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Orientation of mannitol related genes can further differentiate strains of *Vibrio vulnificus* possessing the *vcgC* allele

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Abstract

The bacterium *Vibrio vulnificus* exhibits a high level of strain to strain genetic variation, and can be divided into three biotypes. The main cause of human infections, biotype 1, has been separated into clinical and environmental strains based on either DNA PCR, 16s rRNA sequence, or intergenic spacer regions. The DNA PCR method is based on the *vcg* (virulence correlated gene) locus, with a C-genotype corresponding to clinical isolates and an E-genotype corresponding to environmental isolation. Here we show that C-type strains can be further divided into two subtypes. Strains of the one subtype have been isolated from environmental sources and human cases, while the other subtype has heretofore only been recovered from the environment

Keywords: *Vibrio vulnificus;* mannitol; genotype

Introduction

The Gram-negative halophilic bacterium, *Vibrio vulnificus,* is able to cause grievous wound infections and potentially fatal septicemia in humans [10]. Infections typically result from the consumption and handling of raw or undercooked oysters, with over 85% of infections occurring in males [10]. Infections are primarily associated with individuals that are immunocompromised, diabetic, or have elevated serum iron levels, such as those with liver cirrhosis [16]. This organism is credited by the CDC with causing over 900 infections from 1998 to 2006 [5] and in 2007, 93% of the cases reported by the CDC required hospitalization. *V. vulnificus* carries the highest case fatality rate of any foodborne pathogen [4, 20], with mortality typically over 50% [10].

The species is currently divided into three biotypes, all of which are capable of human disease [1, 3, 16, 21]. Biotype 1 was the first identified and is of the greatest import with regards to human infection [15]. This biotype is subdivided into genotypes, clinical and environmental, by a multitude of methods. One method compares the 16s rRNA sequences of strains and identifies an environmental A-type and a clinical B-type [2, 11, 14]. Another method examines the spacer regions found between the 16S and 23S rRNA genes to evaluate *V. vulnificus* strains [9]. The present study separates *V. vulnificus* isolates into genotypes identified by genetic differences at the *vcg* (virulence correlated gene) site in the *V. vulnificus* genome [20, 24]. Interestingly, we have recently showed that similar differences between the genotypes are not restricted to the *vcg* gene, but occur throughout the genome [19].

A recent National Health Interview Survey estimated the number of Americans with those predisposing health problems that put them at risk for *V. vulnificus* infection to be between 12 and 30 million [17]. With this many high-risk people, one would expect to see far more than the ca. 40 primary septicemia cases that are reported each year [15]. The lack of reported cases can be partially explained by the existence of the two *V. vulnificus* genotypes. The “C-type” with the *vcgC* allele corresponds 90% to clinical isolation, and the “E-type”, containing the *vcgE* allele, corresponds 93% to environmental isolation [20]. *V. vulnificus* cells possessing the C-type DNA sequence are likely more virulent when compared to the *V. vulnificus* population as a whole [15]. Thus, infections may be lower than expected as only some strains of the bacterium are able to cause disease.

Interestingly, it was found that while estuarine water samples contain a mix of almost equal percentages of C-type and E-type cells, the ratio in oysters is 13% C-type to 87% E-type [22]. This finding becomes significant when one considers that most *V. vulnificus* infections originate from the consumption of raw or undercooked oysters [15]. This suggests that the incidence of infection is further decreased because oysters contain fewer of the more virulent C-type strains.

It has been shown that mannitol fermentation is common among C-type strains, but variable in E-types, a phenomenon that was also found to be true with 16S rRNA typing [7-8]. Examination of the complete sequenced genome of *V. vulnificus* strain CMCP6 (GenBank AE016795) revealed an operon (Fig. 1) used in conversion of mannitol to fructose [12]. The genes encoding the IIA domain of the mannitol phosphotransferase system (PTS), mannitol dehydrogenase (*mtlD*), and mannitol operon repressor (*mtlR*) are similar in function and orientation to many species of bacteria that exhibit specific hexitol transport and fermentation [6, 12-13]. Also located in this region, upstream of the mannitol fermentation genes, are two additional genes. These (Fig. 1) code for a putative hemolysin and a TRAP type mannitol transport system [12]. The published genome of *V. vulnificus* strain YJ016 shows a similar genetic arrangement [6], albeit with a 53 amino acid hypothetical protein between the hemolysin and TRAP transporter. As mannitol fermentation has been shown to be correlated with virulence in *V. vulnificus* [7], we decided to study *vcgC* and *vcgE* genotype strains of *V. vulnificus* for the presence of the conserved mannitol operon found in the published clinical isolate genomes [6, 12], to further refine the *vcg* based PCR method of genotyping.

Materials and Methods

Bacterial strains and culture conditions

Fifty-eight *V. vulnificus* strains were used in this study (Table 1), including clinical and environmental isolates and C- and E-genotypes. Strains were grown from freezer stocks overnight in Bacto™ Heart Infusion (HI) broth (BD, New Jersey) or on HI agar plates at 30°C.

DNA extraction for PCR analysis

Bacterial cells were grown overnight in HI broth at 30°C. Cells were centrifuged for five minutes at 16,000 x *g* and the pellet resuspended in phosphate buffered saline (PBS). Cells were lysed by boiling for five minutes and centrifuged again at 16,000 x *g* for five minutes. The supernatants containing the DNA template were used in the PCR reactions.

Mannitol fermentation assay

*V. vulnificus s*trains were streaked onto HI agar plates from freezer stocks and incubated at 30°C for 24 hours. An inoculating needle was used to stab a single colony that was then inoculated into 5 ml of mannitol fermentation broth (16 g of BBL™ Phenol Red broth base [BD] and 1.0% D-mannitol [Sigma Cat. No. M-4124] were added per liter of deionized water then autoclaved at 121° C for 5 minutes). These cultures were incubated at 37° C and examined for mannitol fermentation at 24 and 48 hours.

Strain typing via PCR.

Using the methods developed by Rosche *et al.* [20] and Warner and Oliver [23], each strain was subjected to a multiplex PCR reaction that simultaneously confirmed the isolate as being *V. vulnificus* and identified the

genotype as *vcgC* (C-type) or *vcgE* (E-type). Reactions were performed using GoTaq polymerase (Promega) in a Techne Genius thermal cycler using the parameters suggested by the manufacturer for GoTaq (Promega), but with an annealing temperature of 53°C. PCR products were visualized by gel electrophoresis on 1% agarose gels stained with ethidium bromide.

Arrangement of mannitol fermentation genes

After strain typing, all strains were examined for the presence of the three genes that make up the mannitol fermentation operon (the enzyme II of the phosphotransferase system for mannitol, mannitol specific dehydrogenase, and mannitol operon repressor; Fig. 1). All isolates were also examined, via PCR, for the two genes that are immediately upstream of the mannitol fermentation operon in the previously sequenced, clinically isolated CMCP6 strain. These are a putative hemolysin gene as well as a TRAP-type mannitol transport [12]. The strains were further examined for a fragment of DNA spanning those two genes (Fig. 1, Table 2). Positive PCR results from primers that were located within a gene indicated the presence of the gene, whereas a positive PCR result from primers than spanned two genes indicated that the two genes were adjacent. Primers were designed for each of the lettered areas (A-J) indicated in Fig. 1. PCR was performed as described for strain typing with the annealing temperature and extension time modifications listed with the primer pairs in Table 2.

Statistics

Statistical analyses were performed on the data in Table 1, using Chi-square analysis or Fisher Exact test where appropriate. Analyses were performed using SigmaStat statistical analysis software.

Results

All of the 38 of the C-genotype strains were able to ferment mannitol while only eight out of 20 E-genotype strains exhibited this ability. All strains, regardless of genotype, which were able to ferment mannitol also produced PCR bands representing the three genes of the mannitol fermentation operon (Fig. 1, Table 1).

All 58 tested strains of *V. vulnificus*, regardless of genotype (C or E), had the putative hemolysin gene and the TRAP-type transport gene (Table 4). PCR analysis was subsequently used to clarify the arrangement of the genes under investigation. When PCR reactions performed with primers designed to span these two genes were used, the results varied depending on the genotype and isolation source. While possessing both the TRAP and putative hemolysin genes, none of the 20 E-genotype strains examined were found to have these two genes located adjacent to each other ("published arrangement"; Table 4). In contrast, all but one of the 22 C-type strains that had been isolated from human infections were positive for the gene-spanning fragment. Thus, the hemolysin and TRAP genes were adjacent in these strains, similar to the published genome [12] of the clinically isolated C-type strain (Fig. 1, Table 4). When the environmentally

isolated (oyster or water) C-type strains were examined by the same method, only seven of 16 had these genes adjacent to each other (Table 4).

Discussion

More than 90% of *V. vulnificus* strains recovered from patients are of the C-genotype [20], but this does not necessarily mean that all C-type strains found in the environment are similar or even able to cause disease. Our examination of both clinically- and environmentally-derived C-type strains suggests that the two published *V. vulnificus* genomes [[6, 12]; GenBank AE016795 and GenBank BA000037] may not accurately represent all of the C-type strains, especially those isolated from a water or oyster source (the only published genomes of C-types strains have been from cells isolated from clinical sources). Although all strains (regardless of genotype) were found via PCR to possess both the putative hemolysin and TRAP-type transport genes, many did not share the same arrangement of those genes as seen in the two published *V. vulnificus* genomes (Fig. 1). Not surprisingly, none of the E-type strains were homologous to the available genomic sequence data. Most notably, when testing C-type strains from clinical sources, nearly all were in accord with the published genomic sequences; however many C-type strains of environmental origin have a gene arrangement that is yet unknown.

The human body appears to select for those C-type strains that exist in the environment only when they have genomes similar to the two published *V. vulnificus* genomes. This suggests that the number of *V. vulnificus* strains that are highly virulent is even lower than indicated by possession of the *vcgC* allele. This is somewhat analogous to the situation with *V. parahaemolyticus*, where only a very small percentage of strains in the environment possess the hemolysin genes required to initiate infection, with the latter being found almost exclusively in clinical samples.

A similar phenomenon was uncovered recently by Roig and coworkers [18], who looked at seven environmentally isolated C-type strains of *V. vulnificus.* They found four of these strains to be potential pathogens (having resistance to human serum), while three of the strains were likely non-pathogenic (inhibited by human serum). Coupled with the results of the present study, this suggests that C-type *V. vulnificus* strains may vary enough to warrant a further classification, and can be differentiated using simple PCR.

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Tables and figures

Table 1: Number, genotype, phenotype, and isolation source of strains used in this study.

Type	No. Strains	Genotype	Isolation Source	Mannitol Fermentation	Gene order same as Fig 1
I	21	C-type	Clinical	Yes	Yes
II	7	C-type	Environmental	Yes	Yes
III	9	C-type	Environmental	Yes	No
IV	1	C-type	Clinical	Yes	No
V	1	E-type	Clinical	Yes	No
VI	6	E-type	Clinical	No	No
VII	7	E-type	Environmental	Yes	No
VIII	6	E-type	Environmental	No	No

Table 2. Product predicted by primer pairs employed in this study, with annealing temperatures and extensions times that differ from manufacturer's instructions.

PCR Product (from Fig. 1)	**Annealing Temp. (°C)**	**Extension Time (s)**
A-B	56.1	30
A-D	54.5	90
C-D	54.5	30
E-F	60.8	30
G-H	60.4	30
I-J	54.2	30

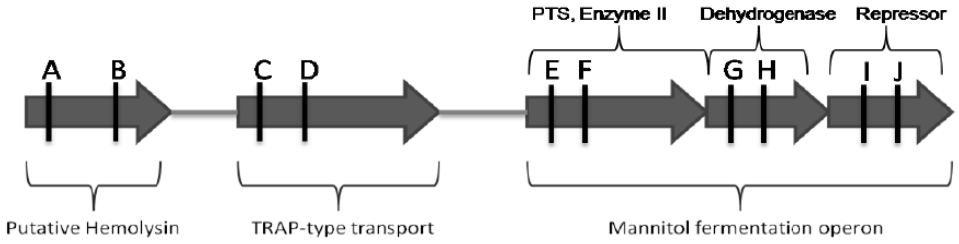
Table 3. Primers used in this study

Primer	Sequence (5' - 3')	Corresponding Location in Fig. 1
Mann Hemo F	ACATTTGACGGATGAGCAG	A
Mann Hemo R	TCCCAGACAAACAGATGATG	B
Mann TRAP F	CGCTGAAGAAATGTCAAACG	C
Mann TRAP R	ACGCATTTTCAACCCTTT	D
Man IIA F	GATGTTGGTGAACAACTTCTCTGC	E
Man IIA R	TCTGAAGCCTGTTGGATGCC	F
Man Dehydro F	CAAAACGCTTTGCCGCTG	G
Man Dehydro R	CAATGGATGGCACTTCGC	H
Man Rep F	CGTCGATGGCTTGGTACAA	I
Man Rep R	TCGGTAAACTCGTATTCTGTG	J

Table 4. PCR results of tested strains. Values represent number of isolates possessing the listed gene (generating a PCR product of the correct size) or gene arrangement (primers overlapping two genes producing a product of the expected size) over the number of strains tested. The "Published arrangement" heading refers to those strains that have the putative hemolysin gene directly upstream of the TRAP-type transport gene, as shown in Fig. 1.

V. vulnificus Isolate	Putative hemolysin	TRAP type transport	Published arrangement
Clinical C-type	22/22	22/22	21/22
Environmental C-type	16/16	16/16	7/16
E-type	20/20	20/20	0/20

Figure 1: Configuration of genes (from *V. vulnificus* CMCP6) examined in this study. Letters A-J represent binding sites for primers listed in Table 3



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