Investigating the Roles of *O*-linked Protein Glycosylation and Type Two Secretion in the Pathogenesis of *Acinetobacter*

by

Rachel Kinsella

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Department of Biological Sciences
University of Alberta

Abstract:

The *Acinetobacter baumannii, A. nosocomialis* and *A. pittii* are Gram-negative opportunistic human pathogens that are of importance to the healthcare system particularily because of their resistance to antibiotics. The thesis below focuses on investigating two virulence attributes of *Acinetobacter* that contribute to its pathogenesis, general *O*-linked protein glycosylation and Type II secretion.

At least seven proteins are *O*-glycosylated in *A. baumannii* ATCC 17978 in an *O*-oligosaccharyltransferase dependent manner. Protein glycosylation is required for biofilm formation, and virulence in an amoeba, insect and murine models of infection. *A. baumannii* utilizes the same glycan to modify glycoproteins as is polymerized into capsular polysaccharide. Capsular polysaccharide is required for resistance to complement mediated killing and therefore also is necessary for colonization in mice. The importance and abundance of this carbohydrate in the pathogenesis of *A. baumannii* as well as the non-linear nature of glycan synthesis, lead us to investigate the diversity, composition and properties of the *Acinetobacter* glycoproteome using various mass spectrometry methods. Twenty-six glycoproteins were identified in the fifteen strains examined, revealing that similar proteins are targeted for glycosylation in *Acinetobacter baumannii, A. calcoaceticus, A. pitti, A. baylyi,* and *A. nosocomialis.* Glycosylation tends to occur at low complexity regions enriched in Proline, Alanine and Serine residues. There was extensive glycan variability between the strains and within the strains examined. All *O*-glycans identified have an N-acetyl hexosamine residue at the reducing end and tended to contain a negatively charged monosaccharide. Glycopeptides modified with more than one subunit of the *O*-glycan were identified in most strains, suggesting that sharing the glycan between protein glycosylation and capsule production is a common feature in *Acinetobacter.*

Most glycoproteins identified required the sensitivity of Mass Spectrometry and Zwitter ionic hydrophilic interaction chromatography glycopeptide enrichment methods to be detected. Two glycoproteins, A1S_3626 andA1S_3744 were identified by two-dimensional differential gel electrophoresis separation of total membrane glycosylated and unglycosylated proteins, implying that these proteins are produced and modified in higher abundance. There is one 30 kDa periodic acid schiff stained glycoprotein band produced by *A. baumannii* ATCC 17978. We hypothesized that either A1S_3626 or A1S_3744 would be required for production of this 30 kDa glycoprotein. A1S_3626 and A1S_3744 are both not essential for production of the 30 kDa glycoprotein, suggesting there may be a yet unidentified glycoprotein. Both A1S_3626 and A1S_3744 are not required for biofilm formation in *A. baumannii*. Strains devoid of protein glycosylation display a virulence defect in *Galleria mellonella*. A1S_3744 is not required for virulence in *G. mellonella*.

A. nosocomialis M2 is a medically relevant member of the *Acinetobacter* genus. Type II secretion is required for virulence and nutrient acquisition by several Gram-negative organisms. Here we show M2 has a functional Type II secretion system that is required for virulence in *G. mellonella* larvae and mice, and is responsible for the secretion of two lipases LipA and LipH as well as a protease, CpaA. LipA and CpaA required membrane anchored chaperones, LipB and CpaB respectively, for their secretion. Bioinformatic analysis revealed putative chaperones adjacent to several characterized Type II substrates, suggesting this may be a wide-spread phenomenon. CpaA is required for full virulence in the *G. mellonella* larvae and splenic colonization in a murine pulmonary infection model. We demonstrate the physical interaction between CpaA and its chaperone CpaB. The C-terminal periplasmic domain of CpaB is adequate for secretion of CpaA. Soluble, periplasmic CpaB is secreted with CpaA, suggesting the purpose

of the membrane anchor may be to retain CpaB and only secrete free CpaA. One quarter of CpaA is sufficient to bind CpaB, indicating there are multiple points of interaction along the length of CpaA. We crystallized the CpaA-CpaB complex and have obtained diffraction data sets ranging from 3.5 to 7 angstrom resolution. There are no crystallized orthologs of CpaB, therefore we are pursuing the heavy-metal derivative in order to solve the crystal structure. The structural data may shed light on the mechanism of CpaB and provide the basis for design of specific CpaA inhibitors.

Preface

Part of the introduction in Chapter 2 is included in a collaborative review on protein glycosylation. I wrote the *O*-glycosylation portion of this review under the supervision of Dr. Feldman. E. Valguarnera wrote the *N*-glycosylation and glycosylating toxins portion of this review. E. Valguarnera, M.F. Feldman and I all contributed to the introduction. This review was published as: Valguarnera E, Kinsella RL, Feldman MF. (2016) Sugar and Spice Make Bacteria Not Nice: Protein Glycosylation and its Influence in Pathogenesis. *J Mol Biol* **428:**3206-20. Part of the type II secretion introduction is my original work and is included in Weber B.S., Kinsella R.L., Harding C.M. and Feldman M.F. (2017) The Secrets of Acinetobacter Secretion. *Trends Microbiol*. **S0966-842X(17)**:30017-3.

The work included in Chapter 3 of this thesis was a collaboration between myself and Dr. Mario Feldman at the University of Alberta and Drs. Nichollas Scott and L.J. Foster at the University of British Columbia. Together with Dr. Mario Feldman we had the idea to investigate the *O*-glycan diversity in *Acinetobacter*. Under the supervision and guidance of Dr. Feldman, I created a directed glycan characterization method called "glycan fishing". I used this method to characterize the *O*-glycan repertoire of strains compatible with *in trans* gene expression. I also performed the periodic acid schiff staining of total membrane extracts and complement killing assay to test for production of capsular polysaccharide and glycoproteins. We established a collaboration with Drs. Scott and Foster to characterize glycan variability in strains incompatible with the "glycan fishing" method. Dr. Scott involved A.V. Edwards, M.R. Larsen, S.M. Dutta and J. Saba in characterization of glycoprotein and *O*-glycan diversity using ZIC-HILIC enrichment and specialized mass spectrometry methods. I prepared the total membrane samples for Dr. Scott, whom then proteolytically digested them and analyzed them using CTD and HTD mass spectrometry analysis. These specialized fragmentation methods were not available to us at the U of A as they require specialized equipment and the data mining requires specific expertise. The work presented in chapter 2 was equally contributed by myself under the supervision of Dr. Feldman and Dr. Scott, and is published as Scott, N.E., R.L. Kinsella, A.V. Edwards, M.R. Larsen, S.M. Dutta, J. Saba, L.J Foster and M.F. Feldman (2014) Diversity within the *O*-linked protein glycosylation systems of *Acinetobacter species. Mol Cell Proteomics* **13**:2354-70. The paper was written by myself, Dr. Feldman and Dr. Scott.

The work presented in Chapter 4 of this thesis is my original work. Experiments were designed and performed under the guidance of Dr. Feldman. Creation of the *pglL* clean deletion strain was performed by Dr. J.A. Iwashkiw. Construction of the pEC-*pglL* plasmid for *in trans* complementation of Δ*pglL* was done by Dr. E. Valguarnera. The remaining work presented in this chapter is my original work.

The work presented in Chapter 4 of this thesis was a collaborative project investigating type II secretion in Acinetobacter. I demonstrated type II secretion systems appear to be active in numerous species of *Acinetobacter* by expressing type II substrate *lipH-his* and monitoring its expression and secretion through Western blot analysis. C.M. Harding performed the experiments characterizing the type II secretion system of *A. nosocomialis* except those investigating the role of type II secretion in pathogenicity. I performed the *A. nosocomialis G. mellonella* larvae infection comparing survival of larvae infected with wildtype, Δ*gspD* or the complemented mutant over time. L.D. Palmer and E.P. Skaar performed the murine pulmonary infection with *A. nosocomialis*. C.M. Harding performed the bioinformatic analysis identifying putative membrane bound type II chaperones. This work has been published as: Harding CM, Kinsella RL, Palmer LD, Skaar EP, and MF Feldman. (2016). Medically Relevant *Acinetobacter* Species Require a Type II Secretion System and Specific Membrane-Associated Chaperones for the Export of Multiple Substrates and Full Virulence. *PLoS Pathog* **12(1):**e1005391. The manuscript was written by C.M. Harding and M.F. Feldman, I was only involved in experiment design, data production and editing.

The data presented in Chapter 5 of this thesis contains some collaborative work. In collaboration with Dr. N.H. Tolia we have crystallized CpaA-CpaB. I cloned, expressed and purified CpaA-CpaB. A member of the Tolia lab, N. Salinas, further purified CpaA-CpaB by FPLC. Under the guidance of N. Salinas I set up 96-well mosquito nanoliter protein crystallization screens and microliter grid-screens. N. Salinas and Dr. Darya Urusova, members of the Tolia lab, looped my CpaA-CpaB crystals and analyzed them by X-ray diffraction. L.D. Palmer and E.P. Skaar performed the murine pulmonary infection to analyze the role of CpaA in mouse colonization. All other work presented in this chapter was performed by myself under the guidance of Dr.

Feldman. Writing of this chapter is my original work. Part of the introduction is included in the section of a protein secretion review that I wrote: Weber BS, Kinsella RL, Harding CM and MF Feldman. (2017) The Secrets of *Acinetobacter* Secretion. *Trends Microbiol*. **S0966-842X(17):**30017-3.

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A huge thank you to my family, Eric's family and my friends for their support throughout all my studies and thesis writing. The encouraging words, prayers, snail-mail and phone calls, especially once I moved away, will always be treasured. Thanks to my friends for the encouragement, always being there for me and all the fun experiences over the last few years. A special thanks to Eric for his support and understanding, especially through my Thesis writing time. I cannot imagine getting through it without you!

Thanks to the Feldman lab members, both past and present. I am grateful for the friendships we have built, the scientific discussions shared and the drive to push one another to become better scientists. It has been a pleasure coming to work, thank you!

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Table of Contents

List of Tables

List of Figures

List of Abreviations

Acb – *Acinetobacter calcoaceticus-baumannii* complex

ACN – acetonitrile

BLAST – Basic Local Alignment Search Tool

CID- collision induced dissociation

BSA – Bovine Serum Albumin

DELTA – Domain Enhanced Lookup Time Accelerated

dHexNAc – deoxy-N-acetyl hexosamine

diNAcBac- di-N-acetylbacillosamine

DNA – Deoxyribonucleic Acid

DTT – Dithiothreitol

ETD – Electron-transfer dissociation

ESI – Electron spray ionization

EDTA – Ethylenediaminetetraacetic Acid

Gal – Galactose

GalNAc- N-Acetylgalactosamine

Glc – glucose

GlcNAc – N-Acetylglucosamine

GlcNAc3NAcA4OAc – Tri-acetylated glucuronic acid

Hex – Hexose

HexNAc – N-acetyl hexosamine

IPTG – Isopropyl β-D-1-thiogalactopyranoside

Leg – Legionaminic

LC-MS/MS – Liquid chromatography - tandem mass spectrometryHCD - Higher-energy collisional dissociation

LPS – Lipopolysaccharide

LOS – Lipoligosaccharide

MALDI – Matrix-assisted laser desorption/ionization QIT – Quadrupole ion trap

MDR – Multi-drug resistantQ-TOF – Quadrupole Time-of-flight

MH+ - molecular ion (positive ion mode)IT – Ion trap

MS – Mass Spectrometry

NulO – nonulosonic acid a nine carbon sugar

O-OTase – *O*-Oligosaccharyltransferase

PAS – Periodic Acid Schiff

PBS – Phosphate Buffered Saline

Pse- Pseudaminic Q-TOF – Quadrupole Time-of-flight

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TCA – Trichloroacetic acidSec – general secretory pathway

Tat – Twin arginine transport systemT3SS – Type Three Secretion System

T2SS – Type Two Secretion System

ZIC-HILIC – Zwitterionic hydrophilic interaction liquid chromatography

2D-DIGE – Two-dimensional difference gel electrophoresis CFU – colony forming units

Chapter 1: Introduction

Part of the introduction in Chapter 1 is included in a collaborative review on protein glycosylation. I wrote the *O*-glycosylation portion of this review under the supervision of Dr. Feldman. E. Valguarnera wrote the *N*-glycosylation and glycosylating toxins portion of this review. E. Valguarnera, M.F. Feldman and I all contributed to the introduction. This review was published as: Valguarnera E, Kinsella RL andFeldman MF. (2016) Sugar and Spice Make Bacteria Not Nice: Protein Glycosylation and its Influence in Pathogenesis. *J Mol Biol* **428:**3206-20. Part of the type II secretion introduction is my original work and is included in Weber B.S., Kinsella R.L., Harding C.M. and Feldman M.F. (2017) The Secrets of Acinetobacter Secretion. *Trends Microbiol*. **S0966-842X(17)**:30017-3.

1.1-Introduction to Acinetobacter

Acinetobacter species are a group of Gram-negative aerobic organisms found in soil, water and the hospital environment (Visca, P. *et al.*, 2011). The opportunistic Gram-negative human pathogen *Acinetobacter baumannii* persists in the healthcare setting because of its ability to survive exposure to various antimicrobials and sterilization agents. *A. baumannii*'s ability to cause multiple infection types complicates diagnosis and treatment (Peleg, A.Y. *et al.*, 2008). *A. baumannii, A. pittii* and *A. nosocomialis* are prevalent in the hospital setting and capable of causing human disease (Visca, P. *et al.*, 2011; Jain, A.L. *et al.*, 2016). *Acinetobacter* can cause a variety of infections such as urinary tract, pneumonia or soft-tissue infections as well as sepsis (Peleg, A.Y. *et al.*, 2008; Dijkshoorn, L. *et al.*, 2007). The antimicrobial resistance combined with the propensity to infect immunocompromised patients complicates treatment options against *Acinetobacter* (Montefour, K. *et al.*, 2008). *Acinetobacter* is among the most prevalent nosocomial infection agents worldwide (Santajit, S. and Indrawattana, N., 2016). According to the CDC, pan-drug resistant isolates of *Acinetobacter* have been identified and over 50% of *Acinetobacter* infections are multi-drug resistant, igniting the need for new treatment options (Santajit, S. and Indrawattana, N., 2016). The study of *Acinetobacter* virulence attributes may shape future treatment methods against *Acinetobacter.*

Research has focused on understanding the mechanisms of antimicrobial resistance and identifying virulence factors of *Acinetobacter*, in hopes of identifying a weakness. Several virulence factors of *Acinetobacter* have been identified including but not limited to OmpA; siderophore iron-acquisition systems; and surface carbohydrates including capsular polysaccharide, poly-beta-1,6-*N*-acetylglucosamine (PNAG) and *O*-linked protein glycosylation (Gaddy, J.A. *et al.*, 2012; Gaddy, J.A. *et al.*, 2009; Lees-Miller, R.G. *et al.*, 2013; Choi, A.H. *et al.*, 2009; Choi, C.H. *et al.*, 2008). Siderophores contribute to *A. baumannii* survival in the iron-poor conditions of a host, where as surface carbohydrates aid in biofilm formation and adhesion leading to environmental survival and host colonization (Gaddy, J.A. *et al.*, 2012; Gaddy, J.A. *et al.*, 2009; Lees-Miller, R.G. *et al.*, 2013)

Acinetobacter, like other Gram-negative bacteria produces PNAG (Choi, A.H. *et al.*, 2009). PNAG specific antibodies provide protection against *A. baumannii* through classical complement mediated killing against *Acinetobacter* PNAG producing strains (Bentancor, L.V.

et al., 2012). PNAG is a cell associated polymerized carbohydrate. PNAG is required for biofilm formation as deletion of the biosynthetic genes results in a strong reduction in biofilm (Choi, A.H. *et al.*, 2009). The ability of *A. baumannii* to colonize abiotic and biotic surfaces likely contributes to its survival in the hospital setting (Tomaras, A.P. *et al.*, 2003). PNAG is a well characterized component of biofilms in *Staphylococcus epidermidis* (Mack, D. *et al.*, 1996), and *Staphylococcus aureus* (Maira-Litran, T. *et al.*, 2002) and is known to have a role in cell-cell adherence in *S. aureus* (Cramton, S.E. *et al.*, 1999) and *S. epidermidis* (Mack, D. *et al.*, 1996). PNAG is a virulence factor and colonization factor for *S. aureus* and *S. epidermidis* (Kropec, A. *et al.*, 2005; Rupp, M.E. *et al.*, 1999; Shiro, H. *et al.*, 1994; Shiro, H. *et al.*, 1995).

1.2-Introduction to Protein Glycosylation:

Protein glycosylation, the covalent attachment of a carbohydrate to an amino acid residue, was formally demonstrated in 1938 and is the most abundant post translational modification in nature (Neuberger, A., 1938; Apweiler, R. *et al.*, 1999). While it was long thought to occur only in eukaryotes, the first identified prokaryotic glycoproteins were the cell surface proteins of the archaeon *Halobacterium* and the bacterial species *Clostridium*, showing that this phenomenon occurs in all the kingdoms of life (Sleytr, U.B., 1975; Mescher, M.F. and Strominger, J.L., 1976). This section introduces you to different forms of protein glycosylation, *N*-linked and *O*-linked, mechanisms of protein glycosylation, as well the roles of protein glycosylation in bacterial pathogenesis.

Protein glycosylation occurs in two main forms, *N*-linked which is the attachment of the carbohydrate on to the nitrogen of an asparagine residue or *O*-linked where the carbohydrate is attached to the oxygen of the hydroxyl group of serine or threonine residues. Beyond the linkage definition, protein glycosylation occurs mainly by two different pathways that differ in the way the sugars are assembled and transferred to the acceptor protein. These are known as oligosaccharyltransferase (OTase) dependent and OTase independent mechanisms. *N*-glycosylation and *O*-glycosylation can occur by OTase dependent and OTase independent mechanisms. OTases are a family of glycosyltransferases (GTases) that catalyze the *en bloc* transfer of an oligosaccharide from a lipid donor to an acceptor molecule, usually a protein. In OTase dependent systems, an initiaiting GTase transfers a monosaccharide from its nucleotide-activated sugar onto the lipid carrier undecaprenyl phosphate (Und-P) at the cytoplasmic face of

the inner membrane (Hug, I. and Feldman, M.F., 2011). Cytoplasmic glycosyltransferases consecutively add nucleotide activated monosaccharides to the growing lipid-linked carbohydrate. The complete Und-PP linked glycan is then flipped to the periplasmic side of the inner membrane by a flippase, where the OTase transfers the glycan to specific residues on the target protein. This mechanism is very similar to eukaryotic *N*-linked glycosylation occurring in the endoplasmic reticulum and shares similarities with the synthesis of lipopolysaccharide (LPS) in Gram-negative bacteria (Hug, I. and Feldman, M.F., 2011; Aebi, M., 2013; Linton, D. *et al.*, 2005; Wacker, M. *et al.*, 2006). In contrast, OTase independent glycosylation occurs in the cytoplasm, where monosaccharides are transferred one at a time onto the acceptor protein by successive action of GTases. These glycoproteins are subsequently transported to the outer membrane or, in the case of flagellin, are secreted forming the flagella filament (Thibault, P. *et al.*, 2001; Schirm, M. *et al.*, 2004a; Taguchi, F. *et al.*, 2006). Protein glycosylation was originally thought to be unique to eukaryotes. Now it is well understood that protein glycosylation is wide-spread in bacteria. Bacteria presentmore protein glycosylation pathways than eukaryotes and archaea, as well as a large diversity of protein-bound carbohydrates (Dell, A. *et al.*, 2010). Some bacterial species carry such a complex array of sugars that many of its components have yet to be identified (Scott, N.E. *et al.*, 2014a). OTase dependent *O*-glycosylation occurs in pathogenic and environmental isolates of *Acinetobacter*, suggesting protein glycosylation has not envolved as a virulence trait in pathogenic organism {Scott, 2014 #5}. Most studies on protein glycosylation are focused on pathogenic bacteria, causing the data to be skewed towards a role in pathogenesis. However, broader studies encompassing pathogenic and environmental isolates demonstrate protein glycosylation is not unique to pathogenic species. Whether this trend holds true for more bacterial genera remains to be studied.

1.2.1-OTase dependent N-glycosylation: *Campylobacter jejuni* as a model organism.

Asparagine-linked glycosylation of proteins, or *N*-linked glycosylation, was not thought to occur in bacteria until it was discovered in *Campylobacter jejuni* over 15 years ago. Currently, *N*-glycosylation is known to be present in all domains of life (Aebi, M., 2013); (Jarrell, K.F. *et al.*, 2014). *N*-glycosylation of proteins in *C. jejuni* is by far the most studied pathway in bacteria (Szymanski, C.M. *et al.*, 1999). *C. jejuni* is a member of the ε-proteobacteria class and is the leading cause of bacterial diarrhea, whose main source are contaminated poultry meat and related

products since birds are the natural reservoirs (Kaakoush, N.O. *et al.*, 2015). Symptoms include severe diarrhea and abdominal pain (Nyati, K.K. and Nyati, R., 2013). Infection with *C. jejuni* can also result in Guillian Barré syndrome, which can cause extreme paralysis. The most complete glycoproteomic study in *C. jejuni* found that more than 60 proteins are *N*-glycosylated, including secreted, periplasmic or inner membrane-bound proteins. This diversity of *N*-glycosylated proteins indicates the importance of a general glycosylation pathway that is central in pathogenesis and virulence of *C. jejuni* but is not essential for cell growth (Scott, N.E. *et al.*, 2011). Loss of *N*-glycosylation inhibits *Campylobacter* in the colonization of mammalian cells both *in vitro* and *in vivo*, increasing the interest in the N-glycosylation machinery as a druggable target (Szymanski, C.M. *et al.*, 2002). Recently it has been shown that N-linked glycosylation of surface proteins may enhance *C. jejuni* fitness by protecting bacterial proteins from cleavage due to gut proteases (Alemka, A. *et al.*, 2013).

The enzymes required for OTase dependent *N*-glycosylation in *C. jejuni* are found in a single locus called *pgl.* The general *N*-glycosylation machinery in *C. jejuni* synthesizes and transfer an heptasaccharide to an asparagine residue on target proteins, whose synthesis begins with the cytoplasmic conversion of N-acetyl glucosamine into N,N'-diacetylbacillosamine, or diNacBac, mediated by the consecutive action of the dehydratase PglF, the aminotransferase PglE, and the acetyltransferase PglD (Morrison, M.J. and Imperiali, B., 2014). A recent structural study on the PglE enzyme shows differences in the way this particular enzyme accommodates its substrate, in comparison to other aminotransferases, emphasizing the importance of structural information as a key to understanding the mechanistic nature of biological processes (Riegert, A.S. *et al.*, 2015). Comparison of X-ray structural data of the aminotransferase PglE, to other aminotransferase structures revealed that the position of the pyranosyl ring within the substrate binding pocket determines the stereochemistry of the resulting product (Riegert, A.S. *et al.*, 2015). The initiating GTase in the pathway, PglC, transfers diNacBac to the inner membrane lipid carrier Und-P. The rest of the *C. jejuni* heptasaccharide is synthetized on the Und-PP bound diNacBac by the GTases PglA, PglJ, PglH, and PglI (Larkin, A. and Imperiali, B., 2011; Nothaft, H. and Szymanski, C.M., 2013). Once the heptasaccharide is complete, a flippase translocates this lipid-linked oligosaccharide to the periplasmic face of the inner membrane. In *C. jejuni*, the flippase PglK has recently been structurally characterized (Perez, C. *et al.*, 2015). Finally, the OTase PglB catalyzes the *en bloc* transfer of the heptasaccharide to periplasmic and membrane-bound target proteins.

The *pglB* gene of *Campylobacter jejuni* was found to be a true homolog of Stt3, the catalytic subunit of eukaryotic *N*-OTases (Aebi, M., 2013; Dell, A. *et al.*, 2010). This similarity was also found in archaeal *N*-OTases, indicating that protein glycosylation is an early event in evolutionary history (Dell, A. *et al.*, 2010).

N-glycans can also be modified by the addition of phosphoethanolamine, mediated by the phosphoethanolamine transferase EptC, already known for modifying Lipid A and the flagellar protein FlgG (Cullen, T.W. and Trent, M.S., 2010; Scott, N.E. *et al.*, 2012). Mutants in *C. jejuni* lacking *eptC* are more sensitive to polymyxin B, are less motile and have a reduced infectivity *in vitro* (Golden, N.J. and Acheson, D.W., 2002). However, it has not been determined how lacking the modification mediated by EptC causes this phenotype. There are clear differences in the glycans that can be found among different *Campylobacter* species, although these differences can only be found in the non-reducing sugars of the glycan, probably an evolutionary trait related to selection by the host immune system and host tropism (Nothaft, H. *et al.*, 2012; Jervis, A.J. *et al.*, 2012).

Other bacterial species in which evidence of N-glycosylation has been found are also members of the ε-proteobacteria class, including *Helicobacter* and *Wolinella* species (Nothaft, H. and Szymanski, C.M., 2013). In the case of *Helicobacter*, *pgl* genes are not found in a single operon, and there seem to be at least two *pglB* homologues. The characterization of the *H. pullorum* glycan found a pentasaccharide that contains *N*-acetylhexosamine (HexNac) as the reducing end sugar and unknown sugars of 203 Da, 216 Da and 217 Da (Jervis, A.J. *et al.*, 2010). Interestingly, the most medically relevant member of the genus *Helicobacter, H. pylori,* does not encode an identifiable *N*-OTase in its genome. However, a homolog of the PglK flippase is involved in LPS synthesis, suggesting evolutionary commonalities between protein glycosylation and LPS synthesis (Hug, I. *et al.*, 2010). In the case of *W. succinogenes*, the reducing sugar is diNAcBac (Jervis, A.J. *et al.*, 2012). Members from the δ-proteobacteria class, mainly *Desulfovibrio*, carry functional *pgl* genes, although *D. desulfuricans* PglB has features of Eukaryotic and Archaeal PglBs (Ielmini, M.V. and Feldman, M.F., 2011a). *D. desulfuricans* PglB recognizes the same glycosylation site, N-X-S/T as the eukaryotic OTase complex, and unlike *C. jejuni* PglB does not require a negatively charged amino acid at the -2 or -3 positions in the glycosylation site. *N*-glycosylation in *Desulfovibrio gigas* was found while studying the structure of the periplasmic cytochrome HmcA, and the presence of an oligosaccharide bound to an asparagine residue was

confirmed by mass spectrometry. As with *Helicobacter*, the *D. gigas* oligosaccharide does not contain diNacBac and it is a trisaccharide formed by *N*-acetylglucosamine as the reducing sugar, *N*-acetylallosamine and *N*-acetylhexosamine (Santos-Silva, T. *et al.*, 2007). Interestingly, CjPglB homologs have been found in bacterial species from deep-sea vents, *Nitratiruptor tergarcus*, and *Sulfurovum lithotrophicum*, both from the ε-proteobacteria class and *Deferribacter desulfuricans*, belonging to the δ-proteobacteria class (Mills, D.C. *et al.*, 2015). The three OTases showed enzymatic activity *in vivo* in *E. coli*, suggesting that these two bacterial classes possess *N*-linked protein glycosylation systems (Mills, D.C. *et al.*, 2015; Feldman, M.F. *et al.*, 2005).

PglB from *C. jejuni* is promiscuous in terms of sugar donors, yet it requires an amino acid sequence (sequon) for glycosylation of its targets, specifically an acidic residue in the – 2 position of the sequon, D/E-X_{-1}-N-X_{+1}-S/T, where X can any amino acid except Proline. However, the -2 position rule is only strictly followed in *C. jejuni*, as diverse PglB orthologs showed glycosylation at other sequons. That is the case of *D. gigas* and *Campylobacter lari* that do not recognize the same bacterial sequon as the *C. jejuni* PglB (Ielmini, M.V. and Feldman, M.F., 2011a; Schwarz, F. *et al.*, 2011a). A recent glycoproteomic study using the *C. jejuni* strain NCTC11168 has also shown glycopeptides on atypical sequons, containing leucine, glutamine or alanine at the -2 position, indicating the diversity of target specificities by PglB orthologs even within strains of *C. jejuni* (Scott, N.E. *et al.*, 2014b).

1.2.2-Glycoengineering

One of the most important events in the field of bacterial OTase-dependent glycosylation was the transfer of the *pgl* locus from *C. jejuni* to *E. coli* to create a functional *N*-glycosylation pathway (Wacker, M. *et al.*, 2002). The reconstitution of an *in vivo* heterologous *N*-glycosylation system in *E. coli* was key for the study of functional and mechanistic aspects of *N*-glycosylation of proteins and its differences with the eukaryotic pathway (Wacker, M. *et al.*, 2006; Kowarik, M. *et al.*, 2006; Gerber, S. *et al.*, 2013). This heterologous system, combined with the glycan relaxed specifity of PglB, enabled the possibility of using *E. coli* as a platform to produce engineered glycoproteins with a vast variety of glycans in applications such as vaccines and diagnostics (Nothaft, H. and Szymanski, C.M., 2013; Feldman, M.F. *et al.*, 2005; Ciocchini, A.E. *et al.*, 2013a; Iwashkiw, J.A. *et al.*, 2012a; Ielmini, M.V. and Feldman, M.F., 2011b; Melli, L.J. *et al.*, 2015). In the case of vaccines, glycoconjugates have been developed for *Burkholderia pseudomallei*

(Garcia-Quintanilla, F. *et al.*, 2014), as well as for *Shigella, Staphylococcus aureus,* and Uropathogenic *E. coli* (Ravenscroft, N. *et al.*, 2016; Kampf, M.M. *et al.*, 2015; Hatz, C.F. *et al.*, 2015; Wetter, M. *et al.*, 2013). Glycoconjugates have also been successfully applied to the diagnosis of Brucellosis and Hemolytic Uremic Syndrome by attaching specific O-antigens from these pathogens onto the AcrA protein from *C. jejuni* in order to detect an immune response from infected animals or patients (Melli, L.J. *et al.*, 2015; Ciocchini, A.E. *et al.*, 2013b; Iwashkiw, J.A. *et al.*, 2012b). Recently, the *in vivo N*-glycosylation system in *E. coli* has been used for the characterization of a series of novel PglB homologs with distinct acceptor-site preferences, including a PglB from *D. gigas* that was able to glycosylate the Fc domain of human immunoglobulin G at its native sequon QYNST (Ollis, A.A. *et al.*, 2015). The diversity of OTases with different specificity is central for expanding the possibilities of glycoengineering because any OTase-dependent *N*-glycosylated protein could potentially be modified at its native sequons. The best characterized PglB is from *C. jejuni,* which requires the extended glycan recognition site, D/E-X_{-1}-N-X_{+1}-S/T. Prior to the discovery of *D. gigas* PglB, the extended glycosylation site of *C. jejuni* PglB would need to be inserted into the target glycoprotein in order for modification to occur. Alternatively, directed mutagenesis strategies may be used to alter glycan site specification of PglB allowing glycosylation of non-native proteins at their native glycosylation site.

1.2.3-OTase dependent *O*-glycosylation.

In eukaryotes, *O*-glycosylation is mediated by GTases in the Golgi apparatus. OTase-mediated glycosylation then thought to be unique to *N*-linked glycosylation. However, it is now clear that many bacterial species *O*-glycosylate proteins in an "*O*-OTase" dependent manner (Iwashkiw, J.A. *et al.*, 2013). *O*-glycosylation of the type IV major pilin from *Pseudomonas aeruginosa* 1244 by the PilO/TfpO system was the first example of OTase mediated *O*-glycosyation and was described more than 2 decades ago (Castric, P., 1995). Type IV pilins are the main components of the retractile pili of several bacterial species. These proteins are also modified in an *O*-OTase dependent manner in *Neisseria meningitidis, N. gonorrhea, Dichelobacter nodosus,* and *Pseudomonas psyringae,* leading to the assumption that pilin proteins were the only target of protein *O*-OTases (Virji, M. *et al.*, 1993; Stimson, E. *et al.*, 1995; Banerjee, A. and Ghosh, S.K., 2003; Cagatay, T.I. and Hickford, J.G., 2008). This hypothesis was disproved by demonstrating that many proteins in addition to pilin are *O*-glycoyslated in *Neisseria* by the *O*-

OTase PglL (Vik, A. *et al.*, 2009a). Since then, general *O*-glycosylation systems that modify multiple target proteins have been characterized in several bacterial species including *Neisseria*, *Acinetobacter baumannii, Porphyromonas gingivalis, Bacteroides fragilis, Francisella tularensis, Burkholderia cenocepacia* and *Ralstonia solanacearum* (Vik, A. *et al.*, 2009a; Egge-Jacobsen, W. *et al.*, 2011; Elhenawy, W. *et al.*, 2015; Fletcher, C.M. *et al.*, 2009; Gallagher, A. *et al.*, 2003; Iwashkiw, J.A. *et al.*, 2012c; Lithgow, K.V. *et al.*, 2014a). *O*-OTases have also been identified and characterized in *Vibrio cholerae* and *Burkholderia thailandensis,* although the protein targets and glycan structures remain to be identified (Gebhart, C. *et al.*, 2012). It has recently been shown that some *Acinetobacter* strains carry two functional OTases, one specific for pilin modification and a general OTase for other glycoproteins. The main difference between these two distinct OTases seems to be the target recognition since both enzymes utilize the same lipid-linked glycan as a substrate (Harding, C.M. *et al.*, 2015a). While PilO/TfpO recognizes terminal serine residues in pilins, PglL OTases recognize low complexity regions rich in serine, proline, and alanine located anywhere in the proteins (Scott, N.E. *et al.*, 2014a; Lithgow, K.V. *et al.*, 2014b).

One of the most interesting aspects of *O*-glycosylation in bacteria is the evolutionary relation with lipopolysaccharide (LPS) synthesis (Hug, I. and Feldman, M.F., 2011). In both processes the lipid-linked glycan is built in a similar fashion and flipped to the periplasmic face by a flippase where it serves as a substrate for its respective enzyme. Both the OTase and the O-antigen ligase WaaL catalyze the transfer of a lipid-linked glycan from the periplasmic face of the inner membrane onto a molecule, which is a protein in the case of an *O*-OTase and the lipid A-core in the case of WaaL (Hug, I. and Feldman, M.F., 2011). Not surprisingly, both enzymes contain domains from the same Wzy_C super-family, making it difficult to predict their functions by bioinformatic analysis (Harding, C.M. *et al.*, 2015b; Power, P.M. *et al.*, 2006). *P. aeruginosa* 1244 pilin is glycosylated with a single O antigen subunit, whereas WaaL transfers the same O antigen, but polymerized, to the lipid A-core. In *Acinetobacter,* the same glycan is employed in protein glycosylation and capsule synthesis (Lees-Miller, R.G. *et al.*, 2013). These examples demonstrate the ability of bacteria to share a lipid-linked glycan for the biosynthesis of different glycoconjugates (Castric, P. *et al.*, 2001). This cross talk between pathways indicates that mechanisms must exist in order to coordinate these processes. When expressed in *E. coli* or *P. aeruginosa*, PilO was able to transfer only O antigen subunits but no polymers to pilin, suggesting that PilO length specificity is what prevents the transfer of long polysaccharides onto the pilin

(Faridmoayer, A. *et al.*, 2007; Horzempa, J. *et al.*, 2006a; Horzempa, J. *et al.*, 2006b; Qutyan, M. *et al.*, 2007). Something similar appears to happen in *A. baumannii* strains. However, in these bacteria, there is no O antigen (Iwashkiw, J.A. *et al.*, 2012d), and the majority of its glycoproteins are modified with a single subunit of the capsule. *Acinetobacter* PglL, unlike PilO from *P. aeruginosa,* is able to transfer glyan polymers to acceptor proteins (Lees-Miller, R.G. *et al.*, 2013; Scott, N.E. *et al.*, 2014a). It has been proposed that the polymerized glycan chains are sequestered by the capsule export machineries, preventing the access of PglL to the longer chains (Faridmoayer, A. *et al.*, 2007).

As with the *N*-OTase PglB, the relaxed glycan specificity of PglL could lead to use in glycoengineering and the production of glycoconjugate vaccines (Faridmoayer, A. *et al.*, 2008). So far, it has not been determined how OTases recognize the lipid-linked glycan substrate and their target proteins. The development of *in vitro* assays has added valuable information on the mechanistic aspects of this process (Musumeci, M.A. *et al.*, 2013a; Musumeci, M.A. *et al.*, 2014). Even more, the ability of OTases to utilize nucleotide activated sugars or glycans bound to foreign lipid carriers *in vitro* suggests that the Und-P is not required for OTase recognition (Musumeci, M.A. *et al.*, 2013b). As mentioned, *O*-glycosylation generally occurs in target proteins at low complexity regions. However, as for *O*-glycosylation in eukaryotes, no glycosylation sequon has been identified, hampering *O*-glycoengineering in eukaryotic proteins and suggesting that OTases would recognize structural information from its targets (Scott, N.E. *et al.*, 2014a; Lithgow, K.V. *et al.*, 2014b; Vik, A. *et al.*, 2009b). While this might be the rule, some exceptions have been found, such as *Bacteroides fragilis* and *Tannerella forsythia*. Both species *O*-glycosylate their target proteins with a complex negatively charged glycan at the specific glycosylation site D(S/T)(A/I/L/V/M/T) (Fletcher, C.M. *et al.*, 2011; Posch, G. *et al.*, 2011; Posch, G. *et al.*, 2013).

In relation with pathogenesis, *O*-glycosylation in *A. baumannii* is required for biofilm formation and pathogenicity in amoeba, insect and murine infection models (Iwashkiw, J.A. *et al.*, 2012c). In the case of *N. gonorrhea*, pilin glycosylation is necessary for adhesion to host cervical tissue (Jennings, M.P. *et al.*, 2011). OTase-dependent *O*-glycosylation is also present in plant pathogens, such as *Ralstonia solanacearum* (Elhenawy, W. *et al.*, 2015). This species has an active general *O*-glycosylation system that modifies 20 proteins including the type IV pilins with a subunit of the LPS O-antigen, HexNAc-(Pen)-$dHex_3$ (Elhenawy, W. *et al.*, 2015). Similar to *Acinetobacter*, the *R. solanacearum* glycoproteome displays extensive glycan heterogeneity

(Scott, N.E. *et al.*, 2014a; Elhenawy, W. *et al.*, 2015). *R. solanacearum* protein glycosylation is required for biofilm formation and pathogenicity in tomato plants. A striking observation is that the absence of protein glycosylation shows no expression changes in the whole proteome of *R. solanacearum* but a decrease in the amount of *R. solanacearum* type IV pilin and an increase in a surface lectin, homologous to *Pseudomonas* PA-IIL (Elhenawy, W. *et al.*, 2015). This finding strongly suggests that glycosylation in this species is not required for homeostasis rather than for specific functions related to host-pathogen interactions (Elhenawy, W. *et al.*, 2015).

1.2.4-OTase independent N-glycosylation: A world of adhesins and autotransporters

An alternative *N*-glycosylation pathway is the OTase-independent system, which appears to be dedicated to autotransporters and has been recently described (McCann, J.R. and St Geme, J.W., 3rd, 2014). Autotransporters are a family of adhesive proteins that belong to the type 5 secretion systems (Leo, J.C. *et al.*, 2012). Autotransporter adhesion proteins are translocated across the inner membrane to the periplasm by the Sec system (Sijbrandi, R. *et al.*, 2003; Brandon, L.D. *et al.*, 2003; Peterson, J.H. *et al.*, 2006). The C-terminal translocator domain inserts into the outer membrane, forming a β-barrel pore for the functional N-terminal passenger domain to travel through the membrane, gaining access to the cell surface where it performs its adhesion function (Konieczny, M.P.J. *et al.*, 2001; Oomen, C.J. *et al.*, 2004). Autotransporters are also involved in bacterial epithelial cell adhesion. This pathway does not employ an *N*-OTase, but instead, the attachment of the sugars to the proteins is mediated by "Protein-*N*-GTases" (PNGTases), a family of cytoplasmic GTases that transfer a monosaccharide directly from the nucleotide-activated sugar to proteins. The most studied PNGTase is *Haemophilus* HMW1G, which transfers hexoses such as glucose and galactose onto the acceptor adhesion protein HMW1 at more than 31 sites (Grass, S. *et al.*, 2003; Grass, S. *et al.*, 2010). Although the sugars are transferred to the Asn-Xaa-Ser/Thr sequon on the HMW1 protein, there is no evidence of common evolutionary origin with the *N*-OTases such as Stt3 (Gross, J. *et al.*, 2008). HMW1 mediates the interaction between the bacterial cell and human epithelial cells, and both HMW1 and HMW1C form part of a two-partner secretion system gene cluster that includes HMW1B, an accessory protein that promotes secretion of HMW1 by forming pores on the outer membrane (St Geme, J.W., 3rd *et al.*, 1993; St Geme, J.W., 3rd and Grass, S., 1998). Lack of glycosylation of HMW1 is detrimental for the permanence of the protein on the bacterial cell surface and for the adherence to epithelial cells, indicating a clear role of this

type of N-glycosylation in pathogenesis (Grass, S. *et al.*, 2003). These types of two partner secretion systems are also present in other pathogens, such as enterotoxigenic *E. coli*, where the PNGTase ortholog EtpC is required for the glycosylation of the HMW1 ortholog, EtpA (Fleckenstein, J.M. *et al.*, 2006). Another HMW1C ortologue is RscC from *Yersinia enterocolitica*, although GTase activity for this protein has not been shown (Nelson, K.M. *et al.*, 2001). The HMW1C homologue from *Actinobacillus pleuropneumoniae*, *Ap*HMW1C, has been used as a model to determine the structure for this type of enzymes and to shed light on the mechanistic nature of cytoplasmic N-glycosylation of proteins in bacteria. (Schwarz, F. *et al.*, 2011b; Kawai, F. *et al.*, 2011; Naegeli, A. *et al.*, 2014a; Naegeli, A. *et al.*, 2014b).

A recent study of the PNGTases in *Kingella kingae* and *Aggregatibacter aphrophilus* has demonstrated that these proteins are required for the modification of adhesive proteins with mono- and di-hexoses. Interestingly, these enzymes modify trimeric autotransporter proteins encoded by genes at distant locations in the genome (Leo, J.C. *et al.*, 2012). Disruption of the PNGTase in both *K. kingae* and *A. aphrophilus* diminished cell adherence and increased auto-aggregation, establishing a central role of unconventional *N*-glycosylation in the pathogenesis of these bacterial species (Rempe, K.A. *et al.*, 2015).

1.2.5-OTase independent *O*-glycosylation: Functions in motility and adhesion.

Flagella, bacterial cell surface appendages utilized for motility are required for host colonization by many bacterial pathogens (Chaban, B. *et al.*, 2015; Rossez, Y. *et al.*, 2015). Flagellins, the structural components of the flagellar filament are commonly *O*-glycoyslated in the bacterial cytoplasm prior to their secretion (Logan, S.M., 2006). OTase-independent flagellin *O*-glycosylation, mediated by "protein *O*-glycosyltransferases" (POGTases) occurs in many Gram-negative organisms and has been identified in Gram-positive genera *Clostridium* and *Listeria* (Schirm, M. *et al.*, 2004b; Twine, S.M. *et al.*, 2008; Twine, S.M. *et al.*, 2009). The degree of glycosylation is highly variable. For example, the POGTase of *Burkholderia* glycosylates its flagellin at a single site while *C. jejuni* and *Listeria monocytogenes* glycosylate their flagellins at multiple sites (Thibault, P. *et al.*, 2001; Schirm, M. *et al.*, 2005; Scott, A.E. *et al.*, 2011). The sugars are also variable. *Helicobacter pylori O*-glycosylates its flagellin with a single monosaccharide known as pseudaminic acid (Josenhans, C. *et al.*, 2002; Schirm, M. *et al.*, 2003). *Campylobacter* modifies its flagellin with derivatives of pseudaminic acid and legionaminic acid,

both are chemically related to sialic acid, an abundant sugar present on many host glycoproteins (Morrison, M.J. and Imperiali, B., 2014). Structural similarity between flagellar glycans and sialic acid led to the idea that glycosylation of flagellins could be involved in host mimicry, athough this has not been shown (Merino, S. and Tomas, J.M., 2014). Flagellin glycosylation is thought to aid in immune evasion, host mimicry, protein stability, adhesion, host recognition and flagella assembly (Rossez, Y. *et al.*, 2015; Logan, S.M., 2006; Merino, S. and Tomas, J.M., 2014; Nothaft, H. and Szymanski, C.M., 2010). Flagellin modification is required for flagellar formation in *C. jejuni* and *H. pylori* but is dispensible in *Burkholderia* and *Pseudomonas* (Thibault, P. *et al.*, 2001; Scott, A.E. *et al.*, 2011; Schirm, M. *et al.*, 2003; Takeuchi, K. *et al.*, 2003). The flagellar glycan composition in *Campylobacter* impacts adherence and invasion of human intestinal epithelial cells, pathogenesis in a ferret model, chick colonization, filament stability and autoagglutination (Ewing, C.P. *et al.*, 2009). *P. aeruginosa* flagellin glycoyslation is not required for flagella assembly or motility, however it is required for virulence in a burn wound infection model (Arora, S.K. *et al.*, 2005). *O*-glycosylation of *Aeromonas hydrophila* polar flagella with a 403 Da glycan is required for polar flagella production, adhesion to Hep-2 cells, biofilm formation and TLR5 stimulation (Fulton, K.M. *et al.*, 2015).

C. jejuni O-glycosylates one protein in an OTase independent manner. This protein is FlaA, the major subunit of its flagella. FlaA is glycosylated at nineteen serine or threonine sites. Flagellar glycosylation is required for flagella formation and therefore motility. Glycosylation of the flagellin is also linked to autoagglutination.

Autotransporter adhesion proteins are *O*-glycosylated by an OTase-independent mechanism, in an analogous manner to *N*-linked glycosylation of autotransporter proteins. *E. coli O*-glycosylated adhesins AIDA-1, Ag43, and TibA have roles in adherence and invasion of epithelial cells, biofilm formation, and autoaggregation (Charbonneau, M.E. *et al.*, 2007a; Diderichsen, B., 1980; Klemm, P. *et al.*, 2004; Knudsen, S.K. *et al.*, 2008; Sherlock, O. *et al.*, 2006; Sherlock, O. *et al.*, 2005). As previously with PNGTases, the POGTases responsible for modifying the autotransporters are encoded adjacent to their target proteins. Nucleotide-activated heptoses borrowed from the LPS biosynthesis pathway are transferred to TibA and AIDA-1 cytoplasmically by the POGTases TibC, or Aah, and Aah2, respectively (Benz, I. and Schmidt, M.A., 2001; Lu, Q. *et al.*, 2014; Moormann, C. *et al.*, 2002). Heptosyltransferases Aah and Aah2 form dodecameric structures and bind the cofactor ferric iron in order to be functionally active

(Lu, Q. *et al.*, 2014). Instead, in the case of the adhesion Ag43, its POGTase is still unknown. (Knudsen, S.K. *et al.*, 2008; Sherlock, O. *et al.*, 2006). Glycosylation of TibA is not required for autoaggregation, biofilm formation or invasion of epithelial cells, it is however required for adhesion to epithelial cells (Cote, J.P. *et al.*, 2013; Cote, J.P. and Mourez, M., 2011). TibA glycosylation impacts folding and oligomerization, allowing for conformational changes between an oligomeric autoaggregation competent conformation and a monomeric adhesion competent conformation (Cote, J.P. *et al.*, 2013). In the case of the rodent pathogen *Citrobacter rodentium*, it has been shown that the Aah homolog recognizes a structural motif on the extracellular domain of an AIDA-like protein and that this POGTase is exchangeable with its homolog from *E. coli* (Charbonneau, M.E. *et al.*, 2007b). Additionally, both expression and heptosylation of the AIDA-like autotransporter CARC are required for colonization of the mouse colon (Lu, Q. *et al.*, 2014).

Similar to Gram-negative autotransporter adhesins, surface-exposed serine-rich repeat proteins (SRRPs) of Gram-positive bacteria are commonly *O*-glycosylated by cytoplasmic POGTases. SRRPs aid in biofilm formation, adherence to host cells and platelets, and autoaggregtion (Bensing, B.A. and Sullam, P.M., 2002; Lizcano, A. *et al.*, 2012; Plummer, C. *et al.*, 2005; Samen, U. *et al.*, 2007; Shivshankar, P. *et al.*, 2009). *Streptococcus sanguinis* SRRP, SrpA, aids in the infection cycle by binding to human platelets (Plummer, C. *et al.*, 2005). Also, glycosylation of Fap1, another SRRP, is required for fimbrial assembly and function, promoting adhesion to the tooth surface and the development of dental plaque (Bu, S. *et al.*, 2008; Zhou, M. and Wu, H., 2009; Zhou, M. *et al.*, 2010; Nobbs, A.H. *et al.*, 2009). Fap1 is known to be heavily *O*-glycosylated with Rha1-3Glc1-(Glc1-3GlcNAc1-)2,6Glc1-6GlcNAc through the activity of several POGTases encoded in the *fap1* locus; however, the exact role of the glycan is unknown (Zhang, H. *et al.*, 2014). In the case of *Aggregatibacter actinomycetemcomitans*, it presents a trimeric autotransporter adhesin, EmaA, that is *O*-glycosylated with a subunit of the LPS O antigen (Tang, G. *et al.*, 2008; Tang, G. and Mintz, K.P., 2010). Interestingly, modification of this adhesin is not mediated by a cytoplasmic POGTases, but rather an LPS WaaL ligase homolog, similar to the phenomenon observed in OTase dependent glycosylation (Tang, G. and Mintz, K.P., 2010; Tang, G. *et al.*, 2012).

O-glycosylation in *Mycobacterium tuberculosis* was originally reported due to reactivity to the mannose binding lectin ConA (Espitia, C. and Mancilla, R., 1989). Several lipoproteins modified with mannose residues have been detected (Espitia, C. *et al.*, 2010; Herrmann, J.L. *et al.*,

1996; Michell, S.L. *et al.*, 2003; Sartain, M.J. and Belisle, J.T., 2009). However, *O*-glycosylation in Mycobacteria follows a path similar to eukaryotic mannosylation, the *M. tuberculosis* transmembrane *O*-mannosyltransferase transfers the mannose from a polyprenol-mono-phosphate carrier (Cooper, H.N. *et al.*, 2002; VanderVen, B.C. *et al.*, 2005). After the initial addition of a mannose residue by the *O*-mannosyltransferase, the glycan is expanded by additional mannosyl transferases (Liu, C.F. *et al.*, 2013). *M. tuberculosis* mutants defective in *O*-mannosylation have decreased survival in macrophages and are less virulent in mice (Liu, C.F. *et al.*, 2013). Bacterial *O*-mannosylation by an *O*-mannosyltransferase is not unique to *Mycobacterium,* as it occurs in other Gram-postive bacterial species such as *Corynebacterium glutamicum* and *Streptomyces coelicolor* (Mahne, M. *et al.*, 2006; Wehmeier, S. *et al.*, 2009; Hartmann, M. *et al.*, 2004).

1.3-Type II secretion

Bacterial protein secretion is one way cells interact with and impact their environment. The type II secretion system (T2SS) is widespread among Gram-negative pathogens as well as environmental species capable of living in a variety of conditions (Nivaskumar, M. and Francetic, O., 2014; Sandkvist, M., 2001a; Sandkvist, M., 2001b). T2SS is a two-step process depicted in figure 1.1, where proteins with an N-terminal secretion signal are translocated across the inner membrane by the general secretory pathway (Sec) or the Twin-arginine (Tat) system to the periplasmic space (Sandkvist, M., 2001a; Sandkvist, M., 2001b; de Keyzer, J. *et al.*, 2003). The Type II secretion system then exports folded proteins from the periplasm to the extracellular environment, through an outer-membrane secretin. The majority of Type II substrates are translocated to the periplasmic space in an unfolded state by the Sec system (Chatzi, K.E. *et al.*, 2013; Korotkov, K.V. *et al.*, 2012). N-terminal secretion signals are recognized by cytoplasmic chaperones such as SecB or trigger factor, and brought to the SecYEG channel through interactions with the ATPase SecA. Alternatively, Sec secretion targets can interact with SecA without the aid of a chaperone. SecA mediates the insertion of each post-translational protein into the SecYEG channel. These proteins may or may not be bound to a cytoplasmic chaperone protein like SecB. SecB binding is thought to help maintain these proteins in an unfolded, translocation competent state. Post-translational translocation is catalyzed by ATP hydrolysis via SecA and the proton-motive force. Co-translational translocation is catalyzed by the ribosome during translation (Chatzi, K.E. *et al.*, 2013). In co-translational translocation, the extremely

hydrophobic N-terminal signal sequence is bound by a signal-recognition-particle and brought to the SecYEG channel. Type II substrates that fold in the cytoplasm or require cytoplasmic post-translational modification are transported to the periplasm by the Tat system (Patel, R. *et al.*, 2014). Two phospholipases from *Pseudomonas aeruginosa* are translocated in a folded state to the periplasm by the Tat system prior to secretion by the T2SS (Voulhoux, R. *et al.*, 2001). Following removal of the secretion signal, folded proteins are then secreted to the extracellular space by the T2SS machinery. Proteins secreted by the T2SS are a diverse set of proteins that tend to have hydrolytic functions.

Type II secretion was initially discovered in *Klebsiella oxytoca*, where the secretion apparatus was required for secretion of pullulanase from *E. coli* (d'Enfert, C. *et al.*, 1987). Cholera toxin is one of the most well known Type II substrates. Secretion of cholera toxin from *Vibrio cholerae* is required for the diarrheal symptoms associated with cholera disease (Holmes, R.K. *et al.*, 1975; Spangler, B.D., 1992). The Type II apparatus depicted in figure 1.1, consists of the outer membrane secretin GspD; the periplasmic pseudopilus made up of major pseudopilin GspG and minor pseudopilins GspHIJK; inner membrane platform consisting of GspC, GspF, GspL and GspM; and, the cytoplasmic ATPase GspE. GspD forms a large 12-14 subunit oligomer in the outer membrane (Sandkvist, M., 2001a, Sandkvist, M., 2001b). The other T2SS components are not required for GspD assembly in the outer membrane (Nivaskumar, M. and Francetic, O., 2014). Inner membrane platform protein, GspC, interacts with the outer membrane secretin GspD and other inner membrane platform proteins GspL and GspM (Korotkov, K.V., *et al.,* 2011, Py, B., *et al.*, 2001). Protein interaction studies involving immunoprecipitation and yeast-two-hybrid analyses have demonstrated interactions between inner membrane platform proteins GspF and GspL with the ATPase GspE. ATP hydrolysis by GspE powers assembly of the pseudopilus allowing protein secretion to occur (McLaughlin, L.S., *et al.*, 2012).

It is not well understood how type II substrates are recognized. They have been shown to bind the outer membrane secretin as well as the pseudopilus (Douzi, B., *et al.*, 2011; Reichow, S.L., *et al*., 2010). Type II substrates are structurally diverse proteins of various sizes and it still is not understood what signal differentiates type II substrates from other soluble periplasmic proteins enabling specific secretion. In order to be secreted the ATPase must power pseudopilus assembly, it has been proposed that the pseudopilus may function in a piston like mechanism

pushing substrates through the outer membrane secretin (Nivaskumar, M. and Francetic, O., 2014). Alternatively, substrates may associated to the pseudopilus and be carried out of the cell.

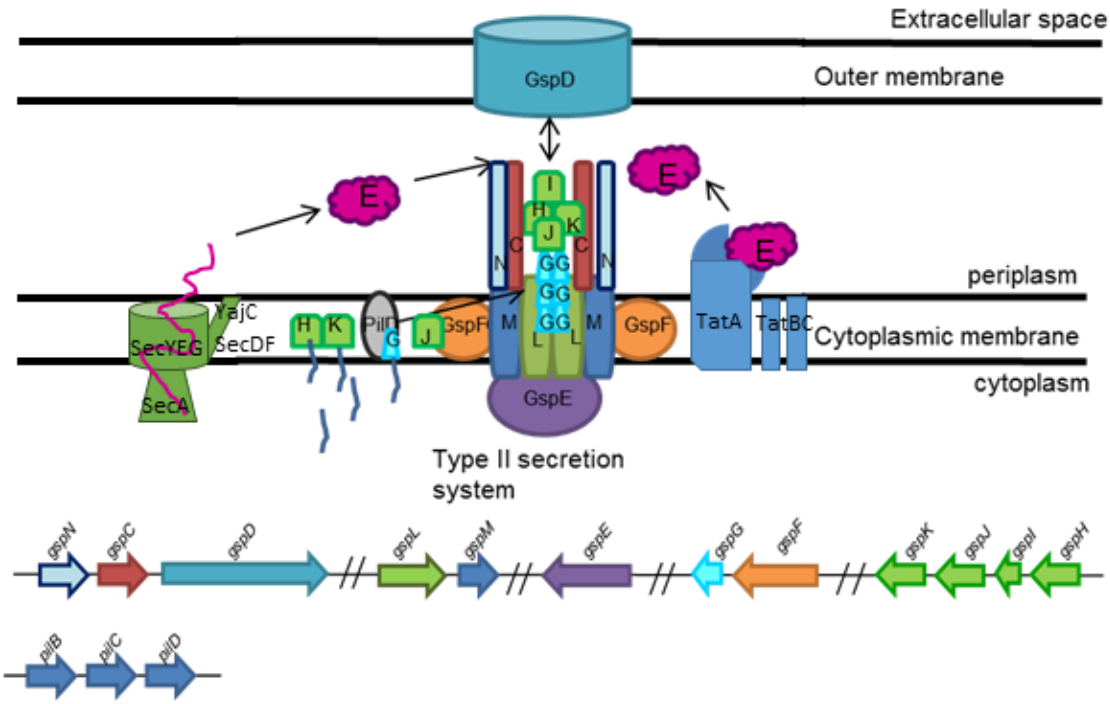

Figure 1.1-The Type II Secretion Apparatus. Type II secretion occurs as a two-step process, proteins are first translocated to the periplasmic space by the Sec secretion system or the Tat translocation machinery. The Sec secretion system translocates proteins in their unfolded state. The Tat secretion system translocates proteins in their full folded state. The Type II apparatus secretes full folded proteins from the periplasm to the extra-cellular space through its outer membrane secretin GspD. The cytoplasmic ATPase GspE powers pseudopilus assembly thereby facilitating protein secretion. The inner membrane platform proteins GspC, GspF, GspM, GspN and GspL connect the cytoplasmic ATPase to the outer membrane secretin.

1.3.1-Essential Components of the Type II Apparatus

Reconsitution of Type II secretion in *E. coli* has been used to determine the essential components of a T2SS. *K. oxytoca pulB, pulH* and *pulN* are not required for reconstitution in *E. coli* (Possot, O.M. *et al.*, 2000). The inner-membrane platform proteins, PulC, PulF, PulL and PulM; outer-membrane secretin, PulD; secretion ATPase, PulE; pre-pilin peptidase PulO, major and minor pseudopilins as well as pilotin PulS are required for secretion in *K. oxytoca* (Possot, O.M. *et al.*, 2000). Several characterized T2SS lack *pulB, pulN* and pilotin homologs, suggesting these

proteins are not required by all T2SS (Korotkov, K.V. *et al.*, 2012). All characterized T2SS have inner-membrane platform proteins, an outer-membrane secretin, a secretion ATPase, pre-pilin peptidase as well as major and minor pseudopilins, implying these are the essential components of a T2SS (Korotkov, K.V. *et al.*, 2012). The inner membrane platform is imbedded in the plasma membrane, and protrudes into the periplasmic space. The N-terminus of the secretin is thought to extend into the periplasm, where it interacts with the inner-membrane platform. The inner-membrane platform is responsible for connecting the cytoplasmic ATPase, pseudopilus and the secretin, thereby coordinating secretion.

1.3.2-Roles of Type II Secretion in bacterial physiology

The T2SS promotes *in vivo* survival and virulence of several pathogens, including *Vibrio cholerae,* enterotoxigenic *Escherichia coli*, *Pseudomonas aeruginosa* and *Legionella pneumophila,* through the activities of secreted toxins and hydrolytic enzymes aiding in nutrient acquisition (DebRoy, S. *et al.*, 2006; McCoy-Simandle, K. *et al.*, 2011; Sandkvist, M. *et al.*, 1993; Sikora, A.E. *et al.*, 2011; Ho, T.D. *et al.*, 2008; Baldi, D.L. *et al.*, 2012; Jyot, J. *et al.*, 2011). Cholera toxin is secreted by the *V. cholerae* T2SS and is responsible for the diarrheal symptoms associated with cholera disease (Holmes, R.K. *et al.*, 1975; Spangler, B.D., 1992). *P. aeruginosa* Type II substrate endotoxin A blocks protein synthesis in host cells. Some T2SS secrete one substrate, where as others secrete numerous proteins. Type II substrates include lipases, phospholipases, proteases and carbohydrate hydrolases.

Some type II substrates require chaperones to become full folded and be secreted by the type II apparatus. In *Burkholderia glumae* a membrane bound chaperone, lipase-specific foldase (Lif), is required for production of enzymatically active lipase, lipA. Lif is also required for secretion of LipA. The transmembrane domain of Lif is not required for its steric chaperone activity (Frenken, L.G. *et al.*, 1993; Rosenau, F. *et al.*, 2004; El Khattabi, M. *et al.*, 1999).

There are type II metalloproteases that require chaperones to become active and be secreted (Bever, R.A. and Iglewski, B.H., 1988). In *Pseudomonas aeruginosa* type II metalloprotease, elastase, is produced as a pre-pro-elastase, where the pro-domain functions as an intramolecular chaperone (Kessler, E., *et al.*, 1992, Shinde, U. and Inouye, M., 2000). The N-terminal sec secretion signal is removed upon translocation into the periplasmic space, leaving inactive pro-elastase in the periplasm (Kessler, E. and Safrin, M., 1994). The pro-domain has chaperone like function as it is required for secretion of elastase and is required for production of

active elastase (McIver, K.S., *et al.*, 2004). The type II apparatus only secretes fully folded protein substrates. The pro-domain complexed with elastase blocks elastase activity (Kessler, E. and Safrin, M., 1994). The elastase pro-domain dissociates from the mature elastase upon secretion.

1.3.3-Type II secretion in *Acinetobacter*

Eijkelkamp *et al.* has reported the presence of a T2SS in *A. baumannii* (Eijkelkamp, B.A. *et al.*, 2014). No work investigating if Type II secretion is active in *Acinetobacter* has been reported. Studies have reported the presence of proteins with N-terminal secretion signals in the *Acinetobacter* secretome, however the mechanism by which these proteins are secreted is unknown (Kwon, S.O. *et al.*, 2009; Mendez, J.A. *et al.*, 2012). These proteomic analyses also identified cytoplasmic proteins, confounding conclusions that the identified N-terminal secretion signal containing proteins are actively secreted.

1.4-Thesis objectives

1.4.1-Characterizing the *O*-glycan variability in *Acinetobacter*

A. baumannii O-glycosylates multiple proteins in an oligosaccharyltransferase dependent manner. The same glycan is used to modify proteins and is polymerized into capsular polysaccharide. My objective was to determine how diverse the *O*-glycans of *Acinetobacter* are. I utilized mass spectrometry glycan sequencing to characterize the glycan repertoire of *Acinetobacter.*

1.4.2-Investigating the role of two abundant glycoproteins in the pathogenesis of *A. baumannii*

A. baumannii appears to produce one prominent glycoprotein. I investigated whether one of the two abundant glycoproteins, A1S_3626 or A1S_3744, constituted this prominent glycoprotein. The objective was to investigate the role of two abundant and highly conserved glycoproteins in *A. baumannii* in biofilm formation and pathogenesis.

1.4.3- Type II Secretion in *Acinetobacter:* Substrates and its Role in Pathogenesis

A. nosocomialis encodes the genes necessary for a T2SS, and sec dependent proteins have been found in the secretome of *Acinetobacter*. This data taken together prompted us to hypothesize that type II secretion is active in *Acinetobacter.* The objective of this study was to determine if *A. nosocomialis* has an active Type II secretion system, and to

identify the Type II substrates. We investigated if Type II secretion was active across medically relevant *Acinetobacter* species or specific for a particular species or strain. We characterized the role of Type II secretion in pathogenesis.

1.4.4- Characterizing Type II Substrate CpaA: Its role in Pathogenesis and Interaction with CpaB

The objective of this study was to investigate the specific role of CpaA in virulence and characterize the interaction between CpaA and its chaperone CpaB.

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Chapter 2-Diversity within the *O*-linked protein glycosylation systems of *Acinetobacter* species

The work included in this chapter was a collaboration between Dr. Mario Feldman at the University of Alberta, Drs. Nichollas Scott and L.J. Foster at the University of British Columbia and myself. Together with Dr. Mario Feldman we had the idea to investigate the *O*-glycan diversity in *Acinetobacter*. Under the supervision and guidance of Dr. Feldman, I created a directed glycan characterization method called "glycan fishing". I used this method to characterize the *O*-glycan repertoire of strains compatible with *in trans* gene expression. I also performed the periodic acid Schiff staining of total membrane extracts and complement killing assay to test for production of capsular polysaccharide and glycoproteins. We established a collaboration with Drs. Scott and Foster to characterize glycan variability in strains incompatible with the "glycan fishing" method. Dr. Scott involved A.V. Edwards, M.R. Larsen, S.M. Dutta and J. Saba in characterization of glycoprotein and *O*-glycan diversity using ZIC-HILIC enrichment and specialized mass spectrometry methods. I prepared the total membrane samples for Dr. Scott, whom then proteolytically digested them and analyzed them using CTD and HTD mass spectrometry analysis. These specialized fragmentation methods were not available to us at the U of A as they require specialized equipment and the data mining requires specific expertise.

The work presented in this chapter was equally contributed by Dr. Scott and myself, under the supervision of Dr. Feldman, and is published as Scott, N.E., R.L. Kinsella, A.V. Edwards, M.R. Larsen, S.M. Dutta, J. Saba, L.J. Foster and M.F. Feldman (2014) Diversity within the *O*-linked protein glycosylation systems of *Acinetobacter* species. *Mol Cell Proteomics* **13**: 2354-70. The paper was written by Dr. Feldman, Dr. Scott and me.

2.1-Introduction:

Acinetobacter baumannii is an emerging opportunistic pathogen of increasing significance to health care institutions worldwide (Gaynes, R. *et al.*, 2005; Cisneros, J.M. and Rodriguez-Bano, J., 2002; Peleg, A.Y. *et al.*, 2008). The growing number of identified multiple drug resistant (MDR) strains (Cisneros, J.M. and Rodriguez-Bano, J., 2002; Peleg, A.Y. *et al.*, 2008; Maragakis, L.L. and Perl, T.M., 2008), the ability of isolates to rapidly acquire resistance (Peleg, A.Y. *et al.*, 2008; Maragakis, L.L. and Perl, T.M., 2008), and the propensity of this agent to survive harsh environmental conditions (Towner, K.J., 2009) account for the increasing number of outbreaks in intensive care, burn, or high dependence health care units since the 1970s (Cisneros, J.M. and Rodriguez-Bano, J., 2002; Peleg, A.Y. *et al.*, 2008; Maragakis, L.L. and Perl, T.M., 2008; Towner, K.J., 2009). The burden on the global health care system of MDR *A. baumannii* is further exasperated by standard infection control measures often being insufficient to quell the spread of *A. baumannii* to high risk individuals and generally failing to remove *A. baumannii* from healthcare institutions (Towner, K.J., 2009). Because of these concerns, there is an urgent need to identify strategies to control *A. baumannii* as well as understand the mechanisms that enable its persistence in health care environments.

Surface glycans have been identified as key virulence factors related to persistence and virulence within the clinical setting (Russo, T.A. *et al.*, 2010; Moffatt, J.H. *et al.*, 2013; Henry, R. *et al.*, 2012). *Acinetobacter* surface carbohydrates were first identified and studied in *A. venetianus* strain RAG-1, leading to the identification of a gene locus required for synthesis and export of the surface carbohydrates (Reisfeld, A. *et al.*, 1972; Nakar, D. and Gutnick, D.L., 2001). These carbohydrate synthesis loci are variable yet ubiquitous in *A. baumannii* (Hu, D. *et al.*, 2013a; Kenyon, J.J. and Hall, R.M., 2013a). Comparison of 12 known capsule structures from *A. baumannii* with the sequences of their carbohydrate synthesis loci has provided strong evidence that these loci are responsible for capsule synthesis with as many as 77 distinct serotypes identified by molecular serotyping (Hu, D. *et al.*, 2013a). Because of the non-linear nature of glycan synthesis, the identification and characterization of the glycans themselves are required to confirm the true diversity. It is very difficult to bioinformatically predict the substrate of a particular glycosyltransferase and the genetic organization of the glycosyltransferases does not indicate the order they act upon the lipid-linked glycan. Glycan structures need to be

determined experimentally. This diversity has wide-spread implications for *Acinetobacter* biology as the resulting carbohydrate structures are not solely used for capsule biosynthesis but can be incorporated and utilized by other ubiquitous systems, such as *O*-linked protein glycosylation (Lees-Miller, R.G. *et al.*, 2013a; Iwashkiw, J.A. *et al.*, 2012a).

Although originally thought to be restricted to species such as *Campylobacter jejuni* (Szymanski, C.M. *et al.*, 1999; Young, N.M. *et al.*, 2002) and *Neisseria meningitidis* (Stimson, E. *et al.*, 1995), bacterial protein glycosylation is now recognized as a common phenomenon within numerous pathogens and commensal bacteria (Iwashkiw, J.A. *et al.*, 2013; Nothaft, H. and Szymanski, C.M., 2010). Unlike eukaryotic glycosylation where robust and high-throughput technologies now exist to enrich (Larsen, M.R. *et al.*, 2007; Hagglund, P. *et al.*, 2004; Zielinska, D.F. *et al.*, 2010) and characterize both the glycan and peptide component of glycopeptides (Parker, B.L. *et al.*, 2013; Kolarich, D. *et al.*, 2012; Jensen, P.H. *et al.*, 2012), the diversity (glycan composition and linkage) within bacterial glycosylation systems makes few technologies broadly applicable to all bacterial glycoproteins. Because of this challenge a deeper understanding of the glycan diversity and substrates of glycosylation has been largely unachievable for the majority of known bacterial glycosylation systems. The recent implementation of selective glycopeptide enrichment methods (Schulz, B.L. *et al.*, 2013; Ding, W. *et al.*, 2009) and the use of multiple fragmentation approaches (Madsen, J.A. *et al.*, 2013; Scott, N.E. *et al.*, 2011) has facilitated identification of an increasing number of glycosylation substrates independent of prior knowledge of the glycan structure (Scott, N.E. *et al.*, 2011; Nothaft, H. *et al.*, 2012a; Scott, N.E. *et al.*, 2012a; Lithgow, K.V. *et al.*, 2014a). These developments have facilitated the undertaking of comparative glycosylation studies, revealing glycosylation is widespread in diverse genera and far more diverse then initially thought. For example, Nothaft *et al.* were able to show *N*-linked glycosylation was widespread in the *Campylobacter* genus and that two broad groupings of the *N*-glycans existed (Nothaft, H. *et al.*, 2012b). The *N*-glycan structure is relatively conserved in thermophilic *Campylobacter* species, but highly variable among non-thermophilic species. In non-thermophilic *Campylobacter* species the only commonality among the glycan structure found was the first two sugars at the reducing end.

During the initial characterization of *A. baumannii O*-linked glycosylation the use of selective enrichment of glycopeptides followed by mass spectrometry analysis with multiple fragmentation technologies was found to be an effective means to identify multiple glycosylated substrates in the strain ATCC 17978 (Iwashkiw, J.A. *et al.*, 2012a). Interestingly in this strain, the glycan utilized for protein modification was identical to a single subunit of the capsule (Lees-Miller, R.G. *et al.*, 2013b) and the loss of either protein glycosylation or glycan synthesis lead to decreases in biofilm formation and virulence (Lees-Miller, R.G. *et al.*, 2013b; Iwashkiw, J.A. *et al.*, 2012b). Because of the diversity in the capsule carbohydrate synthesis loci and the ubiquitous distribution of the PglL *O*-oligosaccharyltransferase required for protein glycosylation, we hypothesized that the glycan variability might be also extended to *O*-linked glycosylation. This diversity, although common in surface carbohydrates such as the lipopolysaccharide of numerous Gram-negative pathogens (Whitfield, C. and Trent, M.S., 2014), has only recently been observed within bacterial protein glycosylation systems that are typically conserved within species (Szymanski, C.M. *et al.*, 2003) and loosely across genus (Nothaft, H. *et al.*, 2012b; Borud, B. *et al.*, 2011).

In this study, we explored the diversity within the *O*-linked protein glycosylation systems of *Acinetobacter* species. Our analysis complements the recent *in silico* studies of *A. baumannii* showing extensive glycan diversity exists in the carbohydrate synthesis loci (Hu, D. *et al.*, 2013a; Kenyon, J.J. and Hall, R.M., 2013b). Employing global strategies for the analysis of glycosylation, we experimentally demonstrate that the variation in *O*-glycan structure extends beyond the genetic diversity predicted by the carbohydrate loci alone and targets proteins of similar properties and identity. Using this knowledge, we developed a targeted approach for the detection of protein glycosylation, enabling streamlined analysis of glycosylation within a range of genetic backgrounds. We determined that: *O*-linked glycosylation is widespread in clinically relevant *Acinetobacter* species; inter- and intra-strain heterogeneity exist within glycan structures; glycan diversity, although extensive results in the generation of glycans with similar properties and that the utilization of a single glycan for capsule and *O*-linked glycosylation is a general feature of *A. baumannii* but may not be a general characteristic of all *Acinetobacter* species such as *A. baylyi*.

2.2-Materials and methods:

2.2.1-Bacterial Strains and Growth Conditions – *Acinetobacter* strains are provided in Table 2-1. All *Acinetobacter* strains were grown in Luria Bertani (LB) broth/agar (10 g Tryptone, 5 g yeast extract, and 10 g of NaCl per 1 L of DH_2O supplemented with 15 g of agar per liter of broth when needed) at 37ºC with shaking at 200 rpm. For protein purification, an additional 50µg/mL of kanamycin was added with 0.2% (w/v) L-arabinose also added for induction when required. For the generation of material for protein purification studies and ZIC-HILIC analysis 1 L cultures were grown overnight as described above. Cells were harvested, washed twice in phosphate buffered saline and either used instantly for protein purification or freeze dried for ZIC-HILIC analysis preparation.

2.2.2-Bioinformatic Analysis of Carbohydrate Synthesis Loci

The Basic Local Alignment Search Tool (BLAST) was used to predict the identity of uncharacterized carbohydrate biosynthesis genes present in the carbohydrate biosynthesis locus.

2.2.3-Glycopeptide Identification from Purified A1S_1193 – The gene encodinh *A. baumannii* glycoprotein, A1S_1193, was previously cloned with a C-terminal Deca-histidine tag into pEXT20 and the L-arabinose inducible *E. coli/Acinetobacter* shuttle vector pEC (Iwashkiw, J.A. *et al.*, 2012a; Madsen, J.A. *et al.*, 2013). *A1S_1193-his* was subcloned from pEXT20 into pBAVMCS, a constitutive *E. coli/Acinetobacter* shuttle plasmid, at BamH1 and SalI (New England Biolabs, Witby, ON). A Qiagen plasmid mini-preparation kit was used to purify the plasmid DNA encoding the Histidine tagged A1S_1193. The purified plasmid DNA was transformed into electro-competent *Acinetobacter* and plated on LB agar plates containing 50 µg/mL kanamycin to select for maintenance of the plasmid. Expression of this recombinant protein in *Acinetobacter* was assessed by Western blot analysis using polyclonal anti-histidine sera (1:4000) (Rockland Immunochemicals, Gilbertsville, PA). The nitrocellulose membrane was then probed with an IRDye conjugated anti-rabbit antibody and an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) was used to visualize the blot. Modifications of the glycoprotein was observed through Western blot analysis as an increase in molecular weight relative to the unglycosylated control produced in the ATCC 17978 Δ*pglL* strain (Iwashkiw, J.A. *et al.*, 2012a).

A1S_1193-His was purified by nickel-affinity chromatography as previously described (Madsen, J.A. *et al.*, 2013). Briefly, overnight cultures were pelleted and suspended in ice-cold PBS

containing complete EDTA-free protease inhibitor cocktail (Roche) and lysed using 3 rounds of disruption at 30 kpsi using a cell disruptor (Constant system ltd.). Unbroken cells and insoluble cell debris were clarified at 10 000 x g for 10 minutes, 4ºC and the resulting supernatant was subjected to ultracentrifugation, 100 000 x g for 60 minutes, 4ºC, yielding a pellet of bacterial membrane. The resulting pellet was solubilized overnight at 4ºC in 1% Triton X-100 in PBS by tumbling. The detergent concentration was decreased to 0.5% by dilution with PBS and insoluble membrane components removed by ultracentrifugation at 100 000 x g, 60 minutes, 4ºC.

Solubilized membrane protein extracts were purified using Ni-sepharose media on an ATKA explorer, previously equilibrated with buffer A (20 mM Tris-HCl pH8.0, 300 mM NaCl, 0.5% Triton X-100) with 10 mM imidazole. Extracts were washed with 5 mL buffer A with 10 mM Imidazole at a flow rate of 1 mL/min to remove unbound proteins. Higher stringency washes were done with buffer A with 30 mM Imidazole at 1 mL/min and elution was performed at 0.5 mL/min with buffer A with 250 mM Imidazole in 1 mL fractions.

Purified proteins were resolved using 10% SDS-PAGE and stained with Coomassie blue to assess purity. In-gel trypsin digestion of the protein band corresponding to glycosylated A1S_1193 was performed according to Shevchenko et al. (2006) (Shevchenko, A. *et al.*, 2006). Briefly, bands were washed with water and dehydrated with 100% acetonitrile (ACN), followed by repeated rehydration with 1:1 (v/v) water and ACN and dehydration with 100% ACN. Next disulfide bonds were reduced through treatment with 10 mM DTT in 50 mM NH_4HCO_3 for 60 minutes at 37ºC. Cysteine thiol groups were then alkylated with 50 mM iodoacetamide in 50 mM NH_4HCO_3 in the dark for 60 minutes at room temperature. Gel pieces were then washed with 50 mM NH_4HCO_3, dehydrated with 100% ACN and dried. A1S_1193 was then digested with 0.02 mg/mL trypsin in 50 mM NH_4HCO_3 (Promega, Madison, WI) at 37ºC for 16 hours. Peptides were eluted from the gel through addition of 100% ACN and water, and lyophilized for mass spectrometry analysis. Peptides were resuspended in 0.1% trifluoroacetic acid and loaded onto a ZipTipC18 (Millipore, USA) column for desalting. Peptides were eluted with 60% ACN, dried down in a Speedvac and resuspended in 0.1% Formic acid (FA). The peptides were analyzed using a Q-TOF Premier (Waters, Manchester, UK) coupled to a nanoACQUITY (Waters) ultra-performance liquid chromatography systems as briefly described (Wang, N. *et al.*, 2007) with MassLynx, v.4.1 (Waters) employed for data analysis.

2.2.4-SDS-PAGE and Periodic Acid Schiff Staining of Total Membrane Proteins – *A. baumannii* strains were grown overnight in 50 mL of LB broth. Cells were pelleted by centrifugation at 10000 rpm for 10 minutes, washed with PBS and resuspended in 10 mL of PBS. Cells were lysed using two rounds of disruption at 30 kpsi using a cell disruptor (Constant System ltd., Kennesaw, GA). Cell lysates were clarified by centrifugation at 15000 rpm for 15 minutes. Total membranes were pelleted from the supernatant by ultracentrifugation at 100 000 x g, 4ºC for 1 hour. Samples were resuspended in laemmli sample buffer and separated by 12% SDS-PAGE. Gels were stained with periodic acid schiff as previously described (Cagatay, T.I. and Hickford, J.G., 2008). Briefly, 0.7% periodic acid in 5% acetic acid oxidizes *cis*-diol sugars sensitizing them to the general carbohydrate stain Schiff reagent (Sigma).

2.2.5-Complement Mediated Killing Assay- *Acinetobacter* complement mediated killing assays were performed as previously reported (Lees-Miller, R.G. *et al.*, 2013c). Briefly, overnight cultures were used to inoculate fresh LB broth and incubated at 37ºC, 200 rpm for 4 hours. Bacterial cells were pelleted at 5000 x g for 10 minutes and washed with PBS a total of two times. Cells were resuspended in 100% horse serum (Gibco) at 1 OD/ml. Ten-fold serial dilutions in 180 µL of serum were performed in a 96-well plate. 10 µL of each dilution series was plated onto LB agar immediately after re-suspension in serum and 3 hours post incubation at 37ºC. Plates were incubated overnight at 37ºC to allow growth of surviving bacteria.

2.2.6-Membrane Preparation for Glycopeptide Enrichment – Lipid free membranes were prepared according to Pessione *et al* (Pessione, E. *et al.*, 2009). Briefly, cells suspended in ice-cold 40 mM Tris (pH 7.4) and lysed using three rounds of disruption at 30 kpsi using a cell disruptor (Constant System ltd., Kennesaw, GA). Lysates were then centrifuged at 100,000 x g for 70 minutes and the resulting pellet resuspended in 1 mL of 50 mM ammonium bicarbonate. Membrane pellets were delipidated using 4 mL of 2:1 v/v trifluoroethanol/chloroform that was allowed to incubate at 4ºC for 10 minutes and the upper phase collected and dried down for use.

2.2.7-Protease Digestion for Glycopeptide Enrichment – Dried membrane proteins were resuspended in 6 M urea, 2 M thiourea, and 40 mM NH_4HCO_3 and reduced/alkylated prior to digestion with LysC (1/200 w/w) and then trypsin (1/50 w/w) as previously described (Scott, N.E. *et al.*, 2011). All peptide digests were dialyzed against ultra-pure water overnight using a Mini Dialysis Kit with a molecular weight mass cut off of 1000 Da (Amersham Biosciences, Buckinghamshire, UK) and on completion were collected and lyophilized.

2.2.8-Identification of Glycopeptides using ZIC-HILIC Enrichment and Reverse Phase LC-MS/MS – ZIC-HILIC enrichment was performed according to (Scott, N.E. *et al.*, 2011) with minor modifications. Micro-columns composed of 10 µM ZIC-HILIC resin (Sequant, Umea, Sweden) packed into p10 tips containing at 1 mm^2 excised C_8 Empore^{TM} disc (Sigma) were packed to a bed length of 0.5 cm. Prior to use, the columns were washed with ultra-pure water, followed by 95% ACN and then equilibrated with 80% ACN and 5% FA. Samples were resuspended in 80% ACN, 5% FA and insoluble material removed by centrifugation at 20,000 X g for 5 minutes at $4^{O}C$. 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 three load volumes of ultra-pure water into low-binding tubes and concentrated using vacuum centrifugation. ZIC-HILIC fractions were resuspended in 0.5% formic acid and separated using EASY-nLC system (Thermo Scientific, San Jose, CA, USA) coupled to either an LTQ-Orbitrap XL with ETD, an LTQ-Orbitrap velos or an Orbitrap Elite (Thermo Scientific). Samples were eluted using a gradient from 100% buffer A (0.5% acetic acid) to 40% buffer B (0.5% acetic acid, 80% ACN) over 148 minutes at a constant flow of 300nl/min. The instrument was operated using Xcalibur v2.2 (Thermo Scientific) in a data-dependent mode automatically switching between MS and HCD/CID on the Orbitrap Elite and Velos whereas CID/ETD was used on the Orbitrap XL. ETD and CID scan events were analyzed using FTMS. On all instruments the 5 most abundant precursor ions were selected and dynamic exclusion of 30 s enabled. MS resolution was set to 60,000 with an ACG target of 1 X 10^6, maximum fill time of 500ms and a mass window of 600 to 2000 m/z. HCD fragmentation (normalized collision energy 40) was carried out with an ACG of 2 X 10^5, maximum fill time of 250 ms, resolution set to 7500 and mass window 200 to 2000 m/z. CID (normalized collision energy 35), while CID fragmentation was carried out with an ACG target of 2 X 10^4 and maximum fill time of 100ms. ETD fragmentation was carried out with an ACG target of 2 X 10^5, ETD reaction time of 100ms. Duplicate enrichments were generated for each glycopeptide analysis.

2.2.9-Glycopeptide data Processing – The raw files were then processed within Proteome Discover version 1.3 (Thermo Scientific) to generate mgf files and searched using Sequest against a composite FASTA database of strain SDF, ATCC 17978 and AYE (NCBI accession: NC_010400.1, NC_009085.1 and NC_010410.1 respectively, obtained from NCBI on

10/07/2012). Scan events that did not result in peptide identification from Sequest searches were exported to Excel (Microsoft, Redmond, WA, USA). To identify possible glycopeptides within this list, the "mgf graph" MS-MS module of GPMAW 8.2 was utilized to identify all scan events within the generated mgfs containing the diagnostic oxonium 301.104 or 204.086 m/z ion. Using Excel, all scan events that were not matched by Sequest and contained a predicted marker of glycosylation were identified. These events were manually inspected and identified as possible glycopeptides based on the presence of the glycan fragment within the CID scan. To facilitate glycopeptide assignments from HCD scans, the 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 that of the deglycosylated peptide and ions corresponding to known carbohydrate oxoniums were removed in a similar approach to post-spectral processing of ETD data (Good, D.M. *et al.*, 2010; Good, D.M., Wenger C. D., McAlister G. C., Bai D. L., Hunt D. F., Coon J. J. , 2009). MASCOT v2.2 searches were using the Walter and Eliza Hall Institute Mascot server (http://sysbio-mascot.wehi.edu.au/mascot/home.html) of the proteobacteria taxonomy of the LugwigNR database. Searches were carried out with a parent ion mass accuracy of 20ppm and a product ion accuracy of 0.02 Da with no protease specificity as well as the fixed modification carbamidomethyl (C) and variable modifications, oxidation (M), deamidation (N), and N-terminal formylation. The instrument setting of MALDI-QIT-TOF was chosen because of previous studies showing quadrupole-like fragmentation within HCD spectra (Olsen, J.V. *et al.*, 2007) (generating a, b, and y ions) and our observation of internal cleavage products that are all included in this setting. All spectra were searched with the decoy option enabled and no matches to this database were detected (FDR 0%). To further validate glycopeptide matches, all spectra HCD spectra were annotated using the Expert Annotation tool (Neuhauser, N. *et al.*, 2012) (http://www.biochem.mpg.de/mann/tools/) with a mass accuracy of 10ppm, whereas ETD data was annotated manually with a mass accuracy of 0.6 Da to ensure al major peaks were match providing further confidence of identity and localization. All annotated spectra are provided within supplemental Tables S2.1, S2.3, S2.4, S2.6A and S2.7-S2.10. Isotopic distribution analysis was accomplished with the aid of the MS-Isotope module of Protein Prospector (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msisotope).

Supplementary material can be found in appendix 1.

Table 2-1 – Strains and plasmids

A. baumannii ATCC 17978	American Type Culture Collection
A. baumannii ATCC 17978 Δ*pglL*	(Iwashkiw, J.A. *et al.*, 2012a)
A. baumannii SDF	Body lice isolate (Vallenet, D. *et al.*, 2008)
A. baumannii AYE	Human isolate (Vallenet, D. *et al.*, 2008)
A. baumannii ATCC 19606	Reference strain (Bouvet P.J.M.G., P.A.D., 1986)
A. baumannii 1224	Clinical isolate, thigh (Weber, B.S. *et al.*, 2013)
A. baumannii 1225	Clinical isolate, coccyx (Weber, B.S. *et al.*, 2013)
A. baumannii 1441 C1	MDR clinical isolate (This study)
A. baumannii 1441 C3	MDR clinical isolate (This study)
A. baumannii Arg1	MDR clinical isolate (This study)
A. baumannii Arg2	MDR clinical isolate (This study)
A. calcoaceticus 1217	Clinical isolate, urine (Weber, B.S. *et al.*, 2013)
A. calcoaceticus 1218	Clinical isolate, leg (Weber, B.S. *et al.*, 2013)
A. pittii 1219	Clinical isolate, urine (Weber, B.S. *et al.*, 2013)
A. baylyi ADP1	Soil isolate (Juni, E., 1972)
A. nosocomialis 1222	Clinical isolate, iscial ulcer (Weber, B.S. *et al.*, 2013)
E. coli DH5α	General cloning and plasmid propagation (Invitrogen)

Plasmids

pEXT20-*A1S_1193His10X*	Cloning and expression vector, IPTG inducible, Amp^r, with *A1S_119310Xhis* inserted at *BamHI and XbaI* (Madsen, J.A. *et al.*, 2013)
pEC-*A1S_1193Hix10X*	Arabinose inducible, Km^r, plasmid used to express *A1S_1193-His* (Inserted at HincII and HindIII) in *Acinetobacter* for glycan fishing (Madsen, J.A. *et al.*, 2013)
pBAVMCS	Km^r pBAV1K-t5-gfp derivative with gfp ORF removed, Constitutive *E. coli/Acinetobacter* shuttle vector (Bryksin, A.V. and Matsumura, I., 2010)
pBAV-*A1S_1193His10X*	Constitutive plasmid, Km^r, used to express *A1S_1193-his* (inserted at BamH1 and SalI) in *Acinetobacter* (This study)

2.3-Results:

2.3.1-*A. baumannii* Strains Produce Diverse *O*-glycan Structures – Protein glycosylation and the carbohydrate synthesis loci thought to be responsible for the production of the *O*-linked glycan are ubiquitous in *Acinetobacter* (Iwashkiw, J.A. *et al.*, 2012a; Lees-Miller, R.G. *et al.*, 2013c). As glycans are produced in a non-template driven manner we began exploring the potential *O*-glycan diversity by undertaking glycopeptide enrichment of commonly used sequenced *A. baumannii* strains as well as clinical isolates. These strains included; ATCC 19606, predicted to produce the same O-glycan as the previously characterized ATCC 17978 glycan (Figure 2.1) (Iwashkiw, J.A. *et al.*, 2012a), two strains thought to produce divergent glycans; AYE and SDF (Figure 2.1); as well as clinical isolates of unknown glycan composition; Arg1, Arg2, 1441 C1, and 1441 C3. ZIC-HILIC enriched preparations of ATCC 19606 resulted in the identification of 50 unique glycopeptides corresponding to 13 unique glycoproteins. Of these glycoproteins, eight were previously unknown. In agreement with their matching carbohydrate synthesis loci, the glycan moieties in ATCC 19606 and ATCC 17978 were identical, consisting of pentasaccharide β-GlcNAc3NAcA4OAc-4-(β-GlcNAc-6-)-α-Gal-6-β-Glc-3-β-GalNAc- (Figure 2.2A).

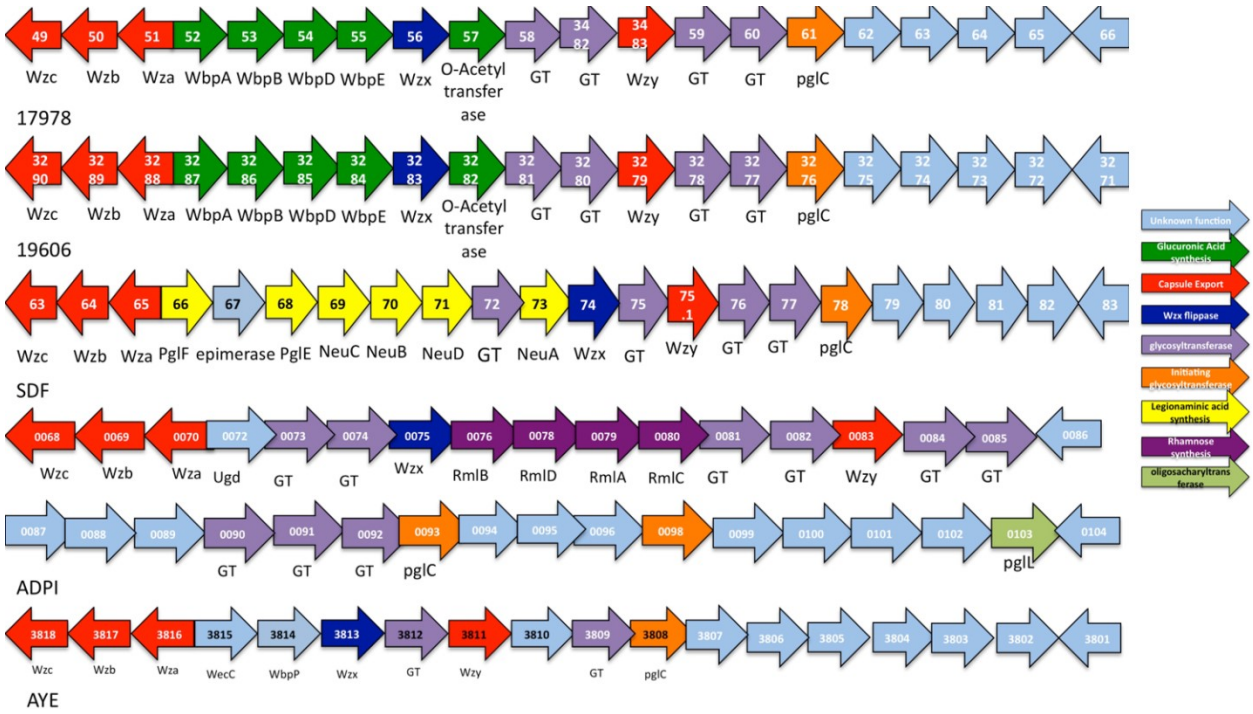


Figure 2.1-Bioinformatic Comparison of *Acinetobacter* Glycosylation Biosynthetic Loci. A) Glycosylation loci from *A. baumannii* ATCC 17978, ATCC 19606, SDF, AYE and *A. baylyi* ADP1. The biosynthesis loci for ATCC 17978 and ATCC 19606 are identical. SDF contain the genes necessary for synthesis of legionaminic acid. *A. baylyi* ADP1 has the genes for rhamnose biosynthesis and has a larger carbohydrate biosynthesis locus than any other sequenced strain. The biosynthesis locus of AYE contains three putative glycosyltransferases and no genes from a distinct carbohydrate synthesis pathway.

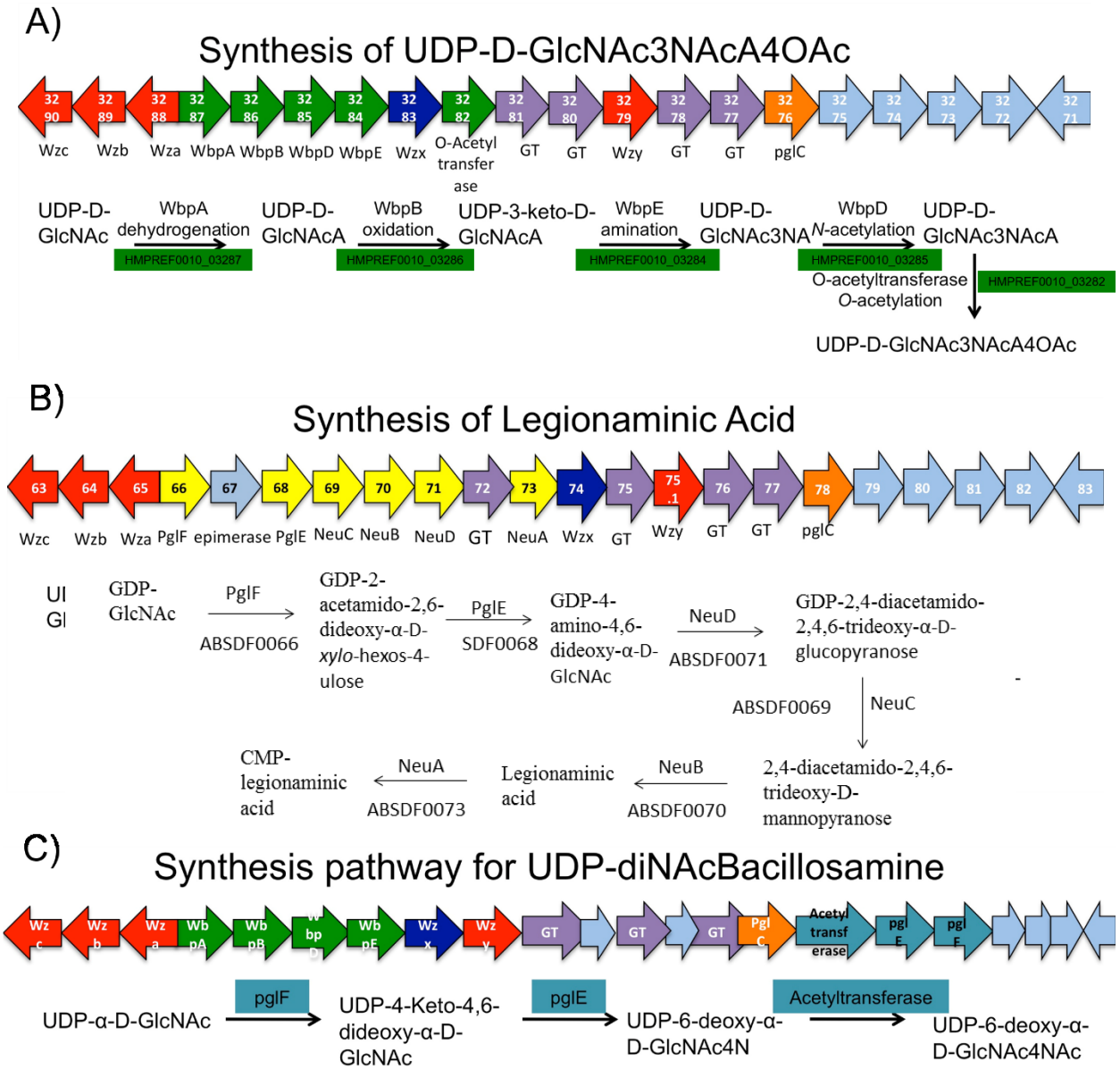


Figure 2.2-Bioinformatic comparison of *Acinetobacter* glycosylation biosynthetic lociExamination of the carbohydrate biosynthesis genes located in this locus provides a basis for predicting the monosaccharide composition of the glycan. The extensive variability in the carbohydrate synthesis genes and the number of glycosyltransferases in each locus suggests *O*-glycan diversity in *Acinetobacter.* B) The *A. baumannii* ATCC 19606 locus is depicted along with the predicted biosynthesis pathway for GlcNAc3NAcA4OAc. We identified a 300 Da monosaccharide in several *O*-glycans including that of ATCC 17978 and ATCC 19606, which

based on previously published NMR data and the presence of this biosynthesis pathway we believe to be GlcNAc3NAcA4OAc. C) The putative biosynthesis pathway for legionaminic acid encoded by the *A. baumannii* SDF locus is depicted. A 316 Da sugar, matching the mass and fragmentation pattern of legionaminic acid was identified in the *O*-glycan of SDF and several clinical isolates. D) The carbohydrate synthesis locus and biosynthesis pathway for diNAcBac encoded in *A. baumannii* clinical isolate 1225 is shown. The *O*-glycan of this strain contains a sugar with a 228 Da mass, which we predict to be UDP-diNAcBac based on the mass and presence of the required biosynthesis genes.

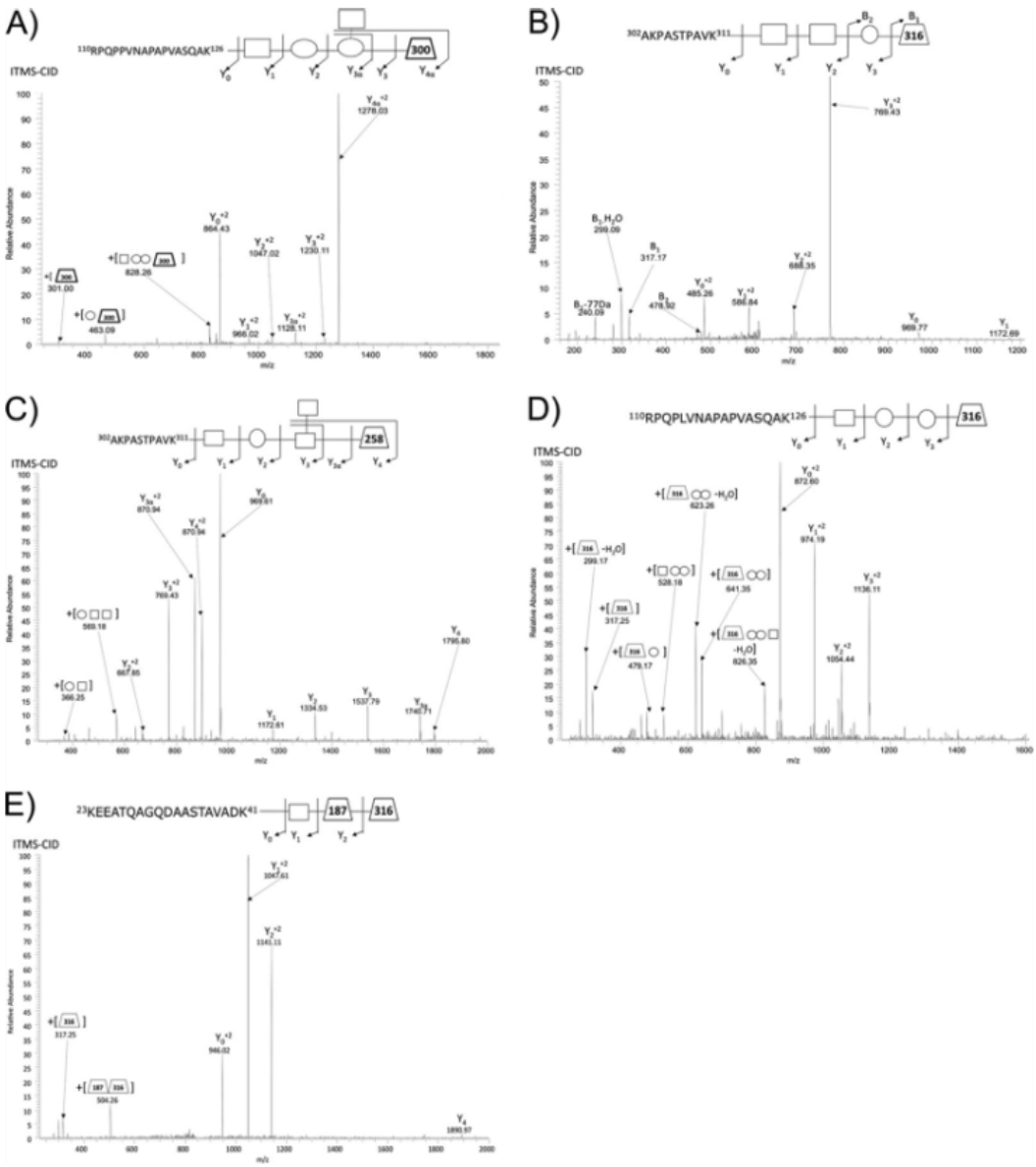


Figure 2.3-Major *O*-glycan structure identified using ZIC-HILIC enrichment. ITMS-CID fragmentation results in near exclusive glycan fragmentation of *A. baumannii* glycopeptides leading to the identification of *A*, the previously pentamer glycan (Iwashkiw, J.A. *et al.*, 2012a)within strain ATCC 19606 (^{110}RPQPPVNAPAPVASQAK^{126} of D0CDA9_ACIBA), *B*, *a* 4-mer glycan containing a 316 Da residue in strain SDF (^{302}AKPASTPAVK^{311} of B0VKN6_ACIBS), *C*, a novel pentamer glycan within strain Arg1 containing a 258 residue (^{302}AKPASTPAVK^{311} of A3M265_ACIBT), *D*, a novel 4-mer glycan within strain Arg2 (^{110}RPQPLVNAPAPVASQAK^{126} of J5IPS4_ACIBA) containing a similar 316 Da residue to SDF, and *E*, the novel trisaccharide identified in strains 1441 C1 and C3

(^{23}KEEATQAGQDAASTAVADK^{41} of A7FB63_ACIBT) containing the 316 Da residue of SDF and Arg 1.

In contrast, *A. baumannii* strains SDF and AYE lack the genes required for the generation of GlcNAc3NAcA4OAc (Figure 2.1), and consequently, no potential glycopeptides could be identified using the GlcNAc3NAcA4OAc oxonium ion. To enable the detection of divergent glycan attachments, we assessed the presence of the oxonium ion 204.086 *m/z,* generated by the presence of HexNAc moieties within ZIC-HILIC enriched samples of A. *baumannii.* Despite multiple attempts, glycosylation could not be identified within strain AYE on either the peptide or protein level, using Periodic Acid Schiff's (PAS) staining. Interestingly, PAS staining also failed to identify the present of capsular polysaccharide (Figure 2.3) suggesting the absence of products from the AYE carbohydrate locus. As the capsular polysaccharide is required for complement resistant (Lees-Miller, R.G. *et al.*, 2013a) we assess the resistance of AYE to complement-mediated killing. Consistent with the lack of the capsular polysaccharide AYE was highly sensitive to complement-mediated killing, compared with ATCC 17978 and all recent clinical isolates examined (Figure 2.4). The levels of sensitivity were also consistent with that of the known capsule mutant ATCC 17978 Δ*pglC* (Figure 2.4) (Lees-Miller, R.G. *et al.*, 2013a), supporting the absence of carbohydrate locus products within AYE. Acapsular *A. baumannii* cells are difficult to evenly resuspend due to a tendancy to stick to one another. This explains the disproportionate ten fold dilutions of both AYE and ATCC 17978 Δ*pglC.*

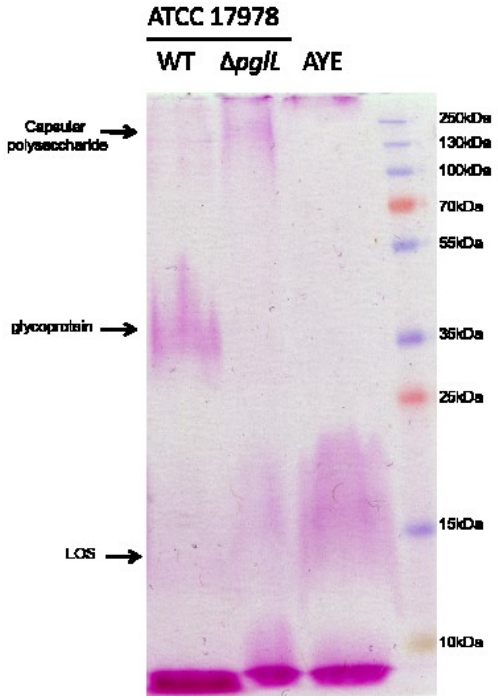


Figure 2.4-Periodic Acid Schiff (PAS) staining of total membrane extracts. PAS staining of total membrane extracts from *A. baumannii* ATCC 17978 wildtype, the *pglL* mutant strain, and AYE. The high molecular weight stained carbohydrate is capsular polysaccharide. The ~35kDa carbohydrate stain is a glycoprotein and the low molecular weight region carbohydrate is likely the lipooligosaccharide (LOS) as seen in the wildtype 17978 lane. The Δ*pglL* strain lacks the glycoprotein smear as it does not have a functional *O*-oligosaccharyltransferase required for protein glycosylation, but still produces both LOS and capsule. AYE lacks both the capsular polysaccharide and the glycoprotein smear, but has LOS.

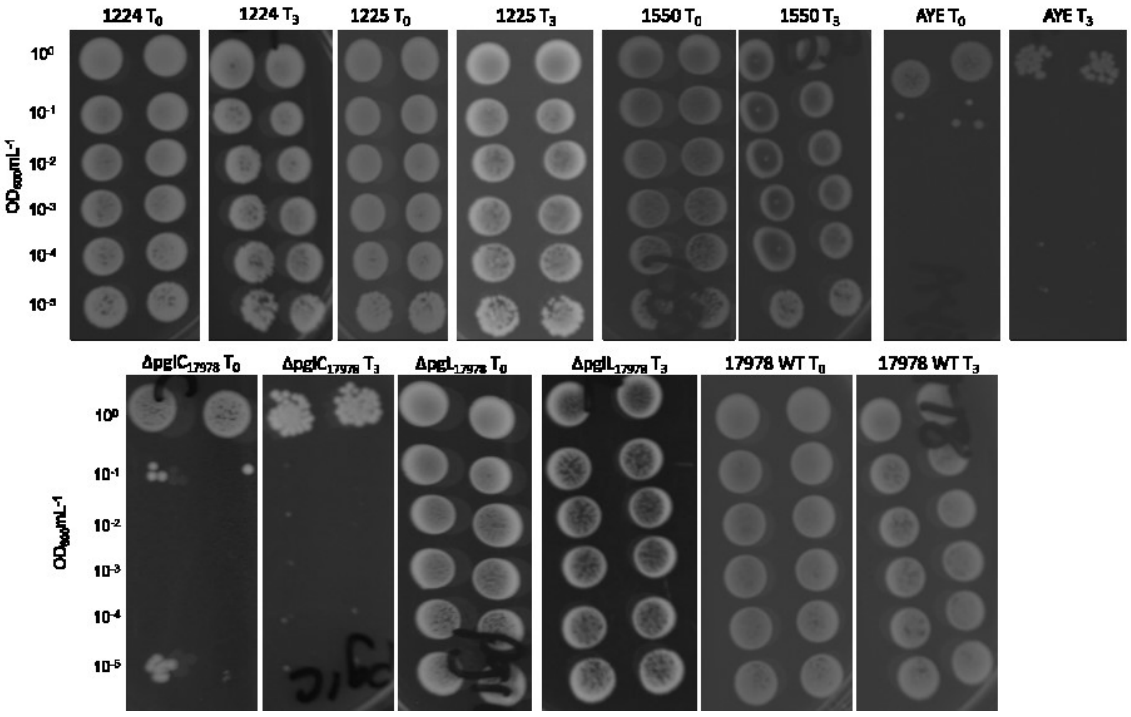


Figure 2.5-Complement mediated killing assay for the presence of capsular polysaccharide in *Acinetobacter.* Mid-log phase bacteria was re-suspended to 1 OD/ml in 100% horse serum and diluted up to 10^{-5} and incubated at 37ºC for 3 hours. Dilutions were spotted on LB agar in 10 µL spots to enumerate the surviving bacteria at time 0 and 3 hours post serum treatment. Acapsular cells are difficult to evenly resuspend due to a tendance to stick to one another. This is evident by the disproportionate ten fold dilutions of AYE and the *ΔpglC*. The bottom panel shows the control strains, where wildtype and the protein glycosylation deficient strain are resistant to complement mediated killing. The acapsular strain, Δ*pglC*, is sensitive to complement. The upper panel shows several *A. baumannii* clinical isolates which are resistant to complement mediated killing. AYE is sensitive to serum, suggesting it is acapsular.

Within SDF the use of ZIC-HILIC enrichment lead to the identification of 13 unique glycopeptides containing a tetrasaccharide glycan composed of $HexNAc_2$-Hex-NulO (mass 884.34 Da) (Figure 2.2B), where NulO corresponds to a 316.13 Da nonulosonic acid sugar. The identification of this unique glycan attached to five unique proteins (Table 2-3) confirmed SDF has an active general O-linked glycosylation system and produces a glycan different to the pentasaccharide characterized in ATCC 17978 and 19606. Analysis of glycan related fragments

within identified glycopeptides of SDF revealed the mass and isotopic distribution of the 317.13 *m/z* oxonium ion (the MH+ of the 316.13 residue, supplemental Figure S2.4), was consistent with legionaminic acid (elemental composition $C_{13}H_{21}O_7N_2$) (Thibault, P. *et al.*, 2001a). To further assess the identity of the 316 Da moiety, analysis of the low mass region of the HCD spectra was undertaken (supplemental Figure S2.4) confirming fragmentation consistent with that of the legionaminic or pseudaminic acid oxonium ion (Thibault, P. *et al.*, 2001a). These observations are in line with the genetic analysis of the glycan locus of SDF, which contains homologs for legionaminic acid biosynthesis as noted previously (Kenyon, J.J. and Hall, R.M., 2013b) (Figure 2.1).

To further understand the O-linked glycosylation diversity and glycan characteristics within A *baumannii* we analyzed four recent MDR clinical isolates (Arg1, Arg2, 1441 C1, and 1441 C3). As with SDF, the analysis of these strains revealed a diverse array of glycans with the major glycopeptides identified containing the glycans HexNAc-Hex_2-HexNAc-258 (mass 988.34 Da), HexNAc-Hexa-NulO (mass 843.31 Da), and HexNAc-dHexNAc-NulO (mass 706.30 Da) within Arg1, Arg2, and 1441 C1 and 1441 C3 respectively (Figures 2.2C-2.2E, and Table 2-3). These glycopeptides corresponded to both previously identified O-glycosylation substrates and a multitude of novel protein acceptors (Supplementary Table S2-2). Inspection of the mass and isotopic distribution of oxonium ions generated by these moieties (corresponding to the 258 and 316 Da residues) supported the assignments of these residues as a deacylated form of GlcNAc3NAcA4OAc, a carbohydrate residue previously identified within the glycan of *Acinetobacter Iwoffii* F78 (Hanuszkiewicz A., K.Z., Lindner B., Goldmann T., Vollmer E., Debarry J., Heine H., Holst O. , 2008), and legionaminic/pseudaminic acid (supplemental Figure S2-5). From these results, we confirm that diverse glycans are utilized in a range of strains, and that similar substrates are targeted for glycosylation across *A. baumannii* strains.

Strain name	Glycans observed	Shared Glycans	Unique Glycans	Glycan 1	Glycan 2	Glycan 3	Glycan 4	Glycan 5	Glycan 6	Glycan 7	Glycan 8	Glycan 9	Glycan 10
A. baumannii ATCC 17978	8	6	2	HexNAc-Hex_2-HexNAc-300*	HexNAc-Hex_2-HexNAc-258	HexNAc-Hex_2-258	HexNAc-Hex_2-HexNAc-314	386-Hex_2-HexNAc-300	HexNAc-Hex_2-300	Double glycan; HexNAc-Hex2-HexNAc-300 and HexNAc-Hex2-300	HexNAc-Hex-$HexNAc_2$-300		
A. baumannii ATCC 19606	7	6	1	HexNAc-Hex_2-HexNAc-300*	HexNAc-Hex_2-HexNAc-258	HexNAc-Hex_2-258	HexNAc-Hex_2-HexNAc-314	Double glycan; HexNAc-Hex2-HexNAc-300 and HexNAc-Hex2-300	Double glycan; 2*(HexNAc-Hex2-HexNAc-300)	HexNAc-Hex_2-300			
A. baumannii SDF	3	0	3	$HexNAc_2$-Hex-316*	$HexNAc_2$	$HexNAc_2$-Hex-358							
A. baumannii Arg1	9	2	7	HexNAc-Hex_2-HexNAc-258*	HexNAc-Hex_2-258	244-Hex_2-HexNAc-258	HexNAc-Hex-$HexNAc_2$-258	Double glycan; HexNAc-Hex2-HexNAc-258	HexNAc-154-Hex_2-HexNAc-258	HexNAc-Hex	HexNAc-Hex_2-HexNAc	Double glycan; HexNAc-Hex2-HexNAc-258-HexNAc-Hex2-258	
A. baumannii Arg2	8	1	7	HexNAc-Hex_3-316*	$HexNAc_2$-Hex_2-316	Double glycan; HexNAc-Hex2-316	HexNAc	HexNAc-Hex-316	HexNAc-Hex-HexNAc-316	HexNAc-Hex_3	$HexNAc_2$-316		
A. baumannii 1441 C1	10	6	4	HexNAc-187-316*	Double glycan; HexNAc-187-316	Triple glycan; HexNAc-187-316	HexNAc-187	HexNAc-187-316-HexNAc-187-331	HexNAc	HexNAc-187-316-HexNAc-187	HexNAc-145-316	HexNAc-187-331	HexNAc-172-316
A. baumannii 1441 C3	5	5	0	HexNAc-187-316*	Double glycan; HexNAc-187-316	Triple glycan; HexNAc-187-316	HexNAc-187	HexNAc-187-316-HexNAc-187					
A. baumannii AYE	0	0	0										
A. calcoaceticus 1217	1	1		HexNAc-176-$HexNAc_2$Hex*									
A. calcoaceticus 1218	1	0	1	HexNAc-HexNAcA-$HexNAc_2$*									
A. pittii 1219	1	1		HexNAc-176-$HexNAc_2$-Hex*									
A. nosocomialis 1222	1	0	1	Triple glycan; HexNAc-Hex_2*									
A. baumannii 1224	1	0	1	HexNAc-Hex-$HexNAc_2$-158-Hex*									
A. baumannii 1225	2	0	2	272-258-HexNAc-258*	diNAcBac-258-HexNAc-258								
A. baylyi ADP1	2	0	2	285-HexNAcA-HexNAcOAc2-HexNAc*	285-HexNAcA-HexNAcOAc-HexNAc2								

Table 2-2- Glycans Identified within *Acinetobacter* species.

2.3.2-A. baumannii Strains Display Glycan Micro-heterogeneity Producing Multiple Related Glycan Structures—In addition to the major glycoform described above, at least three alternative glycoforms were unexpectedly identified within each *A. baumannii* strain, including SDF, Arg1, Arg2, 1441 C1, and 1441 C3 (Figure 2.5A-J, Table 2-2). Glycan diversity appeared to be largely the result of chemical exchange and/or addition of functional groups such as acetyl and methyl groups to the bacterial specific residue observed within each strain, although examples of truncated glycan were also observed (Figure 2.5A-2.5C). Inspection of the mass and isotopic distribution of oxonium ions generated by the modified bacterial specific residues (corresponding to the 331 and 358 Da residues) supported the assignments of these residues as methylated or acetylated forms of the nonulosonic sugar found in the major glycoform (supplemental Figure S2-6). These modified forms although common and found on multiple peptide substrates appear less abundant, based on frequency of identification and ion intensity relative to the major glycoform within each strain (data not shown).

As glycan heterogeneity was not considered during the initial analysis A *baumannii* ATCC 17978 and ATCC 19606, re-inspection of ZIC-HILIC enrichment datasets were carried out leading to the confirmation of glycan heterogeneity within both strains (Figure 2.5D, 2.5E, and Table 2-2). The dominant glycan structure produced by ATCC 17978 and ATCC 19606 is HexNAc-Hex_2-HexNAc-GlcNAc3NAcA4OAc, however, alternative glycans composed of HexNAc-Hex_2-HexNAc-258 and HexNAc-Hex_2-HexNAc-314 were also identified (Figure 2.5D, 2.5E) in both strains. Examination of the low mass region of the HCD spectrum within these alterative glycans revealed the presence of two novel ions corresponding to 259 and 315 *m/z* (consistent with MH+ of the 258.09 and 314.12 Da residues respectively, supplemental Figure S2.7). The observed masses and isotopic distributions of these oxonium ions were consistent with a methylated form and a deacetylated form of GlcNAc3NAcA4OAc, of 315.11733 Da and 259.09112 Da, respectively (supplemental Figure S2.7). Within ATCC 17978 we also noted the existence of two unique glycoforms that differed in the internal carbohydrate of the glycan, one corresponding to the exchange of a HexNAc for a Hex residue (Figure 2.5F) and the other to the exchange of the linking carbohydrate for a moiety of 386 Da (Figure 2.5G). The analysis of the low *m/z* region of the HCD spectra confirmed the presence of a 387.11937 Da MH+ ion and provided insight into the identity of this moiety. By comparing spectra generated from modified and non-modified glycans (supplemental Figures S2.8) a putative characterization was possible suggesting this moiety corresponds to a composition of $C_{13}H_{24}N_1O_{10}S_1$ that is consistent with the mass and isotopic pattern of this residue.

(supplemental Figure S2.8). A molecule with this formula has not been previously reported within a bacterial glycopeptide. This predicted composition does not match any known carbohydrate and appears to represent a minor *O*-linked glycoform of ATCC 17978.

It should be noted that in addition to heterogeneity because of the exchange and/or addition of functional groups within the glycan, polymerized forms of the *O*-linked glycan, glycan oligomers, were also observed within all strains (Table 2-2). These glycan oligomers were present on numerous peptides (Table 2-2) with dimeric glycans (Figure 2.5I) identified in all strains. In addition to glycan dimers, trimers (Figure 2.5J) were also readily detectible within strains 1441 C1 and 1441 C3, which produces the smallest A *baumannii* O-glycan characterized to date, a linear trisaccharide of 706.39 Da.

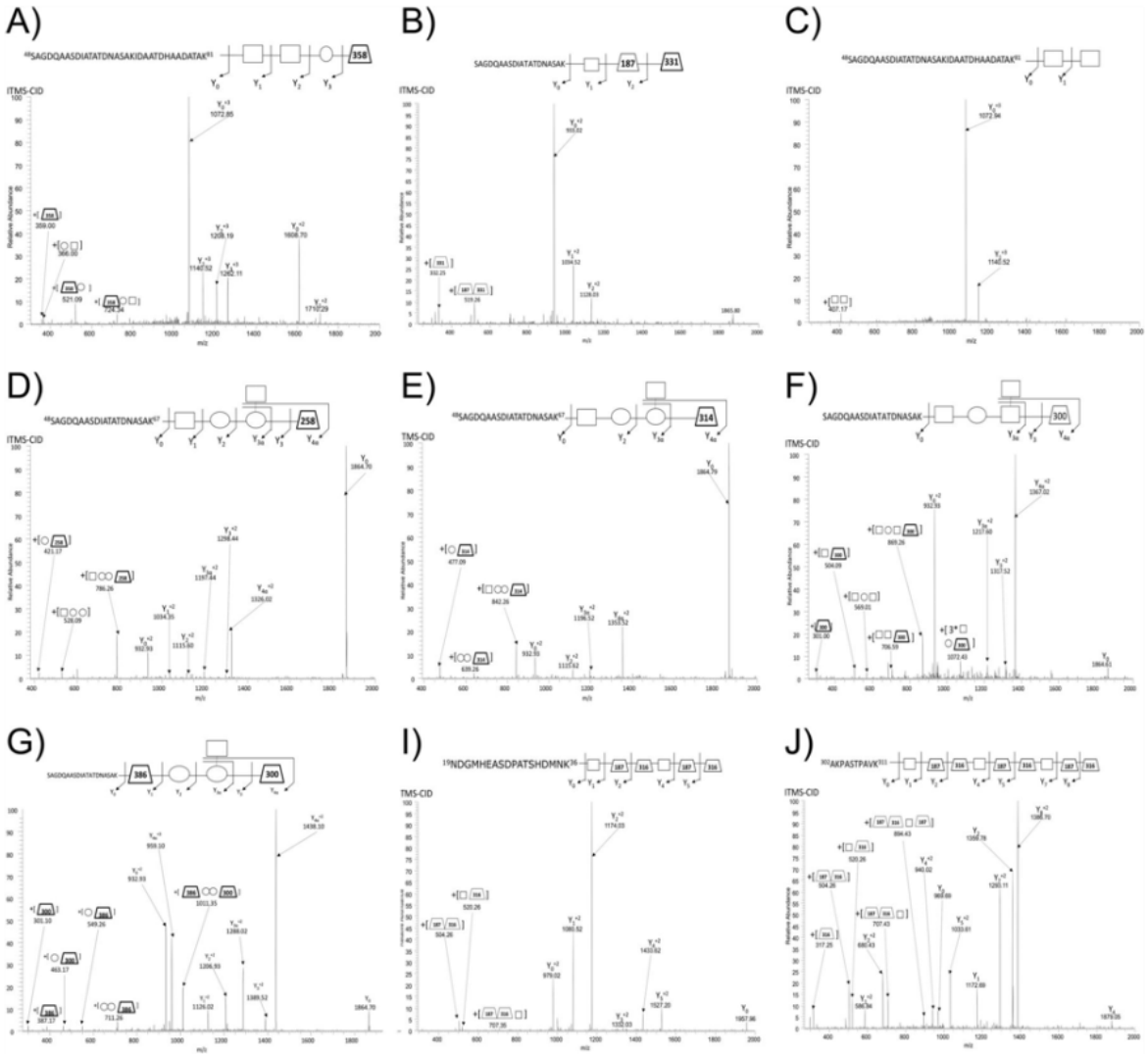


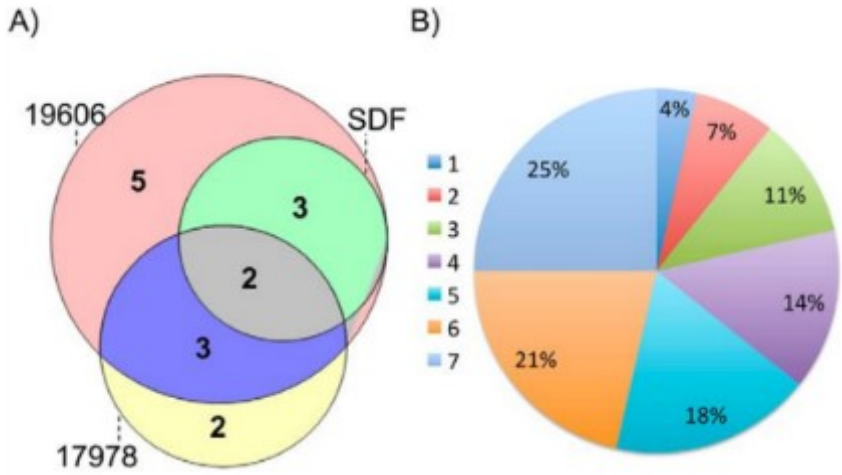
Figure 2.6-Alterative *O*-glycan structure identified using ZIC-HILIC enrichment with *A. baumannii* strains. ITMS-CID fragmentation enabled the identification of *A. baumannii* glycopeptides containing alterative glycoforms within all strains examined including: *A*, A tetramer glycan composed of $HexNAc_2$-Hex-NulOAc within *A. baumannii* SDF (^{48}SAGDQAASDIATATDNASAKIDAATDHAADATAK^{81} of B0VLI0_ACIBS), *B*, A trisaccharide composed of HexNAc-dHexNAc-NulOCH3 within *A. baumannii* 1441 C1 (^{48}SAGDQAASDIATATDNASAK^{67} of A7FB63_ACIBT), *C*, A disaccharide composed of $HexNAc_2$ within *A. baumannii* SDF (^{48}SAGDQAASDIATATDNASAK^{67} of B0VLI0_ACIBS), *D*, A pentamer glycan within *A. baumannii* 19606 composed of HexNAc-Hex_2-HexNAc-GlcNAc3NAcA (^{48}SAGDQAASDIATATDNASAK^{67} of D0CEI7_ACIBA), *E*, A pentamer glycan within *A. baumannii* 19606 and 17978 composed of HexNAc-Hex_2-HexNAc-

GlcNAc3NAcA4OAcCH3 (^{48}SAGDQAASDIATATDNASAK^{67} of A7FB63_ACIBT), *F*, The known pentamer glycan of *A. baumannii* ATCC 17978 where a Hex has been exchanged for a HexNAc residue compared with the previously reported glycan (Iwashkiw, J.A. *et al.*, 2012a) (^{48}SAGDQAASDIATATDNASAK^{67} of A7FB63_ACIBT), *G*, The known pentamer glycan of *A. baumannii* ATCC 17978 where the linking HexNAc residue has been exchanged for the unique 386.11 Da residues compared with the previously reported glycan (Iwashkiw, J.A. *et al.*, 2012a) (^{48}SAGDQAASDIATATDNASAK^{67} of A7FB63_ACIBT), *I*, A dimer of the trisaccharide O-glycan of *A. baumannii* 1441 C1 (^{19}NDGMHEASDPATSHDMNK^{36} of A7FB95_ACIBT), and *J*, A trimer of the trisaccharide O-glycan of *A. baumannii* 1441 C1 (^{302}AKPASTPAVK^{311} of A3M265_ACIBT).

2.3.3-*Acinetobacter baumannii* O-glycosylates Multiple Conserved Protein Substrates via Serine Residues in Low-complexity Regions—Examination of multiple *A. baumannii* strains expanded the repertoire of known glycosylation substrates adding 19 novel glycoproteins to the seven previously identified substrates (supplemental Table S2-2). Interestingly few glycoproteins identified were unique to a single strain; for example, within the laboratory strains examined glycopeptides corresponding to the same eight glycoproteins were observed in SDF, ATCC 17978, and ATCC 19606 (Figure 2.6A), whereas seven were unique to a single strain. This trend was seen across all *A. baumannii* strains examined where ~70% of all glycoproteins were identified within at least two strains (Figure 2.6B). These observations in combination with genetic analyses of multiple genome-sequenced strains of *A. baumannii* confirm that the identified glycoproteins are both conserved and targeted for glycosylation across divergent strains (supplemental Table S2-9). An example of this is the conserved, putative uncharacterized protein D0C6C0_ACIBA (Figure 2.6C), which was identified in five out of the seven strains examined (supplemental Table S2-2). Interestingly, it was also noted that the observed glycoproteins are unique to *Acinetobacter*, with no homologs outside of this genus, and are highly conserved with >97% identity between strains (supplemental Table S2-9). No homologs were found in any other bacterium and therefore their function cannot be assigned based on homology.

To accurately assess the local environment of glycosylation, based on the site of

attachment, the sites of modification were characterized using ETD fragmentation (supplemental Table S2-10). Using this approach a total of seven sites of glycosylation could be localized, which in conjunction with four additional sites localized by the presence of only one hydroxyl containing amino acid in the sequence, lead to the localization of eleven glycosylation sites across the examined *A. baumannii* strains (supplemental Table S2-10). Within this dataset it was noted that only Serine appeared to be glycosylated within the identified glycopeptides, and that the sites of glycosylation seem to have a strong preference for alanine in the −1 position (Figure 2.6D, supplemental Table S2-10). Because previous reports have suggested that glycosylation occurs at disordered regions in other bacterial *O*-linked glycosylation systems rather than at a specific sequon (Vik, A. *et al.*, 2009a; Slynko, V. *et al.*, 2009; Rangarajan, E.S. *et al.*, 2007), we also examined the region surrounding the identified glycosylation sites. Similar to other bacterial *O*-linked systems, sites of *A. baumannii O*-linked glycosylation occur in low complexity regions, rich in proline, alanine, and serine (Figure 2.6E), suggesting that the recognition of substrates by *O*-oligosaccharyltransferases is conserved in different bacteria.



C)

Strains		Acinetobacter baumannii (strain SDF)	Acinetobacter baumannii (strain AYE)	Acinetobacter baumannii ATCC 19606	Acinetobacter baumannii (strain TCDC AB0715)	Acinetobacter baumannii (strain 1656-2)	Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	Acinetobacter baumannii (strain AB307-0294)
	Uniprot #	B0VRK6_ACIBS	B0VTX5_ACIBY	D0C6C0_ACIBA	F0QUR8_ACIBD	F8PBZ1_ACIB1	A3M602_ACIBT	B7H1B6_ACIB3
Acinetobacter baumannii (strain SDF)	B0VRK6_ACIBS	100%	98.00%	99.00%	99.00%	99.00%	97.00%	98.00%
Acinetobacter baumannii (strain AYE)	B0VTX5_ACIBY		100%	98.00%	99.00%	98.00%	97.00%	100.00%
Acinetobacter baumannii ATCC 19606	D0C6C0_ACIBA			100%	99.00%	99.00%	98.00%	98.00%
Acinetobacter baumannii (strain TCDC AB0715)	F0QUR8_ACIBD				100%	99.00%	98.00%	99.00%
Acinetobacter baumannii (strain 1656-2)	F8PBZ1_ACIB1					100%	98.00%	98.00%
Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	A3M602_ACIBT						100%	97.00%
Acinetobacter baumannii (strain AB307-0294)	B7H1B6_ACIB3							100%

Figure 2.7-*A. baumannii* prominently glycosylates Serine residues with Alanine residues in the −1 position. Analysis of glycoproteins and glycosylation sites identified of *A. baumannii* strains. *A*, Comparison of glycoproteins identified within each strain. Showing the high level of overlap between strains. *B*, Analysis of the overlap between strains glycoproteins identified in all seven *A. baumannii* strains. *C*, Comparison of the sequence identity of proteins between strains of *A. baumannii* demonstrating the high level of sequence identity between strains of the identified glycoproteins. *D*, Motif analysis of identified glycosylation sites showing a strong preference for the sequence AS. *E*, Comparison of the region of disorder around the identified

sites of glycosylation, a disordered prediction >0.5 is consisted to be disordered according to PreDisorder, http://casp.rnet.missouri.edu/predisorder.html.

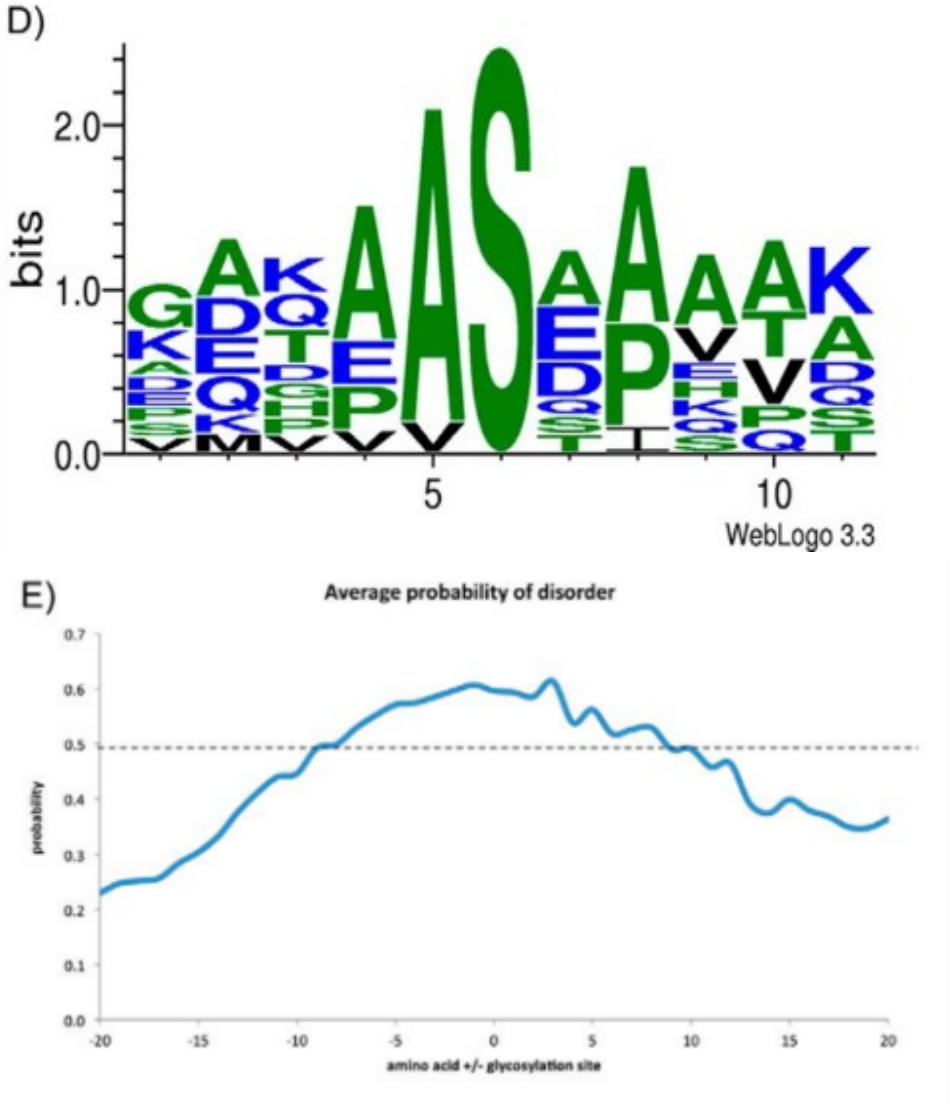


Figure 2.7-*A. baumannii* prominently glycosylates Serine residues with Alanine residues in the −1 position. Analysis of glycoproteins and glycosylation sites identified of *A. baumannii* strains. *A*, Comparison of glycoproteins identified within each strain. Showing the high level of overlap between strains. *B*, Analysis of the overlap between strains glycoproteins identified in all seven *A. baumannii* strains. *C*, Comparison of the sequence identity of proteins between strains of *A. baumannii* demonstrating the high level of sequence identity between strains of the identified glycoproteins. *D*, Motif analysis of identified glycosylation sites showing a strong preference for the sequence AS. *E*, Comparison of the region of disorder around the identified

sites of glycosylation, a disordered prediction >0.5 is consisted to be disordered according to PreDisorder, http://casp.rnet.missouri.edu/predisorder.html.

2.3.4-Targeted Analysis of the Glycan Diversity in Acinetobacter Clinical Isolates—Although the results of the ZIC-HILIC enrichment confirmed our hypothesis of *O*-linked diversity within multiple strains, it also highlighted potential variability in the performance of ZIC-HILIC enrichment. As these methodologies rely on the ability to detect glycosylation based on the presence of diagnostic carbohydrate reporter ions, we reasoned that if strains possessed a glycan lacking known carbohydrate moieties or were ineffectively enriched with ZIC- HILIC, detection and determination of glycosylation would be compromised. In order to refine the analysis of glycan diversity and expand the number of strains analyzed, we developed a targeted approach to assess glycosylation. Based on the observation that the same proteins are glycosylated at sites with similar structural characteristics in multiple strains (Figure 2.6A, 2.6D, and 2.6E), we reasoned that a His-tagged version of one of these conserved proteins could be expressed in other *Acinetobacter* strains and used as bait in order to isolate attached glycans. To achieve this goal, we expressed the *A. baumannii* glycoprotein *A1S_1193* in clinically relevant strains. Previous work from our lab showing the site of glycosylation within A1S_1193 is Serine-205 (Iwashkiw, J.A. *et al.*, 2012a; Madsen, J.A. *et al.*, 2013). , contained within the tryptic peptide ^{203}AA***S***GVEAAAAPATLTLSTDDK^{223} facilitated characterization of the corresponding glycans (14). Expression of *A1S_1193* leads to the decoration of the protein substrate with native glycosylation, enabling the isolation of the protein independent of the chemical properties of the sugars. Importantly, this approach does not require prior knowledge of the genomic sequence or glycan structure, enabling the assessment of glycosylation in unsequenced clinical strains.

To investigate glycan diversity in the clinical setting, six clinical *Acinetobacter* isolates were selected for analysis using this targeted approach. The expression of *A1S_1193* within these *Acinetobacter* clinical isolates lead to a detectible mass shift compared with the non-glycosylated control (Figure 2.7A) suggesting glycosylation of the protein substrates. MS analysis confirmed the addition of glycan structures to A1S_1193 with a total of six novel glycan structures observed across the six strains (Figure 2.7D–2.7J, Table 2-2). From this analysis, it was noted that all glycans were composed of four to six carbohydrates and were largely linear in nature

with few branched sugars observed. As with SDF, ATCC 19606, and ATCC 17978, glycan heterogeneity was also observed within clinical isolates; for example, within *A. baumannii* 1225 two glycans composed of 272–258-HexNAc-258 and 228 –258- HexNAc-258 were identified (Figure 2.7I-2.7J). As with previously identified heterogeneity the alteration in *A. baumannii* 1225 occurred on the bacterial specific residues corresponding to the mass of diNAcBac (228 Da; bacillosamine). Interestingly the glycans of *A. baumannii* 1225 appear similar to the previously characterized glycans of *A. lwoffii* F78 and *A. baumannii* AB307– 0294 where NMR was utilized to confirm the modification of the capsule with the deacetylated form of GlcNAc3NAcA4OAc (258 Da) or diNAcBac (228 Da) and BacNAc modified with 3-OH-butyrate (272 Da) respectively (Hanuszkiewicz A., K.Z., Lindner B., Goldmann T., Vollmer E., Debarry J., Heine H., Holst O. , 2008; Russo, T.A. *et al.*, 2013). Furthermore, sequencing of *A. baumannii* 1225 isolate has confirmed the presence of the genes necessary for GlcNAc3NAcA4OAc and diNAcBac biosynthesis supporting the assignment of these bacterial specific carbohydrates (Figure 2.1D).

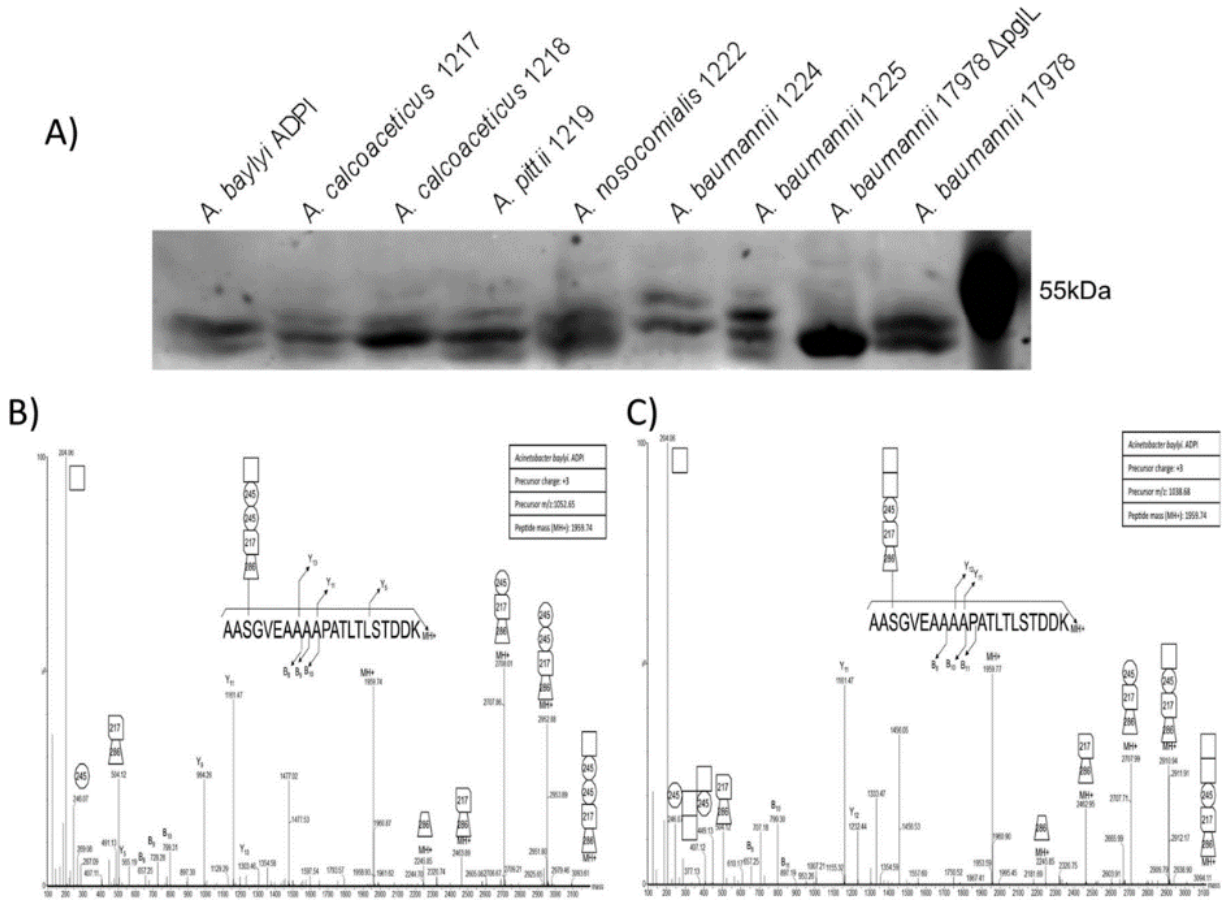


Figure 2.8-Western blot analysis used to resolve the mass difference between glycosylated and unglycosylated A1S_1193. *A*, Anti-Histidine Western blot analysis of *Acinetobacter* strains recombinantly expressing *Acinetobacter* glycoprotein A1S_1193 with a C-terminal Histidine tag. The slight increase in molecular weight indicates the protein has been post-translationally modified. *B-C*, ESI-QTOF MS/MS Analysis of the fished *A. baumannii* glycoprotein A1S_1193 to elucidate the glycan structure. ESI-QTOF-MS and MS/MS was carried out on purified A1S_1193, expressed in various *Acinetobacter* strains, to characterize the posttranslational modification. *B–C*, ESI-QTOF-MS/MS analysis of tryptic peptide ^{203}AA**S**GVEAAAAPATLTLSTDDK^{223} expressed in *A. baylyi* ADP1 revealed either the pentasaccharide 285-217–245$_2$-HexNAc or 285-217–245-HexNAc$_2$ attached to the glycopeptide.

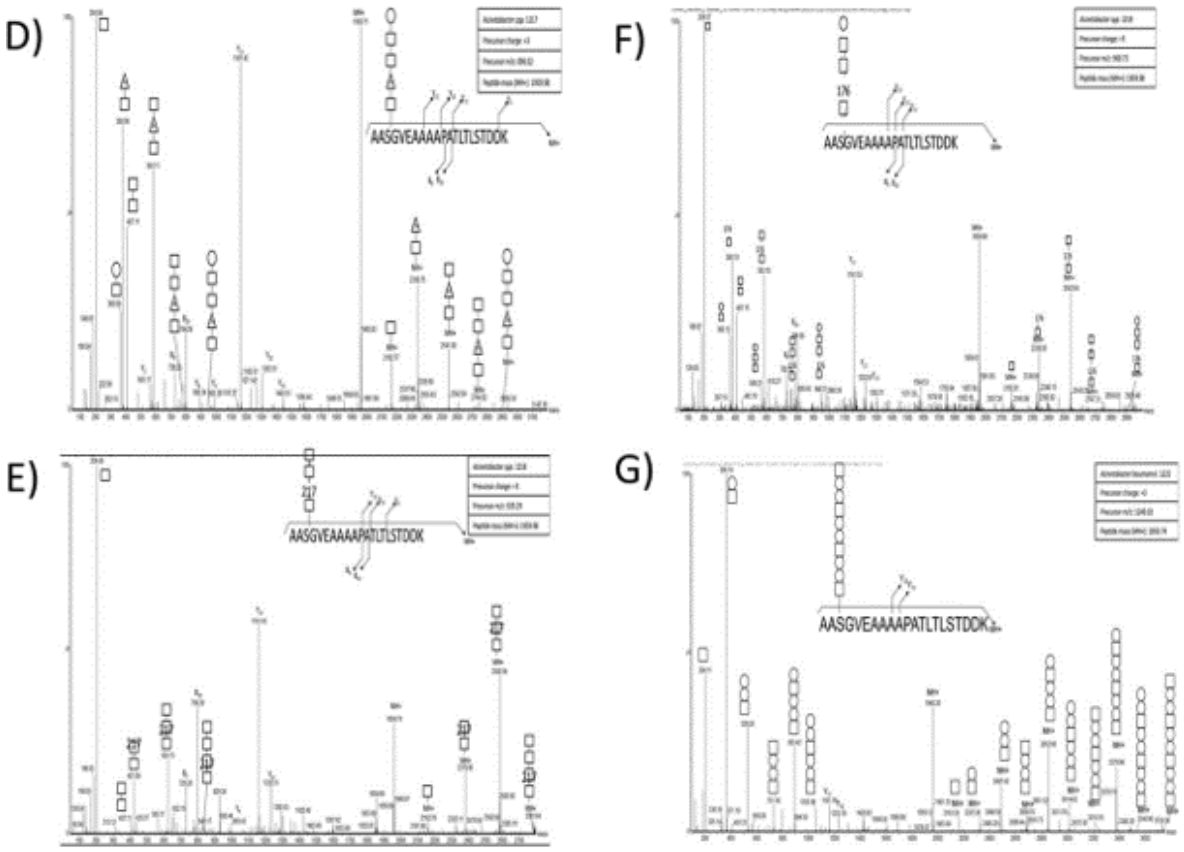


Figure 2.8-Western blot analysis used to resolve the mass difference between glycosylated and unglycosylated A1S_1193 *D–G*, ESI-QTOF MS/MS Analysis of the fished *A. baumannii* glycoprotein A1S_1193 to elucidate the glycan structure. ESI-QTOF-MS and MS/MS was carried out on purified A1S_1193, expressed in various *Acinetobacter* strains, to characterize the posttranslational modification. *D*, MS/MS fragmentation of ^{203}AA**S**GVEAAAAPATLTLSTDDK^{223} expressed in *A. calcoaceticus* 1217 displays modification with the pentasaccharide HexNAc-176-HexNAc$_2$-Hex. *E*, *A. calcoaceticus* 1218 glycosylates the tryptic peptide ^{203}AA**S**GVEAAAAPATLTLSTDDK^{223} with the tetrasaccharide HexNAc-217-HexNAc$_2$. *F*, Fragmentation of tryptic peptide ^{203}AA**S**GVEAAAAPATLTLSTDDK^{223} from A1S_1193 expressed in *A. pittii* 1219 reveals glycosylation with a pentasaccharide identical to 1217, HexNAc-176-HexNAc$_2$-Hex. *G*, *A. nosocomialis* 1222 modifies glycopeptide ^{203}AA**S**GVEAAAAPATLTLSTDDK^{223} with the trisaccharide repeat unit HexNAc-Hex$_2$.

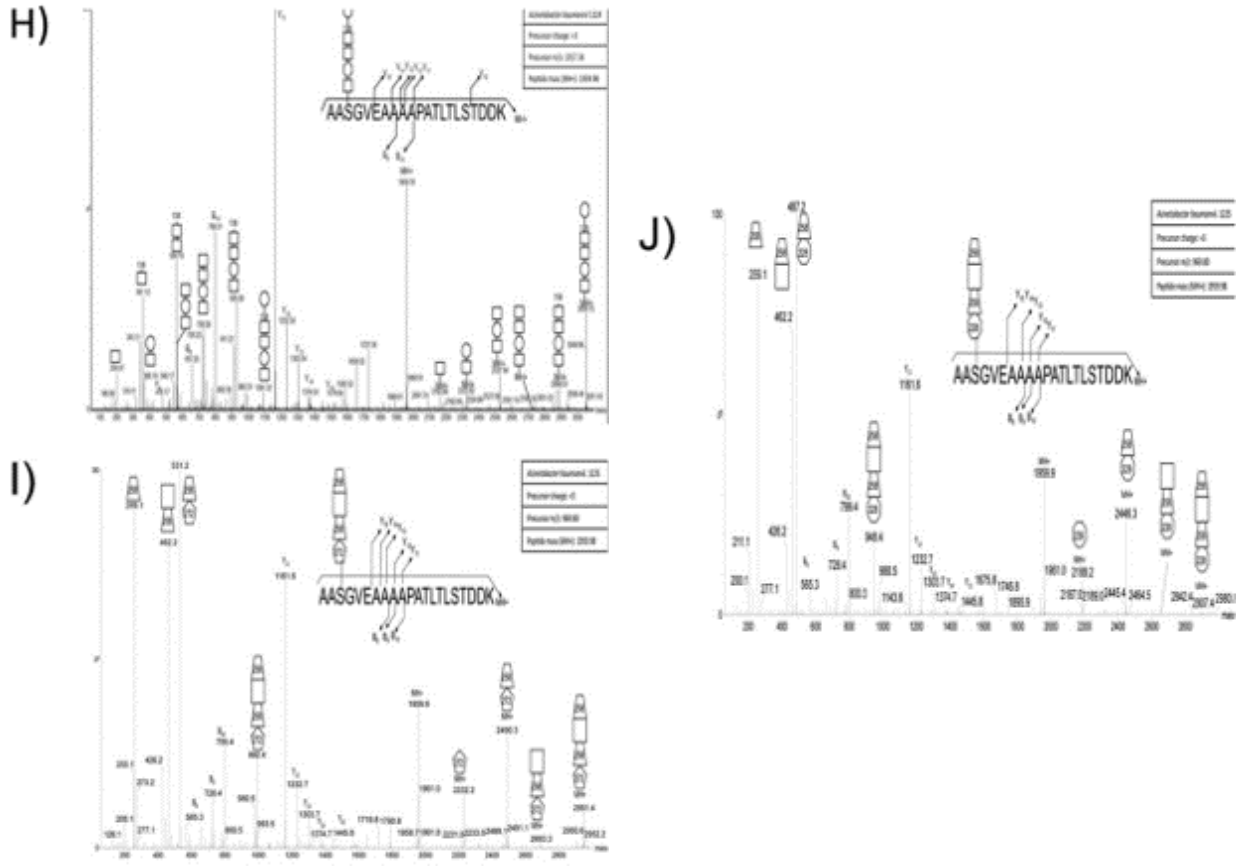


Figure 2.8-Western blot analysis used to resolve the mass difference between glycosylated and unglycosylated A1S_1193. *H-J*, ESI-QTOF MS/MS Analysis of the fished *A. baumannii* glycoprotein A1S_1193 to elucidate the glycan structure. ESI-QTOF-MS and MS/MS was carried out on purified A1S_1193, expressed in various *Acinetobacter* strains, to characterize the posttranslational modification. *H*, *A. baumannii* 1224 uses the hexasaccharide HexNAc-Hex-$HexNAc_2$-158-Hex to glycosylate glycopeptide ^{203}AA**S**GVEAAAAPATLTLSTDDK^{223}. *I–J*, *A. baumannii* 1225 modifies glycopeptide ^{203}AA**S**GVEAAAAPATLTLSTDDK^{223} with one of two tetrasaccharides, 272–258-HexNAc-258 or 228–258-HexNAc-258.

2.3.5-Not all O-glycan Structures within Acinetobacter Match the Predicted Carbohydrate Synthesis Locus: The Investigation of *A. baylyi* ADP1 Glycosylation—With the development of a targeted approach to investigate *Acinetobacter O*-linked glycosylation we also assessed its potential to characterize the *O*-linked glycan of the non-pathogenic model strain *A. baylyi* ADP1. This bacterium is frequently employed as a model *Acinetobacter* strain because of its amenability to genetic manipulation (Metzgar, D. *et al.*, 2004). Interestingly *A. baylyi* ADP1 was recently demonstrated to contain a functional glycosylation system (Schulz, B.L. *et al.*, 2013), although the exact structure has not been elucidated. Using our targeted approach *A. baylyi* ADP1 was found to glycosylate A1S_1193 with a pentasaccharide composed of 285–217-245_2-HexNAc or 285–217-245-$HexNAc_2$, which is distinct from the capsule subunit of its parent strain *A. baylyi* BD4 (Figure 2.7B, 2.7C, Table 2-2). The residues of this glycan included two atypical sugars of mass 217 and 245 Da matching 2-acetamido-2-deoxy-d-hexuronic acid and O-acetyl-N- acetylhexosamine moieties respectively, which have also been recently identified within multiple *Campylobacter species* (Nothaft, H. *et al.*, 2012a). Furthermore within *A. baumannii* AB307– 0294 a form of hexuronic acid, galactosaminuronic acid, has been previously noted (Russo, T.A. *et al.*, 2013). This observation demonstrates the biosynthetic pathways required to generate the underivatized precursor of the 217 Da do exist in *Acinetobacter*. The identification of a shortened glycan composed of chemically unusual moieties demonstrated the importance of experimental analysis of glycan structure, rather than bioinformatic prediction and the convenience of our targeted based approach to rapidly identify glycosylation in the *Acinetobacter* genus.

Glycan Mass	Proposed Glycan Identity
300	GlcNAc3NAcA4OAc
258	GlcNAc3NAcA
314	GlcNAc3NAcA4OAcCH$_3$
228	QuiNAc4NAc / diNAcBac
272	QuiNAc4NBu / BacNAc4NBu
316	NulO
331	NulOCH$_3$
358	NulOAc
176	HexA
187	dHexNAc
217	HexNAcA
245	HexNAcOAc
386	$C_{13}H_{24}N_1O_{10}S_1$
285, 158, 145 and 172	Unknown moieties*

Table 2-3 Carbohydrates identified in the O-linked glycan of Acinetobacter species.

2.4-Discussion

Protein *O*-glycosylation is a common process in bacterial species, and is required for virulence and biofilm formation in *A. baumannii* ATCC 17978. However, the prevalence, diversity, and specific role of the *O*-glycan modification of *A. baumannii* has yet to be determined (Iwashkiw, J.A. *et al.*, 2012a). Lees-Miller *et al.* showed that in the strain ATCC 17978 the building blocks employed for capsular polysaccharide and the *O*-linked glycan are identical and employ the same enzymatic machinery for their synthesis, which is encoded in a single glycan locus (Lees-Miller, R.G. *et al.*, 2013b). Genetic comparison of the capsular loci revealed extensive variability in the predicted capsule structure (Hu, D. *et al.*, 2013b). In this work, we analyzed the glycan structures attached to proteins in the strains most commonly used for molecular studies as well as ten clinical isolates and confirmed the presence of extensive *O*-linked glycan diversity. From this analysis, a diverse array of glycans were observed across all 15 strains examined with a total of 11 unique main glycoforms identified (Tables 2-2). These

observations support the predictions of Hu *et al.* and the presence of extensive glycan diversity in *A. baumannii.* In addition to the prominent structures, alternative *O*-glycans were also identified demonstrating *A. baumannii* is capable of producing multiple glycoforms within a given strain. We observed that the majority of strains produced an array of unique glycans only found within that strain; exceptions to this are the reference strain ATCC 19606 that produced the identical *O*-linked pentasaccharide containing GlcNAc3NAcA4OAc of ATCC 17978 (Figure 2.2A, Table 2-2) and the closely related isolates 1441 C1 and 1441 C3 (Figure 2.2E, Table 2-2). Interestingly, the pentasacccharide of ATCC 17978 and ATCC 19606 has also been identified as the capsule-repeat unit of *A. baumannii* strain SMAL (Fregolino, E. *et al.*, 2011). Although the frequency of the observed GlcNAc3NAcA4OAc containing structure would suggest the commonality of this sugar in *A. baumannii,* both the recent bioinformatics analyses (Hu, D. *et al.*, 2013a; Kenyon, J.J. and Hall, R.M., 2013b) and this work suggest that the pentasacccharide of ATCC 17978 (Iwashkiw, J.A. *et al.*, 2012a; Lees-Miller, R.G. *et al.*, 2013b), ATCC 19606, and SMAL (Fregolino, E. *et al.*, 2011) is just one of the multitude of glycans utilized by *A. baumannii.*

Although the majority of glycans were unique to specific strains, multiple glycans contained common bacterial specific sugars such as in the case of the reference strain SDF and clinical isolates Arg2, 1441 C1, and 1441 C3. Within these strains, multiple glycopeptides were identified decorated with residues matching the mass and fragmentation pattern of the negatively charged sugar, legionaminic or its stereoisomer pseudaminic acid (Figure 2.2B, 2.2D, and 2.2E) (Thibault, P. *et al.*, 2001b). Bioinformatic analysis of the carbohydrate clusters of SDF as well as the unpublished clinical isolates *A. baumannii* 1441 C1 and C3 (Weber, B.S. *et al.*, 2015) supports the presence of the legionaminic acid biosynthesis pathway within these strains (Figures 2.1*C* and S2.4). This finding demonstrates *A. baumannii* is one of the many bacterial species now recognized to utilize legionaminic/pseudaminic acid or their derivatives within protein attached glycans (Thibault, P. *et al.*, 2001b; McNally, D.J. *et al.*, 2007; Twine, S.M. *et al.*, 2008). Within the best characterized of these systems, the *O*-linked glycosylation system of *C. jejuni*, these sialic acid analogs are required for ideal protein function where they are essential for autoagglutination, modulation of the hydrophobicity of the flagellin and dampening of the inflammation response by SigLec-10 binding (Ewing, C.P. *et al.*, 2009; Howard, S.L. *et al.*, 2009; Stephenson, H.N. *et al.*, 2014). Interestingly, the presence of negatively charged sugars

appears to be a common feature for most if not all the *A. baumannii* structures identified (Table 2-2). From this trend, it is tempting to speculate that these negative sugars may be advantages to *A. baumannii* biology and themselves may be important for virulence as seen for other bacterial species where negative surface carbohydrates can provide resistance to complement killing (Lewis, A.L. *et al.*, 2004).

In addition to legionaminic acid, other carbohydrates such as the HexNAcA, HexNAcOAc, and diNAcBac residues, whose identity is assigned based on mass comparison to characterized glycans of bacterial glycosylation systems (Nothaft, H. *et al.*, 2012b), were observed across multiple strains within this study (Table 2-3). Residues with identical masses have been identified as components of glycans utilized within other bacterial protein glycosylation systems (Vik, A. *et al.*, 2009b; Morrison, M.J. and Imperiali, B., 2013; Anonsen, J.H. *et al.*, 2012; Jervis, A.J. *et al.*, 2012) suggesting *A. baumannii* utilizes a similar carbohydrate repertoire as other Gram-negative protein glycosylation systems. Although the identities of these residues cannot be confirmed from the mass alone the convergence of similar residues, irrespective of their stereochemistry, is an observation of significant note. A similar concept of the utilization of a conserved carbohydrate repertoire has been noted within *Neisseria* and *Campylobacter* species where both systems utilize diNAcBac yet the enzymes responsible for the generation of diBacNAc represent two phylogenetically distinct clades (Hartley, M.D. *et al.*, 2011; Morrison, M.J. and Imperiali, B., 2014; Nothaft, H. and Szymanski, C.M., 2013). Widespread use of these unique carbohydrates by multiple bacterial glycosylation systems suggests preference for these sugars in protein modification, although the exact advantages of these residues are unknown. If specific carbohydrates are advantageous for virulence or glycosylation functionality this preference may be exploited by potential antimicrobial therapies and the generation of serological reagents, which may aid in the diagnosis and treatment of *A. baumannii* infections and is currently under investigation within our laboratory (Ciocchini, A.E. *et al.*, 2013; Iwashkiw, J.A. *et al.*, 2012c).

With the exception of AYE, at least one glycan structure could be identified within each of the examined strains, with the presence of multiple alternative glycan forms being a common feature of *A. baumannii* strains. Although bacterial glycan heterogeneity is poorly understood it has been suggested that it could contribute to immune evasion (Borud, B. *et al.*, 2011). Similar to previously observed heterogeneity in the capsule structure of *Acinetobacter* strains

(Hanuszkiewicz A., K.Z., Lindner B., Goldmann T., Vollmer E., Debarry J., Heine H., Holst O. , 2008; Fregolino, E. *et al.*, 2011) glycan diversity appeared to be largely the result of chemical exchange and/or addition of functional groups such as acetyl and methyl groups to the bacterial specific residue observed within each strain, although examples of truncated glycan were also observed (Figures 2.5A–2.5C). These alterations were not uniformly distributed on all carbohydrate residues but appeared to favor the alteration of bacterial specific carbohydrates related to diNAcBac, GlcNAc3NAcA4OAc, and legionaminic/pseudaminic acid. Within these sugars multiple alterations were associated with augmentation of the levels of acetylation (Table 2-2). This observation is of key interest because of the association of acetylation levels in other capsule systems and resistance to complement mediated killing (Lewis, A.L. *et al.*, 2004; Berry, D.S. *et al.*, 2002; Kahler, C.M. *et al.*, 2006). As the capsule is essential in ATCC 17978 for complement resistance (Lees-Miller, R.G. *et al.*, 2013a) variability in capsule structure may alter the levels of resistance, which may influence virulence. In addition to the exchange and addition of chemical functional groups, variations such as the addition or lack of sugars as well as changes in the order of the sugars (Figure 2.5) were also observed across strains. These variations suggest that unlike the archetypical glycosylation system of *C. jejuni*, which only utilizes complete correctly formed glycans, *A. baumannii*, is more promiscuous with glycans utilized for protein modification. This promiscuous nature would be in line with other *O*-linked glycosylation systems such as that of *Neisseria*, which change the *O*-linked glycans because of phase variation of glycosyltransferases involved in the assembly of the glycan to aid in immune evasion (Borud, B. *et al.*, 2010).

In addition to the diversity resulting from the exchange/ alteration of carbohydrates within glycans we also observed heterogeneity in the form of glycan oligomerization (Figure 2.5I and 2.5J, Table 2-2). This finding is in agreement with the previous work of Lees-Miller et al. (Lees-Miller, R.G. *et al.*, 2013b) showing that polymerized capsule subunits can be attached to protein substrates in ATCC 17978, and support the notion that the shared glycan biosynthesis pathway for capsule and protein glycosylation is a general feature of *A. baumannii.* Interestingly the observed diversity within *Acinetobacter* glycans is consistent with the micro-heterogeneity recently noted in the glycosylation systems of other bacteria genera, such as *Campylobacter, Burkholderia,* and *Francisella* (Nothaft, H. *et al.*, 2012a; Whitfield, C. and Trent, M.S., 2014; Szymanski, C.M. *et al.*, 2003; Borud, B. *et al.*, 2011; Shevchenko, A. *et al.*, 2006; Wang, N. *et*

al., 2007; Scott, N.E. *et al.*, 2012b; Lithgow, K.V. *et al.*, 2014b). These observations suggest that most glycosylation systems utilize a range of related glycans with proteins substrates rarely decorated with a homogenous glycan. Although the physiological significance of the glycan micro-heterogeneity is still unknown, the extent of glycan microdiversity seen within *Acinetobacter* is among the highest reported to date.

Within this study glycosylation was detected in all strains examined except the multiple drug resistant strain *A. baumannii* AYE. As previously noted (Hu, D. *et al.*, 2013a; Kenyon, J.J. and Hall, R.M., 2013b), this strain carries a glycan cluster containing three glycosyltransferases and the genes required for the synthesis of diBacNAc, which has been demonstrated to lead to the generation of UDP-diBacNAc in heterogeneous expression systems (Russo, T.A. *et al.*, 2013). Because of the multiple drug resistant nature of AYE targeted glycosylation could not be undertaken leaving only enrichment of glycopeptides by ZIC-HILIC chromatography, which is a variable means to assess glycosylation. We initially reasoned that our inability to identify glycosylation within AYE using ZIC-HILIC enrichment may be the result of technical caveats including; the oligosaccharides may have failed to alter glycopeptides hydrophilicity sufficiently to enable partitioning to the pseudo-water of the ZIC-HILIC stationary phase (Buszewski, B. and Noga, S., 2012); or the resulting glycan may have lacked diagnostics ions used to identify glycosylation within other strains. Conversely it is also possible that the glycosylation machinery in this strain is regulated, and only present under certain growth conditions or functionally inactive. However, the absence of PAS reactive capsular polysaccharide and glycoproteins in this strain, as well as its sensitivity to serum killing provides additional evidence that this strain does not produce surface carbohydrates under the conditions tested. The sensitivity of AYE to serum killing is in contrast to the resistance exhibited by recent clinical isolates (Figures 2.3 and 2.4), suggesting without selective pressure *A. baumannii* may regulate or lose surface carbohydrate expression. The loss of surface carbohydrates in the laboratory has been noted in numerous bacterial species. For example, common *E. coli* K12 strains do not produce O antigen because of the mutation in a rhamnosyltransferase (Liu, D. and Reeves, P.R., 1994) Another example is the human pathogen *Burkholderia cenocepacia* strain J2315 that is extremely sensitive to human serum because of an insertion of IS402 within the glycosyl transferase *wbxE* (*Ortega, X. et al., 2005)*. The requirement of capsular polysaccharide for serum resistance demonstrates the significant biological role for surface carbohydrates in the virulence and infective lifecycle of *A.*

baumannii (Lees-Miller, R.G. *et al.*, 2013b) and the importance for experimental elucidation of both glycans and there phenotypic roles.

Although all strains examined supported our hypothesis of diversity in the *O*-linked glycan, the agreement between the carbohydrate locus and the glycan structure was not always consistent. The large 34 ORF carbohydrate locus of *A. baylyi* ADPI encodes the TDP-rhamnose biosynthetic pathway, which is not necessary for production of the pentasaccharides we detected, that did not contain rhamnose. The locus encodes 10 glycosyltransferases, when only five would be needed for the pentasaccharide (Figure 2.1 and Figure 2.7B–2.7C). The *O*-linked glycan has an unusual sugar composition consisting of monosaccharides that match residues observed in other recently identified *Campylobacter* glycosylation systems as well as the *A. baumannii* capsule glycan of AB307– 0294 (Russo, T.A. *et al.*, 2013; Nothaft, H. *et al.*, 2012c). As the sugars corresponding to the unusual masses are unknown, one possibility is that this strain generates one or multiple residues from a rhamnose based precursor. However, the capsule of the closely related strain *A. baylyi* BD4, which ADP1 is derived from (Vaneechoutte, M. *et al.*, 2006), has been shown to contain L-rhamnose, D-glucose, D-glucuronic acid, and D-mannose (Kaplan, N. *et al.*, 1985). Therefore, it is tempting to speculate that in contrast to *A. baumannii*, ADP1 produces a capsule that is unrelated to the *O*-glycan. Consistent with this, ADP1 was the only strain that did not show evidence of higher oligomer glycan by Western blotting of the A1S_1193 bait (Figure 2.7A). Additionally, the ADPI glycan cluster contains two initiating glycosyltransferase homologs suggesting the potential to produce two unique lipid-linked glycans, one for protein modification and one for capsule (Figure 2.1). Additional work is required to further explore these observations to confirm the segregation of the capsule and *O*-glycan, as it will be interesting to know if and how the two pathways are compartmentalized to avoid possible crosstalk between them.

The ability to assess heterogeneity within this work was because of the use of both targeted and non-targeted technologies for bacterial glycosylation analysis. The ZIC-HILIC approach provides a non-targeted means to assess glycan diversity but requires significant experience in glycopeptide analysis to elucidate sugar structures as well as instrumentation capable of performing multiple fragmentation approaches. To overcome these shortcomings, we developed a method to simplify glycosylation analysis that could be achieved on routine MS instrumentation. This method was developed based on the observation of conservation within

proteins subjected to glycosylation and the structural properties of these proteins within *Acinetobacter* glycosylation. In this approach, a common tagged-glycosylation acceptor protein is introduced as "bait" into the strain of interest. The glycosylated protein is then purified via affinity chromatography, digested and subjected to MS analysis. This approach provides a scalable and optimizable means to produce glycosylated proteins without prior knowledge of the glycan structure, allowing analysis of un-sequenced clinical strains. As the fragmentation pattern of the "bait" is known, the determination of the glycans attached to it is straightforward. This targeted approach was employed here to characterize the sugar composition of seven different *Acinetobacter* strains. We believe that in the future this strategy could be employed to simplify the characterization of other bacterial glycosylation systems. By using both targeted and non-targeted MS approaches we show extensive diversity exists in the *Acinetobacter* glycoproteome; that at least 26 proteins, most of which are unique to *Acinetobacter*, are subjected to *O*-linked glycosylation; and, further demonstrate that the use of a single glycan for both *O*-linked glycosylation and capsule production appears to be a general feature of *A. baumannii*.

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Chapter 3-Investigating the Role of two Abundant Glycoproteins in the Pathogenesis of *A. baumannii*

The work presented in this chapter is my original work. Experiments were designed and performed under the guidance of Dr. Feldman. Creation of the *pglL* clean deletion strain was performed by Dr. J.A. Iwashkiw. Construction of the pEC-*pglL* plasmid for *in trans* complementation of the Δ*pglL* strain was done by Dr. E. Valguarnera. The remaining work presented in this chapter is my original work.

3.1-Introduction:

A. baumannii ATCC 17978 has a general *O*-glycosylation system where an *O*-oligosaccharyltransferase (OTase) PglL transfers a lipid-linked branched pentasaccharide to a serine or threonine of at least 7 glycoproteins (Iwashkiw, J.A. *et al.*, 2012a). This general *O*-glycosylation system is required for biofilm formation; virulence in *Dictyostelium discoideum* and *Galleria mellonella* larvae; as well as, competitive colonization in a murine sepsis model (Iwashkiw, J.A. *et al.*, 2012a). The same pentasaccharide is polymerized and exported to the cell surface as capsular polysaccharide (Lees-Miller, R.G. *et al.*, 2013a). NMR structural analysis determined the capsular polysaccharide and *O*-glycan pentasaccharide to be made of the subunit β-GlcNAc3NAcA4OAc-4-(β-GlcNAc-6-)-α-Gal-6-β-Glc-3-β-GalNAc (Iwashkiw, J.A. *et al.*, 2012a; Lees-Miller, R.G. *et al.*, 2013a). Presence of tri-acetylated glucuronic acid lead to the identification of a carbohydrate biosynthesis locus containing the genes necessary for synthesis of glucuronic acid. This carbohydrate synthesis locus contains one initiating glycosyltransferase further supporting the evidence that protein glycosylation and capsule share the same lipid-linked glycan (Lees-Miller, R.G. *et al.*, 2013a). *O*-glycan variability across *Acinetobacter* is the most diverse reported to-date (Scott, N.E. *et al.*, 2014). Mass spectrometry characterization of the *Acinetobacter O*-glycan repertoire revealed most strains analyzed produced a unique glycan, and that each strain produced multiple glycoforms. Molecular serotyping of the carbohydrate synthesis locus revealed as many as 77 serotypes (Hu, D. *et al.*, 2013). An epidemiological investigation of prevalence and disease severity associated with these serotypes has not been reported.

Some strains of *Acinetobacter* have two *O*-oligosaccharyltransferase (*O*-OTase) genes (Schulz, B.L. *et al.*, 2013; Harding, C.M. *et al.*, 2015a). One of these *O*-OTases is responsible for general protein glycosylation of multiple protein substrates and one of these OTases is responsible for the specific *O*-glycosylation of type IV pilin. In most strains encoding two OTases, the pilin specific OTase is homologous to *Pseudomonas aeruginosa* pilin-specific OTase TfpO/PilO (Harding, C.M. *et al.*, 2015b). In the case of *A. baylyi* ADP1, the pilin specific OTase that glycosylates ComP more similarly resembles PglL the general OTase. Mass spectrometry analysis of the glycopeptides from either general acceptor glycoproteins or pilin demonstrate that the same lipid-linked glycan is transferred to these acceptor proteins by the general OTase or the pilin specific OTase (Harding, C.M. *et al.*, 2015b). In most *Acinetobacter*

strains one glycan is attached to pilin subunits, general *O*-glycosylation glycoproteins and is polymerized as capsular polysaccharide.

Most glycoproteins that have been identified in *Acinetobacter* required glycopeptide enrichment and the sensitivity of mass spectrometry peptide sequencing to be identified (Scott, N.E. *et al.*, 2014; Iwashkiw, J.A. *et al.*, 2012b). Period Acid Schiff staining of total membrane extracts from *A. baumannii* reveal what appears to be one prominent glycoprotein. 2D-DIGE of total membrane extracts from wildtype *A. baumannii* and protein glycosylation deficient *A. baumannii* lead to the identification of two abundant glycoproteins, A1S_3626 and A1S_3744 (Iwashkiw, J.A. *et al.*, 2012c). Despite glycan variability, the proteins modified in *Acinetobacter* are relatively conserved and are glycosylated at low complexity regions enriched in proline, alanine and serine residues. Two proteins that are glycosylated in all strains examined to-date are A1S_3626 and A1S_3744 (Scott, N.E. *et al.*, 2014).

This work focuses on investigating the identity of the PAS reactive glycoprotein band in *A. baumannii.* We demonstrate that abundant glycoproteins A1S_3626 and A1S_3744 are not required for production of the prominent glycoprotein band in *A. baumannii* ATCC 17978. Protein glycosylation is required for full virulence in *G. mellonella* larvae. Glycoprotein, A1S_3744, is not required for pathogenicity in *G. mellonella,* as the percent survival of larvae infected with Δ*A1S_3744* is similar to those infected with wildtype. *A1S_3626* and *A1S_3744* are not required for biofilm formation in *A. baumannii*; however, over expression of either glycoprotein appears to increase adherence to a polystyrene plate.

3.2-Materials and Methods:

3.2.1-Bacterial Strains and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table 3-1. All *Escherichia coli* and *A. baumannii* strains were grown at 37^{o}C and 200 rpm in LB broth/agar (10 g Tryptone, 5 g yeast extract, and 10 g of NaCl per 1 L of dH_2O supplemented with 15 g of agar per liter of broth when needed). Antibiotics were used at the following concentrations unless specified elsewhere: 100 µg/mL ampicillin, 100 µg/mL carbenicillin, 20 µg/mL kanamycin or 5 µg/mL tetracycline. Media was supplemented with 1% arabinose to induce protein expression and 10% sucrose to select against *SacB* when needed.

3.2.2-Generation of Polyclonal Rabbit Anti-glycan sera

1 L of LB was inoculated from an overnight culture of ATCC 17978 and grown over night. Cells were harvested at 10000 x g for 10 minutes and washed three times with PBS, resuspended in 20 ml of mQH2O, and lysed at 35 kpsi using a cell disruptor (Constant System ltd., Kennesaw, GA). Cell lysate was clarified at 10000xg for 20 minutes. Membranes were pelleted by centrifugation at 100000xg for 1 hour. The supernatant was treated with 1µg/mL of DNase and RNase E (Sigma) for 3 hours at 37ºC followed by treatment with 50 µg/mL proteinase K (Sigma) overnight at 60ºC followed by 50 µg/mL pronase E treatment (Sigma) over night at 37ºC before lyophilization. Purified capsule lyophilate was solubilized in mQH2O was sent to SACRI antibody services (University of Calgary, Alberta, Canada) for development of rabbit-derived polyclonal antibodies.

3.2.3-Cloning and Expression of *A. baumannii* Glycoproteins

Primers used to amplify each glycoprotein by PCR are listed in Table 3-2. Briefly Phusion High-fidelity polymerase was used to amplify each open reading frame. The reverse primers were designed to add a C-terminal hexa-histidine tag to each glycoprotein. PCR products were purified, digested with BamH1 and SalI (New England Biolabs) and ligated to pEXT20 at BamH1 and SalI. Ligations were transformed into electrocompetent *E. coli* DH10β and selected on LB supplemented with 100 µg/mL ampicillin. Plasmids were verified by PCR and sequencing. Each gene was subcloned into arabinose inducible *E. coli/ Acinetobacter* shuttle plasmid pEC at HindIII and HincII. *A1S_0556-his* was cloned into pBAVMCS, a constitutive *E.coli/Acinetobacter* shuttle plasmid at BamH1 and SalI. Plasmids were verified by PCR and sequencing. *A1S_3626* was PCR amplified with primes 3626prompvuIfw and 3626rev3flagpvuI (or 3626rev6hispvuI) to include its putative natural promoter and with a C-terminal 3XFlag/hexa-histidine tag. This construct was cloned into pWH1266 at PvuI creating pWH-3626FLAG or pWH-3626His.

3.2.4-Immunoblotting

Strains were for 4 hours in LB broth supplemented with 20 µg/mL kanamycin and 1% arabinose. Whole cell fractions were collected by pelleting by centrifugation and resuspended in 1x Laemmli buffer at 0.01 OD/ul. Samples were boiled at 100ºC for 10 minutes and 0.1 OD was loaded per sample into a 12% SDS-PAGE gel. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Western blot analysis probing with Anti-glycan (polyclonal rabbit derived) (1:2000) and Anti-histidine monoclonal mouse derived (Pierce)

(1:2000) antibodies was performed. Monoclonal mouse derived Anti-FLAG antibody (Sigma) was used at 1:2000. Polyclonal rabbit derived anti-histidine (Pierce) antisera was used at 1:2000. Western blots were probed with IRDye conjugated secondary antibodies and visualized with an Odyssey CLx imaging system (LI-COR Biosciences, Lincoln, NE).

3.2.5-Generation of Unmarked Bacterial Mutants and *in trans* Complementation

An unmarked mutation in *pglL* (Iwashkiw JA, unpublished) was created using the same method as (Lees-Miller, R.G. *et al.*, 2013b) . Briefly, 1000bp of flanking DNA upstream and downstream of *A1S_3176* was amplified from genomic ATCC 17978 DNA. The 3' end of the upstream fragment was designed to be homologous to the 5' end of the downstream region. Equimolar concentrations of the upstream and downstream fragments were stitched together through nested PCR and cloned into *kan-sacB Acinetobacter* suicide vector, pAbKO (Lees-Miller, R.G. *et al.*, 2013b). pAbKO-*pglL* was transformed into electrocompetent ATCC 17978, where single crossover insertions were selected on kanamycin and confirmed by PCR. Colonies were passaged in LB broth for 3 days without antibiotic selection. Double cross-over events were selected for by plating onto LB agar supplemented with 10% sucrose. The unmarked deletion of *pglL* was verified through PCR and sequencing (Iwashkiw JA, unpublished). *pglL* was amplified by PCR and infused into the multiple cloning site of pEC using the In-Fusion HD EcoDry cloning kit (Valguarnera E, unpublished). The pEC-*pglL* construct was verified by sequencing.

Unmarked mutations in *A1S_3626* and *A1S_3744* were done as previously described (Tucker, A.T. *et al.*, 2014). Briefly, a kanamycin resistance cassette was amplified using ~150 bp oligonucleotide primers (3626FRTfwKO and 3626FRTrevKO or 3744FRTrev and 3744FRTfw) (Integrated DNA Technologies) with homology to DNA flanking the target gene and ~20bp of homology to pKD4 FRT site-flanked Kanamycin resistance cassette at the 3' end of the primer. Approximately 1ug of PCR product was transformed into electrocompetent ATCC 17978 carrying the Rec_{Ab} recombinase plasmid pAT04. Mutants were selected on 7.5 μg/mL kanamycin, and confirmed by PCR. FLP recombinase plasmid, pAT03, was transformed into electrocompetent mutants in order to remove the FRT-flanked kanamycin cassette. Clean gene deletions were confirmed through PCR and sequencing analysis.

Construction of *A1S_3626* glycosylation point mutation – Back-to-back PCR amplification of pEC-*A1S_3626-his,* with primers 3626glyptbtobfw and 3626glyptbtobrev, was used to exchange Serine 55 for alanine in *A1S_3626-his*. Primer 3626glyptbtobfw was designed with the codon for serine 55 changed to an alanine. Following PCR amplification with Phusion, the PCR product was purified, DpnI treated to degrade the template pDNA, and ligated to itself. The resulting ligation product was transformed into electrocompetent *E. coli* DH10β. The resulting plasmid, pEC-*A1S_3626S::A-his* was verified by sequencing.

3.2.6-*Galleria mellonella* Infection with *A. baumannii*

The *G. mellonella* infection was performed as previously published (Harding, C.M. *et al.*, 2016; O'Toole, G.A., 2003). Briefly, *A. baumannii* ATCC 17978/pEC, Δ*pglL*/pEC, Δ*A1S_3744*/pEC and Δ*A1S_3744*/pEC-*A1S_3744* were grown overnight in LB broth supplemented with 20 µg/mL kanamycin and 1% arabinose. Overnight cultures were used to inoculate 5 mL cultures at 0.05 OD/mL supplemented with 20 µg/mL kanamycin and 1% arabinose. These cultures were grown to 0.5 OD/ml. 0.5 ODs were pelleted by centrifugation, wash with sterile PBS and resuspended in PBS at 0.5 OD/mL. Larvae were injected with 10 µL of cell suspension equal to $3x10^5$ CFU. Infected larvae were incubated at 37ºC overnight and their survival was monitored over time. Larvae were considered dead if they didn't respond to touch.

3.2.7 -96-well Polystyrene Plate Biofilm Assay – Experiments were performed similarly to (O'Toole, G.A., 2003). Cultures were grown overnight in LB broth supplemented with 20 µg/mL kanamycin and 1% arabinose. Overnight cultures were used to inoculate 200 µL of fresh LB broth at 0.01 OD/mL in a 96-well plate. Plates were incubated in a humidified incubator at 37ºC for 4 hours. Bacterial suspensions were transferred to a sterile 96-well plate and the OD_{600} was measured. Biofilm plates were washed with 250µL of water three times. 250 µL of crystal violet was added to each well and allowed to incubate for 10 minutes. Crystal violet was removed and the wells were washed with 250 µL of water 4 times. The plates were left to dry. 200 µL of 30% acetic acid was added to each well to solubilize the remaining crystal violet. 200 µL of 30% acetic acid was transferred to a sterile 96-well plate and the absorbance at 540 nm was measured. The experiment was performed 3 times each with 6 technical replicates and a representative data set is shown. Biofilm is quantified as A540/OD600.

3.2.8- Purification of A1S_3626 – Wildtype *A. baumannii* ATCC 17978 carrying either pWH-3626Flag or pWH-3626His was grown in 50 mLs of LB broth supplemented with 5 µg/mL

tetracycline overnight. Cells were pelleted at 10 000 x g for 10 minutes. Cells were washed with 30 mM Tris pH8 and resuspended in 15 mLs of 50 mM NaH_2PO_4 300 mM NaCl and 10 mM imidazole pH8. Cells were lysed with a cell disruptor using two rounds at 35 kpsi (Constant System ltd., Kennesaw, GA). Cell lysates were incubated with 0.05% triton X-100, rolling at 4ºC for 1 hour to solubilize lipoproteins (Schnaitman, C.A., 1971). Cell lysates were clarified at 11 000 rpm for 30 minutes. Cell lysate from cells expressing *A1S_3626-his* or *A1S_3626-flag* was passed over a nickel-NTA agarose column (Gold Bio, St. Louis, MO). The load fraction is the total cell lysate. The flow-through was collected as what passed through the column and did not bind the nickel-NTA resin. The column was washed with 20 mL of 50 mM NaH_2PO_4 300 mM NaCl and 20 mM imidazole pH8 and 20 mL of 50 mM NaH_2PO_4 300 mM NaCl and 35 mM imidazole pH8 (Bornhorst JA 2000). Proteins were eluted with 50 mM NaH_2PO_4 300 mM NaCl and 250 mM imidazole pH8. The experimental condition was performed with initially passing cell lysate from *A1S_3626-his* over the Nickel-NTA agarose followed by cell lysate from *A. baumannii* expressing *A1S_3626-flag*. The washing and elution steps were performed as above. Load, flow-through, wash and elution fractions were analyzed by Western blot analysis probing for A1S_3626-FLAG and A1S_3626-His.

Table 3-1-Strains and Plasmids – Glycoprotein Study

pEC-*A1S_1193Hix10X*	Arabinose inducible, Km^r, plasmid used to express *A1S_1193-His* (Inserted at HincII and HindIII) in *Acinetobacter* for glycan fishing (Madsen, J.A. *et al.*, 2013)
pBAVMCS	Km^r pBAV1K-t5-gfp derivative with gfp ORF removed, Constitutive *E. coli/Acinetobacter* shuttle vector (Bryksin, A.V. and Matsumura, I., 2010)
pBAV-*A1S_1193His10X*	Constitutive plasmid, Km^r, used to express *A1S_1193-his* (inserted at BamH1 and SalI) in *Acinetobacter* (This study)
pBAVMCS-*A1S_0556-his*	pBAVMCS carrying *A1S_0556* (inserted at BamH1 and SalI) with a C-terminal hexa-histidine tag (This study)
pEC	Km^r arabinose inducible *E. coli*/*Acinetobacter* shuttle plasmid (Zhang, H. *et al.*, 2007)

pEXT20	Cloning and expression vector, IPTG inducible, Amp^r (Dykxhoorn, D.M. *et al.*, 1996)
pEXT20-*A1S_3626-his*	pEXT20 carrying *A1S_3626* (inserted at BamH1 and SalI) with a C-terminal hexa-histidine tag (This study)
pEXT20-*A1S_3744-his*	pEXT20 carrying *A1S_3744* (inserted at BamH1 and SalI) with a C-terminal hexa-histidine tag (This study)
pEXT20-*A1S_3658-his*	pEXT20 carrying *A1S_3658* (inserted at BamH1 and SalI) with a C-terminal hexa-histidine tag (This study)
pEXT20-*A1S_3580-his*	pEXT20 carrying *A1S_3580* (inserted at BamH1 and SalI) with a C-terminal hexa-hisitidne tag (This study)
pEC-*A1S_3626-his*	pEC carrying *A1S_3626-his* (inserted at HincII and HindIII)
pEC-*A1S_3744-his*	pEC carrying *A1S_3744-his* (inserted at HincII and HindIII)
pEC-*A1S_3658-his*	pEC carrying *A1S_3658-his* (inserted at HincII and HindIII)
pEC-*A1S_3580-his*	pEC carrying *A1S_3580-his* (inserted at HincII and HindIII)
pEC-*pglL*	pEC carrying *A1S_3176* (inserted at HincII)

Strains:

A. baumannii ATCC 17978	ATCC
A. baumannii ATCC 17978 Δ*pglL::kan-sacB*	Insertional mutation through homologous recombination replacing the *pglL* gene with *kan-sacB* (Iwashkiw JA, unpublished)
A. baumannii ATCC 17978 Δ*pglL*	Clean deletion of *pglL* using homologous recombination and sucrose sensitivity of the Kan-SacB (Iwashkiw JA, unpublished)
A. baumannii ATCC 17978 Δ*A1S_3744::FRT*	Clean deletion of *A1S_3744*, where ORF is replaced with an FRT scar
A. baumannii ATCC 17978 *ΔA1S_3626*::FRT	Clean deletion of *A1S_3626*, where ORF is replaced with an FRT scar
pKD4	Kan^r cassette flanked by FRT sites (Datsenko, K.A. and Wanner, B.L., 2000)
pAT03	Amp^r FLP recombinase (Tucker, A.T. *et al.*, 2014)

pAT04 Tc^r Rec_{Ab} system (Tucker, A.T. *et al.*, 2014)

Table3-2 Primer List-*Acinetobacter* Glycoprotein Study

3626FRTfwKO: 5’
GGCTTTTTTAATACGAGAGTTACAACTTTTTAACAATTTTTGATCTTTATTAATCAGT
TACTTTATGTTTATATTTTAGACATCCATAATTTTGGATGCCATGAATTAGAAGGAAA
AACACAATGACATATGAATATCCTCCTTAGTTCCTATTCCG 3’
3626FRTrevKO 5’
TTGCCTTACGTAATAAAGTTGCAGAACGTCAGTCAGTACAAATTGCAATGCGTGATG
AAGGTTTAATTTCATAAGCAATTTTCAAAAGCATTAAAAAAGCCCCTAAGCAATAGG
GGCTTTTTTAATGCTAAAGCTAATTACATATGAATATCCTCCTTAGTTCC 3’
3744FRTrev 5’
CTAAAATGATAGACAGGAGAGCTTTAAAGACAAAAAGTAATAAAATTAAATTTATT
TATATTATGACTATTTATATAAATGCCCAAATTAAAAAAATAATTTGGGCACTCTTG
AACAGAAGCTTACATATGAATATCCTCCTTAGTTCC 3’
3744FRTFw 5’
ACAATAAACAACTGGTTATTCTATCAGCATTTTTAATAAAAAATTAGCTCAATCTGA
CATGATTTTTTCATCGCTATTTACTGTTATAATGCCAATTCCTCAATTAAATGTTGTTT
AGGGCAATGGAGCGATTGTGTAGGCTGGAGC 3’
3626fwBamH1 ATATGGATCCATGAATAAATTACTTGTTGC
3626revSalI
ATATGTCGACTTAGTGGTGGTGGTGGTGGTGGTGTTGAGCATCTTTTTTCACATCAGC
3744revHisSalI 5’
ATATGTCGACTTAATGATGATGATGATGATGGTTCTTAAGTGCTGGTGTTGC 3’
3744FwBamH1 5’ATATGGATCCATGGCAAATAAAAAACTTTTAATCTG 3’
3658revhissalI 5’
ATATGTCGACTTAATGATGATGATGATGATGCGAGTTTTTATTCATGTCATG 3’
3658fwbamh1 5’ ATATGGATCCATGGTAAGAATCTTTTGTGCAGTAAG 3’
3580revhisalI 5’
ATATGTCGACTTAATGATGATGATGATGATGACGAGTTGCAGGAGCAGC 3’
3580fwbamh1 5’ ATATGGATCCATGAAATTAGCTAAAACTTTACTCG 3’
0556HisRevSalI 5’
ATATGTCGACTTAATGATGATGATGATGATGGCTGGCACTTTTTACGGCAGG 3’
0556FwBamHI 5’ ATATGGATCCATGTTAACTTCTAAGGCTTCGCTACATTTAACG 3’
3626rev3flagpvuI 5’
ATATCGATCGTTACTTGTCATCGTCATCCTTGTAATCGATATCATGATCTTTATAATC
ACCGTCATGGTCTTTGTAGTCGTGTTGAGCATCTTTTTTCACATCAGC 3’
3626prompvuIfw 5’
ATATCGATCGTTTATTAATCAGTTACTTTATGTTTATATTTTAGACATCC 3’
3626rev6hispvuI
5’ATATCGATCGTTAGTGGTGGTGGTGGTGGTGGTGTTGAGCATCTTTTTTCACATCA
GC3’
3626glyptbtbrev 5’ 5Phos/CACCAGCAGATTTGATATCTGCTGTTGC 3’
3626glyptbtobfw 5’ 5Phos/ATCAAGCTGCTGCTGATATTGCAACTGCA 3’

RESULTS:

3.3.1-Glycoproteins A1S_3626-His Partially Co-localize with the 30kDa Anti-glycan Reactive Glycoprotein

Previous work identified 7 glycoproteins in ATCC 17978 of various molecular weights (Iwashkiw, J.A. *et al.*, 2012c). 2D-DIGE comparison of wildtype total membrane proteins to the protein glycosylation deficient strain lead to the identification of two glycoproteins, A1S_3626 and A1S_3744, in ATCC 17978 (Iwashkiw, J.A. *et al.*, 2012c). A1S_3626 is predicted to have a molecular weight of 11 kDa, but migrated at 30 kDa in the 2D-DIGE analysis. We hypothesized A1S_3626 to be the anti-glycan reactive glycoprotein band based on its SDS-PAGE migratory pattern and its high abundance evidenced by the ability to detect it in the 2D-DIGE analysis. We predicted the A1S_3626-His signal and anti-glycan glycoprotein signal would co-localize. To analyze this 6 of the 7 previously identified ATCC 17978 glycoproteins were cloned and expressed with C-terminal hexa-histidine tags (Figure 3.1). The seventh glycoprotein gene, *A1S_2371* could not be expressed *in trans*. A1S_2371 is predicted to be peptidoglycan modifying enzyme. Over-expression of this protein may be lethal to *A. baumannii,* explaining our inability to over-express it. A1S_2371 is predicted to be 70kDa in size, and is therefore unlike to be the prominent glycoprotein. The anti-glycan sera was generated against purified capsular polysaccharide from *A. baumannii* ATCC 17978 that was contaminated with some lipooligosaccharide (LOS). The serum recognizes the glycan attached to a 30 kDa glycoprotein, high molecular weight capsular polysaccharide as well as low molecular weight LOS. The antibody against capsular polysaccharide recognizes the modification of glycoproteins because the same glycan that is attached to glycoproteins by PglL is also polymerizes into capsular polysaccharide (Lees-Miller, R.G. *et al.*, 2013).

Glycoprotein constructs were expressed in wildtype and Δ*pglL* ATCC 17978. As expected the 30 kDa anti-glycan reactive band was absent from all Δ*pglL* samples (Figure 3.1). Western blot analysis on whole cell extracts were probed with anti-glycan and anti-histidine antibodies. Expression of A1S_0556-His and A1S_1193-His was detected in all cell expressing these constructs and revealed that both glycoproteins migrate at higher molecular weight than the 30 kDa anti-glycan band (Figure 3.1). Expression of A1S_3580-His and A1S_3658-His was detected in all cells expressing these constructs and revealed that both glycoproteins migrate at lower molecular weights than the 30 kDa anti-glycan band. A1S_3626-His and A1S_3744-His signals

indicate these proteins are being expressed. A1S_3744 migrates slightly above the 30 kDa glycoprotein signal. The anti-glycan serum does not recognize the histidine tagged A1S_3744, therefore A1S_3744 is likely not the main glycoprotein. A1S_3626-His is partly recognized by the anti-glycan sera, leading us to hypothesize that A1S_3626 is the main glycoprotein (Figure 3.1).. A1S_0556, A1S_3580, A1S_3658 and A1S_1193 likely do not contribute to this 30 kDa antiglycan band due to the differential migration of these bands and the 30 kDa protein and the inability of the glycan sera to recognize these proteins.

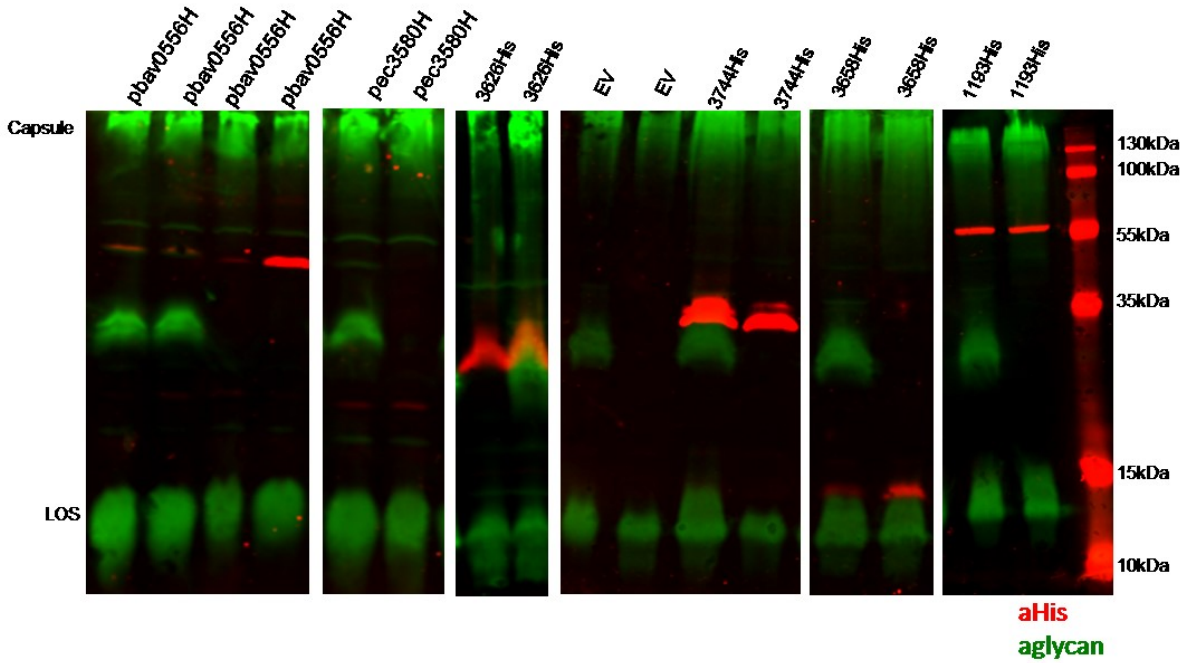

Figure 3.1-Glycoproteins A1S_3626-His Partly Co-localize with the 30kDa Anti-glycan Glycoprotein Signal.

Western blot analysis probing with anti-glycan and anti-histidine sera to investigate the SDS-PAGE migration of ATCC 17978 glycoproteins. Whole cell samples from ATCC 17978 and 17978Δ*pglL* expressing pEC empty vector, pBAV-*A1S_0556-his* pEC-*A1S_3580-his,* pEC-*A1S_3626-his*, pEC-*A1S_3744-his,* pEC-*A1S_3658-his* or pEC-*A1S_1193-his* probing with anti-glycan and anti-histidine sera. Red signals correspond to the histidine tagged glycoprotein and green signal corresponds to the glycan signal including capsular polysaccharide, lipooligosaccharide and a 30kDa glycoprotein. The 30kDa glycoprotein glycan reactive band is absent from all 17978 Δ*pglL* cells. There is some signal co-localization between anti-glycan and anti-His in cells expressing pEC-*A1S_3626-his.* All other histidine tagged glycoproteins migrate at a significant distance from the anti-glycan glycoprotein band.

3.3.2-Cells devoid of *A1S_3744* or *A1S_3626* Produce the 30kDa Anti-glycan Reactive Glycoprotein band.

A1S_3626 and A1S_3744 were the only glycoproteins identifiable by differential 2D gel electrophoresis when comparing the electrophoretic mobility of total membrane proteins from wildtype and protein glycosylation deficient, Δ*pglL*, *A. baumannii* ATCC 17978 (Iwashkiw, J.A. *et al.*, 2012c). A1S_3626 and A1S_3744 are glycosylated in several *Acinetobacter* species and are well conserved across *Acinetobacter* (Scott, N.E. *et al.*, 2014). Due to the abundance of A1S_3744 and A1S_3626 and their SDS-PAGE migratory patterns we hypothesized that either *A1S_3626* or *A1S_3744* would be required to produce the 30 kDa anti-glycan reactive glycoprotein band (Figure 3.1). To test our hypothesis, I deleted the *A1S_3744* gene or the *A1S_3626* gene and probed for production of the 30 kDa anti-glycan reactive band. The 30 kDa anti-glycan reactive band is absent from the Δ*pglL* control and returns in the *pglL*+ complemented strain, indicating this 30 kDa band is *pglL* dependent (Figure 3.2). Cell lysates from wildtype, Δ*A1S_3744* and Δ*A1S_3626* all produce the 30 kDa glycoprotein band, indicating *A1S_3744* and *A1S_3626* are not essential for production of the 30 kDa glycoprotein (Figure 3.2).

To complement the *A1S_3744* or *A1S_3626* mutations, *A1S_3744-his, A1S_3626S*$_{55}$*::A-his* or *A1S_3626-his* were expressed *in trans.* Serine$_{55}$ was previously identified as *O*-glycosylation site of A1S_3626 (Iwashkiw, J.A. *et al.*, 2012a; Scott, N.E. *et al.*, 2014). We hypothesized that by mutating Serine$_{55}$ to Alanine, A1S_3626 will no longer be glycosylated. Expression of A1S_3744-His was detected in strains carrying pEC-*A1S_3744-his* (Figure 3.2). A1S_3744-His is present in two forms, where the higher molecular weight band is indicative of modification, suggesting this band represents glycosylated A1S_3744-His (Scott, N.E. *et al.*, 2014). Expression of A1S_3626-His was detected in strains carrying pEC-*A1S_3626S*$_{55}$*::A-his* or pEC-*A1S_3626-his*. A1S_3626-HisS::A is present in one form, where as there are two forms of A1S_3626-His (Figure 3.2). The shift in electrophoretic mobility indicates the higher molecular weight form is likely modified. Comparison of the mobility of A1S_3626-His and A1S_3626-HisS::A, suggests the lower molecular weight form is unglycosylated and the higher molecular weight form is glycosylated A1S_3626-His (Figure 3.2).

The identity of the 30kDa anti-glycan reactive band is unknown. However, we can conclude that *A1S_3626* and *A1S_3744* are not essential for its production.

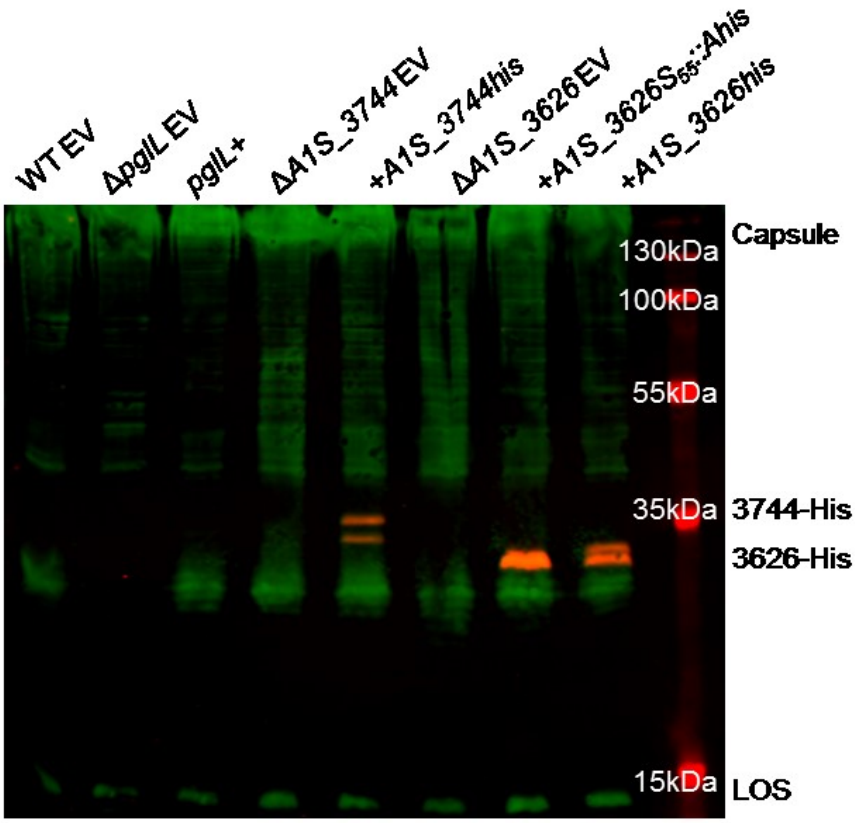

Figure 3.2-Cells Devoid of *A1S_3744* or *A1S_3626* Produce the 30kDa Anti-glycan Reactive Glycoprotein band.

Western blot analysis was performed on whole cell extracts from ATCC 17978 WT, Δ*pglL*, Δ*A1S_3744*, *A1S_3744his+, ΔA1S_3626*, *A1S_3626his+S::A,* or *A1S_3626his+* probing with anti-glycan and anti-histidine sera. Cells expressing A1S_3744-His, A1S_3626-His or A1S_3626S::A-His had anti-histidine reactive bands depicted in red. A1S_3626-His produces two bands, the lower molecular weight band is unglycosylated A1S_3626 which is also detected in cells expressing *A1S_3626S::A*-his where the glycosylation site Serine$_{55}$ has been mutated to Alanine. The higher molecular weight band is glycosylated A1S_3626. A1S_3744-His is detectable in the low molecular weight unglycosylated and the higher molecular weight glycosylated forms. The 30 kDa anti-glycan reactive glycoprotein band is detected in all samples except the Δ*pglL* strain.

3.3.3-Over-expression of Glycoprotein A1S_3626 or A1S_3744 Increases Biofilm Formation in *A. baumannii* ATCC 17978.

Protein glycosylation is required for biofilm formation in several bacterial species (Iwashkiw, J.A. *et al.*, 2013). This is often due to the modification of an important cell-surface exposed adhesion proteins, such as AIDA-I, Ag43 and TibA of *E. coli* (Charbonneau, M.E. *et al.*, 2007; Knudsen, S.K. *et al.*, 2008; Klemm, P. *et al.*, 2004; Diderichsen, B., 1980; Sherlock, O. *et al.*, 2006; Sherlock, O. *et al.*, 2005) . *Ralstonia solanacearum* requires its general PglL dependent *O*-glycosylation system to form biofilms (Elhenawy, W. *et al.*, 2016). It was previously reported that protein glycosylation is required for biofilm formation in *A. baumannii* ATCC 17978 (Iwashkiw, J.A. *et al.*, 2012c). We hypothesized that this protein glycosylation dependent phenotype is likely due to one of these prominent glycoproteins. We set out to test our hypothesis by quantifying biofilm formation using a traditional 96-well polystyrene plate crystal-violet adherence assay (O'Toole, G.A., 2003). In this assay, we compared wildtype *A. baumannii*, Δ*pglL*, Δ*A1S_3636*, Δ*A1S_3744* and the respective complemented strains for their biofilm formation capabilities (Figure 3.4). For this assay a clean deletion of *pglL* was used, rather than the insertional mutation that was originally published on (Iwashkiw, J.A. *et al.*, 2012b). The insertional mutant has a slower growth rate than wildtype, likely contributing to the strong biofilm defect that was observed (Figure 3.3)

The *pglL* mutant had a slight reduction in biofilm relative to wildtype. This is in stark contrast to the strong biofilm defect observed from the insertional mutation (Iwashkiw, J.A. *et al.*, 2012b). Deletion of either *A1S_3626* or *A1S_3744* did not impair biofilm formation (Figure 3.3). All mutations were complemented *in trans* using medium-copy plasmid pEC. Over-expression of *pglL*(pglL+) resulted in a slight increase in biofilm formation compared to wildtype (Figure 3.3). Cells over-expressing either *A1S_3626-his*(3626+) or *A1S_3626S::A-his* (3626S::A+) had increased biofilm formation relative to wildtype (Figure 3.4). Over-expression of *A1S_3744-his* (3744+) caused an extreme increase in biofilm production relative to wildtype (Figure 3.4). The impact of the over-expression of these glycoproteins, suggests that they may have a moderate impact on biofilm formation; however, neither *A1S_3626* or *A1S_3744* are required for biofilm formation (Figure 3.4)

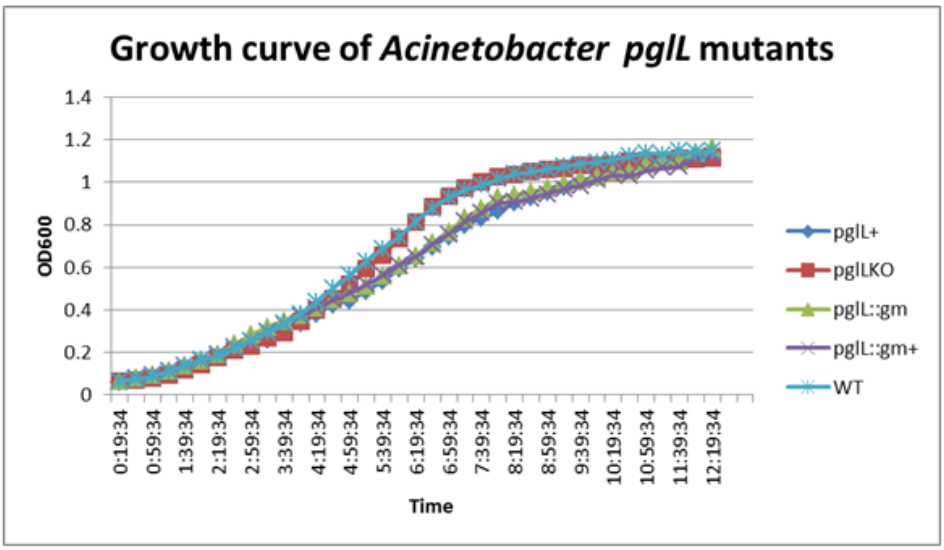

Figure 3.3 – Growth Curve of *A. baumannii* Wildtype, Δ*pglL* and Δ*pglL*::gm Strains

Cultures were grown in LB broth in a 96-well plate for 8 hours. Cultures were inoculated at 0.05 OD/mL and grown at 37ºC for 8 hours. Optical density readings were taken every 30 minutes. The clean *pglL* mutant strain grows at an identical rate to wildtype *A. baumannii* ATCC 17978. The *pglL* insertional mutant used in the original study by Iwashkiw, JA *et al.*, where *pglL* has been replaced by a gentamycin cassette, grows at a slower rate to the wildtype strain.

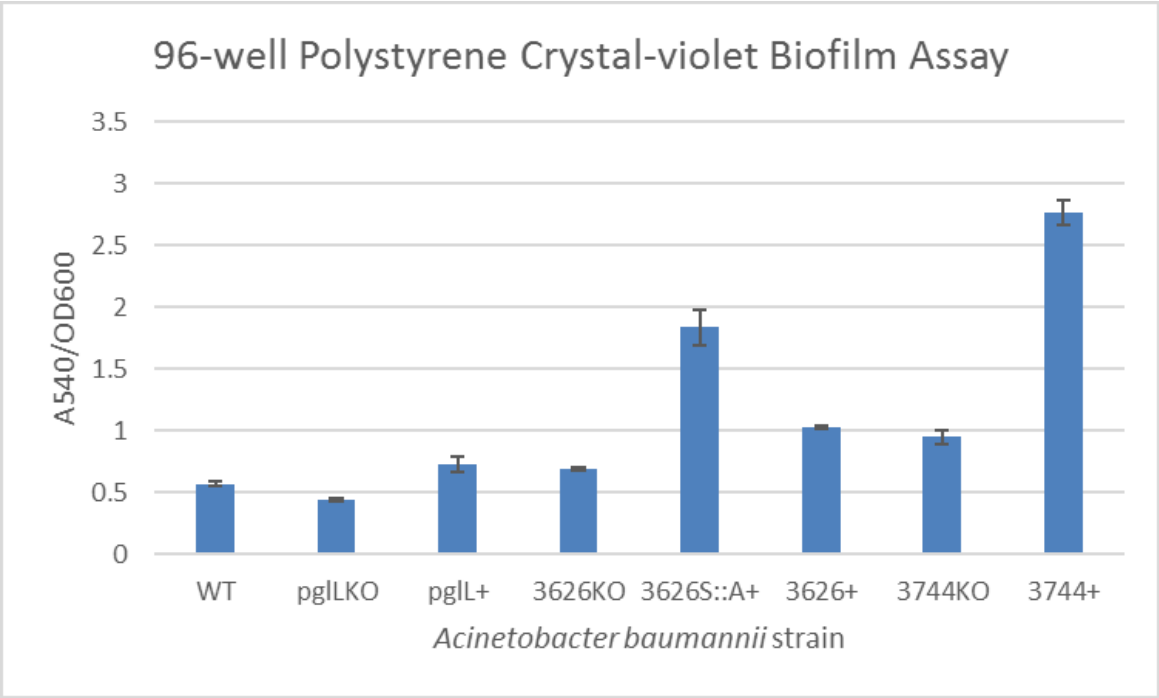

Figure 3.4-*A1S_3744* and *A1S_3626* are not Required for Biofilm Formation but their Over-expression Increases *A. baumannii* Biofilm Formation

Cultures were grown overnight in LB broth supplemented with 20 µg/mL kanamycin and 1% arabinose. Overnight cultures were used to inoculate 200 µL of fresh LB broth at 0.01 OD/ml in a 96-well plate. Plates were incubated in a humidified incubator at 37ºC for 4 hours. Bacterial suspensions were transferred to a sterile 96-well plate and the OD_{600} was measured. Biofilm plates were washed with 250 µL of water three times. 250 µL of crystal violet was added to each well and allowed to incubate for 10 minutes. Crystal violet was removed and the wells were washed with 250 µL of water 4 times. The plates were left to dry. 200 µL of 30% acetic acid was added to each well to solubilize the remaining crystal violet. 200 µL of 30% acetic acid was transferred to a sterile 96-well plate and the absorbance at 540 nm was measured. The experiment was performed 3 times each with 6 technical replicates and a representative data set is shown. Biofilm is quantified as A540/OD600. Deletion of *pglL* lead to a slight decrease in biofilm formation relative to wildtype. Deletion of either *A1S_3626* or *A1S_3744* did not decrease biofilm formation. Over expression of either glycoprotein, A1S_3626 or A1S_3744 resulted in increased biofilm relative to wildtype.

3.3.4-Glycoprotein A1S_3744 has no role in virulence against *Galleria mellonella* larvae.

The original characterization of PglL dependent protein glycosylation in *A. baumannii,* demonstrated a role for protein glycosylation in virulence against *G. mellonella* (Iwashkiw, J.A. *et al.*, 2012b). As discussed above, the *pglL* insertional mutant, used in that study, has a slower growth rate than wildtype, likely accentuating the virulence phenotype observed. We hypothesized that one of the abundant glycoproteins in *A. baumannii* ATCC 17978 may be responsible for this virulence phenotype observed. To test this hypothesis, *G. mellonella* larvae were infected with either 17978/pEC, the Δ*pglL/*pEC mutant, Δ*A1S_3744/*pEC or the complemented Δ*A1S_3744/*pEC-*A1S_3744-his* strain at an inoculum of ~3x10^5 CFU. Infected larvae were incubated at 37^{o}C and monitored for viability over time. Viability of the larvae was determined by melanin accumulation and mobility. Larvae infected with Δ*pglL* had 76% survival after 18 hours (Figure 3.5). Larvae infected with wildtype, Δ*A1S_3744* or the complemented strain had 60%, 58% and 54% survival respectively (Figure 3.5). There was no statistically significant difference in larvae survival over time. We conclude that *A1S_3744* has no role in virulence against *G. mellonella* larvae (Figure 3.5). It is yet to be investigated whether; A1S_3626 or any other specific glycoprotein has a role in virulence.

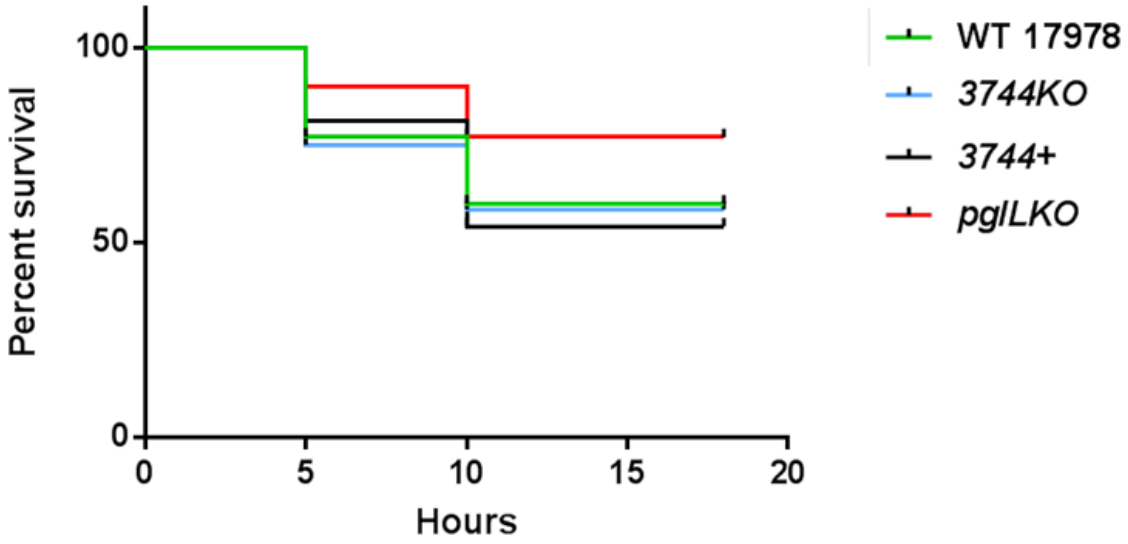

Figure 3.5-*A1S_3744* is not Required for Virulence Against *Galleria mellonella*Larvae

G. mellonella larvae were injected with 10 µL of either the ATCC 17978/pEC, the Δ*pglL*/pEC mutant, Δ*A1S_3744*/pEC or the complemented Δ*A1S_3744*/pEC-*A1S_3744-his* strain at an inoculum of ~3x10^5 CFU. Infected larvae were incubated at 37ºC and monitored for viability over time. Viability of the larvae was determined by melanin accumulation and mobility. Larvae infected with Δ*pglL* had 76% survival after 18 hours. Larvae infected with wildtype, Δ*A1S_3744* or the complemented strain had 60%, 58% and 54% survival respectively. Survival curves were determined to be statistically insignificant using the Mantel-Cox test (P = 0.1912).

3.3.5-Detection of A1S_3626-A1S_3626 Homo-interaction by Co-purification

When purifying large quantities of A1S_3626-His we observed a laddering on SDS-PAGE coomassie brilliant blue stained gels (Figure 3.6) (Iwashkiw, J.A., unpublished). We hypothesized that A1S_3626 may form a homo-polymer. To test our hypothesis, we cloned *A1S_3626* with either a C-terminal histidine or FLAG tag. Attempts at co-expressing pEC-*A1S_3626-his* and pWH-*A1S_3626-flag* were unsuccessful (Data not shown). pWH-*A1S_3626-flag* or pWH-*A1S_3626-his* were expressed *in trans* in wildtype *A. baumannii* ATCC 17978. To assess protein-protein interactions we utilized a pulldown approach, where nickel-NTA affinity chromatography was used to purify A1S_3626-His. Cell lysate from *A. baumannii* expressing *A1S_3626-his* was first passed over the nickel-NTA resin, followed by lysate from *A. baumannii* expressing *A1S_3626-flag*. Control experiments were done where cell lysate from *A. baumannii* expressing

A1S_3626-flag was passed over nickel-NTA resin without exposure to A1S_3626-His as well as purification of A1S_3626-His alone. Expression and purification of A1S_3626-His and A1S_3626-FLAG was assessed by Western blot analysis (Figure 3.7A-B). A1S_3626-His and A1S_3626-FLAG expression was detected in the load fraction corresponding to cells expressing *A1S_3626-his* or *A1S_3626-flag* respectively. A1S_3626-His is enriched for in the elution fraction of pWH-*A1S_3626-his* (Figure 3.7B). A1S_3626-His or A1S_3626-FLAG was detected in the final wash fraction of pWH-*A1S_3626-his* or pWH-*A1S_3626-flag* purifications respectively, indicating the washing step with 50 mM imidazole was not stringent enough to detect specific interactions (Figure 3.7A). A1S_3626-His and A1S_3626-FLAG were enriched in the elution fraction of the co-purification experiment (Figure 3.7A). The enrichment for both forms of A1S_3626 suggests this protein forms a homo-polymer. However, without demonstrating nickel-NTA affinity purification of A1S_3626-FLAG is dependent upon the presence of A1S_3626-His, we cannot draw conclusions about this co-purification.

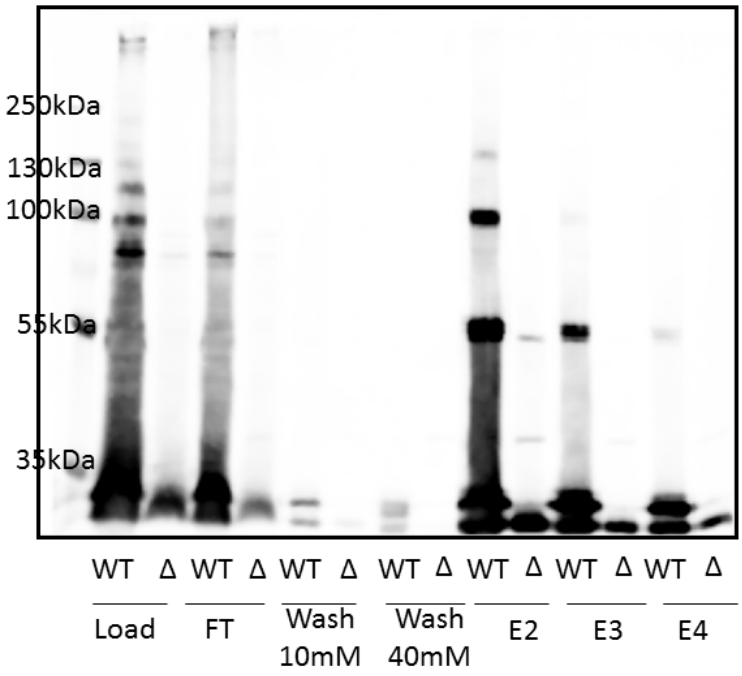

Figure 3.6-A1S_3626-His Purification from *Escherichia coli*

A1S_3626 purified from *E. coli* by nickel-affinity chromatography demonstrates a laddering phenotype when resolved by SDS-PAGE analysis. FT indicates flow through, E indicates elutions of the purified protein.

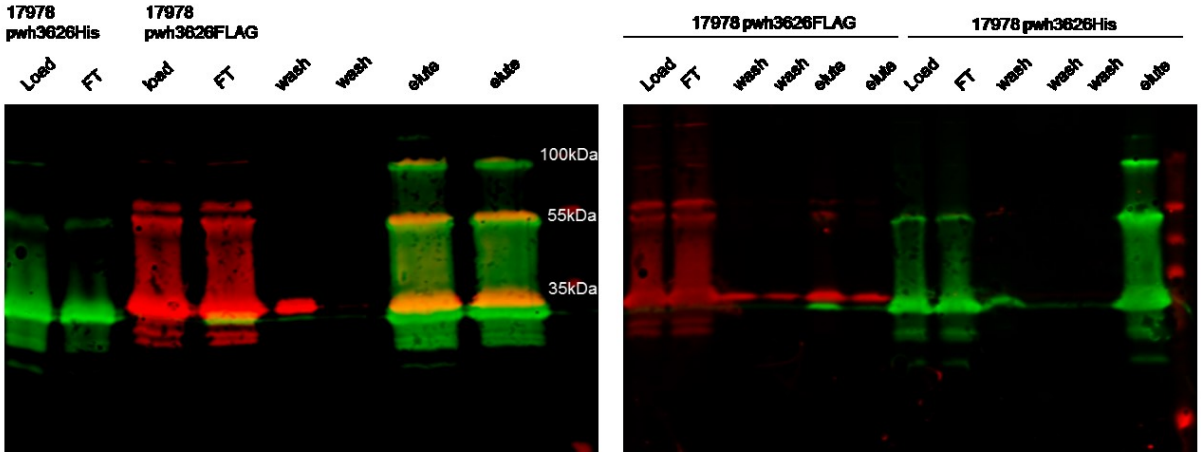

Figure 3.7-A1S_3626-FLAG Co-purifies with A1S_3626-His

(A) Cell lysate from wild type ATCC 17978 expressing pWH-*A1S_3626-his* and ATCC 17978 expressing pWH-*A1S_3626-flag* were separately loaded onto the same nickel-NTA affinity chromatography column for purification of A1S_3626-His. A1S_3626-His is detectable by Western blot analysis in the load, flow-through(FT) and elution fractions. A1S_3626-FLAG is detectable by western blot in the load, flow-through and elution fractions. A1S_3626-His and A1S_3626-FLAG are not detectable in the final wash fraction. (B) In the controls experiment cell lysate from ATCC 17978 expressing pWH-*A1S_3626-flag* or pWH-*A1S_3626-His* were loaded separately onto a nickel-NTA affinity chromatography columns and the purification protocol was followed. A1S_3626-FLAG is detectable by Western blot analysis in the load, flow-through, washes and elution fractions. A1S_3626-FLAG is not enriched in the elution fraction. A1S_3626-His is detectable by Western blot analysis in the load, flow-through, washes and elution fractions. A1S_3626-His is enriched in the elution fraction.

3.4-Discussion:

General *O*-glycosylation is reportedly important for biofilm formation and virulence (Iwashkiw, J.A. *et al.*, 2012b). This system glycosylates at least 7 proteins in *A. baumannii* ATCC 17978 (Iwashkiw, J.A. *et al.*, 2012b). Five of these glycoproteins required glycopeptide enrichment and sensitive mass spectrometry techniques in order to be detected. Two glycoproteins, A1S_3626 and A1S_3744, were identified by macroscopic techniques. The high abundance and extent of glycosylation allowed for identification of these two glycoproteins by 2D-DIGE analysis (Iwashkiw, J.A. *et al.*, 2012b). The acidic nature of the pentasaccharide modifying these proteins

in wildtype *Acinetobacter* as well as the increase in mass upon modification, allowed efficient separation of unglycosyalted and glycosylated A1S_3626 or A1S_3744 (Iwashkiw, J.A. *et al.*, 2012b).

Western blot analysis of whole cell *A. baumannii* with anti-glycan serum revealed a similar staining pattern to PAS stained total membrane extracts, including the ~30 kDa glycoprotein. The 30 kDa band is not detectable in a protein glycosylation deficient strain, indicating this proteinaceous material is a glycoprotein. *In trans* expression of Histidine-tagged glycoproteins revealed A1S_3626 and A1S_3744 migrate at a similar size to the 30 kDa promient glycoprotein. All other tested glycoproteins migrated faster or slower than the 30 kDa glycoprotein. The migratory pattern and the abundance of A1S_3626 and A1S_3744 lead to the hypothesis that either *A1S_3626* or *A1S_3744* may consititute the 30 kDa glycoprotein. *A. baumannii* devoid of either *A1S_3626* or *A1S_3744* still produce the 30 kDa glycoprotein band. A1S_3626 and A1S_3744 are not alone responsible for production of this prominent glycoprotein band. Attempts at *in trans* expression of glycoprotein, *A1S_2371* were unsuccessful. This protein is predicted to be peptidoglycan modifying enzyme. Over-expression of this protein may be lethal to *A. baumannii,* explaining our inability to over-express it. A1S_2371 is predicted to be 70 kDa in size, and is therefore unlike to be the prominent glycoprotein. This prominent glycoprotein may not be amendable to ZIC-HILIC enrichment and Mass spectrometry peptide sequencing, and therefore was not identified in our intial studies. The identity of this 30kDa glycoprotein is yet to be determined.

Biofilm formation contributes to host colonization and persistence in the hospital environment (Gaddy, J.A. and Actis, L.A., 2009). *A1S_3626* and *A1S_3744* are both dispensible for biofilm formation in *A. baumannii* ATCC 17978. Insertional mutatgenesis of *pglL* resulted in a strong biofilm defect relative to wildtype *A. baumannii* ATCC 17978. A clean deletion of *pglL* has a slight biofilm defect, suggesting the antibiotic resistance cassette had an impact on biofilm formation. *In trans* complementation of the Δ*A1S_3626* or Δ*A1S_3744* mutations resulted in increased crystal-violet adherence. Over-production of these proteins, resulting in increased biofilm formation, suggests that they may be involved in adherence to polystyrene or cell-cell adhesion. The strains devoid of these glycoproteins do not have biofilm defects, therefore these proteins are dispensible for biofilm formation. Genetic complementation through over-expression complicates understanding differences in bacterial physiology. If these glycoproteins

interact with a protein that is key in biofilm formation, it is possible that they themselves would be dispensible in biofilm formation, but that their over expression would help stabilize the biofilm leading to a higher crystal-violet retention. Alternatively, performing this strain comparison using flow-cell chamber slides may give a more physiological experiment than the crystal violet retension study.

Larvae infected with *A. baumannii* containing a clean deletion of *pglL* had about 15% increase in survival relative to wildtype, indicating protein glycosylation impacts virulence in *G. mellonella* larvae. *A1S_3744* has no impact on virulence in a *G. mellonella* larvae infection model. The percent survival of laevae infected with wildtype *A. baumannii* and Δ*A1S_3744* were similar. The general protein glycosylation system of ATCC 17978 modifies at least 6 other proteins. One of these other glycoproteins may have a strong impact on virulence in *G. mellonella*. Alternatively, the elusive prominent 30kDa glycoprotein may be responsible for this phenotype.

A1S_3626 appears to self-interact. The results are not conclusive, however affinity purification of A1S_3626-His results in enrichment for A1S_3626-FLAG. This data is supported by a recent publication mapping protein-protein interactions in *A. baumannii* (Wu, X. *et al.*, 2016). *In vivo* crosslinking was performed linking near by lysine residues. Cross-linked proteins were identified by Mass Spectrometry peptide sequencing. The A1S_3626 ortholog, was found cross-linked to itself, the A1S_1193 ortholog and the A1S_3744 ortholog (Wu, X. *et al.*, 2016). A1S_1193, A1S_3626 and A1S_3744 have all been identified as glycoproteins in several *A. baumannii* strains (Scott, N.E., *et al.*, 2014). These glycoproteins do not have any homologs outside of *Acinetobacter*, therefore their biological functions remain elusive. This crosslinking data may prompt some testable hypotheses that may lead to the discovery of their biological functions. This data lead us to the hypothesis that the prominent glycoprotein band visible by PAS staining and anti-glycan immunoblotting may consist of multiple glycoproteins. Immunoprecipitation experiments to confirm this cross-linking data will be the focus of future work. Additionally, creation of clean deletions in multiple glycoprotein genes will be performed to analyze whether this prominent glycoprotein band is the result of a combination of A1S_1193, A1S_3626 and A1S_3744. Perhaps single deletions were not enough to detect a difference in this glycoprotein smear despite the absence of a protein that may contribute to it.

The cross-linking study also revealed an interesting possible interaction between A1S_3626 and both DsbA and DsbC (Wu, X. *et al.*, 2016). In the future immunoprecipitation

studies, will be performed to confirm these protein-protein interactions. DsbA and DsbC are involved in formation of disulfide bonds, which is important for protein folding and stability of membrane and secreted proteins (Heras, B. *et al.*, 2007; Ito, K. and Inaba, K., 2008). A1S_3626 contains one cysteine residue; it is possible that DsbA catalyzes disulfide bond formation between A1S_3626 subunits forming a polymer. Disulfide bond proteins play an important role in virulence in a number of bacterial pathogens (Heras, B. *et al.*, 2009). Biofilm formation is important for *Acinetobacter* colonization, and DsbA play an important role in biofilm formation (Gaddy, J.A. and Actis, L.A., 2009; Chow, J.Y. *et al.*, 2014). Additionally, the hydrogen peroxide resistance of wildtype and Δ*A1S_3626* will be compared to acertain whether A1S_3626, through an interaction with DsbA and/or DsbC, impacts hydrogen peroxide resistance.

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Chapter 4-Medically Relevant *Acinetobacter* Species Require a Type II Secretion System and Specific Membrane-associated Chaperones for the Export of Multiple Substrates and full Virulence:

The work presented in this chapter was a collaborative project investigating type II secretion in *Acinetobacter*. I demonstrated type II secretion systems appear to be active in numerous species of *Acinetobacter* by expressing type II substrate *lipH-His* and monitoring its expression and secretion through Western blot analysis. C.M. Harding performed the experiments characterizing the type II secretion system of *A. nosocomialis* except those investigating the role of type II secretion in pathogenicity. I performed the *A. nosocomialis Galleria mellonella* larvae infection, comparing survival of larvae infected with wildtype, Δ*gspD*, or the complemented strain over time. L.D. Palmer and E.P. Skaar performed the murine pulmonary infection with *A. nosocomialis*. C.M. Harding performed the bioinformatics analysis identifying putative membrane bound type II chaperones.

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4.1-Introduction

Acinetobacter baumannii, *A. nosocomialis*, and *A. pittii* have recently emerged as opportunistic human pathogens capable of causing severe human disease; however, the molecular mechanisms employed by *Acinetobacter* to cause disease remain poorly understood.

Many pathogenic members of the genus *Acinetobacter* contain genes predicted to encode proteins required for the biogenesis of a type II secretion system (T2SS), which have been shown to mediate virulence in many Gram-negative organisms. Here we demonstrate that *Acinetobacter nosocomialis* strain M2 produces a functional T2SS, which is required for full virulence in both the *Galleria mellonella* and murine pulmonary infection models. Importantly, this is the first *bona fide* secretion system shown to be required for virulence in *Acinetobacter*. Using bioinformatics, proteomics, and mutational analyses, we show that *Acinetobacter* employs its T2SS to export multiple substrates, including the lipases LipA and LipH as well as the protease CpaA. Furthermore, the *Acinetobacter* T2SS, which is found scattered amongst five distinct loci, does not contain a dedicated pseudopilin peptidase, but instead relies on the type IV prepilin peptidase, reinforcing the common ancestry of these two systems. Lastly, two of the three secreted proteins characterized in this study require specific chaperones for secretion. These chaperones contain an N-terminal transmembrane domain, are encoded adjacently to their cognate effector, and their disruption abolishes type II secretion of their cognate effector. Bioinformatic analysis identified putative chaperones located adjacent to multiple previously known type II effectors from several Gram-negative bacteria, which suggests that T2SS chaperones constitute a separate class of membrane-associated chaperones mediating type II secretion.

Members of the genus *Acinetobacter* are regarded as opportunistic human pathogens of increasing relevance worldwide due in part to the rapid emergence of multiply-drug resistant strains (Dijkshoorn, L. *et al.*, 2007). In fact, the Center for Disease Control and Prevention has recently categorized multi-drug resistant *Acinetobacter* at the serious hazard level, prompting sustained research and action to further prevent its dissemination. Specifically, *A. baumannii*, *A. pittii*, and *A. nosocomialis* of the *Acinetobacter calcoaceticus-baumannii* (*Acb*) complex have become the most medically relevant members of the genus as they are most frequently isolated from health care facilities as well as human tissues (Sahl, J.W. *et al.*, 2013). Although *A. baumannii* is thought to be the most prevalent and virulent member of the genus *Acinetobacter*,

both *A. pittii* and *A. nosocomialis* are capable of causing severe human disease and are likely under-represented due largely to technological limitations in species identification across clinical laboratories worldwide (Boo, T.W. *et al.*, 2009; Wisplinghoff, H. *et al.*, 2000; Wang, X. *et al.*, 2013).

The ability of *Acinetobacter* to persist in health care facilities has been an active area of investigation; however, it has been mostly limited to the mechanisms utilized to resist antimicrobial therapy, desiccation, and disinfectants. Little is currently known about the virulence factors employed by *Acinetobacter* species (spp.) to colonize and infect different human tissues (Yoon, E.J. *et al.*, 2015; Weber, D.J. *et al.*, 2010; Boll, J.M. *et al.*, 2015; Boost, M.V. *et al.*, 2014). Recent studies have, however, demonstrated that protein glycosylation (Iwashkiw, J.A. *et al.*, 2012; Scott, N.E. *et al.*, 2014), capsule production/modulation (Lees-Miller, R.G. *et al.*, 2013; Geisinger, E. and Isberg, R.R., 2015; Hood, M.I. *et al.*, 2012), metal acquisition strategies (Mortensen, B.L. *et al.*, 2014; Mortensen, B.L. and Skaar, E.P., 2013), outer membrane proteins (Choi, C.H. *et al.*, 2008; Gaddy, J.A. *et al.*, 2009; Lee, J.S. *et al.*, 2010), and alterations in lipid A (Boll, J.M. *et al.*, 2015), all contribute to the ability of medically relevant *Acinetobacter* species to cause disease. It has also been shown that *Acinetobacter* spp. produce both type I pili and type IV pili; however, a definitive role for these pili in virulence has not been determined (Tomaras, A.P. *et al.*, 2003; de Breij, A. *et al.*, 2009; Harding, C.M. *et al.*, 2015a).

Multiple secretion systems have been identified and characterized for their role in the biology and virulence of medically relevant members of the *Acb*. The most comprehensively studied secretion system in *Acinetobacter* is the type VI secretion system (T6SS), which has been functionally identified and studied in the medically relevant species *A. nosocomialis* and *A. baumannii*, as well as in the non-pathogenic species *A. baylyi* (Carruthers, M.D. *et al.*, 2013a; Weber, B.S. *et al.*, 2013). Recently, it was found that several multidrug resistant strains of *A. baumannii* carry a large, self-transmissible plasmid that encodes for the negative regulators of T6SS. It was found that T6SS is silenced in plasmid- containing cells while part of the population loses the plasmid and subsequently activates T6SS (Weber, B.S. *et al.*, 2015). However, unlike *Burkholderia pseudomallei*, which utilizes its T6SS to toxically infect eukaryotic cells (Shalom, G. *et al.*, 2007; Burtnick, M.N. *et al.*, 2014), the *Acinetobacter* T6SS primarily mediates anti-bacterial killing; yet, a recent study identified the *Acinetobacter* T6SS to be required for full virulence in an insect model (Repizo, G.D. *et al.*, 2015). A type V system autotransporter, Ata, has also been characterized and found to mediate biofilm formation, adherence to extracellular matrix proteins,

as well as virulence in a murine systemic model of *Acinetobacter* infection (Bentancor, L.V. *et al.*, 2012). Furthermore, plasmid encoded genes required for the biogenesis of a type IV secretion system (T4SS) in *A. baumannii* and *A. lwoffii* have been bioinformatically identified (Liu, C.C. *et al.*, 2014; Hu, H. *et al.*, 2012); however, no empirical evidence demonstrating their function has been presented. To date, no classical toxins have been described nor have any *bona fide* secretion systems specifically related to disease been discovered in medically relevant *Acinetobacter* members.

Genes encoding proteins predicted to be associated with a type II secretion system (T2SS) have been identified in *A. baumannii* (Wang, N. *et al.*, 2014; Eijkelkamp, B.A. *et al.*, 2014). T2SS are multi-protein complexes, evolutionarily related to type IV pili (T4P) systems, which are responsible for the export of proteins from the periplasmic space to the extracellular milieu or to the outer surface of many Gram- negative bacteria (Korotkov, K.V. *et al.*, 2012; Sandkvist, M., 2001). The T2SS is composed of 12–15 proteins comprising four sub-assemblies: a pseudopilus, an inner-membrane platform assembly, an outer-membrane complex, and a secretion ATPase (Campos, M. *et al.*, 2013). Effector proteins are first translocated to the periplasm by the general secretory (Sec) pathway or the twin arginine transport (Tat) system, where the targeted proteins can then fold into the correct tertiary and/or quaternary structure prior to association with components of the T2SS (Rondelet, A. and Condemine, G., 2013). Competently folded effector proteins can then interact with the different subassemblies of the T2SS and be extruded via interactions with the pseudopilus and the outer-membrane secretin (Nivaskumar, M. and Francetic, O., 2014). Several Gram-negative pathogens, including *Vibrio cholerae* (Sikora, A.E., 2013; Sikora, A.E. *et al.*, 2011), *Legionella pneumophila* (DebRoy, S. *et al.*, 2006; Cianciotto, N.P., 2009), and enterotoxigenic *Escherichia coli* (Tauschek, M. *et al.*, 2002), utilize T2SS for the export of toxins as well as proteins associated with the degradation of biopolymers; thus, T2SS can serve both pathogenic and survival roles for bacteria depending on the environmental niche.

Here, utilizing a proteomics approach coupled with mutational analyses, we demonstrate that *Acinetobacter* spp. carry a functional T2SS. We also present the type II secretome of *A. nosocomialis* strain M2. Using a mutational analysis approach, we further demonstrated that both the type IV pili system and the T2SS share a common prepilin peptidase, PilD. Importantly, we show that two of the three identified effectors required chaperones for secretion by the T2SS, one

of which is a newly characterized protease/chaperone pair. Lastly, we demonstrated that the *Acinetobacter* T2SS contributes to the extracellular lipolytic activity, and the virulence in the both the *Galleria mellonella* infection model and murine pulmonary infection model.

Materials and Methods:

4.2.1-Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids utilized within this study can be located in the Table 4-1. All bacterial strains were grown on L-agar at 37°C. Antibiotic selection for *E. coli* strains was used at the following concentrations: 100 μg ampicillin/mL, 5 μg tetracycline/mL, and 20 μg kanamycin/mL. Antibiotic selection for *Acinetobacter* strains was used at the following concentrations: 200 μg ampicillin/mL, 5 μg tetracycline/mL, 20 μg kanamycin/mL, 12.5 μg chloramphenicol/mL. Sucrose was used at a final concentration of 10% for counter selecting *Acinetobacter* strains that lost the *sacB* cassette.

4.2.2-Generation of Bacterial Mutants and Complemented Mutants

All marked and unmarked mutants were generated using the previously published methodologies found in (Harding, C.M. *et al.*, 2015a; Harding, C.M. *et al.*, 2013) using the In-Fusion HD EcoDry cloning kit. The In-Fusion HD EcoDry cloning kit was used to generate the interrupted gene constructs as described in the supplemental material of (Harding, C.M. *et al.*, 2015a) and introduced into strain M2 via natural transformation as described in (Harding, C.M. *et al.*, 2013). For strains containing the kan-*sacB* cassette, a tri-parental mating strategy was used to transiently introduce the pFLP2 plasmid as described in (Carruthers, M.D. *et al.*, 2013a), in order to replace the cassette with an frt scar. Strains designated with the "::frt" nomenclature contain a frt scar in place of the target gene. Each mutation was complemented using the mTn*7* described in (Harding, C.M. *et al.*, 2015b). A complete list of primers for mutational analyses can be found in Table 4-2.

4.2.3-Bioinformatics

The Basic Local Alignment Search Tool (BLAST) tool was utilized in order to identify known gene homologs of type II secretion system related genes in *Acinetobacter*.

4.2.4-One Dimensional SDS-PAGE analysis of Secreted Proteins

Fifty milliliter cultures of each strain was grown for 18 h in M9 salts supplemented with 1%

casamino acids and 1% glucose with 180 rpm. The secreted proteins were separated from the whole cells by centrifugation at 4000 rpm for 10 mins. The supernatants were then further purified by filtration through 0.22 micron filters. The secreted proteins were then concentrated to ~100 μL using Amicon Ultra Centrifugal Filter units with a 10 kDa cutoff. Laemmli buffer with β-mercaptoethanol was added to each fraction and the samples were heated to 100°C by boiling in water for 10 mins. Twenty microliters of each sample was then separated by SDS-PAGE in a 4–20% gradient gel and subsequently silver stained.

4.2.5-2D-DIGE Analysis

Secreted proteins used for the 2D-DIGE analysis were prepared as described in the above section discussing 1D SDS-PAGE analysis of secreted proteins for both the wild type *A. nosocomialis* strain M2 and its isogenic *gspD*::kan mutant. All 2D-DIGE experiments were performed by the Campus Chemical Instrument Center Mass Spectrometry and Proteomics Facility at The Ohio State University.

4.2.6-Generation of pWH1266 Carrying Effectors and Effector/Chaperone Pairs

In order to validate the 2D-DIGE analysis identifying the putative type II secreted proteins of strain M2, selected effectors and effector/chaperone pairs were cloned into the *Acinetobacter-E. coli* shuttle vector pWH1266. Briefly, *lipA*, *cpaA*, *lipH*, *lipBA*, and *cpaAB* loci were PCR amplified using the primers listed in Table 4-2 using strain M2 genomic DNA as template for PCRs. Each PCR product was purified, digested with PvuI-HF, and ligated into pWH1266 that was predigested with PvuI-HF and treated with phosphatase. The ligations were transformed into *E. coli* TOP10 cells and transformants were selected for on L-agar supplemented with tetracycline. Transformants were sub-cultured and each plasmid was purified and verified by sequencing. The carboxy-terminal His tag was added to *lipA*, *lipH*, and *cpaA* with a second PCR, where the respective forward primer included a 5' overhang encoding for the His-tag using with the primers listed in Table 4-2. The PCR products were purified, DpnI treated, and self-ligated. The ligations were transformed into TOP10 cells and transformants were selected on L-agar supplemented with tetracycline. Transformants were sub-cultured and the plasmids were purified and verified by sequencing. Vectors expressing the His-tagged constructs were electroporated into electrocompetent *Acinetobacter* spp. and transformants were selected for on L-agar supplemented with tetracycline.

4.2.7-Generation of pWH-*gspG*-FLAG

To test for PilD-dependent processing of GspG, the *gspFG* locus including the predicted native promoter was PCR amplified using the primers listed in Table 4-2. The PCR product was purified, digested with PvuI-HF, and ligated into pWH1266 that was predigested with PvuI-HF and treated with phosphatase. The ligations were transformed into TOP10 cells and transformants were selected on L-agar supplemented with tetracycline. Transformants were sub-cultured and the plasmids were purified and verified by sequencing. To remove the *gspF* gene, an inverse PCR strategy was employed to PCR out *gspF* leaving the ATG start codon and the last 21 bp in order to generate an in-frame deletion. The PCR product was purified, treated with kinase, and self-ligated. The ligations were transformed into TOP10 cells and transformants were selected on L-agar supplemented with tetracycline. Transformants were sub-cultured and the plasmids were purified and verified by sequencing. The FLAG tag was PCR amplified onto the carboxy terminus of *gspG* as described above using the primers listed in Table 4-2. The PCR product was purified, treated with kinase, and self-ligated. The ligations were transformed into TOP10 cells and transformants were selected on L-agar supplemented with tetracycline. Transformants were sub-cultured and the plasmids were purified and verified by sequencing. The pWH-*gspG*-FLAG construct was then electroporated into electrocompetent *A. nosocomialis* strains.

4.2.8-Type II Secreted Protein Detection

Strains carrying His-tagged *lipA*, *lipH*, or *cpaA* were screened for active secretion via immunoblotting. Briefly, strains were struck and grown overnight on L-agar supplemented with tetracycline at 37°C. Bacteria were swabbed from the plate, resuspended in LB broth, and used to inoculate 10 mL of LB broth to an OD600 of 0.05 supplemented with tetracycline. The cultures were grown to mid-log phase, normalized to an OD600 of 0.5, then processed for whole cell fractions and secreted fractions. Whole cell fractions were obtained by removing 1 mL of the normalized mid-log cells, pelleting the cells by centrifugation, and removing the supernatant. Bacterial pellets were then resuspended in 50 µL of 1X Laemmli buffer. Secreted fractions were obtained by pelleting the normalized mid-log cultures by centrifugation and carefully removing 1 mL of the supernatant. Secreted proteins were precipitated by the addition of 250 µL of a saturated trichloroacetic acid solution. Precipitated proteins were incubated on ice for 10–30 mins, pelleted by centrifugation, and washed twice with ice-cold acetone. Residual acetone was removed by heating the samples at 95°C. Precipitated proteins were resuspended in 100 µL of 1X Laemmli buffer. Both whole cell fractions and secreted fractions were boiled in 1X Laemmli buffer for 10

mins and subsequently used for immunoblotting. Proteins were separated on a 10% SDS-PAGE gel, transferred to nitrocellulose, and probed for RNA polymerase and 6X-histidine tagged proteins according to our previously published methodologies (Weber, B.S. *et al.*, 2013).

4.2.9-Lipase Assay

In order to determine lipolytic activity of secreted protein fractions, a modified version of the para-nitrophenol palmitate (*p*-NPP) lipase assay was performed. Secreted protein fractions were purified from select strains as described above with slight modifications. Briefly, 2.5 mL of culture supernatant was clarified by centrifugation and then filtered through 0.22µM PVDF filters. The secreted proteins were buffer exchanged into 50 mM Tris and were concentrated to ~250 µL using Amicon Ultra Centrifugal Filter units with a 10 kDa cutoff and promptly used for the lipase assay. Lipase activity was determined by measuring the absorbance at 410nm at 37°C using *p*-NPP as a substrate. The *p*-NPP solution was freshly prepared for each assay by diluting solution A (0.1g *p*-NPP in 100 mL isopropanol) 1:10 with solution B (1 g gum Arabic, 2 g sodium deoxycholate, 5 mL triton X-100, 50 mM Tris-HCl pH 8 in 900 mL). Seventy microliters of the *p*-NPP solution was then added to 30 µL of the concentrated, clarified secreted protein fractions from a respective strain. Kinetic measurements recording the absorbance at 410 nm were then performed over the designated time frame at 37°C with orbital shaking between each absorbance reading. Absorbance measurements were captured using the Synergy HTX multi-mode reader from BioTek. Each experiment was performed in triplicate with three technical replicates per sample.

4.2.10-*Galleria mellonella* Infection

A. nosocomialis M2, the Δ*gspD*::kan mutant and the complemented strain were grown in LB broth overnight in an orbital shaker (37°C, 200 rpm). The overnight cultures were diluted to a starting OD_{600} 0.05 and grown at 37°C with 200 rpm to a final OD_{600} of 0.5. 0.5 ODs was pelleted by centrifugation, washed with filter sterilized PBS and resuspended at and OD of 0.5/ml, 0.158 OD/mL and 0.05 OD/mL in filter sterile PBS. The CFU/mL at 0.5 OD/mL was determined to be 10^9. Serial dilution of the 0.5 OD/mL sample was performed. Larvae were injected with 10 uL of sterile PBS, 10^6 or 10^7 CFU. 3 groups of 10 larvae were injected per experimental group. The larvae were scored as live/dead depending on their response to physical stimulus

approximately every 5 hours. The number of bacterial cells injected into the larvae was determined by plating 10-fold serial dilutions on LB agar and performing CFU counts after over-night incubation at 37°C.

4.2.11-Mouse Model of Pneumonia

All infection experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee. Wild-type C57BL/6 mice, obtained from Jackson Laboratories, were used for single infection experiments with either the wild type *A. nosocomialis* M2, the M2Δ*gspD*::frt mutant, or the respective *gspD* complemented strain. Overnight cultures of each strain were sub-cultured 1/1000 into 50 mL LB broth and grown with shaking at 37°C in 250 mL flasks. Bacterial cells were harvested by centrifugation during logarithmic growth, washed twice with phosphate buffered saline (PBS), and suspended in PBS. Nine-week old male mice were inoculated intranasally with a total of 7–8X 10^8 cfu in 30 μL. At 36 h post- infection, mice were euthanized and CFUs were enumerated from the lungs, livers, and spleens following tissue homogenization and dilution plating to LB agar medium. The data were log transformed and analyzed for Gaussian distribution using the D'Angostino-Pearson omnibus normality test. Data sets displaying Gaussian distribution were then analyzed by One-way ANOVA with Tukey's test for multiple comparisons. Data sets displaying non-Gaussian distribution were analyzed by Kruskal-Wallis test with Dunn's test for multiple comparisons. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA).

Ethics statement

Animal care and experiments were performed in accordance with the NIH "Guide for the Care and Use of the Laboratory Animals" and were reviewed and approved by the Vanderbilt University Institutional Animal Care and Use Committee (Protocol M/10/165). Mice were anesthetized with 2,2,2,-tribromoethanol prior to intranasal inoculation. Mice were euthanized by carbon dioxide.

Supplementary data can be found in Appendix 1.

Table 4-1. Plasmids and bacterial strains included in the study

Plasmid or strain	Relevant characteristic(s)	Reference/ Source
Plasmids		
pKD13	Contains kanamycin resistance gene from Tn5 flanked by FRT sites	(Datsenko, K.A. and Wanner, B.L., 2000)
pFLP2	Encodes Flp recombinase	(Kumar, A. *et al.*, 2010)
pGEM-T-Ez	General cloning plasmid	Promega
pGEM-*gspD*::kan	pGEM containing a *gspD*::kan with 1kb flanking DNA up and downstream	This study
pGEM-*lipA*::kan	pGEM containing *lipA*::kan with 1kb flanking DNA up and downstream	This study
pGEM-*abhA*::kan	pGEM containing *abhA*::kan with 1kb flanking DNA up and downstream	This study
pRSM4063	pSMART-LCkan containing an empty mTn*7* element from pRSM3510 along with 2kb flanking DNA up and downstream of the *att*Tn*7* site in *A. nosocomialis* M2	(Harding, C.M. *et al.*, 2015b)
pRSM4063-*gspNCD*	pRSM4063 containing the *gspNCD* locus with the predicted *gspN* promoter	This study
pRSM4063-*gspN*^{P}-*gspD*	pRSM4063-*gspNCD* lacking *gspNC*	This study
pWH1266	*Acinetobacter-E. coli* shuttle vector	(Hunger, M. *et al.*, 1990)
pWH-*cpaA-his*	pWH1266 containing *cpaA* along with its predicted native promoter	This study
pWH-*cpaA-his*	pWH-*cpaA* containing a hexa-histidine tag on the carboxy terminus of *cpaA*	This study

pWH-*cpaA-cpaB*	pWH1266 containing *cpaA* and *cpaB* along with the predicted native promoter	This study
pWH-*cpaA-his-cpaB*	pWH-*cpaA-cpaB* containing a hexa-histidine tag on the C-terminus of *cpaA*	This study
pWH-*lipA*	pWH1266 containing *lipA* along with the predicted promoter	This study
pWH-*lipA-his*	pWH-*lipA* containing a hexa-histidine tag on the carboxy terminus of *lipA*	This study
pWH-*lipB-lipA*	pWH1266 containing *lipB* and *lipA* along with the predicted native promoter	This study
pWH-*lipB-lipA-his*	pWH-*lipB-lipA* containing a hexa-histidine tag on the carboxy terminus of *lipA*	This study
pWH-*abhA*	pWH1266 containing *abhA* along with its predicted native promoter	This study
pWH-*abhA-his*	pWH-*abhA* containing a hexa-histidine tag on the carboxy terminus of *abhA*	This study
pWH-*gspFG*	pWH1266 containing the *gspFG* locus and the predicted native promoter	This study
pWH-*gspF*^{P}-*gspG*	pWH-*gspFG* with an in-frame deletion of the *gpsF* gene	This study
pWH-*gspF*^{P}-*gspG-FLAG*	pWH-*gspF*^{P}-*gspG* containing a C-terminal FLAG on *gspG*	This study
pRSM4063-*lipA*	pRSM4063 containing the *lipA* gene and its predicted native promoter	This study
pRSM4063-*abhA*	pRSM4063 containing the *abhA* gene and its predicted native promoter	This study
Strains		
A. nosocomialis strain M2	Metro Health Systems Clinical Isolate	(Carruthers, M.D. *et al.*, 2013b; Niu, C. *et al.*, 2008)

M2Δ*gspD*::kan	*A. nosocomialis* strain M2 containing a deletion of *gspD* and replacement with a kanamycin resistance cassette	This study
M2Δ*gspD*::kan (*gspD*+)	M2Δ*gspD*::kan with a mini-Tn*7* element containing the *gspD* gene driven off the predicted *gspN* promoter	This study
M2Δ*gspD*::kan-*sacB*	*A. nosocomialis* strain M2 containing a deletion of *gspD* and replacement with a kanamycin resistance cassette and the *sacB* gene	This study
M2Δ*gspD*::frt	*A. nosocomialis* strain M2 containing an in frame, deletion of *gspD*	This study
M2Δ*gspD*::frt (*gspD*+)	M2Δ*gspD*::frt with a mini-Tn*7* element containing the *gspD* gene driven off the predicted *gspN* promoter	This study
M2Δ*abhA*::kan	*A. nosocomialis* strain M2 containing a deletion of *abhA* and replacement with a kanamycin resistance cassette	This study
M2Δ*abhA*::kan (*abhA*+)	M2Δ*abhA*::kan with a mini-Tn*7* element containing *abhA* driven off the predicted *abhA* promoter	This study
M2Δ*lipA*::kan	*A. nosocomialis* strain M2 containing a deletion of *lipA* and replacement with a kanamycin resistance cassette	This study
M2Δ*lipA*::kan (*lipA*+)	M2Δ*lipA*::kan with a mini-Tn*7* element containing *lipA* driven off the predicted *lipA* promoter	This study
M2Δ*pilA*::frt	*A. nosocomialis* strain M2 containing an in frame deletion of *pilA* and replacement with FLP scar	(Harding, C.M. *et al.*, 2015b)
M2Δ*pilD*::kan	*A. nosocomialis* strain M2 containing a deletion of *pilD* and replacement with a kanamycin resistance cassette	(Harding, C.M. *et al.*, 2013)
M2Δ*pilD*::kan (*pilD*+)	M2Δ*pilD* with a mini-Tn7 element containing the *pilD* gene transcribed from its predicted promoter	(Harding, C.M. *et al.*, 2013)
A. baumannii ATCC 17978	Type strain	ATCC

A. baumannii ATCC 19606	Type strain	ATCC
A. pittii 31132	Clinical isolate	Nationwide Children's Hospital
A. pittii 31357	Clinical isolate	Nationwide Children's Hospital
Acinetobacter spp. 33904	Clinical isolate	Nationwide Children's Hospital
E. coli DH5a	General cloning strain	Invitrogen
E. coli EC100D	General cloning strain, *pir*$^{+}$	Epicentre
E. coli TOP10	General cloning strain	Invitrogen
E. coli HB101(pRK2013)	Conjugation helper strain	(Figurski, D.H. and Helinski, D.R., 1979)

Table 4-2 Primer List

Primer Name	Sequence
5' gspD + 15bp pGEM F	CCGGCCGCCATGGCGatgaagaaaaagtctaagcaatgg
5' gspD + 15bp kan R	acggatccccggaataactcgcgatatttcctaactc
3' gspD + 15bp kan F	cagctccagcctacatagcatgttatgattgataaaaa
3' gspD + 15bp pGEM R	CGCGAATTCACTAGTttagacaattaactcagcaagac
gspD F1	Tggactggtcgcggtaaacaa
gspD R1	Cctgtaaagcttggtaaatctggt
M215_3235 F1	Ttgctggtgatgaatatgctggtc
M215_3235 R1	gttcaggttgggcttggttagttg
5' M215_3235 +15bp pGEM F	CCGGCCGCCATGGCGatgcgtaaacgacaacc
5'M215_3235 +kan R	acggatccccggaataaaatatccttttttatgttatgtgt
3'M215_3235+15bp kan F	cagctccagcctacagttttatttaactagcatttagctt
3'M215_3235+15bp pGEM R	CGCGAATTCACTAGTtctcccatagctcagtcg
M215_10380 F1	gcgcttcacgttcaatctcg
M215_10380 R1	gtagcccatgacaccaacacag
5'M215_10380+15bp pGEM F	CCGGCCGCCATGGCGatgcaaaggatgcagaa
5'M215_10380+15bp kan R	acggatccccggaatttttactcactatttttattttgt

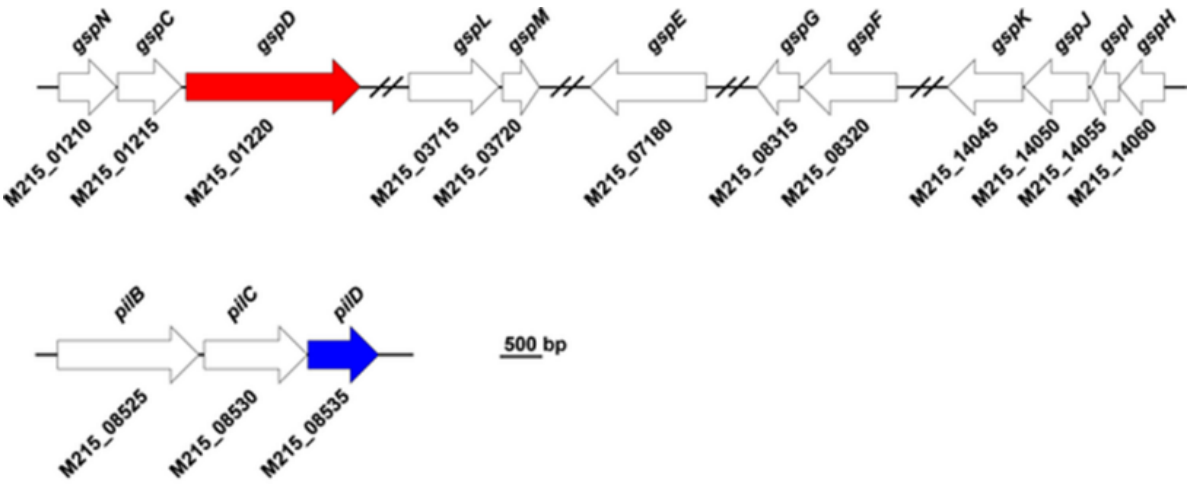
3'M215_10380+15bp kan F	cagctccagcctacattccaaataaaaaaatgcg
3'M215_10380+15bp pGEM R	CGCGAATTCACTAGTgaggagtctgcggtgttt
3235 locus seq 1	gcgtaaccgttctgcgtcta
3235 locus seq 2	cgacaaccagtacttaaacaggaa
3235 locus seq 3	cgacaaggaattactatgacgact
3235 locus seq 4	gtcaattccaatggcctttactgc
3235 locus seq 5	tcgatttcagcattatttcttaca
3235 locus seq 6	tcagttgaaaggcgattggatg
3235 locus seq 7	ttatggtcgtacaggagatgatgg
3235 locus seq 8	ttccttggttattaatggggttgg
3235 locus seq 9	atagggacgtaaacaaacaacaag
5100 locus seq 2	aattttgcactcggtctctggtct
5100 locus seq 3	aatgattgctttggttggctttga
5100 locus seq 4	ctatctcagccatttgtttcttcc
5100 locus seq 5	gacgggttattcagatttagagtt
5100 locus seq 6	ctattcccgagcagttttacatt
5100 locus seq 7	ctcaaggttggacagtagatagtt
5100 locus seq 8	tctagtccatacctttacaatcct
5100 locus seq 9	tattgttcgatggactggtagatt
5100 locus seq 10	caggaacgcgtggtgtaggt
5100 locus seq 11	tagcttatggccagggttct
5100 locus seq 12	agtggtagggctaaatcaacaagt
10380 locus seq 1	gatgcgttttcacttattttatgg
10380 locus seq 2	tgcacaaattcaaccacctcaa
10380 locus seq 3	caacaacaatttagctcatcacag
10380 locus seq 4	tcacgggggaccaacag
10380 locus seq 5	acgagctggtaatttttcacg
10380 locus seq 6	gcattgcttctcgtcttgtgt
10380 locus seq 7	tacaatcaagccgtcataatcaac
10380 locus seq 8	tagcacgtacaaccatcacatca
10380 comp F +PvuI	gcgCGATCGaacaacaatttagctcatcacagg
10380 comp R + PvuI	gcgCGATCGggtgttggtgttgagcgtgtat
10380-His tag F	catcatcatcatcatcactgattccaaataaaaaaatg
10380-His tag R	taatccttgtagttttaaacgat
10380 His seq	tcgtgatgactataactggaacc
5100 comp F + PvuI	gcgCGATCGctatctcagccatttgtttcttcc
5100 comp R + PvuI	gcgCGATCGaacctttctttttctgacggactc
5100 His tag F	catcatcatcatcatcactaatctctatttttagctgtaaata
5100 His tag R	gttatatagagaaatttttgtgc

5100 His seq	caggggatggcggttgtg
3235 comp F + PvuI	caattaaaaccaaagaccgcaacc
3235 comp R + PvuI	ccccattaataaccaaggaacaga
3235 His tag F	catcatcatcatcatcactaagttttatttaactagcattta
3235 His tag R	gaacttacctgtacagtgc
3235 His seq	ctcaagctgggtacgataagg
3235 mTn7 comp F + SacI	cgcGAGCTCcaattaaaaccaaagaccgcaacc
3235 mTn7 comp R + XmaI	gcgCCCGGGccccattaataaccaaggaacaga
10380 mTn7 comp F + XmaI	gcgCCCGGGcaacaacaatttagctcatcacag
10380 mTn7 comp R + KpnI	cgcGGTACCggtgttggtgttgagcgtgtat
5' 10375 +15bp pGEM F	CCGGCCGCCATGGCGctacaaatttaagatacgcttagg
5' 10375 + 15bp Kan R	acggatccccggaatcataaagatatcttccttaagttt
3' 10375 + 15bp Kan F	cagctccagcctacagaactaccatttaactattaggctt
3' 10375 + 15bp pGEM R	CGCGAATTCACTAGTatgagtggtaaacatcctttagtt
10375 tag R	atagttaaatggtagttcacctcc
10375 FLAG tag F	gattacaaggatgacgacgataagtaggcttttctatttcaaagtgag
10375 HIS tag F	catcatcatcatcatcactaggcttttctatttcaaagtgag
5' 5105 + 15bp pGEM F	CCGGCCGCCATGGCGaataaagggtaaaaagagagattta
5' 5105 + 15bp kan R	acggatccccggaattatttacagctaaaaatagagatta
3' 5105 + 15bp kan F	cagctccagcctacaaggaataaatgattttggtag
3' 5105 + 15bp pGEM R	CGCGAATTCACTAGTaatctaaaatggctaacatagaa
5105 F1	ctatctcagccatttgtttcttcc
5105 R1	attatcatttgcagagtcgttaca
gspF comp F + PvuI	gcgCGatCGgcacaggtacggcaacacg
gspG comp R + PvuI	gcgCGatCGcatgatttggctgctaccttctgt
ATG gspF R	catggtaatgttctaattttcc
last 21bp gspF F	cgtaaatatgaacaatatgatttaa
gspG tag R	aggttggtagtaaatatcggc
gspG His F	catcatcatcatcatcactaattaaaatgtaagagagattatcga
gspG FLAG F	gattacaaggatgacgacgataagtaattaaaatgtaagagagattatcga
5100 GTG start R	cacagtagcctcttttatttg
5100 Last 18bp F	atttctctatataactaatctctattttta
5105 comp R + KpnI	cgcGGTACCacctacaccacgcgttcctg
5100 Flag F	gattacaaggatgacgacgataagtaatctctatttttagctgtaaata
10375 comp F + XmaI	gcgCCCGGGcactgatatggcgacgagaaa
10375 comp R + KpnI	cgcGGTACCctgttggtcccccgtga

4.3-Results

4.3.1-Identification of T2SS-associated Loci in Medically Relevant *Acinetobacter* spp

Previous manuscripts have reported the bioinformatic identification of genes predicted to encode proteins required for the biogenesis of a T2SS in *Acinetobacter* spp. (Wang, N. *et al.*, 2014; Eijkelkamp, B.A. *et al.*, 2014). We have also identified homologs of genes associated with the biogenesis of a T2SS in *A. nosocomialis* strain M2. Here we adopt the *gsp* nomenclature for general secretory pathway when defining homologous T2SS associated genes in *Acinetobacter*. Using the Basic Local Alignment Search Tool (BLAST) (Marchler-Bauer, A. *et al.*, 2011) and homologs of known T2SS-associated genes from *V. cholerae*, *P. aeruginosa*, and *E. coli*, we identified several *gsp* homologs in all publically available genomes from medically relevant *Acinetobacter* spp. Unlike many Gram-negative pathogens encoding a T2SS, the genes encoding predicted type II secretion biogenesis proteins were not encoded in a single operon (Sandkvist, M., 2001), but were grouped into five distinct gene clusters separated over large distances on the chromosome (Figure 4.1).



Gene	Predicted/Known* Function	System
gspN	Unknown function	T2SS
gspC	Inner-membrane platform protein	T2SS
gspD	Outer-membrane secretin	T2SS
gspL	Inner-membrane platform protein	T2SS
gspM	Inner-membrane platform protein	T2SS
gspE	Secretion ATPase	T2SS
gspG	Major pseudopilin	T2SS
gspF	Inner-membrane platform protein	T2SS
gspKJIH	Minor pseudopilins	T2SS
pilB	Inner-membrane platform protein	TFP
pilC	Inner-membrane platform protein	TFP
pilD	Prepilin* and pre-pseudopilin peptidase	TFP/ T2SS

Figure 4.1 -The Type II Secretion Associated Gene Loci as Found in *A. nosocomialis* Strain M2. Genes predicted to encode proteins required for the biogenesis of a functioning type II secretion system were clustered into five distinct loci and were distantly spread over the chromosome. A single prepilin/pre-pseudopilin peptidase homolog was identified, which was located in the previously described *pilBCD* cluster.

Differential Secretion of Proteins in a *gspD*-dependent Manner

To test the functionality of the T2SS in *A. nosocomialis* strain M2 we deleted the predicted type II outer membrane secretin gene homolog, *gspD*, from strain M2. GspD secretin monomers form a dodecamer complex in the outer-membrane that is required for the export of periplasmic effector proteins (Figure 4.2A) (Chami, M. *et al.*, 2005; Reichow, S.L. *et al.*, 2010). Using the T2SS deficient M2Δ*gspD*::kan mutant we probed for differentially secreted proteins by one-dimensional sodium dodecyl sulfate poly- acrylamide gel electrophoresis (SDS-PAGE). Furthermore, we complemented the *gspD*::kan mutant and probed for secreted proteins from this genetic background (Figure 4.2A). The secreted protein profiles from all three strains contained

an abundance of proteins; however, differences in the secreted protein profile from the *gspD*::kan mutant were clearly evident when compared to the parental strain. At least 4 silver-reactive protein bands were absent in the secreted profile from the *gspD*::kan mutant when compared to the secreted protein profile from the parental strain (Figure 4.2A). Importantly, the secreted protein profile from the complemented *gspD* strain showed the same profile as the parental strain M2 indicating that these differences observed in the secreted protein profile from the *gspD*::kan mutant were due to the loss of the putative outer membrane secretin and not to the mutational strategy.

4.3.2-2D-DIGE Analysis of Ype II Dependent Secreted Proteins in *A. nosocomialis* Strain M2

Although our 1D SDS-PAGE analysis strongly indicated that *A. nosocomialis* strain M2 did in fact produce a functional T2SS, the abundance of non-type II secreted proteins would interfere with downstream identification. We therefore proceeded with a two-dimensional difference gel electrophoresis (2D-DIGE) analysis to enhance protein separation. The secreted protein fraction from the wild type strain M2 was compared to the secreted protein fraction from the M2Δ*gspD*::kan mutant to generate the preliminary type II secretome of *A. nosocomialis* strain M2 via 2D-DIGE analysis. Analysis of gel images with SameSpots software (TotalLab, New Castle upon Tyne) revealed that 60 spots exhibited a statistically significant average change of at least 4-fold when comparing wild type M2 vs. M2Δ*gspD*::kan samples. A representative gel image from the 2D-DIGE analysis is shown in Figure 4.2B. Gel spots were cored using an Ettan Spot Handling Workstation and prepared for in gel trypsin digestion. Peptides were eluted and analyzed using capillary-liquid chromatography-nanospray tandem mass spectrometry. The complete list of proteins identified for each spot as well as a detailed description of the 2D-DIGE analysis and methodologies can be found in Appendix 1; proteins associated with the largest spot fold change, however, are listed in Figure 4.2C. Three of the proteins identified in Figure 4.2C, M215_05100, M215_10380, and M215_03235, were of particular interest as all contained domains of known function. The remaining proteins listed in Figure 4.2C do not contain any known functional domains, with the exception of M215_02250/M215_02255 pair, which was bioinformatically identified as GlyGly-CTERM and rhomobosortase (Haft, D.H. and Varghese, N., 2011).

The top secreted candidate, M215_05100, is an ortholog of the previously identified CpaA metallopeptidase from the M72 family of peptidases, which was proposed to cleave both factor V and fibrinogen (Tilley, D. *et al.*, 2014). The M72 peptidases are characterized as peptidyl-Asp-endopeptidases containing the HEXXHXXGXX active site, where a zinc ion is predicted to be bound by three histidine residues, and the glutamate is predicted to be the catalytic residue (Rawlings, N.D. *et al.*, 2014). The M215_10380 locus encodes an ortholog of the previously characterized LipA lipase from *A. baylyi* (Kok, R.G. *et al.*, 1995a; Kok, R.G. *et al.*, 1995b), which contains an alpha/beta hydrolase fold from the homologous family abH15.02 (*B. cepacia* lipase-like) within the abH15 superfamily (*Burkholderia* lipase superfamily) as determined by the Lipase Engineering Database (Fischer, M. and Pleiss, J., 2003). These lipases are predicted to have a catalytic triad of a serine, a glutamate or aspartate, and a histidine. Lastly, the M215_03235 locus encodes for another protein containing an alpha/beta hydrolase fold; however, the M215_03235 gene product does not have homology to any know lipases within the Lipase Engineering Database and has yet to be characterized in *Acinetobacter*.

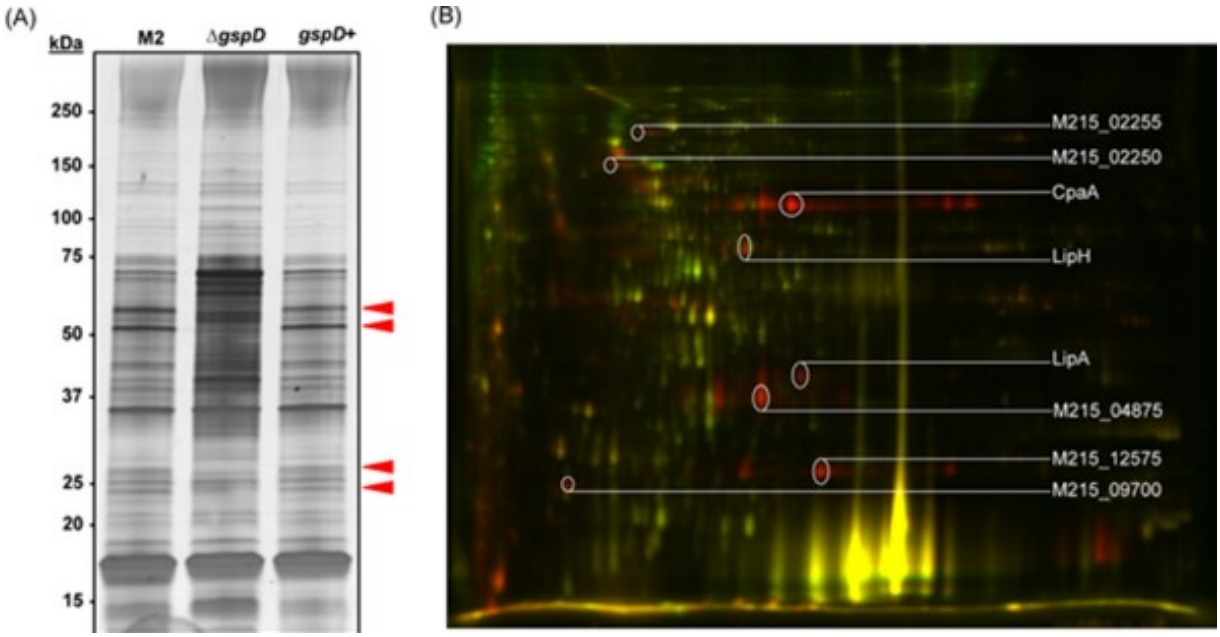


Figure 4.2- Identification of Putative type II secreted

Proteins from *A. nosocomialis* Strain M2. (A) Secreted protein fractions from the parent, Δ*gspD*::kan mutant, and the *gspD* complemented strain were analyzed by one dimensional SDS-PAGE. Red arrows indicate silver reactive bands that were present in both parent and *gspD* complemented strain's secreted fractions. (B) Secreted protein fractions from the parent strain and the Δ*gspD*::kan mutant were analyzed by two-dimensional difference gel electrophoresis (2D-DIGE). A representative gel image showing Cy3 (Δ*gspD*::kan, green) and Cy5-labeled (parent, red) proteins that were isoelectric focused on pH strips (3–10), separated by size using SDS-PAGE, and visualized using a Typhoon 9400 variable mode imager. A merged image of the Cy3 and Cy5-labeled proteins is shown. Proteins with greater abundance in the Δ*gspD*::kan sample appear green and proteins with greater abundance in the parent strain sample appear red. Proteins that did not change relative abundance between the two samples appear yellow.

(C)

Spot Fold Change	S[1]	M[2]	ANOVA (P)	Locus	Putative Product	Signal Peptide[3]
54.2	2686	35	1.57E-09	M215_05100	CpaA metallopeptidase	Sec
16.8	1043	14	1.48E-08	M215_12575	Hypothetical	Sec
15.9	1199	17	2.55E-07	M215_04875	Hypothetical	Sec
10.0	1041	15	1.83E-08	M215_10380	LipA (AB15.02)	Sec
9.6	2217	34	1.92E-04	M215_02255	Membrane protein	Sec
9.3	403	4	5.75E-05	M215_09700	Hypothetical	Sec
8.1	1874	23	1.54E-09	M215_03235	LipH (Alpha/Beta Hydrolase)	Sec
5.9	1781	24	2.75E-07	M215_02250	Rhombotarget A	Sec

Figure 4.2- Identification of Putative type II secreted

Proteins from *A. nosocomialis* Strain M2. (C) Putative T2S-dependent proteins identified via 2D-DIGE analyses. Protein candidates associated with the largest spot fold change were bioinformatically examined for the presence of a signal peptide and putative functions. The protein score is derived from Mascot and provides an indication of how well the peptides matched the indicated protein sequence. The actual score is calculated by the following equation: protein score = -10*Log(P), where P is the probability that the protein match is a random event. Scores above 100 indicate that $p < 0.05$. [2]The protein match score indicates the number of unique peptides that matched the sequence of the identified protein. Two unique peptide matches to a protein sequence confirms the identity of a protein. Signal peptide prediction was bioinformatically predicted using SignalP 4.1.

4.3.3-The Prepilin Peptidase, PilD, is also the Pre-pseudopilin Peptidase of the *Acinetobacter* T2SS

BLAST analysis revealed the presence of only a single prepilin peptidase, *gspO*/*pilD*, which was previously designated PilD and reported to be the major prepilin peptidase for the type IV pili (T4P) system in *Acinetobacter* (Figure 4.1) (Harding, C.M. *et al.*, 2013). Given that only one *gspO*/*pilD* homolog was identified in strain M2's genome as well as in *A. baumannii* ATCC 17978 and 19606, we hypothesized that the previously identified prepilin peptidase, PilD, was also the pre-pseudopilin peptidase required for the T2SS. To this end, we cloned and expressed the predicted major pseudopilin, *gspG*, *in trans* with a carboxy-terminal FLAG tag in the wild type M2 background, the Δ*pilD*::kan mutant, and its respective complement in order to probe for pseudopilin

processing. As expected GspG-FLAG expression was detected in all three backgrounds; however, GspG-FLAG from both the wild type M2 and the complemented *pilD* strain migrated with an increased electrophoretic mobility as compared to GspG-FLAG from the Δ*pilD*::kan strain (Figure 4.3). The increase in electrophoretic mobility was most likely due to the loss of the leader sequence of GspG; furthermore, PilD was required for the processing observed. Lastly, an additional band of intermediate electrophoretic mobility was detected only in the Δ*pilD*:: kan background. We hypothesize this form of GspG-FLAG to be a degradation product.

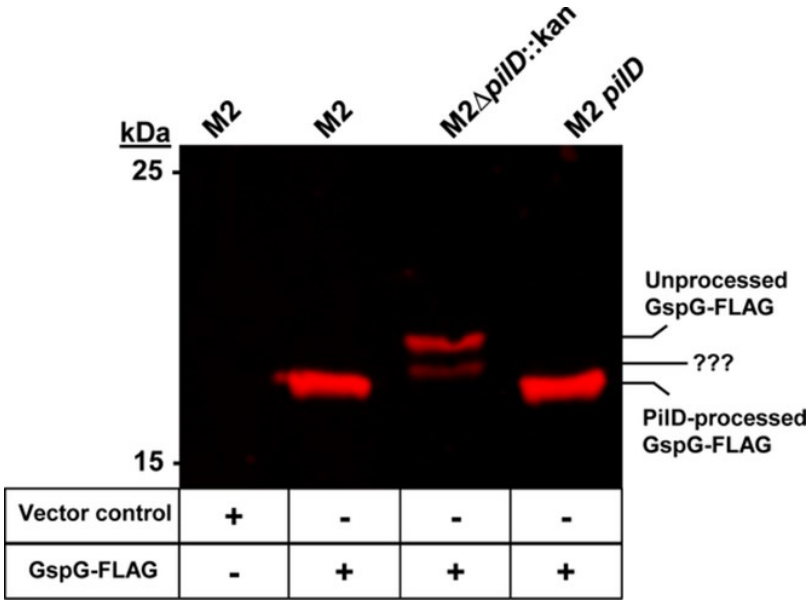


Figure 4.3- The Predicted Major Pseudopilin, GspG, was Processed by the Prepilin Peptidase PilD. Whole cell lysates from strains containing either the empty vector or pWH-*gspG-FLAG* were examined by western blot and probed for processed and unprocessed GspG-FLAG. GspG-FLAG from both the parent and *pilD* complemented strain migrated at a faster electrophoretic mobility when compared to GspG-FLAG from the Δ*pilD*::kan mutant. The theoretical molecular mass of full length GspG and processed GspG is 18,549 Daltons and 14,360 Daltons, respectively.

4.3.4-The type II Secretion of the CpaA Metallopeptidase is Dependent on a Novel Protease Chaperone, CpaB

Our 2D DIGE analysis indicated that the CpaA metallopeptidase was secreted via the T2SS; therefore, we used an immunoblotting approach to verify CpaA secretion was type II dependent. We cloned the *cpaA* gene with its predicted native promoter into the *Acinetobacter-E.*

coli shuttle vector pWH1266 (Hunger, M. *et al.*, 1990), containing a hexa-histidine tag onto the carboxy terminus of *cpaA*. Hexa-histidine tagged CpaA was expressed *in trans* in multiple genetic backgrounds to probe for expression and secretion. CpaA-His expression was detected in all strains tested, however, it was only detected in the secreted fractions from strains predicted to have a fully functioning T2SS (Figure 4.4C). Specifically, neither the Δ*gspD*::kan mutant nor the Δ*pilD*::kan mutant secreted CpaA-His, indicating the dependency of the T2SS for active export of CpaA. As expected secretion was independent of the type IV pilus as the Δ*pilA*::frt mutant displayed active secretion of CpaA-His.

Immediately downstream of *cpaA* is the M215_05105 open reading frame, which when analyzed by BLASTp did not identify any known functional domains. However, when the M215_05105 ORF was analyzed by Domain Enhanced Lookup Time Accelerated (DELTA) BLASTp, which has higher sensitivity than BLASTp (Boratyn, G.M. *et al.*, 2012), the M215_05105 ORF was found to contain a domain from the SRPBCC superfamily (Figure 4.4A and 4.4B). Proteins carrying a domain from the SRPBCC superfamily are predicted to contain a deep hydrophobic ligand-binding pocket and have chaperone-like activity (Lotz, G.P. *et al.*, 2003). We thus hypothesized that the M215_05105 gene product, designated CpaB due to its proximity to CpaA, was a CpaA-specific chaperone. To test our hypothesis, we deleted the *cpaB* gene and probed for CpaA-His expression and secretion. As shown in Figure 4.4D, CpaA-His expression was detected in the Δ*cpaB*::frt mutant; however, CpaA-His was not secreted, indicating that CpaB was required for CpaA secretion. Importantly, we were able to reintroduce the *cpaB* allele and restore the active secretion of CpaA-His.

To further demonstrate the dependency of CpaA secretion on CpaB, we heterologously expressed *cpaA-his* alone or in tandem with *cpaB* in *A. baumannii* ATCC 19606, which does not encode for orthologs of either the CpaA metallopeptidase or the CpaB chaperone, yet is predicted to produce a functional T2SS. As shown in Figure 4.4E, CpaA-His was expressed but not secreted by 19606 cells when the pWH-*cpaA-his* plasmid was introduced, however, when both *cpaA-his* and *cpaB* were co-expressed, CpaA-His was secreted, indicating that CpaA secretion is not only dependent on a functional T2SS, but also on the chaperone activity of CpaB.

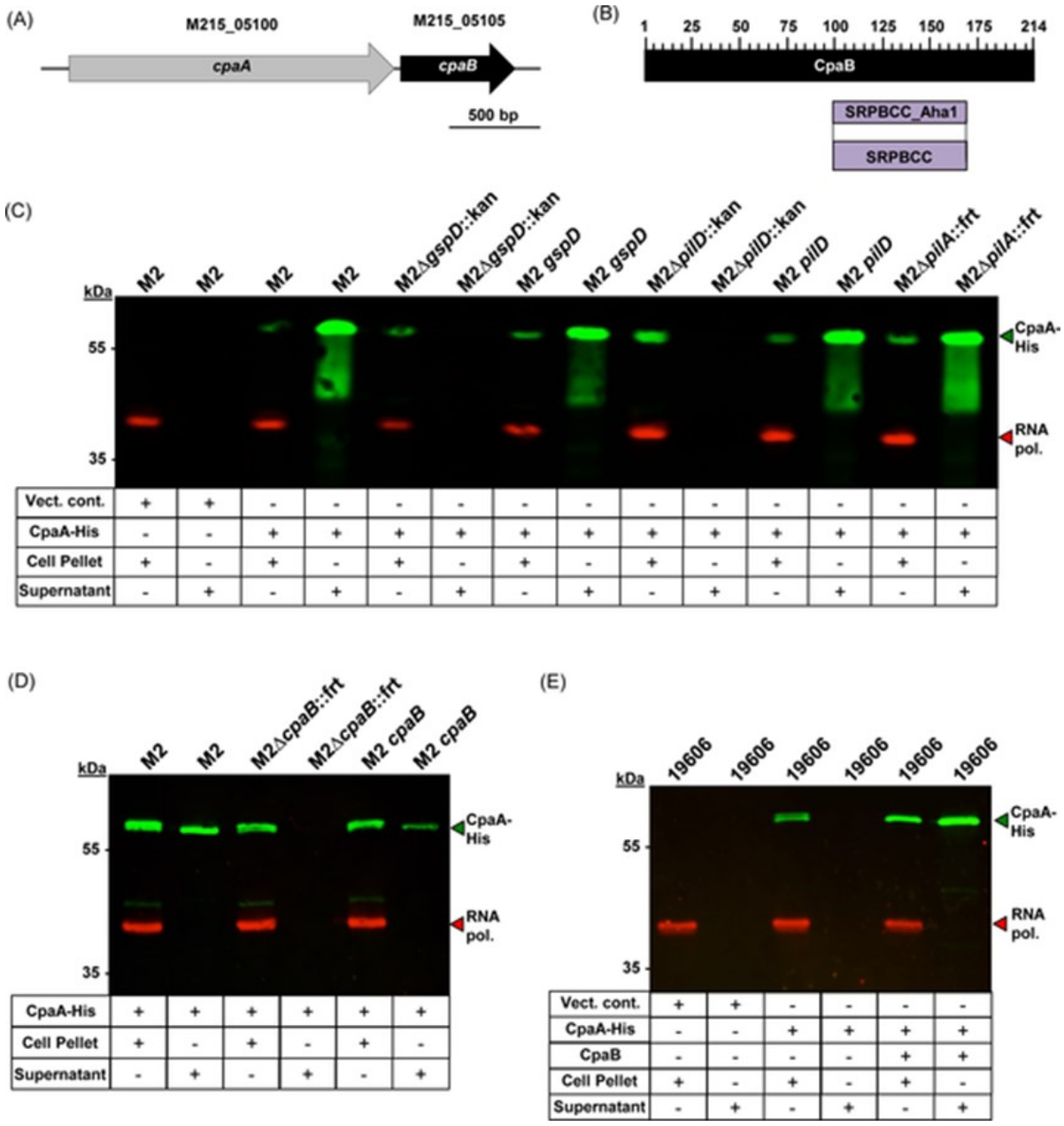


Figure 4.4- Secretion of the CpaA Metallopeptidase was Reliant upon both a Functioning T2SS and the Novel CpaB Chaperone. (A) Gene arrangement of the *cpaAB* gene cluster. (B) DELTA BLASTp analysis of the CpaB amino acid sequence identified a SRPBCC domain. (C) Western blot analysis on whole cell lysates and secreted protein fractions probing for CpaA-His. All strains and fractions were also analyzed for RNA polymerase expression, which served as a lysis control. CpaA-His expression was detected in all strains carrying pWH-*cpaA-his*; however, CpaA-His secretion was only detected in strains predicted to produce a functioning T2SS. (D) Western blot analysis on whole cell lysates and secreted protein fractions probing for CpaA-His

from the parent strain, the Δ*cpaB*::frt mutant, and the *cpaB* complemented strain. CpaA-His was detected in all strains; however, CpaA-His secretion was not detected in the protein fraction from the Δ*cpaB*::frt mutant. (E) Western blot analysis on whole cell lysates or secreted protein fractions from *A. baumannii* ATCC 19606 carrying the empty vector, the pWH-*cpaA-his* vector, or the pWH-*cpaA-his-cpaB* vector. CpaA-His production was detected; however, CpaA-His secretion was only detected when the *cpaB* gene was co-expressed with *cpaA-His*.

4.3.5-The LipA Lipase is Exported by the Type II Secretion System, is Lipolytic Towards Neutral Triglycerides, and is Dependent on the LipB Chaperone for Secretion

The M215_10380 ORF, encoding for a LipA ortholog, was also identified in our 2D-DIGE analysis as a type II effector. It has been previously demonstrated in *A. baylyi* and *Pseudomonas* that secretion and over-expression of LipA orthologs are dependent on a LipB-like chaperone (Kok, R.G. *et al.*, 1995b; Madan, B. and Mishra, P., 2010). In *A. nosocomialis* M2, a *lipB* homolog is adjacent to *lipA* (Figure 4.5A and 4.5B). When LipA-His was over-expressed from the pWH-*lipA-his* plasmid, we did not detect its secretion. However, when we co-expressed the upstream *lipB* gene with *lipA-his*, LipA-His was expressed and secreted in all backgrounds predicted to have a functional T2SS (Figure 4.5C). LipA-His was neither detected in the secreted fraction from the Δ*gspD*::kan mutant nor the Δ*pilD*::kan mutant. Secretion was also independent of the type IV pilus fiber itself (Figure 4.5D). We also confirmed that LipA was secreted in a LipB chaperone-dependent manner by *A. baumannii* ATCC 19606 (Figure 4.5E).

To confirm that LipA is in fact a lipase, we purified culture supernatants from multiple genetic backgrounds and probed for lipolytic activity as determined by a modified para-nitrophenol palmitate (*p*-NPP) assay (Mobarak-Qamsari, E. *et al.*, 2011). As seen in Figure 4.5F, culture supernatants from the wild type M2 exhibited lipolytic activity as demonstrated by an increase in the absorbance at 410 nm (A410) over a 12-hour time period. Culture supernatants from the Δ*gspD*::kan mutant displayed only minimal increases in the A410 indicating almost a complete lack of lipase activity. Importantly, the complemented *gspD* strain displayed very similar increases in the A410 when compared to the wild type, indicating that the lipase activity in culture supernatants was mainly dependent on the T2SS. Culture supernatants from the *lipA*::kan mutant exhibited an approximately 50% reduction in lipase activity; furthermore, the complemented *lipA* strain regained activity; in fact, culture supernatants from the complemented

lipA strain displayed approximately a 30% increase in lipase activity over the wild type strain. Next we purified culture supernatants from the *lipB*::frt mutant and found that it displayed the same profile as the *lipA* mutant when measuring the A410; however, when we reintroduced the *lipB* gene into the *lipB*:: frt mutant, we observed minimal complementation (Figure 4.5F).

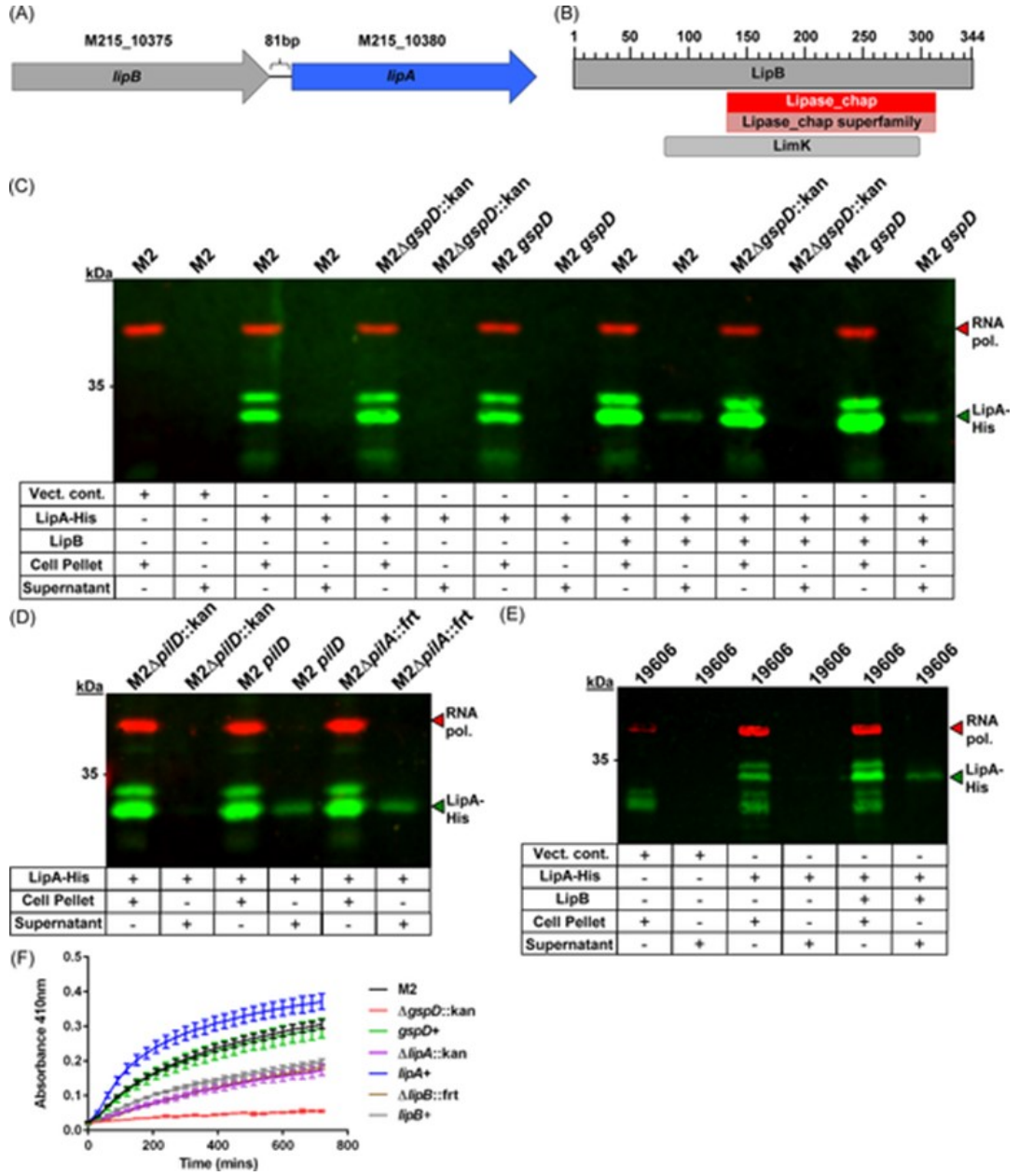


Figure 4.5- Secretion of a LipA Lipase Ortholog was Dependent on the Type II Secretion System and the LipB Chaperone. (A) Gene arrangement of the *lipBA* gene cluster. (B) BLASTp analysis of the LipB amino acid sequence identified multiple domains associated with steric chaperone activity including the LimK domain and a lipase chaperone superfamiliy

domain. (C) Western blot analysis on whole cell lysates and secreted protein fractions probing for LipA-His. All strains and fractions were also analyzed for RNA polymerase expression, which served as a lysis control. LipA-His expression was detected in all strains carrying pWH-*lipA-his* as well as pWH-*lipB-lipA-his*; however, LipA-His secretion was only detected in strains co-expressing the chaperone LipB as well as a predicted functioning T2SS. (D) Secretion of LipA-His was independent of the type IV pilus, as indicated by active secretion in the Δ*pilA*::frt mutant. (E) Western blot analysis on whole cell lysates or secreted protein fractions from *A. baumannii* ATCC 19606 carrying the empty vector, the pWH-*lipA-his* vector, or the pWH-*lipB-lipA-his* vector. LipA-His expression was detected; however, LipA-His secretion was only detected when the *lipB* gene was co-expressed with *lipA-His*. (F) Lipolytic activity of purified culture supernatants from the indicated strains as determined by a modified *p*-NPP assay. Increases in the A_{410} indicate lipolytic activity. A_{410} measurements were recorded every 30 minutes for 12 hours. Three biological replicates with three technical replicates were used for analysis.

4.3.6-The M215_03235 Locus Encodes for a Newly Characterized Lipase, LipH, Which is also Secreted in a Type II Dependent Manner

The 2D-DIGE analysis revealed that the spot corresponding with the M215_03235 protein was associated with an 8.1 fold change when compared to the Δ*gspD*::kan mutant. The M215_03235 gene encodes for a protein containing multiple predicted domains including a LIP domain (pfam03583), a DAP2 domain (COG1506), and two AB hydrolase 5 domains (pfam 12695). Given that all of these domains are associated with predicted lipase/esterase activity, we have designated M215_03235 as *lipH* in order to avoid confusion with previously characterized lipases.

To confirm that LipH was secreted in a T2SS-dependent manner, we utilized a similar approach as described above where we cloned and tagged LipH into pWH1266 with a carboxy-terminal his tag. We then introduced this construct into multiple strains and probed from LipH-His expression and secretion. As seen in Figure 4.6A, LipH-His was detected in whole cell lysates of all strains tested; however, LipH-His was found to only be secreted in strains predicted to express a functional T2SS. We further assessed the ability of a panel of clinical isolates to secrete LipH-His. As shown in Figure 4.6B, LipH-His expression and secretion was detected in all clinical isolates tested.

Because alpha/beta hydrolase domains, such as the one present in LipH, are commonly found in lipases, we verified that LipH has lipolytic activity. We constructed a Δ*lipH*::kan mutant as well as a *lipH* complemented strain and subjected these strains to the *p*-NPP assay utilized above for LipA. As seen in Figure 4.6C, the Δ*lipH*::kan mutant displayed an increase in the A410, indicating lipolytic activity; however, the increase was substantially lower than both the parent strain as well as the *lipH* complemented strain indicating that the LipH protein is a lipase.

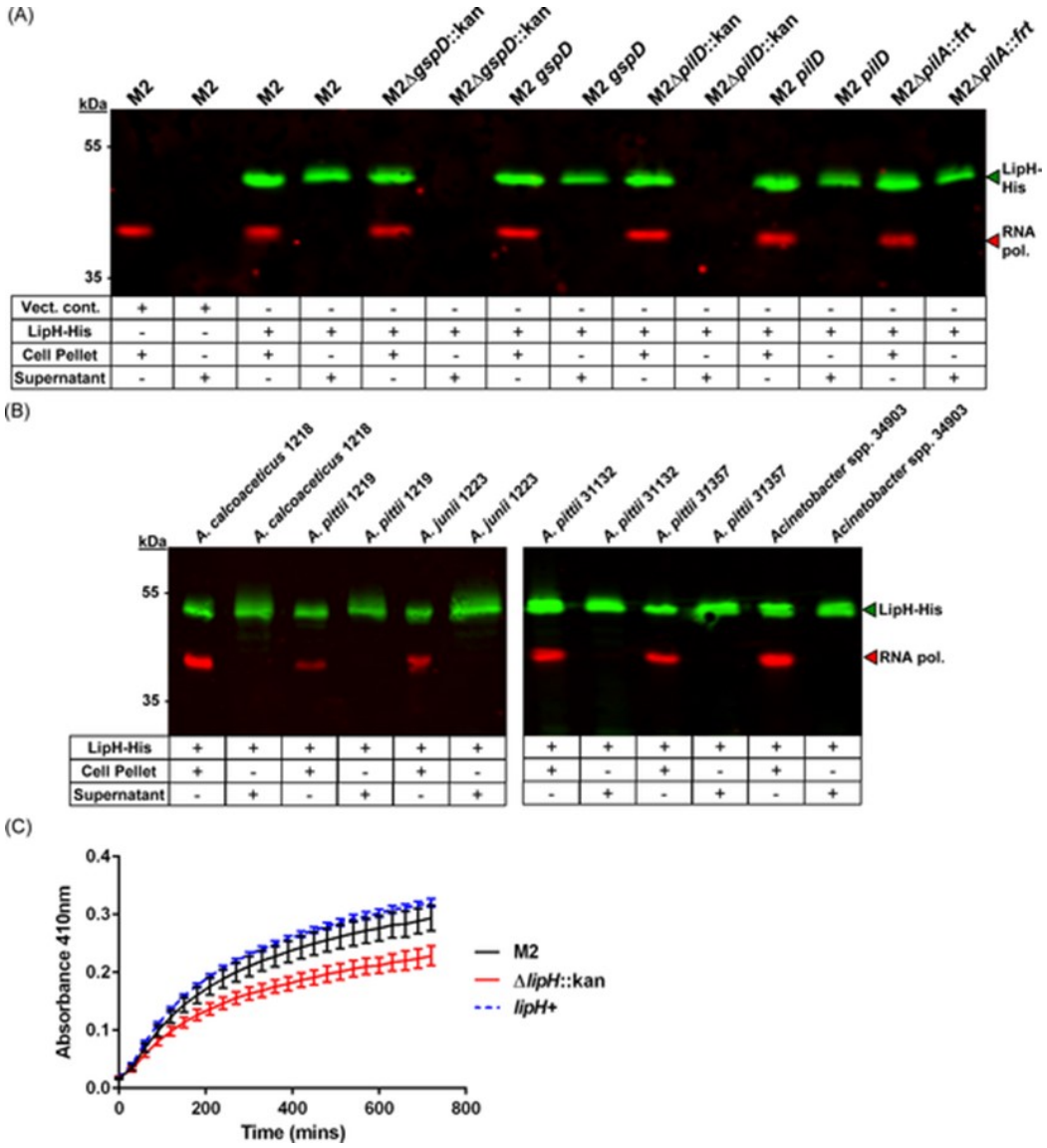


Figure 4.6- The Newly Characterized LipH is Secreted by the T2SS and Displays Lipase Activity. (A) Western blot analysis on whole cell lysates and secreted protein fractions probing for LipH-His. All strains and fractions were also analyzed for RNA polymerase expression, which served as a lysis control. LipH-His expression was detected in all strains carrying the

pWH-*lipH-his*; however, LipH-His secretion was only detected in strains predicted to produce a functioning T2SS. (B) Western blot analysis on whole cell lysates and secreted protein fractions from a range of *Acinetobacter* clinical isolates heterologously expressing LipH-His from the pWH-*lipH-his* plasmid. LipH-His expression and secretion was detected in all clinical isolates tested. (C) Lipase activity of concentrated culture supernatants from the parent strain, the Δ*lipH*::kan mutant, and the *lipH* complemented strain. Increases in the A_{410} indicate lipase activity. A_{410} measurements were recorded every 30 minutes for 12 hours. Three biological replicates with three technical replicates were used for analysis.

4.3.7-The *Acinetobacter* T2SS is Required for Optimal Virulence in the *Galleria mellonella* Model of Infection

The greater wax moth, *Galleria mellonella*, has been routinely used to assess the virulence of *Acinetobacter*(Peleg, A.Y. *et al.*, 2009). Furthermore, strains with attenuated virulence in the *G. mellonella* model have also been shown to have attenuated virulence in murine models of infection (Harding, C.R. *et al.*, 2013). In order to assess the role of the *Acinetobacter* T2SS in the *G. mellonella* model, we first determined the LD_{50} for the wild type *A. nosocomialis* strain M2. Groups of ten larvae were each injected with 10µL of either approximately 10^5, 10^6, or 10^7 total CFU of strain M2, incubated at 37°C for 24 hours, and checked for viability as determined by accumulation of melanin and loss of movement. From these studies, the LD_{50} was determined to be approximately $3X10^6$ CFU and was selected as the inoculation dose for subsequent infections (Appendix 1 Figure S4.1).

The wild type M2, *gspD*::kan mutant, and the *gspD* complemented strain were individually injected into cohorts of *G. mellonella* at the specified dose, incubated at 37°C for 24 hours and checked for viability. As expected, 50% of the larvae injected with either the wild type M2 or complemented *gspD* strain succumbed to the infection (Figure 4.7A); however, only 30% of the larvae injected with the M2Δ*gspD*::kan mutant died after 24 hours. To further demonstrate that the *Acinetobacter* T2SS contributes to the virulence of *Acinetobacter* in the *G. mellonella* model, we injected cohorts of larvae with the pre-determined LD_{50} for the M2Δ*gspD*::kan mutant of 10^7 CFU (Appendix 1 Figure S4.2). As seen in Figure 4.7B, 50% of the larvae injected with M2Δ*gspD*::kan mutant died at the specific dose; however, 80% of the larvae injected with the wild

type M2 died as a result of the infection after 24 hours. Interestingly, almost all of the larvae (~97%) injected with the complemented *gspD* strain died after 24 hours.

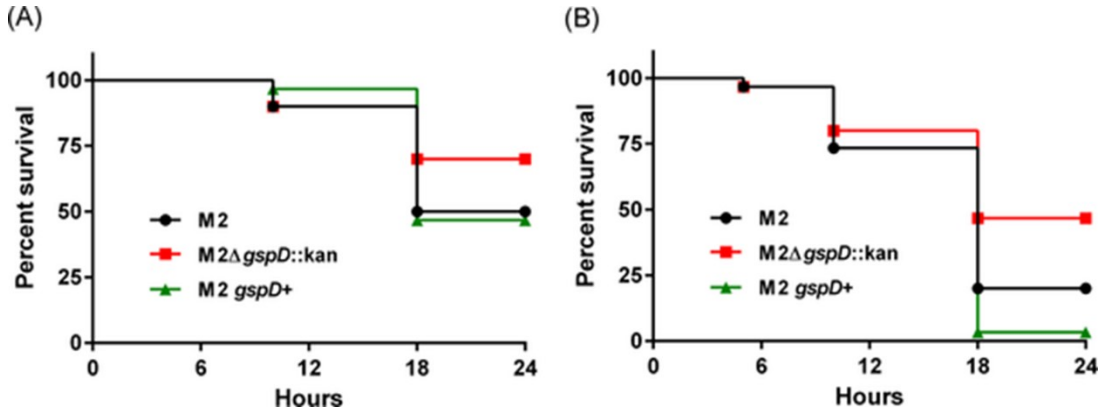


Figure 4.7- The T2SS of *A. nosocomialis* Strain M2 is Required for Optimal Virulence in the *G. mellonella* Infection Model. (A) Groups of *G. mellonella* larvae were injected with 10 µL of either the parent strain, the Δ*gspD*::kan mutant, or the complemented *gspD*::kan strain at an inoculum previously determined to be the equivalent of the LD_{50} for the parent strain. Larvae were checked for viability as determined by melanin accumulation and motility. (B) Groups of *G. mellonella* were injected with 10 µL of either the parent strain, the Δ*gspD*::kan mutant, or the complemented *gspD*::kan strain at an inoculum previously determined to be the LD_{50} for the Δ*gspD*::kan mutant. Larvae were checked for viability as determined by melanin accumulation and motility. Survival curves were determined to be statistically significant using the Mantel-Cox test (P = 0.0147).

4.3.8-The T2SS is Required for Optimal Colonization of both the Lungs and Spleen in a Murine Pulmonary Infection Model

Acinetobacter infections most frequently manifest as pneumonias, specifically, within the mechanically ventilated patient population (McConnell, M.J. *et al.*, 2013). The murine acute pulmonary infection model has therefore been developed to model an active *Acinetobacter* pneumonia clinical presentation. In order to determine a role of the T2SS in *Acinetobacter* virulence, we first constructed a strain of *A. nosocomialis* with an unmarked, in-frame deletion of *gspD*, which encodes for the predicted outer-membrane secretin. Prior to infection studies, we verified that the newly generated M2Δ*gspD*::frt mutant was in fact impaired in secretion of type II effector proteins (Appendix 1 Figure S4.3). Using our previously described murine infection model

(Noto, M.J. *et al.*, 2015), we performed infection experiments with either the wild type *A. nosocomialis* strain M2, the unmarked, isogenic M2Δ*gspD*:: frt mutant, or its respective *gspD* complemented strain. Mice were intranasally inoculated with $1X10^9$ CFU, as we previously determined that inoculating mice with this dose of wild type bacteria resulted in full murine viability, yet, resulted in significant organ-specific bacterial burden (Appendix 1 Figure S4.4). Groups of mice were individually administered an intransal inoculation of either the wild type strain, the Δ*gspD*::frt mutant, or the respective complemented *gspD* strain. Thirty-six hours post-infection, mice were sacrificed and the lungs, spleen, and livers were harvested in order to determine total bacterial burdens. As seen in Figure 4.8A, mice infected with either the wild type strain or the complemented *gspD* strain all had high bacterial burdens in the lungs. Furthermore, bacterial burdens displayed limited variability indicating a full level of complementation for the *gspD* complementation strain. Mice infected with the Δ*gspD*::frt mutant displayed significantly lower bacterial burdens in the lung when compared to either the wild type or complemented *gspD* strain. A similar trend was also observed for bacterial burdens in the spleen, where, mice infected with either the wild type or the complemented *gspD* strain had significantly higher bacterial burdens (Figure 4.8B). We also enumerated bacterial colony forming units from the livers of infected mice and did not observe any significant differences between the cohorts (Figure 4.8C).

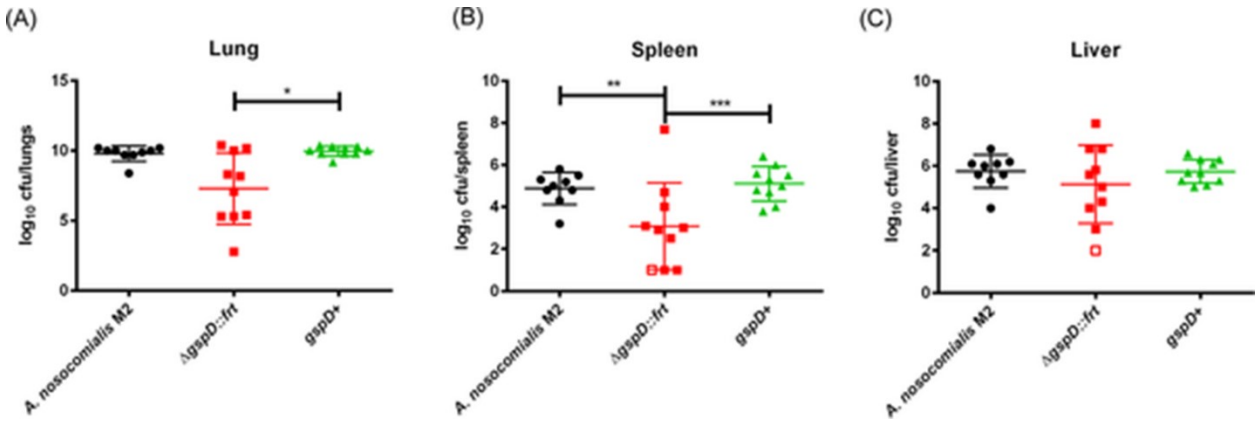


Figure 4.8- *A. nosocomialis* Strain M2 Required its T2SS for Optimal Colonization of both the Lungs and Spleen. Mice were intranasally inoculated with $1X10^9$ CFU of either the wild type, Δ*gspD*::frt mutant, or the *gspD* complemented strain. After 36h post-infection, mice were sacrificed, organs harvested, and CFUs enumerated from homogenized tissue. (A) Total CFUs from the lung demonstrated a statistically significant difference between the complemented *gspD* strain and the Δ*gspD*::frt mutant (Kruskal-Wallis non-parametric with Dunn's multiple

comparison test, ANOVA, *p = 0.0467). (B) Total CFUs from the spleen demonstrated statistically significance comparisons for both comparisons of either the wild type and the Δ*gspD*::frt mutant as well as the complemented *gspD* strain and the Δ*gspD*::frt mutant (One way ANOVA, **p = 0.0230, ***p = 0.0078). Open boxes indicate CFUs that were below the limit of detection. (C) Total CFUs form the liver did not demonstrate any statistically significant differences. Open boxes indicate CFUs that were below the limit of detection.

4.3.9-LipB and CpaB Belong to a Distinct Class of Membrane-bound T2SS Chaperones Found in Gram-negative Bacteria

We have shown that two of the three secreted type II effectors identified in *A. nosocomialis* strain M2 require specific chaperones for secretion. To date only the lipase-specific foldases (Lifs) have been characterized as chaperones for type II effectors (Kok, R.G. *et al.*, 1995b; Frenken, L.G. *et al.*, 1993a; Frenken, L.G. *et al.*, 1993b; Ogierman, M.A. *et al.*, 1997). Indeed, a complex of the *B. glumae* LipA/Lif has been crystallized (Pauwels, K. *et al.*, 2006). The Lifs are unique steric chaperones, which have an N-terminal membrane-anchor and a C-terminal domain that facilitates proper folding of their cognate lipase upon entry into the periplasm (El Khattabi, M. *et al.*, 2000). Furthermore, the first characterization of a chaperone participating in the secretion of a type II secreted protein from *Acinetobacter* was described in 1995. These authors demonstrated that a lipase specific chaperone, designated LipB, was required for secretion of the LipA lipase. They found that the C-terminal domain of the LipB chaperone was located outside of the cytoplasm. Lastly, in contrast to what had been previously found in *Pseudomonas* strains, the authors found that *lipB* was actually encoded upstream of *lipA* (Kok, R.G. *et al.*, 1995a; Kok, R.G. *et al.*, 1995b). We have expanded upon this paradigm with the identification of a novel protease/chaperone pair (CpaA/B). Furthermore, we hypothesized this phenomenon to be more widespread. In order to identify putative chaperones of type II secreted proteins, we first searched for open reading frames (ORFs) encoded adjacently to known type II effectors that were predicted to be part of the same operon. We then narrowed our search to ORFs that encode for proteins with a predicted N-terminal transmembrane domain as this feature is shared both by the Lifs and the newly characterized CpaB chaperone. As found in Table 4.3, we were able to identify several putative chaperones of type II effectors in diverse Gram-negative bacteria such as *V. cholerae*, *P. aeruginosa*, and *B. pseudomallei*, which suggests that CpaB, LipB, and Lifs belong to a family of membrane-bound

chaperones involved in T2SS secretion.

Chaperone/ PutativeChaperone	Genus, species, strain	Type II effector/Function	Nucleotide Separation	Transmembrane Helix	Reference
LipB(M215_10375)	*Acinetobacter nosocomialis* M2	LipA (M215_10380)/Lipase	81bp	7–29	This study
CpaB (M215_05105)	*Acinetobacter nosocomialis* M2	CpaA (M215_05100)/Protease	22bp	7–26	This study
Hypothetical (M215_03240)	*Acinetobacter nosocomialis* M2	LipH (M215_03235)/Lipase	62bp	26–48	This study
LipB (ACIAD3308)	*Acinetobacter baylyi* ADP1	LipA (ACIAD3309)/Lipase	132bp	7–26	[51, 52]
LipB (bglu_2g7740)	*Burkholderia glumae* BGR1	LipA (bglu_2g7730)/Lipase	-1bp	21–40	[63, 64]
Hypothetical (BURPS668_3453)	*Burkholderia pseudomallei* 668	BURPS688_3454/Peptidase S10, serine carboxypeptidase	77bp	7–26	[68]
Hypothetical (BURPS668_1220)	*Burkholderia pseudomallei* 668	BURPS688_1221/ Pectinacetylesterase	62bp	7–24	[68]
Hypothetical (BURPS668_0360)	*Burkholderia pseudomallei* 668	BURPS688_0358/Nonhemolytic phospholipase C	46bp	7–24	[68]
LipB* (PA2863)	*Pseudomonas aeruginosa* PAO1	LipA (PA2862)/Lipase	-17bp	26–44	[69–71]
LipB* (PA2863)	*Pseudomonas aeruginosa* PAO1	LipC (PA4813)/Lipase	2,185,726bp	26–44	[72]
Hypothetical (PA2872)	*Pseudomonas aeruginosa* PAO1	Mep72 (PA2783)/Protease	77bp	5–24	[73]
Hypothetical (A5E_A0255)	*Vibrio cholerae* B33	PrtV (A5E_A0254)/Protease	84bp	5–27	[74]
LipB (VCA0222)	*Vibrio cholera* N16961	LipA (VCA0221)/Lipase	9bp	7–26	[75]

doi:10.1371/journal.ppat.1005391.t001

Table 4-3- Known and Putative type II Chaperones. Open reading frames of unknown function within the same operon as known type II effectors were analyzed for N-terminal transmembrane helices. Transmembrane domains were predicted using the TMHMM Server v. 2.0. *The *lipB* annotation in *P. aeruginosa* PAO1 is truncated, excluding the N-terminal sequence. Here we have included the putative full length open reading frame for analysis.

4.4-Discussion

Acinetobacter spp. have rapidly emerged as significant opportunistic pathogens afflicting healthcare facilities worldwide. Although sophisticated studies track the epidemiology of outbreaks worldwide, our collective understanding of the molecular mechanisms employed by *Acinetobacter* spp. to cause disease is in its infancy. In this work, we combined bioinformatics, proteomics, mutational analyses, and virulence assays to demonstrate that *Acinetobacter* spp. produce a functional T2SS, which is required for the secretion of multiple proteins that are required for full virulence. Importantly, this is the first *bona fide* secretion system required for virulence in a mammalian model identified in *Acinetobacter*. Notably, two of the three secreted proteins characterized in this study require dedicated chaperones for type II secretion. While this paper was under revision, an article reporting the presence of a functioning T2SS in *A. baumannii* ATCC 17978 was published (Johnson, T.L. *et al.*, 2015). In this work, it was found

that 17978 also required a T2SS for the secretion of the LipA lipase and growth on minimal media with olive oil as the sole carbon source. It was also found that both the 17978Δ*gspD* and 17978Δ*lipA* mutants were less fit in a murine septicemia model when competed against the parental strain.

Typically, T2SSs secrete as many as 18–25 proteins and facilitate the delivery of major viru- lence factors to the extracellular environment for many important human pathogens, such as *Legionella pneumophila* and *V*. *cholera* (Sikora, A.E. *et al.*, 2011; Cianciotto, N.P., 2009). Here, we utilized the 2D-DIGE method coupled with mutational analyses to characterize the type II secretome for *A*. *nosocomialis* strain M2. Our analysis identified over 60 spots with a 4-fold difference when comparing the wild type M2 vs. M2Δ*gspD*::kan mutant; however, we concentrated our efforts on three proteins that contain domains of known functions. Other studies will be needed to determine the role of the remaining type 2 effector candidates and of individual secreted proteins in *Acinetobacter* pathobiology given the importance of this system in virulence.

The genetic architecture of T2SSs usually consists of between 12 and 15 genes, most of which appear to be organized in a single operon (Sandkvist, M., 2001). From a regulatory standpoint, the single operon arrangement of T2SS associated genes would seem to be the simplest to transcriptional control. However, as noted above, the T2SS associated genes from *A*. *nosocomialis* strain M2 are found in five distinct genetic loci, a genetic arrangement that resembles the type IVa pilus system (Pelicic, V., 2008). Furthermore, this dispersed genetic arrangement is highly conserved across different *Acinetobacter* species, including the pathogenic species *A*. *baumannii* and the non-pathogenic species *A*. *baylyi*. Closer examination of each T2SS gene cluster does not provide any obvious insights into the regulatory mechanisms as some T2SS genes appear to be in putative operons with other genes not known to be associated with T2SSs. Outside of the genus *Acinetobacter* the same genetic architecture can also be found in bacteria from the genus *Psychrobacter*.

As demonstrated previously, the prepilin peptidase PilD was required for major pilin processing and proper functionality of T4P in *A*. *nosocomialis* strain M2 (Harding, C.M. *et al.*, 2013). Our current data demonstrated that PilD is also required for processing of the predicted major pseudopilin, GspG, and thus secretion of T2S substrates. Given the strong evolutionary relatedness between the T4P system and the T2SS, the phenomenon of sharing protein components between two functionally distinct systems does not seem impractical, nevertheless, it

is uncommon. To date only *D. nodosus* (Han, X. *et al.*, 2007), *P. aeruginosa* (Nunn, D.N. and Lory, S., 1992), *V. cholerae* (Marsh, J.W. and Taylor, R.K., 1998), and *L. pneumophila* (Liles, M.R. *et al.*, 1999; Liles, M.R. *et al.*, 1998) have been demonstrated to share a prepilin peptidase between both the T4P system and a T2SS. Of the three type II effectors studied, only LipA has previously characterized orthologs, which were primarily described in *Pseudomonas* and also require a chaperone (El Khattabi, M. *et al.*, 1999). However, to date, none of these lipases have been connected to pathogenesis. We demonstrated that the LipA lipase was responsible for approximately half of the lipase activity observed from the secreted fraction of the wild type strain M2. As expected, LipA activity was also dependent on the LipB chaperone, as supernatants from the Δ*lipB*::frt mutant displayed nearly identical lipase activity levels as the Δ*lipA*::kan mutation. However, our *lipB* complemented strain only marginally increased the lipase activity of the Δ*lipB*::frt mutant, indicating that even though we constructed an in-frame, unmarked mutation in the *lipB* gene, we may still be observing polar effects on *lipA* transcription. The *lipA* gene is 81bp downstream of the *lipB* gene and therefore could potentially have its own promoter that is partially contained within the 3' region of the *lipB* gene. We and others have observed similar cryptic promoter events during previous studies of the *pilTU* gene cluster, where an in-frame, unmarked mutation of *pilT* still had polar effects on *pilU* expression (Harding, C.M. *et al.*, 2013; Whitchurch, C.B. and Mattick, J.S., 1994).

Even in the absence of *lipA*, culture supernatants retained residual lipase activity as compared to the *gspD* mutant strain. As such, we found that LipH mediated lipase activity of culture supernatants as well. A BLASTp search of LipH orthologs outside of *Acinetobacter* identified similar proteins found in bacteria from the genus *Myriodes*, some of which act as opportunistic human pathogens (Maraki, S. *et al.*, 2012), as well as bacteria from the genus *Bacillus*; however, none of those orthologs have been characterized.

Using *A. nosocomialis* strain M2 as our model system we demonstrated that LipH secretion was indeed dependent on a functional T2SS. We also demonstrated that T2SS is conserved and functional across *Acinetobacter* spp. via immunoblotting of epitope tagged effectors. Specifically, we showed that LipH from M2 was secreted by a panel of *Acinetobacter* clinical isolates, including, *A. calcoaceticus*, *A. baumannii*, *A. pittii*, *and A. junnii*. We also demonstrated that *A. baumannii* ATCC 19606 could secrete both LipA and CpaA; however, as expected the respective chaperones for each protein were required for active secretion. These data strongly suggest the presence of a functional T2SS in the majority of medically relevant *Acinetobacter spp*.

This hypothesis is further supported by the fact that genes predicted to encode proteins required for the biogenesis of the T2SS are highly conserved and distributed amongst *Acinetobacter* spp.

The remaining effector characterized in our study was the CpaA metallopeptidase. CpaA was previously purified from culture supernatants (Tilley, D. *et al.*, 2014); however, its mechanism of secretion was not determined. It was previously shown that CpaA is involved in degradation of Factor V and fibrinogen, which would result in a decrease in clotting activity. Here, we demonstrated that CpaA was secreted in abundance in a type II dependent manner, yet, was also dependent on a novel chaperone, designated CpaB. CpaB is the first characterized T2SS chaperone devoted to the secretion of a protease. Topological modeling of the CpaB chaperone predicts a single N-terminal transmembrane domain with the majority of the protein exposed to the periplasm (Sonnhammer, E.L. *et al.*, 1998; Krogh, A. *et al.*, 2001). The periplasmic exposed C-terminal domain of CpaB was predicted by DELTA BLASTp to contain a domain from the SRPBCC superfamily present in the co-chaperone eukaryotic protein Aha1, the activator of Hsp90 complex (Lotz, G.P. *et al.*, 2003). The SRPBCC domains are predicted to have deep hydrophobic ligand binding pockets. A BLASTp search of CpaB orthologs outside of *Acinetobacter* only identified two weak orthologs from *Lysobacter antibioticus*; however, a DELTA BLASTp search for CpaB orthologs outside of *Acinetobacter* primarily identified Aha1 as the closet ortholog, suggesting a possible eukaryotic ancestry. Currently, we hypothesize that the CpaA metallopeptidase is trafficked through the Sec system, as is the case for most type II secreted substrates. There, CpaA can interact with CpaB as CpaB is predicted to contain a single transmembrane domain with the majority of the protein exposed to the periplasmic space. Upon entry into the periplasmic space of CpaA from the Sec system, CpaB could facilitate proper folding of CpaA due to the requirement of type II secretion systems for competently folded proteins for active secretion.

The potential role of the CpaA metallopeptidase in *Acinetobacter* pathogenesis and evolution is quite intriguing. Firstly, the type strains *A. baumannii* ATCC 17978 and 19606, two of the more primitive *Acinetobacter* spp. used as model organisms do not contain orthologs of the CpaAB system, indicating a horizontal acquisition event within the last 70 years. Analysis of the GC content of the *cpaAB* locus and the surrounding DNA support this hypothesis. It is tempting to speculate, that given the predicted recent acquisition of the CpaAB protease/chaperone system and the role of the T2SS in *Acinetobacter* virulence, CpaA may be one of the major virulence

factors of some pathogenic *Acinetobacter* spp. Future work will be aimed at deciphering the role of CpaA in the virulence assays utilized within this study.

As mentioned above, LipB and CpaB act as specific chaperones for LipA and CpaA respectively. Some effectors secreted via a type III secretion system (T3SS) also require specific chaperones that have collectively been named "T3SS chaperones" (Feldman, M.F. and Cornelis, G.R., 2003). T3SS chaperones do not present sequence similarity, but they are easily identified because they are encoded next to their cognate effector and most of them contain similar molecular weight and isoelectric points. Similarly, we define a "T2SS chaperone" as a protein encoded adjacently and co-regulated with a type II effector, that contains both an N-terminal transmembrane domain, and an exposed C-terminal region to the periplasm, and that is required for secretion of the cognate effectors. We identified "type II chaperones" in multiple Gram-negative species. Interestingly, LipB from *Pseudomonas aeruginosa* is a previously characterized chaperone that serves two T2SS effectors, LipA which is encoded next to LipB as well as LipC, which is encoded more than 2 Mb away (Martinez, A. *et al.*, 1999). This indicates that the T2SS chaperones family may be more widespread than we propose here.

We determined that the *Acinetobacter* T2SS was required for virulence. We first determined that the mutants unable to produce a functioning T2SS were attenuated in the *G. mellonella* infection model. Given the high level of concordance between mutants attenuated in the *G. mellonella* model and mammalian models, we hypothesized a more relevant *in vivo* role for the *Acinetobacter* T2SS. We thus choose to investigate the role of T2S in a murine pulmonary infection model. Specifically, we observed high CFUs for the wild-type strain in the lungs after 36h infection period and also observed dissemination to both the liver and spleen. Using an unmarked, in-frame deletion of *gspD* strain and its respective complemented strain, we were able to demonstrate that the T2SS was indeed required for optimal colonization of both the lungs and spleen, but not the liver. Remarkably, we observed almost a two-log decrease in CFUs in the lungs and spleen of mice infected with the *gspD* mutant strain when compared to either the wild type or the complemented strain. Many studies focusing on *Acinetobacter* pathobiology have utilized a similar murine pneumonia model of infection and also observed differences of around 2 logs; however, these mutants had defects in two-component regulatory systems, metabolism, and/or stress responses, all of which could have more pronounced global effects on *Acinetobacter* biology that mediate defects in colonization (Wang, N. *et al.*, 2014; Elhosseiny, N.M. *et al.*, 2015).

Herein, we have provided evidence of both a functional T2SS in many *Acinetobacter* spp. as well as demonstrated its importance in *Acinetobacter* pathogenicity. However, the exact role for each T2S effector proteins in *Acinetobacter* pathogenicity has yet to be determined. As such we plan to next probe the role of specific effectors in mediating the colonization phenotypes observed, with an emphasis on the most highly secreted protein, the CpaA metallopeptidase. Furthermore, our study highlights the use of other clinically relevant members of the genus *Acinetobacter* outside of *A*. *baumannii* in order to gain insights into the pathogenesis of clinically relevant *Acb* members. Although type strains like *A*. *baumannii* ATCC 17978 and 19606 have served well as model strains for *Acinetobacter* pathogenicity, their relative old age makes them less representative of current epidemic strains, which contain more antibiotic resistance cassettes and possibly novel virulence attributes.

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Chapter 5-Characterizing Type II Substrate CpaA: Its role in Pathogenesis and interaction with CpaB

The data presented in this chapter contains some collaborative work. In collaboration with Dr. N.H. Tolia we have crystallized CpaA-CpaB. I cloned, expressed and purified CpaA-CpaB. A member of the Tolia lab, N. Salinas, further purified CpaA-CpaB by FPLC. Under the guidance of N. Salinas, I set up 96-well mosquito nanoliter protein crystallization screens and microliter grid-screens. N. Salinas and Dr. Darya Urusova, members of the Tolia lab, looped my CpaA-CpaB crystals and analyzed them by X-ray diffraction. L.D. Palmer and E.P. Skaar performed the murine pulmonary infection to analyze the role of CpaA in mouse colonization. All other work presented in this chapter was performed by me under the guidance of Dr. Feldman. Writing of this chapter is my original work. Part of the introduction is included in the section of a protein secretion review I wrote: Weber BS, Kinsella RL, Harding CM and MF Feldman. (2017) The secrets of *Acinetobacter* Secretion. *Trends Microbiol* **S0966-842X (17)**:30017-3.

5.1-Introduction:

Bacterial protein secretion is one way cells interact with and impact their environment. The type II secretion system (T2SS) is widespread among Gram-negative pathogens as well as environmental species capable of living in a variety of conditions (Nivaskumar, M. and Francetic, O., 2014; Sandkvist, M., 2001a; Sandkvist, M., 2001b). The T2SS is a two-step process, where proteins with an N-terminal secretion signal are translocated across the inner membrane by the general secretory pathway (Sec) or the Twin-arginine (Tat) system to the periplasmic space (Sandkvist, M., 2001a; Sandkvist, M., 2001b). Following removal of the secretion signal, folded proteins are then secreted to the extracellular space by the T2SS machinery. The T2SS promotes *in vivo* survival and virulence of several pathogens, including *Vibrio cholerae,* enterotoxigenic *Escherichia coli*, *Pseudomonas aeruginosa* and *Legionella pneumophila,* through the activities of secreted toxins and hydrolytic enzymes aiding in nutrient acquisition (DebRoy, S. *et al.*, 2006; McCoy-Simandle, K. *et al.*, 2011; Sandkvist, M. *et al.*, 1993; Sikora, A.E. *et al.*, 2011; Ho, T.D. *et al.*, 2008; Baldi, D.L. *et al.*, 2012; Jyot, J. *et al.*, 2011).

Eijkelkamp *et al.* were the first to report the presence of T2SS components in *A. baumannii* (Eijkelkamp, B.A. *et al.*, 2014). Subsequently, the T2SS of *A. baumannii* ATCC 17978, a commonly used laboratory strain, was shown to be active (Johnson, T.L. *et al.*, 2015a) Type II dependent secretion of LipA was required for growth in minimal media supplemented with long-chain fatty acids as the sole carbon source, indicating an important role in nutrient acquisition (Johnson, T.L. *et al.*, 2015a) Furthermore, the T2SS and T2SS-dependent lipid utilization were required for competitive colonization of a neutropenic murine model of infection (Johnson, T.L. *et al.*, 2015a).

Harding *et al.* demonstrated T2SS activity in *Acinetobacter* strains *A. nosocomialis* M2 (formerly *A. baumannii* M2), *A. pittii, A. calcoaceticus,* and *A. junii* as well as *A. baumannii,* through heterologous expression and secretion of *A. nosocomialis* T2SS effectors (Harding, C.M. *et al.*, 2016a). Proteomic comparison of supernatants from wildtype *A. nosocomialis* M2 and an outer membrane secretin *gspD* mutant revealed numerous putative effector proteins, all with predicted N-terminal Sec signals (Harding, C.M. *et al.*, 2016a). Three of these effectors, CpaA, LipA and LipH, were confirmed as T2SS effectors, and LipA and LipH were confirmed as lipases through their ability to cleave *para*-nitrophenol palmitate. CpaA was previously shown to

be a secreted zinc-dependent metallo-endopeptidase that was capable of degrading fibrinogen and factor V, deregulating blood coagulation (Tilley, D. *et al.*, 2014). The T2SS of *A. nosocomialis* was required for full virulence in *Galleria mellonella* and pulmonary murine models of infection (Harding, C.M. *et al.*, 2016a). Additionally, the T2SS-deficient strain did not disseminate to the liver or spleen as efficiently as the wildtype or complemented strains, suggesting it plays an important role in virulence. The specific impact of CpaA and LipH in virulence is yet to be elucidated.

Two *Acinetobacter* T2SS effectors, LipA and CpaA, were found to require membrane bound chaperones, LipB and CpaB respectively, for their secretion (Harding, C.M. *et al.*, 2016a). Topological prediction servers and bioinformatic analysis of these membrane bound chaperones suggested that their N-terminal transmembrane domains are imbedded in the inner membrane, with the C-terminal globular portions present in the periplasm. The requirement of T2SS chaperones is reminiscent of type III secretion systems, where the chaperones are soluble cytoplasmic proteins that interact with a specific effector or multiple effectors and aid in folding, stabilization, and/or regulating secretion of these effectors (Page, A.L. and Parsot, C., 2002). CpaB and LipB appear to be specific for CpaA and LipA, as neither was required for the secretion of LipH by various *Acinetobacter* species (Harding, C.M. *et al.*, 2016a). LipB is functionally similar to the *Burkholderia glumae* membrane bound chaperone lipase-specific foldase (Lif), which is required for production of enzymatically active lipase. The transmembrane domain of Lif is not required for its steric chaperone activity (Frenken, L.G. *et al.*, 1993; Rosenau, F. *et al.*, 2004; El Khattabi, M. *et al.*, 1999).

CpaB is the first membrane bound chaperone required for secretion of a T2SS protease. There are other type II metalloproteases that require chaperones to become active and be secreted (Bever, R.A. and Iglewski, B.H., 1988). In *Pseudomonas aeruginosa* type II metalloprotease, elastase, is produced as a pre-pro-elastase, where the pro-domain functions as an intramolecular chaperone (Kessler, E., *et al.*, 1992, Shinde, U. and Inouye, M., 2000). The N-terminal sec secretion signal is removed upon translocation into the periplasmic space, leaving inactive pro-elastase in the periplasm (Kessler, E. and Safrin, M., 1994). The pro-domain has chaperone like function as it is required for secretion of elastase and is required for production of active elastase (McIver, K.S., *et al.*, 1995). The type II apparatus only secretes fully folded protein substrates. The pro-domain complexed with elastase blocks elastase activity (Kessler, E. and Safrin, M.,

1994). The elastase pro-domain dissociates from the mature elastase upon secretion. The mechanism by which CpaB and LipB impact secretion of their cognate substrates is unknown.

Using a combination of mutagenesis, biochemistry and protein crystallography methods we investigated the role of CpaA in *A. nosocomialis* virulence, and characterized the interaction between CpaA and its membrane bound chaperone CpaB. Deletion of *cpaA* results in a virulence defect in *G. mellonella* larvae and decreased splenic colonization in a murine pulmonary infection model. CpaA and CpaB physically interact. The C-terminal globular domain of CpaB is periplasmically exposed and is sufficient for secretion of CpaA. The periplasmic domain of CpaB interacts with CpaA at multiple contact points spanning the length of the protein, rather than a specific chaperone binding domain. We have determined protein crystallization conditions for the CpaA-CpaB complex and have obtained diffraction data sets ranging from 3.5 to 7 angstrom resolution.

5.2-Materials and Methods:

5.2.1-Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study can be found in Table 5-1. All strains were grown in LB broth/agar at 37ºC unless specified elsewhere. Antibiotics for *E. coli* selection were used at the following concentrations: 100 µg/mL carbenicillin, 5 µg/mL tetracycline, 12.5 µg/mL chloramphenicol and 20 µg/mL kanamycin. Antibiotics for *A. nosocomialis* were used at the following concentrations: 20 µg/mL kanamycin, 12.5 µg/mL chloramphenicol, 5 µg/mL tetracycline and 200 µg/mL carbenicillin. To select for the loss of *sacB* supplementation with 10% sucrose was used.

5.2.2-Generation of Bacterial Mutants and Complemented Mutants

Unmarked mutations were made using a previously published method (Harding, C.M. *et al.*, 2015a; Carruthers, M.D. *et al.*, 2013a). The In-Fusion HD EcoDry cloning kit was used in the creation of pGEM-*cpaA::kansacB* and pGEM*cpaAB::kansacB* knockout plasmids similarly to (Harding, C.M. *et al.*, 2015a). Briefly, 1000bp upstream of the target gene was amplified with primers 5510015bpkmsacBrev and 5510015bppgem1fw (or, cpaABKOup15bppgemfw2 and cpaABKOup15bpkmsacBrev1), which produced a product with 15 bp of homology to pGEM at the 5' end and 15bp of homology to *kmsacB* at the 3' end. 1000 bp downstream of the target gene were amplified with primers 3510015bpkmsacB2fw and 3510015bppgem2rev (or,

cpaABKOdw15bppgemrev4 and cpaABKOdw15bpkmsacBfw3), which produced a product with 15bp of homology to pGEM at the 3' end and 15bp of homology to *kmsacB* at the 5' end. The kanamycin resistance cassette and *sacB* gene were amplified using primers Kmsacb2rev and Kmsacb1fw. pGEM was amplified using primers pgem1rev and pgem2fw. The In-Fusion HD EcoDry cloning kit was used to fuse the upstream, downstream, kmsacB and pGEM fragments together, generating pGEM-*cpaA::kmsacB* or pGEM-*cpaAB::kmsacB*. These plasmids were introduced to *A. nosocomialis* through natural transformation (Harding, C.M. *et al.*, 2013). FLP recombinase plasmid, pFLP2, was transiently introduced into M2 *cpaA::kmsacB* and M2 *cpaAB::kmsacB* by tri-parental mating (Carruthers, M.D. *et al.*, 2013a) to replace the resistance cassette with an frt scar. Strains Δ*cpaA* and Δ*cpaAB* were verified by PCR and sequencing. Mutations were complemented with the *cpaAB* locus (amplified with primers kpnIcpaABrevlocus and pstIcpaABfwlocus and cloned into pSMART at KpnI and PstI) under its natural putative promoter using the miniTn7 described in (Harding, C.M. *et al.*, 2015b). MiniTn7 complementation construct, pSMART-*pcpaAB* was introduced into Δ*cpaA* and Δ*cpaAB* through natural transformation as above. Primers can be found in Table 5-2.

5.2.3-Generation of Polyclonal Rabbit sera Against CpaA

The pETDuet vector was used to over-express CpaA and CpaB in the cytoplasm of *E. coli* Rosetta 2 cells for purification. 1 L of LB was inoculated from an overnight culture of Rosetta 2/pETDUET-CpaA-His-CpaB at 0.05 OD/mL, grown to mid-log phase, and induced with 1mM IPTG. The culture was grown for an additional 4 hours. Cells were harvested at 8000 rpm for 10 minutes. Cells were washed with 30 mM Tris pH8 and resuspended in 40 mLs of 50 mM NaH_2PO_4 300 mM NaCl and 10 mM imidazole pH8. Cells were lysed with a cell disruptor using two rounds at 35kpsi (Constant System ltd., Kennesaw, GA). Cell lysates were clarified at 11 000 rpm for 30 minutes. Cell lysates were passed over a nickel-NTA agarose column (Gold Bio, St. Louis, MO). The load fraction is the total cell lysate. The flow-through was collected as what passed through the column and did not bind the nickel-NTA resin. The column was washed with 20 mL of 50 mM NaH_2PO_4 300 mM NaCl and 25 mM imidazole pH8 and 20 mL of 50 mM NaH_2PO_4 300 mM NaCl and 50 mM imidazole pH8 (Bornhorst, J.A. and Falke, J.J., 2000). Proteins were eluted with 50 mM NaH_2PO_4 300 mM NaCl and 250 mM imidazole pH8. Elution fractions were analyzed by SDS-PAGE analysis and coomassie staining. The polyacrylamide gel

band corresponding to CpaA-His was sent to Abore Inc. (Ramona, California, USA) for peptide extraction and development of rabbit-derived polyclonal antibodies.

5.2.4-Generation of pWH1266 *cpaA cpaB* Constructs

The In-Fusion EcoDry cloning kit was used to create the following contructs, pWH-*pcpaA-flag-cpaB-his*, pWH-*pCpaA-flag-cpaB*, pWH-p*cpaB-his* and pWH-p$cpaB_{pelB}$-*his.* pWH-*pcpaA-flag-cpaB-his* was created by fusing *cpaA-flag* (amplified with primers 5cpaAFLAGrev and 3cpaApromfwpwh1), pWH1266 (amplified with 2pwhrev1 and 1pwhfw2) and *cpaB-his* (amplified with primers 8CpaBHisrevpwh and 11cpaBfw15bpcpaAF). pWH-*pcpaA-flag-cpaB* was created by fusing *cpaA-flag* (amplified with primers 5cpaAFLAG rev and 3cpaApromfwpwh1) to pWH1266 (amplified with 2pwhrev1 and 1pwhfw2) and *cpaB* (amplified with CpaBrevNT15bppwh2 and 11cpaBfw15bpcpaAF). pWH-*pcpaB-his* was created by fusing the putative promoter of *cpaAB* (amplified with primers 3cpaApromfwpwh1 and 6cpaABpromrev), pWH1266 and *cpaB-his*. pWH-$pcpaB_{pelB}$-*his* was created by fusing pWH1266 to the *cpaAB* promoter to $cpaB_{pelB}$-*his*, where the N-terminal transmembrane domain has been replaced with an N-terminal Sec secretion signal of PelB, (amplified with 8CpaBHisrevpwh and 9CpaBnoTMpelB15bppromfw). All constructs were verified by PCR and sequencing.

5.2.5-Generation of pBAV-*cpaA-flag*. *CpaA-flag* was PCR amplified with CpaArevHisPstI and CpaAfwBamH1 and cloned into pBAVMCS at PstI and BamH1. pBAV-*cpaA-flag* was confirmed by PCR and sequencing.

5.2.6-Nickel-NTA Affinity Purification of CpaB-His and CpaA-FLAG. The pETDuet vector was used to over-express CpaA and CpaB in the cytoplasm of *E. coli* Rosetta 2 cells for purification and protein crystallization studies. *CpaB* was PCR amplified with CpaBcytorevKpnI and CpaBcytofwduetNdeI, and cloned into mulitiple cloning site 2 at KpnI and NdeI. *CpaA* was cloned into pETDuet using the In-Fusion HD EcoDry cloning kit using petDuetfwinfuse and petDuetrevinfuse to amplify pETDuet-*cpaB* and CpaAcytofwduet15bp and CpaAcytoHisrevduet15bp to amplify *cpaA* with 15bp of homology to pETDuet. The pETDuet-*cpaA-his-cpaB* was confirme through PCR and sequencing.

50 mL cultures of M2 Δ*cpaAB* carrying pWH-*pCpaA-flag-cpaB-his,* pWH-*pCpaA-flag-cpaB* or pWH1266 was grown overnight. Cells were pelleted at 10 000 x g for 10 minutes. Cells were washed with 30 mM Tris pH8 and resuspended in 15 mLs of 50 mM NaH_2PO_4 300 mM

NaCl and 10 mM imidazole pH8. Cells were lysed with a cell disruptor using two rounds at 35kpsi (Constant System ltd., Kennesaw, GA). Cell lysates were incubated with 0.05% triton X-100, rolling at 4ºC for 1 hour to solubilize CpaB-His (Schnaitman CA 1971). Cell lysates were clarified at 11 000 rpm for 30 minutes. Cell lysates were passed over a nickel-NTA agarose column (Gold Bio, St. Louis, MO). The load fraction is the total cell lysate. The flow-through was collected as what passed through the column and did not bind the nickel-NTA resin. The column was washed with 20 mL of 50 mM NaH_2PO_4 300 mM NaCl and 25 mM imidazole pH8 and 20 mL of 50 mM NaH_2PO_4 300 mM NaCl and 50 mM imidazole pH8 (Bornhorst, J.A. and Falke, J.J., 2000). Proteins were eluted with 50 mM NaH_2PO_4 300 mM NaCl and 250 mM imidazole pH8. Load, flow-through, wash and elution fractions were analyzed by Western blot analysis probing for CpaA-FLAG and CpaB-His.

5.2.7-CpaB-His Localization and Limited Proteolysis of *A. nosocomialis* M2 Spheroplasts

Overnight cultures of *A. nosocomialis* M2 carrying pWH-*pcpaB-his* or pWH-*pcpaB*$_{pelB}$-*his* were used to inoculate 50 mL LB broth supplemented with 5 µg/mL tetracycline that were grown at 37ºC 225 rpm. Periplasmic and spheroplast preparations were done as previously reported (Feldman, M.F. *et al.*, 2005). Briefly, cultures grown to 0.5 OD/ml were pelleted by centrifugation at 10 000 rpm for 10 minutes. Cells were resuspended at 1 OD/50µL in 20% sucrose/30 mM Tris-HCl (pH 8.0)/1 mM EDTA/1 mg/mL lysozyme (GoldBio) and incubated on ice for 2 hours. Spheroplasts were pelleted at 16 000 x g for 5 minutes. The supernant fraction was considered the periplasmic fraction. For CpaB-His localization periplasmic and spheroplast fractions were analyzed by SDS-PAGE and immunoblotting. Proteolysis of spheroplasts was performed as follows. Spheroplasts were resuspended at 0.01 OD/µL in 0.5 mg/mL proteinase K (Sigma) in 30 mM Tris-HCl (pH8.0) and incubated at 56ºC for 0 to 20 minutes. Protease activity was stopped by the addition Phenylmethylsulfonyl fluoride and 4X laemmlies buffer and boiling at 100ºC. The equivalent of 0.1 OD of each sample was resolved by SDS-PAGE and analyzed by Western blot probing for CpaB-His and RNA polymerase as a cytoplasmic control.

5.2.8-Immunoblotting

Bacterial secretion whole cell and supernatant samples were prepared as previously published (Harding, C.M. *et al.*, 2016b). Briefly, cultures were grown to 0.5 OD/mL, 0.5OD was pelleted by centrifugation and resuspended in 50 µL of 1X laemmlies buffer for the whole cell samples. Supernatant samples were obtained by TCA precipitating cell-free supernatants as previously

published (Weber, B.S. *et al.*, 2013). Proteins were resolved by SDS-PAGE analysis and transferred to a nitrocellulose membrane by semi-dry transfer and probed with either mono-clonal anti-FLAG (1:2000, Sigma), poly-clonal anti-histidine (1:2000, Pierce), poly-clonal anti-CpaA (1:1000, this study) and/or mono-clonal anti-RNA polymerase (1:2000, Neoclone). Western blots were probed with IRDye conjugated secondary antibodies and visualized with an Odyssey CLx imaging system (LI-COR Biosciences, Lincoln, NE).

5.2.9-*Galleria mellonella* Infection

G. mellonella infections with *A. nosocomialis* M2, Δ*gspD*::frt, Δ*cpaA::*frt and the complemented strains were done as previously published (Harding, C.M. *et al.*, 2016b). Cultures were grown in LB to 0.5 OD/ml. Cells were pelleted by centrifugation, washed with sterile PBS and resuspended in PBS. *G. mellonella* were injected with 10ul of either M2, Δ*gspD*::frt, Δ*cpaA* or the complemented strains at an inoculum of $1x10^7$. Infected larvae were incubated at 37°C and monitored for viability over time. Larvae were considered dead if they did not respond to touch.

5.2.10-Mouse Model of Pneumonia

Vanderbilt University Institutional Animal Care and Use Committee approved these infection experiments. Jackson Laboratories wild-type C57BL/6 mice were infected with either the wild type *A. nosocomialis* M2, M2Δ*gspD*::frt, Δ*cpaA*, or the *cpaA+* complemented strain as previously published (Harding, C.M. *et al.*, 2016b). Nine-week old male mice were inoculated intranasally with $1x10^9$ CFU. Thirty-six hours post-infection mice were euthanized, lung, liver, spleen and heart tissue homogenized and dilutions plated to determine CFUs. Median CFU counts from each mouse are reported and analyzed by Kruskal-Wallis using GraphPad Prism 6 (GraphPad Software Inc, La Jolla, CA).

5.2.11- Generation of GST Tagged CpaA Truncations

In order to determine the chaperone binding domain of CpaA, cytoplasmic truncations of *cpaA* were cloned with an N-terminal Glutathione S Transferase tag and analyzed for their ability to bind cytoplasmic CpaB-His through co-purification analysis. Cytoplasmic *cpaB-his* was cloned into pET28 using In-Fusion HD EcoDry cloning where Pet28 was PCR amplified using Pet28ahisfw and Pet28auprev and *cpaB* was PCR amplified using primers CytocpaBrev and CytocpaBfw. Full length *cpaA* and one quarter fragments of *cpaA* were cloned into pGEX6p1at BamH1 and XhoI. Full length *cpaA* was PCR amplified using CpaAFLrevXhoI and CpaAfwBamH1nosec. The first quarter construct was PCR amplified with CpaAfwBamH1nosec and firstquartRXhoI. The second quarter construct was PCR amplified with secquatfwBamH1

and firsthalfrevXhoI. The third quarter construct was PCR amplified with SecondhalfFBamH1 and 3rdquatRXhoI. The last quarter construct was PCR amplified with 4thquatfwBamH1 and CpaAFLrevXhoI. Rosetta 2 cells expressing pET28-*cpaB-his* and pGEX6p1 or a pGEX-*cpaA* construct were subjected to co-purification experiments as outlined above. Load, flow-through, final wash and elution fractions were analyzed by SDS-PAGE and Western blot probing for GST/GST-CpaA and CpaB-His was performed to determine expression and purification of CpaB-His and GST/GST-CpaA.

5.2.13-CpaA-His-CpaB Co-purification and Protein Crystallization

Six litres of Rosetta 2 carrying pETDuet-*cpaA-his-cpaB* were grown at 30ºC, 225rpm overnight in auto-induction media (1% tryptone, 0.5% yeast extract, 25 mM Na_2HPO_4 25mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 0.5% glycerol 0.05% glucose 0.2% α lactose) (Studier, F.W., 2005). Cells were harvestedat 10 000 rpm for 10 minutes. Cells were resuspended in 50 mM NaH_2PO_4 300 mM NaCl and 10 mM imidazole pH8 at 20 mL/L (Bornhorst, J.A. and Falke, J.J., 2000). Cells were lysed with a cell disruptor using three rounds at 35 kpsi (Constant System ltd., Kennesaw, GA). Cell lysates were clarified at 11000 rpm for 35 minutes. Cell lysate was loaded onto a Nickel-NTA agaraose column and purification proceeded as above. Purified protein was concentrated and buffer exchanged using Amicon Ultra 15ml 10kDa cut off centrifugal filter units. The protein was further purified by gel filtration chromatography using a Hiload 16/60 superdex 200 prepgrade column (GE Healthcare Life Sciences, Pittsburg, PA) with 10mM HEPES 100mM NaCl and the fractions were analyzed by SDS-PAGE and coomassie staining (Salinas, N.D. *et al.*, 2014). Fractions containing the highest and equimolar amounts of Cpa and CpaB were pooled and concentrated using Amicon Ultra 15 mL 10kDa cut off centrifugal filter units to 22 mg/mL.

CpaA-CpaB was screened for protein crystallization conditions in a 96-well 100 nL misquito hanging-drop vapor diffusion screen including JCSG suite I-IV and PEG I and II at 22mg/ml and 17ºC. Six conditions produced protein crystals. Crystallization conditions were narrowed down testing crystal growth at 17ºC using hanging-drop vapor diffusion after mixing 1 µL of 22 mg/mL protein and 1 µL of well solution. Crystals formed in 0.1 M Sodium cacodylate pH6.5 and 1.26 M Ammonium sulfate were cryo-preserved in MiTeGen oil, frozen in liquid nitrogen, and analyzed by X-ray diffraction at the ALS 422 beamline at UC Berkeley.

Table 5-1 -Plasmids and bacterial strains used in this study

pGEM-*gspD::*kan-sacB	Contains kanamycin resistance gene from Tn5 and *sacB* gene flanked by FRT sites (Harding, C.M. *et al.*, 2016a; Datsenko, K.A. and Wanner, B.L., 2000)
pFLP2	Encodes FLp recombinase (Kumar, A. *et al.*, 2010)
pGEM-*cpaA*::kansacB	pGEM containing a *kan-sacB* with 1kb of *cpaA* flanking DNA up and downstream (This study)
pGEM-*cpaAB*::kansacB	pGEM containing a *kan-sacB* with 1kb of *cpaAB* flanking DNA up and downstream (This study)
pRSM4063	pSMART-LCkan containing an empty mTn7 element from pRSM3510 along with 2kb flanking DNA up and downstream of the *att*Tn7 site in *A. nosocomialis* M2 (Harding, C.M. *et al.*, 2015c)
pRSM4063-*pcpaAB*	pRSM4063 containing the *cpaAB* locus with the predicted *cpaA* promoter (This study)
pWH1266	*Acinetobacter-E. coli* shuttle vector (Hunger, M. *et al.*, 1990)
pWH-*cpaA-his*	pWH1266 containing *cpaA* along with its predicted native promoter and a C-terminal hexa-histidine tag (Harding, C.M. *et al.*, 2016b)
pWH-*cpaA-his-cpaB*	pWH1266 containing *cpaA* and *cpaB* along with the predicted promoter and a C-terminal hexa-histidine tag on *cpaA* (Harding, C.M. *et al.*, 2016b)
pWH-*lipH-his*	pWH1266 containing *lipH* and its predicted promoter and a C-terminal hexa-histidine tag on *lipH* (Harding, C.M. *et al.*, 2016a) (Harding, C.M. *et al.*, 2016b)
pWH-*cpaA-flag-cpaB*	pWH1266 containing *cpaA* and *cpaB* along with the predicted native promoter and C-terminal flag tag on *cpaA* (This study)

pWH-*cpaA-flag-cpaB-his*	pWH1266 containing *cpaA* and *cpaB* along with the predicted native promoter and C-terminal flag tag on *cpaA* and a hexa-histidine tag on *cpaB* (This study)
pWH-*cpaB-his*	pWH1266 containing *cpaB* along with the predicted native promoter of *cpaA* and a hexa-histidine tag on the carboxy-terminus (This study)
pWH-*cpaB-pelB-his*	pWH1266 containing *cpaB* with the putative N-terminal transmembrane domain removed and a *pelB* leader sequence and start codon added to the 5' end. A hexa-histidine tag was added at the carboxy-terminus (This study)
pBAV-*cpaA-flag*	pBAVMCS containing *cpaA* with a C-terminal flag tag (This study)
pGEX6p1	GST fusion vector (GE Healthcare)
pGEX-*CpaAFL*	pGEX6p1 containing full length *cpaA* with a N-terminal GST tag (This study)
pGEX-*cpaAFQ*	pGEX6p1 containing the first quarter of *cpaA* with an N-terminal GST tag (This study)
pGEX-*cpaASQ*	pGEX6p1 containing the second quarter of *cpaA* with an N-terminal GST tag (This study)
pGEX-*cpaATQ*	pGEX6p1 containing the third quarter of *cpaA* with an N-terminal GST tag (This study)
pGEX-*cpaALQ*	pGEX6p1 containing the last quarter of *cpaA* with an N-terminal GST tag (This study)
pETDUET	IPTG inducible plasmid designed to co-express two genes Amp^r (Novagen)
pET28a	IPTG inducible, high expression plasmid, Km^r, (Novagen)

pETDUET-*cpaA-his-CpaB*	pETDUET containing cytoplasmic *cpaA* with a C-terminal Histidine tag provided by the vector, and cytoplasmic *cpaB* cloned in at NdeI and KpnI (This study)
pET28-*cpaBHis*	*cpaB* was cloned in frame with the Histidine tag using In-Fusion, the N-terminal transmembrane domain has been removed

Strains

A. nosocomialis M2	Metro Health Systems Clinical Isolate (Niu, C. *et al.*, 2008) (Carruthers, M.D. *et al.*, 2013b)
M2 Δ*cpaA*::frt	*A. nosocomialis* strain M2 containing an in frame, deletion of *cpaA*
M2Δ*cpaAB*::frt	*A. nosocomialis* strain M2 containing an in frame, deletion of *cpaA* and *cpaB*
M2Δ*cpaA::*frt (*cpaAB*+)	M2Δ*cpaA*::frt with a mini-Tn7 element containing the *cpaA-cpaB* locus driven off the predicted *cpaA* promoter
A. baumannii ATCC 17978	Type strain, ATCC
A. baumannii ATCC 19606	Type strain, ATCC
E. coli DH10β	General cloning strain, Invitrogen
E. coli HB101 (pRK2013)	Conjugation helper strain (Figurski, D.H. and Helinski, D.R., 1979)
Rosetta 2	BL21 derivative carrying pRARE2 a plasmid that encodes several rare codonsF$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* pRARE2 (Cam^R) (Novagen)

Table 5-2 Primer List-Investigating Type II Substrate CpaA

petDuetfwinfuse 5’ GAATTCGAGCTCGGCGCGCCTGCAG 3’

petDuetrevinfuse: 5’ GCTGCCCATGGTATATCTCCTTC 3’

CpaBcytorevKpnI 5’ ATATGGTACCCTATGGATGATCATGATGTAACTCC 3’

CpaBcytofwNdeI 5’ ATATCATATGATGCAGCAAAGTTCTTCTGCTTTAACG 3’

CpaAcytofwduet15bp 5’ TATACCATGGGCAGCATGGCAACCGTACTGTCACAAAATCAG 3’

CpaAcytoHisrevduet15bp 5’ GCCGAGCTCGAATTCTTAGTGGTGATGATGGTGATGGTTATATAGAGAAATTTTTTG TGC 3’

pvuI5105HISrev 5’ ATATCGATCGCTAGTGGTGGTGGTGGTGGTGTGGATGATCATGATGTAACTCC 3’

PvuI5105FW 5’ ATATCGATCGATGAAGAAAAATAAGTTGTTAATAGG 3’

CpaAfwbamh1 5’ ATATGGATCCGTGAATTTTAAATTAAAAACATCAC 3’

CpaArevHisPstI 5’ ATATCTGCAGTTAGTGGTGGTGGTGGTGGTGGTTATATAGAGAAATTTTTTGTGCG 3’

CpaBfwnotmpelB 5’ ATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCG GCGATGGCCATGCAGCAAAGTTCTTCTGCTTTAACG 3’

Kmsacb2rev 5’TGTAGGCTGGAGCTGCTTCG3’

Kmsacb1fw 5’ATTCCGGGGATCCGTCGACC3’

Pgem1rev 5’CGCCATGGCGGCCGGGAGCATG 3’

Pgem2fw 5’ACTAGTGAATTCGCGGCCGCCTGCA 3’

3510015bppgem2rev 5’CGCGAATTCACTAGTCGCCTTGCAGGTTGCACAGACTTCAC 3’

3510015bpkmsacB2fw 5’CAGCTCCAGCCTACATAATCTCTATTTTTAGCTGTAAATAATG3’

5510015bpkmsacBrev 5’ACGGATCCCCGGAATCACAGTAGCCTCTTTTATTTGTTTTTAG 3’

5510015bppgem1fw 5’CCGGCCGCCATGGCGACGCCAATGATTGATCTGGAATTG 3’

7CpaBfwpromoverhang 5’ ATAAAAGAGGCTACTTCTCTATTTTTAGCTGTAAATAATGAAG 3’

6cpaABpromrev 5’ AGTAGCCTCTTTTATTTGTTTTTAGATTTTCG 3’

5CpaAFLAGrev 5’ TTACTTATCGTCGTCATCCTTGTAATCGTTATATAGAGAAATTTTTTGTGC 3’

3cpaApromfwpwh1 5’ ACTTACTTCTGACAACGATCGCTATCTCAGCCATTTGTTTCTTCCTG 3’

2Pwhrev1 5’ TTGTCAGAAGTAAGTTGGCCG 3’

1Pwhfw2 5’ GAGGACCGAAGGAGCTAACCGC 3’

9CpaBnoTMpelB15bppromfw 5’
ATAAAAGAGGCTACTCGATCGATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTG
CTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGTCACAGCAAAGTTCTTCTGCTTTA
ACG 3’

8CpaBHisrevpwh2 5’
GCTCCTTCGGTCCTCCGATCGCTAATGATGATGGTGGTGGTGATGATGTGGATGATC
ATGATGTAACTCCTG 3’

11CpaBfw15bpcpaAF 5’
GACGACGATAAGTAATTCTCTATTTTTAGCTGTAAATAATGAAG 3’

cpaBrevNT15bppwh2 5’ GCTCCTTCGGTCCTCCTATGGATGATCATGATGTAACTCC 3’

cpaABKOdw15bppgemrev4 5’
CGCGAATTCACTAGTAAGCTAACTCATGTAATGTTGAAATG 3’

cpaABKOdw15bpkmsacBfw3 5’
CAGCTCCAGCCTACATAGAGGAATAAATGATTTTGGTAGGG 3’

cpaABKOup15bppgemfw2 5’ CCGGCCGCCATGGCGTCTACAGTAGGTGGCGGTG 3’

CpaABkoup15bpkmsacBrev1 5’
ACGGATCCCCGGAATTAAGCACCCAACTATGTGCATAGC3’

SecondhalfFBamH1 5’ TATAGGATCCATGTATTGGCAGCTCATCTCTTC 3’

4thquatfwbamh1 5’ TATAGGATCCATGTATAAAATCCCTGCTGCCAC 3’

CpaAFLRevXhoI 5’ TATACTCGAGTTAGTTATATAGAGAAATTTTTTGTGC 3’

3rdquatRXhoI 5’ TATACTCGAGTTAAGTTATATAACCCACATCTC 3’

secquatfwBamH1 5’ TATAGGATCCATGCAAACCATTGCTTTACCAAG 3’

firstquatRXhoI 5’ TATACTCGAGTTATGCCCATTTGTCATCTTG 3’

CpaAfwbamh1nosecATATGGATCCATGGCAACCGTACTGTCACCAAATCAG

FirsthalfrevXhoI 5’TATACTCGAGTTAGCCTTTGTCGCTTACATACATAAACTC3’

kpnIcpaABrevlocus ATATGGTACCACCTACACCACGCGTTCCTGTAAATAAATAAGC

pstIcpaABfwlocus ATATCTGCAGCTATCTCAGCCATTTGTTTCTTCCTGTTAACTG

Pet28ahisfw CACCACCACCACCACCACTGAGAT

Pet28auprev CATGGTATATCTCCTTCTTAAAGT

CytocpaBrev GTGGTGGTGGTGGTGTGGATGATCATGATGTAACTCCTG

CytoCpaBfw AGGAGATATACCATGCAGCAAAGTTCTTCTGCTTTAACG

5.3-Results:

5.3.1-CpaA is required for Optimal Virulence in *G. mellonella* Larvae

We have previously demonstrated the Type II secretion system of *A. nosocomialis* M2 is required for virulence in *G. mellonella* larvae. Per the 2D-DIGE analysis of M2 type II substrates, CpaA is the most abundant Type II secreted protein under laboratory conditions. We hypothesized that *cpaA* is required for full virulence in *G. mellonella* and the Δ*cpaA* strain would have similar defects in virulence as the Δ*gspD* Type II secretion mutant. To test this hypothesis, an unmarked mutation in *cpaA* was created as well as chromosomal complementation of this mutation, where *pcpaAcpaB* was inserted next to *glmS* using a miniTn7 chromosomal complementation system (Carruthers, M.D. *et al.*, 2013a; Harding, C.M. *et al.*, 2013). There is no difference in growth rate in M9 minimal media supplemented with 1% case amino acids of the mutant or complemented strain relative to wildtype M2 (data not shown). Larvae were infected with either wildtype M2, Δ*gspD*, *gspD*+, Δ*cpaA* or *cpaA*+ and survival was monitored over time. After 24 hours post-infection, 50% of larvae infected with either Δ*gspD* or Δ*cpaA* survived (Figure 5.1). Infection with wildtype M2, *gspD*+ or *cpaA*+ resulted in at least 25% less survival than the mutant strains. The difference in percent survival of larvae infected with either Δ*gspD* or Δ*cpaA* relative to wildtype or the complemented strains is statistically significant (Figure 5.1).

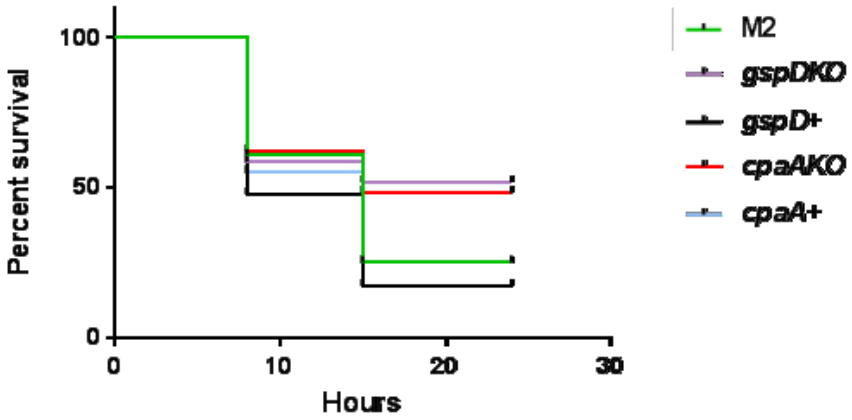

Figure 5.1-Type II secretion and CpaA are required for full virulence in the *G. mellonella* infection model.

G. mellonella larvae were injected with 10uL of either M2, Δ*gspD*, Δ*cpaA,* or the complemented strains at an inoculum of ~1x10^7. Infected larvae were incubated at 37ºC and monitored for viability over time. Viability was determined by melanin accumulation and mobility. Larvae infected with Δ*gspD* or Δ*cpaA* had approximately 50% survival at 24 hours. 25% of the larvae infected with M2 survived after 24 hours. Larvae infected with the Δ*gspD* or Δ*cpaA* complemented strains had 17% survival at 24 hours post-infection. The difference in percent survival of larvae infected with Δ*gspD* or Δ*cpaA* relative to the wildtype or complemented strains is statistically significant (Mantel Cox p=0.0447)

5.3.2-CpaA Aids in Colonization of the Spleen of a Mouse

Deletion of *cpaA* resulted in a virulence defect in *G. mellonella* larvae comparable to the Type II secretion mutation (Figure 5.1). CpaA is reported to have proteolytic activity against fibrinogen and Factor V, suggesting it may impact dissemination from the initial site of infection (Tilley, D. *et al.*, 2014). *A. nosocomialis* M2 Type II secretion system is required for lung colonization and dissemination to the spleen in a murine model of pulmonary infection (Figure 4.9) (Harding, C.M. *et al.*, 2016b). We hypothesized *cpaA* would be required for optimal colonization and dissemination of *A. nosocomialis* in a murine pulmonary infection model. To test this hypothesis, we infected mice intranasally with wildtype M2, Δ*gspD*, Δ*cpaA* or the *cpaA*+ complemented strain. Thirty-six hours post-infection the lungs, spleen, liver and hearts were harvested and analyzed for CFUs.

There is a statistically significant difference in the spleen burden of mice infected with Δ*cpaA* compared to the complemented strain (Figure 5.2). Comparison of the bacterial burden in the lungs of mice infected with Δ*cpaA* compared to the *cpaA*+ complemented strain shows a trend towards decreased colonization by the Δ*cpaA* strain (Figure 5.2B). CpaA has a role in dissemination from the initial site of infection in the lungs, to a distal site in the spleen. The activity of CpaA against coagulation factors fibrinogen and Factor V likely contributes to the defect in Δ*cpaA* to migrate from the initial infection site. This dissemination defect may explain the high variability in CFU counts of Δ*cpaA* in the spleen and lung.

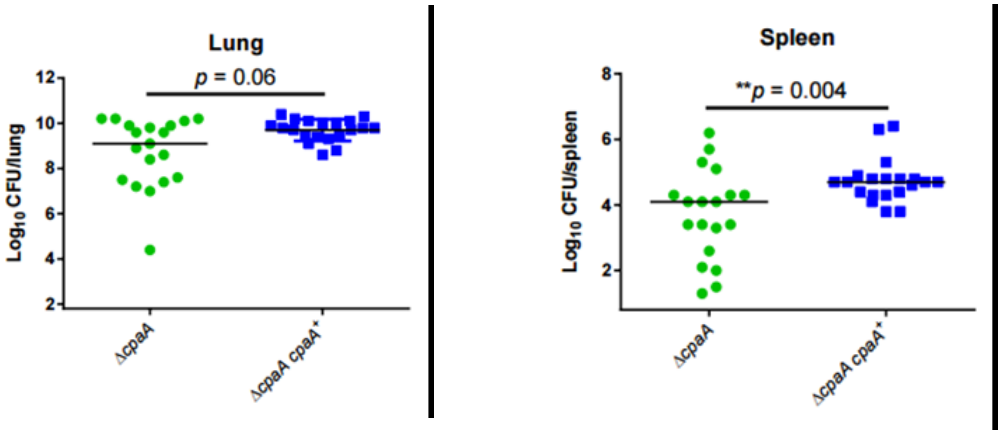

Figure 5.2-CpaA aids in Colonization of the Spleen of a Mouse.
Mice were intranasally inoculated with 1X10^9 CFU of either, Δ*cpaA* or the *cpaA* complemented strain. 36 hours post-infection, mice were sacrificed, organs harvested, and CFUs enumerated from homogenized tissue. Total CFUs from the spleen demonstrate a statistically significant difference between the complemented *cpaA* strain and the Δ*cpaA* mutant (Kruskal-Wallis non-parametric, ***p*=0.004). Total CFUs from the lung demonstrate a non-statistically significant trend towards decreased colonization by the Δ*cpaA* strain (Kruskal-Wallis non-parametric, *p*=0.06).CpaA has a role in dissemination from the initial site of infection in the lungs, to a distal site in the spleen..

5.3.3-CpaA and CpaB are not Required for Secretion of Type II Substrate LipH

Deletion of *cpaA* has a very similar impact on virulence against *G. mellonella* as the loss of Type II secretion (Figure 5.1). Loss of CpaA resulted in statistically significant impairment of spleen colonization realative to the complemented strain (Figure 5.2). Previously we showed that

putative chaperone, CpaB, is required for secretion of CpaA. We hypothesize that deletion of *cpaA* and *cpaB* will only impact secretion of Type II substrate, CpaA, and should not impact secretion of other Type II substrates. To test this hypothesis *cpaA-his*, *lipH-his*, or *cpaA-his-cpaB* were expressed in wildtype M2 or Δ*cpaAB.* Whole cell and supernatant fractions were probed by Western blot analysis for expression and secretion of LipH-His and CpaA-His (Figure 5.3A-B). RNA polymerase was probed for as a cell lysis control. LipH-His expression and secretion were detected in both wildtype and Δ*cpaAB* cells. There is no apparent difference in secretion efficiency of LipH between wildtype and Δ*cpaAB* (Figure 5.3A-B), confirming our hypothesis. CpaA and CpaB are dispensible for secretion of type II substrate LipH. CpaA-His secretion was detected in wildtype M2 carrying pWH-*cpaA-his* or pWH-*cpaA-his-cpaB* (Figure 5.3A). Secretion of CpaA-His was only detected in Δ*cpaAB* cells carrying pWH-*cpaA-his-cpaB* (Figure 5.3B). This confirms that CpaB is required for secretion of CpaA.

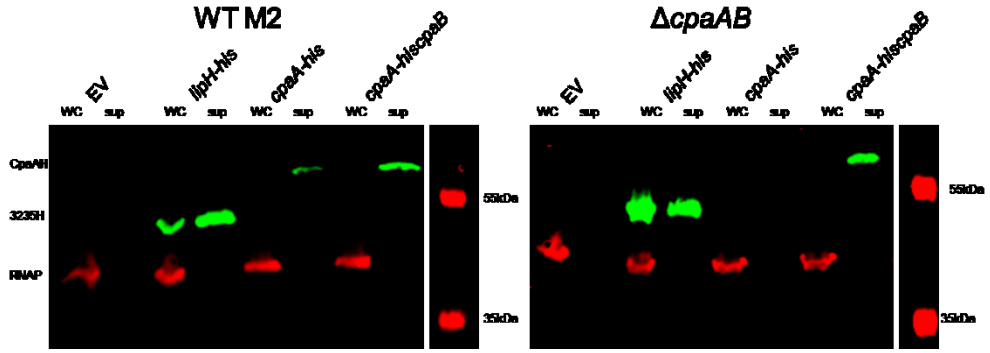

Figure 5.3-M2 *cpaB* is required for secretion of CpaA-His but *cpaA* and *cpaB* are not required for secretion of type II substrate LipH-His

(A)Western blot analysis on whole cell and cell free supernatant fractions of wildtype M2 expressing empty vector, pWH-*lipH-his,* pWH-*cpaA-his,* or pWH-*cpaA-hiscpaB* probing for CpaA-His or LipH-His. All strains and fractions were probed for RNA polymerase as a cell lysis control. CpaA-His secretion was detected in strains carrying pWH-*cpaA-his* or pWH-*cpaA-his-cpaB*. LipH-His expression and secretion was detected in cells carrying pWH-*lipH-his*.
(B)Western blot analysis on whole cell and cell free supernatant fractions of Δ*cpaAB* M2 expressing empty vector, pWH-*lipH-his,* pWH-*cpaA-his,* or pWH-*cpaA-hiscpaB* probing for CpaA-His or LipH-His. All strains and fractions were probed for RNA polymerase as a cell lysis control. CpaA-His secretion was only detected in cells expressing *cpaB*. LipH-His expression

and secretion was detected in cells carrying pWH-*lipH-his*. CpaA and CpaB are not required for secretion of type II substrate LipH. CpaB is required for secretion of CpaA.

5.3.4-CpaA and CpaB Physically Interact

We previously demonstrated that *cpaB* is required for secretion of CpaA. Domain Enhanced Lookup Time Accelerated (DELTA) BLASTp analysis identified a SRPBCC superfamily domain in CpaB leading to the hypothesis that CpaB is a chaperone protein (Harding, C.M. *et al.*, 2016b). Proteins with a SRPBCC domain are predicted to have a deep hydrophobic ligand-binding pocket and have chaperone-like activity. This domain analysis taken together with the requirement of *cpaB* for secretion of CpaA lead us to hypothesize these proteins physically interact and that CpaB likely helps CpaA fold into a secretion competent state. I investigated the physical interaction between CpaB and CpaA through a pulldown method. *CpaA* was cloned with a C-terminal FLAG tag and *cpaB* was cloned with a C-terminal Histitine tag and expressed in M2 Δ*cpaAB*. Nickel-NTA affinity chromatography was performed on cell lysate solubilized with 0.01% triton X-100 co-expressing *cpaA-flag* and *cpaB-his* or *cpaA-flag* and *cpaBnotag* (Schnaitman CA 1971). Western blot analysis probing for CpaA-FLAG and CpaB-His was performed to analyze the purification results. CpaA-FLAG was detected in the load and flow-through of both cell lysates. CpaB-His was detected in the load of cells expressing *cpaB-his*. The final washes of both strains were clear of CpaB-His and CpaA-FLAG signals. CpaB-His and CpaA-FLAG were detected in the elution fraction of cells co-expressing *cpaA-flag* and *cpaB-his* only. CpaB interacts with CpaA. Co-purification of these proteins in the presence of detergent demonstrates a strong interaction.

Figure 5.5 depicts potential mechanisms by which CpaB impacts folding and/or secretion of CpaA. The chaperone (C)is required for secretion of the substrate (E), however the mechanism by which the chaperone impacts secretion of its substrate is not understood. The substrate is translocated to the periplasmic space by the sec translocation system. The substrate must be full folded to be secreted by the type II apparatus. Once in the periplasm, the substrate may interact with the chaperone to become folded gaining a secretion competent state (Figure 5.5 panel A). Alternatively, the substrate may auto-fold, and then interacts with the chaperone in order to block activity of the substrate (Figure 5.5 panel B). It is not well understood how type II substrates are recognized by the type II apparatus. Figure 5.5 panel C depicts a potential ushering

role for the chaperone, where the substrate binds to the chaperone and is guided to the secretion apparatus. LipA and Lif from *B. glumae* forms a one-to-one complex. The ratio of chaperone to substrate of CpaA and CpaB is unknown. To depict the possibility that the chaperone may function as a multimer when binding the effector protein, panel D shows a dimer of the chaperone bound to the substrate.

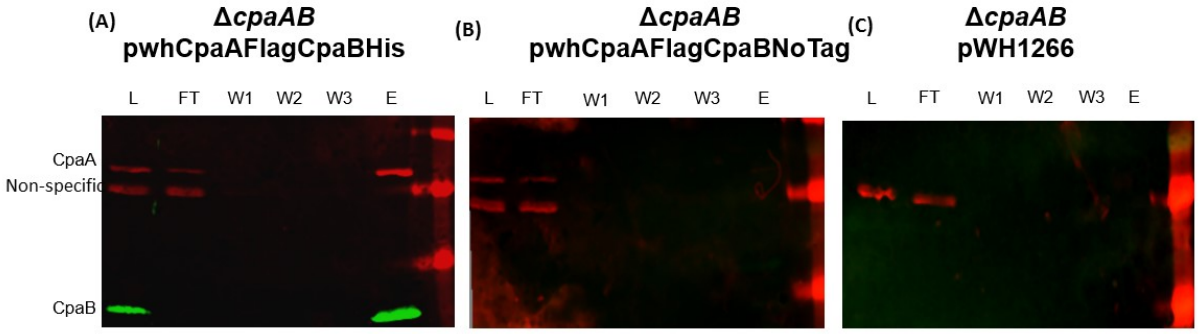

Figure 5.4-The Physical Interaction Between CpaA and CpaB is Demonstrated Through Co-purification

Western blot analysis on purifications from Δ*cpaAB* M2 expressing empty vector (C), pWH-*cpaA-flag-cpaB-his* (A) or pWH-*cpaA-flag-cpaB* (B) probing for CpaA-Flag and CpaB-His. L indicates load fraction, FT is flow-through, W1-3 are washes 1-3 and E indicates elution. CpaA-Flag is detected in the load and flow-through fractions of Δ*cpaAB* expressing *cpaA-flag*. CpaB-His is detected in the load and elution fractions of Δ*cpaAB* expressing *cpaB-his*. CpaA-Flag is only detected in the elution fraction of cells expressing *cpaA-flag* and *cpaB-his*. NeitherCpaA-Flag or CpaB-His are detected in the final wash fraction of any of the conditions.

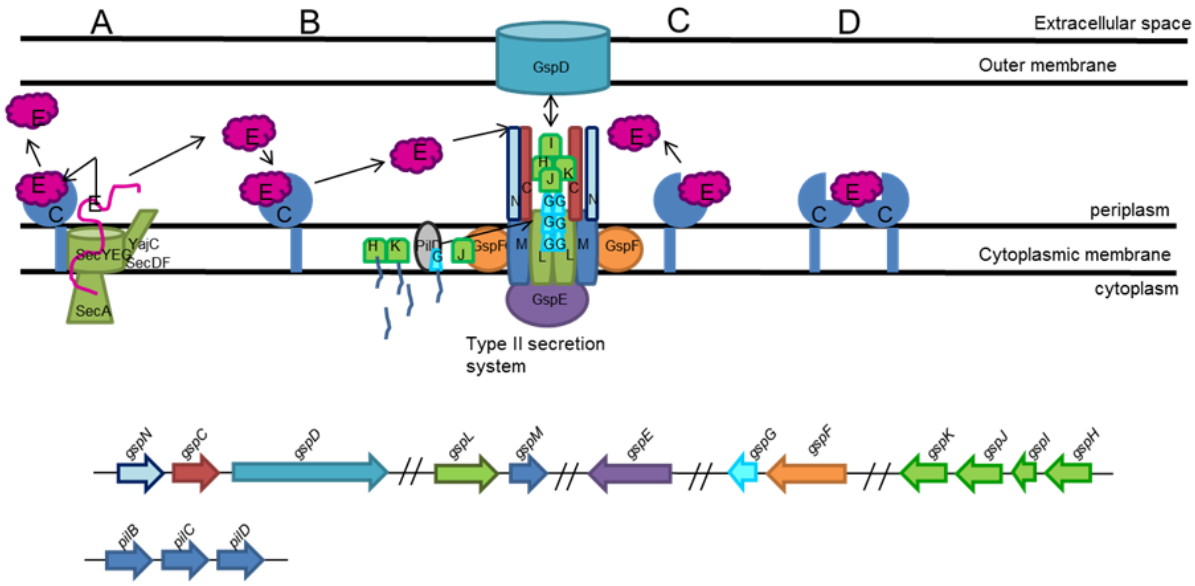

Figure 5.5 -Potential Mechanisms by which Membrane Bound Chaperones Impact Secretion of Type II Substrates. In *A. nosocomialis* M2 secretion of type II effectors, CpaA and LipA, requires specific membrane associated chaperones, CpaB and LipB. How these chaperones (designated C) function in the secretion of their cognate effectors (designated E) is unknown. This figure depicts potential chaperone-effector interaction mechanisms:

A) The effector is transported unfolded from the cytoplasm to the periplasmic space by the Sec secretion system. Once in the periplasm, the chaperone interacts with the effector initiating folding. The effector is then released into the periplasmic space, where it will eventually be secreted to the extracellular space by the T2SS. Alternatively the effector may remain bound to the chaperone until it is released for secretion by the T2SS thereby preventing activity of the effector protein.

B) Upon entering the periplasmic space, through the Sec secretion system the effector folds. Once folded, it interacts with its chaperone. The interaction between the effector and chaperone may block activity of the effector protein through sequestration of the effector until it is released for secretion by the T2SS.

C) The chaperone may function to guide the effector to the T2S machinery, through a secondary interaction with a component of the T2SS. The chaperone may usher type II substrates to the apparatus in addition to a role in protein folding.

D) The chaperone may function as a multimer when binding the effector protein.

5.3.5- The C-terminal Domain of CpaB is Periplasmic

Bioinformatic analysis using transmembrane online prediction server, TMHMM, suggests CpaB has an N-terminal transmembrane domain (data not shown) (Luirink, J. *et al.*, 2005). Whether the C-terminal domain of CpaB interacts with CpaA in the cytoplasm or the periplasmic side of the inner-membrane is unclear. SignalP4.1 predicts there to be an N-terminal Sec secretion signal in *cpaA*, suggesting CpaA is translocated to the periplasm by the Sec secretion system (Tilley, D. *et al.*, 2014) (Fekkes, P. and Driessen, A.J., 1999). We hypothesize that CpaB interacts with CpaA after it has been translocated to the periplasm, aiding it its folding prior to secretion, therefore we predict the C-terminal domain of CpaB will be periplasmically exposed. To test this hypothesis, we used limited proteolysis of either whole cells or spheroplasts containing CpaB-His, where the hexa-histidine tag was added to the C-terminus (Frenken, L.G. *et al.*, 1993) (Feldman, M.F. *et al.*, 2005). Samples were visualized by Western blot analysis probing for CpaB-His and RNA polymerase as a cytoplasmic control. Whole cells treated with proteinase K have equal levels of CpaB-His and RNA polymerase over time (Figure 5.6A). Spheroplasts treated with proteinase K have equal levels of RNA polymerase over time, indicating cytoplasmic proteins were sheltered from proteolysis (Figure 5.6B). The level of CpaB-His in spheroplasts treated with proteinase K decreases over time, suggesting the C-terminal domain is accessible to the protease and therefore must be periplasmic (Figure 5.6B).

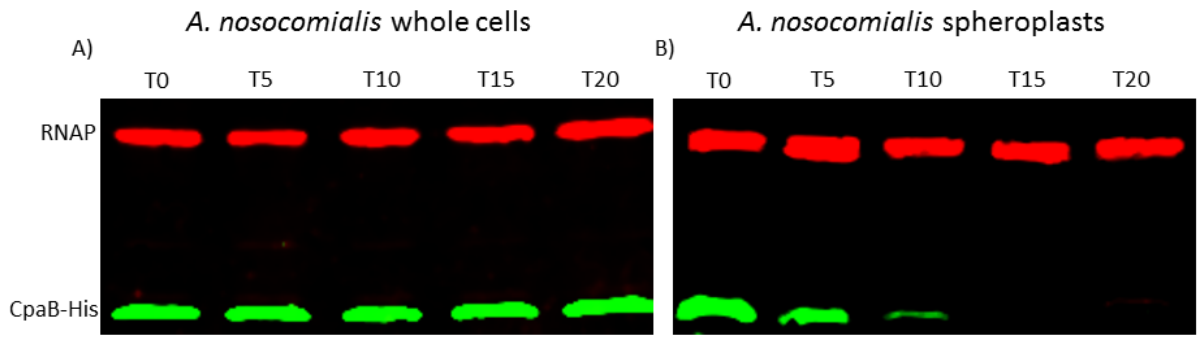

Figure 5.6-The C-terminal domain of CpaB is exposed to the periplasmic space.

Western blot analysis probing for RNA polymerase as a cytoplasmic control and CpaB-His of samples treated with 0.5 µg/mL proteinase K for 0 to 20 minutes at 56ºC. A) Limited proteolysis of M2 whole cells expressing pWH-*cpaB-his*. There was no detectable change in the amount of RNA polymerase over time. CpaB-His was detected in equal proportions over time in whole cells samples treated with proteinase K. B) Limited proteolysis of spheroplasts isolated from M2 expressing pWH-*cpaB-his*. Spheroplasts treated with proteinase K have decreasing amounts of CpaB-His over time.

5.3.6-Soluble Periplasmic CpaB Complements a Δ*cpaB* Mutant and is Secreted with CpaA

The purpose of the N-terminal transmembrane domain, anchoring CpaB to the plasma membrane, is unknown. Whether CpaB needs to be anchored to the plasma membrane to aid in secretion of CpaA is unknown. Soluble, cytoplasmic chaperone, SecB interacts with membrane proteins SecA, the motor protein of the Sec translocation system, through protein-protein interactions (Driessen, A.J., 2001). It is plausible that the transmembrane domain is necessary for interactions with the SecYEG channel or the Type II secretion machinery allowing for acquisition of CpaA from the SecYEG channel or shuttling into the Type II machinery. We hypothesized that CpaB required its transmembrane domain to facilitate secretion of CpaA. To test this hypothesis, we complemented a Δ*cpaAB* mutant with Flag tagged CpaA and either full length CpaB-His or soluble periplasmic CpaB-His (Figure 5.7 -5.8). Soluble periplasmic CpaB-His was obtained by replacing the N-terminal transmembrane domain of *cpaB* with a Sec secretion signal (Figure 5.7) (Luirink, J. *et al.*, 2005; Sockolosky, J.T. *et al.*, 2014). Soluble periplasmic CpaB-His, seen as a lower molecular weight CpaB-His relative to wildtype, was

detected in the periplasmic fraction of cells expressing pWH-*cpaB*$_{pelB}$-*his.* Full length CpaB-His was only detected in the spheroplast fraction, indicating it is membrane bound (Figure 5.7). Expression and secretion of CpaA-FLAG and CpaB-His was monitored by Western blot analysis.

CpaA-FLAG secretion was complemented by either full length CpaB or soluble periplasmic CpaB (Figure 5.8). CpaB does not need to be membrane bound in order for CpaA to be secreted. Interestingly, soluble CpaB-His was also detected in cell free supernatants of cells expressing CpaA-FLAG and CpaB$_{pelB}$-His (Figure 5.8). CpaB$_{pelB}$-His was only detected in supernatants of cells expressing CpaA-FLAG and CpaB$_{pelB}$-His. Soluble periplasmic CpaB is secreted with CpaA.

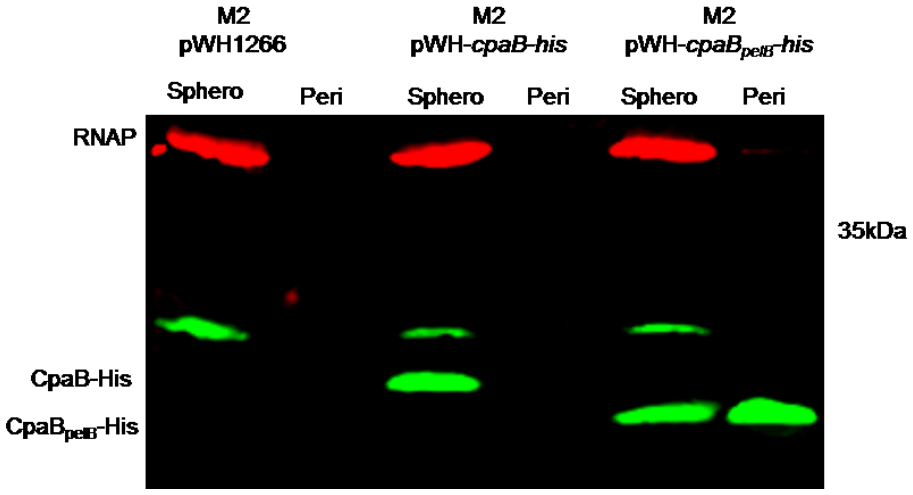

Figure 5.7-Replacement of the N-terminal Transmembrane Domain of CpaB with a Sec Secretion Signal Results in a Soluble Periplasmic Construct of CpaB. Western blot analysis on M2 cells fractionated into periplasm (peri) or spheroplast (sphero) expressing empty vector, pWH-*cpaB-his* or pWH-*cpaB*$_{pelB}$-*his* probing for CpaB-His and RNA polymerase as a lysis control. CpaB-His is detected in the spheroplast fraction of cells expressing full length *cpaB*. CpaB-His is detected in the spheroplast and periplasm fractions of cells expressing the soluble *cpaB*$_{pelB}$ construct where the N-terminal transmembrane domain has been replaced with a Sec secretion signal.

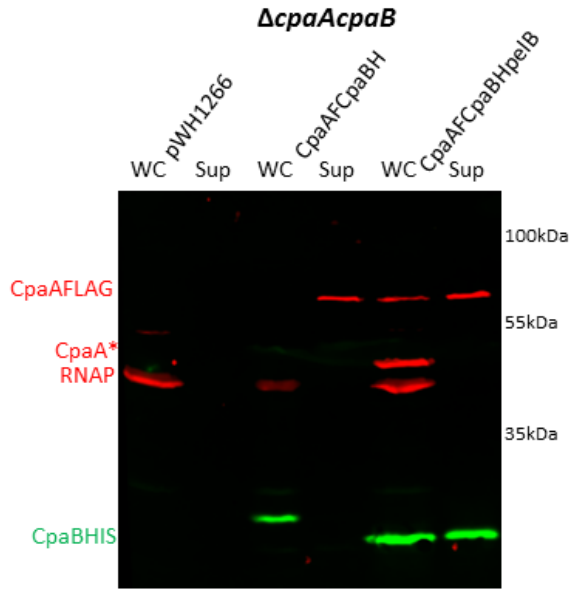

Figure 5.8-Soluble periplasmic CpaB Complements a Δ*cpaB* Mutant and is secreted with CpaA. Western blot analysis on whole cell and cell free supernatant fractions of Δ*cpaAB* M2 expressing empty vector, pWH-*cpaB-his,* pWH-*cpaB*$_{pelB}$-*his*, pWH-*cpaB*$_{pelB}$ -*his* and pbav-*cpaA-flag* was probed for CpaB-His. All fractions were probed for RNA polymerase as a lysis control. CpaB-His was detected in all cells carrying pWH-*cpaB-his* or pWH-*cpaB*$_{pelB}$-*his*. Secretion of CpaB-His was detected in cells carrying pbav-*cpaA-flag* and pWH-*cpaB*$_{pelB}$-*his*. CpaA* is a degredation product from CpaA-FLAG.

5.3.7-Investigating the Chaperone Binding Domain of CpaA

Proteins must fold properly and be delivered to the correct cellular compartment to perform their biological function. A Protein chaperones aid in protein folding, preventing aggregation, maintaining proteins in an unfolded translocation competent state and/or providing a scaffold initiating proper folding (Hendrick, J.P. and Hartl, F.U., 1995; Kim, Y.E. *et al.*, 2013). CpaA is translocated to the periplasm by the SecYEG translocation system in an unfolded state (Green, E.R. and Mecsas, J., 2016). Once in the periplasm CpaA must fold to be secreted by the Type II secretion system (Filloux, A., 2004; Costa, T.R. *et al.*, 2015). CpaB is essential for secretion of CpaA, however the membrane-anchor of CpaB is not required for CpaA secretion (Figure 5.8) (Harding, C.M. *et al.*, 2016a). *Burkholderia glumae* Type II substrate, LipA, requires a membrane bound lipase-specific foldase (Lif), to be folded into its active form and secreted (Rosenau, F. *et al.*, 2004; El Khattabi, M. *et al.*, 2000). The transmembrane-domain of Lif is not required for binding to and folding of LipA (El Khattabi, M. *et al.*, 1999). We predict

that CpaB functions similarly to Lif leading us to hypothesize that the periplasmic domain of CpaB binds CpaA, guiding its folding into an active and secretion competent state (Rosenau, F. *et al.*, 2004; El Khattabi, M. *et al.*, 2000). To investigate the chaperone binding domain of CpaA segments of *cpaA* were cloned with an N-terminal GST tag and CpaB-His co-purification experiments were performed (Figure 5.9A). Cytoplasmic CpaB-His was used in these pulldowns, where the transmembrane domain was removed and a C-terminal hexa-Histidine tag was added. Full-length GST-CpaA co-purified with cyto-CpaB-His (Figure 5.9B). The GST tag alone did not co-purify with cyto-CpaB-His, indicating the interaction with CpaB-His is dependent upon CpaA. GST-CpaA constructs were not purified by Ni-NTA chromatography in the absence of cyto-CpaB-His, demonstrating Histidine tagged CpaB is required for binding the Ni-NTA column. One quarter segments of GST-CpaA regardless of which portion of *cpaA* was expressed, co-purified with cyto-CpaB-His (Figure 5.9B). The data suggests there are multiple contact points between the periplasmic domain of CpaB and CpaA.

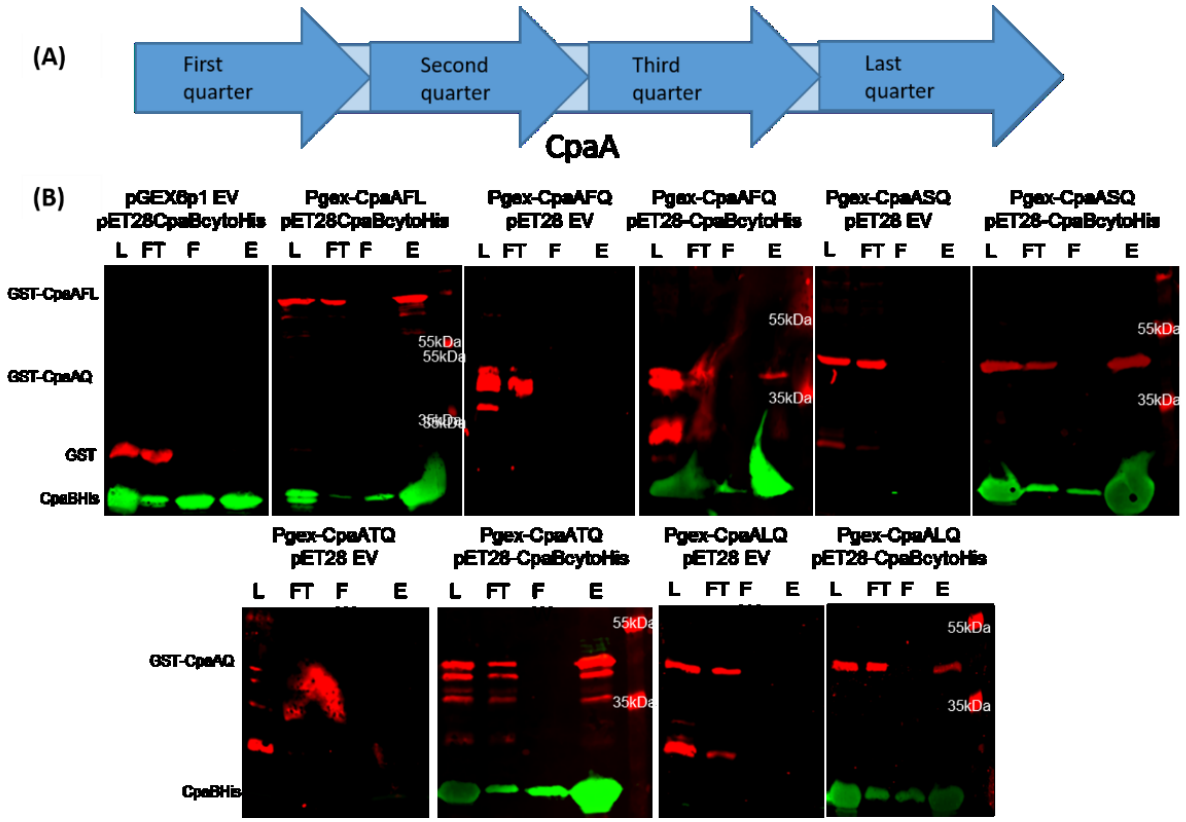

Figure 5.9-Investigating the Chaperone Binding Domain of CpaA

A) Pictorial diagram of the four quarter segments of CpaA that were cloned with an N-terminal GST tag. B) Cytoplasmic pET28-*cpaB-his* was co-expressed with empty pGEX6p1, pGEX-*cpaAFL*, pGEX-*cpaAFQ*, pGEX-*cpaASQ*, pGEX-*cpaATQ* or pGEX-*cpaALQ*. CpaB-His was purified from cell lysates using Nickel-NTA affinity chromatography. L is load fraction, FT is flow-through, F is the final wash and E is the elution fraction. Western blot analysis probing for GST, GST-CpaA and CpaB-His was performed to detect GST tagged CpaA and histidine tagged CpaB present in the load, flow-through, final wash and elution fractions. CpaB-His is detected in all fractions of cells carrying pET28-*cpaB-his*. CpaB-His is present in higher amounts in the elution fraction than the final wash. GST-CpaA is detectable in the load and flow through of all cells carrying a pGEX-*cpaA* plasmid. GST-CpaA is only detectable in the elution fraction of cells co-expressing pGEX-*cpaA* and pET28-*cpaB-his*. GST-CpaA full length, first quarter, second quarter, third quarter and last quarter were all enriched for in the elution fraction of cells expressing CpaB-His. A quarter segment of CpaA is sufficient to be co-purified with CpaB-His.

5.3.9-Towards a CpaA-CpaB 3D Protein Crystal Structure

Since the transmembrane domain of CpaB is not required for the folding and secretion of CpaA (Figure 5.8), cytoplasmic truncated CpaA and CpaB were used in our crystallization study. Over-expression of cytoplasmic CpaA and CpaB was obtained in Rosetta 2 cells grown in auto-induction media at 30ºC 225 rpm. Co-expression of *cpaA-his* and *cpaB* allowed for purification of the CpaA-CpaB complex from *E. coli.* Elution from the size-exclusion column as one peak rather than two distinct peaks suggests these proteins are complexed (Figure 5.10B). CpaA and CpaB are present in equa-molar amounts after size-exclusion chromatography, suggesting they are in complex with one another (Figure 5.10A). After nickel-affinity purification and size-exclusion chromatography we obtained complexed CpaA-CpaB with few contaiminating proteins visible by SDS-PAGE and coomassie staining (Figure 5.10A). Protein crystallization of 1 µL buffer (0.1 M Sodium cacodylate pH6.5 and 1.26 M Ammonium sulfate) and 1 µL 22 mg/mL CpaA-CpaB we obtained numerous crystal clusters with sharpe edges (Figure 5.10C). We collected 3 X-ray diffraction datasets from 0 to 180 degrees at the ALS 422 beamline ranging frome 3.5-7 angstrom resolution (Figure 5.10D).
There are no 3D crystal structures of proteins with high homology to CpaA or CpaB currently available. Without a solved structure to phase our data, we cannot solve the crystal structure. Alternatively, a heavy-metal derivative structure can be acquired and through comparison of these two diffraction patterns the structure can be determined (Jimah, J.R. *et al.*, 2016; Pike, A.C. *et al.*, 2016). Current work is focused on obtaining a protein crystal either soaked in heavy metal derivatives such as Platinum (Jimah, J.R. *et al.*, 2016) or Selinomethionine (Lee, Y.H. *et al.*, 1996) replacement by purifying the protein from auto induction media supplemented with selinomethionine.

To improve the diffraction resolution, we are trying to obtain a single crystal rather than a cluster. Crystals are being grown at 17ºC by hanging-drop diffusion after mixing 1 µL of CpaA-CpaB at 5.25 mg/mL and 1 µL of 0.1 M Sodium cacodylate pH6.5 and 1.26 M Ammonium sulfate. The lower protein concentration should slow down the growth of the crystal, which may result in linear single crystals. Crystal seeding is being used to separate nucleation from crystal growth in hopes of obtaining single linear crystals. Crystals are being grown at 17ºC by hanging-drop vapor diffusion after mixing 1 µL of 22 mg/mL CpaA-CpaB with 1 µL 0.1 M Sodium cacodylate pH6.5 and 1.26 M Ammonium sulfate that has been streak-seeded with CpaA-CpaB

crystals formed in the same condition (Jimah, J.R. *et al.*, 2016). Additonally we will begin optimizing the cryo-preservation conditions (Pflugrath, J.W., 2004). The crystals that have diffracted were all preserved in MiTeGen oil. Some of these crystals had ice rings form despite the presence of MiTeGen oil, demonstrating that cryo-protection was not complete (Pflugrath, J.W., 2004). We will try alternative cryo-preservants such as glycerol, ethidium glycol and PEG solutions.

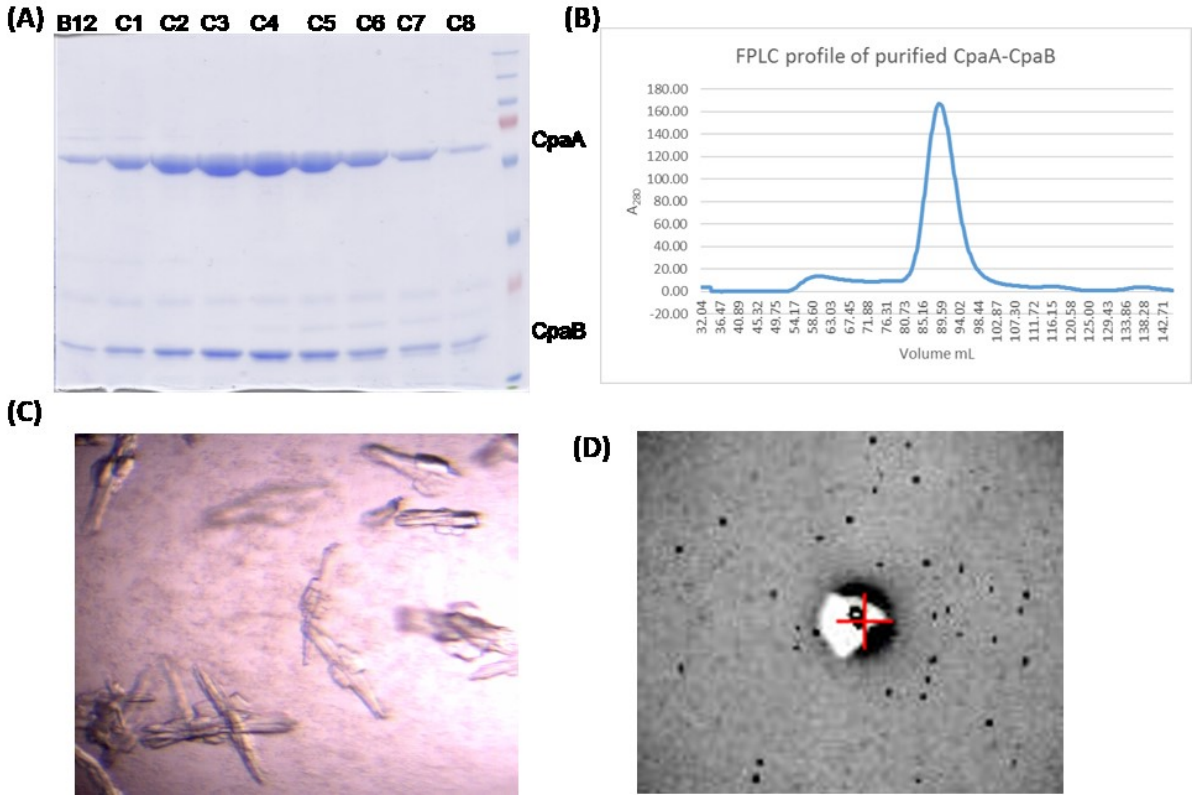

Figure 5.10-Protein X-ray Crystallography Analysis of the CpaA-CpaB protein Complex: A)Size-exclusion chromatography analysis of co-purified CpaA-CpaB. Representative ultra-violet absorbance at 280 nm is plotted against elution volume passed over the column after purified CpaA-CpaB was loaded onto the column. B) Size-exclusion chromatography analysis of co-purified CpaA and CpaB. FPLC fractions of co-purified CpaA/CpaB after size-exclusion chromatography analyzed by SDS-PAGE and commassie brilliant blue staining. Fractions C2-C5 are enriched for CpaA and CpaB. CpaA and CpaB eluted in proportional amounts suggesting they are complexed in solution. Fractions C2-C5 were concentrated to 22 mg/mL and utilized in protein crystallization. C) Representative light microscopy image of CpaA-CpaB protein crystals that formed in 0.1 M Sodium cacodylate pH6.5 and 1.26 M Ammonium sulfate. D)

Representative X-ray diffraction pattern of CpaA-CpaB protein crystals that were analyzed at the ALS 422 beamline. Proteins diffracted at 3.5-7 angstrom resolution.

5.4-Discussion

CpaA was previously shown to be a secreted zinc-dependent metallo-endopeptidase that was capable of degrading fibrinogen and factor V, deregulating blood coagulation (Tilley, D. *et al.*, 2014). Through degrading fibrinogen, CpaA was proposed to have a role in dissemination of *Acinetobacter.* The T2SS of *A. nosocomialis* M2 was required for full virulence in *G. mellonella* and pulmonary murine models of infection (Harding, C.M. *et al.*, 2016a). Additionally, the T2SS-deficient strain did not disseminate to the liver or spleen as efficiently as the wildtype or complemented strains, suggesting it plays an important role in virulence. Type II dependent secretion of LipA in *A. baumannii* ATCC 17978 was required for growth in minimal media supplemented with long-chain fatty acids as the sole carbon source, indicating an important role in nutrient acquisition (Johnson, T.L. *et al.*, 2015a) Furthermore, the T2SS and T2SS-dependent lipid utilization were required for competitive colonization of a neutropenic murine model of infection (Johnson, T.L. *et al.*, 2015a). Type II secretion in *Acinetobacter* is important for pathogenesis and nutrient acquisition.

Deletion of *cpaA* results in a virulence defect in *G. mellonella* larvae and decreased splenic colonization in a murine pulmonary infection model. CpaA has been shown to degrade coagulation factors fibrinogen and factor V, and likely contributes to dissemination of *A. nosocomialis* from the initial site of infection resulting in lower burdens in the spleen relative to the complemented strain (Tilley, D., *et al.,* 2014). This dissemination defect may explain the high variability in CFU counts of Δ*cpaA* in the lung and particularily the spleen.

CpaA is the most abundantly secreted type II substrate in *A. nosocomialis* M2 (Harding, C.M., *et al.*, 2016). Correlation between virulence in *G. mellonella* larvae and mammalian models of infection have been established for *Pseudomonas aeruginosa* and *Cryptococcus neoformans* (Jander, G., *et al.*, 2000, Mylonakis E., *et al.*, 2005).*G. mellonella* are a good insect model for human pathogens as they can be incubated at 37ºC, unlike some invertebrate systems. There are strong similarities between the innate immune system of *G. mellonella* and mammals (Kavanagh K., and Reeves, E.P., 2004). There have been several studies investigating various virulence traits of *Acinetobacter* in *G. mellonella* and murine models of infection. Pathogenic

Acinetobacter strains cause more lethality in *G. mellonella* than non-pathogenic isolates (Peleg, A.Y., *et al.*, 2009). *G. mellonella* hemocytes phagocytose *Acinetobacter* cells in response to infection. Upon infection with *Acinetobacter G. mellonella* larvae deposit melanin in the hemolymph. Melain binds to the bacterial cell and initiates the serine protease cascade similarly to the complement cascade (Kavanagh, K. and Reeves, E.P., 2004). The important role of the innate immune response to *Acinetobacter* infections combined with the similiarites between insect and mammalian innate responses make *G. mellonella* a good infection model. Our investigation into virulence attributed to CpaA demonstrates differences between the insect model and a mammalian infection model. In the insect model, which is focused on the innate response to *A. nosocomialis* infection, we see similar lethality in larvae infected with the Δ*cpaA* or a strain devoid of type II secretion. In the murine model of infection we see stronger colonization defects when the mouse was infected with a strain devoid of type II secretion rather than loss of a single type II substrate. Loss of CpaA clearly impairs *A. nosocomialis* dissemination in the mouse.

Mutation of the Type II secretion system causes a more pronounced decrease in lung and spleen colonization than the loss of CpaA alone. Numerous proteins are secreted in a Type II dependent manner from *A. nosocomialis* M2 (Harding, C.M. *et al.*, 2016a). LipA, a confirmed *Acinetobacter* Type II substrate, is required for *A. baumannii* competative murine colonization (Johnson, T.L. *et al.*, 2015b). LipA likely impacts murine colonization with *A. nosocomialis,* accounting in part of the difference in colonization defect between the Δ*gspD* and Δ*cpaA* strains. Loss of CpaA clearly impairs *A. nosocomialis* dissemination. CpaA has proteolytic activity against coagulation factors fibrinogen and factor V (Tilley, D., *et al.*, 2014). Murine fibrinogen and factor V are similar in sequence to their human orthologs, however the sequences are not identical. It would be interesting to investigate the specificity of CpaA against human and murine coagulation factors. As *A. nosocomialis* is a human pathogen, CpaA may have a more drastic impact on the coagulation cascade during human infection than mice.

CpaA is translocated to the periplasm by the SecYEG translocation system in an unfolded state (Green, E.R. and Mecsas, J., 2016). Once in the periplasm CpaA must fold to be secreted by the Type II secretion system (Filloux, A., 2004; Costa, T.R. *et al.*, 2015). CpaB, a putative membrane anchored chaperone, is required for secretion of CpaA (Harding, C.M. *et al.*, 2016a). This is reminiscent of *B. glumae* Type II substrate, LipA, which requires a membrane bound

lipase-specific foldase (Lif), to be folded into its active form and secreted (Rosenau, F. *et al.*, 2004; El Khattabi, M. *et al.*, 2000). The physical interaction between CpaA and CpaB further supports that notion that CpaB is the chaperone of CpaA. We demonstrated here that similarly to Lif, the C-terminal globular domain of CpaB is periplasmic and sufficient for CpaA secretion (El Khattabi, M. *et al.*, 1999). The strong physical interaction between CpaA and CpaB provides further support that CpaB is a chaperone protein that binds CpaA. CpaA is translocated to the periplasm by the SecYEG translocation system in an unfolded state (Green, E.R. and Mecsas, J., 2016). Once in the periplasm CpaA must fold to be secreted by the Type II secretion system (Filloux, A., 2004; Costa, T.R. *et al.*, 2015). We predicted CpaB functions similarly to Lif, binding CpaA in the periplasm, guiding its folding into an active and secretion competent state (Rosenau, F. *et al.*, 2004; El Khattabi, M. *et al.*, 2000).

B. glumae Lif is a steric chaperone that wraps around LipA helping it obtain an active and secretion competent state. One quarter of CpaA is sufficient to bind CpaB, forming a tight purifiable complex. CpaB forms stable, strong interactions all along the length of CpaA. This is very different from cytoplasmic chaperones SecB or type III secretion system chaperones, which tend to bind specifically to a small chaperone binding domain at the amino terminus. The co-crystal structure of Lif and LipA from *B. glumae* shows Lif wrapped around a large portion of LipA. The ability of CpaB to co-purify fragments of CpaA from the N-terminus to the C-terminus suggest that CpaB binds CpaA at multiple interaction points along the protein. CpaB may function similarly to Lif, wrapping itself along the length of CpaA. The co-crystal structure of CpaA and CpaB in complex will definitively demonstrate their interaction. The structure may also illuminate CpaB's mechanism of action.

Here we have demonstrated CpaA is an important virulence factor for *A. nosocomialis.* Novel treatment options for *Acinetobacter* need to be developed due to increasing rates of multidrug resistance. Specific inhibitors designed to bind and inhibit activity of a virulence factor is plausible option. Structural data from the CpaA-CpaB complex can be used to design specific inhibitors against CpaA. Future work will focus on understanding the impact of the chaperone on activity of CpaA. Some chaperones function as immunity proteins, blocking activity of an effector until it is released from the cell. We have demonstrated the membrane anchor of CpaB is not required for its chaperone function. We will examine whether this membrane anchor facilitates an interaction with either SecYEG or the Type II apparatus, yielding more efficient

secretion of CpaA. Alternatively, the membrane anchor may function to prevent secretion of CpaB with CpaA. Soluble, periplasmic CpaB was secreted with CpaA. If CpaB binding blocks activity of CpaA; *A. nosocomialis* need to ensure only free CpaA is secreted from the cell. Additionally, secretion of both CpaA and CpaB is more energetically costly for the cell than secretion of CpaA alone.

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Chapter 6-Discussion

Part of this discussion on protein glycosylation in *Acinetobacter* was published as R.L. Kinsella, N.E. Scott and M.F. Feldman (2015) Clinical implications of glycoproteomics for *Acinetobacter baumannii*, Exp Rev Prot **12**(1):1-3.

6.1-Protein Glycosylation in *Acinetobacter:*

Epidemiological research on *A. baumannii* may provide antibiotic resistance profiles and tissue tropism associated with specific strains. The development of a serotyping system for *A. baumannii* would aid in rapid detection and strain-to-strain differentiation. This would be clinically beneficial for the rapid and more effective treatment of *A. baumannii*. Because of detectable variation in the polysaccharides presented at Gram-negative cell surfaces, carbohydrate structures have been the basis for technologies used to detect and differentiate bacterial strains for over 40 years. Examples of these are the *Escherichia coli* O antigen polysaccharide-based serotyping system, the *Brucellosis* detection technology based on O antigen structure, and the group B *Streptococci* capsular polysaccharide-based serotyping (Calix, J.J. *et al.*, 2012a; Chong, Y. *et al.*, 1995; Henrichsen, J., 1995; Higgins, R. *et al.*, 1995; Iguchi, A. *et al.*, 2012; Iwashkiw, J.A. *et al.*, 2012a; Ciocchini, A.E. *et al.*, 2013). Unlike many other Gram-negative bacteria, the surface of *A. baumannii* lacks the conventional O antigen attached to lipid A. However, it is made up of other carbohydrate structures, including capsular polysaccharide and *O*-linked glycoproteins. These structures are ideal to be used as diagnostic markers.

O-linked protein glycosylation is active in most strains of *Acinetobacter*. Of all the strains characterized thus far, *A. baumannii* AYE, is the only one that does not produce capsular polysaccharide or glycosylate proteins under the laboratory conditions tested (Scott, N.E. *et al.*, 2014). The proteins modified are quite well conserved and unique to *Acinetobacter*. Bioinformatic analysis of the carbohydrate biosynthesis loci in *Acinetobacter* revealed as many as 77 distinct serotypes (Hu, D. *et al.*, 2013a; Kenyon, J.J. and Hall, R.M., 2013a). Experimental characterization of the glycan diversity in *Acinetobacter* suggests this glycan variability is higher than that predited bioinfomatically. The extensive variability is not only due to genetic diversity between strains but also microheterogeneity within strains. The glycans produced by one strain most commonly differ in acetylation, and the presence or absence of monosaccharides. The non-template driven nature of glycan biosynthesis impacts the resulting glycans produced (Scott, N.E. *et al.*, 2014). Surface exposed glycans such as capsular polysaccharides and O-antigens interact with the extra-cellular environment. The carbohydrate structures can be recognized by the immune system and are often potent immunogens where specific antibodies are developed against the carbohydrate structures. Presure from the immune system can result in evolutionary

adapation through alterations in the carbohydraete structure allowing escape from immune detection. There are over 90 *Streptococcus pneumoniae* serotypes (Henrichsen, J., 1995; Calix, J.J. *et al.*, 2012b)and 184 serotypes of *E. coli* (Iguchi, A. *et al.*, 2015). Genetic variation in *E. coli* O antigen loci come from horizontal gene transfer events (Leopold, S.R. *et al.*, 2009; Iguchi, A. *et al.*, 2011), (Iguchi, A. *et al.*, 2012), point mutations in glycosyltranferase genes as well as acquisition or loss of carbohydrate modification genes (Wang, Q. *et al.*, 2012). *O*-glycan structures produced in *Acinetobacter* are extremely diverse. This variability makes the possibility of a glycan based vaccine less attractive. To-date there has not been a study linking a particular capsular serotype to increased pathogenicity or prevalence. In *S. pneumoniae* there are over 90 serotypes, but only 10 to 15 serotypes cause the majority of disease. If the same trend is established for *Acinetobacter* a glycan based multivalent vaccine approach may be effective.

Similar to other bacteria (Iwashkiw, J.A. *et al.*, 2012b; Iwashkiw, J.A. *et al.*, 2013), all pathogenic *Acinetobacter* strains can post-translationally modify proteins by the addition of diverse glycan structures (Scott, N.E. *et al.*, 2014; Schulz, B.L. *et al.*, 2013; Iwashkiw, J.A. *et al.*, 2012c; Lees-Miller, R.G. *et al.*, 2013). In *Acinetobacter* species *O*-linked glycosylation is mediated by the conserved *O*-oligosaccharyltransferase, PglL, which attaches an *O*-glycan to hydroxylated amino acids (Serine or Threonine) on multiple proteins (Iwashkiw, J.A. *et al.*, 2012c). The biosynthesis of the *O*-glycan is encoded by a single genetic locus also responsible for generation of the capsule. Consistent with the extreme genetic diversity existing both between strains and microheterogeneity within strains (Scott, N.E. *et al.*, 2014). Despite this diversity, *O*-linked glycans from *Acinetobacter* species share similar properties, consisting of 3-5 carbohydrate residues, with limited branching and negatively charged sugars. These common features suggest a shared function of the *O*-linked/capsule monomers across strains. Consistent with this hypothesis, clinical strains of *A. baumannii* are highly resistant to complement-mediated killing in a capsule-dependent manner (Scott, N.E. *et al.*, 2014; Lees-Miller, R.G. *et al.*, 2013), and the attachment of these glycans to protein substrates is also essential for biofilm formation and virulence. Although these findings demonstrate the importance of *O*-glycan biosynthesis and protein glycosylation for virulence, their precise contribution to these phenotypes is unknown.

The use of mass spectrometry and proteomic approaches has been instrumental in identification and characterization of *Acinetobacter* glycosylation and its glycoproteome. The

use of selective enrichment of glycopeptides using zwitterionic hydrophilic interaction liquid chromatography coupled to multiple fragmentation approaches provides a robust platform for the analysis of diverse glycopeptides. This approach enabled the identification of diverse and multimeric forms of the *O*-glycan and the determination of the glycosylation sites using submicrogram amounts of sample (Kenyon, J.J. and Hall, R.M., 2013a; Lees-Miller, R.G. *et al.*, 2013; Hu, D. *et al.*, 2013b). With increasing refinement to these proteomic approaches, it is now possible to assess glycosylation in a quantitative manner using techniques, such as dimethyl labeling (Lithgow, K.V. *et al.*, 2014; Boersema, P.J. *et al.*, 2009). Furthermore, by using ultraviolet photodissociation information on carbohydrate stereochemistry can be gathered from approximately 1/1000 of the sample amount needed for Nuclear Magnetic Resonance spectroscopy (Madsen, J.A. *et al.*, 2013). The application of these developments within the field of bacterial glycosylation analysis paired with continuous refinement of mass spectrometry instrumentation (Hebert, A.S. *et al.*, 2014) enables higher quality data to be generated in shorter periods of time, facilitating the characterization of glycosylation within this genus. With these tools, it is now possible to start addressing the role of protein glycosylation in *Acinetobacter* biology and the potential use of glycoconjugates as diagnostic markers.

Together with others, we have discovered the carbohydrate biosynthesis locus responsible for the production of the *O*-glycan and its polymerization into capsular polysaccharide (Scott, N.E. *et al.*, 2014; Hu, D. *et al.*, 2013a; Lees-Miller, R.G. *et al.*, 2013; Kenyon, J.J. and Hall, R.M., 2013b). As mentioned earlier, the capsular polysaccharide is required for resistance to complement-mediated killing, and therefore survival in the host. A conserved initiating glycosyltransferase, PglC, is responsible for attaching the first sugar of the *O*-glycan to the lipid carrier and is required for protein glycosylation and capsular polysaccharide production (Lees-Miller, R.G. *et al.*, 2013). Deletion of *pglC* results in sensitivity to complement-mediated killing and inability to colonize mice. The increasing prevalence of multidrug resistant *A. baumannii* infections worldwide demands the development of new antimicrobials to fight this pathogen. The conservation of PglC across *Acinetobacter* species makes this enzyme an excellent target for antimicrobial development against *A. baumannii*.

A. baumannii's ability to survive on abiotic and biotic surfaces in the hospital environment provides a potential source of infection to susceptible surgical or immunocompromised patients. Prophylactic vaccination against *A. baumannii* is a potential

solution to this concern. Conjugations of O antigens or capsular polysaccharides to a protein carrier have been successfully used as effective licensed vaccines against *Haemophilus influenza, Neisseria meningitidis, Salmonella* and *Streptococcus pneumoniae* (Astronomo, R.D. and Burton, D.R., 2010). Within these conjugated vaccines, the carbohydrate portion provides serological specific immunity, whereas the protein component initiates long-lasting immunity (Astronomo, R.D. and Burton, D.R., 2010). We suggest that a glycoconjugate vaccine, specific for the *O*-linked glycans produced by each strain makes these antigens ideal targets. Because of the diversity in the *O*-glycan structures produced across *A. baumannii*, further epidemiological studies would be beneficial in determining whether there is a highly prevalent or extreme-disease associated serotype, which would make the best vaccine candidate. In addition, multivalent glycoconjugate vaccinations against highly prevalent *A. baumannii* serotypes may be effective.

Protein glycosylation in bacteria is not essential for growth in laboratory conditions, yet it is central in infection and virulence in bacterial pathogens. Although it is known that glycosylation is required for the correct function of adhesins and flagellins in pathogenesis, there is still no clear mechanistic explaination of how glycosylation impacts the function of these proteins. In the case of some flagellins, glycosylation improves stability and enables flagella assembly, but in general terms, the role of these glycans is still a challenge to be solved.

Advances in key technologies like Mass Spectrometry (MS) and Nuclear Magnetic Resonance have facilitated the characterization of bacterial glycoproteomes. The current MS methods allow the determination of the glycosylated proteins and their preliminary glyan structures without having to purify the glycoproteins. These technological advances and novel developments will have an impact on the discovery of glycosylation pathways, which will help to uncover novel features of the still largely uncharacterized bacterial glycosylation world

Glycoprotein, A1S_3744, was not required for virulence in *G. mellonella*. The protein glycosylation mutant strain was attenuated in *G. mellonella*, demonstrating there is a role for protein glycosylation in pathogenesis. The *A1S_3626* mutant strain will be analyzed for a role in virulence using the larvae model. It is difficult to determine the function of *Acinetobacter* glycoproteins as they tend to be unique with not known homologs. The *in vivo* cross-linking study revealed several protein-protein interactions these proteins are involved in. Homopolymeric interactions between glycoproteins A1S_1193, A1S_3744 and A1S_3626 were detected. Additionally, A1S_3626 was found to interact with A1S_3744, A1S_1193, DsbA and DsbC as

well as several other uncharacterized proteins (Wu, X. *et al.*, 2016). Identification of protein-protein interactions these glycoproteins are involved in may shed light on their apparent functions. Additionally structural determination could reveal structural homologs illuminating the physiological role of these proteins.

6.2-Type II Secretion and the Role of Membrane Anchored Chaperones in *Acinetobacter*:

At least two *Acinetobacter* Type II substrates required membrane anchored chaperones for their secretion. CpaA and LipA are translocated to the periplasmic space by SecYEG in an unfolded state (Green, E.R. and Mecsas, J., 2016). Upon entering the periplasm these proteins must be folded. We predict LipB and CpaB help fold their cognate substrates into an active and secretion competent state (Filloux, A., 2004; Costa, T.R. *et al.*, 2015). Lipase specific foldases are Type II secretion membrane anchored steric chaperones. Lif proteins have been well characterized in *P. aeruginosa* and *B. glumae* (Rosenau, F. *et al.*, 2004; El Khattabi, M. *et al.*, 1999; El Khattabi, M. *et al.*, 2000; Frenken, L.G. *et al.*, 1993a; Frenken, L.G. *et al.*, 1993b; Martinez, A. *et al.*, 1999). Similarily to Lif, the C-terminal globular domain of CpaB is periplasmic and sufficient for CpaA secretion (El Khattabi, M. *et al.*, 1999). The strong physical interaction between CpaA and CpaB provides further support that CpaB is a chaperone protein that binds CpaA.

B. glumae Lif is a steric chaperone that wraps around LipA helping it obtain an active and secretion competent state. Co-crystallization of LipA and Lif revealed Lif wraps itself around LipA, rather than binding at one particular region (Pauwels, K. *et al.*, 2006). CpaB forms stable, strong interactions all along the length of CpaA. The ability of CpaB to bind fragments of CpaA from the N-terminus to the C-terminus suggest that CpaB may function similarly to Lif, wrapping itself along the length of CpaA. The co-crystal structure of CpaA and CpaB in complex will definitively map their interaction. The structure may also illuminate CpaB's mechanism of action.

The mechanism by which membrane anchored chaperones influence secretion of their cognate substrates is not well understood. LipA folded in the presence of Lif is more resistant to proteolytic digestion than LipA that has not been folded by Lif(Pauwels, K. *et al.*, 2012), indicating these chaperones also aid in final stability of their effectors. Whether membrane anchored chaperones impact the secretion efficiency of their substrates has not been reported. It

is plausible that the chaperone may secondarily interact with either the SecYEG translocation system or the Type II apparatus resulting in more efficient export of its cognate effector. Future studies will investigate the protein-interaction network of membrane bound chaperone CpaB.

Here we have demonstrated CpaA is an important virulence factor for *A. nosocomialis*.. Structural data from the CpaA-CpaB complex can be used to design specific inhibitors against CpaA. Future work will focus on understanding the impact of the chaperone on activity of CpaA. Some chaperones function as immunity proteins, blocking activity of an effector until it is released from the cell. We have demonstrated the membrane anchor of CpaB is not required for its chaperone function. We will examine whether this membrane anchor facilitates an interaction with either SecYEG or the Type II apparatus, yielding more efficient secretion of CpaA. Alternatively, the membrane anchor may function to prevent secretion of CpaB with CpaA. Soluble, periplasmic CpaB was secreted with CpaA. If CpaB binding blocks activity of CpaA; *A. nosocomialis* need to ensure only free CpaA is secreted from the cell. Additionally, secretion of both CpaA and CpaB is more energetically costly for the cell than secretion of CpaA alone.

Bioinformatic analysis of characterized Type II substrates revealed the presence of putative membrane associated chaperones. Type II lipases from various bacterial species require membrane anchored chaperones for their secretion (El Khattabi, M. *et al.*, 2000; Frenken, L.G. *et al.*, 1993a; Frenken, L.G. *et al.*, 1993b; Hendrick, J.P. and Hartl, F.U., 1995; Kim, Y.E. *et al.*, 2013). CpaB is the first characterized Type II protease chaperone. Future work will focus on ascertaining if these putative type II chaperones identified bioinformatically are required for secretion of their cognate substrate. If Type II chaperones are required for secretion of important nutrient acquisition enzymes and/or virulence factors they provide a good target for novel antimicrobial design. The structural data mapping the interaction between CpaA and CpaB as well as LipA and Lif can be used to design Type II substrate inhibitors.

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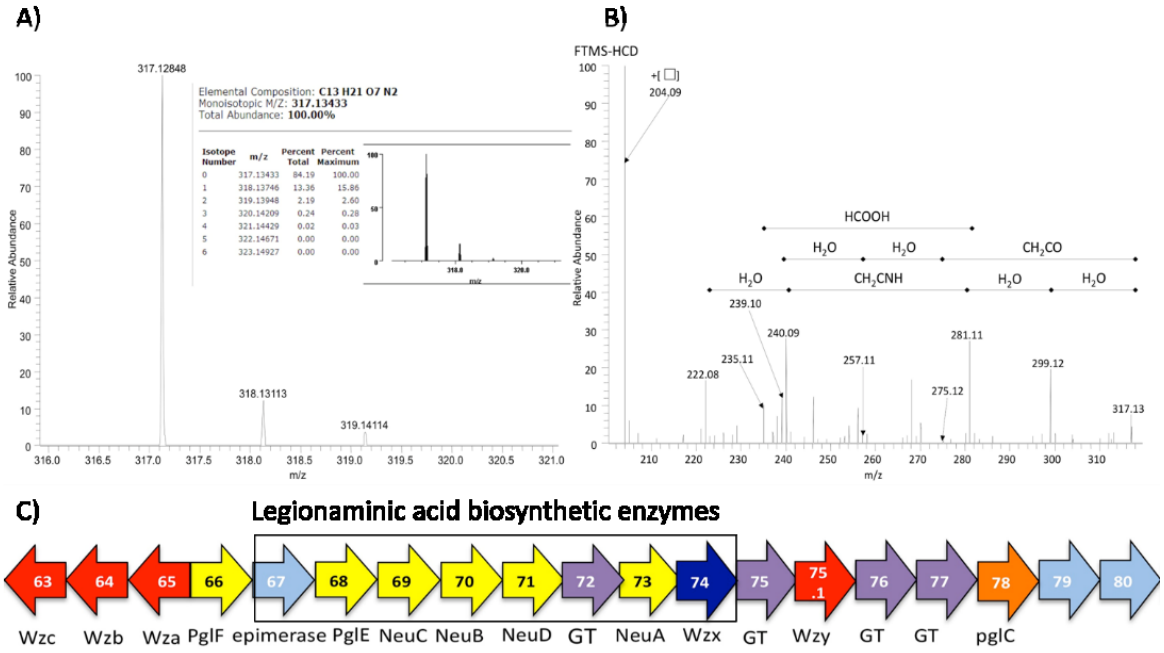
Appendix 1 – Includes supplementary Information from Chapters 2 and 4.

Supplementary data for Chapter 2:

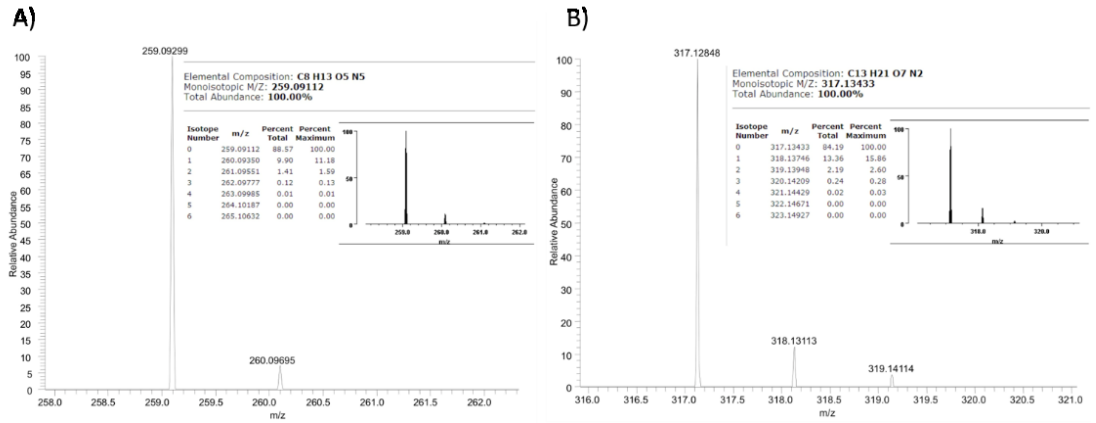
Figure S2.4 MS and bioinformatic analysis of the NulO oxonium ion/ glycosylation cluster of SDF



Supplementary Figure S2.4 – MS and bioinformatic analysis of the NulO oxonium ion/glycosylation cluster of SDF.

HCD spectra of the low mass region of the SDF glycopeptide ^{302}AKPASTPAVK^{311}. A)Isotopic distribution analysis of the 317.13 m/z oxonium ion, the resulting isotopic distribution is identical to the expected chemical composition of legionaminic acid (C12H21O7N2). B)Multiple ions related to the breakdown of 317 can be detected and are consistent with the previously assigned fragmentation of legionaminic acid. C)Bioinformatic analysis demonstrates the presence of the genes responsible for the synthesis of legionaminic acid.

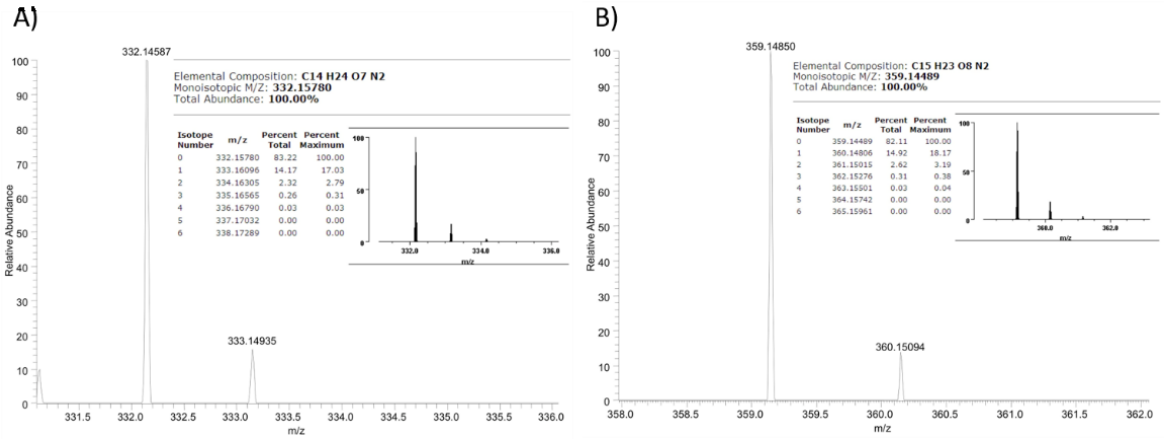
Figure S2.5 Isotopic distribution analysis of the 259.10 and 317.13m/z oxonium ion



Supplementary Figure S2.5-Isotopic distribution analysis of the 259.10 and 317.13 m/z oxonium ion.

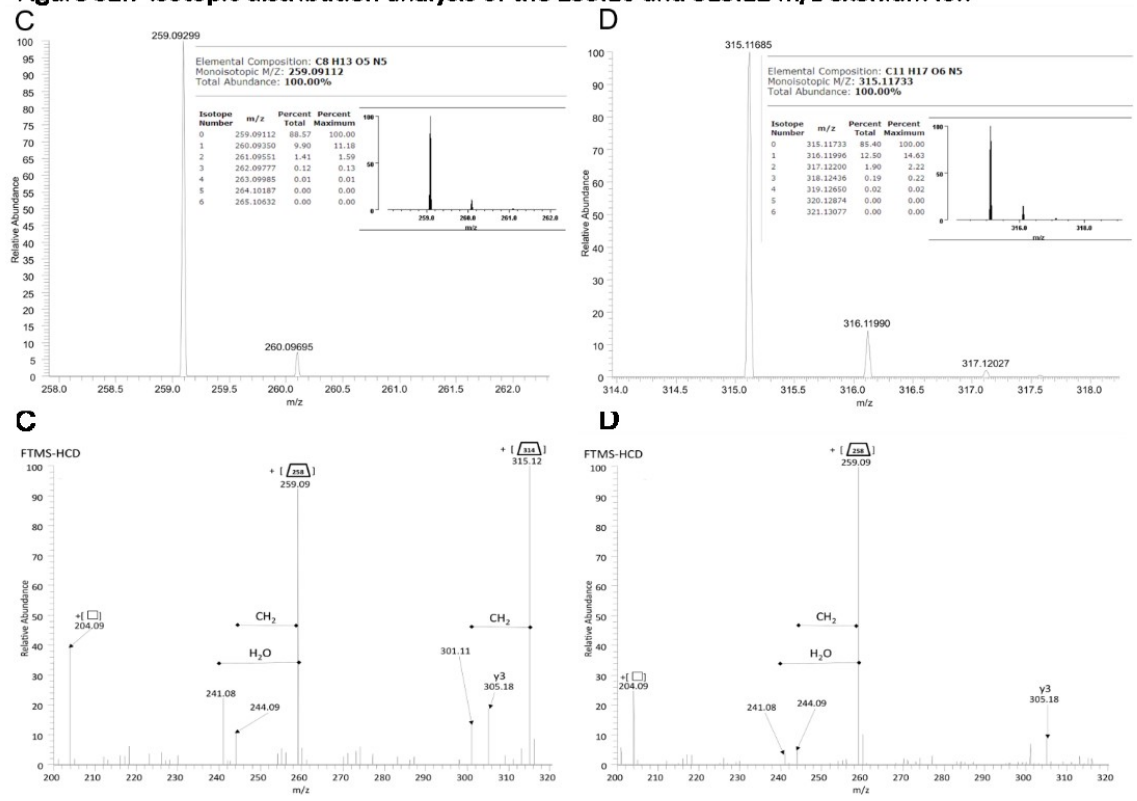
These mass supported the assignments of the 258 DA residues as a deacetylated form of GlcNAc3NAcA4OAc and 316 as a nonulosonic acid (legionaminic/pseudaminic acid) .

Figure S2.6 Isotopic distribution analysis of 332.16 and 359.15 m/z oxonium ion



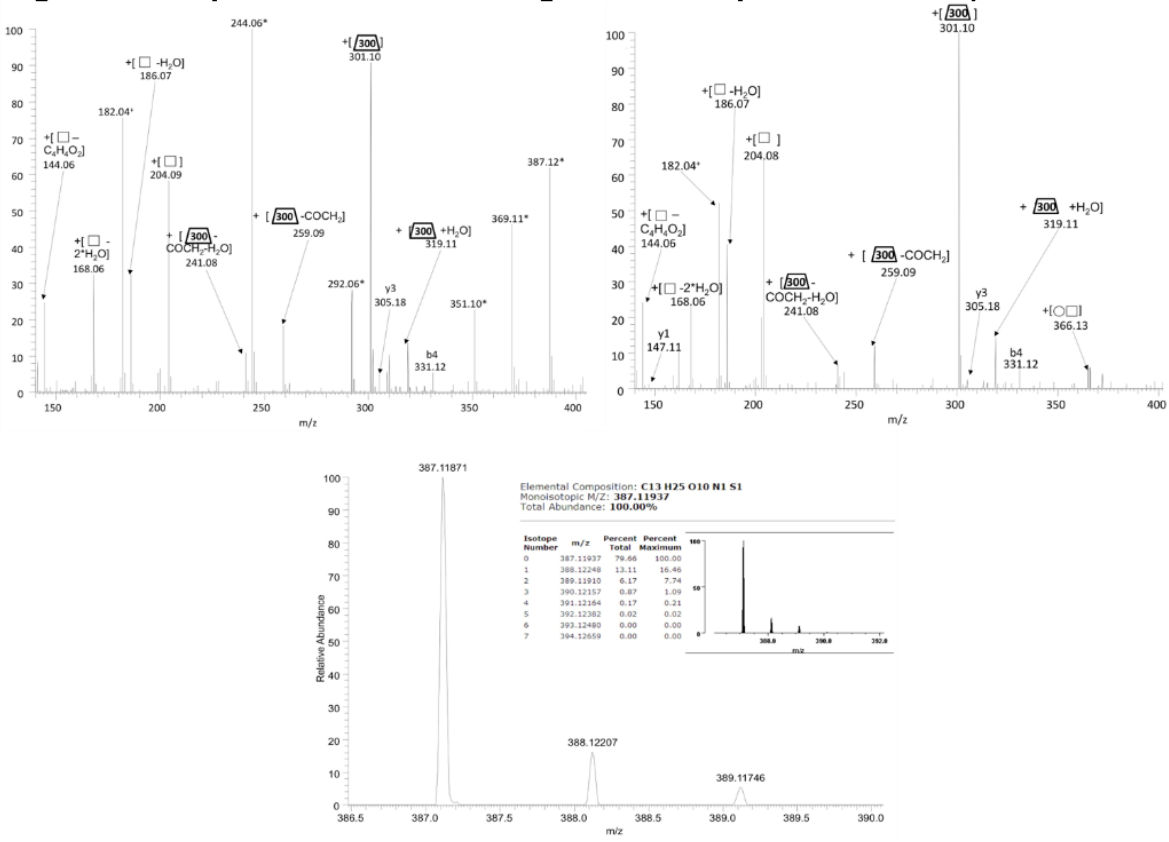
Supplementary Figure S2.6-Isotopic distribution analysis of the 332.16 and 359.15 m/z oxonium ion. These masses supported the assignments of the 332 and 359 Da as modified versions of nonulosonic acid and GlcNAc3NacA4OAc respectively.

Figure S2.7 Isotopic distribution analysis of the 259.10 and 315.12 m/z oxonium ion



Supplementary Figure S2.7-Isotopic distribution analysis of the 259.10 and 315.12 m/z oxonium ion. Both the isotopic distribution (A and B) and the HCD fragmentation of these ions (C and D) supported the assignments of the 258 and 314 Da residues as modified versions of GlcNAc3NacA4OAc.

Figure S2.8 Isotopic distribution and HCD fragmentation analysis of the 387.12 m/z oxoniumion



Supplementary Figure S2.8-Isotopic distribution and HCD fragmentation analysis of the 387.12 m/z oxonium ion. HCD fragmentation supports the 387.12 Da residue fragmenting to generate a 244.06 fragment. A) and B). Isotope analysis supported the assignments of the 387.12 Da as C13H25O10N1S1.

Supplementary Table 2: glycoproteins identified within *Acinetobacter baumannii* strains

ATCC 17978 uniprot accession number	ATCC 17978	SDF	ATCC 19606	Arg 2	Arg 1	1441 C1	1441 C3
A3M128_ACIBT							X
A3M160_ACIBT						X	X
A3M1A0_ACIBT						X	
A3M247_ACIBT			X				
A3M266_ACIBT	X	X	X	X	X	X	X
A3M3E7_ACIBT			X		X	X	X
A3M3X9_ACIBT	X					X	X
A3M647_ACIBT						X	
A3M6D2_ACIBT		X	X		X	X	X
A3M796_ACIBT	X		X	X	X	X	X
A3M886_ACIBT					X		
A3M897_ACIBT		X	X		X		
A3M8P7_ACIBT			X	X	X		
A3M964_ACIBT						X	X
A3M9I2_ACIBT				X		X	X
A3M9I7_ACIBT				X			
A7FAU3_ACIBT		X	X			X	X
A7FB17_ACIBT	X					X	X
A7FB63_ACIBT	X	X	X	X	X	X	X
A7FB95_ACIBT	X		X	X	X	X	X
A7FBB9_ACIBT			X		X	X	
A7FBI1_ACIBT	X		X	X	X	X	X
A7FBQ8_ACIBT						X	X
A7FBW3_ACIBT			X		X	X	X
DNAJ_ACIBT					X		
N8U2Y6_ACIBA*				X			

Supplementary table 2: Glycoproteins identified within Acinetobacter baumannii strains. Identifications are labeled according to the ATCC 17978 Uniprot accesion number, * denotes a protein not found in ATCC 17978

Supplementary Table S2-2

Supplementary Table 9: Bioinformatic analysis of *Acinetobacter baumannii* identified glycoproteins

Uniprotaccession number strain SDF	Uniprot accession number strain ATCC 19606	Uniprot accession number strain ATCC 17967	Page
B0VKN6_ACIBS	D0CAB8_ACIBA	A3M265_ACIBT	2
B0VLI0_ACIBS	D0CEI7_ACIBA	A7FB63_ACIBT	3
B0VRK6_ACIBS	D0C6C0_ACIBA	-	4
B0VSH8_ACIBS	D0CDQ9_ACIBA	-	5
B0VV82_ACIBS	D0CA69_ACIBA	-	6
-	D0C6Q2_ACIBA	A7FBI1_ACIBT	7
-	D0C7T4_ACIBA	-	8
-	D0C8B9_ACIBA	A7FB95_ACIBT	9
-	D0C8Y7_ACIBA	-	10
-	D0CAD2_ACIBA	-	11
-	D0CC33_ACIBA	A3M796_ACIBT	12
-	D0CD31_ACIBA	-	13
-	D0CDA9_ACIBA	-	14

Supplementary table 9: Bioinformatic analysis of identified *A. baumannii* glycoproteins. Identified glycoproteins are labeled according to the Uniprot accesion number for each strain. The corresponding analysis of each protein is shown on the denoted pages

Protein Name: Putative uncharacterized protein
Function: unknown
Molecular weight (Da): 34,608
Localisation[1]: periplasmic
Class[2]: unknown

Sequence Identity between strains

Strains		Acinetobacter baumannii (strain SDF)	Acinetobacter baumannii (strain AYE)	Acinetobacter baumannii ATCC 19606	Acinetobacter baumannii (strain TCDC-AB0715)	Acinetobacter baumannii (strain 1656-2)	Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	Acinetobacter baumannii (strain AB307-0294)
	Uniprot #	B0VKN6_ACIBS	B0V5I4_ACIBY	D0CAB8_ACIBA	F0QNY6_ACIBD	E8PBZ6_ACIB1	A3M265_ACIBT	B7H040_ACIB3
Acinetobacter baumannii (strain SDF)	B0VKN6_ACIBS	100%	97%	97%	97.00%	97.00%	97.00%	96.00%
Acinetobacter baumannii (strain AYE)	B0V5I4_ACIBY		100%	98.00%	99.00%	99.00%	99.00%	99.00%
Acinetobacter baumannii ATCC 19606	D0CAB8_ACIBA			100%	98.00%	98.00%	98.00%	97.00%
Acinetobacter baumannii (strain TCDC-AB0715)	F0QNY6_ACIBD				100%	100.00%	98.00%	99.00%
Acinetobacter baumannii (strain 1656-2)	E8PBZ6_ACIB1					100%	98.00%	99.00%
Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	A3M265_ACIBT						100%	98.00%
Acinetobacter baumannii (strain AB307-0294)	B7H040_ACIB3							100%

[1] based on SignalP, http://www.cbs.dtu.dk/services/SignalP/

[2] based on Psortb, http://www.psort.org/psortb/

Protein Name: Predicted protein
Function: unknown
Molecular weight (Da): 7,395
Localisation[1]: periplasmic
Class[2]: unknown

Sequence Identity between strains

Strains		Acinetobacter baumannii (strain SDF)	Acinetobacter baumannii (strain AYE)	Acinetobacter baumannii ATCC 19606	Acinetobacter baumannii (strain TCDC-AB0715)	Acinetobacter baumannii (strain 1656-2)	Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	Acinetobacter baumannii (strain AB307-0294)
	Uniprot #	B0VV82_ACIBS	B0V5Q3_ACIBY	D0CA69_ACIBA	F0QPJ4_ACIBD	E8PC45_ACIB1	A7FAU3_ACIBT	B7GZZ3_ACIB3
Acinetobacter baumannii (strain SDF)	B0VV82_ACIBS	100%	72.00%	80.00%	80.00%	80.00%	80.00%	72.00%
Acinetobacter baumannii (strain AYE)	B0V5Q3_ACIBY		100%	81.00%	81.00%	81.00%	79.00%	100.00%
Acinetobacter baumannii ATCC 19606	D0CA69_ACIBA			100%	100.00%	100.00%	89.00%	81.00%
Acinetobacter baumannii (strain TCDC-AB0715)	F0QPJ4_ACIBD				100%	100.00%	89.00%	81.00%
Acinetobacter baumannii (strain 1656-2)	E8PC45_ACIB1					100%	89.00%	81.00%
Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	A7FAU3_ACIBT						100%	79.00%
Acinetobacter baumannii (strain AB307-0294)	B7GZZ3_ACIB3							100%

[1] based on SignalP, http://www.cbs.dtu.dk/services/SignalP/

[2] based on Psortb, http://www.psort.org/psortb/

Protein Name: TolA
Function: membrane protein
Molecular weight (Da): 11,226
Localisation[1]: periplasmic
Class[2]: unknown

Sequence Identity between strains

Strains		Acinetobacter baumannii (strain SDF)	Acinetobacter baumannii (strain AYE)	Acinetobacter baumannii ATCC 19606	Acinetobacter baumannii (strain TCDC-AB0715)	Acinetobacter baumannii (strain 1656-2)	Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	Acinetobacter baumannii (strain AB307-0294)
	Uniprot #	B0VLI0_ACIBS	B0VAK5_ACIBY	D0CEI7_ACIBA	F0QJK1_ACIBD	E8PA92_ACIB1	A7FB63_ACIBT	B7GWG8_ACIB3
Acinetobacter baumannii (strain SDF)	B0VLI0_ACIBS	100%	97.00%	97.00%	98.00%	98.00%	97.00%	97.00%
Acinetobacter baumannii (strain AYE)	B0VAK5_ACIBY		100%	100.00%	99.00%	99.00%	100.00%	100.00%
Acinetobacter baumannii ATCC 19606	D0CEI7_ACIBA			100%	99.00%	99.00%	100.00%	100.00%
Acinetobacter baumannii (strain TCDC-AB0715)	F0QJK1_ACIBD				100%	100.00%	99.00%	99.00%
Acinetobacter baumannii (strain 1656-2)	E8PA92_ACIB1					100%	99.00%	99.00%
Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	A7FB63_ACIBT						100%	100.00%
Acinetobacter baumannii (strain AB307-0294)	B7GWG8_ACIB3							100%

[1] based on SignalP, http://www.cbs.dtu.dk/services/SignalP/

[2] based on Psortb, http://www.psort.org/psortb/

Protein Name: Putative uncharacterized protein
Function: unknown
Molecular weight (Da): 42,327
Localisation[1]: periplasmic
Class[2]: CytoplasmicMembrane

Sequence Identity between strains

Strains		Acinetobacter baumannii (strain SDF)	Acinetobacter baumannii (strain AYE)	Acinetobacter baumannii ATCC 19606	Acinetobacter baumannii (strain TCDC-AB0715)	Acinetobacter baumannii (strain 1656-2)	Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	Acinetobacter baumannii (strain AB307-0294)
	Uniprot #	B0VRK6_ACIBS	B0V745_ACIBY	D0C6C0_ACIBA	F0QIR8_ACIBD	E8P8Z1_ACIB1	A3M6D2_ACIBT	B7H1B6_ACIB3
Acinetobacter baumannii (strain SDF)	B0VRK6_ACIBS	100%	98.00%	99.00%	99.00%	99.00%	97.00%	98.00%
Acinetobacter baumannii (strain AYE)	B0V745_ACIBY		100%	98.00%	99.00%	98.00%	97.00%	100.00%
Acinetobacter baumannii ATCC 19606	D0C6C0_ACIBA			100%	99.00%	99.00%	98.00%	98.00%
Acinetobacter baumannii (strain TCDC-AB0715)	F0QIR8_ACIBD				100%	99.00%	98.00%	99.00%
Acinetobacter baumannii (strain 1656-2)	E8P8Z1_ACIB1					100%	98.00%	98.00%
Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	A3M6D2_ACIBT						100%	97.00%
Acinetobacter baumannii (strain AB307-0294)	B7H1B6_ACIB3							100%

[1] based on SignalP, http://www.cbs.dtu.dk/services/SignalP/

[2] based on Psortb, http://www.psort.org/psortb/

Protein Name: Putative uncharacterized protein
Function: unknown
Molecular weight (Da): 30,909
Localisation[1]: unknown
Class[2]: unknown

Sequence Identity between strains

Strains		Acinetobacter baumannii (strain SDF)	Acinetobacter baumannii (strain AYE)	Acinetobacter baumannii ATCC 19606	Acinetobacter baumannii (strain TCDC-AB0715)	Acinetobacter baumannii (strain 1656-2)	Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	Acinetobacter baumannii (strain AB307-0294)
	Uniprot #	B0VSH8_ACIBS	B0VEH4_ACIBY	D0CDQ9_ACIBA	F0QEY1_ACIBD	E8PB50_ACIB1	A3M897_ACIBT	B7GXI1_ACIB3
Acinetobacter baumannii (strain SDF)	B0VSH8_ACIBS	100%	98.00%	99.00%	98.00%	98.00%	98.00%	98.00%
Acinetobacter baumannii (strain AYE)	B0VEH4_ACIBY		100%	99.00%	100.00%	100.00%	99.00%	100.00%
Acinetobacter baumannii ATCC 19606	D0CDQ9_ACIBA			100%	99.00%	99.00%	99.00%	99.00%
Acinetobacter baumannii (strain TCDC-AB0715)	F0QEY1_ACIBD				100%	100.00%	99.00%	100.00%
Acinetobacter baumannii (strain 1656-2)	E8PB50_ACIB1					100%	99.00%	100.00%
Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	A3M897_ACIBT						100%	99.00%
Acinetobacter baumannii (strain AB307-0294)	B7GXI1_ACIB3							100%

[1] based on SignalP, http://www.cbs.dtu.dk/services/SignalP/

[2] based on Psortb, http://www.psort.org/psortb/

Protein Name: Putative uncharacterized protein
Function: unknown
Molecular weight (Da): 18,176
Localisation[1]: periplasmic
Class[2]: unknown

Sequence Identity between strains

Strains		Acinetobacter baumannii (strain SDF)	Acinetobacter baumannii (strain AYE)	Acinetobacter baumannii ATCC 19606	Acinetobacter baumannii (strain TCDC-AB0715)	Acinetobacter baumannii (strain 1656-2)	Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	Acinetobacter baumannii (strain AB307-0294)
	Uniprot #	B0VQI7_ACIBS	B0V5G3_ACIBY	D0C6Q2_ACIBA	F0QGP1_ACIBD	E8PH34_ACIB1	A7FBI1_ACIBT	B7H224_ACIB3
Acinetobacter baumannii (strain SDF)	B0VQI7_ACIBS	100%	99.00%	99.00%	99.00%	99.00%	99.00%	99.00%
Acinetobacter baumannii (strain AYE)	B0V5G3_ACIBY		100%	100.00%	100.00%	100.00%	100.00%	100.00%
Acinetobacter baumannii ATCC 19606	D0C6Q2_ACIBA			100%	100.00%	100.00%	100.00%	100.00%
Acinetobacter baumannii (strain TCDC-AB0715)	F0QGP1_ACIBD				100%	100.00%	100.00%	100.00%
Acinetobacter baumannii (strain 1656-2)	E8PH34_ACIB1					100%	100.00%	100.00%
Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	A7FBI1_ACIBT						100%	100.00%
Acinetobacter baumannii (strain AB307-0294)	B7H224_ACIB3							100%

[1] based on SignalP, http://www.cbs.dtu.dk/services/SignalP/

[2] based on Psortb, http://www.psort.org/psortb/

Protein Name: Putative uncharacterized protein
Function: unknown
Molecular weight (Da): 18,176
Localisation[1]: periplasmic
Class[2]: unknown

Sequence Identity between strains

Strains		Acinetobacter baumannii (strain SDF)	Acinetobacter baumannii (strain AYE)	Acinetobacter baumannii ATCC 19606	Acinetobacter baumannii (strain TCDC-AB0715)	Acinetobacter baumannii (strain 1656-2)	baumannii (strain ATCC 17978 / NCDC KC 755)	Acinetobacter baumannii (strain AB307-0294)
	Uniprot #	B0VMX4_ACIBS	B0VD02_ACIBY	D0C7T4_ACIBA	F0QP13_ACIBD	E8PDM4_ACIB1	A7FBB9_ACIBT	B7H3N7_ACIB3
Acinetobacter baumannii (strain SDF)	B0VMX4_ACIBS	100%	97.00%	95.00%	96.00%	96.00%	96.00%	97.00%
Acinetobacter baumannii (strain AYE)	B0VD02_ACIBY		100%	97.00%	98.00%	98.00%	98.00%	100.00%
Acinetobacter baumannii ATCC 19606	D0C7T4_ACIBA			100%	98.00%	98.00%	98.00%	97.00%
Acinetobacter baumannii (strain TCDC-AB0715)	F0QP13_ACIBD				100%	100.00%	100.00%	98.00%
Acinetobacter baumannii (strain 1656-2)	E8PDM4_ACIB1					100%	100.00%	98.00%
Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	A7FBB9_ACIBT						100%	98.00%
Acinetobacter baumannii (strain AB307-0294)	B7H3N7_ACIB3							100%

[1] based on SignalP, http://www.cbs.dtu.dk/services/SignalP/

[2] based on Psortb, http://www.psort.org/psortb/

Protein Name: Putative uncharacterized protein
Function: unkown
Molecular weight (Da): 5,102
Localisation[1]: periplasmic
Class[2]: Non-Cytoplasmic

Sequence Identity between strains

Strains		Acinetobacter baumannii (strain SDF)	Acinetobacter baumannii (strain AYE)	Acinetobacter baumannii ATCC 19606	Acinetobacter baumannii (strain TCDC-AB0715)	Acinetobacter baumannii (strain 1656-2)	Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	Acinetobacter baumannii (strain AB307-0294)
	Uniprot #	NA	B0VBI1_ACIBY	D0C8B9_ACIBA	NA	E8PC89_ACIB1	A7FB95_ACIBT	B7H463_ACIB3
Acinetobacter baumannii (strain SDF)	NA	-	-	-	-	-	-	-
Acinetobacter baumannii (strain AYE)	B0VBI1_ACIBY		100%	97.00%	-	97.00%	97.00%	100.00%
Acinetobacter baumannii ATCC 19606	D0C8B9_ACIBA			100%	-	100.00%	100.00%	97.00%
Acinetobacter baumannii (strain TCDC-AB0715)	NA				-	-	-	-
Acinetobacter baumannii (strain 1656-2)	E8PC89_ACIB1					100%	100.00%	97.00%
Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	A7FB95_ACIBT						100%	97.00%
Acinetobacter baumannii (strain AB307-0294)	B7H463_ACIB3							100%

[1] based on SignalP, http://www.cbs.dtu.dk/services/SignalP/

[2] based on Psortb, http://www.psort.org/psortb/

Protein Name: Lipoprotein
Function: unkown
Molecular weight (Da): 17,543
Localisation[1]: periplasmic
Class[2]: OuterMembrane

Sequence Identity between strains

Strains		Acinetobacter baumannii (strain SDF)	Acinetobacter baumannii (strain AYE)	Acinetobacter baumannii ATCC 19606	Acinetobacter baumannii (strain TCDC-AB0715)	Acinetobacter baumannii (strain 1656-2)	Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	Acinetobacter baumannii (strain AB307-0294)
	Uniprot #	B0VSC6_ACIBS	B0V9M7_ACIBY	D0C8Y7_ACIBA	F0QH45_ACIBD	E8PFC2_ACIB1	A3M3E7_ACIBT	B7GXU9_ACIB3
Acinetobacter baumannii (strain SDF)	B0VSC6_ACIBS	100%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%
Acinetobacter baumannii (strain AYE)	B0V9M7_ACIBY		100%	100.00%	100.00%	100.00%	100.00%	100.00%
Acinetobacter baumannii ATCC 19606	D0C8Y7_ACIBA			100%	100.00%	100.00%	100.00%	100.00%
Acinetobacter baumannii (strain TCDC-AB0715)	F0QH45_ACIBD				100.00%	100.00%	100.00%	100.00%
Acinetobacter baumannii (strain 1656-2)	E8PFC2_ACIB1					100%	100.00%	100.00%
Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	A3M3E7_ACIBT						100%	100.00%
Acinetobacter baumannii (strain AB307-0294)	B7GXU9_ACIB3							100.00%

[1] based on SignalP, http://www.cbs.dtu.dk/services/SignalP/

[2] based on Psortb, http://www.psort.org/psortb/

Protein Name: Membrane-fusion protein
Function: transmembrane transport
Molecular weight (Da): 48,204
Localisation[1]: unknown
Class[2]: CytoplasmicMembrane

Sequence Identity between strains

Strains		Acinetobacter baumannii (strain SDF)	Acinetobacter baumannii (strain AYE)	Acinetobacter baumannii ATCC 19606	Acinetobacter baumannii (strain TCDC-AB0715)	Acinetobacter baumannii (strain 1656-2)	Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	Acinetobacter baumannii (strain AB307-0294)
	Uniprot #	B0VKQ2_ACIBS	B0V5H5_ACIBY	D0CAD2_ACIBA	F0QNX2_ACIBD	E8PBH5_ACIB1	A3M247_ACIBT	B7H054_ACIB3
Acinetobacter baumannii (strain SDF)	B0VKQ2_ACIBS	100%	99.00%	99.00%	98.00%	98.00%	100.00%	99.00%
Acinetobacter baumannii (strain AYE)	B0V5H5_ACIBY		100%	99.00%	99.00%	99.00%	100.00%	100.00%
Acinetobacter baumannii ATCC 19606	D0CAD2_ACIBA			100%	99.00%	99.00%	99.00%	99.00%
Acinetobacter baumannii (strain TCDC-AB0715)	F0QNX2_ACIBD				100.00%	100.00%	100.00%	99.00%
Acinetobacter baumannii (strain 1656-2)	E8PBH5_ACIB1					100%	100.00%	99.00%
Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	A3M247_ACIBT						100%	100.00%
Acinetobacter baumannii (strain AB307-0294)	B7H054_ACIB3							100.00%

[1] based on SignalP, http://www.cbs.dtu.dk/services/SignalP/

[2] based on Psortb, http://www.psort.org/psortb/

Protein Name: ErfK/YbiS/YcfS/YnhG family
Function: transferase activity
Molecular weight (Da): 44,929
Localisation[1]: periplasmic
Class[2]: Non-Cytoplasmic

Sequence Identity between strains

Strains		Acinetobacter baumannii (strain SDF)	Acinetobacter baumannii (strain AYE)	Acinetobacter baumannii ATCC 19606	Acinetobacter baumannii (strain TCDC-AB0715)	Acinetobacter baumannii (strain 1656-2)	Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	Acinetobacter baumannii (strain AB307-0294)
	Uniprot #	B0VUQ8_ACIBS	B0VBT1_ACIBY	D0CC33_ACIBA	F0QMC7_ACIBD	E8PJ01_ACIB1	A3M796_ACIBT	B7GZK5_ACIB3
Acinetobacter baumannii (strain SDF)	B0VUQ8_ACIBS	100%	99.00%	98.00%	98.00%	99.00%	99.00%	99.00%
Acinetobacter baumannii (strain AYE)	B0VBT1_ACIBY		100%	99.00%	99.00%	100.00%	100.00%	100.00%
Acinetobacter baumannii ATCC 19606	D0CC33_ACIBA			100%	99.00%	99.00%	99.00%	99.00%
Acinetobacter baumannii (strain TCDC-AB0715)	F0QMC7_ACIBD				100.00%	99.00%	99.00%	99.00%
Acinetobacter baumannii (strain 1656-2)	E8PJ01_ACIB1					100%	100.00%	100.00%
Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	A3M796_ACIBT						100%	100.00%
Acinetobacter baumannii (strain AB307-0294)	B7GZK5_ACIB3							100.00%

[1] based on SignalP, http://www.cbs.dtu.dk/services/SignalP/

[2] based on Psortb, http://www.psort.org/psortb/

Protein Name: Putative uncharacterized protein
Function: unknown
Molecular weight (Da): 15,992
Localisation[1]: unknown
Class[2]: unknown

Sequence Identity between strains

Strains		Acinetobacter baumannii (strain SDF)	Acinetobacter baumannii (strain AYE)	Acinetobacter baumannii ATCC 19606	Acinetobacter baumannii (strain TCDC-AB0715)	Acinetobacter baumannii (strain 1656-2)	Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	Acinetobacter baumannii (strain AB307-0294)
	Uniprot #	NA	B0V6T7_ACIBY	D0CD31_ACIBA	F0QHH4_ACIBD	E8PD83_ACIB1	A7FBW3_ACIBT	B7GW36_ACIB3
Acinetobacter baumannii (strain SDF)	NA	-	-	-	-	-	-	-
Acinetobacter baumannii (strain AYE)	B0V6T7_ACIBY		100%	97.00%	99.00%	99.00%	98.00%	100.00%
Acinetobacter baumannii ATCC 19606	D0CD31_ACIBA			100%	97.00%	97.00%	97.00%	97.00%
Acinetobacter baumannii (strain TCDC-AB0715)	F0QHH4_ACIBD				100.00%	100.00%	99.00%	99.00%
Acinetobacter baumannii (strain 1656-2)	E8PD83_ACIB1					100%	99.00%	99.00%
Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	A7FBW3_ACIBT						100%	98.00%
Acinetobacter baumannii (strain AB307-0294)	B7GW36_ACIB3							100.00%

[1] based on SignalP, http://www.cbs.dtu.dk/services/SignalP/

[2] based on Psortb, http://www.psort.org/psortb/

Protein Name: Putative uncharacterized protein
Function: unknown
Molecular weight (Da): 13,936
Localisation[1]: Periplasmic
Class[2]: Periplasmic

Sequence Identity between strains

Strains		Acinetobacter baumannii (strain SDF)	Acinetobacter baumannii (strain AYE)	Acinetobacter baumannii ATCC 19606	Acinetobacter baumannii (strain TCDC-AB0715)	Acinetobacter baumannii (strain 1656-2)	Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	Acinetobacter baumannii (strain AB307-0294)
	Uniprot #	B0VRD8_ACIBS	B0V536_ACIBY	D0CDA9_ACIBA	F0QG99_ACIBD	E8PCH0_ACIB1	A3M8P7_ACIBT	B7GWP0_ACIB3
Acinetobacter baumannii (strain SDF)	B0VRD8_ACIBS	100%	97.00%	99.00%	99.00%	99.00%	98.00%	97.00%
Acinetobacter baumannii (strain AYE)	B0V536_ACIBY		100%	98.00%	98.00%	98.00%	99.00%	100.00%
Acinetobacter baumannii ATCC 19606	D0CDA9_ACIBA			100%	100.00%	100.00%	99.00%	98.00%
Acinetobacter baumannii (strain TCDC-AB0715)	F0QG99_ACIBD				100.00%	100.00%	99.00%	98.00%
Acinetobacter baumannii (strain 1656-2)	E8PCH0_ACIB1					100%	99.00%	98.00%
Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	A3M8P7_ACIBT						100%	99.00%
Acinetobacter baumannii (strain AB307-0294)	B7GWP0_ACIB3							100.00%

[1] based on SignalP, http://www.cbs.dtu.dk/services/SignalP/

[2] based on Psortb, http://www.psort.org/psortb/

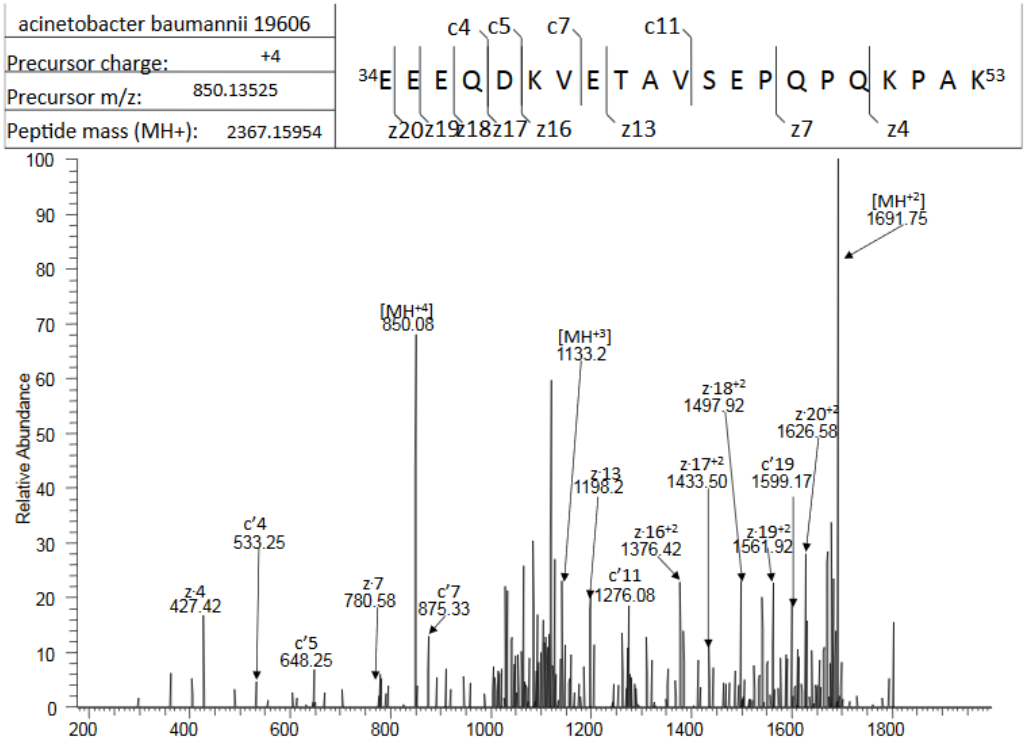
Supplementary table 10A: Glycopeptides identified using ETD in *Acinetobacter baumannii* (strain ATCC19606)

Uniprot accession number	Protein name	parent mass	charge	Precursor MH+	glycan mass	glycan composition	peptide mass	peptide	Site localisation	Page
D0C6Q2_ACIBA	Putative uncharacterized protein [HMPREF0010_00432] [Acinetobacter baumannii ATCC 19606 = CIP 70.34]"	850.14	4	3397.52	1030.36	HexNAc-Hex$_2$-HexNAc-300	2367.160	^{34}EEEQDKVETAVSEPQPQKPAK^{53}	S^{45+}	3
D0C6Q2_ACIBA	Putative uncharacterized protein [HMPREF0010_00432] [Acinetobacter baumannii ATCC 19606 = CIP 70.34]"	866.41	3	2597.19	988.34	HexNAc-Hex$_2$-HexNAc-258	1608.859	^{46}VETAVSEPQPQKPAK^{61}	S^{45+}	4
D0C7T4_ACIBA	Putative uncharacterized protein [HMPREF0010_00814] [Acinetobacter baumannii ATCC 19606 = CIP 70.34]	964.42	3	2891.25	1030.36	HexNAc-Hex$_2$-HexNAc-300	1860.893	^{35}KEEAAQAGQDAASTAVADK^{68}	S^{40+}	5
D0C8B9_ACIBA	Uncharacterized protein [F911_02370] [Acinetobacter baumannii ATCC 19606 = CIP 70.34]	1007.05	3	3019.13	1030.36	HexNAc-Hex$_2$-HexNAc-300	1988.766	^{28}NDGM(+16)HEASDPATSHDM(+16)NK^{45}	S^{35+}	6
D0C8B9_ACIBA	Uncharacterized protein [F911_02370] [Acinetobacter baumannii ATCC 19606 = CIP 70.34]	755.78	4	3020.11	1030.36	HexNAc-Hex$_2$-HexNAc-300	1989.753	^{28}N(+1)DGM(+16)HEASDPATSHDM(+16)NK^{45}	S^{35+}	7
D0C8B9_ACIBA	Uncharacterized protein [F911_02370] [Acinetobacter baumannii ATCC 19606 = CIP 70.34]	755.04	4	3017.15	1044.38	HexNAc-Hex$_2$-HexNAc-314	1972.765	^{28}NDGM(+16)HEASDPATSHDMNK^{45}	S^{35+}	8
D0CAB8_ACIBA	Uncharacterized protein [F911_03368] [Acinetobacter baumannii ATCC 19606 = CIP 70.34]	657.32	3	1969.94	1030.36	HexNAc-Hex$_2$-HexNAc-300	939.562	^{302}AKPASAPAVK^{311}	S^{306+}	9

Supplementary Table S2-9

D0CAB8_ACIBA	Uncharacterized protein [F911_03368] [Acinetobacter baumannii ATCC 19606 = CIP 70.34]	643.31	3	1927.91	988.35	HexNAc-Hex$_2$-HexNAc-258	939.562	^{302}AKPASAPAVK^{311}	S^{306+}	10
D0CAB8_ACIBA	Uncharacterized protein [F911_03368] [Acinetobacter baumannii ATCC 19606 = CIP 70.34]	1128.16	3	3382.47	1030.36	HexNAc-Hex$_2$-HexNAc-300	2352.114	^{116}KTEASAAAATEQQDSFDAQVQR^{136}	S^{120}	11
D0CDQ9_ACIBA	Uncharacterized protein [F911_00789] [Acinetobacter baumannii ATCC 19606 = CIP 70.34]	846.38	3	2536.10	1030.36	HexNAc-Hex$_2$-HexNAc-300	1505.744	^{40}STAKDEQPASSASVK^{54}	S$^{51/50+}$	12
D0CEI7_ACIBA	TolA (Uncharacterized protein) [F911_02604] [Acinetobacter baumannii ATCC 19606 = CIP 70.34]	855.70	3	2565.10	1030.36	HexNAc-Hex$_2$-HexNAc-300	1534.734	^{52}QAASDIATATDNASAK^{67}	S^{55+}	13
D0CEI7_ACIBA	TolA (Uncharacterized protein) [F911_02604] [Acinetobacter baumannii ATCC 19606 = CIP 70.34]	951.74	3	2853.20	988.35	HexNAc-Hex$_2$-HexNAc-300	1864.848	^{48}SAGDQAASDIATATDNASAK^{67}	S^{55+}	14
D0CEI7_ACIBA	TolA (Uncharacterized protein) [F911_02604] [Acinetobacter baumannii ATCC 19606 = CIP 70.34]	965.74	3	2895.21	1030.36	HexNAc-Hex$_2$-HexNAc-300	1864.852	^{48}SAGDQAASDIATATDNASAK^{67}	S^{55+}	15

Supplementary table 10A: Identifications are labeled according to the corresponding Uniprot accession number and protein name. The parent m/z, charge state, deconvoluted mass mass, glycan mass, peptide mass, peptide sequence and glycan site attachment are provided for each identified glycopeptide. All identified glycopeptide ETD spectra are provided on the corresponding pages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Precursor m/z: 850.13525
Peptide mass (MH+): 2367.15954
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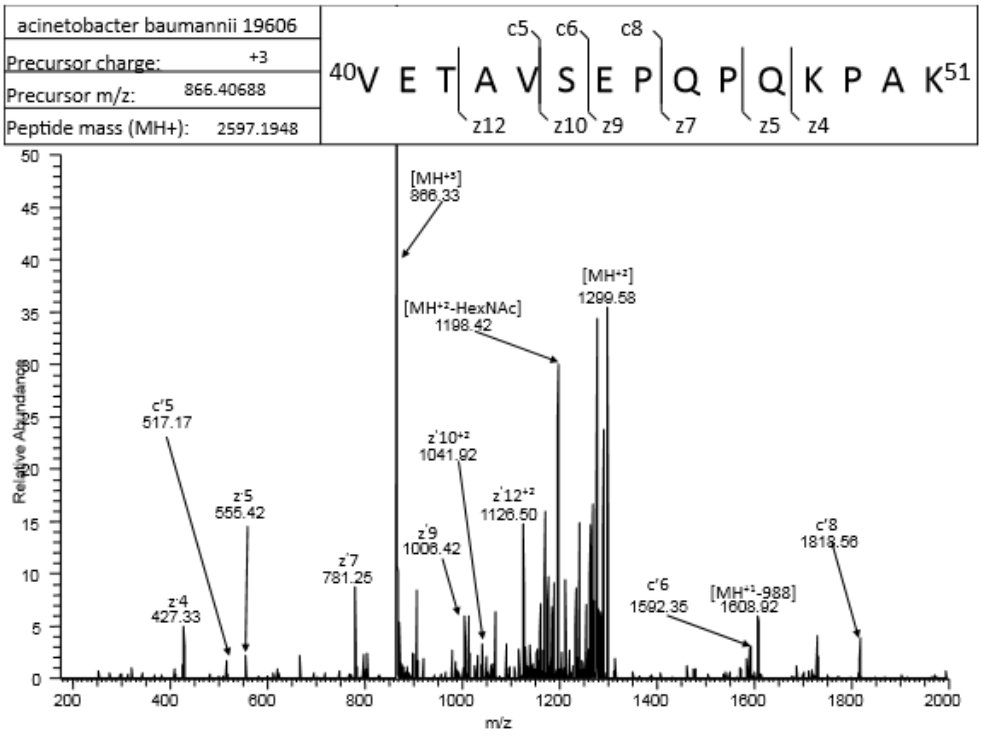
c4 c5 c7 c11
z20 z19 z18 z17 z16 z13 z7 z4
Relative Abundance
[MH+2] 1691.75
[MH+4] 850.08
[MH+3] 1133.2
z·18+2 1497.92
z·20+2 1626.58
z·17+2 1433.50
c'19 1599.17
z·13 1198.2
z·16+2 1376.42
z·19+2 1561.92
c'11 1276.08
c'4 533.25
z·4 427.42
z·7 780.58
c'7 875.33
c'5 648.25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Precursor m/z: 866.40688
Peptide mass (MH+): 2597.1948
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c5 c6 c8
z12 z10 z9 z7 z5 z4
Relative Abundance
m/z
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[MH+2] 1299.58
[MH+2-HexNAc] 1198.42
c'5 517.17
z'10+2 1041.92
z'12+2 1126.50
z'5 555.42
z'9 1006.42
c'8 1818.56
z'7 781.25
c'6 1592.35
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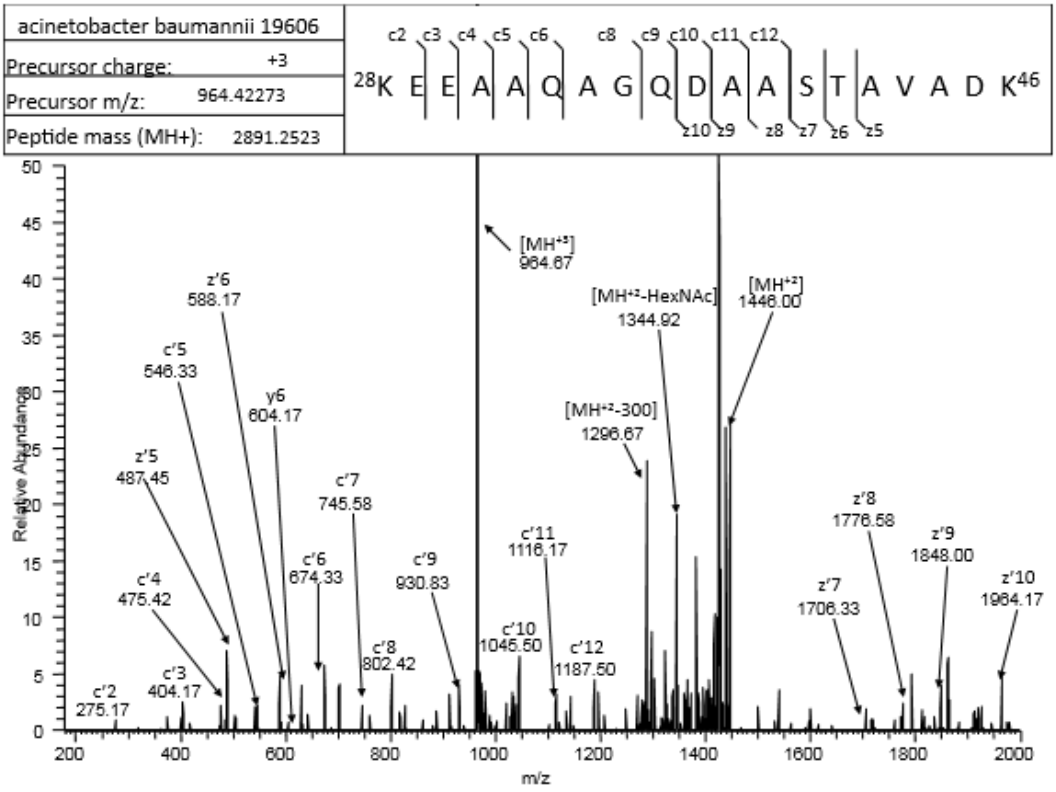
z'4 427.33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Precursor m/z: 964.42273
Peptide mass (MH+): 2891.2523
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Relative Abundance
m/z
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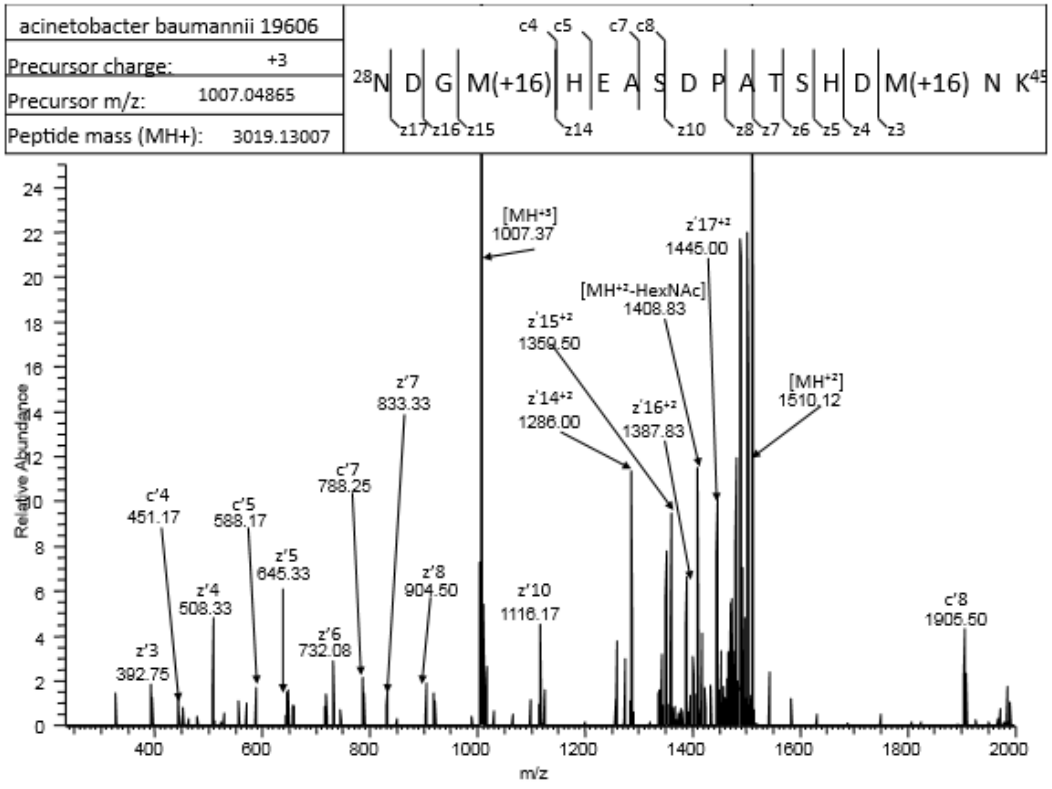
[MH+2-HexNAc] 1344.92
[MH+2] 1446.00
[MH+2-300] 1296.67
z'6 588.17
c'5 546.33
y6 604.17
z'5 487.45
c'7 745.58
c'6 674.33
c'9 930.83
c'11 1116.17
z'8 1776.58
z'9 1848.00
z'7 1706.33
z'10 1964.17
c'4 475.42
c'10 1045.50
c'8 802.42
c'12 1187.50
c'3 404.17
c'2 275.17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Precursor m/z: 1007.04865
Peptide mass (MH+): 3019.13007
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Relative Abundance
m/z
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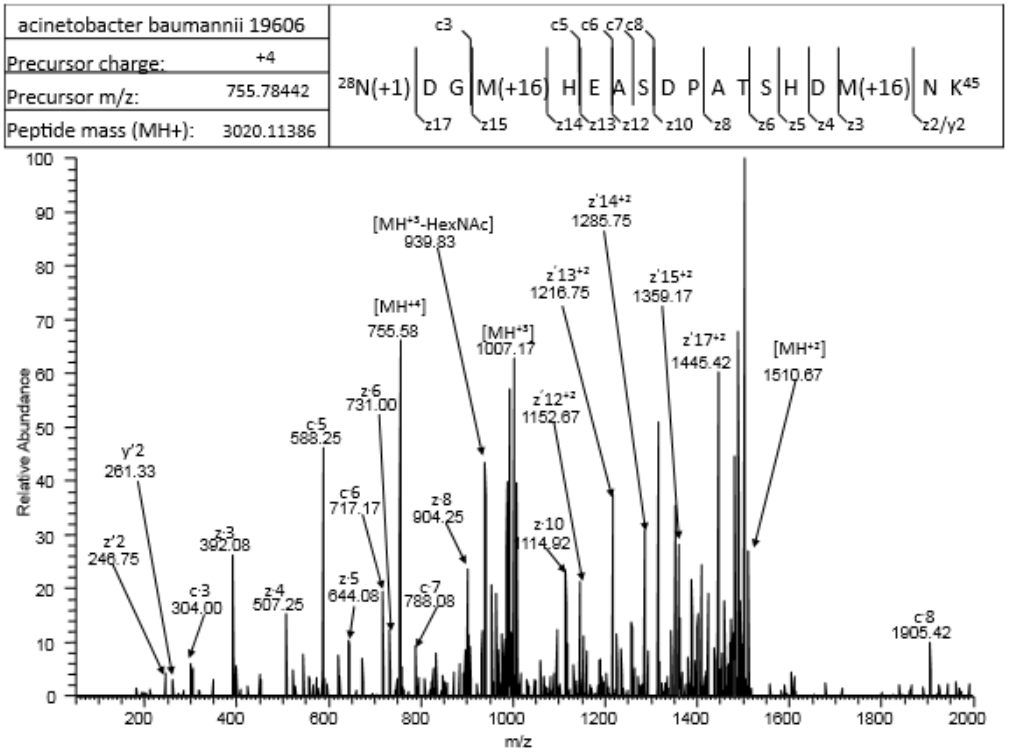
z'17+2 1445.00
[MH+2-HexNAc] 1408.83
z'15+2 1359.50
[MH+2] 1510.12
z'14+2 1286.00
z'16+2 1387.83
z'7 833.33
c'7 788.25
c'4 451.17
c'5 588.17
z'5 645.33
z'8 904.50
z'4 508.33
z'10 1116.17
c'8 1905.50
z'6 732.08
z'3 392.75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Peptide mass (MH+): 3020.11386
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Relative Abundance
m/z
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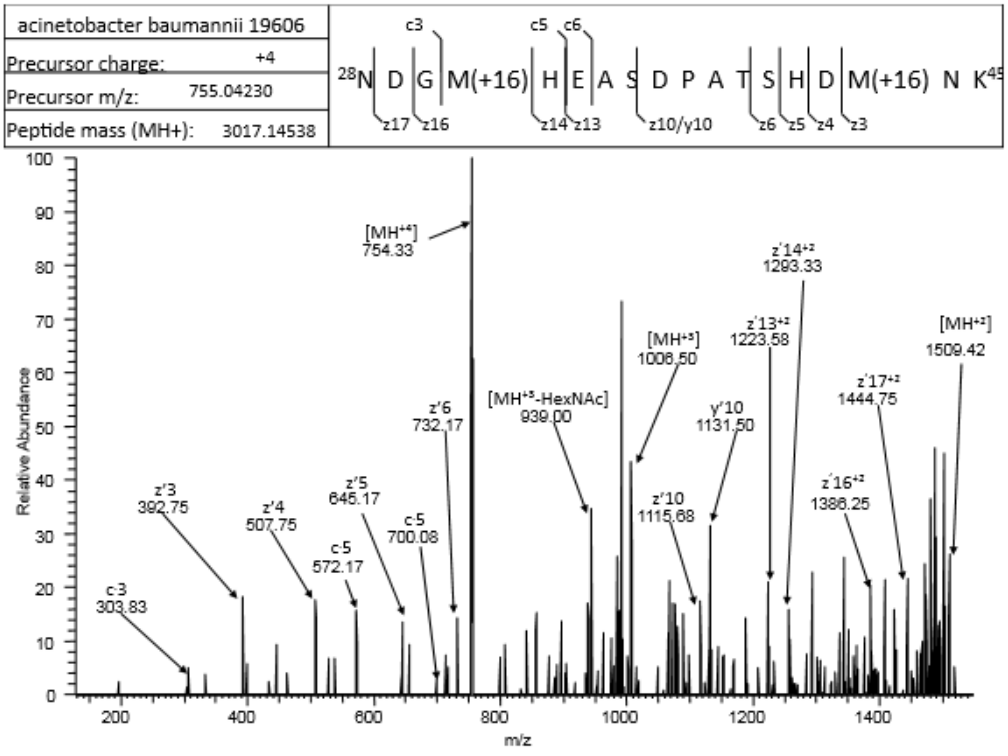
[MH+4] 755.58
[MH+3] 1007.17
[MH+2] 1510.67
z'14+2 1285.75
z'13+2 1216.75
z'15+2 1359.17
z'17+2 1445.42
z'12+2 1152.67
z·6 731.00
c·5 588.25
y'2 261.33
c·6 717.17
z·8 904.25
z·10 1114.92
z'2 246.75
z·3 392.08
c·3 304.00
z·4 507.25
z·5 644.08
c·7 788.08
c·8 1905.42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Precursor m/z: 755.04230
Peptide mass (MH+): 3017.14538
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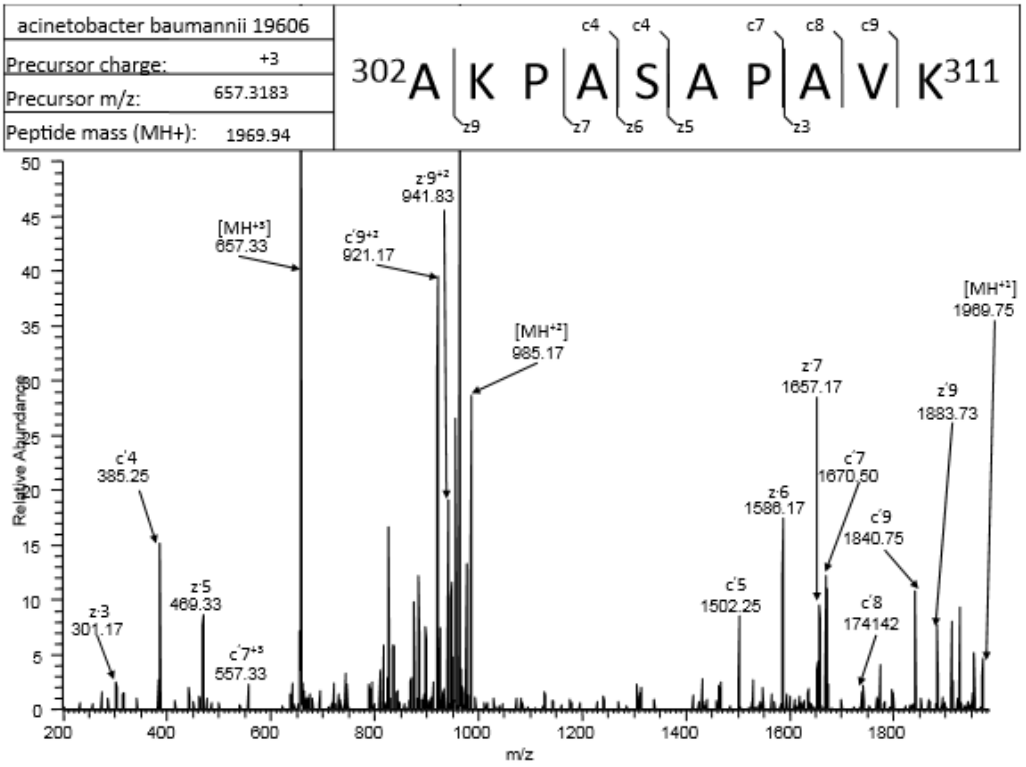
Relative Abundance
m/z
[MH+4] 754.33
z'14+2 1293.33
[MH+3] 1006.50
z'13+2 1223.58
[MH+2] 1509.42
[MH+3-HexNAc] 939.00
y'10 1131.50
z'6 732.17
z'17+2 1444.75
z'10 1115.68
z'16+2 1386.25
z'5 645.17
z'3 392.75
z'4 507.75
c·5 700.08
c·5 572.17
c·3 303.83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Precursor m/z: 657.3183
Peptide mass (MH+): 1969.94
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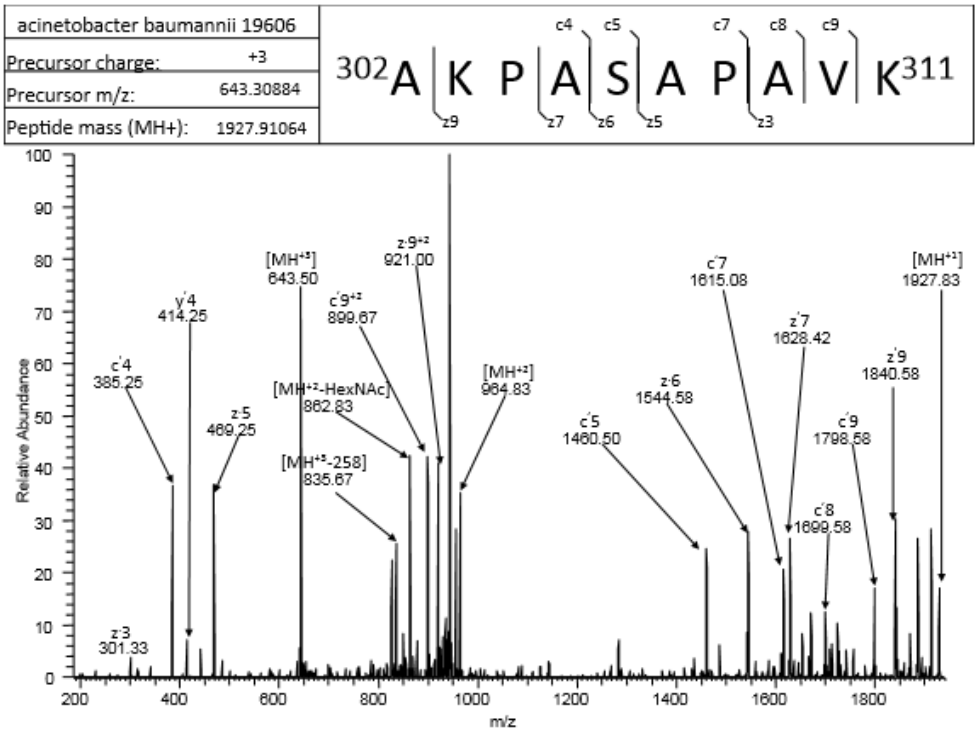
Relative Abundance
m/z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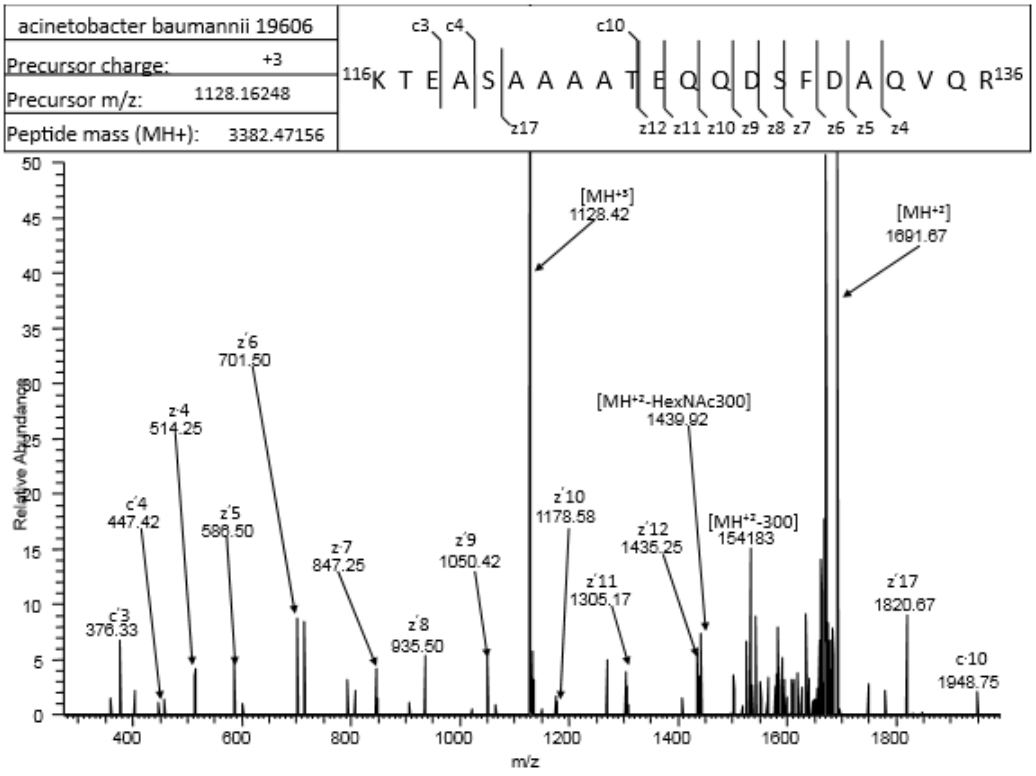
Relative Abundance
m/z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Precursor charge: +3
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Peptide mass (MH+): 3382.47156
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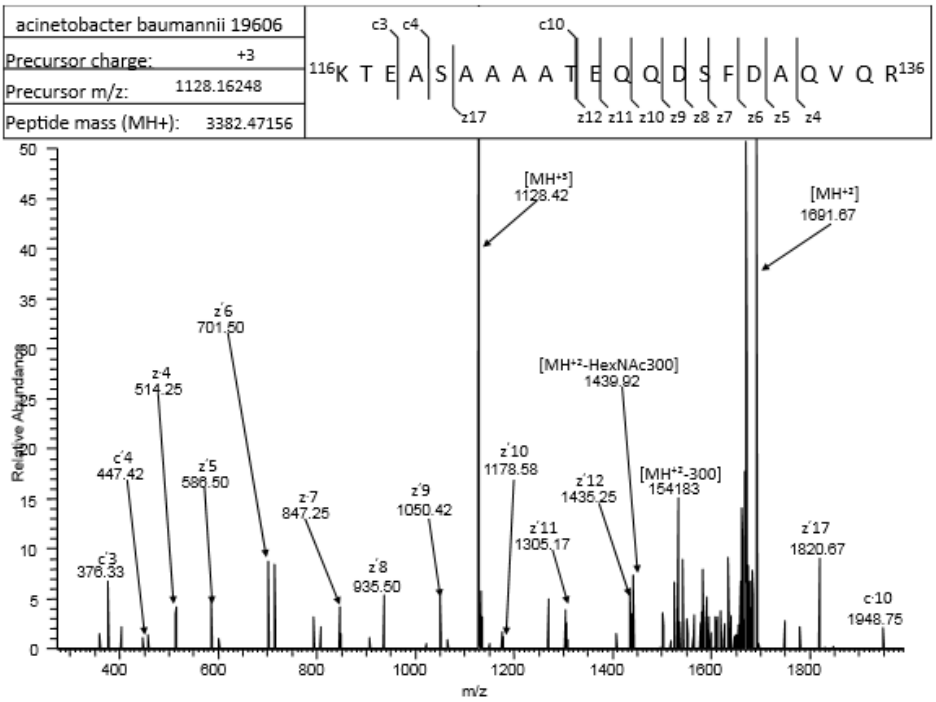
z17 z12 z11 z10 z9 z8 z7 z6 z5 z4
[MH+3] 1128.42
[MH+2] 1691.67
z´6 701.50
z·4 514.25
[MH+2-HexNAc300] 1439.92
c´4 447.42
z´5 586.50
z´10 1178.58
z´12 1435.25
[MH+2-300] 154183
z·7 847.25
z·9 1050.42
z´11 1305.17
z´17 1820.67
c´3 376.33
z´8 935.50
c·10 1948.75
Relative Abundance
m/z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Precursor charge: +3
Precursor m/z: 1128.16248
Peptide mass (MH+): 3382.47156
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c3 c4 c10
z17 z12 z11 z10 z9 z8 z7 z6 z5 z4
[MH+3] 1128.42
[MH+2] 1691.67
z´6 701.50
z·4 514.25
[MH+2-HexNAc300] 1439.92
c´4 447.42
z´5 586.50
z´10 1178.58
z´12 1435.25
[MH+2-300] 154183
z·7 847.25
z·9 1050.42
z´11 1305.17
z´17 1820.67
c´3 376.33
z´8 935.50
c·10 1948.75
Relative Abundance
m/z

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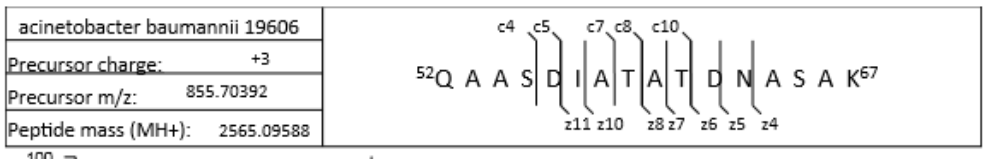
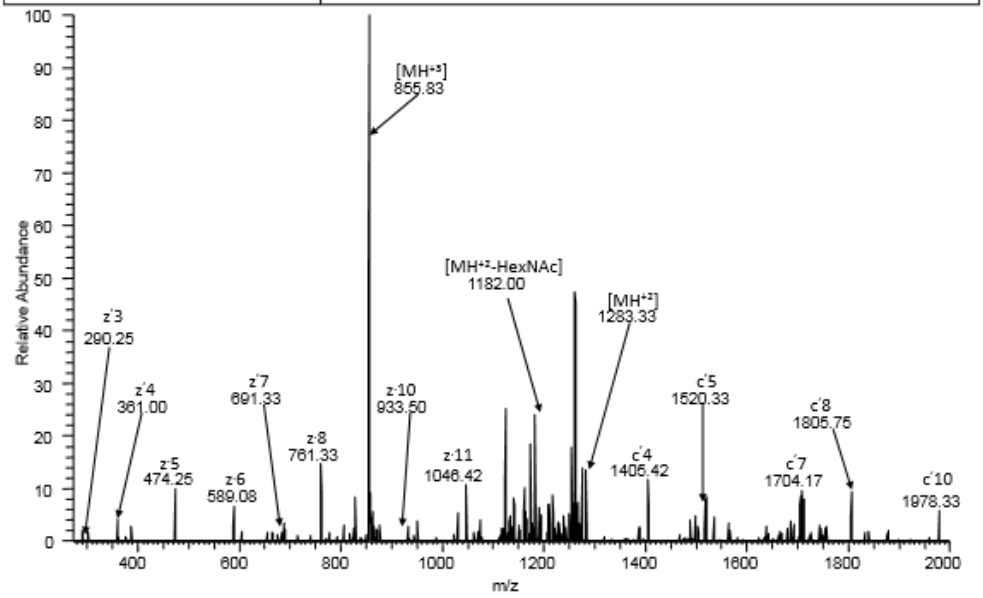
Peptide mass (MH+):	2565.09588





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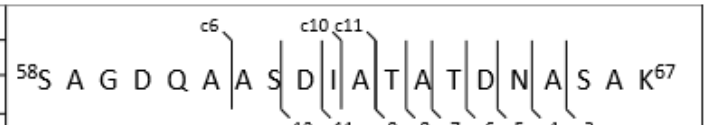
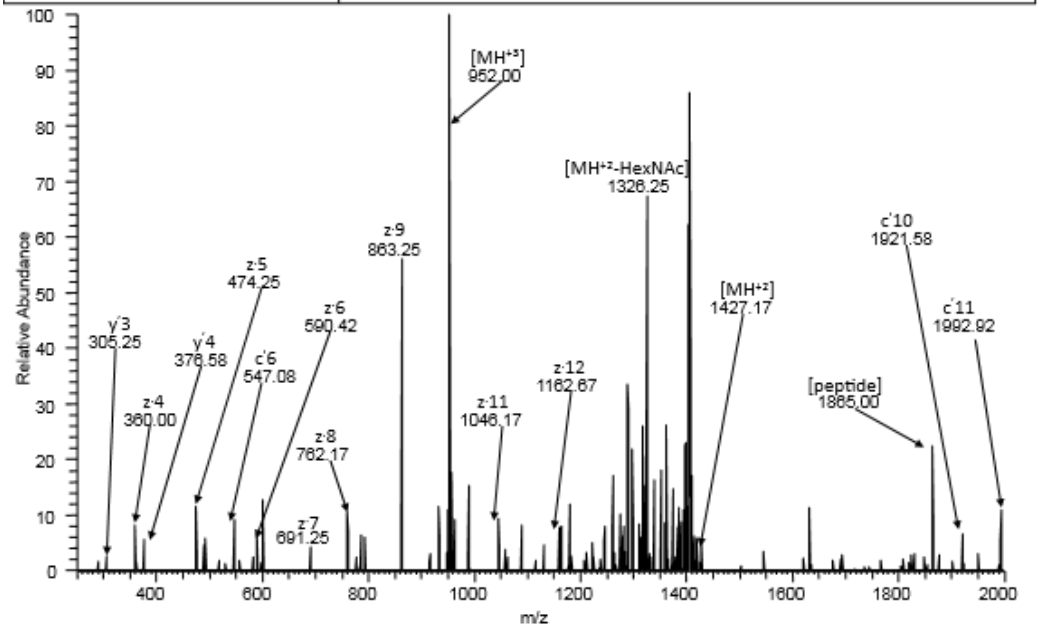
Peptide mass (MH+):	2853.19762





acinetobacter baumannii 19606	
Precursor charge:	+3
Precursor m/z:	965.74292
Peptide mass (MH+):	2895.21398

^{58}S A G D Q A A S D I A T A T D N A S A K^{67}

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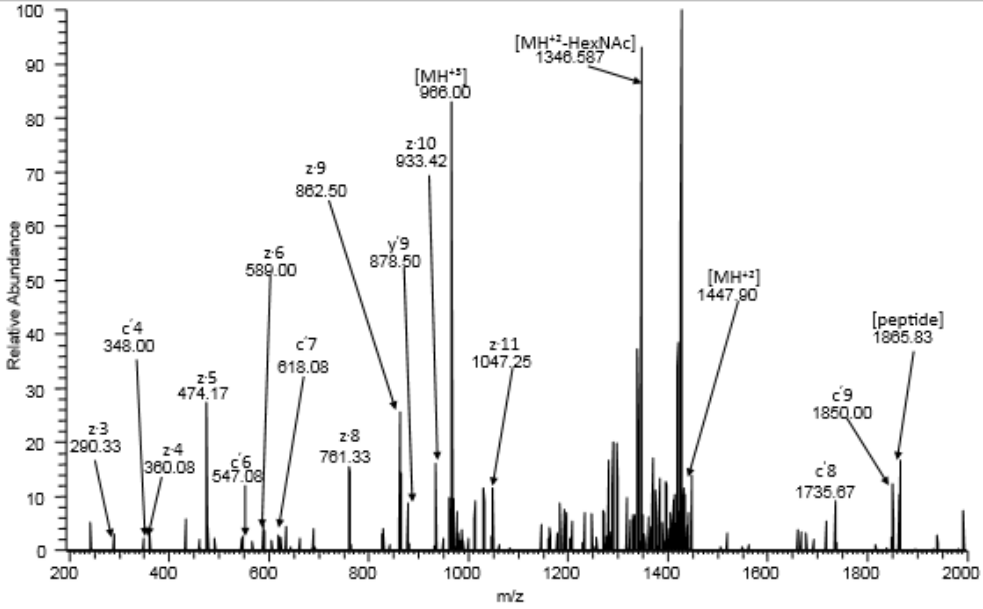

Supplementary Table 10B: Glycosylation site localisation in *Acinetobacter baumannii* (strain ATCC19606)

Uniprot accession number	Site localisation	five amino acid each side of the site
D0C6C0_ACIBA	S_{188}	EQKAASEPEQK
D0C6Q2_ACIBA	S_{45}*	VETAVSEPQPQ
D0C7T4_ACIBA	S_{40}*	GQDAASTAVAD
D0C8B9_ACIBA	S_{34}*	GMHEASDPATS
D0CAB8_ACIBA	S_{306}*	KAKPASAPAVK
D0CA69_ACIBA	S_{24}	AAGAASEAVAA
D0CA69_ACIBA	S_{76}	SDVAASAAH--
D0CAB8_ACIBA	S_{130}*	KKTEASAAAAT
D0CDA9_ACIBA	S_{123}	PAPVASQAK--
D0CDQ9_ACIBA	S_{88}*	DEQPASSASVK
D0CEI7_ACIBA	S_{55}*	GDQAASDIATA

Supplementary table 10B: Glycosylation site localisation in *A. baumannii* ATCC19606. Identifications are labeled according to the corresponding Uniprot accession number within strain ATCC19606. The site and residue of localisation are denoted with * indicating sites localised by ETD. To enable assessment of the local environment of glycosylation five amino acids either side of the site of modification are provided.

Supplementary data for Chapter 4:

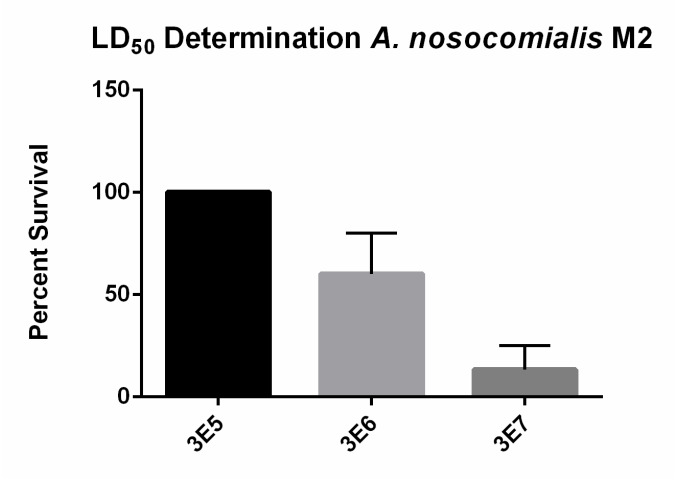

Figure S4.1 LD50 determination for *G. mellonella* larvae infected with *A. nosocomialis* strain M2. Groups of 10 *G. mellonella* were injected with 10µL of *A. nosocomialis* strain M2 at $3X10^5$, $3X10^6$, or $3X10^7$ CFUs. Eighteen hours after injection larvae were checked for viability as determined by melanin accumulation and motility.

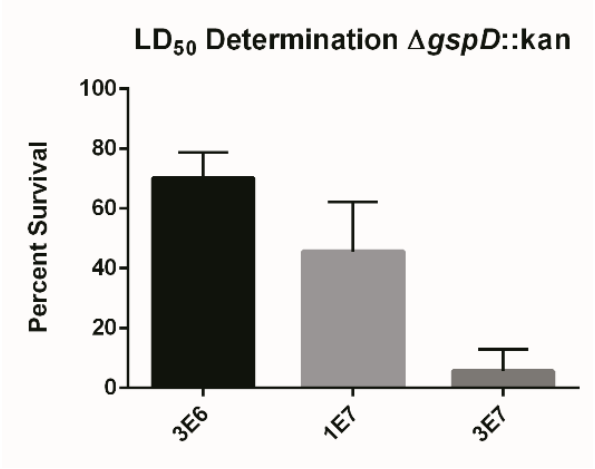

Figure S4.2 LD50 determination for G. mellonella larvae infected with the M2ΔgspD::kan mutant. Groups of 10 *G. mellonella* were injected with 10µL M2Δ*gspD*::kan mutant at $3X10^6$, $1X10^7$, or $3X10^7$ CFUs. Eighteen hours after injection larvae were checked for viability as determined by melanin accumulation and motility.

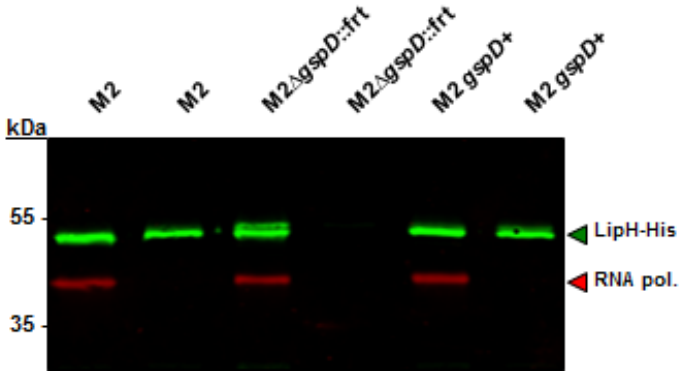

Figure S4.3 Type II secretion is impaired in the M2ΔgspD::frt mutant. Western blot analysis on whole cell lysates and secreted protein fractions probing for LipH-His. All strains and fractions were also analyzed for RNA polymerase expression, which served as a lysis control. LipH-His expression was detected in all strains carrying the pWH-*lipH-his*; however, LipH-His secretion was only detected in the parental M2 strain and the complemented *gspD*::frt strain, but not the Δ*gspD*::frt strain.

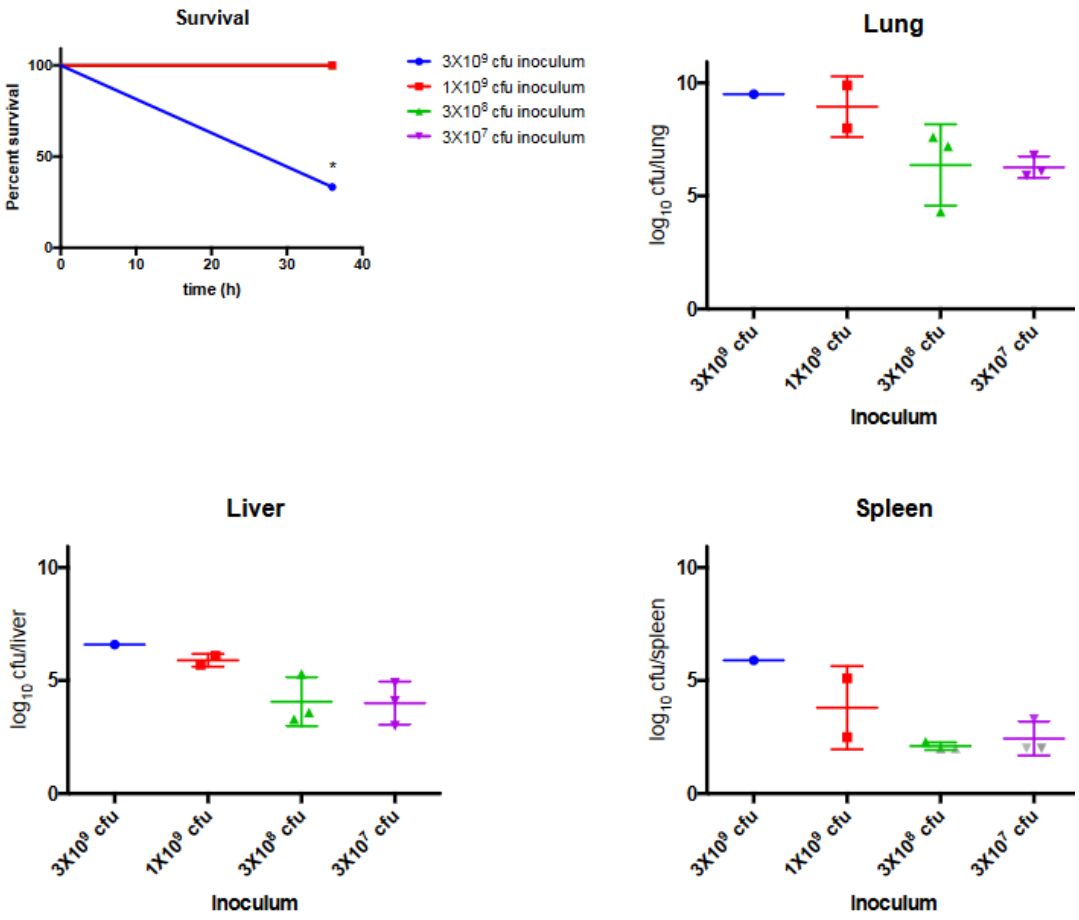

Figure S4.4 Dose determination of A. nosocomialis strain M2 for the murine pulmonary infection experiments. Four groups of three mice were intranasally inoculated with either $3X10^7$, $3X10^8$, $1X10^9$, or $3X10^9$ CFU of *A. nosocomialis* strain M2. Thirty-six hours post infection surviving mice were sacrificed and organs were harvested for CFU enumeration. A single mouse from the $1X10^9$ CFU dose group had to be removed post anesthesia and was excluded from this analysis