Biosynthesis and Assembly of the Vi Antigen Capsule produced by *Salmonella enterica* serovar Typhi

by

Sean Daniel Liston

A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Doctor of Philosophy
in
Molecular and Cellular Biology

Guelph, Ontario, Canada

© Sean Daniel Liston, April, 2018
Capsules are bacterial cell-surface structures composed of hydrated capsular polysaccharides (CPS). The human pathogen Salmonella enterica serovar Typhi produces the ‘Vi antigen’ CPS, which contributes to virulence. This CPS is a component of some current vaccines. Vi antigen is delivered to the cell surface by an ATP-binding cassette (ABC) transporter. CPS ABC transporters participate in heteroligomeric protein complexes, that are proposed to form enclosed translocation conduits to the cell surface. This Thesis describes the identification of Vi antigen biosynthesis genetic loci in the Burkholderiales that are paradoxically distinguished from the S. Typhi by encoding a predicted pectate lyase I named VexL.

Biochemical analyses of a representative VexL from Achromobacter denitrificans demonstrated that it is a Vi antigen-specific endo-lyase enzyme. A 1.2-Å crystal structure of the VexL-Vi antigen complex revealed determinants of specificity and features which distinguish common secreted catabolic pectate lyases from VexL, which participates in cell-surface assembly. When introduced into S. Typhi, VexL localized to the periplasm and degraded nascent Vi antigen. In contrast, a cytosolic derivative had no effect unless Vi antigen export was disrupted. This provides the first evidence that CPS assembled using ABC transporters are periplasm-exposed during translocation.

Vi antigen-producers lack enzymes to build a terminal glycolipid residue that is conserved in all other ABC transporter-dependent CPS assembly systems. VexL was used to depolymerize Vi antigen to facilitate isolation of intact Vi antigen chain termini. Mass spectrometry revealed Vi antigen oligosaccharides with a reducing terminal N-acetylhexosamine residue decorated with two β-hydroxyacyl chains. The VexE protein, which is uniquely encoded by Vi antigen biosynthesis loci, shares similarity with acyltransferases from lipid A biosynthesis. A ΔvexE mutant of S. Typhi produced Vi antigen with altered
physical properties; its export was impaired, the glycan was not attached at the cell surface, and the glycolipid was not identified. Biochemical assays demonstrate that VexE is a UDP-N-acetylglucosamine C6 β-hydroxyacyltransferase that prefers 14-carbon acyl chains. The structure of the terminal glycolipid dictates a unique assembly mechanism and has potential implications in pathogenesis, immunology, and vaccine production. This thesis provides novel insight into mechanisms for CPS production conserved in diverse and clinically-relevant bacteria.
ACKNOWLEDGEMENTS

To Dr. Chris Whitfield and the Whitfield Lab Team, thank you for your mentorship, enthusiasm, and inspiration in both science and life. Thank you to my advisory committee members Drs. Janet Wood and Joseph Lam for your advice and for providing references in grant applications. Thank you to my family and my friends for your comradery and support during my degree.
TABLE OF CONTENTS

List of Tables................................................................................................................ix
List of Figures................................................................................................................x
Abbreviations..............................................................................................................xii

CHAPTER 1: Introduction..............................................................................................1

1.1 Preface....................................................................................................................1
1.2 Gram-negative cell envelope................................................................................1
1.3 ABC transporters..................................................................................................3
1.4 Lipopolysaccharides.............................................................................................5
1.5 Capsular polysaccharides.....................................................................................11
  1.5.1 Biosynthesis of “group 1” CPS employing Wzx flippases..............................12
  1.5.2 Biosynthesis of “group 2” CPS employing KpsMT ABC transporters...........15
  1.5.3 The virulence capsular polysaccharide, a group 2 CPS variant....................20
1.6 Research objectives..............................................................................................22

CHAPTER 2: Materials and Methods...........................................................................23

2.1 Molecular Biology.................................................................................................23
  2.1.1 Cloning.............................................................................................................23
  2.1.2 Generation of chromosomal mutations in S. Typhi........................................23
  2.1.3 Genome sequencing of Achromobacter denitrificans....................................24

2.2 Bioinformatic analyses.........................................................................................24

2.3 Protein purification...............................................................................................25
  2.3.1 Purification of VexL and mutant derivatives....................................................25
  2.3.2 Purification of VexE and mutant derivatives....................................................26
  2.3.3 Purification of LpxA.........................................................................................27
  2.3.4 Purification of holo-acyl carrier protein..........................................................28
  2.3.5 Purification of acyl-ACP synthetase...............................................................29
2.4 Structural Biology .................................................................................................................................30
  2.4.1 Crystallization of VexL .........................................................................................................................30
  2.4.2 Crystallographic data collection, refinement, and analysis .................................................................30

2.5 Examination of protein stabilities .........................................................................................................31
  2.5.1 Melting temperature determination ....................................................................................................31
  2.5.2 Circular dichroism spectroscopy .........................................................................................................31

2.6 Polysaccharide purification and characterization ....................................................................................31
  2.6.1 Purification of cell-associated Vi antigen from S. Typhi .................................................................31
  2.6.2 Purification of secreted Vi antigen from S. Typhi ΔvexE ..................................................................32
  2.6.3 Enzyme digestion of purified Vi antigen ............................................................................................33
  2.6.4 Gel filtration chromatography of purified Vi antigen .........................................................................33
  2.6.5 Chemical de-O-acetylation of purified Vi antigen ...............................................................................33
  2.6.6 Digests of Vi antigen by VexL .............................................................................................................34
  2.6.7 Isolation of the Vi antigen glycolipid terminus ...................................................................................34
  2.6.8 High-resolution mass spectrometry ....................................................................................................34
  2.6.9 Nuclear magnetic resonance spectroscopy of purified polysaccharides ...........................................35
  2.6.10 Purification and mass spectrometry of lipid A ................................................................................35

2.7 Enzyme Assays .......................................................................................................................................36
  2.7.1 Lyase enzyme assay .............................................................................................................................36
  2.7.2 Generation of acyl-ACP donors .........................................................................................................36
  2.7.3 VexE acyltransferase assay .................................................................................................................36
  2.7.4 Characterization of acyltransferase assay product .............................................................................37

2.8 Antibody generation ...............................................................................................................................38
  2.8.1 Generation of VexL-specific polyclonal antibodies ...........................................................................38
  2.8.2 Generation of VexE-specific polyclonal antibodies ...........................................................................38
2.9 Mutant characterization ........................................................................................................39
  2.9.1 Subcellular localization of VexL and mutant derivatives ........................................39
  2.9.2 In vivo lyase-accessibility assay ..........................................................................39
  2.9.3 Bacteriophage-sensitivity assay ..........................................................................40
  2.9.4 Envelope stress response assay ..........................................................................40
  2.9.5 Vi antigen-localization assay ...............................................................................40
  2.9.6 Detection of cell-free Vi antigen in culture supernatants ..................................41
  2.9.7 Examination of spent culture media for VexL activity .........................................41
  2.9.8 Immunofluorescence microscopy .......................................................................41
  2.9.9 High-pressure freezing, freeze substitution, and electron microscopy .............42
  2.9.10 Growth curves ......................................................................................................43

2.10 PAGE and Immunoblotting ..........................................................................................43

CHAPTER 3: A periplasmic depolymerase provides new insight into ABC transporter-dependent
secretion of bacterial capsular polysaccharides ........................................................................45
  3.1 Preface ................................................................................................................................45
  3.2 Rationale ..........................................................................................................................45
  3.3 Results ................................................................................................................................46
    3.3.1 VexL is a pectate lyase homolog conserved in Burkholderiales Vi antigen assembly systems..46
    3.3.2 VexL is a Vi antigen lyase .....................................................................................50
    3.3.3 Structure of VexL ..................................................................................................52
    3.3.4 Vi antigen envelope translocation includes a periplasm-accessible intermediate........60
  3.4 Discussion .........................................................................................................................66
  3.5 Acknowledgements ........................................................................................................70

CHAPTER 4: A unique lipid anchor attaches Vi antigen capsule to the surface of Salmonella enterica
serovar Typhi.................................................................................................................................71
4.1 Preface........................................................................................................................................71
4.2 Rationale.....................................................................................................................................71
4.3 Results.........................................................................................................................................72
  4.3.1 Vi antigen from a ΔvexE mutant has altered physical properties........................................72
  4.3.2 Vi antigen has a unique glycolipid at its reducing terminus.................................................79
  4.3.3 VexE is required for efficient export and cell-surface retention of Vi antigen................81
4.4 Discussion....................................................................................................................................83

CHAPTER 5: VexE is an acyltransferase responsible for assembly of the glycolipid terminus of Vi antigen........................................................................................................................................87
  5.1 Preface.......................................................................................................................................87
  5.2 Rationale.....................................................................................................................................87
  5.3 Results.........................................................................................................................................87
    5.3.1 VexE is homologous to lysophospholipid acyltransferases.................................................87
    5.3.2 VexE is an acyl-ACP-dependent UDP-GlcNAc C-6 β-hydroxyacyltransferase.................91
  5.4 Discussion....................................................................................................................................101

CHAPTER 6: Conclusions....................................................................................................................107
  6.1 A model for ABC-transporter dependent Vi antigen biosynthesis........................................107
  6.2 Future directions........................................................................................................................110
    6.2.1 What is the source of the second acyl chain in the Vi antigen glycolipid terminus?.......110
    6.2.2 What protein is the Vi antigen polymerase?.................................................................112
    6.2.3 What protein is the Vi antigen O-acetyltransferase?....................................................113
    6.2.4 What are the constituents and structure of the Vi antigen biosynthesis complex?........114
  6.3 Closing remarks.........................................................................................................................116

References.......................................................................................................................................117

Appendices.......................................................................................................................................136
LIST OF TABLES

Table 3.1 Crystallographic data collection and refinement statistics.................................................. 53

Table 3.2 Examples of structural homologs of VexL........................................................................ 59

Table 4.1 Chemical shifts observed in $^{13}$C spectra of purified Vi antigens.................................. 79

Table 5.1 $^1$H NMR data (25 °C, D$_2$O)..................................................................................... 100

Table 5.2 $^{13}$C NMR data (25 °C, D$_2$O)..................................................................................... 101

Table A1 Bacterial strains and plasmids......................................................................................... 136

Table A2 Sequences of oligonucleotide primers.......................................................................... 140

Table A3 Species identified in charge-deconvoluted LC-QToF-MS of VexL-digested Vi antigen..... 145

Table A4 Species identified in charge-deconvoluted LC-QToF-MS of S. Typhi Vi antigen glycolipid termini.......................................................... 146
LIST OF FIGURES

Figure 1.1 Conceptual model of the Gram-negative cell envelope..............................................................2

Figure 1.2 Structures of model ABC transporters from LPS biosynthesis in Gram-negative bacteria........4

Figure 1.3 Structures of the lipid components of bacterial glycoconjugates that are substrates for ABC transporters..................................................................................................................5

Figure 1.4 The conserved ‘Raetz pathway’ for Kdo2-lipid A biosynthesis in Gram-negative bacteria.......8

Figure 1.5 Aciyltransferases from Kdo2-lipid A biosynthesis in Gram-negative bacteria..........................9

Figure 1.6 Model for Wzx-dependent CPS assembly in Gram-negative bacteria.......................................14

Figure 1.7 Crystal structure of Wza, the outer membrane translocation channel for the E. coli K30 CPS...15

Figure 1.8 Model for ABC transporter-dependent CPS assembly in Gram-negative bacteria.................17

Figure 1.9 Structure of the MacA-MacB-TolC multidrug-efflux pump.....................................................19

Figure 3.1 Vi antigen biosynthetic genetic loci (viaB) identified in Burkholderiales.................................47

Figure 3.2 VexL is a Vi antigen depolymerizing enzyme.........................................................................48

Figure 3.3 Phylogeny of VexL pectate lyases..........................................................................................49

Figure 3.4 Oligosaccharide profile of VexL reaction products.................................................................51

Figure 3.5 Biochemical characterization of VexL.....................................................................................52

Figure 3.6 Crystal Structure of A. denitrificans VexL-His6 with bound Vi Antigen.....................................55

Figure 3.7 The VexL active site is different from those of classical pectate lyases.....................................57

Figure 3.8 Site-directed replacement of the putative VexL active site residues.......................................58

Figure 3.9 Structural similarity shared by VexL and bacterial pectate lyases........................................60

Figure 3.10 Achromobacter species produce a Vi antigen-like glycan, VexE, and VexL.........................61

Figure 3.11 VexL is located in the periplasm in S. Typhi..........................................................................63

Figure 3.12 Vi antigen is susceptible to degradation by VexL in the periplasm.........................................64

Figure 3.13 The K1 CPS is cytoplasm-exposed during its biosynthesis...................................................65

Figure 4.1 S. Typhi ΔvexE mutant Vi antigen possesses altered physical properties.................................73

Figure 4.2 Mutation of the putative catalytic histidine residue of VexE does not affect folding of the protein...............................................................75
Figure 4.3 Overexpression of VexE does not affect cell growth or lipid A structure.........................76
Figure 4.4 Purification of Vi antigen from S. Typhi ΔwaaG.........................................................78
Figure 4.5 Mass spectra of the Vi antigen glycolipid terminus....................................................80
Figure 4.6 VexE is required for efficient export and surface retention of Vi antigen.......................82
Figure 5.1 Glycolipid substrates for VexE homologs.....................................................................89
Figure 5.2 VexE is homologous to LpxM and PatA, acyltransferases from lipid A and PIM biosynthesis, respectively.........................................................90
Figure 5.3 Site-directed mutagenesis of predicted catalytic residues of VexE.................................91
Figure 5.4 Generation of acyl-ACP donors....................................................................................93
Figure 5.5 VexE is an acyl-ACP-dependent UDP-GlcNAc β-hydroxyacyltransferase....................95
Figure 5.6 VexE prefers UDP-GlcNAc over UDP-GalNAc...............................................................96
Figure 5.7 Mass spectrometry of the VexE reaction product..........................................................97
Figure 5.8 NMR spectroscopy of the VexE reaction product............................................................98
Figure 5.9 Model for VexE activity in assembly of the Vi antigen glycolipid terminus..................105
Figure 6.1 Simplified conceptual model for Vi antigen assembly..................................................109
Figure A1. Supporting MS data for the Vi antigen glycolipid terminus........................................148
Figure A2 Vi-antigen expression data collected in E. coli Top10....................................................149
ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>CPS</td>
<td>Capsular polysaccharide(s)</td>
</tr>
<tr>
<td>CAZy</td>
<td>Carbohydrate-active enzyme</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CMP</td>
<td>Cytidine-monophosphate</td>
</tr>
<tr>
<td>COZY</td>
<td>Correlation spectroscopy</td>
</tr>
<tr>
<td>CV</td>
<td>Column volumes</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPS</td>
<td>Exopolysaccharide</td>
</tr>
<tr>
<td>Gal</td>
<td>α-D-galactose</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetyl-α-D-galactosamine</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetyl-α-D-glucosamine</td>
</tr>
<tr>
<td>GT</td>
<td>Glycosyltransferase</td>
</tr>
<tr>
<td>Heptose</td>
<td>L-glycero-d-manno-heptose</td>
</tr>
<tr>
<td>HexNAc</td>
<td>N-acetyلهexosamine</td>
</tr>
<tr>
<td>HMBC</td>
<td>Heteronuclear multiple bond correlation</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear single quantum coherence</td>
</tr>
<tr>
<td>IMP</td>
<td>Inner membrane protein</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
</tbody>
</table>
LC Liquid chromatography
LPLAT Lysophospholipid acyltransferase
LPS Lipopolysaccharide
MS Mass spectrometry
NBD Nucleotide-binding domain
NDP Nucleotide diphosphate
Neu5Ac \(\text{N-acetylneuraminic acid, sialic acid}\)
NMR Nuclear magnetic resonance
NTA Nitrilotriacetic acid
OMP Outer membrane protein
OPX Outer membrane polysaccharide export
PBS Phosphate-buffered saline
PCR Polymerase chain reaction
PIM Phosphatidyl-\(\text{myo}-\text{inositol}\) mannoside
PVDF Polyvinylidene difluoride
QToF Quadropole time-of-flight
TBE Tris Borate EDTA
TPR Tetrameric peptide repeat
TLC Thin Layer Chromatography
TLR Toll-like Receptor
TMD Transmembrane domain
TOCSY Total correlation spectroscopy
UDP Uridine diphosphate
Und-P Undecaprenyl-phosphate
CHAPTER 1

Introduction

1.1 Preface

1.2 The Gram-negative cell envelope
The Gram-negative cell envelope is composed of two distinct membranes (Fig. 1.1) (reviewed in (Silhavy et al., 2010)). The inner membrane is a cytoplasm-containing glycerophospholipid bilayer that is densely populated with proteins responsible for metabolism and macromolecular trafficking. The outer membrane is atypical in that the inner leaflet is composed of glycerophospholipids and the outer leaflet is composed of predominately lipid A, the membrane-anchoring portion of lipopolysaccharides (LPS). A stress-bearing peptidoglycan layer is contained between these membranes (reviewed in (Vollmer and Holtje, 2004)) and this compartment is known as the periplasm. The outer membrane provides a permeability barrier and is embedded with proteins that mediate transport of select small hydrophilic molecules (Nikaido, 2003). Densely-packed LPS molecules fortify the outer membrane against some antibiotics, detergents, and bile salts (Raetz and Whitfield, 2002). Additionally, some bacteria produce capsules, which are hydrated cell-surface layers composed of high-molecular-weight capsular polysaccharides (CPS) (Reviewed in (Whitfield, 2006)). Glycoconjugates (molecules containing complex carbohydrates) dominate the cell surface and therefore play significant roles in cell-cell and cell-environment interactions.

Sophisticated molecular machines are responsible for assembly and trafficking of cell envelope and secreted macromolecules while maintaining the membrane permeability barrier. Multi-protein complexes drive their secretion, release into the periplasm, and insertion into the outer membrane. These processes often involve inner membrane ATP-binding cassette (ABC) proteins that harness energy from the cytoplasmic ATP pool (Narita, 2011). Examples include SecYEG-dependent protein secretion (Veenendaal
et al., 2004), Bam-dependent assembly of outer membrane β-barrel proteins (Ricci and Silhavy, 2012), Lol-dependent transport of outer membrane lipoproteins (Narita and Tokuda, 2017), and Lpt-dependent transport of LPS (Bowyer et al., 2011; Okuda et al., 2016; Sperandeo et al., 2017; 2011). It is currently unknown how glycerophospholipids are transported to the outer membrane, but an ABC transporter-dependent system that facilitates retrograde transport of phospholipids from the outer leaflet has been identified (Abellón-Ruiz et al., 2017; Ekiert et al., 2017; Malinverni and Silhavy, 2009; Thong et al., 2016). Like these macromolecules, CPS require dedicated biosynthesis and envelope translocation machinery. The research described in this thesis focuses on CPS assembly systems that employ ABC transporters. This introductory Chapter provides a review of these CPS-assembly systems and other bacterial glycoconjugate assembly machinery that have informed current models.

**Figure 1.1 Conceptual model of the Gram-negative cell envelope.** Glycerophospholipids are illustrated as yellow circles with attached fatty acids (zigzags). Glycose residues are represented by hexagons. OMP, outer membrane protein; IMP, inner membrane protein.
1.3 ABC transporters

ABC transporters are found in all domains of life and transfer a remarkable range of substrates into or out of cells (Jones and George, 2013; Locher, 2016). They are classified into distinct subfamilies, but they share a common structural architecture that includes two transmembrane domains (TMD) that form the transport channel and two cytoplasmic nucleotide-binding domains (NBD) that drive the export process at the expense of ATP hydrolysis. NBD share conserved motifs essential for binding and hydrolysis of ATP and for interaction with their cognate TMD. NBD form dimers that sandwich two ATP-binding sites and, in the catalytic cycle, ATP hydrolysis drives the monomers apart. These dynamic transitions separate or push together the TMD via α-helices that interact with the NBD. This converts the transport channel between ‘closed’ and ‘open’ conformations (Fig. 1.2). In bacterial glyco-ABC transporters, each TMD is composed of 6 transmembrane α-helices, although up to 10 have been reported in some examples. The sequences and structural folds of the ABC transporters vary, consistent with the diversity of substrates and direction of transport across the inner membrane. Structures have been solved for several importers and exporters in a range of conformations and ligand-bound states. Despite shared structural features, it is apparent the actual translocation mechanisms are more diverse (Locher, 2016). These aspects, and limitations in interpretation of available structures, are the subject of a recent review (Locher, 2016). Despite the diversity in the oligo- and polysaccharide substrates for glyo-ABC transporters, all characterized examples appear to share a lipid-based and membrane-bound terminal structure. This lipid can be a part of the complete glycan product or represent an intermediate that is removed after transport. Although there are many glycan substrates with diverse chemistries, the essential lipid components represent a limited range of chemical structures (Fig. 1.3).
Figure 1.2 Structures of model ABC transporters from LPS biosynthesis in Gram-negative bacteria. (A) Structure of apo-MsbA, the lipid A-core ABC transporter, in an inward-facing conformation (PDB 3B5X, *Vibrio cholerae*). MsbA is a ‘half-transporter,’ the NBD and TMD are encoded in a single polypeptide (B) MsbA with bound AMP-PNP (pink sticks) in the outward-facing conformation (PDB:3B5Y, *Salmonella enterica* serovar Typhimurium). (C) Structure of the Wzm (TMD)-Wzt (NBD) complex, a putative OPS transporter (PDB: 6AN7; *Aquifex aeolicus*). Wzt contains an additional C-terminal carbohydrate-binding domain that is not shown; the carbohydrate binding-domain was crystallized separately and relative positions in the complex are speculative. In A and B, the α-helices in one TMD are colored in rainbow to illustrate the rearrangement in the TMDs between the closed (A) and open (B) conformations. Both TMD are coloured in C. MsbA does not have an obvious transmembrane channel, that is evident between Wzm subunits in C. This may highlight differences in the mechanism between these ABC transporters.
1.4 Lipopolysaccharides.

LPS are glycolipids that represent a characteristic component of Gram-negative outer membranes. LPS are composed of three conceptual structural components: lipid A, a core oligosaccharide (core), and sometimes an O antigenic polysaccharide (OPS) (Fig. 1.1). Lipid A forms the conserved membrane anchoring component of the molecule, and also has endotoxic properties. LPS are potent immunostimulatory molecules that are recognized by host Toll-like receptors (Alexander and Rietschel, 2016; Bryant et al., 2010; Raetz, 2002; Diks et al., 2016). Lipid A is a di-phosphorylated, tetra-acylated, N-acetylglucosamine disaccharide (Raetz and Whitfield, 2002; Whitfield and Trent, 2014). This structure and the biosynthetic enzymes are essential and conserved in most Gram-negative bacteria (Fig. 1.4), although some bacteria can
survive without LPS (e.g. *Neisseria meningitidis* (Steeghs et al., 1998) and *Acinetobacter baumannii* (Powers and Trent, 2017)) and viable *E. coli* mutants that produce only the tetra-acylated intermediate (lipid IV\(\alpha\)) have been identified (Meredith et al., 2006).

The lipid A biosynthesis system has been extensively characterized and explored as a target for antibacterial development (reviewed in (Raetz and Whitfield, 2002; Whitfield and Trent, 2014)). Lipid A biosynthesis begins with LpxA, a cytoplasmic acyl-acyl carrier protein (ACP)-dependent acyltransferase that transfers a \(\beta\)-hydroxymyristate to C-3 of UDP-GlcNAc (Anderson et al., 1988). LpxA has a \(\beta\)-helical structure with a substrate-binding pocket ‘ruler’ that confers acyl-chain-length specificity ([Fig. 1.5A](#)) (Williams and Raetz, 2007). This reaction is at equilibrium and favours the products (Anderson and Raetz, 1987a); pathway flow is therefore driven by LpxC, an \(N\)-deacetylase that irreversibly removes the acetate from \(\beta\)-hydroxymyristoyl-UDP-GlcNAc in the second (and now committed) step (Jane E Jackman et al., 1999). LpxC has an \(\alpha/\beta\) topology that is unique from human \(N\)-deacetylases (Coggins et al., 2003; Whittington et al., 2003) and multiple potent inhibitors of this enzyme have therefore been developed (Ding et al., 2018). LpxD is an acyl-ACP-dependent acyltransferase that \(N\)-acylates the LpxC product (Bartling and Raetz, 2008). LpxD shares a similar \(\beta\)-helical structure with LpxA ([Fig. 1.5B](#)) (Masoudi et al., 2014).

The remaining steps occur at the cytoplasm-membrane interface due to the localization of the hydrophobic diacyl product. LpxH (Babinski et al., 2002a; 2002b) or in some cases LpxI (Metzger and Raetz, 2010) are pyrophosphatases that remove UMP from UDP-diacyl-GlcN. The sequences and structures of LpxH (Cho et al., 2016) and LpxI (Metzger et al., 2012) are distinct. LpxB is the ‘disaccharide synthase’ which links a de-phosphorylated product of LpxH/LpxI with the phosphorylated product of LpxD (Metzger and Raetz, 2009). LpxB forms a domain-swapped dimer, where each monomer contains a glycosyltransferase domain with a GT-B fold (Bohl et al., 2018); GT-B is one of two major conserved glycosyltransferase folds that are composed of two \(\alpha/\beta/\alpha\) Rossmann-like domains connected by a linker (Lairson et al., 2008). LpxK is a kinase which possesses an unusual \(\alpha\)-helical membrane-interaction domain, and employs ATP to phosphorylate the tetra-acyl-GlcN-disaccharide at C-4’; the product is also known as lipid IV\(\alpha\) (Emptage et al., 2012; 2014; Garrett et al., 1998). WaaA is a GT-B-fold glycosyltransferase that sequentially transfers
two 3-deoxy-α-D-manno-oct-2-ulosonic acid (Kdo) residues from cytidine monophosphate (CMP)-Kdo to the LpxK product (Clementz and Raetz, 1991; Schmidt et al., 2012). This modification is required by the membrane-bound acyl-ACP-dependent acyltransferases that follow. These acyltransferases are ‘secondary’ in that they acylate lipid IVₐ at the β-hydroxyl group of the GlcN-linked acyl chains, creating acyl-oxy-acyl chains on GlcN’ (Clementz et al., 1996; 1997). These enzymes are homologous to glycerophospholipid-acyltransferases (LPLAT) (Six et al., 2008) and their structures do not resemble LpxA or LpxD (Fig. 1.5C) (Dovala et al., 2016). LpxL transfers a laurate residue from lauroyl-ACP to the β-hydroxymyristate at C-2’ (Clementz et al., 1996) forming a penta-acyl product that is then acylated by LpxM, which transfers a myristate residue from myristoyl-ACP to the β-hydroxymyristate at C-3’ (Clementz et al., 1997). These biosynthetic steps complete the ‘Raetz pathway.’ The Kdo₂-lipid A product is extended by glycosyltransferases that build the core oligosaccharide (core) prior to transport (reviewed in (Christian R H Raetz, 2002; Holst, 2011; Whitfield and Trent, 2014)). Lipid A-core is specifically modified in some bacterial pathogens for immunomodulatory effects on the host immune system; this is reflected as diversity in addition or removal of acyl chains, sugar, and non-sugar components (reviewed in (Needham and Trent, 2013; Trent, 2011)). Recent glycoengineering work has explored altering lipid A structure to produce cell-based vaccines with dramatic differences in immune responses (Needham et al., 2013).
Figure 1.4 The ‘Raetz pathway’ for Kdo₂-lipid A biosynthesis in Gram-negative bacteria. Responsible enzymes are labelled in blue and substrates/products in green. Figure was adapted from (Whitfield and Trent, 2014).
**Figure 1.5 Acyltransferases from Kdo2-lipid A biosynthesis in Gram-negative bacteria.** (A) Panel depicts cartoon representation of the *E. coli* LpxA structure (PDB ID: 1LXA). LpxA is composed predominately of a left-handed β-helix; the homo-trimer is shown as a cartoon (wheat) with one protomer coloured in rainbow from N-(blue) to C-terminus (red). (B) Panel depicts cartoon representation of the *E. coli* LpxD structure (PDB ID: 1LXA). The homo-trimer is shown as a cartoon (wheat) with one protomer coloured in rainbow from N-(blue) to C-terminus (red). The three acyl-ACP in the complex are coloured green. *Inset* depicts acyl-ACP binding site; the phosphopantetheine arm of holo-ACP and bound β-hydroxymyristate are shown as sticks. (C) Panel depicts cartoon representation of the *A. baumannii* LpxM structure (PDB ID: 5KN7).

Structural diversity within LPS molecules (produced by different isolates) increases with distance from the conserved lipid A component. Bacteria produce a limited number of core structures; five have been identified in *E. coli*, for example (Caroff and Karibian, 2003). The core is composed of predominately Kdo and L-glycero-d-manno-heptose (heptose) residues, and this structure is important for outer membrane integrity (Raetz and Whitfield, 2002). The core is added at the cytoplasmic face of the inner membrane and then the complete lipid A-core is flipped to the periplasmic face by the ABC transporter MsbA; mutants in
*msbA* accumulate lipid A-core at the cytoplasmic face of the inner membrane (Doerrler et al., 2001; Polissi and Georgopoulos, 1996).

OPS are high-molecular-weight glycans that are linked to the core oligosaccharide in some LPS. These glycans were classically characterized by serotyping, and >180 have been identified in *E. coli* isolates alone (Orskov et al., 1977; Stenutz et al., 2006). The incredible structural diversity in OPS is proposed to have arisen from diverse selective pressures on these surface-exposed molecules (Raetz and Whitfield, 2002). OPS are generally important for serum complement resistance, although their role sometimes differs between glycan structures and chain lengths (Comstock and Kasper, 2006; Joiner, 1988). OPS are synthesized in dedicated biosynthetic pathways on the conserved polyisoprenyl-phosphate (und-P) lipid carrier (Fig. 1.3). This glyco-conjugate is then flipped to the periplasmic face of the inner membrane, in a process that often employs an ABC transporter. This ABC transporter is composed of two copies of the TMD Wzm, and NBD Wzt. The first atomic-resolution structure of a putative Wzm-Wzt ABC transporter has recently been reported, although the precise glycan structure is unknown (Fig. 1.2C) (Bi et al., 2018). In the N-linked protein glycosylation system, the lipid tail of und-PP interacts with the transporter TMD, and remains membrane-embedded during the transport cycle (Locher, 2016; Perez et al., 2015). Structural rearrangements in the TMD expose conserved arginine residues in the ‘outward-facing’ conformation of the transporter that are proposed to interact with the pyrophosphate moiety of und-PP and drive flipping of the glycan across the inner membrane (Perez et al., 2015). The Wzm-Wzt complex, however, contains a direct channel within the TMD, which suggests the flipping mechanism for OPS may differ (Fig. 1.2C) (Bi et al., 2018). OPS are transferred from und-P to LPS-core in the periplasm, by the glycosyltransferase enzyme WaaL (Han et al., 2012). The completed lipid A-core-OPS are then delivered to the outer membrane by the dedicated Lpt machinery (Whitfield and Trent, 2014). This system employs another ABC transporter to extract completed LPS from the inner membrane for Lpt-dependent delivery to the cell surface (Ruiz et al., 2008; Sperandeo et al., 2007).
1.5 Capsular polysaccharides

Selective pressures have led to enormous diversity in CPS chemical structures, which is reflected as variation in monosaccharide constituents, linkage arrangements, and non-sugar decorations. For example, more than 80 CPS structures have been identified in \textit{E. coli} isolates alone (Whitfield, 2006), and these provide influential prototypes for CPS assembly models in other bacteria. CPS aid in resistance to desiccation (Ophir and Gutnick, 1994), allow some commensals to influence homeostasis of the host immune system (Avci and Kasper, 2010), mediate host interactions by plant symbionts (Müller et al., 2009; Sharypova et al., 2006), and are receptors for bacteriophages. CPS are well-established virulence factors; they reduce the efficacy of host immune responses by minimizing and/or masking complement deposition at the cell surface (Crawford et al., 2013; Wilson et al., 2011), sequestering cationic antimicrobial peptides of the humoral immune system (Spinosa et al., 2007), and by masking surface microbe associated molecular patterns such as LPS and flagellin (Marshall and Gunn, 2015; Taylor and Roberts, 2005). Some CPS mimic host glycans, such as sialic acid, hyaluronan, chondroitin, and heparosan, and so are poor immunogens (Cress et al., 2014).

Despite structural diversity of the CPS glycan, the protein machinery for their assembly and secretion is conserved. CPS assembly systems are classified based on mode of translocation across the inner membrane (Whitfield, 2006). In Gram-negative bacteria this step facilitated by either a multidrug/oligosaccharidyl-lipid/polysaccharide export flippase (Wzx) (reviewed in (Hong et al., 2018)), or an ATP-binding cassette (ABC) transporter (reviewed in (Willis and Whitfield, 2013a)). These systems are often referred to by their classification in \textit{E. coli} as “group 1” (Wzx-dependent) or “group 2” (ABC transporter-dependent) (Whitfield, 2006) and this work focuses on the latter. Current models for group 2 CPS assembly draw heavily on structural data uniquely available for group 1 CPS, so both systems are discussed here.
1.5.1 Biosynthesis of “group 1” CPS employing Wzx flippases

The prototype for Wzx-dependent CPS biosynthesis is provided by *E. coli* serotype K30 and the early steps in biosynthesis are essentially identical to Wzx-dependent OPS biosynthesis (Raetz and Whitfield, 2002). These glycans are synthesized as individual repeating units on und-P (Fig. 1.3) at the cytoplasm-membrane interface (Whitfield, 2006). A polyprenol phosphate glycosyltransferase enzyme initiates the synthetic pathway (Eichler and Imperiali, 2018). For *E. coli* K30 CPS, this is performed by WbaP, which transfers galactose-1-phosphate from UDP-galactose to form und-PP-Gal; this reaction is conserved in several glycoconjugates, CPS serotypes, and bacterial species (Drummelsmith and Whitfield, 1999; Saldias et al., 2008). Cytoplasmic glycosyltransferases synthesize a single repeat unit on und-PP-Gal (Drummelsmith and Whitfield, 1999). Individual und-PP-linked repeat units are then flipped to the periplasmic face of the inner membrane by the MOP protein Wzx (Islam and Lam, 2013); in the equivalent OPS biosynthesis pathways, wzx mutants accumulate und-PP linked repeat units at the cytoplasmic face of the inner membrane (Feldman et al., 1999; Liu et al., 1996; Marolda et al., 2004). Periplasmic und-PP-linked repeat units are polymerized by the inner membrane Wzy polymerase (Woodward et al., 2010); wzy mutants do not produce high molecular weight CPS (Drummelsmith and Whitfield, 1999).

CPS export employs a heteroligomeric protein complex including inner-membrane polysaccharide co-polymerase (PCP) and outer-membrane polysaccharide export (OPX) proteins (Fig. 1.6) (Cuthbertson et al., 2009). PCP proteins are diverse group of inner membrane proteins that modify glycan polymerization, and for CPS they provide adaptors for the polysaccharide export apparatus (Cuthbertson et al., 2009; Morona et al., 2000; 2009). These proteins possess N- and C-terminal transmembrane α-helices that flank an extended periplasmic domain. PCP proteins regulate the modal length of OPS formed in Wzy-dependent processes by modulating the activity of the Wzy polymerase, potentially through direct protein-protein interactions and/or interactions with the glycan product (Collins et al., 2017; Kalynych et al., 2015; 2011). The PCP protein Wzc forms a tetrameric complex in synthetic membranes (Collins et al., 2006). Wzc contains a cytosolic tyrosine autokinase domain with a tyrosine-rich C-terminal tail that serves as its substrate (Wugeditsch et al., 2001). Kinase activity of Wzc is opposed by the phosphatase, Wzb; together
they modify the phosphate load on Wzc allowing for production of high-molecular-weight CPS (Paiment et al., 2002; Wugeditsch et al., 2001). The structure of the tyrosine kinase domain (Bechet et al., 2010) and Wzb (Hagelueken et al., 2009) have been solved, but their precise role in CPS biosynthesis has not been resolved.

The OPX protein Wza forms an octameric α-helical barrel that embeds in the outer membrane, and an extended periplasmic domain (Fig. 1.7) (Dong et al., 2006; Drummelsmith and Whitfield, 2000; Ford et al., 2009; Nesper et al., 2003). Mutants of the OPX protein, Wza, do not produce CPS, which suggests the presence of regulatory circuitry that detects properly assembled CPS translocation machinery (Drummelsmith and Whitfield, 1999). The isolated Wza octamer possesses a water-filled lumen that is closed to the periplasm (Dong et al., 2006). Wza binds to Wzc, forming a periplasm-spanning structure (Collins et al., 2007). Conformational changes that occur in this complex are proposed to mediate opening of the periplasmic end of Wza to allow the CPS to access the channel (Collins et al., 2007). Site-specific photo-activated crosslinking studies have trapped CPS within the lumen of Wza, supporting the proposal that Wza does provide the translocation channel to move CPS from the periplasm to the cell surface (Nickerson et al., 2014). The final component, Wzi, is an outer membrane lectin that contributes to cell surface association of the CPS after export (Bushell et al., 2013).
Figure 1.6 Model for Wzx-dependent CPS assembly in Gram-negative bacteria. CPS repeating-units are assembled from NDP-activated glycosyltransferase enzymes at the cytoplasm-membrane interface that act on an und-PP lipid carrier. Lipid-linked repeating-units are flipped across the inner membrane by the MOP protein Wzx. Repeating-units are linked together at the non-reducing end, by the Wzy polymerase in the periplasm. The inner membrane PCP protein Wza interacts with the outer membrane OPX channel, and the nascent CPS transits the cell envelope within this protein complex. Auto-kinase activity of the C-terminal domain of Wzc is opposed by phosphatase (P-ase) activity of Wzb; these activities are required for high-molecular-weight CPS synthesis. Wzi is an outer-membrane lectin implicated in surface association of the secreted CPS.
Figure 1.7 Crystal structure of Wza, the outer membrane translocation channel for the *E. coli* K30 CPS. Cartoon depicts the *E. coli* Wza octamer, with one protomer coloured in rainbow from N-(blue) to C-terminus (red). PDB ID: 2J58.

1.5.2 Biosynthesis of “group 2” CPS employing KpsMT ABC transporters

In *E. coli* prototypes, such as the α-(2→8)-linked N-acetylaneuraminic acid (Neu5Ac; sialic acid) CPS produced by serotype K1, the reducing terminus of the final glycan product is linked to a lysophosphatidylglycerol (lysoPG) residue (Willis et al., 2013) (Fig. 1.3). This lipid serves as an acceptor during glycan synthesis. However, it is uncertain whether the lyso-derivative is the authentic acceptor, or if the physiological acceptor is PG with a single acyl chain being lost later in assembly or during CPS purification. The lysoPG moiety is linked to the reducing terminus of the CPS via an oligosaccharide composed of 5-9 Kdo residues in the uncommon β-configuration (Ovchinnikova et al., 2016a; Willis et al., 2013). CPS synthesis begins with KpsS, a β-Kdo glycosyltransferase that transfers a single Kdo residue from a CMP-Kdo donor to lysoPG (Willis and Whitfield, 2013b). A second CMP-Kdo-dependent polymerizing glycosyltransferase, KpsC, then extends the KpsS product with multiple β-Kdo residues (Ovchinnikova et al., 2016a; Willis and Whitfield, 2013b). The sequences of KpsS and KpsC share motifs and predicted secondary structures with a newly defined family of β-Kdo glycosyltransferases (GT99),
whose prototype is an OPS polymerization-terminating enzyme from *K. pneumoniae* O12 (Ovchinnikova et al., 2016b). KpsC possesses duplicated glycosyltransferase domains and these are responsible for the alternating β-(2→7) and β-(2→4) linkages in the product (Ovchinnikova et al., 2016a). The same poly-Kdo linker has been identified in *E. coli* K1, K5, and *N. meningitidis* serogroup B (Willis et al., 2013) and a wide range of bacteria possess genetic loci for CPS assembly that encode homologs of KpsS and KpsC (Willis and Whitfield, 2013a). Examples from human pathogens include extraintestinal pathogenic *E. coli* (ExPEC), *N. meningitidis*, *C. jejuni*, *Haemophilus influenzae*, *Kingella kingae*, *Pasteurella multocida*, and *Moraxella nonliquefasciens*. The Kdo-containing glycolipid is therefore considered to be a unifying feature of ABC transporter-dependent CPS assembly systems, with only a single exception currently known (see 1.4.3). The serotype-specific CPS glycan is assembled by glycosyltransferases that extend the poly-Kdo linker. In *E. coli* K1, CPS synthesis requires KpsS and KpsC to produce the linker and, NeuE and NeuS proteins (Andreishcheva and Vann, 2006). NeuE is proposed to add the first Neu5Ac residue of the serotype-specific domain to the oligo-Kdo, creating an acceptor for the NeuS polymerase (Willis and Whitfield, 2013b).

The group 2 CPS ABC transporters from different serotypes of *E. coli* are virtually identical in sequence and are functionally exchangeable between isolates (and species) that produce different CPS structures (Cuthbertson et al., 2010). The transporters are composed of two copies each of the TMD (KpsM) and NBD (KpsT) (Bliss and Silver, 1996; Nsahlai and Silver, 2003; Pigeon and Silver, 1994). There are no structures available for these transporters to inform models for secretion. Deletion of *kpsM* or *kpsT* results in accumulation of intracellular CPS near the cytosolic membrane (Bronner et al., 1993; Nsahlai and Silver, 2003; Pavelka et al., 1994), likely reflecting the linkage of the glycan substrate to the membrane-bound lysoPG. The lack of glycan-specificity is interpreted as reflecting the use of the conserved lysoPG-poly-Kdo terminus of the CPS as an export signal. This is potentially supported by the inability to export non-lipidated CPS, which is synthesized on an unknown acceptor in a low-frequency off-pathway reaction in the absence of KpsS or KpsC (Bronner et al., 1993; Vimr et al., 1989; Willis et al., 2013). However, this phenotype must be interpreted with care since it could also reflect loss of the coupling of synthesis and
export due to the absence of KpsC or KpsS, which are important components in the known multi-protein CPS biosynthesis/export complex (McNulty et al., 2006; Steenbergen and Vimr, 2008).

Figure 1.8 Model for ABC transporter-dependent CPS assembly in Gram-negative bacteria. The figure illustrates systems from an *E. coli* group 2 prototype and *S. Typhi* Vi antigen at different conceptual states in the CPS envelope translocation process. CPS glycans are assembled from NDP-activated glycosyl residues by cytosolic glycosyltransferase enzymes at the cytoplasm-membrane interface, prior to recognition and export by the ABC transporter. Binding and hydrolysis of ATP by the cytoplasmic NBD protein dimer drives conformational changes in the TMD that power secretion of CPS across the inner membrane. The transporter is proposed to engage the outer membrane OPX channel via interaction with a PCP adaptor.
The working model, which is strongly influenced by bacterial tripartite multidrug-efflux pumps (Fitzpatrick et al., 2017; Willis and Whitfield, 2013a), includes a continuous export pathway that would exclude a periplasm-exposed CPS intermediate (Fig. 1.8). The export-translocation complex presumably allows the ABC transporter to drive the entire process, while the OPX and PCP components provide a passive conduit that has no selectivity for a particular glycan. OPX homologs exhibit cognate recognition of their interacting PCP partners (Larue et al., 2011). Cryo-EM structures have been reported for group 2 PCP homologs, revealing oligomers with extended periplasmic domains (Larue et al., 2011) that resemble (at least at a superficial level) the assemblies formed by purified drug pump adaptor proteins (Fig. 1.9) (Fitzpatrick et al., 2017; Jeong et al., 2016; Symmons et al., 2009). However there is no structure available for any OPX homolog from an ABC transporter-dependent group 2 CPS assembly system and their role is inferred from structure-function data for OPX proteins from group 1 assembly systems (Fig. 1.6, 1.7). As a result, the comparisons are still tentative. The overall surface delivery mechanism for ABC transporter-dependent CPS differs significantly from lipid A export where additional ATP-driven steps are required following the export step mediated by MsbA (Okuda et al., 2016). However, more work is required to establish the key structure-function details. For example, it is unknown whether the acyl chain(s) of the lipid terminus pass through the ABC transporter lumen with the rest of the CPS substrate. Post-export, the terminal lipid may be instrumental in linking CPS to the cell surface, although outer membrane surface properties participate in CPS association as well (Fresno et al., 2006).
Figure 1.9 Structure of the MacA-MacB-TolC multidrug-efflux pump. The inner-membrane ABC transporter (MacA) interfaces with the outer-membrane porin (TolC) through interactions with the inner membrane-anchored adaptor (MacB). This figure depicts model of the multiprotein complex assembled from single particle cryo-electron microscopy (Fitzpatrick et al., 2017; PDB: 5NIK).
1.5.3 The virulence capsular polysaccharide, a group 2 capsule variant.

*Citrobacter Freundii* and *Salmonella enterica* serovar Typhi produce a CPS also known as the virulence capsular polysaccharide (Vi antigen) (Snellings et al., 1981; Virlogeux et al., 1995). Like other CPS, Vi antigen is involved in defense against the host immune system by masking cell-surface microbe associated molecular patterns (Keestra-Gounder et al., 2015), reducing serum complement deposition and concomitant clearance by host immune cells (Wilson et al., 2011). The Vi antigen is a polymer of α-(1→4)-linked N-acetylgalactosaminuronic acid (GalNAcA) residues that is nonstoichiometrically O-acetylated at C-3 (Heyns and Kiessling, 1967). This glycan is used in some current vaccines for typhoid fever (Klugman et al., 1987; Szu et al., 1987). The O-acetyl groups are the dominant protective epitope, and their presence is essential for a successful vaccine (Szewczyk and Taylor, 1980). Production of a Vi antigen capsule distinguishes *S. Typhi*, which causes systemic infection, from closely-related *Salmonellae* that cause acute gastroenteritis (Keestra-Gounder et al., 2015). As evidence of the importance of this structure, deletion of Vi antigen assembly genes increases the LD$_{50}$ of *S. Typhi* ~10,000-fold in a murine model of typhoid fever (Hone et al., 1988).

The genetic locus for Vi antigen production (*viaB*) resembles those for group 2 CPS assembly. It encodes homologs of the PCP and OPX translocation proteins and a characteristic ABC transporter (Kolyva et al., 1992; Virlogeux et al., 1995; Wetter et al., 2012). The Vi antigen ABC transporter is encoded by two copies of the TMD, VexB, and NBD, VexC. As with group 2 CPS, deletion of either transporter component abrogates Vi antigen secretion and results in the accumulation of cytoplasmic glycan (Wetter et al., 2012). The remaining gene products are thought to be associated with Vi antigen biosynthesis. TviB and TviC are cytoplasmic proteins involved in production of the sugar nucleotide precursor of Vi antigen, UDP-GalNAcA (Zhang et al., 2006). TviB is a UDP-N-acetylgalosamine (UDP-GlcNAc) C-6 dehydrogenase that oxidizes UDP-GlcNAc to UDP-N-acetylgalactosaminuronic acid (UDP-GlcNAcA). TviC is a UDP-GlcNAcA C-4 epimerase that epimerizes UDP-GlcNAcA to UDP-GalNAcA. The Vi antigen polymerizing enzyme(s) have not been characterized. However, deletion of *tviD* or *tviE* abrogates glycan biosynthesis (Wetter et al., 2012). TviE is homologous to glycosyltransferases in family GT4 of the carbohydrate-active
enzymes (CAZy) database (Cantarel et al., 2009), so it is tempting to speculate TviE is the Vi antigen polymerase. TviD contains predicted tetratrico-peptide repeats, which are commonly implicated as protein-protein or protein-ligand interaction modules (D’Andrea and Regan, 2003) but the sequence offers no clues to any catalytic activity. The protein responsible for O-acetylation of the glycan is unknown, but this activity is likely encoded within the viaB locus as the recombinant viaB gene-cluster is sufficient to drive production of O-acetylated Vi antigen in E. coli (Wetter et al., 2012). It is unknown if this modification occurs prior to polymerization or at the polymer level but O-acetylation does precede export (Wetter et al., 2012).

Unusually, the chromosomes of known Vi antigen producing bacteria do not encode homologs of KpsS or KpsC, which precludes the involvement of the lysoPG-Kdo₆ acceptor used for all other group 2 CPS. The viaB locus additionally encodes VexE; Homologs or proteins with related motifs are absent in other CPS biosynthesis genetic loci. E. coli harbouring a plasmid-encoded viaB locus with a transposon mutation in the vexE gene produced and exported Vi antigen but the product lacked cellular association and most was released into the growth medium (Virlogeux et al., 1995; Wetter et al., 2012). Consistent with this effect, this vexE mutant was not susceptible to lysis by a Vi antigen specific bacteriophage (Wetter et al., 2012). The vexE mutant also accumulated cytoplasmic Vi antigen (Wetter et al., 2012), suggesting the mutation may result in export defects similar to kpsSC mutants in E. coli group 2 CPS systems (Bronner et al., 1993). The Vi antigen produced in the vexE mutant also possessed different physical characteristics; it did not bind to a nitrocellulose membrane and its elution profile from size exclusion chromatography indicated that the glycan may be a different apparent molecular weight (Wetter et al., 2012). VexE shares low-levels of similarity with LpxL, an acyl-ACP-dependent acyltransferase that functions in the biosynthesis of LPS lipid A (Fig. 1.4), leading to speculation that altered acylation of Vi antigen underlies the effect of the vexE mutation. However, no analogous terminal lipid had been identified for the Vi antigen.
1.6 Research objectives

ABC transporter-dependent CPS biosynthesis machines are conserved in diverse Gram-negative bacteria that produce structurally-diverse CPS. All known bacterial polysaccharide assembly systems that employ an ABC transporter also include a glycolipid residue that is linked to the terminus of the repeating-unit glycan substrate. These lipids are essential for ‘flipping’ activity of the transporter, and mutants defective in glycolipid assembly accumulate glycan upstream of the transport step. Characterized CPS assembly systems use the conserved KpsS and KpsC enzymes to build the lysoPG-Kdo<sub>n</sub> terminus that serves as (i) the glycan biosynthesis site, (ii) cytosolic export signal, and (iii) contributes to CPS attachment at the cell surface. I hypothesized that glycolipid termini are a unifying feature of ABC transporter-dependent CPS biosynthesis. The conserved enzymes for glycolipid assembly are absent from the genome of S. Typhi, which produces the Vi antigen CPS, indicating a divergence from the usual mechanism. To address this gap in understanding, the overall goal of this thesis research was to characterize the unusual Vi antigen CPS assembly system and determine the role (if any) for a glycolipid terminus. This was achieved in two Aims:

**Aim 1**: Establish a method to purify and then characterize a putative terminal lipid for Vi antigen, of unknown structure. The structural characterization of this molecule is described in Chapter 4 but was dependent on the discovery of a critical enzyme reagent whose characterization and application are described in Chapter 3.

**Aim 2**: Determine the initial biochemical steps in Vi antigen assembly. This is the focus of Chapter 5 and was achieved through *in vitro* dissection of activities of purified Vi antigen biosynthetic enzymes.
CHAPTER 2
Materials and Methods

2.1 Molecular Biology

2.1.1 Cloning

Oligonucleotide primers were obtained from Sigma-Aldrich and are described in Table A1. DNA fragments were generated by PCR employing primers that introduced restriction sites for use in cloning. PCR products were digested using appropriate restriction enzymes (NEB; Invitrogen) and ligated to digested vector DNA using T4 DNA ligase (NEB). Plasmid pWQ892, which encodes both ACP-His\textsubscript{6} and ACPS cloned from \textit{E. coli} W3110, was generated using previously published methods (Masoudi et al., 2014). Briefly, acp-His\textsubscript{6} and acps were independently amplified and then the purified PCR products were ligated by PCR amplification employing the forward primer for acp and reverse primer for acps. The initial PCR introduced complementary sequence 3′ to acp-His\textsubscript{6} and 5′ to acps that facilitated ligation of the PCR products. The ligated PCR product was gel purified, restriction digested, and ligated into pET28a(+) so that expression of both proteins were under control of the T7 promoter. Site-directed mutations of plasmid constructs were generated using KOD HotStart DNA polymerase (Novagen) with complementary primers containing desired point mutations (described in Table A1), by the QuikChange method (Stratagene). Plasmids and PCR products were purified using the PureLink Quick Plasmid Miniprep Kit and PureLink PCR Purification Kit (ThermoFisher), respectively, according to the manufacturer’s instructions. Plasmid construction employed chemically competent \textit{E. coli} prepared by the rubidium chloride method (Hanahan, 1983). \textit{S. Typhi} was transformed with plasmid vectors or PCR products by electroporation (Dower et al., 1988). All DNA constructs were confirmed by Sanger sequencing at the Genomics Facility, Advanced Analysis Centre, University of Guelph, and are described in Table A2.

2.1.2 Generation of chromosomal mutations in \textit{S. Typhi}.

Mutants were generated in \textit{S. Typhi} \textit{aroC} (H251.1) by λ-red recombinase-facilitated homologous recombination (Datsenko and Wanner, 2000; Datta et al., 2006). Cells were transformed with the temperature-sensitive plasmid pSIM6 (which encodes λ-red recombinase genes), followed by linear PCR
products that were amplified from pKD3 or pKD4, using oligonucleotide primers that introduced homology flanking the gene targeted for deletion (described in Table A2). These PCR products contained \textit{frt}-flanked chloramphenicol- or kanamycin-resistance cassettes and recombinants were selected by growth on LB agar containing 25 µg/mL kanamycin or 17 µg/mL chloramphenicol. Antibiotic-resistance cassettes were removed using the Flp recombinase encoded by pCP20, which has a temperature sensitive replicon (Datsenko and Wanner, 2000). Double mutants were constructed sequentially, after removal of the original antibiotic-resistance cassette and pCP20. This cassette was left in the final step in double mutants, as no polar effects were anticipated. This was confirmed by restoration of wildtype phenotypes by complementation with individually-cloned genes.

\textbf{2.1.3 Genome sequencing of \textit{Achromobacter denitrificans}.}

A 5 ml culture of \textit{Achromobacter denitrificans} CWG1240 was grown for 48 hours in BHI broth at 37 °C and genomic DNA was extracted using a PureLink Genomic DNA Mini Kit (ThermoFisher), according to the manufacturer’s instructions. Genomic sequencing was conducted with the help of Drs. Colin Cooper and Brian Coombes, at the Farncombe Metagenomics Facility (Hamilton, On), using Illumina MiSeq technology. A paired-end assembly was generated using MIRA 4 \{Chevreux:ur\}. The genomic DNA sequence from the \textit{Achromobacter denitrificans viaB} locus was submitted to Genbank (Benson et al., 2017) at accession KT99772.

\textbf{2.2 Bioinformatic analyses.}

Homologs of VexL were identified using a position-specific iterative BLAST search of the non-redundant protein sequence database. Initial hits from \textit{Achromobacter} were selected to generate a VexL-specific BLOSUM matrix, which was used in the second iteration (Altschul et al., 1997). The top 500 hits were selected; hypothetical, predicted, ‘low-quality’, and ‘multispecies’ hits were removed. A multiple sequence alignment was generated using MUSCLE (Edgar, 2004). \textit{Dickeya dadanti} PelA, PelC, and \textit{Bacillus sp.} N16-5 PelA were included in multiple sequence alignments due to structural similarity to VexL, although they did not appear as hits in PSI-BLAST. Conserved blocks for phylogenetic analysis were defined using Gblocks (Castresana, 2000). A Maximum-likelihood phylogram was generated from 100 bootstrapped
datasets using PhyML3.0 (Guindon et al., 2010), and visualized by iTOL (Letunic and Bork, 2016). Surface electrostatics were calculated using the Adaptive Poisson-Boltzmann Solver (Jurrus et al., 2017). The VexL PQR file was generated using PDB2PQR (Dolinsky et al., 2007); pKₐ values were calculated using PROPKA at pH 6.5 (pH of crystallization). Conservation scores for amino acid residues were mapped to the VexL structure using ConSurf (Landau et al., 2005), employing the multiple sequence alignment and phylogram described above. Structural homologs of VexL were identified using PDBBeFold (Krissinel and Henrick, 2004). Figures were generated in PyMol (Schrödinger).

For VexE, TPR-motifs were defined using TPRpred (Karpenahalli et al., 2007). Multiple sequence alignments were generated using Clustal Omega (Sievers et al., 2011), and presented using ESPript (Gouet, 2003). Protein secondary structure was predicted using PSIPred (Jones, 1999). Homology models of VexE were generated by one-to-one threading using the Phyre2 server (Kelley and Sternberg, 2009).

2.3 Protein purification

2.3.1 Purification of VexL and mutant derivatives

For crystallography, VexL was expressed and purified from *E. coli* C43 harbouring pWQ791. Bacteria were grown in 6 L of Lysogeny Broth (Miller, 1972), supplemented with 100 µg/mL ampicillin, and inoculated at 1:100 using an overnight culture. Cultures were grown at 37 °C, until the OD₆₀₀ reached 0.6. VexL-His₆ expression was then induced by addition of L-arabinose to 0.2 % (w/v) and growth was continued for 4 h. Cells were collected by centrifugation at 5 000 × g for 20 min and resuspended in Buffer A (100 mM Tris-HCl, 500 mM sucrose, pH 8.0) at a ratio of 0.07 mL buffer A per 1 OD₆₀₀ unit-equivalent of cells. To release periplasmic contents, the cell suspension was incubated on ice for 5 min, lysozyme and EDTA were added to 100 µg/mL and 1 mM, respectively, and incubation was continued for 20 min. MgSO₄ was then added to a final concentration of 20 mM, and the resulting spheroplasts were removed by centrifugation at 12 000 × g for 20 min at 4 °C. The periplasmic fraction (supernatant) was dialyzed against Buffer B (20 mM Tris-HCl, 300 mM NaCl, 10 mM MgCl₂, pH 7.5), using a 3.5 kDa MWCO dialysis membrane (Spectrum Labs). The dialysate was clarified by centrifugation at 15 000 × g for 15 min. Imidazole was then added to a final concentration of 10 mM, and the sample loaded onto a pre-equilibrated 4 mL immobilized metal affinity
column (Super Ni-NTA; Generon). The column was washed successively with 25 CV column volumes (CV) of Buffer C (20 mM Tris-HCl, 350 mM NaCl, pH 7.5), followed by 25 CV each of Buffer C supplemented with 15 mM and 50 mM imidazole. VexL-His<sub>6</sub> was eluted in 5 CV of Buffer C supplemented with 300 mM imidazole. The eluate was concentrated using a 30 kDa MWCO centrifugal concentrator (Vivaspin 20, Sartorius). The sample was then exchanged into Buffer D (100 mM potassium phosphate, pH 6.0) and further purified using an Äkta Pure FPLC equipped with a HiPrep 16/60 Superdex-S200 HR (GE Healthcare) gel filtration column. Elution was monitored by absorbance at 280 nm and by SDS-PAGE. Column fractions containing VexL-His<sub>6</sub> were pooled, concentrated to 20 mg/mL using a 30 kDa MWCO centrifugal concentrator (Vivaspin 20, Sartorius), and stored at -80 °C. Mutant derivatives were purified from 1 L cultures in the same way. Protein concentration was estimated using the predicted extinction coefficient at 280 nm of 44 725 M<sup>-1</sup>cm<sup>-1</sup> (ProtParam (Wilkins et al., 1999)). To confirm folding of the purified VexL-His<sub>6</sub> and mutant derivatives, melting curves were determined using the Protein Thermal Shift kit (see below). For enzyme assay, VexL and mutant derivatives were purified as above from 1 L LB cultures of <i>E. coli</i> Top10 that were induced with 0.02 % (w/v) L-arabinose.

### 2.3.2 Purification of VexE and mutant derivatives

An overnight culture of <i>E. coli</i> Top10 harbouring pWQ787 was grown in LB supplemented with 100 µg/mL ampicillin. Six liters of LB were supplemented with 100 µg/mL ampicillin and inoculated at 1:100 from the overnight culture. The culture was grown at 37 °C. When the culture OD<sub>600</sub> reached 0.6, L-arabinose was added to 0.02 % (w/v) to induce recombinant protein expression and growth was continued for 4 h. Cells were collected by centrifugation at 5 000 × g for 20 min and stored at 80 °C. The cell pellet was resuspended in 200 mL Buffer D (125 mM Na-HEPES, 30 % (w/v) glycerol, pH 7.5), supplemented with 4 cOmplete protease inhibitor tablets (Roche). Cells were lysed by passing the cell suspension through an Emulsiflex homogenizer (Avestin) at 15 000 psi. Unbroken cells and membranes were removed by centrifugation at 100 000 × g, for 1 h, at 4 °C. Four milliliters of pre-equilibrated Ni<sup>2+</sup>-NTA agarose resin (Qiagen) were added to the clarified lysate, and the mixture incubated on a nutator for 1 h at 4 °C. The Ni<sup>2+</sup>-NTA agarose resin was collected in a gravity flow column. The column was washed with 10 CV Buffer D
supplemented with 20 mM imidazole. VexE-His$_6$ eluted in 5 CV Buffer D supplemented with 300 mM imidazole. The eluate was dialyzed into Buffer E (100 mM HEPES, 50 mM L-arginine, 10 % (w/v) glycerol, pH 7.5) using a 3.5 kDa MWCO dialysis membrane (Spectrum Labs), at 4 °C. VexE-His$_6$ was concentrated to 2 mL using a 30 kDa MWCO centrifugal concentrator (Vivaspin 20, Sartorius). VexE-His$_6$ was then further purified by gel filtration chromatography in Buffer E employing an AKTA Pure FPLC equipped with a HiPrep 16/60 Sephacryl-S200 High Resolution gel filtration column. Fractions containing VexE-His$_6$ were determined by SDS-PAGE, pooled, and concentrated using a 30 kDa MWCO centrifugal concentrator (Vivaspin 20, Sartorius). Yield was ~25 mg (50 mg/mL); protein was stored at ‘80 °C in Buffer E. VexE-His$_6$ concentration was estimated based on the theoretical extinction coefficient at 280 nm of 94100 M$^{-1}$cm$^{-1}$ (ProtParam (Wilkins et al., 1999)).

2.3.3 Purification of LpxA

One liter of LB was supplemented with 50 µg/mL kanamycin and inoculated at 1:100 from an overnight culture of E. coli BL21(DE3) harboring pWQ891, which encodes C-terminally hexahistidine-tagged E. coli LpxA. Cultures were grown with 200 rpm shaking at 37 °C, until OD$_{600}$ reached 0.5. Protein expression was induced by addition of IPTG to 1 mM, and growth was continued for 16 h at 20 °C. Cells were collected by centrifugation for 20 min at 5 000 × g, and stored at ‘80 °C. Cells were resuspended in 25 mL Buffer F (20mM sodium phosphate, 350 mM NaCl, pH 7.0), supplemented with 1 Complete protease inhibitor tablet (Roche), 20 µg/mL RNase A, 20 µg/mL DNase I (Roche), and 10 mM imidazole. Cells were lysed by passing the suspension through a French Pressure Cell at 12 000 psi. Unbroken cells were removed by centrifugation for 20 min at 4 000 × g, 4 °C, and membranes were removed by centrifugation for 1 h at 100 000 × g, at 4 °C. Two milliliters of pre-equilibrated Ni$^{2+}$-NTA agarose (Qiagen) were added to the supernatant, which was incubated on a laboratory nutator for 1 h at 4 °C. The resin was collected in a gravity flow column and washed sequentially with 10 CV Buffer F supplemented with 20 and 50 mM imidazole. LpxA-His$_6$ was eluted in 5 CV Buffer F supplemented with 250 mM imidazole. The eluate was concentrated using a 3 kDa MWCO centrifugal concentrator (Vivaspin 20; Sartorius), then further purified by gel filtration chromatography in Buffer F employing an AKTA Pure FPLC equipped with a HiPrep 16/60.
Sephacryl-S200 High Resolution gel filtration column. Fractions containing LpxA-His$_6$ were confirmed by SDS-PAGE, pooled, concentrated using a 3 kDa MWCO centrifugal concentrator (Vivaspin 20, Sartorius), and stored at 80 °C. LpxA-His$_6$ concentration was estimated based on the theoretical extinction coefficient at 280 nm of 9 190 M$^{-1}$cm$^{-1}$ (ProtParam (Wilkins et al., 1999)).

### 2.3.4 Purification of holo-acyl carrier protein

Six liters of LB were supplemented with 50 µg/mL kanamycin and inoculated at 1:100 with an overnight culture of *E. coli* BL21(DE3) harbouring pWQ892. Cultures were grown with 200 rpm shaking at 37 °C, until OD$_{600}$ reached 0.6. Protein expression was then induced by addition of IPTG to 1 mM, and growth was continued for 6 h at 30 °C. Cells were collected by centrifugation for 20 min at 5 000 × g and stored at 80 °C. Cells were resuspended in 100 mL Buffer G (20 mM Na-HEPES, 200 mM NaCl, 2 mM DTT, 10% (w/v) glycerol, pH 8.0) and lysed by passing the suspension through an Emulsiflex homogenizer (Avestin) at 15 000 psi. The lysate was clarified by centrifugation for 1 h at 100 000 × g, 4 °C. The supernatant was applied, by gravity flow, to a 3 mL Ni$^{2+}$-NTA agarose column (Qiagen) that was pre-equilibrated with Buffer G. The column was washed with 10 CV Buffer G supplemented with 20 mM imidazole. Holo-ACP-His$_6$ co-eluted with holo-ACP synthase in 5 CV Buffer G supplemented with 250 mM imidazole. The eluate was dialyzed into Buffer H (25 mM MOPS, 50 mM NaCl, 2 mM DTT, pH 7.5) using a 3.5 kDa MWCO dialysis membrane (Spectrum Labs) and diluted to 50 mL in Buffer H. Holo-ACP-His$_6$ was separated from holo-ACP synthase by ion exchange chromatography on an Akta Pure FPLC equipped with a 5 mL HiTrap QFF column (GE healthcare). The column was pre-equilibrated with 10 CV Buffer H. The dialysate was then applied at a flow rate of 1 mL/min; flow rate was otherwise maintained at 5 mL/min. The column was washed with 10 CV Buffer H. Elution occurred over a 20 CV gradient of 0 - 50% Buffer J (25 mM MOPS, 1 M NaCl, 2 mM DTT, pH 7.5). Fractions containing holo-ACP were monitored by absorbance at 215 nm and confirmed by SDS-PAGE. These fractions were pooled, dialyzed into Buffer K (25 mM MOPS, 300 mM NaCl, pH 7.5) using a 3.5 kDa MWCO dialysis membrane (Spectrum Labs), then diluted to 50 mL in Buffer K. Four milliliters of pre-equilibrated Thiopropyl-Sepharose 6B (GE Healthcare) was added and incubated with mixing on a nutator at 4 °C for 16 h. The
resin was collected in a gravity flow column and washed with 10 CV Buffer K. Holo-ACP-His<sub>6</sub> eluted in 5 CV Buffer K supplemented with 1 mM EDTA and 25 mM DTT. The eluate was concentrated using a 3 kDa MWCO centrifugal concentrator (Vivaspin20, Sartorius), exchanged into Buffer K using a PD10 desalting column (GE Healthcare), and stored at -80 °C. Holo-ACP-His<sub>6</sub> concentration was estimated based on the theoretical extinction coefficient at 280 nm of 1 490 M<sup>-1</sup>cm<sup>-1</sup> (ProtParam (Wilkins et al., 1999)).

### 2.3.5 Purification of acyl-ACP synthetase

One liter of LB was supplemented with 100 µg/mL ampicillin and inoculated at 1:100 from an overnight culture of *E. coli* Top10 harboring pWQ890, which encodes C-terminally hexahistidine-tagged acyl-ACP synthetase (AasS) from *Vibrio harveyi* (Jiang et al., 2006; Masoudi et al., 2014). Cultures were grown with 200 rpm shaking at 37 °C, until OD<sub>600</sub> reached 0.5. Recombinant protein expression was induced by addition of l-arabinose to 0.02 % (w/v), and growth was continued for 16 h at 20 °C. Cells were collected by centrifugation for 20 min at 5 000 × g, and stored at 80 °C. Cells were then resuspended in 25 mL Buffer L (20 mM Tris-HCl, 350 mM NaCl, 10 mM imidazole, pH 7.5), supplemented with a cOmplete mini protease inhibitor tablet, 20 µg/mL RNase A, and 20 µg/mL DNase I (Roche), and lysed by passing the suspension through a French Pressure Cell at 12 000 psi. Unbroken cells were removed by centrifugation for 20 min at 4 000 × g, 4 °C, and membranes were removed by centrifugation for 1 h at 100 000 × g, 4 °C. Two milliliters of pre-equilibrated Ni<sup>2+</sup>-NTA agarose (Qiagen) were added to the supernatant, which was then incubated with mixing on a nutator for 1 h at 4 °C. The resin was collected in a gravity flow column then washed with 10 CV Buffer L supplemented with 20 mM imidazole. AasS-His<sub>6</sub> was eluted in 5 CV Buffer L supplemented with 250 mM imidazole. The eluate was dialyzed into Buffer M (20 mM Tris-HCl, 10 % (w/v) glycerol, 1 mM EDTA, 100 µM DTT, 0.002% (v/v) Triton X-100, pH 7.5), using a 3.5 kDa MWCO dialysis membrane (Spectrum Labs). AasS-His<sub>6</sub> was concentrated to 7 mg/mL using a 30 kDa MWCO centrifugal concentrator (Vivaspin20, Sartorius), and stored at 80 °C. AasS-His<sub>6</sub> concentration was estimated based on the theoretical extinction coefficient at 280 nm of 66 030 M<sup>-1</sup>cm<sup>-1</sup> (ProtParam (Wilkins et al., 1999)).
2.4 Structural Biology

2.4.1 Crystallization of VexL

Prior to crystallization, purified VexL-His$_6$ (see 2.3.1) was diluted to 13.5 mg/mL and incubated with purified and size fractionated Vi antigen (see 2.6.1-4) at 5 mM (estimated based on the molecular weight of a Vi antigen tetrasaccharide, the most abundant product identified in MS; 1 036.27 g/mol (Fig 3.4A)). This mixture was incubated at 37 °C for 1 h, then centrifuged at 16 000 × g, 4 °C for 10 min prior to crystallization. Sparse matrix screening of the sample was performed using a combination of commercial and in-house crystallization screens. All experiments were set up as sitting drops, at 20 °C, using a Crystal Gryphon robot (Art Robbins) at drop ratios of 1:1 and 2:1 protein-precipitant in 300 and 450 nL total drop sizes, respectively. After 1 day, UV-bright crystals were evident in 1.63 M sodium malonate, 0.1 M BisTris HCl pH 6.5, 0.08 M ammonium citrate, and 3.05 % (v/v) 2-methyl-2,4-pentanediol. Larger crystallisation drop experiments were set up at 1.0 and 1.5 µL, and the mother liquor was refined to 1.58 M sodium malonate, 0.1 M BisTris HCl pH 6.5, 0.13 M ammonium citrate, and 2.5 % (v/v) 2-methyl-2,4-pentanediol. To augment Vi antigen binding, one day prior to data collection, crystals were transferred to a drop of mother liquor supplemented with 10 mM Vi antigen and allowed to rest there overnight.

2.4.2 Crystallographic data collection, refinement, and analysis

Crystals were harvested from Vi antigen-supplemented mother liquor and flash cooled directly in liquid nitrogen, without further addition of cryoprotectant. Data were collected at -173 °C on beamline id23-1 at the European Synchrotron Radiation Facility, with automated data processing (Monaco et al., 2013; Nurizzo et al., 2006). The structure was solved by molecular replacement using Balbes (Long et al., 2008) via the online CCP4 (Winn et al., 2011) server. Automatic model building was performed with Buccaneer (CowtanIUCr, 2006), followed by manual rebuilding after interpretation in Coot (Emsley et al., 2004). At this stage, density was visible to place a trisaccharide of Vi antigen. A library for Vi antigen was generated using PRODRG (Schüttelkopf and van Aalten, 2004) and implemented during model refinement in REFMAC5 (Murshudov et al., 1997) and PDBREDO (Joosten et al., 2014). The structure was validated using Molprobity (Chen et al., 2010) and deposited in the PDB (accession code 6FI2).
2.5 Examination of protein stabilities

2.5.1 Melting temperature determination

Thermal melting curves were determined using the Protein Thermal Shift Kit (ThermoFisher Scientific), employing a StepOnePlus Real-Time PCR System (ThermoFisher Scientific). Purified VexL and mutant derivatives were diluted to 1 mg/mL in Buffer N (20 mM Tris-HCl, 350 mM NaCl, pH 7.5), containing 1 × Sypro Orange dye solution. Reaction volumes were 20 µL. Samples were prepared in quadruplicate in 96-well UV-transparent plates. The StepOnePlus Real-Time PCR instrument was set to melt curve, measuring carboxy-X-rhodamine (ROX) reporter. Ramp speed was standard and ramp mode was continuous; 100 % Step 1 (25 °C, 2 min) and 1 % step 2 (99 °C, 2 min). Protein melting temperatures were calculated as the temperature at the minimum of the negative derivative of fluorescence intensity.

2.5.2 Circular dichroism spectroscopy.

Purified VexE and VexE HI466A proteins were dialyzed into Buffer P (5 mM sodium phosphate, pH 7.0) using 3.5 kDa MWCO dialysis membranes. The proteins were diluted to 0.016 mg/mL in Buffer P. CD spectra were collected on a Jasco J-815 spectropolarimeter, using quartz cuvettes with a 1 mm path length at 20.04°C. Six spectra were collected for each sample, averaged, and the spectrum of 5 mM sodium phosphate was subtracted. Data were processed using Spectra Manager (JASCO).

2.6 Polysaccharide purification and characterization

2.6.1 Purification of cell-associated Vi antigen from S. Typhi

Twelve litre LB cultures were supplemented with 100 µg/mL 2,3-dihydroxybenzoic acid, inoculated at 1:100 from an overnight culture of S. Typhi CWG1238, then incubated with shaking at 200 rpm at 37 °C for 16 h. Cells were collected by centrifugation at 10 000 × g for 20 min. and then lyophilized. Polysaccharides were extracted from lyophilized cells by hot aqueous phenol extraction (Westphal and Jann, 1965). The resulting phases were separated by centrifugation at 5 000 × g for 20 min at 4 °C; the aqueous (upper) phase was collected and dialyzed into H2O using a 3.5 kDa MWCO dialysis membrane (Spectrum labs). LPS was also extracted in this procedure and both LPS and Vi antigen were collected in the pellet after centrifugation of the dialysate at 100 000 × g for 16 h. The original purification from LPS
employed gel filtration of this material on a Sephadex G-200 (GE Healthcare) column (Bio-Rad, 25 mm×750 mm) at a flow rate of 50 µL/min. The elution buffer consisted of 10 mM Tris-HCl pH 8 containing 0.25% (w/v) sodium deoxycholate, 200 mM NaCl, 1 mM EDTA, 0.02% (w/v) sodium azide. Fractions that contained Vi antigen were pooled, dialyzed in to the same buffer without deoxycholate, followed by H₂O, and then lyophilized. LPS and Vi antigen elution were monitored by SDS-PAGE, which were visualized by silver staining and immunoblotting, respectively. This gel filtration protocol proved time consuming and ultimately unnecessary, so the following protocol was developed based on cetrimide precipitation (Wetter, et al., 2012). To remove LPS, the pellet from centrifugation at 100 000 × g was resuspended in 100 mL H₂O, and hexadecyltrimethylammonium bromide was added to 0.2 % (w/v) final. The mixture was incubated on a nutator at room temperature for 20 min. Precipitated Vi antigen was collected by centrifugation at 5 000 × g for 10 min. The pellet was resuspended in 20 mL 100 mM NaCl, and insoluble material was removed by centrifugation at 5 000 × g for 10 min. Ethanol precipitation/aqueous solubilization was repeated three times. The final ethanol precipitate was resuspended in 50 mL acetone, collected by centrifugation at 5 000 × g for 10 min, resuspended in 20 mL fresh acetone, and then dried using a rotary evaporator.

2.6.2 Purification of secreted Vi antigen from S. Typhi ΔvexE

The following methods were adapted from those described by Wetter et al., 2012. Twelve liter LB cultures were supplemented with 100 µg/mL 2,3-dihydroxybenzoic acid, inoculated at 1:100 from an overnight culture of S. Typhi CWG1239 (ΔvexE ΔwaaG::kan) then incubated at 37 °C for 16 h. The culture was grown at 37 °C for 16 h. Cells were removed by centrifugation at 5,000 × g for 20 min. To precipitate Vi antigen from the supernatant, hexadecyltrimethylammonium bromide was added to 0.2 % (w/v), and the suspension was stirred for 20 min. Celite 545AW was then added to 10 g/L and the suspension was stirred for 20 min before leaving it to sediment for 20 min. The resultant supernatant was decanted and discarded. Adsorbed polysaccharide was released from the Celite 545AW by 3 sequential washes with 150 mL Buffer Q (50 % (v/v) ethanol, 500mM NaCl). The majority of the adsorbent was removed by centrifugation at 5000 × g for 10 min between washes. Residual adsorbent was removed by passing the suspension through
a sintered glass funnel. Ethanol was added to 80% (v/v) and the mixture was stirred for 20 min. Precipitated polysaccharide/DNA/RNA was collected by centrifugation at 5000 × g for 10 min. The pellet was resuspended in 20 mL 100 mM NaCl, and insoluble material was removed by centrifugation at 5000 × g for 10 min. Ethanol precipitation/water solubilization was repeated three times. The final ethanol precipitate was resuspended in 50 mL acetone, collected by centrifugation at 5000 × g for 10 min, resuspended in 20 mL fresh acetone, and dried using a rotary evaporator (Buchi Corp).

2.6.3 Enzyme digestion of purified Vi antigen.

Dried material from 2.6.1 or 2.6.2 was resuspended in 50 mL Buffer R (20 mM Tris-HCl, 2 mM MgCl₂, pH 8.0). DNase I and RNase A (Roche) were added to 20 µg/mL, and the solution was incubated at 37 °C for 4 h. Proteinase K (Invitrogen) was added to 20 µg/mL, and the solution was then incubated at 55 °C for 2 h. An equal volume of 20 mM Tris-HCl, pH 8.0-saturated phenol was added, and the mixture was stirred for 30 min. The phases were separated by centrifugation at 5000 × g for 20 min at 4 °C; the aqueous (upper) phase was collected and dialyzed into H₂O using a 3.5 kDa MWCO dialysis membrane (Spectrum labs), and lyophilized.

2.6.4 Gel filtration chromatography of purified Vi antigen

Thirty milligrams of dry material from 2.6.3 was resuspended in 2 mL of water and applied to a Sephadex G-50 superfine column (25 mm×750 mm) coupled to a SmartLine 2300 refractive index detector (Knauer), with a flow rate of 0.6 mL/min aqueous 1% (v/v) acetic acid, 0.4 % (v/v) pyridine. The void volume was collected, concentrated using a rotary evaporator, and lyophilized.

2.6.5 Chemical de-O-Acetylation of Vi antigen

Twenty milligrams of purified Vi antigen from 2.6.4 were resuspended in 1.5 mL of 50% (v/v) aqueous ammonium hydroxide. The mixture was incubated at 37 °C for 24 h. Ammonium hydroxide was removed using a stream of nitrogen gas, and the sample was then lyophilized. The dried material was re-purified by gel filtration chromatography, as in 2.6.4. Fractions containing Vi antigen were concentrated using a rotary evaporator and then lyophilized. The absence of 3-O-acetyl groups was confirmed by NMR (see 2.6.9).
2.6.6 Digestion of Vi antigen by VexL

Reactions were performed in 500 µL volumes and contained 1 mg/mL purified Vi antigen, 100 µg/mL VexL-His₆, and 20 mM Tris-HCl pH 6.5. Reactions mixtures were incubated at 37 °C for 16 h, then dried using a vacuum concentrator (SpeedVac; ThermoFisher Scientific). Samples were resuspended in 100 µL H₂O for LC-MS (see 2.6.8).

2.6.7 Isolation of the Vi antigen glycolipid terminus

Twenty milligrams purified Vi antigen from 2.6.4 was resuspended at 1 mg/mL in 50 mM sodium bicarbonate, pH 7.0. Purified VexL-His₆ from 2.3.1 was added to 100 µg/mL and the reaction mixture was incubated at 37 °C for 5 h. A SepPak C18 cartridge (Waters) was pre-equilibrated with 10 mL acetonitrile and then 10 mL H₂O. The Vi antigen digestion was then loaded into the cartridge. The cartridge was washed with 10 mL water, and bound hydrophobic material was eluted in 70 % (v/v) acetonitrile in water. Eluted material was dried by SpeedVac (ThermoFisher Scientific) and then resuspended in 100 µL 25 % (v/v) acetonitrile in water for LC-MS (see 2.6.8).

2.6.8 High-resolution mass spectrometry

LC-MS experiments were performed at the Mass Spectrometry Facility, Advanced Analysis Centre, University of Guelph. These experiments employed an Agilent 1200 high performance liquid chromatograph interfaced with an Agilent UHD 6530 Q-ToF mass spectrometer. For Vi antigen oligosaccharides, an Agilent Extend-C18 column (50 mm × 2.1 mm, 1.8 µm) was used for separation. For purified glycolipids, an Agilent C18 column (Poroshell 120, EC-C18, 50 mm × 3.0 mm, 2.7 µm) was used. The mobile phase consisted of Solvent A (0.1 % (v/v) formic acid in H₂O), and Solvent B (0.1 % (v/v) formic acid in acetonitrile). The mobile phase program was: 1 min 10 % B, increase to 100 % B over 29 min, 5 min 100 % B, and 20 min re-equilibration. Flow rate was 0.4 mL/min. The electrospray capillary voltage was 4.0 kV. Nitrogen was the drying gas (250 °C, 8 L/min), and the nebulizing gas (30 psi). The fragmentor was set to 160 V. The mass-to-charge ratio was scanned in negative ion mode over 100-3000 m/z (2GHz extended dynamic range). Acquisition rate was 2 spectra/s. ESI TuneMix (Agilent) was used for calibration. Injection volume was 2 µL. Data analysis employed Qualitative Analysis software (Agilent).
2.6.9 Nuclear Magnetic Resonance spectroscopy of purified polysaccharides

Purified Vi antigen is of sufficient molecular weight and viscosity that it interferes with structural analysis by NMR. Therefore, the molecular weight was chemically reduced by solvolysis. Fifty milligrams of purified Vi antigen was resuspended in 500 µL of 100 % trifluoroacetic acid and incubated at 100 °C for 16 h in a sealed glass reaction vessel. Trifluoroacetic acid was then evaporated with a stream of nitrogen gas. Dried material was resuspended in water, and lyophilized twice. NMR experiments were performed at the NMR facility in the University of Guelph Advanced Analysis Center. Ten milligrams of dry trifluoroacetic acid-treated Vi antigen was deuterium exchanged by lyophilizing twice from 99.9% D2O and examined as a solution in 99.96% D2O. NMR spectra were collected at 25 °C on a 600 MHz UltraShield™ spectrometer (Bruker), equipped with a cryoprobe. Data was analyzed using Bruker TopSpin software. Sodium 3-trimethylsilylpropanoate-2,2,3,3-d4 provided an internal standard (δH 0, δC –1.6).

2.6.10 Purification and MS of lipid A

Lipid A was isolated from E. coli BKT09 harboring pWQ284 (vector), pWQ794 (vexE), pWQ795 (lpxL), or pWQ796 (lpxM). Two hundred mL of LB supplemented with 0.2 % (w/v) L-arabinose, 25 µg/mL kanamycin, and 37 µg/mL chloramphenicol, were inoculated at 1:100 from 5 mL overnight cultures. Cultures were incubated at 30 °C until culture OD600 reached 1.0, when cells were collected by centrifugation at 4,000 × g for 20 min. Lipid A was isolated by chloroform-methanol extraction and mild-acid hydrolysis as previously described (Henderson et al., 2013). The resultant dried lipid A was resuspended in 400 µL 4:1 (v/v) chloroform-methanol. This solution was diluted 1:5 (v/v) in 50 % (v/v) aqueous isopropanol, before manual infusion in to a Bruker AmaZon SL ion trap mass spectrometer at the Mass Spectrometry Facility in the University of Guelph Advanced Analysis Centre. MS electrospray capillary entrance and exit voltages were set to 4kV and 140V, respectively. Nitrogen was the drying gas which was supplied at 300 °C. The mass-to-charge ratio was scanned across the range of 700-1800 m/z in negative-ion mode. Data was analyzed using Bruker DataAnalysis 4.2.
2.7 Enzyme assays

2.7.1 Lyase enzyme assay

Lyase activities (EC 4.2.2.-) of VexL and its mutant derivatives were determined by spectrophotometric assay employing a Synergy H1 Hybrid Multi-Mode Reader (BioTek Instruments Inc.) with monochromator set to monitor absorbance at 232 nm. Product concentrations were estimated using the extinction coefficient 5 200 M⁻¹ cm⁻¹ (Albersheim, 1966). Reactions were performed at 37 °C in 100 µL volumes, containing 5 µg/mL VexL (or the appropriate mutant derivative) and 0.5 mg/mL purified Vi antigen in 100 mM potassium phosphate, pH 6.0. Reactions were set up in quadruplicate in 96-well plates (Corning 3635, acrylic UV-transparent flat-bottom). Poly-α(1→4)-GalA was purchased from Sigma-Aldrich (81325Sigma).

2.7.2 Generation of acyl-acyl carrier protein donors

ACP acylation reactions contained 100 µM purified holo-ACP, 0.5 µM purified Vibrio harveyi AasS, 300 µM fatty acid (Sigma; stocked in 100 % ethanol), 100 mM Tris-HCl, pH 7.5, 10 mM ATP, and 1 mM MgCl₂. The reaction volume was usually 1 mL. Reactions were incubated at 37 °C for 1 h, then concentrated using a 3 kDa MWCO centrifugal concentrator (Vivaspin20, Sartorius). Reaction progress was monitored by SDS-free Tris-Glycine PAGE in 2.5M Urea at pH 9.5 (Rock et al., 1981) and typically occurred to completion (Fig. 5.4). Acyl-ACPs were purified and exchanged into Buffer S (25 mM MOPS, 300 mM NaCl, pH 7.5) by gel filtration, at 0.5 mL/min, using an AKTA Pure FPLC equipped with a HiPrep 16/60 Sephacryl-S200 High Resolution column (GE Lifesciences). Fractions containing ACP were identified by absorbance at 215 nm and confirmed by SDS-PAGE. Acyl-ACPs were concentrated to ~20 mg/mL, and stored at 80 °C. Acyl-ACP concentrations were estimated using a theoretical extinction coefficient at 280 nm of 1 490 M⁻¹ cm⁻¹ (ProtParam (Wilkins et al., 1999)).

2.7.3 VexE acyltransferase assay

VexE acylation reactions were typically performed in 20 µL and contained 45 µM UDP-[1−¹⁴C]GlcNAc (ARC0151; 55 mCi/mmol, 0.1 mCi/µL), 100 µM purified acyl-ACP, 10 µM purified VexE, and 100mM Na-HEPES, pH 7.5. Reactions were incubated at 25 °C for 16 h and analyzed by thin layer chromatography
(TLC) and MS. For TLC, 1.5 µL aliquots of reaction mixture were spotted and dried on TLC plates (Fluka Analytical; silica gel on Al foil, 60 Å medium pore diameter). The plates were developed in ethyl acetate-butanol-glacial acetic acid-water (10:10:8:5). After drying, the plates were exposed on phosphor storage screens (Kodak) for 2 d, then imaged using a Personal FX Phosphor Imager (Bio-Rad). For MS, reactions were typically 50 µL and contained 10 mM unlabeled UDP-GlcNAc (Sigma). MS was performed as described in 2.6.8.

2.7.4 Characterization of acyltransferase assay product

To isolate product in amounts sufficient for structural determination, the VexE acylation reaction was scaled up to 20 mL and contained 10 mM UDP-GlcNAc, 100 µM holo-ACP, 0.5 µM AasS, 25 µM VexE, 300 µM β-hydroxymyristate, 10 mM ATP, 1 mM MgCl₂, and 100 mM Na-HEPES, pH 7.5. The reaction was incubated at 25 °C for 2 h. Protein was removed using a 3 kDa MWCO centrifugal concentrator (Vivaspin20, Sartorius); the flow-through was collected and concentrated to 1 mL using a SpeedVac vacuum concentrator (ThermoFisher Scientific). This sample was then purified by HPLC employing an Agilent 1260 Infinity II chromatograph, equipped with a semi-preparative reverse-phase liquid chromatography column (Phenomenex 250 × 10 mm, Synergi 4 µm Fusion-RP 80 Å). Mobile phases were H₂O (A) and acetonitrile (B). Flow rate was 3.4 mL/min. The mobile phase program was 2 % B for 5 min, increase to 80 % B over 20 min, hold 80 % B for 5 min, then re-equilibrate at 2 % B for 10 min. Injection volume was 100 µL. Elution was monitored by absorbance at 254 nm (Agilent VWD 1260). Fractions were dried using a SpeedVac vacuum concentrator (ThermoFisher Scientific). Contents were confirmed by MS, as described in 2.6.8. ~0.5 mg HPLC-purified VexE product (β-hydroxymyristoyl-UDP-GlcNAc) was deuterium exchanged by lyophilizing twice from 99.9% D₂O and examined in a solution of 99.96% D₂O. NMR spectra were collected at 25 °C on a 600 MHz UltraShield™ spectrometer (Bruker), equipped with a cryoprobe. Data were analyzed using Bruker TopSpin software. Sodium 3-trimethylsilylpropanoate-2,2,3,3-d₄ was used as an internal standard (δH 0, δC –1.6).
2.8 Antibody generation

2.8.1 Generation of VexL-specific polyclonal antibodies

Two hundred micrograms of purified VexL-His<sub>6</sub> were diluted in to 750 µL PBS, then emulsified in 750 µL Freund’s incomplete adjuvant (Sigma). This suspension was used to immunize a New Zealand white rabbit at the Central Animal Facility, University of Guelph. Immunization was repeated after two weeks, and serum was collected two weeks later. Blood cells were removed by centrifugation at 1100 × g, for 20 min at 4 °C, and serum was stored at 80 °C until needed. VexL-His<sub>6</sub>-specific antibodies were purified from serum by affinity chromatography, as follows. Three grams of CNBr-Activated Sepharose 4B (GE Healthcare), were hydrated and washed with 750 mL 1 mM HCl, in a sintered glass funnel. Eighteen milligrams of purified VexL-His<sub>6</sub> were diluted into 36 mL Buffer T (100 mM NaHCO<sub>3</sub>, 500 mM NaCl, pH 8.3), mixed with the resin, and incubated with mixing for 16 h at 4 °C. The VexL-His<sub>6</sub>-loaded resin was collected in a sintered glass funnel and remaining unreacted groups were blocked by incubation with 50 mL 100 mM Tris-HCl, pH 8.0, for 2 h at 25 °C. The resin was then sequentially washed three times each with 100 mL Buffer U (100 mM acetic acid-sodium acetate, 500 mM NaCl, pH 4.0), and Buffer V (100 mM Tris-HCl, 500 mM NaCl, pH 8.0). The resin was collected, washed with 50 mL PBS, resuspended in 50 mL serum, and incubated with mixing for 16 h at 4 °C. The resin, with bound antibodies, was collected in a gravity flow column (Bio-Rad), and washed with 50 mL PBS. Antibodies were eluted in 5 mL Buffer W (100mM glycine, pH 2.4). 1 mL fractions were collected; collection tubes contained 60 µL 1.5M Tris-HCl, pH 8.8, and 20 µL 5 M NaCl to immediately neutralize the eluate. Precipitate was removed by centrifugation at 13 000 × g for 10 min at 4 °C and the eluate was concentrated to 1 mL using a 3 kDa MWCO centrifugal concentrator (Vivaspin 20, Sartorius), and stored at 80 °C for later use in immunoblotting.

2.8.2 Generation of VexE-specific polyclonal antibodies

Two hundred micrograms purified VexE-His<sub>6</sub> were diluted in 750 µL phosphate buffered saline, and then emulsified in 750 µL Freund’s incomplete adjuvant (Sigma) and used to immunize a New Zealand white rabbit using the procedure described in 2.8.1. Serum was collected and stored as described.
2.9 Mutant characterization

2.9.1 Subcellular localization of VexL-His\(_6\) and mutant derivatives

*S. Typhi* H251.1 was transformed with pWQ791, pWQ935, or pWQ939. Fifty milliliter LB cultures were supplemented with 100 \(\mu\)g/mL ampicillin and 100 \(\mu\)g/mL 2,3-dihydroxybenzoic acid, and were inoculated at 1:100 from overnight cultures of each transformant. MalE (encoded by pMAL-p2) was used as an internal control for a confirmed periplasmic protein; leaky expression from the *tac* promotor was sufficient for detection of the protein. Cultures were grown at 37 °C until OD\(_{600}\) reached 0.5. Expression of the *vexL* derivatives was then induced with 0.02 % (w/v) final l-arabinose, and growth was continued for 30 min. Cells were then collected by centrifugation at 5 000 \(\times\) g for 20 min at 4 °C. Cells were resuspended in Buffer A (100 mM Tris-HCl, 500 mM sucrose, pH 8.0) at 0.07 mL per 1 OD\(_{600}\) unit-equivalent of cells. The cell suspension was incubated on ice for 5 min. Lysozyme and EDTA were then added to 100 \(\mu\)g/mL and 1 mM, respectively, and incubation was continued for 20 min. MgSO\(_4\) was then added to 20 mM, a total protein sample was taken, and the cell suspension was centrifuged at 12 000 \(\times\) g for 20 min at 4 °C. A sample from the supernatant (i.e. released periplasmic contents) was collected. The pellet (spheroplast fraction) was resuspended in an equal volume of Buffer A and sonicated on ice for 3 min (15 s on, 15 s off). Large debris were removed by centrifugation at 4 000 \(\times\) g for 20 min at 4 °C. A total spheroplast sample was taken from the supernatant. Membranes were then collected by centrifugation at 100 000 \(\times\) g for 1 h at 4 °C and samples of soluble protein and membrane protein were taken from the supernatant and pellet, respectively; these were resuspended in an equal volume of Buffer A.

2.9.2 *In vivo* lyase accessibility assay

Five milliliter LB cultures, were supplemented with 100 \(\mu\)g/mL ampicillin, 0.2 % (w/v) glucose, and 100 \(\mu\)g/mL 2,3-dihydroxybenzoic acid, inoculated with *S. Typhi* H251.1 harbouring either pWQ791, pWQ935, or pWQ939, and grown for 16 h, at 37 °C. Samples corresponding to 1 OD\(_{600}\) unit-equivalent of cells were collected and washed twice with 1 mL sterile PBS, and used to inoculate (at 1:1000) 5 mL fresh growth media without glucose. Cultures were grown at 37 °C until OD\(_{600}\) reached 0.5. Expression of the VexL
derivatives was then induced with the indicated final concentrations of L-arabinose, and growth was continued for 30 min. Samples corresponding to 1 OD$_{600}$ unit-equivalent of cells were collected by centrifugation at 10 000 × g for 1 min, immediately resuspended in SDS-PAGE loading buffer (Laemmli, 1970), and incubated at 100 °C for 10 min. Samples were then used for immunoblotting using Vi antigen-specific antibodies.

2.9.3 Bacteriophage-sensitivity assays

Half of an LB Agar plate was inoculated with $3 \times 10^8$ pfu bacteriophage Vi II (HER#39; Félix d’Hérelle Reference center for Bacterial Viruses, Université Laval, Québec, Canada), or $2 \times 10^9$ pfu bacteriophage K1F (Vimr and Troy, 1985). LB agar plates contained 100 µg/mL ampicillin and 0.02% (w/v) L-arabinose or 0.5 mM IPTG, to induce plasmid-encoded gene expression. Plates were dried at room temperature. Then, 8 µL of stationary-phase cultures were dropped onto the phage-free side of the plate, and the plate was then tipped, allowing the inoculum to run on to the phage-inoculated area. Plates were incubated at 37 °C for 5 h, then imaged using a flatbed scanner (EPSON Perfection 2450 Photo).

2.9.4 Envelope stress response assay

Luciferase assays were performed using S. Typhi and mutant derivatives transformed with pNLP15 (Price and Raivio, 2009). This plasmid contains the spy (Spheroplast protein Y) promoter upstream of the luxCDABE cassette. Cells were inoculated at 1:100 in 5 mL LB then grown until OD$_{600}$ reached 0.5. At this time, 200 µL aliquots of culture were transferred in triplicate into 96 well plates for luminance and OD$_{600}$ determination. Data are presented as luminance counts per second normalized to culture OD$_{600}$; values represent mean ± standard error of three independent experiments.

2.9.5 Vi antigen localization assay

LB cultures of S. Typhi H251.1 and mutant derivatives were inoculated at 1:1000 from an overnight culture, and were grown with shaking at 37 °C until culture OD$_{600}$ reached 0.5. One OD$_{600}$ unit equivalent of cells was collected by centrifugation. Cells were resuspended in phosphate buffered saline (PBS) supplemented with, and without, VexL-His$_6$ (100 µg/mL final). Cell suspensions were incubated at 37 °C for 1 h and then collected by centrifugation. The cells were solubilized in SDS-PAGE loading buffer and analyzed by
Western immunoblotting. To ensure that undigested Vi antigen resulted only from its inaccessibility, aliquots of cells were lysed using a French pressure cell, unbroken cells were removed by centrifugation, and VexL-His$_6$ was added, incubated, and samples analyzed as above.

**2.9.6 Detection of cell-free Vi antigen in culture supernatants**

Fifty milliliter LB cultures were grown at 37 °C until OD$_{600}$ reached 0.5. Cells were then collected by centrifugation at 5,000 × g for 15 min. The supernatant was dialyzed in to H$_2$O using a 3.5 kDa MWCO dialysis membrane. The dialysate was lyophilized, resuspended in 1 mL water, and then examined by western immunoblotting at 50 × culture concentration.

**2.9.7 Examination of spent culture media for VexL activity**

Two hundred and fifty mL cultures of S. Typhi H251.1, harbouring either pBAD24, pWQ791 (VexL-His$_6$), or pWQ935 (VexL$_{24-402}$-His$_6$) were grown as described above. Cells were removed by centrifugation at 5,000 × g for 20 min at 4 °C. Thirty milliliters of the supernatant were passed through a syringe filter (Filtropur S 0.2 µm; Sarstedt), dialyzed against water using a 3.5 kDa MWCO dialysis membranes (Spectrum labs), and lyophilized. Dried material was resuspended in 600 µL H$_2$O, to create a 50 × concentrated sample for SDS-PAGE and immunoblotting using antibodies specific for VexL. To test for VexL activity, 30 microliters of 10 mg/mL purified Vi antigen were incubated with 270 µL of filtered spent culture medium at 37°C for 16 h. The samples were then analyzed by PAGE, which was stained with alcian blue/silver (see 2.11).

**2.9.8 Immunofluorescence microscopy.**

Five milliliter LB cultures were inoculated at 1:100 from overnight cultures and grown at 37 °C until OD$_{600}$ reached 0.5. One A$_{600}$ unit-equivalent of cells was collected by centrifugation at 5,000 × g for 10 min. Cells were washed 3 times in 1 mL PBS and incubated with 100 µL mouse Vi antigen-specific monoclonal antibody (P2B1G2/A9) (Qadri et al., 1990) diluted 1:100 in PBS containing 1 % (w/v) bovine serum albumin (PBS-BSA). The cells were then washed 3 times with PBS and incubated with 100 µL rhodamine red-conjugated Goat anti-mouse IgG (Jackson ImmunoResearch) diluted 1:50 in PBS-BSA. The labeled
cells were then washed 3 times and resuspended in 100 µL PBS. Ten µL of cell suspension was applied to a 2 % (w/v) agarose pad and then imaged on a Zeiss Axiovert 200 microscope using the 100 × objective. Images were processed using Volocity software (PerkinElmer). To permeabilize cell membranes, 1 OD\textsubscript{600} unit-equivalent of cells were fixed in 5 % (v/v) formaldehyde in PBS for 16 h at 4 °C. Cells were washed 3 times and resuspended in 100 µL PBS. Ten µL of cell suspension was applied to poly-1-lysine coated glass slides and incubated at 20 °C for 10 min. The immobilized cells were incubated with 10 µL 0.5 mg/mL lysozyme, 10 mM EDTA, 25 mM Tris-HCl pH 8.0 for 15 min, followed by incubation with 0.1 % (v/v) Triton X-100 in PBS for 15 min. Slides were blocked with PBS-BSA for 15 min, and labeled with 10 µL aliquots of antibodies, mounted in Vectashield, and imaged as above.

**2.9.9 High-pressure freezing, freeze substitution, and electron microscopy.**

* S. Typhi and mutant derivatives were grown in 1 L of LB medium supplemented with 100 µg/mL 2,3-dihydroxybenzoic acid at 37 °C until OD\textsubscript{600} reached 0.4. Cells were collected by centrifugation at 4 000 × g for 5 min then washed with 1 mL fresh growth media. 2 µL of cell culture was mixed with 1 µL of 250 mM sucrose and then immediately frozen using a Leica EM HPM100 high-pressure freezer. Cryofixed cells were transferred to vials containing 1 mL substitution media (1% OsO₄, 0.1% uranyl acetate, in acetone) and placed into a Leica AFS2 freeze substitution unit for substitution under controlled temperatures. Following substitution, cells were washed three times in 100 % HPLC-grade acetone and infiltrated with 10-15% (v/v) Epon 812 in acetone overnight. Samples were further infiltrated with 25 % (v/v) Epon 812 in acetone for 3 h, followed by 50 % (v/v) Epon 812 in acetone overnight. The Epon/acetone mix was exchanged with fresh 50% (v/v) Epon 812 in acetone and the acetone was allowed to evaporate overnight before samples were embedded in 100% Epon 812 and polymerized at 60°C for 48 h. Ultrathin sections were cut using a ultramicrotome (Reichert UltraCut E) and placed on 100-mesh platinum/copper grids for viewing. Ultra-thin sections were negatively stained with 2 % (w/v) uranyl acetate for 7 min, washed with water, and then stained with Reynold’s lead citrate for 3 min. Images were acquired using a FEI Tecnai G2
F20 transmission electron microscope at 200kV coupled to a bottom mount Gatan 4k CCD camera in the Molecular and Cellular Imaging Facility at the University of Guelph Advanced Analysis Center.

2.9.10 Growth Curves

Viable cell counts were performed using *E. coli* Top10 harboring pBAD24 (vector), pWQ787 (VexE), or pWQ789 (VexE<sup>H466A</sup>). Five mL overnight cultures were used to inoculate 100 mL LB medium (in 500 mL flasks), supplemented with 25 µg/mL kanamycin and 37 µg/mL chloramphenicol, to give an initial OD<sub>600</sub> of 0.01. Cultures were incubated at 37 °C with shaking at 200 rpm. L-Arabinose was added to culture media to a final concentration of 0.02 % (w/v) at 2.5 h. Aliquots of cells were removed each hour and dilutions plated on LB-agar supplemented with 25 µg/mL kanamycin and 37 µg/mL chloramphenicol.

2.10 PAGE and Immunoblotting

Whole-cell lysates were prepared by suspending 1 OD<sub>600</sub> unit-equivalent of cells in 100 µL SDS-PAGE buffer (Laemmli, 1970). SDS-PAGE samples were incubated at 100 °C for 10 min, prior to electrophoresis (Tris-Glycine, 10 % (w/v) acrylamide). Proteins were stained with Coomassie Brilliant Blue R-250. For immunoblotting, protein samples were transferred to nitrocellulose membranes (Amersham Protran, 0.45µm). Primary antibodies were murine monoclonal anti-His<sub>5</sub> (Qiagen; diluted 1:3000), murine monoclonal anti-*E. coli* RNA polymerase α (Santa Cruz Biotechnology, sc-101597; diluted 1:2000), murine anti-MalE (NEB; diluted 1:20,000), rabbit anti-OmpA (a gift from Dr. Thomas Silhavy (Harms et al., 1999); diluted 1:3000), or purified polyclonal rabbit anti-VexL-His<sub>6</sub> (this work; diluted 1:3000), polyclonal rabbit anti-VexE-His<sub>6</sub> (this work; diluted 1:3000). Secondary antibodies were Horseradish peroxidase (HRP)-conjugated goat anti-mouse (Qiagen; diluted 1:3000), or HRP-conjugated goat anti-rabbit (Qiagen; diluted 1:3000). Detection employed HRP-substrate Luminata Classico (Millipore).

To analyze polysaccharides in whole-cell lysates, samples were prepared as above and then incubated with 50 µg proteinase K for 1 h at 55 °C. The lysates were then separated by SDS-PAGE and transferred to PVDF (Amersham HyBond P 0.45 µm) or nylon membranes (BioDyne B; Pall). Membranes were probed with murine monoclonal anti-Vi antigen antibody P2B1G2/A9 ((Qadri et al., 1990); diluted 1:350), or anti-polysialic acid-NCAM antibody (MAB5324 clone 2-2b, Millipore Sigma; diluted 1:1000),
followed by alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Qiagen; diluted 1:3000). Detection employed nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche).

For purified polysaccharide, 1 µL of 2 M sucrose was added to 5 µL of sample (typically 1mg/mL Vi antigen), and loaded into 15% (w/v) acrylamide, 89 mM Tris, pH 7.6, 89 mM boric acid, 2 mM EDTA mini-gels, for electrophoresis at 250 V for 70 min. Gels were stained for 16 h with 0.125 % (w/v) Alcian blue, 10 % (v/v) acetic acid, 25 % (v/v) ethanol, destained with 10% (v/v) acetic acid, 10 % (v/v) ethanol for 1 h, then developed using the Pierce Silver Stain kit.
CHAPTER 3

A periplasmic depolymerase provides new insight into ABC transporter-dependent secretion of bacterial capsular polysaccharides

3.1 Preface

This chapter has been submitted for publication in a modified form as Liston, S.D., McMahon, S.A., Le Bas, A., Suits, M.D.L., Naismith, J.H., and Whitfield, C. (2018) A periplasmic depolymerase provides new insight into ABC transporter-dependent secretion of bacterial capsular polysaccharides. *Proc Natl Acad Sci U S A. in press*. For the purpose of this chapter, some overlapping published data have been drawn from Liston, S.D., Ovchinnikova, O., and Whitfield, C. (2016) Unique lipid anchor attaches Vi antigen capsule to the surface of *Salmonella enterica* serovar Typhi. *Proc Natl Acad Sci U S A.* 113(24); 6719-6724, which is the focus of Chapter 4.

All of the bioinformatics, protein purification, biochemical assays, and figures presented in this chapter were performed or generated by myself. During a 1-semester visit to Dr. J. Naismith’s laboratory, I worked with S.A.M. and A.L.B. to screen crystallization conditions for the purified VexL protein. During this time, I was able to generate crystals that diffracted to 2.8 Å and used Robetta (Kim et al., 2004a) to generate a homology model of sufficient quality to solve a portion of the VexL structure. Returning to Guelph, I purified polysaccharide that S.A.M. and A.L. used to optimize crystallization conditions and solve the 1.22 Å VexL-Vi antigen complex structure presented here. I gratefully thank J.H.N and M.D.L.S for their support and expert assistance in processing and interpretation of these structural data.

3.2 Rationale

Analysis of the terminal chemistry of high-molecular-weight glycans is thwarted by overwhelming contributions from the repeating-unit glycan itself (i.e. the molar ratio of ends to repeating unit-residues is vanishingly small). Previous work on ‘group 2’ CPS from *E. coli* and *N. meningitidis* employed bacteriophage-derived CPS-specific endoglycanase enzymes to trim repeating units from the terminal residue(s), which facilitated downstream purification and biophysical characterization (Willis et al., 2013). Unfortunately, a similar approach could not be used for the Vi antigen CPS; all known (and diverse) Vi
antigen-specific bacteriophages instead encode O-acetylerase proteins (Pickard et al., 2010). This Chapter describes the identification and characterization of a novel Vi antigen-specific endoglycanase enzyme that I identified in Vi antigen biosynthesis genetic loci from bacteria belonging to the Burkholderiales. This enzyme provided an essential tool for characterization of the Vi antigen glycolipid terminus, described in Chapter 4. However, the enzyme possessed unique structural and catalytic features which are described in this chapter. In addition, I leveraged the discovery of this periplasmic CPS-depolymerizing enzyme to interrogate the broader model for group 2 capsule assembly, where nascent CPS molecules are proposed to transit the bacterial periplasm within a conserved multiprotein complex.

3.3 Results

3.3.1 VexL is a pectate lyase homolog conserved in Burkholderiales Vi antigen assembly systems

BLAST searches using the S. Typhi viaB locus as a query identified similar loci in several Achromobacter species. While examining the sequence of the Achromobacter denitrificans viaB locus, an additional open reading frame downstream of vexE was identified (Fig. 3.1A). The predicted gene product was homologous to the ambrosia allergen protein family (Fig. 3.2A), which contains pectate lyase enzymes (Conserved Domain Database(Marchler-Bauer et al., 2011)). Bacterial pectate lyases are most often secreted virulence factors produced by plant pathogens, that function to degrade structural glycans of plant tissues (Hugouvieux-Cotte-Pattat et al., 2014). VexL contained a predicted signal sequence (Mean D score residues 1-23 = 0.73, cutoff = 0.57, SignalP4.1, (Petersen et al., 2011)), which suggested VexL is an exported protein but gave no insight into its final cellular location or biological function. The structure of Vi antigen superficially resembles pectin (which has the repeat unit structure \([\rightarrow 4]\)-D-GalA-\(\alpha\)-(1→6)), and I hypothesized that this protein was a pectate lyase homolog with Vi antigen-depolymerizing activity. To test this prediction, the open reading frame was cloned, expressed, its gene product purified, then tested for activity against purified Vi antigen (Fig. 3.2B). The results showed a time-dependent reduction in the apparent size of the Vi antigen, confirming the depolymerase activity. The corresponding gene was therefore renamed vexL.
B

Glc-6-P

Fruc-6-P

PGI

Fruc-6-P

GlcN-6-P

GlcM

GlcN-1-P

GlcN-ac-1-P

Vi antigen Biosynthesis

UDP-GalNAcA

UDP-GlcNAcA

TviC

TviB

TviD

TviE

VexL

WaaF

TviA

GlmU+MFS

Lrp/AsnC

GlmS

Figure 3.1 Vi antigen biosynthetic genetic loci (via B) identified in Burkholderiales. (A) Open reading frames adjacent to vexL are annotated as arrows indicating direction of transcription. Vi antigen biosynthesis genes (tviE, and vexA-L) are color-coded to match their homologs. Protein sequence identity to Achromobacter denitrificans VexL is indicated within the arrows. tviA (colored brown) is a transcriptional regulator found in S. Typhi that renders Vi antigen production under control of the rcs regulatory system. ORFs encoding predicted proteins homologous to transcriptional regulators and glycoactive enzymes adjacent to Vi antigen biosynthesis genes are colored gray; predicted transcriptional regulators are labeled in green. Some Burkholderiales Vi antigen biosynthesis loci have non-canonical gene organizations, and some include duplications of genes for biosynthesis of UDP-GlcNAc, the starting substrate to generate UDP-GalNAcA, the precursor for Vi antigen polymerization. The pathway for UDP-GalNAcA biosynthesis is shown in panel (B). Production of UDP-GlcNAc is an essential housekeeping function in these bacteria because of its role in the biosynthesis of peptidoglycan (Barreteau et al., 2008) and lipopolysaccharide lipid A (Whitfield and Trent, 2014). Achromobacter xylosoxidans and Paraburkholderia susongensis ‘via B’ encode GlmU (bifunctional acetyl-CoA:GlcN-1-phosphate 2-N-acetyltransferase and UDP- GlcNAc synthase) and GlmS (GlcN-6-phosphate synthase). For example, P. susongensis GlmU (WP_085480480.1) and GlmS (WP_085480481.1) are 79 and 67% identical to housekeeping copies of GlmU (WP_085483265.1) and GlmS (WP_085483267.1) located elsewhere in the chromosome, respectively. Many of the Burkholderiales ‘via B’ clusters do not contain TviC (UDP-GlcNAc A C-4 epimerase), which is present between tviB and tviD in S. Typhi (Virlogeux et al., 1995;
Wetter et al., 2012) and *Citrobacter freundii* (Houng et al., 1992). Instead, many have a *tviC* homolog inserted between *vexE* and *vexL*. Others have TviC homologs encoded elsewhere in their genomes; *Achromobacter spanius*, for example, has a duplicate copy of *tviB* (WP_050448110.1) and *tviC* (WP_050448075.1), in a different operon that contains predicted glycosyltransferases of unknown function.

![Figure 3.2](image)

**Figure 3.2 VexL is a Vi antigen depolymerizing enzyme.** (A) *A. denitrificans* VexL contains an N-terminal signal sequence and an ‘ambrosia allergen’ domain, which is homologous to pectate lyases. (B) The purified VexL lyase protein depolymerizes purified Vi antigen, as evident by a decrease in its apparent molecular weight profile in PAGE, visualized by staining with alcian blue and silver.

I used position-specific iterative BLAST (Altschul et al., 1997) to identify additional homologs of VexL; hits were plant pectate lyases or bacterial enzymes within the Order *Burkholderiales* (Fig. 3.3). The *Burkholderiales* homologs shared 65-89% identity with *A. denitrificans* VexL. Interestingly, known and characterized bacterial pectate lyases were not identified (e.g. PelA and PelC from the plant pathogen *Dickeya dadantii* (Scavetta et al., 1999; Thomas et al., 2002) and PelC from *Bacillus subtilis* (Zheng et al., 2012b)). When the phylogeny of these enzymes was investigated, bacterial VexL homologs and known bacterial pectate lyases clustered separately from representatives from plants (Fig. 3.3). However, *Burkholderiales* VexL form a distinct clade, separate from characterized bacterial pectate lyases. The genes encoding VexL-clade enzymes were all located adjacent to genes encoding homologs of Vi antigen biosynthesis and export proteins (Fig. 3.1A), supporting the hypothesis that VexL is specifically involved in Vi antigen assembly and/or processing rather than a catabolic pectinase.
Figure 3.3 Phylogeny of VexL pectate lyases. Figure depicts phylogram of VexL homologs, identified by position-specific iterative BLAST (Altschul et al., 1997) of VexL from *Achromobacter denitrificans*. The maximum-likelihood phylogram was generated from 100 bootstrapped datasets using PhyML3.0 (Guindon et al., 2010), and visualized using iTOL (Letunic and Bork, 2016). GenBank (Benson et al., 2017) accession numbers for VexL homologs are listed. Bootstraps are labeled as purple circles.
3.3.2 VexL is a Vi antigen lyase

To investigate VexL activity in more detail, reaction products were analyzed by high-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS). Mass spectra revealed species that differed by 217.059 \( m/z \), which corresponds to oligosaccharides of GalNAcA, 2-7 residues in length (Fig. 3.4A; Table A3). No monosaccharides were detected, indicative of an endo-acting enzyme. MS also revealed species that differed by -42.011 and -18.011 \( m/z \), which represent non-stoichiometric \( O \)-acetylation of the polysaccharide, and water loss, respectively. No hydrated species (i.e. hydrolase products) were detected. In all of the products, VexL creates a non-reducing 4-deoxy-2-\( N \)-acetyl-\( \alpha \)-\( D \)-galact-4-enuronosyl residue, which is characteristic of the lyase mechanism, which involves \( \text{trans-} \beta \)-elimination rather than hydrolysis (Yip and Withers, 2006). This modification appears in MS as an anhydro-residue at the non-reducing terminus (Fig. 3.4A; Table A3). Cross-ring fragmentation products in MS\(^2\) were consistent with this terminal structure (Fig. 3.4B).

Formation of enuronosyl residues, which absorb at 232nm, offered the opportunity to follow the reaction by spectrophotometry (Albersheim, 1966) and additionally confirmed that VexL is an authentic lyase enzyme. \textit{In vitro}, VexL degraded Vi antigen, generating 3.2 \( \mu \)mol min\(^{-1}\) mg\(^{-1}\) non-reducing termini at its pH optimum of 5.5 (Fig. 3.5B). Product formation was dependent on \( O \)-acetylation of the glycan; no activity was detected for de-\( O \)-acetylated Vi antigen or pectin (Fig. 3.5A). VexL activity was unaffected by EDTA, indicating no divalent cation(s) was required for catalysis (Fig. 3.5A).
Figure 3.4 Oligosaccharide profile of VexL reaction products. (A) Shown is a charge-deconvoluted Q-ToF Mass Spectrum of Vi antigen oligosaccharides generated by VexL depolymerization. Ions correspond to polymers of O-Acetylated HexNAcA residues, 2 to 7 residues in length (yellow box symbols), with a non-reducing terminal 4-deoxy-α-D-galact-4-enuronosyl residue (gray box). Species with non-stoichiometric O-acetylation (Δ42.010 amu) and/or water-loss (Δ18.010 amu) are indicated with red and green bars, respectively. (B) QToF MS² data for a representative [M-H]⁺ ion at 1035.2689 m/z, which represents a tetrasaccharide of acetylated HexNAcA residues, with a non-reducing terminal 4-deoxy-2-N-acetyl-α-D-galact-4-enuronosyl residue.
Figure 3.5 Biochemical characterization of VexL. (A) Panel depicts specific activity of VexL-His$_6$ at 37°C for 0.5 mg/mL (final) Vi antigen in 100 mM potassium phosphate pH 6.0, determined by spectrophotometric assay at 232 nm. EDTA was included at 5 mM where indicated. De-$O$-acetylated Vi antigen and poly-$\alpha$(1→4)-galacturonic acid (pectin) were also included at 0.5 mg/mL (final). (B) VexL has optimal activity at pH 5.5. Specific activities were determined in 100 mM potassium phosphate adjusted to the indicated pH. Reactions buffers at the pH extremes (pH < 5.8 and > 7.4) were measured but reside outside of the buffering capacity of phosphate, and therefore must be interpreted with care.

3.3.3 Structure of VexL

To further characterize VexL, its three-dimensional structure was determined by X-ray crystallography. The gene construct encoded the entire *A. denitrificans* VexL pre-protein, including the predicted N-terminal signal sequence and a C-terminal affinity tag. VexL-His$_6$ was produced in *E. coli* and purified from the periplasm to ensure proper folding of the mature protein, that might be affected by the oxidizing periplasmic environment and signal peptidase processing. Initial optimized VexL crystals diffracted to ≥ 2.8Å (space group $P\ 3_1$). To improve resolution, VexL-His$_6$ was incubated with purified Vi antigen and then used to screen for new crystallization conditions. The resulting tetragonal crystals diffracted to 1.22 Å (space group $P\ 4_2\ 2_1\ 2$). These data were phased by molecular replacement and discussion focuses on this structure (Table 3.1). The asymmetric unit contains one copy of VexL, a malonate ion (buffer), and a trisaccharide of Vi antigen ([→4]-d-GalNAcA3Ac-$\alpha$(1→3)). Amino acid residues of VexL are hereafter numbered based on
the full-length pre-protein sequence. VexL currently represents the most complete and highest-resolution structure for any active pectate lyase in the PDB.

<table>
<thead>
<tr>
<th>Table 3.1 Crystallographic data collection and refinement statistics.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data Collection</strong></td>
</tr>
<tr>
<td>Space group</td>
</tr>
<tr>
<td>Cell dimensions</td>
</tr>
<tr>
<td>a, b, c (Å)</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
</tr>
<tr>
<td>Resolution (Å)</td>
</tr>
<tr>
<td>(High resolution)</td>
</tr>
<tr>
<td>Rmerge</td>
</tr>
<tr>
<td>I / σI</td>
</tr>
<tr>
<td>Completeness (%)</td>
</tr>
<tr>
<td>Average Redundancy</td>
</tr>
<tr>
<td>Vm (Å³/Da)</td>
</tr>
<tr>
<td>Solvent (%)</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
</tr>
<tr>
<td>No. of Unique Reflections</td>
</tr>
<tr>
<td>R_work/R_free (%)</td>
</tr>
<tr>
<td>No. of Atoms (non-H)</td>
</tr>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>Vi antigen</td>
</tr>
<tr>
<td>Malonate</td>
</tr>
<tr>
<td>B-factors (Å)</td>
</tr>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>Vi antigen</td>
</tr>
<tr>
<td>Malonate</td>
</tr>
<tr>
<td>R.m.s. deviations</td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
</tr>
<tr>
<td>Bond angles (°)</td>
</tr>
<tr>
<td>Ramachandran</td>
</tr>
<tr>
<td>Favoured (%)</td>
</tr>
<tr>
<td>Disallowed (%)</td>
</tr>
<tr>
<td>Molprobity score / centile</td>
</tr>
<tr>
<td>PDB</td>
</tr>
</tbody>
</table>
The VexL structure contains density for residues Cys$^{28}$-Pro$^{392}$, Arg$^{395}$-Gly$^{402}$ and two His residues from the affinity tag. There are 3 disulfide linkages, Cys$^{28}$-Cys$^{35}$, Cys$^{279}$-Cys$^{296}$, and Cys$^{364}$-Cys$^{400}$. The majority of VexL is composed of a right-handed parallel-β-helix with three ‘faces’ composed of parallel β-sheets (Fig. 3.6AB). Each β-strand is connected to the adjacent one by a short 2-4 residue loop. This forms a compact ‘core,’ which is packed with ordered arrays of aliphatic and hydrophobic amino acids, contributed by each ‘rung’ in the β-helix (labeled 1-10; Fig. 3.6B). The regular pattern is interrupted by insertions that comprise a loop with an α-helix (rung 6; Leu$^{199}$-Arg$^{218}$), a long loop with an α-helix (rung 9; Asn$^{273}$-Arg$^{305}$) that is stabilized by a disulfide linkage (Cys$^{279}$-Cys$^{296}$) and salt bridge (Asp$^{252}$-Arg$^{277}$), and a loop with an α-helix (rung 9; Asn$^{314}$-Asp$^{330}$) (Fig. 3.8A). These insertions result in two faces of the β-helix being buried and the third face forming the base of an extensive groove. This groove is populated by conserved (Fig. 3.6C) and positively-charged amino acids (Fig. 3.6D). The N-terminus (Cys$^{28}$-Pro$^{85}$) comprises two α-helices, a β-strand, and a long loop. The long loop and long α-helix pack against one face of the β-helix. The shorter α-helix and β-strand pack against the top end the β-helix. The C-terminus (Gly$^{343}$-Gly$^{402}$) forms loops and a short α-helix that cap the bottom end of the β-helix then pack against the same β-sheet as the N-terminus and its neighbor (Fig. 3.6AB). This parallel-β-helix architecture is shared by polysaccharide lyase enzymes (Table 3.2 and Fig. 3.9).
Figure 3.6 Crystal Structure of *A. denitrificans* VexL-His₆ with bound Vi Antigen. (A) Cartoon representation of VexL was generated from a 1.22 Å diffraction dataset collected from a single crystal of VexL-His₆ bound to a trisaccharide of purified Vi antigen. The cartoon is colored in rainbow from N-terminus (blue) to C-terminus (red). (B) Topology of *A. denitrificans* VexL. Secondary structure elements are coloured turquoise (β-sheets) or orange (α-helices). Disulfide linkages are coloured red. Thick (3 pt) lines represent β-sheets and loops above the plane of the page. (C) Amino acid conservation of VexL with the bacterial and plant pectate lyases described in Fig. 3.3. Conservation scores were mapped to the VexL structure using ConSurf, and displayed on the solvent-accessible surface (Landau et al., 2005). (D) Electrostatic surface potential for VexL was calculated at pH 6.5 using the Adaptive Poisson-Boltzmann Solver (Jurus et al., 2017) and is displayed from -3 kT/e (red) to +3 kT/e (blue). (E) VexL structure with bound Vi antigen depicted as yellow sticks. The inset depicts the 2F₀ - Fc electron density map contoured at 1σ for the bound trisaccharide, as grey mesh.
The co-complex reveals clear electron density for 3 sugar residues of Vi antigen, positioned within the large groove highlighted above (Fig. 3.6E). There is additional density that suggests a longer polymer, but it was too ambiguous to model beyond the trisaccharide. The long axis of the trisaccharide molecule and the VexL β-helix align; the reducing sugar sits at the C-terminal end of the β-helix, essentially level with the ‘base’ of the structure. The carboxylate group of the non-reducing sugar makes a bidentate salt contact with the side chain of Arg^{235}, whilst the oxygen of the N-acetyl group is hydrogen bonded to Lys^{195} (Fig. 3.7A). The O4 of this sugar makes a hydrogen bond with Arg^{232} and the pyranose ring oxygen (O5) hydrogen bonds to Arg^{232} and water. There was no additional difference density for an O-acetyl group of this sugar. The importance of these amino acids was probed by site-directed mutagenesis. Purified periplasmic VexL K195A, R232K, R232A, and R235A (Fig. 3.8A) were inactive in in vitro assays monitoring depolymerization or formation of unsaturated termini (Fig. 3.8CD), despite remaining folded (Fig. 3.8B). VexL R235K had 5 % of wildtype activity. Gln^{237} hydrogen bonds to Arg^{235}, while Arg^{172} and Asp^{171} hydrogen bond to Arg^{232} through water (Fig. 3.7A). VexL R172K had 44 % of wildtype activity. Replacement of these ‘supporting’ residues to alanine reduced lyase activity by ~50 % (Fig. 3.8D), except for Arg^{172}, which was inactive (Fig. 3.8CD).

The carboxylate of the central sugar is hydrogen bonded to both Gln^{231} and Tyr^{295} and the oxygen atom of the N-acetyl group is hydrogen bonded to water (Fig. 3.7A). The terminal oxygen atom of the O-acetyl group in this sugar is bridged to the protein by hydrogen bonds to a water molecule. The reducing sugar makes two hydrogen bonds to the protein, Arg^{305} with oxygen atom of the N-acetyl group and Tyr^{298} with the terminal oxygen atom of the O-acetyl group. In addition to the hydrogen bonds, all three sugars make extensive van der Waals contacts with the protein. Of particular note is the methyl group of the O-acetyl of the middle sugar which sits in a hydrophobic pocket bounded by Tyr^{254} and Leu^{308}. Altering this pocket reduced depolymerase activity; VexL Y254F has 47 % of wildtype activity (Fig. 3.8D).
**Figure 3.7 The VexL active site is different from those of classical pectate lyases.** Panels A and B depict models of glycan binding sites in *A. denitrificans* VexL, and *Dickeya dadantii* PelC, respectively. Amino acids side chains are shown for glycan-interacting residues and numbered based on pre-protein sequences. Water molecules and Ca\(^{2+}\) are shown as red and blue spheres, respectively. Lys\(^{240}\) (green) is a point mutant from the WT Arg\(^{240}\).
**Figure 3.8 Site-directed replacement of the putative VexL active site residues.** (A) Production of mutant VexL derivatives. The panel depicts SDS-PAGE of VexL-His<sub>6</sub> and mutant derivatives purified from the periplasm. Gels were stained with Coomassie Brilliant Blue R-250; the vertical bar indicates separate gels. (B) All VexL-his<sub>6</sub> mutants, except for E249A, are folded. The panel depicts melting temperatures of purified VexL-His<sub>6</sub> and mutant derivatives, determined by monitoring protein unfolding-induced fluorescence of Sypro Orange dye (ThermoFisher). Melting temperature was defined as the temperature at the minimum of the derivative of fluorescence intensity over a temperature gradient. Error bars represent standard deviation of 4 independent experiments. Inset depicts representative melt curves for purified VexL-His<sub>6</sub> and VexL R232A-His<sub>6</sub>. (C) VexL R172K, K195A, R232K, R232A, R235A, E249A are inactive. The panel depicts Tris-boric acid-EDTA PAGE of purified Vi antigen which was incubated with VexL-His<sub>6</sub> or the indicated mutant derivative for 2 h at 37 °C prior to electrophoresis. Vi antigen in PAGE was visualized by staining with alcian blue and silver. (D) Specific activities of VexL-His<sub>6</sub> and mutant derivatives. Error bars represent mean ± SE of 3 independent experiments. Mean specific activities are significantly different from WT (P ≤ 0.0001) for all mutant derivatives except VexL Y225F-His<sub>6</sub> (P=0.0007) calculated using Dunnett’s multiple comparison test.
Table 3.2 Examples of structural homologs of VexL.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Protein</th>
<th>PDB</th>
<th>N_RES</th>
<th>%_SSE</th>
<th>RMSD</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. denitrificans</em></td>
<td>VexL</td>
<td>6FI2</td>
<td>375</td>
<td>-</td>
<td>-</td>
<td>This Study</td>
</tr>
<tr>
<td><em>Juniperus ashei</em></td>
<td>JunA1</td>
<td>1PXZ</td>
<td>346</td>
<td>64</td>
<td>1.59</td>
<td>(Czerwinski et al., 2005)</td>
</tr>
<tr>
<td><em>Bacillus sp. N16-5</em></td>
<td>PelA</td>
<td>3VMV</td>
<td>324</td>
<td>87</td>
<td>1.6</td>
<td>(Zheng et al., 2012b)</td>
</tr>
<tr>
<td><em>Bacillus sp. N16-5</em></td>
<td>PelA</td>
<td>3VMW</td>
<td>324</td>
<td>82</td>
<td>1.68</td>
<td>(Zheng et al., 2012a)</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Pel</td>
<td>2NZM</td>
<td>399</td>
<td>69</td>
<td>1.74</td>
<td>(Seyedarabi et al., 2010)</td>
</tr>
<tr>
<td><em>Dickeya dadanti</em></td>
<td>PelC</td>
<td>2EWE</td>
<td>352</td>
<td>73</td>
<td>1.75</td>
<td>(Scavetta and Jurnak, 2005)</td>
</tr>
<tr>
<td><em>Dickeya dadanti</em></td>
<td>PelA</td>
<td>1JTA</td>
<td>361</td>
<td>71</td>
<td>1.77</td>
<td>(Thomas et al., 2002)</td>
</tr>
<tr>
<td><em>Thermotoga maritima</em></td>
<td>Pel</td>
<td>3ZSC</td>
<td>329</td>
<td>67</td>
<td>1.87</td>
<td>(McDonough et al., 2012)</td>
</tr>
<tr>
<td><em>Dickeya dadanti</em></td>
<td>PelC</td>
<td>1PLU</td>
<td>352</td>
<td>76</td>
<td>1.89</td>
<td>(Yoder and Jurnak, 1995)</td>
</tr>
<tr>
<td><em>Dickeya dadanti</em></td>
<td>PelC</td>
<td>1AIR</td>
<td>352</td>
<td>73</td>
<td>1.91</td>
<td>(Lietzke et al., 1997)</td>
</tr>
<tr>
<td><em>Dickeya dadanti</em></td>
<td>PelC</td>
<td>2PEC</td>
<td>352</td>
<td>76</td>
<td>1.91</td>
<td>(Yoder and Jurnak, 1995)</td>
</tr>
<tr>
<td><em>Dickeya dadanti</em></td>
<td>PelC</td>
<td>1O8L</td>
<td>352</td>
<td>73</td>
<td>1.92</td>
<td>(Herron et al., 2003)</td>
</tr>
</tbody>
</table>
**Figure 3.9 Structural similarity shared by VexL and bacterial pectate lyases.** (A) Cartoon representation of VexL-His<sub>6</sub>, with loop ‘insertions’ indicated in blue (rung 6; Leu<sup>199</sup>-Arg<sup>218</sup>), green (rung 9; Asn<sup>273</sup>-Arg<sup>305</sup>), and red (rung 9; Asn<sup>314</sup>-Asp<sup>330</sup>). Panels B-F show structural alignments of VexL (wheat, orange loops) superimposed with structural homologs identified using PDBfOLD (Green, blue loops) (Krissinel and Henrick, 2004); (B) Juniperus ashei JunA1 (PDB: 1PXZ), (C) Dickeya dadanti PelC (PDB: 2EWE), (D) Bacillus sp. N16-5 PelA (PDB: 3VMV), (E) Dickeya dadanti PelA (PDB: 1JTA), and (F) Bacillus subtilis pectate lyase (PDB: 2NZM).

### 3.3.4 Vi antigen envelope translocation includes a periplasm-accessible intermediate

A predicted signal sequence suggested VexL is an exported protein but did not indicate its final location. Proteins reactive with VexL-specific antibodies were found associated with cells in 3 *Achromobacter* species (Fig. 3.10). However, no protein or lyase activity was detected in the spent media from cultures of these bacteria, indicating that VexL likely resides in the periplasm of its natural host, rather than being a
substrate for a protein secretion system (Fig. 3.11). Material reactive with Vi antigen-specific antibody was identified in lysates of these Achromobacter species, suggesting that these Achromobacter do produce a glycan resembling Vi antigen (Fig. 3.10). However, addressing the role of VexL in Vi antigen production in Burkholderiales is complicated by the absence of information concerning both the diversity of polysaccharides it produces and the underlying biology (i.e. glycan function and growth conditions that affect Vi antigen synthesis). An additional barrier is the resistance of Achromobacter isolates to antibiotics used as markers in molecular-genetic tools (Bador et al., 2013). The impact of VexL was therefore investigated in the characterized S. Typhi system. These organisms possess essentially identical Vi antigen biosynthesis and export proteins (Fig. 3.1A). However, S. Typhi does not encode VexL, and A. denitrificans does not encode TviA, a transcriptional activator of the Vi antigen genetic locus unique to S. Typhi (Virlogeux et al., 1995; Winter et al., 2009).

![Figure 3.10 Achromobacter species produce a Vi antigen-like glycan, VexE, and VexL.](image)

The figure depicts immunoblots of whole cell lysates of Achromobacter spanius, Achromobacter xylosoxidans, Achromobacter denitrificans grown on LB Agar at 20 °C probed with VexL-, VexE-, and Vi antigen-specific antibodies.
S. Typhi was transformed with inducible plasmid vectors encoding *A. denitrificans* VexL-His$_6$, VexL R232A-His$_6$, or VexL$^{24-402}$-His$_6$; the latter lacks the predicted signal sequence. To confirm localization of these constructs in *S. Typhi*, released periplasmic contents were examined by immunoblotting employing VexL-specific antibodies. As expected, VexL and VexL R232A appeared in the periplasmic fraction, whereas VexL$^{24-402}$ was confined to the cytoplasm and remained within spheroplasts (Fig. 3.11A). Expression of VexL-His$_6$ reduced the average molecular weight of Vi antigen (Fig. 3.12A), while the catalytically-inactive VexL R232A-His$_6$ (Fig. 3.12A) did not, confirming that the change in molecular weight is a specific catalytic effect rather than a structural consequence of overexpression of periplasmic VexL-His$_6$. In contrast, cytoplasmic VexL$^{24-402}$-His$_6$ had no effect on Vi antigen profiles (Fig. 3.12A), indicating that any degradation of product occurred post-export from the cytosol. The Vi antigen produced by cells expressing VexL variants was located on the cell surface in *S. Typhi* as judged by susceptibility to the Vi antigen-specific bacteriophage ViII (Fig. 3.12B). Growth media from these cultures was examined, but no VexL protein was detected in the spent media by immunoblotting (Fig. 3.11B). Moreover, no degradation of purified Vi antigen was evident after incubation with this media (Fig. 3.11C). These data ruled out any unanticipated effect occurring at the cell surface from small amounts of VexL released during growth. These experiments are entirely consistent with a model in which Vi antigen is protected within the cytoplasm during biosynthesis but exposed in the periplasm during translocation.
Figure 3.11 **VexL is located in the periplasm in S. Typhi**. Panel (A) depicts Western immunoblots of subcellular fractionation of *S. Typhi* that harbored plasmid-encoded VexL-His₆, VexL-His₆ R232A, or VexL²⁴⁻⁴⁰²-His₆. Periplasmic contents were released by osmotic shock. The resulting spheroplasts were collected, lysed, and separated based on centrifugation at 100 000 × g for 1 h (Cytosolic, supernatant; Membranes, pellet). RNA polymerase, OmpA, and MalE provided controls for cytosolic, membrane, and periplasmic proteins, respectively. (B) VexL is not secreted in to culture supernatants. The figure depicts Western immunoblots of 50 × concentrated cell-free spent culture media, from identical cultures to (A). An equivalent culture was lysed by sonication prior to removing cells and included as a positive control for cell lysis. (C) VexL depolymerase activity is not detected in cell-free spent culture media of *S. Typhi* harboring plasmid-encoded VexL (and derivatives). Purified Vi antigen was resuspended at 1 mg/mL in cell-free culture media, incubated at 37 °C for 16 h, then resolved by Tris-boric acid-EDTA PAGE. Vi antigen in PAGE was visualized with alcian blue and silver staining. Identical cultures were lysed by sonication prior to removing cells, to provide positive controls for lysis.
The inability of cytosolic VexL<sup>24-402</sup>-His<sub>6</sub> to affect the Vi antigen profiles was surprising. To examine this phenotype in more detail, VexL<sup>24-402</sup>-His<sub>6</sub> was expressed in <i>S. Typhi</i> ΔvexC, which lacks the ABC transporter NBD. This mutation interrupts Vi antigen export and leads to accumulation of glycan in the cytosol (Wetter et al., 2012). As expected, this strain is resistant to infection by bacteriophage ViII (Fig. 3.12B). In contrast to WT <i>S. Typhi</i>, vexC mutant Vi antigen was susceptible to degradation by VexL<sup>24-402</sup> (Fig. 3.12A), which confirmed that VexL<sup>24-402</sup> is active <i>in vivo</i> and can access its substrate if the normal assembly pathway is perturbed.

**Figure 3.12 Vi antigen is susceptible to degradation by VexL in the periplasm.** (A) Expression of VexL in <i>S. Typhi</i> reduces the apparent molecular weight of Vi antigen in SDS-PAGE, whereas VexL R232A and signal sequence-truncated VexL<sup>24-402</sup> do not. Deletion of the Vi antigen transporter NBD (<i>vexC</i>), renders (now cytoplasmic) Vi antigen susceptible to degradation by VexL<sup>24-402</sup>. The figure depicts immunoblots of whole cell lysates, probed with Vi antigen- or VexL-specific antibodies. Cultures were grown to mid-log phase, prior to induction of plasmid-encoded protein expression with indicated final concentration of L-arabinose for 30 min. (B) Expression of VexL or VexL<sup>24-402</sup> does not eliminate susceptibility to lysis by a Vi antigen-specific bacteriophage (Vi phage II, HER#39). LB agar contained 0.02% (w/v) L-arabinose to induce plasmid-encoded gene expression. <i>S. Typhi</i> and <i>S. Typhi</i> ΔvexC, provide positive and negative controls for surface-exposed Vi antigen, respectively.
With the intention to probe the periplasmic-accessibility of other group 2 CPS, I turned to *E. coli* K1. A published study suggested that this CPS was protected from a cytoplasmic K1 CPS-specific endoglycanase, EndoN (Steenbergen and Vimr, 2008). These experiments were recapitulated with *E. coli* EV36 (K1) and showed that it retained susceptibility to the CPS-specific bacteriophage when EndoN was expressed in the cytoplasm (as reported (Steenbergen and Vimr, 2008); **Fig. 3.13A**). However, when the CPS was examined by immunoblotting, most was degraded (**Fig. 3.13B**). This suggested that the K1 CPS was not cytoplasm-protected and precluded ability to assess the effects of an engineered periplasmic EndoN.

**Figure 3.13 The K1 CPS is cytoplasm-exposed during its biosynthesis.** (A) Published cytoplasmic accessibility experiments for the K1 CPS were recapitulated by expression of the K1-specific endosialidase (EndoN) as a cytoplasmic maltose-binding protein fusion in *E. coli* EV36 (Steenbergen and Vimr, 2008). EndoN expression eliminated K1 CPS in immunoblots of whole cell lysates, prepared identically to S. Typhi experiments. EndoN expression was monitored using an antibody specific to the MalE fusion. These results indicated that the K1 CPS exposed to EndoN in the cytoplasm. (B) As previously described (Steenbergen and Vimr, 2008), expression of this protein did not abrogate susceptibility to the K1 CPS-specific bacteriophage K1F, indicating sufficient CPS remained to generate the required phage receptor (Vimr et al., 1984). *E. coli* EV36 and NBD-mutant EV36 (∆kpsT) provide positive and negative controls for surface exposed K1 CPS, respectively. LB agar contained 100 µg/mL ampicillin and 0.5 mM IPTG to induce plasmid-encoded gene expression.
3.4 DISCUSSION

VexL shares a conserved β-helical structure with diverse bacterial and plant catabolic polysaccharide lyases found in family PL1 of the Carbohydrate-Active Enzyme (CAZy) database (Cantarel et al., 2009; Hugouvieux-Cotte-Pattat et al., 2014). Families PL3 and PL9 also share this structure but were not identified in sequence or structure-based searches. The representative structures of these protein families differ predominately in the position of loops inserted at turns that frame the glycan-binding site(s) but have little variation within the β-helix itself, suggesting that these elaborations are important for substrate recognition. Close structural homologs of VexL (Fig. 3.9, Table 3.2) belong to family PL1, which contains metal-dependent, endo-acting, pectin and pectate lyases with alkaline pH optima (Hugouvieux-Cotte-Pattat et al., 2014; Yip and Withers, 2006). Despite the existence of dramatically different polysaccharide lyase folds (e.g. (α/α)_3 barrel, PL10), their catalytic centers for trans-β-elimination are conserved (Charnock et al., 2002).

The pectate lyase reaction employs an arginine residue positioned to extract a proton from C4 of the hexuronic acid residue in the +1 subsite (Yip and Withers, 2006). Arg^{232} likely fills this role in VexL. This is supported by inactivating replacements in VexL R232K (conservative) and R232A and the position of this residue relative to C4 of GalA_4 in the +1 subsite of the *Dickeya dadanti* PelC-GalA_4 complex (Fig. 3.7B) (Scavetta et al., 1999). *D. dadanti* PelC includes an additional conserved arginine (Arg^{245}; Fig. 3.7B) that is involved in a ligand-positioning bidentate salt contact with the carboxylate of the GalA residue in the -1 subsite. In VexL, Arg^{235} fills this role (Fig. 3.7A), and, as such, the trisaccharide of Vi antigen in the VexL structure likely represents glycan-binding subsites -3, -2, and -1. The hydrogen-bonding network involving Vi antigen N- and O-acetyl decorations (Fig. 3.7A) is a critical component of substrate recognition; all point mutants of interacting residues had reduced lyase activity (Fig. 3.8D). The specific requirement for the O-acetyl group is achieved not through a single interaction but rather through a network involving bridging water molecules. Using a network, as opposed to a single interaction, likely underpins the tolerance of VexL for non-stoichiometric acetylation of its substrate. Notably, the VexL-binding groove lacks multiple DXD motifs, which coordinate divalent cations essential for glycan-binding and activity in
conventional pectate lyases (Hugouvieux-Cotte-Pattat et al., 2014), such as *D. dadanti* PelC (Fig. 3.7B). However bound Ca$^{2+}$ may only be recruited to the Michaelis-Menten complex, making it necessary to inactivate VexL to observe these ions by crystallography. The lack of sensitivity to EDTA supports the conclusion that VexL does not require a divalent cation(s) for catalysis. VexL has a signal peptide, a pH optimum more acidic than other relative enzymes (pH 5.5 vs ~8.0-9.5), and presence of multiple disulfide linkages that stabilize the N- and C-termini of the protein. Because of these unique properties, it appears VexL has evolved unique structural changes and catalytic features that facilitate function in the more acidic (Yang et al., 2014) and oxidizing environment of the periplasm; VexL is a distinctive and unusual addition to the polysaccharide lyase family. Based on these data, VexL defines a unique subfamily within PL1 that possesses a novel activity for a PL enzyme (Dr. Bernard Henrissat, Head of Glycogenomics, Head of the CAZy database, personal communication).

To my knowledge, VexL is the first confirmed example of a CPS-degrading enzyme associated with an ABC transporter-dependent CPS assembly system. In contrast, periplasmic glycan-modifying proteins participate in production of some secreted exopolysaccharides (EPS) that feature entirely different export systems. The *Pseudomonas aeruginosa* PEL polysaccharide provides an example. Unlike CPS, PEL does not possess a terminal lipid (Franklin et al., 2011); its secretion requires a multidrug and toxic compound extrusion transporter and a periplasmic scaffold that guides PEL to an outer membrane channel. PelA is periplasmic PEL acety lesterase and hydrolase that binds the scaffold (Marmont et al., 2017a) and is required for PEL production (Marmont et al., 2017b). Similarly, *E. coli* poly-N-acetylglucosamine EPS employs a periplasmic acetyl esterase (PgaB) that is required for export but not synthesis (Itoh et al., 2008). In contrast, production of the (Wzx-dependent) *P. aeruginosa* PSL EPS does not require the cognate PslG periplasmic hydrolase, although PslG overexpression decreases PSL production and biofilm formation (Baker et al., 2015).

EPS systems are associated with biofilm formation and this seems the most likely role for the Vi antigen-like glycan produced by the *Burkholderiales. Achromobacter* isolates with vexL-containing Vi antigen assembly loci are emerging drug-resistant human pathogens (Bador et al., 2013; Gross et al., 2008;
but the role for Vi antigen in these organisms has yet to be examined. Vi antigen is a major constituent of biofilm matrices in S. Typhi biofilms grown on gall stones \textit{ex vivo} (Marshall et al., 2014) but is apparently dispensable for biofilm formation in this setting (Prouty et al., 2002). In contrast, Vi antigen production in S. Typhi is unequivocally linked to decreasing complement deposition and immune clearance (Keestra-Gounder et al., 2015). Vi production is upregulated when S. Typhi enters intestinal epithelial cells (Tran et al., 2010), reduces complement and Toll-like receptor-dependent detection, and decreases recruitment of immune effectors to the site of infection (Wangdi et al., 2012; Wilson et al., 2011; 2008). Vi antigen purified from S. Typhi is viscous; this property is related to glycan chain length and diminished by VexL treatment. It is therefore tempting to speculate that the gain or loss of an enzyme that alters the size, release, and/or quantity of CPS produced may be a critical factor in the function(s) of Vi antigen in organisms with different physiologies and niches.

VexL provides important new insight into the secretion machinery for Vi antigen and, given the overall similarities in their export machineries, the findings are potentially relevant for other ABC transporter-dependent CPS assembly systems (Willis and Whitfield, 2013a). Understanding the details of these processes is vital in considering CPS as a therapeutic target in anti-virulence strategies. These data establish the Vi antigen is protected from VexL degradation during its synthesis in the cytoplasm. This could be accomplished by a complex of biosynthetic enzymes creating a protected environment for the glycan, or more likely by tight coupling of polymerization and export, such that the glycan is exported as it is polymerized. Such coupling occurs in the synthesis of some O antigenic polysaccharides which couple a multiprotein synthesis complex to an ABC-transporter (Kos and Whitfield, 2010; Kos et al., 2009). Coupling synthesis and export of O antigen regulates product chain length and a similar effect is evident in the increased molecular weight of Vi antigen in the \textit{vexC} mutant (Fig. 3.11A). The observation that the Vi antigen becomes susceptible to cytoplasmic degradation in the absence of export suggests there is normally a protected cytosolic ‘compartment’ but does not on its own distinguish between an enveloping protein complex or coupling-mediated effect. However, the susceptibility of cytosolic Vi antigen accumulating in export-deficient mutants leads me to favoring the latter explanation.
Previous studies used recombinant bacteriophage-derived endoglycanase enzymes to examine the cytoplasmic accessibility of CPS during its synthesis in *E. coli* K1 (Steenbergen and Vimr, 2008), and K5 (Hudson et al., 2009). In both cases, CPS degradation was assessed by susceptibility of the bacteria to lysis by the corresponding CPS-specific bacteriophage. In K5, CPS production was also directly quantified (Hudson et al., 2009). Surprisingly, the studies concluded that K1 was synthesized in a protected cellular compartment but K5 was not. The immunoblotting data presented here suggest K1 CPS is not in fact protected in the cytoplasm and this is more consistent with results obtained with *E. coli* K5. The different published conclusions may reflect the different approaches and bacteriophages used to assess phenotypes. Neither system offers the cytoplasmic protection seen for Vi antigen and this may reflect the differences in biosynthetic machinery and glycolipid acceptors used by *E. coli* and *S. Typhi*. The organization of the reported biosynthesis/export complex (McNulty et al., 2006; Rigg et al., 1998; Steenbergen and Vimr, 2008) would therefore appear to differ from that for Vi antigen.

These results demonstrate that the Vi antigen is degraded by VexL during the translocation phase in the periplasm. This finding is inconsistent with the current assembly model (Fig. 1.7). This model was influenced by the ABC transporter-based tripartite drug efflux pumps (Willis and Whitfield, 2013a), such as the MacA-MacB-TolC complex (Fig. 1.8) (Fitzpatrick et al., 2017). A prototype OPX protein from the Wzx-dependent export of *E. coli* ‘Group 1’ CPS, forms a multimeric channel across the outer membrane (Fig. 1.6) (Dong et al., 2006) and interacts with its inner membrane (periplasm-exposed) PCP partner (Collins et al., 2007). In the group 2 *E. coli* K5 system, a multiprotein complex has been detected that contains the ABC transporter (KpsMT), the PCP protein (KpsE) and the OPX protein (KpsD) (McNulty et al., 2006). Furthermore, genetic data supports cognate interaction between PCP-OPX protein pairs in other group 2 systems (Larue et al., 2011). Although there is currently no structural data for KpsD, the PCP protein KpsE forms a membrane-bound multimeric structure in cryo-EM whose periplasmic domain resembles the adaptor complex of tripartite drug efflux pumps (Larue et al., 2011). While the size and complexity of the CPS substrate and structures of efflux pumps has made the concept of a protected translocation pathway attractive as a working model, the accessibility of Vi antigen to degradation in the
periplasm indicates this must be re-assessed. In group 1 systems, CPS polymerization occurs in the periplasm external to the PCP-OPX complex and the glycan is proposed to access the lumen of the complex laterally (Collins et al., 2007). Site-directed crosslinking experiments established that CPS glycans transit the outer membrane within the OPX pore (Nickerson et al., 2014). The results presented here indicate that periplasmic access to the translocation pathway is also required in group 2 systems. Periplasmic exposure of the glycan may offer interesting avenues for CPS structural diversification. For example, LPS O antigens exported by ABC transporters are exposed during assembly/translocation and can be substrates for post-polymerization periplasmic glycosylation systems, creating new structures and altered antigenic epitopes (Mann and Whitfield, 2016).

3.5 Acknowledgements

I gratefully acknowledge support from the Natural Sciences and Engineering Research Council in the form of an Alexander Graham Bell Canada Graduate Scholarship and Michael Smith Foreign Study Supplement. With this support, I travelled to Dr. James Naismith’s laboratory at the University of St. Andrews, UK.
CHAPTER 4

A unique lipid anchor attaches Vi antigen capsule to the surface of Salmonella enterica serovar Typhi

4.1 Preface

This chapter has been published in a modified form as Liston, S.D., Ovchinnikova, O., and Whitfield, C. (2016) Unique lipid anchor attaches Vi antigen capsule to the surface of Salmonella enterica serovar Typhi. Proc Natl Acad Sci U S A. 113(24); 6719-6724.

I performed all of the experiments concerning the purification of the lipid terminus, purification and biochemical analysis of VexE, and the biological assays. NMR spectra were recorded in the Advanced Analysis Centre with technical support from Dr. Andy Lo and were interpreted with the help of Dr. Olga Ovchinnikova. MS data were collected in the Advance Analysis Center with technical support from Drs. Dyanne Brewer and Armen Charchoglyan; I independently performed the data analysis. Immunofluorescence microscopy experiments were completed by myself but Dr. Michaela Strüder-Kypke and Mr. Robert Harris performed electron microscopy.

4.2 Rationale

Polysaccharide ABC transporters typically employ terminal glycolipids in ‘flipping’ the glycan across cell membranes. Prototypical CPS ABC transporters engage a conserved terminal lysoPG-Kdo₉ residue which is linked to the reducing terminus of diverse CPS glycans and is necessary for transport (Willis and Whitfield, 2013a). However, Vi antigen capsule-producing bacteria lack the conserved enzymes to assemble this terminal residue. This Chapter describes the isolation and biophysical characterization of a terminal glycolipid that is unique to the Vi antigen, employing the Vi antigen depolymerase described in Chapter 3. The structure of this residue resembled lipid A and Vi antigen biosynthesis genetic loci encode a unique acyltransferase homolog, VexE. This drove the hypothesis that VexE was responsible for assembly of the Vi antigen glycolipid terminus, and the consequences of vexE mutation were therefore investigated in S. Typhi.
4.3 Results

4.3.1 Vi antigen from a ΔvexE mutant has altered physical properties.

Despite the absence of kpsS and kpsC from the chromosomes of Vi antigen producing bacteria, I hypothesized that a glycolipid terminus of some form is a unifying feature for all ABC transporter-dependent glycan biosynthesis systems. Other bacterial surface glycoconjugates exported by ABC transporters frequently employ undecaprenyl diphosphate carrier lipids. To test the possibility that these lipids participated in Vi antigen production, the viaB locus was introduced into E. coli CWG1214 (ΔwecA), which is unable to make und-PP-GlcNAc in the obligatory first step in biosynthesis of E. coli LPS O antigens and enterobacterial common antigen (Raetz and Whitfield, 2002). E. coli lacks wbaP, whose gene product produces undecaprenyl diphospho-galactose in the corresponding initiation step for most O antigens in Salmonellae (Raetz and Whitfield, 2002). Western immunoblots of cell lysates E. coli CWG1214 transformed with the viaB locus displayed robust Vi antigen production (Fig. 4.1D), ruling out the logical candidates for undecaprenyl-active enzymes in Vi antigen assembly.

The viaB loci all encode a predicted VexE protein containing a potential C-terminal lysosphospholipid acyltransferase (LPLAT) domain (Fig. 4.1A). This domain is also found in E. coli LpxL (Fig. 1B), an acyl-acyl carrier protein (ACP)-dependent secondary acyltransferase involved in the biosynthesis of lipid A (see Fig. 1.4). LpxL and VexE share only 18% identity overall (e-value: 1.08×10⁻³) but the conserved LPLAT domain (cd07984) shares higher similarity (e-value: 1.67×10⁻⁸). The putative LPLAT domain in VexE led to the hypothesis that this protein is an acyltransferase that creates a different type of lipid terminus on Vi antigen chains.
Fig. 4.1 S. Typhi ΔvexE mutant Vi antigen possesses altered physical properties. (A) Achromobacter denitrificans VexE contains a predicted N-terminal region of tetratricopeptide repeats (TPR), which form α-helical superstructures implicated in protein-protein interactions (D’Andrea and Regan, 2003), and a C-terminal lysophospholipid acyltransferase (LPLAT) domain. (B) Multiple sequence alignment of the LPLAT domain of VexE from S. Typhi, A. denitrificans, and E. coli LpxL. Motifs historically characteristic of LPLAT are highlighted in yellow and the putative role of particular residues is noted (Lewin et al., 1999). Residues that were replaced are boxed in blue. (C) In immunoblots, Vi antigen produced by S. Typhi bound PVDF and positively-charged nylon membranes, whereas Vi antigen from the ΔvexE mutant bound only nylon. The panels show immunoblots of proteinase K-digested whole cell lysates probed with anti-Vi antigen antibody. PVDF binding was restored when the ΔvexE mutant was complemented with either S. Typhi or A. denitrificans vexE. The corresponding putative catalytic mutants of VexE from either S. Typhi (H487A) or A. denitrificans (H466A) failed to restore PVDF binding. A Y471F mutation in the A. denitrificans enzyme (at position 6 of HX₃D/E motif in VexE) had no discernible effect on its activity. VexE expression was monitored by Western blotting of hexahistidine tagged VexE constructs from identical cell cultures. (D) Vi antigen is produced in E. coli Top10 and a ΔwecA mutant derivative.
Previous analyses of Vi antigen phenotypes in viaB gene mutants were performed using recombinant E. coli transformed with plasmid-encoded viaB, but the possibility of a lipid terminus and the precise role of VexE had not been examined (Virlogeux et al., 1995; Wetter et al., 2012). To avoid complications arising from multicopy gene expression and the unnatural host background, the role of VexE was examined by generating chromosomal mutations in S. Typhi H251.1. In Western immunoblots, Vi antigen in cell lysates of the parent strain bound to both hydrophobic PVDF and positively-charged nylon membranes (Fig. 4.1C). In contrast, Vi antigen in lysates from the ΔvexE mutant bound only to nylon, indicating a change in the physical properties of Vi antigen produced by the mutant. Wildtype binding properties were restored in the mutant by expression of VexE homologs from S. Typhi or A. denitrificans (Fig. 4.1C). LPLAT enzymes possess a conserved HX₄D/E motif, which contains the essential catalytic His/Asp pair (Lewin et al., 1999) (Fig. 4.1B). The corresponding H→A mutant in E. coli LpxL results in >1,000-fold reduction in lauroyltransferase activity (Six et al., 2008). VexE shares the putative catalytic His residue, which was replaced in the VexE homologs from S. Typhi and A. denitrificans, and these were expressed in S. Typhi ΔvexE. Vi antigen from these transformants did not bind to PVDF, despite robust expression of the enzymes (Fig. 4.1C). Proper folding of VexE H466A was confirmed by comparing circular dichroism spectra of purified wildtype and mutant proteins (Fig. 4.2). The catalytic activity of VexE is therefore linked to alterations in the physical properties of Vi antigen, resulting in differential binding to membranes with varying chemistries. Based on homology to LpxL, the possibility that VexE interfered with normal lipid A biosynthesis in E. coli was examined. Expression of VexE slightly slowed growth but this effect was likely due to protein overexpression and was independent of VexE catalytic activity (Fig. 4.3A). In addition, VexE was unable to modify lipid A in an E. coli mutant with deletions of all known secondary acyltransferases from lipid A biosynthesis (Fig. 4.3B). This suggested that VexE played a direct role in Vi antigen assembly.
Figure 4.2 Replacement of the putative catalytic histidine residue of VexE does not affect folding of the protein. The circular dichroism spectra of purified VexE and VexE^{H466A} from *A. denitrificans* are identical.
Figure 4.3 Overexpression of vexE does not affect cell growth or lipid A structure. (A) Viable-cell-count growth curves of E. coli Top10 harboring plasmids encoding VexE and VexE<sup>H466A</sup>. Overexpression of vexE, induced by addition of L-arabinose (final concentration 0.02% (w/v)) at 2.5 h (arrow), slightly hindered growth of E. coli, independent of catalytic activity, because a similar profile was obtained with VexE<sup>H466A</sup>. Data represents mean ± standard error of three independent experiments. (B) Charge-deconvoluted ESI-MS of lipid A species isolated from E. coli BKT09, which possesses mutations in lpxL, lpxM, lpxP, and pagP, and therefore produces only tetraacylated lipid A. Signals below 1% of the maximum were excluded from the deconvolution. Overexpression of the secondary acyltransferases LpxL and LpxM resulted in the expected additions of laurate and palmitate modifications, respectively, generating pentaacylated lipid A. Overexpression of VexE did not alter lipid A structure.
To determine whether the altered binding properties reflected differences in the repeat-unit structure of the parental and mutant Vi antigens, such as alterations in O-acetylation, the glycans were purified and their structure was examined by NMR. Initial preparations of wildtype Vi antigen were consistently contaminated with LPS and substantial amounts of wildtype Vi antigen sedimented with LPS micelles in centrifugation. Deletion of vexE abrogated this property (Fig. 4.4B), adding weight to the contention that VexE influenced physical properties of Vi antigen. To obtain Vi antigen free of LPS, ΔwaaG mutants were generated. waaG mutants produce truncated LPS molecules due to the loss of most of the core oligosaccharide and O antigen (reviewed in (Whitfield and Trent, 2014) (Fig. 4.4A) and could be separated from Vi antigen by gel filtration chromatography. $^{13}$C NMR spectra of Vi antigens from ΔwaaG and ΔwaaG ΔvexE double mutants were identical (Fig. 4.4CD), and comparable to those predicted (Table 4.1) and previously published (Szu et al., 1987). The altered properties of the Vi antigen produced by the vexE mutant were therefore not due to changes in the polysaccharide backbone structure but could be explained by alterations in a putative acylated terminus.
Fig. 4.4 Purification of Vi antigen from S. Typhi ΔwaaG. Wildtype S. Typhi produced Vi antigen that was contaminated by LPS, so a waaG mutant was constructed to generate truncated LPS molecules that could be separated from Vi antigen by gel filtration chromatography. WaaG is a glycosyltransferase that adds the first glucose residue to the outer core of LPS (Kadam et al., 1985), so ΔwaaG mutants lack the attachment site for the O antigen. (A) Phenotypes of ΔwaaG mutants. The samples are proteinase K-digested whole-cell lysates immunoblotted with Vi antigen-specific antibody (upper panel) and the corresponding gels were stained with silver to visualize LPS (lower panel). The ΔwaaG mutants were unaffected in their Vi antigen production. The ΔvexE mutants resulted in Vi antigen with increased average sizes and this is independent of the waaG mutation. The lower amounts of O antigen-substituted LPS in complemented waaG mutants compared to the parent strain reflects the efficiency of complementation (B) The association between LPS and extracted Vi antigen is eliminated in the ΔvexE mutant. The panels show material extracted by phenol-water and then collected by centrifugation at 100,000 × g (P) or material that remained in the supernatant (S). Vi antigen was detected by western immunoblotting (upper panels) and LPS by silver-staining the corresponding SDS-PAGE gel (lower panels). (C) 13C NMR spectra of purified Vi antigens from S. Typhi ΔwaaG and the ΔwaaG ΔvexE double mutant. (D) 13C NMR spectra of the same samples after chemical de-O-acetylation by base treatment. As expected, chemical removal of O-acetyl groups resulted in loss of the O-acetyl signals at 20.06 ppm (Me), and 173.90 (CO).
Table 4.1 Chemical shifts observed in $^{13}$C spectra of purified Vi-antigens. Chemical shifts were predicted using CASPER (Lundborg and Widmalm, 2015) for GalNAcA.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vi-CPS</th>
<th>δC (ppm)</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
<th>NAc (Me)</th>
<th>NAc (CO$_2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>Predicted</td>
<td>98.95</td>
<td>51.35</td>
<td>68.44</td>
<td>78.89</td>
<td>72.59</td>
<td>175.87</td>
<td>22.91</td>
<td>175.43</td>
<td></td>
</tr>
<tr>
<td>$E. coli$ [viaB]</td>
<td>Observed</td>
<td>99.65</td>
<td>50.71</td>
<td>67.88</td>
<td>79.05</td>
<td>72.49</td>
<td>176.21</td>
<td>23.63</td>
<td>174.95</td>
<td></td>
</tr>
<tr>
<td>$E. coli$ [viaBAvexE]</td>
<td>Observed</td>
<td>99.39</td>
<td>50.56</td>
<td>68.01</td>
<td>78.78</td>
<td>72.61</td>
<td>176.06</td>
<td>23.55</td>
<td>175.57</td>
<td></td>
</tr>
<tr>
<td>S. Typhi</td>
<td>Observed</td>
<td>99.71</td>
<td>50.69</td>
<td>67.53</td>
<td>78.98</td>
<td>72.11</td>
<td>176.16</td>
<td>23.55</td>
<td>174.06</td>
<td></td>
</tr>
<tr>
<td>S. Typhi ΔvexE</td>
<td>Observed</td>
<td>99.68</td>
<td>50.72</td>
<td>67.80</td>
<td>79.04</td>
<td>72.54</td>
<td>176.23</td>
<td>23.61</td>
<td>174.73</td>
<td></td>
</tr>
</tbody>
</table>

4.3.2 Vi antigen has a unique glycolipid at its reducing terminus.

Structural investigation of the termini of high-molecular-weight polysaccharides requires a method that reduces the degree of polymerization while preserving linkages between terminal residues and the repeating-unit glycan. Previous works exploited endoglycanase enzymes from CPS-specific bacteriophages to identify the terminal glycolipid structure from the CPS of $E. coli$ K1 and K5, and meningococcal serotype b (Willis et al., 2013). However, all known Vi antigen-specific bacteriophage encode $O$-acetylesterase tail spike proteins, not depolymerases (Pickard et al., 2010). Purified $A. denitrificans$ VexL was therefore used to depolymerize purified Vi antigen. Hydrophobic products released in these reactions were then collected by solid phase extraction and analyzed by LC-MS.

LC-MS of these molecules revealed a series of species that differed by 217.059 amu, representing increments of one GalNAcA residue (Fig. 4.5A; Table A4). Decorations by one or more $O$-acetyl groups ($\Delta$amu = 42.011) were also evident. As for total oligosaccharides seen in Chapter 3, these products possessed a non-reducing terminal 4-deoxy-2-$N$-acyetyl-$\alpha$-$D$-galact-4-enuronosyl residue. This modification was evident in MS$^2$ fragmentation products (Fig. 4.5B). The Vi antigen oligosaccharides were linked to a reducing terminal $N$-acetylhexosamine (HexNAc) residue modified with either two $\beta$-hydroxymyristate chains, or one $\beta$-hydroxymyristate and one $\beta$-hydroxypalmitate chain. Fragmentation of the [M-H]$^-$ ion at
1333.666 \text{ m/z} \) produced products (including cross-ring cleavages), consistent with a structure comprising 3 GalNAcA residues linked to a single reducing terminal HexNAc possessing β-hydroxymyristate and β-hydroxypalmitate modifications (Fig. 4.5B) but were not informative for linkage position of these acyl chains. As confirmation, the isotopic distribution of the \([\text{M-H}]^+\) ion at 1333.666 \text{ m/z} \) agreed with that predicted for the glycolipid (Fig. A1A), and fragmentation of the \([\text{M-2H}]^2^-\) ion at \text{m/z} = 774.858 revealed the same structure extended with an additional GalNAcA residue (Fig. A1B). No ions corresponding to this glycolipid were identified when the procedure was repeated for CPS purified from \textit{S. Typhi} Δ\textit{ vexE}.

![Diagram A](image1)

![Diagram B](image2)

**Figure 4.5 Mass spectra of the Vi antigen glycolipid terminus.** (A) Charge deconvoluted LC-ESI-QToF MS spectrum for Vi antigen termini purified from \textit{S. Typhi}. All ions correspond to a di-β-hydroxyacylated HexNAc residue, linked to two or more variably \(O\)-acylated HexNAcA residues. (B) LC-MS/MS for the singly-charged (blue) and doubly-charged (red) ions which correspond to a GalNAcA\(\alpha\) oligosaccharide attached to a reducing terminal diacyl-HexNAc. Overlapping signals are colored purple. Fragmentations are illustrated in green. \text{m/z} = mass-to-charge.
4.3.3 VexE is required for efficient export and cell-surface retention of Vi antigen.

The role of the glycolipid terminus in Vi antigen export and surface assembly of the Vi capsule was investigated in S. Typhi by its susceptibility to degradation by VexL. VexL degraded almost all detectable Vi antigen in the wildtype, indicating minimal amounts of intracellular (untransported) glycan. In contrast, Vi antigen profiles from the ΔvexC (lacking the ABC transporter ATPase) or ΔvexE mutant were unaffected by the presence of the enzyme (Fig. 4.6A). Permeabilization of the mutant cells facilitated complete digestion of the glycan, indicating its stability in whole cells was solely due to inaccessibility to the lyase. Furthermore, a Vi antigen-specific bacteriophage infected the wildtype but formed no plaques on the ΔvexC or ΔvexE mutants, confirming no phage receptor is available on the mutant cell surfaces (Fig. 4.6C). Complementation of the mutations with the respective genes restored phage sensitivity. Wildtype S. Typhi possessed a Vi antigen capsule on the cell surface that was detectable by immunofluorescence microscopy (Fig. 4.6D) and complementation of the ΔvexC and ΔvexE mutants with the respective genes restored the Vi antigen capsule. The ΔvexC and ΔvexE mutants both possessed inclusion bodies (Fig. 4.6D), which were labeled with Vi antigen-specific antibodies in immunofluorescence microscopy of permeabilized cells. Electron microscopy, performed by Dr. Michaela Strüder-Kypke and Mr. Robert Harris, support that the inclusions were cytosolic in both ΔvexC and ΔvexE mutants (Fig. 4.6E). To examine possible deleterious effects of these inclusions on cell physiology, the Cpx envelope stress response was assessed in S. Typhi and mutant derivatives (Fig. 4.6F). Surprisingly, the Cpx response was only significantly upregulated in the ΔvexC mutant, where no export occurs, and this increase was eliminated in a ΔvexC ΔvexE mutant, indicating that activation of a stress response by the inclusions was dependent on acylation.

These results are consistent with intracellular accumulation of Vi antigen in the ΔvexC and ΔvexE mutants, suggesting that both mutations resulted in export defects. However, published mutant phenotypes indicated extensive Vi antigen export in a vexE mutant of an E. coli recombinant containing viaB (Wetter et al., 2012). Since lyase-treatment and immunofluorescence microscopy of whole cells cannot account for Vi antigen released into the growth medium, cell-free supernatants from early exponential phase cultures of S. Typhi and its mutant derivatives were examined for Vi antigen release (Fig. 4.6B). Wildtype cells
released some Vi antigen into the media, as expected with any encapsulated bacterium and consistent with published observations (Virlogeux et al., 1995; Wetter et al., 2012). The ΔvexC mutant released only a trace of Vi antigen, which could be explained by small amounts of lysis during growth, consistent with the release of cytosolic RNA polymerase in the same cultures (Fig. 4.6B). Export and release of Vi antigen was restored when the ΔvexC mutation was complemented with vexC. In contrast, ΔvexE cells released large quantities of Vi antigen. This material was eliminated in a ΔvexCE double mutant (Fig. 4.6BD), indicating an active process involving the ABC transporter, rather than elevated leakage resulting from the vexE defect.
**Figure 4.6 VexE is required for efficient export and surface retention of Vi antigen.** (A) Purified VexL was unable to access Vi antigen within intact cells of the ΔvexE mutant. Whole or lysed cells of S. Typhi and mutant derivatives were incubated with purified VexL, collected by centrifugation, digested with proteinase K, and then samples were probed for Vi antigen. VexL was able to degrade Vi antigen of the WT but not in S. Typhi ΔvexC, providing positive and negative controls for export, respectively. (B) S. Typhi ΔvexE was able to export Vi antigen in a transporter-dependent manner. Growth media from early-exponential phase cultures was collected and probed for Vi antigen and (cytosolic) RNA polymerase by Western immunoblotting. (C) S. Typhi ΔvexE is not sensitive to Vi phage II (HER#39; Félix d’Hérelle Reference Center for Bacterial Viruses, Université Laval, Québec, Canada), indicating the absence of Vi antigen bacteriophage receptor on the cell surface. Six microliters of overnight cultures were dropped on a petri dish, in which one half of the plate had been inoculated with $7.5 \times 10^6$ pfu bacteriophage (arrow). Cells are unable to grow on the phage-coated plate if they express surface-associated Vi antigen. (D) Immunofluorescence microscopy of cells probed with anti-Vi antigen antibodies illustrated that the ΔvexE, ΔvexC, and ΔvexCE mutant possessed no Vi antigen on their surfaces but accumulated intracellular Vi antigen in inclusion bodies, which became accessible to antibody in permeabilized cells. Complementation with plasmids containing vexC and vexE restored surface expression of Vi-antigen. Scale bars represent 10 µm. Insets are enlarged to show a representative cell. (E) ΔvexE and ΔvexC mutants of S. Typhi accumulate cytosolic Vi antigen. Electron micrographs of thin sections of freeze-substituted S. Typhi and mutant derivatives. The hydrated Vi-antigen capsule in the WT strain collapses during preparation (black arrowheads). The absence of export in the ΔvexC mutant, and proper acylation in the ΔvexE mutant, result in electron transparent inclusions that accumulate in the cytoplasm. Some inclusion bodies appear to interfere with cell division, as invaginations of the outer membrane are visible in the ΔvexC mutant surrounding an inclusion body (black arrows). Scale bars represent 400 nm. (F) The Cpx cell envelope stress response is upregulated in ΔvexC mutant, but not ΔvexE mutant S. Typhi. S. Typhi and mutant derivatives were transformed with pNLP15, which contains the spy promoter upstream of the luxCDABE cassette. Luciferase activity was monitored as luminescence counts per second normalized to optical density of the cell culture. Data represents mean ± standard error of three independent experiments. Statistical differences were determined using a student’s t-test; significant differences are indicated: *, p<0.01; ** p<0.001.

### 4.4 Discussion

Vi antigen has a lipid terminus that differs from those of any other known CPS assembled in ABC transporter-dependent pathway. It is composed of a reducing terminal HexNAc residue modified with two β-hydroxy fatty acids and resembles one half of the structure of lipid A (**Fig. 4.5**). This structure, together with similarity shared by VexE and LpxL, a secondary acyl-ACP-dependent acyltransferase from lipid A biosynthesis is consistent with the proposal that VexE is an acyltransferase that transfers a β-hydroxymyristate or β-hydroxypalmitate chain to the terminus of Vi antigen. The action of VexE would be comparable to the secondary acyltransferases in lipid A biosynthesis, although LpxL and LpxM transfer non-hydroxylated fatty acids (Whitfield and Trent, 2014) (**Fig. 1.4**). VexE is the only acyltransferase encoded by the viaB locus. There is no precedent for such enzymes being able to transfer both acyl chains and this would require radically different acceptor specificities in a single catalytic site. A logical origin of
this terminal moiety involves secondary acylation of the UDP-activated β-hydroxyacyl-GlcNAc product of LpxA in lipid A biosynthesis (Anderson and Raetz, 1987a; Anderson et al., 1988). Such a reaction could divert this intermediate for use in Vi antigen biosynthesis and would represent the first off-pathway use of an Lpx-pathway intermediate. The inability of VexE activity to influence lipid A biosynthesis is perhaps not surprising given the regulation of the essential Raetz pathway process. The LpxA reaction equilibrium favors the reverse reaction and the first committed step of lipid A biosynthesis (LpxC) is tightly regulated (Anderson and Raetz, 1987b; Raetz and Whitfield, 2002; Whitfield and Trent, 2014) so pathway flow is regulated according to lipid A requirement. The possibility that ΔvexE Vi antigen possesses a single acyl chain (the product of LpxA) at its reducing terminus was pursued. However, diacyl- or monoacyl-HexNAc were not identified in either extracellular or intracellular (accumulated) Vi antigen from the ΔvexE mutant. This negative result could reflect an absolute requirement for diacylated UDP-GlcNAc, offering an additional means of separation from the lipid A pathway. This does not rule out technical issues where monoacylated Vi antigen termini may lack sufficient hydrophobicity for the separation protocol (and is consistent with altered PVDF binding).

The apparent ability to synthesize Vi antigen in the absence of the acylated terminus could reflect the mutations creating conditions that facilitate polymer synthesis on non-physiological acceptors, as is the case in the E. coli and N. meningitidis kpsS and kpsC mutants (Willis et al., 2013). However, this assumes that the diacyl-HexNAc actually serves as an acceptor, but the identity and mechanism of the Vi antigen polymerase is unknown. Vi antigen could potentially be synthesized by growth at the reducing terminus in a process similar to class I hyaluronan synthases. These enzymes use UDP-GlcNAc or UDP-glucuronic acid (GlcA) as acceptors and the nascent [3]-GlcNAc-β-(1→4)-GlcA-β-(1→3)-UDP chain as the donor during chain extension (Weigel et al., 2015). As an additional complexity, hyaluronan synthase activity requires a self-made chitin-UDP primer ([GlcNAc-β-(1→4)-]2.3-GlcNAc-α-1→UDP) which is retained at the non-reducing end of the hyaluronan product (Weigel et al., 2017). It is unknown how the reducing terminal UDP moiety is removed in the final glycan product. In such a scenario, addition of diacyl-HexNAc could represent the last step in Vi antigen biosynthesis before export, explaining the ability to synthesize
Vi antigen in the absence of VexE. Biochemical characterization of the Vi antigen polymerase(s) is required to resolve this question.

In *E. coli* group 2 CPS assembly, export is dependent on the presence of the glycolipid terminus (Liston et al., 2017; Willis and Whitfield, 2013a; Willis et al., 2013). In contrast, defective acylation of the Vi antigen in the ΔvexE mutant does not prevent export but accumulation of intracellular Vi antigen (which is not seen in the wildtype) also occurs. This could reflect altered recognition by the export machinery. For example, the LPS ABC transporter MsbA from *E. coli* is highly selective for completed (hexaacylated) LPS molecules. Export of tetraacylated precursors occurs only at low levels (reviewed in (Christian R H Raetz, 2002)). Alternatively, the export defect in the ΔvexE mutant could reflect alteration of essential interactions that couple synthesis to export in a multi-protein complex. However, this is unlikely because the phenotype resulting from the VexE catalytic-site mutation (which should preserve protein-protein interactions) is indistinguishable from the vexE deletion (Fig. 4.1C). Interestingly, intracellular Vi antigen in transport-defective mutants showed an increase in the average chain length (Fig. 4.1C, 4.6A), while overexpression of VexE caused a reduction. Lowering chain length requires VexE catalytic activity, rather than a simple structural requirement for the protein, because the size reduction was not evident in ΔvexE cells expressing VexE^{H466A}. Altered chain lengths can be explained by an elongation phase differing from the normal assembly process occurring with molecules with a complete glycolipid terminus. There is precedent for the modulation of glycan chain length by competition between export and extension in other bacterial systems with ABC transporters (Kos et al., 2009; Larue et al., 2011).

The use of a conserved intermediate from the lipid A-biosynthesis pathway to create the lipid terminus potentially facilitates Vi antigen production in diverse Gram-negative bacteria by horizontal transfer of the *viaB* locus with a limited gene complement. This is evident in the possession of the locus in *Achromobacter*, *Bordetella* and *Citrobacter* sp. and expression in *E. coli* but why some Vi antigen-producers possess the additional VexL component is unknown. It is also unknown whether the terminal lipid itself is important in interaction of Vi antigen with the host immune system. In the context of Vi
antigen-based vaccines, a production strain lacking vexE may offer advantages since it exports Vi antigen with altered micellar properties and a reduced association with LPS.
CHAPTER 5

VexE is an acyltransferase responsible for assembly of the glycolipid terminus of Vi antigen

5.1 Preface

This chapter contains currently unpublished data for the *in vitro* characterization of the putative Vi antigen acyltransferase VexE, which was initially described in Chapter 4. The biological and biochemical assays, HPLC, and MS described within were performed by myself. I prepared samples for NMR spectroscopy, and the interpretation of the data was led by Dr. Olga Ovchinnikova.

5.2 Rationale

Vi antigen has a unique reducing-terminal glycolipid whose functional role seems to be analogous to the lysoPG-Kdo₉ employed by prototypical group 2 CPS assembly systems. This glycolipid is composed of a HexNAc residue that is decorated by two β-hydroxyacyl chains. Although mass spectra of purified glycolipid termini were diagnostic for the presence of these acyl chains, fragmentation products in MS² were not conclusive in defining their linkage position. The assembly of this glycolipid appears to involve the predicted acyltransferase, VexE, that is conserved in Vi antigen biosynthesis loci and is absent from prototypical group 2 CPS assembly systems. The glycolipid terminus was not found in Vi antigen purified from *S. Typhi* ΔvexE. These results led to the proposal that VexE is a secondary acyltransferase that acylates the UDP-β-hydroxymyristoyl-GlcNAc product of LpxA, potentially diverting this intermediate for use in Vi antigen biosynthesis, however, this hypothesis remained untested. This chapter therefore describes further biochemical characterization of VexE and its role in Vi antigen assembly.

5.3 Results

5.3.1 VexE is homologous to lysosphospholipid acyltransferases

Initial bioinformatic efforts (described in Chapter 4) defined a C-terminal LPLAT domain in *S. Typhi* and *A. denitrificans* VexE proteins. This LPLAT domain is homologous to the acyl-ACP-dependent secondary acyltransferases LpxL and LpxM from lipid A biosynthesis (Fig. 1.4). LPLAT proteins contain a characteristic H(X)₄D/E motif, that was proposed to contain the catalytic His-Asp pair (Six et al., 2008). A histidine to alanine replacement at this position reduces acyltransferase activity of LpxL >1,000-fold (Six
et al., 2008). Based on multiple sequence alignment, *S. Typhi* and *A. denitrificans* VexE proteins share this histidine residue (Fig. 4.1B), and VexE H466A did not complement a ΔvexE mutation (Fig. 4.1C), despite remaining folded (Fig. 4.2). This phenotype was assessed based on differential binding of the glycan to immunoblotting membranes; Vi antigen from wildtype *S. Typhi* binds both PVDF and Nylon membranes, but Vi antigen from a vexE mutant only binds Nylon (see Chapter 4.1C). VexE includes a tyrosine residue at the sixth position of the H(X)₄D/E motif that tolerated mutagenesis to phenylalanine, and the consequence(s) of this divergence from D/E were unknown at the time these experiments were completed.

Our understanding of the LPLAT protein family was recently advanced by the simultaneous release of structures for two LPLAT-family enzymes, *A. baumannii* LpxM (Dovala et al., 2016) and *Mycobacterium smegmatis* PatA (Albesa-Jové et al., 2016). LpxM acylates an intermediate in lipid A biosynthesis (Fig. 5.1, Fig. 1.4), while PatA acylates phosphatidyl-myoinositol mannosides (PIM) (Fig. 5.1), which are a family of cell-surface glycolipids produced by Mycobacteria (Angala et al., 2014). *A. baumannii* LpxM is ‘secondary’ in that it myristoylates β-hydroxyacyl groups of lipid A (Clementz et al., 1997; Dovala et al., 2016), whereas PatA directly acylates a mannose residue at C-6, and is therefore ‘primary’ (Fig. 5.1). PatA uses coenzyme A (CoA)-activated acyl donors, unlike LpxL or LpxM which use acyl-ACP donors (Albesa-Jové et al., 2016). Despite these differences, both LpxM and PatA possess complete H(X)₄D/E motifs, and their acyltransferase activities/specificities have been defined (Albesa-Jové et al., 2016; Dovala et al., 2016; Tersa et al., 2017). Both contain a deep active-site pocket that functions as a ‘molecular ruler’ (i.e. the length of the pocket defines acyl chain length specificity) (Albesa-Jové et al., 2016; Dovala et al., 2016; Tersa et al., 2017). For PatA, molecular dynamic simulations and co-structures with substrates/products suggest that the histidine residue of the H(X)₄D/E motif directly deprotonates the C-6 hydroxyl of the mannose acceptor, which then attacks the acyl-thioester donor (Tersa et al., 2017). The Asp residue hydrogen bonds to this histidine and therefore plays a supporting role in protonation/deprotonation. Despite differences in acyl donors and acceptors, LpxM and PatA share similar structures (2.0 r.m.s.d., PDBeFold (Krissinel and Henrick, 2004)). This suggests that the overall
acyltransferase fold/mechanism is conserved between these enzymes, and that comparison of VexE to these LPLAT enzymes may be informative.

Although both LpxM and PatA showed low sequence identity with VexE and contain complete H(X)₄D/E motifs, their secondary structures (Jones, 1999) are remarkable similar to that predicted for VexE based on its amino acid sequence (Fig. 5.2A). When the sequence of VexEₐₜ was threaded on to the PatA structure, the model revealed, surprisingly, that the catalytic D/E of VexE was most likely contributed by D₅₃₂, which resides outside of the H(X)₄D/E motif (Fig. 5.2AB). This supports the conclusion that VexE contains a catalytically-competent active site, despite encoding a tyrosine at the sixth position in the H(X)₄D/E motif. VexE D₅₃₂A only partially complemented ΔvexE mutant S. Typhi, based on restoration of PVDF-binding of the Vi antigen product. VexE H₄₆₆A D₅₃₂A was inactive, despite robust expression of this VexE derivative (Fig. 5.3).

**Figure 5.1 Glycolipid substrates for VexE homologs.** LpxL and LpxM are secondary lauroyl and myristoyl transferases, respectively, from Kdo₂-lipid A biosynthesis. PatA is an acyltransferase that palmitylates PI-Man₂ (Albesa-Jové et al., 2016; Tersa et al., 2017). PIM are membrane glycolipids produced by *Mycobacteria tuberculosis* and *M. smegmatis*, for example.
Figure 5.2 VexE is homologous to LpxM and PatA, acyltransferases from lipid A and PIM biosynthesis, respectively. (A) The panel depicts multiple sequence alignment of the LPLAT domain of S. Typhi and A. denitrificans VexE, with PatA from M. smegmatis, and LpxM from A. baumannii. Secondary structure elements from structures of PatA (PDB ID: 5F34) and LpxM (PDB ID: 5KN7) are shown as green cylinders (α-helices) and yellow arrows (β-sheets). Turns are indicated by a red ‘T.’ Secondary structure elements displayed for S. Typhi and A. denitrificans VexE were predicted using PSIPred (Jones, 1999), based on amino acid sequence and were independent of structural modelling by Phyre². (B) The panel depicts a homology model of the LPLAT domain of A. denitrificans VexE, generated by one-to-one threading on to the PatA structure (PDB ID: 5F34) employing the Phyre² server (Kelley and Sternberg, 2009). The cartoon is coloured in rainbow from N-(blue) to C-terminus (red). Inset shows the predicted active site, with important residues labelled. The corresponding residues in PatA structure are shown as black sticks. The β-hydroxymyristate residue (pick sticks) was manually modelled based on the position of a palmitate residue in the PatA structure.
**Figure 5.3 Site-directed mutagenesis of predicted catalytic residues of VexE.** Wildtype Vi antigen bound both PVDF and Nylon membranes, whereas Vi antigen produced by a ΔvexE mutant possessed increased apparent molecular weight and bound only to Nylon. Expression of WT VexE restores PVDF binding and lowers apparent molecular weight of the Vi antigen. VexE D532A restored some PVDF-binding, whereas VexE H466A and VexE H466A D532A did not. The figure depicts western immunoblots of whole-cell lysates prepared from *S*. Typhi or *S*. Typhi ΔvexE expressing plasmid-encoded VexE or site-directed mutant derivatives. Immunoblots were probed with Vi antigen- or VexE-specific antibodies.

### 5.3.2 VexE is an acyl-ACP-dependent UDP-GlcNAc C-6 β-hydroxyacyltransferase

The Vi antigen contains a reducing terminal HexNAc residue that possesses two hydroxylated acyl chains. This structure was determined by high-resolution MS of hydrophobic oligosaccharides liberated by the Vi antigen depolymerase VexL (see Chapter 4). However, these mass spectra were not informative to the acyl linkage position or configuration of the HexNAc residue. Unfortunately, attempts to sufficiently increase the yield and purity of these glycolipids, to observe linkage positions by NMR spectroscopy, were
unsuccessful. To understand the biosynthesis of the glycolipid residue, the activity of the putative VexE acyltransferase was therefore investigated biochemically.

Acyltransferase enzymes use activated acyl donors, which are generated by covalent thiol linkage to Coenzyme A (CoA) or holo-ACP. The purified glycolipid terminus contains two β-hydroxyacyl chains and, unfortunately, these acyl donors are not commercially available in either form. LpxL and LpxM, which share higher sequence identity with VexE in comparison to PatA, use acyl-ACP donors (Six et al., 2008). To test the possibility that VexE also used acyl-ACP, a series of acyl-ACP donors were generated in vitro. ACP and holo-ACP synthase (AcpS) were co-expressed and purified from *E. coli*; AcpS transfers a phosphopantetheine functional group to ACP, generating the holo-enzyme (holo-ACP) (Beld et al., 2014; Flugel, 2000). Purification of holo-ACP followed a scheme adapted from previously published methods (Masoudi et al., 2014) and employed IMAC and AEC chromatography (Fig. 5.4A), together with covalent chromatography using resin that binds free sulfhydryl residues. This protocol facilitated separation of holo-ACP from the acyl- and apo-protein, which do not contain a free thiol group. Purified soluble acyl-ACP synthetase (AasS) from *Vibrio harveyi* (Fig. 5.4B) was then used to load holo-ACP with commercially available fatty acids in vitro (Jiang et al., 2006; Masoudi et al., 2014). The loading reaction was monitored by SDS-free Tris-Glycine PAGE in the presence of 2.5 M urea (Fig 5.4C). Under these conditions, electrophoretic mobility of purified holo-ACP increases dramatically when loaded with its fatty acid cargo (Rock et al., 1981). Based on shifts in electrophoretic mobility of the acyl-ACP product, AasS efficiently loaded holo-ACP with acyl and β-hydroxyacyl chains up to 16 carbons in length (Fig 5.4D). The presence of two bands in PAGE of the β-hydroxyacyl-ACPs likely reflects the racemic mixture of D.L-3-hydroxy-fatty acids used.
Figure 5.4 Generation of acyl-ACP donors. (A) The panel depicts SDS-PAGE (upper) and the corresponding western immunoblot probed with His₅-specific antibody (lower) of fractions from the holo-ACP purification. ACP-His₅ and holo-ACP synthase (AasS) were co-expressed and then co-purified from E. coli by immobilized metal-affinity chromatography (IMAC). AcpS was then removed by anion-exchange chromatography (AEC). Although the molecular weight of ACP-His₅ is lower than AcpS, it consistently shows lower relative mobility in SDS-PAGE. FT: flow-through (B) The panel shows SDS-PAGE of fractions from the purification of V. harveyi AasS. (C) V. harveyi AasS specifically acylates holo-ACP in vitro with high-efficiency. The panel depicts SDS-free Tris-glycine PAGE in 2.5 M urea at pH 9.5 stained with Coomassie Brilliant Blue R250. Acylation reactions contained d,l-β-hydroxymyristic acid, purified AasS, and purified holo-ACP where indicated. The electrophoretic mobility of ACP increases when loaded with acyl-cargo (Rock et al., 1981), which only occurred when all reagents were included. (D) AasS effectively loads holo-ACP with fatty acids and their d,l-β-hydroxylated derivatives from 8-16 carbons in chain length. Lanes 1 and 8 contain negative control reactions containing holo-ACP and AcpS incubated with fatty acid-'free' stock solvent. The panel shows PAGE of acylation reactions prepared as in B. Chain-length of the acyl-donors are indicated. The vertical line marks two separate gels.
Figure 5.5 VexE is an acyl-ACP-dependent UDP-GlcNAc β-hydroxyacyltransferase. (A) The panel depicts SDS-PAGE of purified VexE-His<sub>6</sub>. (B) VexE acylates UDP-GlcNAc <i>in vitro</i>, with an absolute requirement for β-hydroxylation of the acyl donor, and preference for 12-14-carbon chain lengths. The panel shows TLC analysis of acyltransferase reactions containing purified VexE, UDP-[1-<sup>14</sup>C]-GlcNAc, and indicated purified acyl-ACP donor. (C) Product formation was dependent on catalytically-active VexE; No product was evident for purified VexE H466A or VexE H466A/D532A. TLC plates were developed in ethyl acetate-butanol-glacial acetic acid-water (10:10:8:5), exposed on a phosphor storage screen, then imaged on a Personal FX Phosphor Imager (Bio-Rad).
Figure 5.6 VexE prefers UDP-GlcNAc over UDP-GalNAc. The traces depict extracted ion chromatograms of HPLC-MS/MS separation of VexE reaction products. The vertical axis represents counts in MS² for the diagnostic ion at 526.242 m/z, which is the expected [M-H] value for β-hydroxymyristoyl-HexNAc-1-phosphate. No products were detected unless VexE, acyl-ACP, and UDP-GlcNAc were all included.

As the diacyl-HexNAc terminus resembled intermediates of lipid A biosynthesis, it was originally proposed that VexE was a secondary acyltransferase that β-hydroxyacylates the UDP-β-hydroxymyristoyl-GlcNAc product of LpxA (see Fig. 1.4). This modification would divert the LpxA reaction product from the Raetz pathway for use in Vi antigen assembly. However, when this candidate system was reconstituted in vitro, purified VexE (Fig. 5.5A) was able to directly modify UDP-GlcNAc independent of the presence of LpxA (Fig. 5.5B). This reaction was followed by thin-layer chromatography (TLC) and detection employed UDP-\([1^{14}C]\)GlcNAc. A single product with a higher relative mobility was evident in reactions that contained β-hydroxyacyl-ACP donors from 10-14 carbons in length; the most abundant product was obtained with β-hydroxymyristoyl-ACP (C14) (Fig. 5.5B). Formation of this product was dependent on catalytic activity of VexE, since no products were observed with purified VexE H466A or VexE H466A D532A (Fig. 5.5C). However, a small amount of product was detected with purified VexE D532A, consistent with the observation that this derivative was able to partially complement a vexE mutant in vivo (Fig. 5.3). Because the configuration of the terminal hexose residue in the glycolipid terminus was unknown, acylation reactions were also performed with UDP-GalNAc. However, no products were evident in HPLC-MS of these reactions (Fig. 5.6). These data indicate that VexE is an acyl-ACP dependent β-
hydroxyacyltransferase and suggest that the HexNAc residue in the authentic glycolipid terminus is GlcNAc.

To gain more insight into the structure of the reaction product, scaled-up acylation reactions containing VexE, β-hydroxymyristoyl-ACP, and unlabelled UDP-GlcNAc were performed and the hydrophobic product was purified by HPLC (Fig. 5.7A). The mass spectrum of this fraction contained predominately singly- and doubly-charged ions that corresponded to UDP-β-hydroxymyristoyl-GlcNAc (Fig. 5.7B). Like the authentic glycolipid terminus described in the previous chapter, fragmentation products supported this structure, but did not inform linkage position of the acyl chain (Fig. 5.7CD).
Figure 5.7 Mass spectrometry of the VexE reaction product. (A) HPLC separation of VexE acylation reactions employing UDP-GlcNAc and β-hydroxymyristoyl-ACP substrates, monitored by spectrophotometry at 254 nm. (B) Negative-mode direct-infusion ESI-MS of the pooled and concentrated fractions indicated in A. (C) MS² fragmentation of the ion at 415.63 m/z, which corresponds to doubly-charged UDP-β-hydroxymyristoyl-GlcNAc. (D) MS² fragmentation of the ion at 832.43 m/z, which corresponds to singly-charged UDP-β-hydroxymyristoyl-GlcNAc. Expected m/z are indicated in parentheses adjacent to those observed. Fragmentation products are illustrated by green arrows. The linkage position of the acyl chain reflects NMR data collected from this material and was not informed by MS.
Figure 5.8 NMR spectroscopy of the VexE product. (A) The structure of VexE product (1) and borate diol monoester of UDP-sugar (2). In the samples, a mixture of borate diol monoester and two diastereomeric diesters is expected, however, our NMR data could not distinguish between these complexes. (B) Selected parts of the $^1$H,$^1$C HSQC spectrum of VexE product. C/H pairs of moieties are labelled as follows: U, uracil; R, ribose, G, glucosamine, M, β-hydroxymyristoyl group. Blue signals denote uracil and ribose participating in the borate complex. Signals for contaminating glycerol (*) and PEG (**) are indicated.
To determine the position of the β-hydroxymyristoyl group, the purified product of VexE was analyzed by \(^1\)H, \(^{13}\)C, and \(^{31}\)P NMR spectroscopy (Fig. 5.8). The presence of the β-hydroxymyristoyl moiety was evident from the characteristic triplet at \(\delta_H 0.87\) for the terminal methyl protons (H-14), which appeared to be roughly the same integral intensity as the signal for the N-acetyl group of GlcNAc (\(\delta_H 2.08\)). The heteronuclear \(^1\)H, \(^{13}\)C single quantum coherence (HSQC) spectrum contained additional characteristic signals at \(\delta_H/\delta_C 2.55/43.0\) and 2.69/43.0 for \(\alpha\)-methylene protons (H-2) adjacent to the carbonyl group. The \(^{13}\)C NMR chemical shift for the carbonyl carbon (C-1) was obtained from heteronuclear multiple bond correlation (HMBC) spectroscopy data. The remaining signals for the β-hydroxymyristoyl group were assigned based on 2D correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), HSQC, and HMBC experiments, and by comparison with published data (Sillerud et al., 1986; Tulloch and Mazurek, 1976). \(^1\)H and \(^{13}\)C NMR chemical shifts are summarized in Tables 5.1 and 5.2.

The anomeric signal (H-1''/C-1'') in the HSQC spectrum at \(\delta 5.50/95.7\) was employed as a starting point to assign signals for the GlcNAc residue. A small \(J_{1''-2''}\) coupling constant of \(\sim 3\) Hz and \(J_{1''-3''}\) of \(7.2\) Hz support \(\alpha\)-D-configuration of the GlcNAc residue. The TOCSY spectrum demonstrated correlations between H-1'' and H-2''–H-6'' protons, which is characteristic for sugars with gluco configuration, and correlations traced in the COSY spectrum were used to distinguish protons within the spin system. \(^{13}\)C NMR chemical shifts for the GlcNAc residue were obtained from HSQC and HMBC spectra. Since a three-bond correlation was not observed between C-1 of the β-hydroxymyristoyl group and any of the GlcNAc protons in the HMBC spectrum, the position of acyl substituent was inferred based on \(^1\)H and \(^{13}\)C chemical shifts of the GlcNAc residue. Acylation of a sugar hydroxyl group results in downfield displacement of the signal for the proton at the acylation site (Anderson and Raetz, 1987a). This is consistent with the low field position observed for H-6''a,b at \(\delta 4.41\) and 4.44, compared with their position at \(\delta 3.81\) and 3.86 in UDP-GlcNAc (Table 5.2). This was due to the deshielding effect of the O-acyl substituent located at position 6 and was confirmed by a downfield shift (+2.5 ppm) of the signal for C-6'' and an upfield shift (−2.2 ppm) of the signal for the neighboring carbon C-5'' (Table 5.2).
Table 5.1. $^1$H NMR data (25 °C, D$_2$O)

<table>
<thead>
<tr>
<th>Moiety</th>
<th>VexE product (free, minor)(^a)</th>
<th>VexE product–borate complex(^a)</th>
<th>UDP-GlcNAc</th>
<th>UDP-GlcNAc–borate complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uracil</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-5</td>
<td>5.95</td>
<td>5.93</td>
<td>5.97</td>
<td>5.93</td>
</tr>
<tr>
<td>H-6</td>
<td>7.93</td>
<td>7.81</td>
<td>7.97</td>
<td>7.82</td>
</tr>
<tr>
<td>Ribose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-1’</td>
<td>5.99</td>
<td>5.96</td>
<td>5.99</td>
<td>5.97</td>
</tr>
<tr>
<td>H-2’</td>
<td>4.36</td>
<td>4.50</td>
<td>4.38</td>
<td>4.51</td>
</tr>
<tr>
<td>H-3’</td>
<td>4.36</td>
<td>4.49</td>
<td>4.37</td>
<td>4.49</td>
</tr>
<tr>
<td>H-4’</td>
<td>4.28</td>
<td>4.29</td>
<td>4.29</td>
<td>4.29</td>
</tr>
<tr>
<td>H-5’</td>
<td>4.21, 4.23</td>
<td>4.14, 4.22</td>
<td>4.20, 4.25</td>
<td>4.14, 4.22</td>
</tr>
<tr>
<td>Pyranose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-1’’</td>
<td>5.50</td>
<td>5.52</td>
<td>5.52</td>
<td></td>
</tr>
<tr>
<td>H-2’’</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
<td></td>
</tr>
<tr>
<td>H-3’’</td>
<td>3.83</td>
<td>3.81</td>
<td>3.82</td>
<td></td>
</tr>
<tr>
<td>H-4’’</td>
<td>3.61</td>
<td>3.56</td>
<td>3.56</td>
<td></td>
</tr>
<tr>
<td>H-5’’</td>
<td>4.15</td>
<td>3.94</td>
<td>3.94</td>
<td></td>
</tr>
<tr>
<td>H-6’’</td>
<td>4.41, 4.44</td>
<td>3.81, 3.86</td>
<td>3.81, 3.86</td>
<td></td>
</tr>
<tr>
<td>Acetyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH$_3$</td>
<td>2.08</td>
<td>2.08</td>
<td>2.08</td>
<td></td>
</tr>
<tr>
<td>β-hydroxymyristoyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-2</td>
<td>2.55, 2.69</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-3</td>
<td>4.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-4</td>
<td>1.52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-5</td>
<td>1.33, 1.40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-6–H-11</td>
<td>1.27–1.33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-12</td>
<td>1.27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-13</td>
<td>1.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-14</td>
<td>0.87</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) One set of signals was observed for GlcNAc and β-hydroxymyristoyl moieties
Table 5.2 $^{13}$C NMR data (25 °C, D$_2$O)

<table>
<thead>
<tr>
<th>Moiety</th>
<th>VexE product (free, minor)$^a$</th>
<th>VexE product in borate complex$^a$</th>
<th>UDP-GlcNAc$^b$</th>
<th>UDP-GlcNAc in borate complex$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uracil</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-2</td>
<td>154.5</td>
<td>153.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-4</td>
<td>169.4</td>
<td>168.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-5</td>
<td>104.0</td>
<td>103.8</td>
<td>103.9</td>
<td>103.8</td>
</tr>
<tr>
<td>C-6</td>
<td>142.6</td>
<td>143.9</td>
<td>142.9</td>
<td>144.0</td>
</tr>
<tr>
<td>Ribose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-1’</td>
<td>89.8</td>
<td>93.3</td>
<td>89.7</td>
<td>93.3</td>
</tr>
<tr>
<td>C-2’</td>
<td>75.1</td>
<td>81.8</td>
<td>75.1</td>
<td>81.7</td>
</tr>
<tr>
<td>C-3’</td>
<td>70.9</td>
<td>77.2</td>
<td>70.9</td>
<td>77.2</td>
</tr>
<tr>
<td>C-4’</td>
<td>84.3</td>
<td>86.4</td>
<td>84.5</td>
<td>86.5</td>
</tr>
<tr>
<td>C-5’</td>
<td>66.3</td>
<td>67.2</td>
<td>66.2</td>
<td>67.3</td>
</tr>
<tr>
<td>Pyranose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-1’’</td>
<td>95.7</td>
<td>95.8</td>
<td>95.8</td>
<td>95.8</td>
</tr>
<tr>
<td>C-2’’</td>
<td>54.9</td>
<td>55.0</td>
<td>55.0</td>
<td>55.0</td>
</tr>
<tr>
<td>C-3’’</td>
<td>72.2</td>
<td>72.2</td>
<td>72.2</td>
<td>72.2</td>
</tr>
<tr>
<td>C-4’’</td>
<td>70.7</td>
<td>70.8</td>
<td>70.8</td>
<td>70.8</td>
</tr>
<tr>
<td>C-5’’</td>
<td>72.1</td>
<td>74.3</td>
<td>74.3</td>
<td></td>
</tr>
<tr>
<td>C-6’’</td>
<td>64.1</td>
<td>61.6</td>
<td>61.6</td>
<td></td>
</tr>
<tr>
<td>Acetyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH$_3$</td>
<td>23.4</td>
<td>23.4</td>
<td>23.4</td>
<td></td>
</tr>
<tr>
<td>CO</td>
<td>176.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-hydroxymyristoyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-1</td>
<td>175.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-2</td>
<td>43.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-3</td>
<td>69.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-4</td>
<td>37.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-5</td>
<td>25.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-6–C-11</td>
<td>30.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-12</td>
<td>32.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-13</td>
<td>23.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-14</td>
<td>14.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ One set of signals was observed for GlcNAc and β-hydroxymyristoyl moieties

$^b$ Data from HSQC experiment

The remaining signals for the uracil and ribose moieties of the VexE product were split. The minor signal series was virtually identical to published values for uracil and ribose signals in UDP-GlcNAc; whereas the major series differed from UDP-GlcNAc, free UDP, and UMP. The $^{31}$P spectrum contained two signals for the diphosphate group at δ $–10.8$ and $–12.7$, which were assigned based on correlations with ribose H-5’, GlcNAc H-1’’ and H-2’’ in the $^1$H,$^{31}$P HMBC spectrum. Both $^{31}$P signals were split, which reflects structural heterogeneity in the uridine moiety.
In the major signal series, ribose carbons C-1’–C-5’ all resonated downfield of those in UDP-GlcNAc, with the largest difference being for C-2’ and C-3’ (~6.5 ppm shift downfield). This was indicative of a diester borate complex. A similar change in chemical shift upon borate esterification of 6-7 ppm was reported for C-2 and C-3 of apiofuranosides (Ishii and Ono, 1999), which have the same relative stereochemistry at C-2 and C-3 as ribose. The formation of borate complexes with cis-diol groups of ribose residues have been reported (Kim et al., 2004b), but NMR data for borate-nucleotide complexes have not. To confirm the presence of a borate complex with the VexE product, a UDP-GlcNAc–borate complex was prepared by mixing equimolar amounts of boric acid and UDP-GlcNAc in D$_2$O and adjusting pH to 8, as described for apiofuranosides (Ishii and Ono, 1999). $^1$H NMR and HSQC spectra of this mixture contained an additional set of signals, when compared to UDP-GlcNAc alone. The ratio of free to borate-esterified UDP-GlcNAc in this sample was ~ 5.6:1, based on the ratio of the integral intensities of the uracil $^1$H-6 signals. The $^1$H and $^{13}$C NMR chemical shifts of the uracil and ribose in the UDP-GlcNAc–borate complex were essentially identical to those observed in the major signal series in the HSQC spectrum of the VexE product (Tables 5.1, 5.2). Lowering the pH of the VexE product to 1 disrupted the borate complex; the corresponding signals in the HSQC spectrum disappeared, and the intensity of the non-esterified ribose signals increased. In addition, the signal for GlcNAc H-6”a,b shifted upfield to δ 3.80 and 3.86, respectively, indicating that position 6 was no longer O-acylated.

5.4 Discussion

Chemical analyses of the authentic glycolipid terminus and VexE reaction product were consistently hampered by low yields. For the glycolipid terminus, this was anticipated because terminal residues represent a small fraction of total mass in polysaccharide purifications. Combined with loss during the purification process, the collection of definitive structural information was limited. Nevertheless, the biochemical data reported in this chapter clearly support earlier proposals that VexE is a novel acyltransferase that acylates the Vi antigen glycolipid terminus.
These *in vitro* data indicate that VexE is a β-hydroxyacyl-ACP dependent acyltransferase that acylates UDP-GlcNAc at C-6 to form UDP-6-O-[β-hydroxy-myristoyl]-α-D-GlcNAc. This suggests that the structure initially proposed for the glycolipid terminus was incorrect but does not conflict with reported MS data for the purified glycolipid. That is, the terminal HexNAc residue is likely β-hydroxyacylated at two positions: C-6 and (hypothetically) C-3. MS data for the glycolipid terminus were not diagnostic for the acylation positions; the proposed acylation pattern had been influenced by the structure of lipid A and the secondary acyltransferase activities of the VexE homologs, LpxL and LpxM. Tandem MS of the VexE product reported in this chapter (Fig. 5.7) also failed to generate fragmentation products diagnostic for acylation position. They did, however, unequivocally indicate that the β-hydroxyacyl chain was covalently linked to UDP-GlcNAc, despite the lack of direct evidence for this in NMR spectra of the same sample, which indirectly demonstrated the acyl chain was linked at C-6. Unusually, NMR spectra indicated that the ribose moiety of the VexE product formed a complex with borate. This complex is likely an *in vitro* artefact, and likely reflects the low sample concentration; this residue likely originated from the borosilicate glass vials used during purification.

The LPLAT protein PatA acylates a mannose residue (in PIM) at C-6, which suggests that the primary activity seen for VexE is not beyond reason for a LPLAT enzyme. PatA was not initially identified based on sequence similarity to VexE but was instead found later in the development of this thesis research due to shared predicted secondary structure when the PatA structure was deposited. The finding that H^{466} but not D^{532} was absolutely required for activity corresponds with the mechanism proposed for PatA (Tersa et al., 2017). Informed by this data, a refined catalytic model for VexE can be proposed, where H^{466} extracts a proton from C-6 of UDP-GlcNAc, allowing for nucleophilic attack on the thioester-linked acyl cargo of ACP. D^{532} hydrogen bonds to H^{466} supporting its protonation/deprotonation during the reaction. The preference for 14>12>10 carbon-long acyl chains (but not 16) suggests that VexE contains a substrate-binding pocket with ‘ruler’ function, similar to those seen in both LpxM (Dovala et al., 2016) and PatA (Albesa-Jové et al., 2016; Tersa et al., 2017). The absolute requirement for β-hydroxylated acyl donors
supports the structure proposed for the terminal glycolipid, and preference for UDP-GlcNAc suggests that this forms the configuration of the HexNAc residue.

The additional structural information reported in this Chapter indicates a revision is needed in the model for Vi antigen glycolipid assembly (Fig. 5.9). The model described in Chapter 4 proposed that VexE acylates the β-hydroxyl group of UDP-3-0-[β-hydroxyacyl]-GlcNAc, which is the product of LpxA and is conserved in Gram-negative bacteria. The finding that VexE directly acylates UDP-GlcNAc at C-6 in vitro does not change this proposal but refines the linkage position to C-6 of the GlcNAc moiety. Regulation of pathways consuming UDP-GlcNAc is important for bacterial fitness due to the use of this precursor in the assembly of essential glycoconjugates including lipid A (Whitfield and Trent, 2014) and peptidoglycan (Barreteau et al., 2008). LpxA is essential in most Gram-negative bacteria, and catalyzes a reversible reaction with an equilibrium that ‘protects’ the UDP-GlcNAc pool by favouring the substrates (Anderson and Raetz, 1987a). All of the lipid A acyltransferases have exquisite specificity for acyl chain length and substitution position (Bainbridge et al., 2008; Bednarski, 1996; Clementz et al., 1997; Williams and Raetz, 2007). It is therefore more likely that VexE acylates the UDP-3-0-[β-hydroxymyristoyl]-GlcNAc product of LpxA, rather than LpxA acting on the UDP-6-0-[β-hydroxymyristoyl]-GlcNAc product of VexE. However, the LpxA-VexE reaction was not successfully reconstituted in vitro, so this pathway is speculative.
Figure 5.9 Model for VexE activity in assembly of the Vi antigen glycolipid terminus. LpxA is an acyltransferase from lipid A biosynthesis in Gram-negative bacteria. LpxA transfers a β-hydroxymyristate from β-hydroxymyristoyl-ACP, to C-3 of UDP-GlcNAc. This reaction is at equilibrium that favours the substrates. VexE is an acyltransferase that transfers a β-hydroxymyristate from β-hydroxymyristoyl-ACP, to C-6 of UDP-GlcNAc. The glycolipid terminus is linked to Vi antigen in an unknown reaction; the Vi antigen polymerase(s) is not characterized. At some point in this process the UDP-moiety is removed.

One possibility for the inability to reconstitute the proposed pathway is that the *in vitro* reaction conditions did not support the formation of a doubly-acylated product. LpxA and VexE could require different conditions to function together *in vitro*. The physical properties of wildtype and ΔvexE Vi antigens differ and this is proposed to reflect differences in acylation state of the glycan (di- vs. mono- or non-acylated). For comparison, the di-β-hydroxyacylated-GlcN product of LpxD (*Fig. 1.4*) partitions in to membranes and forms micelles at low µM concentrations (Bartling and Raetz, 2008; Radika and Raetz, 1988). The properties of the enzymes involved in the pathway may be attuned to these differences and the VexE *in vitro* assay did not include membranes or detergents, which may be important for product formation and/or stability. This can only be resolved by extensive further research on the enzymes and their physical requirements.

An obvious hurdle is the relatively low VexE reaction efficiency *in vitro*. This could be due to acylation by VexE only occurring as required in Vi antigen biosynthesis. This is consistent with observation that overexpression of VexE does not affect growth of *E. coli* (*Fig. 4.3*). This occurs with LpxA, whose reaction equilibrium favours the substrates (Anderson and Raetz, 1987b); the reaction is driven by product consumption by the upstream N-acetylesterase LpxC, according to LPS requirement (Young et al., 1995).
Including LpxA in *in vitro* reactions did not drive the VexE reaction forward, potentially for this reason. Attempts to improve yield by ‘pushing’ the VexE reaction from behind, by including AasS to re-generate acyl-ACP *in situ*, had little effect. It was not possible to test ‘pulling’ the VexE reaction forward as the Vi antigen polymerase(s) and glycolipid attachment mechanism are unknown. Characterization of the Vi antigen polymerizing proteins may be required to further inform the biosynthesis model and to test these hypotheses *in vitro*.

Finally, the physiological relevance of the *in vitro* reaction product must be confirmed. This will require analysis of acyl and Vi antigen linkage positions in the authentic glycolipid terminus. Confirming the proposed biochemical pathway will require isolation of putative monoacylated intermediates in mutants of implicated acyltransferase genes. These experiments are not trivial and are discussed in Chapter 6. Due to the lack of information for the Vi antigen biosynthesis proteins, many steps in the assembly pathway remain speculative for now. These biochemical data provide a starting point to address this puzzle.
CHAPTER 6

Conclusions

6.1 A model for ABC-transporter dependent Vi antigen biosynthesis

ABC transporters participate in the secretion of diverse cell-surface glycoconjugates. These transporters share some overall structural principles, but subtle differences result in diversity in the details of their transport mechanisms (Locher, 2016). The research described in this thesis provides a significant contribution to the understanding of a glycoconjugate substrate and export pathway that involves a subset of ABC transporters, which participate in the assembly of polysaccharide capsules in Gram-negative bacteria. In this work, a novel glycolipid was identified at the terminus of the Vi antigen CPS that is proposed to play an analogous role to the lysoPG-Kdo residue conserved in prototype ABC transporter-dependent CPS assembly systems (Willis and Whitfield, 2013a). Similar to these systems, interruption of Vi anchor-assembly (through deletion of vexE) resulted in glycan export defects. Furthermore, any glycan that was exported was not retained on the cell surface, so the protective capsule virulence factor was lost. This discovery supports the broad hypothesis that a glycolipid residue is a requisite feature of ABC transporter-dependent glycoconjugate assembly and begins to define mechanistic steps in assembly of the unusual Vi antigen CPS.

The reduced transport efficiency in a ΔvexE mutant is apparent as cytoplasmic Vi antigen-containing aggregates and increased molecular weight of the Vi antigen product. These phenotypes are also observed in an ABC transporter ATPase mutant (ΔvexC) and could be explained by an extended polymerization phase concomitant with decreased transport. These data suggest that the Vi antigen ABC transporter possesses specificity for complete glycolipid termini. For comparison, the ABC transporter for lipid A-core, MsbA, has specificity for hexa-acylated (complete) lipid A molecules, and this serves as a ‘quality-control’ point in LPS assembly (Fujishima et al., 2002; Karow and Georgopoulos, 1993; Mamat et al., 2008; Meredith et al., 2006; Reynolds and Raetz, 2009). Purified lipid A stimulates MsbA ATPase activity in vitro and this does not occur with di- and tetra-acylated intermediates (Doerrler and Raetz, 2002). However, mutant bacteria that produce only tetra-acylated lipid A are viable only if the MsbA transporter
is overexpressed (Karow and Georgopoulos, 1993). This suggests that some transport of tetra-acylated lipid A occurs albeit at a rate too slow to sustain growth under physiological conditions. VexE is proposed to be one of two acyltransferases important for generation of the terminal glycolipid residues. Theoretically, the ΔvexE mutant should produce Vi antigen with one β-hydroxyacyl modification, which appears to be a substrate for the ABC-transporter, albeit a poor one when compared to the complete di-acylated glycolipid. However, this intermediate was not detected in Vi antigen isolated from S. Typhi ΔvexE. This could result either from low hydrophobicity of this residue that impaired its purification, or lack of any acylation in the absence of VexE. Future work to establish the biosynthetic route for this molecule is discussed below.

Although the Vi antigen biosynthesis genes had been identified in S. Typhi (Virlogeux et al., 1995) and C. freundii (Snellings et al., 1981) and appeared to be an confined to these bacteria, this work identified Vi antigen biosynthesis genetic loci in many Burkholderiales. These organisms represent potential emerging human pathogens (Aisenberg et al., 2004; Igra-Siegman et al., 1980) of which Achromobacter xylosoxidans is most frequently associated with disease (Aisenberg et al., 2004). A. xylosoxidans colonises the cystic fibrosis lung and implanted medical devices, and is innately multi-drug resistant (Bador et al., 2013). These bacteria produce a periplasmic Vi antigen-specific depolymerase, distinguishing them from the S. Typhi prototype, and this provides the first documented example of a glycan depolymerizing enzyme that participates in an ABC transporter-dependent CPS assembly system. When expressed in S. Typhi, VexL localized to the periplasm, where it degraded nascent Vi antigen. This provides direct evidence that the Vi antigen does not transit the periplasm in a contiguous channel and that current models for ABC transporter-dependent envelope translocation must therefore be re-evaluated. One possible explanation is that the Vi antigen transiently resides exterior to the PCP-OPX complex before entering the conduit (Fig. 6.1). This model is attractive as it is more consistent with models for group 1 CPS assembly; these CPS are polymerized in the periplasm, so the glycan must enter the PCP-OPX complex laterally but the molecular details for this have not been resolved.
Figure 6.1 Conceptual model for Vi antigen assembly. The sugar nucleotide precursor, UDP-GalNAcA, is synthesized from UDP-GlcNAc by the cytoplasmic enzymes TviB and TviC (Zhang et al., 2006). These precursors are polymerized in the cytoplasm, potentially by the action of TviD and/or TviE (Wetter et al., 2012). The glycan is O-acetylated prior to export (Wetter et al., 2012), potentially by TviD. VexE-acylates UDP-GlcNAc at C-6 (see Chapter 4 and 5). In the current hypothetical pathway, the other acyl chain is added at C-3 by LpxA, and this occurs prior to modification by VexE. This glycolipid is important for transport and either provides a site for extension by the CPS glycosyltransferase(s), similar to lyxPG-Kdo in other CPS assembly systems that use ABC transporters, or is incorporated post-polymerization. ATP hydrolysis in the NBD (VexC) drives conformational changes in the TMD (VexB), that power flipping of the glycan to the periplasmic face of the inner membrane. There, the CPS is accessible to the periplasmic glycanase, VexL (see Chapter 3). In the Burkholderiales, VexL-mediated alterations in glycan chain length may affect function of the capsule, and/or allow for release of a percentage of the glycan from the cell surface. The nascent glycan transits the outer membrane within a translocation complex formed by PCP and OPX proteins. The glycolipid terminus is involved in attachment of the CPS, once it reaches its cell surface destination.
6.2 Future directions

Although this work has provided substantial original insight into the structure and function of glycolipid termini in CPS assembly, several important questions remain concerning the overall Vi antigen biosynthetic pathway. Many of these experiments will require structural characterization of multiprotein complexes and therefore represent a significant challenge. The primary open questions are outlined below.

6.2.1 What is the source of the second acyl chain in the Vi antigen glycolipid terminus?

The Vi antigen glycolipid terminus consists of a HexNAc residue decorated with two β-hydroxyacyl chains. However, MS data for these molecules could not define the linkage position of the acyl and Vi antigen CPS substituents. *In vitro* reactions with the acyltransferase VexE suggest that the HexNAc residue has *gluco* configuration and that one of the acyl chains resides at HexNAc C-6. However, the linkage position of the second acyl chain and glycan backbone remain untested. Therefore, the most pressing need is to define these linkages in the physiological glycolipid, and this will require NMR spectroscopy. Improving yields for Vi antigen will be instrumental in this process. For reference, the typical yield was ~4 mg Vi antigen per L of stationary-phase bacterial culture. From this, terminal residues (and impurities) represented < 0.1 mg of material, which was sufficient for detection in HPLC-MS but not for NMR spectroscopy. NMR spectroscopy will require approximately 0.5 mg of material, at higher purity, which is less than would be required for chemical methylation/neutral sugar composition-GC-MS methods. The original HPLC-MS experiments were pioneering in that the chemistry of the glycolipid was unknown and therefore were performed using glycan produced by *S. Typhi*. It will therefore be useful (and perhaps necessary) to purify Vi antigen from *E. coli* that is harbouring the Vi antigen biosynthesis locus on a multicopy plasmid. These bacteria produce substantially higher quantities of Vi antigen (*Fig. A2*). This material is associated with the cell surface and binds PVDF membranes; these properties suggest that the glycolipid terminus has the authentic structure in the background. It will also be important to improve yield of glycolipid termini. The pH optimum of the VexL depolymerase was ~5.5 in potassium phosphate, which is significantly different from that used in the original glycolipid purification protocol (pH 7.5). Preforming digestions at this pH will allow for use of less enzyme, which could be binding/aggregating with released glycolipid termini and...
decreasing yield. The lower enzyme concentration will also allow for removal of a protein-removing ultrafiltration step; released glycolipids may bind these dialysis membranes similar to what is seen for the glycan with immunoblotting membranes.

A complete structure of the authentic glycolipid terminus will inform biosynthesis models, but these will require conformation in vivo. The most direct evidence for the proposed biosynthetic route will be demonstration of monoacylated terminal intermediates in deletion mutants of candidate acyltransferase genes. Monoacylated termini were not detected in S. Typhi ΔvexE. It is conceivable that the loss of both acylation activities reflects disruption of the Vi antigen biosynthesis complex in the absence of critical protein-protein interactions provided by VexE. This possibility could be addressed by using a system with catalytically inactivated VexE instead of one lacking the protein entirely. The proposed model includes LpxA, which generates the UDP-β-hydroxymyristoyl-GlcNAc intermediate in lipid A assembly. As VexE can directly acylate UDP-GlcNAc in vitro, it may be possible to isolate mono-acylated Vi antigen that contains only VexE-generated acyl chains from a ΔlpxA mutant. However, this experiment is not straightforward. In S. Typhi and E. coli lpxA is essential, so this hypothesis will require expression of the Vi antigen biosynthesis locus in bacteria where LPS is not essential. N. meningitidis (Steeghs et al., 1998) and A. baumannii (Powers and Trent, 2017) provide candidates, but whether the recombinant Vi antigen biosynthesis locus is sufficient for Vi antigen production in these bacteria remains to be tested. Abrogation of LPS, and therefore perturbation of membrane properties, may affect the structure and function of the Vi antigen assembly apparatus. If the second acylation is not contributed by LpxA, candidate acyltransferases will be difficult to identify. S. Typhi ΔlpxL, ΔlpxM, and ΔlpxO all produce wildtype glycolipid anchors (data not shown), and E. coli ΔlpxLMP (BKT09) produces acylated Vi antigen, although the glycolipid was not examined in this background. There are many acyltransferases encoded by bacteria, that play diverse roles in glycoconjugate assembly and metabolism. These proteins have diverse folds and may share several key catalytic residues, however, sequence similarity between these enzymes is low. This perhaps reflects diversity in donor and acceptor substrates and confounds bioinformatic identification.
6.2.2 What protein is the Vi antigen polymerase?

It will also be important to define when and how the glycolipid terminus is incorporated into the Vi antigen glycan, but the identity and mode of action of the Vi antigen polymerase(s) are currently unknown. Because VexL leaves an unsaturated bond at the non-reducing terminus of its products and this modification was evident in preparation of Vi antigen glycolipid termini, there is convincing data that the Vi antigen glycolipid terminus resides at the reducing end of the glycan. However, this does not necessarily inform the direction of glycan-chain growth and cannot be taken as proof that the chain extends on the glycolipid terminus. Resolving the direction of Vi antigen glycan chain growth and timing for incorporation of the terminal glycolipid first requires identification and characterization of the Vi antigen polymerase(s).

Most polymerizing glycosyltransferases require an initiating glycosyltransferase to first build an acceptor that the polymerase then extends, and this is due to the dramatic differences in specificities required of these proteins. This is the case for E. coli K1, where the lysoPG-Kdo₉ acceptor is thought to be capped by NeuE, which forms an acceptor for the NeuS polymerase to extend (Willis and Whitfield, 2013a). If the di-β-hydroxyacyl-GlcNAc terminus is the acceptor for Vi antigen synthesis, two glycosyltransferases might be expected for Vi antigen assembly, yet the Vi antigen biosynthesis locus only encodes one protein with homology to known glycosyltransferases, TviE. An alternative explanation is that a single glycosyltransferase is sufficient to drive Vi antigen synthesis. ‘Self-priming’ polymerizing glycosyltransferases do exist, and ‘Class 1’ hyaluronan synthase provides an example (Weigel et al., 2015; 2017). Streptococcal hyaluronan synthase initiates glycan synthesis on UDP-GlcNAc and forms an oligosaccharide of GlcNAc residues (chitin) before switching to assembly of the [3)]-GlcNAc-β-(1→4)-GlcA-β-(1→] hyaluronan backbone that makes up the majority of the molecule (Weigel et al., 2017). How this switch is achieved is not resolved. In hyaluronan biosynthesis, glycosylation occurs at the reducing terminus but the direction of Vi antigen chain growth is still unknown. If Vi antigen chain growth occurs at the reducing end, it is possible that random incorporation of di-β-hydroxyacylated-HexNAc during polymerization terminates chain growth. Terminating residues are frequently employed by ABC transporter-dependent OPS biosynthesis systems, where they are important for regulation of glycan chain.
length (Clarke et al., 2009; Hagelueken et al., 2015; King et al., 2014); the NBD of the corresponding ABC transporter contains a cognate carbohydrate-binding domain that selects only terminated glycans for transport (Bi et al., 2018; Cuthbertson et al., 2007; Mann et al., 2016). The Vi antigen ABC transporter does not contain an obvious specificity-conferring module, however similar specificity could be mediated through TMD-glycolipid interactions at the cytoplasm-membrane interface. It is also possible that a single Vi antigen polymerase begins glycan synthesis on UDP-GlcNAc, before ‘switching’ to addition of UDP-GalNAcA at the non-reducing end. In this case, the nascent (reducing-terminal) GlcNAc residue could be acylated by VexE post-polymerization. How the UDP-moiety is removed is also unknown. For hyaluronan, it remains attached to the final product (Weigel et al., 2015).

Elucidation of the precise polymerization pathway requires identification of the central payers and activities. TviE is homologous to glycosyltransferases in family 4 of the CAZy database, which contains distributive polymerases (i.e. enzymes that associate and dissociate from the glycan between successive glycose addition (Lairson et al., 2008)). The ‘self-priming’ hyaluronan synthases discussed earlier belong to the distinct family-2 (Lairson et al., 2008; Weigel et al., 2017). Some GT-4 enzymes build repeating-unit glycans (e.g. WbdA, the polymerase from OPS biosynthesis in E. coli O9a (Greenfield et al., 2012)). This makes TviE an attractive target for in vitro assays employing UDP-GlcNAc and UDP-GalNAcA donors that could establish whether it is the Vi antigen polymerase, and if it is able to ‘self-prime.’ In the case TviE is not able to initiate synthesis on UDP-GlcNAc or UDP-GalNAcA, one could employ the protocols described in this thesis to purify UDP-acyl-GlcNAc or Vi antigen oligosaccharides and test if TviE can act on these acceptors. Chemical synthesis of defined Vi antigen oligosaccharide substrates is an alternative option (Kossaczka et al., 1997; Yang et al., 2011). Additional complications to these assays may arise if O-acetylation is necessary for glycan polymerization, and this aspect is described below.

6.2.3 Which protein is the Vi antigen O-acetyltransferase?

The importance of O-acetyl modification in Vi antigen-based vaccine preparations (Szewczyk and Taylor, 1980) reinforces the need to discover of the Vi antigen O-acetyltransferase. The recombinant Vi antigen biosynthesis locus is sufficient to build O-acetylated Vi antigen in E. coli, and O-acetylation occurs prior
to transport (Wetter et al., 2012). However, it is unknown if O-acetylation occurs at the precursor or polymer level. For comparison, the analogous O-acetyltransferase NeuO modifies the K1 polysialic acid CPS after polymerization (Deszo et al., 2005), rather than individual nucleotide-activated CMP-NeuNAc precursors. None of the genes in the locus encode a protein with homology to known acetyltransferases. Vi antigen remained O-acetylated in S. Typhi ΔvexE; VexE represented an early candidate for this activity because O-acetyl and O-acyltransferase reactions are essentially identical. The acceptor specificity and reaction location excludes candidate acetyltransferases that are encoded by the E. coli chromosome (Wang et al., 2010). The TviD protein is a strong candidate as it is the only remaining ORF encoded by the viaB locus without a functional annotation, and it is thought to be required for Vi antigen assembly (Wetter et al., 2012). However, the published data is complicated by its sole dependence on enzyme-linked immunosorbent assays that employed monoclonal antibodies unable to detect O-acetylated Vi antigen (Wetter et al., 2012). In this assay, a Vi antigen O-acetyltransferase mutant would appear not to synthesize glycan at all. TviD is cytoplasmic, consistent with O-acetylation preceding export, but the predicted protein shares no similarity with any catalytic domains to inform functional investigations. In vitro reconstitution of O-acetylation employing purified TviD, UDP-GalNAcA/Vi antigen oligosaccharide acceptors, and acetyl-CoA donor will be required to answer this question.

6.2.4 What are the constituent and structure of the Vi antigen assembly complex?

The inability of VexL to degrade Vi antigen in the cytoplasm suggests that it is ‘protected’ from digestion. This has been explained by a cytoplasmic biosynthesis protein complex (excluding the VexL probe), or by glycan synthesis being coupled to transport such that the nascent glycan enters the transporter as it is built. Examples for both features exist in other bacterial glycan assembly systems and these possibilities are not mutually exclusive. Many of the proteins in the Vi antigen biosynthesis locus contain predicted tetratricopeptide repeat (TPR) domains. TPR motifs commonly define protein-protein and protein-substrate interaction modules (D'Andrea and Regan, 2003), and their presence suggests interaction(s) between the Vi antigen biosynthesis proteins. The presence of large TPR-containing domains in the cytoplasmic proteins TviD and acyltransferase VexE specifically suggest that these Vi antigen assembly proteins may interact.
The predicted glycosyltransferase TviE may also contain several TPR at the N-terminus based on homology modelling. In Synthase-dependent EPS biosynthesis, periplasmic TPR-proteins form scaffolds for glycan secretion (reviewed in (Whitney and Howell, 2013)). These TPR proteins are proposed to direct the glycan to the outer membrane pore (Marmont et al., 2017a) and mediate interactions with glycan-modifying enzymes (Colvin et al., 2013; Marmont et al., 2017b). Testing the interactions of the cytoplasmic Vi antigen biosynthesis proteins by bacterial two-hybrid assay and by direct (pull down) methods will provide an important next step in beginning to define members and organization of this VexL-exclusive complex.

With respect to the PCP-OPX translocation channel, all CPS assembly models have been based on structural data from group 1 systems, which do not use ABC transporters. There are currently no atomic-resolution structures available for CPS ABC transporters (KpsMT) or their PCP and OPX counterparts. PCP proteins from group 1 systems contain additional cytoplasmic catalytic domains that are important for CPS synthesis (Cuthbertson et al., 2009; Morona et al., 2009) and these are notably absent in PCP proteins from ABC transporter-dependent systems. The OPX proteins also differ significantly in size, subcellular localization (Diao et al., 2017), and presence of a palmitoylation site (Cuthbertson et al., 2009). These observations suggest significant structural differences between group 1 and group 2 envelope translocation machinery that could underpin different organization and functional details in the hetero-complexes. As a starting point, it would be interesting to test whether classical ABC transporters that flip \textit{lysoPG-Kdo}_{\text{n}}-linked CPS are able to transport the di-acyl-HexNAc containing Vi antigen (and vice versa). It may be possible to test ATPase activity of the Vi antigen transporter \textit{in vitro} with mono-and di-acylated glycan substrates, similar to experiments performed with the lipid A transporter MsbA (Doerrler and Raetz, 2002). Resolving mechanistic details of the translocation process will require structural characterization of purified translocation proteins by X-ray crystallography, and Cryo EM has established utility in characterization of architectural features of these oligomeric complexes (Collins et al., 2007).
6.3 Closing remarks

The Vi antigen represents an important virulence factor for *S. Typhi* (Keestra-Gounder et al., 2015), and may play a similar role for Vi antigen-encapsulated Achromobacter isolates that represent emerging multidrug-resistant human pathogens (Aisenberg et al., 2004; Bador et al., 2013). Addressing the significance of the Vi antigen capsule in isolates from the *Burkholderiales* will be important in rationalizing potential therapeutic strategies that might draw on resources developed for *S. Typhi*. The acyltransferase VexE may represent a therapeutic target, as interrupting VexE-dependent acylation abrogates antigen presentation on the cell surface and concomitant antibody formation (Li et al., 2018) and humans do not possess a VexE homolog. The data presented in this thesis show that inactivating VexE in *S. Typhi* leads to the complete loss of a coherent capsule. This structure normally reduces complement deposition and clearance in the host, and plays a significant role in infection outcomes (Keestra-Gounder et al., 2015; Wilson et al., 2011).

Glycoconjugates play essential roles in biology. These macromolecules dominate the cell surface and therefore play instrumental roles in diverse cell-cell and cell-environment interactions. Their synthesis and trafficking mechanisms are as complex as the glycoconjugates themselves. Understanding the mechanisms of glycoconjugate assembly is critical for their application in glyco-engineering strategies (Baker et al., 2013), such as generation of precise glycoprotein vaccines (Cuccui and Wren, 2015; Ihssen et al., 2015). Some glycoconjugate assembly systems provide validated antibacterial targets (Sewell and Brown, 2014; Swoboda et al., 2009). The structures and mechanisms for CPS glycan polymerases, ABC transporters, and associated translocation complexes for important classes of glycans (OPS, CPS, and wall teichoic acids) remain unknown. The research presented in this thesis provides initial insight into the substrate(s) of an ABC transporter that represents an ‘outlier’ from prototypical CPS. However, the conserved nature of the assembly and translocation apparatus makes these findings relevant to many encapsulated pathogens with clinical significance.
REFERENCES


Weigel, P.H., Baggenstoss, B.A., and Washburn, J.L. (2017). Hyaluronan synthase assembles hyaluronan on a [GlcNAc(β1,4)],-GlcNAc(α1→)UDP primer and hyaluronan retains this residual chitin oligomer as a cap at the nonreducing end. Glycobiology 27, 536–554.


**APPENDICES**

**Table A1:** Bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Strain, Phage, or Plasmid</th>
<th>Genotype or Property</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Top10</td>
<td>F·, mcrA, Δ(mrr-hsdRMS-mcrBC), φ80, lacZΔM15, ΔlacX74, deoR, mupG, recA1, araD139, Δ(ara-leu)7697, galU, galK, rpsL(Su&lt;sup&gt;R&lt;/sup&gt;), endA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>BKT09</td>
<td>Δ(araD-araB)567, ΔlacZ4787(:rrnB-3), λ&lt;sup&gt;+&lt;/sup&gt;, rph-1, Δ(rhaD-rhaB)568, hsdR514, ΔpagP, ΔlpxP, ΔlpxM, ΔlpxL::Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(2012)</td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td>B F&lt;sup&gt;−&lt;/sup&gt;ompT gal dcm lon hsdS&lt;sub&gt;8&lt;/sub&gt;(r&lt;sub&gt;B&lt;/sub&gt; m&lt;sub&gt;B&lt;/sub&gt;) Δ&lt;sup&gt;−&lt;/sup&gt;λ(DE3) [lacI lacUV5-T7p07 ind1 sam7 nin5] [malB]&lt;sup&gt;−&lt;/sup&gt;Δ&lt;sup&gt;−&lt;/sup&gt;λ&lt;sup&gt;+&lt;/sup&gt;(λ&lt;sup&gt;B&lt;/sup&gt;)&lt;sup&gt;−&lt;/sup&gt;Δ&lt;sup&gt;−&lt;/sup&gt;λ&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>C43</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;ompT gal dcm hsdS&lt;sub&gt;8&lt;/sub&gt;(r&lt;sub&gt;B&lt;/sub&gt; m&lt;sub&gt;B&lt;/sub&gt;)Δ(λ&lt;sup&gt;B&lt;/sup&gt;)&lt;sup&gt;−&lt;/sup&gt;Δ&lt;sup&gt;−&lt;/sup&gt;λ&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Lucigen</td>
</tr>
<tr>
<td>CWG1241</td>
<td>Top10 ΔwecA::cat; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>EV36</td>
<td>K-12/K1 hybrid</td>
<td>(Vimr and Troy, 1985)</td>
</tr>
<tr>
<td>RS2436</td>
<td>EV36 ΔkpsT</td>
<td>(Pavelka et al., 1994)</td>
</tr>
<tr>
<td>W3110</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; lambda&lt;sup&gt;+&lt;/sup&gt; IN(rrnD-rrnE)I rph-</td>
<td>ATCC</td>
</tr>
<tr>
<td><strong>Salmonella enterica</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H251.1</td>
<td>serovar Typhi Ty2 trp, cys, ΔaroC1019</td>
<td>(Hone et al., 1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K.E. Sanderson, <em>Salmonella</em> Genetic Stock Centre, University of Calgary</td>
</tr>
<tr>
<td>CWG1235</td>
<td>S. Typhi H251.1 ΔvexC</td>
<td>This study</td>
</tr>
<tr>
<td>CWG1236</td>
<td>S. Typhi H251.1 ΔvexE</td>
<td>This study</td>
</tr>
<tr>
<td>CWG1237</td>
<td>S. Typhi H251.1 ΔvexC ΔvexE</td>
<td>This study</td>
</tr>
<tr>
<td>CWG1238</td>
<td>S. Typhi H251.1 ΔwaaG::kan; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>CWG1239</td>
<td>S. Typhi H251.1 ΔvexE ΔwaaG::kan; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>CWG###</td>
<td>S. Typhi H251.1 ΔlpxL::kan</td>
<td>This study</td>
</tr>
<tr>
<td>CWG###</td>
<td>S. Typhi H251.1 ΔlpxM::kan</td>
<td>This study</td>
</tr>
<tr>
<td>CWG###</td>
<td>S. Typhi H251.1 ΔvexE ΔlpxL::kan</td>
<td>This study</td>
</tr>
<tr>
<td>CWG###</td>
<td>S. Typhi H251.1 ΔvexE ΔlpxM::kan</td>
<td>This study</td>
</tr>
<tr>
<td>CWG###</td>
<td>S. Typhi H251.1 ΔlpxO::kan</td>
<td>This study</td>
</tr>
<tr>
<td>CWG###</td>
<td>S. Typhi H251.1 ΔlpxO::kan ΔwaaG::cat</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Achromobacter**

| CWG1240 | A. denitrificans; Sm<sup>R</sup>, Amp<sup>R</sup>, Kan<sup>R</sup> | This study |
| CWG1347 | A. xylosoxidans | G.D. Wright |
| CWG1348 | A. spanius | G.D. Wright |

**Plasmid**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACYC184</td>
<td>Cloning vector containing tetracycline and chloramphenicol resistance cassettes; Tet&lt;sup&gt;R&lt;/sup&gt;, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Chang and Cohen, 1978)</td>
</tr>
<tr>
<td>pBAD24</td>
<td>Plasmid vector with L-arabinose-inducible promoter; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Guzman et al., 1995)</td>
</tr>
<tr>
<td>pCP20</td>
<td>Source of Flp recombinase, temperature sensitive replicon; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Datsenko and Wanner, 2000)</td>
</tr>
<tr>
<td>pET28a(+)</td>
<td>Plasmid vector with IPTG-inducible promoter; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Novagen</td>
</tr>
<tr>
<td>pGVXN158</td>
<td>pLAFR1 derivative encoding S. Typhi BRD948 viaB locus; Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Wetter et al., 2012)</td>
</tr>
<tr>
<td>pKD3</td>
<td>Source of frt-flanked chloramphenicol resistance cassette; Ap&lt;sup&gt;R&lt;/sup&gt;, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Datsenko and Wanner, 2000)</td>
</tr>
<tr>
<td>pKD4</td>
<td>Source of frt-flanked kanamycin resistance cassette; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Datsenko and Wanner, 2000)</td>
</tr>
<tr>
<td>pMALc2</td>
<td>Plasmid vector with IPTG-inducible promoter, for generation of cytoplasmic N-terminal MalE-fusion proteins; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>pMALp2</td>
<td>Plasmid vector with IPTG-inducible promoter, for generation of periplasmic N-terminal MalE-fusion proteins; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>pNLP15</td>
<td><em>spy</em> (Spheroplast protein Y) reporter; <em>spy</em> promoter fused to promoterless luxCDABE operon; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Price and Raivio, 2009)</td>
</tr>
<tr>
<td>Construct</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------------------------------------------------------------------------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>pSIM6</td>
<td>λ-red recombinase helper plasmid, temperature sensitive replicon; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Datta et al., 2006)</td>
</tr>
<tr>
<td>pWQ284</td>
<td>pBAD24 containing a chloramphenicol resistance cassette; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Cuthbertson et al., 2007)</td>
</tr>
<tr>
<td>pWQ782</td>
<td>pACYC184 derivative containing an oligocassette containing AscI and SpeI restriction sites; Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ783</td>
<td>pWQ782 containing the <em>Salmonella</em> Typhi viaB operon; Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ784</td>
<td>Derived from pWQ783; viaB containing a deletion of vexE; Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ785</td>
<td>Derived from pWQ783; viaB containing a deletion of vexC; Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ786</td>
<td>pBAD24 derivative encoding <em>Salmonella</em> Typhi VexE-His&lt;sub&gt;6&lt;/sub&gt;; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ787</td>
<td>pBAD24 derivative encoding <em>Achromobacter denitrificans</em> VexE-His&lt;sub&gt;6&lt;/sub&gt;; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ788</td>
<td>pWQ786 containing a H487A mutation; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ789</td>
<td>pWQ787 containing a H466A mutation; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ790</td>
<td>pWQ787 containing a Y471F mutation; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ791</td>
<td>pBAD24 derivative encoding <em>Achromobacter denitrificans</em> VexL-His&lt;sub&gt;6&lt;/sub&gt;; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ792</td>
<td>pBAD24 derivative encoding VexC-His&lt;sub&gt;10&lt;/sub&gt;; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ793</td>
<td>pWQ284 derivative encoding VexC-His&lt;sub&gt;10&lt;/sub&gt;; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ794</td>
<td>pWQ284 derivative encoding <em>Achromobacter denitrificans</em> VexE-His&lt;sub&gt;6&lt;/sub&gt;; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ795</td>
<td>pWQ284 derivative encoding <em>Salmonella</em> Typhi LpxL-His&lt;sub&gt;6&lt;/sub&gt;; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ796</td>
<td>pWQ284 derivative encoding <em>Salmonella</em> Typhi LpxM; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ889</td>
<td>GeneArt cloning plasmid encoding <em>Vibrio harveyi</em> AasS-His&lt;sub&gt;6&lt;/sub&gt;; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This Study</td>
</tr>
<tr>
<td>pWQ890</td>
<td>pBAD24 derivative encoding <em>Vibrio harveyi</em> AasS-His&lt;sub&gt;6&lt;/sub&gt;; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This Study</td>
</tr>
<tr>
<td>pWQ891</td>
<td>pET28a(+) derivative encoding <em>E. coli</em> LpxA-His&lt;sub&gt;6&lt;/sub&gt;; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This Study</td>
</tr>
<tr>
<td>pWQ892</td>
<td>pET28a(+) derivative encoding <em>E. coli</em> ACP and ACPS; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This Study</td>
</tr>
<tr>
<td>pWQ893</td>
<td>pWQ787 containing D532A mutation; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWQ894</td>
<td>pWQ789 containing D532A mutations; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>
pWQ903: pBAD24 derivative encoding WaaG from *E. coli* F470; *Ap*<sup>R</sup> (Heinrichs et al., 1998)

pWQ935: pBAD24 derivative encoding *Achromobacter denitrificans* VexL<sub>24-402</sub>-His<sub>6</sub> (truncated signal sequence); *Ap*<sup>R</sup> This Study

pWQ936: pMALc2 derivative encoding signal sequence-truncated MalE-endoN; *Ap*<sup>R</sup> This Study

pWQ937: pMALp2 derivative encoding MalE-endoN; *Ap*<sup>R</sup> This Study

pWQ938: pWQ791 derivative encoding VexL-His<sub>6</sub>R232K; *Ap*<sup>R</sup> This Study

pWQ939: pWQ791 derivative encoding VexL-His<sub>6</sub>R232A; *Ap*<sup>R</sup> This Study

pWQ940: pWQ791 derivative encoding VexL-His<sub>6</sub>R235K; *Ap*<sup>R</sup> This Study

pWQ941: pWQ791 derivative encoding VexL-His<sub>6</sub>R235A; *Ap*<sup>R</sup> This Study

pWQ942: pWQ791 derivative encoding VexL-His<sub>6</sub>E267A; *Ap*<sup>R</sup> This Study

pWQ943: pWQ791 derivative encoding VexL-His<sub>6</sub>R172K; *Ap*<sup>R</sup> This Study

pWQ944: pWQ791 derivative encoding VexL-His<sub>6</sub>R172A; *Ap*<sup>R</sup> This Study

pWQ945: pWQ791 derivative encoding VexL-His<sub>6</sub>K195A; *Ap*<sup>R</sup> This Study

pWQ946: pWQ791 derivative encoding VexL-His<sub>6</sub>Y254F; *Ap*<sup>R</sup> This Study

pWQ947: pWQ791 derivative encoding VexL-His<sub>6</sub>E319A; *Ap*<sup>R</sup> This Study

pWQ948: pWQ791 derivative encoding VexL-His<sub>6</sub>E249A; *Ap*<sup>R</sup> This Study

pWQ949: pWQ791 derivative encoding VexL-His<sub>6</sub>Y225F; *Ap*<sup>R</sup> This Study

pWQ959: pWQ791 derivative encoding VexL-His<sub>6</sub>D171A; *Ap*<sup>R</sup> This Study

pWQ960: pWQ791 derivative encoding VexL-His<sub>6</sub>Q237A; *Ap*<sup>R</sup> This Study
## Table A2. Sequences of oligonucleotide primers.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Primer</th>
<th>Sequence&lt;sup&gt;a,b&lt;/sup&gt; (5′→3′)</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>pWQ782</td>
<td>SL024</td>
<td>gatggggcgccGTTACCAetagtAGCAATCTACGAGGCAAG</td>
<td>Forward primer for amplification of pACYC184 introducing AscI and SpeI restriction sites</td>
</tr>
<tr>
<td></td>
<td>SL025</td>
<td>gatggggcgccCATCCGGAAATCCGATATGGCAAATG</td>
<td>Reverse primer for amplification of pACYC184 introducing AscI and SpeI restriction sites</td>
</tr>
<tr>
<td>pWQ783</td>
<td>SL035</td>
<td>cattgggggccccATTATCCCGAGAGCAAGTCATTT</td>
<td>Forward primer for amplification of S. Typhi <em>viAB</em>; AscI restriction site</td>
</tr>
<tr>
<td></td>
<td>MM17</td>
<td>aacagtagCTTGGGATGCTGGGTGCTAGGCTAGGG</td>
<td>Reverse primer for the amplification of S. Typhi <em>viAB</em>; SpeI restriction site.</td>
</tr>
<tr>
<td>pWQ784</td>
<td>SL026</td>
<td>gatggggcgccTTCATATTGCAAGTCAGCACG</td>
<td>Reverse primer for the amplification of S. Typhi <em>viiABCDEvexABCDE</em>; SpeI restriction site.</td>
</tr>
<tr>
<td>pWQ785</td>
<td>MNL05</td>
<td>AGGAGAGACGCATTCTaaaTTAGTAAATATACGGATAGAGTAGGG</td>
<td>Forward mutagenesis primer for <em>vexC</em> deletion in pWQ783</td>
</tr>
<tr>
<td></td>
<td>MNL06</td>
<td>CTCTATCCGTATATTTACATTTAAGTGTGG</td>
<td>Forward mutagenesis primer for <em>vexC</em> deletion in pWQ783</td>
</tr>
<tr>
<td>pWQ786</td>
<td>AZG1</td>
<td>geccccggaatcactACAGACTGCACTCTACATTT</td>
<td>Forward primer for the amplification of S. Typhi <em>vexE</em>-His&lt;sub&gt;6&lt;/sub&gt;; EcoRI restriction site</td>
</tr>
</tbody>
</table>
|         | AZG2-2 | gcgegetcagctagctttgttgttgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtg
pWQ791  SL079  cattgaacctATGCAGCTTCCGAAAGACAGTGATGG  Forward primer for amplification of *A. denitrificans* vexL-His$_6$; EcoRI restriction site

SL081  cattaagcttttaagttggttaggtgg  Reverse primer for amplification of *A. denitrificans* vexL-His$_6$; HindIII restriction site

pWQ792  SL083  caggaatctAAGAAGATGTTATTAGTTG  Forward primer for the amplification of *S. Typhi* vexC-His$_{10}$; EcoRI restriction site

SL084  gatcaagcttttaagttggttaggtgg  Reverse primer for amplification of *S. Typhi* vexC-His$_{10}$; HindIII restriction site

pWQ795  SL102  cagggaatctATGACGAAGTTGCCTAAGTTC  Forward primer for amplification of *S. Typhi* LpxL-His$_6$; EcoRI restriction site

SL103  gatcaagcttttaagttggttaggtgg  Reverse primer for amplification of *S. Typhi* LpxL-His$_6$; HindIII restriction site

pWQ796  SL119  cagccggfacctATGGAAACCA  Forward primer for amplification of *S. Typhi* LpxM; KpnI restriction site

SL120  gatcaagcttttaagttggttaggtgg  Reverse primer for amplification of *S. Typhi* LpxM; HindIII restriction site

pWQ891  SL111  cattctctaaATAATTTTGTAACTTTAGAAGAGATATACCACTCCGACCCGCGGATGTAGTGGATAAAATCCAACCTTTG  Forward primer for amplification of *E. coli* lpxA-His$_6$; XbaI restriction site

SL112  cattaagccttaACGAATCAGACGCGCGTGTG  Reverse primer for amplification of *E. coli* lpxA-His$_6$; HindIII restriction site

pWQ892  ACP-forward  gcgcgcgcgcatATGAGCACAATCAGAACAAGAGCTTTCGCCCTGTGAGCTTTTG  Forward primer for amplification of *E. coli* acp; NdeI restriction site. Reverse primer for amplification of *E. coli* acp

ACP-Reverse  cggggccgccccgaaacggtgctttaaAATGGAATCTTTAGGTTTGAGGC  Forward primer for amplification of *E. coli* acpS

ACPS-Forward  gcgcgcgcgcgcatATGAGCACAATCAGAACAAGAGCTTTCGCCCTGTGAGCTTTTG  Reverse Primer for amplification of *E. coli* acpS, introduces XhoI site

ACPS-Reverse  gcgcgcgcgcgcatATGAGCACAATCAGAACAAGAGCTTTCGCCCTGTGAGCTTTTG  Forward primer for amplification of *E. coli* acpS, introduces XhoI site

pWQ893,  SL144  pWQ894  CATTGCTCTTGCCATCg  Forward mutagenesis primer for VexE D532A

SL145  CGGGATCCGCAGCCGCCGATCGGATCCCG  Reverse mutagenesis primer for VexE D532A

pWQ935  SL080  cattgaacttttaggtgttggttg  Forward primer for amplification of VexL$_{24-408}$-His$_{10}$; EcoRI restriction site

SL081  cattgaacttttaggtgttggttg  Reverse primer for amplification of VexL$_{24-408}$-His$_{10}$; HindIII restriction site

pWQ936  SL150  gagggaactttcGCTAAGGAGATGGGTGTCACCTG  Forward primer for amplification of K1F endosialidase for cloning into pMAL-c2/p2; EcoRI restriction site
<table>
<thead>
<tr>
<th>SL</th>
<th>Reverse primer for amplification of K1F endosialidase for cloning into pMAL-c2/p2; SalI restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL151</td>
<td>ggcggagtcgaccTATTACTTC TGGTCAAGAGCAGAAAGTC CTATTTCTTCAACACCGTGCAAGaaaAATCCGCGCGC GCAATTCGGCCTTTCGAGTTCTGCACGAGGTGCAGGGTTCGGCGAGAAATAG</td>
</tr>
<tr>
<td>SL156</td>
<td>Forward mutagenesis primer for VexL R232K</td>
</tr>
<tr>
<td>SL157</td>
<td>Reverse mutagenesis primer for VexL R232K</td>
</tr>
<tr>
<td>SL158</td>
<td>Forward mutagenesis primer for VexL R232A</td>
</tr>
<tr>
<td>SL159</td>
<td>Reverse mutagenesis primer for VexL R232A</td>
</tr>
<tr>
<td>SL160</td>
<td>Forward mutagenesis primer for VexL R235K</td>
</tr>
<tr>
<td>SL161</td>
<td>Reverse mutagenesis primer for VexL R235K</td>
</tr>
<tr>
<td>SL162</td>
<td>Forward mutagenesis primer for VexL R235A</td>
</tr>
<tr>
<td>SL163</td>
<td>Reverse mutagenesis primer for VexL R235A</td>
</tr>
<tr>
<td>SL164</td>
<td>Forward mutagenesis primer for VexL E266A</td>
</tr>
<tr>
<td>SL165</td>
<td>Reverse mutagenesis primer for VexL E266A</td>
</tr>
<tr>
<td>SL166</td>
<td>Forward mutagenesis primer for VexL R172K</td>
</tr>
<tr>
<td>SL167</td>
<td>Reverse mutagenesis primer for VexL R172K</td>
</tr>
<tr>
<td>SL168</td>
<td>Forward mutagenesis primer for VexL R172A</td>
</tr>
<tr>
<td>SL169</td>
<td>Reverse mutagenesis primer for VexL R172A</td>
</tr>
<tr>
<td>SL170</td>
<td>Forward mutagenesis primer for VexL K195A</td>
</tr>
<tr>
<td>SL171</td>
<td>Reverse mutagenesis primer for VexL K195A</td>
</tr>
</tbody>
</table>
pWQ946  SL172  GCTCGAAAAACTGGGACT
TCttGGCATGAGTTTTCAG
CCTG
SL173  CAGGCTGAAACTCTATGC
CaaAgAAGTCAGCTTT
CGAGC
Forward mutagenesis primer for VexL
Y254F
Reverse mutagenesis primer for VexL
Y254F

pWQ947  SL176  CTCCGATCGCGGCCT
ATGccAAGCGCAAGGCG
GATCAGC
SL177  CGTGTACCGCTCTGC
TTGccATAGGCCGCCGA
TCGGAG
Forward mutagenesis primer for VexL
E319A
Reverse mutagenesis primer for VexL
E319A

pWQ948  SL178  CCTGTTCACAACAAGCTGC
TCGccAACCTGGGACTTCT
ACGGC
SL179  GCCGTAGAAAGTCCCAGT
TGGcGAGCAGGTTGTTGA
ACAGG
Forward mutagenesis primer for VexL
E249A
Reverse mutagenesis primer for VexL
E249A

pWQ949  SL180  GTGACGCTGCATCACAA
CttTTTTACCAAACCTGC
ACGC
SL181  GCTGCACGGTGTTGAAG
AAaaaGTTGTGATGCAGC
GTCAC
Forward mutagenesis primer for VexL
Y225F
Reverse mutagenesis primer for VexL
Y225F

pWQ959  SL182  GACCTGTCGCCGATGT
CGgccCGCCTGCTGAACG
TGAAGAAC
SL183  GTTCTTCACGTTCAGCA
GGCGggcCGACATCCGCG
ACAGGTCC
Forward mutagenesis primer for VexL
D171A
Reverse mutagenesis primer for VexL
D171A

pWQ960  SL184  CAGCGCAATCCGGGGC
GgccCTCGGCCTCAGC
GTCAG
SL185  CAGGTGGAAGGTGCCGA
AggcCGCGCGGATTGC
GCTG
Forward mutagenesis primer for VexL
Q237A
Reverse mutagenesis primer for VexL
Q237A

CWG1236  SL089  CCTGATAAACCTCAGGC
GGATCGCTGAATAAGG
TGAAGGAAATATAg
gtagctgagctccttc
SL090  ATTTTTCGTCCTGAAGT
ACAAATTTCATCTACC
GCAATTAAATCGCTTAcat
tatatctctctctcttttag
\(\lambda\)-red recombination primer for vexE
deletion (S. Typhi)
\(\lambda\)-red recombination primer for vexE
deletion (S. Typhi)
\(\lambda\)-red recombination primer for vexC
deletion (S. Typhi)
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Function</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL093</td>
<td>CATTTTTTAATACGTTCTGAATTITCCACACTCTATCCGTATATTACTAAcatagaatatctctcttag</td>
<td>λ-red recombination primer for vexC deletion (S. Typhi)</td>
<td></td>
</tr>
<tr>
<td>CWG1239, CWG1240</td>
<td>SL096 GAAAAAAATGCTGCCGCA TGAGGCACGCCCATAGATTTGGACAGCCTGsctaggctggagctgtc</td>
<td>λ-red recombination primer for waaG deletion (S. Typhi)</td>
<td></td>
</tr>
<tr>
<td>SL097</td>
<td>CCTCAAAAGCATCTTTATCCGCGCATAGTGTGTTAACGGCCGTTCAGCaratgaatatctctcttag</td>
<td>λ-red recombination primer for waaG deletion (S. Typhi)</td>
<td></td>
</tr>
<tr>
<td>CWG1241</td>
<td>LRWec A-f GGTCTTCTGGTTATAC TTCTGCTAAATAATTCTCTGAGGCATGCAAtgtgtacctggtagctgtcattgagctggagctgtc</td>
<td>λ-red recombination primer for wecA deletion (E. coli)</td>
<td></td>
</tr>
<tr>
<td>LRWec A-r AGCGTCTTCGCGCCGTTTCCAGGCATGATGGTGTCATACATCCTCAcatatgaatatctctcttag</td>
<td>λ-red recombination primer for wecA deletion (E. coli)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aRestriction sites are underlined.
bNon-chromosomal sequences are lowercase.
### Table A3: Species identified in charge-deconvoluted LC-QToF-MS of VexL-digested Vi antigen.

<table>
<thead>
<tr>
<th>GalNAcA</th>
<th>O-Ac</th>
<th>Molecule</th>
<th>Expected</th>
<th>Observed</th>
<th>Difference</th>
<th>Error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>M</td>
<td>476.1278</td>
<td>476.1270</td>
<td>0.0008</td>
<td>1.68</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>M-H2O</td>
<td>458.1173</td>
<td>458.1166</td>
<td>0.0007</td>
<td>1.53</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>M</td>
<td>518.1384</td>
<td>518.1379</td>
<td>0.0005</td>
<td>0.96</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>M-H2O</td>
<td>675.1759</td>
<td>675.1738</td>
<td>0.0021</td>
<td>3.11</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>M</td>
<td>693.1865</td>
<td>693.1850</td>
<td>0.0015</td>
<td>2.16</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>M</td>
<td>735.1970</td>
<td>735.1953</td>
<td>0.0017</td>
<td>2.31</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>M-H2O</td>
<td>717.1865</td>
<td>717.1849</td>
<td>0.0016</td>
<td>2.23</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>M</td>
<td>777.2076</td>
<td>777.2058</td>
<td>0.0018</td>
<td>2.32</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>M+H2O</td>
<td>795.2182</td>
<td>795.2156</td>
<td>0.0026</td>
<td>3.27</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>M-H2O</td>
<td>892.2346</td>
<td>892.2311</td>
<td>0.0035</td>
<td>3.92</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>M</td>
<td>910.2451</td>
<td>910.2426</td>
<td>0.0025</td>
<td>2.75</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>M-H2O</td>
<td>934.2451</td>
<td>934.2432</td>
<td>0.0019</td>
<td>2.03</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>M</td>
<td>952.2557</td>
<td>952.2526</td>
<td>0.0031</td>
<td>3.26</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>M-H2O</td>
<td>976.2557</td>
<td>976.2545</td>
<td>0.0012</td>
<td>1.23</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>M</td>
<td>994.2663</td>
<td>994.2641</td>
<td>0.0022</td>
<td>2.21</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>M</td>
<td>1036.2768</td>
<td>1036.2748</td>
<td>0.002</td>
<td>1.93</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>M+H2O</td>
<td>1054.2874</td>
<td>1054.2833</td>
<td>0.0041</td>
<td>3.89</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>M-H2O</td>
<td>1151.3038</td>
<td>1151.3017</td>
<td>0.0021</td>
<td>1.82</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>M</td>
<td>1169.3143</td>
<td>1169.3118</td>
<td>0.0025</td>
<td>2.14</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>M-H2O</td>
<td>1193.3143</td>
<td>1193.3134</td>
<td>0.0009</td>
<td>0.75</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>M</td>
<td>1211.3249</td>
<td>1211.3235</td>
<td>0.0014</td>
<td>1.16</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>M-H2O</td>
<td>1235.3249</td>
<td>1235.3240</td>
<td>0.0009</td>
<td>0.73</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>M</td>
<td>1295.3355</td>
<td>1295.3344</td>
<td>0.0011</td>
<td>0.85</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>M+H2O</td>
<td>1271.3460</td>
<td>1271.3424</td>
<td>0.0036</td>
<td>2.83</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>M</td>
<td>1295.3460</td>
<td>1295.3451</td>
<td>0.0009</td>
<td>0.69</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>M-H2O</td>
<td>1368.3624</td>
<td>1368.3583</td>
<td>0.0041</td>
<td>3.00</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>M-H2O</td>
<td>1410.3730</td>
<td>1410.3708</td>
<td>0.0022</td>
<td>1.56</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>M</td>
<td>1428.3835</td>
<td>1428.3818</td>
<td>0.0017</td>
<td>1.19</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>M-H2O</td>
<td>1452.3835</td>
<td>1452.3799</td>
<td>0.0036</td>
<td>2.48</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>M</td>
<td>1603.4316</td>
<td>1603.4268</td>
<td>0.0048</td>
<td>2.99</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>M</td>
<td>1645.4422</td>
<td>1645.4411</td>
<td>0.0011</td>
<td>0.67</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>M</td>
<td>1687.4527</td>
<td>1687.4505</td>
<td>0.0022</td>
<td>1.30</td>
</tr>
</tbody>
</table>
Table A4 Species identified in charge-deconvoluted LC-QToF-MS of S. Typhi Vi antigen glycolipid termini.

<table>
<thead>
<tr>
<th>GalNAcA residues</th>
<th>Ac</th>
<th>Compounds</th>
<th>species</th>
<th>Calculated mass (u)</th>
<th>Observed mass (u)</th>
<th>Diff</th>
<th>Error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0</td>
<td>anhydroHexNAcA-HexNAcA-HexNAcA-(C14OH/C14OH)</td>
<td>[M]</td>
<td>1089.583</td>
<td>1089.587</td>
<td>0.004</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anhydroHexNAcA-HexNAcA-HexNAcA-(C14OH/C16OH)</td>
<td>[M-H+]Na</td>
<td>1328.623</td>
<td>1328.623</td>
<td>0.000</td>
<td>-0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anhydroHexNAcA-(HexNAcA)−HexNAc-(C14OH/C14OH)</td>
<td>[M-2H+2Na]</td>
<td>1350.605</td>
<td>1350.606</td>
<td>0.001</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>anhydroHexNAcA-(HexNAcA)−HexNAc-(C14OH/C15OH)</td>
<td>[M]</td>
<td>1320.658</td>
<td>1320.658</td>
<td>0.000</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anhydroHexNAcA-(HexNAcA)−HexNAc-(C14OH/C16OH)</td>
<td>[M-H+]Na</td>
<td>1342.639</td>
<td>1342.639</td>
<td>0.000</td>
<td>-0.4</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>anhydroHexNAcA-(HexNAcA)−HexNAc-(C14OH/C16OH)</td>
<td>[M]</td>
<td>1334.673</td>
<td>1334.678</td>
<td>0.005</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anhydroHexNAcA-(HexNAcA)−HexNAc-(C14OH/C14OH)</td>
<td>[M-H+]Na</td>
<td>1356.655</td>
<td>1356.659</td>
<td>0.004</td>
<td>2.6</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>anhydroHexNAcA-(HexNAcA)−HexNAc-(C14OH/C16OH)</td>
<td>[M]</td>
<td>1523.701</td>
<td>1523.703</td>
<td>0.002</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anhydroHexNAcA-(HexNAcA)−HexNAc-(C14OH/C14OH)</td>
<td>[M-H+]Na</td>
<td>1545.682</td>
<td>1545.683</td>
<td>0.001</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anhydroHexNAcA-(HexNAcA)−HexNAc-(C14OH/C14OH)</td>
<td>[M-H2O]</td>
<td>1505.692</td>
<td>1505.692</td>
<td>0.000</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>anhydroHexNAcA-(HexNAcA)−HexNAc-(C14OH/C16OH)</td>
<td>[M]</td>
<td>1551.732</td>
<td>1551.730</td>
<td>-0.002</td>
<td>-1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anhydroHexNAcA-(HexNAcA)−HexNAc-(C14OH/C14OH)</td>
<td>[M-H+]Na</td>
<td>1573.714</td>
<td>1573.715</td>
<td>0.001</td>
<td>0.9</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>anhydroHexNAcA-(HexNAcA)−Ac-HexNAc-(C14OH/C14OH)</td>
<td>[M]</td>
<td>1565.713</td>
<td>1565.712</td>
<td>-0.001</td>
<td>-0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anhydroHexNAcA-(HexNAcA)−Ac-HexNAc-(C14OH/C16OH)</td>
<td>[M]</td>
<td>1587.693</td>
<td>1587.692</td>
<td>-0.001</td>
<td>-0.9</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>anhydroHexNAcA-(HexNAcA)−HexNAc-(C14OH/C14OH)</td>
<td>[M]</td>
<td>1740.759</td>
<td>1740.761</td>
<td>0.002</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anhydroHexNAcA-(HexNAcA)−HexNAc-(C14OH/C16OH)</td>
<td>[M-H+]Na</td>
<td>1762.741</td>
<td>1762.742</td>
<td>0.001</td>
<td>0.7</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>anhydroHexNAcA-(HexNAcA)−HexNAc-(C14OH/C16OH)</td>
<td>[M]</td>
<td>1768.790</td>
<td>1768.786</td>
<td>-0.004</td>
<td>-2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anhydroHexNAcA-(HexNAcA)−Ac-HexNAc-(C14OH/C14OH)</td>
<td>[M-H2O]</td>
<td>1750.780</td>
<td>1750.776</td>
<td>-0.004</td>
<td>-2.0</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>anhydroHexNAcA-(HexNAcA)−Ac-HexNAc-(C14OH/C16OH)</td>
<td>[M]</td>
<td>1782.770</td>
<td>1782.772</td>
<td>0.002</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anhydroHexNAcA-(HexNAcA)−Ac-HexNAc-(C14OH/C16OH)</td>
<td>[M-H+]Na</td>
<td>1804.752</td>
<td>1804.755</td>
<td>0.003</td>
<td>1.9</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>anhydroHexNAcA-(HexNAcA)−Ac-HexNAc-(C14OH/C16OH)</td>
<td>[M]</td>
<td>1810.801</td>
<td>1810.796</td>
<td>-0.005</td>
<td>-2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anhydroHexNAcA-(HexNAcA)−Ac-HexNAc-(C14OH/C14OH)</td>
<td>[M-H+]Na</td>
<td>1832.783</td>
<td>1832.778</td>
<td>-0.005</td>
<td>-2.7</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>anhydroHexNAcA-(HexNAcA)−Ac-HexNAc-(C14OH/C14OH)</td>
<td>[M]</td>
<td>1824.780</td>
<td>1824.782</td>
<td>0.002</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anhydroHexNAcA-(HexNAcA)−Ac-HexNAc-(C14OH/C16OH)</td>
<td>[M-H+]Na</td>
<td>1846.762</td>
<td>1846.763</td>
<td>0.001</td>
<td>0.4</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>anhydroHexNAcA-(HexNAcA)−Ac-HexNAc-(C14OH/C16OH)</td>
<td>[M]</td>
<td>1852.812</td>
<td>1852.807</td>
<td>-0.005</td>
<td>-2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anhydroHexNAcA-(HexNAcA)−Ac-HexNAc-(C14OH/C16OH)</td>
<td>[M-H+]Na</td>
<td>1874.794</td>
<td>1874.786</td>
<td>-0.008</td>
<td>-3.8</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>anhydroHexNAcA-(HexNAcA)−HexNAc-(C14OH/C15OH)</td>
<td>[M]</td>
<td>1971.833</td>
<td>1971.829</td>
<td>-0.004</td>
<td>-2.3</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>anhydroHexNAcA-(HexNAcA)−HexNAc-(C14OH/C16OH)</td>
<td>[M]</td>
<td>1985.849</td>
<td>1985.853</td>
<td>0.004</td>
<td>1.8</td>
</tr>
<tr>
<td>#</td>
<td>Anhydro HexNAcA-(HexNAcA)</td>
<td>[M]</td>
<td>1999.828</td>
<td>1999.830</td>
<td>0.002</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>-----------------------------</td>
<td>-------</td>
<td>----------</td>
<td>----------</td>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>6 1</td>
<td>Ac-HexNAc-(C14OH/C14OH) anhydro HexNAcA-(HexNAcA)</td>
<td>[M]</td>
<td>2013.844</td>
<td>2013.838</td>
<td>-0.006</td>
<td>-2.8</td>
<td></td>
</tr>
<tr>
<td>6 1</td>
<td>Ac-HexNAc-(C14OH/C15OH) anhydro HexNAcA-(HexNAcA)</td>
<td>[M]</td>
<td>2027.860</td>
<td>2027.852</td>
<td>-0.007</td>
<td>-3.6</td>
<td></td>
</tr>
<tr>
<td>6 2</td>
<td>Ac2-HexNAc-(C14OH/C14OH) anhydro HexNAcA-(HexNAcA)</td>
<td>[M]</td>
<td>2041.839</td>
<td>2041.840</td>
<td>0.008</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>6 2</td>
<td>Ac2-HexNAc-(C14OH/C15OH) anhydro HexNAcA-(HexNAcA)</td>
<td>[M]</td>
<td>2055.855</td>
<td>2055.862</td>
<td>0.007</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>6 2</td>
<td>Ac2-HexNAc-(C14OH/C16OH) anhydro HexNAcA-(HexNAcA)</td>
<td>[M]</td>
<td>2069.870</td>
<td>2069.868</td>
<td>-0.002</td>
<td>-0.9</td>
<td></td>
</tr>
<tr>
<td>6 3</td>
<td>Ac3-HexNAc-(C14OH/C16OH) anhydro HexNAcA-(HexNAcA)</td>
<td>[M]</td>
<td>2111.881</td>
<td>2111.883</td>
<td>0.002</td>
<td>1.2</td>
<td></td>
</tr>
</tbody>
</table>
Figure A1. Supporting MS data for the Vi antigen glycolipid terminus. (A) observed (blue) and expected (red) isotopic distributions for the 1333.663 m/z ion, corresponding to 3 GalNAcA residues attached to the reducing terminal diacyl-HexNAc (B) Fragmentation of the Vi antigen terminal fragment with m = 1551.730 u. LC-ESI-QToF-MS/MS spectrum for the doubly-charged ion ([M-2H]^2+, m/z = 774.858), corresponding to 4 GalNAcA residues linked to a reducing terminal diacyl-HexNAc. Observed m/z values are indicated and the expected m/z values from the fragmentation pattern are shown in brackets. m/z = mass to charge.
Figure A2 Vi-antigen expression data collected in *E. coli* Top10. (A) Vi-antigen produced by *E. coli* harboring viaB on a plasmid bound hydrophobic PVDF and negatively-charged nylon membranes, whereas Vi antigen from the corresponding ΔvexE mutant was only bound by nylon. The panels show immunoblots of proteinase K-digested whole cell lysates probed with anti-Vi-antigen antibody. PVDF binding was restored when the ΔvexE mutant was complemented with either *S. Typhi* vexE or *A. denitrificans* vexE. The corresponding putative catalytic mutants of VexE from either *S. Typhi* (H487A) or *A. denitrificans* (H466A) failed to restore PVDF binding. A Y471F mutation in the *A. denitrificans* enzyme had no discernible effect on its activity. VexE expression was monitored by Western immunoblotting of hexahistidine tagged VexE constructs from identical cell cultures. (B) The H466A mutation did not significantly affect VexE secondary structure. Circular dichroism spectra of purified VexE and VexE<sup>H466A</sup> from *A. denitrificans*. (C) Immunofluorescence microscopy images of live or fixed and permeabilized *E. coli* harboring plasmid-encoded viaB or its mutant derivatives, probed with anti-Vi antigen antibodies. All *E. coli* recombinants accumulated Vi antigen in intracellular inclusion bodies. vexE and vexC mutants possessed no Vi antigen on their cell surfaces. Scale bars represent 10 µm. Insets are enlarged to show a single representative cell. (D) Vi antigen is synthesized in a ΔwecA mutant of *E. coli* Top10, prepared and immunoblotted as above.