Abstract

The gram-negative bacterium *Vibrio vulnificus* is a deadly seafood-borne pathogen whose virulence has been associated with expression of the capsule, a mucoid extracellular polysaccharide. The capsule of clinical strain MO6-24 is a tetrasaccharide comprised of three L-\(N\)-acetylquinovosamine (L-\(\text{QuiNAc}\)) residues and one \(N\)-acetylgalactosamineuronic acid (GalNAcA) residue. Eight genes in the capsule polysaccharide operon code for enzymes responsible for modifying these residues before polymerization. The remaining four enzymes are responsible for sequentially transferring the four sugar residues to a \(C_{55}\) isoprenoid membrane anchor, bactoprenyl phosphate (BP). The functional characterization of these enzymes is essential to determine their role in capsule biosynthesis. In this study, the function of the sugar-modifying enzymes will be established. The enzymes WbjB, RmlD, and WbjD are proposed to convert uridine diphosphate-\(N\)-acetylglucosamine (UDP-GlcNAc) to UDP-L-\(\text{QuiNAc}\). The enzymes WbpP and WecC, as well as WbfT and WbfV, and the multi-function enzyme WbfY are believed to act in parallel to convert UDP-GlcNAc to UDP-GalNAcA. Functional assays that take advantage of the UV absorbance of the nucleotide-linked sugars will be used to characterize the function of the UDP-GalNAcA producing enzymes. Assays will be used to determine kinetic parameters for the UDP-\(\text{QuiNAc}\) producing enzymes. These products will be isolated using high-performance liquid chromatography and characterized by mass spectrometry and multi-dimensional NMR to delineate the roles of these enzymes in capsule synthesis. Understanding this metabolic pathway will be an important step in illuminating the biosynthesis of the capsule of *V. vulnificus*. 
Research Proposal

A. Background

*Vibrio vulnificus* is a gram-negative bacterium found in raw shellfish that poses serious health risks. It is an opportunistic pathogen that causes more seafood-borne fatalities in the US than any other bacterium, and has recently been implicated in outbreaks of necrotizing fasciitis.\(^1\)\(^-\)\(^2\)

Virulence has been linked to expression of a capsule polysaccharide (CPS).\(^3\) The CPS is a mucoid layer expressed on the exterior surface of a bacterium, and is thought to aid in defense against the host immune reaction.\(^4\)\(^-\)\(^5\) The CPS of clinical strain MO6-24 had previously been isolated and characterized as a repeating tetrasaccharide composed of three L-\(N\)-acetyl-quinovosamine (L-QuiNAc) residues and one \(N\)-acetyl-galactosamineuronic acid (GalNAcA) residue, as seen in figure 1.\(^6\) The genetic cluster responsible for the expression pathway of the CPS had also previously been deduced in the MO6-24 strain of *V. vulnificus*.\(^7\) Function of those genes in producing the polysaccharide was proposed by knocking out the CPS operon resulting in reduced expression of the polysaccharide.\(^8\) Yet the role of individual enzymes in the expression pathway is not well understood.

![Figure 1: Tetrasaccharide subunit of the *V. vulnificus* CPS (strain MO6-24).](image-url)
There are twelve genes thought to be responsible for the biosynthesis of this tetrasaccharide monomer. Eight of these are thought to code for sugar-modifying enzymes. Three of these enzymes, WbjB, RmlD, and WbjD, are proposed to catalyze the transformation of uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) to UDP-L-QuiNAc, and general function of WbjB and RmlD has previously been shown by members of the Troutman lab. Five of these enzymes, WbpP, WecC, WbfT, WbfV, and WbfY, are proposed to catalyze the transformation of UDP-GlcNAc to UDP-GalNAcA. Interestingly, the enzymes WbpP and WecC are homologous to WbfT and WbfV respectively, indicating redundancy in the biosynthetic pathway. Furthermore, the enzyme WbfY is thought to catalyze both the dehydration and the epimerization reaction as well, converting UDP-GlcNAc to UDP-GalNAcA. The proposed scheme for these modifications is shown in figure 2. After modification, the monosaccharide residues are then sequentially transferred to a bactoprenyl phosphate (BP) C_{55} isoprenoid membrane anchor by the remaining four enzymes: the initiating hexose-1-phosphate transferase WbfU, as determined by another member of the Troutman lab, and the glycosyltransferases: RfaG, WbuB, and GTB.

Figure 2: Proposed metabolic pathway of the eight sugar-modifying enzymes of the CPS operon.
Since the modification of these sugars and the sequential transfer to the BPP are essential first steps for synthesis of the CPS, the characterization of these biosynthetic enzymes will elucidate mechanisms for virulence. This study will seek to characterize the function of each of those sugar-modifying enzymes by isolating and characterizing products, determining substrate specificity, and defining kinetic parameters.

**B. Aims**

**Specific Aim 1:** Expression and isolation of *V. vulnificus* CPS operon enzymes

The twelve genes in the CPS operon have been successfully cloned into plasmid vectors, creating a genomic library of this operon. These genes have been transformed into *Escherichia coli* cell lines for enzyme overexpression. Ten of these enzymes have been overexpressed and isolated. In addition to this, the primary sequence of the multi-function enzyme WbfY shows that there are likely four hydrophobic transmembrane domains in the tertiary structure, giving rise to potential solubility issues.9 Two constructs of this gene have been built: one full-length enzyme and one truncated version with the four transmembrane domains removed. These two separate constructs will be overexpressed as well to probe function.

**Specific Aim 2:** Characterization of the CPS sugar-modifying enzymes

Preliminary function of four of the sugar-modifying enzymes has been shown: the epimerases WbpP and WbfT, as well as the dehydratase WbjB and the reductase RmlD. Function of the remaining four enzymes WecC, WbfV, WbfY, and WbjD will be determined either by separating reaction components via capillary electrophoresis and monitoring UV absorption of the nucleotide-linked sugars or continuous 96-well plate assay tracking fluorescence of NAD(P)H. Differing temperature, pH, and buffer conditions will be used to determine optimal conditions
for these enzymes. Once the reaction conditions have been determined, substrate specificity will be probed using different nucleotide-linked monosaccharides. Kinetic parameters will be determined for the first two enzymes producing UDP-QuNAc: WbjB and RmlD. Catalytic efficiency (k_cat/K_m) of these enzymes will be determined by first determining the Michaelis constant (K_m), and the turnover rate (k_cat).

**Specific Aim 3: Isolation and characterization of reaction products**

The products of the reactions of WbpP and WecC, or another WecC-like enzyme, will first be isolated via high-performance liquid chromatography (HPLC). New methods will be developed to resolve some structurally similar nucleotide-linked sugars. After sufficient quantities of these products are isolated, they will be characterized using mass spectrometry (MS) and structures determined using multidimensional nuclear magnetic resonance (NMR) spectroscopy. These isolated nucleotide-linked sugars will be used as substrates to probe the function of other enzymes in the sugar-modifying pathway.

**Specific Aim 4: Determination of the order of the CPS metabolic pathway**

Once these enzymes are characterized and products isolated, several enzymatic reactions will be combined to determine the order and function of the enzymes in the sugar-modifying pathway. Specifically, NAD(P) turnover or recycling will be examined to see if coupling the oxidation of UDP-GalNAc by WecC and reduction of UDP-4-keto-6-deoxyhexopyranose by RmlD leads to increased activity. Understanding the order of reactions in this pathway is essential to deliniating the overall nature of capsule metabolism. This will also lead to the development of a one-pot multi-enzyme biosynthesis of UDP-GalNAcA and UDP-L-QuNAc, two important substrates that will be necessary for future projects probing the function of glycosyltransferases.
C. References