Identification and Exploitation of Oligosaccharyltransferase Dependent Bacterial Glycosylation Systems

by

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Abstract

Protein glycosylation, the covalent attachment of carbohydrates to amino acids, was once thought to be a unique attribute of Eukaryotes and select bacteria. However, both *N*- (attached to asparagine) and *O*- (attached to serine or threonine) glycosylation systems have been identified in an ever increasing number of bacteria, including various important human pathogens. Two distinct classes of glycosylation are categorized based on the presence of an oligosaccharyltransferase (OTase). For OTase-independent glycosylation, individual glycosyltransferases (GTs) transfer individual monosaccharides to proteins cytoplasmically. The other class has a more complex pathway, where individual GTs transfer monosaccharides sequentially onto a lipid carrier on the cytoplasmic face of the inner membrane, and when completed, translocated to the periplasmic face, where the OTase transfers the glycan *en bloc* to acceptor proteins. By exploiting the exponentially increasing number of sequenced bacterial genomes available, *in silico* analysis has revealed several potential OTase-dependent glycosylation systems.

A homologue of *Neisseria* OTase (PglL) was identified in the emerging Gram negative pathogen *Acinetobacter baumannii*. This bacterium has been isolated from healthcare facilities for over 40 years, but was not dangerous because it was easily controllable with antibiotics. In the last decade, numerous *A. baumannii* isolates have developed extreme resistance to antibiotics, desiccation, and disinfectants, leading its classification as a “superbug” and one of the greatest threats to the modern healthcare system. In this thesis, a combination of genetics, molecular and cell biology, mass spectroscopy, and virulence model systems identify and characterize the general *O*-glycosylation system in *A. baumannii*. Analysis of the glycoproteome of *A. baumannii* revealed seven different glycoproteins modified with a pentasaccharide with a unique

terminal subunit dependent on the OTase $PglL_{Ab}$. Several of these glycoproteins were recombinantly expressed and further analyzed by Western blot and mass spectroscopy. The glycosylation system of *A. baumannii* can be functionally reconstituted in *E. coli*, and $PglL_{Ab}$ was able to transfer mono-, oligo, and polysaccharides with different structures, with relaxed glycan specificity.

Bacteria require protein glycosylation for a variety of functions, including motility and pathogenesis. *A. baumannii* does not require *O*-glycosylation for swarming motility, but is essential for biofilms. The glycosylation deficient strain was non pathogenic towards *Dictyostellium discoideum* (macrophage model), *Galleria mellonella* (innate immunity response model), and was unable to compete with the wild type in colonizing and killing mice. Additionally, *O*-glycosylation appears to be important in infecting and killing human alveolar epithelial cells. *A. baumannii* was observed to cause Eukaryotic cell rounding in a glycosylation dependent manner, but did not cross link actin *in vivo*.

It appears that *O*-glycosylation is highly conserved in *Acinetobacter*. All sequenced strains to date have a homologue of $PglL_{Ab}$, and a genetic locus responsible for synthesis of the *O*-glycan. Additionally, in this locus are the genes required for capsular polysaccharide export leading to the hypothesis a dual use for glycan. These results demonstrate *A. baumannii* possesses an active *O*-glycosylation conserved in *Acinetobacter*, and it is required for pathogenesis.

In a related applied project, a glycoconjugate was produced by reconstituting the *Campylobacter jejuni* N-glycosylation system in *Yersinia enterocolitica* O:9. The glycosylated carrier protein was purified and the glycan was identified as the *Y. enterocolitica* O:9 O antigen, which is identical to the *Brucella* O antigen. Sera obtained

from mice injected with the glycoconjugate was immunoreactive against both species purified LPS, but no protection was observed against *Brucella* infection. However, by conjugating the glycoconjugate to magnetic beads, a novel diagnostic system against brucellosis was generated.

Preface

(Mandatory due to Collaborative Work)

Some of the research conducted for this thesis forms part of an international research collaboration. Portions of Chapter 1 of this thesis have been published as:

Iwashkiw JA, Vozza NF, Kinsella RL, and Feldman MF. 2013. **Pour some sugar on it: the expanding world of bacterial protein O-linked glycosylation**. Mol Microbiol. Jul;89(1):14-28. In this publication, I was responsible for research, writing, and editing of the manuscript. Both NF Vozza and RL Kinsella were responsible for writing one section each. MF Feldman was responsible for writing, editing, and proofreading the manuscript.

Portions of Chapter 2 of this thesis have been published as:

Iwashkiw J.A., Seper, A., Weber B.S., Scott N.E., Vinogradov E., Stratilo C., Reiz B., Cordwell S.J., Whittal R., Schild S., Feldman M.F. 2012. **Identification of a general *O*-linked protein glycosylation system in *Acinetobacter baumannii* and it role in virulence and biofilm formation**. PLoS Pathog 8(6): e1002758. In this publication, I was responsible for performing experimental work for molecular biology, mass spectrometry, 96 well biofilm assays, virulence assays, preparing samples for ZIC-HILIC and NMR analysis. A. Seper was responsible for characterization of biofilms in a flow cell system. N.E. Scott was responsible for performing mass spectroscopy on ZIC-HILIC enriched samples and identification of additional glycoproteins. E. Vinogradov was responsible for performing and analyzing the NMR characterizing the structure of the *O*-glycan. C. Stratilo was responsible for performing the mice virulence assay. B.S. Weber was responsible for species identification of *Acinetobacter* clinical isolates.

Lees-Miller R.G., Iwashkiw J.A., Scott N.E., Seper A., Vinogradov E., Schild S., Feldman M.F. 2013. **A common pathway for *O*-linked protein glycosylation and synthesis of capsule in *Acinetobacter baumannii***. Mol Micro 89(5) 816-830 In this publication, I was responsible for identifying the genetic cluster responsible for *O*-glycosylation, the hypothesis that *O*-glycosylation and capsular polysaccharides utilize a common glycan, and assisted in experimental design. R.G. Lees-Miller was responsible for experimental work. N.E. Scott was responsible for ZIC-HILIC mass spectroscopy, A. Seper was responsible for characterizing biofilms in a flow cell system, and E. Vinogardov was responsible for NMR analysis.

Portions of Chapter 3 of this thesis have been published as:

Iwashkiw, J.A., Fentabil, M.A., Faridmoayer, A., Mills, DC., Peppler, M., Czibener, C., Ciocchini, A.E., Comerci, D.J., Ugalde, J.E., Feldman, M.F. **Exploiting the *Campylobacter jejuni* Protein Glycosylation System for Glycoengineering Vaccines and Diagnostic Tools Directed Against Brucellosis**. *Microbial Cell Factories* 2012, **11**:13.

In this publication I was responsible for the molecular biology, mass spectroscopy analysis, protein purification, and ELISA analysis. M.A. Fentabil was responsible for injection of samples into the mass spectrometer. Czibener, C., Ciocchini, A.E., Comerci, D.J., Ugalde were responsible for the murine virulence model and spleen colony counts, in addition to development of the Magnetic bead-based immunoassay.

Portions of Appendix A of this thesis have been published as:

Karen V. Lithgow, Nichollas E. Scott, Jeremy A. Iwashkiw, Euan L. S. Thomson, Leonard J. Foster, Mario F. Feldman and Jonathan J. Dennis. **A general protein *O*-glycosylation**

system within the *Burkholderia cepacia* complex is involved in motility and virulence Mol Micro 92(1) 116-137.

In this publication I was responsible for assisting Karen V. Lithgow with *in vivo* glycosylation assays, and Western blot visualization of whole cell extracts. Additionally, I was responsible for protein purification and mass spectrometry characterization of DsbA with the *C. jejuni N*-glycan, and expression and analysis of the pilin identified as glycosylated but not expressed in the conditions tested.

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I would like to thank my supervisor Dr. Mario Feldman for initially accepting me into his research group, and never giving up on me. Without his belief and resolve, I would not have had the opportunity to pursue this passion. I also would like to thank Mario for the training and mentorship he provided with my work, in addition to the special and unique lab atmosphere that all past, present, and future members of the group will cherish.

Thanks to the Feldman research group, all members past and present. Without the support and team attitude of everyone, this would not have been possible. Special thanks to Amir Faridmoayer for taking me under his wing and mentoring me in both science and life. Everyone else, every day in the lab was made better by each of you, and I thank you for being a major part of my life for my time here.

Thanks to all the people on campus that significantly assisted my studies with help, instruction, and advice. Tom, Tony, Troy, Michelle, Ches, Cheryl, Melany, Carol, Arlene, as well as everyone else who had a helping hand in my time here from the department. Bela, Randy, Jing, and Messele from the Chemistry, and Steve from MMI, every time I had questions you were always willing to provide solutions and help in any way possible.

Thanks to my family for being there everyday, anytime, and no supporting me unconditionally. None of this would have been possible without your love, and I could never ask for a better father, mother, or sister.

A huge thanks goes out to my friends, especially Corey, Harald, Ash, Anushka, Mo, Vanessa, Keith, Justin, and so many more for being there for both the bad and good days, and making them all better in a smile or a hug. I also would like to thank all alumni of S.S. Lazio Soccer club, for all the work and friendship both on and off the soccer pitch.

None of this would have been possible without all of the mentioned people above, and for that I will never forget any of you!

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List of Abbreviations

ABC transporter	ATP-binding cassette transporter
bp	Base pair
Bac	Bacillosamine (2,4-diacetamido-2,4,6-trideoxyglucose)
CFU	Colony forming units
CID	Collision induced dissociation
CPS	Capsular polysaccharide
DATDH	2,4-diacetamido-2,4,6 trideoxyglucose
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DIGE	Differential in Gel Electrophoresis
DNA	Deoxyribonucleic acid
DolP	Dolichyl phosphate
DolP-P	Dolichyl pyrophosphate
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
fgl	Flagellar glycosylation locus
Fuc	Fucose
FucNAc	*N*-Acetylfucosamine
Gal	Galactose
GalNAc	*N*-Acetylgalactosamine
GI	Glycosylation island
GlcNAc	*N*-Acetylglucosamine
Gm^R	Gentamicin resistance
GT	Glycosyltransferase

HCD	High collision dissociation
Hex	Hexose
HexNAc	*N*-Acetylhexosamine
HP-	Homopolymer negative *Y. enterocolitica* O9 Strain producing outercore only
IAA	Iodoacetamide
Ig	Immunoglobulin
iGT	Initiating Glycosyltransferase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kan	Kanamycin
kD	Kilodalton
LB	Luria-Bertani
LC ESI-Q-TOF	Liquid chromatography electrospray ionization quadrupole time-of-flight
LLO	Lipid-linked oligosaccharide
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MALDI	Matrix-assisted laser desorption ionization
mM	Millimolar
MS	Mass spectrometry
MS/MS	Tandem MS
Ni-NTA	Nickel-nitriloacetic acid
NMR	Nuclear magnetic resonance
OC-	Outer core negative *Y. enterocolitica* O9 strain only producing

	homopolymeric O antigen
OD	Optical density
OST	Eukaryotic oligosaccharyltransferase
OTase	Oligosaccharyltransferase
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.1% Tween 20
PCR	Polymerase chain reaction
PMT	Protein *O*-mannosyltransferase
Rha	Rhamnose
Ser	Serine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
Thr	Threonine
UDP	Uridine di-phosphate
UndP	Undecaprenyl phosphate
UndP-P	Undecaprenyl pyrophophate
WT	Wild type

Chapter 1

Introduction

Portions of this chapter have been published.
Iwashkiw JA, Vozza NF, Kinsella RL, and Feldman MF. 2013. Pour some sugar on it: the expanding world of bacterial protein O-linked glycosylation. Mol Microbiol. Jul;89(1):14-28.

Glycobiology is the field of studying of the structure, synthesis, and function of carbohydrates or saccharides, which are paramount for biological life (Varki and Sharon 2009). Carbohydrates are macromolecules consisting of carbon, hydrogen, and oxygen with the empirical formula of $C_x(H_20)_y$, and are categorized based on the number of glycans covalently attached from individual subunits (monosaccharides), to short chains (oligosaccharides), to larger structures (polysaccharides). Glycans can be covalently attached to lipids or proteins and are essential to bacteria as glycoconjugates such as peptidoglycan, exopolysaccharides, capsule, lipopolysaccharide (LPS), lipooligosaccharide (LOS), and glycoproteins (Hug 2010). Interestingly, the biosynthetic pathways are similar for many of these structures. A specialized initiating glycosyltransferase (iGT) covalently attaches a nucleotide-activated monosaccharide onto a membrane-bound lipid carrier, and additional glycosyltransferases sequentially transfer nucleotide-activated monosaccharides onto this structure until completed. The lipid-linked oligosaccharide (LLO) is subsequently translocated across the membrane, where additional modification or polymerization steps can occur, the glycan is finally removed from the lipid carrier and transferred to other structures such as Lipid A or protein acceptors (Hug 2010).

Glycosylation is one of the most important protein modifications. It is estimated that over 50% of all proteins in nature are glycosylated (Apweiler *et al.*, 1999). Protein glycosylation is thought to be involved in protein stability, cellular motility and adherence, as well as immunogenicity or immune evasion (Nothaft & Szymanski, 2010). While protein glycosylation is probably widespread throughout the bacterial kingdom, there seems to be an association between glycosylation systems and the pathogenicity towards humans. Most commonly, glycoproteins are exposed on the cell surface (i.e. pilin or flagella), which

led researchers to believe that the main function of protein glycosylation was immune evasion in bacteria (Ku *et al.*, 2009). However, recent research has shown that glycoproteins can be localized in the inner membrane and periplasmic space of Gram negative species (Vik *et al.*, 2009, Fletcher *et al.*, 2009). In almost all cases, loss of protein glycosylation in these pathogenic strains correlates with a decrease in virulence, but to date the exact role has not been determined to date.

In this thesis the objective was to further expand the knowledge of bacterial protein glycosylation by identifying and characterizing new systems in addition to exploiting previously characterized systems. The first project was the discovery and characterization of a general *O*-glycosylation system in the multidrug resistant bacterium *Acinetobacter baumannii*. The second project was utilization of the characterized *N*-protein glycosylation machinery of *Campylobacter jejuni* for the synthesis of glycoconjugates with vaccine and diagnostic potential. Increasing our knowledge of both known and novel protein glycosylation systems could have value for pharmaceutical applications such as vaccine development, designer antibiotic drugs for multidrug resistant bacteria, and diagnostic tools.

1.1 Bacterial Protein Glycosylation

Protein glycosylation, the covalent attachment of a saccharide to an amino acid, is the most common posttranslational modification identified to date with more than two thirds of all eukaryotic proteins hypothesized to be glycosylated (Apweiler et al., 1999). Initially identified in 1938 (Neuberger, 1938), glycosylation was thought of as a uniquely eukaryotic until the identification of S-layer glycoproteins in *Halobacterium* and *Clostridium* (Mescher & Strominger, 1976, Sleytr, 1975). Since then, protein glycosylation systems have been

identified in all forms of life. Protein glycosylation can occur by three main different mechanisms. Carbohydrates can be covalently attached to the amine nitrogen of asparagine residues (*N*-glycosylation), to the hydroxyl oxygen of serine or threonine residues (*O*-glycosylation), or the indole C2 carbon of tryptophan residues (*C*-mannosylation). However, to date, C-mannosylation has not been identified in bacteria and will not be covered any further.

As with other fields of study, glycobiology has undergone an exponential growth in knowledge due an exponential increase in the sensitivity of new technologies. The evolution of analytical methods from the rudimentary initial colorimetric assay (Neuberger, 1938) to state of the art techniques of tandem mass spectrometry (MS/MS) (Azadi & Heiss, 2009, Karlsson *et al.*, 2009) nuclear magnetic resonance (NMR) (Slynko *et al.*, 2009), and genomic sequencing has provided more powerful and reliable methodologies. Countless genes, pathways, and conjugated proteins have been identified and characterized, creating a broader understanding of the world of glycobiology. Unlike Eukaryotes, prokaryotic glycoproteins structures and biosynthetic pathways are much more complex and more variable, increasing the difficulty in characterizing the enzymes involved (Nothaft & Szymanski, 2010). With an ever expanding database of sequenced prokaryotic genomes, enzymes required for carbohydrate synthesis and attachment to proteins (ex. glycosyltransferases and oligosaccharyltransferases) are being characterized in a vast array of bacteria. For the majority of understood glycosylation systems, the genetic loci for the machinery required to synthesize the sugar-substrate are located close together on the genome and are collectively known as the protein glycosylation locus (*pgl*) (Linton *et al.*, 2005, Tabei *et al.*, 2009, Schirm *et al.*, 2004a, Arora *et al.*, 2001). There is a significant amount of genetic and structural homology between *pgl* and other carbohydrate

synthesis pathways such as O-antigen, capsular, and S-layer synthesis (Szymanski *et al.*, 1999, Feldman *et al.*, 2005, Faridmoayer *et al.*, 2007), which leads to important questions about pathway cross talk, which will be discussed later.

1.2 *O*-Glycosylation

Bacterial *O*-glycosylation can occur by two different mechanisms, based on the presence of a key enzyme, an Oligosaccharyltransferase (OTase) (Fig 1.1). OTases are enzymes that transfer a preassembled glycan *en bloc* to a protein acceptor. OTase-dependent *O*-glycosylation has not been described in Eukaryotes. The pathway is initiated by a specialized initiating glycosyltransferase that transfers a nucleotide-activated monosaccharide to an undecaprenylphosphate (UndP) lipid carrier on the inner face of the plasma membrane (Hug & Feldman, 2011). Glycosyltransferases subsequently attach additional monosaccharides to the first sugar residue on Und-PP and, when the carbohydrate is completed, the UndP-P-linked glycan is flipped to the periplasmic face, where an OTase transfers the carbohydrate to selected serine or threonine residues in acceptor proteins. This pathway is reminiscent of the *N*-glycosylation process that occurs in the endoplasmic reticulum of eukaryotes and in the periplasm of bacteria such as *Campylobacter jejuni*, and also shares some similarities with the synthesis of lipopolysaccharide (LPS) in Gram negative bacteria (Aebi *et al.*, 1996, Linton et al., 2005, Hug & Feldman, 2011). Alternatively, during OTase-independent *O*-glycosylation glycosyltransferases sequentially transfer individual monosaccharides to a protein acceptor from their nucleotide-activated forms. This system is primarily utilized to decorate flagellar structural proteins and adhesins, and has been heavily studied in the past two decades (Dell *et al.*, 2010, Logan, 2006).

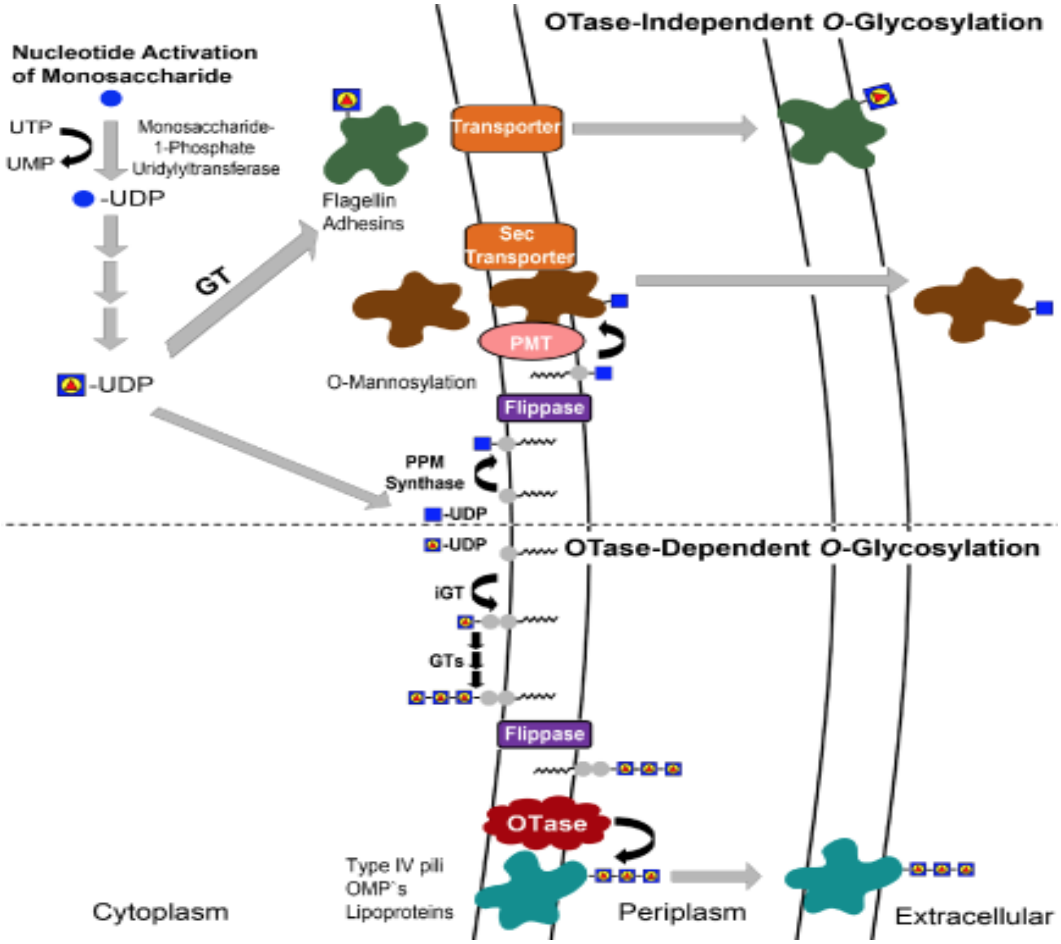

Figure 1.1. Model for the Proposed Mechanisms of *O*-glycosylation in Bacteria. Uridylyltransferases modify free monosaccharides to form UDP-glycans, which can be modified by a variety of processes (methylation, phosphorylation, sulphation, acetylation, etc.). In OTase-independent *O*-glycosylation, glycosyltransferases (GTs) attach individual monosaccharides to acceptor proteins in the cytoplasm and they are then transported to the outer membrane as shown, or secreted by the flagellum. In mycobacterial *O*-mannosylation, the individual sugars are attached to a polyprenol-phosphate (PPM synthase) and transferred to the target proteins via a mannosyltransferase (PMT) acting synchronously with protein secretion. OTase-dependent *O*-glycosylation is initiated by a specialized initiating GT (iGT) that attaches the nucleotide-activated monosaccharide to an undecaprenolphosphate (Und-P) lipid carrier on the inner face of the plasma membrane. Several GTs complete the oligosaccharide onto Und-PP carrier. Then the Und-PP linked glycan is flipped to the periplasmic space, where an OTase transfers the carbohydrate to Ser or Thr residues in acceptor proteins. Both forms of glycosylation also occur in Gram-positive bacteria (Table 1.1), but for simplicity, only Gram-negative is depicted.

1.2.1 OTase-independent *O*-Glycosylation

1.2.1.1 Flagellar Glycosylation

Many bacteria utilize a rigid, helical protein filament called a flagellum for locomotion (Berg & Anderson, 1973). The flagellum is composed of approximately 30 different proteins, and is divided into three components: basal body, hook, and filament. Flagellated bacteria have been classified into four groups based on the surface arrangement of the structures: a single polar flagellum (monotrichous), one flagellum present at each pole (amphitrichous), several flagella at one pole (lophotrichous), or several flagella randomly distributed on the cell surface (peritrichous). Many pathogenic bacteria require flagella to colonize and cause disease (Logan, 2006). Interestingly, animals, plants, and insects have developed innate immune recognition of flagellin monomers, with mammals possessing both extracellular and intracellular mechanisms to recognize flagellin (Chinchilla *et al.*, 2007, Miao *et al.*, 2007). This suggests that flagella are important virulence factors for bacterial pathogenesis. In this apparent molecular arms race, bacteria seem to have devised new mechanisms to subvert the innate immune response. Some bacteria (e.g. *Vibrio parahaemolyticus, Helicobacter pylori*) cover their flagella with an extension of the outer membrane, called a sheath (Doig & Trust, 1994, Fuerst, 1980, Geis *et al.*, 1993). Other bacteria add carbohydrates to their flagellins, the individual subunits of the flagellar filament (Table 1.1).

Early studies on protein glycosylation utilized lectin-blots, antibodies, enzymatic digestion, and chemical techniques such as periodic acid/hydrazide labeling to detect glycan structures (Alm *et al.*, 1992, Doig *et al.*, 1996, Ansorg, 1978, Sadoff *et al.*, 1985, Balonova *et al.*, 2009). While these techniques proved valuable as an initial screen for further studies and characterization, they are not conclusive and prone to false positives

(Longwell & Dube, 2013). For example, it was proposed that *Borrelia* spp. glycosylated several proteins (Sambri *et al.*, 1992, Sambri *et al.*, 1993), but later work demonstrated that this conclusion was based on the unspecific staining of membrane preparations (Sterba *et al.*, 2008). Techniques such as MS and NMR have had significant improvements in sensitivity and automation, allowing the definitive identification and characterization of bacterial glycoproteins from complex samples (Ashline *et al.*, 2005, Brisson *et al.*, 2010). Both bottom-up MS, the enzymatic digestion of a glycoprotein to identify glycosylation site and glycan structure, and top-down MS, where intact proteins are assayed for increase in molecular weight from the predicted primary sequence indicating modification, have been successfully employed for bacterial glycomics (Longwell & Dube, 2013). A novel approach for identifying glycoproteins is metabolic oligosaccharide engineering using a chemically modified monosaccharide that can be incorporated into bacterial glycoproteins, and subsequently detected and purified for further characterization (Laughlin & Bertozzi, 2007, Longwell & Dube, 2013). In flagellar glycosylation, no known recognition sequence has been identified. However, *O*-glycans appear to be localized in the highly variable central region of the flagellin, and exposed to the environment (Beatson *et al.*, 2006, Malapaka *et al.*, 2007, Westerlund-Wikstrom, 2000). To date, only *O*-glycosylation of flagellin in bacteria has been conclusively demonstrated, whereas *N*-glycosylation of these proteins has been suggested but not confirmed.

Table 1.1 Summary of OTase Independent *O*-Glycosylation in Bacteria

OTase-Independent Glycosylation					
Flagellar Glycosylation					
Organism	**Glyco-protein**	**Number of Sites**	**Glycan structure**	**Role of Glycosylation**	**Reference**
Gram Negative					
Aeromonas caviae UU 51 (Sch3)	FlaA/ FlaB	6/7	Pse5Ac7Ac8Ac (Pse5Ac7Ac)	Flagellar formation, Motility, Adherence to Eukaryotic Cells	(Schirm *et al.*, 2005, Tabei et al., 2009)
Aeromonas hydrophilia AH-3	FlaA/ FlaB	6 (FlaB T161)	376 Da-Hex-Hex-HexNAc-HexNAc-HexNAc-102 Da	Flagellar formation, Motility, Adherence to Eukaryotic Cells	(Wilhelms *et al.*, 2012)
	LafA	3	376 Da (pseudaminic acid derivative)	Flagellar formation, Motility	(Wilhelms et al., 2012)
Azospirillum brasilense Sp7	Laf1	N/D	(→3)-α-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→)$_{n=3-4}$	N/D	(Belyakov *et al.*, 2012)
Burkholderia pseudomallei K96243	FliC	1	582.4 Da (2x 291 Da)	Motility	(Scott *et al.*, 2011a)
Burkholderia thailandensis E264	FliC	1	342 Da	Motility	(Scott et al., 2011a)
Campylobacter jejuni 81-176	FlaA	19	Pse5Ac7Ac, PseAm, Pse8OAc PseAmGlnAc	Autoagglutination, flagellar biosynthesis, motility	(Thibault *et al.*, 2001, Schirm et al., 2005)
Campylobacter coli VC167	FlaA	16	Pse5Ac7Ac, PseAm, Pse/PseAm-deoxypentose,Leg 5Ac7Ac, Leg5Am7Ac,Leg5 AmNMe7Ac	N/D	(Logan *et al.*, 2002)
Helicobacter pylori	FlaA/ FlaB	7/10	Pse5Ac7Ac	Motility and mouse colonization	(Schirm *et al.*, 2003, Schirm et al., 2005)
Pseudomonas aeruginosa PAK	FliC	2	11 residues (pentose, hexose, deoxyhexose, hexuronic) attached via L-rhamnose	IL-8 stimulation, virulence	(Schirm et al., 2004a)
Pseudomonas aeruginosa PAO1	FliC	2	356 Da (209 Da and rhamnose phosphate)	N/D	(Verma *et al.*, 2006, Lindhout *et al.*, 2009)
Pseudomonas syringae pv. tabaci	FliC	6	ß-D-Quip4N(3-hydroxy-1-oxobutyl)2Me-(133)-α-L-Rha*p*-(132)-α-L-Rha*p*	Host specificity	(Taguchi *et al.*, 2006b, Takeuchi *et al.*, 2007)
Gram Positive					
Clostridium botulinum Langeland (group I)	FlaA	7	Legionaminic acid, di/tri-acetamido hexuronic acid	N/D	(Twine *et al.*, 2008)

Clostridium botulinum (group II)	FlaA	7	696 Da	N/D	(Twine et al., 2008)
Clostridium difficile 630	FliC	7	HexNAc-Phosphate-Methylated aspartic (398 Da)	Motility	(Twine *et al.*, 2009)
Clostridium difficile Bi-1	FliC	7	204 (HexNAc), 146 (deoxyHex), 160 (methylated deoxyHex), and 192 (hept)	Motility	(Twine et al., 2009)
Listeria monocytogenes	FlaA	6	*ß-O*-GlcNAc	Unknown	(Schirm *et al.*, 2004b)
Adhesin Glycosylation					
Aggregatibacter actinomycetemcomitans	EmaA	N/D	O-polysaccharide / contains fucose	Protein localization and collagen binding	(Tang & Mintz, 2010)
Escherichia coli	AIDA-1	~19	Heptose	Adherence to HeLa cells, conformation and protein stability	(Benz & Schmidt, 2001, Charbonneau *et al.*, 2007)
	TibA	N/D	Heptose	Adherence and invasion of mammalian cells	(Lindenthal & Elsinghorst, 1999, Moormann *et al.*, 2002, Sherlock *et al.*, 2005)
	Ag43	16	Heptose	Thermostability, increases collagen binding, decreases eukaryotic cell adhesion and autoaggregation	(Knudsen *et al.*, 2008, Reidl *et al.*, 2009)
Staphylococcus gordonii M99	GspB	N/D	GlcNAc, glucose, galactose, GalNAc, fucose and mannose	Binding to human platelets, GspB stability,	(Bensing & Sullam, 2002, Bensing *et al.*, 2004)
Staphylococcus parasanguis FW213	Fap1	N/D	Glucose, GlcNAc, galactose, GalNAc and rhamnose (mix of oligosaccharides)	Assembly, adherence to Saliva-coated hydroxyapatite and biofilm formation	(Stephenson *et al.*, 2002, Peng *et al.*, 2008)
Other Glycosylation					
Corynebacterium glutamicum	Rpf2	N/D	Unknown, Man and maybe Gal	Signaling, reactivation of growth	(Hartmann *et al.*, 2004)
Flavobacterium meningosepticum	P40	1	(2-OMe)Man-GlcNAcU-GlcU-Glc(2-OMe)-GlcU-[(2-OMe)Rha]Man	Secreted protease	(Reinhold *et al.*, 1995)
Mycobacterium tuberculosis	Apa	4	**α**-D-Man*p*(1 - 2)	Secreted antigen, putative Host-cell binding	(Dobos *et al.*, 1996, Espitia *et al.*, 2010)
	SodC	6	1 to 3 α-mannose	Localization and stability	(Sartain & Belisle, 2009)
Streptomyces coelicolor	PstS	1	Up to 3 hexoses **α**-D-Manp(1 - 2)	Phosphate uptake Lipoprotein	(Wehmeier *et al.*, 2009)
Lactobacillus plantarum	Acm2	5	Up to 3 HexNAc (GlcNac according to sWGA lectin blot)	*N*-Acetylglucosaminidase Autolysin	(Fredriksen *et al.*, 2012)

1.2.1.1.1 *Clostridium*

Clostridium sp. are spore-forming anaerobic bacteria possessing a polar flagella. Certain strains, like *C. botulinum*, *C. difficile,* and *C. tetani* produce extremely potent toxins, which can be fatal to humans. The first evidence of flagella glycosylation in these species was the presence of additional high molecular weight bands in flagellar extracts sensitive to periodic acid and ß-elimination in *C. tyrobutryricum, C. acetobutylicum*, and *C. difficile* (Arnold *et al.*, 1998) (Bedouet *et al.*, 1998, Tasteyre *et al.*, 2000, Lyristis *et al.*, 2000). Mass spectrometry and NMR techniques demonstrated *C. botulinum* group I strains glycosylate FlaC up to seven times with either legionaminic acid, Leg5GluNMe7Ac, or a di/tri-acetamido-hexuronic acid monosaccharide, whereas group II strains utilize a glycan of 696 Da (Twine et al., 2008). Bioinformatic analysis of the *C. botulinum* strain Langeland revealed a flagellar glycosylation island (FGI) located in close proximity to flagellar structural genes, believed to be involved in the biosynthesis of legionaminic acid due to homology to previously characterized genes from *C. jejuni* and *H. pylori* (Twine et al., 2008). Genetic variability has been observed in the FGI, supporting the hypothesis of variability of glycan residues attached to flagellins (Carter *et al.*, 2009). In *C. difficile* 630 seven sites were observed glycosylated on FliC with a HexNAc residue modified with a methylated aspartic acid linked via a phosphate bond, and glycosylation was dependent on a putative glycosyltransferase (CD0240) (Twine et al., 2009). Several other isolates glycosylated FliC with a heterogeneous glycan comprised of five monosaccharides with masses of 204 Da (HexNAc), 146 Da (deoyhexose), 160 Da (methylated deoxyhexose), and 192 Da (heptose) (Twine et al., 2009). All characterized *C. difficile O*-glycans are linked to the protein backbone via a HexNAc residue. Motility of *C. difficile* has been previously established in stab agar tubes and insertional mutants of *CD0240* and *fliC* are both non-motile (Tasteyre et al., 2000, Twine et al., 2009).

1.2.1.1.2 *Listeria monocytogenes.*

Listeria monocytogenes is a food borne pathogen and is responsible for listeriosis, which can range from severe gastrointestinal, systemic, or central nervous system infections (Williams *et al.*, 2012). *L. monocytogenes* can survive at high salt levels, in addition to a wide pH, and temperature range (O'Driscoll *et al.*, 1996, Walker *et al.*, 1990). The bacterium is motile and produces 4-6 peritrichous flagella at temperatures less than 30^{o}C, but at 37^{o}C, motility is significantly reduced and only minor quantities of flagella could be detected by electron microscopy (Peel *et al.*, 1988b). Purified flagellin was detected in several distinct bands when visualized by Western Immunoblot, and further analysis determined FlaA was glycosylated with ß-linked O-GlcNAc residues attached variably to three to six different sites (Peel *et al.*, 1988a, Schirm et al., 2004b, Shevchenko *et al.*, 1996). Glycosyltransferases have been identified in close proximity to the flagellins they modify and this approach identified Lmo0688 (GmaR) as the first flagellin and prokaryotic *O*-GlcNAc glycosyltransferase (OGT), which is common in eukaryotic systems (Shen *et al.*, 2006). Interestingly, GmaR functions as an anti-repressor, allowing the expression of FlaA, its substrate for activity (Shen et al., 2006). Further studies have demonstrated GmaR is the first example of a protein demonstrated to function as a thermo-sensing anti-repressor for flagella expression at high temperatures (Kamp & Higgins, 2011). *L. monocytogenes* requires flagella for motility and forming biofilms, but glycosylation does not appear to be required for flagellin formation (Lemon *et al.*, 2007). Additionally, glycosylation of FlaA is not involved with activation of NF-κB (Shen et al., 2006). Flagellar glycosylation has not been demonstrated to play a specific role in *L. monocytogenes* to date, however it is possible its role may be important for environments aspects of its lifestyle.

1.2.1.1.3 *Burkholderia sp.*

Burkholderia pseudomallei, the causative agent for meliodosis, is an environmental saprophyte found in wet soils and standing water as well as a facultative intracellular pathogen (Galyov *et al.*, 2010). Meliodosis can present as septic shock and/or acute pneumonia is endemic in Southeast Asia, Australia, the Middle East, Africa, India, and South America (Cheng & Currie, 2005). It has been demonstrated that *fliC* is an important virulence factor for *B. pseudomallei* (Chua *et al.*, 2003). Preliminary work suggested *B. pseudomallei* and *B. thailandensis* possess glycoproteins (Kondo *et al.*, 1994, Kondo *et al.*, 1991, Khrapova *et al.*, 1998, Zou *et al.*, 2008). Mass spectrometry analysis determined FliC was *O*-glycosylated in both *B. pseudomallei* K96243 and *B. thailandensis* E264, and the carbohydrate was identified as either 582.4 Da, (two monosaccharides of 291 Da), or 300/342 Da (*N*-acetyl hexuronic acid ± acetyl group) in *B. pseudomallei* K96243 and *B. thailandensis* respectively (Scott et al., 2011a). Analysis of one additional strain of each species revealed the glycan was conserved, suggesting some species-specific utilization. Interestingly, *rmlB* (BPSL2686) was required for flagellar glycosylation in addition to O antigen production in *B. pseudomallei*. A ∆*rmlB* mutant still produced WT flagellar filaments on its surface, demonstrating that flagellar glycosylation is required for motility, but is not involved in flagellar assembly (Scott et al., 2011a).

1.2.1.1.4 *Aeromonas spp.*

The mesophilic aeromonads are motile, have an optimum growth temperature well between 35^{o}C to 37^{o}C and are associated with human infections. (Janda & Abbott, 2010). Mesophilic *Aeromonas* are associated with gastrointestinal syndromes and soft tissue wound infections in healthy individuals, as well as septicemia and hemolytic-uremic

syndrome (Janda & Abbott, 2010). These bacteria produce two forms of flagella; one, single, polar flagella when grown in liquid culture, and peritrichous lateral flagella when grown on solid media, both of which are essential for adherence and virulence (Rabaan *et al.*, 2001, Gavin *et al.*, 2002). A flagellar glycosylation locus was identified in *Aeromonas* consisting of the main structural polar flagellar proteins FlaA and FlaB adjacent to a locus consisting of homologues of *N*-acetylneuraminic acid (NeuA) biosynthesis (Gryllos *et al.*, 2001, Tabei et al., 2009). Deletion of two different glycan biosynthetic genes resulted in a loss of polar flagella and a decrease in molecular weight of LPS (Gryllos et al., 2001, Tabei et al., 2009, Rabaan et al., 2001). Mass spectrometry analysis of *A. caviae* Sch3N revealed *O*-glycosylation of both FlaA (6 sites) and FlaB (7 sites) with an unusual 373 Da monosaccharide, which the authors proposed to be Pse5Ac7Ac9Ac (Schirm et al., 2005). Glycosylation of the polar flagella appears to be required for flagella formation and function, as insertional inactivation of *neuA* or *neuB* in *A. cavaie* Sch3N resulted in the absence of polar flagella, a decrease adherence to eukaryotic cells, and a loss of motility (Gryllos et al., 2001). *A. hydrophila* strain AH-3 glycosylates FliC with a heptasaccharide comprised of three HexNAc (variably modified with 0-2 phosphate groups and 0-2 methyl groups on each), two Hex, and two unknown monosaccharides of 376 and 102 Da (S/T-376-Hex-Hex-HexNAc-HexNAc-HexNAc-102) (Wilhelms et al., 2012). It has recently been suggested that *maf1* is a glycosyltransferase responsible for transferring the pseudaminic acid onto the flagellin (Parker *et al.*, 2012).

Glycosylation of lateral flagella and genetic locus responsible (*laf*) of *A. caviae* Sch3N and *A. hydrophila* AH-3 was determined similarly in a similar fashion as the polar flagellin (Gavin et al., 2002). Mass spectrometry analysis of *A. hydrophila* AH-3 demonstrated LafA is *O*-glycosylated with a monosaccharide of 376 Da in at least three

different sites (Wilhelms et al., 2012). The monosaccharide was confirmed to be the same pseudaminic acid derivative as the polar flagellar glycan, and was required for lateral flagellar formation and cell motility. Interestingly, in *A. hydrophila*, the pseudaminic acid mutants can still transcribe the flagellar DNA similar to the wild type, but cannot produce significant amounts of protein in the cytoplasm (Wilhelms et al., 2012). It has been suggested that different mechanisms are responsible for glycosylation of each flagellin, but further studies are required to fully understand the dual glycosylation systems of *Aeromonas* (Canals *et al.*, 2006a, Canals *et al.*, 2006b)

1.2.1.1.5 *Campylobacter jejuni*

Campylobacter sp is a microaerophilic bacterium that is one of the leading causes of human gastroenteritis worldwide (Allos, 2001). *C. jejuni* is a zoonotic mucosal pathogen that is commonly found as a commensal organism in most animals (Guerry & Szymanski, 2008). Flagellar *O*-glycosylation in *Campylobacter* has been extensively reviewed in several other publications (Guerry & Szymanski, 2008, Szymanski *et al.*, 2003a, Szymanski & Wren, 2005, Nothaft & Szymanski, 2010, Logan, 2006). It was initially hypothesized to exist with the identification of a posttranslational modification in *C. coli* (Alm et al., 1992, Logan *et al.*, 1989). A total of 19 *O*-glycosylation sites were identified on FlaA in a centrally located, exposed domain via Ser/Thr residues, making FlaA one of the most glycosylated proteins analyzed with carbohydrate accounting for ~10% of the total mass (Thibault et al., 2001). However, contrary to the initial suggestion of glycosylation of FlaA with sialic acid, tandem MS/MS and NMR analysis identified the modification as pseudaminic acid (Pse5Ac7Ac), a derivative of sialic acid (Thibault et al., 2001). Further work has demonstrated glycosylation of the *Campylobacter* flagella with several derivatives of both pseudaminic and legionaminic acid residues suggesting heterogeneity

might play a role in immune evasion against host defenses (Logan et al., 2002, Schirm et al., 2005, Thibault et al., 2001, McNally *et al.*, 2007, Logan *et al.*, 2009).

A significant amount of effort has focused on characterization of the genetic components responsible for glycosylation of FlaA and its function in *C. jejuni*. Sequencing of the *C. jejuni* NCTC 11168 genome revealed a flagellar glycosylation locus (fgl) containing ~50 genes, containing both *flaA* and *flaB*, but the full glycan structure has yet to be described (Logan et al., 2009, Parkhill *et al.*, 2000, Howard *et al.*, 2009). *C. jejuni* 81-176 has a reduced simpler fgl, consisting of only 26 genes, and two major glycoforms were identified (Guerry *et al.*, 2006). The full biosynthetic pathway has been determined for the main *O*-glycans of *C. jejuni* and has been described in the following review (Nothaft & Szymanski, 2010). Functional characterization of the *O*-glycosylation sites on FlaA of *C. jejuni* 81-176 revealed five sites are required for auto-agglutination, and three others are required for bacterial motility (Ewing *et al.*, 2009).

1.2.1.1.6 *Helicobacter*

The *Helicobacter* sp. possess two polar, sheathed, flagella composed of two different flagellins, FlaA and FlaB (Geis et al., 1993, Josenhans *et al.*, 2002). Initial evidence suggested *H. felis* glycosylated both FlaA and FlaB demonstrated by periodic acid treatment, antibody labeling, and western blot analysis (Josenhans *et al.*, 1999). Further analysis revealed *H. pylori* glycosylates both proteins, and two genes with homology to the *Campylobacter* genes *neuA* and *flmD* (HP0326a and HP0326b) were required (Josenhans et al., 2002). Mass spectrometry techniques identified seven *O*-glycosylation sites on FlaA and ten on FlaB modified with pseudaminic acid (Pse) (Schirm et al., 2005, Schirm et al., 2003). Unlike *Campylobacter* flagellar *O*-glycan, no

heterogeneity has been observed. One hypothesis for this difference is the flagellar sheath reduces the need for variation of the glycan (Logan, 2006). Bioinformatic analyses support the lack of heterogeneity, identifying several genes required for flagellar glycosylation in *Helicobacter* (Creuzenet *et al.*, 2000, Merkx-Jacques *et al.*, 2004, Schirm et al., 2003, Schoenhofen *et al.*, 2006). Examination of mutants of these genes demonstrated a significant decrease in both bacterial motility and colonization of mice stomachs, suggesting that glycosylation is required for pathogenesis of *H. pylori* (Schirm et al., 2003).

1.2.1.1.7 *Pseudomonas aeruginosa.*

Pseudomonas sp are considered ubiquitous environmental microorganisms, and have been identified as pathogens of both plants and humans. *P. aeruginosa* is a opportunistic pathogen, causing high rates of disease and death in immunocompromised patients (i.e. cystic fibrosis) (Bodey *et al.*, 1983, Kus *et al.*, 2004). *P. aeruginosa* expresses a single, polar flagellum required for motility, and has two distinct flagellin proteins encoded by *fliC* (type A and B), distinguishable by molecular weight and reactivity to specific antibodies (Ansorg, 1978, Montie & Anderson, 1988).

Type A flagella was first characterized from *P. aeruginosa* PAK and, although when expressed in *E. coli* had a molecular weight of ~39 kDa, was observed with a mass between 45-52 kDa (Totten & Lory, 1990). Initial evidence for glycosylation was demonstrated in three different strains of *P. aeruginosa*, and MS demonstrated strains PAK and JJ692 *O*-glycosylated FliC with different carbohydrate structures (Schirm et al., 2004a, Tsai Cm, 1982). Tandem mass spectrometry revealed $FliC_{PAK}$ is *O*-glycosylated with up to 11 monosaccharides attached via a reducing residue of *L*-rhamnose (Schirm et

al., 2004a). The exact composition of the remainder of the glycan remains unknown due to glycan heterogeneity, although it is composed of pentoses, hexoses, deoxyhexoses, hexuronic acids, and possibly deoxyhexoses with amino and formyl substitutions. The type A flagella of JJ692 strain was only *O*-glycosylated by single deoxyhexose at each glycosylation site (Schirm et al., 2004a).

It was initially accepted that *P. aeruginosa* type B flagella were not glycosylated since no shift in molecular mass was observed, and type A flagellar glycosylation strains were unable to glycosylate type B flagella (Arora et al., 2001). However, *P. aeruginosa* PAO1 glycosylates type B flagella with an *O*-linked oligosaccharide (Verma et al., 2006). Two different amino acids (S191 and S195) of FliC are glycosylated with a glycan of 356 Da, which appears to be composed of a unique residue with a mass of 209Da containing a phosphate moiety and a deoxyhexose suggested to be rhamnose by genetic and molecular biology techniques (Verma et al., 2006, Lindhout et al., 2009). Although both flagellar structures are *O*-glycosylated by a reducing deoxyhexose residue, there is significantly less glycan heterogeneity in type B than in type A flagellar glycosylation (Verma et al., 2006).

P. aeruginosa PAK (type A) possesses a glycosylation island (GI) which encodes for a series of biosynthetic genes shown to be important in the glycosylation (Arora et al., 2001). Comparison of the GI of JJ692 to PAK revealed a significantly reduced size in the region in JJ692, with the absence of 5 open reading frames (*orfI, orfJ, orfK, orfL,* and *orfM*) as well as a truncation of *orfE* (a serine O-acetyltransferase) (Arora et al., 2001, Schirm et al., 2004a). Mass spectrometry characterization of purified FliC determined that *orfN* was responsible for attaching the first deoxyhexose, and *orfA* was responsible for the addition of the second monosaccharide. (Schirm et al., 2004a). Further investigation into the

biosynthesis of the type A flagellar glycan revealed *wpbO* (dehydrogenase; converts UDP-D-GlcNAc → UDP-D-GlcNAcA), previously demonstrated to be involved in O antigen biosynthesis, is also required for *O*-glycosylation (Miller *et al.*, 2008).

The type B flagellar strain PAO1 GI resembles strain JJ692, with only four genes present (PA1088, PA1089, PA1090, and PA1091) (Verma et al., 2006). PA1091 is responsible for glycosylation of the reducing residue, but cross species complementation with OrfN (transfers the first glycan in strain JJ692) did not restore glycosylation of ΔPA1091, suggesting unique specificities for each enzyme (Verma et al., 2006, Schirm et al., 2004a). Mass spectrometry of purified FliC from an insertional mutant of PA1088 showed the protein was only modified with deoxyhexose, suggesting the gene was required for either biosynthesis or addition of the 209 Da residue (Verma et al., 2006).

P. aeruginosa requires its flagella for the pathogenesis in mice (Montie *et al.*, 1982, Feldman *et al.*, 1998). While glycosylation does not play a role in flagella formation or motility, glycosylated flagella invoke a significantly higher IL-8 response compared to the unglycosylated (Verma *et al.*, 2005, Arora *et al.*, 2005). A key study demonstrated glycosylation plays a significant role in pathogenesis for both types of flagella, with non-glycosylated flagellar strains having an LD_{50} 35 to >10,000 fold greater than WT strains (Arora et al., 2005). It was proposed that the glycan structure could be responsible for increased binding of flagella to host cell receptors, and increasing the inflammatory response. This result suggests that in *P. aeruginosa*, *O*-glycosylation increases the immunogenic property of flagella, thus increasing the bacteria's virulence towards host cells.

1.2.1.1.8 *Pseudomonas syringae.*

The phytopathogenic species *P. syringae* possesses several polar flagella, can cause a range of speck, spot, and blight disease on several different agriculturally important plants, and are classified into over fifty pathovars based on their specific host (O'Brien *et al.*, 2011). Purified flagellin (FliC) from *P. syringae* pv. glycinea, tabaci, and tomato, although over 95% identical, induced hypersensitive cell death in non-host plants, but pv. tabaci and tomato had no detrimental effects to their natural host plant (Taguchi *et al.*, 2003). FliC glycosylation in *P. syringae* was first observed by lectin blot, and confirmed by glycan staining of purified FliC from *P. syringae* pv. tabaci (Taguchi et al., 2003, Takeuchi *et al.*, 2003). Mass spectrometry identified six serine residues glycosylated with a 540 Da glycan, and further analysis revealed both *P. syringae* pv. tabaci and glycinea utilized a trisaccharide with two rhamnose residues and a 4-amino-4,6-dideoxyglucosyl (viosamine) residue ß-D-Qui*p*4N(3-hydroxy-1-oxobutyl)2Me-(1→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p* (Takeuchi et al., 2007). (Taguchi et al., 2006b). Interestingly, only L-rhamnose was found in the *O*-glycan of *P. syringae* pv. tabaci, whereas a 4:1 molar ratio of L-Rha to D-Rha was identified in the *O*-glycan of *P. syringae* pv. glycinea, and this difference in chirality was suggested to be important for host recognition (Takeuchi et al., 2007).

A GI similar to the type B flagellar GI of *P. aeruginosa* was identified in *P. syringae* pv. glycinea, with three ORF's found between the flagellar structural genes *flgL* and *fliC* (Takeuchi et al., 2003). Deletion of *fgt1* and *fgt2* (glycosyltransferases; 32 and 38% identity to *fgtA* of *P. aeruginosa* PAK) resulted in unglycosylated FliC, with no glycosylation observed in the absence of Fgt1, and a partially glycosylated protein in the absence of Fgt2 suggesting a cooperative effect between the two glycosyltransferases (Takeuchi et

al., 2003, Taguchi *et al.*, 2006a). While no glycosylation defect was observed in Δ*orf3*, bioinformatic analysis and subsequent characterization demonstrated *orf3* to be important for production of *N*-acyl homoserine lactones (AHLs) and quorum sensing (Taguchi et al., 2006a). More recently, a genetic locus responsible for the synthesis (*vioA* and vioB) and transfer of the viosamine monosaccharide (*vioT*) was identified, and when mutated, FliC was found to be glycosylated with 2-5 rhamnose residues at each glycosylation site, suggesting the viosamine acts as a capping mechanism (Nguyen *et al.*, 2009).

Similarly to *P. aeruginosa* PAK and PAO1, *P. syringae* requires flagellar *O*-glycosylation for virulence towards its host organism (Takeuchi et al., 2003, Westman *et al.*, 2007). P. *syringae* also requires proper glycosylation for swimming and swarming motility, in addition to AHL production (Westman *et al.*, 2008, Westman et al., 2007). Unglycosylated flagella filaments were tightly associated that was absent in WT *P. syringae* pv. tabaci 6605, suggesting glycosylation facilitates the formation of suprastructures, and in turn, bacterial motility (Westman et al., 2008). Recently, it was shown that both *fgt1* and *fgt2* mutants exhibit an increase in surfactant production (3-(3-hydroxyalkanoyloxy) alkanoic acid; HAA) and upregulation of genes involved in flagellin and chemotaxis (Westphal O, 1965, Xu *et al.*, 2012). The authors suggest that the surfactant is important for flagellar lubrication to minimize flagellar breakage and sticking, and must be upregulated in the absence of flagellar glycosylation in an attempt to maintain motility.

1.2.1.2 *O*-glycosylation in Mycobacteria and other Actinomycetes

Glycoproteins in *Mycobacterium tuberculosis* were first described in 1989, based on reactivity towards ConA lectin (Espitia & Mancilla, 1989). Since then, several proteins

were found to be *O*-glycosylated with mannose residues (Herrmann *et al.*, 1996, Michell *et al.*, 2003, Sartain & Belisle, 2009, Espitia et al., 2010). The mannosylated proteins were identified as lipoproteins and it was proposed that sugar addition might play a role in regulating specific proteolysis of the signal peptide, contributing to the proper localization of these lipoproteins (Herrmann et al., 1996, Pethe *et al.*, 2001).

Membrane-associated mannosyltransferase activity was found to be dependent on polyprenol-mono-phosphate-mannose (Cooper *et al.*, 2002). Homology searches identified a protein *O*-mannosyltransferase (PMT) with similarity to *Saccharomyces cerevisiae* and other eukaryotic PMTs (VanderVen *et al.*, 2005). A PMT homologue and several glycoproteins were also identified in *Corynebacterium glutamicum*, another species belonging to the genus *Actinomycetes*, indicating that glycoproteins are not exclusive to mycobacteria (Hartmann et al., 2004, Mahne *et al.*, 2006). In *Streptomyces coelicolor* the glycosylation machinery consists of a putative polyprenol-P-mannose synthase (PPM), responsible for the transfer of mannose to polyprenol phosphate, and a PMT homologue, which transfers the mannose to the PstS protein (Wehmeier et al., 2009). The commonalities between Eukaryotic and bacterial mannosylation systems include a PMT that encompasses 10 or more transmembrane domains, the protein acceptors (usually lipoproteins), and the fact that the reactions employ polyprenol-P-mannose, which was confirmed by inhibition with amphomycin, an inhibitor of mannosylphosphoryldolichol synthesis in eukaryotes. In these cells, the activated mannosyl precursor is linked to dolichol, but in *Actinobacteria*, the sugar is linked to shorter polyprenols, although the length of the polyprenol may be variable (Gurcha *et al.*, 2002). In one of the models proposed in *Mycobacterium*, PMT might act with partially folded or unfolded proteins that

are translocated through the Sec pathway (VanderVen et al., 2005), homologous to the endoplasmic reticulum translocation.

In *M. tuberculosis* glycosylation consists of one to four sugars per glycosylation site, although evidence of up to 10 sugars per residue has been found. Glycosylation occurs through an initial mannosylation step by a PMT, with the subsequent elongation of mannosyl chains through stepwise addition via one to two mannosyl transferases (Liu *et al.*, 2013). Some glycoproteins contain a single glycosylated residue, and others, like the Apa adhesin and SodC lipoprotein from *M. tuberculosis* were found to be glycosylated at several residues in a definite region (Sartain & Belisle, 2009, Espitia et al., 2010). In other organisms like *Corynebacterium*, *Streptomyces* or *Mycobacterium smegmatis*, PMT mutants are viable (Mahne et al., 2006, Wehmeier et al., 2009, Liu et al., 2013). In *M. tuberculosis*, the mutagenesis of PMT resulted in decreased survival in macrophages and attenuated virulence in mice (Liu et al., 2013). The antigenic nature of *O*-glycopeptides in *Mycobacterium* and its potential role in host cell surface adhesion (Ragas *et al.*, 2007) along with some structural differences with eukaryotic homologues (Mahne et al., 2006) makes PMTs an interesting target for potential therapies against these pathogens (Lengeler *et al.*, 2008).

1.2.1.3 Bacterial Adhesins

Autotransporter adhesion proteins (adhesins) are characterized by their structural organization and mechanism of export from the cell. The N-terminal signal sequence, recognized by the Sec secretion system, mediates transport across the inner membrane; upon translocation the signal sequence is cleaved (Brandon *et al.*, 2003, Sijbrandi *et al.*, 2003, Peterson *et al.*, 2006). The C-terminal translocator domain then inserts into the outer

membrane forming a β-barrel pore that facilitates translocation of the functional passenger domain to the cell surface (Konieczny *et al.*, 2001, Oomen *et al.*, 2004). Adhesins are often glycosylated and frequently associated with functions such as adherence, invasion, autoaggregation and/or biofilm formation (Cote & Mourez, 2011).

Three glycosylated adhesins identified in *Escherichia coli*, AIDA-I, Ag43 and TibA, aid in biofilm formation, autoaggregation as well as adherence and invasion of epithelial cells (Diderichsen, 1980, Klemm *et al.*, 2004, Sherlock et al., 2005, Sherlock *et al.*, 2006, Charbonneau et al., 2007, Knudsen et al., 2008). TibA and AIDA-I are cytoplasmically glycosylated by the heptosyltransferases TibC and Aah, encoded upstream of their target autotransporters. The nucleotide-activated heptoses derive from the LPS biosynthesis pathway (Benz & Schmidt, 2001, Moormann et al., 2002). The heptosyltransferase responsible for modification of Ag43 has yet to be identified (Sherlock et al., 2006, Knudsen et al., 2008).

Little is known about the glycosylation site recognition for adhesin *O*-linked protein glycosylation. Comparison of multiple adhesin glycoproteins revealed the presence of a 19-amino-acid glycosylation consensus sequence, predicted to form a β-strand-short acceptor loop-β-strand motif, with the glycosylated serine or threonine residue residing in the loop (Charbonneau *et al.*, 2012). Either the consensus sequence or the structural motif are sufficient for *O*-OTase-independent glycosylation by the *E. coli* heptosyltransferase Aah, indicating acceptor recognition is mediated through the structural motif rather than the amino acid sequence (Charbonneau et al., 2012).

Streptococci and *Staphylococci* species produce cytoplasmically glycosylated adhesins involved in adherence to host cells and platelets, fimbrial assembly and biofilm formation (Bensing & Sullam, 2002, Plummer *et al.*, 2005, Samen *et al.*, 2007, Shivshankar *et al.*, 2009, Zhou & Wu, 2009). Maturation and glycosylation are done in a coordinated effort by glycosyltransferases, secretion proteins and accessory proteins (APs) encoded downstream of the adhesin (Bensing & Sullam, 2002). Interaction between APs and secretion system components is required for maturation and export of fully glycosylated Fap1 and GspB (Chen *et al.*, 2004, Peng et al., 2008, Zhou *et al.*, 2012). APs appear to provide chaperone and quality control activities, stabilizing protein–protein interactions between accessory Sec system components and monitoring the glycosylation status of the adhesin (Li *et al.*, 2008, Peng et al., 2008, Seepersaud *et al.*, 2012, Echlin *et al.*, 2013).

EmaA, a trimeric autotransporter adhesin of the human oral pathogen *Aggregatibacter actinomycetemcomitans* associated with collagen binding, is *O*-glycosylated with the *O*-polysaccharide of its LPS (Tang *et al.*, 2008, Tang & Mintz, 2010). This is the first report of an autotransporter modified by an LPS WaaL ligase homologue, likely at the periplasmic side of the membrane, rather than a cytoplasmic glycosyltransferases (Tang & Mintz, 2010, Tang *et al.*, 2012).

1.2.2 OTase-dependent *O*-Glycosylation

For a long time, *N*-glycosylation was thought to be the only glycosylation pathway that operated via an OTase-dependent pathway. However, although it has not been described yet in Eukaryotes, *O*-OTases are now known to be present in many bacterial species (Table 1.2). These enzymes catalyze the transfer of an oligosaccharide from a

lipid carrier to Ser or Thr residues of the protein substrates. Power and Jennings identified a domain that is present in all the *O*-OTases, annotated as Wzy_C domain (Power *et al.*, 2006). This domain is also found in the WaaL ligases, enzymes that attach the O-antigens to lipid A-core during LPS synthesis. Therefore LPS synthesis and *O*-linked protein glycosylation are evolutionarily related (Hug & Feldman, 2011). Previously, experimentation was the only way to distinguish between ligases and OTases. However, recent work by Schulz *et al* has identified two conserved motifs that are solely found in PglL homologues and not in WaaL ligases (Schulz *et al.*, 2013). Initial *in silico* analysis of sequenced genomes suggests that OTase-dependent *O*-glycosylation is significantly more common than previously hypothesized (Schulz et al., 2013).

Table 1.2 Summary of OTase Dependent *O*-glycosylation in Bacteria

OTase-Dependent Glycosylation					
Organism	**OTase**	**Number of target proteins**	**Glycan Structure**	**Role of Glycosylation**	**Reference**
Bacteroides fragilis	N/D	>100	1550.56 Da (3Hex, 2Hexuronic acids, 2 HexNAc, deoxyhexose)	Growth, Mouse intestine colonization	(Fletcher et al., 2009, Fletcher *et al.*, 2011, Posch *et al.*, 2013)
Bacteroides cellulosolvens	N/D	N/D	D-Gal*f*-α-(1-->3)-D-Glc*p*NAc-α-(1-->2)-D-Gal*f*-α-(1-->2)-[D-Gal*f*- β-(1-->3)]-D-Gal	N/D	(Gerwig *et al.*, 1993)
Clostridium thermocellum	N/D	N/D	3-O-Me-D-Glc*p*NAc-α-(1-->2)-[D-Gal*p*-α-(1-->3)]-D-Gal*f*-α-(1-->2)-D-Gal	N/D	(Gerwig et al., 1993)
Francisella tularensis	PglA	>3	144-PO3-HexNAc-HexNAc-Hex- Hex-HexNAc	N/D	(Balonova *et al.*, 2012)
Geobacillus stearothermophilus NRS 2004/3a	N/D	1	2-OMe-α-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→[2)-α-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→]$_{n = 13-18}$ 2)-α-L-Rha*p*-(1→[3)-α-L-Rha*p*]$_{n = 1-2}$-(1→3)-β-D-Gal*p*-(1→	N/D	(Schaffer *et al.*, 2002, Steiner *et al.*, 2006)

Neisseria spp.	PglL	>12	(OAc)Gal-Gal-DATDH and (OAc)Gal-Gal-GATDH	Antigenicity, immunogenicity, activation of complement receptor 3, adherence, pilus assembly and retraction dynamics	(Marceau *et al.*, 1998, Power *et al.*, 2003, Borud *et al.*, 2011, Jennings *et al.*, 2011)
Paenibacillus alvei	WsfB	1	([→3)-β-D-Gal*p*-(1[α-D-Glc*p*-(1→6)]→4)-β-D-Man*p*NAc-(1→])$_{n=\sim23}$ +adaptor -[GroA-2→OPO_2→4-β-D-Man*p*NAc-(1→4)]→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→	N/D	(Altman *et al.*, 1991, Messner *et al.*, 1995, Zarschler *et al.*, 2009)
Pseudomonas aeruginosa 1244	PilO	1 (PilA)	α-5NβOH$C_4$7NFmPse-(2→4)β-Xyl-(1→3)-β-FucNAc-(1→3)-β-	Pilin hydrophobicity, Competitive virulence	(Castric, 1995, Castric *et al.*, 2001)
Pseudomonas aeruginosa Pa5196	TpfW	1 (PilA)	1 to 3 monosaccharides of D-Araf	N/D	(Voisin *et al.*, 2007, Kus *et al.*, 2008)
Tannerella forsythia	N/D	4	4-Me-β-Man*p*NAcCONH(2)-(1→3)-[Pse5Am7Gc-(2→4)-]-β-Man*p*NAcA-(1→4)-[4-Me-α-Gal*p*-(1→2)-]-α-Fuc*p*-(1→4)-[-α-Xyl*p*-(1→3)-]-β-Glc*p*A-(1→3)-[-β-Dig*p*-(1→2)-]-α-Gal*p*	N/D	(Posch *et al.*, 2011, Posch et al., 2013)
Thermoanaerobacter thermohydrosulfuricus strains LIII-6	N/D	1	3-OMe-L-Rha*p*-α1-[4-D-Man*p*-α1-3-L-Rha*p*α1]$_{n\sim27}$,-4-D-Man*p*-α1-3-L-Rha*p*-*α*-1-3-L-Rhap-α1-3-L-Rha*p*-αl3~Gal*pβ1*	N/D	(Bock *et al.*, 1994)
Thermoanaerobacterium thermosaccharolyticum D120-70	N/D	1	-->4)[α-D-Gal*p*-(1-->2)]-α-L-Rha*p*-(1-->3)[beta-D-Glcp-(1-->6)]-β-D-Man*p*-(1-->4)-α-L-Rha*p*-(1-->3)-α-D-Glc*p*-(1-->	N/D	(Schaffer *et al.*, 2000)
Vibrio Cholerae/ Burkholderia thailandensis	PglL	N/D	N/D	N/D	(Gebhart *et al.,* 2012)

1.2.2.1 *Pseudomonas aeruginosa*

A key virulence factor of *Pseudomonas* spp. is the polar type IV pili required for bacterial-host interactions, biofilms, motility, and adherence to eukaryotic cells (Hahn, 1997, Bradley, 1980, O'Toole & Kolter, 1998, Farinha *et al.*, 1994). Early work suggested the Type IV pili and LPS structures to be immunologically similar due to cross reactivity with monoclonal antibodies (Sadoff et al., 1985), but no pilin glycosylation was observed in *P. aeruginosa* PAK and PAO strains (Frost & Paranchych, 1977, Paranchych *et al.*, 1979). Genetic analysis of the *pilA* locus in several *P. aeruginosa* genomes revealed heterogeneity, resulting in a classification of strains into five distinct phylogenetic groups (Kus et al., 2004). Group II (strains PAK and PAO) possess only *pilA*, Groups III and V have accessory genes involved in pili assembly/disassembly, and Groups I and IV have accessory genes with homology to carbohydrate related genes (Kus et al., 2004, Asikyan *et al.*, 2008). Pioneering work from Castric *et al.* demonstrated PilA is modified with an oligosaccharide, and the gene *pilO*, located adjacent to *pilA*, was required for this modification in *P. aeruginosa* strain 1244 (group I) (Castric, 1995). Further analysis of strain 1244 confirmed PilA was *O*-glycosylated with a trisaccharide identical to a single LPS O-antigen repeat (α-5NßOHC$_4$7NFmPse-(2→4)-ß-Xyl-(1→3)-ß-FucNAc-(1→3)-ß-Ser) (Castric et al., 2001). PilA is glycosylated on S148, located in close proximity to a disulfide loop (DSL), a region previously demonstrated to be important for adhesion (Comer *et al.*, 2002, Lee *et al.*, 1989). While mutation of S148T and deletion of the DSL did not hinder glycosylation, a minimum tail length and localized surface charge were required for glycosylation of strain 1244 PilA (Horzempa *et al.*, 2006a). Due to the similarity to LPS biosynthesis, it was suggested PilA glycosylation by PilO utilized the O-antigen biosynthetic pathway (DiGiandomenico *et al.*, 2002). Two genes *wbpM* and *wbpL* are involved in O-antigen biosynthesis and also required for PilA glycosylation in strain 1244,

suggesting a common glycan pool was utilized for both structures (DiGiandomenico et al., 2002). Additionally, when PilA and PilO were heterologously expressed in different *P. aeruginosa* strains and *E. coli* O157:H7, glycosylation of PilA was observed with one subunit of eight structurally distinct O-antigens (DiGiandomenico et al., 2002). Bioinformatic characterization of PilO revealed similarities to Wzx O-antigen ligases; both localize on the periplasmic face of the cytoplasmic membrane, transfer carbohydrates *en bloc*, utilize undecaprenyl pyrophosphate-linked oligosaccharides, recognize and only require a reducing end moiety, and possess relaxed glycan specificity, although PilO can only transfer single O-antigen subunits (Faridmoayer et al., 2007, Qutyan *et al.*, 2007, Horzempa *et al.*, 2006b). PilO can glycosylate engineered non-pilin proteins, suggesting therapeutic uses for vaccine development may be possible (Qutyan *et al.*, 2010).

While glycosylation of PilA is not required for twitching motility, pili formation, or phage sensitivity in *P. aeruginosa* strain 1244, it significantly decreases the pili hydrophobicity and has a competitive advantage over a glycosylation deficient strain in a murine model (Castric, 1995, Smedley *et al.*, 2005). Interestingly, a significantly higher percentage of group I *P. aeruginosa* isolates have been identified in human cystic fibrosis patients than glycosylation negative groups II and III, but no role has been identified to date (Kus et al., 2004). It has been suggested that the glycan acts as a shield against neutralizing antibodies or proteolytic degradation (Castric et al., 2001, Comer et al., 2002).

P. aeruginosa (group IV) Pa5196 type IV pili, unlike group I, it does not utilize O antigen subunits for PilA glycosylation (Voisin et al., 2007). Group IV strains possess two genes, *tfpX* and *tfpW,* that are required for glycosylation of PilA (Kus et al., 2004). A combination of mass spectrometry and NMR techniques determined PilA to be *O*-

glycosylated with up to 16 subunits of a homo-oligomer of α-1,5-linked D-Ara*f*, similar to the oligosaccharides found in the cell walls of mycobacteria (Kus et al., 2008, Voisin et al., 2007). Whereas pilin from group I strains only had a single glycosylation site, PilA from group IV strains was shown to be glycosylated on Thr64 and Thr66 with trisaccharides, and four Ser residues were each glycosylated with 1-2 monosaccharides (Voisin et al., 2007, Kus et al., 2008). Despite low sequence identity, TfpW resembles PilO based on membrane association and topology, is required for glycosylation of PilA, but PilO is unable to complement a *tfpW* mutant (Kus et al., 2008). Deletion of *tfpW* caused a reduction in twitching motility, and a similar result was observed in site directed mutants of each of the glycosylated amino acids, suggesting that *O*-glycosylation affects pilus assembly or the extension/retraction mechanism (Kus et al., 2008).

1.2.2.2 *Neisseria*

The *Neisseria* genus is composed of several species, but only *N. meningitidis* and *N. gonorrhoeae* are considered to be pathogenic in humans and are the agent of meningococcal disease and the sexually transmitted disease gonorrhoeae respectively (Power & Jennings, 2003). The earliest evidence of *Neisseria* glycosylation was the observation of a reduction in electrophoretic mobility of pilin when treated with periodate or galactosidase, suggesting the glycan was *O*-linked and galactose residues were present (Robertson *et al.*, 1977, Gubish *et al.*, 1982). Definitive evidence of glycosylation was the crystallographic structure of *N. gonorrhoeae* pilin, and demonstrated an *O*-linked disaccharide linked to Ser63 of GlcNAc-α1,3-Gal (Parge *et al.*, 1995). In the same year, characterization of the *N. meningitidis* C311 Type IV pili revealed the pili was *O*-glycosylated with a trisaccharide of Hex_2-DATDH (2,4 diacetylamido-2,4,6 trideoxyhexose) (Stimson *et al.*, 1995). A significant number of studies have demonstrated the *O*-glycan of

Neisseria glycoproteins seem to be highly variable (Stimson et al., 1995, Hegge *et al.*, 2004) (Banerjee *et al.*, 2002). Much of this variation is due to phase variation of glycosyltransferases involved in the process (Johannessen *et al.*, 2012, Viburiene *et al.*, 2013, Borud et al., 2011). The pathway for the biosynthesis of the *O*-glycan has been characterized (Fig 1.2). The initiating glycosyltransferase PglB transfers bacillosamine to the undecaprenyl lipid carrier on the cytoplasmic face of the inner membrane, followed by PglA and PglE transferring the second and third monosaccharide respectively (Power *et al.*, 2000, Jennings *et al.*, 1998, Power et al., 2003, Aas et al. 2007). The undecaprenyl pyrophosphate linked glycan is subsequently translocated to the periplasmic face by the flippase PglF, where the OTase transfers the *O*-glycan to the pilin acceptor.

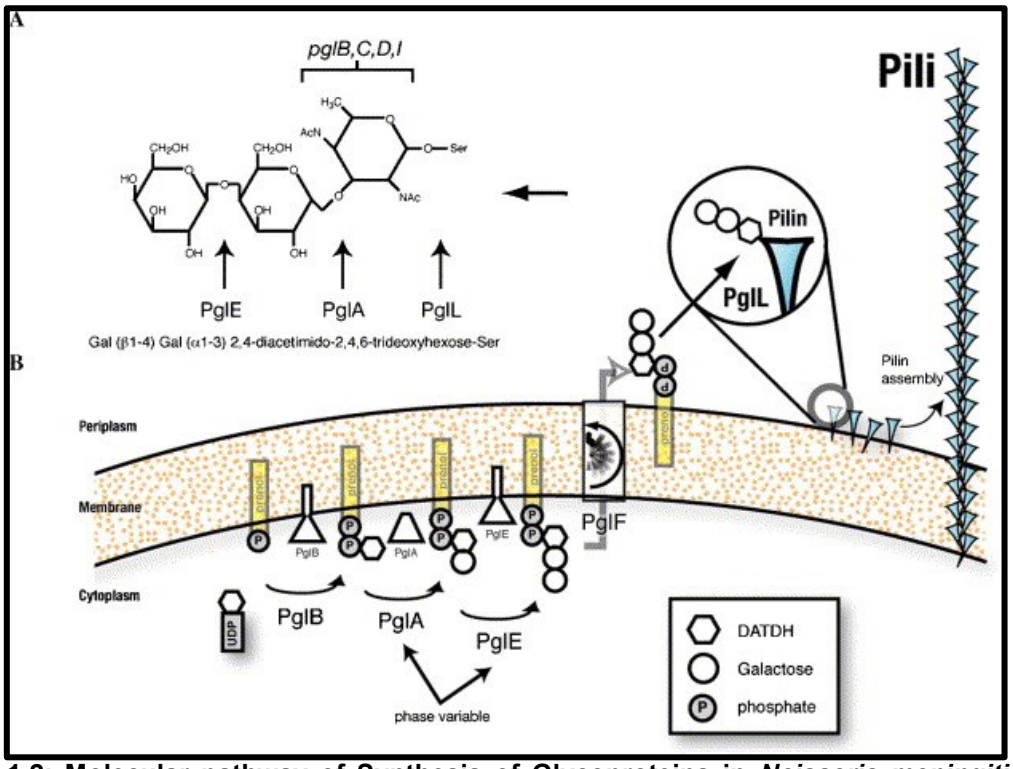

Figure 1.2: Molecular pathway of Synthesis of Glycoproteins in *Neisseria meningitidis*. A) Structure of the characterized *O*-glycan and genes responsible for synthesis. B) Biosynthetic pathway of *O*-glycosylation. PglB initiates glycan synthesis by transfering the reducing monosaccharide to the undecaprenyl lipid carrier, and the next two monosaccharides are added by phase variable GTs PglA and PglE. PglF translocates the completed glycan to the periplasmic face of the inner membrane, where the *O*-OTase PglL transfers the glycan to serine/threonine residues of acceptor proteins. Adapted from (Power et al., 2006)

All *N. meningitidis* and *N. gonnorrhoeae* species appear to carry an *O*-OTase, which in spite of being almost identical, were named PglL and PglO in each of these species respectively. For simplicity, we will call both enzymes PglL. Power and Jennings first demonstrated that mutagenesis of PglL changed the electrophoretic mobility of the pilin (Power et al., 2006). Faridmoayer *et al*. expressed the enzyme in *E. coli* and showed that PglL is an *O*-OTase able to glycosylate pilin in the bacterial periplasm, and suggested PilO and PglL were the first members of the *O*-OTase family (Faridmoayer et al., 2007). It

was later demonstrated PglL glycosylates several proteins, besides the pilins in *Neisseria* (Vik et al., 2009). The variability of the sugars implies that the *O*-OTase must recognize and transfer diverse glycan structures to proteins. When expressed in *E. coli*, PglL did not exhibit sugar specificity and appeared to be able to transfer virtually any glycan structure, including mono-, oligo- and polysaccharides derived from capsule and O-antigen biosynthetic pathways and from other glycosylation systems (Faridmoayer *et al.*, 2008).

Even though multiple proteins have been identified as *O*-glycosylated by PglL, no obvious consensus amino acid sequence for glycosylation has been identified (Vik et al., 2009). However, there are clues including an over-represented tetrapeptide (Ser-Ala-Pro-Ala) in low complexity regions (LCR; homopolymeric runs or short repeats), the acceptor site is confined to interdomain regions, clustering of multiple acceptor sites, and an affinity for tandem repeat elements that might help elucidate the requirement for glycosylation (Vik et al., 2009). Another possible requirement for PglL-dependent *O*-glycosylation may be the acceptor protein must have an association with membranes, as no soluble glycoproteins have been identified to date.

The role of O-glycosylation in *Neisseria* may not be well understood, but recent work has demonstrated it is important for immunogenicity, activation of complement receptor 3, adherence, pilus assembly and retraction dynamics (Marceau et al., 1998, Power et al., 2003, Borud et al., 2011, Jennings et al., 2011) However, due to the high frequency of phase variability in the biosynthetic pathway of the trisaccharide, a role in immune evasion has been hypothesized. (Ku et al., 2009). Overall, the role of *O*-glycosylation in *Neisseria* has not been elucidated, and this is partly because no known

animal model has been identified to examine changes in virulence. Unlike glycosylation in *P. aeruginosa* flagella, no role in pilin subunit stability has been identified.

1.2.2.3 *Bacteroides fragilis*

Bacteroides are Gram negative, obligate anaerobic bacteria that are normally symbionts of the human gastrointestinal flora. These bacteria are able to produce eight different capsular polysaccharides, and can utilize exogenous L-fucose taken from human glycoproteins and incorporate it into their capsules (Krinos *et al.*, 2001, Coyne *et al.*, 2005). Interestingly, during characterization of genetic elements responsible for capsular polysaccharide synthesis by detecting ^{3}H-L-Fucose incorporation, Coyne *et al.* also detected fucose attached to proteins in several different species of *Bacteroides* (Coyne et al., 2005). While both carbohydrates utilize fucose, they are not the same structure (Coyne *et al.*, 2008). Identification of glycoproteins was achieved by lectin enrichment and 2D SDS-PAGE, followed by MS. Eight distinct proteins were identified as glycoproteins, and an acceptor sequon of D-(S/T)-(A/I/L/V/M/T) was proposed (Fletcher et al., 2009). Computational analysis and confirmation by experimentation confirmed this three-residue motif, increasing the number of confirmed glycoproteins to 20, with the potential for 100's of more (Fletcher et al., 2011). Mass spectrometry showed the *B. fragilis* O-glycan consists of 9 monosaccharides, has a mass of 1550.6 Da (Posch et al., 2013). Instead of the normal OTase dependent glycosylation system seen previously, it is proposed that *B. fragilis* synthesizes its *O*-glycan in two stages (Fig 1.3) (Coyne *et al.*, 2013). A core glycan consisting of a reducing end hexose and methylated deoxyhexose are initially attached to the glycosylation site, while the outer glycan is synthesized separately, and attached to the core glycan in the periplasmic space (Coyne et al., 2013). It appears this unique system is highly conserved in the *Bacteroidales* order, with general *O*-glycosylation systems being

identified in *Tannerella forsythensis* (Posch et al., 2011, Posch et al., 2013), demonstration of immunological reactivity towards an antibody against the *B. fragilis* outer glycan, and direct glycosylation of a recombinantly expressed protein expressed in different genera (Coyne et al., 2013). Additionally, *O*-glycosylation is conserved in a the phylum Bacteroidetes, with 18 different species confirmed to glycosylate multiple proteins (Coyne et al., 2013).

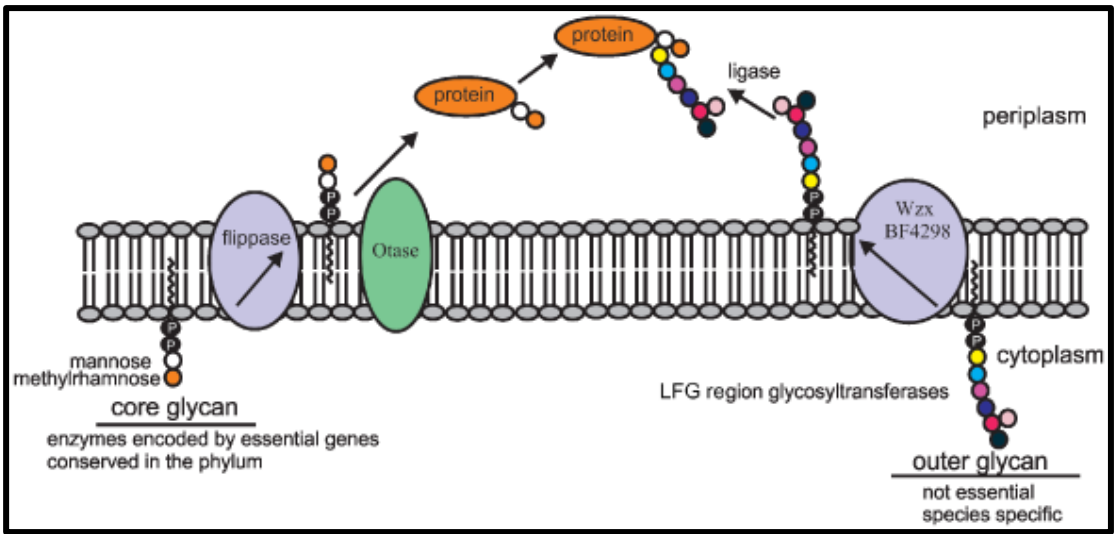

Fig 1.3. Proposed Model for *O*-Glycan Synthesis and Attachment in *B. fragilis*. Two different glycan structures (core and outer glycan) are built separately on Und-P on the cytoplasmic face of the inner membrane, and translocated to the periplasmic face. Next, an unknown *O*-OTase transfers the core glycan to acceptor proteins, and a ligase homologue transfers the outer glycan to the core glycan. Adapted from (Coyne et al., 2013)

Initial reports suggested that while incorporation of the fucose residue was not required for *B. fragilis* colonization of gnotobiotic mice, it was essential for competitive fitness in a mouse model (Coyne et al., 2005, Coyne et al., 2008). However, the mutant utilized for these experiments had a significant growth defect *in vitro*, which could explain the observed phenotype (Fletcher et al., 2009).

1.2.3 Gram Positive Bacteria

OTase-dependent *O*-glycosylation also occurs in Gram-positive bacteria, although the glycosylation machineries in these organisms are less characterized. S-layer proteins are glycosylated in several members of *Bacillaceae* and *Clostridiaceae* family (Messner *et al.*, 2008). Although the mechanism has not been studied, it is likely that the *O*-glycoproteins identified in the cellulosome of *Clostridium thermocellum*are modified by an *O*-OTase (Gerwig et al., 1993). The secreted protein Msp1 from *Lactobacillus rhamnosus* has been shown to be glycosylated (Lebeer *et al.*, 2012). However if this modification employs an *O*-OTase is unknown. PglL homologues can be found in other Gram-positive bacteria, and therefore it should not be a surprise to find OTase-dependent *O*-glycosylation in these organisms.

1.3 *N*-Glycosylation

N-glycosylation is defined as the covalent attachment of a glycan to the amide nitrogen of asparagine residues (Nothaft & Szymanski, 2010). The β-glycosylamine linkage between N-acetylglucosamine (GlcNAc) and the asparagine residue is the most common glycan-protein bond identified (Spiro, 2002). The initial discovery of *N*-glycosylation was observed on ovalbumin (Johansen *et al.*, 1961) and was subsequently found in a vast array of eukaryotic protein structures (Spiro, 2002). It is predicted that over 50% of eukaryotic proteins have an N-linked glycan present (Apweiler et al., 1999).

1.3.1 *N*-Glycosylation in Eukaryotes

The biosynthetic pathway of all Eukaryotic *N*-glycans has similarities to the bacterial system. First, initiation occurs on the cytoplasmic face of the endoplasmic reticulum with the transfer of a GlcNAc-P from the activated sugar nucleotide UDP-GlcNAc

to a lipid-like dolicol-monophosphate (Dol-P), to create DolP-P-GluNAc (Kelleher & Gilmore, 2006). A series of glycosyltransferases transfer seven other saccharides sequentially to the GlcNAc before the glycan is translocated to the luminal face of the ER by a flippase where synthesis and modification of the glycan is completed (Helenius & Aebi, 2004). After completed, the oligosaccharide is transferred onto the asparagine residue by an OTase complex (Helenius & Aebi, 2004). *Saccharomyces cerevisiae* was employed to help characterize the eukaryotic *N*-glycosylation OTase. It is composed of eight subunits, of which the catalytic subunit, Stt3p, is responsible for the transfer of the glycan (Kelleher & Gilmore, 2006). Stt3p is the most highly conserved protein in all eukaryotic OTase complexes, with all sequenced *N*-glycosylating Eukaryotic genomes having at least one homologous Stt3p family member (Kelleher & Gilmore, 2006). Another important discovery in *N*-glycosylation was the identification of a minimum protein motif recognised by the OTase. It was postulated that the consensus sequence in eukaryotes as Asn-X-Ser/Thr (X= any amino acid but proline) over forty years ago (Marshall, 1974), and was proven by various studies (Spiro, 2002). Overall, the general *N*-linked protein glycosylation mechanism is fairly well studied and understood in eukaryotes.

1.3.2 *N*-Glycosylation in Bacteria

Since the initial discovery of *N*-glycosylation in *C. jejuni*, several other bacteria have been identified with *N*-glycoproteins (Nothaft & Szymanski, 2013). Nearly all bacteria in the ε-subdivision of Proteobacteria have a homologue of the OTase PglB, and several have been characterized including other *Campylobacter, Helicobacter,* and *Wolinella* species (Fig 1.4). To date, the best characterized *N*-glycosylation system is the originally identified system in *C. jejuni*.

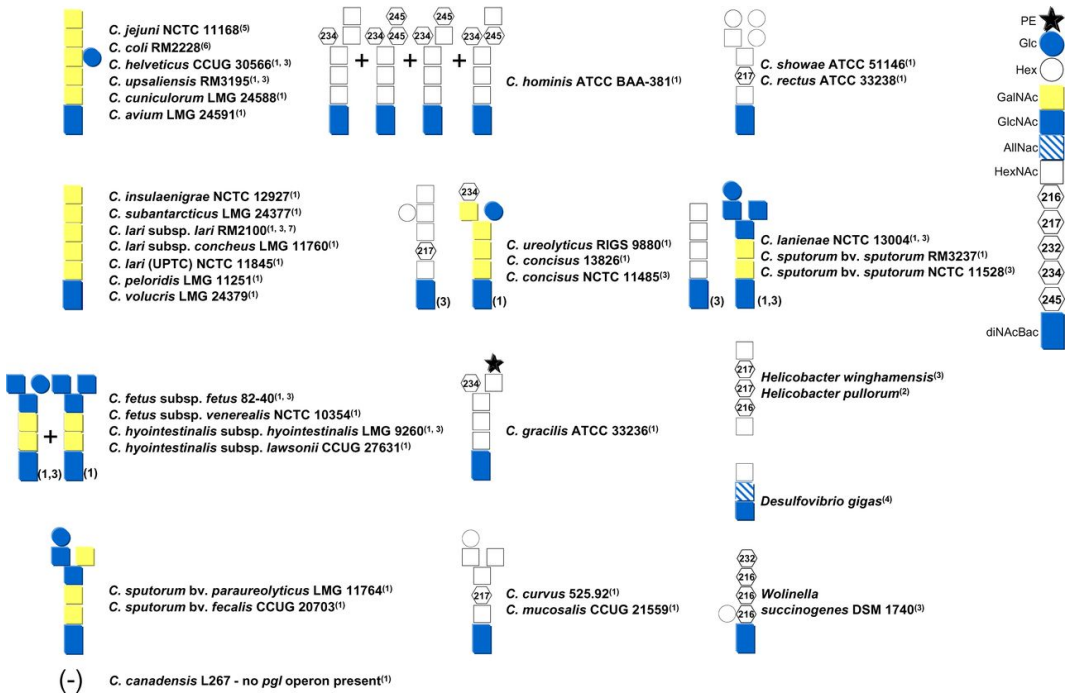

Figure 1.4 Characterized *N*-Glycan Structures from Bacterial Strains Possessing a Homologue of the *C. jejuni N*-OTase PglB. Original manuscripts describing each of the *N*-glycan structures are indicated beside the strain identification number: 1 (Nothaft *et al.*, 2012), 2 (Jervis *et al.*, 2010), 3 (Jervis *et al.*, 2012), 4 (Santos-Silva *et al.*, 2007), 5 (Young *et al.*, 2002), 6 (Szymanski *et al.*, 2003b), 7 (Schwarz *et al.*, 2011b). Modified from (Nothaft & Szymanski, 2013)

1.3.2.1 *Campylobacter jejuni*

Campylobacter jejuni is one of the most common pathogens in humans, as well as the leading causative agent of bacterial gastroenteritis and food-borne illness in North America (Ruiz-Palacios, 2007). The bacterium is a Gram negative, non-spore forming, motile curved rod which is found in water and animal reservoirs (Ruiz-Palacios, 2007). It was originally isolated in 1906 from an aborted sheep fetus, and is commonly a commensal in cattle, birds, and swine (Young *et al.*, 2007). Normally *Campylobacter* infections in humans results in fever, diarrhea, vomiting and abdominal pain, but can also cause more severe symptoms like arthritis, acute appendicitis, and Guillian-Barré syndrome (Young et al., 2007).

An interesting study in 1998 described a genetic locus with similarity to LPS biosynthesis genes from *C. jejuni* 81116 was able to modify host LPS when cloned and expressed in *E. coli* (Fry *et al.*, 1998). Due to these observations, genetic locus was implicated in LPS biosynthesis. However, further analysis revealed these genes were involved in glycosylation of several soluble and membrane associated proteins in *C. jejuni* (Szymanski et al., 1999). This work was the first example of a glycosylation system in bacteria and this locus was named the protein glycosylation locus (*pgl*) (Szymanski et al., 1999).

In the last decade, extensive research has been done to characterize the sugar moiety and enzymes responsible for the *N*-glycosylation system of *C. jejuni* (Fig 1.5). Mutagenesis of the *pgl* locus, a 17 kb region, only effected protein glycosylation, not other carbohydrate biosynthesis pathways (LPS, capsule), demonstrating its specific role in the bacterium (Szymanski et al., 1999, Szymanski *et al.*, 2002). Two glycoproteins, PEB3 and CgpA, were identified and purified using a specific lectin soybean agglutinin, which recognizes GalNAc residues (Linton *et al.*, 2002). MS/MS and NMR allowed characterization of the oligosaccharide determined it to be a heptasaccharide with a structure of GalNAc-α1,4-GalNAc-α1,4-(Glcβ1,3)-GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-Bac-β1- (Young et al., 2002). Glycosylated proteins were identified in the periplasm, suggested a localization of the glycosylation machinery (Young et al., 2002). The current proposed model of the biosynthesis of the glycan and attachment to the protein initiates with UDP-GlcNAc being attached to an inner membrane lipid (Und-PP) by PglC (Fig 1.5B) (Nothaft & Szymanski, 2010). Next, the Und-PP-GlcNAc is converted to Bac by three sequential enzymatic steps beginning with PglF, a dehydratase, followed by PglE, an

aminotransferase, and finally PglD, an acetyltransferase (Nothaft & Szymanski, 2010). Due to genetic homology with a *Neisseria meningitidis* glycosyltransferase that attaches α-1,3-linked galactose to DATDH (diacetamido-trideoxy hexose; a structural homologue of bacillosamine), PglA was hypothesized to transfer α-1,3 linked GalNAc to the Und-PP bacillosamine. The next residue is transferred by PglJ, an α-1,4-GalNAc glycosyltransferase (Glover *et al.*, 2005). PglH, has significant genetic homology with PglJ, is a processive α-1,4-GalNAc glycosyltransferase which adds the next 3 GlcNAc residues (Kelly *et al.*, 2006). PglI completes the structure with the addition of the branched glucose (Kelly et al., 2006). After the biosynthesis has been completed, a flippase (PglK, an ABC transporter) translocates the heptasaccharide to the periplasmic face of the inner membrane (Alaimo *et al.*, 2006), where the OTase, PglB, transfers the completed glycan *en bloc* to an Asparagine residue (Wacker *et al.*, 2002). More recent work has demonstrated at least 16 different *N*-glycan structures in the *Campylobacter* genus, and at least 65 different proteins are modified (Nothaft et al., 2012, Jervis et al., 2012).

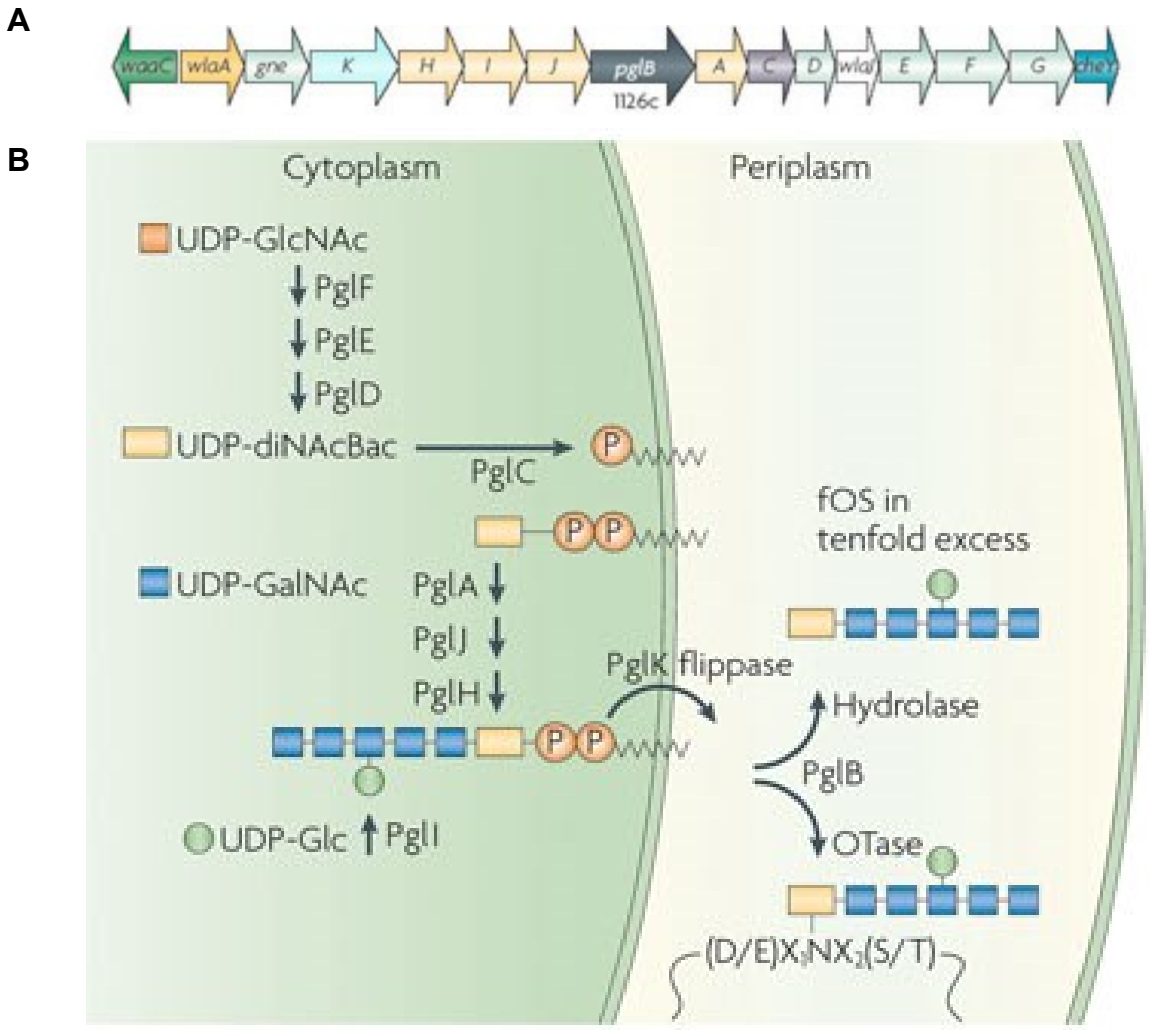

Figure 1.5: Summary of N-Linked Glycosylation in *C. jejuni*. A) The *pgl* locus of *C. jejuni*. Each of the genes in the biosynthetic mechanism of N-linked protein glycosylation are in a single locus, and are categorized based on enzymatic function. Adapted from ((Nothaft & Szymanski, 2010). B) The biosynthetic pathway for the heptasaccharide of *C. jejuni*. Briefly, a series of glycosyltransferases act in a sequential manner to form a completed oligosaccharide on undecaprenyl phosphate, where PglK translocates the carbohydrate to the periplasm. Finally, PglB transfers the glycan *en bloc* to an appropriate acceptor protein (Chen *et al.*, 2007).

1.3.2.1.1 PglB: Oligosaccharyltransferase Responsible for Transfer *en bloc*

The key enzyme in the N-linked glycosylation pathway of *C. jejuni* is the integral membrane OTase PglB. PglB has high homology with the catalytic subunit of the Eukaryotic OTase Stt3p, and contains the conserved catalytic domain WWDXG (Kelleher

& Gilmore, 2006). Unlike the Eukaryotic *N*-glycosylation system, PglB alone is sufficient for the attachment of carbohydrates to the asparagine residue (Feldman et al., 2005). PglB transfers completed sugar structures *en bloc* to protein backbones, similar to the WaaL ligase of the LPS biosynthesis pathway of Gram negative bacteria. Pioneering work by Wacker *et al.* demonstrated that the *N*-glycosylation system of *C. jejuni* could be functionally reconstituted in *E. coli* (Wacker et al., 2002). Feldman *et al.* demonstrated that PglB has a relaxed specificity towards the lipid linked carbohydrate, by showing its ability to transfer a variety of O-antigens to a protein acceptor when expressed in *E. coli* (Fig 1.6) (Feldman et al., 2005). PglB has two specific limitations in regard to the transferable O antigens; an inability to transfer the *E. coli* K30 capsule and *Salmonella enterica* LT2 antigen due to the absence of an acetamido group at the C-2 position of the reducing end monosaccharide (Wacker *et al.*, 2006), and it has extremely low efficiency transferring β-(1,4) linked reducing residues to the protein (Chen et al., 2007). When PglB was purified and tested *in vitro* with lipid-linked glycans and an acceptor protein, glycosylation was detected on folded proteins (Kowarik *et al.*, 2006a). This result was important because it demonstrates PglB mediated glycosylation occurs post translationally and PglB could be used to glycosylate purified proteins *in vitro*. Due to the relaxed glycan specificity, PglB is hypothesized to be a useful tool for the creation of novel therapeutic glycoconjugate proteins such as vaccines (Wacker et al., 2006). Recent work has defined the crystal structure of PglB from *C. lari*, providing further inside into the mechanism of *N*-glycosylation in bacteria (Lizak *et al.*, 2011)

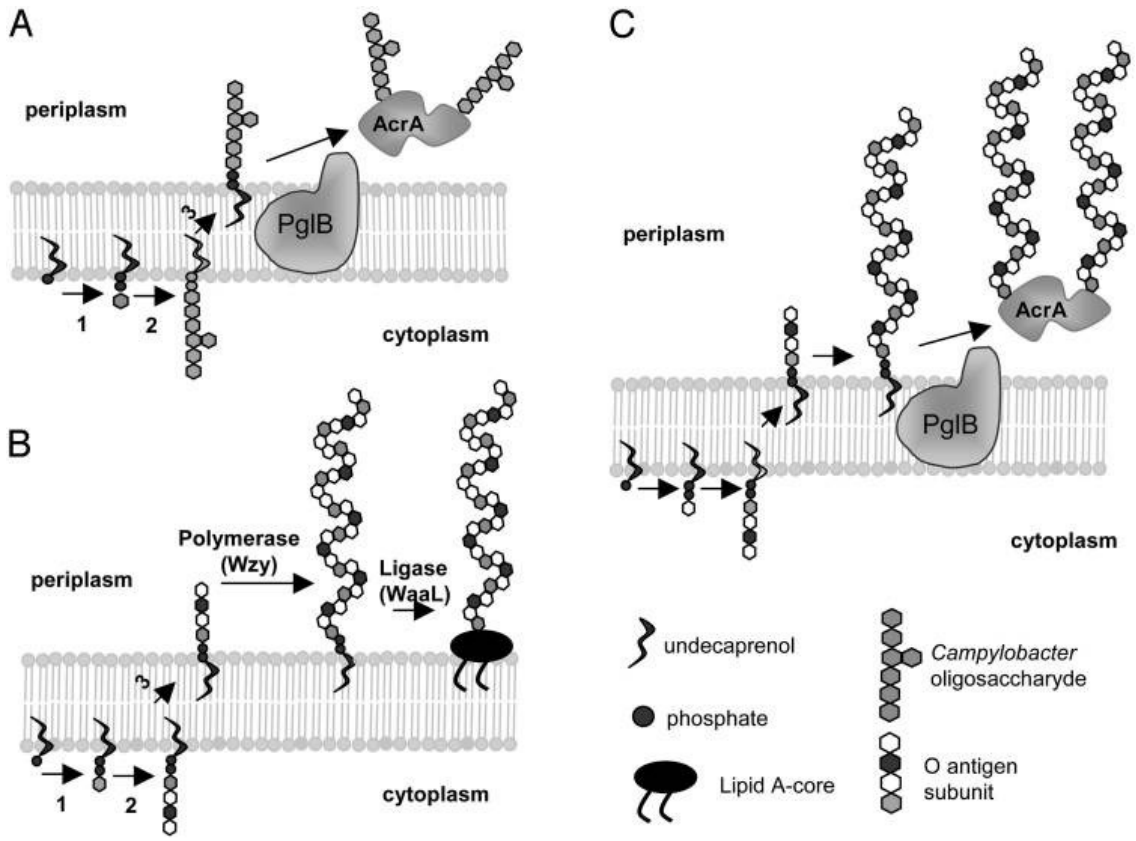

Figure 1.6: **Comparison of *N*-Glycosylation and *wzy*-Dependent LPS Biosynthesis Pathways and Convergence**. A) Schematic representation of N-linked protein glycosylation in *C. jejuni* with the completed glycan transferred from undecaprenyl pyrophosphate to an acceptor protein by PglB. B) Schematic representation of the *wzy*-dependent LPS biosynthetic pathway. Briefly, the O-antigen subunits are synthesized on undecaprenyl pyrophosphate and translocated to the periplasmic face by Wzx flippase. The O-antigen is polymerized by Wzy and Wzz and when completed, transferred to the Lipid A core by WaaL ligase. C) Cross talk between the two pathways leading to *N*-glycosylation of carrier proteins with O-antigens catalyzed by PglB. Adapted from (Feldman et al., 2005)

1.3.2.1.2 Amino Acid Acceptor Sequence for PglB

In Eukaryotic *N*-glycosylation, an acceptor sequence of N-X-S/T was identified (X≠ proline) (Moens & Vanderleyden, 1997). However, in *C. jejuni*, this motif was insufficient for glycosylation suggesting an additional requirement (Kowarik *et al.*, 2006b). Mutational

analysis of a native *C. jejuni* lipoprotein AcrA was used to determine the minimum recognised sequence for glycosylation to occur (Fig 1.7). An additional consensus requirement of a negatively charged amino acid at the -2 location is essential for N-linked glycosylation in *C. jejuni* (D/E-Z-N-X-S/T; Z,X≠P) (Kowarik et al., 2006b).

Figure 1.7: **Identification of the N-Linked Glycosylation Acceptor Sequence for PglB.** Manipulation of the amino acid sequence of AcrA, a glycosylated lipoprotein, revealed an additional requirement of a negatively charged amino acid at the -2 position for N-glycosylation by PglB as compared to the eukaryotic acceptor sequence. Adapted from (Kowarik et al., 2006b)

1.3.2.1.3 Role in Virulence

The *N*-glycosylation system of *C. jejuni* has been concretely linked to the virulence of the bacterium (Zilbauer *et al.*, 2008). Since it is highly conserved, its function must have a significant importance to the bacterium but since the locus post translationally modifies several proteins (>65), the role in virulence is not well understood (Scott *et al.*, 2011b, Young et al., 2007). It has been observed that *pgl* mutations decrease *C. jejuni* ability for adherence and invasion in intestinal epithelial cell lines *in vitro* (Larsen *et al.*, 2004). Also, a decrease in the colonization of *C. jejuni* lacking the *N*-glycosylation machinery in mouse and chick models has been demonstrated (Karlyshev *et al.*, 2004, Kakuda & DiRita, 2006). This data suggests that *N*-glycosylation is important for adherence and colonization of host

tissues, but because of the wide range of glycosylated proteins, no exact role has been identified.

Overall, the *C. jejuni* general N-linked glycosylation system has been well characterized; from initial monosaccharide synthesis, to transfer of the complete heptasaccharide *en bloc* by PglB to an acceptor protein with the consensus sequence D/E-Z-N-X-S/T. Although an absolute understanding of the role in virulence has not been determined, strong evidence suggest that the *pgl* cluster is importance for colonization, invasion, competence, and transformation of *C. jejuni*.

1.3.3 Other OTase Dependent *N*-Glycosylation Systems

Homology searches revealed that almost all bacteria in epsilonproteobacteria possess at least one homologue of $PglB_{Cj}$ (Nothaft & Szymanski, 2013). Unlike *C. jejuni*, where all the genes required for the synthesis of the *N*-glycan are located in a single locus, most sequenced genomes of *Campylobacter* and *Helicobacter* have the genes dispersed throughout the genome (Nothaft & Szymanski, 2013). The only other characterized PglB *N*-glycosylation system is from *H. pullorum*, where one of two $PglB_{Cj}$ homologues is required for glycosylation (Jervis et al., 2010). *In vitro* experiments with $PglB_{Hp}$ demonstrated the same utilization of acceptor sequon, and the *H. pullorum N*-glycan is a linear pentasaccharide *N*-glycan (Fig 1.4) (Jervis et al., 2010). *N*-glycan structures have been determined for *Wolinella succinogenes* and *Helicobacter winghamenis* (Fig 1.4); supporting the hypothesis that PglB-dependent glycosylation is highly prevalent in epsilonproteobacteria (Jervis et al., 2012, Nothaft et al., 2012). Additionally, a $PglB_{Cj}$ homologue was identified in the deltaproteobacterium *Desulfovibrio desulfuricans* (Nothaft & Szymanski, 2010), and an *N*-glycan was observed in the crystal structure of HmcA from

D. gigas (Santos-Silva et al., 2007). Functional expression of $PglB_{Dd}$ in *E. coli* confirmed its OTase activity, with an ability to transfer mono-, oligo-, and polysaccharides to the *C. jejuni* protein AcrA with a recognized sequon of N-X-S/T, similar to the Eukaryotic OTase (Ielmini & Feldman, 2011).

1.3.4 Non-OTase Dependent *N*-Glycosylation in Bacteria

A unique *N*-glycosylation system has been characterized in both *Haemophilus influenzae* and *Actinobacillus pleuropneumoniae.* Instead of the traditional periplasmic glycosylation by an OTase as seen previously, *N*-glycosylation is occurs in an OTase-independent manner cytoplasmically (Grass *et al.*, 2003, Schwarz *et al.*, 2011a). In *H. influenzae*, a high molecular weight adhesin (HMW1A) was shown to be glycosylated cytoplasmically with glucose, galactose, or di-glucose on 31 individual sites with the eukaryotic glycosylation sequon N-X-S/T by HMW1C glycosyltransferase (Gross *et al.*, 2008, Grass *et al.*, 2010, Grass et al., 2003). These findings are unique because of the lack of a N-acetyl group on the reducing hexose, suggesting a glycosyltransferase with novel properties is utilized (Gross et al., 2008). *In vitro* assays confirmed that HMW1C transfers UDP-Gluc/Gal directly to HMW1A (Grass et al., 2010). *N*-Glycosylation of HMW1A is required for protein tethering to HMW1B the *H. influenzae* cell surface and adherence to Chang epithelial cells (Grass et al., 2003, Grass et al., 2010). *In silico* analysis revealed the presence of HMW1C homologues in a number of pathogenic Gram negative bacteria including Enterotoxigenic *Escherichia coli*, *Yersinia pseudotuberculosis, Y. enterocolitica, Y. pestis, H. ducreyi, Actinobacillus pleuropneumoniae, Mannheimia spp., Xanthomonas spp.*, and *Burkholderia spp*, (Choi *et al.*, 2010). The closest homologue of $HMW1C_{Hi}$ was found in *A. pleuropneumoniae,* and recombinant expression and *in vitro* studies of $HMW1C_{Ap}$ confirmed *N*-glycosylation activity analogous to $HMW1C_{Hi}$

of the *H. influenzae* glycoprotein HMW1A (Choi et al., 2010, Schwarz et al., 2011a). Further insights into the enzymatic activity were obtained by analyzing the HMW1CAp crystal structure (Kawai *et al.*, 2011). Interestingly, genomic analysis of *A. pleuropneumoniae* revealed several carbohydrate related genes in close proximity to $HMW1C_{Ap}$ including an ORF adjacent encoding a putative glycosyltransferase (APP7_1697) (Schwarz et al., 2011a). *In vitro* analysis of APP7_1697 revealed it to be able to add up to 6 α-1,6 glucose monosaccharides solely to peptides previously modified with glucose (Schwarz et al., 2011a), confirming it to be a polymerizing α-1,6-glucosyltransferase. The glycosylation system of *A. pleuropneumoniae* was functionally reconstituted in *E. coli*, and several native autotransporters and outer membrane adhesins were *N*-glucosylated at N-X-S/T sites, suggesting relaxed peptide specificity, but a preference for the specific type of protein (Naegeli *et al.*, 2014).

1.4 Lipopolysaccharide

LPS is a defining characteristic of Gram negative bacteria, and as such, has been studied extensively. It is the major molecule of the outer leaflet of the bacterial outer membrane, and is comprised of three domains: a Lipid A anchor, a core oligosaccharide, and a variable length polysaccharide (O-antigen) (Trent *et al.*, 2006, Needham & Trent, 2013). Many bacteria produce large polysaccharides consisting of repeating units attached to the core oligosaccharide, but some, such as *Neisseria* sp, produce a very short LOS (Wang *et al.*, 2010, Schneider *et al.*, 1984). While the Lipid A anchor is fairly conserved between different bacteria, the O antigen is highly variable, and this diversity is thought to be important for a survival advantage for specific niches (Wang et al., 2010). LPS acts as a protective barrier for the bacteria, shielding the cell from bacteriophages, environmental changes, and host immune defenses during infection (Wang et al., 2010, Lerouge &

Vanderleyden, 2002). Additionally, it is highly immunogenic and an important virulence factor, and the variability in structure has been exploited by scientists for identifying and serotyping many Gram negative bacteria (Wang et al., 2010). Some pathogens, such as *Helicobacter pylori* and *Campylobacter jejuni*, mimic human glycan structures with their O antigens to modulate the immune system and avoid immune detection (Bergman *et al.*, 2004, Yuki *et al.*, 1993)

The LPS biosynthetic pathway is now well understood. Genes for O antigen biosynthesis are usually identified chromosomally as a locus, with three main functions: nucleotide glycan synthesis, glycan transferase, and processing (Reeves & Wang, 2002). Three different biosynthetic pathways have been identified, and the main differences are based on the polymerization and transport of the O antigen to the periplasmic space (Fig 1.8) (Raetz & Whitfield, 2002). However, all of the pathways assemble the O antigen subunits on a lipid carrier on the cytoplasmic face of the inner membrane, then translocated to the periplasmic face of the membrane, where the O antigen is ligated to the Lipid A-core structure by WaaL to form mature LPS (Raetz & Whitfield, 2002), and finally actively transported to the outer membrane (Polissi & Sperandeo, 2014). The majority of O antigens appear to be synthesized by the Wzy-dependent pathway, where subunits of the polysaccharide are synthesized first, translocated by Wzx to the periplasmic leaflet and polymerized by Wzy to form the polysaccharide with the final length of carbohydrate being regulated by Wzz (Wang et al., 2010). Most other O antigens are synthesized by the ABC transporter pathway, which differs as the complete polysaccharide being synthesized cytoplasmically, and is subsequently translocated to the periplasm by Wzm and Wzt (ABC transporters) (Wang et al., 2010). The least common system utilizes a single protein called a synthase, which is responsible for both addition and translocation of the

polysaccharide across the membrane (Wang et al., 2010). To date, only *Salmonella enterica* O54 has been identified with a functional synthase pathway for building O antigens (Keenleyside *et al.*, 1994).

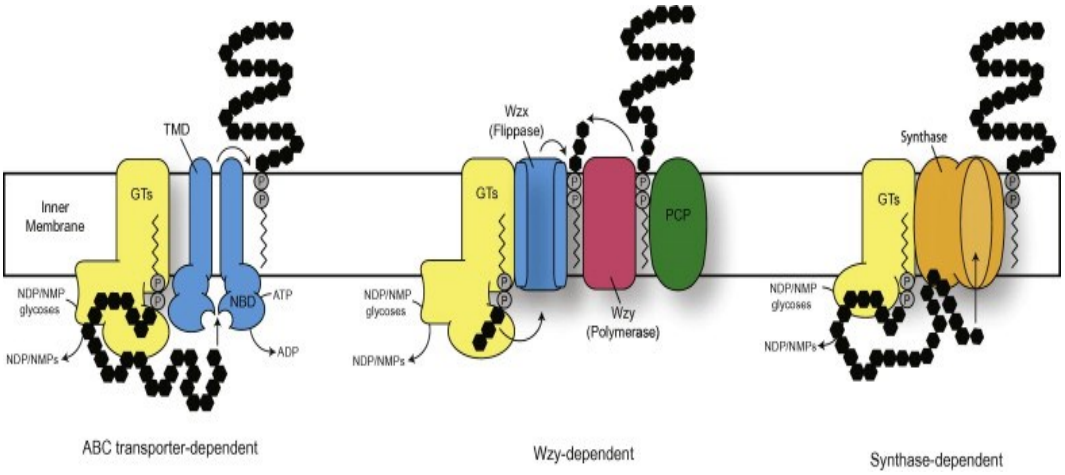

Figure 1.8 Mechanisms of O polysaccharide biosynthesis in bacteria. ABC transporter dependent synthesis polymerizes the complete polysaccharide in the cytoplasm, and then it is translocated to the periplasmic face by an ABC transporter. In Wzy-dependent systems, individual subunits of the polysaccharide are synthesized on the cytoplasmic face, translocated to the periplasmic face by Wzx, where a Wzy polymerase catalyzes the attachment of repeat subunits to build the polysaccharide. Although not well understood, the synthase dependent pathway is hypothesized to utilize a single enzyme to polymerize the polysaccharide on the cytoplasmic membrane and is involved in the export of the glycan. Adapted from (Greenfield & Whitfield, 2012)

1.5 Capsular Polysaccharides

Bacterial capsular polysaccharides (CPS) are comprised of high molecular weight polysaccharides that encase the cell and are attached to the cells surface in both Gram negative and Gram positive bacteria (Yother, 2011, Whitfield, 2006). CPS has been demonstrated to be an important virulence factor for pathogens by protecting the cell against complement-mediated killing, opsonophagocytosis, in addition to environmental survival (Whitfield, 2006, Yother, 2011). Like the bacterial O antigen, there is an immense

variation in structures, with over 80 identified in *E. coli* and 93 in *Steptococcus pneumoniae* alone (Whitfield, 2006, Yother, 2011).

The biosynthetic and assembly pathways for CPS have recently been elucidated. Interestingly, there are significant number of similarities between LPS and CPS biosynthesis (Greenfield & Whitfield, 2012). As seen previously for O antigens, CPS biosynthetic genes normally reside in a chromosomal locus containing the required genes and synthesis of the CPS occurs by similar Wzy, ABC transporter, or synthase dependent pathways in both Gram negative and positive bacteria (Fig 1.8) (Whitfield, 2006).

For Gram negative bacteria, instead of the mature polysaccharide being attached to a Lipid A-core acceptor, the carbohydrate is exported to the cell surface. In the Wzy-dependent mechanism it is hypothesized an additional protein, Wzc, plays a role in the polymerization and chain length in *E. coli* (Cuthbertson *et al.*, 2009, Reid & Whitfield, 2005). Once the CPS is synthesized, an unknown mechanism cleaves it from the lipid carrier, and it is exported through a transmembrane channel of Wzc and Wza (Whitfield, 2006). For ABC transporter-dependent synthesis, the full length CPS is synthesized cytoplasmically, and exported directed to the cell surface via the ABC transporter (KpsM and KpsT) (Whitfield, 2006).

Due to the lack of an outer membrane in Gram positive bacteria, there is no requirement for a transmembrane channel for CPS export. Both Wzy and synthase dependent pathways have been characterized in *Streptococcus pneumoniae* (Yother, 2011). For the Wzy pathway, the initial steps are homologous to the previous systems mentioned, with the glycan subunits being polymerized on the outer leaflet of the inner

membrane, with a Wzc homologue having a role in chain length regulation (Yother, 2011). Similarly to synthase dependent LPS biosynthesis, *S. pneumoniae* can utilize a single protein to synthesize the full length CPS on the cytoplasmic leaflet of the cell membrane, and export the CPS to the cell surface (Yother, 2011).

1.6 Exploitation of Bacterial Glycosylation Systems

Identification and characterization of bacterial glycosylation pathways has stimulated significant interest in the possibility to engineer novel glycoconjugate proteins for therapeutic uses such as vaccination. Three main classes of vaccines are commercially produced. Live attenuated bacteria that have been shown to be highly effective as vaccine candidates, however drawbacks such as case to case overreactions, stability, and manufacturing remain problematic (Galen & Curtiss, 2013). Whole cell killed bacterial vaccines are easy to commercially manufacture, but have been shown to have problems with long term protection. Purified surface carbohydrates have been utilized as a vaccine candidates, but typically only produce short term protection and are not effective in children or mature individuals (Lockhart, 2003). Traditional conjugate vaccines, where bacterial surface polysaccharides are chemically conjugated to a carrier protein, have been demonstrated to be highly effective, best demonstrated by the *Haemophilus influenzae* type b conjugate vaccine, which has nearly eliminated infections in most parts of the world (Pollard *et al.*, 2009, Makela & Kayhty, 2002, V. Verez-Bencomo, 2004). However, synthesis of glycoconjugate vaccines by complex synthetic chemistry has proven to be difficult, laborious, as well as ineffective (Jansen & Snippe, 2004). During the chemical synthesis and attachment, the glycans are prone to modifications at multiple sites and are coupled randomly to proteins in a cluster-like form and are difficult to reproduce (Fig 1.9A) (Wang *et al.*, 2003). Chemical conjugation of the polysaccharide may alter the

natural epitopes of the antigens and results in the conjugates not eliciting the appropriate antibodies to the antigen (Jansen & Snippe, 2004). Additionally, chemical conjugation with larger polysaccharides may cause precipitation, which limits traditional methods to using shorter oligosaccharide glycans (Wang et al., 2003).

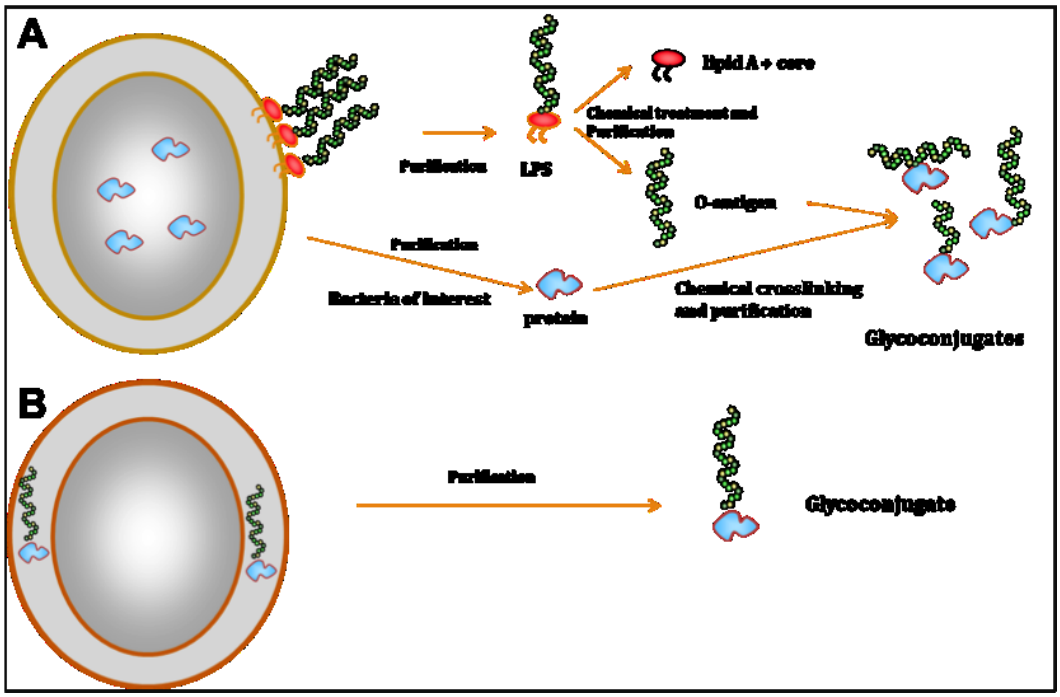

Figure 1.9 Comparison of Methods for Producing Glycoconjugate Vaccines. A) The traditional synthetic methodology for producing glycoconjugate proteins. LPS is purified from the bacterium of interest and the lipid A (and core) are removed from the O-antigen. A suitable protein is also purified from the bacterium, and via harsh chemical crosslinking, the two structures are randomly linked. B) Our proposed novel methodology of producing glycoconjugate proteins. By functionally transferring an OTase, an engineered acceptor protein, and an appropriate O-antigen cluster into *E. coli*, the glycoprotein could be purified with simple, reproducible methodology. This novel method would eliminate the harsh chemicals, which have been shown to damage the native structure of glycans.

Researchers hypothesize a more effective method of producing glycoconjugate vaccines could be the exploitation of bacterial protein glycosylation systems (Fig 1.9B). As previously described, Wzy dependent LPS and capsular biosynthesis is highly homologous to OTase-dependent protein glycosylation biosynthetic pathways (Feldman et al., 2005, Faridmoayer et al., 2007, Whitfield, 2006). To determine if these pathways could be combined and exploited, Feldman *et al.* expressed $PglB_{Cj}$ and AcrA in *E. coli* CLM24 (*waaL*$^-$), a strain hypothesized to increase the pool of lipid-linked glycan substrates, and improve glycosylation efficiency (Feldman et al., 2005). PglB has relaxed glycan specificity, and can transfer a diverse array of oligo and polysaccharides to acceptor proteins via *N*-glycosylation as described in section 1.3.2.2 (Feldman et al., 2005). Due to the prior knowledge of a defined acceptor sequence for PglB glycosylation, this result opened up the possibility to bioengineer novel glycoproteins. Further characterization of the glycan specificity of $PglB_{Cj}$ revealed a specific requirement of a C-2 acetamido group on the reducing sugar as well as an inability to transfer glycans containing a β-(1,4) linkage between the first two reducing end residues limits the usefulness of the *N*-OTase to a small class of O antigens (Chen et al., 2007, Wacker et al., 2006).

The identification of *O*-glycosylation systems in both *P. aeruginosa* and *Neisseria* sp. led to the notion PilO and PglL could also be exploited in a similar manner. Both *O*-OTases were functionally expressed in *E. coli* with their respective pili genes (*pilA* and *pilE*) (Faridmoayer et al., 2007). Both enzymes were able to transfer a diverse array of undecaprenol pyrophosphate linked glycans to their pilin, although $PglL_{Nm}$ was able to transfer both oligo and polysaccharides similar to $PglB_{Cj}$, while PilO was only able to transfer oligosaccharides, up to 2 O-antigen subunits (Faridmoayer et al., 2007). Further study into the glycan specificity of $PglL_{Nm}$ showed relaxed specificity toward the lipid

moiety of the glycan donor and additionally can transfer nucleotide activated monosaccharides, which suggests a relaxed specificity towards the glycan donor (Musumeci *et al.*, 2013a, Faridmoayer et al., 2008). It has also been demonstrated that $PglL_{Nm}$ can glycosylate protein *in vitro,* allowing further characterization of its enzymatic properties (Musumeci et al., 2013a, Musumeci *et al.*, 2013b, Musumeci *et al.*, 2014). These characteristics could be exploited for developing the next generation of glycoconjugate vaccines based on bacterial exposed glycans, such as *Streptococcus pneumoniae* capsule, since there are no limitations on reducing end residues (unlike $PglB_{Cj}$). Currently, the major limitation of $PglL_{Nm}$ for use in commercial applications is a lack of defined consensus sequence and soluble protein acceptor (Vik et al., 2009).

Recently, additional OTases have been discovered and partially characterized (Table 1.4). A homologue of the $PglB_{Cj}$ was identified in *Desulfovibrio desulfuricans* and functionally expressed in *E. coli* (Ielmini & Feldman, 2011). $PglB_{Dd}$ was able to glycosylate AcrA with mono-, oligo-, and polysaccharides as previously seen with other OTases, but was highly inefficient with larger carbohydrate structures (Ielmini & Feldman, 2011). Interestingly, while $PglB_{Cj}$ required a negatively charged amino acid in the -2 position of its consensus sequon (D/E-X-N-Y-S/T), $PglB_{Dd}$ did not. This suggests that *N*-OTases could be engineered to recognise different consensus sequences. Two additional *O*-OTases have also been recently identified and characterized from *Vibrio cholerae* and *Burkholderia thailandensis* (Gebhart et al., 2012). Both $PglL_{Vc}$ and $PglL_{Bt}$ were functionally expressed in *E. coli*, with similar relaxed glycan specificity to the previously characterized $PglL_{Nm}$. An interesting observation was that it appears that different *O*-OTases have protein acceptor and glycan structure combination specificities, which has a significant relevance for glycoengineering applications (Gebhart et al., 2012).

Table 1.3 Summary of the Attributes of Characterized OTases in Bacteria

Host Species	OTase	Linkage	Acceptor Sequence	Known Glyco-Proteins	Reducing Residue Specificity	Limitations
C. jejuni	PglB	N	(D/E)-Y-N-X-(S/T)	>65, mainly periplasmic	-Acetamido group on C-2 -low efficiency for β(1,4) linked reducing sugars	Only Acetamido substituted residues transferred
Desulfovibrio desulfuricans	PglB	N	N-X-S/T	AcrA (*C. jejuni)*	Unknown	Unknown, very inefficient for polysaccharides
P. aeruginosa	PilO	O	Unknown-hydrophobic patches?	PilA	Relaxed Specificity	Only transfer oligosaccharides (up to 2 O-antigen subunits)
Neisseria sp.	PglL	O	Unknown-hydrophobic patches?	>15, PilE, AniA, and mainly periplasmic or membrane associated	Very Relaxed specificity	-No identified acceptor sequence -Possibly only can glycosylated membrane associated proteins
Vibrio cholerae	PglL	O	Unknown	$PilE_{Nm}$, $DsbA_{Nm}$	Very Relaxed Specificity	-No identified acceptor sequence - Only can DsbA
B. thailandensis	PglL	O	Unknown	$PilE_{Nm}$, $DsbA_{Nm}$	Very Relaxed Specificity	-No identified acceptor sequence -Possibly only can glycosylated membrane associated proteins

To further demonstrate the usefulness of bacterial glycosylation systems for developing novel glycoproteins, it was showed it is possible to combine glycosyltransferases from different organisms to synthesize a novel glycan (Schwarz *et al.*, 2010, Hug *et al.*, 2011). By being able to isolate enzymes with specific activities and express in a recombinant manner, almost any glycan structure could be synthesized by bacteria instead of the traditional method of complex synthetic chemistry. This further demonstrates the usefulness and value of bacterial glycosylation systems for the production of glycoconjugate proteins that could be useful as therapeutics such as vaccines.

Overall, to date the most useful OTase is $PglB_{Cj}$ for the production of therapeutic glycoconjugates. All characterized *O*-OTases can only recognise integral membrane or membrane associated acceptor proteins, and the cost and labour intensive work to purify membrane proteins is inhibitory for commercial applications. No acceptor sequon has been identified for *O*-OTases, limiting the ability to engineer acceptor proteins. The exception to this is the acceptor sequon of *B. fragilis*, but no OTase has been identified. However, the limitation of $PglB_{Cj}$ is the reducing monosaccharide requirement of a C-2 N-acetyl group, and the inability to transfer β-(1,4) linked reducing sugars.

1.7 Thesis Objectives

This thesis has two main topics, focusing on the discovery and characterization of an *O*-glycosylation system in a pathogenic bacterium and exploitation of bacterial glycosylation systems for the development of vaccines and diagnostics.

1.7.1 Discovery and Characterization of *O*-Glycosylation in *A. baumannii*

To date, only a few *O*-OTase dependent general glycosylation systems have been identified and characterized in bacteria including *Neisseria* sp. and *Bacteroides fragilis* (Vik et al., 2009, Fletcher et al., 2009). Preliminary evidence that *A. baumannii* might possess an *O*-glycosylation system was determined by *in silico* searches revealing homology of the *Neisseria meningitidis* PglL to a hypothetical protein A1S_3176.

The first objective of this thesis was to determine if *A. baumannii* could produce glycoproteins dependent on A1S_3176, and if present, to characterize the glycoproteome. If A1S_3176 is an *O*-OTase, another objective was to characterize its glycan specificity to see if the enzyme would be a potential tool for glycoengineering. Additionally, since *A. baumannii* is an emerging pathogen and difficult to eliminate from hospital settings (Hanlon, 2005), the final objective was to characterize if *O*-glycosylation was important for environmental survival and pathogenic phenotypes.

1.7.2 Synthesis of a Glycoconjugate Vaccine Against *Brucella*

With the knowledge of the relaxed glycan specificity of OTases, it has been hypothesized that bacterial cells could synthesize designer glycoconjugates *in vivo*. Reconstitution of the *C. jejuni N*-glycosylation system in *E. coli* demonstrated O-antigens of bacteria of interest could be transferred to a soluble acceptor protein (Feldman et al., 2005).

Three *Brucella* species, *B. abortus*, *B. melitensis,* and *B. suis* are the common species that cause human brucellosis. Brucellosis is the most common bacterial zoonosis with over half a million new cases annually and high levels of abortions in cattle in

developing countries (Franco *et al.*, 2007, Seleem *et al.*). In addition, *Brucella* sp. are considered highly effective biological weapons (Pappas *et al.*, 2006), and there is no currently available vaccine for humans.

The second objective of this thesis was to generate a glycoconjugate vaccine against *Brucella* by reconstituting the *N*-glycosylation system of *C. jejuni* in *Yersinia enterocolitica* O:9. The O antigens of *Brucella* sp and *Y. enterocolitica* O:9 have both been determined to be homopolymers of N-formylperosamine, and are immunologically cross reactive (Bundle & Perry, 1985, Bundle *et al.*, 1984). *Brucella* sp are considered class III biosafety hazardous organism, while *Y. enterocolitica* O:9 is a Class II biosafety hazard organism and is easily manipulated and cultured, making it a suitable host for the production of the glycoconjugate protein we hypothesize could cross-protect against brucellosis.

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Chapter 2

Identification and Characterization of a General *O*-Glycosylation System in *Acinetobacter baumannii*

Portions of this chapter have been published.

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Lees-Miller R.G., Iwashkiw J.A., Scott N.E., Seper A., Vinogradov E., Schild S., Feldman M.F. 2013. **A common pathway for *O*-linked protein glycosylation and synthesis of capsule in *Acinetobacter baumannii***. Mol Micro 89(5) 816-830

2.1 Introduction

Acinetobacter baumannii is a strictly aerobic Gram negative, non-fermentative, opportunistic pathogen. Since the 1970's, this organism has frequently been isolated from healthcare facilities, but was easily controlled with antibiotics (Towner, 2009, Peleg *et al.*, 2008). However, many clinical isolates of *A. baumannii* have recently emerged with extreme resistance to antibiotics, disinfectants, and desiccation, which has permitted *A. baumannii* to disseminate throughout healthcare facilities worldwide (Rello & Diaz, 2003, Hanlon, 2005, Smith *et al.*, 2007, Fournier & Richet, 2006, Giamarellou *et al.*, 2008). Additionally, *A. baumannii* infections have emerged in long-term care facilities, in the community, and in wounded military personnel (Anstey *et al.*, 2002, Sebeny *et al.*, 2008, Sengstock *et al.*, 2010). One recent study showed that from 2001 to 2008 the percentage of *A. baumannii* isolates resistant to at least three classes of antibiotics increased from 4% to 55%, and 17% of all isolates were resistant to at least four drug classes (Sengstock et al., 2010). Panresistant strains of *A. baumannii* have also been isolated (Arroyo *et al.*, 2009). *A. baumannii* infections present in an impressing array of forms, including; pneumonia (hospital and community acquired), endocarditis, meningitis, soft tissue and skin infections, and urinary tract infections (Peleg et al., 2008). Because of its importance as an emerging pathogen, attention towards *A. baumannii* has increased considerably. Most of the efforts have focused on antibiotic resistance mechanisms, but little is known about its virulence factors. A significant amount of work has been done to characterize biofilm formation, which seems to play a role in pathogenesis (Choi *et al.*, 2008, Tomaras *et al.*, 2008). The best characterized virulence factor identified to date is an outer membrane protein OmpA (Choi *et al.*, 2005). It has been demonstrated that purified OmpA localized to the mitochondria and was required for apoptosis of human laryngeal epithelial cells (Choi et al., 2005). Other suggested virulence factors for *A. baumannii*

include the capsule, exopolysaccharide, pili and lipopolysaccharide (LPS) (Choi *et al.*, 2009, Gordon & Wareham, 2010, Gonzalez *et al.*, 2001, Tomaras et al., 2008). Undoubtedly, more research is needed in order to understand *A. baumannii* pathogenesis.

Genomic analysis of all sequenced *A. baumannii* strains revealed the presence of homologous genes to those encoding enzymes involved in the *Neisseria meningitidis* protein *O*-glycosylation system. Many different mucosal pathogenic bacteria require protein glycosylation for virulence, and glycoproteins seem to play a role in adhesion, motility, DNA uptake, protein stability, immune evasion, and animal colonization (Nothaft & Szymanski, 2010). Whereas *N*-glycosylation seems to be restricted to epsilon and a few delta proteobacteria, *O*-glycosylation appears to be more widespread among bacteria. Gram negative bacteria including *Neisseria spp.* and *Bacteroides fragilis* employ OTase-dependent *O*-glycosylation as a general system to modify multiple proteins (Vik *et al.*, 2009, Fletcher *et al.*, 2009). OTase-dependent *O*-glycosylation is initiated by a specialized glycosyltransferase that attaches a nucleotide-activated monosaccharide-1P to an undecaprenolphosphate (Und-P) lipid carrier on the inner face of the inner membrane. A series of glycosyltransferases subsequently attach additional monosaccharides to the first sugar residue on Und-PP. When the carbohydrate structure is completed, the Und-PP linked glycan is flipped to the periplasmic face, where an *O*-oligosaccharyltransferase (*O*-OTase) transfers the carbohydrate to selected Ser or Thr residues in acceptor proteins (Alaimo *et al.*, 2006, Faridmoayer *et al.*, 2007). *Campylobacter jejuni* employs a similar *N*-glycosylation pathway to modify about 65 proteins (Scott *et al.*, 2011b).

This work demonstrates the existence of a general *O*-glycosylation system in *A. baumannii* ATCC 17978, which is required for efficient biofilm formation and pathogenesis

in the *Dictyostelium discoideum*, *Galleria mellonella*, murine septicemia, and eukaryotic cell line virulence models. We identified seven glycoproteins carrying a branched pentasaccharide, the structure of which has been characterized by MS and NMR techniques. *O*-glycosylation appears to be ubiquitous in the *Acinetobacter* genus, which suggests that this system might be a possible target for novel antimicrobial treatments.

2.2 Molecular Characterization of an *O*-glycosylation System in *A. baumannii*

2.2.1 Identification of an *O*-OTase Homologue in *A. baumannii* ATCC 17978

Initial screening of the *A. baumannii* ATCC 17978 genome for homologues of known *O*-OTases was performed. Via a BLAST analysis, we identified a homolog to the *N. meningitidis O*-OTase PglL (A1S_3176; E-value 1e-9) that contained a Wzy_C motif (Fig 2.1). This motif is conserved in all *O*-OTases, but is also found in WaaL ligases, which catalyze the transfer of the O antigen to the Lipid A core (Gebhart *et al.*, 2012, Power *et al.*, 2006). To date, only experimental determination allows the definitive assignment of an ORF containing the Wzy_C motif as either an *O*-OTase or a ligase (Hug & Feldman, 2011). No other ORFs contained a Wzy_C motif in the *A. baumannii* ATCC 17978 genome. A1S_3176 is not predicted to be part of an operon (Dehal *et al.*, 2010).

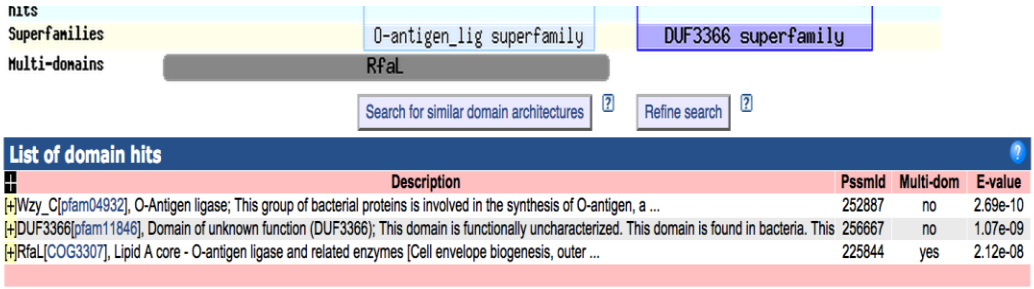

Figure 2.1. Conserved Domains of Hypothetical Protein A1S_3176 of *A. baumannii* ATCC 17978. BLAST search analysis of *A. baumannii* ATCC 17978 with the *N. meningitidis* PglL protein yielded a single protein with a significant match. Analysis of A1S_3176 showed it possesses a Wzy_C domain that is conserved in both *O*-OTases and WaaL ligases. (Altschul *et al.*, 1997)

2.2.2 Construction of an In-Frame Knockout of A1S_3176

Mutagenesis was achieved of the A1S_3176 gene by homologous recombination to evaluate if its encoded protein is an *O*-OTase or a WaaL ligase. Approximately 1000 bps were cloned on both the 3' and 5' flanking regions of A1S_3176 and a gentamycin cassette was inserted between them in pEXT20 cloning plasmid in *E. coli.* This knockout cassette was subcloned into pFLP2 (pMUT), a plasmid that contains a *sacB* gene that is lethal to cells when exposed to sucrose, and does not replicate in *A. baumannii*. Electrocompetent *A. baumannii* cells were transformed with this plasmid and plated on LB supplemented with gentamycin. Colonies were assayed by colony PCR to determine if a single cross over event had occurred (Fig 2.2). When the external primers designed for the construction of the cassette were used (Fig 2.2B), a band was observed with a lower molecular weight than the WT DNA extract (~500 bp's smaller; lane 1-15 vs. lane G) in all of the colonies tested. The band in the candidate colonies was the same size as observed in the lane using the pMUT as the template. Testing the candidate colonies with the same forward primer with an internal primer for the GmR cassette only yielded a band in the colonies and the positive control (pMUT), supporting the hypothesis that a single cross over event had occurred. However, when an internal primer for A1S_3176 was used, a

band was observed in all of the colonies and the WT DNA, suggesting that A1S_3176 was still in the chromosome, further supporting that a single cross over had occurred.

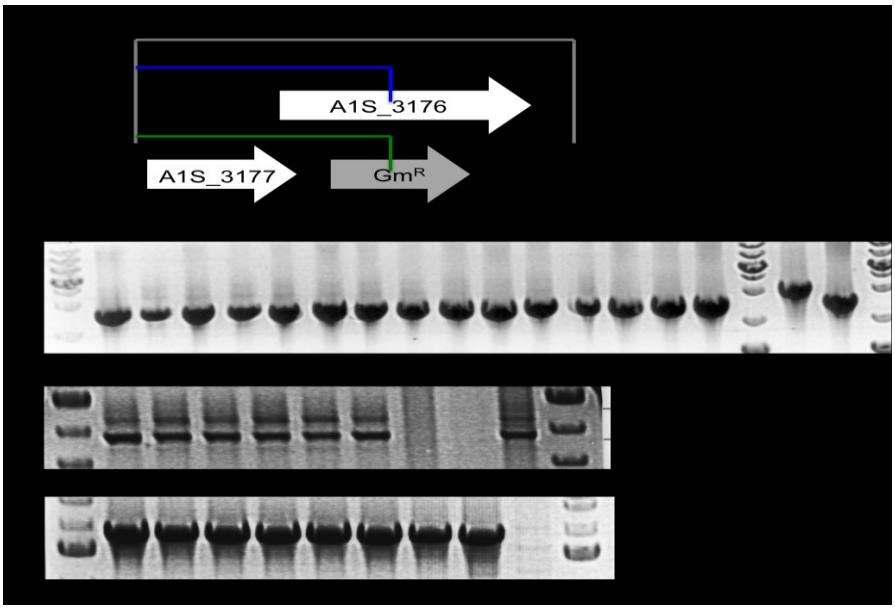

Figure 2.2 Colony PCR Analysis of Individual *A. baumannii* Colonies Transformed with pMUT and Gm^R. A) Schematic representation of knockout construct for the homologous recombination event removing A1S_3176 from the genome of *A. baumannii* B) Candidate colonies produced a band of a lower molecular weight as compared to the WT extract and the same size and the knockout construct. C) Candidate colonies produced a band showing that that the GmR cassette is present in the genome of *A. baumannii*. D) Candidate colonies produce a band demonstrating that A1S_3176 is still present for A1S_3176 being present.

Colonies 2 and 15 were selected for further work to create *A. baumannii* ΔA1S_3176. Both of these colonies were grown for 24 hours in selection free LB broth, and reinnoculated 3 times for a total of 72 hours of growth to allow for the double recombination event to occur. Aliquots of the cultures were plated on LB agar supplemented with gentamycin and 10% sucrose, as a double selection pressure for a double recombination event to occur. The knockout plasmid pMUT contains *sacB* on the backbone, which is lethal to bacteria when grown in sucrose. DNA extracts from the

positive colonies were tested by PCR with primers outside of the cloned region to ensure no false positives could occur and were analyzed on an agarose gel (Fig 2.3). The WT DNA produced a band as expected at about 3.2 kb, whereas all three of the candidate colonies produced a band with a size of ~2.5 kb, consistent with the replacement of A1S_3176 with the gentamycin cassette. No band was observed when the pMUT was used as the template, ensuring that no false positives could exist. We therefore concluded that a double recombination event had occurred, creating the strain $Ab_{\Delta A1S\_3176}$.

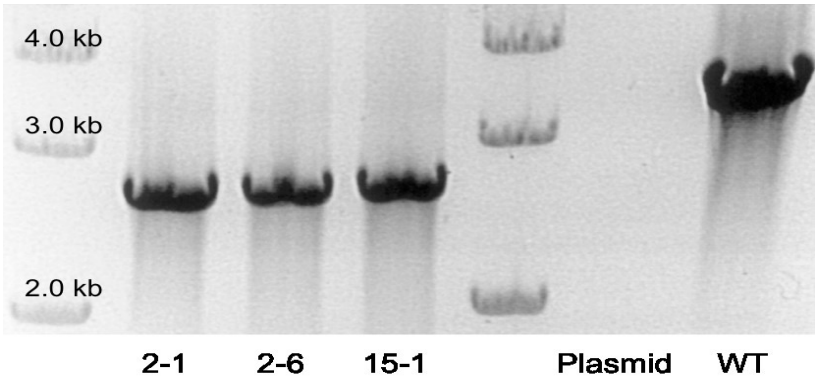

Figure 2.3 PCR Analysis of Genomic Extracts from ΔA1S_3176 Candidate Colonies. PCR was done with primers outside of the cloned construct and products were observed on a 1% agarose gel.

There was no significant difference between the growth curves of the WT and the A1S_3176 mutant strains at 37° C, indicating that growth in these conditions was not affected by the mutation (Fig 2.4).

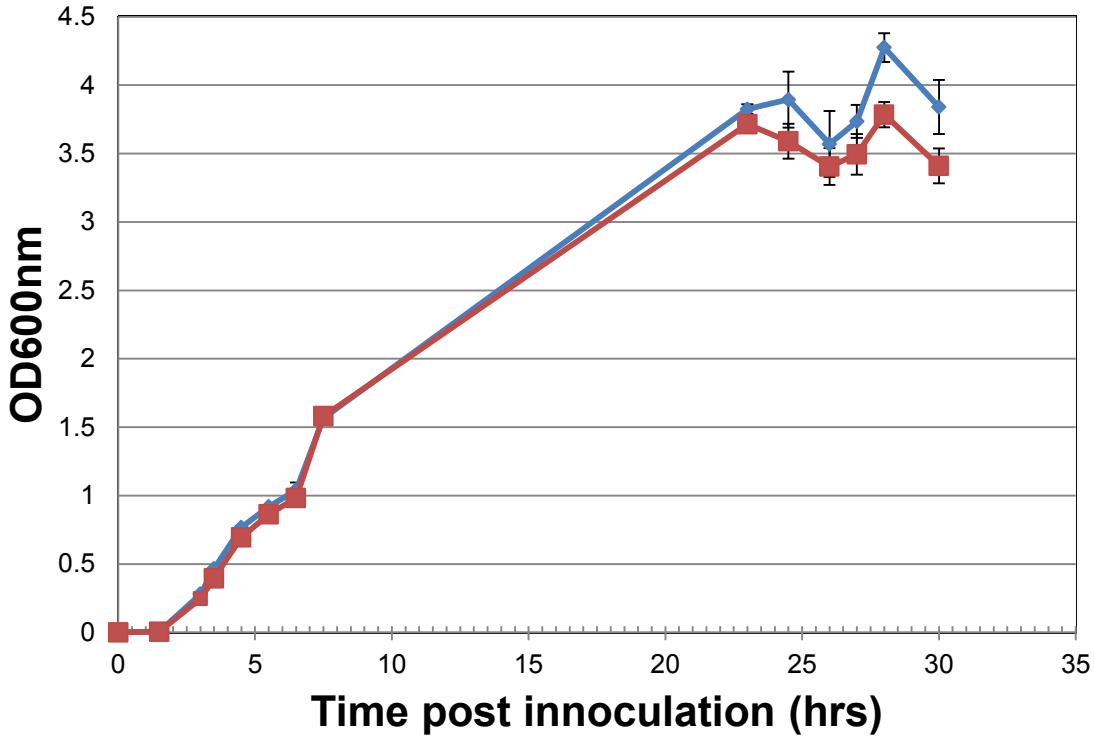

Figure 2.4 Growth Curves of *A. baumannii* WT and ΔA1S_3176 at 37°C in LB. Bacterial growth was observed by optical density in a spectrometer at 600nm wavelength. No significant difference could be observed between the WT (blue diamonds) and the mutant strain (red triangles) when grown without selective pressure.

2.2.3 A1S_3176 ($PglL_{Ab}$) is Required for Glycosylation of Membrane Proteins

Most of the *Neisseria* *O*-glycoproteins identified to date are associated to membranes (Vik et al., 2009). Membrane extracts from wild type and ΔA1S_3176 *A. baumannii* strains were analyzed by SDS-PAGE followed by PAS staining, a technique that is specific for detecting glycans, but presents low sensitivity (Fig 2.5). A broad band migrating from 25 to 35 kDa was visualized in the extract of *A. baumannii* WT. Although the membrane protein profile between the WT and the ΔA1S_3176 strains appeared similar, the band detected via PAS stain was not visible in the mutant strain, suggesting that A1S_3176 is required for glycosylation of at least one protein (Fig 2.5B). The PAS-reactive band disappeared upon treatment with proteinase K, associating the glycan signal

with proteinaceous material. Complementation of A1S_3176 was achieved *in trans*, and analysis of *A. baumannii* ΔA1S_3176-pWH1266-*pglL* membrane extract showed the reappearance of the PAS stained band. Due to the aforementioned similarity between *O*-OTases and ligases, we carried out a conventional LPS extraction and analyzed the extract of the different strains via SDS-PAGE. Silver stain showed no obvious differences in the carbohydrate pattern, suggesting that A1S_3176 is not involved in LPS synthesis (Fig 2.5C). To further determine if A1S_3176 effected LPS biosynthesis, whole cells were digested with proteinase K and analyzed by Silver stain and no differences were observed. However, it has been reported that the O-antigen chains of certain *A. baumannii* strains are not detectable by Silver stain and therefore we cannot conclusively exclude a role of A1S_3176 in LPS synthesis (Pantophlet *et al.*, 1998). Together these results suggest that A1S_3176 is an *O*-OTase responsible for *O*-glycosylation in *A. baumannii* and will be referred from here on as $PglL_{Ab}$, as per its *N. meningitidis* ortholog.

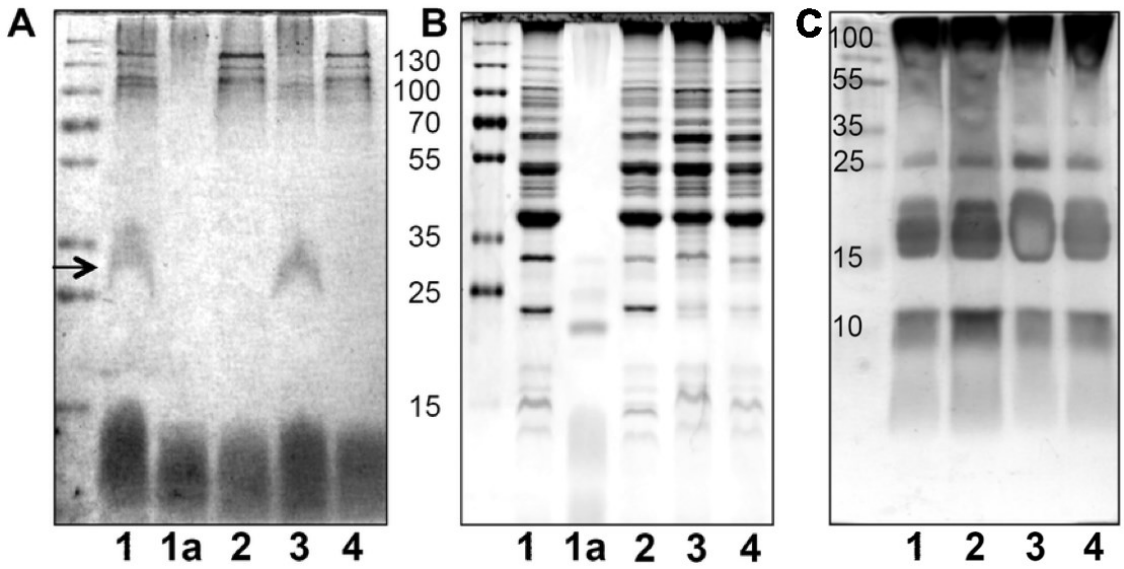

Figure 2.5. *A. baumannii* Requires $PglL_{Ab}$ to Glycosylate Membrane Proteins and is not Involved in LPS Biosynthesis. Ten µg of membrane extract from *A. baumannii* strains (lanes 1,2,3,4) were resolved by SDS-PAGE. A) Carbohydrates were detected by PAS stain. B) Proteins were detected by Coomassie staining. C) Analysis of LPS extraction of *A. baumannii* strains resolved by SDS-PAGE and visualized by Silverstain.Samples were as follows: lane 1 WT; lane 1a Proteinase K treated WT; lane 2 Δ*pglL*, lane 3 Δ*pglL*, pWH1266-*pglL*, 4-Δ*pglL*, pWH1266 control.

2.2.4 Exploratory Lectin Reactivity of WT and Δ*pglL* Membrane Extracts

With no previous knowledge of glycosylation in *A. baumannii*, we sought to identify the modified proteins from *A. baumannii* membrane extracts. Previous work has exploited glycan specific lectins to identify several different glycoproteins in bacteria (Taguchi *et al.*, 2003, Fletcher et al., 2009, Linton *et al.*, 2002). Membrane extracts from both WT and Δ*pglL* strains were separated on SDS-PAGE and subjected to different lectins to detect any differences in reactivity (Fig 2.6). Despite the different substrates of the lectins, we observed similar bands in all tested. In an attempt to explain this result, we tested the streptavidin-IRDye conjugate against the membrane extracts to look for a lack of specificity, and observed the same banding pattern seen with the different lectins. This

result suggests that there was no detection of any glycoproteins by the lectins, and all the observed bands are due to non-specific binding of streptavidin to the membrane extracts.

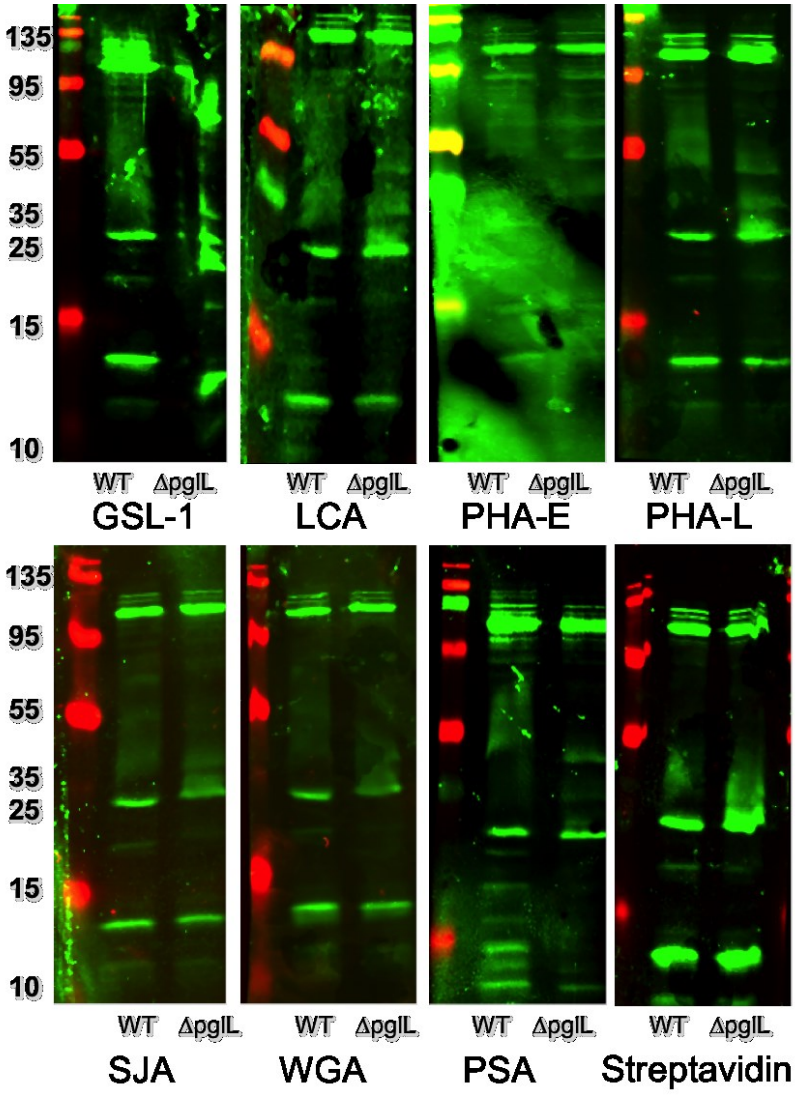

Figure 2.6 Lectin Immunoblot Analysis of *A. baumannii* Membrane Extracts. Samples were separated by SDS-PAGE and blotted with different lectins conjugated to biotin, and visualized with streptavidin conjugated to IRDye for detection. The vast majority of the observed bands are due to a lack of specificity of the streptavidin.

2.2.5 Enrichment of Glycoproteins by Anion Exchange Chromatography

We next attempted to enrich for glycoprotein(s) from solubilized membrane extracts by employing anion exchange chromatography (AEC). Membranes were collected from 1 L of *A. baumannii* culture grown overnight, were solubilized in 1% Triton X-100, and loaded onto a MonoQ column in 20mM Tris pH 8.0 (Fig 2.7). As observed by PAS staining, all of the glycosylated protein bound to the column, and elution by a linear gradient up to 2M NaCl showed that the PAS reactive band eluted off the column at a low (~100mM) concentration in the first peak (Fig 2.7A). Further analysis of this first peak had 10x 1mL fractions containing PAS reactive material, with fractions B5 and B6 having the highest levels (Data not shown). However, due to the high amount of protein in these fractions, mass spectrometric analysis did not yield any positive data to the glycoprotein or glycan structure (Data not shown).

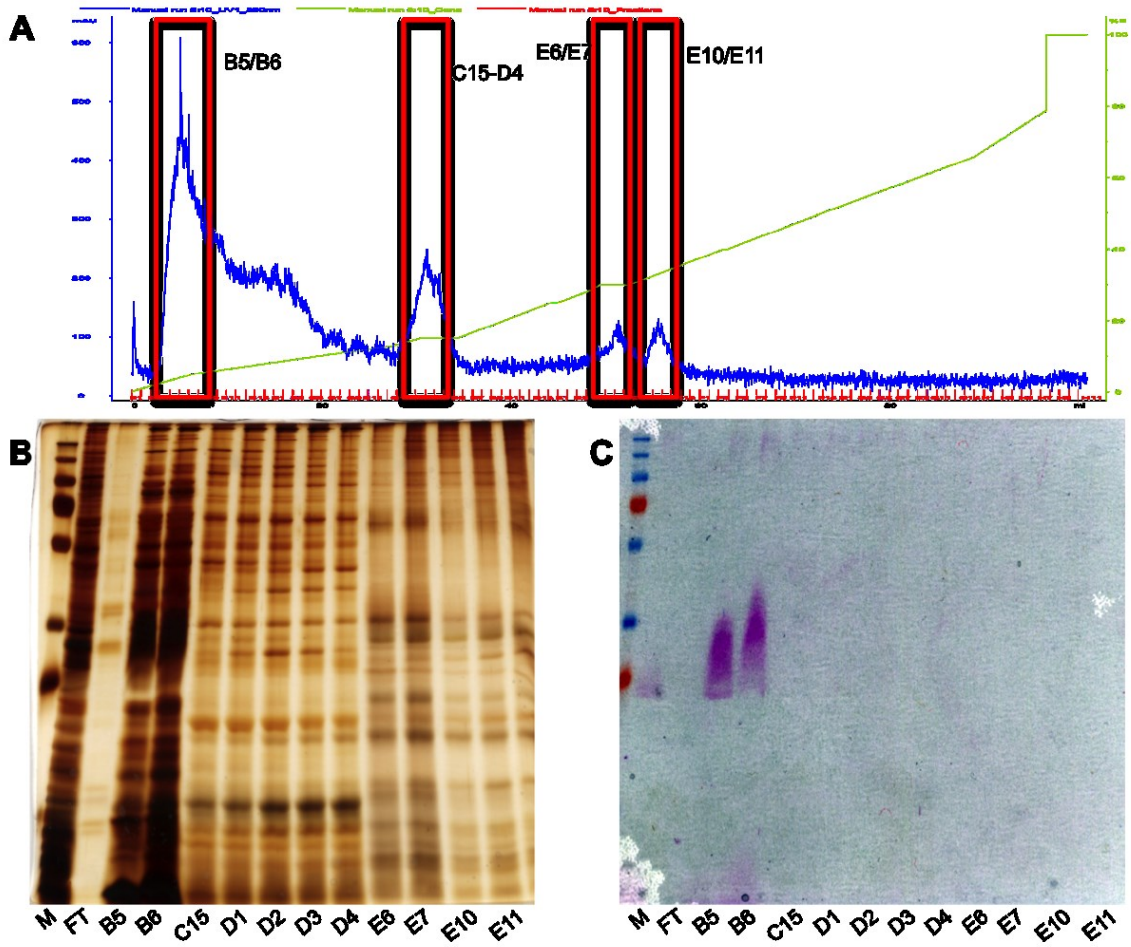

Figure 2.7 AEC of Solublized Membrane Extracts of *A. baumannii* WT. Sample was loaded in 20mM Tris pH 8 +1% Triton X-100 onto a MonoQ column and eluted with a linear gradient to a final concentration of 2M NaCl using the ATKA explorer HPLC. A) Chromatogram of the relative protein quantity eluted in each fraction. B) Protein silverstain of selected 1mL fractions. C) PAS stain of selected 1 mL fractions. Both gels were loaded with 20 µL of sample.

2.2.6 Two Dimensional Gel Electrophoresis of the Enriched PAS Reactive Material and Identification of A1S_3626 as a Glycoprotein

In order to isolate any glycoproteins from the AEC enriched PAS reactive sample, we employed two dimensional gel electrophoresis. The 1 mL fraction of B6 was loaded onto two 7 cm Immobiline DryStrip IEF strip pH 4-7 (GE Healthcare) overnight, subjected

to IEF for a total of 37,000 V h over 8 hours, and the proteins were separated with a 12% acrylamide SDS-PAGE. The gels were either visualized with protein Silverstain or PAS stain (Fig 2.8). We observed a PAS reactive spot (Fig 2.9B), but could not definitively correlate it to an individual spot on the Silverstain. Each of the 4 spots identified on the Silverstain were excised, digested with trypsin, and subjected to LC-ESI Q-TOF MS/MS for protein identification and *de novo* sequencing.

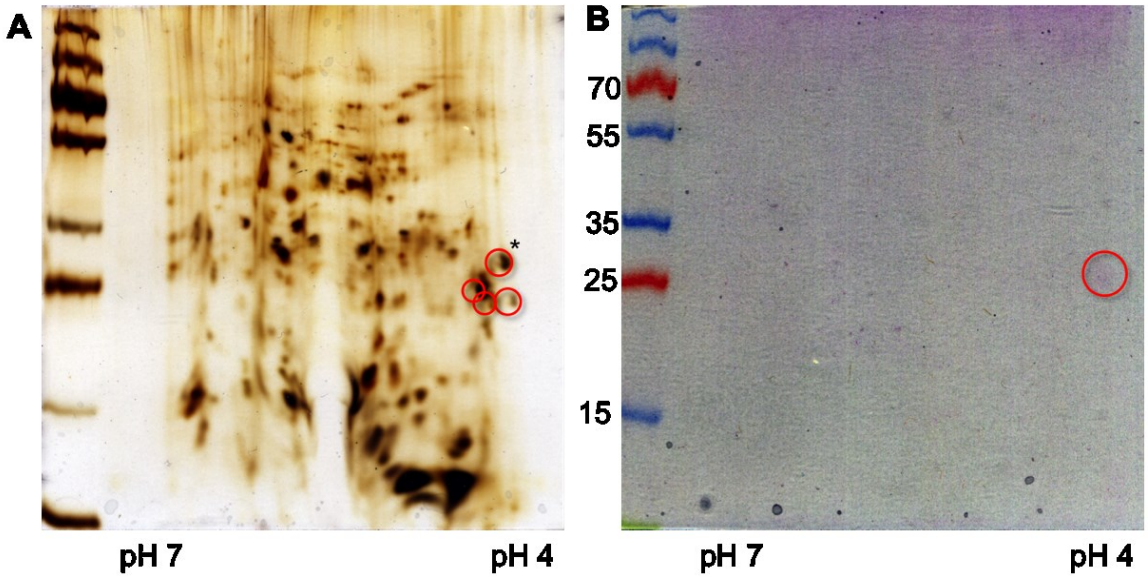

Figure 2.8 2-DE Gels of the Enriched Glycoprotein Fraction of *A. baumannii*. Sample B6 from the previous experiment was separated by 2DE and visualized by either protein silverstain (A) or PAS (B). A PAS reactive spot was observed, and silver stained proteins were excised from the vicinity of the PAS spot.

Analysis of the mass spectrometry data revealed that all 4 samples had uncharacterized membrane proteins. Interestingly, one spot (denoted with the asterisk; Fig 2.8A) was identified as A1S_3626 and has a theoretical molecular weight of 11 kDa, but was observed at ~30 kDa on the gel. Manual analysis of the raw MS/MS data of A1S_3626 found peptide triply charged peptide of 951.81 *m/z* (2895.4 Da) possessing the tryptic peptide **SAGDQAASDIATATDNASAK** (1865.0 Da), and a modification of 1030.4

Da (Fig 2.9). Due to a lack of sample quantity, we were unable to definitively identify the modification structure in the high or low molecular weight regions. However, we did observe a trisaccharide of HexNAc-Hex-Hex in the low molecular weight region, but the full glycan structure remained elusive (Fig 2.9B). With the identification of the peptide and a trimeric glycan, we concluded that *A. baumannii* does synthesize glycoproteins, but were unable to unequivocally identify the glycan structure or linkage to the peptide due to the presence of asparagine, threonine, and serine residues in the peptide.

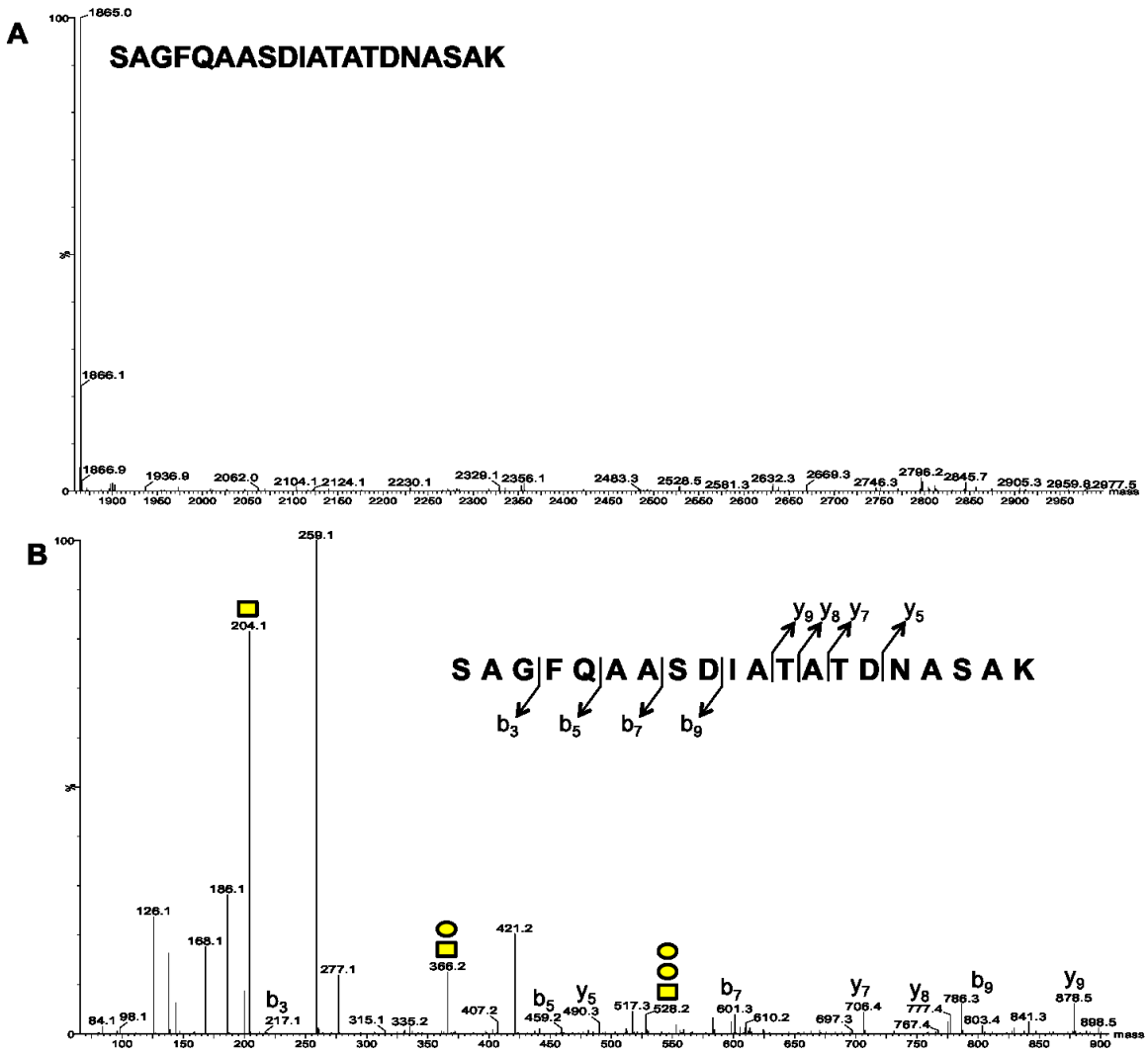

Figure 2.9 Identification of A1S_3626 as a Glycopeptide by LC-ESI Q-TOF MS/MS. Mass spectrometric analysis of a spot on a 2D SDS-PAGE revealed the peptide **SAGFQAASDIATATDNASAK** with a glycan modification. A) Identification of the full peptide mass with an additional 1030.4 Da of uncharacterized modification on the parental peak 951.81^{3+} with a mass of 2895.4 Da. B) Low molecular weight region of the parental peak 951.81^{3+} reveals fragmentation of the peptide in addition to the trisaccharide HexNAc-Hex-Hex

2.2.7 Cloning, Expression, and Purification of A1S_3626 from *A. baumannii*

With the identification of A1S_3626 as a glycoprotein, we next wanted to characterize this protein of unknown function. The gene was cloned into pEXT20 with a deca-histidine tag with the restriction sites BamHI/XbaI (Fig 2.10A). Expression of

A1S_3626 in pEXT20 resulted in aggregation of the protein in inclusion bodies, resulting in high molecular weight smears when observed by Western blot (data not shown). The gene was subsequently subcloned into two separate plasmids, pEC and pBBR-MCS2, which were experimentally shown to replicate in *A. baumannii*. Expression of A1S_3626 was observed by Western blot of whole cell lysates in both plasmids (Fig 2.10B). The theoretical mass of A1S_3626 is predicted to be 11.2 kDa based on amino acid sequence, but we observed expression of the protein at ~25 kDa on a SDS-PAGE gel. This suggests that multimers of the protein are highly stable. Electrocompetent *A. baumannii* WT and Δ*pglL* cells were transformed with pEC-A1S_3626, and the protein was purified from membrane extracts by Ni^{2+}-NTA immobilized metal ion affinity chromatography. Fractions were analyzed by α-His Western blot (Fig 2.10C), and in the elution fractions a single band was observed around 25 kDa in the mutant while a double band was observed for the protein purified from the WT strain. This suggested that the protein was glycosylated. Additionally, several higher molecular weight bands were observed in the WT elution fractions, suggesting that glycosylation may be important for the stability of a self associating structure. To confirm that the higher molecular weight bands were glycosylated forms of A1S_3626, we excised, tryptically digested, and performed MALDI-TOF-TOF MS/MS on the two bands identified with red boxes from Fig 2.10C (2.10D). We identified the same peptide **SAGDQAASDIATATDNASAK** (1865 Da) modified with the same glycan mass of 1030 Da. We were able to positively sequence the glycan due to strong high molecular weight peaks, and two different forms were observed, either HexNAc-Hex-Hex-300-HexNAc or HexNAc-Hex-Hex-HexNAc-300. We were unable to discriminate between the two structures, suggesting either two different glycoforms existed, or a branched glycan was attached to A1S_3626.

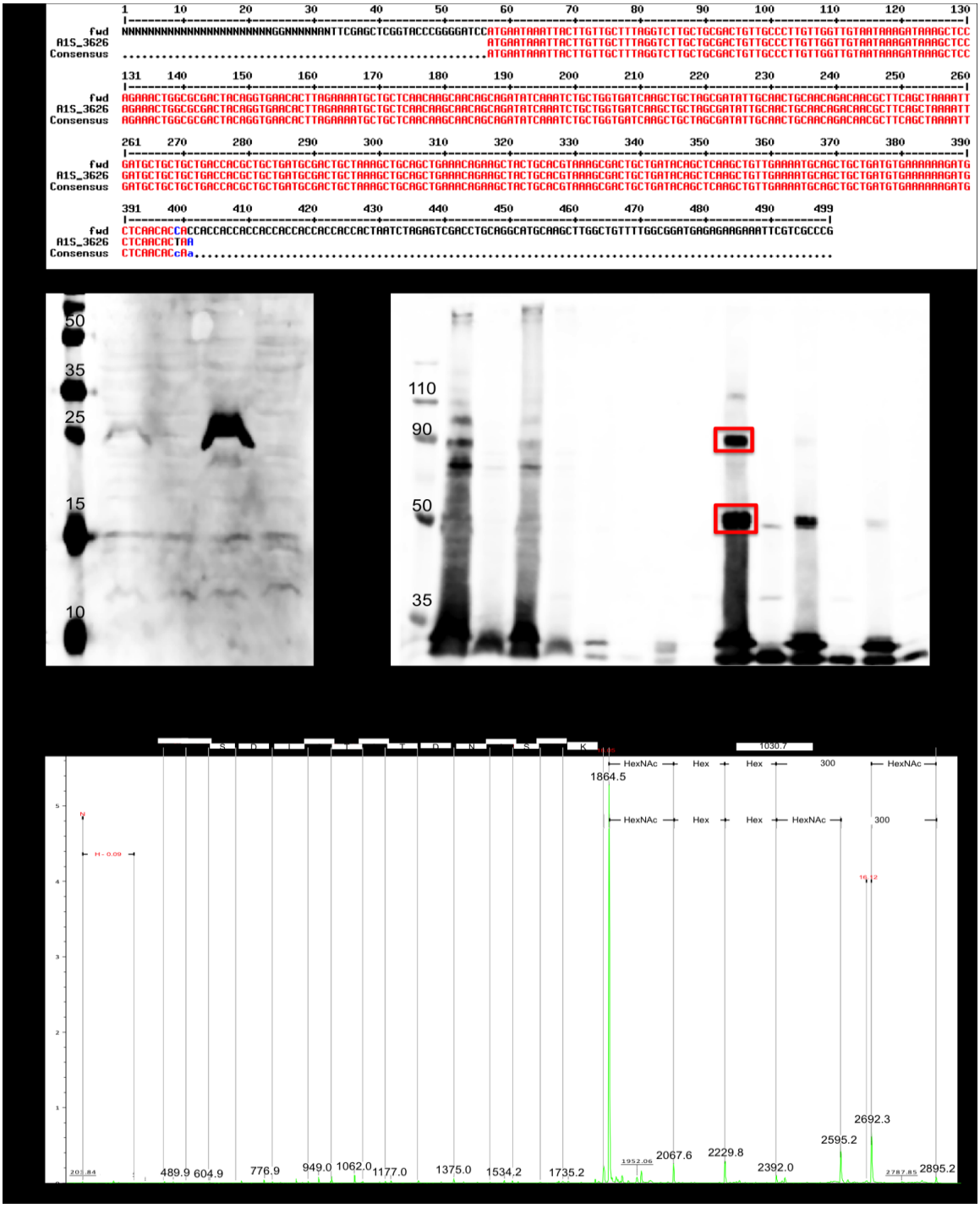

Figure 2.10 Cloning, Expression, and Purification of A1S_3626. A) Sequencing of a positive pEXT20-A1S_3626 colony. Total coverage of the gene was observed demonstrating the correct cloning of the gene. B) Expression of A1S_3626 subcloned into either pEC or pBBR-MCS2 visualized by α-His Western blot. C) Purification of A1S_3626 from either WT or Δ*pglL A. baumannii* strains visualized by α-His Western blot. D) MALDI TOF-TOF MS/MS of the peak 2895.2

m/z from WT purified A1S_3626 shows glycosylation of the peptide **SAGDQAASDIATATDNASAK** with a 1030 Da glycan

2.2.8 Identification of Two Glycoproteins in *A. baumannii* via 2D-DIGE and Preliminary Characterization of the *O*-Glycan by MALDI-TOF/TOF MS and MS/MS Analysis.

To identify additional glycoprotein(s) in *A. baumannii*, we performed two dimensional in-gel electrophoresis (2D-DIGE) experiments (Cabral *et al.*, 2011). Membrane samples of both WT and Δ*pglL* were isolated by ultracentrifugation and the lipidic components were removed as previously described (Pessione *et al.*, 2009). Most of the signals corresponding to the wild type (Fig 2.11A, green) and Δ*pglL* (Fig 2.11B, red) proteins co-localized in the gel (Fig 2.11C, yellow), indicating that these proteins were likely not glycosylated. However, a few proteins exhibited differential electrophoretic behavior (Fig 2.11). The gel was subsequently stained with Coomassie, and these proteins spots were excised, in-gel digested, and analyzed by MALDI-TOF/TOF MS and MS/MS. We identified two separate pairs of proteins, which according to their electrophoretic migration, appeared to be larger and more acidic in the WT strain (WT1 and WT2) than in the Δ*pglL* strain (MT1 and MT2). Mass spectrometric analysis determined WT1 and MT1 samples to be A1S_3626 protein, whereas WT2 and MT2 were identified as A1S_3744 protein. Both, A1S_3626 and A1S_3744 are annotated as hypothetical proteins, and BLAST searches yielded homologues exclusively within the *Acinetobacter* genus.

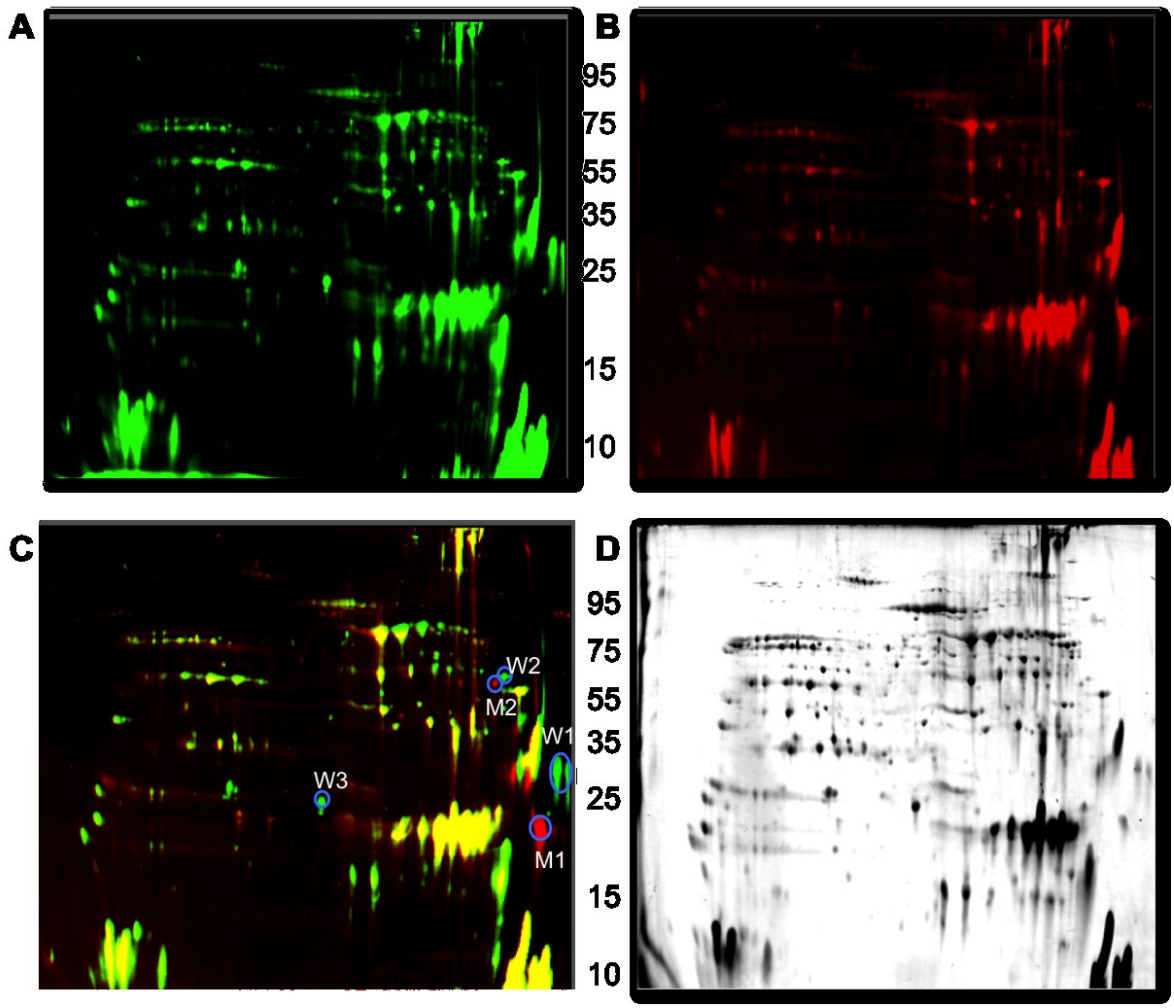

Figure 2.11 Comparison of *A. baumannii* WT and Δ*pglL* Membrane Extracts by 2D-DIGE. Analysis of the membrane proteome of *A. baumannii* WT strain (A), Δ*pglL* strain (B), merge (C), and Coomassie stained (D). Spots WT1 and WT2 only present in the WT strain (green) whereas MT1 and MT2 were only present in the Δ*pglL* strain (red). MALDI-TOF MS analysis identified WT1 and MT1 spots as A1S_3626 protein and WT2 and MT2 spots as A1S_3744 protein.

Analysis of the MALDI-TOF MS spectra of a tryptic digest of WT1 (A1S_3626) revealed a peptide fragment of 2895.24 Da that was absent in MT1 (Fig 2.12A). MALDI-TOF-TOF MS/MS of this ion determined that in the wild-type strain the peptide **SAGDQAASDIATATDNASAK** was linked to the glycan HexNAc-Hex-Hex-(HexNAc)-300, where 300 corresponded to an unknown residue of *m/z* 300, whereas the same peptide

was unmodified in Δ*pglL* sample (Fig 2.13A). Similarly, MALDI-TOF MS analysis of a tryptic digest of WT2 (A1S_3744) revealed a peptide fragment of 3852.69 Da that was absent in MT2 (Fig 2.11C). MALDI-TOF-TOF MS/MS of the 3852.69 Da peak revealed the same pentasaccharide identified on A1S_3626 on the peptide **ETPKEEEQDKVETAVSEPQPQKPAK** (2822.33 Da), whereas the same peptide was unmodified in Δ*pglL* sample (Fig 2.12B). We next purified membranes from *A. baumannii*, digested the sample with Pronase E, and enriched glycosylated peptides using activated charcoal microspin columns. We identified a peak in the MALDI-TOF MS of 1358.4 m/z that was subsequently analyzed by MALDI-TOF/TOF MS/MS (Fig 2.13C). Manual peak annotation identified the previously characterized pentasaccharide attached to a sodiated tripeptide containing the amino acids A, T and D. Overall, these results demonstrate that **PglL$_{Ab}$ glycosylates at least two different proteins with a pentasaccharide** with a preliminary structure of HexNAc-Hex-Hex-(HexNAc)-300.

We observed other spots possibly corresponding to proteins migrating differently in *A. baumannii* WT and Δ*pglL* strains. The most prominent was marked as WT3, and was observed only in the WT extract (Fig 2.11C). Mass spectroscopy analysis determined this spot corresponded to OmpA (A1S_2840). However, manual analysis using MS/MS of WT3 indicated that OmpA was not glycosylated. Western blot analysis of whole cell extracts of the WT and Δ*pglL* strains revealed no difference in OmpA expression levels, which implies that manipulation of membrane samples could account for apparent differences observed in expression levels of proteins detected by 2D-DIGE (Fig 2.14).

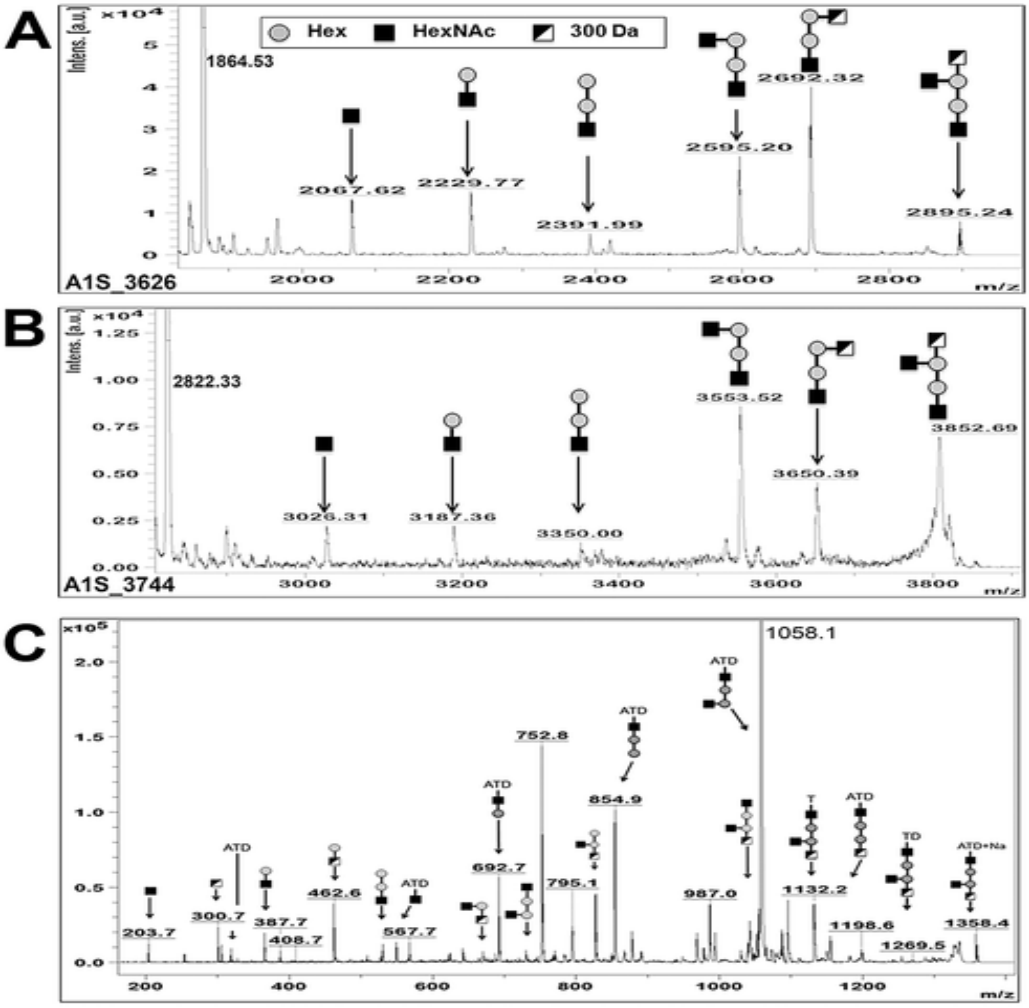

Figure 2.12 MS/MS of A1S_3626 and A1S_3744 Showing Glycosylation in *A. baumannii* with a Pentasaccharide. Spots excised from the 2D DIGE, digested with trypsin, and analyzed by MALDI-TOF-MS. Peaks not corresponding to peptide fragmentation were analyzed for glycosylation. A) MS/MS of the precursor ion peak at *m/z* 2895.165 from A1S_3626 revealed the peptide **SAGDQAASDIATATDNASAK** with a pentasaccharide of HexNAc-Hex-Hex-(HexNAc)-300 attached. B) MS/MS of the precursor ion peak at *m/z* 3852.76 from A1S_3744 revealed the peptide **ETPKEEEQDKVETAVSEPQPQKPAK** with the same pentasaccharide attached. C) MALDI-TOF MS of Pronase E digested membrane proteins showed a precursor ion peak of *m/z* 1358.4 which MS/MS analysis demonstrated to be the previously identified *O*-glycan (HexNAc-Hex-Hex-(HexNAc)-300 attached to the peptide fragment "ATD".

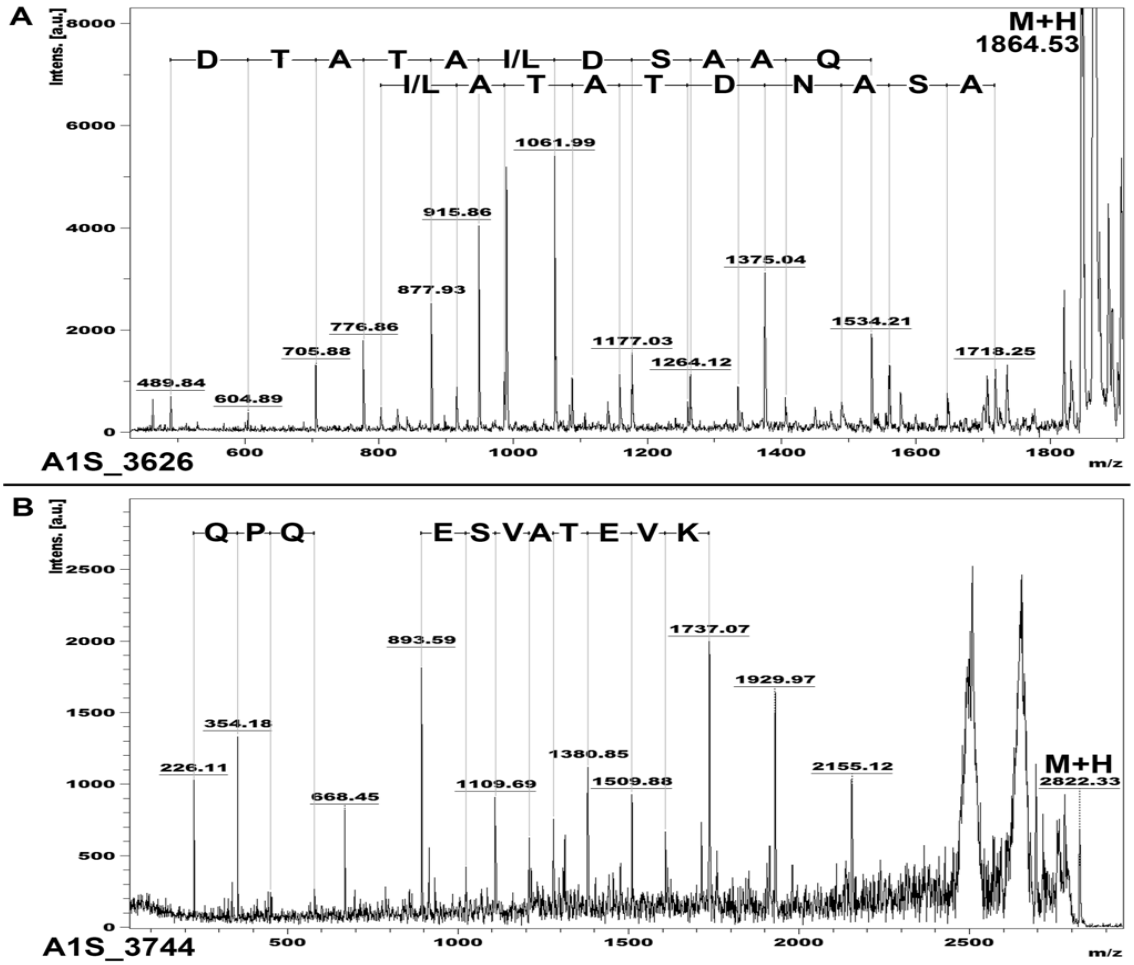

Figure 2.13. **MALDI-TOF/TOF MS/MS Fragmentation Analysis of Glycosylated Peptides in *A. baumannii***. A) Sequencing of the peptide **SAGDQAASDIATATDNASAK** from the parental peak 2895.24 Da demonstrates the peptide matches the expected sequence of A1S_3626. B) Sequencing of the peptide **ETPKEEEQDKVETAVSEPQPQKPAK** from the parental peak 2822.33 Da demonstrates the peptide matches the expected sequence of A1S_3744.

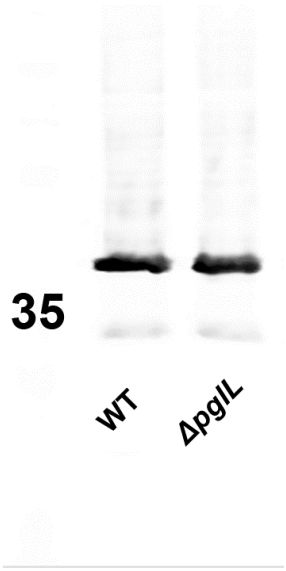

Figure 2.14 Western Immunoblot of OmpA (A1S_2840) in Whole Cell Extracts of *A. baumannii* ATCC 17978 Strains. Each lane was loaded with 0.2 OD_{600} of sample, and probed with OmpA monoclonal antibody with no observable differences between the WT and *ΔpglL*.

2.2.9 Characterization of the *O*-Glycan by Permethylation

We further characterize the *O*-glycan of *A. baumannii* ATCC 17978 by permethylation of the carbohydrate and analysis with mass spectrometry. Carbohydrate permethylation replaces hydrogen element on hydroxyl groups, amine groups, and carboxyl groups with methyl groups, making the glycan hydrophobic (Ruhaak *et al.*, 2010). This has significant advantages for several reasons including enhancing the glycans signal strength in ESI and MALDI MS, providing easier removal of salts and contaminants from the glycan, and allows for elucidate interglycosidic bonds between monosaccharides (Morelle & Michalski, 2007, Ruhaak et al., 2010). We previously demonstrated that Pronase E digested lipid free membrane extracts yield the *A. baumannii O*-glycan attached to small peptides (Fig 2.12C). By exploiting this knowledge, we enriched for more of this sample, and treated it by ß-elimination to remove any amino acids still attached, performed permethylation on the free *O*-glycan, and analyzed the sample by MALDI MS/MS. Manual

analysis of the spectra yielded a peak of 1267.5 *m/z* that was subsequently analyzed by MALDI-TOF-TOF MS/MS (Fig 2.15). We observed the first HexNAc with a mass of 299.7 Da, due to permethylation of a reducing end residue with sodium adduct (245.2+31.0+22.9). The next residue, a hexose, was observed with the predicted mass of 204.1 Da, but we only observed a single methylation event on the second Hex residue, which signifies a branching event. Due to the branching of the *O*-glycan, there are two different options for the next monosaccharide, either a HexNAc, or a monosaccharide with a permethylated mass of 314.3 Da. Only a single methylation event occurred on this residue, since we observed the unmodified mass was 300 Da (Fig 2.12C). This suggests that the monosaccharide is highly substituted, since only one free hydrogen site was modified. Overall, we determined that the *O*-glycan of *A. baumannii* ATCC 17978 is a branched pentasaccharide with a structure of HexNAc-Hex-Hex-(HexNAc)-300 Da.

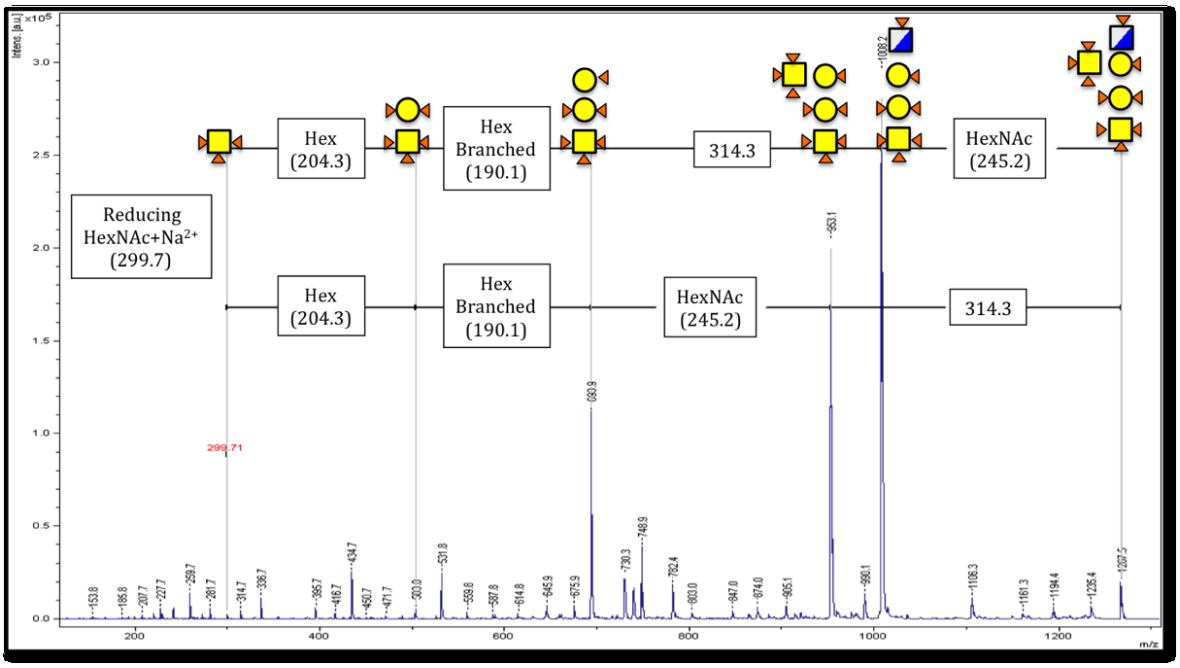

Figure 2.15 Analysis of the *A. baumannii* *O*-glycan by Permethylation. Membrane proteins were purified digested with Pronase E and glycosylated peptides were enriched by activated charcoal. The sample underwent ß-elimination and permethylation, and was analyzed. The MS/MS of the parental peak 1267.5 M/Z revealed the pentasaccharide with the second Hex lacking one permethylation due to second monosaccharide attached to it. Additionally, the unknown 300 Da residue was only had a single permethylation.

2.2.10 Identification of Additional *O*-glycosylated Proteins by Zwitterionic Hydrophilic Interaction Chromatography (ZIC-HILIC) MS/MS

To determine if additional glycoproteins were present in *A. baumannii* ATCC 17978, we employed ZIC-HILIC glycopeptide enrichment. Utilizing membrane extracts previously shown to contain A1S_3626 and A1S_3744 putative glycopeptides were enriched and analyzed using an LTQ-Orbitrap Velos (Mass spectrometry was performed by Dr. Nicholas Scott, UBC). HCD scans containing oxonium ion were manually inspected and searched using MASCOT resulting in the identification of at least 9 different glycosylation sites on 7 different glycoproteins in *A. baumannii* ATCC 17978 (Table 2.1; Fig 2.16). This peptide-centric approach enabled multiple novel glycoproteins to be

identified of which six of the seven proteins are annotated as uncharacterized hypothetical proteins, with the remaining being annotated as MotB (A1S_1193). (Table 1). This demonstrates that $PglL_{Ab}$ is able to glycosylate multiple proteins in *A. baumannii* ATCC 17978.

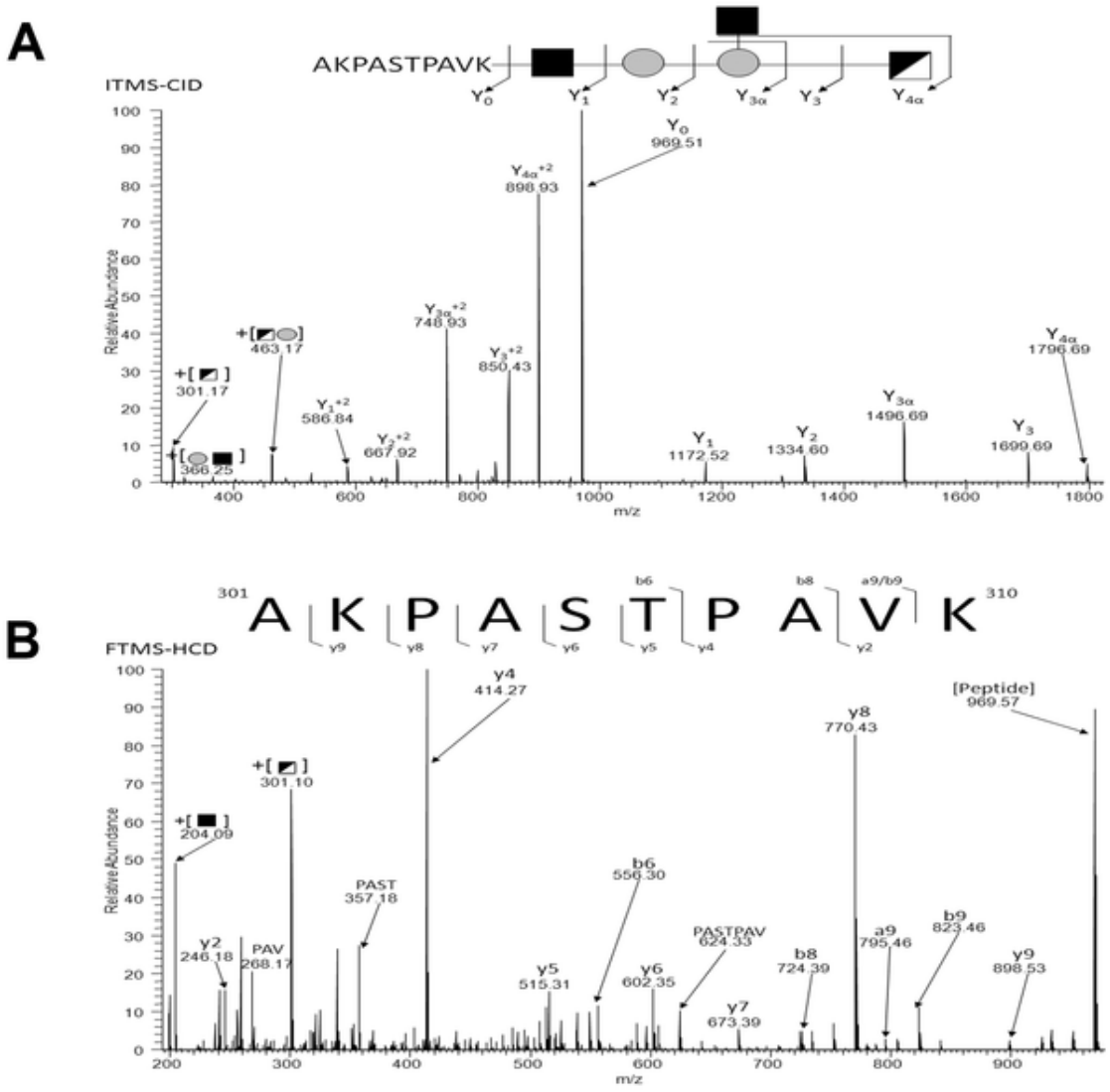

Figure 2.16. Identification of Additional glycoproteins in *A. baumannii* ATCC 17978. Tryptically digested membranes were enriched via ZIC-HILIC and analyzed by LC-MS and HCD MS-MS. All spectra were analyzed for the diagnostic oxonium ion of 301.10 m/z, and positive spectra were analyzed manually to identify the glycopeptide. This spectra is representative of each glycopeptide identified in Table 2. A) ITMS-CID of the precursor ion at *m/z* 1999.943 reveals the pentasaccharide attached to the peptide **AKPASTPAVK**. B) FTMS-HCD of the precursor ion at *m/z* 1999.943 reveals the peptide sequence **AKPASTPAVK.**

Table 2.1. Identification of Additional Glycoproteins modified with the same *O*-glycan. Seven *O*-glycosylated proteins with nine unique glycosylation sites by ZIC HILIC enrichment of tryptically digested *A. baumannii* ATCC 17978 membrane extracts

MH+ [Da]	Peptide mass [Da]	Sequence	Protein name	Annotation for *A. baumannii* ATCC 17978
2245.0	1214.6	^{301}AKPASTPAVKSAS^{313}	Putative uncharacterized protein	A1S_0556
1999.9	969.5	^{301}AKPASTPAVK^{310}	Putative uncharacterized protein	A1S_0556
3996.7	2966.4	^{108}AAQADKKTEASAAATTEQQDSFDAQVQR^{135}	Putative uncharacterized protein	A1S_0556
5448.6	4418.2	^{203}AASGVEAAAAPATLTLSTDDKGAVSQCQAGIGDQGFLATLQTQVK^{247}	OmpA/MotB	A1S_1193
3430.5	2400.2	^{78}NLQKAEDQNADSGIAASTPVATAK^{101}	Putative uncharacterized protein	A1S_2371
4031.8	3001.5	^{72}ASTTLQNLQKAEDQNADSGIAASTPVATAK^{101}	Putative uncharacterized protein	A1S_2371
4405.1	3374.7	^{22}ASTTEQPLNPNKVSAPVEDPIDPLAVDAASTVK^{54}	Putative uncharacterized protein	A1S_2371
2420.0	1389.6	^{52}SDAVGSASEAAPATR^{66}	Putative uncharacterized protein	A1S_3580
2562.1	1531.7	^{50}AASDAVGSASEAAPATR^{66}	Putative uncharacterized protein	A1S_3580
2565.1	1534.7	^{52}QAASDIATATDNASAK^{67}	Putative uncharacterized protein	A1S_3626
4217.8	3187.4	^{48}SAGDQAASDIATATDN(+1)ASAKIDAAADHAADATAK^{81}	Putative uncharacterized protein	A1S_3626
2895.2	1864.8	^{48}SAGDQAASDIATATDNASAK^{67}	Putative uncharacterized protein	A1S_3626
4216.8	3186.4	^{48}SAGDQAASDIATATDNASAKIDAAADHAADATAK^{81}	Putative uncharacterized protein	A1S_3626
3019.1	1988.7	^{28}NDGM(+16)HEASDPATSHDM(+16)NK^{45}	Putative uncharacterized protein	A1S_3658
2987.1	1956.7	^{28}NDGMHEASDPATSHDMNK^{45}	Putative uncharacterized protein	A1S_3658
3188.2	2157.8	^{28}NDGMHEASDPATSHDMNKNS^{47}	Putative uncharacterized protein	A1S_3658
3003.1	1972.7	^{28}NDGM(+16)HEASDPATSHDMNK^{45}	Putative uncharacterized protein	A1S_3658
3397.5	2367.1	^{29}EEEQDKVETAVSEPQPQKPAK^{49}	Putative uncharacterized protein	A1S_3744
2639.2	1608.8	^{35}VETAVSEPQPQKPAK^{49}	Putative uncharacterized protein	A1S_3744

2.2.11 Cloning and Expression of A1S_1193 and A1S_0556

To confirm that protein glycosylation occurred with several proteins in *A. baumannii* ATCC 17978, we cloned and expressed two different proteins in both WT and Δ*pglL* strains. The only glycoprotein with significant homology was A1S_1193, with homology to OmpA/MotB, due to the presence of an OmpA_C-like domain at the C-terminus. This domain is thought to be involved in peptidoglycan binding. The protein was initially cloned into pEXT20 with BamHI/XbaI restriction sites with a C-terminal decahistidine tag, and sequenced (Fig 2.17). Subsequently, the gene was subcloned into pEC, which can express in *A. baumannii*, and transformed into both WT and Δ*pglL* strains. Colonies from both strains were tested for expression of A1S_1193, and the protein was purified by Ni-NTA^{2+} affinity chromatography and analyzed by Coomassie and α-His Western blot (Fig 2.17). A1S_1193 purified from Δ*pglL* migrated as two bands (Fig 2.17B Lanes E1 and E2), and three bands were observed with a slightly higher molecular weight in the WT sample. This suggested that glycosylation of A1S_1193 occurred in the WT samples and further mass spectrometric analysis was required to confirm.

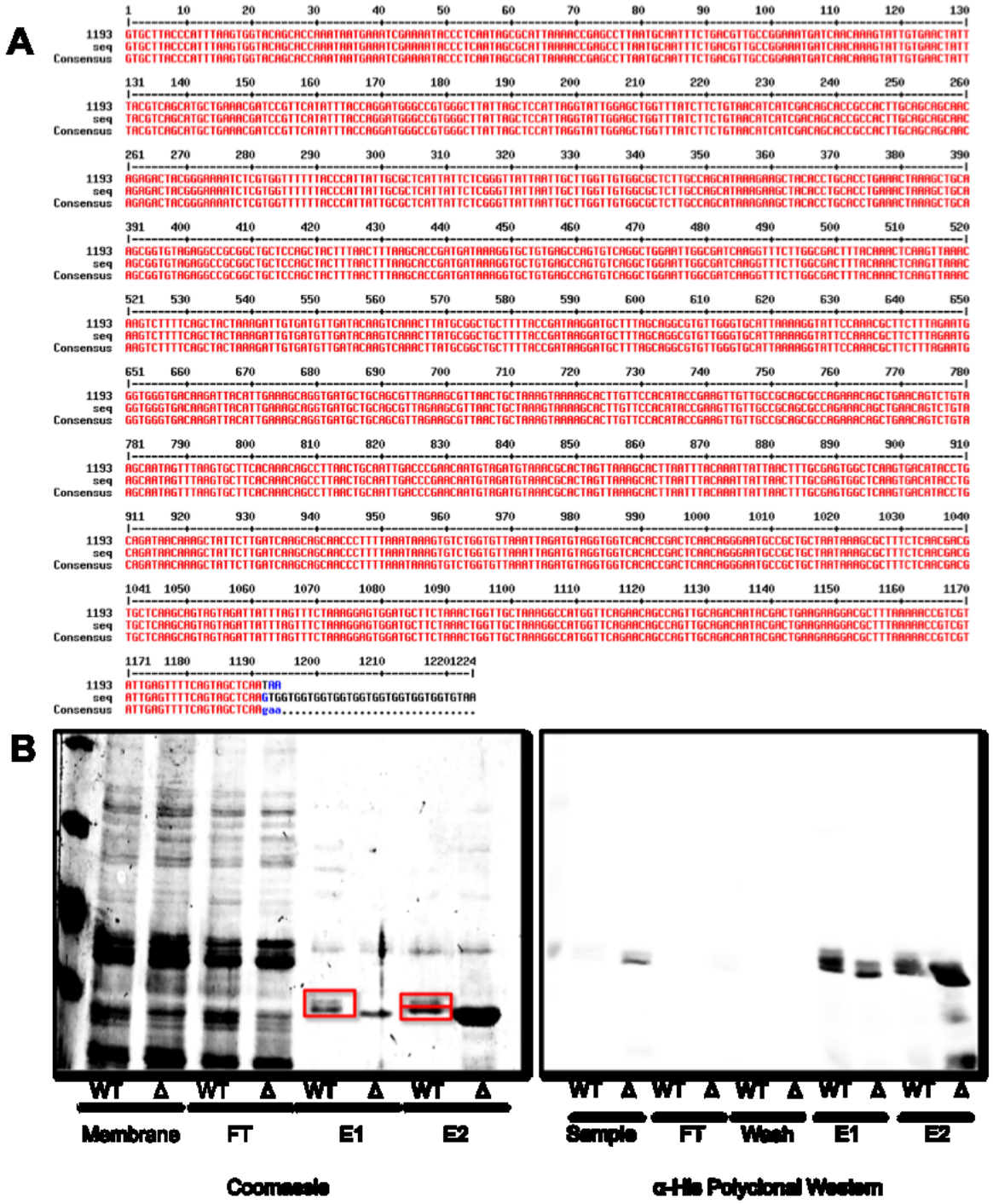

Figure 2.17 Cloning, Expression, and Purification of A1S_1193. A) Sequencing of a pEXT20-A1S_1193 positive colony shows correct DNA sequence B) Different fractions of the purification of A1S_1193 from WT and Δ*pglL* strains were isolated was separated on a 10% acrylamide SDS-PAGE gel and probed with Coomassie staining or an α-His antibody.

To confirm that A1S_1193 was glycosylated, each of the bands identified were excised from the Coomassie stained gel (Fig 2.17B), tryptically digested, and analyzed by both LC-ESI Q-TOF MS/MS and MALDI TOF-TOF MS/MS (Fig 2.18). Manual analysis of the LC-ESI-Q-TOF MS spectra revealed a 997.48^{3+} *m/z* peak possessing the previously identified glycopeptide (AASGVEAAAAPATLTLSTDDK; 1959.8 Da) with the 1030 Da *O*-glycan (Table 2.1). MS/MS sequencing of the peak strongly showed the peptide fragmentation, but no high molecular weight peaks were observed with enough strength for positive identification of the *O*-glycan. Therefore, we performed MALDI TOF-TOF MS/MS of parental ion of 2990.4 Da, and positively identified the *O*-glycan attached to the glycopeptide of A1S_1193 (Fig 2.18B). Therefore we concluded that A1S_1193 is glycosylated in *A. baumannii* ATCC 17978

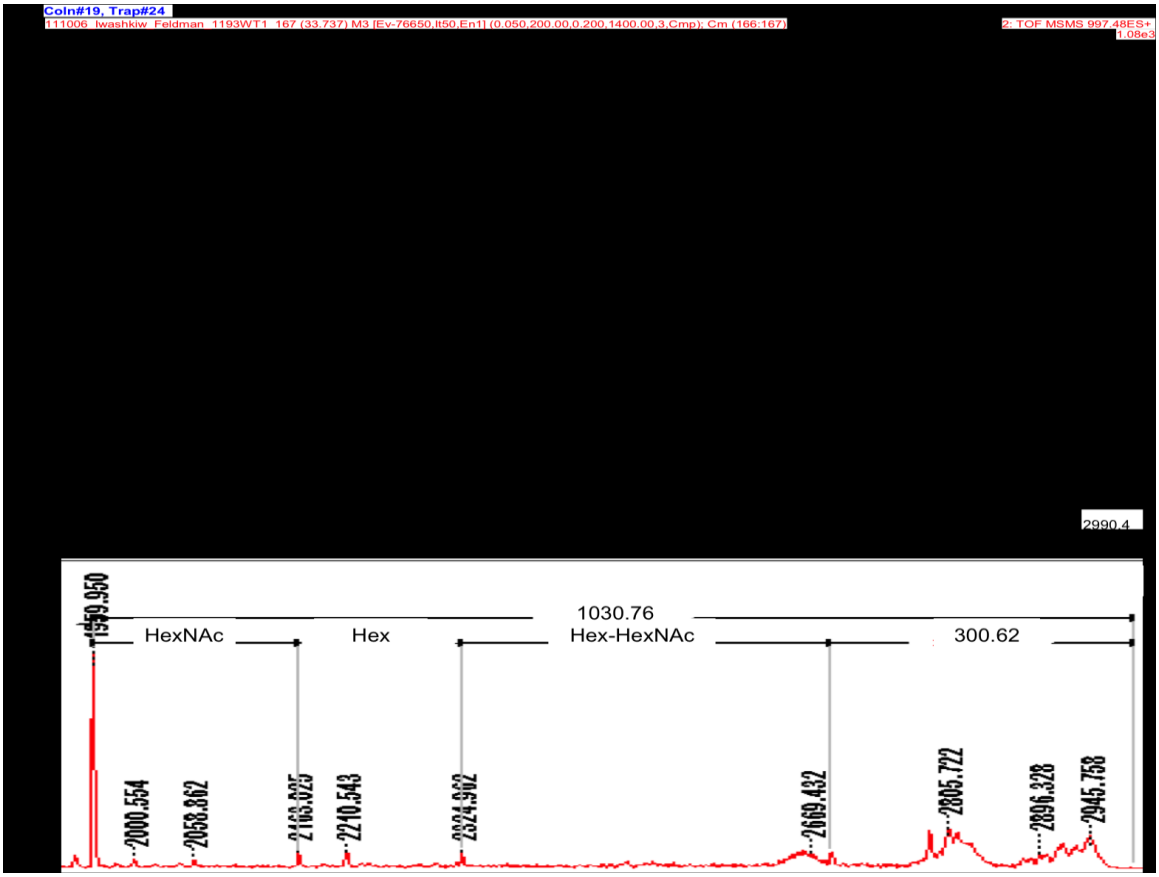

Fig 2.18 Mass Spectrometric Analysis of the Glycoprotein A1S_1193 Purified from *A. baumannii* WT. A) LC-ESI MS/MS of the parental peptide *m/z* 997.48^{3+} showed both b and y ions corresponding to the fragmentation of A1S_1193 peptide AASGVEAAAAPATLTLSTDDK with the addition of a 1030.6 modification B) MALDI TOF-TOF MS/MS of the same parental peak determine the glycan structure to be HexNAc-Hex-Hex-HexNAc-300 Da.

An additional glycoprotein, A1S_0556 was analyzed. This protein has homology to putative lipoproteins, and possesses a DUF3298 domain that has been hypothesized to be endo-1,3-beta-xylanase-like, but no definitive evidence has been demonstrated (Marchler-Bauer *et al.*, 2011). A1S_0556 was cloned in a similar fashion as A1S_1193 into pEXT20 with a decahistidine tag, sequenced (Fig 2.19), and subsequently subcloned into pEC for expression in both *A. baumannii* WT and Δ*pglL* strains. Positively expressing colonies were selected for in each strain by α-His Western blot of whole cell lysates, and subsequently examined on a single SDS-PAGE gel (Fig 2.19B). In agreement with the

ZIC-HILIC data of A1S_0556, two additional bands of higher molecular weight were observed when the protein was expressed in the WT strain as compared to Δ*pglL*. This result, in conjunction with the previous molecular characterization of the glycoproteome of *A. baumannii* ATCC 17978, confirms that 4 glycoproteins are positively identified as being glycosylated with the same *O*-glycan.

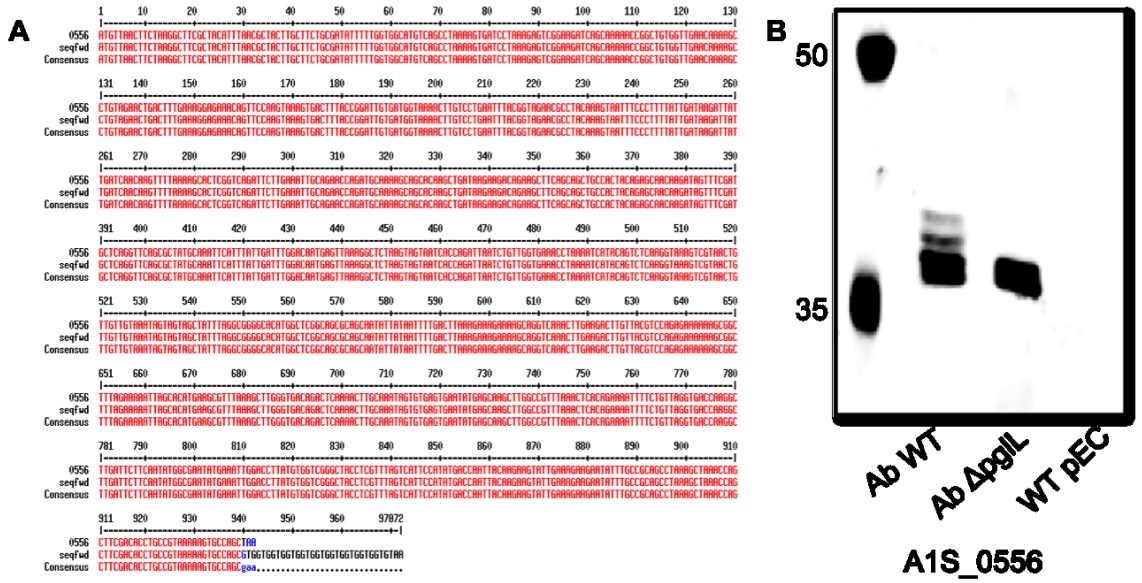

Figure 2.19 Sequencing and Expression of A1S_0556 in Different *A. baumannii* strains. A) Sequencing of a pEXT20-A1S_0556 positive colony shows correct DNA sequence B) Whole cell lysates from each of the strains was separated on a 10% acrylamide SDS-PAGE gel and probed with an α-His antibody. Two additional higher molecular weight bands were observed in the WT sample as compared to the Δ*pglL* extract, confirming the ZIC-HILIC data that A1S_0556 has two glycosylation sites. No bands were observed in the vector control whole cell lysate.

2.2.12 Structure Determination of the *O*-Linked Glycan by 2D NMR

Identification of the *O*-glycan of *A. baumannii* ATCC 17978 was achieved by 2D NMR analysis. The Pronase E digested membrane protein extracts characterized in Fig 3C were analyzed by ^{1}H:^{13}C HSQC 2D NMR (NMR analysis done by Dr. Evgueny Vinogradov, NRC) and revealed the structure of the pentasaccharide to be β-GlcNAc3NAcA4OAc-4-(β-

GlcNAc-6-)-α-Gal-6-β-Glc-3-β-GalNAc-, with the amino acids S, E, and A attached in any combination (Fig 2.20, Table 2.2). β-GlcNAc3NAcA4OAc (corresponding to m/z 300) is an *O*-acetylated derivative of glucuronic acid, and can account for the more acidic migration of the WT glycoproteins compared to the Δ*pglL* in the 2D-DIGE analysis.

Table 2.2. $^{1}H:^{13}C$ HSQC 2D NMR data for the characterization of the *A. baumannii* 17978 *O*-glycan.

Unit		H/C-1	H/C-2	H/C-3	H/C-4	H/C-5	H/C-6a;b
Gal A	H	4.95	3.68	3.94	4.12	4.02	3.78; 4.12
	C	99.3	69.8	70.6	79.1	70.8	72.3
GlcNNA B	H	4.84	3.92	4.20	4.96	3.99	
	C	103.4	54.5	53.9	71.9	76.0	174.4
GlcNNA B'	H	4.81	3.79	3.99	3.64	3.88	
	C	103.5	55.0	56.1	71.1		
GalNAc C	H	4.58	4.07	3.87	4.14	3.70	3.77; 3.80
	C	101.9	52.0	81.4	69.0	76.1	62.2
Glc D	H	4.53	3.30	3.47	3.50	3.61	3.69; 3.88
	C	105.7	74.0	76.9	70.6	75.5	66.8
GlcNAc E	H	4.50	3.70	3.52	3.45	3.45	3.77; 3.94
	C	103.1	56.7	75.2	71.0	77.0	61.8
Ser	H		4.30	4.14; 4.14			
	C	178.2	54.3	68.1			
Glu	H		4.45	2.02; 2.14	2.51; 2.51		
	C	178.4	54.2	27.7	31.4		
Ala	H		4.42	1.42			
	C	175.9	51.0	17.7			
Ala*	H		4.31	1.42			
	C	178.4	51.0	17.7			

Indicates impurity.

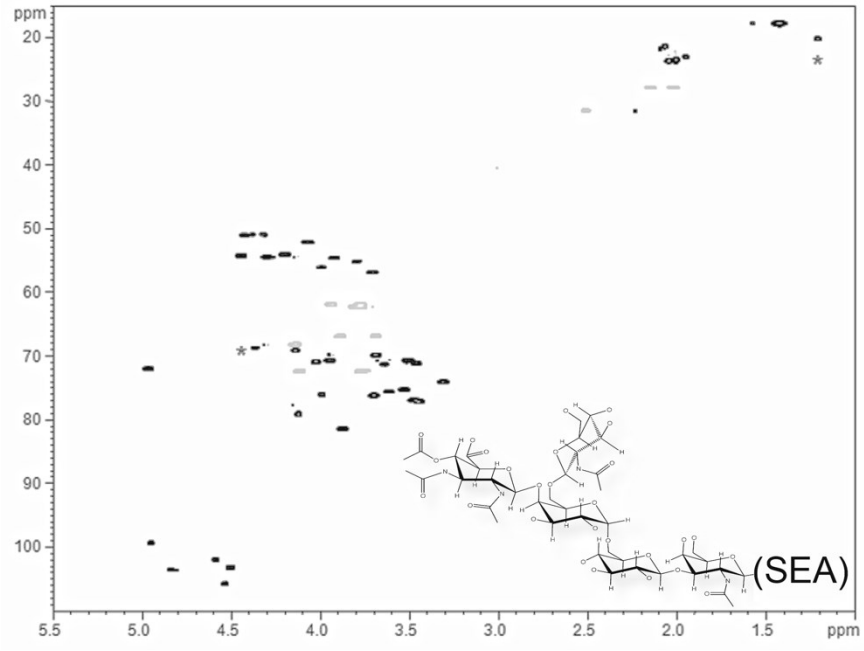

Figure 2.20. **^{1}H:^{13}C HSQC 2D NMR Spectra of the *A. baumannii O*-Glycan**. NMR data for the *A. baumannii O*-glycan (D_2O, 28 °C, 600 MHz). NAc: 1.95/23.0; 2.00/23.3; 2.04/23.5; 2.04/23.5 ppm, all C-1 at 175.9 ppm. OAc: 2.06/ 21.3, 174.0 ppm. The amino acids attached to the pentasaccharide were determined to be S-E-A (order not determined). * indicates impurity.

2.2.13 Protein Glycosylation Appears to be Ubiquitous in *Acinetobacter*

To determine the degree of conservation of the *O*-glycosylation system in *Acinetobacter* sp., we searched for the presence of $PglL_{Ab}$ homologues in different species within the genus. We obtained eight clinical isolates from the University of Alberta Hospital. The isolates were identified by 16S rRNA and *recA* sequencing to be different species within the *Acinetobacter* genus (*A. baumannii, A. nosocomialis, A. pittii,* and *A. calcoaceticus*; identification of the isolates was performed by Brent Weber). Membranes of these strains were purified and analyzed by PAS staining for the presence of glycoproteins (Fig 2.21A). While there appears to be variation in the size and intensity of the PAS stained band, all the isolates were positive for glycoproteins, demonstrating that $PglL_{Ab}$ was active in all these strains. This genomic search showed that $PglL_{Ab}$ was present in all

the genomes analyzed with high sequence homology (Fig 2.21B). This indicates that despite the plasticity of *Acinetobacter* sp. genomes (Snitkin *et al.*, 2011), there is a strong evolutionary pressure to retain a functional *O*-glycosylation system.

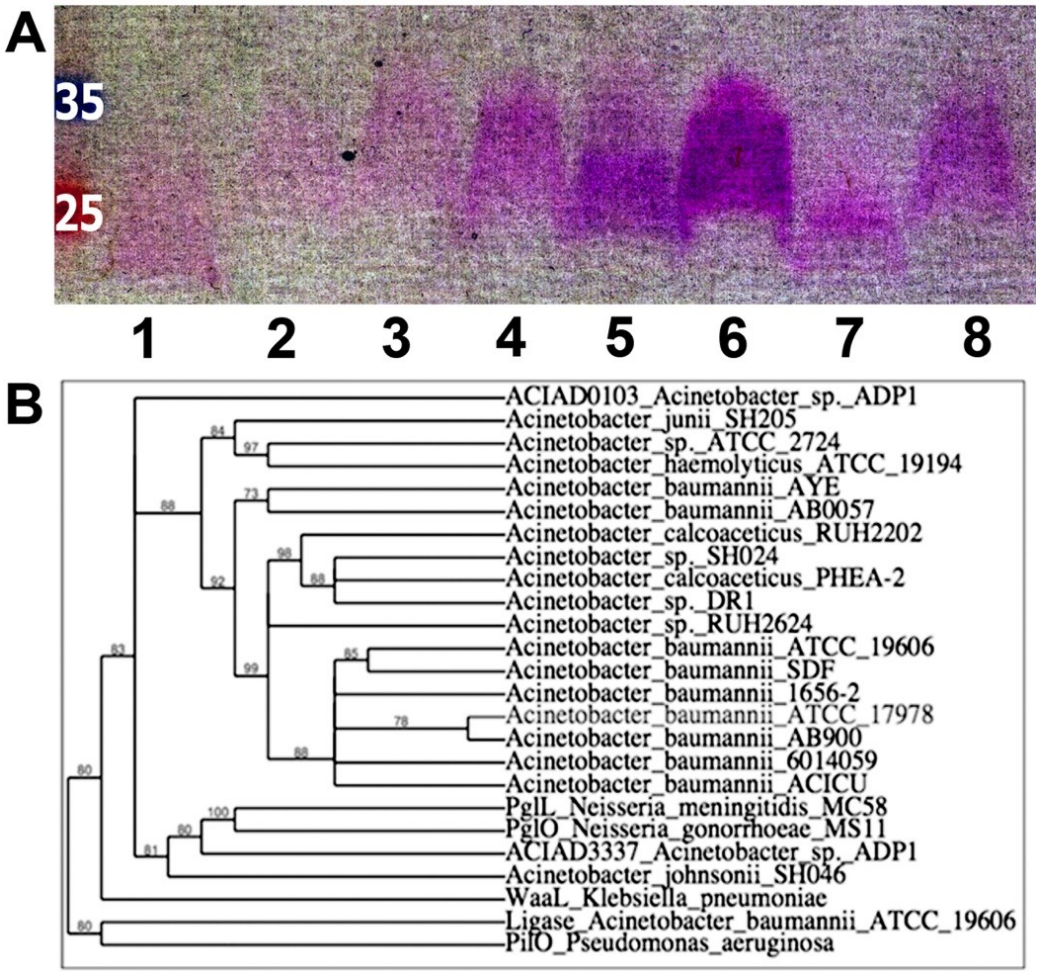

Figure 2.21. **Protein Glycosylation Appears to be Highly Conserved in *A. baumannii*.** A) Ten µg of membrane extract from *A. baumannii* clinical isolates obtained from the University of Alberta Hospital were resolved by SDS-PAGE and detection of carbohydrates was performed by PAS stain. 8/8 isolates have a similar PAS reactive band to *A. baumannii* ATCC 17978. Isolates were putatively identified by sequencing 16S rRNA and *recA* and are as follows: lane 1 *A. calcoaceticus,* lane 2 *A. calcoaceticus*, lane 3 *A. pittii*, lane 4 *A. pittii*, lane 5 *A. nosocomialis*, lane 6 *A. nosocomialis*, lane 7 *A. junii*, lane 8 *A. baumannii.* B) Phylogenetic tree of hypothetical *O*-OTases of *Acinetobacter* sp. and known *O*-OTases and *O*-antigen ligases. Protein identification numbers are as follows: *N. meningitidis* M58 PglL (NP_273640.1); *N. gonorrhoeae* MS11 PglO

(ZP_04726765.1); *P. aeruginosa* PAO1 PilO (AAP43787.1); *A. baumannii* ATCC 17978 (YP_001086175.1); *A. baumannii* AB900 (ZP_04661261.1); *A. baumannii* ACICU (YP_001848035.1); *A. baumannii* ATCC 19606 (ZP_05829147.1), *A. baumannii* ATCC 19606 Ligase (YP_001713790.1); *A. baumannii* SDF (YP_001705998.1); *A. baumannii* 1656-2 (ADX05061.1); *A. baumannii* 6014059 (ZP_08441731.1); *A. calcoaceticus* RUH2202 (ZP_06059388.1); *A. baumannii* AYE (YP_001712289.1); *A. baumannii* AB0057 (YP_002320930.1); *A. calcoaceticus* PHEA-2 (ADY83231.1); *Acinetobacter* sp. SH024 (ZP_06693023.1); *Acinetobacter* sp. DR1 (YP_003730587.1); *Acinetobacter* sp. RUH2624 (ZP_05825054.1); *Acinetobacter* sp. ATCC_2724 (ZP_03824224.1); *A. junii* SH205 (ZP_06066893.1); *A. haemolyticus* ATCC 19194 (ZP_06729056.1); *Acinetobacter* sp. ADP1 ACIAD0103 (YP_044903.1); *Acinetobacter* sp. ADP1 ACIAD3337 (YP_047828.1); *A. lwoffii* SH145 (ZP_06070298.1); *Klebsiella pneumoniae* WaaL (AAX20101.1). Phylogenetic tree was built using http://www.phylogeny.fr/version2_cgi/index.cgi (Dereeper *et al.*, 2008)

2.2.14 *A. baumannii O*-Glycosylation System can be Reconstituted in *E. coli* and $PglL_{Ab}$ has Relaxed Glycan Specificity

Previous work has demonstrated that both *N*- and *O*-glycosylation systems can be functionally reconstituted in *E. coli* (Faridmoayer et al., 2007, Wacker *et al.*, 2002, Gebhart et al., 2012). We therefore wished to test the activity of the *A. baumannii* glycosylation system in a similar fashion. We co-expressed the *O*-OTase from *A. baumannii* ATCC 1978 and the glycoprotein A1S_1193 with different lipid-linked carbohydrates in *E. coli* CLM24 (*waaL*-). CLM24 is a convenient strain for testing glycosylation systems since it does not synthesize its native O-antigen due to an inactivation in a rhamnosyltransferase (Liu & Reeves, 1994), and cannot transfer any lipid liked glycans to lipid A due to an inactivation of the WaaL ligase (Feldman *et al.*, 2005). We first tested if $PglL_{Ab}$ can transfer a foreign reducing monosaccharide, bacillosamine, found in *Neisseria* sp. and *C. jejuni* (Young *et al.*, 2002, Power *et al.*, 2003). Whole cell lysates expressing the three components were isolated from overnight cultures, separated by SDS-PAGE, and analyzed by Western Blot (Fig 2.22A). No signal was observed with the α-glycan antibody in the absence of pEF33

(bacillosamine expressing plasmid), whereas two bands were observed when pEF33 was present and expressed. No difference in mass of the protein was observed with the α-His antibody between the different samples, which is expected since the mass of a monosaccharide would not affect the electrophoretic motility of a protein of 50 kDa. When the α-glycan and α-His antibodies channels were overlaid, co-localization of the signals, signified by the yellow merging of the signals, demonstrates that $PglL_{Ab}$ transferred bacillosamine to A1S_1193.

To test if $PglL_{Ab}$ can transfer oligosaccharides with different structures, we utilized a plasmid that expresses the *Campylobacter jejuni N*-glycan ($pACYC_{pglBmut}$). Similarly to the previous experiment, Western blot analysis of whole cell lysates was visualized with both α-glycan and α-His antibodies (Fig 2.22B). No signal was observed in the vector control samples, while a strong band was observed in the glycosylation test samples. A second higher molecular weight band was only observed in the glycosylation test samples, and co-localization of the two antibodies was observed, confirming that $PglL_{Ab}$ can transfer oligosaccharides with different structures.

The ability of $PglL_{Ab}$ to transfer both mono- and oligosaccharides to A1S_1193 impelled us to assay if it could also transfer larger polysaccharides. CLM24 naturally expresses the *E. coli* K12 O16 O-antigen, but is prevented by an insertion in the rhamnosyltransferase (*wbbL*) gene. This can be complemented by expression *in trans* of WbbL from the plasmid pMF19 (Faridmoayer *et al.*, 2008, Gebhart et al., 2012). CLM24 cells expressing $PglL_{Ab}$, A1S_1193, with or without pMF19 were grown overnight, harvested, separated by SDS-PAGE, and visualized by Western blot (Fig 2.22C). The sample lacking pMF19 did not produce the O16 O-antigen, and only a single band was

observed corresponding to unglycosylated A1S_1193. Interestingly, when pMF19 was introduced, the O-antigen was observed by the α-glycan, and the α-His signal was shifted significantly higher molecular weight. This is strong evidence that $PglL_{Ab}$ can glycosylate protein with high molecular weight polysaccharides, in conjunction with the ability to transfer mono- and oligosaccharides, shows that it has low glycan specificity, making it a possible tool for future glycoengineering projects.

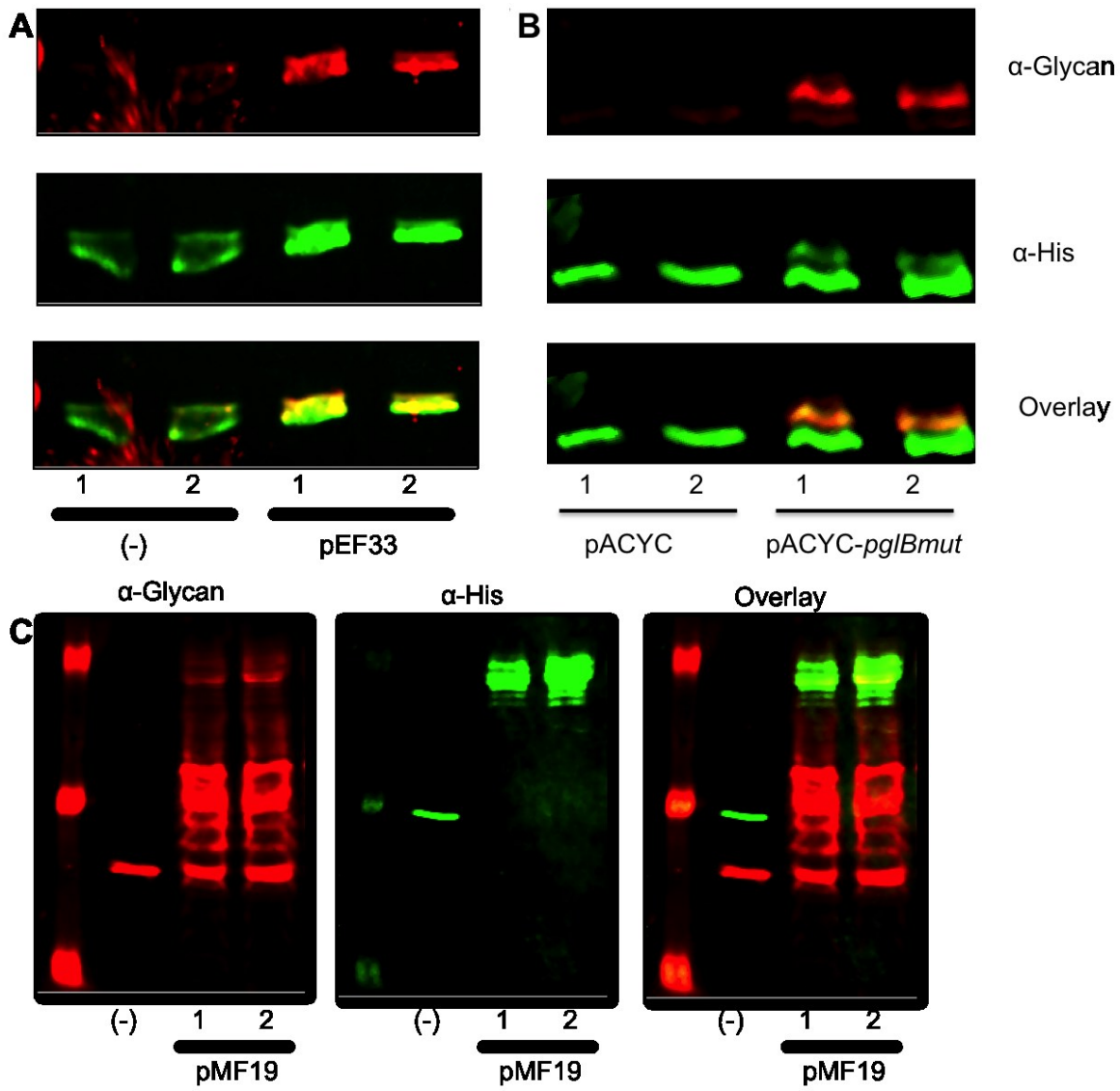

Figure 2.22 Reconstitution of the *A. baumannii* Glycosylation System in *E. coli* CLM24 and Glycan Promiscuity of $PglL_{Ab}$. Whole cell lysates of the different samples were separated by SDS-PAGE and analyzed by Western blot. Proteins were visualized with a monoclonal α-His antibody, and the different carbohydrates were visualized with glycan-specific polyclonal antibodies.

2.2.15 Identification of a Genetic Locus Responsible for the Biosynthesis of the *O*-Glycan of *A. baumannii* ATCC 17978

With the identification of the *O*-glycan possessing the unique monosaccharide tri-acetylated glucuronic acid (GlcNAc3NAcA4OAc), we investigated the genome of *A. baumannii* ATCC 17978 for a genetic locus responsible for its biosynthesis. Previous work has characterized the biosynthetic pathway responsible for the formation of a similar derivative of glucuronic acid in *Pseudomonas aeruginosa* and *Bordetella pertussis* (Larkin & Imperiali, 2009, Westman *et al.*, 2008). *In silico* analysis revealed a gene cluster (A1S_0052-A1S_0057) with homology to the *wbp* and *wld* gene clusters in *P. aeruginosa* and *B. pertussis*. Intercalated between the genes necessary for the synthesis of GlcNAc3NAcA4OAc we identified a *wzx* flippase homologue, A1S_0056, a typical component of the Wzy-dependent *en bloc* glycan synthesis pathway (Fig 2.23). Genetic analysis of this locus also identified genes putatively encoding four glycosyltransferases (A1S_0058, A1S_3482, A1S_0059, A1S_0060); an iGT (A1S_0061); a UTP-glucose-1-phosphate uridylyltransferase (A1S_0062); a UDP-Glc dehydrogenase (A1S_0063); a phosphoglucoisomerase (A1S_0064); an epimerase (A1S_0065), and a mutase (A1S_0066) (Fig 2.24). All these genes would have a predicted role in the assembly of the *O*-glycan. However, other genes with a predicted role in capsule biosynthesis [*wzc* (A1S_0049), *wzb* (A1S_0050), *wza* (A1S_0051), and *wzy* (A1S_3483)] were also encoded within or adjacently to this locus. A homologue of *wzi*, thought to be involved in mediating surface association of the capsule, was found separately in the *A. baumannii* chromosome (A1S_0999). The genetic organization of this gene cluster prompted us to hypothesize that it codes for the synthesis of the previously described *O*-glycan and the Type I capsule, both shown to be important virulence factors for *A. baumannii*. Further work by Lees-Miller *et al*. confirmed that the type I capsular

polysaccharide and O-glycoproteins are synthesized by this genetic locus (Lees-Miller *et al.*, 2013).

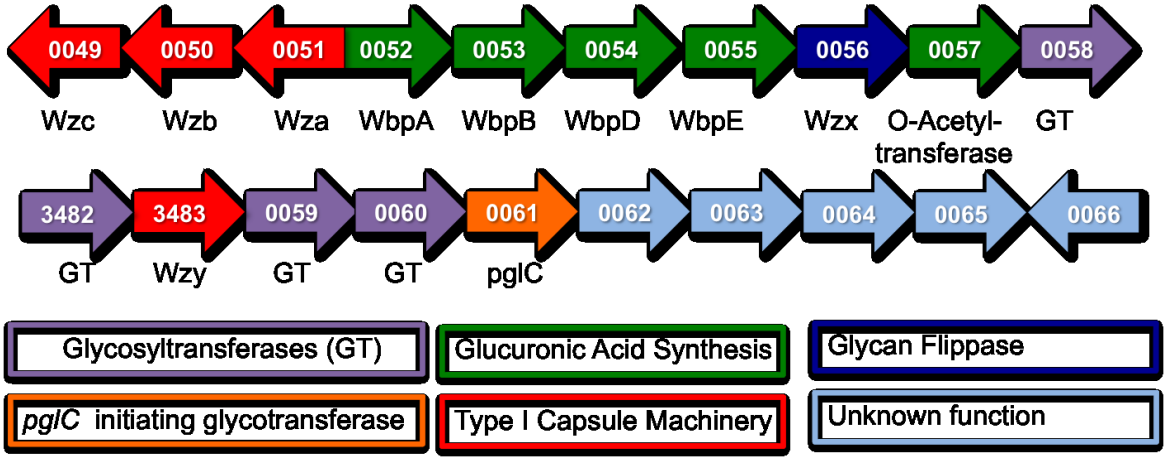

Fig 2.23 A Genetic Locus Dedicated to Glycan Synthesis is Present in *A. baumannii* ATCC 17978. The locus appears to contain genes required for *O*-glycosylation and wzy-dependent capsule synthesis. Genes were annotated with gene names or functions identified by homology though BLAST searches. GT1 and GT2 are the CAZY designation of the hypothetical glycosyltransferase genes, but it is not entirely clear which specific transfer reactions they catalyse. Colours correspond to: Capsule synthesis (red), GlcNAc3NAcA4OAc synthesis (green), flippase (dark blue), glycosyltransferases (purple), iGT (orange), and associated genes (blue). Not to scale, arrow direction is orientation within the genome, numbers in arrows represent Open Reading Frame annotation.

2.2.16 *A. baumannii* uses a Common Glycan for Both *O*-glycosylation and Capsular Polysaccharides

The identification of a locus responsible for both *O*-glycan and capsule biosynthesis led us to propose a bifurcated pathway model where the synthesis of these structures is common at early stages, diverging in the periplasm of the cell (Fig. 2.24). We propose that the pentasaccharide is initiated by the attachment of GalNAc to Und-P by PglC (A1S_0061) on the cytoplasmic face of the inner membrane. Four GTs (A1S_0058,

A1S_3482, A1S_0059, and A1S_0060) complete the oligosaccharide. Four genes (A1S_0052–A1S_0057) are responsible for the synthesis of GlcNAc3NAcA4OAc. After flipping to the periplasm by Wzx (A1S_0056), the pentasaccharide is then conjugated to proteins by the PglL OTase (A1S_3176), or polymerized by Wzy (A1S_3483) and exported through the Wza (A1S_0051) channel as CPS.

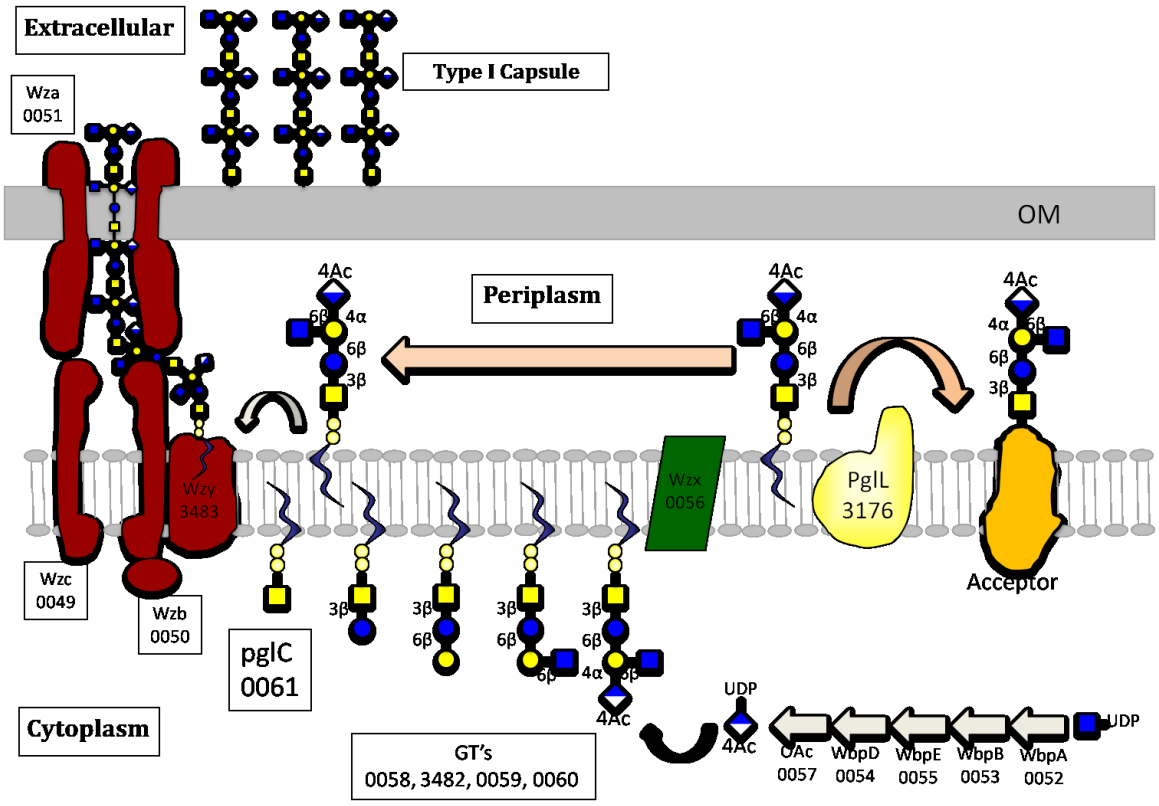

Figure 2.24 Proposed model for the bifurcated pathway for protein glycosylation and capsule biosynthesis in *A. baumannii* ATCC 17978 showing relevant enzymes for cytoplasmic synthesis and periplasmic assembly.

2.2.17 Conservation of the *O*-glycan Genetic Loci in the *Acinetobacter*

With the identification of homologues of the characterized PglL of *A. baumannii* ATCC 17978 in other *Acinetobacter* species, and of a locus responsible for both *O*-glycosylation and capsular polysaccharide, we searched *in silico* for the presence of the locus in the *Acinetobacter* genus. Interestingly, we observed the glycan synthesis locus flanked by a capsule export and UDP-glycan biosynthesis gene throughout several distinct *Acinetobacter* species (Fig 2.25). We observed different species possessing similar genetic elements to the reference strain ATCC 17978, which produces a branched pentasaccharide with the unique tri-acetylated glucuronic acid. *A. baumannii* ATCC 19606 has a genetically identical locus to ATCC 17978, whereas strain ACICU has a completely distinct set of genes with homology to those responsible for neuraminic acid biosynthesis. Kenyon *et al* recently described in more detail the genetic variation of the *Acinetobacter baumannii* glycan loci, suggesting bioinformatically that 9 distinct structures could exist (Kenyon & Hall, 2013). Further work on the characterization of different *O*-glycans in *Acinetobacter* is currently being studied by Rachel Kinsella (submitted). Interestingly, all of the different structures appear to have a negatively charged monosaccharide in the structure, which could have an important function for *Acinetobacter* species.

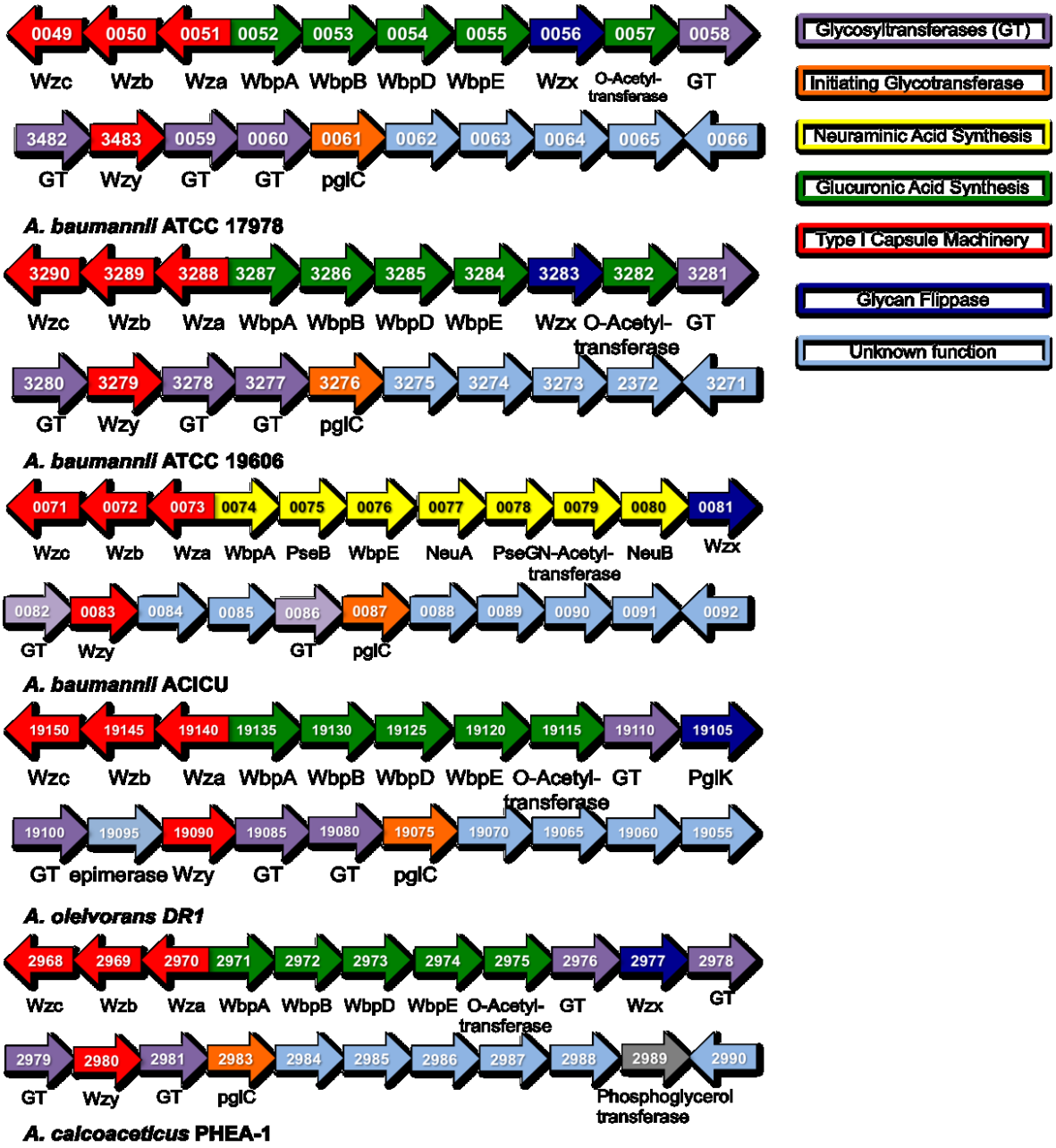

Figure 2.25 Identification of a Conserved Glycan Locus in *Acinetobacter* Species. Horizontal arrows represent genes displaying the direction of transcription. Genes are colour coded based on predicted function, and described by the most homologous identified genes below. Gene identification numbers are shown inside the arrows based on NCBI annotation.

2.3 Phenotypic Characterization of Protein Glycosylation in *A. baumannii* ATCC 17978

2.3.1 Protein Glycosylation does not have a Role in Swarming Motility

Protein glycosylation has been shown to be important for bacterial motility for several different bacteria (Wilhelms *et al.*, 2012, Scott *et al.*, 2011a, Twine *et al.*, 2009). Although it was previously thought *A. baumannii* was non-motile, it has been recently shown *A. baumannii* utilize swimming or swarming motility (Mussi *et al.*, 2010). Further work demonstrated that LPS and exopolysaccharides are involved in surface associated motility (McQueary *et al.*, 2012). Therefore, we sought to test if the *O*-glycosylation system was also involved. Bacterial cultures grown to mid-log phase were spotting in triplicate on 0.3% agar LB plates and grown overnight at 37^{o}C. We observed that both the WT and Δ*pglL* strains had equal amount of surface associated motility (Fig 2.26). Interestingly, both the vector control in the WT and the complemented Δ*pglL* strains were non-motile, suggesting that the presence of the plasmid pWH1266 (+/- $pglL_{Ab}$) was inhibitory for bacterial motility. Consequently, we concluded that *O*-glycosylation did not play a role in motility in *A. baumannii*.

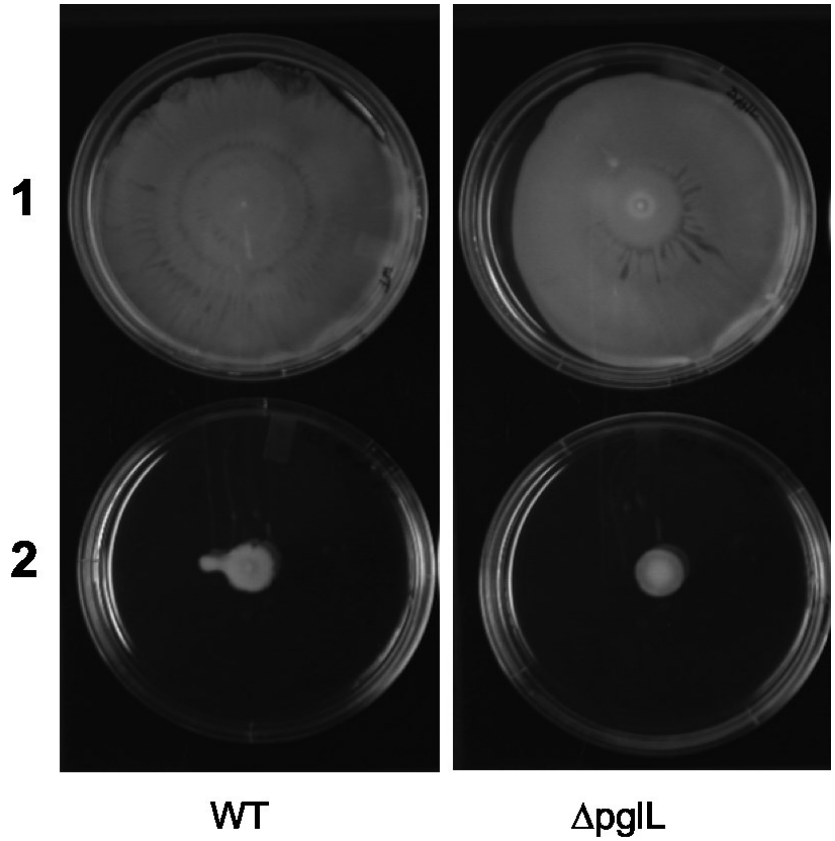

Figure 2.26 Swarming Motility in *A. baumannii* is not Affected by Protein Glycosylation. Plates (0.3% agar LB) were spotted with 5µL of mid log bacterial cultures, grown overnight at 37°C, and observed for swimming motility. In the absence of pWH1266 (lane 1), both WT and Δ*pglL* strains were highly motile, but when pWH1266 (+/-) pglL was introduced (2), both strains were inhibited for motility.

2.3.2 $PglL_{Ab}$ is Required for Efficient Biofilm Formation

It has been suggested that biofilm formation is important for *A. baumannii* virulence (Gaddy & Actis, 2009). We tested if *O*-glycosylation has an impact on biofilm formation in this organism. Biofilm formation was detected using crystal violet staining and quantitatively analyzed by comparing the ratio between cell growth (OD_{600}) and biofilm formation (OD_{580}) at 30°C after 48 hours incubation (Fig 2.27). High absorbance values corresponding to a strong ability to create biofilms (1.23 ± 0.48 and 1.12 ± 0.40) were

obtained for the WT strain and the Δ*pglL* strain complemented *in trans* respectively. On the contrary, the Δ*pglL* strain and the Δ*pglL* strain transformed with pWH1266 exhibited severely reduced levels of absorbance (0.18 ±0.07 and 0.20 ± 0.04). Similar results were also observed at 37^{o}C (data not shown). We further characterized the role of *O*-glycosylation in biofilm formation by employing a flow cell system (Experiment done by Andrea Seper, Institut fuer Molekulare Biowissenschaften, Karl-Franzens-Universitaet Graz, Graz, Austria). *A. baumannii* strains were stained with the green fluorescent stain SYTO 9, visualized by confocal laser scanning microscopy, and quantitative analysis of the biofilms was performed with COMSTAT. Assessment of the initial attachment after 2 hours shows that Δ*pglL* strain and vector control had significantly less surface coverage (4.12% and 2.32% respectively) than the WT and *in trans* complemented strain (6.41% and 6.45% respectively; Fig 2.27). Confocal microscopy and subsequent analysis of biofilms biomass, as well as average and maximal thickness after 24 hours showed significantly higher levels for the WT compared to the Δ*pglL* strain, and the phenotype was restored to WT levels when $pglL_{Ab}$ was complemented *in trans* (Fig 2.27 C, D, E, F; *P< 0.05). These data indicate that the *A. baumannii* strain defective in *O*-glycosylation has a severely diminished capacity to form biofilms.

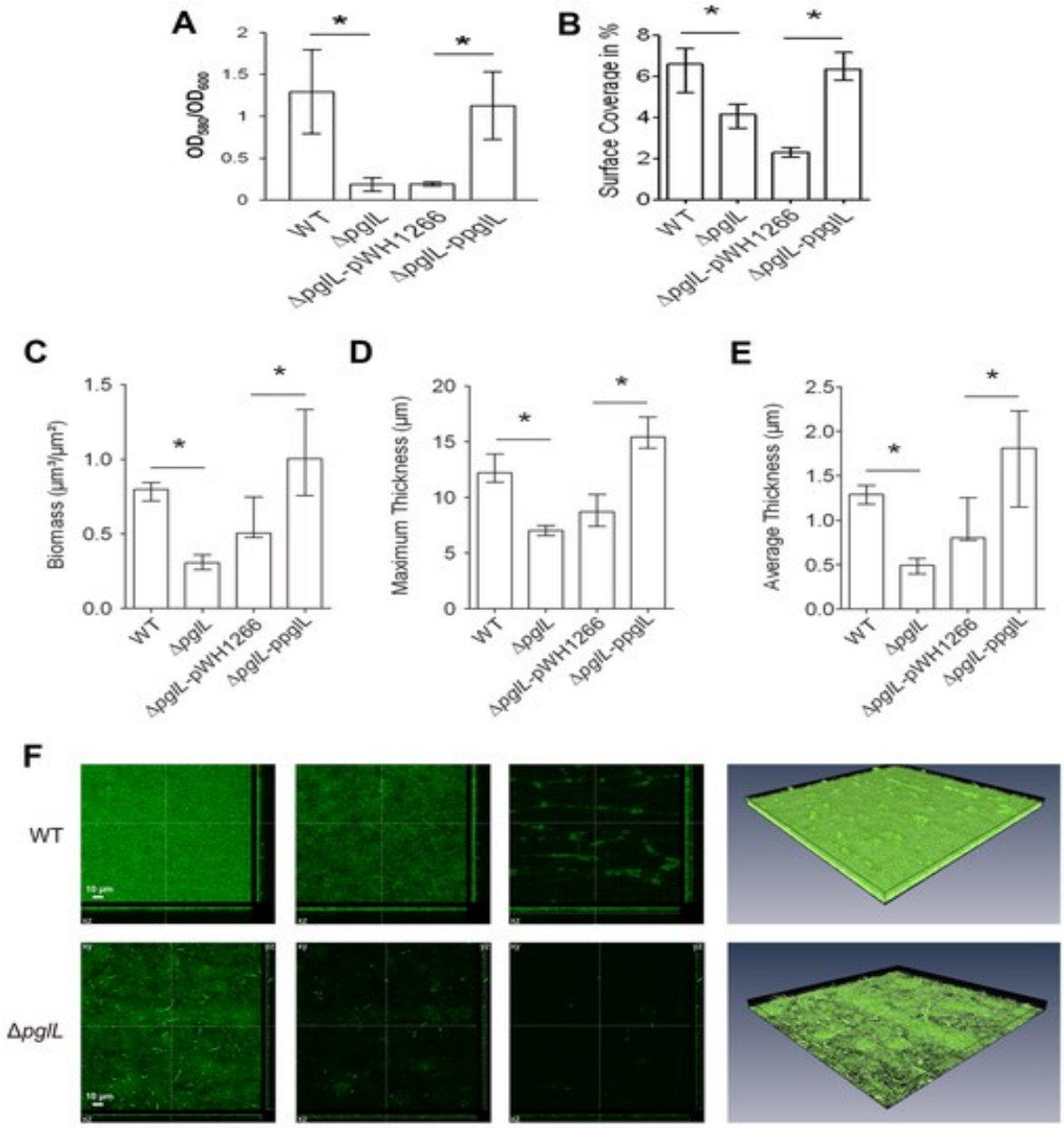

Figure 2.27 ***A. baumannii*** **Requires PglL$_{Ab}$ for Biofilm Formation**. A) Quantitative biofilm formation on polystyrene 96 well plates by strains incubated without perturbation in LB at 30°C. The bars indicate the means for 8 replicates. The error bars indicate the standard deviation of the means. Asterisks indicate significant differences (*, $P < 0.005$ [*t* test; $n = 8$]; **, $P < 0.001$ [*t* test; $n = 8$]). B) The median surface coverage after incubation for 2 h in flow cell chambers of the WT, Δ*pglL*, Δ*pglL* pWH1266 and Δ*pglL* ppglL was determined by the COMSTAT software. For each strain at least six micrographs from three independent experiments were analyzed. The error bars indicate the interquartile range. Asterisks indicate significant differences (*, $P < 0.05$ [Mann-Whitney U test; $n = 6$]). C) - E) Image stacks of the WT, Δ*pglL*, Δ*pglL* pWH1266 and Δ*pglL* ppglL biofilms grown in

flow cells for 24 h were analyzed for the biomass as well as the maximum and average thickness using the COMSTAT software. Shown are the medians of at least six image stacks from three independent experiments for each strain. The error bars indicate the interquartile range. Asterisks indicate significant differences (*, $P < 0.05$ [Mann-Whitney U test; $n = 6$]). F) Shown are representative confocal laser scanning microscopy images of the WT (upper row) and Δ*pglL* mutant (lower row) biofilms grown in flow cells for 24 h. The first three images represent horizontal (xy, large panel) and vertical (xz and yz, side panels) projections at different z-levels (from left to right 0.2 µm, 3 µm and 6 µm). The fourth micrograph of each row represents a three-dimensional image analyzed by the AMIRA software package of the WT and Δ*pglL* mutant biofilms, respectively.

2.3.3 $PglL_{Ab}$ is Required for Virulence Towards *Dictyostelium discoideum* and *Galleria mellonella*

Two well-established virulence models for *A. baumannii* are the *D. discoideum* predation and the *G. mellonella* infection models (Gaddy *et al.*, 2012, Wand *et al.*, 2011, Antunes *et al.*, 2011, Smith et al., 2007, Peleg *et al.*, 2009, Hornsey & Wareham, 2011). *D. discoideum* is an unicellular amoeba that feeds on bacteria and previous work has demonstrated similarity between phagocytosis of the amoebae and mammalian phagocytes (Hasselbring *et al.*, 2011). We examined if protein glycosylation was required for virulence towards *D. discoideum* by co-incubation of *A. baumannii* strains with the amoebae on SM/5 nutrient agar. *A. baumannii* was previously shown to inhibit amoebae growth in the presence of 1% ethanol (Smith et al., 2007). The WT strain was virulent and inhibited all *D. discoideum* growth in the presence of 1% ethanol, which resulted in no plaque being formed. However the Δ*pglL* strain was avirulent towards the amoeba, which resulted in plaque formation in the bacterial lawn within 48 hours and clearing of the plate within 4-5 days (Fig 2.28). *G. mellonella* have been used to study many host-pathogen interactions, and have several advantages over other virulence models including the presence of both humoral (ie. antimicrobial peptides) and cellular immune response systems (phagocytic cells) (Peleg et al., 2009). Most importantly, a correlation has been

established between the virulence of several bacteria in *G. mellonella* and mammalian models (Jander *et al.*, 2000, Mylonakis *et al.*, 2005). For the *G. mellonella*, while a similar bacterial load (2.31±1.13 x10^5 CFU) was injected for each of the strains, only the WT and complemented strains were able to kill the wax moth larvae after 36 hours, (20% and 0% survival), whereas larvae injected with Δ*pglL* and the Δ*pglL* vector control strains had significantly higher survival rates (100% and 80%; Fig 2.29). The LD_{50} of the WT and complemented strains were determined to be approximately 2.6x10^4 and 1.4x10^4 respectively after 36 hours. No additional killing was observed in the Δ*pglL* or vector control strains up to 96 hours. A PBS injected control maintained 100% survival throughout the length of the virulence assay. These results demonstrate a critical role for *O*-glycosylation in the virulence of *A. baumannii* in these two model systems.

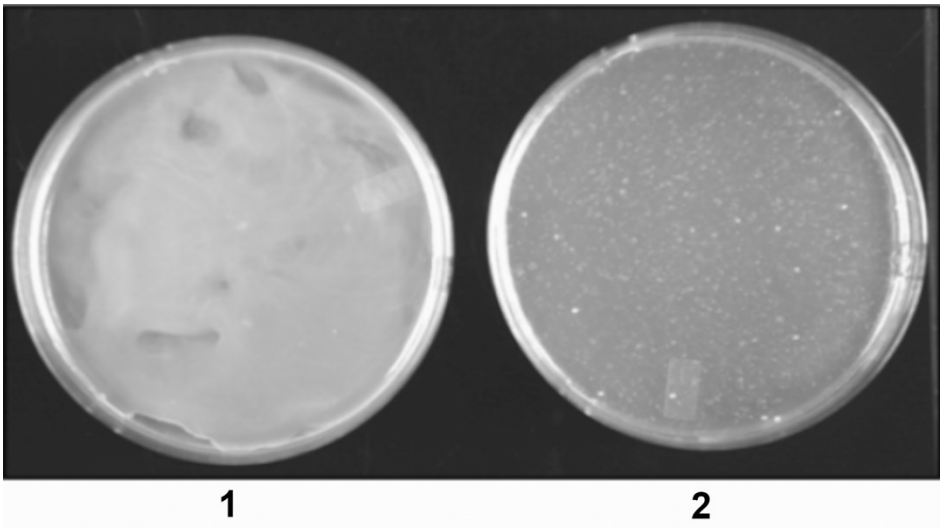

Figure 2.28. ***Dictyostelium discoideum*** **Plaque Assay Comparing Virulence of *A. baumannii* strains**. Bacteria were mixed with ~500 amoebae, plated on SM/5 agar with 1% ethanol, and incubated at room temperature for 72 hours to allow for plaque formation. Results are representative of four independent experiments with 1 representing WT and 2 representing Δ*pglL*.

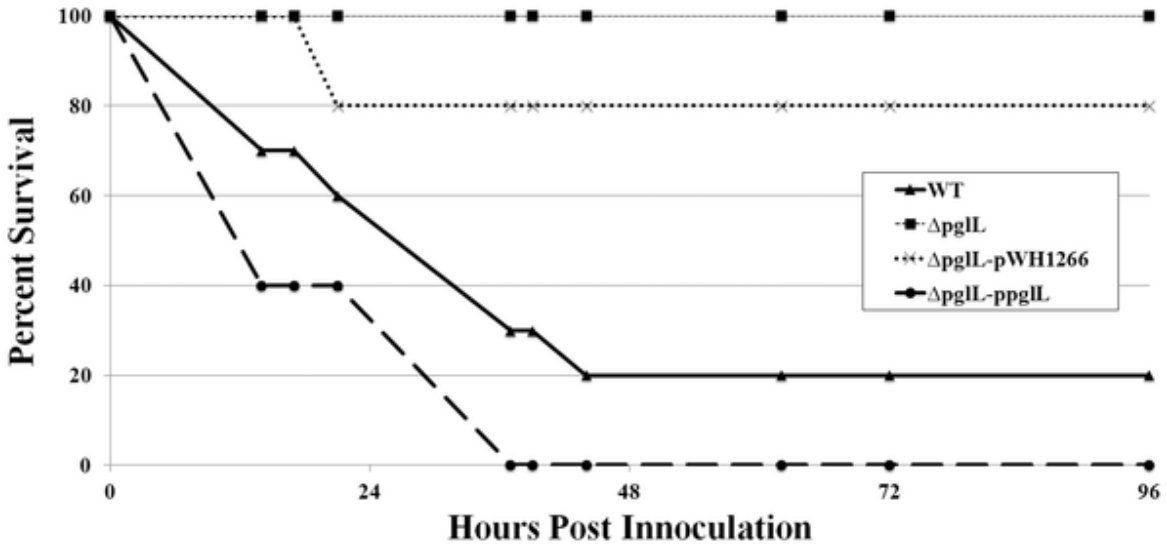

Figure 2.29. ***A. baumannii*** **Pathogenesis is Dependent on $PglL_{Ab}$ in the *Galleria mellonella* Virulence Model**. Representative data of survival rate of 3 biological replicates of 10 individual *G. mellonella* injected with 2.31±1.13 x10^5 CFU of each strain in 5 µL of sterilized PBS and incubated @ 37°C. Survival was assayed by response to touch or discoloration. While killing by WT and Δ*pglL*-pglL was observed, no killing was observed in the Δ*pglL* and 20% killing was observed in the Δ*pglL*-pWH1266 strains up to 96 hours. No killing was observed in the PBS injection control for the length of the experiment.

2.3.4 $PglL_{Ab}$ is Required for Competitive Fitness in BALB/c Mice

Next, We tested *A. baumannii* Δ*pglL* virulence *in vivo* using a previously described murine septicemia competition model (Experiment performed by Dr. Chad Stratilo, Defence Research and Development Canada Suffield) (Lopez-Rojas *et al.*, 2010, Smani *et al.*, 2011, Lau *et al.*, 2004). We first determined the LD_{50} of *A. baumannii* ATCC 17978 strain by injecting groups of 5 BALB/c mice with serially diluted bacteria cultures (Fig 2.30). A very small dose range between full survival and full killing was observed, and the LD_{50} was determined to be 6.49 x10^4 CFU/mouse. The competition index (CI) was defined as the number of Δ*pglL* CFUs recovered/number of WT CFUs recovered, divided by the number of Δ*pglL* CFUs inoculated/number of WT CFUs inoculated. Cultures of each strain were mixed at a ratio of 1:1, serial diluted, and plated to determine the initial CI. 1x10^5

CFU of the mixed strains were injected intraperitoneally into the BALB/c mice, which were subsequently sacrificed 18 hrs post injection. The spleens were aseptically harvested, serial diluted, and plated. All of the mice had a high spleen CFU load of 3.75 ± 2.37x10^8 CFU/gram and were moribund at the time of sacrifice. While the initial prescreen showed a CI of 1.18 ± 0.21 favoring the Δ*pglL* mutant, the spleen counts after 18 hrs showed a CI of 0.10 ± 0.03 (Fig 2.31). This data suggests that Ab Δ*pglL* has a competitive disadvantage as compared to the WT strain. Together, these results indicate that *A. baumannii* strains lacking *O*-glycosylation are attenuated in mice.

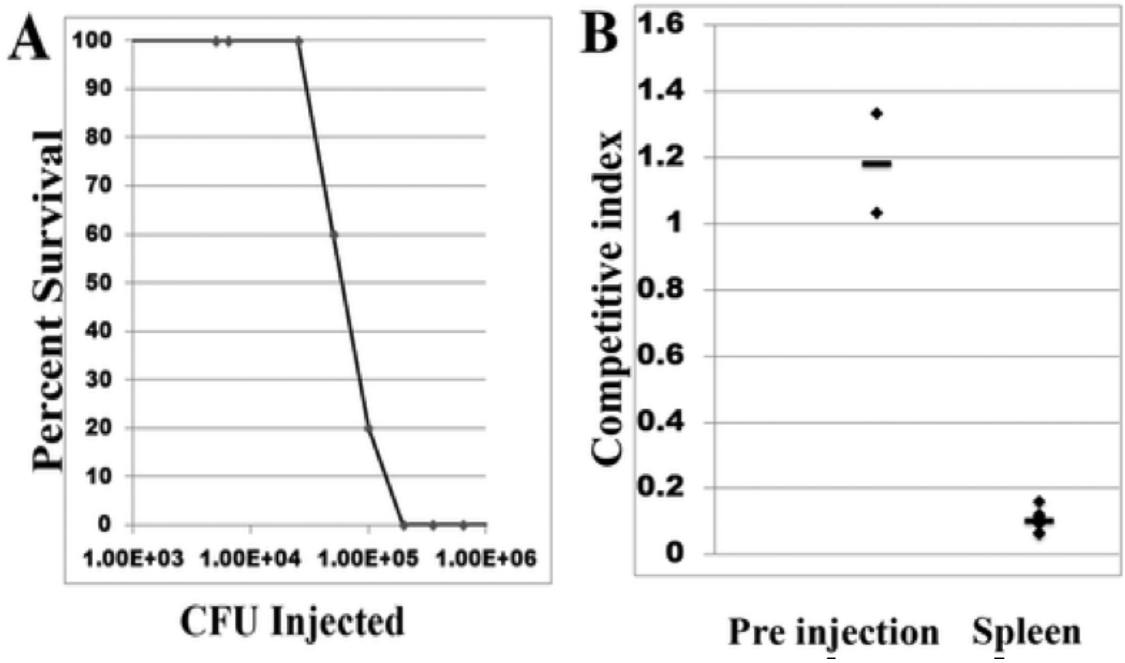

Figure 2.30. Characterization of *A. baumannii* ATCC 17978 Pathogenesis in a Murine Septicemia Model. A) Determination of the LD_{50} of *A. baumannii* ATCC 17978. Groups of 5 mice were injected with serial dilutions of *A. baumannii* WT to determine the LD_{50} which was calculated to be 6.49 x10^4 CFU @ 18 hrs post infection. B) Murine competition septicemia between *A. baumannii* WT and Δ*pglL*. Groups of 3 mice were injected with ~ 1:1 WT to Δ*pglL* CFU's and were sacrificed after 18 hrs, spleens were harvested, and bacterial load determined

2.3.5 Protein Glycosylation Appears to Have a Role in A549 Apoptosis and Necrosis

Our next approach to further understand the role of the general *O*-glycosylation system of *A. baumannii* was virulence against Eukaryotic cells. The bronchial alveolar epithelial cell line A549 was selected due to its robust growth, and previous work demonstrating *A. baumannii* being able to attach, invade, and cause A549 cell rounding (Gaddy *et al.*, 2009, Gaddy et al., 2012). We tested WT and Δ*pglL* strains for their ability to cause apoptosis and/or necrosis of A549 cells by using a counter strain technique using acridine orange and ethidium bromide (AO/EB) (Kasibhatla *et al.*, 2006, Ribble *et al.*, 2005). Acridine orange stains both live and dead cells, whereas ethidium bromide will only stain cells that have lost membrane integrity (Kasibhatla et al., 2006). When visualized by fluorescent microscopy, live cells appear uniformly green, early apoptotic cells stain green and contain bright green dots in the nuclei due to chromatin condensation and nuclear fragmentation, and necrotic cells stain orange but have no chromatin condensation (green spots; Fig 2.31A) (Kasibhatla et al., 2006). For our experiment, Eukaryotic cells (A549) will be grown on coverslips for 48h at 37^{o}C and which point bacterial strains will be added at an MOI of ~1:100. Bacteria will be co-incubated for 2h, and then all extracellular bacteria will be killed by the addition of 200 µg/mL gentamycin. At set time points, slides will be removed, stained at different time intervals with 1 µL of AO/EB solution (100µL each), mixed, and immediately evaluated by fluorescent microscopy (Fig 2.31). We observed the three described phenotypes when A549 cells were incubated with *A. baumannii* WT (Fig 2.31A). Interestingly, when the experiment was done as a time course over 24 hours, A549 cells incubated with WT bacteria showed ~25% apoptotic phenotype, whereas Δ*pglL* and DH5α strains only presented 5% of cells in a similar fashion (Fig 2.32B). We saw a similar result when we tested A549 cells for necrosis, and at 24 hours post infection we observed ~20% of cells presenting with necrotic phenotype, with a significantly smaller

proportion of cells (~5%) observed in the Δ*pglL* and DH5α samples (Fig 2.32C). Overall, these results suggest that *O*-glycosylation is important for *A. baumannii* to cause apoptosis and necrosis in human alveolar epithelial cells with the experimental conditions assayed.

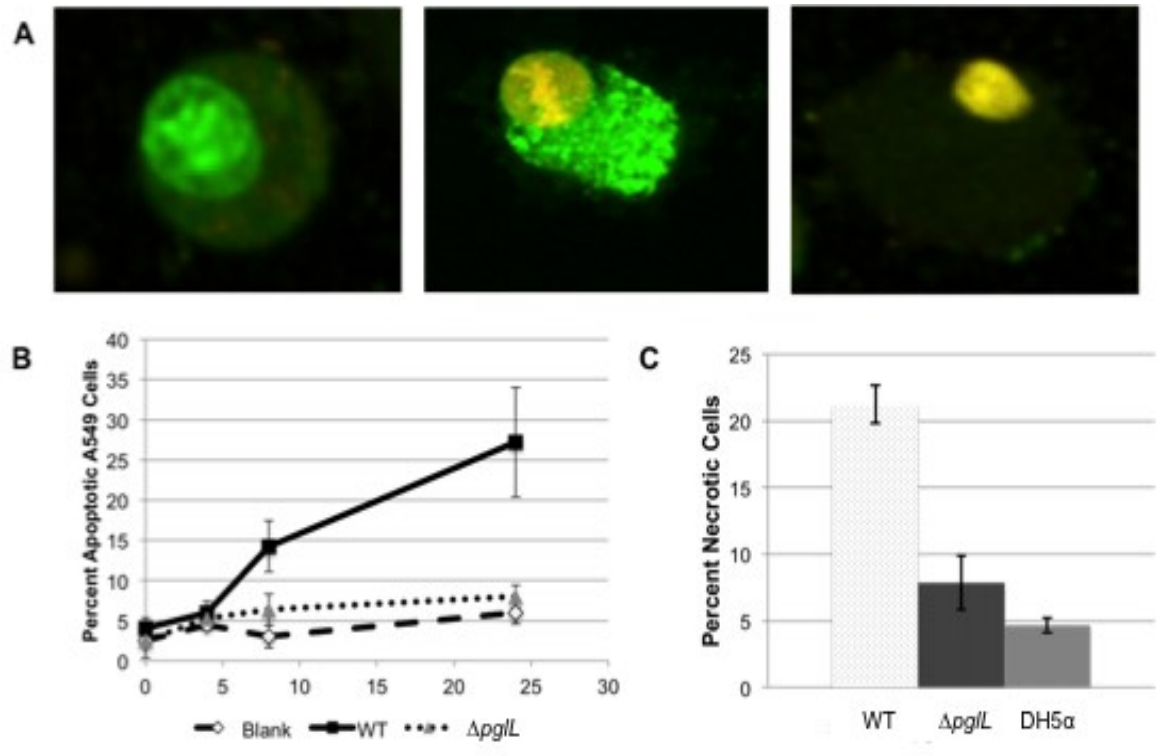

Figure 2.31 Apoptosis and Necrosis of A549 cells due to Different Bacterial Strains Visualized by Acridine Orange/ Ethidium Bromide (AO/EB). A549 cells were incubated with different bacteria for two hours, and extracellular bacteria were killed with gentamycin. A) Eukaryotic cell apoptosis and necrosis were visualized AO/EB staining with healthy cells staining solely green (left), apoptotic cell staining both colours with chromatin condensation in the nuclei seen as spots (center), and necrotic cells nuclei only visible staining orange. B) Time course of infection with bacterial strains detecting for apoptosis of A549 cells. C) A549 cell counts at 24 hours for necrosis. Both B) and C) were repeated 3 times and a minimum of 200 cells were counted for each time point.

2.3.6 Preliminary Characterization of the Role of *O*-Glycosylation in Cell Tissue Cultures

We tested to see if *A. baumannii* ATCC 1978 WT and Δ*pglL* strains could attach and invade A549 cells by traditional bacterial cell counts. A549 cells were grown to ~70% confluence, and different bacterial strains were introduced to the media at an MOI of 100. After two hours of incubation at 37^{o}C, the well was washed with PBS three times, and all bacteria cells were collected by washing the well with 1mL of 1% Triton X-100. We observed similar cell counts for both the WT and Δ*pglL* strains for both adhesion and invasion for samples done in duplicate, on three separate occasions (Fig 2.32). We did observe an unexpectedly high level of adhesion counts for the DH5α negative control, but no colonies were observed for invasion. This data suggested that protein glycosylation does not have an effect on the ability of *A. baumannii* ATCC 17978 to adhere or infection A549 epithelial cells in the conditions tested.

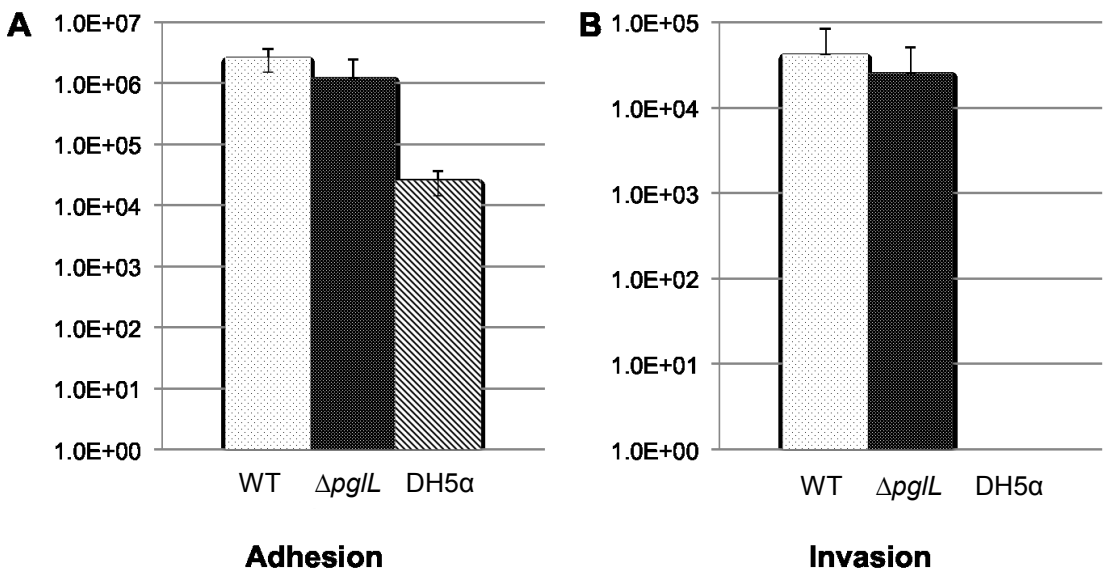

Fig 2.32 Adhesion and Invasion of A549 Cells by Different Bacterial Strains. A) Colony counts of bacteria that were associated with A549 cells after two hours of incubation. B) As described in A) with the exception that after 2 hours, extracellular bacteria were killed by gentamycin (200μg/mL) for 1 hour, and bacteria were harvested and counted by serial dilution and colony count.

2.3.7 Protein Glycosylation Appears to be Important for Causing Eukaryotic Cell Rounding, but does not Crosslink Actin *in vivo*

To study the role of glycosylation in causing A549 cell death, we performed fluorescent microscopy experiments. A549 cells were seeded at $1x10^4$ cells per coverslip in 6 well plates and grown for 24h. Mid-log phase *Ab* strains expressing GFP were introduced for the infection of A549 cells. At different time points, the cover slip was removed from the well and cells were washed with PBS, fixed, permeabilized, and analyzed by fluorescent microscopy. The eukaryotic cell structure was visualized through actin staining with rhodamine fluorescent dye. As a counterstain, DNA was visualized by DAPI. Bacteria, actin, and DNA were fluorescently labeled in green, red, and blue, respectively (Fig. 2.33A). We compared WT and Δ*pglL A. baumannii* strains for an effect on A549 cells. As shown in figure 2.33A, WT bacteria caused rounding of the eukaryotic

cells, which may suggest the wild-type bacteria are causing actin rearrangements and cellular death. This effect has not been previously described for *A. baumannii*. The cells infected with the Δ*pglL* bacteria did not show a detectable phenotype, as A549 cells look identical to the uninfected control (Fig 2.33A). The rounding may indicate early stages of apoptosis or rearrangement of the cytoskeleton. To test if *A. baumannii* can cause actin polymerization as previously seen with other bacteria including *V. cholerae* (Sheahan *et al.*, 2004), we performed the same infection experiment, but instead harvested all cellular material, separated it on an SDS-PAGE, and visualized with an α-Actin antibody (Fig 2.33B). A single band corresponding to a monomer of actin (~37 kDa) was observed in all samples containing A549 cells, but not in the sample only containing bacteria. When the A549 cells were infected with *V. cholerae* V52, a strain previously demonstrated to cross-link actin, higher molecular weight bands were observed. However, these additional higher molecular weight bands were not observed in any of the other samples, suggesting that *A. baumannii* cannot cross-link actin of Eukaryotic cells in the conditions tested. Further study will be required to fully understand how *A. baumannii* interacts with Eukaryotic cells and causes cellular apoptosis and necrosis.

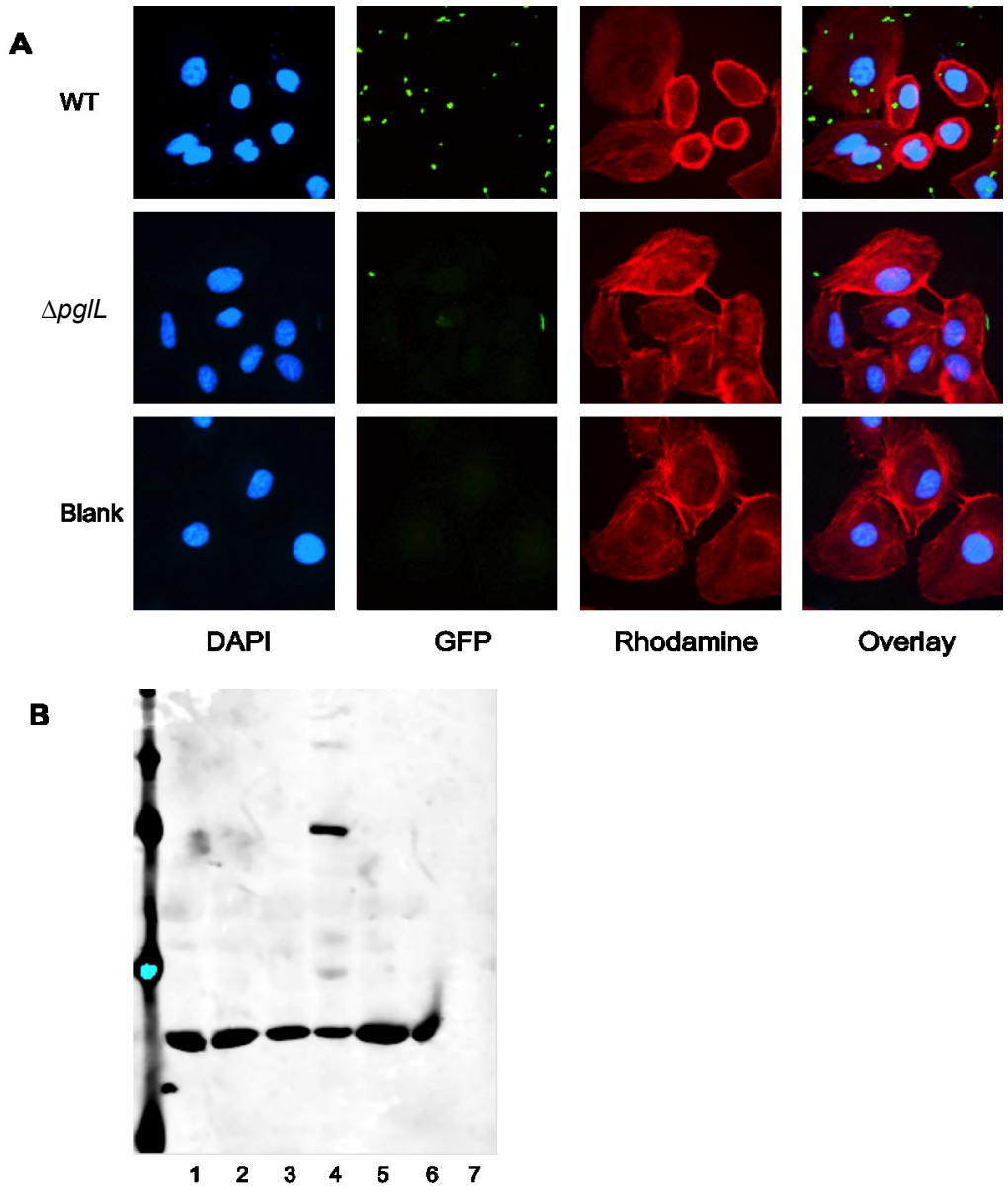

Figure 2.33 ***A. baumannii*** **WT Appears to Cause Cell Rounding of A549 Cells, but does not Cross-Link Actin** ***in vivo***. A) Eukaryotic cells seeded on coverslips were stained with rhodamine-phallodin (actin) and DAPI (DNA) and infected with GFP *A. baumannii* strains (Green). A549 cells infected with wild-type *A. baumannii* cells for 4 hours show significant cell rounding, while A549 cells infected with glycosylation deficient *A. baumannii* show no difference in cellular structure than uninfected. Uninfected A549 cells show normal epithelial morphology with large filamentous actin structures. B) Western blot of A549 cells infected with different bacterial strains. Strains are as follows: 1-WT, 2-Δ*pglL*, 3- Δ*vasK*, 4- *V. cholerae* V52, 5- *E. coli* DH5α, 6- uninfected A549 cells, 7-WT *A. baumannii* only

2.4 Discussion

Isolation of MDR strains of *A. baumannii* is increasing at impressive rates. Despite its growing incidence as nosocomial pathogen, only a few *A. baumannii* virulence factors have been characterized. In this article we describe a general *O*-glycosylation system in *A. baumannii* ATCC 17978. In most bacterial species known to synthesize glycoproteins, glycosylation is restricted to a few proteins including adhesins, flagellins or pilins (Nothaft & Szymanski, 2010, Iwashkiw *et al.*, 2013). Only a few general glycosylation systems in which more than a single protein is glycosylated have been characterized. In *C. jejuni* more than 65 proteins are *N*-glycosylated with the same heptasaccharide. Here, inactivation of the glycosylation pathway does not have an effect on growth *in vitro*, but does reduce adhesion and invasion to cells in culture, and affects chicken and mice colonization (Szymanski *et al.*, 2002). In *Neisseria gonorrhoeae* at least 12 proteins were shown be to *O*-glycosylated with a highly variable glycan structure (Borud *et al.*, 2011). The glycan has recently been shown to be important for infection of cervical epithelial cells (Jennings *et al.*, 2011). *Bacteroides fragilis* also has a general *O*-glycosylation system, where hundreds of proteins are predicted to be glycosylated (Fletcher *et al.*, 2011). Inactivation of this glycosylation system results in severe growth defects *in vitro* (Fletcher et al., 2009). It was then not surprising to see that the glycosylation mutant strain was outcompeted by the wild-type strain in gnotobiotic mice colonization experiments. In *A. baumannii*, seven proteins are *O*-glycosylated by the PglL OTase encoded by the A1S_3176 gene. Cells unable to perform protein glycosylation do not show any differential growth phenotype *in vitro*, while exhibiting a diminished capacity to form biofilms and reduced virulence in *D. discoideum, G. mellonella*, and murine septicemia pathogenesis models systems.

Two glycoproteins were identified using 2D-DIGE. To our knowledge, this is the first time this technique is applied to study bacterial glycoproteomics. The structure of the glycan used to decorate these proteins in *A. baumannii* was determined by a combination of MS and NMR techniques. The sugar was determined to be a pentasaccharide of the formula β-GlcNAc3NAcA4OAc-4-(β-GlcNAc-6-)-α-Gal-6-β-Glc-3-β-GalNAc-S/T (Fig 2.20). The glycan contains a terminal *O*-acetylated glucuronic acid derivative that is negatively charged and has not previously been described. A similar monosaccharide was found in *Pseudomonas aeruginosa* and *Bordetella pertussis* (Knirel & Kochetkov, 1994). Of the glycoproteins identified, only one (A1S_1193; MotB) has any significant homology outside of the genus *Acinetobacter*, with the remaining being annotated as hypothetical proteins. MotB has homology with proteins such as Pal from *Haemophilus influenzae* that have been shown to bind to peptidoglycan and stabilize the outer membrane (Parsons *et al.*, 2006). Functional characterization of *A. baumannii* glycoproteins will be crucial to explain the phenotypes associated with lack of glycosylation.

We identified the genomic locus responsible for the synthesis of the *O*-glycan of *A. baumannii* ATCC 17978, and this locus is conserved throughout the *Acinetobacter* genus. Further work has demonstrated that this glycan is also utilized to produce a Type I capsular polysaccharide (Lees-Miller et al., 2013). Our proposed bifurcated pathway model (Fig 2.22) poses an interesting challenge regarding the regulation of these two processes in *A. baumannii*: how are these two pathways synchronized in the cell? Likely, there is a mechanism leading to the selective *O*-glycosylation of proteins with a single pentasaccharide. In *P. aeruginosa*, O antigen and pilin glycosylation employ the same glycan structure (Castric *et al.*, 2001). In this system, the enzymatic activity of the PilO *O*-OTase is restricted to monomers of the O-antigen oligosaccharide, and regulation is due to

the enzyme being unable to transfer long polysaccharides onto pilin (Faridmoayer et al., 2007). In *E. coli* O9, single subunits of the K30 capsular polysaccharide antigen can be found as O-antigen in the LPS (Drummelsmith & Whitfield, 1999). In this strain, polymerized K antigen is detected as a component of the LPS only in a Wza mutant strain, suggesting that polymers of K antigen are not accessible to the WaaL ligase by sequestration in the CPS channel export machinery (Drummelsmith & Whitfield, 1999). By functionally reconstituting the *A. baumannii O*-glycosylation system in *E. coli,* we demonstrated that PglLAb has relaxed glycan specificity, and is able to transfer glycans with different compositions, reducing monosaccharides, and mono- and polysaccharides (Fig 2.22). We also observed in *A. baumannii* that glycoproteins can be modified with up to ~10 monosaccharides (2x the *O*-glycan in strain ATCC 17978), suggesting that the CPS export machinery sequesters UDP-linked oligosaccharides with more than this size. We hypothesize that once the glycan is larger than 10 monosaccharides, the carbohydrate structure would be interacting with the channel, and thus not available for $PglL_{Ab}$ to utilize for protein glycosylation.

Biofilms are proposed to be a virulence factor that is associated with increased antibiotic resistance, pathogenicity, and persistence of a bacterial population (Johnson, 2008, Bazaka *et al.*, 2011, Hoiby *et al.*, 2011). We have found that *O*-glycosylation enhances biofilm formation by *A. baumannii* ATCC 17978. Biofilm formation is a multistep process that involves an initial weak association leading to an irreversible attachment, which leads eventually to a complex maturation into sophisticated superstructures (Sauer *et al.*, 2002). We observed by flow cell and confocal imaging that glycosylation enhances the initial attachment as well as mature biofilm mass and density. It is tempting to speculate that glycans of the glycoproteins may have a function in cell-to-cell adhesion

(O'Toole & Kolter, 1998). Further work will elucidate in which aspect protein glycosylation is required for efficient biofilm formation.

The basic mechanisms of phagocytic cells are used in both amoebae and mammalian macrophages. As an infection model, the amoebae *D. discoideum* is considered a primitive macrophage. *D. discoideum* cells were unable to predate on *A. baumannii* WT lawns, but were able to efficiently predate on lawns of the glycosylation-deficient bacteria (Fig 2.27). It is uncertain how protein *O*-glycosylation protects *A. baumannii* from *D. discoideum* but we can hypothesize that glycosylation may help in the inhibition of phagocytosis by the amoebae, and/or prevent bacterial lysis by reactive oxygen species produced by the amoebae (Cosson & Soldati, 2008). Another possibility is that glycosylation of certain proteins is required to interfere with bacterial degradation and intracellular vesicle transport and/or fusion, as shown for *Legionella* (Bozue & Johnson, 1996). We also analyzed if protein *O*-glycosylation plays a role in pathogenesis in *G. mellonella* larvae (Fig 2.28). This model system has been recently shown to recreate the mammalian humoral immune system, with similar antimicrobial peptides, toll-like receptors, and the complement-like mechanism of melanization (Kavanagh & Reeves, 2004). Similar to the *D. discoideum* model, *A. baumannii* Δ*pglL* strain was unable to kill *G. mellonella*. *O*-glycosylation could mediate killing of the larvae by stabilizing the bacterial outer membrane of *A. baumannii*, which could prevent killing by antimicrobial peptides. The negative charges of the glycan chains could play a role in this process. Alternatively, glycosylation could mask signals detected by the larvae or prevent phagocytosis by *G. mellonella* haemocytes, among other possibilities. The involvement of glycoproteins in virulence is further supported by the demonstration that the Δ*pglL* strain is outcompeted by wild type bacteria in a murine septicemia model.

Since *A. baumannii* is primarily a human pathogen, we assayed its ability to affect the human alveolar epithelial cell line (A549) in cell tissue experiments. Since it has been shown *A. baumannii* can kill eukaryotic cells by similar work, we hypothesized protein glycosylation could be involved in several different aspects of bacterial virulence (Fig 2.34). We determined that protein glycosylation did have a role in virulence towards A549 cells by using acridine orange/ Ethidium bromide counter staining. Invasive bacteria were able to kill ~30% of the A549 cells after 24 hours incubation, compared to ~5% for the glycosylation deficient strain. Previous work observed *A. baumannii* ATCC 17978 cells to be able to kill ~50% of A549 cells by monitoring mitochondrial reduction activity, which may be a more sensitive assay (Smani *et al.*, 2013). Regardless, protein glycosylation deficient bacteria were not able to cause apoptosis and/or necrosis at a similar level as the WT, suggesting that glycosylation could be important for one of the proposed steps in our model. We observed no difference between the WT and Δ*pglL* strains for ability to adhere and invade A549 cells by collecting and doing plate counts of surviving bacterial cells. The levels of adhesion and invasion for the WT strain are similar to previously published results with a similar strain (ATCC 19606) (Gaddy et al., 2009, Gaddy et al., 2012). We did not observe any significant difference for the levels of adherence and invasion for the Δ*pglL* strain, suggesting that glycosylation isn't important for these steps of virulence. We next tested the *A. baumannii* strains for their ability to cause Eukaryotic cell rounding and actin polymerization by immunofluorescence. It has been shown that bacteria that possess a Type VI secretion system can cause this phenotype, and recent work demonstrated that *A. baumannii* has a functional type VI secretion system (Weber *et al.*, 2013). We observed that A549 cells infected with WT bacteria had a pronounced cellular rounding phenotype, with thicker actin walls surrounding the nucleus. Since we did not observe this phenotype

in the Δ*pglL* or uninfected controls, we hypothesized that *A. baumannii* could cause actin polymerization in eukaryotic cells after internalization. However, Western blot analysis of the A549 cells showed no evidence for this phenotype, and actin polymerization was only observed when the A549 cells were infected with *V. cholerae* V52. It appears that *A. baumannii* requires protein glycosylation for causing apoptosis, necrosis, and eukaryotic cell rounding, but is not required for attachment or invasion. Further work will be required to elucidate the importance of glycosylation in *A. baumannii* virulence. Further work using strains carrying mutations in individual glycoproteins will help to elucidate the exact role of protein glycosylation in pathogenesis.

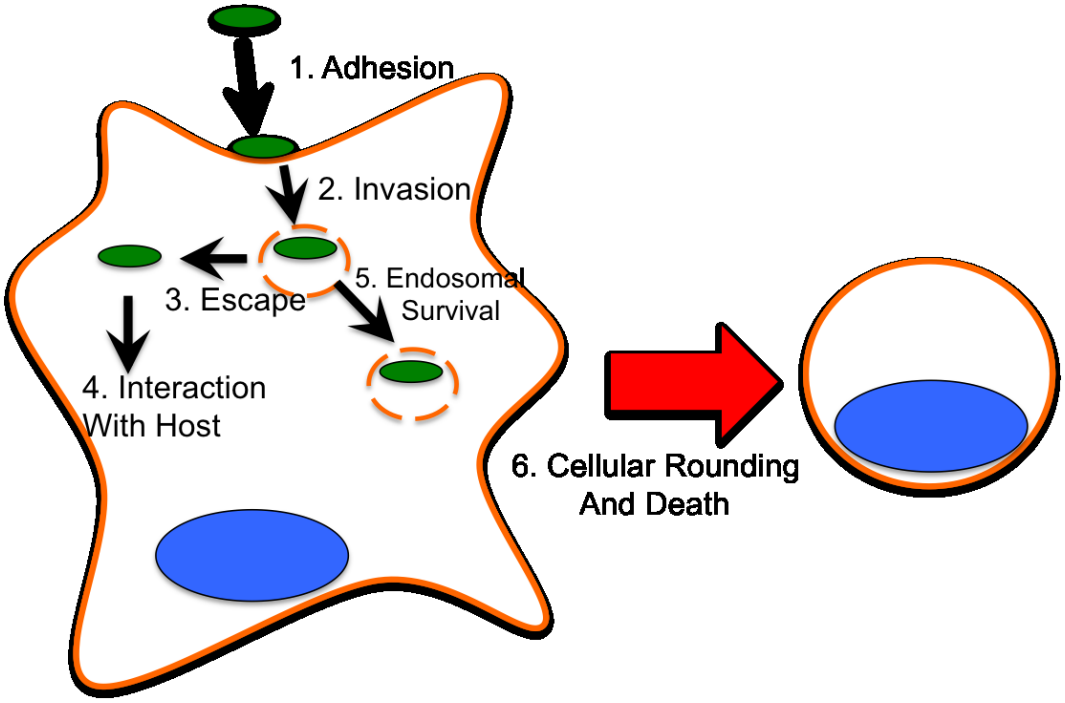

Figure 2.34 Proposed Model for *A. baumannii* Pathogenesis Towards Eukaryotic Cells. Since *A. baumannii* appears to require *O*-glycosylation to cause Eukaryotic cell rounding and death, we hypothesize that it would be required for one of the described steps of infection. Experimental evidence suggests that *O*-glycosylation is not required for adhesion or invasion of A549 cells. Therefore, it appears that glycosylation is important for either endosomal survival or escape, and possibly interaction with the host to cause cellular death.

Glycoproteins are usually immunodominant in bacteria, and therefore, the glycoproteins identified in this study may be the base of future vaccine formulations and diagnostic methods. The prevalence of the *O*-glycosylation machinery in *Acinetobacter* sp., together with its role in virulence in the three different pathogenesis models, suggest that protein *O*-glycosylation represents a novel target for the development of antibiotics that could be key to prevent further dissemination of this emerging human pathogen, which has become a major threat to our healthcare systems.

2.5 Materials and Methods

2.5.1 Bacterial strains, plasmids, growth conditions, and antimicrobial agents.

The bacterial strains and plasmids used in this study are listed in Table 3. *A. baumannii* strains were grown in Luria Bertani broth/agar at 37°C. The antibiotics ampicillin (Ap) 100 µg/mL, gentamicin (Gm) 50 µg/mL, and tetracycline (Tc) 5 µg/mL were added for selection as needed.

Table 2.3. List of Strains, Plasmids, and Primers.

Strains	**Reference**
A. baumannii ATCC 17978	(Piechaud & Second, 1951)
A. baumannii ATCC 17978 Δ*pglL*	This work
Plasmid	**Reference**
pEXT20	(Dykxhoorn *et al.*, 1996)
pWH1266	(Hunger *et al.*, 1990)
pFLP2	(Hoang *et al.*, 1998)
pSPG1	(Ugalde *et al.*, 2000)
Primers	**Sequence (5'-3')**
pglL$_{fwd}$ EcoRI	gcgaattcatgttttctagtgaaatgggtgcctttgcagcag
pglL$_{rev}$ XbaI 10His	gaggaattacattcggctttggatacaccaccaccaccaccaccaccaccaccacca ctaatctagaaatt
aacC1$_{int\ rev}$	catcgcgcttgctgccttcgaccaagaagc
K/O *pglL*$_{fwd}$ EcoRI	gagaattcatgaatgcacaaaaaggttttacattaattgaactcatg
K/O *pglL*$_{rev}$ XbaI	ccgagttgttcaattccccaatcattaaacatacgagtctagatgt

2.5.2 Construction of *A. baumannii* Δ*pglL* knockout

In order to create a Δ*pglL* via homologous recombination, we cloned a ~3500 bp fragment consisting of ~1000 bp upstream and downstream of A1S_3176 into pEXT20 using primers K/O *pglL*$_{fwd}$ and K/O *pglL*$_{rev}$ from *A. baumannii* ATCC 17978 genomic DNA (Table 3). The construct was subsequently subcloned from pEXT20 into pFLP2. We then digested pFLP2-*pglL* with PsiI and replaced A1S_3176 with a SmaI excised Gentamicin resistance cassette (*aacC*1) from pSPG1 (Ugalde et al., 2000). The plasmid pFLP2 does not replicate in *A. baumannii* ATCC 17978. This final construct was transformed into electro-competent *A. baumannii* WT cells and selection for a single recombination event was analyzed using media supplemented with gentamicin. Positive colonies were grown in 5 mL LB at 37^{o}C for 72 hours, with 1/1000 re-inoculations into fresh LB media every 24 hour period. After 72 hours, the liquid culture was plated on LB agar supplemented with gentamicin and 10% sucrose to select for a double recombination event. Colony PCR

using both internal and external primers showed the allelic exchange of A1S_3176 with *aacC1*, generating a knockout mutant of *A. baumannii pglL*.

2.5.3 SDS-PAGE and Periodic acid stain (PAS) analysis of membrane extracts

Bacterial cultures were pelleted by centrifugation for 15 mins at 10,000 x g, washed with PBS, resuspended in PBS, and subsequently lysed by French Press. Unbroken cells were pelleted by centrifugation for 15 mins @ 5,000 x g. The supernatant was ultracentrifugated for 1hr @ 100,000 x g (4^{o}C) to pellet cell membrane. Samples were quantified by Bradford protein quantification (Biorad) and analyzed on a 12% SDS-PAGE. The PAS stain protocol used was previously described (Cagatay & Hickford, 2008).

2.5.4 LPS extraction

LPS was extracted according to Marolda *et al* (Marolda *et al.*, 2006). Samples were resuspended in 50 µL of dH_20 and analyzed by Silverstain on a 15% SDS-PAGE.

2.5.5 Lectin Blots of membrane extracts

Membrane extracts were isolated from both WT and Δ*pglL* strains, separated by 15% acrylamide SDS-PAGE, and transferred to a nitrocellulose membrane as previously described. Membranes were blocked with Odyssey blocking buffer (Licor) for 1 hour at room temperature. Lectins were incubated with the membrane (1:500 dilution) for 1 hours in TBS (10mM Tris pH 7.4, 150mM NaCl)+ 0.1% Triton X-100 with 0.1mM $CaCl_2$, $MgCl_2$, and $MnCl_2$. Membranes were washed 3x 10 mins with TBS +0.1% Triton X-100, and incubated with streptavidin IR-800 (Licor, 1:4000 dilution) for 1 hour at room temperature. Membranes were washed 2x 10 mins with TBS +0.1% Triton X-100 and visualized with Licor Odyssey Imaging system.

2.5.6 Anion Exchange Chromatography enrichment of glycoproteins by MonoQ column.

Membrane extracts were obtained from overnight cultures of *A. baumannii* strains by cell disruption and ultracentrifugation of the supernatant. Membrane proteins were solubilized overnight in PBS+ 1% Triton X-100, and lipids were removed by ultracentrifugation at 100,000 x g for 1 hr. Soluble membrane proteins were diluted to a concentration of 0.5% Triton X-100, and loaded onto a MonoQ column at a flow rate of 1 mL/min (ATKA explorer). After all of the sample was loaded, bound proteins were eluted with a linear gradient to a maximum concentration of 1M NaCl. Fractions were assayed for protein and glycosylated material by silverstain and PAS glycan stain.

2.5.7 2D-DIGE analysis of *A. baumannii* membrane extracts

Lipid-free membranes were obtained for 2D-DIGE analysis according to (Pessione et al., 2009). The material was resuspended in: 6.5 M Urea, 2.2 M thiourea, 1% w/v ASB-14, 5 mM Tris-HCl pH 8.8, 20 mM DDT, 0.5 % IPG buffer. The samples were labeled using CyDye minimal labeling protocol (Amersham Biosciences). *A. baumannii* WT membranes were labeled with Cy5 and Δ*pglL* were labeled with Cy3. Samples were quantified by 2D-Quant kit (GE Healthcare) and 600 µg of each WT and Δ*pglL* membranes were mixed in Destreak solution (GE Healthcare) to a final volume of 450 µL. 24 cm pH 3-11 NL IPG strips were simultaneously rehydrated and sample loaded for 24 hrs at room temperature in the dark. Isoelectric focusing was done using the Ettan IPGphor system for a total of 56,000 Vhr in the dark. The strip was then incubated in 10 mL of equilibration solution (2% SDS, 50mM Tris-HCl, 6 M Urea, 30% (v/v) glycerol, 0.002% bromophenol blue) for 15 mins with 100 mg DTT and then 10 mL equilibration solution with 250 mg iodoacetamide.

The strip was then sealed into a DALT 12.5 precast gel with 0.5% agarose. The system was run at 2.5 W/gel for 30 mins, the 17 W/gel until the dye front exited the bottom. The gel was visualized using FLA-5000 (FujiFilm) and the images analyzed by ImageQuant 5.0. The gel was subsequently stained with Coomassie brilliant blue, and individual spots excised and prepared for mass spectrometry.

2.5.8 MALDI-TOF/TOF MS and MS/MS analysis of glycoproteins

Samples were in gel tryptically-digested and the peptides were desalted using C_{18} Zip-Tips and eluted with 60% CH_3CN / 40% H_2O. Samples were spotted on a Bruker Daltonics MTP ground steel or Bruker Daltonics MTP AC600 Anchorchip target plate and air dried. 1µL for ground steel and 0.4 µL for the AC600 target of 2,5-dihydroxybenzoic acid (DHB, 10mg/mL in 30% H_2O, 70% CH_3CN) was spotted on top and allowed to dry. Mass spectra were obtained in the positive mode of ionization using a Bruker Daltonics (Bremen, GmbH) UltrafleXtreme MALDI TOF/TOF mass spectrometer. The FlexAnalysis software provided by the manufacturer was used for analysis of the mass spectra.The MS/MS spectra were obtained manually. The exact m/z used as the precursor m/z for MS/MS was determined first on a Bruker Daltonics (Billerica, MA) Apex Qe MALDI FTICR MS instrument and the MS/MS spectrum was automatically re-calibrated based upon this m/z.

2.5.9 MALDI TOF-TOF MS characterization of the *A. baumannii O*-glycan from membrane extracts

Lipid free membrane extracts were digested for 72 hrs at 37^{o}C with 2 µL Pronase E (20 mg/mL) being freshly added every 24 hrs. Glycosylated peptides were enriched using Active Charcoal Micro SpinColumn (HARVARD Apparatus) Briefly, the column was prewashed 3x with 400 µL of 0.1% TFA in of 80% ACN and 20% ddH2O and centrifuged

at 500 RCF for 2 minutes. The column was equilibrated 3x using 400 µL of H_2O. The sample was loaded 3x at 500 RCF for 2 minutes. The column was washed 2x with 200 µL of ddH_2O at 500 RCF for 2 minutes. The glycan was eluted 3x with 100µL 0.1% TFA in 50% ACN and 50% H2O at 1000 RCF for 2 minutes. The sample was dried by vacuum centrifugation and analyzed by MALDI-TOF/TOF MS and MS/MS.

2.5.10 ß-Elimination and Permethylation of *A. baumannii O*-glycan

The *A. baumannii O*-glycan obtained from Pronase E digested membrane was released by ß-elimination, and the free glycan was modified by permethylation (Kang *et al.*, 2008, Alley *et al.*, 2010). Briefly, NaOH beads were suspended in ACN and loaded into a spin column (HARVARD Apparatus) up to about 1 cm from the top. The column was washed with DMF and centrifuged for 2 minutes at 500 RCF. The previously lyophilized glycan was resuspended in 100 µL of DMR/CH_3I/H_20 (70:25:5), loaded onto the column, allowed to react statically for 15 mins, then centrifuged for 2 mins at 500 RCF. Another 25 µL of CH_3I was added to the sample, and was reapplied to the column statically for another 15 mins, and eluted by centrifugation. The column was washed twice with 100 µL of ACN, and the elutant was to the sample. The permethylated glycan was extracted by liquid/liquid extraction. 300 µL of dichloromethane was added to the sample in a glass vial, and 1 mL of 0.5M NaCl was added. The solution was vortexed, then centrifuged to separate the organic and aqueous phases. The aqueous phase was removed, and the procedure was repeated twice. The same procedure was repeated twice with HPLC grade H20, and the pooled aqueous phase was dried in a speedvac, and analyzed by MALDI TOF-TOF MS and MS/MS.

2.5.11 Purification of glycans for NMR analysis.

For NMR analysis glycoproteins were digested with a large excess of proteinase K at pH 8 (adjusted by addition of ammonia) at 37 °C for 48 hours. Products of digestion or free oligosaccharides were separated on Sephadex G-15 column (1.5x60 cm) and each fraction eluted before salt peak was dried and analyzed by ^{1}H NMR. Fractions containing desired products were separated by anion exchange chromatography on Hitrap Q column (5 mL size, Amersham) and glycan eluted with a linear gradient of NaCl (0-1 M, 1 h). Desalting was performed on Sephadex G15 prior to analysis by NMR.

2.5.12 NMR spectroscopy analysis

NMR experiments were carried out on a Varian INOVA 600 MHz (^{1}H) spectrometer with 3 mm gradient probe at 25 °C with acetone internal reference (2.225 ppm for ^{1}H and 31.45 ppm for ^{13}C) using standard pulse sequences DQCOSY, TOCSY (mixing time 120 ms), ROESY (mixing time 500 ms), HSQC and HMBC (100 ms long range transfer delay). AQ time was kept at 0.8-1 sec for H-H correlations and 0.25 sec for HSQC, 256 increments was acquired for t1. Assignment of spectra was performed using Topspin 2 (Bruker Biospin) program for spectra visualization and overlap. Monosaccharides were identified by COSY, TOCSY and NOESY cross peak patterns and ^{13}C NMR chemical shifts. Aminogroup location was concluded from high field signal position of aminated carbons (CH at 45-60 ppm). Connections between monosaccharides were determined from transglycosidic NOE and HMBC correlations.

2.5.13 Protease digestion and enrichment of glycopeptides by ZIC-HILIC

Dried membrane protein-enriched fractions were resuspended in 6 M urea, 2 M thiourea, 40 mM NH_4HCO_3. Samples were reduced, alkylated, digested with Lys-C (1/200 w/w) and

then trypsin (1/50 w/w) as described previously (Scott et al., 2011b). Digested samples were then dialyzed against ultra-pure water overnight using a Mini Dialysis Kit with a molecular mass cut off of 1000 Da (Amersham Biosciences, Buckinghamshire, UK) and on completion were collected and lyophilized. ZIC-HILIC enrichment was performed according to (Scott et al., 2011b) with minor modifications. Micro-columns composed of 10 µm ZIC-HILIC resin (Sequant, Umeå, Sweden) were packed into P10 tips on a stage of Empire C_8 material (Sigma) to a bed length of 0.5 cm and washed with ultra-pure water prior to use. Dried digested samples were resuspended in 80% acetonitrile (ACN), 5% formic acid (FA) and insoluble material removed by centrifugation at 20,000 x *g* for 5 min at 4ºC. Samples were adjusted to a concentration of 2 µg/µL and 100 µg of peptide material loaded onto a column and washed with 10 load volumes of 80% ACN, 5% FA. Peptides were eluted with 3 load volumes of ultra-pure water into low-bind tubes and concentrated using vacuum centrifugation.

2.5.14 Identification of glycopeptides using reversed phase LC-MS and HCD MS-MS

ZIC-HILIC fractions were resuspended in 0.1% FA and loaded onto a Acclaim PepMap 100 µm C18 Nano-Trap Column (Dionex Corporation, Sunnyvale, CA) for 10 min using a UltiMate 3000 intelligent LC system (Dionex Corporation). Peptides were eluted and separated on 20 cm, 100 µm inner diameter, 360 µm outer diameter, ReproSil – Pur C_{18} AQ 3 µm (Dr. Maisch, Ammerbuch-Entringen, Germany) in house packed column. Enriched peptides derived from tryptic digests were analysed using an LTQ-Orbitrap Velos (Thermo Scientific, San Jose CA). Samples were eluted using a gradient from 100% buffer A (0.5% acetic acid) to 40% buffer B (0.5% acetic acid, 80% MeCN) over 120 mins at a constant flow of 200 nL / min enabling the infusion of sample in the instrument using ESI. The LTQ-Orbitrap Velos was operated using Xcalibur v2.2 (Thermo Scientific) with a

capillary temperature of 200°C in a data-dependent mode automatically switching between MS ion trap CID and HCD MS-MS. For each MS scan, the three most abundant precursor ions were selected for fragmentation with collision induced dissociation (CID), activation time 30ms and normalized collision energy 35, followed by high collision dissociation (HCD), activation time 30ms and normalized collision energy 45. MS resolution was set to 60,000 with an ACG of $1e^6$, maximum fill time of 500 ms and a mass window of *m/z* 600 to 2000. MS-MS fragmentation was carried out with an ACG of $3e^4/2e^5$ for CID/HCD and maximum fill time of 100ms/500ms CID/HCD. For HCD events an MS resolution of 7500 was set. A total of six HILIC enrichments were performed and analysis by the above protocol.

2.5.15 Database interrogation of identified glycopeptides

Raw files were processed within Proteome Discover version 1.0 Build 43 (Thermo Scientific) to generate .mgf files. To identify possible glycopeptides within exported scans, the MS-MS module of GPMAW 8.2 called 'mgf graph' was utilized. This module allowed the identification of all scan events within the generated .mgf files containing the diagnostic oxonium *m/z* 301.10 ion. These scan events were manually inspected and identified as possible glycopeptides based on the presence of the deglycosylated peptide ion with a tolerance of 20 ppm. To facilitate glycopeptide assignments from HCD scan events, ions below the mass of the predicted deglycosylated peptides were extracted with Xcalibur v2.2 using the Spectrum list function. Ions with a deconvoluted mass above the deglycosylated peptide mass and ions corresponding to known carbohydrate oxonium ions such as 204.08 and 366.14 were removed in a similar approach to post-spectral processing of ETD data (Good *et al.*, Good *et al.*, 2009). MASCOT v2.2 searches were conducted via the Australasian Proteomics Computational Facility (www.apcf.edu.au) with the Proteobacteria

taxonomy selected. Searches were carried out with a parent ion mass accuracy of 20 ppm and a product ion accuracy of 0.02 Da with no protease specificity, instrument selected as MALDI-QIT-TOF (use of this instrumentation setting was due to the observation of multiple internal cleavage products, extensive NH_3 and H_2O loss from *a*, *b*, *y* ions, which are all included within this scoring setting) as well as the fixed modification carbamidomethyl (C) and variable modifications, oxidation (M) and deamidation (N). An ion score cut-off of 20 was accepted and all data were searched with the decoy setting activated generating a zero false positive rate generated against the decoy database.

2.5.16 *In vivo* protein glycosylation Assay and Western Blot Analysis

All protein glycosylation assays were performed in the *E. coli* strain CLM24 transformed with plasmids expressing $PglL_{Ab}$, A1S_1193, and the biosynthetic machinery for the glycan of interest. Cultures were grown at 37°C to an optical density of 0.4-0.6 OD_{600nm}, and induced with 0.1mM IPTG and/or 0.2% arabinose as required. Cultures induced with arabinose were re-induced again after 4 hours. Cultures were harvested after overnight growth in stationary phase, and whole cell lysates were solubilized with Urea buffer, and a total of 0.2 OD was separated by 12% acrylamide SDS-PAGE gel. Separated proteins were transferred to a nitrocellulose membrane by semi-wet electroblot transfer, and probed with both a monoclonal α-His and polyclonal α-glycan antibody. Membranes were visualized with the Odyssey® Infrared Imaging System (Li-Cor Biosciences, USA), allowing for the direct fluorescent detection at 685nm and 785nm wavelengths simultaneously. Secondary antibodies used were IRDye 800 Goat anti-Rabbit (polyclonal;red) and IRDye 680 Goat anti-Mouse antibodies.

2.5.17 Biofilm analysis using 96 well Polystyrene Plates

Cultures were grown overnight and re-inoculated at an OD_{600nm} 0.05 in 100 µL into replicates in a 96 well polystyrene plate (Costar). The cultures were subsequently grown without shaking for 48 hours at 30°C. Bacterial growth was determined by measuring the absorbance at OD_{600nm}. The cultures were removed and the wells washed with ddH_20, followed by the addition of 100 µL of 1% crystal violet in ethanol to stain the cells. The plate was incubated for 30 mins with gentle agitation, then thoroughly washed with ddH_20, and the stained biofilms solubilized with 100 µL of 2% SDS for 30 minutes with gentle agitation. The amount of biofilm formed was quantified by measuring the absorbance at OD_{580nm}. The data was normalized using the ratio between OD_{580nm}/OD_{600nm}.

2.5.18 Flow cell biofilm experiments, fluorescent staining and confocal laser scanning microscopy.

Flow cell experiments and fluorescent staining were performed as described previously by Seper et al. (Seper *et al.*, 2011). Briefly, the respective overnight cultures were adjusted to OD_{600} = 0.1 using 50-fold diluted LB (2%). Per channel, approximately 250 µl of the dilutions were inoculated. After static incubation for 2 h, flow of pre-warmed 2% LB (37°C) was initiated (3 ml/h). Biofilms were allowed to form for a time period of 24 h and were stained with SYTO 9 (Invitrogen) for visualization. Images of attached bacteria or biofilms were acquired using a Leica SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) with spectral detection and a Leica HCX PL APO CS 40x oil immersion objective (NA 1.25). For the SYTO 9 signal, the excitation wavelength was set at 488 nm and fluorescence emission was detected between 500-530 nm. Optical sections were recorded in 0.2 µm steps. For two-dimensional image visualization the Leica LAF and for

three-dimensional image processing the AMIRA software (direct volume rendering with VOLREN module) was used. Quantification of image stacks was performed using COMSTAT (http://www.comstat.dk) (Heydorn *et al.*, 2000) (M. Vorregaard et al., pers. comm.). For COMSTAT analysis at least six image stacks from three independent experiments were used.

2.5.19 *Dictyostelium discoideum* virulence assay

This assay was performed essentially as described by (Pukatzki *et al.*, 2002). Briefly, midlogarithmic cultures of *D. discoideum* were mixed with overnight cultures of bacteria to a final concentration of 1×10^3 amoebae ml^{-1}. 0.2 ml of the suspension was then plated on SM/5 agar containing 1% ethanol. Plates were incubated at room temperature and monitored for *D. discoideum* plaques for 3-5 days. Wild type bacteria are toxic to the amoebae. Appearance of plaques indicates attenuation.

2.5.20 *Galleria mellonella* virulence assay

This assay was performed as previously described (Peleg et al., 2009). *Galleria mellonella* larvae were bred in sterile conditions at 37°C by Dr. Andrew Kedde (University of Alberta). After injection of bacteria, larvae were incubated at 37°C, and the number of dead larvae were scored every 5 hours. Larvae were considered dead when they were nonresponsive to touch. This experiment is a representative of 3 biological replicates.

2.5.21 Murine bacterial competition model assay

A murine model of disseminated sepsis using BALB/c mice (16-20 grams) was used for bacterial challenge (Ko *et al.*, 2004, Fattahian *et al.*, 2011). *A. baumannii* strains were grown for 18 h at 37°C in Luria broth with appropriate antibiotics and adjusted to the

appropriate concentration in physiologic saline. Inoculums were prepared by mixing the bacterial suspensions 1:1 (v:v) with a 10% solution (w/v) of porcine mucin (Sigma, St. Louis, MO) which increases the infectivity of *A. baumannii*, allowing for a lower concentration of bacteria to be used (McConnell *et al.*, 2011, Batson *et al.*, 1950, McConnell & Pachon, 2010). Mice were injected intraperitoneally with 0.2 ml of the bacterial / mucin inoculums. Bacterial concentrations were determined by plating dilutions on Luria agar. The wild type strain lethal dose for 50% of animals was determined by the limit test where groups of 5 mice were infected with dilutions of bacteria, at a range of concentrations within 2 logs of a concentration of bacteria that had previously been shown to be lethal with this species of bacteria using a disseminated sepsis model.

An *in vivo* competition assay was used to compare fitness between the wt and Δ*pglL* strains (Lopez-Rojas et al., 2010, Smani et al., 2011, Lau et al., 2004). Liquid cultures containing individual strains were diluted and plated on LB agar. Mixed inoculums were established by mixing equal proportions of strains based on the OD_{600nm}. Once mixed the inoculums were serially diluted and plated on LB agar and LB agar with gentamycin to select for the Δ*pglL*. The expected ratio of CFU on LB compared to CFU on LB with gentamycin was 2:1.

For bacterial competition experiments *in vivo* an animal model of sepsis was used. Groups of 3 BALB/c female 16–20-g mice were inoculated intraperitoneally with 1 $x10^5$ CFUs of mixed inoculums (50% of each strain). Groups of 3 mice were sacrificed at 18 h after inoculation. Mice at 18 hours of infection were showing clinical signs of illness and were often moribund. Spleens were aseptically removed, weighed, and homogenized via passage through a cell strainer (BD falcon 70um cell strainer) in physiological saline before plating serial log dilutions on Luria agar plates for bacterial quantification. If the two

strains had equal fitness in vivo the ratio established prior to infection should be maintained.

2.5.22 A549 adhesion and invasion assays with *A. baumannii* WT and ∆*pglL* strains.

To determine bacterial adhesion, A549 human alveolar epithelial cells were grown in 5% CO_2 at 37°C in DMEM (Sigma Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS) until 70% confluence was observed in six-well plates with or without glass coverslips placed in the six-well plates. Confluent monolayers were washed twice with PBS before infection with 2×10^6 bacterial cells per well resuspended in DMEM 10% FBS (MOI of 10) and incubated for 2 hours at 37°C in 5% CO_2. For quantification of bacteria, the infected monolayers were washed three times with PBS and then lysed in 1 mL of PBS+ 0.1% Triton X-100. Lysates were serially diluted, plated on LB agar, and incubated at 37°C for 24 h. The following day, the colonies were counted to determine the number of bacteria that attached to A549 cells.

To determine bacterial invasion, A549 monolayers were grown in six-well plates, infected, and washed as described above for the adhesion assays. The monolayers were then treated with 2 mL of PBS supplemented with 200 µg/mL gentamycin for 30 mins. The cells were washed three times with PBS, and the cells were trypsinized and resuspended in PBS. A549 cells were subsequently lysed as previously described. Dilutions of the lysates were plated onto LB agar and incubated at 37°C for 24 h. The following day, the colonies were counted to determine the number of bacteria that invaded the A549 cells. The adhesion and invasion assays were repeated three times using fresh biological samples each time.

2.5.23 Acridine Orange/ Ethidium Bromide staining of A549 cells

To test is glycosylation if required for adhesion, A549 cells will be grown at 37^{o}C with different bacterial strains at an MOI of ~1:100. After 2h, the wells will be thoroughly washed with PBS, and all adhesive and invading bacterial cells will be removed from the eukaryotic cells by solubilisation with PBS +0.1% Triton X-100. The bacteria will be serially diluted and plated on agar to determine CFUs. To determine bacterial invasion, the same experiment will be repeated with the addition of media containing 200 µg/mL gentamicin after the 2h incubation to kill all non-intracellular bacteria. The eukaryotic cells will incubate for 2h, and then solubilized and quantified the same way as previously described.

2.5.24 Immunofluorescence of A549 cells infected with different bacterial strains

A549 cells were innoculated at 4x10^{5} cells/ml into 10 mL in a 10mm petri dish for 24 hrs in 10% FBS in DMEM. 45 mins before infection, media was removed and replaced with 1% FBS/DMEM. Bacterial cultures were grown till midlog phase and standardized to 1 OD600 per infection (works out to ~ 1x10^{8} CFU/mL). Slides were removed at specified time points, fixed with 3.7% formaldehyde solution, and kept in PBS until all time points were fixed. Tissue solubilized with 1% Triton X-100 in sterile PBS for 20 mins with gentle rocking. All slides were blocked with PBST +1% goat serum. Slides washed 3x10 mins in PBS. slides incubated with rhodamine (3 U) in blocking buffer for 35 mins. Slides washed 3 x 10 mins, then DAPI staining (1 in 15,000 of 5 mg/mL stock) for 2 mins, washed with PBS. Slides mounted using PPD solution and sealed with clear nail polish.

2.6 References

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Chapter 3

Exploiting *N*-Glycosylation for the Production of Glycoengineered Vaccines and Diagnostic Tools Directed Against Brucellosis

Portions of this chapter have been published.
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3.1 Introduction

Brucella sp., the causative agents of brucellosis, are Gram-negative, facultative intracellular α-proteobacteria (Pappas *et al.*, 2006, Fugier *et al.*, 2007, Ada & Isaacs, 2003). Three *Brucella* species, *B. abortus*, *B. melitensis,* and *B. suis* are the common species that cause human brucellosis (Table 3.1). They can also infect domestic livestock, causing miscarriages and sterility leading to significant economic loss (Young *et al.*, 2000, Corbel, 1997). Brucellosis is the most common bacterial zoonosis with over half a million new cases annually and high levels of abortions in cattle in developing countries (Franco *et al.*, 2007, Seleem *et al.*). In addition, *Brucella* sp. are considered highly effective biological weapons (Pappas et al., 2006). *B. abortus* is the causative agent for brucellosis in cattle and the second most common cause of human infections (Pappas, 2010). The current commercially available vaccines against *B. abortus* are attenuated strains, which are effective in livestock, but retain virulence to humans (Schurig *et al.*, 2002). Due to this and other disadvantages, such as the impossibility to discriminate between infected and vaccinated animals during immune-screening procedures, new vaccines against brucellosis are required. Among several promising vaccine candidates is a live attenuated strain lacking the phosphoglucomutase gene (Δ*pgm*), which is unable to assemble the O polysaccharide (Ugalde *et al.*, 2003)

Table 3.1: Summary of characteristics of *Brucella* infections in animals and humans (Woods, 2005)

***Brucella* spp.**	**1° reservoir**	**2° reservoir**	**Geographic Distribution**	**Human Exposure Activity**	**Pathogenicity to Humans**
Abortus	Cattle, Bison	Goats, Sheep, Dog, Human	Worldwide	Raw Dairy Foods, Animal Husbandry, Laboratory	Moderate
Melitensis	Goats, Sheep	Dog, Human	Latin America, Asia, Middle East	Raw Dairy Foods, Animal Husbandry, Laboratory	Highest
Suis	Pig (wild, Domestic)	Dog, Human, Cattle	SE Asia, Midwest USA, South America, Scattered	Pork Slaughter, Processing, Wild Pig Hunting, Laboratory	High

Immune responses directed towards surface polysaccharides are effective in preventing colonization and infection against several bacterial pathogens (Jones, 2005). However, to generate long-term protection in children, the polysaccharides must be covalently attached to an appropriate protein carrier (Jones, 2005, Mond *et al.*, 1995). The efficacy of conjugating bacterial polysaccharides to proteins is best exemplified by the *Haemophilus influenzae* type b conjugate vaccine, which has virtually eradicated the infections caused by this organism in most parts of the world (Jones, 2005). Indeed, glycoconjugate vaccines have also been used for the prevention and treatment of a diverse array of bacterial, viral, protozoan, parasitic, and cancerous diseases (Jones, 2005). Presently, the production of these conjugate vaccines requires intricate synthetic chemistry for obtaining, activating, and attaching the polysaccharides to protein carriers (Fig 1.9A) (Jones, 2005). The polysaccharides are either obtained from the target pathogen, or by laborious synthesis. Extraction of the polysaccharides from pathogenic organisms usually requires large cultures, which constitutes a major health hazard (Astronomo & Burton, 2010). Furthermore, when purifying O antigens, chemical removal of the endotoxin is required to prevent fever (Jones, 2005). In most cases, bacterial

polysaccharides are too complex to be synthesized efficiently by chemical methods, which make this process economically unfavorable (Astronomo & Burton, 2010). In the final stage of conjugation, chemical attachment of the carbohydrate to the protein often results in large and heterogeneous conjugates. In addition, a considerable amount of toxic waste is generated during the conjugation process (Astronomo & Burton, 2010). For these reasons, production of conjugate vaccines using conventional procedures is complex and the costs are prohibitive for global vaccination programs.

The O antigen of *B. abortus* and *B. suis* is a homopolymer of N-formylperosamine (Meikle *et al.*, 1989, Bundle & Perry, 1985). Only a few studies evaluating the suitability of conjugate vaccines against *Brucella* have been published. A conjugate vaccine obtained by covalently coupling the O-polysaccharide obtained from *B. melitensis* to bovine serum albumin (BSA) induced antibodies and was protective in mice (Jacques *et al.*, 1991). Nevertheless, because *Brucella* sp. requires class III biosafety facilities, production of glycoconjugates containing *Brucella* glycans in its native host is challenging and possibly unsafe. It has recently been established that conjugates containing polysaccharide from pathogenic bacteria can be produced in *E. coli* by exploitation of bacterial glycosylation systems. Bacterial OTases are enzymes capable of transferring glycan chains, including polysaccharides, from lipid carriers to proteins, in a process called OTase-dependent protein glycosylation (Nothaft & Szymanski, 2010). Previous work has demonstrated that *C. jejuni* PglB can transfer an array of glycans, including O antigens, from the lipid donors to carrier proteins (Feldman *et al.*, 2005). Due to their versatility, bacterial glycosylation systems can be seen as toolboxes for engineering novel glycoconjugates (Fig 1.9B). Conjugates produced by this method may constitute a new generation of vaccines,

circumventing most of the disadvantages of the conventional chemical methods, significantly reducing costs, and improving the reproducibility of the product obtained.

3.2 Results

3.2.1 Cross Reactivity Between *Brucella* and *Y. enterocolitica* O:9

The *B. abortus* and *B. suis* O antigens were previously characterized by genomic analysis, NMR, and serological assays and they appear to be identical to that of *Yersinia enterocolitica* O:9 (Figure 3.2) (Bundle & Perry, 1985, Bundle D.R., 1989, Bundle *et al.*, 1984). *Y. enterocolitica* O:9 is a Class II biosafety hazard organism and is easily manipulated and cultured, making it a suitable host for the production of the glycoconjugate protein with the N-formylperosamine homopolymer, which we hypothesize could cross-protect against brucellosis (Skurnik *et al.*, 2007). In some *Y. enterocolitica* strains, an additional "outer core" (OC) consisting of a shorter glycan chain is assembled onto the Und-PP carrier and subsequently ligated to lipid A. To confirm cross reactivity of the *Y. enterocolitica* O:9 (Table 3.3) and the *B. abortus* O antigens, LPS of both species were analyzed by SDS-PAGE and immunoblot (Fig 3.3). Our analysis included the WT *Y. enterocolitica* O:9 strain, plus three derivatives lacking the OC, the O antigen, or both glycan structures. LPS samples from of the *Y. enterocolitica* O:9 strains exhibited a different electrophoretic pattern according to the mutation carried by each strain. The double mutant strain only displayed a band corresponding to lipid A core (lane 1). The O antigen deficient strain exhibited a unique band that migrated slower than the lipid A core, as expected for the presence of the low molecular weight OC structure attached to the lipid A (lane 2). The OC minus strain only produced the high molecular weight homopolymer (lane 3), and the WT strain produced both glycan structures (lane 4; Fig 3.3A). Analysis of the LPS extracts using monoclonal antibodies (Fig 3.3B, C) demonstrated that only the

high molecular weight carbohydrate of Ye O:9 was reactive towards the Yst9-2 (anti-Ye O:9 antigen) monoclonal antibody (mAb; Fig 3.3B, lanes 3 and 4). The Yst9-2 mAb antibody also recognized the O antigen of *B abortus*, *B. melitensis* and *B suis*, confirming the cross reactivity of the LPS between the species (Fig 3.3B, Lanes 5-7). The M84 mAb directed against *B. abortus* O antigen also reacted with the Ye O:9 polysaccharide (Fig 3.3C, lanes 3 and 4). Interestingly, *B. melitensis* LPS did not react towards the M84 antibody (Fig 3.3C, lane 6). This result was not totally unexpected because although the *B. abortus* and *B. melitensis* polysaccharides have a similar composition, there are structural differences between their O antigens (Bundle *et al.*, 1987). These results confirmed that the O antigens of Ye 0:9 and *B. abortus* have a similar structure, and suggested that a conjugate carrying the Ye O:9 antigen could mount an immune response that may be cross-protective against *B. abortus*.

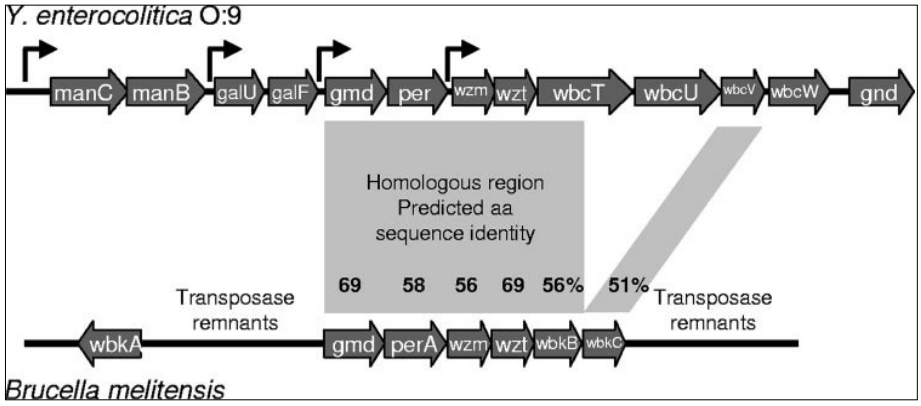

Figure 3.1. **Genetic Homology of LPS Synthesis Pathways Between *Y. enterocolitica* O:9 and *Brucella melitensis***. There is a significant homology between the LPS synthesis pathway genes for the two species, which suggests that the O-antigens are identical. Adapted from (Skurnik et al., 2007)

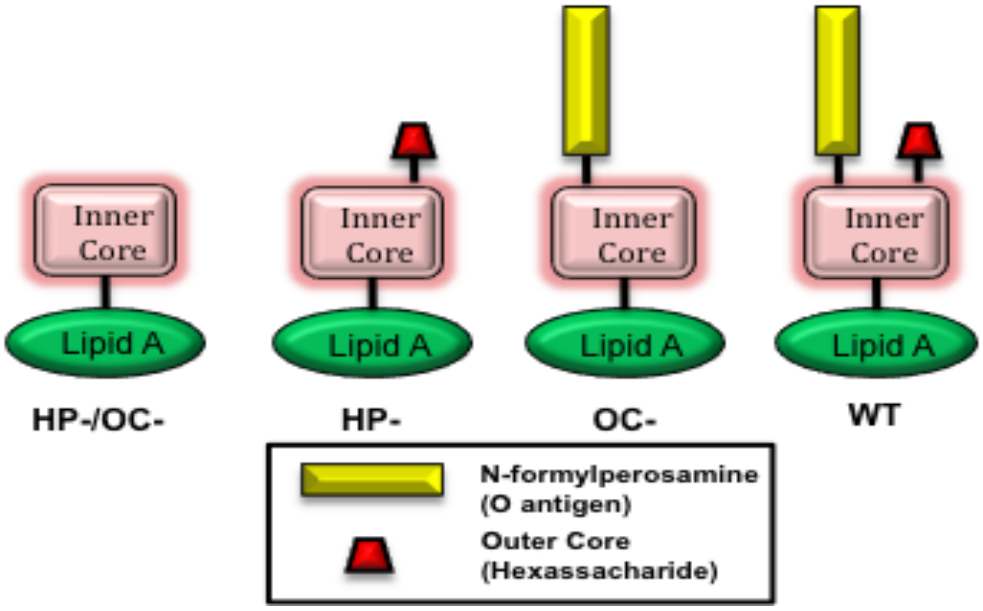

Figure 3.2 Schematic Representation of the Different Strains of *Y. enterocolitica* O:9 LPS production. *Y. enterocolitica* O:9 produces two different O antigens; Outer core (OC) and a homopolymeric O antigen (HP). Four different strains were obtained with different LPS profiles. For HP-/OC-, both glycan structures are not produced, in HP- only the outer core is produced, in OC- only the O antigen is synthesized, and in the WT both antigens are simultaneously expressed.

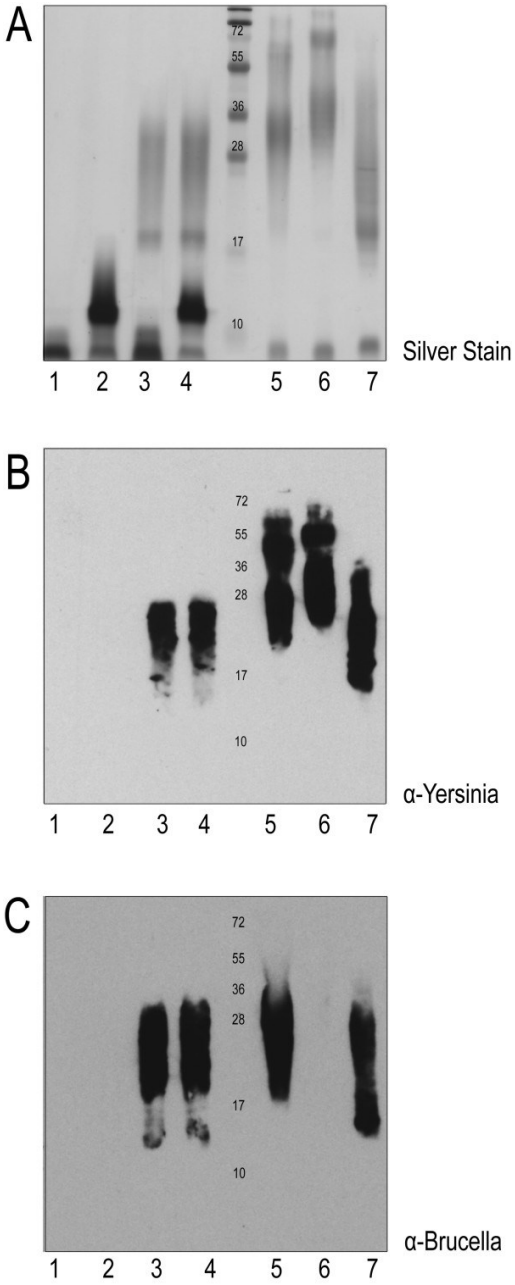

Figure 3.3 Cross Reactivity Between *Y. enterocolitica* O:9 and *Brucella* spp. LPS Samples. (0.2 OD/ sample loaded) on 15% SDS-PAGE: A) LPS silver stain analysis of samples of *Y. enterocolitica* 1) OC-/HP-, 2) HP-, 3) OC-, 4) WT; *Brucella* 5) *B. abortus*, 6) *B. melintensis*, and 7) *B. suis*. B) Immunoblot against the same samples with monoclonal α-*Yersinia* (Yst 9-2). C) Immunoblot of the same samples using monoclonal α-*Brucella* (M84). Cross reactivity between the two different genus' LPS is observed by both monoclonal antibodies reacting against the higher molecular weight homopolymeric N-formylperosamine polysaccharide.

3.2.2 Purification of Glycosylated AcrA from *Y. enterocolitica* O:9 Strains

In earlier work, *N*-glycosylated AcrA was synthesized in *E. coli* by co-expression of *C. jejuni* PglB and AcrA with an appropriate carbohydrate structure (Wacker *et al.*, 2006). In order to determine if we could transfer the Ye 0:9 carbohydrate structures to AcrA in Ye O:9, we transformed each of the strains with pMAF10, expressing PglB under an arabinose-inducible promoter, and pMH5, expressing a Hexa-His-tagged version of AcrA. Cultures of each transformed strain were grown and induced as required, and AcrA was purified from periplasmic extracts by affinity chromatography and analyzed by SDS-PAGE (Fig 3.4). Protein concentration was determined by Bradford Assay, and standardized the loading (Table 3.2). The single band visualized by Coomassie stain (Fig. 3.4A) suggested that AcrA was unglycosylated in the double mutant strain (lane 1), while the two glycosylation sites of AcrA were modified with OC in the O antigen mutant strain producing an additional two bands (lane 2). The large molecular weight O antigen was transferred to AcrA in the OC mutant strain (lane 3, and 3*), and both glycan structures were bound to AcrA in the Ye O:9 WT (lane 4). Additionally, the purified AcrA samples were analyzed by immunoblot using α-AcrA antibodies (Fig 3.4B). The different pattern observed in each sample confirmed that different glycans were attached in each strain. Interestingly, unglycosylated AcrA appeared as a double band (lane 1). The OC mutant strain displayed a pattern compatible with a poorly glycosylated form of AcrA with the O antigen (Fig 3.4A and 3.4B, lanes 3 and 3*). These conclusions were further supported by a Immunoblot of the same samples using the α-Yersinia O:9 (Yst9-2) and the α-Brucella O antigen (M84) monoclonal antibodies (Fig 3.4C and 3.4D, respectively). These results indicated the *C. jejuni* glycosylation system was efficiently reconstituted in *Y. enterocolitica* O:9. Although the OC- mutant would be the ideal strain for the generation of the conjugate carrying the O:9 antigen, the unexpectedly low AcrA glycosylation levels in this strain prevented its

utilization for the production of the conjugate. For this reason, the conjugate containing the Ye O:9 antigen was purified from the wild-type strain.

Table 3.2. Quantification of Glycoprotein Quantity Produced from Different *Y. enterocolitica* Strains

Strain	**Quantity of Glycoprotein Purified from 1 L starting culture (µg)**
WT	667.4
OC+ HP-	259.9
OC- HP-	12.4
OC- HP+	519.9

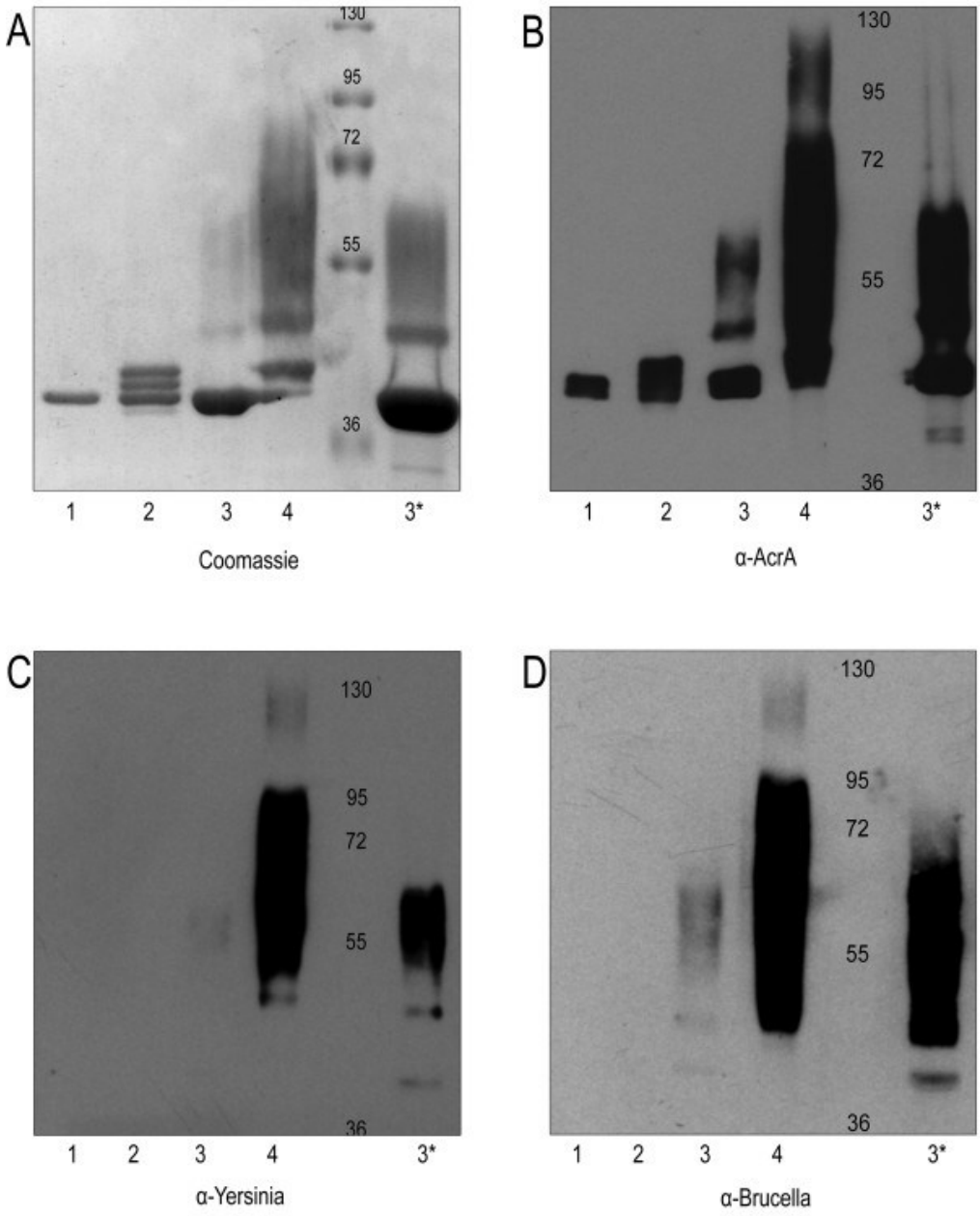

Figure 3.4 Proteins Carrying *Y. enterocolitica* O:9 O-Antigens are Immunoreactive Against α-*Yersinia* and α-*Brucella* Monoclonal Antibodies. Each strain was transformed with pMAF10 (*pglB*$_{Cj}$) and pMH5 (*acrA*), and glycosylated AcrA was purified from 1L of culture using Ni^{2+} affinity chromatography. After purification, samples were loaded onto a 10% SDS-PAGE gel and analyzed by: A) Coomassie brilliant blue (5μg/sample), immunoblot (2μg/sample) using B) α-AcrA, C) α-Ye O:9 (Yst9-2) mAb, or D) α-*Brucella* O antigen M84 mAb. Samples were purified from the following strains: 1) OC-/HP-, 2) HP-, 3) OC-, 4) WT, and 3*) 10x loaded volume of 3 (OC-).

3.2.3 Identification of the Carbohydrates Attached to AcrA by Mass Spectrometry

In order to fully characterize the carbohydrates attached to AcrA, mass spectrometry techniques were employed. AcrA was purified from Ye O:9 strains, separated by SDS-PAGE, the bands of interest were excised and in-gel digested with trypsin, and the resulting peptides analyzed by ESI-Q-TOF MS and MS/MS. Examination of the higher molecular weight smear from the Ye O:9 WT glycosylated AcrA by MS revealed a peak at 1954.7^{1+} *m/z*, and subsequent analysis of this peak by MS/MS identified the known glycopeptide DFNR modified with a carbohydrate moiety (Fig 3.5A). We identified a HexNAc-Hex disaccharide followed by a 173 *m/z* repeat, which corresponds to the N-formylperosamine homopolymer, attached to the tetrapeptide DFNR, which represents one of the known glycosylation sites of AcrA (Fig 3.5A). MS analysis of the glycoprotein produced by O antigen deficient strain revealed a peak of 1284.6^{3+} *m/z*. MS/MS analysis of this peak identified the second known glycosylated site of AcrA (AVFDNNNSTLLPGAFATITSEGFIQK) modified with the hexasaccharide HexNAc-HexNAc-Hex-Hex-HexNAc-Hex (Fig 3.5B). This hexasaccharide is known as the outer core and is also present in *Y. enterocolitica* O:3. Contrary to previously published work, the outer core of Ye O:9 is therefore not structurally homologous to that of *Y. enterocolitica* O:3, despite genetic homology (Skurnik, 2004, Muller-Loennies *et al.*, 1999).

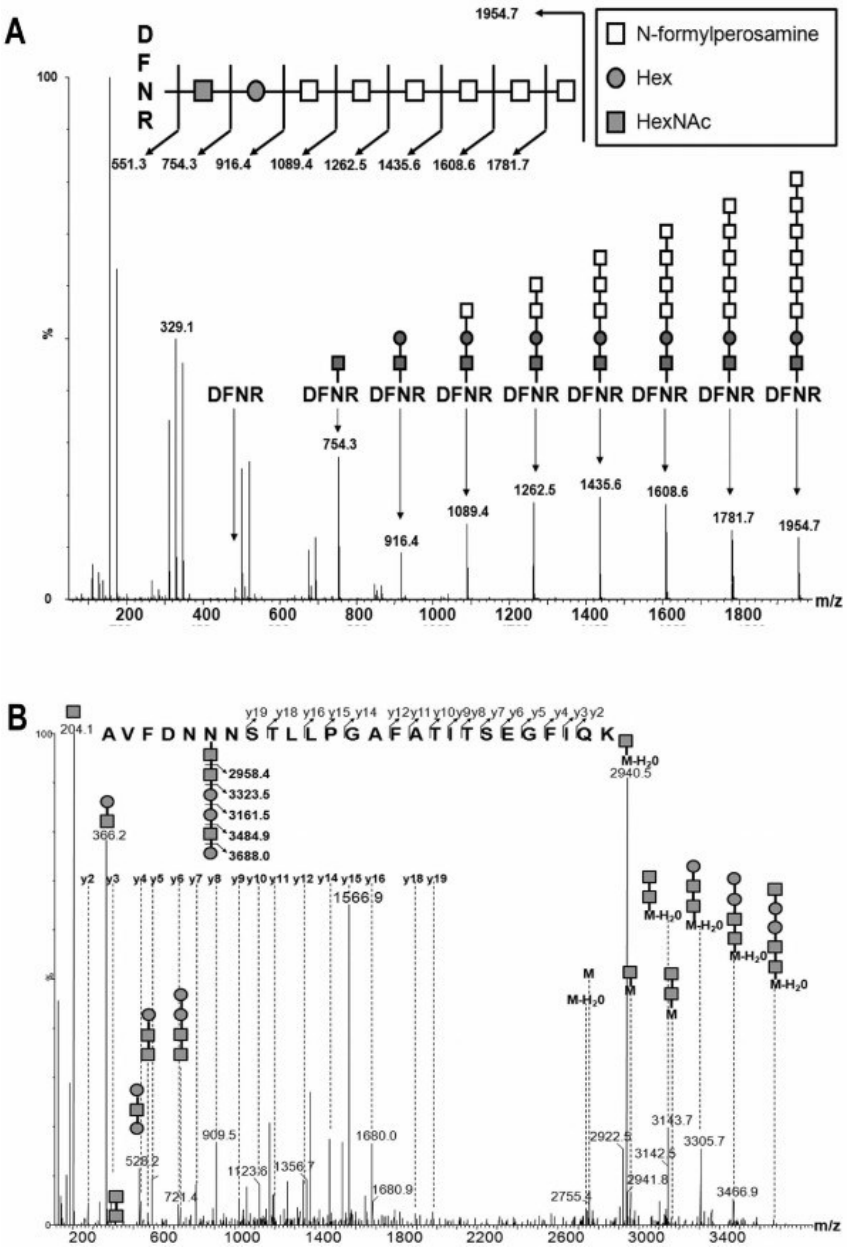

Figure 3.5. ESI-Q-TOF MS and MS/MS Analysis of *Y. enterocolitica* O:9 Glycobioconjugates. A) MS of high molecular weight glycosylated AcrA purified from Ye O:9 WT revealed the peak 1954.7 M/Z. MS/MS of this peak showed a disaccharide of HexNAc-Hex linking a characteristic 173 M/Z pattern corresponding to the N-formylperosamine subunit to the known glycopeptide DFNR. B) MS of high molecular weight glycosylated AcrA purified from Ye O:9 O antigen mutant revealed the peak 1284.6^{3+} M/Z. MS/MS of this peak shows the second known glycosylated site of AcrA (AVFDNNNSTLLPGAFATITSEGFIQK) modified with the hexasaccharide HexNAc-HexNAc-Hex-Hex-HexNAc-Hex.

3.2.4 Removal of Unglycosylated and Outer Core Glycosylated AcrA by Gel Filtration.

In order to further purify and concentrate the AcrA glycosylated with N-formylperosamine, gel filtration chromatography was used in an attempt to remove the outer core glycosylated AcrA. The glycosylated AcrA was concentrated using the Amicon® Ultra Centrifugal Filter Device (10 kDa MW cutoff) and was centrifuged at 4000 x g for 10 minutes. The concentrated sample was injected into a Superdex 200 gel filtration column, and was eluted in 1X PBS. Upon analysis of the chromatograph, two peaks were observed and the fractions were separated by SDS-PAGE and analyzed by Western Immunoblot and Coomassie staining (Fig 3.6). Samples A7-A9 showed no separation of AcrA glycosylated with the outer core and homopolymer, whereas samples B1 and B2 showed no non-glycosylated AcrA and limited quantities of the Outer core glycosylated AcrA. Previous unpublished work has shown that the engineered soluble AcrA does form multimers under non-denaturing conditions (Data not shown). Overall, we were able to decrease the quantity of non-glycosylated and OC-glycosylated AcrA from the desired N-formylperosamine glycosylated AcrA.

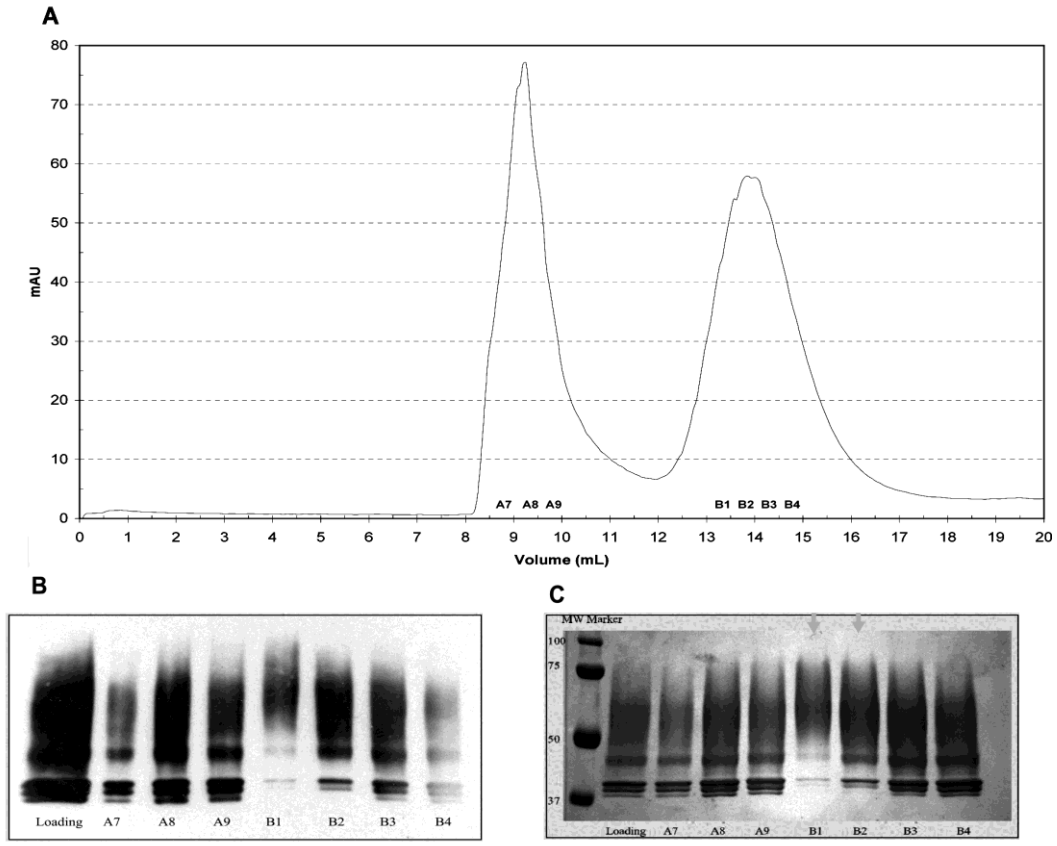

Figure 3.6. **Gel Filtration Purification of YeO9 Glycosylated AcrA and Analysis**. Two distinct peaks were observed, and the sample was not separated optimally because of multimeric forms of AcrA A) Chromatograph of gel-filtration resulting in two distinct peaks with 7 samples of protein (A7-A9, B1-B4). B) α-AcrA Western Immunoblot analysis of the gel purified samples. C) Coomassie Staining of gel purified samples

3.2.5 Immune Response in BALB/c Mice Vaccinated with YeO9-Glycosylated AcrA

To evaluate the potential use of the glycoprotein as conjugate vaccine, the purified AcrA containing the Ye O:9 antigen was injected intraperitoneally into mice to measure the immune response as well as test subsequent protection against a challenge with *B. abortus*. The purified glycosylated AcrA was quantified for protein (1.77 mg/mL) and carbohydrate (0.71 mg/mL) concentration, giving a protein: carbohydrate ratio of 2.48. Three separate groups of mice were injected, one with unglycosylated AcrA and two with different amounts (1.5 μg and 3 μg equivalents of N-formylperosamine per mouse) of

glycosylated AcrA, respectively. After a second injection of glycoprotein, sera obtained from the control (Fig 3.7A, C, and E) and 3 µg group (Fig 3.7B, D, and F) were analyzed for IgG immune response by Immunoblot. As expected, both sets of sera reacted strongly against AcrA (Fig 3.7A, B). However, when the sera were assayed against the purified *Y. enterocolitica* LPS, only the groups injected with glycosylated AcrA were reactive against purified LPS samples from *Y. enterocolitica* O:9 OC mutant and WT strains, indicating that IgG antibodies against the N-formylperosamine homopolymer were generated (Fig 3.7C, D). No reactivity was observed for the control sera towards the *Brucella* LPS, whereas the sera from the mice injected with glycosylated AcrA showed a strong immunoreactivity towards the *B. abortus* and *B. suis* LPS (Fig 3.7E, F). A very weak response was observed against the *B. melintensis* LPS.

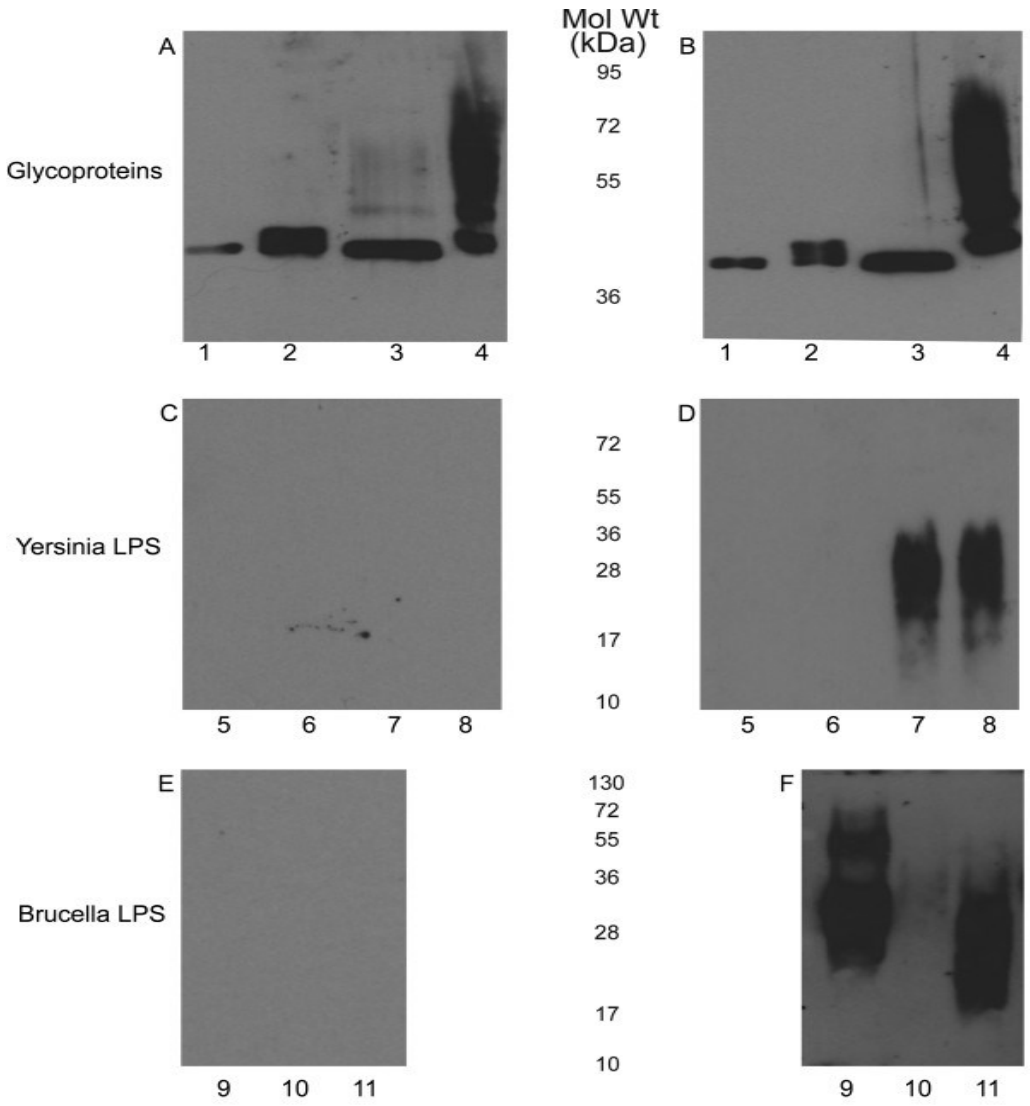

Figure 3.7. Sera of BALB/c Mice Immunized with Bioconjugate Shows a Directed IgG Immune Response Against N-formylperosamine of *Y. enterocolitica* O:9 and *Brucella* spp. A) Control sera and B) Immune sera raised by injecting purified glycoproteins containing 3 μg glycan: 1) Unglycosylated AcrA, 2) HP-, 3) OC-, 4) WT. Both sets of sera react with each glycoprotein due to the high immunostimulatory characteristic of AcrA. C) Control sera and D); immune serum (IgG response) blotted against *Y. enterocolitica* 0:9 LPS from different strains from Fig 1. 5) OC-/HP-, 6) HP-, 7) OC-, 8) WT. Only the test serum was reactive against the higher molecular weight portion corresponding to the homopolymer of N-formylperosamine. E) Control sera and F) immune serum blotted against *Brucella* spp. LPS: 9) *B. abortus*, 10) *B. melitensis*, and 11) *B. suis*. Only the

immune sera are reactive against the *Brucella* LPS. Interestingly, although each LPS is comprised of N-formylperosamine, different linkages are present which may cause the difference in reactivity of the sera.

3.2.6 LPS Purification and ELISA Optimization.

In order to analyze the sera from the different mouse groups, we optimized an ELISA protocol for each of the different purified LPS samples. A sample of *B. melitensis* LPS was kindly provided by Dr. Bundle, and *Y. enterocolitica* O:9 LPS was purified from 1L of stationary culture by the Phenol-Chloroform LPS extraction technique (Westphal O, 1965). The ELISA protocol (Bundle *et al.*, 1984) was optimized against *Y. enterocolitica* O:9 LPS, *B. melitensis* LPS, as well as against the carrier protein AcrA (Data not shown). Optimization of the 2^{o} antibody, α-mouse alkaline-phosphatase conjugated antibody (IgG and IgM, Biorad) was also completed (Data not shown). The optimal concentration of each variable is shown in Table 3.3.

Table 3.3 Optimization of Variables for ELISA of Mice Sera

Antigen	Optimal Concentration
Y. enterocolitica O:9 LPS	12.5 µg/mL
B. melitensis LPS	50 µg/mL
AcrA	1 µg/mL
α-mouse AP conjugated IgG	1:3000
α-mouse AP conjugated IgM	1:3000

3.2.7 ELISA Analysis of Immune Response Against the Glycoconjugate Protein in Mice.

All 3 samples of sera were analyzed for immune response according to Bundle *et al.,* (1984). All samples were tested in triplicate, in order to ensure accuracy. The initial (control) bleed and second bleed were tested for IgM response against *Y. enterocolitica*

O:9 LPS by ELISA to determine if the glycoconjugate could produce an immune response in mice. Sera samples were tested at a 1/200 dilution, there is a positive correlation between quantity of sugar injected and immune response detected for the second bleed whereas there was no statistical difference between the first bleed injection groups (Fig. 3.8).

Statistical analysis of the IgM fraction of the sera against *Y. enterocolitica* O:9 LPS was also done using ANOVA (single factor). No significant difference was observed between the different mice groups IgM response for the initial bleed. Additionally, no significant increase in IgM response was observed in the control group after 6 weeks (P=0.6). Analysis of the second bleed IgM response to *Y. enterocolitica* O:9 LPS showed a statistical difference between both the 1.5 µg and 3 µg groups when compared to the 0 µg group (P=0.008 and P=$7.99e^{-5}$ respectively). However, there was no statistical difference between the 1 µg and 2.5 µg injection sample groups (P=0.379).

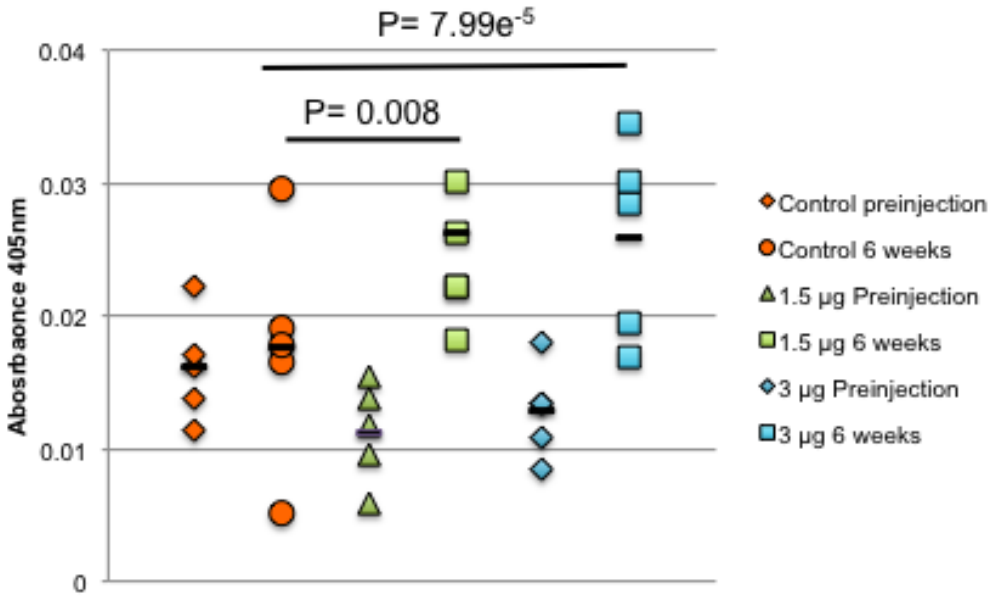

Figure 3.8: **BALB/c Mice Elicit an IgM Immune Response Against *Y. enterocolitica* O:9 LPS After Primary Vaccination**. ELISA response of the first bleed sera (1/200 dilution) of the different mouse groups against *Y. enterocolitica* O:9 LPS. Microtiter plates were coated with 12.5 µg of *Y. enterocolitica* O:9 LPS. Each datum point represents the average of three replicate wells. Response was read after 1 hr @ 37°C at OD_{405nm}. The bar in each set of data corresponds to the average of each group.

The first and third bleed sera samples were tested against the carrier protein for IgG response. All three injection groups had a significant increase in response against AcrA, with a positive correlation between higher sugar injection groups and immune response detected when analyzed by the ELISA method (Fig 3.9). There was one mouse in the 0 µg injection group that had a significantly higher response than the rest. Previous work has demonstrated that AcrA has a high immunogenicity, and our results are in agreement, with all of the mice groups having a high IgG immune response towards the AcrA carrier protein.

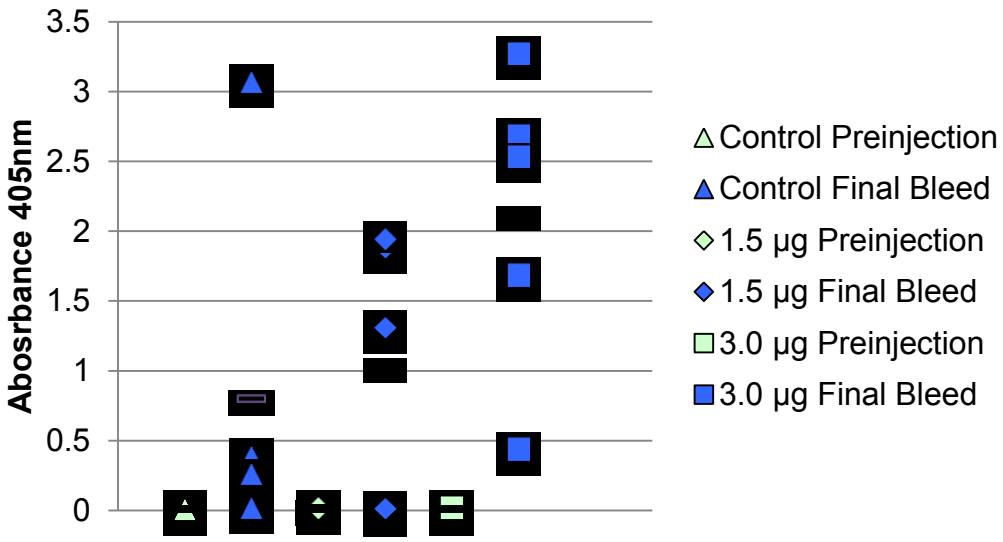

Figure 3.9: **BALB/c Mice Elicit an IgG immune Response Against AcrA**. ELISA response of the third bleed sera (1/200 dilution) of the different mouse groups against purified unglycosylated AcrA. Microtiter plates were coated with 1 µg of AcrA. Each datum point represents the average of three replicate wells. Response was read after 1 hr @ 37°C at OD_{405nm}. The bar in each set of data corresponds to the average of each group.

The generation of antibodies against the Ye O:9 antigen was further analyzed by ELISA. Each well was coated with 12.5 µg of Ye O:9 LPS (Fig 3.10). Of the three groups of mice, only the sera from mice belonging to the two groups inoculated with glycosylated AcrA showed an IgG response directed towards the polysaccharide. However, a high level of variation in the absorbance values was observed, with some animals showing no significant response. Interestingly, the group of mice inoculated with the lower amount of glycoprotein (1.5 µg) exhibited a higher average OD_{405nm} than the group inoculated with 3 µg. Nevertheless, the immune response was insufficient or inefficient in protecting the mice against a challenge with *B. abortus*, as no statistical difference was observed in bacterial load in the spleen of infected mice irrespective of whether they were injected with glycosylated or unglycosylated AcrA (Table 3.4).

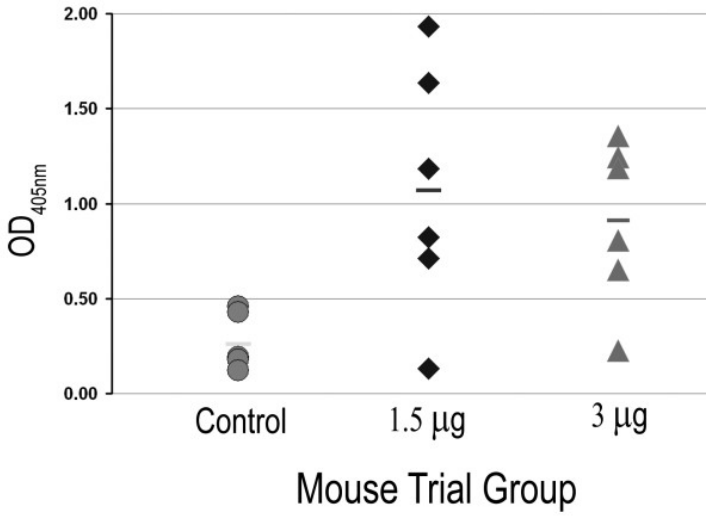

Figure 3.10. BALB/c Mice Elicit an IgG immune Response Against *Y. enterocolitica* LPS. ELISA response of the third bleed sera (1/200 dilution) of the different mouse groups against *Y. enterocolitica* O:9 LPS. Microtiter plates were coated with 12.5 µg of *Y. enterocolitica* O:9 LPS. Each datum point represents the average of three replicate wells. Response was read after 1 hr @ 37^{o}C at OD_{405nm}. The bar in each set of data corresponds to the average of each group.

Table 3.4. Bacterial Spleen Counts of *B. abortus* for Mice Vaccinated with YeO9-AcrA. Log CFU per spleen of mice vaccinated with differing quantities of either unglycosylated (control) or glycosylated AcrA.

Mouse	**Control**	**1.5 µg Glycosylated**	**3 µg Glycosylated**
1	4.68	3.87	5.66
2	5.90	5.24	5.15
3	5.30	5.92	5.60
4	5.74	4.60	4.95
5	4.64	5.92	5.26
6	4.38	5.97	5.60
Average +/- ST Dev	**5.11+/- 0.63**	**5.26 +/- 0.87**	**5.37+/- 0.29**

3.2.8 Glycoconjugates as Novel Antigens for the Diagnosis of Brucellosis

Because vaccination with the glycosylated AcrA induced the production of a specific IgG immune response against the O antigen, we asked if this glycoconjugate could be used as an antigen for the diagnosis of the infection in cows. To test this, we immobilized AcrA (control) or AcrA-O:9 on paramagnetic microbeads (see Materials and Methods) and tested the reactivity towards sera from non-infected animals, as well as from cows vaccinated with the *B. abortus* Δ*pgm* or infected with *B. abortus* 2308 strain (conjugation and sera assay was performed by Cecilia Czibener, Andres Ciocchini, and Diego Comerci, Instituto de Investigaciones Biotecnologicas, Buenos Aires, Argentina). These animals are part of an efficacy trial to test the protective capacity of the Δ*pgm* strain (Ugalde *et al.*, 2000, Ugalde et al., 2003). As mentioned earlier, Δ*pgm* is a rough strain that does not induce the production of anti-O-antigen specific immunoglobulin titers in mice. As can be observed in Figure 3.11A, the assay clearly differentiates non-infected from infected animals and does not react with sera from animals vaccinated with a strain that lacks a complete LPS. Additionally, it is shown that none of these sera reacted against the non-glycosylated form of AcrA in an immunoblot indicating that the IgG response detected is directed specifically towards the carbohydrate moiety of the antigen (Fig 3.11B). Taken together, these results strongly suggest that this novel antigen could be used for the development of new diagnostic tools for brucellosis.

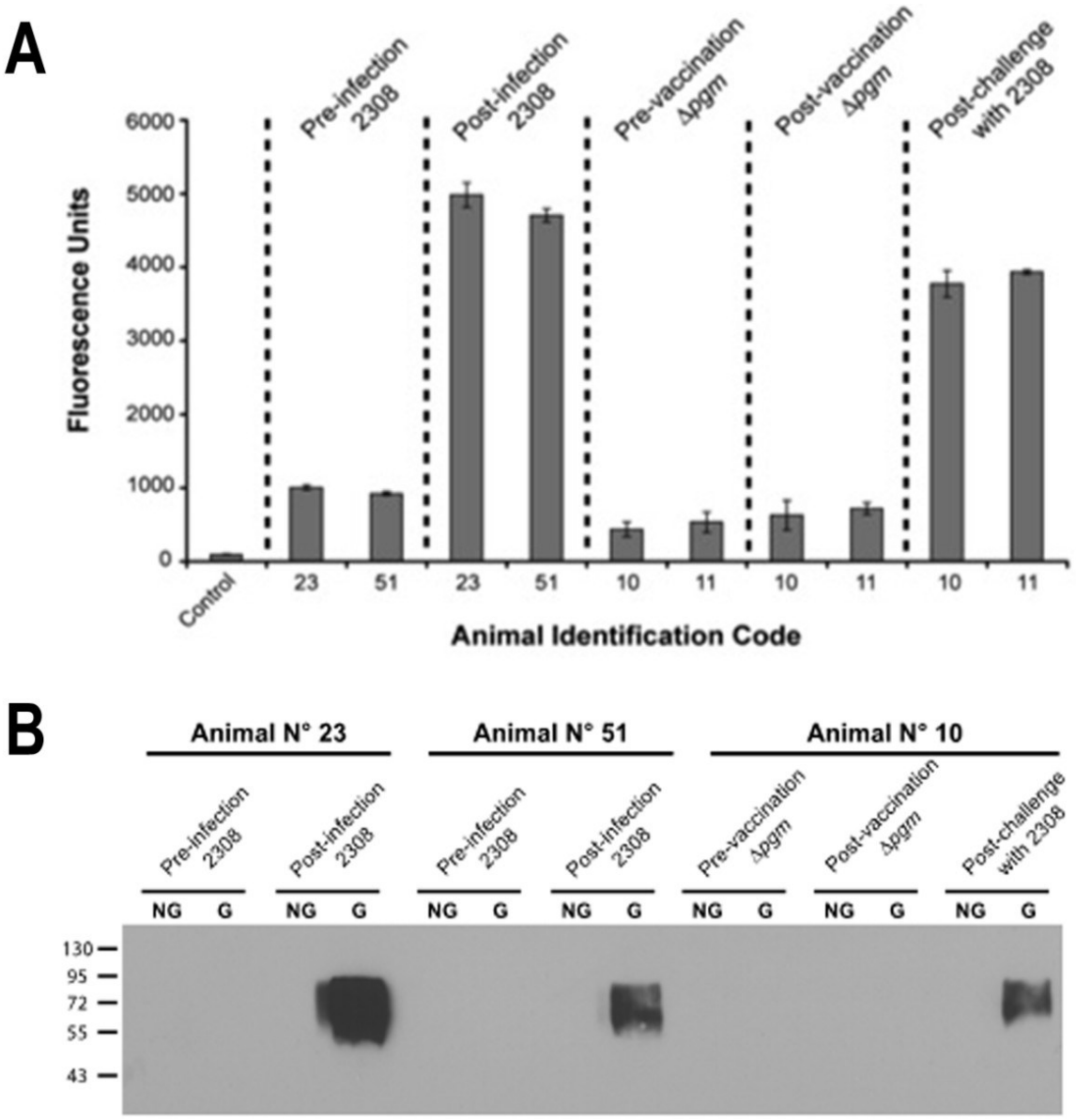

Figure 3.11. *Y. enterocolitica* O:9 Bioconjugate as a Promising Antigen for the Diagnosis of Bovine Brucellosis. A) Magnetic bead-based immunoassay for detection of antibodies against *Brucella abortus* O-antigen. Magnetic beads coated with the AcrA-OAg glycoconjugate were incubated with the indicated bovine serum samples (dilution 1/200). Bound antibodies were detected using a Cy5-conjugated goat anti-bovine IgG. The bar graph data represents the means and standard deviation for two separate determinations. Control: magnetic beads incubated with PBS-Tween 0.1 %. B) Immunoblot of the same bovine serum samples

3.3 Discussion

Because the *Y. enterocolitica* O:9 and *B. abortus* LPSs share the same structure, we hypothesized that a glycoprotein carrying the Ye O:9 glycan would be able to generate antibodies recognizing *B. abortus* O polysaccharide, with the overall goal of eliciting a protective immune response against infection by this organism. We also explored if such a glycoprotein could be used for the diagnosis of brucellosis. We confirmed, using Immunoblot, that mAb raised against *Yersinia* O:9 and *B. abortus* O antigens can cross-react with both structures. A glycoprotein consisting of the *Y. enterocolitica* O:9 antigen attached to the carrier protein AcrA was obtained *in vivo* by expression of the *N*-linked protein glycosylation OTase PglB of *C. jejuni* in *Y. enterocolitica* strains. The glycoprotein was purified from Ye O:9 cells and characterized by MS techniques. We confirmed the attachment of a polymer of N-formylperosamine to AcrA, which was reactive towards two mAbs directed against Ye O:9 and *B. abortus* O antigens, respectively. However, we also identified a previously unreported disaccharide of the formula HexNAc-Hex acting as a linker between the protein and the Ye O:9 homopolymer. A similar linker has been described in many homopolymeric O antigens in different bacterial species (Raetz & Whitfield, 2002) and also in other polysaccharides such as the arabinogalactan of *M. tuberculosis* and the teichoic acids in Gram-positive bacteria (Qu *et al.*, 2007). This indicates that disaccharides acting as linkers are a common feature in the synthesis of bacterial polysaccharides via the polymerase (wzy) independent pathway. Additionally, MS/MS analysis demonstrated the attachment of shorter glycan to AcrA in the wild type strain, with a structure of HexNAc-HexNAc-Hex-Hex-HexNAc-Hex. This hexasaccharide is known as the outer core and is also present in *Y. enterocolitica* O:3. Based on genetic similarities within the O:9 and the O:3 strains, it was hypothesized that the structures in

both strains would be identical. However, our results demonstrate that a different structure is generated in the O:9 strain (Skurnik & Bengoechea, 2003, Muller-Loennies et al., 1999).

Injection of AcrA-O9 in mice was able to elicit an IgG immune response against the O:9 polysaccharide. Sera of inoculated mice reacted with Ye O:9 LPS, and *B. abortus* and *B. suis* O antigens. However, the reaction towards *B. melitensis* O polysaccharide was practically undetectable. *B. abortus* has both an A (α-1,2-linked homopolymer of N-formyperosamine) and an M (pentasaccharide with four α-1,2 and one α-1,3-linked polymers of the same sugar) epitope (Meikle et al., 1989). *Y. enterocolitica* O:9 antigen is comprised solely of α-1,2-linked N-formylperosamine, *B. abortus* has ~98% A epitope, *B. suis* has a unique 1:7 ratio of α1,3- α1,2 linked polymer, whereas *B. melitensis* has only the M antigen of the pentasaccharide repeat (Meikle et al., 1989). These structural details help to explain why the M84 mAb against *B. abortus* does not recognize *B. melitensis* LPS, as this mAb is likely directed to an epitope absent in *B. melitensis.* However, the three *Brucella* strains reacted against the mAb α-*Yersinia* antibody, reflecting common epitopes that exist in the four structures (Fig 3.3B). We therefore expected that these common epitopes present in the AcrA-O9 glycoprotein would elicit antibodies that would also cross react with *B. melitensis* LPS, but the sera of the mice injected with AcrA-O9 failed to recognize B. *melitensis* LPS. This indicates that the common epitopes in all the structures are not the immunodominant ones. Interestingly, although AcrA was glycosylated with both the Ye O:9 and the OC glycan structures, only the O:9 antigen was detected by the mice sera, suggesting that the outer core is not immunogenic (Fig 3.4D).

A previous report suggested that a conjugate containing BSA and the O polysaccharide of *B. melitensis* was protective in mice (Jacques et al., 1991). In

preliminary experiments we found that passive immunization with the Yst9 mAb was protective against *B. abortus* challenge (data not shown). These results prompted us to test the efficacy of our recombinant glycoconjugate against *B. abortus* challenge. An elevated dispersion in the titers of the vaccinated mice was obtained. However, no correlation between antibody titers and bacterial load in the spleen was found, resulting in the absence of difference in bacterial colonization of the three groups. Lack of protection could possibly be explained by the fact that *B. abortus* is an intracellular pathogen and that antibodies against this bacterium may not be able to encounter the microorganism once the infection is established. Alternatively, higher antibody titers may be necessary to elicit a protective immune response. The antibody titers were higher in the animals vaccinated with the lowest amount of glycoconjugate. It is possible that lower amounts of antigen may have to be injected to obtain protective antibody titers.

Our AcrA-O9 conjugate showed promising applications in the diagnostics of brucellosis. Diagnostics of brucellosis using lipopolysaccharide (LPS) as an antigen have been previously explored (Lindberg *et al.*, 1982). LPS are large molecules that also contain a core polysaccharide and a lipid A moiety, as well as the O antigen. LPS-based assays often suffer from false positives due to the presence of antibodies against common core antigen and lipid A, generated by other bacterial species. Here, we showed that coating magnetic beads with the AcrA-O:9 glycoprotein allows the distinction between infected and uninfected cows. The assay will be particularly useful in conjunction with vaccines like the RB51 or the Δ*pgm* strain, which do not have O antigen, as our assay will allow the distinction between vaccinated and infected animals. Further studies will be carried out to confirm the suitability, i.e. sensitivity and specificity of this assay for detection of bovine and human brucellosis.

Ten years have gone by since the demonstration that bacterial glycosylation systems can be successfully transferred into *E. coli*. Since then we have learned that the bacterial OTases have a relaxed specificity and are able to transfer a variety of glycans, including O antigens, to suitable protein acceptors. The experiments presented here demonstrate that an IgG immune response can be mounted against the glycan moieties in bacterial glycoproteins. Further work will expand these efforts for the generation of novel vaccines against other important bacterial pathogens. Furthermore, we also expect that the platform presented here for the detection of brucellosis will also be applied in the future for the design of additional bacterial-glycoprotein based diagnostic methods.

3.4 Materials and Methods

3.4.1 Bacterial Strains, Plasmids, and Growth Conditions

Yersinia enterocolitica O:9 strains were grown in LB media @ 37^{o}C. Trimethoprim (100µg/mL) and chloramphenicol (20µg/mL) were used for plasmid selection as required. The strains and plasmids used in this study are listed in Table 3.5.

Table 3.5. List of Strains and Plasmids

Strain/Plasmid	Description	Source
pMAF10	HA-tagged $PglB_{Ci}$ cloned in pMLBAD, Tmp^R	(Feldman et al., 2005)
pMH5	Soluble periplasmic hexa-His-tagged AcrA under control of Tet promoter, in pACYC, Cm^R	(Feldman et al., 2005)
YeO9-c-OC (OC-)	Δ (*wzx-wbcL*)::KmGB; OC negative; derivative of Ruokola/71-c	(Skurnik et al., 2007)
YeO9-OC-R (OC-/HP-)	Phage ϕR1-37-resistant spontaneous OC-negative derivative of YeO9-R1	(Skurnik et al., 2007)
YeO9-R1 (HP-)	Δ*per*::KmGB; rough (O-antigen negative); Km^r derivative of Ruokola/71	(Skurnik, 1984, Skurnik et al., 2007)
Ruokola/71-c (WT)	Spontaneous virulence plasmid-cured derivative of Ruokola/71	(Skurnik, 1984)

3.4.2 Electrocompetent Cell Preparation

Cultures were grown in 50mL LB to mid long phase (OD=0.6-0.8) and harvested by centrifugation (5 minutes @ 5000 x g). The cells are washed with 50mL of ice cold ddH_20, and then washed with 50mL of ice cold 10% glycerol (centrifuged both times as previously stated). The cells are resuspended in 1mL of 10% glycerol, aliquoted (100 ul/tube) and frozen in liquid Nitrogen. Electrocompetent cells are stored at -86^{o}C. Protocol was based on Guerry *et al* (1994).

3.4.3 Production and Purification of Glycosylated AcrA

Yersinia enterocolitica O:9 strains transformed with *C. jejuni* PglB (pMAF10) and AcrA (pMH5) were grown overnight at 37^{0}C. Cultures were reinnoculated 1/100 into fresh LB media and grown at 37^{0}C for 2.5 hrs (OD_{600} ~0.5) and $PglB_{Cj}$ expression was induced with addition of arabinose to a final concentration of 0.2% (w/v). Four hrs after induction at 37°C, $PglB_{Cj}$ was re-induced by a second addition of arabinose to maximize glycosylation of AcrA. Cells were harvested by centrifugation after a 20 h induction period and periplasmic extracts were prepared by lysozyme treatment as described elsewhere (Feldman et al., 2005). Subsequently, the periplasmic fraction was equilibrated with 1/9 vol 10x loading buffer (0.1M Imidizole, 3M NaCl, 0.2M Tris-HCl pH 8.0) and subjected to a Ni^{2+} affinity chromatography. The column was equilibrated with 10 column volumes of 1x loading buffer and loaded on a HisTrap HP column (Amersham Pharmacia Biosciences) at a flow rate of 1mL/min. The column was washed with 25 column volumes of wash buffer (0.02M Imidazole, 0.3M NaCl, 0.02M Tris-HCl pH 8.0), and eluted from the column by elution buffer (0.250M Imidazole, 0.3M NaCl, 0.02M Tris-HCl pH 8.0).

3.4.4 Methanol Free Coomassie Staining

Samples run on 10% Polyacrylamide gels and were incubated in 50mL of methanol free destain solution (7% acetic acid) for 10 minutes to equilibrate the gel. The gel was subsequently incubated in 50 mL of Coomassie staining solution (0.02% (w/v) Coomassie Brilliant Blue R-250, 2% Methanol, 7% Acetic acid) was incubated for 2 hours (constant rocking). The staining solution was discarded and the gel was washed with methanol free-destain solution, which was changed every 10 min to increase the rate of destaining.

3.4.5 Protein Quantification

Protein quantification was done by Bradford assay (Biorad) using microplate analysis. Five µL of sample was added to 250 µL of Protein Assay Dye Reagent Concentrate and mixed by pipet. After 20 mins at room temperature, the colour development was determined by reading the 595nm colourmetric response and the protein concentration was determined against BSA standards.

3.4.6 Protein Sample Dialysis

Removal of the elution buffer from purified protein samples was achieved by utilizing a PD-10 Desalting column (GE Healthcare). The PD-10 column was equilibrated with 25 mL PBS by gravity filtration. After all the PBS had passed through the column, 1 mL elution fraction was added to the column and allowed to completely enter, followed by 1.5 mL of PBS. 5 mL of PBS was added to the column, and fractions were collected and analyzed for protein by Bradford assay.

3.4.7 LPS Extraction

LPS was extracted using the hot phenol-water method as described by (Westphal and Jann, 1965). Cells were cultured and harvested by centrifugation (8000 rpm, 10 minutes, 4°C). The pellet was then washed with buffer A (50mM phosphate, 150mM NaCl, pH 7.4). The cells were resuspended in PCP (phenol-chloroform-petroleum ether 1:2.5:4) at a concentration of 2g/50mL and disrupted using a potter homogenizer. Samples were centrifuged (7,000 rpm, 10 minutes, 4°C) and the supernatant incubated at 55°C until the complete evaporation of the chloroform-ether phase. LPS was precipitated by the addition of 1 volume of distillated H_20 and centrifuged (7,000 rpm, 10 minutes, 4°C). At this stage, 3 separate layers were visible in the suspension (H_20 in the upper phase, LPS in the middle phase, and phenol in the lower phase). The phenol phase was removed by Pasteur pipet, and a second precipitation was done by the addition of 1 volume of distillated H_20, and centrifugation was repeated. Both LPS phases were combined and centrifuged (12,000 rpm, 10 minutes, 4°C). The pellet (containing LPS) was washed with 20mL of methanol, centrifuged for 15 minutes at 12,000 rpm, and the supernatant was discarded (by suction). The LPS pellet was vacuum-dried, resuspended in 10mL of 0.1mM $MgCl_2$ (in distillated H_20) and centrifuged at 15°C, 100,000 x g for 16 hrs. The final pellet was resuspended in 2mL of distillated H_20.

3.4.8 LPS Silver Staining Visualization of SDS-PAGE Gel

Samples were separated on 15% acrylamide SDS-PAGE gels and the LPS was visualized following the method of Tsai and Frasch (1982). Gels were incubated overnight in EtOH:Acetic acid:H_20 (8:1:11). The carbohydrates were oxidized with periodic acid (0.7% (w/c)) in EtOH:acetic acid:H_20 for 45 mins. The gel was subsequently washed three times for 10 mins with ddH_20, then incubated for 10 mins in staining solution (mixed fresh

in the following order: 28mL 0.1M NaOH, 2mL NH_4OH, 5mL 20% (w/v) $AgNO_3$ added drop wise, 115 mL H_20). After three 10 min washes with ddH20, developer buffer (10mg citric acid and 100µL formaldehyde (37%) in 200 mL H_20) was added until visualization of saccharides. Gels were imaged with a CanoScan 8400F.

3.4.9 Sugar Quantification of Glycoproteins

Protocol was adapted from the total sugar quantification protocol (Dubois M, 1956). Briefly, mix in a glass tube 90 µL ddH_20, 10 µL of sample, and 100 µL of 5% phenol (freshly made) in ddH_20. Briskly add 1mL of conc. H_2SO_4 into the mixture and immediately vortex the solution for several seconds. An orange color with intensity proportional concentration will begin to develop immediately and reach a maximum after 2 h @ 30°C. Read against glucose standards @ OD_{500nm}.

3.4.10 Western Immunoblot

Western blotting was performed based on the procedure described in (Aebi *et al.*, 1996). Samples were separated on 10% or 15% SDS-PAGE gels and transferred to a nitrocellulose membrane via semi-dry membrane transfer and analyzed with a variety of antibodies. α-AcrA antibody (Wacker *et al.*, 2002), Yst9-2 antibody (Bundle et al., 1984), and Brucella antibodies M84 (Nielsen *et al.*, 1995) were employed as previously described.

3.4.11 Mass Spectrometry

The nickel column purified protein/glycoprotein was subsequently separated by SDS-PAGE, and the bands corresponding to the desired protein and glycoprotein were in-

gel digested with trypsin (Promega) using the protocol of Shevchenko *et al.* with modification (Shevchenko *et al.*, 1996). Briefly, the bands corresponding to the glycoproteins were excised and transferred to 1.5ml Eppendorf vials. After destaining with 50mM ammonium bicarbonate in 50% acetonitrile/water, the gel pieces were dehydrated with acetonitrile and rehydrate with 10µl of (~2µg) trypsin. Then the sample was left in 37^{o}C oven for overnight digestion after addition of 50µl ammonium bicarbonate (50mM) aqueous. The samples were extracted using Zip-Tips (Millipore), and analyzed using a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Waters, UK) equipped with ananoACQUITY Ultra Performance liquid chromatography system (Waters, Milford, MA). Briefly, 2µl of the peptide solution was injected on to a VanGuard micro precolumn C18 cartridge that is connected to a 75um i.d. x 150um Atlantis dC18 column (Waters, Milford, MA). Solvent A consisted of 0.1% formic acid in water, and solvent B consisted of 0.1% formic acid in acetonitrile. After 1 min trap wash in the precolumn with solvent A at flow rate of 10µl/min, peptides were separated using solvent gradient and electrosprayed to the mass spectrometer at a flow rate of 350nl/min. The collision energy used to perform MS/MS was varied according to the mass and charge state of the eluting peptides. The instrument is calibrated every 1 min with GFP and LecErK using the LockSpray. For the data acquisition and analysis, MassLynx (Waters MassLynx V4.1) was used.

3.4.12 Mouse Trials Experimental Design

A group of 18 BALB/c female mice were divided into 3 groups and injected with differing quantities of N-formylperosamine per injection intraperitoneally. The mice were bled via tail bleed method, resulting in a blood sample of 5-25 µL. The blood was allowed to coagulate @ 24^{o}C for 4 hrs, after which the samples were centrifuged for 10 minutes @

13,000 rpm to isolate the blood sera. The sample was stored at -20°C until analyzed. Injections were done according to the TiterMax®Kontes Pellet Pestle® Homogenizer method using TiterMax® Gold. A second bleeding of the mice occurred 6 weeks after the initial injection, resulting in similar sera yields. The mice were given a second injection with the same quantities of sample. The third and final bleed occurred 4 weeks after the second injection, and was also stored @ -20°C until required for analysis.

3.4.13 ELISA Analysis of Mouse Sera

ELISA analysis was done (Costar® polystyrene High Binding plate) and optimized from (5). 100 µl of each of *Y. enterocolitica* O:9 (12.5 µg/mL LPS), *Brucella melitensis* (50 µg/mL LPS), and purified AcrA (1 µg/mL) was determined to give the optimal colorimetric response in 0.05M sodium carbonate buffer (pH 9.8). Wells were blocked using 2.5% (wt/vol) skim milk in PBS buffer for 1.5 hrs. Sera from the mice were diluted in PBS buffer, and 100 µL was placed in each well. Plates were washed 3 times with PBST. The 2° antibodies conjugated to alkaline phosphatase (Biorad Laboratories) were incubated at a dilution of 1:3000 for 1 hour at room temperature. Plates were washed 5 times with PBST, and were incubated with 100 µL *p*-nitrophenolphosphate substrate (1mg/mL) in 0.05mM sodium carbonate buffer (pH 9.8) for 1 hour @ 37°C. Plates were read at OD_{405nm}.

3.4.14 *Brucella* Challenge Against Injected BALB/c Mice

Groups of 6 Balb/c female mice intraperitonelly vaccinated with either 1.5 or 3 □g of the glycoconjugate were challenged four weeks after the second dose with $5x10^4$ CFUs intraperitoneally of wild type *B. abortus* 2308 and, two weeks post-infection, the bacterial load in the spleens determined as previously described (Ugalde et al., 2003).

3.4.15 Magnetic-Bead Based Immunoassays

Superparamagnetic COOH-modified microbeads (Bangs's Laboratories) were coated with the AcrA-OAg glycoconjugate in one step using EDAC [1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride] and NHS [N-hydroxy succinimide] reactives. Functionalized microbeads were incubated with bovine serum samples (dilution 1/200) and bound antibodies were detected using a Cy5-conjugated goat anti-bovine IgG (Biomeda). Fluorescence reading was performed using a plate fluorometer (DTX 880 Multimode Detector, Beckman Coulter).

3.5 References

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Chapter 4

Discussion

Portions of this chapter have been published.
Iwashkiw JA, Vozza NF, Kinsella RL, and Feldman MF. 2013. **Pour some sugar on it: the expanding world of bacterial protein O-linked glycosylation**. Mol Microbiol. Jul;89(1):14-28.

Protein glycosylation, once thought of as solely a eukaryotic feature, has been identified in all forms of life. Bacteria possess both *N*- and *O*-glycosylation systems, and different variations of both have been identified. In particular, *O*-glycosylation was initially regarded as a peculiarity of a few bacterial species. However, in recent years, a panoply of *O*-glycosylation mechanisms have been identified in a diverse genera of bacteria, including various important human pathogens. *N*-glycosylation appears to be mostly limited to epsilonproteobacteria, however, the identification of a novel mechanism in *H. influenzae* and *Actinobacillus pleuropneumoniae* opens up the possibility that *N*-glycosylation may be more widespread in bacteria than previously hypothesized.

This chapter is a general discussion and future perspectives regarding bacterial protein glycosylation, and specifically the role of *O*-glycosylation in *Acinetobacter.* Additionally, the discovery of bacterial OTases has opened the possibilities of developing bioengineered products that could be utilized as commercial and therapeutic products.

4.1 Bacterial Protein Glycosylation

Significant advances in technology have triggered the rapid expansion of the field of bacterial glycobiology. Initial studies identified glycoproteins by lectin blots, glycan specific antibodies, and chemical reagents including periodic acid (Balonova *et al.*, 2009). While invaluable in detection of abundant glycoproteins, these techniques have limitations including: low sensitivity, high specificity of glycan antibodies, and the unspecific labeling of chemical methods. High throughput genomic sequencing, mass spectrometry, NMR, and metabolic glycan labeling techniques have significantly increased the ability to detect and characterize glycosylation systems. Interestingly, several of the prototype glycosylation systems in bacteria including *C. jejuni, H. influenzae, D. gigas, Neisseria* sp.,

and *Bacteroides* were discovered indirectly through attempts to characterize other components of bacteria (Szymanski *et al.*, 1999, Grass *et al.*, 2003, Santos-Silva *et al.*, 2007, Parge *et al.*, 1995, Coyne *et al.*, 2008). With advances in technology, general glycosylation systems have been identified and characterized, increasing the number of known glycan structures. However, without these initial discoveries, the field of glycobiology would most probably still be in its infantile stage, since most of the diversity is now characterized based on genetic homology to the previously identified systems. In *N*-glycosylation, additional systems were hypothesized to exist based on homology to the OTase $PglB_{Cj}$, and several have been characterized in *Campylobacter* sp., *Helicobacter* and *Desulfovibrio* (Ielmini & Feldman, 2011, Szymanski et al., 1999, Jervis *et al.*, 2010). Until the identification of the *N*-glycosylation system in *H. influenzae*, it was hypothesized $PglB_{Cj}$-dependent glycosylation was the only system present in bacteria. Now, over ten different species are thought to utilize this novel system, and the key enzyme HMW1C has been crystalized allowing enzymatic characterization (Kawai *et al.*, 2011). In *O*-glycosylation, both OTase independent and dependent systems have been characterized. Several bacteria can glycosylate their adhesins and/or flagellins with mono- or oligosaccharides by cytoplasmic glycosyltransferases. More recently, the discovery of the OTase mediated *O*-glycosylation has led to significant focus regarding these systems. PilO, the first OTase identified, can only transfer short glycans to a single protein in *Pseudomonas aeruginosa*, while PglL in *Neisseria* has a very relaxed glycan specificity, and glycosylates several proteins (Faridmoayer *et al.*, 2007). Bioinformatic analysis of sequenced genomes shows several homologues of $PglL_{Nm}$ in bacteria, and active OTases have been characterized from *Vibrio cholerae, Burkholderia thailandensis, B. cepacia*, *Acinetobacter baumannii*, and *A. baylyi* (Gebhart *et al.*, 2012, Lithgow *et al.*, 2014, Iwashkiw *et al.*, 2012, Schulz *et al.*, 2013). However PglL and O antigen ligases possess a

common domain (Wzy_C), and distinguishing between the two enzymatic functions was only possible by experimental characterization. A recently published paper has identified protein domains specific to *O*-OTases, which could expedite the process of characterizing new systems (Schulz et al., 2013). Interestingly, a novel general *O*-glycosylation system has been identified in the Bacteroidetes phyla, but no OTase has been identified to date. Overall, most identified glycosylation systems in bacteria are based on the previously characterized systems, and are dependent on homology to key enzymes. However, novel *N*- and *O*-glycosylation systems have been recently described, leading to the question of how many more different systems exist in bacteria that have yet to be discovered.

Advances in technology, specifically in sensitivity of techniques like mass spectrometry, have been utilized to identify additional glycoproteins and glycan structures. However, the validity of these additional glycoproteins has come into question, as seen in *Neisseria* (Schulz et al., 2013). Eleven proteins were previously identified as glycoproteins by mass spectrometry (Vik *et al.*, 2009), but further analysis suggests that only three are physiologically relevant glycoproteins, and the remainder are either glycosylated at low levels or glycosylation is a mistake made by the OTase (Schulz et al., 2013). An additional unexpected result of the high sensitivity of mass spectrometric techniques has been the identification of glycan variation within a specific strain, as seen in *Campylobacter* (Nothaft *et al.*, 2012) and *Acinetobacter baumannii* (Kinsella RL *et al.,* submitted). Whether these additional glycoproteins and glycan micro-variations are physiologically important, or if the mass spectrometry techniques are simply catching bacterial “mistakes” has yet to be determined.

Many enzymes involved in bacterial *O*-glycosylation are not present in eukaryotes, and because some of these glycans play a crucial role in pathogenesis, they may represent novel targets for antibiotic development. Compounds inhibiting flagellin or adhesin glycosylation could act as antimicrobials. Although precise glycan structures and glycosylation targets cannot be predicted from genome sequences, the availability of the genomic information for multiple species contributed to the initial identification of *O*-glycosylation systems. Advances in key technologies like MS and NMR have facilitated the characterization of bacterial glycoproteomes. The current MS methods allow the determination of the glycosylated proteins and their preliminary glycan structures without having to purify the glycoproteins. These technological advances will have an impact on the discovery of novel glycosylation pathways, which will help to uncover novel features of the still largely uncharacterized bacterial glycosylation world.

4.2 *O*-Glycosylation in *Acinetobacter*

A. baumannii is an emerging opportunistic pathogen threatening modern healthcare facilities due to its acquisition of numerous antibiotic resistance genes, in addition to an innate resistance to desiccation and disinfectants. Recent work has identified several virulence factors, including biofilm formation, OmpA, capsule, exopolysaccharides, pili, and lipopolysaccharide (Choi *et al.*, 2005, Gordon & Wareham, 2010, Gaddy *et al.*, 2009, Tomaras *et al.*, 2008).

Identification of a homologue of $PglL_{Nm}$ in *A. baumannii* led to functional characterization of a general *O*-glycosylation system. Similar to previously characterized *O*-glycosylation systems, several different glycoproteins were identified. Interestingly, while these glycoproteins were highly conserved in *Acinetobacter*, only one had significant

homology outside of the genus. It is tempting to speculate these proteins may be key to *Acinetobacter* physiology. The most prevalent glycoprotein A1S_3626 appears to be required for survival, as attempts to create a mutant strain was unsuccessful (data not shown) and is essential in *A. baylyi* (de Berardinis *et al.*, 2008). Pilin glycosylation has been identified in other Gram negative *O*-OTase dependent glycosylation systems, while no pili homologue has been identified in the glycoproteome of *A. baumannii* to date. In *Neisseria*, the validity of a "general" *O*-glycosylation system has recently been challenged (Schulz et al., 2013). In *A. baumannii* ATCC 17978, seven different glycoproteins were identified by mass spectrometry, but 2D-DIGE analysis of membrane extracts only identified two (Fig 2.11). Recombinant expression of three different proteins demonstrated all were glycosylated, but a significant percentage of each was unmodified (Fig 2.10, 2.17, 2.19). Whether this is due to overexpression of the glycoproteins overwhelming the OTase with abnormally high quantities, or if these glycoproteins are "mistakes" made by a promiscuous OTase will require further study.

Bacterial glycosylation has been demonstrated to be important for pathogenesis. Similar to *Neisseria* and *Bacteroides fragilis* (Fletcher *et al.*, 2009, Jennings *et al.*, 2011), *A. baumannii* requires *O*-glycosylation for pathogenicity in several models, including competitive fitness in mice. *O*-glycosylation is not involved in swimming motility in *A. baumannii*, but is required for efficient biofilm formation. It is tempting to speculate that one of the identified glycoproteins is important for either adhesion to abiotic surfaces or cell-to-cell adherence. Additionally, glycosylation appears to be required for killing of Eukaryotic cells, so further work to understand the roles of the individual glycoproteins may reveal novel virulence factors.

Bioinformatic analysis of the *A. baumannii* ATCC 17978 genome revealed a locus responsible for *O*-glycan biosynthesis. Interestingly, this locus also contained genes responsible for the export of capsular polysaccharide, and the same glycan is utilized for both structures (Lees-Miller *et al.*, 2013). Previous work has described the evolutionary connection between LPS and protein glycosylation (Hug & Feldman, 2011), and it appears capsular polysaccharide is also highly related to these two pathways. Both $PglL_{Ab}$ and this locus are conserved throughout the *Acinetobacter* genus. Variation is observed in the glycan biosynthetic genes, suggesting that different *O*-glycans and capsules could be strain specific. A recent bioinformatic analysis of *A. baumannii* genomes identified nine different *O*-glycan serotypes (Kenyon & Hall, 2013), and mass spectrometry analysis by Kinsella *et al.* identified several different structures, but all pathogenic *Acinetobacter* species appear to conserve a negatively charged terminal monosaccharide (submitted). Having a negatively charged capsule has been demonstrated to be important for resistance to cationic antimicrobial peptides in *Neisseria* (Morgenthau *et al.*, 2014). The negatively charged capsule could also contribute to desiccation resistance in *A. baumannii* isolates by attracting water to the bacterial surface. Additionally, it appears that *O*-glycosylation is conserved in environmental isolates of *Acinetobacter.* While similar carbohydrate biosynthetic loci are present in pathogenic strains, *A. oleivorans* DR1 has a sulfatase homologue (AOLE_19045) in this locus suggesting a unique modification of the *O*-glycan and CPS, which may be important for its ability to binding and growing on diesel-oil (Kang *et al.*, 2011). The importance of charged carbohydrates in *Acinetobacter* requires further study, but with the high degree of conservation, it would appear to be necessary for *Acinetobacter* physiology.

4.3 Future Perspectives in Glycoengineering

The discovery of OTases and their relaxed specificity expanded the current toolboxes for glycoengineering novel recombinant glycoproteins. As shown for the *N*-OTases from *C. jejuni* and related organisms, *O*-OTases can be employed to generate glycoconjugates composed of different proteins attached to polysaccharides derived from LPS or capsule biosynthesis (Feldman *et al.*, 2005, Faridmoayer *et al.*, 2008, Gebhart et al., 2012, Wetter *et al.*, 2013, Lees-Miller et al., 2013). These recombinant glycoproteins can be exploited for vaccines and diagnostics of bacterial infections. With the limited number of characterized enzymes to date, both *N*- and *O*- OTases have limitations for their usefulness for *in vivo* biosynthesis of these therapeutics. *O*-OTases have only been shown to glycosylate integral or membrane associated proteins. Lithgow *el al*. demonstrated glycosylation of LolA (T0FJU1_9BURK), a putative periplasmic chaperone involved in the translocation of lipoproteins to the outer membrane in *B. cenocepacia* (Lithgow et al., 2014). This suggests that $PglL_{Bc}$ could be able to glycosylate soluble proteins, a major hindrance to the applicability of *O*-OTases for glycoengineering therapeutics.

An interesting new advance for engineered glycoproteins is their application for diagnostics. Ciocchini *et al*. demonstrated the effectiveness of this technique by developing immunoassays for detecting brucellosis in humans (Ciocchini *et al.*, 2013). Diagnosing human diseases by traditional methods can be expensive, slow, and require culturing of potentially hazardous pathogens. However, immunoassay of sera extracts from infected patients against engineered glycoproteins with different bacterial, viral, proteozoic, and even cancer glycan antigens would be significantly simpler, quicker, highly accurate, and low cost. This diagnostic tool is also attractive for use in facilities lacking

infrastructure or trained technicians. Further development of diagnostic tools utilizing engineered glycoproteins could have a significant positive impact on the medical field.

4.4 Concluding Remarks

The field of bacterial glycobiology has expanded exponentially in the last decade and glycosylation systems are being identified in an ever-increasing range of environmental and pathogenic species. LPS biosynthesis, capsule polysaccharide production, and protein glycosylation appear to be highly related, allowing exploitation of these pathways for development of therapeutic products. However, it is tempting to speculate additional glycosylation mechanisms might exist in nature. The identification of novel enzymes and pathways might increase the biotechnological applicability. Further investigation is required to fully understand the value of bacterial glycobiology in pathogenesis, vaccine development, and other industrial applications.

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Appendix A

Characterization of *O*-Glycosylation in the *Burkholderia cepacia*-Complex

Portions of this chapter have been published.
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A-1 Introduction

This investigation and characterization of the *O*-glycosylation system of *Burkholderia cepacia*-complex was part of collaboration with the labs of Dr. John Dennis and Dr. Nichollas Scott (UBC). Previous work had identified a homologue of the *O*-OTase PglL from *Neisseria meningitidis* in *B. cepacia*, and the gene was knocked out by homologous recombination to prove the functionality and phenotypes associated with protein glycosylation. The objective of my part of the investigation was to help reconstitute the glycosylation system in *E. coli*, prove glycosylation by mass spectrometry, and show that a pilin adjacent to $PglL_{Bc}$ was glycosylated in *B. cepacia*

A-2 Results

A-2.1 Bcal0960 Exhibits *O*-OTase Activity When Expressed in *E. coli*

To assess whether Bcal0960 is a functional *O*-OTase capable of *O*-glycosylation, we utilized an enzymatic activity assay previously employed to characterize *O*-OTases of *B. thailandensis* and *V. cholerae* (Gebhart *et al.*, 2012). Bcal0960 was recombinantly expressed with a C-terminal decahistidine tag in *E. coli* CLM24, a strain lacking a WaaL O antigen ligase. Two additional plasmids encoding a C-terminal hexahistidine-tagged acceptor protein, *Neisseria meningitidis* DsbA1 (Ng_1706) (Vik *et al.*, 2009), and the biosynthesis genes for an undecaprenyl-linked glycan, *C. jejuni* heptasaccharide (LLO_{Cj}), were coexpressed (Linton *et al.*, 2005). Whole-cell lysates were separated via SDS-PAGE and analysed by Western immunoblot (Fig. A.1A). Polyclonal HR6 antibody (red) detects the LLO_{Cj} glycan, monoclonal anti-His6 antibody (green) detects the DsbA1 acceptor protein, and colocalization (yellow) represents DsbA1 with the heptasaccharide modification. In the presence of Bcal0960, the *C. jejuni* heptasaccharide was transferred to DsbA1, demonstrating OTase activity (Fig. A.1A). Glycosylated DsbA1 was purified from

solubilized membrane extracts using nickel affinity chromatography, separated by SDS-PAGE, visualized by Coomassie staining, and bands predicted to be the glycosylated form of DsbA1 were excised and tryptically digested for MS/MS analysis. Manual analysis of LC-ESI Q-TOF MS data revealed a parental peak 941.69^{3+} *m/z*, and subsequent evaluation of the MS/MS fragmentation of this peak identified the *C. jejuni* heptasaccharide [Hex(HexNAc)6] (Linton et al., 2005, Reid *et al.*, 2009) attached to the previously identified glycopeptide (Vik et al., 2009) of DsbA1 (Fig. A.1B), confirming *O*-OTase activity in this recombinant strain. Although the native *Campylobacter jejuni* 81116 *N*-glycan utilizes bacillosamine as the reducing monosaccharide, in *E. coli* HexNAc can be utilized instead (Linton et al., 2005). These results suggest that Bcal0960 is an *O*-OTase responsible for *O*-glycosylation, and will be denoted from here on as $PglL_{Bc}$, as per its *N. meningitidis* orthologue

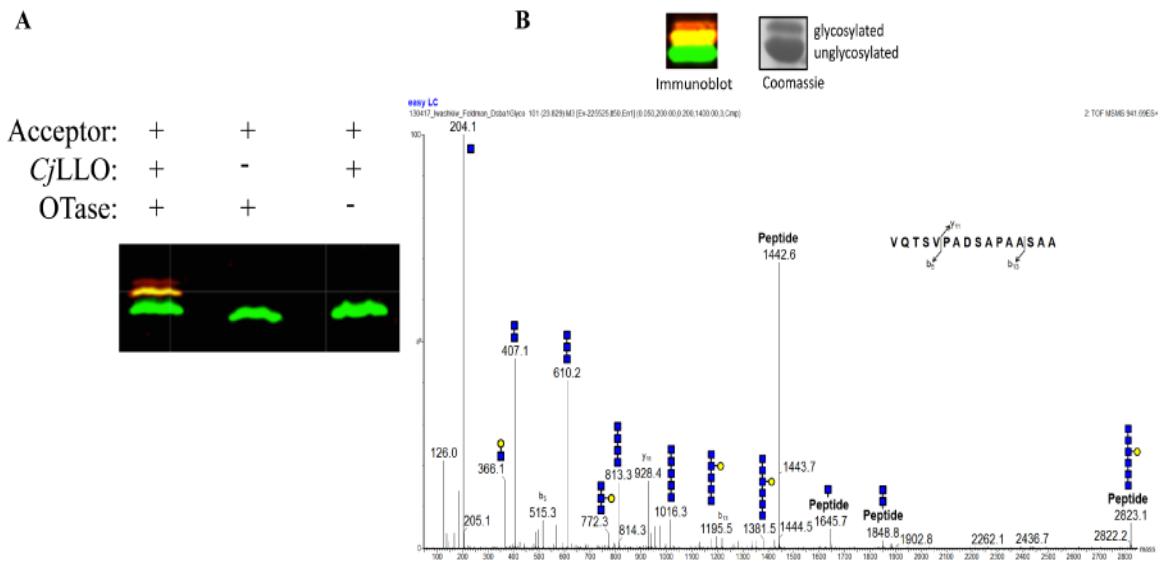

Figure A.1 Bcal0960 has *O*-OTase Activity. A) *In vivo* glycosylation confirming enzymatic activity of Bcal0960 as an *O*-OTase, as previously employed in *B. thailandensis* (Gebhart et al., 2012). *E. coli* whole-cell lysates expressing Bcal0960 co-transformed with His_6-tagged DsbA1 and the lipid linked *C. jejuni* heptasaccharide (*Cj*LLO). Polyclonal HR6 antibody (red) detects the LLO_{Cj} glycan, monoclonal anti-His_6 antibody (green) detects the acceptor protein, and colocalization (yellow) represents the His-tagged protein harbouring the heptasaccharide modification. B) Glycosylated DsbA1 was purified from solubilized membrane extracts using nickel affinity chromatography, separated by SDS-PAGE and visualized by Coomassie staining and Western immunoblotting. Bands corresponding to the glycosylated form of DsbA1 were excised and tryptically digested for MS/MS analysis. MS/MS mass spectra were obtained from an analyzed using a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Waters, UK) equipped with ananoACQUITY Ultra Performance liquid chromatography system (Waters, Milford, MA). Squares are representative of *N*-acetylhexosamine residues consistent with *N*-acetylgalactosamine residues in the CjLLO structure, while circles correspond to hexose residues.

A-2.2 *In trans* Expression of a Pilin Homologue Adjacent to $PglL_{Bc}$ is a Glycoprotein

As pilin proteins are commonly glycosylated in other Gram-negative bacteria, we were somewhat surprised that the Bcal0959 pilin was not a target protein for $PglL_{Bc}$ as determined by our MS results. To further understand why Bcal0959 was not among the target glycoproteins, we cloned a His-tagged *bcal0959* pilin gene into plasmid pSCRhaB2 and transformed it into both wild-type K56-2 and the *O*-OTase mutant *bcal0960*::*tet*.

Western blot analysis with antibodies to the hexa-histidine tag showed two pilin protein bands produced in the wild-type strain, corresponding to the newly expressed pilin protein as well as a glycosylated form of the pilin protein, whereas the *O*-OTase mutant only produced the unglycosylated form of the pilin (Fig A.2). However, electron micrographs of the K56-2 pSCRhaB2, K56-2 with cloned *bcal0959*, mutant *bcal0960*::*tet* pSCRhaB2, or *bcal0960*::*tet* with cloned *bcal0959* exhibited no surface expression of pili (data not shown), suggesting that the defect in K56-2 pili production relates to something more than pilin protein expression or glycosylation. These data indicate that when properly expressed, the Bcal0959 protein is a target for $PglL_{Bc}$ glycosylation, but that in wild-type *B. cenocepacia* K56-2 under the conditions tested, the pilin gene *bcal0959* is not expressed.

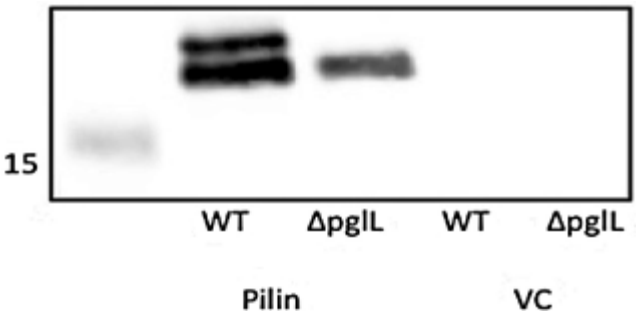

Figure A.2. Bcal0959 Pilin is Glycosylated by *pglL*$_{Bc}$ but not Expressed in *B. cenocepacia* K56-2. The cloned and His-tagged *bcal0959* pilin gene was expressed in K56-2 and the *pgl*L_{Bc} insertion mutant, and the resulting proteins were separated by SDS-PAGE and immunoblotted to identify the pilin isoforms (Pilin). With anti-His antibody, a band containing the cloned (unglycosylated) pilin protein was visible in the Δ*pgl*L_{Bc} lane, whereas two bands (glycosylated and unglycosylated) of pilin protein were evident in the wild-type K56-2 lane. In the lanes with proteins isolated from cells containing vector controls (VC) no His-tagged expressed proteins were observed. A 15 kDa molecular mass standard is shown in the left lane.

A-3 Discussion

We were able to confirm that $PglL_{Bc}$ was active, recognizes the *O*-glycosylation site of DsbA1 of *Neisseria meningitidis*, and *O*-OTase can be reconstituted in *E. coli*. Additionally, although a pilin adjacent to $PglL_{Bc}$ was not identified in ZIC-HILIC MS/MS as a glycoprotein, recombinant expression demonstrates that it is glycosylated. This suggests that the pilin was not expressed in the conditions tested.

A-4 Materials and Methods

A-4.1 Glycosylation of DsbA1 in *E. coli* and Mass Spectrometry Analysis

In vivo glycosylation assays in *E. coli*, including culture conditions, immunoblotting, purification and tryptic digestion, and MS/MS analysis of DsbA1 were carried out as described in (Gebhart et al, 2012). Briefly, membrane protein purification was achieved as follows: bacterial cells were harvested during stationary phase at 5000 *g* for 15 min at 4°C and were washed with phosphate-buffered saline (PBS). The bacterial pellets were resuspended in a ratio of 1 g of pellet to 10 ml of buffer #1 (1 μg ml^{-1} DNase I, complete EDTA-free protease inhibitor cocktail, 2% Triton X-114 in PBS). Bacterial lysis occurred during incubation on ice for 4 h. Cellular debris was removed by centrifugation at 10 000 *g* for 10 min at 4°C. The supernatant was incubated at 37°C for a minimal time to induce phase separation. The aqueous phase was removed after centrifugation at 10 000 *g* for 10 min at 30°C. The remaining detergent layer containing the glycosylated lipoproteins was diluted to the original solution volume with buffer #1 without detergent. Ice incubation and aqueous phase removal were repeated twice, with the ice incubation reduced to 1 h. To the detergent phase, 10 mM imidazole and 300 mM NaCl were added prior to the addition of an appropriate volume of 50% nickel-nitrilotriacetic acid (Ni-NTA) resin equilibrated in buffer #2 (2% Triton X-114, and 30 mM NaCl in PBS). This solution

was allowed to mix overnight at 4°C. The Ni-NTA resin was packed and then washed three times with buffer #2 containing 50 mM imidazole. The selected His-tagged lipoproteins were eluted twice with buffer #2 containing 300 mM imidazole and were concentrated by TCA precipitation.

A-4.2 Protein manipulation and Western Immunoblot

Samples were manipulated as previously described in earlier chapters.

A-5 References

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Appendix B

Production of a Recombinant Vaccine against *Burkholderia pseudomallei*

B-1 Introduction

This investigation on the ability to exploit *N*-glycosylation machinery of *C. jejuni* reconstituted in *E. coli* for the production of a glycoconjugate vaccine against *B. pseudomallei* was started by Dr. Fatima Garcia Quintanilla in the lab of Dr. Mario Feldman. As we have previously shown, glycoconjugates can be produced with exogenous O antigens by expressing the OTase PglB and engineered acceptor glycoprotein AcrA in *E. coli*. Here, Fatima was able to clone and express the OPS II of *B. pseudomallei* in *E. coli*. Glycosylation did not occur in the traditional *E. coli* host strains, so Fatima created the strain SDB1, (*wecA*$^{-}$ and *waaL*$^{-}$). The objective of my part of the investigation was to perform protein purification and mass spectrometry to confirm glycosylation of AcrA with the *B. pseudomallei* OPS II structure.

B-2 Results

B-2.1 Reconstitution of the *C. jejuni N*-Glycosylation System in *E. coli* to Synthesize a Glycoconjugate with the *B. pseudomallei* OPS II

Previous work cloned a region consisting of 21 potential open reading frames, and further investigation identified a cluster of 15 genes required for the biosynthesis of *B. pseudomallei* K96243 OPS II (DeShazer *et al.*, 1998). A previous study demonstrated by NMR analysis the structure of the OPS II to be a polymer of a disaccharide repeating structure composed of -3-)-β-D-glucopyranose-(1-3)-α-L-6-deoxy-talopyranose-(1-, with variable *O*-methyl and *O*-acetyl modifications (Perry *et al.*, 1995). In order to recombinantly express the *B. pseudomallei* OPS II in *E. coli*, essential 15 genes were subcloned (genes *rmlB* to *wbil*) from the plasmid pCC1FOS-BPF16β_E10 by restriction digest into the arabinose inducible expression vector pBAD24, generating pEQ3. Expression of the *B. pseudomallei* OPS II in *E. coli* was visualized by Western blot with a

polymer of immunoreactive bands being observed, confirming the production of the carbohydrate structure. Next, we attempted to generate a *N*-linked glycoprotein with the OPS II by exploiting the *C. jejuni N*-glycosylation system as previously described (Ihssen *et al.*, 2010, Iwashkiw *et al.*, 2012, Cuccui *et al.*, 2013, Wetter *et al.*, 2013). In earlier work, *N*-glycosylated AcrA was synthesized in *E. coli* by co-expression of *C. jejuni* PglB and AcrA with an appropriate carbohydrate structure. We therefore expressed PglB (pMAF10), AcrA (pIH18), and the *B. pseudomallei* OPS II antigen (pEQ3) in both an expression (EPI300) and *wecA*- strain (CLM37) and tested for glycosylation by Western blot. We were unable to detect any evidence of glycosylation (Fig B.1)

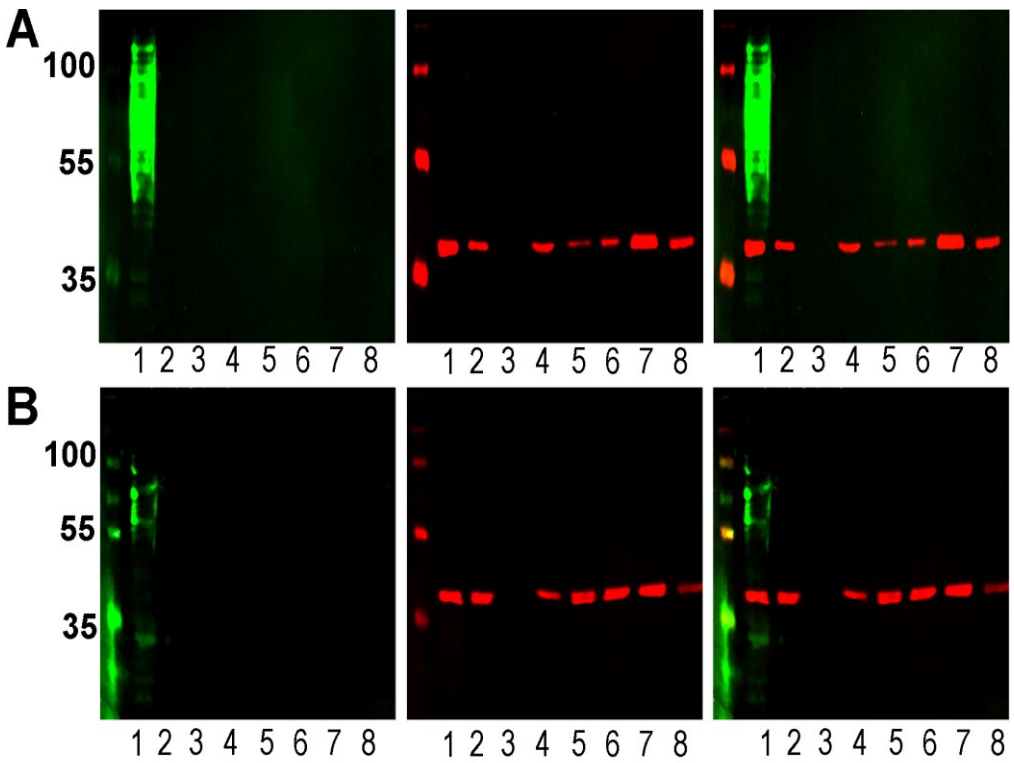

Figure B.1. **Western Blot of AcrA Purified From Either EPI300 or CLM37 Expressing PglB (pMAF10), OPS II (pEQ3), and AcrA (pIH18) Reveals No Glycoprotein Produced.** AcrA was purified from either EPI300 (A) or CLM37 (B) by affinity chromatography and fractions were loaded on 10% SDS-PAGE and visualized by Western immunoblot (left α-OPSII, middle α-His, and right overlay). Lanes are as follows: 1- Whole cell lysate, 2-Periplasmic extract, 3- Flow through, 4- Wash, 5 -> 8- elutions. OPS II is only observed in whole cell lysates (Lane 1), while AcrA is observed in all lanes except the flow-through waste.

B-2.2 *In vivo* Synthesis and Purification of a *N*-Linked Glycoconjugate with the *B. pseudomallei* OPS II Glycan

In order to create a *N*-linked glycoconjugate, we transformed the *E. coli* strain SDB1 with the pEQ3 (OPS II), pMAF10 (PglB), and pIH18 (AcrA). Cultures of each transformed strain were grown and induced as required, and AcrA was purified from periplasmic extracts by Ni^{2+} affinity chromatography. To determine if AcrA was

glycosylated, we analyzed the purified protein by Western blot with antibodies specific to either AcrA (Fig 5A) or OPS II (Fig 5B) and when visualized together, we observe an overlap of the signal, highly suggesting glycosylation (Fig B.2).

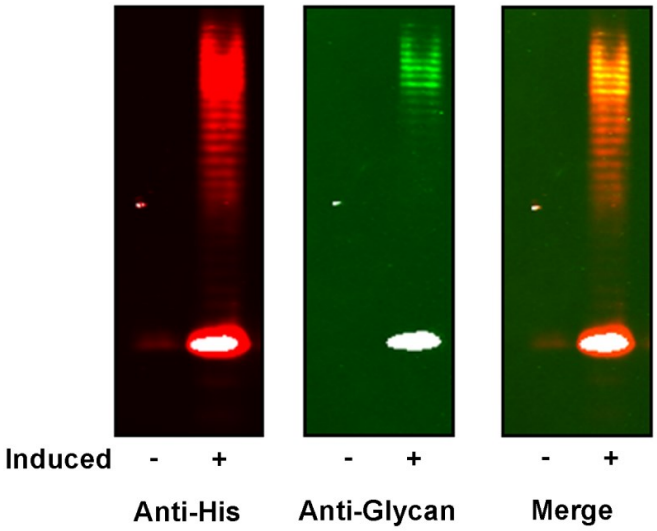

Figure B.2 Analysis of Purified AcrA by Western Blot. SDB1 transformed with pMAF10 (*pglB*), pIH18 (*acrA*), and pEQ3 (OPS II) were grown (+/-) induction of OPS II, harvested, and AcrA was purified by Ni2+-NTA affinity chromatography. A single band is only observed by anti-His Western blot at ~35 kDa without induction of pEQ3, whereas a high molecular weight ladder is co-detected by both anti-His and anti-Glycan antibodies in cultures induced with 0.2% arabinose, confirming glycosylation of AcrA.

B-2.3 Mass Spectrometry Analysis of the Purified Glycoprotein Confirms OPS II Attached to AcrA

To confirm that AcrA was glycosylated with the *B. pseudomallei* OPS II carbohydrate, we employed mass spectrometry techniques. The purified glycoprotein was tryptically digested in-solution, and the resulting peptides were examined by LC-ESI-Q-TOF MS and MS/MS. Manual analysis of the MS data revealed a peak with a *m/z* 1152.06^{3+}, and further inspection of this peak by MS/MS revealed a glycopeptide that corresponded to the previously identified second glycosylation site (AVFDNNNSTLLPGAFATITSEGFIQK; *m/z* 2754.1) of AcrA with the addition of an *m/z* 700.2 modification (Fig B.3). Manual peak annotation identified the modification to be a tetramer of 188-162-188-162. The mass of 188 Da is consistent with an O-acetyl deoxyhexose residue, and the 162 Da is consistent with a Hexose residue. We were also able to identify in the low molecular region both an individual O-acetyl deoxyhexose (189.0 Da), and a subunit of the dimer with a mass of 351.1 Da. Our mass spectrometry characterization of the *B. pseudomallei* OPS II glycan is consistent with the previously published data identifying it to be a polymer of dimers of O-acetylated deoxytalose and glucose (Perry, 1995). This data combined with the immunoreactivity of our glycoconjugate to the *B. pseudomallei* OPS II antibody confirms that we were able to synthesize the correct glycan structure in *E. coli* SDB1.

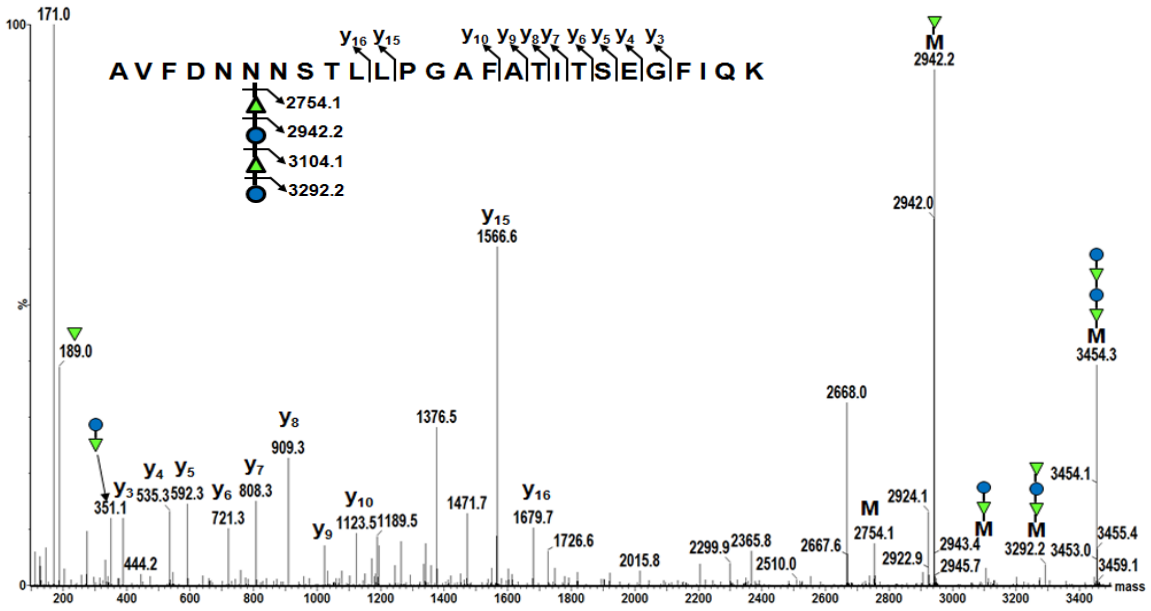

Figure B.3 Mass Spectrometry Identification of the *B. pseudomallei* OPS II Glycan Attached to AcrA. MS analysis of tryptically digested glycosylated AcrA revealed a peak of *m/z* 1152.06^{3+}. MS/MS of this peak showed a previously characterized glycosylation site of AcrA (AVFDNNNSTLLPGAFATITSEGFIQK; 2754.1 Da) with a modification of 700.2 Da. Analysis of the modification revealed a tetrameric glycan, with a structure of 188-162-188-162. This structure is consistent with the previously determined structure of *B. pseudomallei* OPS II being a polymer of dimers of O-acetylated deoxytalose (188 Da) and glucose (162 Da).

B-3 Discussion

We have demonstrated that the OPS II glycan of *B. pseudomallei* can be functionally expressed in *E. coli*. Additionally, this glycan can be utilized by the *N*-glycosylation system of *C. jejuni* to produce a glycoconjugate, and our novel *E. coli* strain SDB1 has a higher efficiency of production as compared to previously used strains.

B-4 Materials and Methods

B-4.1 Bacterial strains, plasmids, and growth conditions

Escherichia coli strains were grown on LB broth at 37°C. Trimethoprim (100 µg/ml), spectinomycin (80 µg/ml), and ampicillin (100 µg/ml) were added to the media for plasmid selection as needed. The strains and plasmids used are listed in Table A-4.1.

Table B.1 List of Strains and Plasmids Used in this Work

Strain	**Genotype or description**	**Reference**
EPI300	F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL (StrR) nupG trfA dhfr	Epicentre
CLM37	W3110, ΔwecA	(Linton *et al.*, 2005)
SDB1	W3110, Δ*waaL* ligase, Δ*wecA* GalNAc transferase	This study
Plasmids		
pEQ3	*B. pseudomallei* type II OPS, Ap^R	This study
pIH18	Soluble periplasmic *C. jejuni* $acrA_{6xHis}$ cloned into pEXT21, Sp^R	(Hug *et al.*, 2010)
pMAF10	*C. jejuni pgl*B cloned into pMLBAD, Tp^R	(Feldman *et al.*, 2005)

B-4.2 SDS-PAGE and Western Immunoblot

Glycan expression and protein glycosylation were separated by SDS-PAGE (10% acrylamide), transferred nitrocellulose membranes with transfer buffer MeOH:10x gel running buffer:H_20 (2:1:7) for 40 mins, using a Trans-blot SD semi-dry transfer cell (Biorad). Membranes were blocked with Odyssey blocking buffer (Licor Biosciences) for 1h, and analyzed with primary antibodies α-His (Roche) and α-BPs OPS II glycan (1:1,000) (DeShazer et al., 1998). Blots were shortly washed 3x 10 mins with PBST and subsequently incubated with a secondary goat α-mouse IgG IRDye-800 or goat α-rabbit IgG IRDye-680 antibody (Licor Biosciences). Membranes were washed 2x 10 mins with

PBST and 1x 10 mins PBS and dried. Membranes were visualized using the Odyssey Infrared Imaging System (Li-Cor Biosciences, USA).

B-4.3 Production and Purification of Glycosylated AcrA

SDB1 strain transformed with *C. jejuni* PglB (pMAF10), AcrA (pIH18) and BPs type II O-antigen (pEQ3) was grown overnight at 37°C. Culture was reinnoculated 1/33 into fresh LB media using a culture/flask ratio 1:10. After 2 hours at 37°C with shaking at 200 rpm the cultures were induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside and 0.2% (w/v) arabinose. To increase the glycosylation yield in SDB1 we also added $MnCl_2$ (4 mM). Five hours after induction at 37°C, arabinose was added again to ensure PglB expression. Cells were harvested by centrifugation after an overnight induction period and the periplasmic extract containing the glycoproteins was extracted using a lysozyme treatment as described previously (Iwashkiw et al., 2012). For purification, the periplasmic fraction was equilibrated with 1/9 vol 10 X loading buffer (0.1 M Imidazole, 3 M NaCl, 0.2 M Tris-HCl pH 8.0) and subjected to a Ni^{2+}affinity chromatography as described (Iwashkiw et al., 2012).

B-4.4 Mass Spectrometric Analysis of Glycosylated AcrA

This technique was described in earlier chapters

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