

Cloning Sequencing and Characterization of Lipopolysaccharides Genes of *Vibrio alginolyticus*

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Abstract: Bacterial lipopolysaccharides are the major outer surface membrane components present in almost all Gram-negative bacteria and act as extremely strong stimulators of innate or natural immunity in diverse eukaryotic species ranging from insects to humans. The DNA sequence of the O-antigen biosynthesis cluster of a putative probiotic and pathogenic strain, *Vibrio alginolyticus* has been determined. Here, we report the sequence of the LPS biosynthesis genes, *wzm*, *wzt* and *wbil* and the analysis of the genes using Biology Workbench 3.2. From the study, it shows that the sequences of LPS genes in *V. alginolyticus* are highly homologous to the LPS genes in *Vibrio cholerae* isolates with more 80% homology. However, several variants of the *wbil* sequence have been found in the *V. alginolyticus* isolates compared to the other genes, *wzm* and *wzt*.

Key words: *Vibrio alginolyticus*, lipopolysaccharides, cloning, sequencing, primer, genes

INTRODUCTION

Lipopolysaccharide (LPS) is one of the major components of the bacterial outer membrane (Nikaido and Vaara, 1985). It is composed of lipid A and a core oligosaccharide and in many species of bacteria also has a polysaccharide chain termed the O-specific antigen (Whitfield and Valvano, 1993). The O-antigen is a major contributor to the antigenic variability of the bacterial cell surface. The O-antigen promoted the activation of complement and formation of the complement membrane attack complex away from its site of insertion in the outer membrane (Joiner, 1988). The early research has been focused on the role of LPS as a virulence determinant and on its use as a vaccine candidate. Since that time, studies have expanded to include analysis of the chemistry and biosynthesis of the O-antigenic region due to its immunogenicity, serotype specificity and serum resistance properties.

Therefore, the understanding of its mechanism of synthesis may unravel new targets for antimicrobial therapy that may contribute to the control of infections by gram-negative bacteria. The biosynthesis of LPS is very complex and requires the activity of many genes, most of which are clustered in different regions of the

chromosomal map. In this study, *Vibrio alginolyticus*, as halophilic vibrios (Janda *et al.*, 1988; Wong *et al.*, 1992; Holt *et al.*, 1994) was selected to characterize the LPS biosynthesis genes. Thus, here we report the amplification of LPS biosynthesis genes (*wzm*, *wzt* and *wbil*) in *Vibrio alginolyticus* and comparison of the sequences to the LPS gene sequence in other gram negative bacteria (*Vibrio cholerae*, *Escherichia coli* and *Burghedoria pseudomallei*).

MATERIALS AND METHODS

DNA procedures: Ten isolates of *Vibrio alginolyticus* isolated from the fish and 2 clinical isolates of *Vibrio cholerae* (as a comparison) were used in this study. Chromosomal DNA was isolated from *V. alginolyticus* and *V. cholerae* using Genispin (BST Techlab) followed the manufacturer's instructions.

Primer designing: The primers for *wzm* and *wzt* genes were designed according to the published sequence of *Vibrio cholerae* in the Genbank under accession number X59554 and *wbil* gene from AB012956. The primers were designed using DNASIS (Hitachi Software Corporation) program.

PCR amplification and cloning: PCR amplification was carried out on a DNA thermal cycler PCR System (Biometra-TRIO Thermoblock) using 10 pmol of each primer, 100 ng μL^{-1} of DNA template, 1.8 MgCl₂, 200 μM dNTPs and 1U Taq DNA polymerase (BST). DNA amplification steps performed were initial denaturation at 96°C for 2 min, followed by 35 cycles of amplification steps consisting of denaturation at 94°C for 1 min, annealing at 57°C for 1 min and elongation at 72°C for 2 min. The amplification was ended with a single final extension at 72°C for 7 min. An aliquot of 10 μL of the PCR product mixture was electrophoresed through 1.0% agarose gel. Following amplification, the PCR products (Table 1) corresponding to *wzt*, *wzm* and *wbiI* regions were purified using the QIAquick[®] PCR Purification Kit (QIAGEN, Germany) according to the manufacturer's instructions. The purified PCR product obtained was cloned into 2.1 TOPO TA vector (Invitrogen, USA, cat. No. K370-01) and transformed into TOP10 *E. coli* host strain.

Plasmid preparation, DNA sequencing and analysis:

Small-scale plasmid DNA preparations were performed using QIAprep[®] Spin Miniprep kit (QIAGEN, Germany). The extracted plasmid was digested by EcoRI enzyme (Gibco-BRL Boehringer Mannheim) to confirm the

presence of the insert. The plasmid containing insert was sent for commercial automated sequencing to confirm the DNA target sequence and the orientation of the gene. DNA sequence homologies were analysed by using Biology Workbench 3.2 (<http://workbench.sdsc.edu/CGI/bw.cgi>) and protein sequence databases through the National Center for Biotechnology Information blast network server (Altschul *et al.*, 1997).

Reverse transcriptase-PCR: The preparation of mRNA from the recombinant colonies, were carried out using Micro-FastTrack 2.0 kit (Invitrogen Corp) following the manufacturer's instructions with slight modifications. The isolation of cDNA from both recombinants was carried out using a cDNA Cycle kit (Invitrogen Corp.) following the manufacturer's instructions. Two μL of each cDNA product was subjected to PCR using the primers, which were used to verify the gene by using the same protocol.

RESULTS

PCR amplification: The total genomic DNA of high molecular weight was successfully extracted from *Vibrio alginolyticus* and *Vibrio cholerae* isolates using the Genispin kit (BST Techlab). The Polymerase Chain Reactions (PCR) with specific primers were sensitive enough to detect 100 ng μL^{-1} genomic DNA. This study demonstrates that the *wzm* and *wbiI* genes have been successfully amplified and isolated from *V. alginolyticus* and *V. cholerae*. The amplification of *wzt* and *wbiI* genes produced a single band of 471 (Fig. 1) and 1069 bp (Fig. 2), respectively.

Table 1: Sequence of primers used in the study

Gene	Primer sequence 5'-3'	Product size
<i>wzt</i>	TGGTGGTGTACGGT	433 bp
	GCAAGATTGGTGTTC	
<i>wzm</i>	AAATGGAGAAATGGGAGTAGATG	471 bp
	AAIAGCGAAGC AAAATGGA	
<i>wbiI</i>	CCIACGC IACCICACITTC	1169 bp
	GAACCACAGACCCGATG	

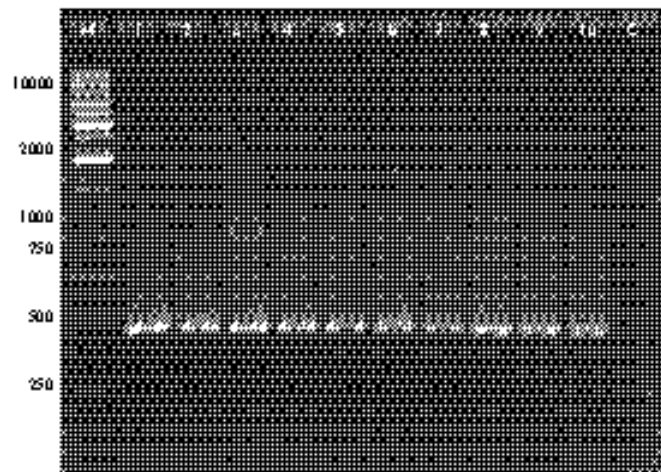


Fig. 1: Amplification of *wzm* gene by PCR. Lanes 1-10, a single band of size 471 bp showing the presence of *wzt* gene, lane C is a negative control and lane M is a 1 kb DNA ladder (Fermentas)

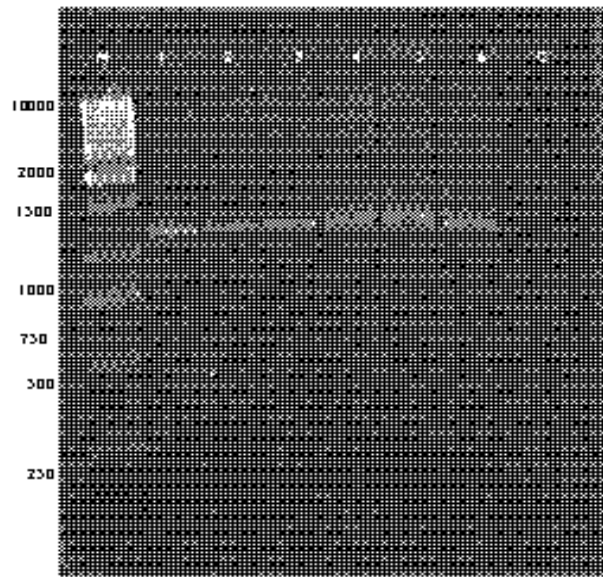


Fig. 2: Amplification of *wbil* gene by PCR. The *wbil* gene positive isolates have a single band present in the region between the ladders of 1000-1500 bp (lanes 1-6). The actual band position is at a 1069 bp. Lane C negative control and lane M is a 1 kb DNA ladder (Fermentas)



Fig. 3: Restriction endonuclease digestion of 2.1 IOPO IA- positive clones. The positive clones show two bands at sizes 3.9 kb and 471 bp after digestion with *EcoR*I. Lane 5 is a negative control that produced band at size 3.9 kb and lane M is a 1 kb DNA marker (Fermentas, Germany)

Cloning and transformation of *wzm* gene: The PCR product containing the *wzm* gene was successfully ligated to 2.1 IOPO IA vector (Invitrogen, USA), a IA cloning system. The recombinant *wzm* gene was transformed into IOP10 and confirmatory PCR (cPCR) was carried out for screening the insert (*wzm*), which were selected randomly. The primers annealed to the inserted DNA and the expected amplified products of 417 bp were obtained. Restriction enzyme analysis was carried out on the plasmid DNA containing the *wzm* gene of interest, using *EcoR*I enzymes. As expected, the positive transformants

produced 2 fragments with the sizes approximately 3.9 kb and 417 bp (Fig. 3). The results indicated that the *wzm* gene had been successfully cloned and transformed into *E. coli* I OP10 host. The positive clones were chosen and sent for the automated sequencing for further confirmation.

Analysis of sequences was performed using The Biology Workbench 3.2 under CLUSTAL W program (Thompson *et al.*, 1994). The *wzm* gene sequence was aligned to the *wzm* gene sequence of *V. cholerae* under accession number X59554 and is shown in Fig. 4. The

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4V305      GCGGCCGCCAGTGTGATGGATATCTGCAGAATTCG-CCCTTAATATGGAGAAATGGGAGTA
5V8        GCGGCCGCCAGTGTGATGGATATCTGCAGAATTCG-CCCTTAATATGGAGAAATGGGAGTA
8V14       -----ATATGGAGAAATGGGAGTA
10V1003    -----ATATGGAGAAATGGGAGTA
8V11       -----ATATGGAGAAATGGGAGTA
9V14       -----AATTCG-CCCTTAATATGGAGAAATGGGAGTA
x59554     ATGTTCTGTACAAATCTCTGTATAGGGCTAATTTGGTCTTTAATATGGAGAAATGGGAGTA
                                           *****

4V305      GATGATTATTTACCATATGTCGGTGTGGACACATAATTTACTTGTTTTATAC TCAAACA
5V8        GATGATTATTTACCATATGTCGGTGTGGACACATAATTTACTTGTTTTATAC TCAAACA
8V14       GATGATTATTTACCATATGTCGGTGTGGACACATAATTTACTTGTTTTATAC TCAAACA
10V1003    GATGATTATTTACCATATGTCGGTGTGGACACATAATTTACTTGTTTTATAC TCAAACA
8V11       GATGATTATTTACCATATGTCGGTGTGGACACATAATTTACTTGTTTTATAC TCAAACA
9V14       GATGATTATTTACCATATGTCGGTGTGGACACATAATTTACTTGTTTTATAC TCAAACA
x59554     GATGATTATTTACCATATGTCGGTGTGGACACATAATTTACTTGTTTTATAC TCAAACA
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4V305      ATTAACGAAAGCACAGGAATCTTCGTTGCAGATGCTCGAGTATACCTAAATGATAGGCAA
5V8        ATTAACGAAAGCACAGGAATCTTCGTTGCAGATGCTCGAGTATACCTAAATGATAGGCAA
8V14       ATTAACGAAAGCACAGGAATCTTCGTTGCAGATGCTCGAGTATACCTAAATGATAGGCAA
10V1003    ATTAACGAAAGCACAGGAATCTTCGTTGCAGATGCTCGAGTATACCTAAATGATAGGCAA
8V11       ATTAACGAAAGCACAGGAATCTTCGTTGCAGATGCTCGAGTATACCTAAATGATAGGCAA
9V14       ATTAACGAAAGCACAGGAATCTTCGTTGCAGATGCTCGAGTATACCTAAATGATAGGCAA
x59554     ATTAACGAAAGCACAGGAATCTTCGTTGCAGATGCTCGAGTATACCTAAATGATAGGCAA
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4V305      CCTTTATGCTTTTCAGTCGGTCTCACATATATCGGAACGTGCTGATTCCTTCATAAT
5V8        CCTTTATGCTTTTCAGTCGGTCTCACATATATCGGAACGTGCTGATTCCTTCATAAT
8V14       CCTTTATGCTTTTCAGTCGGTCTCACATATATCGGAACGTGCTGATTCCTTCATAAT
10V1003    CCTTTATGCTTTTCAGTCGGTCTCACATATATCGGAACGTGCTGATTCCTTCATAAT
8V11       CCTTTATGCTTTTCAGTCGGTCTCACATATATCGGAACGTGCTGATTCCTTCATAAT
9V14       CCTTTATGCTTTTCAGTCGGTCTCACATATATCGGAACGTGCTGATTCCTTCATAAT
x59554     CCTTTATGCTTTTCAGTCGGTCTCACATATATCGGAACGTGCTGATTCCTTCATAAT
*****

4V305      ATACCAACCATATTTTGCTTGCATATGGTCTAGCTCGGCAACTTTGAATTTAGTTTT
5V8        ATACCAACCATATTTTGCTTGCATATGGTCTAGCTCGGCAACTTCGAATTTAGTTCT
8V14       ACACCAACCATATTTTGCTTGCATATGGTCTAGCTCGGCAACTTTGAATTTAGTTTT
10V1003    ATACCAACCATATTTTGCTTGCATATGGTCTAGCTCGGCAACTTTGAATTTAGTTTT
8V11       ATACCAACCATATTTTGCTTGCATATGGTCTAGCTCGGCAACTTTGAATTTAGTTTT
9V14       ATACCAACCATATTTTGCTTGCATATGGTCTAGCTCGGCAACTTTGAATTTAGTTTT
x59554     ATACCAACCATATTTTGCTTGCATATGGTCTAGCTCGGCAACTTTGAATTTAGTTTT
*****

4V305      ATGTTTGTCAATTTCGTTGAGTTTAAAGTCTGTTTTTCGTCCTATTTGCTAGCTATTTTGT
5V8        ATGTTTGTCAATTTCGTTGAGTTTAAAGTCTGTTTTTCGTCCTATTTGCTAGCTATTTTGT
8V14       ATGTTTGTCAATTTCGTTGAGTTTAAAGTCTGTTTTTCGTCCTATTTGCTAGCTATTTTGT
10V1003    ATGTTTGTCAATTTCGTTGAGTTTAAAGTCTGTTTTTCGTCCTATTTGCTAGCTATTTTGT
8V11       ATGTTTGTCAATTTCGTTGAGTTTAAAGTCTGTTTTTCGTCCTATTTGCTAGCTATTTTGT
9V14       ATGTTTGTCAATTTCGTTGAGTTTAAAGTCTGTTTTTCGTCCTATTTGCTAGCTATTTTGT
x59554     ATGTTTGTCAATTTCGTTGAGTTTAAAGTCTGTTTTTCGTCCTATTTGCTAGCTATTTTGT
*****

4V305      GCTGTTATTTCAACGAGATTTAGAGACTTAATCCAGCTGATGGGCTTTTGAATGAGTTA
5V8        GCTGTTATTTCAACGAGATTTAGAGACTTAATCCAGCTGATGGGCTTTTGAATGAGTTA
8V14       GCTGTTATTTCAACGAGATTTAGAGACTTAATCCAGCTGATGGGCTTTTGAATGAGTTA
10V1003    GCTGTTATTTCAACGAGATTTAGAGACTTAATCCAGCTGATGGGCTTTTGAATGAGTTA
8V11       GCTGTTATTTCAACGAGATTTAGAGACTTAATCCAGCTGATGGGCTTTTGAATGAGTTA
9V14       GCTGTTATTTCAACGAGATTTAGAGACTTAATCCAGCTGATGGGCTTTTGAATGAGTTA
x59554     GCTGTTATTTCAACGAGATTTAGAGACTTAATCCAGCTGATGGGCTTTTGAATGAGTTA
*****

4V305      GCTTCTTTTGAAGTCCGTCATGTTGAAAGTAAGCTTTCTACCCGAACAATATCAAAC
5V8        GCTTCTTTTGAAGTCCGTCATGTTGAAAGTAAGCTTTCTACCCGAACAATATCAAAC
8V14       GCTTCTTTTGAAGTCCGTCATGTTGAAAGTAAGCTTTCTACCCGAACAATATCAAAC
10V1003    GCTTCTTTTGAAGTCCGTCATGTTGAAAGTAAGCTTTCTACCCGAACAATATCAAAC
8V11       GCTTCTTTTGAAGTCCGTCATGTTGAAAGTAAGCTTTCTACCCGAACAATATCAAAC
9V14       GCTTCTTTTGAAGTCCGTCATGTTGAAAGTAAGCTTTCTACCCGAACAATATCAAAC
x59554     GCTTCTTTTGAAGTCCGTCATGTTGAAAGTAAGCTTTCTACCCGAACAATATCAAAC
*****

4V305      TATGTGTACATAAATCCATTTGCTTCGCTATT
5V8        TATGTGTACATAAATCCATTTGCTTCGCTATT
8V14       TATGTGTACATAAATCCATTTGCTTCGCTATT
10V1003    TATGTGTACATAAATCCATTTGCTTCGCTATT
8V11       TATGTGTACATAAATCCATTTGCTTCGCTATT
9V14       TATGTGTACATAAATCCATTTGCTTCGCTATT
x59554     TATGTGTACATAAATCCATTTGCTTCGCTATT
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Fig. 4: Alignment of *wzm* genes by Biology Workbench 3.2. The nucleotide analysis by CLUSTAL W (multiple sequence alignments) shows that the vector system had altered the base sequence of the insert

alignments showed that the wzm gene sequence obtained were highly homologous (97-99%). On the other hand, the sequence that obtained from wbiI gene was compared to the sequence in *V. cholerae* from the database (AB012956). The alignments shows that the wbiI gene sequences in *V. alginolyticus* were 81-82% homologous compared to *V. cholerae* sequence.

DISCUSSION

In this study, the LPS biosynthesis genes which are wzt, wzm and wbiI were amplified in *Vibrio alginolyticus* isolates and *Vibrio cholerae* as a control. The wzm and wzt genes which are similar to a subfamily of ATP-binding cassette (ATP) transporter involved in the export of polysaccharides. ABC-2 transport systems are composed of 2 proteins, an inner membrane protein (wzm) and a cytoplasmic ATP-binding protein (wzt). Two subunits of each protein comprise a functional ABC-2 transport system (Fath and Kolter, 1993). On the other hand, the wbiI gene, which is the last gene in the type II O-PS (O antigenic polysaccharide) biosynthetic gene cluster (DeShazer *et al.*, 1998). This protein is required for the biosynthesis of surface polysaccharide in numerous bacterial species and may function as nucleotide sugar epimerase or dehydratase. The wzm gene was successfully amplified with the product of a size 471 bp and cloned into 2.1 TOPO TA vector. Analysis of sequencing for this gene using Biology Workbench 3.2 showed that the sequence was highly homologous (99%) to *V. cholerae* published sequence (X59554). From the study, it was found that the sequences of wzt and wzm genes are similar to the particular genes in *V. cholerae* isolates. Therefore, it can be concluded that the LPS biosynthesis genes within the genus are same. Furthermore, in Fig. 1 (Artemis software), the location of genes (wzm and wzt) in *V. alginolyticus* was assumed according to the database of *V. cholerae*. However, the sequence of the gene (wzt) in other family such as *S. marcescens* (AF038816), *P. aeruginosa* (U63722), *B. pseudomallei* (AF064070), *Synechocystis* (PCC6803), *K. pneumoniae* (L41518) were different, even though the name and the functional of the gene is the same (ABC-transporter). According to DeShazer *et al.* (1998) and Reeves (1992) wzm gene was also successfully detected in mRNA samples by RT-PCR. The wbiI gene was also successfully amplified by PCR, suggesting that wbiI is essential for type II O-PS biosynthesis because it is the last gene in the type II O-PS biosynthesis gene cluster.

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