping; Variable number tandem repeats

1. Introduction

Vibrio vulnificus, a gram-negative bacterium is an autochthonous inhabitant of the coastal waters. Epidemiological data indicate that *V. vulnificus* is commonly concentrated in gut and other tissues of filter feeders such as oysters and clams and infection has been associated with the ingestion of these contaminated shellfish particularly oysters, leading to illness such as gastroenteritis and invasive fatal septicemia (Mascola et al., 1996; Strom and Paranjpye, 2000; Samir et al., 2005). The organism is also associated with life-threatening wound infections acquired through contact with shellfish or waters where the organism is present (Kumamoto and Vukich 1998). Individuals who are either immunocompromised or suffering from underlying chronic disorders are at a greater risk of infection by this organism (Hlady, 1997; Strom and Paranjpye, 2000). The prevalence of this organism in coastal waters and shellfish in India has been reported earlier (Karunasagar et al., 1987, 1990; Thampuran and Surendran, 1998; Parvathi et al., 2005).

Genotyping of bacterial strains, essential to several disciplines such as taxonomy, epidemiology and evolution has been the focus of research since the last decade. Several independent studies employing genetic typing techniques such as ribotyping using 16 S rRNA (DePaola et al., 2003), pulsed-field gel electrophoresis (Tamplin et al., 1996); RAPD PCR (Warner and Oliver, 1999; Rosche et al., 2005); *gyr*B gene analysis (Venkateswaran et al., 1998; Parvathi et al., 2005) and repetitive extragenic palindromic PCR analysis (Chatzidaki-Livanis et al., 2006) have clearly established that there exists considerable genetic diversity among *V. vulnificus* strains. Bisharat et al.

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(2007) used multi-locus sequence typing to show that recombination contributes more substantially than mutation to generate strain diversity in *V. vulnificus*.

In this study we try to assess the genetic diversity in *V. vulnificus* strains based on variable number tandem repeats (VNTRs). Simple sequence repeats (SSRs) or microsatellites are defined as iterations of short motifs of 1–7 bases. Tandem repeat loci exhibiting variability in their copy numbers are referred to as 'variable number tandem repeats' (VNTRs). Such repeats have been found in various organisms (vanBelkaum, 1998) and are now increasingly recognized as informative markers for studying genotypic variations among strains and in genomic evolutionary studies.

VNTR alleles have found several applications such as in DNA fingerprinting, gene mapping both in humans (Jeffreys et al., 1985, Nakamura et al., 1987) and several microorganisms (Kim et al., 2001). In bacteria, in addition to studying strain variation (vanBelkaum et al., 1997; Sun et al., 2004) VNTRs also serve as potential markers for the identification of pathogenic bacteria (Hood et al., 1996; Peak et al., 1996) and the virulence factors associated with their pathogenicity (Saunders et al., 2000).

In this study, we analyzed a VNTR loci for polymorphism, which could be used in subtyping strains of this pathogen to enable differentiation.

2. Materials and methods

2.1. VNTR search and primer design

V. vulnificus whole genome consists of two circular chromosomes, chromosome 1 and chromosome 2. The complete DNA sequence of chromosome 1 and 2 respectively of each of the two *V. vulnificus* isolates CMCP6 (accession numbers (acc. no.) NC_004459, NC_004460) and YJ016 (acc. no. NC_005139, NC_005140) available in GenBank were downloaded. Tandem repeats were identified using the standalone version of tandem repeat finder (TRF) program (Bensen, 1999). Parameters for repeat search were set to +2, −7, −7 (matches, mismatches, and indels), with a minimum alignment score of 50. Potential VNTR loci were identified by comparative analysis of all the repeats obtained. The presence of VNTR loci within coding or non-coding regions were identified based on annotation of *V. vulnificus* genomes in GenBank. To amplify a DNA fragment containing the VNTR loci, we designed primers using the Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) software. The forward and reverse primers were as follows: 5′-tctacgcttttgggctcagt-3′ and 3′-cgaaaaacccaaacctcaaa-5′.

2.2. Bacterial strains

A total of 30 *V. vulnificus* strains isolated from oysters collected from their natural beds were used in this study. Briefly, 25 g of homogenized oyster meat was taken in 250 ml of Alkaline Peptone water containing 1×10^4 U of polymyxin. This enrichment broth with the sample was incubated at 37 °C for 16 h. A loopful of the inoculum was streaked onto mCPC (modified cellobiose-polymyxin colistin) agar plates and incubated overnight at 42 °C. Golden yellow colonies were picked and purified on TSAS 1% and were identified based on biochemical tests for *V. vulnificus* (McFaddin, 1980). The isolates were further confirmed by PCR using *vvh*A1 and *vvh*A2 nested primer sets (Lee et al., 1998).

2.3. DNA extraction, PCR amplification and data analysis

Each of the 30 isolates of *V. vulnificus* used in this study was inoculated to 5 ml of pre-sterilized Luria Bertani broth tubes and incubated at 37 °C for 18 h. One ml of the culture was centrifuged at 10,000 ×g for 10 min. The pellet was washed twice in sterile distilled water, resuspended in 100 µl sterile TE buffer (10 mM Tris–Cl, pH 8.0, 1 mM EDTA) and heated at 95 °C for 15 min in a hot water bath. The tubes were snap cooled on ice and centrifuged again to sediment cell debris. 5 µl of the DNA extract was amplified in a 50 µl PCR mixture consisting of 1× PCR buffer (10 mM Tris–HCl pH 8.3, 2 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin), 200 µM of each of the four deoxyribonucleotide triphosphate (dATP, dTTP, dGTP and dCTP), 50 pmol of each primer and 1.25 U of *Taq* polymerase (Bangalore Genie, Bangalore, India). Thirty cycles were carried out in a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA) with denaturation at 94 °C for 1 min, annealing at

Table 1
Potential VNTR loci on chromosome 2 of *Vibrio vulnificus*

Designation	Repeat unit size	Repeat sequence	*V. vulnificus* genome	Genomic start position	Copy nos.	Strand	Associated ORF
*Vv*1	6	TGTTGC	CMCP6	1716498	44.7	−	VV21562
		TGTTGC	YJ016	441865	33.7	−	VVA0375
*Vv*2	7	CTAGGTT	CMCP6	1059528	23.6	+	VV20982
		CTAGGTT	YJ016	1622343	19.6	+	VVA1474
*Vv*3	7	AGG(A/G)GCT	CMCP6	950419	10.4	+	NC
		AGGGGCT	YJ016	1492991	23.6	+	NC
*Vv*4	7	TCTAGG(T/C)	CMCP6	391956	10.7	+	NC
		TCTAGGG	YJ016	1023042	18.7	+	NC
*Vv*5	7	GGGACTA	CMCP6	900731	7.0	+	NC
		GGGACTA	YJ016	1437650	24.0	+	NC

NC: repeats present in non-coding regions of the genome.
'−' minus strand and '+' positive strand.

60 °C for 30 s and extension at 72 °C for 1 min. The cycling included an initiation delay for 5 min at 95 °C and a final delay for 5 min at 72 °C. PCR products were visualized using gel documentation system (Herolab RH-2.1, Wiesloch, Germany) after electrophoresis in 1.5% agarose gel containing 0.5 μg/ml ethidium bromide.

2.4. Amplicon size

The amplicon size was determined using Kodak Digital Science 1D (KSD1D 2.0, USA) software. The number of repeat units (RUs) were calculated as [amplicon size − 259] / 6. The RUs were adjusted to the nearest whole number considering that

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      1                                                                  60
 Vv15 TCTACGCTTT TGGGCTCAGT CTCT..TTCT TGCGCGGGTT TATCGCTCGG CTTAACTTGT
CMCP6 TCTACGCTTT TGGGCTCAGT CTCTCTTTCT TGCGCGGGTT TATCGCTCGG CTTAACTTGC
 Vv36 TCTACGCTTT TGGGCTCAGT CTCT.TTTCT TGCGCGGGTT TATCGCTCGG CTTA.CTTGT
YJ016 TCTACGCTTT TGGGCTCAGT CTCTCTTTCT TGCGCGGGTT TATCGCTCGG CTTAACTTGT
  Vv7 TCTACGCTTT TGGGCTCAGT CTCTCTTTCT TGCGCGGGTT TATCGCTCGG CTTAACTTGC
 Vv70 TCTACGCTTT TGGGCTCAGT CTCTCTTTCT TGCGCGGGTT TATCGCTCGG CTTAACTTGC
      61                                                                120
 Vv15 TCTTCACCCT GTTCGGATGG TTTTTTCTGT CGTTGAGCAT CGCCTTGTTC ATTGTTTTCT
CMCP6 TCTTCACCCT GTTCGGATGG TTTTTTCTGT CGTTGAGCAT TGCCTTGTTC ACTGTTTTCT
 Vv36 TCTTCACCCT GTTCGGATGG TTTTTTCTGT CGTTGAGCAT CGCCTTGCTC ATTGTTTTCT
YJ016 TCTTCACCCT GTTCGGATGG TTTTTTCTGT CGTTGAGCAT TGCCTTGTTC ACTGTTTTCT
  Vv7 TCTTCACCCT GTTCGGATGG TTTTTTCTGT CGTTGAGCAT TGCCTTGTTC ACTGTTTTCT
 Vv70 TCTTCACCCT GTTCGGATGG TTTTTTCTGT CGTTGAGCAT CGCCTTGTTC ACTGTTTTCT
      121                                                               180
 Vv15 TGAGACTTTT CTTTCGCCTC TTTTGGCGCA TTTTCGCGAT TTTCTGATTG ACTCTGATTA
CMCP6 TGAGACTTTT CTTTTGCCTT ATTTGGCGCA TTTTCGCGAT TTTCTGATTG ACTCTGATTA
 Vv36 TGAGACTTTT CTTTCGCCTC TTTTGGCGCA TTTTCGCGAT TTTCTGATTG ACTCTGATTA
YJ016 TGAGACTTTT CTTTTGCCTT ATTTGGCGCA TTTTCGCGAT TTTCTGATTG ACTCTGATTA
  Vv7 TGAGACTTTT CTTTTGCCTT ATTTGGCGCA TTTTCGCGAT TTTCTGATTG ACTCTGATTA
 Vv70 TGAGACTTTT TTTTCGCCTC TTTTGGCGCA TTTTCGCGAT TTTCTGATTG ACTCTGATTA
      181                                                               240
 Vv15 TCGCGATTGG AAGACTTTTG TTGCTGTTGC TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC
CMCP6 TCGCGATTGG AAGACTTTTG TTGCTGTTGC TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC
 Vv36 TCGCGATTGG AAGACTTTTG TTGCTGTTGC TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC
YJ016 TCGCGATTGG AAGACTTTTG TTGCTGTTGC TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC
  Vv7 TCGCGATTGG AAGACTTTTG TTGCTGTTGC TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC
 Vv70 TCGCGATTGG AAGACTTTTG TTGCTGTTGC TGTTGCTGTT GCTGTT.... ..........
      241                                                               300
 Vv15 TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC
CMCP6 TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC
 Vv36 TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC
YJ016 TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC
  Vv7 TGTT...... .......... .......... .......... .......... ..........
 Vv70 .......... .......... .......... .......... .......... ..........
      301                                                               360
 Vv15 TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC
CMCP6 TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC
 Vv36 TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC
YJ016 TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC
  Vv7 .......... .......... .......... .......... .......... ..........
 Vv70 .......... .......... .......... .......... .......... ..........
      361                                                               420
 Vv15 TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC
CMCP6 TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC
 Vv36 TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC TGTTGCTGTT GCTGTT.... ..........
YJ016 TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC TGTTGCTGTT .......... ..........
  Vv7 .......... .......... .......... .......... .......... ..........
 Vv70 .......... .......... .......... .......... .......... ..........
      421                                                               480
 Vv15 TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC TGTTGCTGTT GCTGTTGCTG TTTTGCTTTT
CMCP6 TGTTGCTGTT GCTGTTGCTG TTGCTGTTGC TGTTGCTGTT GCTGTT.... ..TTGCTTTT
 Vv36 .......... .......... .......... .......... .......... ..TTGCTTTT
YJ016 .......... .......... .......... .......... .......... ..TTGCTTTT
  Vv7 .......... .......... .......... .......... .......... ..TTGCTTTT
 Vv70 .......... .......... .......... .......... .......... ..TTGCTTTT
      481                                                   530
 Vv15 TCTACTATTT CGCGATTGGT TTGTGCTTGT TTGAGGTTTG GGTTTTTCG
CMCP6 TCTACTATTT CGCGATTGGT TTGTGCTTGT TTGAGGTTTG GGTTTTTCG
 Vv36 TCTACAATGT CGCGATTGGT TTGTGCTTGT TTGAGGTTTG GGTTTTTCG
YJ016 TCTACAATTT CGCGATTGGT TTGTGCTTGT TTGAGGTTTG GGTTTTTCG
  Vv7 TCTACAATGT CGCGATTGGT TTGTGCTTGT TTGAGGTTTG GGTTTTTCG
 Vv70 TCTACTATTT CGCGATTGGT TTGTGCTTGT TTGAGGTTTG GGTTTTTCG
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Fig. 1. Multiple sequence alignment for the *V. vulnificus Vv*1 loci. CMPC6 (acc.no. NC_004460) and YJ016 (NC_005140) are sequences for the *Vv*1 loci on chromosome 2. Vv7 (EU429298), Vv15 (EU429299), Vv36 (EU429300), Vv70 (EU429301) are sequences of our samples. Text in bold indicates repeat regions. Forward and reverse primers are underlined.

the *Vv*1 loci of the two *V. vulnificus* completely sequenced genomes studied had 44.7 and 33.7 repeats for this locus.

2.5. Cloning and sequencing of PCR products

Randomly selected four representative PCR products were purified using PCR purification kit (QIAGEN, USA). These purified products were subsequently cloned into pSC-A cloning vector (Strataclone, USA) and sequenced using T3 and T7 primers to confirm the region of amplification and repeat patterns.

2.6. Sequence analysis

The sequences obtained were analyzed for the presence of tandem repeats using the TRF program. DNA sequences were aligned using the MultAlin program (Corpet, 1988) at http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html.

3. Results

We identified five potential VNTR loci in chromosome 2 of *V. vulnificus* genome and designated as *Vv*1–*Vv*5 (Table 1). No VNTR loci were identified in chromosome 1. Two of the VNTR loci, *Vv*1 and *Vv*2 were within coding region while the remaining three were associated with non-coding regions. For studying polymorphism, we chose the VNTR locus *Vv*1, which was a hexanucleotide motif (TGTTGC) showing the highest copy variation of 44.7 and 33.7 repeats in CMCP6 and YJ016 genomes respectively (Table 1, Fig. 1). Our analysis with *V. vulnificus* strains from oysters from South India revealed high polymorphism for the *Vv*1 repeat region in *V. vulnificus* strains. Twenty six out of the thirty samples tested showed amplification with *Vv*1 specific primers. All samples tested showed the presence of a single band, except in the case of one, which showed the presence of two bands (Fig. 2, lane 14). This could probably be due to the presence of two variants of the strain in the culture. The number of repeat units varied from 4–55 making a total of 17 distinct polymorphic groups for *V. vulnificus* (Table 2). Further, sequencing results of the four random samples positive for the *Vv*1 locus, validated our results predicted by band size (Fig. 1). These sequences have been submitted to GenBank and assigned accession numbers: EU429298, EU429299, EU429300, EU429301. The BLAST program http://www.ncbi.nlm.nih.gov/blast available at NCBI was used for similarity search for the four sequences with available sequences in GenBank database and generated 'no hits' except partial similarity to the *V. vulnificus* genome available in database.

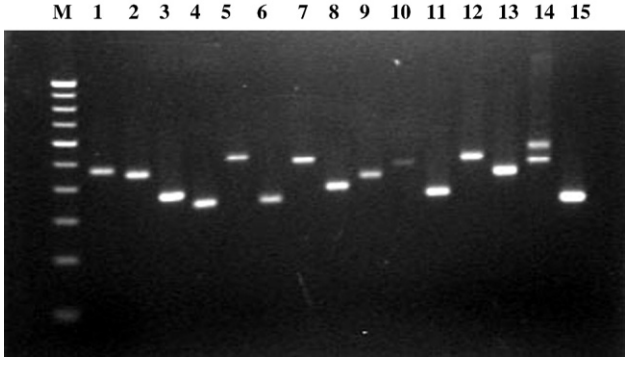

Fig. 2. Representative agarose gel picture showing repeat polymorphism for the *Vv*1 region. Lane M: 100 bp marker; lanes 1–15: *V. vulnificus* samples.

Table 2
Repeat polymorphism observed for *Vv*1 locus in *V. vulnificus* isolates

Strain	Amplicon size (bp)	Repeat number[a]
Vv 48	283	4
Vv 70	283	4
Vv 67	289	5
Vv 66	325	11
Vv 4	349	15
Vv 68	355	16
Vv 7	361	17
Vv 3	373	19
Vv 41	373	19
Vv 69	373	19
Vv 14	391	22
Vv 46	391	22
Vv 47	391	22
Vv 43	397	23
Vv 42	403	24
Vv 9	409	25
Vv 2	451	32
Vv 10	451	32
Vv 1	463	34
Vv 36	463	34
Vv 8	505	41
Vv 13	505	41
Vv 84	511	42
Vv 6	529	45
Vv 15	529	45
Vv 40	589	55

[a] Calculated as (amplicon size − 259)/6.

4. Discussion

The VNTRs detected in this study present a valuable addition to the various DNA procedures that are available for typing *V. vulnificus* strains. Repetitive regions have been used as genetic markers for strain differentiation and epidemiology of several bacterial species (Li et al., 2004). The SSR variation for *Vv*1 locus observed in this study shows that this locus can be used as a genetic marker for polymorphism studies and strain discrimination of this organism. SSRs show high mutation rates and several mechanisms have been proposed for the genesis of tandem repeats including slipped–stand mispairing (Levinson and Gutman, 1987) wherein base mispairing during replication gives rise to addition or deletion of repeat units. In bacteria, such phase-variations are known to be an adaptive process, wherein they have evolved the ability to undergo frequent and reversible phenotypic changes at particular genomic locus, without increasing the overall mutability of the rest of the genome (Moxon et al., 1994). There is also growing evidence that when these phase-variable genes are located within coding regions they can directly alter gene expression (vanBelkaum, 1998) and gene regulation, affecting gene products and bringing

about phenotypic changes (Yeramian and Buch, 1999). Such genes referred to as phase-variable or 'contingency' genes are crucial for the survival of pathogens and adaptation of bacterial population to changing environmental conditions (Hallet, 2001). Earlier studies have shown that virulence in *V. vulnificus*, is correlated with the polymorphism associated with the 16 S rRNA (Nilsson et al, 2003) and the heptanucleotide repeat sequences present downstream of ORF Vv0401 (*V. vulnificus* CMCP6 genome) (Rosche et al, 2005). These revelations demonstrate the role of polymorphism and repeat sequence as indicative of potential virulence of the bacterium.

DePaola et al. (2003) in their study based on RAPD-PCR genotyping and plasmid analysis to differentiate oyster and clinical isolates concluded that all oyster isolates were virulent. However in our study, we used only environmental isolates since clinical cases have not been reported and thus isolates are not available. It therefore remains to be seen whether the repeat variability associated with the hexanucleotide motif within the coding ORF of *V. vulnificus* genome in our study has any implication as a 'contingency gene' involved in host adaptation and virulence of this organism.

*Vv*1 locus in our study is a part of a gene which is 1953 residues in size, annotated as containing two domains: a tetratricopeptide repeat (TPR) and a von Willebrand factor type A (vWA) domain. While TPR repeats are known to be involved in a variety of functions including protein–protein interactions (D'Andrea and Regan, 2003), vWA is a part of a large multimeric glycoprotein associated with important cellular functions such as blood coagulation in humans (Ginsburg and Bowie, 1992) and microbial adhesion (Bjerketorp et al., 2002). The tandem repeats of the hexanucleotide TGTTGC occur downstream of the TPR repeat and when translated into its amino acid sequence, gives a homopolymeric tract of glutamine repeats (polyQ). Significance of the glutamine homo repeats in *Vibrio* proteins is not clear. However, it is known that polar glutamine repeats are primarily present in proteins that are involved in transcription-translational activities especially proteins interacting with DNA and other proteins (Tanaka et al., 2003) and therefore we assume that the glutamine stretches seen within the gene could be in some way involved with the functional aspects of this gene. Understanding of this gene and the involvement of this gene if any, in the virulence mechanism of this bacterium and the role of glutamine repeats in specific interactions need to be further studied.

This study provides a substantial number of polymorphic loci in *V. vulnificus* genome. The high diversity of polymorphism for the *Vv*1 loci in the present may give us a clue on the degree of pathogenicity or genetic variation in the *V. vulnificus* strains in the west coast of India. It would be interesting to see the effect of copy number on the virulence gene expression in *V. vulnificus*.

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