Exploiting novel *Acinetobacter* Oligosaccharyltransferases for the development of glycoconjugate vaccines against the Pneumococcus

by

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Abstract

Vaccines bring many diseases under control, an example of which is the *Streptococcus pneumoniae*, or the Pneumococcus. Pneumococcus is one of the leading causes of meningitis in children under 5 years, and is responsible for almost a million child deaths annually, with more than 90 serotypes identified. The discovery of antigenicity of the capsular polysaccharide (CPS) is the basis for current Pneumococcus vaccines, as it is used as a serotype specific polysaccharide-only vaccine. These were reported to be effective in adults, but not in young children under 5 years. Glycoconjugate vaccines, where CPS subunits are conjugated to immunogenic proteins, revolutionized immunization against the Pneumococcus, as their efficacy was demonstrated in young children. A Pneumococcus conjugate vaccine was first introduced in the year 2000 against 7 serotypes. This vaccine has shown exceptional efficacy and is produced by chemically conjugating CPS to an immunogenic protein. This process has a lot of drawbacks including product heterogeneity and high costs. Bacterial glycosylation systems have been used as an alternative to overcome these drawbacks and produce affordable vaccines. A problem with that is the glycan and protein specificity of the conjugating enzymes, the oligosaccharyltransferases (OTases). Continuous probing for OTases with different specificities is essential for driving the glycoconjugate industry forward.

In this thesis, we characterize the protein *O*-linked glycosylation system of members of the genus *Acinetobacter*. *O*-linked glycosylation is mediated by *O*-OTases, enzymes that transfer glycans from a lipid carrier to serine and threonine residues of acceptor proteins. We employ the clinical isolate *A. nosocomialis* M2 and the environmental isolate *A.*

baylyi ADP1 to show that most *Acinetobacter* spp. encode two *O*-OTases with different acceptor protein specificities, contrary to previously characterized *O*-glycosylation systems which encode a single *O*-OTase. One of these OTases is specific for Type IV pilin (Tfp) glycosylation and the other one glycosylates multiple proteins. Pilin-specific OTases in *Acinetobacter* resemble the TfpO *O*-OTase from *Pseudomonas aeruginosa* whereas general OTases resemble the *Neisseria meningitidis* PglL. *A. baylyi* is an exception however, as both OTases are closely related to PglL with one being a general OTase and the other specific for glycosylation of the Tfp-like protein ComP.

In the second part of this thesis, we attempt to exploit the *A. baylyi* PglLs in the glycoconjugate vaccine industry. We characterize the glycan specificity which is believed to depend on the sugar residue at the reducing end of the glycan, the first sugar added to the lipid carrier and the one in contact with serine or threonine residues of acceptor proteins. We show that both PglLs have very similar glycan specificity to the well-characterized PglL from *Neisseria meningitidis*. We also show that only one of the PglLs, designated $PglL_{ComP}$, successfully transfers Pneumococcus CPS subunits to the acceptor protein ComP. Pneumococcus CPS have a glucose residue at the reducing end and were never demonstrated to be transferred by the previously identified OTases. This is an unprecedented finding that greatly expands the tools available for glycoengineering.

Preface

Chapter 2 of this thesis has been published as Harding CM, Nasr MA, Kinsella RL, Scott NE, Foster LJ, Weber BS, Fiester SE, Actis LA, Tracy EN, Munson RS, Jr., Feldman MF. 2015. *Acinetobacter* strains carry two functional oligosaccharyltransferases, one devoted exclusively to type IV pilin, and the other one dedicated to *O*-glycosylation of multiple proteins. Mol Microbiol, 96:1023-1041. In this work, *A. nosocomialis* experiments were carried out by Harding CM including Pilin and OmpA purification. Kinsella RL prepared and analyzed MS data for purified glycosylated OmpA and PilA. I have conducted all *A. baylyi* experiments under the supervision of Feldman MF. As well, I have performed *A. baylyi* total membrane purifications for proteomic analyses conducted by Scott N.E. and Foster L.J. I was responsible for manuscript preparation along with Harding CM, Kinsella RL, Scott NE, Feldman MF and Munson RS, Jr.

Chapter 3 of this work is my original work. I was responsible for conducting experiments and data collection and conclusions were drawn under the supervision of Feldman MF.

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Last but not least, to my family. Words cannot do justice to how I feel about you. You have always been so kind, caring and encouraging and for that I will be eternally grateful.

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Acb	*Acinetobacter calcoaceticus-baumannii* complex
ACN	Acetonitrile
APC	Antigen presenting cells
Bac	Bacillosamine
BCR	B cell receptors
CjPglB	*Campylobacter jejuni* PglB
CPS	Capsular polysaccharide
CPS14	Pneumococcus serotype 14 capsular polysaccharide
CPS15b	Pneumococcus serotype 15b capsular polysaccharide
DATDH	2,4-diacetamido-2,4,6-trideoxyhexose
diNAcBac	di-*N*-acetyl bacillosamine
DDM	n-dodecyl-β-D-maltoside
DTT	Dithiothreitol
ECA	Enterobacterial common antigen
FA	Formic acid
FucNAc	*N*-acetyl fucosamine
Gal	Galactose
GalNAc	*N*-acetyl galactosamine
Glc	Glucose
GlcNAc	*N*-acetyl glucosamine
GT	Glycosyltransferase
IAA	Iodoacetamide
LCR	Low complexity regions
LLO	Lipid-linked oligosaccharide
LOS	Lipooligosaccharide
LPS	Lipopolysachharide
MHCII	Major histocompatibility complex class II
MPA	Major Polysaccharide Antigen
MurNAc	*N*-Acetylmuramic acid

NmPglL	*Neisseria meningitidis* PglL
OmpA	Outer membrane protein A
OMV	Outer membrane vesicles
OTase	Oligosaccharyltransferase
PBS	Phosphate buffered saline
PCV7	Pneumococcus conjugate vaccine covering 7 serotypes
PCV13	Pneumococcus conjugate vaccine covering 13 serotypes
$PglL_{Vc}$	*Vibrio cholerae* PglL
PgyA	Putative glycosylase A
SAAT	Surface-associating autotransporters
S-layers	Surface layers
T6SS	Type IV secretion system
TFA	Trifluoro acetic acid
Tfp	Type IV pilin
Und-P	Undecaprenyl phosphate
Und-PP	Undecaprenyl pyrophosphate
ZIC-HILIC	Zwitterionic Hydrophilic Interaction Liquid Chromatography

1. Introduction

1.1 Vaccines in the post-antibiotic era

World public health organizations have announced that the world is heading towards a post antibiotic era and collaborative action must be taken. Antibiotics are becoming less effective at combatting infections, which threatens to reverse all gains made on many disease fronts. Of particular significance are bacteria belonging to the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), which are gaining drug resistance mechanisms at alarming speeds and are major causes of infections in hospitals (Pendleton *et al.*, 2013). At the same time, pharmaceutical companies have deprioritized spending on antibiotic research and development, as antibiotics perform poorly in the current economical models (Power, 2006). Alternatives to antibiotics are being sought after to combat infections as well as preserve the efficacy of the remaining effective antibiotics, and vaccines are one of these alternatives.

The World Health Organization defines a vaccine as a biological preparation that improves immunity to a particular disease (WHO | Vaccines, n.d.). A vaccine is made of pathogen-derived components that will initiate an immune response against the pathogen and, ideally, develop memory for subsequent infections. Vaccines could be based on live attenuated, killed whole cells, toxoid vaccines, DNA vaccines or subunit vaccines that are made of antigenic surface proteins or polysaccharides. As well, bacterial outer membrane vesicles have successfully been used as vaccines against type B meningococcus (Delany *et al.*, 2014).

The first vaccination practice was demonstrated by Edward Jenner, who vaccinated against small pox in 1796, with several reports suggesting undocumented vaccination

trials prior to that (Riedel, 2005; Delany *et al.*, 2014). Eradication of small pox was declared in 1980, and was achieved through collective efforts by vaccination (WHO | Statue commemorates smallpox eradication, n.d.). Polio, which has seen a 99% reduction in cases since 1988, is under control by vaccines as well. (WHO | Poliomyelitis, n.d.). These vaccines are either inactivated (killed) or oral (live attenuated) polio vaccines, developed by Jonas Salk and Albert Sabine respectively (Chumakov and Ehrenfeld, 2008). Another example of a major disease controlled by vaccines is meningitis, caused by the encapsulated bacteria *Streptococcus pneumoniae* (Pneumococcus), *Neisseria meningitidis* (meningococcus) and *Haemophilus influenza* (Pace, 2013)

1.2 The Pneumococcus

Streptococcus pneumoniae, or the Pneumococcus, is an encapsulated Gram-positive bacterium that is one of the leading causes of bacterial meningitis in infants and children (Watson *et al.*, 1995). Louis Pasteur in France and George Sternberg in the US isolated it independently and simultaneously in 1881. It was first designated the name *Diplococcus pneumoniae* because of its diplococcus cell morphology, and was later changed in the 1970s to *Streptococcus pneumoniae* (Watson *et al.*, 1993).

Reports show that asymptomatic nasopharyngeal carriage of the pneumococcus has reached 60% of healthy preschool children in some areas (Korona-Glowniak and Malm, 2012). Pneumococci inhabit the nasopharynx as commensals in single or multispecies biofilms, without causing pathologies (Shak *et al.*, 2013; Chao *et al.*, 2014). They become invasive after dispersal from biofilms to reach other parts of the respiratory system, the bloodstream or the ear to cause meningitis, bacteremia, middle ear infections

and pneumonia. This invasiveness was found to be due to increased virulence factor expression after release from biofilms (Sanchez *et al.*, 2011; Chao *et al.*, 2014).

More than 90 serotypes have been identified for the Pneumococcus, each possessing a structurally and immunogenically unique polysaccharide capsule that is essential for virulence and is the target of vaccines in the market today (Lund, 1970; Kadioglu *et al.*, 2008). All serotypes are capable of causing invasive pneumococcal disease, with varying serotype geographical distribution (Hausdorff *et al.*, 2000; Jauneikaite *et al.*, 2012).

1.2.1 Epidemiology

Children under 5, the elderly and immunocompromised are at a particular high risk of community-acquired pneumococcal infections. In 2000, conservative estimates of Pneumococcus infections predicted 14.5 million cases. Pneumococcus-associated mortalities in children under 5 years are estimated to be 826000 annually, accounting for approximately 11% of mortalities in children under 5 years. Mortalities from pneumococcal-associated meningitis reach 73% in some parts of the world (O'Brien *et al.*, 2009). Individuals with underlying diseases or compromised immune systems are at a higher risk of pneumococcal-associated infections. Pneumococcus is transmitted by droplets from asymptomatic carriers, which then either asymptomatically colonize the nasopharynx or cause disease by invading the lungs or other sites (Donkor, 2013). Incubation period is not clearly defined and varies depending on the type of infection.

1.2.2 Antibiotic resistance

Since penicillin has been introduced in 1943, it had been the drug of choice for treating Pneumococcus infections. The first report of a penicillin resistant Pneumococcus was in Australia in 1967 (Hansman and Bullen, 1967), which has spread to reach 30% of the isolates in the 1980s (Campbell, Jr. and Silberman, 1998). By the late 1990s, the incidence of drug resistant pneumococci has dramatically increased and has become variable, with as high as 80% penicillin resistance reported in Korea and only 4% reported in India (Song *et al.*, 1999). Nowadays, 40% of Pneumococcus isolates display drug resistant phenotypes, with the majority being to penicillin or macrolides (Van Bambeke *et al.*, 2007). Drug resistant phenotypes have been reported to a lesser extent against tetracycline, folate synthesis inhibitors (sulphamethoxazole/trimethoprim), kanamycin, tetracycline, fluoroquinolones and chloramphenicol (Doern *et al.*, 2001; Jenkins *et al.*, 2008; Donkor and Badoe, 2014). Pneumococci remain susceptible to vancomycin, although tolerance has been reported, were they are able to survive but not grow in the presence of the antibiotic (Novak *et al.*, 1999). Drug resistance epidemiological studies in the Pneumococcus are still lacking (Ortega *et al.*, 2003; Gillis *et al.*, 2005; Sung *et al.*, 2006).

Since vaccines have been introduced, the incidence of drug resistant isolates of Pneumococcus in the US has decreased (Wroe *et al.*, 2012; Kempf *et al.*, 2015). In Canada, drug resistant pneumococci remained under control ever since the 13-valent conjugate vaccine PCV13 (against 13 serotypes) has been introduced in 2010. Vancomycin-resistance isolates were negligible, and less than 3% of isolates were

resistant to carbapenem and meropenem (Canadian Antimicrobial Resistance Surveillance System Report 2015, 2015).

1.3 Pneumococcal vaccines

1.3.1 A timeline

Pneumococcus capsule antigenicity was first reported in mice followed shortly by humans, where serotype specific immunity was observed (Francis and Tillett, 1930; Downie, 1937). The first documented pneumococcal vaccine controlled trial was in the 1940s, where the efficacy of a quadrivalent vaccine in US military recruits was demonstrated (Macleod *et al.*, 1945). Since then, a consumer-ready capsular polysaccharide-based vaccine has been slowly evolving, due to the success and efficacy of penicillin as therapy for the Pneumococcus at the time (Pinkbook | Pneumococcal | Epidemiology of Vaccine Preventable Diseases | CDC, n.d.). The need for a vaccine resurfaced with the rise of pneumococcal antibiotic resistance. Robert Austrian, a pioneer in Pneumococcus microbiology and vaccinology, has conducted clinical trials on South African gold miners in the 1970s using 6 and 12-valent polysaccharide vaccines to demonstrate their safety and efficacy (Grabenstein and Klugman, 2012). The first polysaccharide vaccine was a 14-valent vaccine developed in 1977 by Merck, with more serotypes subsequently added (Pace, 2013). A major drawback of polysaccharide vaccines is the poor response of infants, which has been noted on multiple occasions (Davies, 1937; Borgono *et al.*, 1978).

Conjugate vaccines are made by covalently conjugating polysaccharides to carrier proteins (Feldman and Anderson, 2014). The first conjugate vaccine was developed in 1980 against *Haemophilus influenzae* type B, with its efficacy for infants later demonstrated (Schneerson *et al.*, 1980; Eskola *et al.*, 1987; Eskola *et al.*, 1990). A conjugate vaccine for the Pneumococcus was developed in 2000, and like other conjugate vaccines, has revolutionized immunization against this disease especially in infants and the elderly. This in turn has led to a decline in pneumococcal disease burden caused by vaccine serotypes (Pilishvili *et al.*, 2010; Desai *et al.*, 2015). The first pneumococcal conjugate vaccine (PCV7) was licensed against 7 serotypes, with 6 more serotypes added in PCV13, introduced in 2010 (Figure 1) (Pace, 2013).

Table 3. Formulations of different pneumococcal vaccines that are/were in use.

Year of first licensure	Valency	Vaccine serotypes	Licensed formulations
1977	14-valent polysaccharide	1, 2, 3, 4, **6A**, 7F, 8, 9N, 12F, 14, 18C, 19F, 23F, **25F**	Pneumovax (Merck & Co., Inc.) Pnu-Immune (Lederle-Praxis Biologicals)
1983	23-valent polysaccharide	1, 2, 3, 4, **5**, **6B**, 7F, 8, 9N, **9V**, **10A**, **11A**, 12F, 14, **15B**, **17F**, 18C, **19A**, 19F, **20**, **22F**, 23F, **33**	Pneumovax 23 (Merck & Co., Inc.) Pneumo 23 (Sanofi Pasteur)
1986	17-valent polysaccharide	1, 2, 3, 4, 6A, 7F, 8, 9N, 11A, 12F, 14, 15F, 17F, 18C, 19F, 23F, 25	Moniarix (SmithKline Beecham) (used from 1986 – 88 in Belgium)
2000	7-valent CRM_{197} glycoconjugate: PCV7-CRM_{197}	4, 6B, 9V, 14, 18C, 19F, 23F	Prevenar (Wyeth, now Pfizer)
Clinical trial use only	9-valent CRM_{197} glycoconjugate : PCV9-CRM_{197}	1, 4, 5, 6B, 9V, 14, 18C, 19F, 23F	Not licensed (Wyeth, now Pfizer)
Clinical trial use only	11-valent DT/TT glycoconjugate : PncD/T11	1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F	Not licensed (Aventis, Pasteur: now Sanofi Pasteur)
2009	10-valent protein D (*H. influenzae*) glycoconjugate : PHiD-CV	**1,** 4, **5,** 6B, **7F,** 9V, 14, 18C, 19F, 23F	Synflorix (GlaxoSmithKline)
2010	13-valent CRM_{197} glycoconjugate: PCV13-CRM_{197}	1, **3**, 4, 5, **6A**, 6B, 7F, 9V, 14, 18C, **19A**, 19F, 23F	Prevenar 13 (Pfizer)
Clinical trial use only	15- valent CRM_{197} glycoconjugate: PCV15-CRM_{197}	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, **22F**, 23F, **33F**	Under development (Merck & Co., Inc.)

Figure 1.1: Timeline of polysaccharide and conjugate vaccine development against the Pneumococcus. The first polysaccharide vaccine was introduced in 1977 and covered 14 serotypes. PCV7, the first Pneumococcus conjugate vaccine, was introduced in 2000 and covered seven serotypes. Six more serotypes were added for the vaccine introduced in 2010, PCV13 (Figure modifed without permission from Pace, 2013).

1.3.2 Pneumococcal polysaccharide vaccines

The charge of capsular polysaccharides (CPS) is a detrimental factor in the immune response elicited. The majority of CPS are negatively charged, and elicit T cell-independent immune responses as they are not processed by MHCII and not presented to T cells. Zwitterionic polysaccharides, which carry both positive and negative charges, were found to elicit T cell-dependent immune responses. This is seen in polysaccharide A of *Bacteroides fragilis* and only the CPS of *Streptococcus pneumoniae* serotype 1 (Tzianabos *et al.*, 1993). Later research shows that these zwitterionic polysaccharides are processed differently by a nitric oxide-dependent mechanism and then associate with MHCII followed by presentation to T cells (Cobb *et al.*, 2004; Velez *et al.*, 2009). Furthermore, negatively charged CPS could be chemically altered to be zwitterionic, which was found to change the immune response to be T cell-dependent (Tzianabos *et al.*, 1993).

Briefly, the T cell-independent immune response to negatively charged CPS is characterized by complement factor C3 activation by an alternative pathway, where complement protein C3d forms a complex with the polysaccharide. Complement receptor 2 (CD21) of marginal-zone B cells in the spleen recognizes and interacts with the C3d-polysaccharide complex leading to B cell activation (Dintzis *et al.*, 1983; Griffioen *et al.*, 1991). It has long been believed that B cell activation leads to differentiation into plasma cells only, with no memory cells being formed. However, several reports demonstrate B cell differentiation into phenotypically different memory cells, called B1b lymphocytes, that produce only IgM antibodies (Hosokawa, 1979; Alugupalli *et al.*, 2004; Obukhanych, 2006; Tarlinton, 2006). In a T cell-independent response, plasma cells

produce predominantly IgM antibodies and some switching to IgG2 is seen, both of which are relatively low affinity antibodies (Barrett and Ayoub, 1986; Goldblatt, 2000). Furthermore, affinity maturation of these antibodies, which is the increase in antibody affinity to antigens during the course of an immune response does not occur (Kim and Siskind, 1978; Konradsen, 1995)

The efficacy of the polysaccharide pneumococcal vaccine in protection has been well documented in adults under 55 years (Shapiro *et al.*, 1991). Children <2 respond poorly, if at all, to polysaccharide based vaccines, which has been attributed to the low expression of CD21 on the surface of B cells in the spleen and blood at this age (Griffioen *et al.*, 1991). In the elderly, polysaccharide vaccines are less effective due to the physiological age-associated atrophy of haematopoietic tissue and primary lymphoid organs, causing a decreased production of B and T cells. This increases their susceptibility to infections and decreases the efficacy of vaccines (Simell *et al.*, 2008; Siegrist and Aspinall, 2009).

1.3.3 Glycoconjugate vaccines:

Pneumococcal conjugate vaccines have greatly reduced the incidence of invasive pneumococcal disease caused by vaccine serotypes, especially in children under 5 years (Black, 2010). Conjugation of CPS to proteins having T cell epitopes induces T cell-dependent immune responses to the polysaccharide antigen. Examples of proteins used are the diphtheria toxoid, tetanus toxoid and the modified diphtheria toxin CRM_{197} (Mäkelä and Käyhty, 2014).

To date, the exact molecular mechanisms of glycopeptide presentation to T cells are poorly understood. A proposed model is that glycoproteins are internalized by antigen presenting cells (APC) resulting in the *de novo* synthesis of MHCII, which then gets sorted to the endosomes and forms complexes with the carrier protein. These MHCII-peptide complexes undergo exocytosis for surface presentation of the MHCII-peptide complex to CD4+ T helper cells for their activation (Robinson and Delvig, 2002; Avci *et al.*, 2011). However, since the strong peptide-glycan covalent bond is not likely to be broken in endosomes, another model has been proposed where the peptide forms a complex with MHCII to present the glycopeptide to T cells (Avci *et al.*, 2011).

Independently, circulating naïve B cells recognize exogenous glycoproteins through surface IgM receptors and migrate to T cell zones in the lymph nodes and spleen, where B cell differentiation is stimulated by the activated CD4+ T helper cells. Unlike with T cell-independent responses, B cells are differentiated into non-antibody secreting memory cells and plasma cells, which primarily produce the high affinity IgG1 and IgG3 antibodies as well as IgG2 (Wykes *et al.*, 1998; Soininen, 1999). Upon subsequent exposure to the antigen through an infection or by a vaccine booster dose, memory cells differentiate into antibody-secreting plasma cells faster than naïve B cells (Tangye *et al.*, 2003; Horikawa *et al.*, 2007). The major advantage of conjugate vaccines is their efficacy in children <5 years, which could be attributed to the fact that infant T cells show adult immunophenotypes and mount equally robust immune responses to conjugate vaccine antigens (Timens *et al.*, 1989).

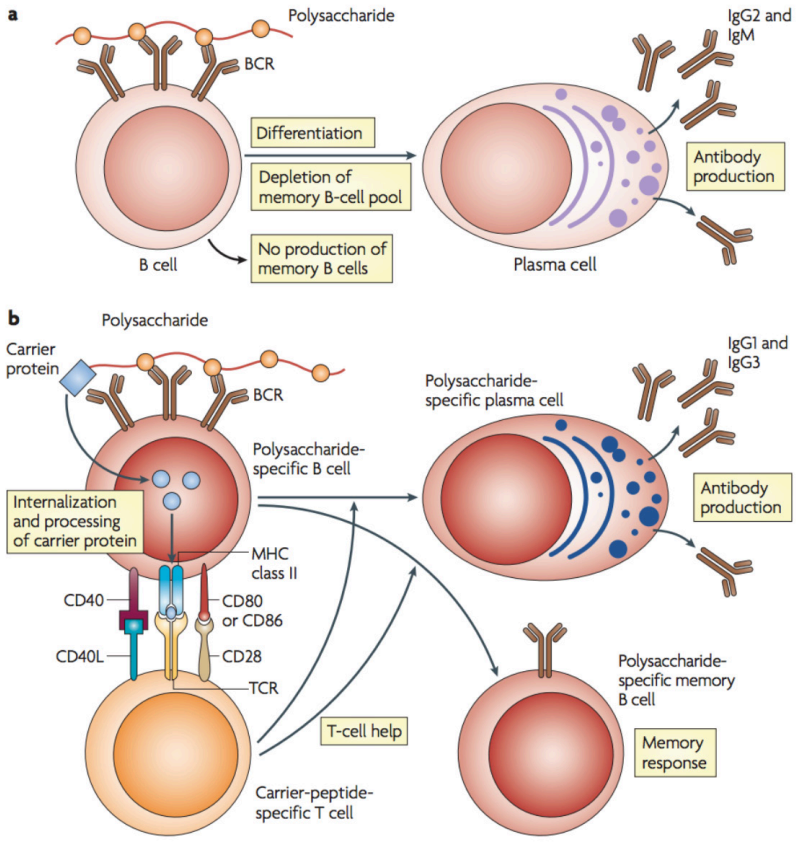

Figure 1.2: **A.** T cell-independent immune response to polysaccharide vaccines. Polysaccharides act by cross-linking with B cell receptors (BCR) leading to the differentiation of B cells into plasma cells, with only the low affinity IgG2 and IgM antibodies secreted.

B. T cell-dependent immune response to glycoconjugate vaccines, where cross-linking between the glycan and BCR is followed by internalization of the glycoprotein. A peptide-MHCII complex is formed in endosomes that undergo exocytosis to be presented to CD4+ T helper cells, causing the differentiation of B cells to high affinity antibody-producing plasma cells and memory cells (Figure modifed without permission from Pollard *et al.*, 2009).

1.3.4 Serotype replacement

Most studies involving serotype replacement looked at the effects of vaccinating using the PCV7 conjugate vaccine introduced in 2000, which covers only 7 serotypes. Hicks and colleagues suggest that by using vaccines that target a subset of serotypes, we are unintentionally applying a selective pressure against these serotypes and selecting for non-vaccine serotypes. They report a slow incremental annual increase in infections caused by non-vaccine serotypes, including serotype 19A (which was later covered by the introduction of PCV13 in 2010) (Hicks *et al.*, 2007). Another report from 2010 declares a complete serotype replacement in Massachusetts has taken place, predominantly by serotype 19A (Hanage *et al.*, 2010).

The same cycle is predicted to occur for every serotype-specific vaccine introduced, where a decline in vaccine serotypes within 4-6 years of launching the vaccine is followed by the introduction of new serotypes to fill the empty ecological niche we have introduced by our selective vaccination. Therefore, non-PCV13 serotypes are expected to expand with the next few years, and it is impossible to predict which non-vaccine serotypes would be more prevalent. A solution to this problem would be the rapid development and introduction of vaccines covering as many serotypes as possible and continuous surveillance and vaccine program evaluation to monitor the prevalence of serotype replacement (Weinberger *et al.*, 2011). Another solution would be prioritizing non-serotype specific vaccine research.

1.3.5 Non-serotype specific vaccines

Due to the success of polysaccharide and conjugate vaccines, protein and whole-cell vaccines have not been a research priority. Protein vaccines based on conserved surface proteins could potentially overcome the serotype replacement issue of polysaccharide and conjugate vaccines. Surface antigens tested as potential protein vaccines include the pneumococcal histidine triad protein D (PhtD), pneumococcal surface protein A (PspA) and Pneumococcal choline-binding protein A (PcpA). It has been suggested that surface proteins alone do not elicit an appreciable immune response in humans, which was seen before with meningococcal surface proteins (Santolaya *et al*., 2012). However, pneumococcal protein-only vaccines have shown promising results and multiple candidates have proceeded to clinical trials (Ochs *et al*., 2008; Vadesilho *et al*., 2012; Bologa *et al*., 2012; Darrieux *et al*., 2015).

A whole cell pneumococcal vaccine candidate is currently undergoing clinical trials. The vaccine is comprised of an ethanol-killed unencapsulated mutant of serotype 2 expressing conserved surface antigens. Animal model testing shows a T cell-dependent immune response with antibodies against multiple surface proteins, which was shown before to provide enhanced protection over vaccines composed of single antigenic components (Ogunniyi *et al.*, 2000; Malley *et al.*, 2001; Malley *et al.*, 2004).

1.3.6 Limitations of chemical conjugations

Pneumococcus glycoconjugate vaccines in the market today are synthesized by chemically conjugating glycans to proteins (Terra *et al.*, 2012). Despite the vaccine's exceptional efficacy, it is significantly more expensive than polysaccharide vaccines.

According to the CDC vaccine price list, the 13-valent conjugate vaccine PCV13 costs almost 3 times as much as the 23-valent polysaccharide vaccine PPSV23 (VFC | Current CDC Vaccine Price List | CDC, n.d.). This has led the slow uptake and implementation of vaccine programs by low-income countries without external aid (Wenger, 2001; Weinberger *et al.*, 2011). PATH, an international health organization, is currently working on a low-cost conjugate vaccine with less serotypes included, focusing only on serotypes more prevalent in developing countries (Vaccine development – Vaccine Development Global Program - PATH, n.d.). In the case of pneumococcal conjugate vaccines, CPS are chemically conjugated to CRM_{197}, a non-toxic diphtheria toxin mutant (Uchida *et al.*, 1973; Pace, 2013). This chemical conjugation is a complicated process that involves the purification of the polysaccharide, its chemical activation, conjugation to a protein and purification of the glycoconjugate from reaction components and other impurities (Dick and Beurret, 1989; Peeters *et al.*, 2003; Lees *et al.*, 2006). This process is plagued with low yields and batch-to-batch variations (Frasch, 2009). An alternative to chemical conjugations is biological conjugations. These have recently come into consideration after the identification of multiple bacterial protein glycosylation systems that could be exploited for glycan-protein conjugations.

1.4 Protein glycosylation

Protein glycosylation, or the covalent attachment of carbohydrate moieties to proteins, is a ubiquitous post-translational protein modification that is seen in all domains of life. Glycosylation was first discovered in eukaryotes in the 1930s (Neuberger, 1938), and, long believed to be exclusive to eukaryotes, was described in archaea and bacteria almost

40 years later (Sleytr, 1975; Mescher and Strominger, 1976). The most common types of protein glycosylation are *N*-linked, where the glycan is attached to the amide nitrogen of asparagine residues and *O*-linked where the glycan is attached to hydroxyl groups of serine or threonine residues (Nothaft and Szymanski, 2010).

1.4.1 Bacterial glycosylation systems

Bacterial glycoproteins were first described in the Surface layers (S-layers) of thermophilic *Clostridium* species in the 1970s (Sleytr, 1975; Sleytr and Thorne, 1976). Around 20 years later, *O*-linked glycosylation of type IV pili (Tfp) in *Pseudomonas aeruginosa* and *Neisseria meningitidis* were described, followed shortly by the description of the first *N*-linked protein glycosylation system in *Campylobacter jejuni* (Castric, 1995; Stimson *et al.*, 1995; Szymanski *et al.*, 1999). Since then glycosylation systems have been reported in both pathogenic and non-pathogenic bacteria and our understanding of the system has been rapidly improving, in part due to the development of molecular and biochemical tools (Nothaft and Szymanski, 2010). Iwashkiw and colleagues propose bacterial *O*- and *N*-linked glycosylation to be further classified based on the need for a periplasmic Oligosaccharyltransferase (OTase) to OTase-dependent and OTase-independent glycosylation (Iwashkiw *et al.*, 2013).

1.4.2 OTase-independent protein glycosylation:

OTase-independent protein glycosylation occurs in the cytoplasm, where nucleotide activated monosaccharides are directly or sequentially attached to acceptor proteins by

soluble glycosyltransferases. Flagella and autotransporters adhesins are examples of proteins glycosylated in this manner (Nothaft and Szymanski, 2010) .

Flagella are glycosylated either with a monosaccharide as seen in *C. jejuni* and *Helicobacter pylori*, or with a heterogeneous glycan that varies from one strain to another and ranges from a monosaccharide to an oligosaccharide such as in *P. aeruginosa* (Thibault *et al.*, 2001; Josenhans *et al.*, 2002; Schirm *et al.*, 2004). Single and multiple flagellar glycosylation sites have also been described in spp and *C. jejuni* respectively, and are mostly surface exposed residues of the flagellar protein (Samatey *et al.*, 2000; Thibault *et al.*, 2001; Andrew E Scott *et al.*, 2011; Hanuszkiewicz *et al.*, 2014). The flagellar glycan is essential for flagellar filament assembly and stability, host colonization, and plays a role in immunomodulation (Nothaft and Szymanski, 2010; Merino and Tomás, 2014; Stephenson *et al.*, 2014).

The self-associating autotransporters (SAATs) such as AIDA, TibA and Ag43 from diarrhoea-causing *E. coli* are *O*-glycosylated by cytoplasmic glycosyltransferases in a similar manner. In case of AIDA and TibA, glycosylation was shown to be required for protein stability and maintaining protein conformation, whereas glycosylated Ag43 displayed enhanced binding to a human epithelial cell line (Sherlock *et al.*, 2006; Charbonneau *et al.*, 2007; Côté *et al.*, 2013). The non-pilus adhesin HMW1 from *Haemophilus influenza* is also glycosylated in an OTase-independent manner with an *N*-linked glycan (Grass *et al.*, 2003; Gross *et al.*, 2008). Glycosylation was shown to play a role in HMW1 stability, tethering to the surface and therefore adhesin-mediated adherence (Grass *et al.*, 2003). Moreover, the platelet-binding adhesin GspB from the Gram-positive *Streptococcus gordonii* is *O*-glycosylated in the cytoplasm with GlcNAc

and Glc residues, and unglycosylated GspB was demonstrated to be highly unstable. However, the glycosyltransferase is yet to be identified (Bensing *et al.*, 2004; Bensing *et al.*, 2004). Additionally, the trimeric adhesin EmaA of the oral pathogen *Aggregatibacter actinomycetemcomitans* is glycosylated with O antigen subunits by a WaaL O antigen ligase homologue and was shown to be critical for collagen binding and resistance to proteolytic cleavage. This evidence suggests that EmaA glycosylation occurs on the periplasmic side of the inner membrane (Tang and Mintz, 2010; Tang *et al.*, 2012).

1.4.3 OTase-dependent glycosylation:

OTases are periplasmic proteins that transfer a preassembled lipid-linked glycan to acceptor proteins and are classified into *N*- and *O*-OTases, depending on the site of glycan attachment (Iwashkiw *et al.*, 2013). The glycan first has to be sequentially assembled on the cytoplasmic side of the inner membrane, where a specific initiating glycosyltransferase attaches a nucleotide-activated monosaccharide to an undecaprenyl phosphate (Und-P) lipid carrier. Other cytoplasmic glycosyltransferases then sequentially attach additional nucleotide monosaccharides to und-PP to build the oligosaccharide, which is then flipped to the periplasmic side of the inner membrane by a flippase. Periplasmic *O*-OTases or *N*-OTases then transfer this lipid-linked glycan *en bloc* to acceptor proteins (Figure 1.3) (Nothaft and Szymanski, 2010; Iwashkiw *et al.*, 2013).

The first *N*-glycosylation system described was in *Campylobacter jejuni*, and was later shown to be ubiquitous throughout the genus, with structurally diverse glycans identified (Szymanski *et al.*, 1999; Jervis *et al.*, 2012; Nothaft *et al.*, 2012). *N*-glycosylation in *C. jejuni* plays roles in attachment, virulence, colonization and protein stability (Nothaft and

Szymanski, 2013). Functional *N*-glycosylation systems have also been described in *Helicobacter* spp., *Wolinella succinogenes* and *Desulfovibrio desulfuricans* among many others (Jervis *et al.*, 2010; Ielmini and Feldman, 2011; Jervis *et al.*, 2012). The *C. jejuni* *N*-OTase was found to glycosylate asparagine residues in the consensus sequence D/E-X_1-*N*-X_2-S/T, where X_1 and X_2 can be any amino acid except proline (Kowarik *et al.*, 2006). In addition to the glycosylation activity of PglB, osmolarity-dependent hydrolytic activity was described as well in *Campylobacter*, with the release of free oligosaccharides (fOS) observed (Nothaft *et al.*, 2009; Dwivedi *et al.*, 2013).

O-glycosylation systems were first described in *Pseudomonas aeruginosa* and *Neisseria meningitidis* (Castric, 1995; Stimson *et al.*, 1995). The type IV pilin (Tfp) PilA from *P. aeruginosa*, which is critical for virulence, is *O*-glycosylated by an OTase-dependent mechanism. PilA is encoded upstream of the OTase TfpO and is the only known protein glycosylated by this OTase, with the pilin glycosylation site and glycan structure later identified (Castric, 1995; Castric *et al.*, 2001; Comer *et al.*, 2002). Glycosylation deficient mutants of *P. aeruginosa* were outcompeted by the wild type in a mouse respiratory model (Smedley *et al.*, 2005). The *P. aeruginosa* pilin glycan was later demonstrated to be essential for resisting opsonization by a pulmonary surfactant protein (Tan *et al.*, 2015). In *Neisseria meningitidis* and *N. gonorrhoeae*, the *O*-OTase is called PglL, and is a general OTase that glycosylates other proteins in addition to the Tfp PilE (Ku *et al.*, 2009; Vik *et al.*, 2009). The biological significance of *N. meningitidis* glycosylation is yet to be identified. For *N. gonorrhoeae*, the pilin glycan was shown to be essential for invading cervical epithelial cells by an unknown mechanism (Jennings *et al.*, 2011). In addition to *Pseudomonas* and *Neisseria* spp., *O*-glycosylation systems have

been described in other pathogenic and non-pathogenic bacteria. Examples include *Francisella tularensis*, *Acinetobacter* spp., *Burkholderia* spp., *Bacteroides* spp. and *Ralstonia solanacearum* (Egge-Jacobsen *et al.*, 2011; Jeremy A. Iwashkiw *et al.*, 2012; Gebhart *et al.*, 2012; Coyne *et al.*, 2013; Lithgow *et al.*, 2014; Scott *et al.*, 2014; Elhenawy *et al.*, 2015). *O*-glycosylation has been linked to motility, virulence, biofilm formation and bacterial growth and colonization, the precise mechanisms of which remain unknown (Jeremy A. Iwashkiw *et al.*, 2012; Coyne *et al.*, 2013; Lithgow *et al.*, 2014). Contrary to *N*-OTases, the exact mechanism by which *O*-OTases function is poorly understood, as its crystal structure is yet to be identified. Only conserved amino acid residues critical for *O*-OTase activity have been identified (Musumeci *et al.*, 2014). Moreover, *O*-glycosylation sites have been identified to be serine or threonine residues that lie in regions of low complexity that are rich in proline, alanine and serine residues, with no consensus sequence identified similar to *N*-glycosylation (Vik *et al.*, 2009).

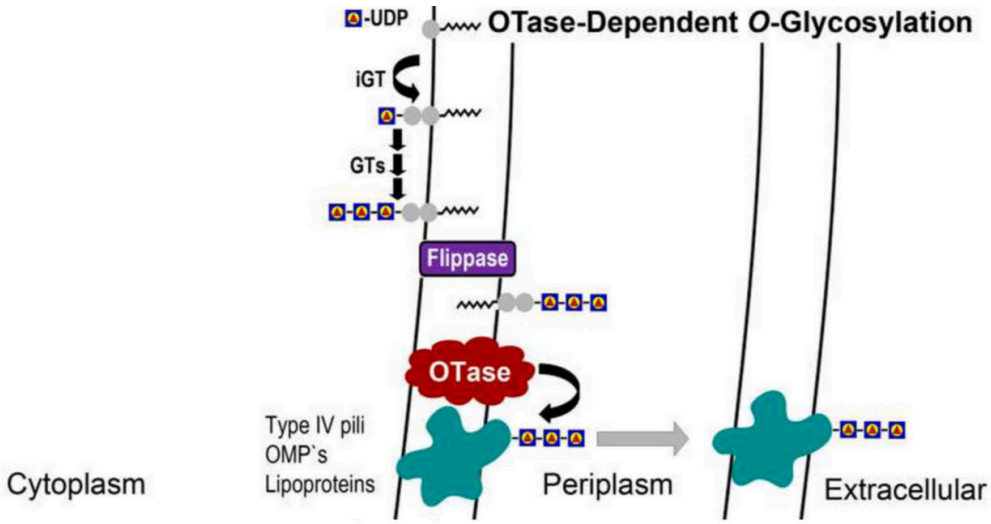

Figure 1.3: A model of bacterial OTase-dependent *O*-glycosylation, where a nucleotide-activated monosaccharide is added by an initiating glycosyltransferase to the undecaprenyl phosphate (Und-P) lipid carrier. Cytoplasmic glycosyltransferases build the oligosaccharide by attaching additional monosaccharides to Und-PP. The oligosaccharide is then flipped to the periplasmic side of the inner membrane where *O*-OTases transfer the glycan to serine or threonine residues of acceptor proteins. The same mechanism is seen in OTase-dependent *N*-linked glycosylation, except that the glycan is transferred by *N*-OTases to asparagine residues of acceptor proteins (Figure modifed without permission from Iwashkiw *et al.*, 2013).

1.4.4 *O*-linked glycosylation: Evolutionary connections

O-OTases regardless of acceptor protein specificity share limited sequence homology, with only the Wzy_c domain being conserved (Power *et al.*, 2006). O antigen ligases also contain the same Wzy_c domain and were found to be evolutionarily related to *O*-OTases, since both transfer lipid linked glycans either to acceptor proteins for glycosylation or lipid A for lipopolysaccharide (LPS) synthesis (Hug and Feldman, 2011). Interestingly, proteins with a Wzy_c domain were shown to perform other functions. O antigen polymerases required for CPS and/or LPS synthesis in *Vibrio vulnificus* and *Klebsiella pneumonia* contain Wzy_c domains (Nakhamchik *et al.*, 2007; Lin *et al.*, 2012). Therefore, experimentation is required to identify the function of a Wzy_c containing protein. Computational tools have been proposed for function elucidation, although later proven insufficient (Schulz *et al.*, 2013; Harding *et al.*, 2015). Additionally, lipid-linked glycan assembly is shared by multiple pathways, such as exopolysaccharide synthesis, Wzy-dependent CPS synthesis, peptidoglycan synthesis, LPS synthesis and as mentioned here, *N*- and *O*-linked protein glycosylation (Sutherland, 2001; Whitfield, 2006; Bouhss *et al.*, 2008; Hug and Feldman, 2011). These evolutionary relationships are more evident in *P. aeruginosa*, where the same lipid-linked trisaccharide was identified to be the O antigen repeating subunit in LPS and the glycan decorating Tfp subunits (Figure 1.4). As expected, immunization with glycosylated *P. aeruginosa* Tfp resulted in O antigen-specific protection (Castric *et al.*, 2001; Horzempa *et al.*, 2008)

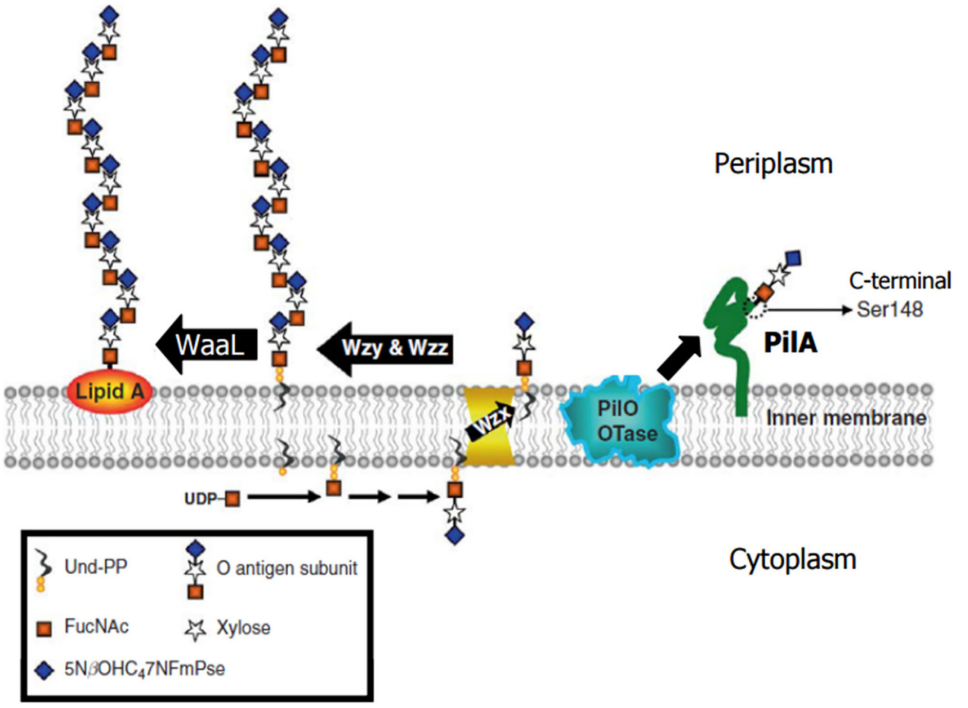

Figure 1.4 Protein glycosylation and LPS synthesis in *P. aeruginosa* share the same lipid-linked glycan. The same trisaccharide is assembled on an undecaprenol lipid carrier, flipped to the periplasm and either added directly to pilin by TfpO (PilO) or polymerized and added to lipid A by the WaaL O antigen ligase. (Figure modifed without permission from Faridmoayer and Feldman, 2010).

1.4.5 Glycosylation in other domains of life

As mentioned earlier, functional protein glycosylation systems have been identified in the three domains of life, with lots of similarities between these systems. With some variations, bacterial OTase-dependent *N*-glycosylation could be seen in archaea and eukaryotes where the glycan is first sequentially assembled on a lipid carrier by glycosyltransferases. Unlike the smaller undecaprenol lipid carrier in bacteria, eukaryotes and most archaea employ a larger dolichol lipid carrier (Magidovich and Eichler, 2009). The lipid-linked glycan is then flipped across either the cytoplasmic membrane in case of archaea or the endoplasmic reticulum membrane in eukaryotes, to be transferred to acceptor proteins by OTases (Dempski and Imperiali, 2002; Jarrell *et al.*, 2014). In higher eukaryotes, *N*-OTases are octameric protein complexes, the catalytic subunit being the STT3 protein (Dempski and Imperiali, 2002; Yan and Lennarz, 2002). Archaeal *N*-OTases (AglB), like their bacterial orthologues, are monomeric proteins that share structural and architectural similarities with STT3 (Maita *et al.*, 2010; Wei and Zhang, 2013).

Proteins equivalent to bacterial *O*-OTases have not been identified in archaea and eukaryotes to date. Archaeal *O*-linked glycans are seen in S-layers, and not much is known about their biosynthesis (Calo *et al.*, 2010). In eukaryotes, the most studied *O*-linked glycosylation system is the mucin-type *O*-glycosylation, where glycosyltransferases add monosaccharides to serine and threonine residues of proteins in the ER lumen, with further modification by glycosyltransferases taking place in the golgi apparatus (Hang and Bertozzi, 2005).

Protein glycosylation was found to be necessary for a plethora of processes in eukaryotes, which was later explained when glycosylation was linked to the proper folding and kinetic and thermodynamic stability of proteins (Varki, 1993; Helenius and Aebi, 2004). More than 40 congenital disorders of glycosylation have been identified in humans (Jaeken, 2013). As well, Alzheimer's disease and defective protein glycosylation have been correlated (Schedin-Weiss *et al.*, 2014). Furthermore, aberrant glycosylation is seen in cancer cells due to differential glycosyltransferase expression, and these cancer-associated glycoproteins could be utilized as cancer biomarkers (Meany and Chan, 2011; Kuzmanov *et al.*, 2013).

In archaea, protein glycosylation was first described in the S-layers of *Halobacterium salinarum,* where glycan moieties are linked to asparagine residues of proteins (Mescher and Strominger, 1976). Glycosylation in archaea is believed to aid in surviving the extreme environments they inhabit (Eichler, 2013). For example, glycosylation deficient *Haloferax volcanii* are incapable of surviving in high salt concentrations and have a different S-layer architecture that is less resistant to proteolysis (Abu-Qarn *et al.*, 2007; Yurist-Doutsch *et al.*, 2008; Yurist-Doutsch *et al.*, 2010). Remarkably, growing *H. volcanii* in different salt concentrations caused S-layer proteins to be decorated by variable glycan structures at different glycosylation sites (Guan *et al.*, 2012).

1.4.6 Exploiting bacterial OTases for conjugate vaccine synthesis

Biological conjugations of glycans to proteins using OTases are an alternative to chemical conjugations due to their many limitations. These conjugations involve the transfer of a glycan by an OTase to an acceptor protein in a suitable background, mostly

E. coli. This process involves the successful expression of a glycan synthesis locus, an OTase and an acceptor protein in engineered *E. coli* strains (Terra *et al.*, 2012). The *C. jejuni N*-OTase has been successfully used previously for developing glycoconjugate vaccines against *Shigella* spp., *Francisella tularensis* and *Burkholderia pseudomallei* (Ihssen *et al.*, 2010; Cuccui *et al.*, 2013; Garcia-Quintanilla *et al.*, 2014; Kämpf *et al.*, 2015). However, biological conjugations have limitations pertaining to glycan and protein specificity of OTases, which will be thoroughly discussed in Chapter 3.

1.5 Thesis outline:

The *O*-glycosylation system of the nosocomial pathogen *Acinetobacter baumannii* is essential for virulence and biofilm formation, where a single OTase and 9 glycoproteins were identified in the strain ATCC17978 (Jeremy A. Iwashkiw *et al.*, 2012). This system was shown later to be ubiquitous in the genus *Acinetobacter* (Scott *et al.*, 2014). However, the majority of members of *Acinetobacter* encode two Wzy_c domain-containing proteins, mainly characteristic of *O*-OTases and O antigen ligases. Previous work employed a computational model to predict the functions of both proteins in the environmental strain *A. baylyi*, where one was suggested to be an OTase while the other an O antigen ligase (Schulz *et al.*, 2013). However, *Acinetobacter* is known to form only lipooligosaccharide (LOS) (Kenyon *et al.*, 2014). This in itself suggests that an O antigen ligase is not required by *Acinetobacter*, and proposes a new role for the second Wzy_C domain-containing protein.

Objectives:

- Experimentally identifying the role of both Wzy_c superfamily proteins in *Acinetobacter*, by employing the model organism *A. baylyi* ADP1 and the clinical isolate *A. nosocomialis* M2 as representative strains.

- Evaluating the potential of these proteins, if OTase activity is demonstrated, in the glycoconjugate vaccine industry, which is always in search of new OTases with different glycan and protein specificities. In particular, the ability to transfer the Pneumococcus CPS to acceptor proteins will be tested, which was never successfully demonstrated before by any OTases.

1.6 References:

Abu-Qarn, M., Yurist-Doutsch, S., Giordano, A., Trauner, A., Morris, H.R., Hitchen, P., Medalia, O., Dell, A., and Eichler, J. (2007) *Haloferax volcanii* AglB and AglD are Involved in N-glycosylation of the S-layer Glycoprotein and Proper Assembly of the Surface Layer. *J Mol Biol* **374**: 1224–1236.

Alugupalli, K.R., Leong, J.M., Woodland, R.T., Muramatsu, M., Honjo, T., and Gerstein, R.M. (2004) B1b lymphocytes confer T cell-independent long-lasting immunity. *Immunity* **21**: 379–90.

Avci, F.Y., Li, X., Tsuji, M., and Kasper, D.L. (2011) A mechanism for glycoconjugate vaccine activation of the adaptive immune system and its implications for vaccine design. *Nat Med* **17**: 1602–9.

Bambeke, F. Van, Reinert, R.R., Appelbaum, P.C., Tulkens, P.M., and Peetermans, W.E. (2007) Multidrug-Resistant *Streptococcus pneumoniae* Infections. *Drugs* **67**: 2355–2382.

Barrett, D.J., and Ayoub, E.M. (1986) IgG2 subclass restriction of antibody to pneumococcal polysaccharides. *Clin Exp Immunol* **63**: 127–34.

Bensing, B.A., Gibson, B.W., and Sullam, P.M. (2004) The *Streptococcus* gordonii Platelet Binding Protein GspB Undergoes Glycosylation Independently of Export. *J Bacteriol* **186**: 638–645.

Bensing, B.A., López, J.A., and Sullam, P.M. (2004) The *Streptococcus* gordonii surface proteins GspB and Hsa mediate binding to sialylated carbohydrate epitopes on the platelet membrane glycoprotein Ibalpha. *Infect Immun* **72**: 6528–37.

Black, S. (2010) The volatile nature of pneumococcal serotype epidemiology: potential for misinterpretation. *Pediatr Infect Dis J* **29**: 301–3.

Bologa, M., Kamtchoua, T., Hopfer, R., Sheng, X., Hicks, B., Bixler, G., Hou, V., Pehlic, V., Yuan, T., and Gurunathan, S. (2012) Safety and immunogenicity of pneumococcal protein vaccine candidates: monovalent choline-binding protein A (PcpA) vaccine and bivalent PcpA-pneumococcal histidine triad protein D vaccine. *Vaccine* **30**: 7461–8.

Borgono, J.M., McLean, A.A., Vella, P.P., Woodhour, A.F., Canepa, I., Davidson, W.L., and Hilleman, M.R. (1978) Vaccination and Revaccination with Polyvalent Pneumococcal Polysaccharide Vaccines in Adults and Infants. *Exp Biol Med* **157**: 148–154.

Børud, B., Viburiene, R., Hartley, M.D., Paulsen, B.S., Egge-Jacobsen, W., Imperiali, B., and Koomey, M. (2011) Genetic and molecular analyses reveal an evolutionary trajectory

for glycan synthesis in a bacterial protein glycosylation system. *Proc Natl Acad Sci U S A* **108**: 9643–8.

Bouhss, A., Trunkfield, A.E., Bugg, T.D.H., and Mengin-Lecreulx, D. (2008) The biosynthesis of peptidoglycan lipid-linked intermediates. *FEMS Microbiol Rev* **32**: 208–233.

Calo, D., Kaminski, L., and Eichler, J. (2010) Protein glycosylation in Archaea: sweet and extreme. *Glycobiology* **20**: 1065–76.

Campbell, Jr., G.D., and Silberman, R. (1998) Drug-Resistant *Streptococcus pneumoniae*. *Clin Infect Dis* **26**: 1188–1195.

Canadian Antimicrobial Resistance Surveillance System Report 2015 (2015) .

Castric, P. (1995) pilO, a gene required for glycosylation of *Pseudomonas aeruginosa* 1244 pilin. *Microbiology* **141 (Pt 5**: 1247–54.

Castric, P., Cassels, F.J., and Carlson, R.W. (2001) Structural Characterization of the *Pseudomonas aeruginosa* 1244 Pilin Glycan. *J Biol Chem* **276**: 26479–26485.

Chao, Y., Marks, L.R., Pettigrew, M.M., and Hakansson, A.P. (2014) *Streptococcus pneumoniae* biofilm formation and dispersion during colonization and disease. *Front Cell Infect Microbiol* **4**: 194.

Charbonneau, M.-E., Girard, V., Nikolakakis, A., Campos, M., Berthiaume, F., Dumas, F., Lépine, F., and Mourez, M. (2007) O-linked glycosylation ensures the normal conformation of the autotransporter adhesin involved in diffuse adherence. *J Bacteriol* **189**: 8880–9.

Chumakov, K., and Ehrenfeld, E. (2008) New generation of inactivated poliovirus vaccines for universal immunization after eradication of poliomyelitis. *Clin Infect Dis* **47**: 1587–92.

Cobb, B.A., Wang, Q., Tzianabos, A.O., and Kasper, D.L. (2004) Polysaccharide processing and presentation by the MHCII pathway. *Cell* **117**: 677–87.

Comer, J.E., Marshall, M.A., Blanch, V.J., Deal, C.D., and Castric, P. (2002) Identification of the *Pseudomonas aeruginosa* 1244 pilin glycosylation site. *Infect Immun* **70**: 2837–45.

Côté, J.-P., Charbonneau, M.-È., and Mourez, M. (2013) Glycosylation of the *Escherichia coli* TibA self-associating autotransporter influences the conformation and the functionality of the protein. *PLoS One* **8**: e80739.

Coyne, M.J., Fletcher, C.M., Chatzidaki-Livanis, M., Posch, G., Schaffer, C., and Comstock, L.E. (2013) Phylum-wide general protein O-glycosylation system of the Bacteroidetes. *Mol Microbiol* **88**: 772–83.

Cuccui, J., Thomas, R.M., Moule, M.G., D'Elia, R. V, Laws, T.R., Mills, D.C., Williamson, D., Atkins, T.P., Prior, J.L., and Wren, B.W. (2013) Exploitation of bacterial N-linked glycosylation to develop a novel recombinant glycoconjugate vaccine against *Francisella tularensis*. *Open Biol* **3**: 130002.

Cuccui, J., and Wren, B.W. (2013) Bacteria like sharing their sweets. *Mol Microbiol* **89**: 811–5.

Darrieux, M., Goulart, C., Briles, D., and Leite, L.C. de C. (2015) Current status and perspectives on protein-based pneumococcal vaccines. *Crit Rev Microbiol* .

Davies, J.A. V. (1937) The Response of Infants to Inoculation with Type I Pneumococcus Carbohydrate. *J Immunol* **33**: 1–7.

Delany, I., Rappuoli, R., and DeGregorio, E. (2014) Vaccines for the 21st century. *EMBO Mol Med* **6**: 708–20.

Dempski, R.E., and Imperiali, B. (2002) Oligosaccharyl transferase: gatekeeper to the secretory pathway. *Curr Opin Chem Biol* **6**: 844–850.

Desai, A.P., Sharma, D., Crispell, E.K., Baughman, W., Thomas, S., Tunali, A., Sherwood, L., Zmitrovich, A., Jerris, R., Satola, S., Beall, B., Moore, M.R., Jain, S., and Farley, M.M. (2015) Decline in Pneumococcal Nasopharyngeal Carriage of Vaccine Serotypes After the Introduction of the 13-Valent Pneumococcal Conjugate Vaccine in Children in Atlanta, Georgia. *Pediatr Infect Dis J* .

Dick, W.E., and Beurret, M. (1989) Glycoconjugates of bacterial carbohydrate antigens. A survey and consideration of design and preparation factors. *Contrib Microbiol Immunol* **10**: 48–114.

Dintzis, R.Z., Middleton, M.H., and Dintzis, H.M. (1983) Studies on the immunogenicity and tolerogenicity of T-independent antigens. *J Immunol* **131**: 2196–203.

Doern, G. V, Heilmann, K.P., Huynh, H.K., Rhomberg, P.R., Coffman, S.L., and Brueggemann, A.B. (2001) Antimicrobial resistance among clinical isolates of *Streptococcus pneumoniae* in the United States during 1999--2000, including a comparison of resistance rates since 1994--1995. *Antimicrob Agents Chemother* **45**: 1721–9.

Donkor, E.S. (2013) Understanding the pneumococcus: transmission and evolution. *Front Cell Infect Microbiol* **3**: 7.

Donkor, E.S., and Badoe, E. V. (2014) Insights into Pneumococcal Pathogenesis and Antibiotic Resistance. *Adv Microbiol* **04**: 627–643.

Downie, A.W. (1937) The specific capsular polysaccharide of pneumococcus type I and the immunity which it induces in mice. *J Pathol Bacteriol* **45**: 131–147.

Dwivedi, R., Nothaft, H., Reiz, B., Whittal, R.M., and Szymanski, C.M. (2013) Generation of free oligosaccharides from bacterial protein N-linked glycosylation systems. *Biopolymers* **99**: 772–83.

Egge-Jacobsen, W., Salomonsson, E.N., Aas, F.E., Forslund, A.-L., Winther-Larsen, H.C., Maier, J., Macellaro, A., Kuoppa, K., Oyston, P.C.F., Titball, R.W., Thomas, R.M., Forsberg, Å., Prior, J.L., and Koomey, M. (2011) O-linked glycosylation of the PilA pilin protein of *Francisella tularensis*: identification of the endogenous protein-targeting oligosaccharyltransferase and characterization of the native oligosaccharide. *J Bacteriol* **193**: 5487–97.

Eichler, J. (2013) Extreme sweetness: protein glycosylation in archaea. *Nat Rev Microbiol* **11**: 151–6.

Elhenawy, W., Scott, N.E., Tondo, M.L., Orellano, E.G., Foster, L.J., and Feldman, M.F. (2015) Protein O-linked glycosylation in the plant pathogen *Ralstonia solanacearum*. *Glycobiology* cwv098.

Eskola, J., Käyhty, H., Takala, A.K., Peltola, H., Rönnberg, P.-R., Kela, E., Pekkanen, E., McVerry, P.H., and Mäkelä, P.H. (1990) A Randomized, Prospective Field Trial of a Conjugate Vaccine in the Protection of Infants and Young Children against Invasive Haemophilus influenzae Type b Disease. *N Engl J Med* **323**: 1381–1387.

Eskola, J., Peltola, H., Takala, A.K., Käyhty, H., Hakulinen, M., Karanko, V., Kela, E., Rekola, P., Rönnberg, P.R., and Samuelson, J.S. (1987) Efficacy of Haemophilus influenzae type b polysaccharide-diphtheria toxoid conjugate vaccine in infancy. *N Engl J Med* **317**: 717–22.

Faridmoayer, A. and Feldman, M.F. (2010) Comprehensive Natural Products II. Elsevier.

Feldman, C., and Anderson, R. (2014) Review: current and new generation pneumococcal vaccines. *J Infect* **69**: 309–25.

Francis, T., and Tillett, W.S. (1930) Cutaneous reactions in pneumonia. The development of antibodies following the intradermal injection of type-specific polysaccharide. *J Exp Med* **52**: 573–85.

Frasch, C.E. (2009) Preparation of bacterial polysaccharide-protein conjugates: analytical and manufacturing challenges. *Vaccine* **27**: 6468–70.

Garcia-Quintanilla, F., Iwashkiw, J.A., Price, N.L., Stratilo, C., and Feldman, M.F. (2014) Production of a recombinant vaccine candidate against *Burkholderia pseudomallei* exploiting the bacterial N-glycosylation machinery. *Front Microbiol* **5**: 381.

Gebhart, C., Ielmini, M.V., Reiz, B., Price, N.L., Aas, F.E., Koomey, M., and Feldman, M.F. (2012) Characterization of exogenous bacterial oligosaccharyltransferases in *Escherichia coli* reveals the potential for O-linked protein glycosylation in Vibrio cholerae and *Burkholderia thailandensis*. *Glycobiology* **22**: 962–74.

Gillis, L.M., White, H.D., Whitehurst, A., and Sullivan, D.C. (2005) Vancomycin-tolerance among clinical isolates of *Streptococcus pneumoniae* in Mississippi during 1999-2001. *Am J Med Sci* **330**: 65–8.

Goldblatt, D. (2000) Conjugate vaccines. *Clin Exp Immunol* **119**: 1–3.

Grabenstein, J.D., and Klugman, K.P. (2012) A century of pneumococcal vaccination research in humans. *Clin Microbiol Infect* **18 Suppl 5**: 15–24.

Grass, S., Buscher, A.Z., Swords, W.E., Apicella, M.A., Barenkamp, S.J., Ozchlewski, N., and St Geme, J.W. (2003) The *Haemophilus influenzae* HMW1 adhesin is glycosylated in a process that requires HMW1C and phosphoglucomutase, an enzyme involved in lipooligosaccharide biosynthesis. *Mol Microbiol* **48**: 737–751.

Griffioen, A.W., Rijkers, G.T., Janssens-Korpela, P., and Zegers, B.J. (1991) Pneumococcal polysaccharides complexed with C3d bind to human B lymphocytes via complement receptor type 2. *Infect Immun* **59**: 1839–45.

Gross, J., Grass, S., Davis, A.E., Gilmore-Erdmann, P., Townsend, R.R., and St Geme, J.W. (2008) The *Haemophilus influenzae* HMW1 adhesin is a glycoprotein with an unusual N-linked carbohydrate modification. *J Biol Chem* **283**: 26010–5.

Guan, Z., Naparstek, S., Calo, D., and Eichler, J. (2012) Protein glycosylation as an adaptive response in Archaea: growth at different salt concentrations leads to alterations in *Haloferax volcanii* S-layer glycoprotein N-glycosylation. *Environ Microbiol* **14**: 743–53.

Hanage, W.P., Finkelstein, J.A., Huang, S.S., Pelton, S.I., Stevenson, A.E., Kleinman, K., Hinrichsen, V.L., and Fraser, C. (2010) Evidence that pneumococcal serotype replacement in Massachusetts following conjugate vaccination is now complete. *Epidemics* **2**: 80–4.

Hang, H.C., and Bertozzi, C.R. (2005) The chemistry and biology of mucin-type O-linked glycosylation. *Bioorg Med Chem* **13**: 5021–34.

Hansman, D., and Bullen, M.M. (1967) A RESISTANT PNEUMOCOCCUS. *Lancet* **290**: 264–265.

Hanuszkiewicz, A., Pittock, P., Humphries, F., Moll, H., Roa Rosales, A., Molinaro, A., Moynagh, P.N., Lajoie, G., and Valvano, M.A. (2014) Identification of the flagellin glycosylation system in *Burkholderia cenocepacia* and the contribution of glycosylated flagellin to evasion of human innate immune responses. *J Biol Chem* M114.562603–.

Harding, C.M., Nasr, M.A., Kinsella, R.L., Scott, N.E., Foster, L.J., Weber, B.S., Fiester, S.E., Actis, L.A., Tracy, E.N., Munson, R.S., and Feldman, M.F. (2015) *Acinetobacter* strains carry two functional oligosaccharyltransferases, one devoted exclusively to type IV pilin, and the other one dedicated to O-glycosylation of multiple proteins. *Mol Microbiol* .

Harding, C.M., Tracy, E.N., Carruthers, M.D., Rather, P.N., Actis, L.A., and Munson, R.S. (2013) *Acinetobacter baumannii* strain M2 produces type IV pili which play a role in natural transformation and twitching motility but not surface-associated motility. *MBio* **4**: e00360–13–.

Hausdorff, W.P., Bryant, J., Paradiso, P.R., and Siber, G.R. (2000) Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. *Clin Infect Dis* **30**: 100–21.

Helenius, A., and Aebi, M. (2004) Roles of N-linked glycans in the endoplasmic reticulum. *Annu Rev Biochem* **73**: 1019–49.

Hicks, L.A., Harrison, L.H., Flannery, B., Hadler, J.L., Schaffner, W., Craig, A.S., Jackson, D., Thomas, A., Beall, B., Lynfield, R., Reingold, A., Farley, M. M., Whitney, C. G. (2007) Incidence of Pneumococcal Disease Due to Non–Pneumococcal Conjugate Vaccine (PCV7) Serotypes in the United States during the Era of Widespread PCV7 Vaccination, 1998–2004. *J Infect Dis* **196**: 1346–1354.

Horikawa, K., Martin, S.W., Pogue, S.L., Silver, K., Peng, K., Takatsu, K., and Goodnow, C.C. (2007) Enhancement and suppression of signaling by the conserved tail of IgG memory-type B cell antigen receptors. *J Exp Med* **204**: 759–69.

Horzempa, J., Held, T.K., Cross, A.S., Furst, D., Qutyan, M., Neely, A.N., and Castric, P. (2008) Immunization with a *Pseudomonas aeruginosa* 1244 pilin provides O-antigen-specific protection. *Clin Vaccine Immunol* **15**: 590–7.

Hosokawa, T. (1979) Studies on B-cell memory. II. T-cell independent antigen can induce B-cell memory. *Immunology* **38**: 291–9.

Hug, I., and Feldman, M.F. (2011) Analogies and homologies in lipopolysaccharide and glycoprotein biosynthesis in bacteria. *Glycobiology* **21**: 138–51.

Ielmini, M. V, and Feldman, M.F. (2011) Desulfovibrio desulfuricans PglB homolog possesses oligosaccharyltransferase activity with relaxed glycan specificity and distinct protein acceptor sequence requirements. *Glycobiology* **21**: 734–42.

Ihssen, J., Kowarik, M., Dilettoso, S., Tanner, C., Wacker, M., and Thöny-Meyer, L. (2010) Production of glycoprotein vaccines in *Escherichia coli*. *Microb Cell Fact* **9**: 61.

Iwashkiw, J.A., Seper, A., Weber, B.S., Scott, N.E., Vinogradov, E., Stratilo, C., Reiz, B., Cordwell, S.J., Whittal, R., Schild, S., and Feldman, M.F. (2012) Identification of a General O-linked Protein Glycosylation System in *Acinetobacter baumannii* and Its Role in Virulence and Biofilm Formation. *PLoS Pathog* **8**: e1002758.

Iwashkiw, J.A., Vozza, N.F., Kinsella, R.L., and Feldman, M.F. (2013) Pour some sugar on it: the expanding world of bacterial protein O-linked glycosylation. *Mol Microbiol* **89**: 14–28.

Jaeken, J. (2013) Congenital disorders of glycosylation. *Handb Clin Neurol* **113**: 1737–43.

Jarrell, K.F., Ding, Y., Meyer, B.H., Albers, S.-V., Kaminski, L., and Eichler, J. (2014) N-linked glycosylation in Archaea: a structural, functional, and genetic analysis. *Microbiol Mol Biol Rev* **78**: 304–41.

Jauneikaite, E., Jefferies, J.M., Hibberd, M.L., and Clarke, S.C. (2012) Prevalence of *Streptococcus pneumoniae* serotypes causing invasive and non-invasive disease in South East Asia: a review. *Vaccine* **30**: 3503–14.

Jenkins, S.G., Brown, S.D., and Farrell, D.J. (2008) Trends in antibacterial resistance among *Streptococcus pneumoniae* isolated in the USA: update from PROTEKT US Years 1-4. *Ann Clin Microbiol Antimicrob* **7**: 1.

Jennings, M.P., Jen, F.E.-C., Roddam, L.F., Apicella, M.A., and Edwards, J.L. (2011) *Neisseria gonorrhoeae* pilin glycan contributes to CR3 activation during challenge of primary cervical epithelial cells. *Cell Microbiol* **13**: 885–96.

Jervis, A.J., Butler, J.A., Lawson, A.J., Langdon, R., Wren, B.W., and Linton, D. (2012) Characterization of the structurally diverse N-linked glycans of *Campylobacter* species. *J Bacteriol* **194**: 2355–62.

Jervis, A.J., Langdon, R., Hitchen, P., Lawson, A.J., Wood, A., Fothergill, J.L., Morris, H.R., Dell, A., Wren, B., and Linton, D. (2010) Characterization of N-linked protein glycosylation in *Helicobacter pullorum*. *J Bacteriol* **192**: 5228–36.

Josenhans, C., Vossebein, L., Friedrich, S., and Suerbaum, S. (2002) The neuA/flmD gene cluster of *Helicobacter pylori* is involved in flagellar biosynthesis and flagellin glycosylation. *FEMS Microbiol Lett* **210**: 165–72.

Kadioglu, A., Weiser, J.N., Paton, J.C., and Andrew, P.W. (2008) The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. *Nat Rev Microbiol* **6**: 288–301.

Kämpf, M.M., Braun, M., Sirena, D., Ihssen, J., Thöny-Meyer, L., and Ren, Q. (2015) In vivo production of a novel glycoconjugate vaccine against Shigella flexneri 2a in recombinant *Escherichia coli*: identification of stimulating factors for in vivo glycosylation. *Microb Cell Fact* **14**: 12.

Kempf, M., Varon, E., Lepoutre, A., Gravet, A., Baraduc, R., Brun, M., Chardon, H., Cremniter, J., Croizé, J., Dalmay, F., Demachy, M. C., Fosse, T., Grelaud, C., Hadou, T., Hamdad, F., Koeck, J. L., Luce, S., Mermond, S., Patry, I., Péchinot, A., Raymond, J., Ros, A., Segonds, C., Soullié, B., Tandé, D., Vergnaud, M., Vernet-Garnier, V., Wallet, F., Gutmann, L., Ploy, M. C., Lanotte, P. (2015) Decline in antibiotic resistance and changes in the serotype distribution of *Streptococcus pneumoniae* isolates from children with acute otitis media; a 2001-2011 survey by the French Pneumococcal Network. *Clin Microbiol Infect* **21**: 35–42.

Kenyon, J.J., Nigro, S.J., and Hall, R.M. (2014) Variation in the OC locus of *Acinetobacter baumannii* genomes predicts extensive structural diversity in the lipooligosaccharide. *PLoS One* **9**: e107833.

Kim, Y.T., and Siskind, G.W. (1978) Studies on the control of antibody synthesis. XII. Genetic influences on antibody affinity. *Immunology* **34**: 669–78.

Konradsen, H.B. (1995) Quantity and avidity of pneumococcal antibodies before and up to five years after pneumococcal vaccination of elderly persons. *Clin Infect Dis* **21**: 616–20.

Korona-Glowniak, I., and Malm, A. (2012) Characteristics of *Streptococcus pneumoniae* strains colonizing upper respiratory tract of healthy preschool children in Poland. *ScientificWorldJournal* **2012**: 732901.

Kowarik, M., Young, N.M., Numao, S., Schulz, B.L., Hug, I., Callewaert, N., Mills, D. C., Hernandez, M., Kelly, J. F., Wacker, M. and Aebi, M. (2006) Definition of the bacterial N-glycosylation site consensus sequence. *EMBO J* **25**: 1957–66.

Ku, S.C., Schulz, B.L., Power, P.M., and Jennings, M.P. (2009) The pilin O-glycosylation pathway of pathogenic *Neisseria* is a general system that glycosylates AniA, an outer membrane nitrite reductase. *Biochem Biophys Res Commun* **378**: 84–9.

Kuzmanov, U., Kosanam, H., and Diamandis, E.P. (2013) The sweet and sour of serological glycoprotein tumor biomarker quantification. *BMC Med* **11**: 31.

Lee, E.-Y., Bang, J.Y., Park, G.W., Choi, D.-S., Kang, J.S., Kim, H.-J., Park, K.-S., Lee, J.-O., Kim, Y.-K., Kwon, K.-H., Kim, K.-P., and Gho, Y.S. (2007) Global proteomic profiling of native outer membrane vesicles derived from *Escherichia coli*. *Proteomics* **7**: 3143–53.

Lees, A., Sen, G., and LopezAcosta, A. (2006) Versatile and efficient synthesis of protein–polysaccharide conjugate vaccines using aminooxy reagents and oxime chemistry. *Vaccine* **24**: 716–729.

Lin, T.-L., Yang, F.-L., Yang, A.-S., Peng, H.-P., Li, T.-L., Tsai, M.-D., Wu, S.-H., and Wang, J.-T. (2012) Amino acid substitutions of MagA in Klebsiella *pneumoniae* affect the biosynthesis of the capsular polysaccharide. *PLoS One* **7**: e46783.

Lithgow, K. V., Scott, N.E., Iwashkiw, J.A., Thomson, E.L.S., Foster, L.J., Feldman, M.F., and Dennis, J.J. (2014) A general protein O -glycosylation system within the *Burkholderia cepacia* complex is involved in motility and virulence. *Mol Microbiol* n/a–n/a.

Lund, E. (1970) On the nomenclature of the pneumococcal types. *Int J Syst Bacteriol* **20**: 321–323.

Macleod, C.M., Hodges, R.G., Heidelberger, M., and Bernhard, W.G. (1945) Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides. *J Exp Med* **82**: 445–65.

Magidovich, H., and Eichler, J. (2009) Glycosyltransferases and oligosaccharyltransferases in Archaea: putative components of the N-glycosylation pathway in the third domain of life. *FEMS Microbiol Lett* **300**: 122–30.

Maita, N., Nyirenda, J., Igura, M., Kamishikiryo, J., and Kohda, D. (2010) Comparative structural biology of eubacterial and archaeal oligosaccharyltransferases. *J Biol Chem* **285**: 4941–50.

Mäkelä, P.H., and Käyhty, H. (2014) Evolution of conjugate vaccines. *Expert Rev Vaccines* .

Malley, R., Lipsitch, M., Stack, A., Saladino, R., Fleisher, G., Pelton, S., Thompson, C., Briles, D., and Anderson, P. (2001) Intranasal Immunization with Killed Unencapsulated Whole Cells Prevents Colonization and Invasive Disease by Capsulated Pneumococci. *Infect Immun* **69**: 4870–4873.

Malley, R., Morse, S.C., Leite, L.C.C., Areas, A.P.M., Ho, P.L., Kubrusly, F.S., Almeida, I.C., and Anderson, P. (2004) Multiserotype protection of mice against pneumococcal colonization of the nasopharynx and middle ear by killed nonencapsulated cells given intranasally with a nontoxic adjuvant. *Infect Immun* **72**: 4290–2.

Meany, D.L., and Chan, D.W. (2011) Aberrant glycosylation associated with enzymes as cancer biomarkers. *Clin Proteomics* **8**: 7.

Merino, S., and Tomás, J.M. (2014) Gram-negative flagella glycosylation. *Int J Mol Sci* **15**: 2840–57.

Mescher, M.F., and Strominger, J.L. (1976) Structural (shape-maintaining) role of the cell surface glycoprotein of Halobacterium salinarium. *Proc Natl Acad Sci U S A* **73**: 2687–91.

Musumeci, M.A., Faridmoayer, A., Watanabe, Y., and Feldman, M.F. (2014) Evaluating the role of conserved amino acids in bacterial O-oligosaccharyltransferases by in vivo, in vitro and limited proteolysis assays. *Glycobiology* **24**: 39–50.

Nakhamchik, A., Wilde, C., and Rowe-Magnus, D.A. (2007) Identification of a Wzy polymerase required for group IV capsular polysaccharide and lipopolysaccharide biosynthesis in Vibrio vulnificus. *Infect Immun* **75**: 5550–8.

Neuberger, A. (1938) Carbohydrates in protein: The carbohydrate component of crystalline egg albumin. *Biochem J* **32**: 1435–51.

Nothaft, H., Liu, X., McNally, D.J., Li, J., and Szymanski, C.M. (2009) Study of free oligosaccharides derived from the bacterial N-glycosylation pathway. *Proc Natl Acad Sci U S A* **106**: 15019–24.

Nothaft, H., Scott, N.E., Vinogradov, E., Liu, X., Hu, R., Beadle, B., Fodor, C., Miller, W.G., Li, J., Cordwell, S.J., and Szymanski, C.M. (2012) Diversity in the protein N-glycosylation pathways within the *Campylobacter* genus. *Mol Cell Proteomics* **11**: 1203–19.

Nothaft, H., and Szymanski, C.M. (2010) Protein glycosylation in bacteria: sweeter than ever. *Nat Rev Microbiol* **8**: 765–78.

Nothaft, H., and Szymanski, C.M. (2013) Bacterial protein N-glycosylation: new perspectives and applications. *J Biol Chem* **288**: 6912–20.

Novak, R., Henriques, B., Charpentier, E., Normark, S., and Tuomanen, E. (1999) Emergence of vancomycin tolerance in *Streptococcus pneumoniae*. *Nature* **399**: 590–3.

O'Brien, K.L., Wolfson, L.J., Watt, J.P., Henkle, E., Deloria-Knoll, M., McCall, N., Lee, E., Mulholland, K., Levine, O.S., and Cherian, T. (2009) Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet* **374**: 893–902.

Obukhanych, T. V. (2006) T-independent type II immune responses generate memory B cells. *J Exp Med* **203**: 305–310.

Ochs, M.M., Bartlett, W., Briles, D.E., Hicks, B., Jurkuvenas, A., Lau, P., Ren, B., and Millar, A. (2008) Vaccine-induced human antibodies to PspA augment complement C3 deposition on *Streptococcus pneumoniae*. *Microb Pathog* **44**: 204–14.

Ogunniyi, A.D., Folland, R.L., Briles, D.E., Hollingshead, S.K., and Paton, J.C. (2000) Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. *Infect Immun* **68**: 3028–33.

Ortega, M., Marco, F., Soriano, A., García, E., Martínez, J.A., and Mensa, J. (2003) Lack of vancomycin tolerance in *Streptococcus pneumoniae* strains isolated in Barcelona, Spain, from 1999 to 2001. *Antimicrob Agents Chemother* **47**: 1976–8.

Pace, D. (2013) Glycoconjugate vaccines. *Expert Opin Biol Ther* **13**: 11–33.

Peeters, C.C.A.M., Lagerman, P.R., Weers, O. de, Oomen, L.A., Hoogerhout, P., Beurret, M., Poolman, J.T., and Reddin, K.M. (2003) Preparation of polysaccharide-conjugate vaccines. *Methods Mol Med* **87**: 153–74.

Pendleton, J.N., Gorman, S.P., and Gilmore, B.F. (2013) Clinical relevance of the ESKAPE pathogens. *Expert Rev Anti Infect Ther* **11**: 297–308.

Pilishvili, T., Lexau, C., Farley, M.M., Hadler, J., Harrison, L.H., Bennett, N.M., Reingold, A., Thomas, A., Schaffner, W., Craig, A.S., Smith, P.J., Beall, B.W., Whitney, C.G., and Moore, M.R. (2010) Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. *J Infect Dis* **201**: 32–41.

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Pollard, A.J., Perrett, K.P., and Beverley, P.C. (2009) Maintaining protection against invasive bacteria with protein-polysaccharide conjugate vaccines. *Nat Rev Immunol* **9**: 213–20.

Power, E. (2006) Impact of antibiotic restrictions: the pharmaceutical perspective. *Clin Microbiol Infect* **12**: 25–34.

Power, P.M., Seib, K.L., and Jennings, M.P. (2006) Pilin glycosylation in *Neisseria meningitidis* occurs by a similar pathway to wzy-dependent O-antigen biosynthesis in *Escherichia coli*. *Biochem Biophys Res Commun* **347**: 904–8.

Riedel, S. (2005) Edward Jenner and the history of smallpox and vaccination. *Proc (Bayl Univ Med Cent)* **18**: 21–5.

Robinson, J.H., and Delvig, A.A. (2002) Diversity in MHC class II antigen presentation. *Immunology* **105**: 252–62.

Ruan, X., Loyola, D.E., Marolda, C.L., Perez-Donoso, J.M., and Valvano, M.A. (2012) The WaaL O-antigen lipopolysaccharide ligase has features in common with metal ion-independent inverting glycosyltransferases. *Glycobiology* **22**: 288–99.

Samatey, F.A., Imada, K., Vonderviszt, F., Shirakihara, Y., and Namba, K. (2000) Crystallization of the F41 Fragment of Flagellin and Data Collection from Extremely Thin Crystals. *J Struct Biol* **132**: 106–111.

Sanchez, C.J., Kumar, N., Lizcano, A., Shivshankar, P., Dunning Hotopp, J.C., Jorgensen, J.H., Tettelin, H., and Orihuela, C.J. (2011) *Streptococcus pneumoniae* in biofilms are unable to cause invasive disease due to altered virulence determinant production. *PLoS One* **6**: e28738.

Santolaya, M.E., O'Ryan, M.L., Valenzuela, M.T., Prado, V., Vergara, R., Muñoz, A., Toneatto, D., Graña, G., Wang, H., Clemens, R., and Dull, P.M. (2012) Immunogenicity and tolerability of a multicomponent meningococcal serogroup B (4CMenB) vaccine in healthy adolescents in Chile: a phase 2b/3 randomised, observer-blind, placebo-controlled study. *Lancet (London, England)* **379**: 617–24.

Schedin-Weiss, S., Winblad, B., and Tjernberg, L.O. (2014) The role of protein glycosylation in Alzheimer disease. *FEBS J* **281**: 46–62.

Schirm, M., Arora, S.K., Verma, A., Vinogradov, E., Thibault, P., Ramphal, R., and Logan, S.M. (2004) Structural and Genetic Characterization of Glycosylation of Type a Flagellin in *Pseudomonas aeruginosa*. *J Bacteriol* **186**: 2523–2531.

Schneerson, R., Robbins, J.B., Barrera, O., Sutton, A., Habig, W.B., Hardegree, M.C., and Chaimovich, J. (1980) *Haemophilus influenzae* type B polysaccharide-protein conjugates: model for a new generation of capsular polysaccharide vaccines. *Prog Clin Biol Res* **47**: 77–94.

Schulz, B.L., Jen, F.E.C., Power, P.M., Jones, C.E., Fox, K.L., Ku, S.C., Blanchfield, J.T., and Jennings, M.P. (2013) Identification of bacterial protein O-oligosaccharyltransferases and their glycoprotein substrates. *PLoS One* **8**: e62768.

Scott, A.E., Twine, S.M., Fulton, K.M., Titball, R.W., Essex-Lopresti, A.E., Atkins, T.P., and Prior, J.L. (2011) Flagellar glycosylation in *Burkholderia pseudomallei* and *Burkholderia thailandensis*. *J Bacteriol* **193**: 3577–87.

Scott, N.E., Kinsella, R.L., Edwards, A.V.G., Larsen, M.R., Dutta, S., Saba, J., Foster, L.J., and Feldman, M.F. (2014) Diversity Within the O-linked Protein Glycosylation Systems of *Acinetobacter* Species. *Mol Cell Proteomics* **13**: 2354–70.

Shak, J.R., Vidal, J.E., and Klugman, K.P. (2013) Influence of bacterial interactions on pneumococcal colonization of the nasopharynx. *Trends Microbiol* **21**: 129–35.

Shapiro, E.D., Berg, A.T., Austrian, R., Schroeder, D., Parcells, V., Margolis, A., Adair, R.K., and Clemens, J.D. (1991) The protective efficacy of polyvalent pneumococcal polysaccharide vaccine. *N Engl J Med* **325**: 1453–60.

Sherlock, O., Dobrindt, U., Jensen, J.B., Munk Vejborg, R., and Klemm, P. (2006) Glycosylation of the Self-Recognizing *Escherichia coli* Ag43 Autotransporter Protein. *J Bacteriol* **188**: 1798–1807.

Siegrist, C.-A., and Aspinall, R. (2009) B-cell responses to vaccination at the extremes of age. *Nat Rev Immunol* **9**: 185–94.

Simell, B., Lahdenkari, M., Reunanen, A., Kayhty, H., and Vakevainen, M. (2008) Effects of Ageing and Gender on Naturally Acquired Antibodies to Pneumococcal Capsular Polysaccharides and Virulence-Associated Proteins. *Clin Vaccine Immunol* **15**: 1391–1397.

Sleytr, U.B. (1975) Heterologous reattachment of regular arrays of glycoproteins on bacterial surfaces. *Nature* **257**: 400–402.

Sleytr, U.B., and Thorne, K.J. (1976) Chemical characterization of the regularly arranged surface layers of *Clostridium thermosaccharolyticum* and *Clostridium thermohydrosulfuricum*. *J Bacteriol* **126**: 377–83.

Smedley, J.G., Jewell, E., Roguskie, J., Horzempa, J., Syboldt, A., Stolz, D.B., and Castric, P. (2005) Influence of pilin glycosylation on *Pseudomonas aeruginosa* 1244 pilus function. *Infect Immun* **73**: 7922–31.

Soininen, A. (1999) IgG subclass distribution of antibodies after vaccination of adults with pneumococcal conjugate vaccines. *Vaccine* **17**: 1889–1897.

Song, J.H., Lee, N.Y., Ichiyama, S., Yoshida, R., Hirakata, Y., Fu, W., Chongthaleong, A., Aswapokee, N., Chiu, C.H., Lalitha, M.K., Thomas, K., Perera, J., Yee, T.T., Jamal, F., Warsa, U.C., Vinh, B.X., Jacobs, M.R., Appelbaum, P.C., and Pai, C.H. (1999) Spread of drug-resistant *Streptococcus pneumoniae* in Asian countries: Asian Network for Surveillance of Resistant Pathogens (ANSORP) Study. *Clin Infect Dis* **28**: 1206–11.

Stephenson, H.N., Mills, D.C., Jones, H., Milioris, E., Copland, A., Dorrell, N., Wren, B.W., Crocker, P.R., Escors, D., and Bajaj-Elliott, M. (2014) Pseudaminic acid on *Campylobacter jejuni* flagella modulates dendritic cell IL-10 expression via Siglec-10 receptor: a novel flagellin-host interaction. *J Infect Dis* **210**: 1487–98.

Stimson, E., Virji, M., Makepeace, K., Dell, A., Morris, H.R., Payne, G., Saunders, J.R., Jennings, M.P., Barker, S., and Panico, M. (1995) Meningococcal pilin: a glycoprotein substituted with digalactosyl 2,4-diacetamido-2,4,6-trideoxyhexose. *Mol Microbiol* **17**: 1201–14.

Sung, H., Shin, H.B., Kim, M.-N., Lee, K., Kim, E.-C., Song, W., Jeong, S.H., Lee, W.-G., Park, Y.-J., and Eliopoulos, G.M. (2006) Vancomycin-Tolerant *Streptococcus pneumoniae* in Korea. *J Clin Microbiol* **44**: 3524–3528.

Sutherland, I.W. (2001) Microbial polysaccharides from Gram-negative bacteria. *Int Dairy J* **11**: 663–674.

Szymanski, C.M., Yao, R., Ewing, C.P., Trust, T.J., and Guerry, P. (1999) Evidence for a system of general protein glycosylation in *Campylobacter jejuni*. *Mol Microbiol* **32**: 1022–1030.

Tan, R.M., Kuang, Z., Hao, Y., Lee, F., Lee, T., Lee, R.J., and Lau, G.W. (2015) Type IV pilus glycosylation mediates resistance of *Pseudomonas aeruginosa* to opsonic activities of the pulmonary surfactant protein A. *Infect Immun* **83**: 1339–46.

Tang, G., and Mintz, K.P. (2010) Glycosylation of the collagen adhesin EmaA of Aggregatibacter actinomycetemcomitans is dependent upon the lipopolysaccharide biosynthetic pathway. *J Bacteriol* **192**: 1395–404.

Tang, G., Ruiz, T., and Mintz, K.P. (2012) O-Polysaccharide Glycosylation Is Required for Stability and Function of the Collagen Adhesin EmaA of Aggregatibacter actinomycetemcomitans. *Infect Immun* **80**: 2868–2877.

Tangye, S.G., Avery, D.T., Deenick, E.K., and Hodgkin, P.D. (2003) Intrinsic differences in the proliferation of naive and memory human B cells as a mechanism for enhanced secondary immune responses. *J Immunol* **170**: 686–94.

Tarlinton, D. (2006) B-cell memory: are subsets necessary? *Nat Rev Immunol* **6**: 785–90.

Terra, V.S., Mills, D.C., Yates, L.E., Abouelhadid, S., Cuccui, J., and Wren, B.W. (2012) Recent developments in bacterial protein glycan coupling technology and glycoconjugate vaccine design. *J Med Microbiol* **61**: 919–926.

Thibault, P., Logan, S.M., Kelly, J.F., Brisson, J.R., Ewing, C.P., Trust, T.J., and Guerry, P. (2001) Identification of the carbohydrate moieties and glycosylation motifs in *Campylobacter jejuni* flagellin. *J Biol Chem* **276**: 34862–70.

Timens, W., Boes, A., Rozeboom-Uiterwijk, T., and Poppema, S. (1989) Immaturity of the human splenic marginal zone in infancy. Possible contribution to the deficient infant immune response. *J Immunol* **143**: 3200–6.

Tzianabos, A., Onderdonk, A., Rosner, B., Cisneros, R., and Kasper, D. (1993) Structural features of polysaccharides that induce intra-abdominal abscesses. *Science (80-)* **262**: 416–419.

Uchida, T., Pappenheimer, A.M., and Greany, R. (1973) Diphtheria toxin and related proteins. I. Isolation and properties of mutant proteins serologically related to diphtheria toxin. *J Biol Chem* **248**: 3838–44.

Vaccine development – Vaccine Development Global Program - PATH http://sites.path.org/vaccinedevelopment/pneumonia-and-pneumococcus/vaccine-development/. Accessed October 6, 2015.

Vadesilho, C.F.M., Ferreira, D.M., Moreno, A.T., Chavez-Olortegui, C., Machado de Avila, R.A., Oliveira, M.L.S., Ho, P.L., and Miyaji, E.N. (2012) Characterization of the antibody response elicited by immunization with pneumococcal surface protein A (PspA) as recombinant protein or DNA vaccine and analysis of protection against an intranasal lethal challenge with *Streptococcus pneumoniae*. *Microb Pathog* **53**: 243–9.

Varki, A. (1993) Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* **3**: 97–130.

Velez, C.D., Lewis, C.J., Kasper, D.L., and Cobb, B.A. (2009) Type I *Streptococcus pneumoniae* carbohydrate utilizes a nitric oxide and MHC II-dependent pathway for antigen presentation. *Immunology* **127**: 73–82.

VFC | Current CDC Vaccine Price List | CDC http://www.cdc.gov/vaccines/programs/vfc/awardees/vaccine-management/price-list/. Accessed October 6, 2015.

Vik, A., Aas, F.E., Anonsen, J.H., Bilsborough, S., Schneider, A., Egge-Jacobsen, W., and Koomey, M. (2009) Broad spectrum O-linked protein glycosylation in the human pathogen *Neisseria gonorrhoeae*. *Proc Natl Acad Sci U S A* **106**: 4447–52.

Watson, D.A., Musher, D.M., Jacobson, J.W., and Verhoef, J. (1993) A Brief History of the Pneumococcus in Biomedical Research: A Panoply of Scientific Discovery. *Clin Infect Dis* **17**: 913–924.

Watson, D.A., Musher, D.M., and Verhoef, J. (1995) Pneumococcal virulence factors and host immune responses to them. *Eur J Clin Microbiol Infect Dis* **14**: 479–490.

Wei, Z., and Zhang, M. (2013) Decorating proteins with “sweets” is a flexible matter. *Structure* **21**: 1–2.

Weinberger, D.M., Malley, R., and Lipsitch, M. (2011) Serotype replacement in disease after pneumococcal vaccination. *Lancet (London, England)* **378**: 1962–73.

Wenger, J. (2001) Vaccines for the developing world: current status and future directions. *Vaccine* **19**: 1588–1591.

Whitfield, C. (2006) Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. *Annu Rev Biochem* **75**: 39–68.

WHO | Poliomyelitis

WHO | Statue commemorates smallpox eradication

WHO | Vaccines

Wroe, P.C., Lee, G.M., Finkelstein, J.A., Pelton, S.I., Hanage, W.P., Lipsitch, M., Stevenson, A.E., Rifas-Shiman, S.L., Kleinman, K., Dutta-Linn, M.M., Hinrichsen, V.L., Lakoma, M., and Huang, S.S. (2012) Pneumococcal Carriage and Antibiotic Resistance in Young Children Before 13-valent Conjugate Vaccine. *Pediatr Infect Dis J* **31**: 249–254.

Wykes, M., Pombo, A., Jenkins, C., and MacPherson, G.G. (1998) Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. *J Immunol* **161**: 1313–9.

Yan, Q., and Lennarz, W.J. (2002) Studies on the function of oligosaccharyl transferase subunits. Stt3p is directly involved in the glycosylation process. *J Biol Chem* **277**: 47692–700.

Yurist-Doutsch, S., Chaban, B., VanDyke, D.J., Jarrell, K.F., and Eichler, J. (2008) Sweet to the extreme: protein glycosylation in Archaea. *Mol Microbiol* **68**: 1079–84.

Yurist-Doutsch, S., Magidovich, H., Ventura, V. V, Hitchen, P.G., Dell, A., and Eichler, J. (2010) N-glycosylation in Archaea: on the coordinated actions of *Haloferax volcanii* AglF and AglM. *Mol Microbiol* **75**: 1047–58.

2. *Acinetobacter* strains carry two functional oligosaccharyltransferases, one devoted exclusively to type IV pilin, and the other one dedicated to *O*-glycosylation of multiple proteins.

Preface:

This chapter has been published as Harding CM, Nasr MA, Kinsella RL, Scott NE, Foster LJ, Weber BS, Fiester SE, Actis LA, Tracy EN, Munson RS, Jr., Feldman MF. 2015. *Acinetobacter* strains carry two functional oligosaccharyltransferases, one devoted exclusively to type IV pilin, and the other one dedicated to *O*-glycosylation of multiple proteins. Mol Microbiol, 96:1023-1041. In this work, *A. nosocomialis* experiments were carried out by Harding CM including Pilin and OmpA purification. Kinsella RL prepared and analyzed MS data for purified glycosylated OmpA and PilA. I have conducted all *A. baylyi* experiments under the supervision of Feldman MF. As well, I have performed *A. baylyi* total membrane purifications for proteomic analyses conducted by Scott N.E. and Foster L.J. I was responsible for manuscript preparation along with Harding CM, Kinsella RL, Scott NE, Feldman MF and Munson RS, Jr.

2.1 Introduction

Acinetobacter baumannii and *A. nosocomialis* are clinically relevant members of the *Acinetobacter calcoaceticus*- *A. baumannii* (*Acb*) complex and important opportunistic nosocomial pathogens (Wisplinghoff *et al*., 2012). These species have emerged as troublesome pathogens due in part to their remarkable resistance to disinfection, desiccation, as well as their ability to acquire multiple drug resistant phenotypes, all of which promote their survivability in the hospital setting. Furthermore, pan-resistant strains within the *Acb* are continuously being isolated from hospitals worldwide (Arroyo *et al*., 2009; Göttig *et al*., 2014). While the mechanisms of antibiotic resistance of *Acb* members has been intensively studied (Gordon *et al.*, 2010), our understanding of their virulence mechanisms is unclear. Identified virulence factors include an outer membrane protein A (OmpA), the ability to form biofilms, exopolysaccharide, lipopolysaccharide (LPS), protein glycosylation systems and capsule (Choi *et al*., 2008; Choi *et al.,* 2009; Gordon *et al*., 2010; Iwashkiw *et al*., 2012; Lees-Miller *et al*., 2013). A type VI secretion system (T6SS) has been also identified, although a role in pathogenesis has not been demonstrated (Carruthers *et al*., 2013; Weber *et al*., 2013).

Acinetobacter baylyi is a non-pathogenic member of the genus *Acinetobacter*, characterized by its genetic tractability and natural competence. For these properties, *A. baylyi* is widely used as a model organism for molecular and genetic studies of the genus *Acinetobacter* (Vaneechoutte *et al*., 2006; de Berardinis *et al*., 2008; Brzoska *et al*., 2013) and is also utilized in bioremediation (Abd-El-Haleem *et al*., 2002; Mara *et al*., 2012). All members of the *Acinetobacter* genus, independent of their pathogenicity, carry a protein glycosylation system (Jeremy A. Iwashkiw *et al.*, 2012).

Protein glycosylation, the covalent attachment of carbohydrate moieties to protein substrates, is the most abundant post-translational modification of proteins (Varki, 1993) and occurs in all domains of life (Neuberger, 1938; Sleytr, 1975; Mescher & Strominger, 1976). The major types of protein glycosylation are *N*- and *O*-glycosylation. Both processes can be classified as oligosaccharyltransferase (OTase)-dependent and OTase-independent (Nothaft & Szymanski, 2010; Iwashkiw *et al*., 2013). OTases are enzymes that catalyze the transfer of a glycan, previously assembled by cytoplasmic glycosyltransferases (GT) onto an undecaprenyl pyrophosphate lipid carrier, to target proteins. The development of sensitive analytical techniques has led to the identification of OTase-dependent protein glycosylation in numerous bacterial species. These include members of the genera *Campylobacter, Neisseria, Pseudomonas, Francisella, Vibrio, Burkholderia* and *Bacteroides* (Szymanski *et al*., 1999; Faridmoayer *et al*., 2007; Egge-Jacobsen *et al*., 2011; Balonova *et al*., 2012; Gebhart *et al*., 2012; Coyne *et al*., 2013; Lithgow *et al*., 2014). Glycosylation frequently affects protein stability, bacterial adhesion, flagellar filament assembly, biofilm formation, and virulence in general (Logan, 2006; Iwashkiw *et al*., 2013). An OTase-dependent, ubiquitous *O*-linked protein glycosylation system has been recently discovered within the genus *Acinetobacter*. This system was required for biofilm formation and pathogenicity of *A. baumannii* (Jeremy A. Iwashkiw *et al.*, 2012). The glycan structures for several strains of *A. baumannii* have also been characterized and extensive carbohydrate diversity has been established (Scott *et al.*, 2014).

OTases involved in *O*-glycosylation (*O*-OTases) do not share extensive primary amino acid sequence homologies; yet, all *O*-Otases contain domains from the Wzy_C

superfamily (Power and Jennings, 2003). Orthologs of PglL general *O*-Otases and WaaL O-antigen ligases are two of the most well characterized enzymes from the Wzy_C superfamily. It has proven challenging to identify *O*-OTases based solely on bioinformatic methodologies as *O*-OTases and WaaL ligases catalyze similar reactions, i.e. the transfer of lipid-linked glycans to acceptor proteins or lipid A respectively (Hug and Feldman, 2011). The two enzymes appear to be evolutionarily and mechanistically related as mutagenesis of topologically similar conserved histidine residues of the *E. coli* O-antigen ligase (H337) and *N. meningitidis O*-OTase (H349) results in the loss of glycan transfer activities (Pérez *et al.*, 2008; Ruan *et al*., 2012; Musumeci *et al., 2014*). Recently, the PglL_A and PglL_B hidden Markov models (HMM) were defined to better resolve orthologs of PglL *O*-OTases from other enzymes of the Wzy_C superfamily (Power *et al.*, 2006; Schulz *et al*., 2013).

O-OTases are often encoded downstream of their cognate target protein. This genetic arrangement is often found in Gram-negative organisms encoding type IV pili (Tfp) systems, where the major pilin subunit gene is immediately 5' of the cognate OTase gene (Schulz *et al*., 2013). For example, in *P. aeruginosa* strain 1244 the major pilin, PilA, is glycosylated by PilO (later renamed TfpO), an *O*-OTase encoded immediately downstream of *pilA* (Castric, 1995; Kus *et al*., 2004). This modification is believed to play a role in virulence as glycosylation-deficient mutants showed decreased twitching motility and were out-competed by the wild type in a mouse respiratory infection model (Kus *et al.*, 2004; Smedley *et al.*, 2005). The same genetic arrangement and glycosylation phenotype has also been found in *P. syringae* (Nguyen *et al.*, 2012).

Pilin post-translational modification has also been identified in *Acinetobacter* species. In *A. baylyi* ADP1, two Wzy_C superfamily domain-containing proteins are encoded in the genome. One gene is found immediately downstream of the gene encoding the pilin-like protein ComP, whereas the other gene is found within a distant glycan biosynthesis gene cluster. Mutation of the predicted OTase encoded downstream of the *comP* gene affected the electrophoretic mobility of ComP, indicating this gene may encode for a ComP-specific OTase (Porstendorfer *et al*., 2000; Schulz *et al*., 2013). Additionally, during the course of our previous study demonstrating the functional production of Tfp by the medically relevant *A. nosocomialis* strain M2, we also identified two molecular forms of PilA differing by apparent molecular weight leading us to hypothesize that the pilins of *Acb* members may also be post-translationally modified (Harding *et al*., 2013; Carruthers *et al*., 2013)

A genomic analysis of sequenced genomes of *Acinetobacter* spp. revealed that, in addition to *A. baylyi* ADP1, multiple strains within the genus *Acinetobacter* encode two OTases. In this work we employed genetic and proteomic techniques to demonstrate that both OTases are functional and that one of these enzymes acted as a pilin-specific OTase, whereas the other OTase was able to glycosylate a wide range of proteins. In addition, using mass spectrometry, we characterized the glycan structure of *A. nosocomialis* strain M2 and defined the glycoproteome of *A. baylyi*.

2.2 Materials and Methods

2.2.1 Strains, plasmids, and growth conditions

A list of bacterial strains and plasmids can be found in appendix A. All bacteria were grown on L-agar or in LB-broth at 37°C unless otherwise noted. When appropriate, antibiotics were added to the *A. nosocomialis* or *A. baumannii* cultures at the following concentrations except when noted otherwise: 100 µg ampicillin/mL, 20 µg kanamycin/mL, or 12.5 µg chloramphenicol/mL. When appropriate, *E. coli* cultures were supplemented with antibiotics at the following concentrations: 50 µg ampicillin/mL for *E. coli* strains containing plasmids other than pGEM derivatives, 100 µg ampicillin/mL for *E. coli* strains containing pGEM derivatives, or 20 µg kanamycin/mL. *R. solanacearum* was grown at 30˚C in Boucher gelose (BG) media (Boucher *et al*.,1985).

2.2.2 Bioinformatic analysis of *Acinetobacter* OTases

Protein sequences for *Acinetobacter* specific OTases were analyzed using NCBI's Basic Local Alignment Search Tool (BLAST) and protein domains identified using the Conserved Domain Database for the annotation of proteins (Marchler-Bauer *et al.,* 2004, Marchler-Bauer *et al*., 2009, Marchler-Bauer *et al*., 2011).

2.2.3 Plasmid, mutant, and complement construction

Methodologies for the generation of all plasmid constructs, mutants, and complemented mutants can be found in appendix A.

2.2.4 Truncated recombinant, soluble PilA (rsPilAM2) purification and generation of Anti-rsPilA antisera

Methodologies for the purification of rsPilA and the generation of anti-rsPilA antisera can be found in appendix A.

2.2.5 Western blot analyses

Western blot analyses were performed according to our previously described methodologies (Harding *et al*., 2013). Primary antibodies used were Anti-rsPilA_{M2} or Penta-His Antibody (Qiagen). Secondary antibodies used were Goat anti-rabbit IgG (H+L), alkaline phosphatase antibody (Molecular Probes) and Goat-anti-mouse IgG (H+L), alkaline phosphatase antibody (Molecular Probes). Membranes were developed with the BCIP/NBT Liquid Substrate System (Sigma).

2.2.6 Pili Shear Preparations

Pili shear preparations were prepared as previously described with the following modifications. Briefly, bacterial lawns were removed from the agar surface and resuspended in 5 mL of ice cold DPBS supplemented with 1X protease inhibitors (Roche). The bacterial suspensions were normalized to an optical density at A_{600nm} equal to 70. To shear surface exposed proteins, bacterial suspensions were vortexed on high for 1 minute. Bacteria were pelleted at 10,000 x *g* for 10 minutes at 4°C. The supernatants were collected and again centrifuged at 10,000 x *g* for 10 minutes at 4°C. The supernatants were collected and further clarified by centrifugation at 20,000 x *g* for 5 mins at 4°C. The sheared surface proteins were precipitated with ammonium sulfate at a

final concentration of 30%. Precipitated proteins were pelleted by centrifugation at 20,000 x *g* for 10 minutes at 4°C. The supernatants were discarded and the pellets were resuspended in 100 µL of 1X Laemmli buffer. Preparations were boiled for 10 minutes, run on SDS-PAGE, coomassie-stained, and bands were excised and prepared for mass spectrometric analysis according to Shevchenko *et al.* (2006). Briefly, bands were washed with water and dehydrated with acetonitrile (ACN) repeatedly. Disulfide bonds were reduced with 10mM DTT in 50mM NH_4HCO_3 for 60 minutes at 37°C followed by alkylation of cysteine thiol groups with 50mM iodoacetamide in 50mM NH_4HCO_3 for 60 minutes in the dark at room temperature. Gel pieces were then washed with 50mM NH_4HCO_3, dehydrated with 100% ACN and dried. Pilin was digested with 0.02mg/mL trypsin in 50mM NH_4HCO_3 (Promega) at 37°C for 16 hours. Peptides were eluted with 100% ACN and water and lyophilized. Tryptic peptides were resuspended in 0.1% Trifluoroacetic acid and desalted with a C18 ZipTip (Millipore, USA). 60% ACN was used to elute the peptides, which were dried in a speedvac and resuspended with 0.1% Formic Acid. The analysis was done using a Q-TOF Premier (Waters, Manchester, UK) coupled to a nanoACQUITY (Waters) ultra-performance liquid chromatography system as previously described (Wang *et al*., 2007). MassLynx, v. 4.1 (Waters) was used to analyze the data. OmpA-His was purified from strain M2 and prepared for ESI-QTOF-MS/MS analysis as described above.

2.2.7 LPS extraction and silver staining

LPS from *A. baylyi* and *R. solanacearum* was extracted from overnight cultures by the TRI-reagent method as described previously (Yi and Hackett, 2000). Equal amounts of

LPS were loaded on 12.5% SDS-PAGE gels for LPS separation followed by silver staining as previously described (Tsai & Frasch, 1982).

2.2.8 DsbA1 glycosylation in *E. coli*

E. coli CLM24 cells were co-transformed with three plasmids: one plasmid encoding the *C. jejuni* glycosylation locus, another plasmid encoding a single *O*-OTase gene, and the last plasmid, pAMF22, encoding *dsbA1-His*. Ampicillin (100 μg/ml), trimethoprim (50 μg/ml) and chloramphenicol (10 μg/ml) were added as required for plasmid selection. Cells were grown at 37°C to an OD_{600} of 0.4-0.6 and then were induced with 0.1 mM IPTG and/or 0.2% arabinose. Cultures requiring arabinose induction were given a second dose of induction after 4 hours. Whole cell lysates were obtained at stationary phases and western blot analyses were employed to determine DsbA1 modification.

2.2.9 Digestion of membrane enriched samples of *A. baylyi* ADP1

Peptide lysates for glycopeptide enrichment and quantitative analysis were prepared according to Lithgow *et al*. (2014) with minor modifications. Cells grown overnight at 37°C were washed with phosphate buffered saline (PBS) buffer and resuspended in the same buffer. Cells were lysed by two rounds of cell disruption using a French press (Constant systems Ltd) followed by the addition of a protease inhibitor cocktail (Roche). Lysates were centrifuged for 30 minutes at 10000 x g to get rid of cell debris and supernatants were then ultracentrifuged at 100000 x g for 60 minutes to pellet total membranes followed by lyophilization. Subsequently, 2 mg of lyophilized membrane enriched protein samples were solubilized in 6 M urea, 2 M thiourea, 40 mM NH_4HCO_3

and reduced with 10mM Dithiothreitol (DTT). Reduced, solubilized peptides were alkylated with 25mM iodoacetamide (IAA) for one hour in the absence of light. The resulting alkylated protein mixture was digested with Lys-C (1/100 w/w) for 4 hours at 25°C, diluted 1:5 in 40 mM NH_4HCO_3, then digested with trypsin (1/50 w/w) overnight at 25°C. Digestion was terminated with the addition of 1% trifluoroacetic acid (TFA). Peptide digests were purified using the C_{18} empore (Sigma-Aldrich, St. Louis MO) STop And Go Extraction (STAGE) tips (Rappsilber *et al*., 2007) to remove primary amide and salts.

2.2.10 Enrichment of *A. baylyi* ADP1 glycopeptides using ZIC-HILIC purification

ZIC-HILIC (Zwitterionic hydrophilic interaction liquid chromatography) enrichment was performed according to Scott *et al*., 2011 with minor modifications. Micro-columns composed of 10 µm ZIC-HILIC resin (Sequant, Umeå, Sweden) packed into p10 tips containing a 1 mm^2 excised C_8 Empore™ 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% acetonitrile (ACN), and then equilibrated with 80% ACN and 5% formic acid (FA). Samples were resuspended in 80% ACN and 5% FA and insoluble material was removed by centrifugation at 16,100 × *g* for 5 min at 4ºC. Samples were adjusted to a concentration of 3 µg/µL and 150 µg of peptide material was loaded onto a column and washed with 10 load volumes of 80% ACN, 5% FA. Unbound fractions were collected, pooled, and dried by vacuum centrifugation. ZIC-HILIC bound peptides were eluted with 3 load volumes of ultra-pure water and concentrated using vacuum centrifugation.

Biological replicates were subjected to ZIC-HILIC enrichment independently using freshly prepared reagents.

2.2.11 Identification of glycopeptides using reversed-phase LC-MS, CID MS-MS and HCD MS-MS

Purified glycopeptides/peptides were resuspended in Buffer A (0.5% acetic acid) and separated using reversed-phase chromatography on either an Agilent 1290 Series HPLC (Agilent Technologies, Mississauga, ON) coupled to LTQ-Orbitrap Velos (Thermo Scientific, San Jose CA) for qualitative analysis of glycopeptides or an EASY-nLC1000 system coupled to a Q-exactive for quantitative studies. For qualitative analysis of *A. baylyi* ADP1 glycopeptides, a packed in-house 20 cm, 75 µm inner diameter, 360 µm outer diameter, ReproSil – Pur C_{18} AQ 1.9µm (Dr. Maisch, Ammerbuch-Entringen, Germany) column was used. For quantitative studies a house packaged 45 cm, 50 µm inner diameter, 360 µm outer diameter, ReproSil – Pur C_{18} AQ 1.9µm column was used. In both systems, samples were loaded onto a trap column, an in-house packed 2 cm, 100 µm inner diameter, 360 µm outer diameter column containing Aqua 5 µm C18 (Phenomenex, Torrance, CA), at 5 µL/min prior to gradient separation and infused for mass spectrometry. A 180 min gradient was run from 0% buffer B (80% ACN, 0.5% acetic acid) to 32% B over 140 min, next from 32% B to 40% B in the next 5 min, then increased to 100% B over 2.5 min period, held at 100% B for 2.5 min, and then dropped to 0% B for another 20 min. Unbound fractions from ZIC-HILIC glycopeptide enrichment were subjected to analysis using the same instrumental set up as qualitative analysis of glycopeptides. Both instruments were operated using Xcalibur v2.2 (Thermo

Scientific) with a capillary temperature of 275°C in a data-dependent mode automatically switching between MS, CID MS-MS and HCD MS-MS for qualitative analysis as previously described (Nichollas E Scott *et al.*, 2011) and using a top 10 data-dependent approach switching between MS (resolution 70k, AGC target of $1x10^6$), and HCD MS-MS events (resolution 17.5k AGC target of $1x10^6$ with a maximum injection time of 60ms, NCE 28 with 20% stepping) for quantitative studies.

2.2.12 Identification of *A. baylyi* ADP1 glycopeptides

Raw files for qualitative glycosylation analysis were processed as previously described (Scott *et al*., 2011, 2012). Briefly, Proteome Discoverer v. 1.2 (Thermo Scientific) was used to search the resulting glycopeptide data using MASCOT v2.4 against the *A. baylyi* ADP1 database (obtained from UNIPROT, http://www.uniprot.org/, 2014-06-10, Taxon identifier: 62977 containing 3263 protein sequences). Mascot searches were preformed using the following parameters: peptide mass accuracy 20 ppm; fragment mass accuracy 0.02 Da; no enzyme specificity, fixed modifications - carbamidomethyl, variable modifications - methionine oxidation and deamidated N, Q. The instrument setting of MALDI-QUAD-TOF was chosen as previous studies show quadrupole–like fragmentation within HCD spectra (Olsen *et al.*, 2007). Scan events that did not result in peptide identifications from MASCOT searches were exported to Microsoft Excel (Microsoft, Redmond WA). To identify possible glycopeptides within exported non-match scans, the MS-MS module of GPMAW 8.2 called 'mgf graph' was used to identify HCD scan events that contained the 204.08 *m/z* oxonium of HexNAc. All scan events containing the oxonium 204.08 *m/z* ion were manually inspected to identify possible

glycopeptides. To facilitate glycopeptide assignments HCD scan events containing the 204.08 oxonium were manual inspected to identify potential deglycosylated peptides ions. Within these HCD scans the MS features (*m/z*, charge and intensity), which corresponded to masses below that of the deglycosylated peptide were extracted using the Spectrum list function of Xcalibur v2.2. The resulting numerical values of the detected MS features were scripted into mgf files and the peptide mass set to that of the deglycosylated peptide mass. The resulting mgf files were then searched using the MASCOT setting described above. All spectra were searched with the decoy option enabled and no matches to this database were detected; the false discovery rate (FDR) was 0%.

Quantitative analysis of dimethylated *A. baylyi* ADP1 and mutant glycopeptides was preformed as previously reported (Lithgow *et al.*, 2014). Briefly, dimethylated *A. baylyi* ADP1 glycopeptides were identified as above and quantified by manually extracting the area under the curve of the monoisotopic peak using Xcalibur v2.2. Triplex (wild type ADP1 vs ADP1 $\Delta pglL_{ComP}$ vs ADP1$\Delta pglL_{ADP1}$).

2.3 Results

2.3.1 The two OTase homologs encoded by *Acinetobacter* contain different pfam domains

It was previously reported that *A. baylyi* ADP1 encodes two proteins containing domains from the Wzy_C superfamily (Schulz *et al.*, 2013). We wondered if other strains within the *Acinetobacter* genus encoded two Wzy_C domain-containing proteins. Through bioinformatic analyses we identified several *Acinetobacter* spp. with two open reading frames (ORFs) immediately downstream of the major type IVa pilin subunit *pilA* (Figure 2.1, panel A) that are predicted to encode proteins that contain evolutionarily related domains from the Wzy_C superfamily.

In *A. nosocomialis* strain M2, the *pilA* gene is immediately upstream of two genes, M215_10480 and M215_10475, both of which encode members of the Wzy_C superfamily. M215_10480 and M215_10475 contain the pfam13425 and the pfam04932 domains, respectively (Figure 2.1, panels A and B). At the time of this study, the same genetic arrangement was found in 12 of the 17 completed genomes for *A. baumannii* strains, 7 of 8 *A. nosocomialis* genomes, and 3 of 5 *A. pittii* genomes demonstrating the conservation of this locus amongst medically relevant members of the *Acb* complex (Data not shown). Previously we designated the gene encoding the pfam13425 domain-containing protein (ORF M215_10480) as the **p**utative **g**l**y**cosylase **A** (*pgyA*) (Harding *et al.*, 2013). Given that the gene encoding M215_10480 is immediately downstream of *pilA,* together with the functional data provided in this paper which demonstrates that this

protein is a pilin glycosylase, we have renamed the gene encoding ORF M215_10480 as a **t**ype **f**our **p**ilin specific *O*-**O**ligosaccharyltransferase gene (***tfpO***).

The second ORF, M215_10475, encodes a predicted protein that contains a domain from the pfam04932 family, a domain that has been found in all previously characterized PglL orthologs as well as in O-antigen ligases. The PglL_A and the PglL_B domains were also identified in M215_10475. Together with data presented in this paper, we conclude that this protein is an ortholog of the PglL general OTases; thus, we have designated ORF M215_10475 as $pglL_{M2}$ (Figure 2.1, panels A and B).

In the *A. baylyi* ADP1 genome, the *comP* gene, encoding a pilin-like protein, is followed by ACIAD3337 encoding a pfam04932-containing OTase-like protein, which was designated *pglL* by Schulz *et al*., (2013). In order to avoid confusion, we have designated ACIAD3337 as $pglL_{ComP}$ due to its proximity to *comP* (Figure 2.1, panels C and D) and the previously reported evidence demonstrating its requirement for post-translational modification of ComP (Schulz *et al.*, 2013).

A second pfam13425 domain containing ORF (ACIAD0103) predicted to encode a WaaL ligase ortholog was also identified. ACIAD0103 was not located near the pilin gene homolog, but instead was found within a glycan biosynthetic locus. We have designated ACIAD0103 as the $pglL_{ADP1}$ (Figure 2.1, panels C and D). *A. baylyi* was the only strain containing two genes encoding proteins with domains from the Wzy_C superfamily that were not encoded by adjacent genes.

In *A. baumannii* ATCC 17978, a well-studied strain with respect to its glycosylation, only one general *O*-OTase was identified, which was previously designated PglL (Jeremy A. Iwashkiw *et al.*, 2012; Lees-Miller *et al.*, 2013). The novelty of two putative *O*-OTase

homologs encoded by most *Acinetobacter* strains prompted us to investigate their function and protein specificity.

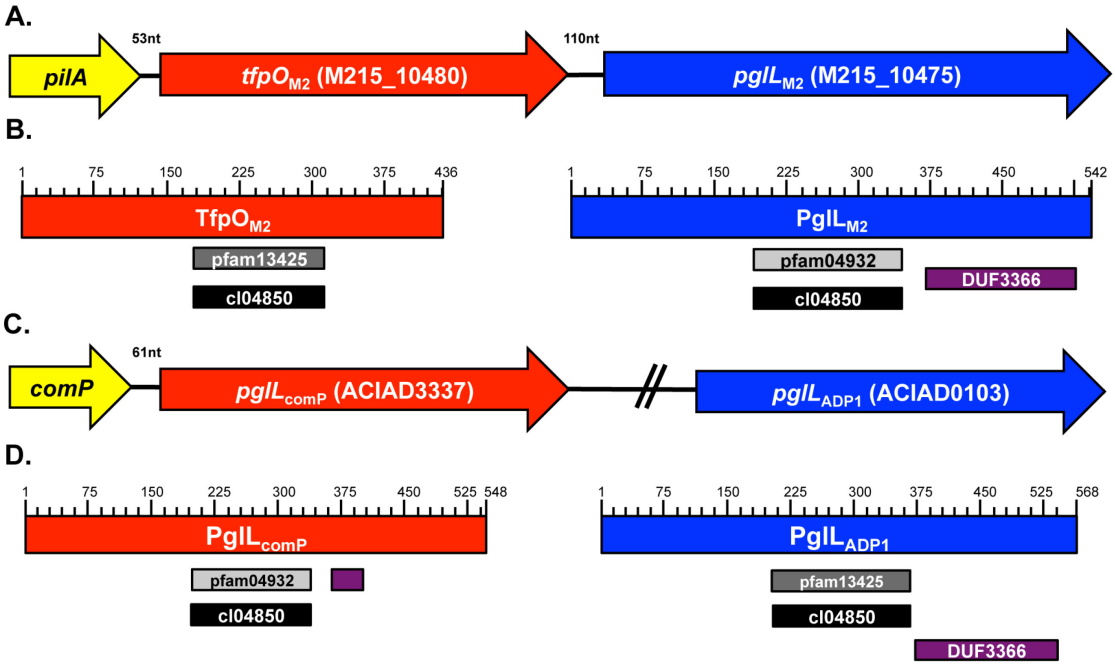

Figure 2.1 Genomic and domain organization of putative *O*-OTases of *Acinetobacter* spp. encoding two OTase genes. (A) Genomic context of OTases encoded by the *A. nosocomialis* strain M2 chromosome. (B) Wzy_C super family (cl04850) and DUF3366 domains present in $TfpO_{M2}$ and $PglL_{M2}$. (C) Genomic context of OTases encoded by the *A. baylyi* ADP1 chromosome. (D) Wzy_C super family (cl04850) and DUF3366 domains present in $PglL_{ComP}$ and $PglL_{ADP1}$. The purple rectangle below $PglL_{ComP}$ indicates a portion of the DUF3366 domain.

2.3.2 TfpO is required for post-translational modification of pilin in *A. nosocomialis* strain M2

Western blot analysis of whole cell lysates from strain M2, the isogenic *pilA* mutant, and the complemented *pilA* mutant strain confirmed our previous findings that PilA existed in two molecular forms differing by apparent molecular weight (Figure 2.2A). The more abundant, higher molecular weight form of PilA was likely a post-translationally modified species of PilA while the lower molecular weight form of PilA was an unmodified species. To determine the effects of TfpO on PilA post-translational modification, we constructed an isogenic *tfpO* mutant and probed for PilA expression. PilA from the strain lacking *tfpO* existed only in the lower molecular weight form (Figure 2.2A). The increase in PilA's electrophoretic mobility is consistent with the loss of a post-translational modification. Furthermore, PilA from the complemented *tfpO* mutant strain existed primarily in the higher molecular weight form confirming that TfpO was required for post-translational modification of PilA.

Immediately downstream of *tfpO* in strain M2 is $pglL_{M2}$, which encodes a homolog of the general *O*-OTases responsible for glycosylation of many membrane-associated proteins in *Neisseria gonorrhoeae* and *N. meningiditis* (Vik *et al*., 2009; Børud *et al*., 2011). To determine if $PglL_{M2}$ also played a role in post-translational modification of PilA, we generated an isogenic mutant strain lacking $pglL_{M2}$. Western blot analysis of whole cell lysates from the $pglL_{M2}$ mutant demonstrated that PilA existed primarily in the modified form, indicating that $PglL_{M2}$ is not required for the post-translational modification of PilA we observed (Figure 2.2A).

2.3.3 $PilA_{M2}$ was glycosylated in a $TfpO_{M2}$-dependent manner with a tetrasaccharide containing $(HexNAc)_2$, hexose and *N*-acetyl-deoxy hexose

To confirm that PilA was glycosylated by TfpO, PilA was purified from surface shear preparations from strain M2, a hyper-piliated M2Δ*pilT* mutant, and a hyper-piliated M2Δ*tfpO*::kanΔ*pilT*::strep mutant. The *pilT* gene encodes for the predicted retraction ATPase; therefore, mutants lacking *pilT* have a hyper-piliated phenotype, which results in an abundance of surface exposed PilA. Proteins in the shear preparations were separated by SDS-PAGE, coomassie-stained, excised and subjected to mass spectrometric analysis. MS/MS analysis of PilA from both strains M2 and M2Δ *pilT* identified the presence of a tetrasaccharide, comprised of two HexNAc residues, a Hexose and *N*-acetyl-deoxyHexose, on PilA (Figure 2.2B). MS/MS analysis revealed that the tetrasaccharide was present on the carboxy-terminal tryptic $_{119}$NSGTDTPVELLPQSFVAS$_{136}$ peptide. PilA from the M2Δ *tfpO*::kanΔ*pilT*::strep mutant was unmodified confirming that TfpO was required for PilA glycosylation (data not shown).

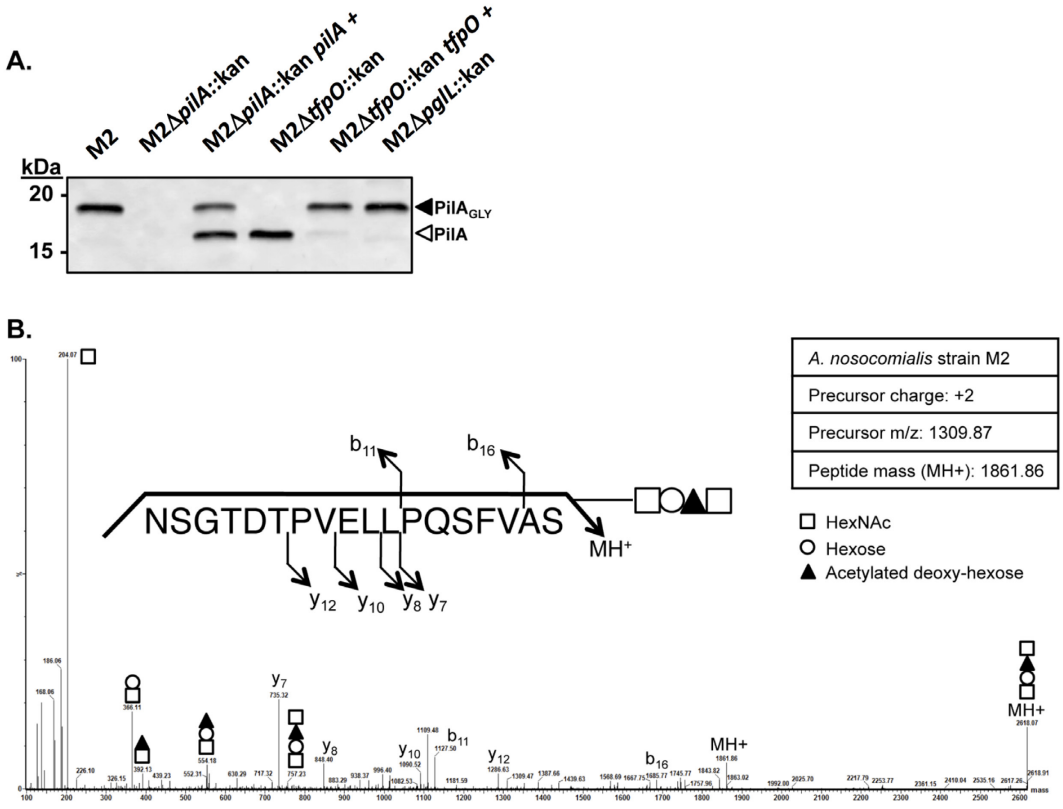

Figure 2.2 $PilA_{M2}$ was glycosylated in a TfpO-dependent manner with a tetrasaccharide containing $(HexNAc)_2$, Hexose and *N*-acetyl-deoxyHexose. (A) Surface proteins from the indicated strains were prepared by shearing, as described in the materials and methods, followed by separation by SDS-PAGE and western blot analysis of whole cell lysates. $PilA_{M2}$ from strain M2 was identified employing rabbit anti-$PilA_{M2}$. $PilA_{M2}$ from the M2Δ*tfpO*::kan mutant existed only as a lower molecular form indicating TfpO was required for $PilA_{M2}$ post-translational modification. Strains M2 *pilA*+ and M2 *tfpO*+ were complemented *pilA* and *tfpO* mutants, respectively. (B) $PilA_{M2}$ was sheared from the surface of strain M2 and a hyper-piliated mutant, precipitated, separated by SDS-PAGE, and visualized by Coomassie staining. Bands associated with $PilA_{M2}$ were excised and tryptically digested for MS/MS analysis.

2.3.4 The carboxy-terminal serine$_{136}$ of PilA$_{M2}$ was required for pilin modification

In *P. aeruginosa* 1244 the pilin protein PilA is glycosylated in a TfpO-dependent manner (Castric, 1995). The glycosylation site was later determined to be at the carboxy-terminal serine 148 (Comer *et al*., 2002). Amino acid sequences of PilA proteins from *Acinetobacter* spp., including *A. nosocomialis* M2, were compared to the *P. aeruginosa* 1244 PilA. Although the sequences share limited homology, strain M2's PilA sequence also contains a C-terminal serine, which was included in the glycopeptide identified by MS (Figure 2.2B). In fact, all *Acinetobacter* spp. containing two consecutive genes encoding *O*-OTase homologs contain a carboxy-terminal serine in their respective PilA amino acid sequences (Figure 2.3A).

In order to determine if the carboxy-terminal serine$_{136}$ was required for PilA post-translational modification we generated the M2(*pilA*[S136A])+ strain. First, we generated a strain with an in-frame deletion of the *pilA* gene so as to not affect the transcription of the downstream *tfpO* gene. We then complemented the M2Δ *pilA* strain with an allele of *pilA* where the carboxy-terminal serine was mutated to an alanine residue generating an M2(*pilA*[S136A])+ strain. Western blot analysis of whole cell lysates from the M2(*pilA*[S136A])+ strain demonstrated that PilA only existed in the unmodified, lower molecular weight form indicating that the carboxy-terminal serine was required for PilA post-translational modification (Figure 2.3B). Another highly conserved serine was found at position 132. We constructed the M2(*pilA*[S132A])+ strain in order to determine if this site was also required for glycosylation. Western blot analysis of whole cell lysates from the M2(*pilA*[S132A])+ strain demonstrated that PilA existed in the modified form indicating that serine 132 was not required for glycosylation (Figure 2.3B). The carboxy-

terminal serine to alanine point mutation did not affect Tfp functionality as the M2(*pilA*[S136A])+ strain was naturally transformable (Figure 2.3C).

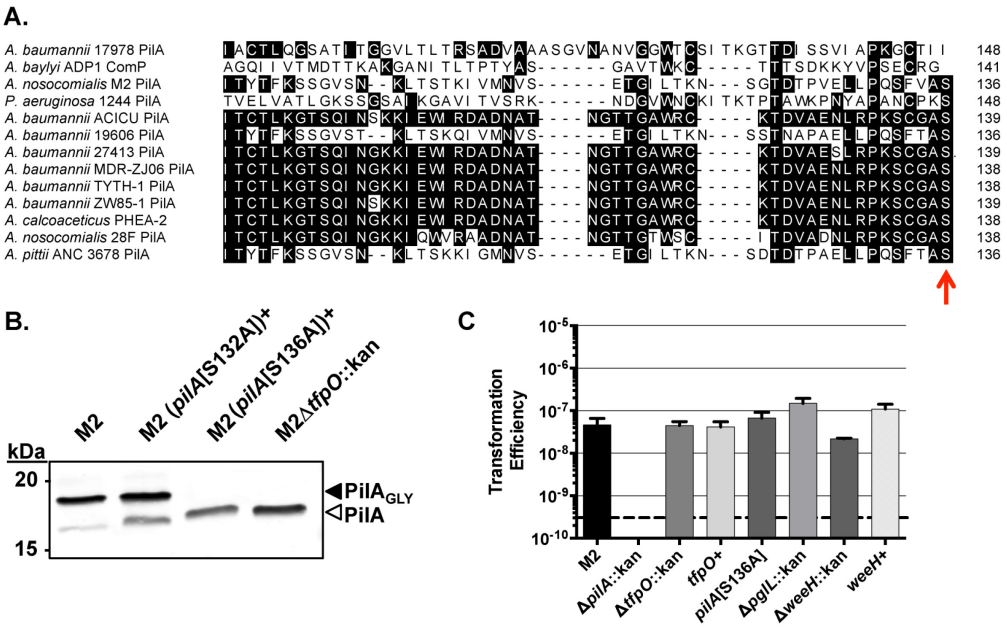

Figure 2.3 PilA$_{M2}$-like glycosylation was dependent on a conserved carboxy-terminal serine. (A) Alignment of the carboxy terminal region of PilA proteins from *P. aeruginosa* strain 1244 and selected *Acinetobacter* strains. All *Acinetobacter* strains encoding *tfpO* homologs contain a carboxy-terminal serine on their respective PilA proteins. (B) Western blot analysis of whole cell extracts probing for PilA$_{M2}$ expression and electrophoretic mobility. Strain M2 derivatives expressing PilA[S132A] and PilA[S136A] were constructed and extracts chararacterized. Serine 132 was not required for glycosylation while serine 136, the C-terminal serine was required for glycosylation. (C) Pilin glycosylation in strain M2 is not required for natural transformation. Mutants that were unable to glycosylate PilA$_{M2}$ were still naturally transformable.

2.3.5 The Major Polysaccharide Antigen (MPA) Locus is required for post-translational modification of $PilA_{M2}$

Hu *et al*. recently developed a molecular serotyping scheme for *Acinetobacter* spp. containing a major polysaccharide antigen (MPA) locus. The MPA Locus, found between the conserved *fkpA* and *lldP* genes, was identified in all sequenced *Acinetobacter* strains included in their study and was also present in *A. nosocomialis* strain M2 (Hu *et al*., 2013, Carruthers *et al*., 2013).

The MPA Locus from *A. nosocomialis* strain M2 contains three predicted glycosyl-transferases (designated *wafY*, *wafZ*, and *wagB*) and one predicted initiating glycosyl-transferase (designated *weeH* or *pglC*) (Figure 2.4A). To determine if the MPA Locus was required for post-translational modification of $PilA_{M2}$, we constructed individual isogenic mutants lacking each of the predicted glycosyltransferases. Western blot analysis of whole cell lysates from the strain lacking *weeH* demonstrated that PilA existed in the lower molecular weight form indicating that WeeH is required for glycosylation of PilA (Figure 2.4B). Deletion of the other three glycosyltransferases yielded PilA proteins with intermediate electrophoretic mobilities. PilA from the *wafY*::kan mutant migrated closest to the WT PilA mobility, then PilA from the *wafZ*::kan mutant, followed by PilA from the *wagB*::kan mutant (Figure 2.4B). Interestingly, both partially modified and unmodified forms of PilA were identified from the *wafZ*::kan and *wagB*::kan mutant backgrounds. All mutant strains were successfully complemented, indicating that the products of *wafY*, *wafZ*, *wagB*, and *weeH* genes were all required to produce fully modified PilA.

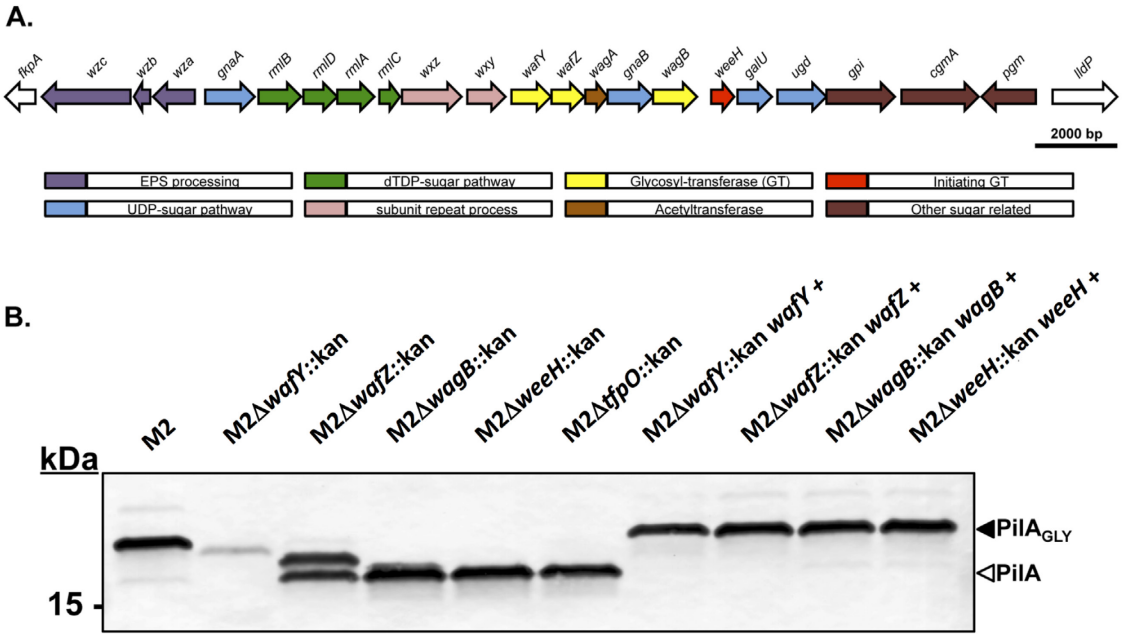

Figure 2.4 The major polysaccharide antigen locus (MPA) was required for pilin glycosylation. (A) Genetic organization of the strain M2 MPA Locus which is located between the conserved *fkpA* and *lldP* genes. Adapted from Hu *et al*., 2013. (B) Western blot analysis of whole cell extracts probing for $PilA_{M2}$ expression and electrophoretic mobility from MPA Locus mutants. $PilA_{M2}$ from the Δ*weeH*::kan mutant ran at the same electrophoretic mobility as $PilA_{M2}$ from the *tfpO*::kan mutant indicating it was not glycosylated. Deletion of the other three glycosyltransferases yielded $PilA_{M2}$ proteins with intermediate electrophoretic mobilities. PilA from the *wafY*::kan mutant migrated closest to the WT PilA mobility, then PilA from the *wafZ*::kan mutant, followed by PilA from the *wagB*::kan mutant. Mutants that were complemented all glycosylated $PilA_{M2}$.

2.3.6 *pglL*$_{M2}$ encodes a PglL-like *O*-OTase in *A. nosocomialis* strain M2 and uses the same tetrasaccharide precursor as a donor for general protein glycosylation

In figure 2.2A we showed that *pglL*$_{M2}$, the second ORF containing the Wzy_C domain, was not required for pilin glycosylation. We hypothesized that *pglL*$_{M2}$ could be a general *O*-OTase that, like the previously characterized PglL in *A. baumannii* ATCC 17978, could glycosylate non-pilin target proteins. We recently demonstrated that A1S_1193-His, encoding for the protein OmpA from *A. baumannii* ATCC 17978, could serve as a bait acceptor protein in order to isolate and identify *Acinetobacter* strain specific glycans, as it is recognized by PglLs from different strains (Scott *et al.*, 2014). We expressed OmpA-His, containing a carboxy terminal His-tag, in strains M2, ΔM2 *tfpO*::kan, M2Δ*pglL::*kan, and M2Δ *weeH*::kan. Western blot analysis demonstrated that all four strains expressed OmpA-His; however, OmpA-His from the M2Δ*pglL::*kan and the M2Δ*weeH*::kan backgrounds migrated at an increased electrophoretic mobility, consistent with the lack of a post-translational modification (Figure 2.5A). ESI-TOF-MS/MS analysis of OmpA-His purified from strain M2 revealed glycosylation with two subunits of a branched tetrasaccharide. These results indicated that M215_10475 is a general *O*-OTase providing functional evidence for the PglL$_{M2}$ designation. Furthermore, this branched tetrasaccharide was the same tetrasaccharide found on PilA, indicating that TfpO$_{M2}$ and PglL$_{M2}$ both utilize the same lipid-linked glycan precursor as the substrate for protein glycosylation (Figure 2.5B). This observation was expected given that WeeH was required for both PilA and OmpA-His post-translational modification, indicating a common glycan precursor pathway

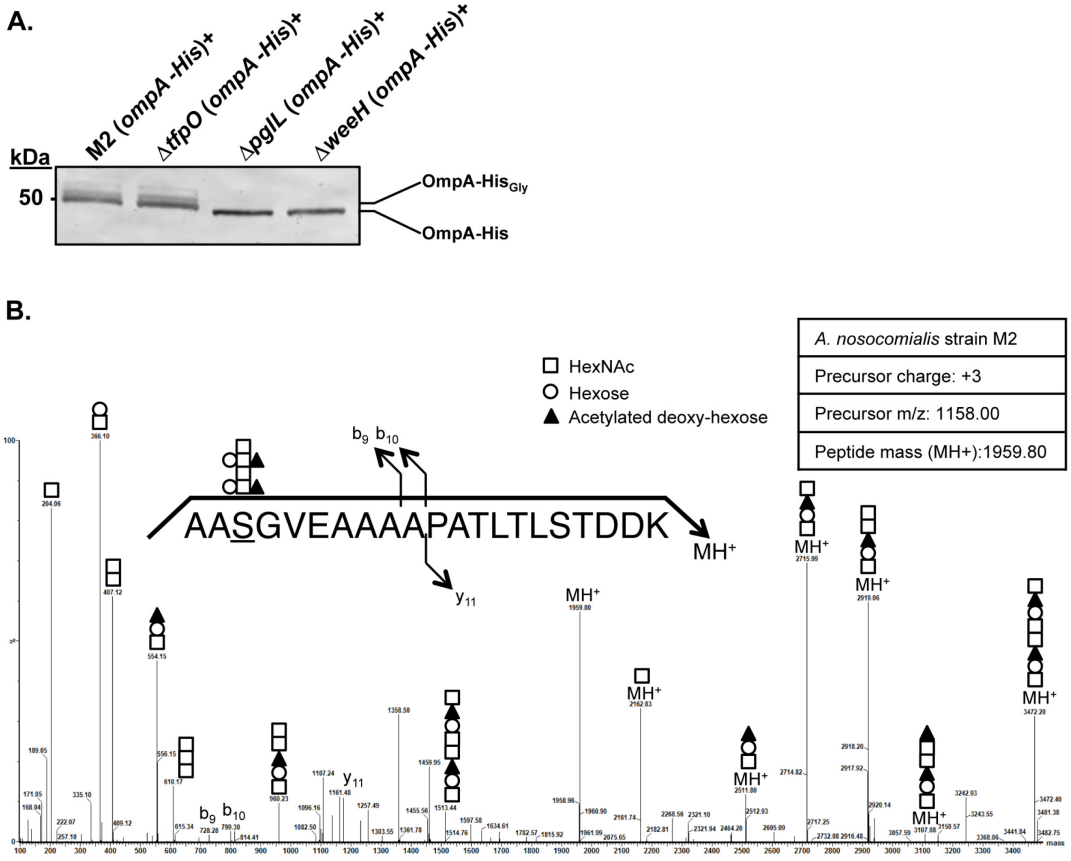

Figure 2.5 PglL$_{M2}$ is a general *O*-OTase and utilizes the same lipid-linked glycan donor as TfpO$_{M2}$ (A) Western blot analysis of whole cell extracts probing for OmpA-His expression and electrophoretic mobility. OmpA-His served as bait protein for glycosylation by strain M2 as well as the isogenic *tfpO*$_{M2}$::kan and *pglL*$_{M2}$::kan mutants. All strains expressed OmpA-His; however, OmpA-His from the *pglL*$_{M2}$::kan mutant ran at an increased electrophoretic mobility indicating the lack of glycosylation. (B) Glycosylated OmpA-His was purified from solubilized membranes using nickel affinity chromatography, separated by SDS-PAGE, and visualized by Coomassie staining. OmpA-His was excised from the gel and characterized by MS/MS analysis.

2.3.7 ACIAD0103 is not a WaaL O-antigen ligase and is not required for ComP modification

As noted above, two OTase-like proteins containing domains from the Wzy_C superfamily are encoded in the *A. baylyi* ADP1 genome. One of these, *pglL*$_{ComP}$ (ACIAD3337), is located adjacent to *comP*. Schulz *et al.* (2013) determined, and we independently confirmed, that *pglL*$_{ComP}$ (ACIAD3337) is required for ComP modification (Figure 2.6A). Furthermore, western blot analysis probing for ComP-His expression from an isogenic *pglL*$_{ADP1}$ (ACIAD0103) mutant strain demonstrated that PglL$_{ADP1}$ is not required for ComP post-translational modification (Figure 2.6A).

Schulz *et al.* (2013) speculated that the other Wzy_C superfamily domain-containing protein, PglL$_{ADP1}$ (ACIAD0103), encoded a WaaL O-antigen ligase. For LPS biosynthesis, the O-antigen repeat unit is sequentially assembled on the same lipid carrier as the *O*-glycan on the cytoplasmic side of the inner membrane, flipped to the periplasm, polymerized to form the O-antigen chain and transferred to the lipid A-core polysaccharide by the O-antigen ligase (Hug and Feldman, 2011). Differences in the number of O-antigen subunit repeats in LPS molecules appear as a ladder-like banding pattern in LPS silver stains (Whitfield, 1995). In order to determine if *pglL*$_{ADP1}$ was acting as an O-antigen ligase, we purified LPS from *A. baylyi* ADP1, the ADP1Δ*pglL*$_{ComP}$::kan, and ADP1Δ*pglL*$_{ADP1}$::kan mutants and silver stained the SDS-PAGE-separated preparation. LPS silver stained SDS polyacrylamide gels showed identical banding patterns, with no O-antigen subunits observed in the LOS compared to O-antigen-containing LPS obtained from the plant pathogen *Ralstonia solanacearum*

(Figure 2.6B). Given that $PglL_{ADP1}$ was not acting as an O-antigen ligase, we hypothesized that $PglL_{ADP1}$ could encode a second *O*-OTase.

2.3.8 ACIAD0103 encodes the general *O*-OTase, $PglL_{ADP1}$, in *A. baylyi* ADP1

We next tested whether $PglL_{ADP1}$ was able to glycosylate DsbA1 from *N. meningitidis* and OmpA from *A. baumannii* ATCC 17978, which are also modified by general *O*-OTases in their respective strains, and were previously employed as models to study glycosylation (Vik *et al*., 2009; Iwashkiw *et al*., 2012; Gebhart *et al*., 2012; Lithgow *et al*., 2014) These two proteins were independently expressed in wild-type, $\Delta pglL_{ComP}$ and $\Delta pglL_{ADP1}$ *A. baylyi* strains. DsbA1-His (Figure 2.6C) and OmpA-His (Figure 2.6D) displayed an increased electrophoretic mobility in the $\Delta pglL_{ADP1}$ background relative to wild-type and $\Delta pglL_{ComP}$ backgrounds. These experiments support the role of $PglL_{ADP1}$ as a general *O*-OTase.

In vivo glycosylation assays in *E. coli* were performed to further confirm the OTase activity of $PglL_{ADP1}$ (Gebhart *et al.*, 2012). We employed *E. coli* CLM24, a strain lacking the WaaL O-antigen ligase, which leads to the accumulation of lipid-linked glycan precursors that then are able to serve as substrates for heterologous *O*-OTase activity (Feldman *et al.*, 2005). *E. coli* CLM24 was transformed with plasmids encoding an acceptor protein (DsbA1), a glycan donor (the *Campylobacter jejuni* lipid-linked oligosaccharide (CjLLO)), and one OTase, as previously described (Faridmoayer *et al*., 2007; Ielmini & Feldman, 2011). We also included $PglL_{ComP}$ and employed $TfpO_{19606}$ and $PglL_{19606,}$ encoding the pilin-specific and the general *O*-OTase from *A. baumannii* ATCC 19606, as controls. DsbAl-His was detected with an anti-histidine antibody and

glycosylation was detected employing the hR6 antibody, which is reactive against the *C. jejuni* heptasaccharide. A band reacting with both antibodies, corresponding to DsbA1-His modified by the *C. jejuni* heptasaccharide, was only present in *E. coli* co-expressing DsbA1-His, the CjLLO and either $PglL_{ADP1}$ or the general *O*-OTase $PglL_{19606,}$ (Figure 2.7). Together this data supports the role of $PglL_{ADP1}$ as a general *O*-OTase.

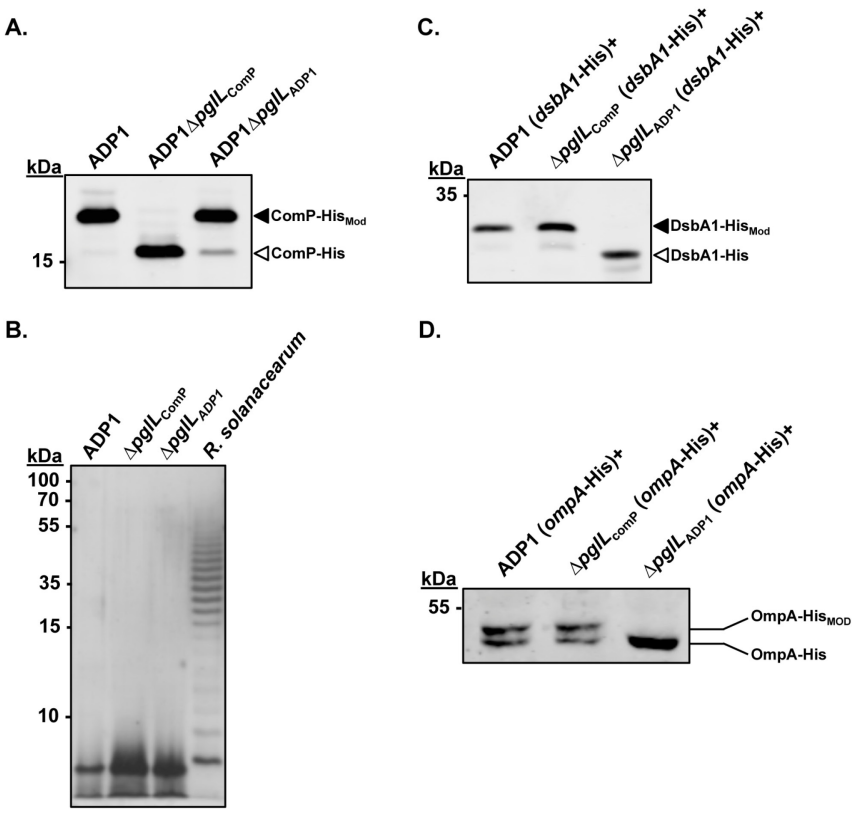

Figure 2.6 Activity of *O*-OTases in *A. baylyi* ADP1 (A) Western blot analysis probing for ComP-His expression in whole cell lysates of *A. baylyi* ADP1 as well as the isogenic Δ*pglL*$_{\text{ComP}}$ and Δ*pglL*$_{\text{ADP1}}$ mutants. The increase in ComP-His electrophoretic mobility seen in the Δ*pglL*$_{\text{ComP}}$ mutant indicates the absence of pilin glycosylation in this strain. (B) Silver stain of LPS obtained from *A. baylyi* ADP1, the isogenic Δ*pglL*$_{\text{ComP}}$, and Δ*pglL*$_{\text{ADP1}}$ mutants as well as *Ralstonia solanacearum*. *A. baylyi* lacks O-antigen, as seen by the lack of laddering observed with *R. solanacearum* LPS. (C, D) Western blot analysis of whole cell lysates from *A. baylyi* ADP1, the isogenic Δ*pglL*$_{\text{ComP}}$ and Δ*pglL*$_{\text{ADP1}}$ mutants recombinantly expressing his-tagged proteins Dsba1 (C) or OmpA-His (D). The increases in the relative mobility of the His-tagged proteins produced in the Δ*pglL*$_{\text{ADP1}}$ background indicate that expression of PglL$_{\text{ADP1}}$ was required for glycosylation of these proteins.

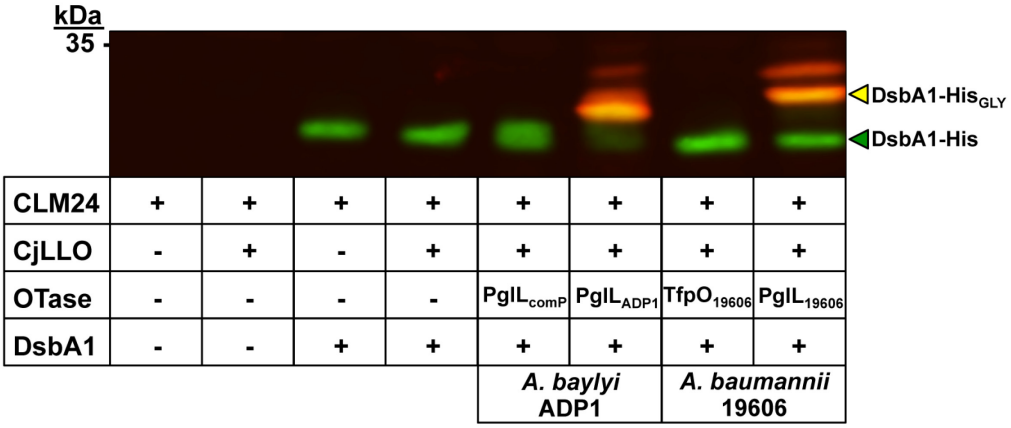

Figure 2.7 Heterologous expression of TfpO and PglL OTases in *E. coli* Western blot analysis of whole cell lysates of *E. coli* CLM24 expressing, as indicated, the *C. jejuni* lipid linked oligosaccharide (CjLLO) and His-tagged DsbA1 together with an *A. baylyi* or *A. baumannii* ATCC 19606 OTase. His-tagged DsbA1 was detected using the polyclonal anti-his antibody (green) and CjLLO was detected using the hR6 antibody (red). Co-localization of both signals, seen in yellow, indicates glycosylation of DsbA1 by the *Campylobacter* oligosaccharide. This is seen only when $PglL_{ADP1}$ or $PglL_{19606}$ were expressed in *E. coli* CLM24 along with CjLLO and His-tagged DsbA1.

2.3.9 Comparative proteomic analysis of *A. baylyi* ADP1 wild-type, Δ*pglL*$_{ComP}$ and Δ*pglL*$_{ADP1}$ strains

To unequivocally determine the role of both putative *O*-OTases in *A. baylyi* ADP1 glycosylation, we compared the glycoproteome of *A. baylyi* ADP1 to either the ADP1Δ*pglL*$_{ComP}$ mutant or the ADP1 *pglL*$_{ADP1}$ mutant. Using ZIC-HILIC for glycopeptide enrichment (Iwashkiw *et al*., 2012; Nothaft *et al*., 2012; Scott *et al*., 2014; Lithgow *et al*., 2014) and multiple MS/MS fragmentation approaches (Nichollas E Scott *et al.*, 2011), 21 unique glycopeptides from eight protein substrates were identified within *A. baylyi* ADP1 (Appendix A Table S1.3). Similar to the diversity observed within other *Acinetobacter* spp. (Scott *et al.*, 2014), *A. baylyi* ADP1 generated unique glycans with glycopeptides decorated with one of four pentasaccharide glycoforms composed of 286-217-HexNAc$_3$ (1112.41 Da, Figure 2.8A and 2.8D), 286-217-245-HexNAc$_2$ (1154.41 Da, Figure 2.8B and 2.8F), 286-217-HexNAc-245-HexNAc (1154.41 Da, Figure 2.8B and 2.8G) and 286-217-245$_2$-HexNAc (1196.41 Da, Figure 2.8C and 2.8E). Glycopeptide analysis of membrane proteins from *A. baylyi* ADP1Δ*pglL*$_{ComP}$ enabled the identification of identical glycopeptides suggesting the glycoproteome was unaffected by the loss of this gene. In contrast none of 21 glycopeptides observed within wild type *A. baylyi* ADP1 could be detected within extracts of *A. baylyi* ADP1Δ*pglL*$_{ADP1}$ (data not shown), confirming that *pglL*$_{ADP1}$ was responsible for general protein glycosylation within *A. baylyi* ADP1. Furthermore, quantitative dimethylation labeling enabled comparison of all three strains simultaneously providing an internal positive control for glycopeptide enrichment and led to the detection of seven unique glycopeptides (Appendix A Table S1.4).

Consistent with the requirement of $PglL_{ADP1}$ for glycosylation, no glycopeptides derived from the Δ*pglL*$_{ADP1}$ mutant (Appendix A figure S1.1 A-C) could be detected, while non-glycosylated peptides within the samples were observed at a ~1:1:1 ratio (Appendix A Figure S1.1 D-E, table S1.4).

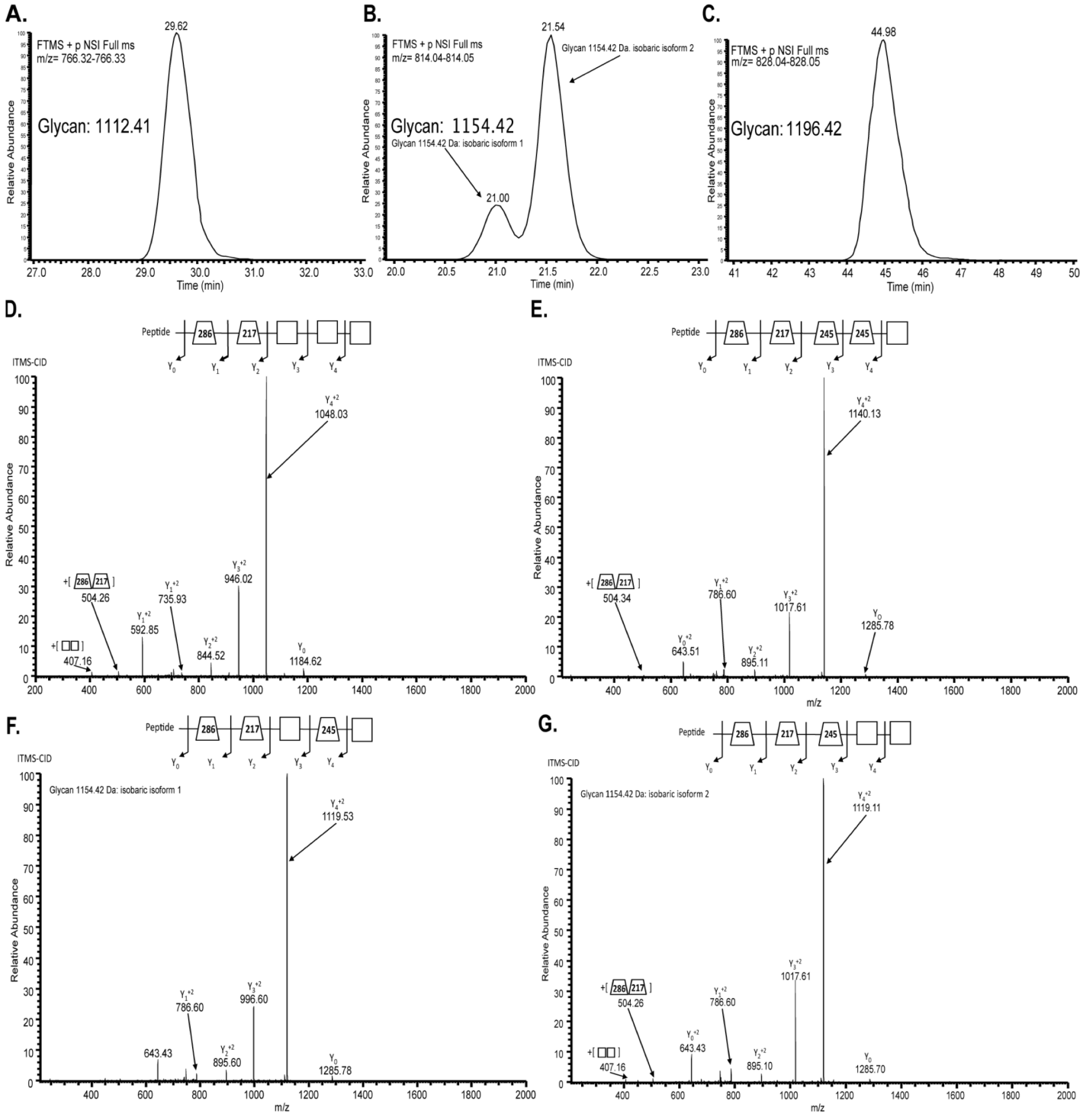

Figure 2.8 *O*-glycan structure identified using ZIC-HILIC enrichment of *A. baylyi* ADP1 glycoproteins. ITMS-CID fragmentation results in near exclusive glycan fragmentation of *A. baylyi* ADP1 glycopeptides enabling the identification of four unique

glycans on multiple protein substrates corresponding to: (A and D) a pentasaccharide composed of 286-217-HexNAc3 (1112.41 Da, 92DAAHDAAASVEK 103 of Q6FCV1_ACIAD); (B, F and G) two isobaric glycoforms composed of 286-217-245-HexNAc2 and 286-217-HexNAc-245-HexNAc (1154.41 Da, 344NTAASSVAATHKK356 of Q6F814_ ACIAD) and (C and E) a pentasaccharide composed of 286-217-2452-HexNAc (1196.41 Da, 344NTAASSVAATHKK356 of Q6F814_ACIAD).

2.3.10 $PglL_{ComP}$ acceptor protein specificity distinguishes it from TfpO and general *O*-OTases

Given the strong genetic linkage between the major type IVa pilin genes and downstream *O*-OTase genes on *Acinetobacter* chromosomes, we sought to determine whether these *O*-OTases were specific for their cognate pilin protein. To test this hypothesis, we introduced a plasmid expressing $PilA_{M2}$ from *A. nosocomialis* strain M2 into different *Acinetobacter* spp., and then conducted western blot analysis probing for the expression and electrophoretic mobility of the pilin protein. $PilA_{M2}$ was modified by *A. baumannii* ATCC 19606 and the clinical isolate *A. baumannii* 27413, both of which encode *tfpO* homologs and pilins containing terminal serine residues, as evidenced by the presence of both the higher molecular weight and lower molecular forms of $PilA_{M2}$ (Figure 2.9A). We recently demonstrated that the glycan associated with *A. baumannii* ATCC 19606 was identical to the pentasaccharide identified in *A. baumannii* ATCC 17978. As expected, $PilA_{M2}$ expressed in *A. baumannii* ATCC 19606 ran with the slowest electrophoretic mobility indicative of a larger glycan associated with $PilA_{M2}$ (Scott *et al*., 2014). Both *A. baumannii* ATCC 17978 and *A. baylyi* ADP1, which lack a *tfpO* homolog, were unable to glycosylate $PilA_{M2}$.

On the contrary, when we heterologously expressed ComP-His in different *Acinetobacter* spp. we found that it was glycosylated only in *A. baylyi* ADP1. Strains encoding *tfpO* homologs were unable to modify ComP-His, with the exception of *A. baumannii* ATCC 19606, which appeared to have a marginal capacity to modify ComP-His (Figure 2.9B). We then analyzed if $PglL_{ComP}$ was able to modify *A. baumannii* ATCC 17978 pilin, which does not carry a terminal serine residue. $PilA_{17978}$ was not glycosylated by

$PglL_{ComP}$, but was glycosylated by both its cognate $PglL_{17978}$ and the $PglL_{ADP1}$ general *O*-OTases (Figure 2.9C). These results clearly distinguish $PglL_{ComP}$ from the other pilin-specific TfpO OTases that recognize terminal serine residues, and from the general PglL *O*-OTases. The sequence of $PglL_{ComP}$ is more similar to PglL-like OTases but, unexpectedly, its activity is specific for ComP, which is a pilin-like protein that does not have a terminal serine residue. $PglL_{ComP}$ appears to belong to a new class of *O*-OTases.

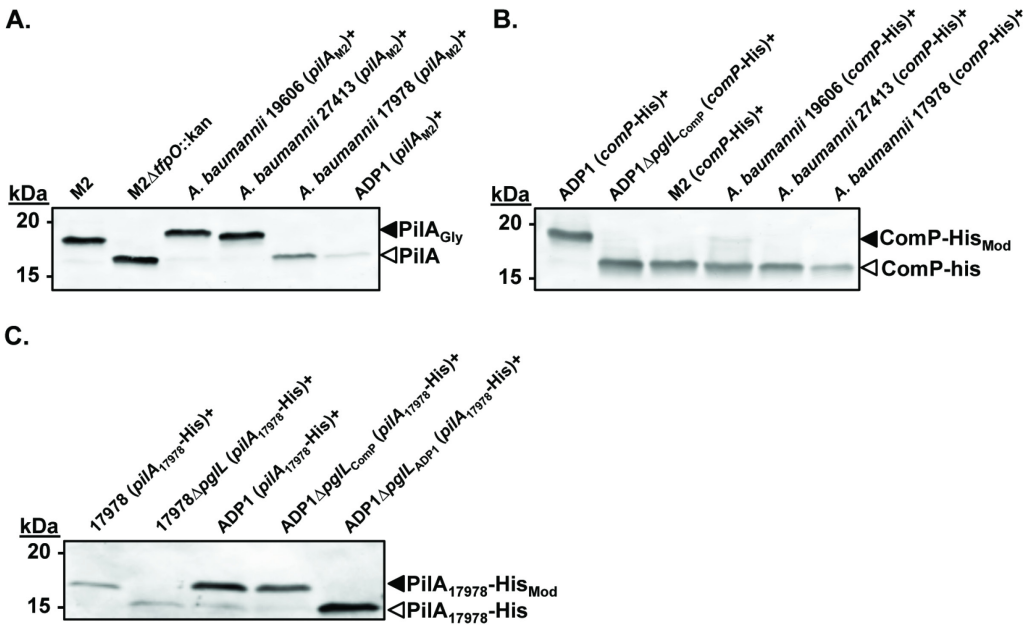

Figure 2.9 PglL$_{ComP}$, but not TfpO$_{M2}$, is specific for its cognate pilin protein. (A) Western blot analysis of whole cell extracts probing for heterologous PilA$_{M2}$ expression and electrophoretic mobility. PilA$_{M2}$ was glycosylated in *A. baumannii* ATCC 19606 and *A. baumannii* 27413, both of which encode *tfpO* homologs. Strains lacking *tfpO* homologs (*A. baumannii* ATCC 17978 and *A. baylyi* ADP1) were unable to glycosylate PilA$_{M2}$. (B) Western blot analysis probing for heterologous ComP-His expression and electrophoretic mobility. ComP-His was only modified in *A. baylyi* ADP1 indicating that PglL$_{ComP}$ is specific for ComP. (C) Western blot analysis probing for heterologous PilA$_{17978}$ expression and electrophoretic mobility. PilA$_{17978}$ was glycosylated in its native strain by PglL$_{17978}$ and in *A. baylyi* ADP1 by PglL$_{ADP1}$, but not by PglL$_{ComP}$.

2.3 Discussion

In the recent years, a panoply of glycosylation pathways have been identified in bacteria. Irrespective of the pathway utilized, both *N*- and *O*-glycans often decorate cell surface adhesins in both Gram-negative and Gram-positive bacteria. Examples of glycosylated surface-associated proteins include the *O*-glycosylation of AIDA-I and TibA in *E. coli* (Charbonneau *et al*., 2007; Côté *et al*., 2013), the type IV pilins of *Pseudomonas*, *Neisseria*, *Dichelobacter nodosus*, and *Fransicella tularensis* (Castric *et al*., 1995, Aas *et al*., 2006; Faridmoayer *et al*., 2007, Voisin *et al*. 2007, Cagatay *et al*., 2008, Egge-Jacobsen *et al*., 2011), the flagellins in multiple bacterial species (Nothaft & Szymanski, 2010; Iwashkiw *et al*., 2013), the serine rich adhesins in *Streptococcus* spp. (Zhou and Wu, 2009), and the *N*-glycosylation of HMWG in *Haemophilus* (Gross *et al.*, 2008) and *Aggregatibacter actinomycetemcomitans* (Tang and Mintz, 2010). Medically relevant *Acb* members are not the exception and our present and previous work show that pilin and multiple outer membrane proteins are *O*-glycosylated, which could provide an adherence advantage either to host cells, in bacterial communities, or to abiotic surfaces. However, as of yet, we have not elucidated the biological role for glycosylation of *Acinetobacter* pilin subunits. Specifically, the *A. nosocomialis* strain M2Δ *tfpO*::kan mutant was equally as transformable as the parental strain (Figure 2.3C). Furthermore, we did not find a condition in which *A. baylyi* ADP1 would attach to abiotic surfaces or form biofilms (Appendix A figure S1.3). As mentioned earlier, pilin glycosylation in *P. aeruginosa* and *P. syringae*, mediated by PilO and TfpO, respectively, was essential for motility, biofilm formation and virulence (Smedley *et al*., 2005; Nguyen *et al*., 2012). Pilin glycosylation had no effect on natural competence in *A. baylyi* (Porstendorfer *et al.*,

2000) or *A. nosocomialis* (Figure 2.3C). Nevertheless, the ubiquitous nature of *O*-linked protein glycosylation within the genus *Acinetobacter* suggests a key, still unknown role for this post-translational modification.

In this paper we demonstrate that some *Acinetobacter* strains encode two OTase homologs, one of which is required for general *O*-glycosylation and the other that specifically modifies pilin. The majority of the medically relevant *Acinetobacter* strains, including *A. nosocomialis* strain M2, encode two contiguous OTases, which are located immediately downstream of a type IVa major pilin subunit gene. At the time of manuscript preparation, 76% of *A. baumannii* isolates with completed genomes encoded a PilA protein containing a carboxy-terminal serine. All isolates containing a gene encoding a PilA protein with a carboxy-terminal serine also encode for a *tfpO* homolog found immediately downstream of *pilA*. This finding is congruous with findings reported for the group I pilins ($PilA_I$) found in *P. aeruginosa* (Kus *et al*., 2004). Thus there appears to be multiple lineages of pilin genes, specifically, a lineage that contains an allele that encodes for a PilA with a carboxy-terminal serine and the downstream accessory gene *tfpO* and lineages that are not glycosylated by a TfpO-like activity. All isolates lacking a carboxy-terminal serine on the major pilin protein, including ATCC 17978, do not encode for a *tfpO* homolog consistent with the separate evolution of *Acinetobacter* pilin lineages.

In contrast to the contiguous organization of OTases in the medically relevant *Acinetobacter* spp., the two OTases of the environmental isolate *A. baylyi* ADP1 are distantly separated on the chromosome. We confirmed the findings of Schulz *et al.* (2013), who showed that the OTase homolog $pglL_{ComP}$, which is encoded adjacent to

comP, is responsible for ComP modification. Mutational analysis coupled with an *in vivo* glycosylation assay as well as the characterization of the glycoproteome demonstrated that $pglL_{ComP}$ is a ComP-specific OTase. On the other hand, the second OTase, $PglL_{ADP1}$, is not an O-antigen ligase as previously suggested but rather a general *O*-OTase glycosylating multiple protein targets.

Our study also demonstrated that $PglL_{M2}$, encoded by M215_10475**,** is able to recognize the same motif that the general *O*-OTase PglL found in all other *A. baumannii* strains recognizes, as evidenced by the ability of *A. nosocomialis* M2 to glycosylate OmpA-His. In *A. nosocomialis* strain M2, both $PglL_{M2}$ and $TfpO_{M2}$ utilize the same lipid-linked tetrasaccharide to modify their target proteins. We found that $PglL_{M2}$ was able to transfer two subunits of the glycan, whereas $TfpO_{M2}$ only transferred a single glycan chain. This result is in agreement with our previous studies showing two subunits of the glycans being transferred by general OTases (Scott *et al*., 2014); and with our previous demonstration that TfpO is unable to transfer long sugar chains to *P. aeruginosa* pilin (Faridmoayer *et al*., 2007). Furthermore, we identified the MPA Locus as the source of the genes encoding the proteins responsible for the synthesis of the shared lipid-linked tetrasaccharide. Incorporating all of the data led us to propose the model depicting *O*-glycan synthesis by the MPA cluster and the shared usage of this lipid-linked glycan by $TfpO_{M2}$ and $PglL_{M2}$ (Figure 2.10).

Although many protein glycosylation systems have been identified, how *O*-OTases, such as the ones from *A. baumannii*, *Neisseria* spp. and *Burkholderia* spp., recognize the acceptor sequences in their protein targets is still not clear. It has been established that OTases recognize low complexity regions (LCR), rich in serine, alanine and proline (Vik

et al., 2009). The pilin specific TfpO enzymes described here recognize a peptide of about 15 amino acids containing many serine and proline residues.

Similarly to *P. aeruginosa* TfpO, our evidence suggests that the carboxy-terminal serine of $PilA_{M2}$ serves as the site of $TfpO_{M2}$-dependent glycosylation.

Bacterial species carrying two functional *O*-OTases, a PglL-general OTase and a pilin-specific OTase have not previously been identified. TfpO is the only OTase present in *Pseudomonas* (Smedley *et al*., 2005; Nguyen *et al*., 2012), while PglL is the only OTase identified in *Neisseria* (Faridmoayer *et al*., 2007), *A. baumannii* ATCC 17978 (Jeremy A. Iwashkiw *et al.*, 2012), *B. cenocepacia* K56-2 (Lithgow *et al.*, 2014) and *R. solanacearum* (manuscript in preparation). Three possible *O*-OTases have been identified in *V. cholerae*, but the activity of only one of these has been shown in *E. coli* (Gebhart *et al.*, 2012), and no glycoproteins have been identified in *V. cholerae*. In *N. meningitidis* and *N. gonorrhoeae*, the OTase PglL is able to glycosylate pilin and several other proteins (Aas *et al*., 2006; Faridmoayer *et al*., 2007). Although PglL can recognize three glycosylation sites in pilin when the system is reconstituted in *E. coli*, none of them contain the typical LCR domain found in the remaining *Neisseria* glycoproteins, indicating that PglL can recognize more than one motif (Musumeci *et al.*, 2014). In *Francisella* spp. the OTase is closely related to PilO/TfpO and it appears to be responsible for both pilin and general glycosylation (Balonova *et al.*, 2012). Why *Acinetobacter* strains require two different OTases to glycosylate pilin and other proteins remains unclear as some pathogenic strains of *A. baumannii* carry only PglL, which is required for optimal biofilm formation and virulence (Jeremy A. Iwashkiw *et al.*, 2012).

It is important to note that non-pathogenic *A. baylyi* ADP1 also contains two *O*-OTases. However there are several differences between the ComP-specific OTase $PglL_{ComP}$ of *A. baylyi* and the pilin-specific OTases TfpO of the medically relevant *Acinetobacter* spp. Although both OTases are encoded immediately downstream of their cognate protein acceptors (Figure 2.1 panels A and C), TfpO OTases are hypothesized to be specific for the carboxy-terminal serine present on PilA, as a carboxy-terminal serine to alanine point mutant was unable to produce glycosylated pilin. Interestingly, all *Acinetobacter* strains encoding a *tfpO* gene homolog also contained the carboxy-terminal serine on their respective PilA sequences. Furthermore, our experiments demonstrated that *Acinetobacter* TfpO homologs are functionally exchangeable as $PilA_{M2}$ was modified by each *tfpO* encoding strain tested (Figure 2.9A). The variable electrophoretic mobility of $PilA_{M2}$ is likely due to glycan variability between these strains (Scott, *et al.*, 2014). Although the site of ComP glycosylation has not been identified, it is predicted to be at an internal residue as ComP does not contain a carboxy terminal serine or any carboxy-terminal residue associated with post-translational modification. BLAST analysis of the ComP-specific OTases also demonstrated that $PglL_{ComP}$ is more closely related to the general OTase $PglL_{M2}$ than to TfpO. Although the pilin-specific TfpO OTase could cross glycosylate different pilins containing carboxy-terminal serines, $PglL_{ComP}$ was unable to glycosylate the pilins recognized by TfpO.

For these reasons we conclude that three different classes of OTases are present in *Acinetobacter*: the pilin-specific TfpO enzymes that glycosylate pilins containing carboxy-terminal serine residues; the general PglL OTases that recognize LCR in multiple proteins; and $PglL_{ComP}$, which is the first PglL-like protein that specifically

glycosylates one protein. These enzymes have different biochemical characteristics, expanding our current glycoengineering toolbox for the synthesis of novel glycoconjugates with biotechnological applications. The differentiation between these enzymes is not trivial, and cannot be accurately predicted just by the presence of pfam domains. For example, despite having the highest degree of sequence similarity and being functionally homologous, PilO/TfpO from *P. aeruginosa* strain 1244 contains the pfam04932 domain, whereas *tfpO* from *A. nosocomialis* strain M2 contains the pfam13425 domain. Moreover, the general PglL OTases of the medically relevant *Acinetobacter* spp., including strain M2 and *A. baumannii* ATCC 17978, contain domains from the pfam04932 family and the *A. baylyi* general $PglL_{ADP1}$ OTases contain a pfam13425 domain. Adding to the complexity is the fact that the general PglL OTases from medically relevant *Acinetobacter* spp. and the *A. baylyi* ComP-specific $PglL_{ComP}$ contain the same pfam04932 domains yet recognize different sequons. In addition this pfam domain is present in the WaaL O-antigen ligases. While bioinformatic analyses can be powerful tools to initially locate and identify ORFs encoding proteins predicted to be involved in glycan transfer events, our data collectively reinforces the concept that the activity of bioinformatically identified *O*-OTases must be experimentally determined and reveals a complex and fascinating evolutionary pathway for bacterial *O*-OTases.

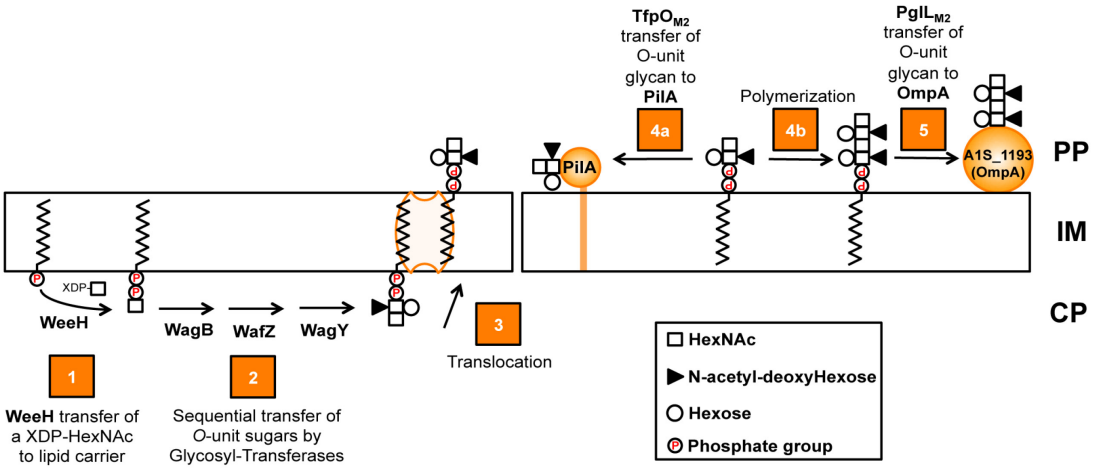

Figure 2.10 Model of lipid-linked oligosaccharide synthesis, TfpO$_{M2}$-dependent pilin glycosylation, and PglL$_{M2}$ general *O*-glycosylation in *A. nosocomialis* strain M2 The proteins encoded by the genes from the major polysaccharide antigen locus synthesize the tetrasaccharide (HexNAc)-(Hex)-(deoxy-Hex)-(HexNAc) on an undecaprenyl lipid carrier, which is then transferred to the periplasm. The lipid-linked oligosaccharide can then be transferred to the major pilin protein, PilA, by the pilin-specific OTase TfpO or further processed and transferred to other proteins, such as, OmpA by the general OTase PglL$_{M2}$.

2.4 References

Aas, F.E., Egge-Jacobsen, W., Winther-Larsen, H.C., Løvold, C., Hitchen, P.G., Dell, A., and Koomey, M. (2006) *Neisseria gonorrhoeae* type IV pili undergo multisite, hierarchical modifications with phosphoethanolamine and phosphocholine requiring an enzyme structurally related to lipopolysaccharide phosphoethanolamine transferases. *J Biol Chem* **281**: 27712–23.

Abd-El-Haleem, D., Moawad, H., Zaki, E.A., and Zaki, S. (2002) Molecular characterization of phenol-degrading bacteria isolated from different Egyptian ecosystems. *Microb Ecol* **43**: 217–24.

Arroyo, L.A., Mateos, I., González, V., and Aznar, J. (2009) In vitro activities of tigecycline, minocycline, and colistin-tigecycline combination against multi- and pandrug-resistant clinical isolates of *Acinetobacter baumannii* group. *Antimicrob Agents Chemother* **53**: 1295–6.

Balonova, L., Mann, B.F., Cerveny, L., Alley, W.R., Chovancova, E., Forslund, A.-L., Salomonsson, E.N., Forsberg, A., Damborsky, J., Novotny, M. V, Hernychova, L., and Stulik, J. (2012) Characterization of protein glycosylation in *Francisella tularensis* subsp. holarctica: identification of a novel glycosylated lipoprotein required for virulence. *Mol Cell Proteomics* **11**: M111.015016.

Børud, B., Viburiene, R., Hartley, M.D., Paulsen, B.S., Egge-Jacobsen, W., Imperiali, B., and Koomey, M. (2011) Genetic and molecular analyses reveal an evolutionary trajectory for glycan synthesis in a bacterial protein glycosylation system. *Proc Natl Acad Sci U S A* **108**: 9643–8.

Boucher, C.A., Barberis, P.A., and Demery, D.A. (1985) Transposon Mutagenesis of *Pseudomonas solanacearum*: Isolation of Tn5-Induced Avirulent Mutants. *Microbiology* **131**: 2449–2457.

Brzoska, A.J., Hassan, K.A., Leon, E.J. de, Paulsen, I.T., and Lewis, P.J. (2013) Single-step selection of drug resistant *Acinetobacter baylyi* ADP1 mutants reveals a functional redundancy in the recruitment of multidrug efflux systems. *PLoS One* **8**: e56090.

Cagatay, T.I., and Hickford, J.G.H. (2008) Glycosylation of type-IV fimbriae of Dichelobacter nodosus. *Vet Microbiol* **126**: 160–7.

Carruthers, M.D., Nicholson, P.A., Tracy, E.N., and Munson, R.S. (2013) *Acinetobacter baumannii* utilizes a type VI secretion system for bacterial competition. *PLoS One* **8**: e59388.

Castric, P. (1995) pilO, a gene required for glycosylation of *Pseudomonas aeruginosa* 1244 pilin. *Microbiology* **141 (Pt 5**: 1247–54.

Charbonneau, M.-E., Girard, V., Nikolakakis, A., Campos, M., Berthiaume, F., Dumas, F., Lépine, F., and Mourez, M. (2007) O-linked glycosylation ensures the normal conformation of the autotransporter adhesin involved in diffuse adherence. *J Bacteriol* **189**: 8880–9.

Choi, A.H.K., Slamti, L., Avci, F.Y., Pier, G.B., and Maira-Litrán, T. (2009) The pgaABCD locus of *Acinetobacter baumannii* encodes the production of poly-beta-1-6-N-acetylglucosamine, which is critical for biofilm formation. *J Bacteriol* **191**: 5953–63.

Choi, C.H., Hyun, S.H., Lee, J.Y., Lee, J.S., Lee, Y.S., Kim, S.A., Chae, J.-P., Yoo, S.M., and Lee, J.C. (2008) *Acinetobacter baumannii* outer membrane protein A targets the nucleus and induces cytotoxicity. *Cell Microbiol* **10**: 309–19.

Comer, J.E., Marshall, M.A., Blanch, V.J., Deal, C.D., and Castric, P. (2002) Identification of the *Pseudomonas aeruginosa* 1244 pilin glycosylation site. *Infect Immun* **70**: 2837–45.

Côté, J.-P., Charbonneau, M.-È., and Mourez, M. (2013) Glycosylation of the *Escherichia coli* TibA self-associating autotransporter influences the conformation and the functionality of the protein. *PLoS One* **8**: e80739.

Coyne, M.J., Fletcher, C.M., Chatzidaki-Livanis, M., Posch, G., Schaffer, C., and Comstock, L.E. (2013) Phylum-wide general protein O-glycosylation system of the Bacteroidetes. *Mol Microbiol* **88**: 772–83.

de Berardinis, V., Vallenet, D., Castelli, V., Besnard, M., Pinet, A., Cruaud, C., Samair, S., Lechaplais, C., Gyapay, G., Richez, C., Durot, M., Kreimeyer, A., Le Fèvre, F., Schächter, V.,Pezo, V., Döring, V., Scarpelli, C., Médigue, C., Cohen, G. N., Marlière, P., Salanoubat, M. and Weissenbach, J.,. (2008) A complete collection of single-gene deletion mutants of *Acinetobacter baylyi* ADP1. *Mol Syst Biol* **4**: 174.

Egge-Jacobsen, W., Salomonsson, E.N., Aas, F.E., Forslund, A.-L., Winther-Larsen, H.C., Maier, J., Macellaro, A., Kuoppa, K., Oyston, P.C.F., Titball, R.W., Thomas, R.M., Forsberg, Å., Prior, J.L., and Koomey, M. (2011) O-linked glycosylation of the PilA pilin protein of *Francisella tularensis*: identification of the endogenous protein-targeting oligosaccharyltransferase and characterization of the native oligosaccharide. *J Bacteriol* **193**: 5487–97.

Faridmoayer, A., Fentabil, M.A., Mills, D.C., Klassen, J.S., and Feldman, M.F. (2007) Functional characterization of bacterial oligosaccharyltransferases involved in O-linked protein glycosylation. *J Bacteriol* **189**: 8088–98.

Feldman, M.F., Wacker, M., Hernandez, M., Hitchen, P.G., Marolda, C.L., Kowarik, M., *et al.* (2005) Engineering N-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli*. *Proc Natl Acad Sci U S A* **102**: 3016–21.

Gebhart, C., Ielmini, M.V., Reiz, B., Price, N.L., Aas, F.E., Koomey, M., and Feldman, M.F. (2012) Characterization of exogenous bacterial oligosaccharyltransferases in *Escherichia coli* reveals the potential for O-linked protein glycosylation in Vibrio cholerae and *Burkholderia thailandensis*. *Glycobiology* **22**: 962–74.

Gordon, N.C., and Wareham, D.W. (2010) Multidrug-resistant *Acinetobacter baumannii*: mechanisms of virulence and resistance. *Int J Antimicrob Agents* **35**: 219–26.

Göttig, S., Gruber, T.M., Higgins, P.G., Wachsmuth, M., Seifert, H., and Kempf, V.A.J. (2014) Detection of pan drug-resistant *Acinetobacter baumannii* in Germany. *J Antimicrob Chemother* **69**: 2578–9.

Gross, J., Grass, S., Davis, A.E., Gilmore-Erdmann, P., Townsend, R.R., and St Geme, J.W. (2008) The *Haemophilus influenzae* HMW1 adhesin is a glycoprotein with an unusual N-linked carbohydrate modification. *J Biol Chem* **283**: 26010–5.

Harding, C.M., Tracy, E.N., Carruthers, M.D., Rather, P.N., Actis, L.A., and Munson, R.S. (2013) *Acinetobacter baumannii* strain M2 produces type IV pili which play a role in natural transformation and twitching motility but not surface-associated motility. *MBio* **4**: e00360–13–.

Hu, D., Liu, B., Dijkshoorn, L., Wang, L., and Reeves, P.R. (2013) Diversity in the major polysaccharide antigen of *Acinetobacter baumannii* assessed by DNA sequencing, and development of a molecular serotyping scheme. *PLoS One* **8**: e70329.

Hug, I., and Feldman, M.F. (2011) Analogies and homologies in lipopolysaccharide and glycoprotein biosynthesis in bacteria. *Glycobiology* **21**: 138–51.

Ielmini, M. V, and Feldman, M.F. (2011) *Desulfovibrio desulfuricans* PglB homolog possesses oligosaccharyltransferase activity with relaxed glycan specificity and distinct protein acceptor sequence requirements. *Glycobiology* **21**: 734–42.

Iwashkiw, J.A., Seper, A., Weber, B.S., Scott, N.E., Vinogradov, E., Stratilo, C., Reiz, B., Cordwell, S.J., Whittal, R., Schild, S., and Feldman, M.F. (2012) Identification of a General O-linked Protein Glycosylation System in *Acinetobacter baumannii* and Its Role in Virulence and Biofilm Formation. *PLoS Pathog* **8**: e1002758.

Iwashkiw, J.A., Vozza, N.F., Kinsella, R.L., and Feldman, M.F. (2013) Pour some sugar on it: the expanding world of bacterial protein O-linked glycosylation. *Mol Microbiol* **89**: 14–28.

Kus, J. V, Tullis, E., Cvitkovitch, D.G., and Burrows, L.L. (2004) Significant differences in type IV pilin allele distribution among *Pseudomonas aeruginosa* isolates from cystic fibrosis (CF) versus non-CF patients. *Microbiology* **150**: 1315–26.

Lees-Miller, R.G., Iwashkiw, J.A., Scott, N.E., Seper, A., Vinogradov, E., Schild, S., and Feldman, M.F. (2013) A common pathway for O-linked protein-glycosylation and synthesis of capsule in *Acinetobacter baumannii*. *Mol Microbiol* **89**: 816–30.

Lithgow, K. V., Scott, N.E., Iwashkiw, J.A., Thomson, E.L.S., Foster, L.J., Feldman, M.F., and Dennis, J.J. (2014) A general protein O -glycosylation system within the *Burkholderia cepacia* complex is involved in motility and virulence. *Mol Microbiol* n/a–n/a.

Logan, S.M. (2006) Flagellar glycosylation - a new component of the motility repertoire? *Microbiology* **152**: 1249–62.

Mara, K., Decorosi, F., Viti, C., Giovannetti, L., Papaleo, M.C., Maida, I., Perrin, E., Fondi, M., Vaneechoutte, M., Nemec, A., Barselaar, M. van den, Dijkshoorn, L., and Fani, R. (2012) Molecular and phenotypic characterization of *Acinetobacter* strains able to degrade diesel fuel. *Res Microbiol* **163**: 161–72.

Marchler-Bauer, A., Anderson, J.B., Chitsaz, F., Derbyshire, M.K., DeWeese-Scott, C., Fong, J.H., *et al.* (2009) CDD: specific functional annotation with the Conserved Domain Database. *Nucleic Acids Res* **37**: D205–10

Marchler-Bauer, A., and Bryant, S.H. (2004) CD-Search: protein domain annotations on the fly. *Nucleic Acids Res* **32**: W327–31

Marchler-Bauer, A., Lu, S., Anderson, J.B., Chitsaz, F., Derbyshire, M.K., DeWeese-Scott, C., *et al.* (2011) CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res* **39**: D225–9

Mescher, M.F., and Strominger, J.L. (1976) Structural (shape-maintaining) role of the cell surface glycoprotein of Halobacterium salinarium. *Proc Natl Acad Sci U S A* **73**: 2687–91.

Musumeci, M.A., Faridmoayer, A., Watanabe, Y., and Feldman, M.F. (2014) Evaluating the role of conserved amino acids in bacterial O-oligosaccharyltransferases by in vivo, in vitro and limited proteolysis assays. *Glycobiology* **24**: 39–50.

Neuberger, A. (1938) Carbohydrates in protein: The carbohydrate component of crystalline egg albumin. *Biochem J* **32**: 1435–51.

Nguyen, L.C., Taguchi, F., Tran, Q.M., Naito, K., Yamamoto, M., Ohnishi-Kameyama, M., Ono, H., Yoshida, M., Chiku, K., Ishii, T., Inagaki, Y., Toyoda, K., Shiraishi, T., and Ichinose, Y. (2012) Type IV pilin is glycosylated in *Pseudomonas syringae* pv. tabaci 6605 and is required for surface motility and virulence. *Mol Plant Pathol* **13**: 764–74.

Nothaft, H., Scott, N.E., Vinogradov, E., Liu, X., Hu, R., Beadle, B., Fodor, C., Miller, W.G., Li, J., Cordwell, S.J., and Szymanski, C.M. (2012) Diversity in the protein N-glycosylation pathways within the *Campylobacter* genus. *Mol Cell Proteomics* **11**: 1203–

19.

Nothaft, H., and Szymanski, C.M. (2010) Protein glycosylation in bacteria: sweeter than ever. *Nat Rev Microbiol* **8**: 765–78.

Olsen, J. V, Macek, B., Lange, O., Makarov, A., Horning, S., and Mann, M. (2007) Higher-energy C-trap dissociation for peptide modification analysis. *Nat Methods* **4**: 709–12.

Pérez, J.M., McGarry, M.A., Marolda, C.L., and Valvano, M.A. (2008) Functional analysis of the large periplasmic loop of the *Escherichia coli* K-12 WaaL O-antigen ligase. *Mol Microbiol* **70**: 1424–40.

Porstendorfer, D., Gohl, O., Mayer, F., and Averhoff, B. (2000) ComP, a Pilin-Like Protein Essential for Natural Competence in *Acinetobacter* sp. Strain BD413: Regulation, Modification, and Cellular Localization. *J Bacteriol* **182**: 3673–3680.

Power, P., and Jennings, M. (2003) The genetics of glycosylation in Gram-negative bacteria. *FEMS Microbiol Lett* 218: 211–222

Power, P.M., Seib, K.L., and Jennings, M.P. (2006) Pilin glycosylation in *Neisseria meningitidis* occurs by a similar pathway to wzy-dependent O-antigen biosynthesis in *Escherichia coli*. *Biochem Biophys Res Commun* **347**: 904–8.

Rappsilber, J., Mann, M., and Ishihama, Y. (2007) Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat Protoc* **2**: 1896–906.

Ruan, X., Loyola, D.E., Marolda, C.L., Perez-Donoso, J.M., and Valvano, M.A. (2012) The WaaL O-antigen lipopolysaccharide ligase has features in common with metal ion-independent inverting glycosyltransferases. *Glycobiology* **22**: 288–99.

Schulz, B.L., Jen, F.E.C., Power, P.M., Jones, C.E., Fox, K.L., Ku, S.C., Blanchfield, J.T., and Jennings, M.P. (2013) Identification of bacterial protein O-oligosaccharyltransferases and their glycoprotein substrates. *PLoS One* **8**: e62768.

Scott, N.E., Kinsella, R.L., Edwards, A.V.G., Larsen, M.R., Dutta, S., Saba, J., Foster, L.J., and Feldman, M.F. (2014) Diversity Within the O-linked Protein Glycosylation Systems of *Acinetobacter* Species. *Mol Cell Proteomics* **13**: 2354–70.

Scott, N.E., Nothaft, H., Edwards, A.V.G., Labbate, M., Djordjevic, S.P., Larsen, M.R., Szymanski, C.M., and Cordwell, S.J. (2012) Modification of the *Campylobacter jejuni* N-linked glycan by EptC protein-mediated addition of phosphoethanolamine. *J Biol Chem* **287**: 29384–96.

Scott, N.E., Parker, B.L., Connolly, A.M., Paulech, J., Edwards, A.V.G., Crossett, B., Falconer, L., Kolarich, D., Djordjevic, S.P., Højrup, P., Packer, N.H., Larsen, M.R., and

Cordwell, S.J. (2011) Simultaneous glycan-peptide characterization using hydrophilic interaction chromatography and parallel fragmentation by CID, higher energy collisional dissociation, and electron transfer dissociation MS applied to the N-linked glycoproteome of Campylobact. *Mol Cell Proteomics* **10**: M000031–MCP201.

Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V, and Mann, M. (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* **1**: 2856–60

Sleytr, U.B. (1975) Heterologous reattachment of regular arrays of glycoproteins on bacterial surfaces. *Nature* **257**: 400–402.

Smedley, J.G., Jewell, E., Roguskie, J., Horzempa, J., Syboldt, A., Stolz, D.B., and Castric, P. (2005) Influence of pilin glycosylation on *Pseudomonas aeruginosa* 1244 pilus function. *Infect Immun* **73**: 7922–31.

Szymanski, C.M., Yao, R., Ewing, C.P., Trust, T.J., and Guerry, P. (1999) Evidence for a system of general protein glycosylation in *Campylobacter jejuni*. *Mol Microbiol* **32**: 1022–1030.

Tang, G., and Mintz, K.P. (2010) Glycosylation of the collagen adhesin EmaA of Aggregatibacter actinomycetemcomitans is dependent upon the lipopolysaccharide biosynthetic pathway. *J Bacteriol* **192**: 1395–404.

Tsai, C.M., and Frasch, C.E. (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* **119**: 115–9.

Vaneechoutte, M., Young, D.M., Ornston, L.N., Baere, T. De, Nemec, A., Reijden, T. Van Der, Carr, E., Tjernberg, I., and Dijkshoorn, L. (2006) Naturally Transformable *Acinetobacter* sp. Strain ADP1 Belongs to the Newly Described Species *Acinetobacter baylyi*. *Appl Environ Microbiol* **72**: 932–936.

Varki, A. (1993) Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* **3**: 97–130.

Vik, A., Aas, F.E., Anonsen, J.H., Bilsborough, S., Schneider, A., Egge-Jacobsen, W., and Koomey, M. (2009) Broad spectrum O-linked protein glycosylation in the human pathogen *Neisseria gonorrhoeae*. *Proc Natl Acad Sci U S A* **106**: 4447–52.

Voisin, S., Kus, J. V, Houliston, S., St-Michael, F., Watson, D., Cvitkovitch, D.G., Kelly J., Brisson J.R. and Burrows L.L.. (2007) Glycosylation of *Pseudomonas aeruginosa* strain Pa5196 type IV pilins with mycobacterium-like alpha-1,5-linked d-Araf oligosaccharides. *J Bacteriol* **189**: 151–9.

Wang, N., Mackenzie, L., Souza, A.G. De, Zhong, H., Goss, G., and Li, L. (2007) Proteome profile of cytosolic component of zebrafish liver generated by LC-ESI MS/MS combined with trypsin digestion and microwave-assisted acid hydrolysis. *J Proteome Res*

6: 263–72

Weber, B.S., Miyata, S.T., Iwashkiw, J.A., Mortensen, B.L., Skaar, E.P., Pukatzki, S., and Feldman, M.F. (2013) Genomic and functional analysis of the type VI secretion system in *Acinetobacter*. *PLoS One* **8**: e55142.

Whitfield, C. (1995) Biosynthesis of lipopolysaccharide O antigens. *Trends Microbiol* **3**: 178–185.

Wisplinghoff, H., Paulus, T., Lugenheim, M., Stefanik, D., Higgins, P.G., Edmond, M.B., Wenzel, R.P., and Seifert, H. (2012) Nosocomial bloodstream infections due to *Acinetobacter baumannii*, *Acinetobacter pittii* and *Acinetobacter nosocomialis* in the United States. *J Infect* **64**: 282–290.

Yi, E.C., and Hackett, M. (2000) Rapid isolation method for lipopolysaccharide and lipid A from Gram-negative bacteria. *Analyst* **125**: 651–656.

Zhou, M., and Wu, H. (2009) Glycosylation and biogenesis of a family of serine-rich bacterial adhesins. *Microbiology* **155**: 317–27.

3. Exploiting *Acinetobacter* OTases in glycoengineering

3.1 Introduction

The advantages that glycoconjugate vaccines have over polysaccharide vaccines have been acknowledged for some time, particularly in high-risk populations of children under 5 (Pace, 2013). Glycoconjugate vaccines available in the market today are produced by chemical conjugation, which has many drawbacks (Frasch, 2009). In an attempt to address these drawbacks, bacterial glycosylation systems have been utilized to produce glycoconjugates. At the time of thesis preparation, biologically conjugated glycoconjugate vaccines were still in clinical trials (Cuccui and Wren, 2015).

For successful production of recombinant glycoconjugates in bacteria, a bacterial surface glycan-encoding locus must be coexpressed with an oligosaccharyltransferase (OTase) and an acceptor protein in a suitable strain of *E. coli*, with the recombinant glycoprotein subsequently purified (Terra *et al.*, 2012).

3.1.1 *E. coli*: The host strain

Glycoproteins could be recombinantly produced in engineered strains of *E. coli* K12, such as CLM24, CLM37 and SDB1. Most K12 strains cannot synthesize O antigen due to an inactivating insertion in the rhamnosyltransferase *wbbL*, which adds the second sugar to the glycan assembled on the undecaprenol lipid carrier (Liu and Reeves, 1994). In addition to *wbbL*, the strain CLM24 has a *waaL* O antigen ligase deletion. Since lipid-linked glycan is a substrate for *O*-glycosylation and LPS synthesis, this deletion results in the accumulation of lipid-linked glycan to be targeted only for *O*-glycosylation (Feldman *et al.*, 2005). In the strain CLM37, the initiating glycosyltransferase *wecA* has been deleted. This glycosyltransferase adds specifically a GlcNAc at the reducing end of the

oligosaccharide, and knocking it out eliminates any reduction in efficiency associated with synthesizing glycans lacking GlcNAc at the reducing end, since initiating glycosyltransferases are highly specific (Linton *et al.*, 2005). As well, knocking *wecA* out eliminates contamination of the glycoconjugate with another glycan moiety that is also assembled on the undecaprenol lipid carrier, the *E. coli* enterobacterial common antigen (ECA) (Erbel *et al.*, 2003; Wetter *et al.*, 2013). Another strain that is used for glycoconjugate synthesis is the strain SDB1. This strain was constructed by deleting *wecA* in CLM24, thereby being a double mutant of the *waaL* O antigen ligase and the *wecA* glycosyltransferase (Garcia-Quintanilla *et al.*, 2014). Furthermore, glycoconjugates have been previously produced in their native backgrounds by expressing an OTase and an acceptor protein in *Salmonella enterica* serovar Typhimurium and *Yersinia enterocolitica* (Wacker *et al.*, 2006; Iwashkiw *et al.*, 2012)

3.1.2 Expression of the glycan synthesis loci

Glycan synthesis loci have been heterologously expressed in *E. coli* and many were demonstrated to be substrates for OTases, as seen by glycan transfer to acceptor proteins. The glycan must be assembled on the undecaprenol lipid carrier, which could act as a glycan donor for OTases to glycosylate acceptor proteins. Examples of these glycan synthesis loci include the *Campylobacter jejuni* heptasaccharide which decorates surface proteins and is encoded by the *pgl* locus (Wacker *et al.*, 2002). CPS and O antigen synthesis loci have also been expressed in *E. coli*. CPS from *Staphylococcus aureus* and O antigens from *Pseudomonas aeruginosa* O11, *Salmonella enterica* serovar Typhimurium, *Shigella dysenteriae* and *Burkholderia pseudomallei* were glycan donors

for OTases to glycosylate proteins (Moreau *et al.*, 1990; Goldberg *et al.*, 1992; Neal *et al.*, 1993; Wacker *et al.*, 2006; Faridmoayer *et al.*, 2008; Ihssen *et al.*, 2010; Garcia-Quintanilla *et al.*, 2014).

3.1.3 OTase expression and glycan specificity

Numerous *N*-OTases have been successfully expressed in *E. coli*, the first being the *C. jejuni* PglB (CjPglB) (Wacker *et al.*, 2002). Other *N*-OTases expressed in *E. coli* include those of *Helicobacter pullorum*, *Desulfovibrio desulfuricans* and *C. lari* (Jervis *et al.*, 2010; Ielmini and Feldman, 2011; Lizak *et al.*, 2011). Glycan specificity of PglBs is believed to depend on the sugar at the reducing end of the glycan (Wacker *et al.*, 2006). Identified PglBs to date require an acetamido group at C-2 of the reducing end sugar of the glycan substrate, and it is believed to play a role in glycan substrate recognition. Therefore, PglBs could transfer glycans with GlcNAc, Bac, FucNAc and GalNAc at the reducing end, but not the *S. enterica* S. Typhimurium O antigen, which has a Gal residue without an acetamido group at the reducing end (Table 3.1) (Wacker *et al.*, 2006). Additionally, CjPglB was demonstrated to transfer *B. pseudomallei* O polysaccharide II, the equivalent of O antigen. This glycan is a polymer of disaccharide repeating subunits composed of glucose and deoxytalose (Garcia- Quintanilla *et al.*, 2014).

As well, *N*-OTases from the three domains of life were demonstrated to require divalent cations for activity, usually Mg^{2+} or Mn^{2+}, and they were shown bound to the catalytic pocket of the crystallized *C. lari* PglB (Lairson *et al.*, 2008; Lizak *et al.*, 2011; Cohen-Rosenzweig *et al.*, 2014). However, CjPglB activity was demonstrated without exogenous supplementation of Mn^{2+} (Garcia-Quintanilla *et al.*, 2014).

O-OTases have also been expressed in *E. coli*, with examples including the *P. aeruginosa* TfpO as well as PglLs from *Neisseria meningitidis*, *Vibrio cholerae*, *Burkholderia* spp. and the phytopathogen *Ralstonia solanacearum* (Faridmoayer *et al.*, 2007; Gebhart *et al.*, 2012; Lithgow *et al.*, 2014; Elhenawy *et al.*, 2015). Like *N*-OTases, the glycan specificity of *O*-OTases is believed to depend on the glycan at the reducing end (Horzempa *et al.*, 2006). *N. meningitidis* PglL (NmPglL) is the best characterized *O*-OTase to date, and was demonstrated to have a relaxed specificity towards glycan substrates. NmPglL transferred almost all glycans to target proteins, including the *Salmonella* O antigen, which has a hexose at the reducing end that could not be transferred by CjPglB (Table 3.1). However, glycans containing a Glc residue at the reducing end such as the *Streptococcus pneumoniae* CPS could not be transferred by NmPglL (Table 3.1) (Feldman MF, Personal communication). Intriguingly, PglL transferred also peptidoglycan subunits to target proteins, as they are also assembled on the undecaprenol lipid carrier (Bouhss *et al.*, 2008; Faridmoayer *et al.*, 2008). Furthermore, NmPglL also transferred glycans *in vitro* from the much shorter synthetic lipid carrier farnesyl pyrophosphate, demonstrating a relaxed specificity towards the lipid carrier (Faridmoayer *et al.*, 2008). As well, NmPglL was able to transfer nucleotide activated monosaccharides to acceptor proteins *in vitro* (Musumeci *et al*., 2013). Another example is $PglL_{Vc}$ from *V. cholerae*, which was never demonstrated to be active in its native strain. However, OTase activity in *E. coli* was demonstrated, as $PglL_{Vc}$ transferred *C. jejuni* LLO and diNAcBac to acceptor proteins, and rather unusually, to itself (Gebhart *et al.*, 2012).

TfpO from *P. aeruginosa* strain 1244 transferred multiple *Pseudomonas* O antigens from other strains to pilin subunits, all of which are either tri or tetrasaccharides with FucNAc

at the reducing end. TfpO also transferred the *E. coli* O157 antigen, a tetrasaccharide with GalNAc at the reducing end (DiGiandomenico *et al.*, 2002; Horzempa *et al.*, 2006). However, a later study showed that TfpO could only transfer short chain oligosaccharides of the *E. coli* O7 antigen, a pentasaccharide with GlcNAc at the reducing end. This was demonstrated by comparing O7 antigen synthesis clusters from the wild type and a *wzz* chain length regulator mutant, which would lead to an abundance of short chain oligosaccharides with few repeating subunits (Marolda *et al.*, 1999). TfpO successfully transferred up to two subunits of the O7 antigen from the *wzz* mutant, but not from the wild type cluster (Faridmoayer *et al.*, 2007). This feature is a serious limitation for the use of TfpO for vaccine purposes.

In addition to having different glycan and protein specificities, PglLs and TfpOs are sequentially and phylogenetically distinct, which suggests that PglL and TfpO comprise two distinct classes of *O*-OTases. However, PglL-like OTases appear to be far more superior and with more potential in glycoengineering than TfpO-like OTases, given their ability to transfer longer oligosaccharides and their more relaxed glycan specificity.

3.1.4 Acceptor proteins

N-OTases transfer glycans to asparagine residues of acceptor proteins that lie in a specific sequon. For CjPglB, it was found that the asparagine (N) residue must reside in the sequon D/EXNXS/T, with X being any amino acid except proline, and the residue in the -2 position being acidic, namely glutamic acid (D) or aspartic acid (E) (Kowarik *et al.*, 2006). Another example is the *D. desulfuricans* PglB which, similar to its eukaryotic counterpart, glycosylates asparagine residues in a NXS/T sequon without an acidic -2

residue (Ielmini and Feldman, 2011). For CjPglB, the DQNAT sequon was identified to be the optimal sequon for glycosylation (Chen *et al.*, 2007). Insertion of this sequon, termed "glycotag", at N or C termini of unglycosylated proteins such as *E. coli* maltose-binding protein MalE induced its glycosylation by CjPglB (Fisher *et al.*, 2010).

On the other hand, no consensus sequence has been identified for the glycosylation site of *O*-OTases to date. Instead, the glycosylated residues identified were in low complexity regions, with an abundance of serine, proline and alanine residues (Vik *et al.*, 2009). This limits the tools available for using PglLs in glycoengineering, as the acceptor proteins are limited to the natural substrates of PglLs. However, a similar idea to the *N*-glycosylation glycotags has been employed in *P. aeruginosa*, where TfpO only glycosylates PilA at the terminal serine. Other proteins with the C terminal 15 residues of PilA (where the glycosylation site is) fused to their C terminal were successfully glycosylated by TfpO (Qutyan *et al.*, 2010)

3.2 Objectives

As mentioned in the previous chapter, most members of *Acb* encode 2 functional *O*-OTases, one being a TfpO-like OTase that only glycosylates Type IV pilin (Tfp). The other is a PglL-like OTase that has relaxed specificity and glycosylates multiple proteins. However, the environmental isolate *A. baylyi* is an exception, as both OTases are homologous to PglL. Both PglLs have the same protein specificities as other *Acb* OTases, as $PglL_{ComP}$ is only specific for glycosylating the Tfp ComP. $PglL_{ADP1}$ on the other hand glycosylates multiple proteins, some of which have been identified in the previous chapter. In this work, we characterize the glycan specificity of $PglL_{ComP}$ and Pgl_{ADP1} to

realize their potential in the glycoconjugate vaccine industry. We particularly focus on the Pneumococcus capsular polysaccharide, which could not be transferred by previously identified *N*- and *O*-OTases.

Reducing end glycan	**Source**	**CjPglB**	**NmPglL**
DATDH	*C. jejuni* LLO	Yes	Yes
Gal	*S. enterica* S. Typhimurium	No	Yes
GalNAc	*E. coli* O86	Yes	Yes
FucNAc	*P. aeruginosa* O11	Yes	Yes
Glc	*S. pneumoniae* CPS	No	No
GlcNAc	*E. coli* O7	Yes	Yes

Table 3.1 Reducing end glycan specificities of the *N*-OTase CjPglB and the *O*-OTase NmPglL. *N*-OTases require an acetamido group in C-2 of the reducing end sugar, and therefore could not transfer glycans with Glc and Gal residues at the reducing end. NmPglL has more relaxed glycan specificity and was demonstrated to transfer all glycans including hexoses, but not the *S. pneumoniae* CPS, which has a Glc residue at its reducing end. Based on published and unpublished data (Feldman *et al.*, 2005; Wacker *et al.*, 2006; Faridmoayer *et al.*, 2008)

3.3 Materials and methods:

3.3.1 Strains and plasmids

A list of the Bacterial strains and plasmids used in this study could be found in table 3.2. *A. baylyi* genomic DNA was isolated using the DNeasy blood and tissue kit (Qiagen). For amplifying the gene *aciad3337* (*pglL*$_{ComP}$) with its upstream non-coding region, primers igrF (ACTGGTCGACTAGTAGTACTATATGGCTTTAAA) and igrR (ACTGCTGCAGTTAATATTCTATTGAACAAAATTTTAAC) were used. The resulting PCR product was digested with *SalI* and *PstI* and inserted in the same sites of pEXT20, creating pMN8. Plasmid pMN1 was constructed by subcloning ComP from pBAV-ComP-his into *BamHI* and *SalI* sites of pEXT20. Plasmid pMN2 was constructed by subcloning *pglL*$_{ComP}$ with its upstream region from the *SalI* and *PstI* sites of pMN8 to pMN1.

Table 3.2: Bacterial Strains and plasmids used in this study

Strain/Plasmid	Description	Reference/ Source
Strains		
E. coli CLM24	W3110, Δ*waaL* ligase	Feldman *et al*. (2005)
E. coli CLM37	W3110, Δ*wecA* glycosyltransferase	Linton *et al*. (2005)
E. coli SDB1	W3110, Δ waaL ligase, ΔwecA glycosyltransferase	Garcia-Quintanilla *et al*. (2014)
E. coli DH5α	General cloning strain	Invitrogen
Plasmids		
pEXT20	Cloning vector, Amp^R, IPTG inducible	Dykxhoorn *et al.*, (1996)
pBAV-ComP-his	C-6X His-tagged ComP cloned in *BamHI* and *SalI* sites of pBAVmcs, Kan^R, constitutive expression	Harding *et al*., (2015)
pMN1	C-6X His-tagged ComP cloned in *BamHI* and *SalI* sites of pEXT20, Amp^R, IPTG inducible	This work
pMN2	Non-coding region and $PglL_{ComP}$ cloned in *SalI* and *PstI* sites of pMN1, Amp^R, IPTG inducible	This work
pMN3	ComP S69A in pMN2 background	This work
pMN4	$PglL_{ComP}$ H325A in pMN2 background	This work
pMN8	Non-coding region and $PglL_{ComP}$ cloned in *SalI* and *PstI* sites of pEXT20, Amp^R, IPTG inducible	This work
pMAF10	HA-tagged PglB cloned in pMLBAD, TpR, Arabinose inducible	Feldman *et al*. (2005)
pAMF10	C-10× His-tagged NmPglL cloned inyo pEXT20, AmpR, IPTG inducible	Faridmoayer *et al.*, (2008)
pIH18	C-6X His-tagged AcrA from C. cloned into pEXT21, SpR, IPTG inducible	Hug *et al*., 2010
pAMF22	C-6X His-tagged *dsbA1* from *N. meningitidis* MC58 cloned into pMLBAD, Tp^R Arabinose inducible	Faridmoayer *et al.*, (unpublished)
pACYCpglBmut	pACYC184-based plasmid encoding the *C. jejuni* pgl locus with mutations W458A and D459A in PglB. Cm^R, IPTG inducible.	Wacker *et al*., (2002)
pEXT20-$pglL_{ADP1}$	pEXT20 expressing C-6X His-tagged $pglL_{ADP1}$ from *A. baylyi* inserted at BamHI and SalI, Amp^R, IPTG inducible	Harding *et al*., (2015)
pLPS2	*P. aeruginosa* O11 antigen synthesis cluster, Tet^R	Goldberg *et al.*, (1992)
pJHCV32	*E. coli* O7 antigen synthesis cluster, Tet^R	Feldman *et al.*, (1999)
pNLP80	*S. pneumoniae* CPS14 cluster on pWSK129, Kan^R	Price *et al*., unpublished
pOSH59	*S. pneumoniae* CPS15b cluster on pACT3, Cm^R	Posch *et al*., unpublished
pPR1347	*S. enterica* serovar Typhimurium O antigen synthesis cluster, Kan^R	Neal *et al.*, (1993)

3.3.2 Glycosylation in *E. coli*

Electrocompetent *E. coli* CLM24, CLM37 or SDB1 were prepared as per the protocol described by Dower and colleagues (Dower *et al.*, 1988). Cells were transformed with plasmids encoding the glycan synthesis loci, acceptor protein and OTase. For plasmid selection, ampicillin (100 μg/ml), tetracycline (20 μg /ml), trimethoprim (50 μg /ml), chloramphenicol (12.5 μg /ml), kanamycin (20 μg /ml) and spectinomycin (80 μg /ml) were added as needed. Cells were grown at 37°C in LB broth to an OD_{600} of 0.4-0.6, induced with 0.05 mM or 0.1 mM IPTG or 0.2% arabinose as required and left overnight at 37°C. Cultures requiring arabinose induction received a second dose of arabinose after 4 hours. Whole cell lysates were obtained at stationary phases, of which 0.1 OD were loaded on 12.5% SDS-PAGE gels, which were then transferred to nitrocellulose membranes. Western blot analysis was employed to determine protein modification and antibodies used are outlined in table 3.2. Nitrocellulose membranes were visualized using the Odyssey Infrared Imaging System (LiCor Biosciences, USA). A list of the antibodies used could be found in table 3.3.

Table 3.3: Antibodies employed in this study

Antibody	Description	Reference/ Source
Anti His Poly	Rabbit polyclonal, 1:4000	Rockland
Anti His Mono	Mouse monoclonal, 1.5000	Abcam
Anti CPS14	Rabbit polyclonal, 1:1000	Statens
Anti CPS15b	Rabbit polyclonal, 1:1000	Statens
Anti *Pseudomonas* O11 antigen	Rabbit polyclonal, 1:500	Lam lab, University of Guelph
Anti *Salmonella* LT2 O antigen	Rabbit polyclonal, 1:1000	Statens
hR6	Rabbit polyclonal, 1:200000	Aebi lab
Goat Anti rabbit IR680	1:15000	Li-Cor
Goat Anti mouse IR800	1:15000	Li-Cor

3.3.3 Total membrane preparation

Cells grown overnight at 37°C were washed with phosphate buffered saline (PBS) buffer and resuspended in the same buffer. Cells were lysed by two rounds of cell disruption using a French press (Constant systems Ltd) followed by the addition of a protease inhibitor cocktail (Roche). Lysates were centrifuged for 30 minutes at 10000 x *g* to get rid of cell debris and supernatants were then ultracentrifuged at 100000 x *g* for 60 minutes to pellet total membranes. The pellet was resuspended in a buffer containing 0.1% Triton x100 or 1% n-dodecyl-β-D-maltoside (DDM) in 10 ml PBS and membrane proteins were solubilized by tumbling overnight. An equal volume of PBS was added to the suspension to reduce detergent concentration and the suspension was ultracentrifuged at 100000 G for 60 minutes. Supernatants, which correspond to solubilized membrane proteins, were loaded on columns for nickel affinity protein purifications.

3.3.4 Nickel affinity protein purifications

Hexa-histidine-tagged proteins were purified from solubilized total membranes by nickel affinity chromatography. Briefly, total membranes were loaded on nickel-nitrilotriacetic acid (Ni-NTA) agarose columns (Qiagen) previously equilibrated with a buffer containing 20 mM imidazole. To remove unbound proteins, the column was washed four times each with buffers containing 20 mM and 30 mM imidazole. His-tagged proteins bound to the column were eluted over six fractions with an elution buffer containing 250 mM imidazole.

We have also employed the ÄKTA purifier (Amersham Biosciences, Sweden) for protein purifications. Solubilized membrane proteins were first filtered through 0.45 μm and 0.22

μm filters, and then loaded on a His-Trap HP column (GE Healthcare) previously equilibrated with a buffer containing 20 mM imidazole. Unbound proteins were removed by washing the column ten times each with buffers containing 20 mM and 30 mM imidazole. To elute proteins bound to the column, gradient elutions with an incremental increase in imidazole concentration of the elution buffer were used. The majority of ComP was found eluted between 180 mM and 250 mM imidazole.

3.3.5 Site-directed mutagenesis

Site-directed mutagenesis was carried out to mutate the residues H325 in PglLComP and S69 of ComP as previously described (Fisher and Pei, 1997). Mutagenic primers were designed using Primer X, a web-based primer design program (http://www.bioinformatics.org/primerx/). Primers used for H325A $PglL_{ComP}$ were H325AF (GAGAATGGTTTACATACTCAGCGAATTTGTTCTTAGATTTAATG) and H325AR (CATTAAATCTAAGAACAAATTCGCTGAGTATGTAAACCATTCTC). For S69A ComP, primer sets S69AF (GAGCCTGCAGTAGCAGGAGTACC) and S69AR (CAGGTACTCCTGCTACTGCAGGC) were used. PCR reactions were done using *Pfu* polymerase and 2 ng of pMN2 as template. The PCR reaction consisted of an initial denaturation of 30 s at 95˚c followed by 16 cycles of 30 s at 95˚c, 60 s at 55˚c, 360 s at 68˚c, with no final extension. The PCR reactions were *Dpn* digested for 1 hour to remove the template plasmid, then transformed into electrocompetent DH5α cells and grown on ampicillin for plasmid selection. Colonies were sequenced to confirm knockouts.

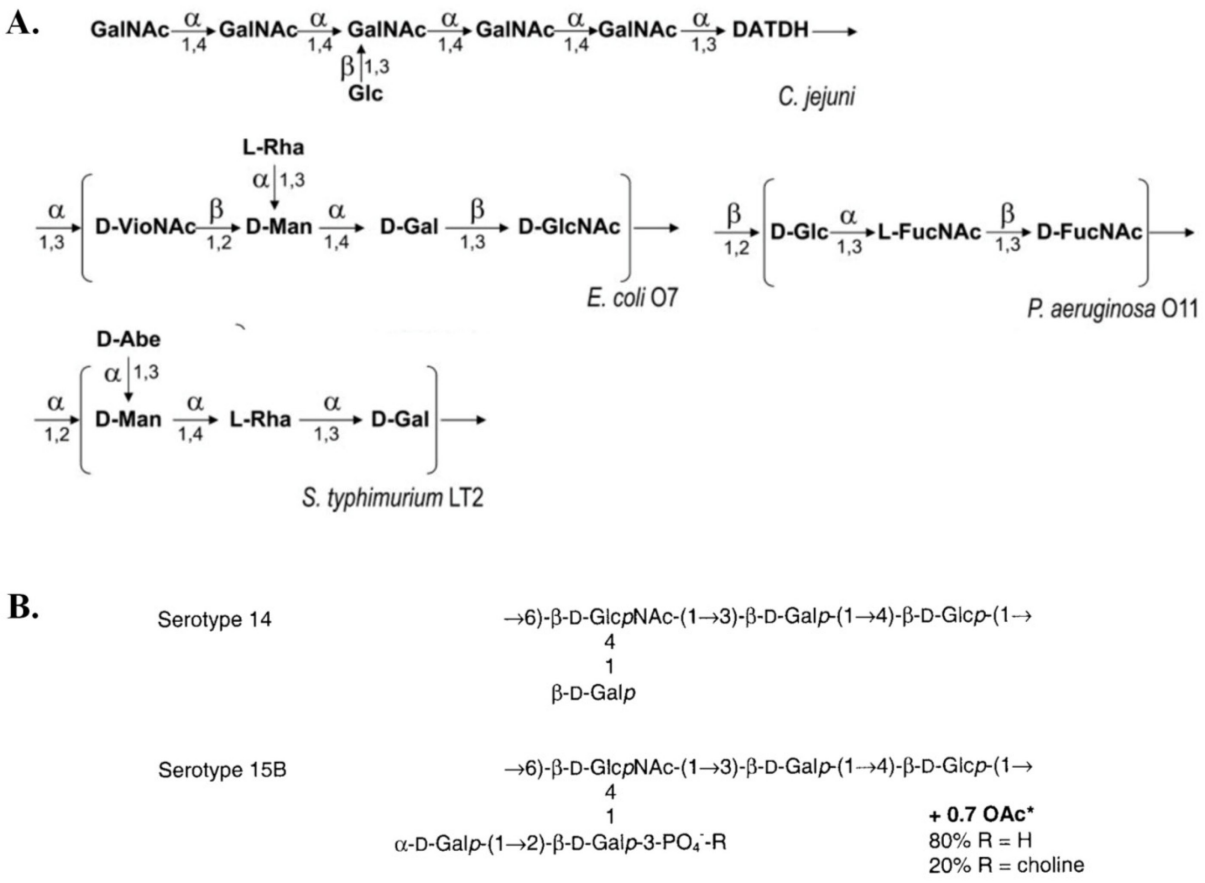

Figure 3.1 A. Structures of the *C. jejuni* LLO, *P. aeruginosa* O11 antigen, *Salmonella. enterica* s. Typhimurium O antigen and *E. coli* O7 antigen, modified from Faridmoayer *et al.*, (2008).

B. Structures of CPS from *S. pneumoniae* serotypes 14 and 15b, modified from van Selm *et al.*, (2003).

3.4 Results:

3.4.1 In the absence of other LLO, $PglL_{ComP}$ and $PglL_{ADP1}$ transfer an unknown glycan moiety in *E. coli*

It was previously shown that NmPglL interferes with the peptidoglycan biosynthetic machinery, as it transfers incomplete peptidoglycan subunits of MurNAc(GlcNAc) linked to S63 of the *N. meningitidis* Tfp PilE (Faridmoayer *et al.*, 2008). We sought to determine if $PglL_{ComP}$ and $PglL_{ADP1}$ transfer a similar glycan moiety to their acceptor proteins. Western blot analysis on whole cell lysates of *E. coli* CLM24 or SDB1 expressing his-tagged ComP with or without $PglL_{ComP}$ show bands with lower electrophoretic mobility than the unglycosylated form in lanes 2 and 3 where ComP and $PglL_{ComP}$ are coexpressed (Figure 3.2). This band disappears upon Proteinase K digestion (Lane 4), suggesting that it is a modified form of ComP. For $PglL_{ADP1}$, we have employed the protein DsbA1 from *N. meningitidis* as an acceptor protein, as it was shown to be compatible with a number of PglLs (Gebhart *et al.*, 2012; Lithgow *et al.*, 2014; Harding *et al.*, 2015; Elhenawy *et al.*, 2015). Similar to $PglL_{ComP}$, lower electrophoretic mobility bands could be seen in lanes 6 and 7 where DsbA1 and $PglL_{ADP1}$ are coexpressed, which disappear after Proteinase K digestion (Lane 7). This suggests a PglL-dependent modification of acceptor proteins in *E. coli*. Since this occurs in both CLM24 and SDB1, the possibility of this unknown moiety being enterobacterial common antigen (ECA) is less likely, as *wecA*, which is required for ECA synthesis is knocked out in SDB1. This finding is useful in understanding the activity of $PglL_{ComP}$ and $PglL_{ADP1}$, and mass spectrometry is required to correctly identify this unknown moiety.

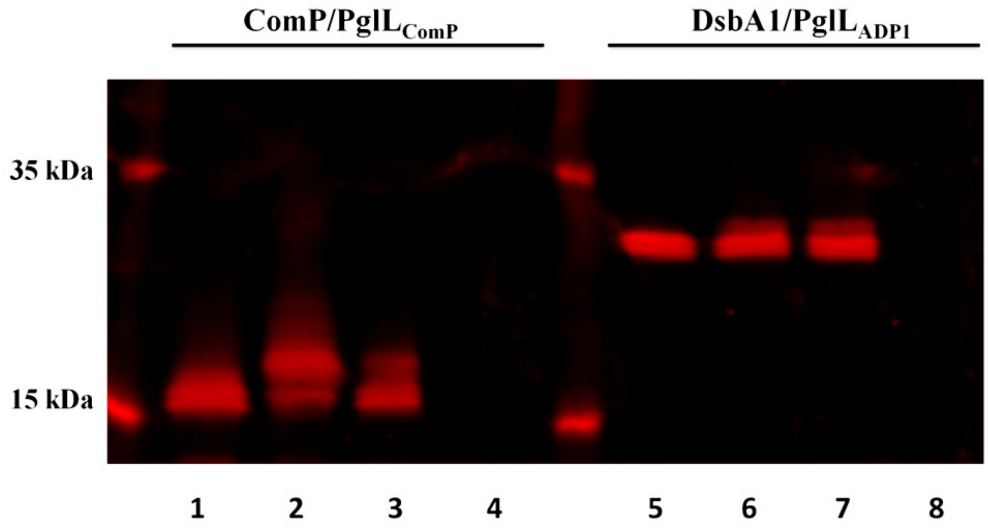

Figure 3.2 Western blot analysis probing for expression of his-tagged ComP (lanes 1-4) and DsbA1 (lanes 5-8) with a monoclonal anti-his antibody. ComP and DsbA1 are expressed in *E. coli* CLM24 in lanes 1 and 5 respectively. Coexpression of ComP with PglL$_{ComP}$ in CLM24 (lane 2) and SDB1 (lane 3) indicate its modification in a PglL$_{ComP}$-dependent manner. DsbA1 is also modified in a PglL$_{ADP1}$-dependent manner in CLM24 (lane 6) and SDB1 (lane 7). Modifications of ComP and DsbA1 disappear after Proteinase K digestion in lanes 4 and 8 respectively.

3.4.2 H325 of $PglL_{ComP}$ is required for OTase activity

A conserved residue in the large periplasmic loop of *O*-OTases and O antigen ligases has been demonstrated to be essential for their activity, as it was suggested to play a role in binding phosphate groups of Und-PP (Schild *et al.*, 2005; Pérez *et al.*, 2008). The importance of the equivalent residue H349 of NmPglL has been established, as its mutation leads to abolishing OTase activity both *in vivo* and *in vitro* (Musumeci *et al.*, 2014). The residue H325 of $PglL_{ComP}$ is the homologous residue to H349 of NmPglL, and coexpressing H325A $PglL_{ComP}$ and ComP (pMN4) in *E. coli* CLM24 shows that $PglL_{ComP}$ has indeed lost its activity. Western blot analyses show only the high electrophoretic mobility band of ComP, indicating the lack of glycosylation with the *E. coli* glycan moiety in lane 3 (Figure 3.3).

3.4.3 Deleting S69 of ComP did not abolish glycosylation

Tfp proteins display a high degree of sequence variation, especially towards the C-terminal (Nassif *et al.*, 1993; Power *et al.*, 2004). The Tfp PilE from *Neisseria* spp. are glycosylated at Serine 63, and additional glycosylation sites have later been identified for *N. meningitidis* (Parge *et al.*, 1995; Hegge *et al.*, 2004; Faridmoayer *et al.*, 2007; Musumeci *et al.*, 2014). This residue appears to be conserved, and recently the Tfp PilA from the phytopathogen *Ralstonia solanacearum* was shown to be glycosylated in a peptide encompassing the S63 region, with the exact glycosylation site yet to be identified (Elhenawy *et al.*, 2015). The residue S69 in ComP is homologous to the *Neisseria* S63 residue, and we hypothesized that it would be the glycosylation site. We have substituted the residue with alanine, and expressed S69A ComP with $PglL_{ComP}$

(pMN3) in *E. coli* CLM24. Western blot analyses reveal that this substitution did not abolish ComP glycosylation by the unknown *E. coli* glycan moiety in lane 2 (Figure 3.3).

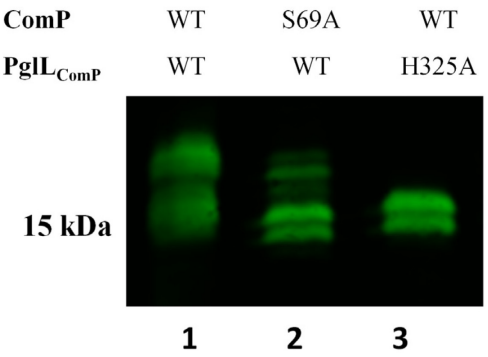

Figure 3.3 Western blot analyses of *E. coli* CLM24 whole cell lysates coexpressing S69A ComP with PglL$_{ComP}$ (lane 2) show the same electrophoretic mobility as wild type glycosylated ComP (lane 1). H325A mutation of PglL$_{ComP}$ abolishes glycosylation by the *E coli* glycan moiety, seen in the lack of low electrophoretic mobility bands when H325A PglL$_{ComP}$ and ComP are coexpressed in *E. coli* CLM24 (lane 3). Expression was probed for with a monoclonal anti-his antibody.

3.4.4 *N*-acetylated glycans are substrates for $PglL_{ComP}$ and $PglL_{ADP1}$

An acetamido group at C-2 of the reducing end sugar is a prerequisite for glycan transfer by PglB to acceptor proteins. PglB was demonstrated to transfer glycans with FucNAc, GalNAc, GlcNAc and Bac residues at the reducing end (Feldman *et al.*, 2005; Wacker *et al.*, 2006). As well, NmPglL was able to transfer these glycans (Faridmoayer *et al.*, 2008). To determine if $PglL_{ComP}$ and $PglL_{ADP1}$ could transfer these glycans, $PglL_{ComP}$ and ComP (pMN2) or $PglL_{ADP1}$ (pEXT20-$pglL_{ADP1}$) and DsbA1 (pAMF22) were coexpressed in *E. coli* with glycan synthesis loci. These glycan synthesis loci encode the synthesis of *P. aeruginosa* O11 antigen (pLPS2), *E. coli* O7 antigen (pJHCV32) and *C. jejuni* LLO (pACYC$pglB_{mut}$), which have FucNAc, GlcNAc and Bac residues at the reducing end respectively (Figure 3.1A). Western blot analyses using antibodies against expression of O11 antigen or *C. jejuni* LLO (red) and the protein his-tag (green) show glycosylated ComP and DsbA1 in lane 2, visualized as an overlay of both signals (yellow) (Figure 3.4 panels A and B). Panel C of figure 3.4 shows the glycosylation by O7 antigen subunits with only protein expression probed for (green), as an anti O7 antigen was not available in the lab at the time of thesis preparation. Treatment with Proteinase K in lane 3 removes the lower electrophoretic mobility bands corresponding to glycosylated ComP (Figure 3.4).

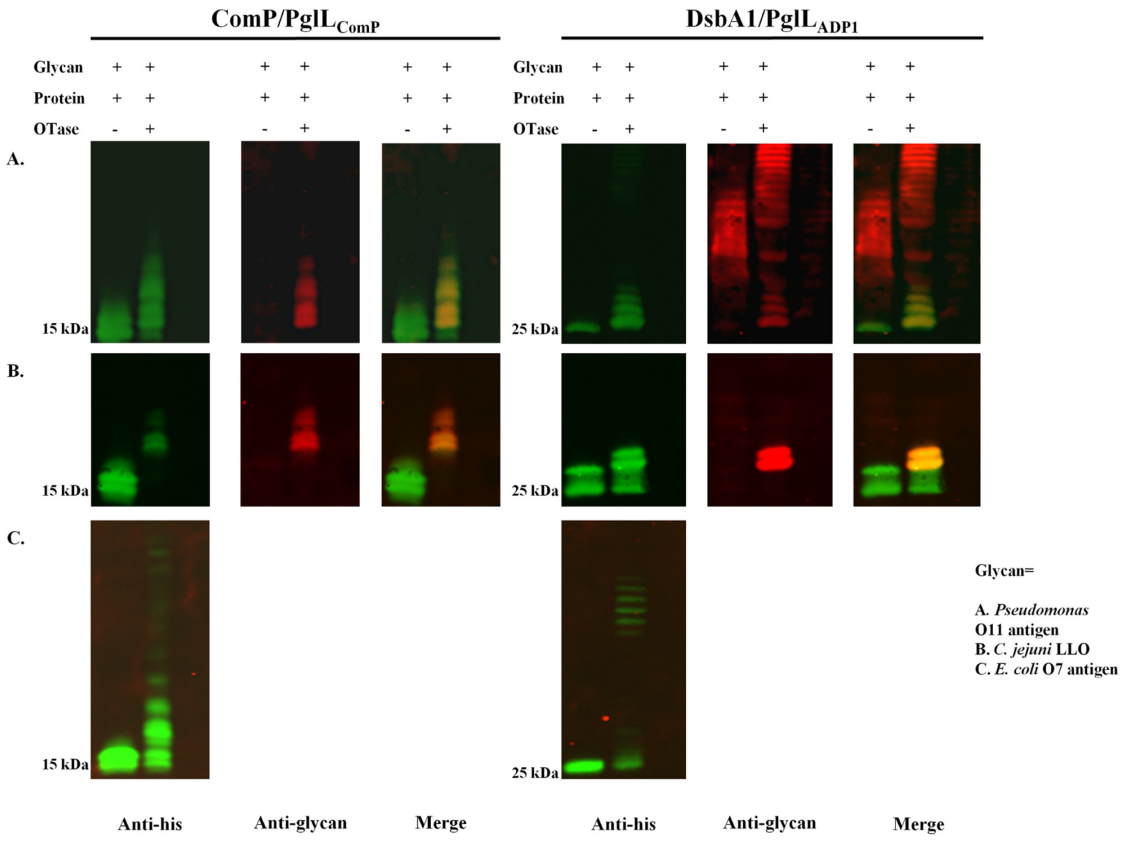

Figure 3.4 Western blot analyses on *E. coli* CLM24 whole cell lysates expressing as indicated ComP/PglL$_{ComP}$ or DsbA1/PglL$_{ADP1}$ coexpressed with (A) *P. aeruginosa* O11 antigen, (B) *C. jejuni* LLO and (C) *E. coli* O7 antigen. Lane 1 corresponds to unglycosylated acceptor protein, with no OTases expressed. In lane 2, acceptor proteins and OTases are coexpressed and glycosylation is detected by the lower electrophoretic mobility bands that react to both monoclonal anti-his (green) and polyclonal anti-glycan antibodies (red), seen as yellow when signals are merged. Glycoprotein signals disappear upon Proteinase K digests (lane 3). Expression from panel C was probed for with anti-his antibody only (green).

3.4.5 $PglL_{ComP}$ and $PglL_{ADP1}$ transfer glycans with Gal residues at the reducing end

PglB could not transfer glycans with hexose residues at the reducing end, as evident by the lack of transfer of the *S. enterica* S. Typhimurium O antigen to AcrA (Wacker *et al.*, 2006). However, directed evolution of PglB by engineering the glycan-binding pocket has led to identifying a mutant that could transfer this glycan (Ihssen *et al.*, 2015). NmPglL was shown to transfer this *Salmonella* O antigen to PilE previously, as it has a more relaxed glycan specificity than PglB (Faridmoayer *et al.*, 2008). Either $PglL_{ComP}$ and ComP or $PglL_{ADP1}$ and DsbA1 were coexpressed with the plasmid pPR1347 encoding the *S. enterica* S. Typhimurium O antigen. Expression was probed for with antibodies against the O antigen (red) and the protein his-tag (green). Colocalization of both signals in lane 2 and the absence of a signal after Proteinase K digestion in lane 3 indicates that $PglL_{ComP}$ and $PglL_{ADP1}$ glycosylate ComP and DsbA1 respectively with the *Salmonella* O antigen (Figure 3.5).

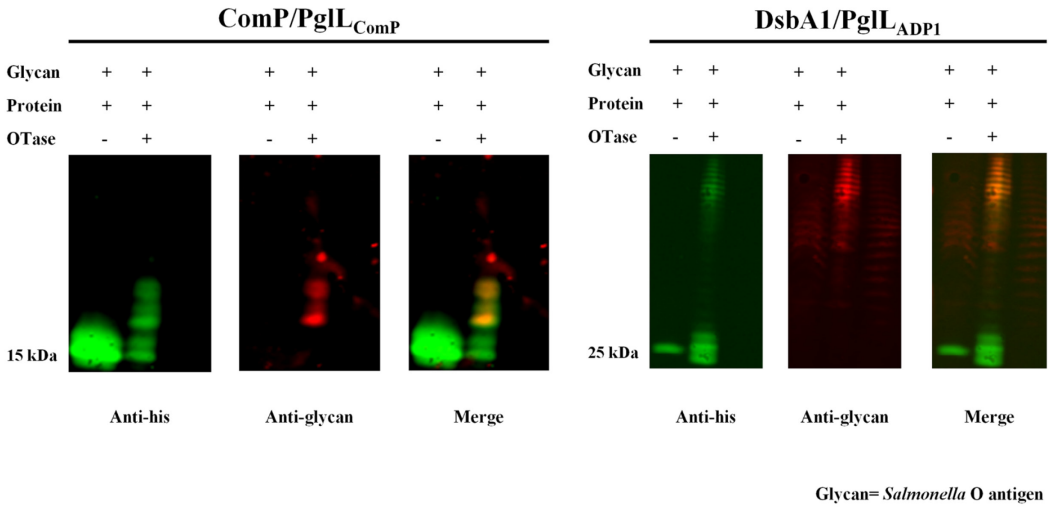

Figure 3.5 Western blot analyses of *E. coli* CLM24 whole cell lysates expressing the *S. enterica* S. Typhimurium O antigen with either ComP and PglL$_{ComP}$ or DsbA1 and PglL$_{ADP1}$ as indicated. Lane 2, which corresponds to OTase coexpressed with acceptor protein, shows bands with lower electrophoretic mobility than when acceptor protein only is expressed in lane 1. These slower bands react to both monoclonal anti-his (green) and polyclonal anti-*Salmonella* O antigen (red) seen by signal overlay (yellow) indicating glycosylation of ComP and DsbA1 by O antigen subunits. Glycoprotein signal disappears upon Proteinase K digestion (lane 3).

3.4.6 Only $PglL_{ComP}$ can transfer glycans with Glc residues at the reducing end

Glycans with Glc residues at the reducing end, characteristic of the Pneumococcus CPS, are not substrates of PglB due to the lack of the C-2 acetamido group (Wacker *et al.*, 2006; Appendix B figure S2.1). Furthermore, NmPglL could not transfer these glycans (unpublished results; Appendix B figure S2.2), despite transferring glycans with Gal residues at the reducing end (Faridmoayer *et al.*, 2008). Here we tested if $PglL_{ComP}$ and $PglL_{ADP1}$ would be able to transfer these glycans to ComP and DsbA1 respectively. We hypothesized that $PglL_{ComP}$ might have a different glycan specificity from $PglL_{ADP1}$ and NmPglL, as it appears to constitute its own class of *O*-OTases, and could be able to transfer Pneumococcus CPS subunits to ComP. To test this hypothesis, we coexpressed pNLP80, which encodes the CPS synthesis cluster of Pneumococcus serotype 14 (CPS14), with $PglL_{ComP}$ and ComP in *E. coli* SDB1. Western blot analyses probing for expression from *E. coli* whole cell lysates with antibodies against CPS14 and the his-tag did not detect glycosylation of ComP with CPS14 (Figure 3.6). The lower electrophoretic mobility bands in these lanes most likely belong to acceptor proteins glycosylated with the unknown *E. coli* glycan moiety described earlier, as no colocalization between the signals of both antibodies could be seen.

We proceeded to detect protein glycosylation in purified protein samples. Ni-NTA agarose beads were used to purify proteins from total membranes solubilized by Triton X-100, which solubilizes inner membrane proteins (Schnaitman, 1971). Western blot analyses on purified ComP show bands with lower electrophoretic mobility with signal colocalization from the CPS14 and his-tag antibodies (Figure 3.7A). For purifications with fewer impurities, we switched the detergent Triton X-100 to DDM for solubilizing

membrane proteins and utilized a His-trap column connected to the ÄKTA FPLC system. This yielded elution fractions that show the same lower electrophoretic mobility bands of ComP modified by CPS14 subunits (Figure 3.7B). These bands disappeared after Proteinase K digests, suggesting the transfer of multiple subunits of CPS14 to ComP in a $PglL_{ComP}$-dependent manner (Figure 3.7C).

DsbA1 purifications from *E. coli* SDB1 coexpressing $PglL_{ADP1}$ with DsbA1 and CPS14 show a band around the 25 kDa mark. This band corresponds to unglycosylated DsbA1, although capsule expression is markedly reduced. As well, faint lower electrophoretic mobility bands corresponding to glycosylation by the *E. coli* glycan moiety that do not react to the anti CPS14 antibody could be seen. This indicates that CPS14 subunits were not transferred to DsbA1 by $PglL_{ADP1}$ (Figure 3.8)

As a proof of principle, we tested if $PglL_{ComP}$ would be able to transfer CPS from serotype 15b (CPS15b), which also has a Glc residue at the reducing end (Figure 3.1B). A similar result to CPS14 is seen with ComP purified from *E. coli* SDB1 coexpressing the CPS15b synthesis locus (pOSH59), $PglL_{ComP}$ and ComP. Lower electrophoretic mobility bands that react to antibodies against CPS15b and the protein his-tag are seen (Figure 3.9A), which disappear upon Proteinase K digestion (Figure 3.9B), suggesting the transfer of multiple CPS15b subunits.

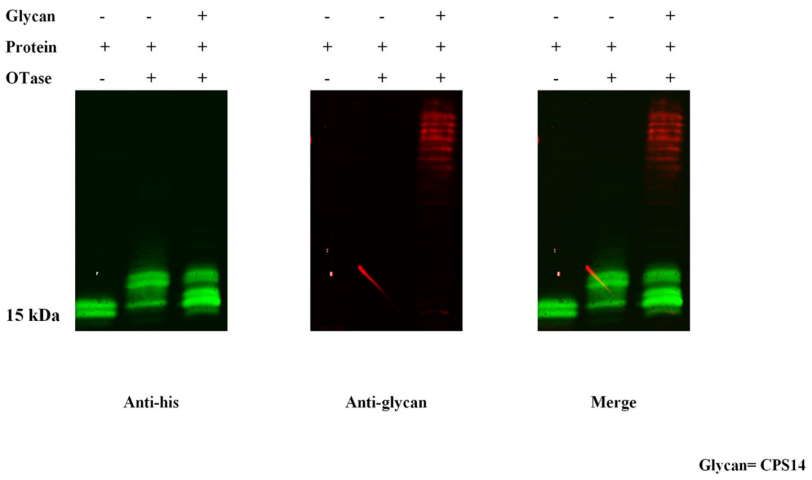

Figure 3.6 Western blot analyses of whole cell lysates of *E. coli* SDB1 expressing different combinations of the Pneumococcus CPS14 synthesis locus with ComP and $PglL_{ComP}$. Lane 3, where ComP, $PglL_{ComP}$ and CPS14 are coexpressed shows bands with lower electrophoretic mobility relative to ComP only (lane 1). These bands have a similar mobility to lane 2 where ComP and $PglL_{ComP}$ are coexpressed, and only react to anti-his (green) but not anti-CPS14 (red). This indicates protein modification in a PglL-dependent manner, but not with CPS14 subunits.

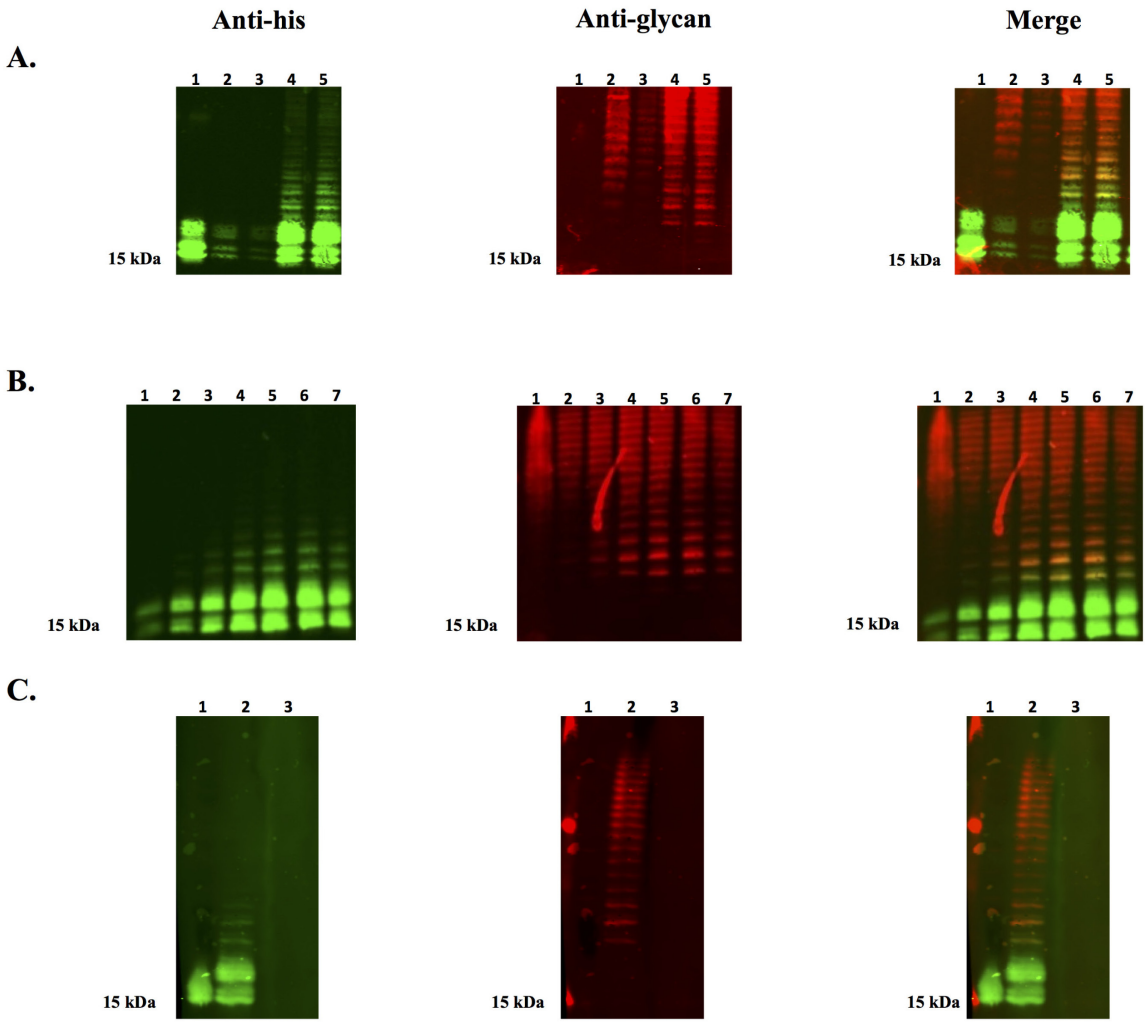

Figure 3.7

A. Western blot analyses on Ni-NTA purifications of his-tagged ComP coexpressed with $PglL_{ComP}$ and the Pneumococcus CPS14 synthesis locus in *E. coli* SDB1 (lanes 2-5). Lanes 2 and 3 represent column washes. Lanes 4 and 5 are elution fractions, showing bands with lower electrophoretic mobility than ComP glycosylated with the *E. coli* glycan moiety (lane 1). These bands react to both monoclonal anti-his (green) and polyclonal anti CPS14 antibodies (red), seen as a signal merge (yellow), which indicates ComP glycosylation with CPS14 subunits.

B. Western blot analyses of ÄKTA-purified his-tagged ComP coexpressed with $PglL_{ComP}$ and the Pneumococcus CPS14 synthesis locus in *E. coli* SDB1. Lanes 2-7 are gradient elutions with incremental increases in imidazole concentrations. The majority of glycosylated ComP is eluted in lanes 5 and 6 that show lower electrophoretic mobility bands in comparison with unpurified whole cell lysates (lane 1). These bands react to both monoclonal anti-his (green) and polyclonal anti CPS14 antibodies (red), seen as a signal merge (yellow).

C. Purified ComP glycosylated with CPS14 subunits in lane 2 has a lower electrophoretic mobility than unglycosylated ComP that is coexpressed with the inactive H325A $PglL_{ComP}$ (lane 1). Proteinase K digests of CPS14-glycosylated ComP in lane 3 show a loss of signal.

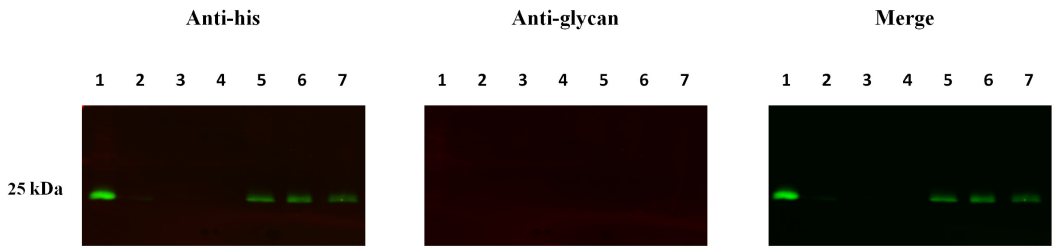

Figure 3.8 Western blot analyses on Ni-NTA purifications of his-tagged DsbA1 coexpressed with $PglL_{ADP1}$ and the Pneumococcus CPS14 synthesis locus in *E. coli* SDB1 (lanes 2-7). Lanes 2-4 represent column washes. Lanes 5-7 are elution fractions of purified DsbA1, showing bands with the same electrophoretic mobility as DsbA1 expressed from the whole cell sample. Faint lower electrophoretic mobility bands could be seen, that do not react to anti-CPS15b which are characteristic of modification with the *E. coli* glycan moiety. Expression was probed for with monoclonal anti-his (green) and polyclonal anti CPS14 (red).

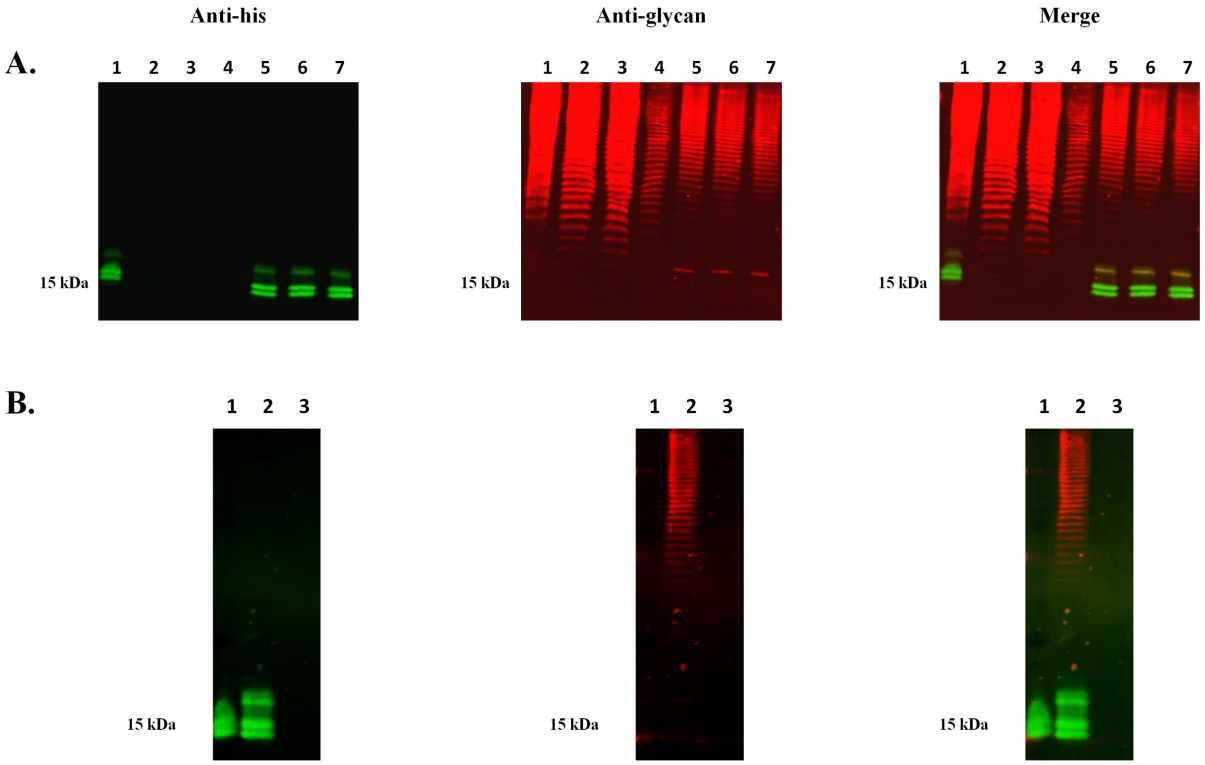

Figure 3.9

A. Western blot analyses on Ni-NTA purifications of his-tagged ComP coexpressed with $PglL_{ComP}$ and a Pneumococcus CPS15b synthesis locus in *E. coli* SDB1 (lanes 2-7). Lanes 2-4 represent column washes, with no ComP signal seen. Lanes 5-7 are elution fractions, showing a band with lower electrophoretic mobility when compared with unpurified whole cell lysates (lane 1). This band reacts to both monoclonal anti-his (green) and polyclonal anti CPS15b antibodies (red), seen on merging signals (yellow), indicating ComP glycosylation by Pneumococcus CPS15b subunits.

B. Purified ComP glycosylated with CPS15b subunits (lane 2) has a lower electrophoretic mobility than ComP coexpressed with the inactive H325A $PglL_{ComP}$ (lane 1). Proteinase K digests of purified CPS15b-glycosylated ComP show a loss of signal (lane 3).

Reducing end glycan	**Source**	**CjPglB**	**NmPglL**	**$PglL_{ComP}$**	**$PglL_{ADP1}$**
DATDH	*C. jejuni* LLO	Yes	Yes	Yes	Yes
Gal	*S. enterica* S. Typhimurium	No	Yes	Yes	Yes
GalNAc	*E. coli* O86	Yes	Yes	?	?
FucNAc	*P. aeruginosa* O11	Yes	Yes	Yes	Yes
Glc	*S. pneumoniae* CPS	No	No	Yes	No
GlcNAc	*E. coli* O7	Yes	Yes	Yes	Yes

Figure 3.10:

Summary of the current knowledge of the glycans transferred by CjPglB and NmPglL to their acceptor proteins in comparison with $PglL_{ComP}$ and $PglL_{ADP1}$.

3.5 Discussion

Bacterial protein glycosylation systems could be exploited for synthesizing glycoconjugate vaccines to potentially overcome the problems associated with chemical conjugations (Wacker *et al.*, 2002; Frasch, 2009; Terra *et al.*, 2012). However, drawbacks of this process are the glycan and acceptor protein specificity of the OTases, and continuous probing for novel OTases with different specificities is essential for advancing the glycoconjugate vaccine industry.

Similar to *N*-OTases, the glycan specificity of *O*-OTases is believed to depend on the sugar at the reducing end of the glycan (DiGiandomenico *et al.*, 2002; Wacker *et al.*, 2006; Horzempa *et al.*, 2006). In this work, we sought to characterize the glycan specificity of the two *O*-OTases identified in the environmental isolate *Acinetobacter baylyi* ADP1, designated $PglL_{ADP1}$ and $PglL_{ComP,}$ by reconstituting the *Acinetobacter* glycosylation system in *E. coli*. Our results show $PglL_{ComP}$ and $PglL_{ADP1}$ to be very similar to the well-characterized NmPglL in terms of glycan specificity (Figure 3.10). Both could transfer an *E. coli* glycan moiety that was shown to be peptidoglycan subunits in case of NmPglL (Faridmoayer *et al.*, 2008). As well, we show that glycans with *N*-acetylated sugars at the reducing end such as GlcNAc, Bac and FucNAc residues are substrates of $PglL_{ComP}$ and $PglL_{ADP1}$. Furthermore, both PglLs could transfer the *S. enterica* S. Typhimurium O antigen that has a Gal residue at its reducing end. We proceeded to test if Pneumococcus CPS subunits would be substrates to either PglL, since NmPglL does not transfer it to acceptor proteins (unpublished data). To our surprise, $PglL_{ComP}$ but not $PglL_{ADP1}$ transferred CPS subunits from Pneumococcus serotypes 14 and 15b to ComP, which we could only visualize with purified glycoproteins and not

with whole cell lysates. CPS from these serotypes have a Glc residue at the reducing end, and conjugating these glycans to acceptor proteins by an OTase is -to our knowledge- unprecedented. $PglL_{ComP}$ appears to constitute a novel class of *O*-OTases. It is phylogenetically and sequentially homologous to PglLs. However, similar to TfpOs, its only known acceptor protein is ComP. Unlike the Tfp-specific OTase TfpO, which transfers short oligosaccharides, $PglL_{ComP}$ was shown here to transfer longer oligosaccharides similar to other PglLs.

PglLs in general are superior to PglBs in terms of glycan specificity as they do not require an acetamido group in C-2 of the reducing end glycan and therefore have more relaxed glycan specificity (Wacker *et al.*, 2006; Faridmoayer *et al.*, 2008). The identification of the *C. lari* PglB crystal structure, which is 56% identical to CjPglB facilitated the structure-guided engineering of CjPglB (Lizak *et al.*, 2011). Mutagenesis of non-conserved residues in the glycan binding site of CjPglB, specifically the N311 residue, yielded a beneficial, albeit less stable mutant of CjPglB. This mutant displayed glycan-specific increased glycosylation efficiency and could successfully transfer the *Salmonella* O antigen, which lacks a C-2 acetamido group, to acceptor proteins (Ihssen *et al.*, 2015). The induced relaxed glycan specificity of this mutant is believed to be glycan-specific, with no reports of a CjPglB mutant that could transfer Pneumococcus CPS to date (Ihssen *et al.*, 2015).

A major limitation that is hindering the use of *O*-OTases in the glycoconjugate industry is the lack of a consensus sequence for the acceptor protein glycosylation site similar to that of *N*-OTases (Kowarik *et al.*, 2006). It is suggested that *O*-OTases glycosylation sites are regions of low complexity that are rich in serine, alanine and proline residues, similar to

what was seen with NmPglL (Vik *et al.*, 2009). As a result, it would be challenging to engineer non-native targets of *O*-OTases to be acceptor proteins. However, a promising result was shown with TfpO, where the transfer of the C-terminal 14 residues preceding the glycosylation site of PilA to C-termini of other proteins induced their glycosylation by TfpO (Qutyan *et al.*, 2010). This means that the identification of the glycosylation site of ComP is crucial if $PglL_{ComP}$ is to be further utilized in the glycoconjugate vaccine industry. A glycotag comprised of the residues surrounding the glycosylation site could be added to exposed protein loops to hypothetically induce their glycosylation by $PglL_{ComP}$ (Qutyan *et al.*, 2010; Fisher *et al.*, 2010). Our preliminary results show that the conserved Tfp residue that is homologous to *Neisseria* spp. S63 of PilE either is not the glycosylation site of ComP or more than one glycosylation site exist. Furthermore, identifying the unknown glycan moiety that decorates ComP in *E. coli*, which is most likely incomplete peptidoglycan subunits, is essential (Faridmoayer *et al.*, 2008). More studies are required to determine any vaccine efficacy issues or adverse effects arising from this unavoidable conjugation. An additional round of lectin affinity chromatography could be employed for glycoprotein purification from all other forms, or could entirely substitute the nickel affinity chromatography used here to purify CPS-conjugated ComP (Celik *et al.*, 2010; Schwarz *et al.*, 2011).

Identifying an OTase that conjugates a glycan with a Glc residue at the reducing end to acceptor proteins greatly expands the current tools available for glycoengineering. Manufacturing Pneumococcus glycoconjugates by this method would be less complicated and less costly than with chemical conjugations. $PglL_{ComP}$ could potentially be used to manufacture vaccines covering more of the >90 Pneumococcus serotypes, as the majority

of them have Glc residues at the reducing end (Bentley *et al.*, 2006). Using biological conjugations for manufacturing glycoconjugate vaccines would make them more affordable as chemically conjugated vaccines are currently three times more expensive than polysaccharide only vaccines (VFC | Current CDC Vaccine Price List | CDC, n.d.). This would lead to a more widespread use of the vaccine in low-income countries and ultimately to a reduction in pneumococcal disease burden. As well, vaccines targeting more Pneumococcus serotypes will address the serotype replacement issue, where vaccine serotypes are eliminated to be replaced by other non-vaccine serotypes (Weinberger *et al.*, 2011). Furthermore, $PglL_{ComP}$ could be further exploited in animal vaccines against other *Streptococcus* spp. like the swine pathogen *S. suis*, which also has Glc residues at the reducing end of its CPS (Van Calsteren *et al.*, 2010; Van Calsteren *et al.*, 2013).

3.6 References:

Bentley, S.D., Aanensen, D.M., Mavroidi, A., Saunders, D., Rabbinowitsch, E., Collins, M., Donohoe, K., Harris, D., Murphy, L., Quail, M.A., Samuel, G., Skovsted, I.C., Kaltoft, M.S., Barrell, B., Reeves, P.R., Parkhill, J., and Spratt, B.G. (2006) Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. *PLoS Genet* **2**: e31.

Bouhss, A., Trunkfield, A.E., Bugg, T.D.H., and Mengin-Lecreulx, D. (2008) The biosynthesis of peptidoglycan lipid-linked intermediates. *FEMS Microbiol Rev* **32**: 208–233.

Calsteren, M.-R. Van, Gagnon, F., Calzas, C., Goyette-Desjardins, G., Okura, M., Takamatsu, D., Gottschalk, M., and Segura, M. (2013) Structure determination of *Streptococcus suis* serotype 14 capsular polysaccharide. *Biochem Cell Biol* **91**: 49–58.

Calsteren, M.-R. Van, Gagnon, F., Lacouture, S., Fittipaldi, N., and Gottschalk, M. (2010) Structure determination of *Streptococcus suis* serotype 2 capsular polysaccharide. *Biochem Cell Biol* **88**: 513–25.

Celik, E., Fisher, A.C., Guarino, C., Mansell, T.J., and DeLisa, M.P. (2010) A filamentous phage display system for *N*-linked glycoproteins. *Protein Sci* **19**: 2006–13.

Chen, M.M., Glover, K.J., and Imperiali, B. (2007) From peptide to protein: comparative analysis of the substrate specificity of *N*-linked glycosylation in *C. jejuni*. *Biochemistry* **46**: 5579–85.

Cohen-Rosenzweig, C., Guan, Z., Shaanan, B., and Eichler, J. (2014) Substrate promiscuity: AglB, the archaeal oligosaccharyltransferase, can process a variety of lipid-linked glycans. *Appl Environ Microbiol* **80**: 486–96.

Cuccui, J., and Wren, B. (2015) Hijacking bacterial glycosylation for the production of glycoconjugates, from vaccines to humanised glycoproteins. *J Pharm Pharmacol* **67**: 338–50.

DiGiandomenico, A., Matewish, M.J., Bisaillon, A., Stehle, J.R., Lam, J.S., and Castric, P. (2002) Glycosylation of *Pseudomonas aeruginosa* 1244 pilin: glycan substrate specificity. *Mol Microbiol* **46**: 519–30.

Dower, W.J., Miller, J.F., and Ragsdale, C.W. (1988) High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res* **16**: 6127–45.

Dykxhoorn, D.M., Pierre, R. St., and Linn, T. (1996) A set of compatible tac promoter expression vectors. *Gene* **177**: 133–136.

Elhenawy, W., Scott, N.E., Tondo, M.L., Orellano, E.G., Foster, L.J., and Feldman, M.F. (2015) Protein *O*-linked glycosylation in the plant pathogen *Ralstonia solanacearum*. *Glycobiology* cwv098.

Erbel, P.J.A., Barr, K., Gao, N., Gerwig, G.J., Rick, P.D., and Gardner, K.H. (2003) Identification and biosynthesis of cyclic enterobacterial common antigen in *Escherichia coli*. *J Bacteriol* **185**: 1995–2004.

Faridmoayer, A., Fentabil, M.A., Haurat, M.F., Yi, W., Woodward, R., Wang, P.G., and Feldman, M.F. (2008) Extreme substrate promiscuity of the *Neisseria* oligosaccharyl transferase involved in protein *O*-glycosylation. *J Biol Chem* **283**: 34596–604.

Faridmoayer, A., Fentabil, M.A., Mills, D.C., Klassen, J.S., and Feldman, M.F. (2007) Functional characterization of bacterial oligosaccharyltransferases involved in *O*-linked protein glycosylation. *J Bacteriol* **189**: 8088–98.

Feldman, M.F., Marolda, C.L., Monteiro, M.A., Perry, M.B., Parodi, A.J., and Valvano, M.A. (1999) The Activity of a Putative Polyisoprenol-linked Sugar Translocase (Wzx) Involved in *Escherichia coli* O Antigen Assembly Is Independent of the Chemical Structure of the O Repeat. *J Biol Chem* **274**: 35129–35138.

Feldman, M.F., Wacker, M., Hernandez, M., Hitchen, P.G., Marolda, C.L., Kowarik, M., Morris, H.R., Dell, A., Valvano, M.A., and Aebi, M. (2005) Engineering N-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli*. *Proc Natl Acad Sci U S A* **102**: 3016–21.

Fisher, A.C., Haitjema, C.H., Guarino, C., Celik, E., Endicott, C.E., Reading, C.A., Merritt, J.H., Ptak, A.C., Zhang, S., and DeLisa, M.P. (2010) Production of Secretory and Extracellular N-Linked Glycoproteins in *Escherichia coli*. *Appl Environ Microbiol* **77**: 871–881.

Fisher, C.L., and Pei, G.K. (1997) Modification of a PCR-based site-directed mutagenesis method. *Biotechniques* **23**: 570–1, 574.

Frasch, C.E. (2009) Preparation of bacterial polysaccharide-protein conjugates: analytical and manufacturing challenges. *Vaccine* **27**: 6468–70.

Garcia-Quintanilla, F., Iwashkiw, J.A., Price, N.L., Stratilo, C., and Feldman, M.F. (2014) Production of a recombinant vaccine candidate against *Burkholderia pseudomallei* exploiting the bacterial *N*-glycosylation machinery. *Front Microbiol* **5**: 381.

Gebhart, C., Ielmini, M.V., Reiz, B., Price, N.L., Aas, F.E., Koomey, M., and Feldman, M.F. (2012) Characterization of exogenous bacterial oligosaccharyltransferases in *Escherichia coli* reveals the potential for *O*-linked protein glycosylation in *Vibrio cholerae* and *Burkholderia thailandensis*. *Glycobiology* **22**: 962–74.

Goldberg, J.B., Hatano, K., Meluleni, G.S., and Pier, G.B. (1992) Cloning and surface expression of *Pseudomonas aeruginosa* O antigen in *Escherichia coli*. *Proc Natl Acad Sci U S A* **89**: 10716–20.

Harding, C.M., Nasr, M.A., Kinsella, R.L., Scott, N.E., Foster, L.J., Weber, B.S., Fiester, S. E., Actis, L. A., Tracy, E. N., Munson, R. S., Feldman, M. F. (2015) *Acinetobacter* strains carry two functional oligosaccharyltransferases, one devoted exclusively to type IV pilin, and the other one dedicated to *O*-glycosylation of multiple proteins. *Mol Microbiol* .

Hegge, F.T., Hitchen, P.G., Aas, F.E., Kristiansen, H., Løvold, C., Egge-Jacobsen, W., Panico, M., Leong, W.Y., Bull, V., Virji, M., Morris, H.R., Dell, A., and Koomey, M. (2004) Unique modifications with phosphocholine and phosphoethanolamine define alternate antigenic forms of *Neisseria gonorrhoeae* type IV pili. *Proc Natl Acad Sci U S A* **101**: 10798–803.

Horzempa, J., Dean, C.R., Goldberg, J.B., and Castric, P. (2006) *Pseudomonas aeruginosa* 1244 pilin glycosylation: glycan substrate recognition. *J Bacteriol* **188**: 4244–52.

Ielmini, M. V, and Feldman, M.F. (2011) *Desulfovibrio desulfuricans* PglB homolog possesses oligosaccharyltransferase activity with relaxed glycan specificity and distinct protein acceptor sequence requirements. *Glycobiology* **21**: 734–42.

Ihssen, J., Haas, J., Kowarik, M., Wiesli, L., Wacker, M., Schwede, T., and Thöny-Meyer, L. (2015) Increased efficiency of *Campylobacter jejuni N*-oligosaccharyltransferase PglB by structure-guided engineering. *Open Biol* **5**: 140227.

Ihssen, J., Kowarik, M., Dilettoso, S., Tanner, C., Wacker, M., and Thöny-Meyer, L. (2010) Production of glycoprotein vaccines in *Escherichia coli*. *Microb Cell Fact* **9**: 61.

Iwashkiw, J.A., Fentabil, M.A., Faridmoayer, A., Mills, D.C., Peppler, M., Czibener, C., Ciocchini, A.E., Comerci, D.J., Ugalde, J.E., and Feldman, M.F. (2012) Exploiting the *Campylobacter jejuni* protein glycosylation system for glycoengineering vaccines and diagnostic tools directed against brucellosis. *Microb Cell Fact* **11**: 13.

Jervis, A.J., Langdon, R., Hitchen, P., Lawson, A.J., Wood, A., Fothergill, J.L., Morris, H. R., Dell, A., Wren, B., Linton, D. (2010) Characterization of *N*-linked protein glycosylation in *Helicobacter pullorum*. *J Bacteriol* **192**: 5228–36.

Kowarik, M., Young, N.M., Numao, S., Schulz, B.L., Hug, I., Callewaert, N., Mills D. C., Watson, D. C., Hernandez, M., Kelly, J. F., Wacker, M., Aebi, M. (2006) Definition of the bacterial *N*-glycosylation site consensus sequence. *EMBO J* **25**: 1957–66.

Lairson, L.L., Henrissat, B., Davies, G.J., and Withers, S.G. (2008) Glycosyltransferases: structures, functions, and mechanisms. *Annu Rev Biochem* **77**: 521–55.

Linton, D., Dorrell, N., Hitchen, P.G., Amber, S., Karlyshev, A. V, Morris, H.R., Dell, A., Valvano, M.A., Aebi, M., and Wren, B.W. (2005) Functional analysis of the *Campylobacter jejuni* N-linked protein glycosylation pathway. *Mol Microbiol* **55**: 1695–703.

Lithgow, K. V., Scott, N.E., Iwashkiw, J.A., Thomson, E.L.S., Foster, L.J., Feldman, M.F., and Dennis, J.J. (2014) A general protein *O*-glycosylation system within the *Burkholderia cepacia* complex is involved in motility and virulence. *Mol Microbiol* n/a–n/a.

Liu, D., and Reeves, P.R. (1994) *Escherichia coli* K12 regains its O antigen. *Microbiology* **140 (Pt 1**: 49–57.

Lizak, C., Gerber, S., Numao, S., Aebi, M., and Locher, K.P. (2011) X-ray structure of a bacterial oligosaccharyltransferase. *Nature* **474**: 350–5.

Marolda, C.L., Feldman, M.F., and Valvano, M.A. (1999) Genetic organization of the O7-specific lipopolysaccharide biosynthesis cluster of *Escherichia coli* VW187 (O7:K1). *Microbiology* **145 (Pt 9**: 2485–95.

Moreau, M., Richards, J.C., Fournier, J.M., Byrd, R.A., Karakawa, W.W., and Vann, W.F. (1990) Structure of the type 5 capsular polysaccharide of *Staphylococcus aureus*. *Carbohydr Res* **201**: 285–97.

Musumeci, M.A., Faridmoayer, A., Watanabe, Y., and Feldman, M.F. (2014) Evaluating the role of conserved amino acids in bacterial *O*-oligosaccharyltransferases by in vivo, in vitro and limited proteolysis assays. *Glycobiology* **24**: 39–50.

Musumeci, M.A., Hug, I., Scott, N., Ielmini, M.V., Foster, L.J., Wang, P.G., and Feldman, M.F. (2013) In vitro activity of *Neisseria meningitidis* PglL *O*-oligosaccharyltransferase with diverse synthetic lipid donors and a UDP-activated sugar. J Biol Chem 288: 10578–10587.

Nassif, X., Lowy, J., Stenberg, P., O'Gaora, P., Ganji, A., and So, M. (1993) Antigenic variation of pilin regulates adhesion of *Neisseria meningitidis* to human epithelial cells. *Mol Microbiol* **8**: 719–725.

Neal, B.L., Brown, P.K., and Reeves, P.R. (1993) Use of *Salmonella* phage P22 for transduction in *Escherichia coli*. *J Bacteriol* **175**: 7115–8.

Pace, D. (2013) Glycoconjugate vaccines. *Expert Opin Biol Ther* **13**: 11–33.

Parge, H.E., Forest, K.T., Hickey, M.J., Christensen, D.A., Getzoff, E.D., and Tainer, J.A. (1995) Structure of the fibre-forming protein pilin at 2.6 A resolution. *Nature* **378**: 32–8.

Pérez, J.M., McGarry, M.A., Marolda, C.L., and Valvano, M.A. (2008) Functional analysis of the large periplasmic loop of the *Escherichia coli* K-12 WaaL O-antigen ligase. *Mol Microbiol* **70**: 1424–40.

Power, P.M., Roddam, L.F., Rutter, K., Fitzpatrick, S.Z., Srikhanta, Y.N., and Jennings, M.P. (2004) Genetic characterization of pilin glycosylation and phase variation in *Neisseria meningitidis*. *Mol Microbiol* **49**: 833–847.

Qutyan, M., Henkel, M., Horzempa, J., Quinn, M., and Castric, P. (2010) Glycosylation of Pilin and Nonpilin Protein Constructs by *Pseudomonas aeruginosa* 1244. *J Bacteriol* **192**: 5972–5981.

Schild, S., Lamprecht, A.-K., and Reidl, J. (2005) Molecular and functional characterization of O antigen transfer in *Vibrio cholerae*. *J Biol Chem* **280**: 25936–47.

Schnaitman, C.A. (1971) Solubilization of the Cytoplasmic Membrane of *Escherichia coli* by Triton X-100. *J Bacteriol* **108**: 545–552.

Schwarz, F., Lizak, C., Fan, Y.-Y., Fleurkens, S., Kowarik, M., and Aebi, M. (2011) Relaxed acceptor site specificity of bacterial oligosaccharyltransferase *in vivo*. *Glycobiology* **21**: 45–54.

Terra, V.S., Mills, D.C., Yates, L.E., Abouelhadid, S., Cuccui, J., and Wren, B.W. (2012) Recent developments in bacterial protein glycan coupling technology and glycoconjugate vaccine design. *J Med Microbiol* **61**: 919–926.

van Selm, S., van Cann, L.M., Kolkman, M.A.B., van der Zeijst, B.A.M., and Putten, J.P.M. van (2003) Genetic basis for the structural difference between *Streptococcus pneumoniae* serotype 15B and 15C capsular polysaccharides. *Infect Immun* **71**: 6192–8.

VFC | Current CDC Vaccine Price List | CDC http://www.cdc.gov/vaccines/programs/vfc/awardees/vaccine-management/price-list/. Accessed October 6, 2015.

Vik, A., Aas, F.E., Anonsen, J.H., Bilsborough, S., Schneider, A., Egge-Jacobsen, W., and Koomey, M. (2009) Broad spectrum *O*-linked protein glycosylation in the human pathogen *Neisseria gonorrhoeae*. *Proc Natl Acad Sci U S A* **106**: 4447–52.

Wacker, M., Feldman, M.F., Callewaert, N., Kowarik, M., Clarke, B.R., Pohl, N.L., Hernandez, M., Vines, E.D., Valvano, M.A., Whitfield, C., and Aebi, M. (2006) Substrate specificity of bacterial oligosaccharyltransferase suggests a common transfer mechanism for the bacterial and eukaryotic systems. *Proc Natl Acad Sci U S A* **103**: 7088–93.

Wacker, M., Linton, D., Hitchen, P.G., Nita-Lazar, M., Haslam, S.M., North, S.J., Panico, M., Morris, H.R., Dell, A., Wren, B.W., and Aebi, M. (2002) N-linked glycosylation in *Campylobacter jejuni* and its functional transfer into E. *coli*. *Science*

298: 1790–3.

Weinberger, D.M., Malley, R., and Lipsitch, M. (2011) Serotype replacement in disease after pneumococcal vaccination. *Lancet (London, England)* **378**: 1962–73.

Wetter, M., Kowarik, M., Steffen, M., Carranza, P., Corradin, G., and Wacker, M. (2013) Engineering, conjugation, and immunogenicity assessment of *Escherichia coli* O121 O antigen for its potential use as a typhoid vaccine component. *Glycoconj J* **30**: 511–22.

4. Conclusions

Vaccines play a major role in disease control and are one of the few alternatives to antibiotics once resistance arises. Glycoconjugate vaccines are immunologically superior to polysaccharide only vaccines especially in children under 5 years, as first demonstrated with the *Haemophillus influenza* type b vaccine (Eskola *et al.*, 1987; Eskola *et al.*, 1990; Mäkelä and Käyhty, 2014). Chemically conjugated vaccines have demonstrated an exceptional efficacy in Pneumococcus control in young children, but producing them remains troublesome and too expensive for low-income countries (VFC | Current CDC Vaccine Price List | CDC, n.d.; Frasch, 2009). The description of bacterial protein glycosylation systems revolutionized the field of conjugate vaccines due to the possibility of exploiting these systems for glycoconjugate production. A major issue that is still hindering this technology is the glycan and protein specificity of the conjugating enzymes, the Oligosaccharyltransferases (OTases). Therefore, continuous probing for OTases with different glycan and protein specificities is essential for advancing the glycoconjugate vaccine industry. In this work, we identify novel OTases from the genus *Acinetobacter* and attempt to exploit them for producing glycoconjugate vaccines, specifically against the Pneumococcus.

4.1 Protein glycosylation in *Acinetobacter*

The genus *Acinetobacter*, particularly species belonging to the *Acinetobacter calcoaceticus baumannii* complex (*Acb*) are known for causing nosocomial infections (Dijkshoorn *et al.*, 2007). *A. baumannii* possesses a protein *O*-glycosylation system which was shown to be critical for biofilm formation and pathogenesis in a number of animal models, and was later found to be ubiquitous within the genus *Acinetobacter*

(Iwashkiw *et al.*, 2012; Scott *et al.*, 2014). *O*-glycosylation and LPS synthesis are evolutionarily related processes that share many homologies, such as *O*-OTases and O antigen ligases being members of the Wzy_c superfamily. Using *in silico* methods for discerning them is challenging and only experimentation truly identifies the function of a Wzy_c superfamily protein (Hug and Feldman, 2011). Most members of *Acb*, as well as the environmental isolate *A. baylyi* ADP1, encode two proteins with Wzy_C domains. In *A. baylyi*, a computational model suggested one of them to be an *O*-OTase and the other an O antigen ligase (Schulz *et al.*, 2013). Here, we have used *A. baylyi* and a representative of *Acb*, the clinical isolate *A. nosocomialis* M2, to experimentally determine the function of both proteins.

Our data show that both Wzy_c superfamily proteins are functional *O*-OTases with different acceptor protein specificities, which was never shown before in any previously described *O*-glycosylation systems. One of these OTases is specific for pilin glycosylation while the other is a general one that glycosylates multiple proteins. The roles of these glycosylation systems in pathogenesis are yet to be identified, and we could not determine any roles *in vitro* in this work. The wild type and knockouts of either OTase displayed similar natural competence abilities, biofilm formation, growth and twitching motility in the conditions tested. However, differences in twitching motility between wild type and pilin glycosylation mutants of *P. aeruginosa* were recently reported in ionic strengths comparable to host infection sites (Allison *et al.*, 2015). Furthermore, as previously shown with the *N. gonorrhoeae* and *P. aeruginosa* glycosylation systems, roles of the pilin glycan in pathogenesis were identified with cell line infection models (Jennings *et al.*, 2011; Tan *et al.*, 2015). For *Acb*, given that the

respiratory tract is the most common site of infections, a number of models could be used to determine the effects of the lack of either OTase on fitness and virulence. Relevant models include adherence to alveolar epithelial cells (A549) (Gaddy *et al.*, 2009) or apoptosis of laryngeal HEp-2 epithelial cells (Choi *et al.*, 2005). Future work also includes comparative proteomic profiling of wild type *Acb* strains and their OTase knockouts in conditions mimicking the host i.e. artificial sputum media (Sriramulu *et al.*, 2005; Reen *et al.*, 2012). Furthermore, *in vivo* proteomic profiling of *Acb* would give a more realistic image of the glycoproteome within the host and could identify novel virulence-associated glycoproteins. This could be done by labeling proteins with non-radioactive isotopes or unnatural amino acids (Ong and Mann, 2006; Dieterich *et al.*, 2007).

4.2 Exploiting the *A. baylyi* glycosylation system for glycoconjugate synthesis

As shown in this work, *A. baylyi* encodes two functional OTases, where one glycosylates only the Tfp ComP while the other glycosylates multiple proteins. The ComP-specific OTase, designated $PglL_{ComP}$, appears to constitute a novel class of OTases, as it combines features of both TfpO-like and PglL-like OTases. This OTase is phylogenetically and sequentially homologous to PglL-like OTases yet glycosylates only the Tfp ComP, similar to TfpO-like OTases. $PglL_{ComP}$ is not restricted to transferring only short oligosaccharides like TfpO as it transfers all glycans that could be transferred by PglL. Additionally, $PglL_{ComP}$ is shown here to transfer Pneumococcus capsular polysaccharides (CPS) of serotypes 14 and 15b, which have Glc residues at the reducing end of the glycan. This is an unprecedented finding that will revolutionize the glycoconjugate

vaccine industry as no previously identified OTases were demonstrated to transfer Pneumococcus CPS subunits to target proteins. Future directions for using $PglL_{ComP}$ in the glycoconjugate vaccine industry include:

4.2.1 Improving the efficiency of glycan transfer

Identifying the crystal structure of $PglL_{ComP}$ or other *O*-OTases/O antigen ligases is critical, as it would lead to identifying the glycan-binding pocket. This would pave the way for directed evolution and structure-guided engineering of $PglL_{ComP}$ to isolate mutants that could potentially be less efficient at transferring peptidoglycan, or be more efficient at transferring CPS subunits. This has been previously shown with the structure-guided engineering of the glycan-binding pocket of CjPglB (Lizak *et al.*, 2011; Ihssen *et al.*, 2015). Furthermore, previous work probing for factors stimulating *N*-glycosylation by CjPglB identified exogenous supplementation of the reducing sugar to culture broth to be one, the mechanism of which remains unknown (Kämpf *et al.*, 2015). Similar factors stimulating OTase-dependent *O*-glycosylation were never established. To help identify factors or conditions that increase *O*-glycosylation efficiency *in vivo*, a quantitative assay has to be employed. Only *in vitro* assays have been demonstrated and no *in vivo* assays were successfully shown to our knowledge (Musumeci *et al.*, 2013).

4.2.2 Expanding the panel of acceptor proteins for $PglL_{ComP}$

Identifying the crystal structure of *O*-OTases would help with the structure guided engineering of the acceptor protein-binding site of $PglL_{ComP}$. This could potentially lead to identifying advantageous mutants with relaxed acceptor protein specificity, which has

never before been demonstrated with any OTases. Another method for expanding the panel of acceptor proteins for $PglL_{ComP}$ is to use the residues surrounding the ComP glycosylation site as a "glycotag". This tag would be added to exposed protein residues to induce their glycosylation, similar to what was done previously with CjPglB and *P. aeruginosa* TfpO (Chen *et al.*, 2007; Qutyan *et al.*, 2010; Fisher *et al.*, 2010).

4.2.3 The effect of the conjugation site on vaccine efficacy

Seldom studied, the choice of the site where glycans are conjugated to acceptor proteins appears to have an effect on vaccine efficacy. This was recently demonstrated with a *S. enterica* conjugate vaccine where O antigen was chemically conjugated to CRM_{197} at different sites, which resulted in different anti O antigen antibody titers (Stefanetti *et al.*, 2015). This should be expected, since the glycan affects protein folding and thermodynamic stability and therefore potentially T cell epitope exposure (Shental-Bechor and Levy, 2008). More studies have to be done in this area to improve our understanding of the role the glycan conjugation site plays in vaccine stability and immunology.

4.2.4 Glycoconjugate trafficking to outer membrane vesicles

Vaccines require adjuvants to stimulate an immune response (Marciani, 2003). Given that only a few adjuvants are licensed for human use, vaccines based on outer membrane vesicles (OMVs) have been proposed as inherently immunogenic molecules in OMVs could act as adjuvants (Acevedo *et al.*, 2014). Bexsero® is an example of a successful OMV-based vaccine against *N. meningitidis* serogroup B (Gorringe and Pajón, 2012).

Using OMVs as vaccine delivery systems is more cost-effective, which is especially appealing when designing livestock vaccines.

Engineering glycoconjugates for secretion via OMVs was successfully demonstrated in *E. coli* where the OMV-secreted protein ClyA was engineered to be *N*-glycosylated prior to secretion by introducing glycotags to its C-terminus (Fisher *et al.*, 2010). We have attempted to apply this concept to *O*-glycosylation, which was challenging due to the lack of knowledge of an *O*-glycosylation site consensus sequence or the ComP glycosylation site. We have employed a strategy similar to that used for engineering surface display of proteins, which was done by anchoring the whole protein to truncated *E. coli* outer membrane proteins. This has successfully led to the surface display of proteins such as lipases, endoxylanases and alkaline phosphatases to the *E. coli* surface (Yim *et al.*, 2013; Lee *et al.*, 2013). We have fused mature ComP, lacking the signal peptide, to proteins that get secreted via vesicles, namely the *E. coli* outer membrane proteins OmpW and OmpX and the *C. jejuni* HisJ (Lee *et al.*, 2007; Elmi *et al.*, 2012). Our preliminary results show that these fusion constructs are stably expressed, with the exception of OmpW. Future work will involve probing for glycosylation and OMV secretion of these fusion constructs, as well as determining OMV localization. Interestingly, surface display of proteins on OMVs, which was long believed to be a prerequisite of successful OMV vaccines, is not necessary. Despite being insufficiently studied, OMVs with antigenic components in their lumen were recently demonstrated to successfully elicit antigen-specific protective immune responses (Fantappiè *et al.*, 2014).

4.3 Concluding remarks

This work clearly shows that continuous bioprospecting for novel OTases with different glycan and protein specificities is an essential pillar for the glycoconjugate vaccine industry. As seen here, *Acinetobacter baylyi*, a non-pathogenic model organism has provided a solution to conjugating Pneumococcus CPS with glucose residues at the reducing end to acceptor proteins. Biological conjugations are more cost-effective than chemical conjugations and therefore will make these vaccines more affordable for low-income countries, ultimately reducing Pneumococcus disease burden.

4.4 References

Acevedo, R., Fernández, S., Zayas, C., Acosta, A., Sarmiento, M.E., Ferro, V.A., Rosenqvist, E., Campa, C., Cardoso, D., Garcia, L., and Perez, J.L. (2014) Bacterial outer membrane vesicles and vaccine applications. *Front Immunol* **5**: 121.

Allison, T.M., Conrad, S., and Castric, P. (2015) The group I pilin glycan affects type IVa pilus hydrophobicity and twitching motility in *Pseudomonas aeruginosa* 1244. *Microbiology* **161**: 1780–9.

Benjamin J. Gross, Jonathan G. Swoboda, and, and Walker*, S. (2007) A Strategy to Discover Inhibitors of *O*-Linked Glycosylation. .

Chen, M.M., Glover, K.J., and Imperiali, B. (2007) From peptide to protein: comparative analysis of the substrate specificity of *N*-linked glycosylation in *C. jejuni*. *Biochemistry* **46**: 5579–85.

Choi, C.H., Lee, E.Y., Lee, Y.C., Park, T.I., Kim, H.J., Hyun, S.H., Kim, S.A., Lee, S.-K., and Lee, J.C. (2005) Outer membrane protein 38 of *Acinetobacter baumannii* localizes to the mitochondria and induces apoptosis of epithelial cells. *Cell Microbiol* **7**: 1127–1138.

Dieterich, D.C., Lee, J.J., Link, A.J., Graumann, J., Tirrell, D.A., and Schuman, E.M. (2007) Labeling, detection and identification of newly synthesized proteomes with bioorthogonal non-canonical amino-acid tagging. *Nat Protoc* **2**: 532–40.

Dijkshoorn, L., Nemec, A., and Seifert, H. (2007) An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nat Rev Microbiol* **5**: 939–51.

Elmi, A., Watson, E., Sandu, P., Gundogdu, O., Mills, D.C., Inglis, N.F., Manson, E., Imrie, L., Bajaj-Elliott, M., Wren, B.W., Smith, D.G.E., and Dorrell, N. (2012) *Campylobacter jejuni* outer membrane vesicles play an important role in bacterial interactions with human intestinal epithelial cells. *Infect Immun* **80**: 4089–98.

Eskola, J., Käyhty, H., Takala, A.K., Peltola, H., Rönnberg, P.-R., Kela, E., Pekkanen, E., McVerry, P. H.,Mäkelä, P. H. (1990) A Randomized, Prospective Field Trial of a Conjugate Vaccine in the Protection of Infants and Young Children against Invasive *Haemophilus influenzae* Type b Disease. *N Engl J Med* **323**: 1381–1387.

Eskola, J., Peltola, H., Takala, A.K., Käyhty, H., Hakulinen, M., Karanko, V., Kela, E., Rekola, P., Rönnberg, P. R., Samuelson, J. S. (1987) Efficacy of *Haemophilus influenzae* type b polysaccharide-diphtheria toxoid conjugate vaccine in infancy. *N Engl J Med* **317**: 717–22.

Fantappiè, L., Santis, M. de, Chiarot, E., Carboni, F., Bensi, G., Jousson, O., Margarit, I., and Grandi, G. (2014) Antibody-mediated immunity induced by engineered *Escherichia coli* OMVs carrying heterologous antigens in their lumen. *J Extracell vesicles* **3**.

Fisher, A.C., Haitjema, C.H., Guarino, C., Celik, E., Endicott, C.E., Reading, C.A., Merritt, J.H., Ptak, A.C., Zhang, S., and DeLisa, M.P. (2010) Production of Secretory and Extracellular N-Linked Glycoproteins in *Escherichia coli*. *Appl Environ Microbiol* **77**: 871–881.

Frasch, C.E. (2009) Preparation of bacterial polysaccharide-protein conjugates: analytical and manufacturing challenges. *Vaccine* **27**: 6468–70.

Gaddy, J.A., Tomaras, A.P., and Actis, L.A. (2009) The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. *Infect Immun* **77**: 3150–60.

Gorringe, A.R., and Pajón, R. (2012) Bexsero: a multicomponent vaccine for prevention of meningococcal disease. *Hum Vaccin Immunother* **8**: 174–83.

Hug, I., and Feldman, M.F. (2011) Analogies and homologies in lipopolysaccharide and glycoprotein biosynthesis in bacteria. *Glycobiology* **21**: 138–51.

Ihssen, J., Haas, J., Kowarik, M., Wiesli, L., Wacker, M., Schwede, T., and Thöny-Meyer, L. (2015) Increased efficiency of *Campylobacter jejuni N*-oligosaccharyltransferase PglB by structure-guided engineering. *Open Biol* **5**: 140227.

Iwashkiw, J.A., Seper, A., Weber, B.S., Scott, N.E., Vinogradov, E., Stratilo, C., Reiz, B., Cordwell, S. J., Whittal, R., Schild, S., Feldman, M. F. (2012) Identification of a General O-linked Protein Glycosylation System in *Acinetobacter baumannii* and Its Role in Virulence and Biofilm Formation. *PLoS Pathog* **8**: e1002758.

Jennings, M.P., Jen, F.E.-C., Roddam, L.F., Apicella, M.A., and Edwards, J.L. (2011) *Neisseria gonorrhoeae* pilin glycan contributes to CR3 activation during challenge of primary cervical epithelial cells. *Cell Microbiol* **13**: 885–96.

Kämpf, M.M., Braun, M., Sirena, D., Ihssen, J., Thöny-Meyer, L., and Ren, Q. (2015) *In vivo* production of a novel glycoconjugate vaccine against *Shigella flexneri* 2a in recombinant *Escherichia coli*: identification of stimulating factors for *in vivo* glycosylation. *Microb Cell Fact* **14**: 12.

Lee, E.-Y., Bang, J.Y., Park, G.W., Choi, D.-S., Kang, J.S., Kim, H.-J., Park, K.-S., Lee, J.-O., Kim, Y.-K., Kwon, K.-H., Kim, K.-P., and Gho, Y.S. (2007) Global proteomic profiling of native outer membrane vesicles derived from *Escherichia coli*. *Proteomics* **7**: 3143–53.

Lee, H., Park, S.J., Han, M.-J., Eom, G.T., Choi, M.-J., Kim, S.H., Oh, Y.H., Song, B.K., and Lee, S.H. (2013) Expression of a lipase on the cell-surface of *Escherichia coli* using

the OmpW anchoring motif and its application to enantioselective reactions. *Biotechnol Lett* **35**: 1677–83.

Li, D., Chiu, H., Zhang, H., and Chan, D.W. (2013) Analysis of serum protein glycosylation by a differential lectin immunosorbant assay (dLISA). *Clin Proteomics* **10**: 12.

Lizak, C., Gerber, S., Numao, S., Aebi, M., and Locher, K.P. (2011) X-ray structure of a bacterial oligosaccharyltransferase. *Nature* **474**: 350–5.

Mäkelä, P.H., and Käyhty, H. (2014) Evolution of conjugate vaccines. *Expert Rev Vaccines* .

Marciani, D.J. (2003) Vaccine adjuvants: role and mechanisms of action in vaccine immunogenicity. *Drug Discov Today* **8**: 934–943.

Musumeci, M.A., Ielmini, M. V, and Feldman, M.F. (2013) *In vitro* glycosylation assay for bacterial oligosaccharyltransferases. *Methods Mol Biol* **1022**: 161–71.

Ong, S.-E., and Mann, M. (2006) A practical recipe for stable isotope labeling by amino acids in cell culture (SILAC). *Nat Protoc* **1**: 2650–60.

Qutyan, M., Henkel, M., Horzempa, J., Quinn, M., and Castric, P. (2010) Glycosylation of Pilin and Nonpilin Protein Constructs by *Pseudomonas aeruginosa* 1244. *J Bacteriol* **192**: 5972–5981.

Reen, F.J., Woods, D.F., Mooij, M.J., Adams, C., and O'Gara, F. (2012) Respiratory pathogens adopt a chronic lifestyle in response to bile. *PLoS One* **7**: e45978.

Schulz, B.L., Jen, F.E.C., Power, P.M., Jones, C.E., Fox, K.L., Ku, S.C., Blanchfield, J.T., and Jennings, M.P. (2013) Identification of bacterial protein O-oligosaccharyltransferases and their glycoprotein substrates. *PLoS One* **8**: e62768.

Scott, N.E., Kinsella, R.L., Edwards, A.V.G., Larsen, M.R., Dutta, S., Saba, J., Foster, L.J., and Feldman, M.F. (2014) Diversity Within the O-linked Protein Glycosylation Systems of *Acinetobacter* Species. *Mol Cell Proteomics* **13**: 2354–70.

Shental-Bechor, D., and Levy, Y. (2008) Effect of glycosylation on protein folding: a close look at thermodynamic stabilization. *Proc Natl Acad Sci U S A* **105**: 8256–61.

Sriramulu, D.D., Lünsdorf, H., Lam, J.S., and Römling, U. (2005) Microcolony formation: a novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung. *J Med Microbiol* **54**: 667–76.

Stefanetti, G., Hu, Q.-Y., Usera, A., Robinson, Z., Allan, M., Singh, A., Imase, H., Cobb, J., Zhai, H., Quinn, D., Lei, M., Saul, A., Adamo, R., MacLennan, C.A., and Micoli, F. (2015) Sugar-Protein Connectivity Impacts on the Immunogenicity of Site-Selective

Salmonella O-Antigen Glycoconjugate Vaccines. *Angew Chemie Int Ed* **54**: n/a–n/a.

Tan, R.M., Kuang, Z., Hao, Y., Lee, F., Lee, T., Lee, R.J., and Lau, G.W. (2015) Type IV pilus glycosylation mediates resistance of *Pseudomonas aeruginosa* to opsonic activities of the pulmonary surfactant protein A. *Infect Immun* **83**: 1339–46.

VFC | Current CDC Vaccine Price List | CDC http://www.cdc.gov/vaccines/programs/vfc/awardees/vaccine-management/price-list/. Accessed October 6, 2015.

Yim, S.S., An, S.J., Han, M.-J., Choi, J.W., and Jeong, K.J. (2013) Isolation of a potential anchoring motif based on proteome analysis of *Escherichia coli* and its use for cell surface display. *Appl Biochem Biotechnol* **170**: 787–804.

Bibliography

Aas, F.E., Egge-Jacobsen, W., Winther-Larsen, H.C., Løvold, C., Hitchen, P.G., Dell, A., and Koomey, M. (2006) *Neisseria gonorrhoeae* type IV pili undergo multisite, hierarchical modifications with phosphoethanolamine and phosphocholine requiring an enzyme structurally related to lipopolysaccharide phosphoethanolamine transferases. *J Biol Chem* **281**: 27712–23.

Abd-El-Haleem, D., Moawad, H., Zaki, E.A., and Zaki, S. (2002) Molecular characterization of phenol-degrading bacteria isolated from different Egyptian ecosystems. *Microb Ecol* **43**: 217–24.

Abu-Qarn, M., Yurist-Doutsch, S., Giordano, A., Trauner, A., Morris, H.R., Hitchen, P., Medalia, O., Dell, A., and Eichler, J. (2007) *Haloferax volcanii* AglB and AglD are Involved in N-glycosylation of the S-layer Glycoprotein and Proper Assembly of the Surface Layer. *J Mol Biol* **374**: 1224–1236.

Acevedo, R., Fernández, S., Zayas, C., Acosta, A., Sarmiento, M.E., Ferro, V.A., Rosenqvist, E., Campa, C., Cardoso, D., Garcia, L., and Perez, J.L. (2014) Bacterial outer membrane vesicles and vaccine applications. *Front Immunol* **5**: 121.

Actis, L.A., Potter, S.A., and Crosa, J.H. (1985) Iron-regulated outer membrane protein OM2 of Vibrio anguillarum is encoded by virulence plasmid pJM1. *J Bacteriol* **161**: 736–42.

Allison, T.M., Conrad, S., and Castric, P. (2015) The group I pilin glycan affects type IVa pilus hydrophobicity and twitching motility in *Pseudomonas aeruginosa* 1244. *Microbiology* **161**: 1780–9.

Alugupalli, K.R., Leong, J.M., Woodland, R.T., Muramatsu, M., Honjo, T., and Gerstein, R.M. (2004) B1b lymphocytes confer T cell-independent long-lasting immunity. *Immunity* **21**: 379–90.

Aranda, J., Poza, M., Pardo, B.G., Rumbo, S., Rumbo, C., Parreira, J.R., Rodríguez-Velo, P., and Bou, G. (2010) A rapid and simple method for constructing stable mutants of *Acinetobacter baumannii*. *BMC Microbiol* **10**: 279.

Arroyo, L.A., Mateos, I., González, V., and Aznar, J. (2009) In vitro activities of tigecycline, minocycline, and colistin-tigecycline combination against multi- and pandrug-resistant clinical isolates of *Acinetobacter baumannii* group. *Antimicrob Agents Chemother* **53**: 1295–6.

Avci, F.Y., Li, X., Tsuji, M., and Kasper, D.L. (2011) A mechanism for glycoconjugate vaccine activation of the adaptive immune system and its implications for vaccine design. *Nat Med* **17**: 1602–9.

Balonova, L., Mann, B.F., Cerveny, L., Alley, W.R., Chovancova, E., Forslund, A.-L., Salomonsson, E.N., Forsberg, A., Damborsky, J., Novotny, M. V, Hernychova, L., and Stulik, J. (2012) Characterization of protein glycosylation in *Francisella tularensis* subsp. holarctica: identification of a novel glycosylated lipoprotein required for virulence. *Mol Cell Proteomics* **11**: M111.015016.

Bambeke, F. Van, Reinert, R.R., Appelbaum, P.C., Tulkens, P.M., and Peetermans, W.E. (2007) Multidrug-Resistant *Streptococcus pneumoniae* Infections. *Drugs* **67**: 2355–2382.

Barrett, D.J., and Ayoub, E.M. (1986) IgG2 subclass restriction of antibody to pneumococcal polysaccharides. *Clin Exp Immunol* **63**: 127–34.

Benjamin J. Gross, Jonathan G. Swoboda, and, and Walker*, S. (2007) A Strategy to Discover Inhibitors of *O*-Linked Glycosylation. .

Bensing, B.A., Gibson, B.W., and Sullam, P.M. (2004) The *Streptococcus* gordonii Platelet Binding Protein GspB Undergoes Glycosylation Independently of Export. *J Bacteriol* **186**: 638–645.

Bensing, B.A., López, J.A., and Sullam, P.M. (2004) The *Streptococcus* gordonii surface proteins GspB and Hsa mediate binding to sialylated carbohydrate epitopes on the platelet membrane glycoprotein Ibalpha. *Infect Immun* **72**: 6528–37.

Bentley, S.D., Aanensen, D.M., Mavroidi, A., Saunders, D., Rabbinowitsch, E., Collins, M., Donohoe, K., Harris, D., Murphy, L., Quail, M.A., Samuel, G., Skovsted, I.C., Kaltoft, M.S., Barrell, B., Reeves, P.R., Parkhill, J., and Spratt, B.G. (2006) Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. *PLoS Genet* **2**: e31.

Black, S. (2010) The volatile nature of pneumococcal serotype epidemiology: potential for misinterpretation. *Pediatr Infect Dis J* **29**: 301–3.

Boersema, P.J., Raijmakers, R., Lemeer, S., Mohammed, S., and Heck, A.J.R. (2009) Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. *Nat Protoc* **4**: 484–94.

Bologa, M., Kamtchoua, T., Hopfer, R., Sheng, X., Hicks, B., Bixler, G., Hou, V., Pehlic, V., Yuan, T., and Gurunathan, S. (2012) Safety and immunogenicity of pneumococcal protein vaccine candidates: monovalent choline-binding protein A (PcpA) vaccine and bivalent PcpA-pneumococcal histidine triad protein D vaccine. *Vaccine* **30**: 7461–8.

Borgono, J.M., McLean, A.A., Vella, P.P., Woodhour, A.F., Canepa, I., Davidson, W.L., and Hilleman, M.R. (1978) Vaccination and Revaccination with Polyvalent Pneumococcal Polysaccharide Vaccines in Adults and Infants. *Exp Biol Med* **157**: 148–154.

Børud, B., Viburiene, R., Hartley, M.D., Paulsen, B.S., Egge-Jacobsen, W., Imperiali, B., and Koomey, M. (2011) Genetic and molecular analyses reveal an evolutionary trajectory for glycan synthesis in a bacterial protein glycosylation system. *Proc Natl Acad Sci U S A* **108**: 9643–8.

Boucher, C.A., Barberis, P.A., and Demery, D.A. (1985) Transposon Mutagenesis of *Pseudomonas solanacearum*: Isolation of Tn5-Induced Avirulent Mutants. *Microbiology* **131**: 2449–2457.

Bouhss, A., Trunkfield, A.E., Bugg, T.D.H., and Mengin-Lecreulx, D. (2008) The biosynthesis of peptidoglycan lipid-linked intermediates. *FEMS Microbiol Rev* **32**: 208–233.

Brzoska, A.J., Hassan, K.A., Leon, E.J. de, Paulsen, I.T., and Lewis, P.J. (2013) Single-step selection of drug resistant *Acinetobacter baylyi* ADP1 mutants reveals a functional redundancy in the recruitment of multidrug efflux systems. *PLoS One* **8**: e56090.

Cagatay, T.I., and Hickford, J.G.H. (2008) Glycosylation of type-IV fimbriae of Dichelobacter nodosus. *Vet Microbiol* **126**: 160–7.

Calo, D., Kaminski, L., and Eichler, J. (2010) Protein glycosylation in Archaea: sweet and extreme. *Glycobiology* **20**: 1065–76.

Calsteren, M.-R. Van, Gagnon, F., Lacouture, S., Fittipaldi, N., and Gottschalk, M. (2010) Structure determination of *Streptococcus suis* serotype 2 capsular polysaccharide. *Biochem Cell Biol* **88**: 513–25.

Campbell, Jr., G.D., and Silberman, R. (1998) Drug-Resistant *Streptococcus pneumoniae*. *Clin Infect Dis* **26**: 1188–1195.

Canadian Antimicrobial Resistance Surveillance System Report 2015 (2015) .

Carruthers, M.D., Harding, C.M., Baker, B.D., Bonomo, R.A., Hujer, K.M., Rather, P.N., and Munson, R.S. (2013) Draft Genome Sequence of the Clinical Isolate *Acinetobacter nosocomialis* Strain M2. *Genome Announc* **1**: e00906–13

Carruthers, M.D., Nicholson, P.A., Tracy, E.N., and Munson, R.S. (2013) *Acinetobacter baumannii* utilizes a type VI secretion system for bacterial competition. *PLoS One* **8**: e59388.

Castric, P. (1995) pilO, a gene required for glycosylation of *Pseudomonas aeruginosa* 1244 pilin. *Microbiology* **141 (Pt 5**: 1247–54.

Castric, P., Cassels, F.J., and Carlson, R.W. (2001) Structural Characterization of the *Pseudomonas aeruginosa* 1244 Pilin Glycan. *J Biol Chem* **276**: 26479–26485.

Celik, E., Fisher, A.C., Guarino, C., Mansell, T.J., and DeLisa, M.P. (2010) A filamentous phage display system for *N*-linked glycoproteins. *Protein Sci* **19**: 2006–13.

Chao, Y., Marks, L.R., Pettigrew, M.M., and Hakansson, A.P. (2014) *Streptococcus pneumoniae* biofilm formation and dispersion during colonization and disease. *Front Cell Infect Microbiol* **4**: 194.

Charbonneau, M.-E., Girard, V., Nikolakakis, A., Campos, M., Berthiaume, F., Dumas, F., Lépine, F., and Mourez, M. (2007) O-linked glycosylation ensures the normal conformation of the autotransporter adhesin involved in diffuse adherence. *J Bacteriol* **189**: 8880–9.

Chen, M.M., Glover, K.J., and Imperiali, B. (2007) From peptide to protein: comparative analysis of the substrate specificity of *N*-linked glycosylation in *C. jejuni*. *Biochemistry* **46**: 5579–85.

Choi, A.H.K., Slamti, L., Avci, F.Y., Pier, G.B., and Maira-Litrán, T. (2009) The pgaABCD locus of *Acinetobacter baumannii* encodes the production of poly-beta-1-6-N-acetylglucosamine, which is critical for biofilm formation. *J Bacteriol* **191**: 5953–63.

Choi, C.H., Hyun, S.H., Lee, J.Y., Lee, J.S., Lee, Y.S., Kim, S.A., Chae, J.-P., Yoo, S.M., and Lee, J.C. (2008) *Acinetobacter baumannii* outer membrane protein A targets the nucleus and induces cytotoxicity. *Cell Microbiol* **10**: 309–19.

Choi, C.H., Lee, E.Y., Lee, Y.C., Park, T.I., Kim, H.J., Hyun, S.H., Kim, S.A., Lee, S.-K., and Lee, J.C. (2005) Outer membrane protein 38 of *Acinetobacter baumannii* localizes to the mitochondria and induces apoptosis of epithelial cells. *Cell Microbiol* **7**: 1127–1138.

Choi, K.-H., Gaynor, J.B., White, K.G., Lopez, C., Bosio, C.M., Karkhoff-Schweizer, R.R., and Schweizer, H.P. (2005) A Tn7-based broad-range bacterial cloning and expression system. *Nat Methods* **2**: 443–8.

Chumakov, K., and Ehrenfeld, E. (2008) New generation of inactivated poliovirus vaccines for universal immunization after eradication of poliomyelitis. *Clin Infect Dis* **47**: 1587–92.

Cobb, B.A., Wang, Q., Tzianabos, A.O., and Kasper, D.L. (2004) Polysaccharide processing and presentation by the MHCII pathway. *Cell* **117**: 677–87.

Cohen-Rosenzweig, C., Guan, Z., Shaanan, B., and Eichler, J. (2014) Substrate promiscuity: AglB, the archaeal oligosaccharyltransferase, can process a variety of lipid-linked glycans. *Appl Environ Microbiol* **80**: 486–96.

Comer, J.E., Marshall, M.A., Blanch, V.J., Deal, C.D., and Castric, P. (2002) Identification of the *Pseudomonas aeruginosa* 1244 pilin glycosylation site. *Infect Immun* **70**: 2837–45.

Côté, J.-P., Charbonneau, M.-È., and Mourez, M. (2013) Glycosylation of the *Escherichia coli* TibA self-associating autotransporter influences the conformation and the functionality of the protein. *PLoS One* **8**: e80739.

Coyne, M.J., Fletcher, C.M., Chatzidaki-Livanis, M., Posch, G., Schaffer, C., and Comstock, L.E. (2013) Phylum-wide general protein O-glycosylation system of the Bacteroidetes. *Mol Microbiol* **88**: 772–83.

Cuccui, J., and Wren, B. (2015) Hijacking bacterial glycosylation for the production of glycoconjugates, from vaccines to humanised glycoproteins. *J Pharm Pharmacol* **67**: 338–50.

Cuccui, J., and Wren, B.W. (2013) Bacteria like sharing their sweets. *Mol Microbiol* **89**: 811–5.

Cuccui, J., Thomas, R.M., Moule, M.G., D'Elia, R. V, Laws, T.R., Mills, D.C., Williamson, D., Atkins, T.P., Prior, J.L., and Wren, B.W. (2013) Exploitation of bacterial N-linked glycosylation to develop a novel recombinant glycoconjugate vaccine against *Francisella tularensis*. *Open Biol* **3**: 130002.

Darrieux, M., Goulart, C., Briles, D., and Leite, L.C. de C. (2015) Current status and perspectives on protein-based pneumococcal vaccines. *Crit Rev Microbiol* .

Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**: 6640–5.

Davies, J.A. V. (1937) The Response of Infants to Inoculation with Type I Pneumococcus Carbohydrate. *J Immunol* **33**: 1–7.

de Berardinis, V., Vallenet, D., Castelli, V., Besnard, M., Pinet, A., Cruaud, C., Samair, S., Lechaplais, C., Gyapay, G., Richez, C., Durot, M., Kreimeyer, A., Le Fèvre, F., Schächter, V.,Pezo, V., Döring, V., Scarpelli, C., Médigue, C., Cohen, G. N., Marlière, P., Salanoubat, M. and Weissenbach, J.,. (2008) A complete collection of single-gene deletion mutants of *Acinetobacter baylyi* ADP1. *Mol Syst Biol* **4**: 174.

Delany, I., Rappuoli, R., and DeGregorio, E. (2014) Vaccines for the 21st century. *EMBO Mol Med* **6**: 708–20.

Dempski, R.E., and Imperiali, B. (2002) Oligosaccharyl transferase: gatekeeper to the secretory pathway. *Curr Opin Chem Biol* **6**: 844–850.

Desai, A.P., Sharma, D., Crispell, E.K., Baughman, W., Thomas, S., Tunali, A., Sherwood, L., Zmitrovich, A., Jerris, R., Satola, S., Beall, B., Moore, M.R., Jain, S., and Farley, M.M. (2015) Decline in Pneumococcal Nasopharyngeal Carriage of Vaccine Serotypes After the Introduction of the 13-Valent Pneumococcal Conjugate Vaccine in Children in Atlanta, Georgia. *Pediatr Infect Dis J* .

Dick, W.E., and Beurret, M. (1989) Glycoconjugates of bacterial carbohydrate antigens. A survey and consideration of design and preparation factors. *Contrib Microbiol Immunol* **10**: 48–114.

Dieterich, D.C., Lee, J.J., Link, A.J., Graumann, J., Tirrell, D.A., and Schuman, E.M. (2007) Labeling, detection and identification of newly synthesized proteomes with bioorthogonal non-canonical amino-acid tagging. *Nat Protoc* **2**: 532–40.

DiGiandomenico, A., Matewish, M.J., Bisaillon, A., Stehle, J.R., Lam, J.S., and Castric, P. (2002) Glycosylation of *Pseudomonas aeruginosa* 1244 pilin: glycan substrate specificity. *Mol Microbiol* **46**: 519–30.

Dijkshoorn, L., Nemec, A., and Seifert, H. (2007) An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nat Rev Microbiol* **5**: 939–51.

Dintzis, R.Z., Middleton, M.H., and Dintzis, H.M. (1983) Studies on the immunogenicity and tolerogenicity of T-independent antigens. *J Immunol* **131**: 2196–203.

Doern, G. V, Heilmann, K.P., Huynh, H.K., Rhomberg, P.R., Coffman, S.L., and Brueggemann, A.B. (2001) Antimicrobial resistance among clinical isolates of *Streptococcus pneumoniae* in the United States during 1999--2000, including a comparison of resistance rates since 1994--1995. *Antimicrob Agents Chemother* **45**: 1721–9.

Donkor, E.S. (2013) Understanding the pneumococcus: transmission and evolution. *Front Cell Infect Microbiol* **3**: 7.

Dower, W.J., Miller, J.F., and Ragsdale, C.W. (1988) High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res* **16**: 6127–45.

Downie, A.W. (1937) The specific capsular polysaccharide of pneumococcus type I and the immunity which it induces in mice. *J Pathol Bacteriol* **45**: 131–147.

Dwivedi, R., Nothaft, H., Reiz, B., Whittal, R.M., and Szymanski, C.M. (2013) Generation of free oligosaccharides from bacterial protein N-linked glycosylation systems. *Biopolymers* **99**: 772–83.

Dykxhoorn, D.M., Pierre, R. St., and Linn, T. (1996) A set of compatible tac promoter expression vectors. *Gene* **177**: 133–136.

Egge-Jacobsen, W., Salomonsson, E.N., Aas, F.E., Forslund, A.-L., Winther-Larsen, H.C., Maier, J., Macellaro, A., Kuoppa, K., Oyston, P.C.F., Titball, R.W., Thomas, R.M., Forsberg, Å., Prior, J.L., and Koomey, M. (2011) O-linked glycosylation of the PilA pilin protein of *Francisella tularensis*: identification of the endogenous protein-targeting oligosaccharyltransferase and characterization of the native oligosaccharide. *J Bacteriol* **193**: 5487–97.

Eichler, J. (2013) Extreme sweetness: protein glycosylation in archaea. *Nat Rev Microbiol* **11**: 151–6.

Elhenawy, W., Scott, N.E., Tondo, M.L., Orellano, E.G., Foster, L.J., and Feldman, M.F. (2015) Protein O-linked glycosylation in the plant pathogen *Ralstonia solanacearum*. *Glycobiology* cwv098.

Elmi, A., Watson, E., Sandu, P., Gundogdu, O., Mills, D.C., Inglis, N.F., Manson, E., Imrie, L., Bajaj-Elliott, M., Wren, B.W., Smith, D.G.E., and Dorrell, N. (2012) *Campylobacter jejuni* outer membrane vesicles play an important role in bacterial interactions with human intestinal epithelial cells. *Infect Immun* **80**: 4089–98.

Erbel, P.J.A., Barr, K., Gao, N., Gerwig, G.J., Rick, P.D., and Gardner, K.H. (2003) Identification and biosynthesis of cyclic enterobacterial common antigen in *Escherichia coli*. *J Bacteriol* **185**: 1995–2004.

Eskola, J., Käyhty, H., Takala, A.K., Peltola, H., Rönnberg, P.-R., Kela, E., Pekkanen, E., McVerry, P. H.,Mäkelä, P. H. (1990) A Randomized, Prospective Field Trial of a Conjugate Vaccine in the Protection of Infants and Young Children against Invasive *Haemophilus influenzae* Type b Disease. *N Engl J Med* **323**: 1381–1387.

Eskola, J., Peltola, H., Takala, A.K., Käyhty, H., Hakulinen, M., Karanko, V., Kela, E., Rekola, P., Rönnberg, P.R., and Samuelson, J.S. (1987) Efficacy of Haemophilus influenzae type b polysaccharide-diphtheria toxoid conjugate vaccine in infancy. *N Engl J Med* **317**: 717–22.

Fantappiè, L., Santis, M. de, Chiarot, E., Carboni, F., Bensi, G., Jousson, O., Margarit, I., and Grandi, G. (2014) Antibody-mediated immunity induced by engineered *Escherichia coli* OMVs carrying heterologous antigens in their lumen. *J Extracell vesicles* **3**.

Faridmoayer, A. and Feldman, M.F. (2010) Comprehensive Natural Products II. Elsevier.

Faridmoayer, A., Fentabil, M.A., Haurat, M.F., Yi, W., Woodward, R., Wang, P.G., and Feldman, M.F. (2008) Extreme substrate promiscuity of the *Neisseria* oligosaccharyl transferase involved in protein *O*-glycosylation. *J Biol Chem* **283**: 34596–604.

Faridmoayer, A., Fentabil, M.A., Mills, D.C., Klassen, J.S., and Feldman, M.F. (2007) Functional characterization of bacterial oligosaccharyltransferases involved in O-linked protein glycosylation. *J Bacteriol* **189**: 8088–98.

Feldman, C., and Anderson, R. (2014) Review: current and new generation pneumococcal vaccines. *J Infect* **69**: 309–25.

Feldman, M.F., Marolda, C.L., Monteiro, M.A., Perry, M.B., Parodi, A.J., and Valvano, M.A. (1999) The Activity of a Putative Polyisoprenol-linked Sugar Translocase (Wzx) Involved in *Escherichia coli* O Antigen Assembly Is Independent of the Chemical Structure of the O Repeat. *J Biol Chem* **274**: 35129–35138.

Feldman, M.F., Wacker, M., Hernandez, M., Hitchen, P.G., Marolda, C.L., Kowarik, M., *et al.* (2005) Engineering N-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli*. *Proc Natl Acad Sci U S A* **102**: 3016–21.

Figurski, D.H., and Helinski, D.R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci* **76**: 1648–1652.

Fisher, A.C., Haitjema, C.H., Guarino, C., Celik, E., Endicott, C.E., Reading, C.A., Merritt, J.H., Ptak, A.C., Zhang, S., and DeLisa, M.P. (2010) Production of Secretory and Extracellular N-Linked Glycoproteins in *Escherichia coli*. *Appl Environ Microbiol* **77**: 871–881.

Fisher, C.L., and Pei, G.K. (1997) Modification of a PCR-based site-directed mutagenesis method. *Biotechniques* **23**: 570–1, 574.

Francis, T., and Tillett, W.S. (1930) Cutaneous reactions in pneumonia. The development of antibodies following the intradermal injection of type-specific polysaccharide. *J Exp Med* **52**: 573–85.

Frasch, C.E. (2009) Preparation of bacterial polysaccharide-protein conjugates: analytical and manufacturing challenges. *Vaccine* **27**: 6468–70.

Gaddy, J.A., Tomaras, A.P., and Actis, L.A. (2009) The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. *Infect Immun* **77**: 3150–60.

Garcia-Quintanilla, F., Iwashkiw, J.A., Price, N.L., Stratilo, C., and Feldman, M.F. (2014) Production of a recombinant vaccine candidate against *Burkholderia pseudomallei* exploiting the bacterial N-glycosylation machinery. *Front Microbiol* **5**: 381.

Gebhart, C., Ielmini, M.V., Reiz, B., Price, N.L., Aas, F.E., Koomey, M., and Feldman, M.F. (2012) Characterization of exogenous bacterial oligosaccharyltransferases in *Escherichia coli* reveals the potential for O-linked protein glycosylation in Vibrio cholerae and *Burkholderia thailandensis*. *Glycobiology* **22**: 962–74.

Gillis, L.M., White, H.D., Whitehurst, A., and Sullivan, D.C. (2005) Vancomycin-tolerance among clinical isolates of *Streptococcus pneumoniae* in Mississippi during 1999-2001. *Am J Med Sci* **330**: 65–8.

Goldberg, J.B., Hatano, K., Meluleni, G.S., and Pier, G.B. (1992) Cloning and surface expression of *Pseudomonas aeruginosa* O antigen in *Escherichia coli*. *Proc Natl Acad Sci U S A* **89**: 10716–20.

Goldblatt, D. (2000) Conjugate vaccines. *Clin Exp Immunol* **119**: 1–3.

Gordon, N.C., and Wareham, D.W. (2010) Multidrug-resistant *Acinetobacter baumannii*: mechanisms of virulence and resistance. *Int J Antimicrob Agents* **35**: 219–26.

Gorringe, A.R., and Pajón, R. (2012) Bexsero: a multicomponent vaccine for prevention of meningococcal disease. *Hum Vaccin Immunother* **8**: 174–83.

Göttig, S., Gruber, T.M., Higgins, P.G., Wachsmuth, M., Seifert, H., and Kempf, V.A.J. (2014) Detection of pan drug-resistant *Acinetobacter baumannii* in Germany. *J Antimicrob Chemother* **69**: 2578–9.

Grabenstein, J.D., and Klugman, K.P. (2012) A century of pneumococcal vaccination research in humans. *Clin Microbiol Infect* **18 Suppl 5**: 15–24.

Grass, S., Buscher, A.Z., Swords, W.E., Apicella, M.A., Barenkamp, S.J., Ozchlewski, N., and St Geme, J.W. (2003) The *Haemophilus influenzae* HMW1 adhesin is glycosylated in a process that requires HMW1C and phosphoglucomutase, an enzyme involved in lipooligosaccharide biosynthesis. *Mol Microbiol* **48**: 737–751.

Griffioen, A.W., Rijkers, G.T., Janssens-Korpela, P., and Zegers, B.J. (1991) Pneumococcal polysaccharides complexed with C3d bind to human B lymphocytes via complement receptor type 2. *Infect Immun* **59**: 1839–45.

Gross, J., Grass, S., Davis, A.E., Gilmore-Erdmann, P., Townsend, R.R., and St Geme, J.W. (2008) The *Haemophilus influenzae* HMW1 adhesin is a glycoprotein with an unusual N-linked carbohydrate modification. *J Biol Chem* **283**: 26010–5.

Guan, Z., Naparstek, S., Calo, D., and Eichler, J. (2012) Protein glycosylation as an adaptive response in Archaea: growth at different salt concentrations leads to alterations in *Haloferax volcanii* S-layer glycoprotein N-glycosylation. *Environ Microbiol* **14**: 743–53.

Hanage, W.P., Finkelstein, J.A., Huang, S.S., Pelton, S.I., Stevenson, A.E., Kleinman, K., Hinrichsen, V.L., and Fraser, C. (2010) Evidence that pneumococcal serotype replacement in Massachusetts following conjugate vaccination is now complete. *Epidemics* **2**: 80–4.

Hang, H.C., and Bertozzi, C.R. (2005) The chemistry and biology of mucin-type O-linked glycosylation. *Bioorg Med Chem* **13**: 5021–34.

Hansman, D., and Bullen, M.M. (1967) A RESISTANT PNEUMOCOCCUS. *Lancet* **290**: 264–265.

Hanuszkiewicz, A., Pittock, P., Humphries, F., Moll, H., Roa Rosales, A., Molinaro, A., Moynagh, P.N., Lajoie, G., and Valvano, M.A. (2014) Identification of the flagellin glycosylation system in *Burkholderia cenocepacia* and the contribution of glycosylated flagellin to evasion of human innate immune responses. *J Biol Chem* M114.562603–..

Harding, C.M., Nasr, M.A., Kinsella, R.L., Scott, N.E., Foster, L.J., Weber, B.S., Fiester, S. E., Actis, L. A., Tracy, E. N., Munson, R. S., Feldman, M. F. (2015) *Acinetobacter* strains carry two functional oligosaccharyltransferases, one devoted exclusively to type IV pilin, and the other one dedicated to *O*-glycosylation of multiple proteins. *Mol Microbiol* .

Harding, C.M., Tracy, E.N., Carruthers, M.D., Rather, P.N., Actis, L.A., and Munson, R.S. (2013) *Acinetobacter baumannii* strain M2 produces type IV pili which play a role in natural transformation and twitching motility but not surface-associated motility. *MBio* **4**: e00360–13–.

Hausdorff, W.P., Bryant, J., Paradiso, P.R., and Siber, G.R. (2000) Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. *Clin Infect Dis* **30**: 100–21.

Hegge, F.T., Hitchen, P.G., Aas, F.E., Kristiansen, H., Løvold, C., Egge-Jacobsen, W., Panico, M., Leong, W.Y., Bull, V., Virji, M., Morris, H.R., Dell, A., and Koomey, M. (2004) Unique modifications with phosphocholine and phosphoethanolamine define alternate antigenic forms of *Neisseria gonorrhoeae* type IV pili. *Proc Natl Acad Sci U S A* **101**: 10798–803.

Helenius, A., and Aebi, M. (2004) Roles of N-linked glycans in the endoplasmic reticulum. *Annu Rev Biochem* **73**: 1019–49.

Hicks, L.A., Harrison, L.H., Flannery, B., Hadler, J.L., Schaffner, W., Craig, A.S., Jackson, D., Thomas, A., Beall, B., Lynfield, R., Reingold, A., Farley, M. M., Whitney, C. G. (2007) Incidence of Pneumococcal Disease Due to Non–Pneumococcal Conjugate Vaccine (PCV7) Serotypes in the United States during the Era of Widespread PCV7 Vaccination, 1998–2004. *J Infect Dis* **196**: 1346–1354.

Horikawa, K., Martin, S.W., Pogue, S.L., Silver, K., Peng, K., Takatsu, K., and Goodnow, C.C. (2007) Enhancement and suppression of signaling by the conserved tail of IgG memory-type B cell antigen receptors. *J Exp Med* **204**: 759–69.

Horzempa, J., Dean, C.R., Goldberg, J.B., and Castric, P. (2006) *Pseudomonas aeruginosa* 1244 pilin glycosylation: glycan substrate recognition. *J Bacteriol* **188**: 4244–52.

Horzempa, J., Held, T.K., Cross, A.S., Furst, D., Qutyan, M., Neely, A.N., and Castric, P. (2008) Immunization with a *Pseudomonas aeruginosa* 1244 pilin provides O-antigen-specific protection. *Clin Vaccine Immunol* **15**: 590–7.

Hosokawa, T. (1979) Studies on B-cell memory. II. T-cell independent antigen can induce B-cell memory. *Immunology* **38**: 291–9.

Hu, D., Liu, B., Dijkshoorn, L., Wang, L., and Reeves, P.R. (2013) Diversity in the major polysaccharide antigen of *Acinetobacter baumannii* assessed by DNA sequencing, and development of a molecular serotyping scheme. *PLoS One* **8**: e70329.

Hug, I., and Feldman, M.F. (2011) Analogies and homologies in lipopolysaccharide and glycoprotein biosynthesis in bacteria. *Glycobiology* **21**: 138–51.

Hunger, M., Schmucker, R., Kishan, V., and Hillen, W. (1990) Analysis and nucleotide sequence of an origin of an origin of DNA replication in *Acinetobacter calcoaceticus* and its use for *Escherichia coli* shuttle plasmids. *Gene* **87**: 45–51.

Ielmini, M. V, and Feldman, M.F. (2011) *Desulfovibrio desulfuricans* PglB homolog possesses oligosaccharyltransferase activity with relaxed glycan specificity and distinct protein acceptor sequence requirements. *Glycobiology* **21**: 734–42.

Ihssen, J., Haas, J., Kowarik, M., Wiesli, L., Wacker, M., Schwede, T., and Thöny-Meyer, L. (2015) Increased efficiency of *Campylobacter jejuni N*-oligosaccharyltransferase PglB by structure-guided engineering. *Open Biol* **5**: 140227.

Ihssen, J., Kowarik, M., Dilettoso, S., Tanner, C., Wacker, M., and Thöny-Meyer, L. (2010) Production of glycoprotein vaccines in *Escherichia coli*. *Microb Cell Fact* **9**: 61.

Iwashkiw, J.A., Fentabil, M.A., Faridmoayer, A., Mills, D.C., Peppler, M., Czibener, C., Ciocchini, A.E., Comerci, D.J., Ugalde, J.E., and Feldman, M.F. (2012) Exploiting the *Campylobacter jejuni* protein glycosylation system for glycoengineering vaccines and diagnostic tools directed against brucellosis. *Microb Cell Fact* **11**: 13.

Iwashkiw, J.A., Seper, A., Weber, B.S., Scott, N.E., Vinogradov, E., Stratilo, C., Reiz, B., Cordwell, S.J., Whittal, R., Schild, S., and Feldman, M.F. (2012) Identification of a General O-linked Protein Glycosylation System in *Acinetobacter baumannii* and Its Role in Virulence and Biofilm Formation. *PLoS Pathog* **8**: e1002758.

Iwashkiw, J.A., Vozza, N.F., Kinsella, R.L., and Feldman, M.F. (2013) Pour some sugar on it: the expanding world of bacterial protein O-linked glycosylation. *Mol Microbiol* **89**: 14–28.

Jaeken, J. (2013) Congenital disorders of glycosylation. *Handb Clin Neurol* **113**: 1737–43.

Jarrell, K.F., Ding, Y., Meyer, B.H., Albers, S.-V., Kaminski, L., and Eichler, J. (2014) N-linked glycosylation in Archaea: a structural, functional, and genetic analysis. *Microbiol Mol Biol Rev* **78**: 304–41.

Jauneikaite, E., Jefferies, J.M., Hibberd, M.L., and Clarke, S.C. (2012) Prevalence of *Streptococcus pneumoniae* serotypes causing invasive and non-invasive disease in South East Asia: a review. *Vaccine* **30**: 3503–14.

Jenkins, S.G., Brown, S.D., and Farrell, D.J. (2008) Trends in antibacterial resistance among *Streptococcus pneumoniae* isolated in the USA: update from PROTEKT US Years 1-4. *Ann Clin Microbiol Antimicrob* **7**: 1.

Jennings, M.P., Jen, F.E.-C., Roddam, L.F., Apicella, M.A., and Edwards, J.L. (2011) *Neisseria gonorrhoeae* pilin glycan contributes to CR3 activation during challenge of primary cervical epithelial cells. *Cell Microbiol* **13**: 885–96.

Jervis, A.J., Butler, J.A., Lawson, A.J., Langdon, R., Wren, B.W., and Linton, D. (2012) Characterization of the structurally diverse N-linked glycans of *Campylobacter* species. *J Bacteriol* **194**: 2355–62.

Jervis, A.J., Langdon, R., Hitchen, P., Lawson, A.J., Wood, A., Fothergill, J.L., Morris, H. R., Dell, A., Wren, B., Linton, D. (2010) Characterization of *N*-linked protein glycosylation in *Helicobacter pullorum*. *J Bacteriol* **192**: 5228–36.

Josenhans, C., Vossebein, L., Friedrich, S., and Suerbaum, S. (2002) The neuA/flmD gene cluster of *Helicobacter pylori* is involved in flagellar biosynthesis and flagellin glycosylation. *FEMS Microbiol Lett* **210**: 165–72.

Kadioglu, A., Weiser, J.N., Paton, J.C., and Andrew, P.W. (2008) The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. *Nat Rev Microbiol* **6**: 288–301.

Kämpf, M.M., Braun, M., Sirena, D., Ihssen, J., Thöny-Meyer, L., and Ren, Q. (2015) In vivo production of a novel glycoconjugate vaccine against Shigella flexneri 2a in recombinant *Escherichia coli*: identification of stimulating factors for in vivo glycosylation. *Microb Cell Fact* **14**: 12.

Kempf, M., Varon, E., Lepoutre, A., Gravet, A., Baraduc, R., Brun, M., Chardon, H., Cremniter, J., Croizé, J., Dalmay, F., Demachy, M. C., Fosse, T., Grelaud, C., Hadou, T., Hamdad, F., Koeck, J. L., Luce, S., Mermond, S., Patry, I., Péchinot, A., Raymond, J., Ros, A., Segonds, C., Soullié, B., Tandé, D., Vergnaud, M., Vernet-Garnier, V., Wallet, F., Gutmann, L., Ploy, M. C., Lanotte, P. (2015) Decline in antibiotic resistance and changes in the serotype distribution of *Streptococcus pneumoniae* isolates from children with acute otitis media; a 2001-2011 survey by the French Pneumococcal Network. *Clin Microbiol Infect* **21**: 35–42.

Kenyon, J.J., Nigro, S.J., and Hall, R.M. (2014) Variation in the OC locus of *Acinetobacter baumannii* genomes predicts extensive structural diversity in the lipooligosaccharide. *PLoS One* **9**: e107833.

Kim, Y.T., and Siskind, G.W. (1978) Studies on the control of antibody synthesis. XII. Genetic influences on antibody affinity. *Immunology* **34**: 669–78.

Konradsen, H.B. (1995) Quantity and avidity of pneumococcal antibodies before and up to five years after pneumococcal vaccination of elderly persons. *Clin Infect Dis* **21**: 616–20.

Korona-Glowniak, I., and Malm, A. (2012) Characteristics of *Streptococcus pneumoniae* strains colonizing upper respiratory tract of healthy preschool children in Poland. *ScientificWorldJournal* **2012**: 732901.

Kowarik, M., Young, N.M., Numao, S., Schulz, B.L., Hug, I., Callewaert, N., Mills D. C., Watson, D. C., Hernandez, M., Kelly, J. F., Wacker, M., Aebi, M. (2006) Definition of the bacterial *N*-glycosylation site consensus sequence. *EMBO J* **25**: 1957–66.

Ku, S.C., Schulz, B.L., Power, P.M., and Jennings, M.P. (2009) The pilin O-glycosylation pathway of pathogenic *Neisseria* is a general system that glycosylates AniA, an outer membrane nitrite reductase. *Biochem Biophys Res Commun* **378**: 84–9.

Kumar, A., Dalton, C., Cortez-Cordova, J., and Schweizer, H.P. (2010) Mini-Tn7 vectors as genetic tools for single copy gene cloning in *Acinetobacter baumannii*. *J Microbiol Methods* **82**: 296–300.

Kus, J. V, Tullis, E., Cvitkovitch, D.G., and Burrows, L.L. (2004) Significant differences in type IV pilin allele distribution among *Pseudomonas aeruginosa* isolates from cystic fibrosis (CF) versus non-CF patients. *Microbiology* **150**: 1315–26.

Kuzmanov, U., Kosanam, H., and Diamandis, E.P. (2013) The sweet and sour of serological glycoprotein tumor biomarker quantification. *BMC Med* **11**: 31.

Lairson, L.L., Henrissat, B., Davies, G.J., and Withers, S.G. (2008) Glycosyltransferases: structures, functions, and mechanisms. *Annu Rev Biochem* **77**: 521–55.

Lee, E.C., Yu, D., Martinez de Velasco, J., Tessarollo, L., Swing, D.A., Court, D.L., Jenkins, N.A., and Copeland, N.G. (2001) A highly efficient *Escherichia coli*-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* **73**: 56–65.

Lee, E.-Y., Bang, J.Y., Park, G.W., Choi, D.-S., Kang, J.S., Kim, H.-J., Park, K.-S., Lee, J.-O., Kim, Y.-K., Kwon, K.-H., Kim, K.-P., and Gho, Y.S. (2007) Global proteomic profiling of native outer membrane vesicles derived from *Escherichia coli*. *Proteomics* **7**: 3143–53.

Lee, H., Park, S.J., Han, M.-J., Eom, G.T., Choi, M.-J., Kim, S.H., Oh, Y.H., Song, B.K., and Lee, S.H. (2013) Expression of a lipase on the cell-surface of *Escherichia coli* using the OmpW anchoring motif and its application to enantioselective reactions. *Biotechnol Lett* **35**: 1677–83.

Lees, A., Sen, G., and LopezAcosta, A. (2006) Versatile and efficient synthesis of protein–polysaccharide conjugate vaccines using aminooxy reagents and oxime chemistry. *Vaccine* **24**: 716–729.

Lees-Miller, R.G., Iwashkiw, J.A., Scott, N.E., Seper, A., Vinogradov, E., Schild, S., and Feldman, M.F. (2013) A common pathway for O-linked protein-glycosylation and synthesis of capsule in *Acinetobacter baumannii*. *Mol Microbiol* **89**: 816–30.

Li, D., Chiu, H., Zhang, H., and Chan, D.W. (2013) Analysis of serum protein glycosylation by a differential lectin immunosorbant assay (dLISA). *Clin Proteomics* **10**: 12.

Lin, T.-L., Yang, F.-L., Yang, A.-S., Peng, H.-P., Li, T.-L., Tsai, M.-D., Wu, S.-H., and Wang, J.-T. (2012) Amino acid substitutions of MagA in Klebsiella *pneumoniae* affect the biosynthesis of the capsular polysaccharide. *PLoS One* **7**: e46783.

Linton, D., Dorrell, N., Hitchen, P.G., Amber, S., Karlyshev, A. V, Morris, H.R., Dell, A., Valvano, M.A., Aebi, M., and Wren, B.W. (2005) Functional analysis of the *Campylobacter jejuni* N-linked protein glycosylation pathway. *Mol Microbiol* **55**: 1695–703.

Lithgow, K. V., Scott, N.E., Iwashkiw, J.A., Thomson, E.L.S., Foster, L.J., Feldman, M.F., and Dennis, J.J. (2014) A general protein *O*-glycosylation system within the *Burkholderia cepacia* complex is involved in motility and virulence. *Mol Microbiol* n/a–n/a.

Liu, D., and Reeves, P.R. (1994) *Escherichia coli* K12 regains its O antigen. *Microbiology* **140 (Pt 1**: 49–57.

Lizak, C., Gerber, S., Numao, S., Aebi, M., and Locher, K.P. (2011) X-ray structure of a bacterial oligosaccharyltransferase. *Nature* **474**: 350–5.

Logan, S.M. (2006) Flagellar glycosylation - a new component of the motility repertoire? *Microbiology* **152**: 1249–62.

Lund, E. (1970) On the nomenclature of the pneumococcal types. *Int J Syst Bacteriol* **20**: 321–323.

Macleod, C.M., Hodges, R.G., Heidelberger, M., and Bernhard, W.G. (1945) Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides. *J Exp Med* **82**: 445–65.

Magidovich, H., and Eichler, J. (2009) Glycosyltransferases and oligosaccharyltransferases in Archaea: putative components of the N-glycosylation pathway in the third domain of life. *FEMS Microbiol Lett* **300**: 122–30.

Maita, N., Nyirenda, J., Igura, M., Kamishikiryo, J., and Kohda, D. (2010) Comparative structural biology of eubacterial and archaeal oligosaccharyltransferases. *J Biol Chem* **285**: 4941–50.

Mäkelä, P.H., and Käyhty, H. (2014) Evolution of conjugate vaccines. *Expert Rev Vaccines* .

Malley, R., Lipsitch, M., Stack, A., Saladino, R., Fleisher, G., Pelton, S., Thompson, C., Briles, D., and Anderson, P. (2001) Intranasal Immunization with Killed Unencapsulated Whole Cells Prevents Colonization and Invasive Disease by Capsulated Pneumococci. *Infect Immun* **69**: 4870–4873.

Mara, K., Decorosi, F., Viti, C., Giovannetti, L., Papaleo, M.C., Maida, I., Perrin, E., Fondi, M., Vaneechoutte, M., Nemec, A., Barselaar, M. van den, Dijkshoorn, L., and Fani, R. (2012) Molecular and phenotypic characterization of *Acinetobacter* strains able to degrade diesel fuel. *Res Microbiol* **163**: 161–72.

Marchler-Bauer, A., and Bryant, S.H. (2004) CD-Search: protein domain annotations on the fly. *Nucleic Acids Res* **32**: W327–31
Marchler-Bauer, A., Anderson, J.B., Chitsaz, F., Derbyshire, M.K., DeWeese-Scott, C., Fong, J.H., *et al.* (2009) CDD: specific functional annotation with the Conserved Domain Database. *Nucleic Acids Res* **37**: D205–10
Marchler-Bauer, A., Lu, S., Anderson, J.B., Chitsaz, F., Derbyshire, M.K., DeWeese-Scott, C., *et al.* (2011) CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res* **39**: D225–9

Marciani, D.J. (2003) Vaccine adjuvants: role and mechanisms of action in vaccine immunogenicity. *Drug Discov Today* **8**: 934–943.

Marolda, C.L., Feldman, M.F., and Valvano, M.A. (1999) Genetic organization of the O7-specific lipopolysaccharide biosynthesis cluster of *Escherichia coli* VW187 (O7:K1). *Microbiology* **145 (Pt 9**: 2485–95.

Meany, D.L., and Chan, D.W. (2011) Aberrant glycosylation associated with enzymes as cancer biomarkers. *Clin Proteomics* **8**: 7.

Merino, S., and Tomás, J.M. (2014) Gram-negative flagella glycosylation. *Int J Mol Sci* **15**: 2840–57.

Mescher, M.F., and Strominger, J.L. (1976) Structural (shape-maintaining) role of the cell surface glycoprotein of Halobacterium salinarium. *Proc Natl Acad Sci U S A* **73**: 2687–91.

Moreau, M., Richards, J.C., Fournier, J.M., Byrd, R.A., Karakawa, W.W., and Vann, W.F. (1990) Structure of the type 5 capsular polysaccharide of *Staphylococcus aureus*. *Carbohydr Res* **201**: 285–97.

Musumeci, M.A., Faridmoayer, A., Watanabe, Y., and Feldman, M.F. (2014) Evaluating the role of conserved amino acids in bacterial O-oligosaccharyltransferases by in vivo, in vitro and limited proteolysis assays. *Glycobiology* **24**: 39–50.

Musumeci, M.A., Hug, I., Scott, N., Ielmini, M.V., Foster, L.J., Wang, P.G., and Feldman, M.F. (2013) In vitro activity of *Neisseria meningitidis* PglL *O*-oligosaccharyltransferase with diverse synthetic lipid donors and a UDP-activated sugar. J Biol Chem 288: 10578–10587.

Musumeci, M.A., Ielmini, M. V, and Feldman, M.F. (2013) *In vitro* glycosylation assay for bacterial oligosaccharyltransferases. *Methods Mol Biol* **1022**: 161–71.

Nakar, D., and Gutnick, D.L. (2001) Analysis of the wee gene cluster responsible for the biosynthesis of the polymeric bioemulsifier from the oil-degrading strain *Acinetobacter* lwoffii RAG-1. *Microbiology* **147**: 1937–1946.

Nakhamchik, A., Wilde, C., and Rowe-Magnus, D.A. (2007) Identification of a Wzy polymerase required for group IV capsular polysaccharide and lipopolysaccharide biosynthesis in Vibrio vulnificus. *Infect Immun* **75**: 5550–8.

Nassif, X., Lowy, J., Stenberg, P., O'Gaora, P., Ganji, A., and So, M. (1993) Antigenic variation of pilin regulates adhesion of *Neisseria meningitidis* to human epithelial cells. *Mol Microbiol* **8**: 719–725.

Neal, B.L., Brown, P.K., and Reeves, P.R. (1993) Use of *Salmonella* phage P22 for transduction in *Escherichia coli*. *J Bacteriol* **175**: 7115–8.

Neuberger, A. (1938) Carbohydrates in protein: The carbohydrate component of crystalline egg albumin. *Biochem J* **32**: 1435–51.

Nguyen, L.C., Taguchi, F., Tran, Q.M., Naito, K., Yamamoto, M., Ohnishi-Kameyama, M., Ono, H., Yoshida, M., Chiku, K., Ishii, T., Inagaki, Y., Toyoda, K., Shiraishi, T., and Ichinose, Y. (2012) Type IV pilin is glycosylated in *Pseudomonas syringae* pv. tabaci 6605 and is required for surface motility and virulence. *Mol Plant Pathol* **13**: 764–74.

Niu, C., Clemmer, K.M., Bonomo, R.A., and Rather, P.N. (2008) Isolation and characterization of an autoinducer synthase from *Acinetobacter baumannii*. *J Bacteriol* **190**: 3386–92.

Nothaft, H., and Szymanski, C.M. (2010) Protein glycosylation in bacteria: sweeter than ever. *Nat Rev Microbiol* **8**: 765–78.

Nothaft, H., and Szymanski, C.M. (2013) Bacterial protein N-glycosylation: new perspectives and applications. *J Biol Chem* **288**: 6912–20.

Nothaft, H., Liu, X., McNally, D.J., Li, J., and Szymanski, C.M. (2009) Study of free oligosaccharides derived from the bacterial N-glycosylation pathway. *Proc Natl Acad Sci U S A* **106**: 15019–24.

Nothaft, H., Scott, N.E., Vinogradov, E., Liu, X., Hu, R., Beadle, B., Fodor, C., Miller, W.G., Li, J., Cordwell, S.J., and Szymanski, C.M. (2012) Diversity in the protein N-glycosylation pathways within the *Campylobacter* genus. *Mol Cell Proteomics* **11**: 1203–19.

Novak, R., Henriques, B., Charpentier, E., Normark, S., and Tuomanen, E. (1999) Emergence of vancomycin tolerance in *Streptococcus pneumoniae*. *Nature* **399**: 590–3.

O'Brien, K.L., Wolfson, L.J., Watt, J.P., Henkle, E., Deloria-Knoll, M., McCall, N., Lee, E., Mulholland, K., Levine, O.S., and Cherian, T. (2009) Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet* **374**: 893–902.

Obukhanych, T. V. (2006) T-independent type II immune responses generate memory B cells. *J Exp Med* **203**: 305–310.

Ochs, M.M., Bartlett, W., Briles, D.E., Hicks, B., Jurkuvenas, A., Lau, P., Ren, B., and Millar, A. (2008) Vaccine-induced human antibodies to PspA augment complement C3 deposition on *Streptococcus pneumoniae*. *Microb Pathog* **44**: 204–14.

Ogunniyi, A.D., Folland, R.L., Briles, D.E., Hollingshead, S.K., and Paton, J.C. (2000) Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. *Infect Immun* **68**: 3028–33.

Olsen, J. V, Macek, B., Lange, O., Makarov, A., Horning, S., and Mann, M. (2007) Higher-energy C-trap dissociation for peptide modification analysis. *Nat Methods* **4**: 709–12.

Ong, S.-E., and Mann, M. (2006) A practical recipe for stable isotope labeling by amino acids in cell culture (SILAC). *Nat Protoc* **1**: 2650–60.

Ortega, M., Marco, F., Soriano, A., García, E., Martínez, J.A., and Mensa, J. (2003) Lack of vancomycin tolerance in *Streptococcus pneumoniae* strains isolated in Barcelona, Spain, from 1999 to 2001. *Antimicrob Agents Chemother* **47**: 1976–8.

Pace, D. (2013) Glycoconjugate vaccines. *Expert Opin Biol Ther* **13**: 11–33.

Parge, H.E., Forest, K.T., Hickey, M.J., Christensen, D.A., Getzoff, E.D., and Tainer, J.A. (1995) Structure of the fibre-forming protein pilin at 2.6 A resolution. *Nature* **378**: 32–8.

Peeters, C.C.A.M., Lagerman, P.R., Weers, O. de, Oomen, L.A., Hoogerhout, P., Beurret, M., Poolman, J.T., and Reddin, K.M. (2003) Preparation of polysaccharide-conjugate vaccines. *Methods Mol Med* **87**: 153–74.

Pendleton, J.N., Gorman, S.P., and Gilmore, B.F. (2013) Clinical relevance of the ESKAPE pathogens. *Expert Rev Anti Infect Ther* **11**: 297–308.

Pérez, J.M., McGarry, M.A., Marolda, C.L., and Valvano, M.A. (2008) Functional analysis of the large periplasmic loop of the *Escherichia coli* K-12 WaaL O-antigen ligase. *Mol Microbiol* **70**: 1424–40.

Pilishvili, T., Lexau, C., Farley, M.M., Hadler, J., Harrison, L.H., Bennett, N.M., Reingold, A., Thomas, A., Schaffner, W., Craig, A.S., Smith, P.J., Beall, B.W., Whitney, C.G., and Moore, M.R. (2010) Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. *J Infect Dis* **201**: 32–41.

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Pollard, A.J., Perrett, K.P., and Beverley, P.C. (2009) Maintaining protection against invasive bacteria with protein-polysaccharide conjugate vaccines. *Nat Rev Immunol* **9**: 213–20.

Porstendorfer, D., Gohl, O., Mayer, F., and Averhoff, B. (2000) ComP, a Pilin-Like Protein Essential for Natural Competence in *Acinetobacter* sp. Strain BD413: Regulation, Modification, and Cellular Localization. *J Bacteriol* **182**: 3673–3680.

Power, E. (2006) Impact of antibiotic restrictions: the pharmaceutical perspective. *Clin Microbiol Infect* **12**: 25–34.

Power, P., and Jennings, M. (2003) The genetics of glycosylation in Gram-negative bacteria. *FEMS Microbiol Lett* 218: 211–222

Power, P.M., Roddam, L.F., Rutter, K., Fitzpatrick, S.Z., Srikhanta, Y.N., and Jennings, M.P. (2004) Genetic characterization of pilin glycosylation and phase variation in *Neisseria meningitidis*. *Mol Microbiol* **49**: 833–847.

Power, P.M., Seib, K.L., and Jennings, M.P. (2006) Pilin glycosylation in *Neisseria meningitidis* occurs by a similar pathway to wzy-dependent O-antigen biosynthesis in *Escherichia coli*. *Biochem Biophys Res Commun* **347**: 904–8.

Qutyan, M., Henkel, M., Horzempa, J., Quinn, M., and Castric, P. (2010) Glycosylation of Pilin and Nonpilin Protein Constructs by *Pseudomonas aeruginosa* 1244. *J Bacteriol* **192**: 5972–5981.

Rappsilber, J., Mann, M., and Ishihama, Y. (2007) Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat Protoc* **2**: 1896–906.

Reen, F.J., Woods, D.F., Mooij, M.J., Adams, C., and O'Gara, F. (2012) Respiratory pathogens adopt a chronic lifestyle in response to bile. *PLoS One* **7**: e45978.

Rekola, P., Rönnberg, P. R., Samuelson, J. S. (1987) Efficacy of *Haemophilus influenzae* type b polysaccharide-diphtheria toxoid conjugate vaccine in infancy. *N Engl J Med* **317**: 717–22.

Riedel, S. (2005) Edward Jenner and the history of smallpox and vaccination. *Proc (Bayl Univ Med Cent)* **18**: 21–5.

Robinson, J.H., and Delvig, A.A. (2002) Diversity in MHC class II antigen presentation. *Immunology* **105**: 252–62.

Ruan, X., Loyola, D.E., Marolda, C.L., Perez-Donoso, J.M., and Valvano, M.A. (2012) The WaaL O-antigen lipopolysaccharide ligase has features in common with metal ion-independent inverting glycosyltransferases. *Glycobiology* **22**: 288–99.

Samatey, F.A., Imada, K., Vonderviszt, F., Shirakihara, Y., and Namba, K. (2000) Crystallization of the F41 Fragment of Flagellin and Data Collection from Extremely Thin Crystals. *J Struct Biol* **132**: 106–111.

Sanchez, C.J., Kumar, N., Lizcano, A., Shivshankar, P., Dunning Hotopp, J.C., Jorgensen, J.H., Tettelin, H., and Orihuela, C.J. (2011) *Streptococcus pneumoniae* in biofilms are unable to cause invasive disease due to altered virulence determinant production. *PLoS One* **6**: e28738.

Santolaya, M.E., O'Ryan, M.L., Valenzuela, M.T., Prado, V., Vergara, R., Muñoz, A., Toneatto, D., Graña, G., Wang, H., Clemens, R., and Dull, P.M. (2012) Immunogenicity and tolerability of a multicomponent meningococcal serogroup B (4CMenB) vaccine in healthy adolescents in Chile: a phase 2b/3 randomised, observer-blind, placebo-controlled study. *Lancet (London, England)* **379**: 617–24.

Schedin-Weiss, S., Winblad, B., and Tjernberg, L.O. (2014) The role of protein glycosylation in Alzheimer disease. *FEBS J* **281**: 46–62.

Schild, S., Lamprecht, A.-K., and Reidl, J. (2005) Molecular and functional characterization of O antigen transfer in *Vibrio cholerae*. *J Biol Chem* **280**: 25936–47.

Schirm, M., Arora, S.K., Verma, A., Vinogradov, E., Thibault, P., Ramphal, R., and Logan, S.M. (2004) Structural and Genetic Characterization of Glycosylation of Type a Flagellin in *Pseudomonas aeruginosa*. *J Bacteriol* **186**: 2523–2531.

Schnaitman, C.A. (1971) Solubilization of the Cytoplasmic Membrane of *Escherichia coli* by Triton X-100. *J Bacteriol* **108**: 545–552.

Schneerson, R., Robbins, J.B., Barrera, O., Sutton, A., Habig, W.B., Hardegree, M.C., and Chaimovich, J. (1980) *Haemophilus influenzae* type B polysaccharide-protein conjugates: model for a new generation of capsular polysaccharide vaccines. *Prog Clin Biol Res* **47**: 77–94.

Schulz, B.L., Jen, F.E.C., Power, P.M., Jones, C.E., Fox, K.L., Ku, S.C., Blanchfield, J.T., and Jennings, M.P. (2013) Identification of bacterial protein O-oligosaccharyltransferases and their glycoprotein substrates. *PLoS One* **8**: e62768.

Schwarz, F., Lizak, C., Fan, Y.-Y., Fleurkens, S., Kowarik, M., and Aebi, M. (2011) Relaxed acceptor site specificity of bacterial oligosaccharyltransferase *in vivo*. *Glycobiology* **21**: 45–54.

Scott, A.E., Twine, S.M., Fulton, K.M., Titball, R.W., Essex-Lopresti, A.E., Atkins, T.P., and Prior, J.L. (2011) Flagellar glycosylation in *Burkholderia pseudomallei* and *Burkholderia thailandensis*. *J Bacteriol* **193**: 3577–87.

Scott, N.E., Kinsella, R.L., Edwards, A.V.G., Larsen, M.R., Dutta, S., Saba, J., Foster, L.J., and Feldman, M.F. (2014) Diversity Within the O-linked Protein Glycosylation Systems of *Acinetobacter* Species. *Mol Cell Proteomics* **13**: 2354–70.

Scott, N.E., Nothaft, H., Edwards, A.V.G., Labbate, M., Djordjevic, S.P., Larsen, M.R., Szymanski, C.M., and Cordwell, S.J. (2012) Modification of the *Campylobacter jejuni* N-linked glycan by EptC protein-mediated addition of phosphoethanolamine. *J Biol Chem* **287**: 29384–96.

Scott, N.E., Parker, B.L., Connolly, A.M., Paulech, J., Edwards, A.V.G., Crossett, B., Falconer, L., Kolarich, D., Djordjevic, S.P., Højrup, P., Packer, N.H., Larsen, M.R., and Cordwell, S.J. (2011) Simultaneous glycan-peptide characterization using hydrophilic interaction chromatography and parallel fragmentation by CID, higher energy collisional dissociation, and electron transfer dissociation MS applied to the N-linked glycoproteome of Campylobact. *Mol Cell Proteomics* **10**: M000031–MCP201.

Shak, J.R., Vidal, J.E., and Klugman, K.P. (2013) Influence of bacterial interactions on pneumococcal colonization of the nasopharynx. *Trends Microbiol* **21**: 129–35.

Shapiro, E.D., Berg, A.T., Austrian, R., Schroeder, D., Parcells, V., Margolis, A., Adair, R.K., and Clemens, J.D. (1991) The protective efficacy of polyvalent pneumococcal polysaccharide vaccine. *N Engl J Med* **325**: 1453–60.

Shental-Bechor, D., and Levy, Y. (2008) Effect of glycosylation on protein folding: a close look at thermodynamic stabilization. *Proc Natl Acad Sci U S A* **105**: 8256–61.

Sherlock, O., Dobrindt, U., Jensen, J.B., Munk Vejborg, R., and Klemm, P. (2006) Glycosylation of the Self-Recognizing *Escherichia coli* Ag43 Autotransporter Protein. *J Bacteriol* **188**: 1798–1807.

Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V, and Mann, M. (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* **1**: 2856–60

Siegrist, C.-A., and Aspinall, R. (2009) B-cell responses to vaccination at the extremes of age. *Nat Rev Immunol* **9**: 185–94.

Simell, B., Lahdenkari, M., Reunanen, A., Kayhty, H., and Vakevainen, M. (2008) Effects of Ageing and Gender on Naturally Acquired Antibodies to Pneumococcal Capsular Polysaccharides and Virulence-Associated Proteins. *Clin Vaccine Immunol* **15**: 1391–1397.

Sleytr, U.B. (1975) Heterologous reattachment of regular arrays of glycoproteins on bacterial surfaces. *Nature* **257**: 400–402.

Sleytr, U.B., and Thorne, K.J. (1976) Chemical characterization of the regularly arranged surface layers of *Clostridium thermosaccharolyticum* and *Clostridium thermohydrosulfuricum*. *J Bacteriol* **126**: 377–83.

Smedley, J.G., Jewell, E., Roguskie, J., Horzempa, J., Syboldt, A., Stolz, D.B., and Castric, P. (2005) Influence of pilin glycosylation on *Pseudomonas aeruginosa* 1244 pilus function. *Infect Immun* **73**: 7922–31.

Soininen, A. (1999) IgG subclass distribution of antibodies after vaccination of adults with pneumococcal conjugate vaccines. *Vaccine* **17**: 1889–1897.

Song, J.H., Lee, N.Y., Ichiyama, S., Yoshida, R., Hirakata, Y., Fu, W., Chongthaleong, A., Aswapokee, N., Chiu, C.H., Lalitha, M.K., Thomas, K., Perera, J., Yee, T.T., Jamal, F., Warsa, U.C., Vinh, B.X., Jacobs, M.R., Appelbaum, P.C., and Pai, C.H. (1999) Spread of drug-resistant *Streptococcus pneumoniae* in Asian countries: Asian Network for Surveillance of Resistant Pathogens (ANSORP) Study. *Clin Infect Dis* **28**: 1206–11.

Sriramulu, D.D., Lünsdorf, H., Lam, J.S., and Römling, U. (2005) Microcolony formation: a novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung. *J Med Microbiol* **54**: 667–76.

Stefanetti, G., Hu, Q.-Y., Usera, A., Robinson, Z., Allan, M., Singh, A., Imase, H., Cobb, J., Zhai, H., Quinn, D., Lei, M., Saul, A., Adamo, R., MacLennan, C.A., and Micoli, F. (2015) Sugar-Protein Connectivity Impacts on the Immunogenicity of Site-Selective *Salmonella* O-Antigen Glycoconjugate Vaccines. *Angew Chemie Int Ed* **54**: n/a–n/a.

Stephenson, H.N., Mills, D.C., Jones, H., Milioris, E., Copland, A., Dorrell, N., Wren, B.W., Crocker, P.R., Escors, D., and Bajaj-Elliott, M. (2014) Pseudaminic acid on

Campylobacter jejuni flagella modulates dendritic cell IL-10 expression via Siglec-10 receptor: a novel flagellin-host interaction. *J Infect Dis* **210**: 1487–98.

Stimson, E., Virji, M., Makepeace, K., Dell, A., Morris, H.R., Payne, G., Saunders, J.R., Jennings, M.P., Barker, S., and Panico, M. (1995) Meningococcal pilin: a glycoprotein substituted with digalactosyl 2,4-diacetamido-2,4,6-trideoxyhexose. *Mol Microbiol* **17**: 1201–14.

Sung, H., Shin, H.B., Kim, M.-N., Lee, K., Kim, E.-C., Song, W., Jeong, S.H., Lee, W.-G., Park, Y.-J., and Eliopoulos, G.M. (2006) Vancomycin-Tolerant *Streptococcus pneumoniae* in Korea. *J Clin Microbiol* **44**: 3524–3528.

Sutherland, I.W. (2001) Microbial polysaccharides from Gram-negative bacteria. *Int Dairy J* **11**: 663–674.

Szymanski, C.M., Yao, R., Ewing, C.P., Trust, T.J., and Guerry, P. (1999) Evidence for a system of general protein glycosylation in *Campylobacter jejuni*. *Mol Microbiol* **32**: 1022–1030.

Tan, R.M., Kuang, Z., Hao, Y., Lee, F., Lee, T., Lee, R.J., and Lau, G.W. (2015) Type IV pilus glycosylation mediates resistance of *Pseudomonas aeruginosa* to opsonic activities of the pulmonary surfactant protein A. *Infect Immun* **83**: 1339–46.

Tan, R.M., Kuang, Z., Hao, Y., Lee, F., Lee, T., Lee, R.J., and Lau, G.W. (2015) Type IV pilus glycosylation mediates resistance of *Pseudomonas aeruginosa* to opsonic activities of the pulmonary surfactant protein A. *Infect Immun* **83**: 1339–46.

Tang, G., and Mintz, K.P. (2010) Glycosylation of the collagen adhesin EmaA of Aggregatibacter actinomycetemcomitans is dependent upon the lipopolysaccharide biosynthetic pathway. *J Bacteriol* **192**: 1395–404.

Tang, G., Ruiz, T., and Mintz, K.P. (2012) O-Polysaccharide Glycosylation Is Required for Stability and Function of the Collagen Adhesin EmaA of Aggregatibacter actinomycetemcomitans. *Infect Immun* **80**: 2868–2877.

Tangye, S.G., Avery, D.T., Deenick, E.K., and Hodgkin, P.D. (2003) Intrinsic differences in the proliferation of naive and memory human B cells as a mechanism for enhanced secondary immune responses. *J Immunol* **170**: 686–94.

Tarlinton, D. (2006) B-cell memory: are subsets necessary? *Nat Rev Immunol* **6**: 785–90.

Terra, V.S., Mills, D.C., Yates, L.E., Abouelhadid, S., Cuccui, J., and Wren, B.W. (2012) Recent developments in bacterial protein glycan coupling technology and glycoconjugate vaccine design. *J Med Microbiol* **61**: 919–926.

Thibault, P., Logan, S.M., Kelly, J.F., Brisson, J.R., Ewing, C.P., Trust, T.J., and Guerry, P. (2001) Identification of the carbohydrate moieties and glycosylation motifs in *Campylobacter jejuni* flagellin. *J Biol Chem* **276**: 34862–70.

Timens, W., Boes, A., Rozeboom-Uiterwijk, T., and Poppema, S. (1989) Immaturity of the human splenic marginal zone in infancy. Possible contribution to the deficient infant immune response. *J Immunol* **143**: 3200–6.

Tsai, C.M., and Frasch, C.E. (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* **119**: 115–9.

Tzianabos, A., Onderdonk, A., Rosner, B., Cisneros, R., and Kasper, D. (1993) Structural features of polysaccharides that induce intra-abdominal abscesses. *Science (80-)* **262**: 416–419.

Uchida, T., Pappenheimer, A.M., and Greany, R. (1973) Diphtheria toxin and related proteins. I. Isolation and properties of mutant proteins serologically related to diphtheria toxin. *J Biol Chem* **248**: 3838–44.

Vaccine development – Vaccine Development Global Program - PATH http://sites.path.org/vaccinedevelopment/pneumonia-and-pneumococcus/vaccine-development/. Accessed October 6, 2015.

Vadesilho, C.F.M., Ferreira, D.M., Moreno, A.T., Chavez-Olortegui, C., Machado de Avila, R.A., Oliveira, M.L.S., Ho, P.L., and Miyaji, E.N. (2012) Characterization of the antibody response elicited by immunization with pneumococcal surface protein A (PspA) as recombinant protein or DNA vaccine and analysis of protection against an intranasal lethal challenge with *Streptococcus pneumoniae*. *Microb Pathog* **53**: 243–9.

van Selm, S., van Cann, L.M., Kolkman, M.A.B., van der Zeijst, B.A.M., and Putten, J.P.M. van (2003) Genetic basis for the structural difference between *Streptococcus pneumoniae* serotype 15B and 15C capsular polysaccharides. *Infect Immun* **71**: 6192–8.

Vaneechoutte, M., Young, D.M., Ornston, L.N., Baere, T. De, Nemec, A., Reijden, T. Van Der, Carr, E., Tjernberg, I., and Dijkshoorn, L. (2006) Naturally Transformable *Acinetobacter* sp. Strain ADP1 Belongs to the Newly Described Species *Acinetobacter baylyi*. *Appl Environ Microbiol* **72**: 932–936.

Varki, A. (1993) Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* **3**: 97–130.

Velez, C.D., Lewis, C.J., Kasper, D.L., and Cobb, B.A. (2009) Type I *Streptococcus pneumoniae* carbohydrate utilizes a nitric oxide and MHC II-dependent pathway for antigen presentation. *Immunology* **127**: 73–82.

VFC | Current CDC Vaccine Price List | CDC http://www.cdc.gov/vaccines/programs/vfc/awardees/vaccine-management/price-list/. Accessed October 6, 2015.

Vik, A., Aas, F.E., Anonsen, J.H., Bilsborough, S., Schneider, A., Egge-Jacobsen, W., and Koomey, M. (2009) Broad spectrum *O*-linked protein glycosylation in the human pathogen *Neisseria gonorrhoeae*. *Proc Natl Acad Sci U S A* **106**: 4447–52.

Voisin, S., Kus, J. V, Houliston, S., St-Michael, F., Watson, D., Cvitkovitch, D.G., Kelly J., Brisson J.R. and Burrows L.L.. (2007) Glycosylation of *Pseudomonas aeruginosa* strain Pa5196 type IV pilins with mycobacterium-like alpha-1,5-linked d-Araf oligosaccharides. *J Bacteriol* **189**: 151–9.

Wacker, M., Feldman, M.F., Callewaert, N., Kowarik, M., Clarke, B.R., Pohl, N.L., Hernandez, M., Vines, E.D., Valvano, M.A., Whitfield, C., and Aebi, M. (2006) Substrate specificity of bacterial oligosaccharyltransferase suggests a common transfer mechanism for the bacterial and eukaryotic systems. *Proc Natl Acad Sci U S A* **103**: 7088–93.

Wacker, M., Linton, D., Hitchen, P.G., Nita-Lazar, M., Haslam, S.M., North, S.J., Panico, M., Morris, H.R., Dell, A., Wren, B.W., and Aebi, M. (2002) N-linked glycosylation in *Campylobacter jejuni* and its functional transfer into E. *coli*. *Science* **298**: 1790–3.

Wang, N., Mackenzie, L., Souza, A.G. De, Zhong, H., Goss, G., and Li, L. (2007) Proteome profile of cytosolic component of zebrafish liver generated by LC-ESI MS/MS combined with trypsin digestion and microwave-assisted acid hydrolysis. *J Proteome Res* 6: 263–72

Watson, D.A., Musher, D.M., and Verhoef, J. (1995) Pneumococcal virulence factors and host immune responses to them. *Eur J Clin Microbiol Infect Dis* **14**: 479–490.

Watson, D.A., Musher, D.M., Jacobson, J.W., and Verhoef, J. (1993) A Brief History of the Pneumococcus in Biomedical Research: A Panoply of Scientific Discovery. *Clin Infect Dis* **17**: 913–924.

Weber, B.S., Miyata, S.T., Iwashkiw, J.A., Mortensen, B.L., Skaar, E.P., Pukatzki, S., and Feldman, M.F. (2013) Genomic and functional analysis of the type VI secretion system in *Acinetobacter*. *PLoS One* **8**: e55142.

Wei, Z., and Zhang, M. (2013) Decorating proteins with “sweets” is a flexible matter. *Structure* **21**: 1–2.

Weinberger, D.M., Malley, R., and Lipsitch, M. (2011) Serotype replacement in disease after pneumococcal vaccination. *Lancet (London, England)* **378**: 1962–73.

Wenger, J. (2001) Vaccines for the developing world: current status and future directions. *Vaccine* **19**: 1588–1591.

Wetter, M., Kowarik, M., Steffen, M., Carranza, P., Corradin, G., and Wacker, M. (2013) Engineering, conjugation, and immunogenicity assessment of *Escherichia coli* O121 O antigen for its potential use as a typhoid vaccine component. *Glycoconj J* **30**: 511–22.

Whitfield, C. (1995) Biosynthesis of lipopolysaccharide O antigens. *Trends Microbiol* **3**: 178–185.

Whitfield, C. (2006) Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. *Annu Rev Biochem* **75**: 39–68.

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WHO | Vaccines

Wisplinghoff, H., Paulus, T., Lugenheim, M., Stefanik, D., Higgins, P.G., Edmond, M.B., Wenzel, R.P., and Seifert, H. (2012) Nosocomial bloodstream infections due to *Acinetobacter baumannii*, *Acinetobacter pittii* and *Acinetobacter nosocomialis* in the United States. *J Infect* **64**: 282–290.

Wroe, P.C., Lee, G.M., Finkelstein, J.A., Pelton, S.I., Hanage, W.P., Lipsitch, M., Stevenson, A.E., Rifas-Shiman, S.L., Kleinman, K., Dutta-Linn, M.M., Hinrichsen, V.L., Lakoma, M., and Huang, S.S. (2012) Pneumococcal Carriage and Antibiotic Resistance in Young Children Before 13-valent Conjugate Vaccine. *Pediatr Infect Dis J* **31**: 249–254.

Wykes, M., Pombo, A., Jenkins, C., and MacPherson, G.G. (1998) Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. *J Immunol* **161**: 1313–9.

Yan, Q., and Lennarz, W.J. (2002) Studies on the function of oligosaccharyl transferase subunits. Stt3p is directly involved in the glycosylation process. *J Biol Chem* **277**: 47692–700.

Yi, E.C., and Hackett, M. (2000) Rapid isolation method for lipopolysaccharide and lipid A from Gram-negative bacteria. *Analyst* **125**: 651–656.

Yim, S.S., An, S.J., Han, M.-J., Choi, J.W., and Jeong, K.J. (2013) Isolation of a potential anchoring motif based on proteome analysis of *Escherichia coli* and its use for cell surface display. *Appl Biochem Biotechnol* **170**: 787–804.

Yurist-Doutsch, S., Chaban, B., VanDyke, D.J., Jarrell, K.F., and Eichler, J. (2008) Sweet to the extreme: protein glycosylation in Archaea. *Mol Microbiol* **68**: 1079–84.

Yurist-Doutsch, S., Magidovich, H., Ventura, V. V, Hitchen, P.G., Dell, A., and Eichler, J. (2010) N-glycosylation in Archaea: on the coordinated actions of *Haloferax volcanii* AglF and AglM. *Mol Microbiol* **75**: 1047–58.

Zhou, M., and Wu, H. (2009) Glycosylation and biogenesis of a family of serine-rich bacterial adhesins. *Microbiology* **155**: 317–27.

Appendix A:

Supplementary data for Chapter 2:

Materials and Methods:

Generation of a strain with an in-frame deletion of *pilA*. The Δ*pilA* mutant was constructed, generating an in-frame deletion of *pilA*, according to the methodology published previously by our group (Harding *et al*., 2013). Primer sets 1 and 2 were used and can be found in the primer table in appendix A.

Construction of the Δ*tfpO*::kan mutant. We constructed the Δ*tfpO*::kan mutant for our previous publication (Harding *et al*., 2013), however, at the time the gene was designated as the *pgyA* gene, not the *tfpO* gene.

Complementation of the Δ *tfpO*::kan mutant. To complement the Δ *tfpO*::kan mutant, we cloned the *tfpO* gene with the predicted *pilA* promoter into a mini-Tn7 element as previously described. Briefly, gDNA from the Δ *pilA* mutant was used as template with primer set 3 to generate an amplicon containing the predicted *pilA* promoter, the ATG start codon of the *pilA* open reading frame, a FLP scar, the last 21bp of *pilA*, the 53bp intergenic region, and the entire *tfpO* open reading frame. A four-parental mating strategy was used to introduce the mini-Tn*7* element containing *tfpO* driven off the predicted *pilA* promoter into the Δ *tfpO*::kan mutant as previously described (Harding *et al*., 2013). A correct clone was verified by sequencing and designated as the *tfpO*+ complement.

Plasmid construction and transfer into *E. coli*. The *pilA* gene from strain M2 was PCR amplified from gDNA using primer set 4 and cloned into the EcoRV site of pWH1266. A correct clone containing the pWH-$pilA_{M2}$ plasmid was verified by restriction digestion and sequencing. pWH-$pilA_{M2}$ was purified from *E. coli* harboring the plasmid, then electroporated into *Acinetobacter* isolates. *Acinetobacter* isolates were made electrocompetent according to the methods previously described (Aranda *et al*., 2010)

The pBAV-*comP*-His plasmid was built by using primer set 32. The sticky-ended amplicon was digested with the respective restriction enzymes and ligated into the vector pBAVmcs in the same sites. The ligation was then electroporated into DH5α-E with transformants being selected for on L-agar plates supplemented with kanamycin.

The pEXT-$pglL_{ComP}$, pEXT-$pglL_{ADP1}$, pEXT-$tfpO_{19606}$ and pEXT-$pglL_{19606}$ plasmids were built using primer sets 33, 34, 35 and 36 respectively. The resulting amplicons were digested with BamHI and SalI and inserted in the same sites of pEXT20. Ligations were electroporated into DH5α-E and transformants were selected for on L-agar plates supplemented with ampicillin.

pWH-pilA17978-His construction and western blot analysis. The pilA allele from A. baumaniii ATCC 17978 was PCR amplified using primer set 37. The PCR product was purified and End-it repaired to phosphorylate the 5' ends. The vector pWH1266 was linearized with EcoRI, End-it repaired, and then treated with alkaline phosphatase. The linearized pWH1266 and the PCR purified pilA allele were ligated, transformed into DH5α, and transformants were selected on L-agar supplemented with ampicillin. The pWH-pilA17978 plasmid was sequenced verified and used as template for an inverse

PCR to add a C-terminal hexa-histidine tag using primer set 38. The PCR product was purified, DpnI treated, End-it repaired, and ligated. The ligation was electroporated into DH5α and transformants were selected on L-agar supplemented with ampicillin. The pWH-pilA17978-His electroporated into A. *baumannii* strains as previously described and naturally transformed into A. *baylyi* strains according to our previously described methods for transforming A. *nosocomialis* strain M2. *Acinetobacter* transformants were selected on L-agar supplemented with ampicillin. Western blot analysis on whole cell lysates was conducted as described above with the following exceptions. Strains were grown in LB broth without NaCl and were normalized to an OD600 = 2.0.

Construction of a strain M2 Δ*pglL*::kan mutant. The entire *pilA-tfpO-pglL* locus along with 1kb of flanking DNA from *A. nosocomialis* strain M2 was amplified using primer set 6. The PCR product was ligated to pCC1 (Epicentre) and transformed into *E. coli* EPI300. A correct clone containing the pCC1-*pilA-tfpO-pglL* vector was verified by restriction digestion and sequencing. To replace the *pglL* gene with a kanamycin cassette, a modified recombineering protocol was used as previously described (Harding *et al*., 2013). To introduce the mutation into *A. nosocomialis* strain M2, the plasmid pCC1-Δ*pgl*::kan was linearized and transformed via natural transformation. Transformants were selected on L-agar supplemented with kanamycin. The M2Δ*pglL*::kan region in the mutant was verified by sequencing.

Generation of strains containing point mutations in *pilA* in strain M2. To generate a strain with a carboxy-terminal serine to alanine point mutation in the *pilA* gene of strain

M2, the M2Δ*pilA* mutant was complemented with a mini-Tn*7* element containing a variant of the *pilA* allele, where the carboxy-terminal serine was mutated to an alanine (pRSM3510-*pilA*[S136A]). The pRSM3510-*pilA*[S136A] plasmid was constructed using the Quikchange Site Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol using primer set 8. A correct clone carrying the pRSM3510-*pilA*[S136A] plasmid was verified by restriction digest and sequencing. The mini-Tn*7* construct containing the *pilA*[S136A] allele was transposed into the *att*Tn7 of strain M2 via a four-parental mating strategy previously described above. The same protocol was used to generate the *pilA*[S132A] except primer set 39 was used.

Construction of glycosyl-transferase mutants in the strain M2 background. In order to replace each glycosyl-transferase gene with a kanamycin resistance cassette, an In-Fusion HD EcoDry cloning kit was used according to the manufacturers protocol (ClonTech). The following protocol describing the construction of the M2 *weeH*::kan mutation was used for each glycosyl-transferase mutant, except the 15bp overhangs were added to the primers which amplified the 5' and 3' flanking regions of each respective gene. Briefly, the upstream and downstream flanking DNA regions around *weeH* were PCR amplified with primer sets 9 and 10 respectively. The Tn*5* kanamycin cassette and pGEM vector were PCR amplified with 15bp overhangs homologous to the DNA in which they were to be recombined using primer sets 11 and 12 respectively. The PCR amplicons were gel extracted and ethanol precipitated. One hundred nanograms of each product was added to the In-fusion EcoDry cloning tube according to the manufacturer's protocol and incubated at 37 for 15 mins then at 50˚C for 15 mins. The newly

generated vector was transformed into chemically competent Stellar cells (Clontech) according to the manufacturer's protocol. Transformants were selected for on L-agar plates supplemented with kanamycin. A correct clone containing the pGEM-*weeH*::kan plasmid was sequence verified. The *weeH*::kan cassette was PCR amplified using the forward primer of primer set 9 with reverse primer of primer set 10. The amplicon was DpnI treated, ethanol precipitated, then transformed into strain M2 according to our previously published methodologies. A correct clone designated ΔM2 *weeH*::kan was sequence verified.

The upstream and downstream regions of *wafY* were amplified using primer sets 17 and 18, linearized pGEM was amplified using primer set 15, and the kanamycin cassette was amplified with primer set 16. The above protocol was used to In-Fuse all four PCR products. pGEM-*wafY*::kan was linearized with EcoRI and introduced into *A. nosocomialis* strain M2 via natural transformation as previously described. A correct clone designated M2Δ*wafY*::kan was sequence verified.

The upstream and downstream regions of *wafZ* were amplified using primer sets 19 and 20 and the upstream and downstream regions of *wagB* were amplified using primer sets 21 and 22. Mutants were then constructed as described for the *wafY* mutant. Clones designated M2Δ *wafZ*::kan and M2Δ *wagB*::kan were identified and sequence verified.

Construction of pRSM4063. To generate the pRSM4063 vector, we first introduced an empty mini-Tn*7* element into strain M2 via a four-parental mating strategy previously described. Transposition of the empty mini-Tn*7* element into the *att*Tn*7* was sequence

verified generating the strain M2attTn*7*::MCS_Empty. Genomic DNA was purified from this strain and used as a template in a PCR using primer set 13. The forward primer of primer set 13 is approximately 2kb upstream of the mini-Tn7 element and the reverse primer of primer set 13 is approximately 2kb downstream of the mini-Tn7 element. The ensuing PCR product was ligated into the pSMART-LCKan vector, sequence verified, and designated pRSM4063.

Construction of the *weeH* complemented mutant. To complement the Δ *weeH*::kan mutant, *weeH* plus 375bp of upstream DNA was PCR amplified using primer set 14. The amplicon was digested with XmaI and KpnI, cloned into pRSM4063 and electroporated into DH5α. To complement the Δ *weeH*::kan mutant, pRSM4063-*weeH* was linearized with NdeI and introduced into M2 *weeH*::kan via natural transformation according to our previously published procedure (Harding *et al*., 2013).

Construction of the *wafY, wafZ*, and *wagB* complemented mutants. Given the lack of an obvious promoter driving expression of each of the glycosyl-transferase genes and the over-lapping nature of the open reading frames, the upstream *wxy* promoter was selected to drive expression of each of the three genes. Each glycosyl-transferase mutant was complemented by returning the deleted gene driven off the predicted *wxy* promoter to the chromosome using the mini-Tn*7* system. Briefly, the glycosyl-transferase locus was PCR amplified using primer set 23, End-It repaired (Epicentre) and ligated into pCC1 (Epicentre). Transformants were selected on chloramphenicol and the pCC1-GT plasmid was verified by restriction digest. The pCC1-GT plasmid contained the predicted

promoter of the *wxy* gene (329 bp upstream), *wxy*, *wafY*, *wafZ*, *wagA*, *gnaB*, and *wagB*. To generate the *wxy* promoter-*wafY* construct, an inverse PCR strategy was employed to remove the *wxy* gene and join the *wxy* promoter to the ATG start codon of the *wafY* gene using primer set 24 and pCC1-GT as template. The subsequent PCR product was End-It repaired (Epicentre) and ligated to itself generating the pCC1-wxy^P-*wafY* construct. The wxy^P-*wafY* DNA fragment was PCR amplified using primer set 27, which contained XmaI and KpnI restriction overhangs. The PCR product was digested and ligated to pre-digested pRSM3510 then transformed into EC100D cells. Transformants were selected for on L-agar supplemented with kanamycin. The mini-Tn7 element containing wxy^P-*wafY* was introduced into the M2Δ*wafY*::kan mutant using a four-parental mating strategy previously described.

The same process was used to generate pRSM3510- wxy^P-*wafY* except primer set 25 and primer set 28 were used. The mini-Tn7 element containing wxy^P-*wafY* was introduced to the M2Δ*wafZ*::kan mutant via a four-parental mating strategy.

The wxy^P-*wagB* fragment was generated using primer set 26 and primer set 29; but, was cloned into pRSM4063. The pRSM4063- wxy^P-*wagB* vector was linearized with XhoI and introduced into M2 *wagB*::kan via natural transformation as previously described.

Construction and transfer of p4063-A1S_1193-5X into *Acinetobacter* strains. The A1S_1193 open reading frame along with its predicted promoter was PCR amplified to include a C-terminal 5X His tag using primer set 30, which also contained XmaI and KpnI restriction overhangs. The PCR product was digested and ligated to pre-digested

pRSM4063. The pRSM4063-A1S_1193-5X vector was linearized with XhoI and introduced to *Acinetobacter* strains via natural transformation as previously described.

Construction of pET-15b-rsPilA$_{M2}$. A truncated His-tagged **r**ecombinant, **s**oluble derivative of *pilA* (rsPilA$_{M2}$) was amplified using gDNA from *A. nosocomialis* strain M2 as template with primer set 31 deleting the first 28 amino acids of the PilA protein. This PCR product was then used as template for a second PCR where the forward primer of primer set 15 contained an NdeI site and the reverse contained a BamHI site to aid in directional cloning into pET-15b. The amplicon was digested with NdeI and BamHI then ligated into the expression vector pET-15b, which was digested with NdeI and BamHI generating a first codon fusion driven off of the T7 promoter with an N-terminal His tag followed by a thrombin cleavage site. Ligation products were electroporated into DH5α-E (Invitrogen), transformants were subcloned and verified to contain the vector with insert by restriction digestion and sequencing. A correct clone was transformed into *E. coli* strain Origami B (DE3) (Novagen) for expression of the recombinant protein.

rsPilA$_{M2}$ purification. Origami B(DE3) (Millipore) containing pET15b-rsPilA$_{M2}$ was inoculated into 100 mL of LB broth to an A_{600nm} optical density of 0.05 and grown at 37˚C with 180 rpm to mid-log phase at which point rsPilA$_{M2}$ expression was induced with IPTG at a final concentration of 500 µM. Cells were transitioned to 16˚C with 180 rpm and grown for 18 h. Cells were harvested by centrifugation into two equal pellets and resolved in 4 mL each of 1X Ni-NTA Bind Buffer (Novagen) with protease inhibitors (Roche). Resuspended pellets were added to 15 mL TeenPrep Lysing matrix B tubes (MP

Biomedicals) and lysed in a Fast Prep 24 homogenizer (MP Biomedicals) with two rounds at 6.0 m/s for 40 seconds with a 5 minute incubation on ice between each round. Supernatants were separated from the unlysed bacteria and the lysing matrix by centrifugation at 4000 rpm for 10 mins at 4C. Supernatants were further clarified with 1 hour of ultracentrifugation at 100,000 x *g* for 1 hour at 4C. Clarified supernatants were incubated with 1 mL of Ni-NTA His bind resin (Novagen) for 2 hours at 4C with gentl e rocking followed by two 4 mL washes with 1X Ni-NTA wash buffer. His-tag rsPilA$_{M2}$ was eluted from the resin with three washes of Ni-NTA elution buffer and dialyzed overnight in phosphate buffered saline. The N-terminal His tag on rsPilA$_{M2}$ was thrombin cleaved with 0.04 units/µL of biotinylated-thrombin (Novagen) for 2 hours at room temperature. The biotinylated-thrombin was captured with streptavidin-agarose beads for 30 minutes and the rsPilA$_{M2}$ was collected with a centrifugation in a spin filter at 500 x *g* for 5 minutes. To remove any small peptides containing the cleaved His-tag or uncleaved His-tag rsPilA$_{M2}$, the filtrate was run over Ni-NTA bind resin and the flow through was collected as pure, cleaved rsPilA$_{M2}$. The pure protein was dialyzed in phosphate buffer saline with 50% glycerol then normalized to 1 mg/mL using a BCA total protein assay kit (Pierce).

Generation of polyclonal antiserum against rsPilA$_{M2}$. Polyclonal antiserum against rsPilA$_{M2}$ was raised following our previously described methods (Actis *et al*., 1985). Briefly, 100 µg of purified rsPilA$_{M2}$ emulsified in one milliliter of Freund's complete adjuvant was injected using a 23 gauge needle at ten intracutaneous sites into the haunch of a 6-month old female New Zealand white rabbit (Charles River Laboratories

International, Inc., Wilmington, MA). Injections consisting of 100 µg rsPilA$_{M2}$ emulsified in Freund's incomplete adjuvant were subsequently delivered at 15-day intervals, and serum was collected at 10-day intervals following the initial injection. The specificity and reactivity of the anti-rsPilA$_{M2}$ antibodies were confirmed by immunoblotting rsPilA$_{M2}$ and *A. nosocomialis* strain M2 whole-cell lysates after proteins were size-fractionated by SDS-PAGE.

Transformation efficiency assays. Natural transformation was assayed as described previously (Harding *et al*., 2013) Transformation efficiency was calculated by dividing the CFU of transformants by the total CFU. Experiments were conducted on at least three separate occasions.

Crystal violet biofilm assay:

Overnight cultures were grown at 37˚C then diluted to OD_{600} 0.05 and 100 µl of that were inoculated in each well of a non-tissue culture treated 96 well plate. The plate was then incubated at 30˚C for 48 hours without shaking and bacterial growth was determined by measuring the absorbance at OD_{600} nm. Afterwards, cultures were removed from the wells and the plate was washed with distilled water followed by the addition of 100 mL of 1% crystal violet in ethanol to stain the bound cells. The plate was incubated for 30 minutes with gentle agitation at room temperature and then washed with distilled water. Stained biofilms were solubilized by adding 100 µl of 2% SDS per well and incubating for 30 minutes with gentle agitation at room temperature. Biofilms were quantified by measuring absorbance at OD_{580} nm. OD readings were normalized using the ratio between OD_{580}/OD_{600}.

Quantitative dimethylation of *A. baylyi* ADP1 membrane extracts

Quantitative dimethylation of lysates from *A. baylyi* ADP1, the ADP1Δ*pglL*$_{ComP}$ mutant, and the ADP1Δ*pglL*$_{ADP1}$ mutant was performed as outlined previously (Boersema *et al*., 2009). Briefly, 1 mg of peptide lysate from each strain was resuspended in 30 µl of 100 mM tetraethylammonium bromide and mixed with the following combinations of 200 mM formaldehyde (30 µl) and 1M sodium cyanoborohyride (3 µl) isotopologues: ADP1 samples were labeled with light formaldehyde (CH_2O) and light sodium cyanoborohyride ($NaBH_3CN$), ADP1Δ*pglL*$_{ADP1}$ samples with medium formaldehyde (CD_2O) and light sodium cyanoborohyride, and ADP1Δ*pglL*$_{ComP}$ with heavy formaldehyde ($^{13}CD_2O$) and heavy sodium cyanoborodeuteride ($NaBD_3CN$). Reagents were mixed and samples incubated at room temperature for 1 h. Dimethylation reactions were repeated twice to ensure complete labeling of all amine groups. Dimethylation reactions were terminated by the addition of 30 µl of 1M NH_4Cl for 20 minutes at room temperature. Samples were acidified by addition of 5% (v/v) acetic acid and allowed to equilibrate in the dark for 1 h before pooling the three samples at 1:1:1 ratio. Pooled samples were then STAGE tip purified, lyophilized, and stored at -20ºC.

Table S1.1: Plasmids and bacterial strains included in the study

Plasmid or strain	Relevant characteristic(s)	Reference/ Source
PLASMIDS		
pFLP2	Encodes FLP recombinase	Kumar *et al*., (2010)
pKD13	Contains kanamycin resistance gene from Tn5 flanked by FRT sites	Datsenko & Wanner, (2000)
pRSM3542	pKD13 containing *kan-sacB*	Carruthers *et al*., (2013)
pGEM-T-Ez	General cloning plasmid	Promega
pCC1	Single copy, general cloning plasmid	Epicentre
pSMART-LCKAN	Low copy blunt cloning vector	Lucigen
pGEM-*pilA*	pGEM containing *pilA* with 1 kb flanking DNA	Harding *et al*. (2013)
pGEM-*pilA*::kan-*sacB*	pGEM-*pilA* containing *pilA*::kan	This study
pCC1-*pilA-tfpO-pglL*	pCC1 containing the *pilA-tfpO-pglL* locus with approximately 1 kb of flanking DNA	This study
pRSM3510	pKNOCK derivative with a mini-Tn*7* element containing a multiple cloning site	Harding *et al*. (2013)
pRSM3510-*pilA*	pRSM3510 containing *pilA* with expression driven from the predicted *pilA* promoter	Harding *et al*. (2013)
pRSM3510-*pilA*[S136A]	pRSM3510-*pilA* with a carboxy terminal serine to alanine point mutation	This study
pRSM3510-*pilA*[S132A]	pRSM3510-*pilA* with a serine 132 to alanine point mutation	This study
pRSM3510-$pilA^{P}$-*tfpO*	pRSM3510 containing the predicted *pilA* promoter, the ATG of *pilA*, a FLP scar, the last 21bp of *pilA*, and the *tfpO* gene including the 48bp intergenic region between *pilA* and *tfpO*	This study
pCC1-*pglL*::kan	pCC1-*pilA-tfpO-pglL* containing *pglL*::kan	This study
pGEM-*weeH*::kan	pGEM-T-Ez containing *weeH*::kan	This study
pRSM4063	pSMART-LCKan containing an the empty mini-Tn*7* element from pRSM3510 along with 2kb of flanking DNA up and downstream of the *att*Tn*7* from strain M2	This study
pRSM4063-*weeH*	pRSM4063 containing the *weeH* gene with its predicted promoter	This study
pWH1266	*E. coli – Acinetobacter* shuttle vector	Hunger *et al*., (1990)
pGEM-*wafY*::kan	pGEM-T-Ez containing *wafY*::kan	This study
pGEM-*wafZ*::kan	pGEM-T-Ez containing *wafZ*::kan	This study
pGEM-*wagB*::kan	pGEM-T-Ez containing *wagB*::kan	This study
pCC1-GT	pCC1 containing the predicted promoter of the *wxy* gene (329 bp upstream), *wxy*, *wafY*, *wafZ*, *wagA*, *gnaB*, and *wagB*	This study
pCC1-wxy^{P}-*wafY*	pCC1-GT lacking the *wxy* open reading frame	This study
pCC1-wxy^{P}-*wafZ*	pCC1-GT lacking the *wxy* and *wafY* open reading frames	This study
pCC1-wxy^{P}-*wagB*	pCC1-GT lacking the *wxy*, *wafY*, *wagA*, and *gnaB* open reading frames	This study
pRSM4063-wxy^{P}-*wafY*	pRSM4063 containing *wafY* driven off the predicted *wxy* promoter	This study
pRSM4063-wxy^{P}-*wafZ*	pRSM4063 containing *wafZ* driven off the predicted *wxy* promoter	This study
pRSM4063-wxy^{P}-*wagB*	pRSM4063 containing *wagB* driven off the predicted *wxy* promoter	This study
pWH-$pilA_{M2}$	pWH1266 expressing $pilA_{M2}$ driven by the predicted *pilA*	This study

	promoter	
pRSM3510-A1S_1193-his	pRSM3510 containing A1S_1193 driven off its predicted native promoter	This study
pET-15b	General plasmid for expression and cloning of recombinant proteins based on the T7-promoter driven system	Novagen
pET-15b-rsPilA$_{M2}$	pET-15b expressing a truncated *pilA* from the T7 promoter	This Study
pEXT20	Amp^r cloning and expression vector, IPTG inducible.	Dykxhoorn *et al*., (1996)
pBAVMCS	Km^r pBAV1K-T5-gfp derivative with *gfp* ORF removed. Constitutive *E. coli*/*Acinetobacter* shuttle vector	Nakar & Gutnick, (2001)
pBAV-*comP*-his	Km^r pBAVmcs constitutively expressing C-6X His-tagged *comP* from *A. baylyi*, inserted at BamHI and SalI.	This Study
pBAV-*pilA*$_{17978}$	Km^r pBAVmcs constitutively expressing C-6X His-tagged *pilA* from *A. baumannii* ATCC 17978, inserted at XmaI and SalI.	This study
pEXT-*pglL*$_{comP}$	Amp^r pEXT20 expressing C-6X His-tagged *pglL*$_{ComP}$ from *A. baylyi* inserted at BamHI and SalI, IPTG inducible.	This Study
pEXT-*pglL*$_{ADP1}$	Amp^r pEXT20 expressing C-6X His-tagged *pglL*$_{ADP1}$ from *A. baylyi* inserted at BamHI and SalI, IPTG inducible.	This Study
pEXT-*tfpO*$_{19606}$	Amp^r pEXT20 expressing C-10X His-tagged *tfpO*$_{19606}$ from *A. baumannii* ATCC 19606 inserted at BamHI and SalI, IPTG inducible.	This Study
pEXT-*pglL*$_{19606}$	Amp^r pEXT20 expressing C-10X His-tagged *pglL*$_{19606}$ from *A. baumannii* ATCC 19606 inserted at BamHI and SalI, IPTG inducible.	This Study
pAMF22	Tp^r C-10X His-tagged *dsbA1* from *N. meningitidis* MC58 cloned into pMLBAD, Arabinose inducible.	Faridmoayer A. and Feldman MF. (unpublished)
pBAV-*dsbA1*-His	C-6X His-tagged *dsbA1* subcloned into pBAVMCS, Km^r, at BamHI and HindIII. Constitutively expressing.	This Study
pACYC*pglB*	Cm^r pACYC184-based plasmid encoding the *C. jejuni* protein glycosylation locus cluster with mutations W458A and D459A in PglB, IPTG inducible.	Wacker *et al*., (2002)
pBAVMCS-A1S_1193His10X	Km^r pBAVMCS constitutively expressing C-10X hist-tagged A1S_1193 inserted at BamHI and SalI.	Scott *et al*., (2014)
STRAINS		
Acinetobacter nosocomialis strain M2	Metro Health Systems Clinical Isolate	Niu *et al*., (2008)
M2Δ*pilA*::kan	Strain M2 containing a deletion of *pilA* and replacement with a kanamycin resistance cassette	Harding *et al*. (2013)
M2Δ*pilA*::kan-*sacB*	Strain M2 containing a deletion of *pilA* and replacement with a kan-*sacB* cassette	This study
M2Δ*pilA*	Strain M2 containing an unmarked, in-frame deletion of *pilA*	This study
M2Δ*pilT*	Strain M2 containing an unmarked, in-frame deletion of *pilA*	Harding *et al*. (2013)
M2Δ*tfpO*::kan	Strain M2 containing a deletion of *tfpO* and replacement with a kanamycin resistance cassette	This study
M2Δ*tfpO*::kanΔ*pilT*::strep	M2Δ*tfpO*::kan containing a deletion of *pilT* and replacement with a streptomycin resistance cassette	This study
M2Δ*pglL*::kan	Strain M2 containing a deletion of *pglL* and replacement with a kanamycin cassette	This study
M2Δ*pilA* (*pilA*[S136A]+)	M2Δ*pilA*::kan with a mini-Tn*7* element containing an allele	This study

	of *pilA* with a carboxy-terminal serine to alanine point mutation	
M2Δ*wafY*::kan	Strain M2 containing a deletion of *wafY* and replacement with a kanamycin resistance cassette	This study
M2Δ*wafZ*::kan	Strain M2 containing a deletion of *wafZ* and replacement with a kanamycin resistance cassette	This study
M2Δ*wagB*::kan	Strain M2 containing a deletion of *wagB* and replacement with a kanamycin resistance cassette	This study
M2Δ*weeH*::kan	Strain M2 containing a deletion of *weeH* and replacement with a kanamycin resistance cassette	This study
M2Δ*pilA*::kan (*pilA*+)	M2Δ*pilA*::kan with a mini-Tn7 element containing the *pilA* gene transcribed from its predicted promoter	Harding *et al.* (2013)
M2Δ*tfpO*::kan (*tfpO+)*	M2Δ*tfpO*::kan with a mini-Tn7 element containing the *tfpO* gene transcribed from the *pilA* predicted promoter	This study
M2Δ*wafY*::kan (*wafY*+)	M2Δ*wafY*::kan with a mini-Tn7 element containing the *wafY* gene under control of the predicted *wxy* promoter	This study
M2Δ*wafZ*::kan (*wafZ*+)	M2Δ*wafZ*::kan with a mini-Tn7 element containing the *wafY* gene under control of the predicted *wxy* promoter	This study
M2Δ*wagB*::kan (*wagB*+)	M2Δ*wagB*::kan with a mini-Tn7 element containing the *wafY* gene under control of the predicted *wxy* promoter	This study
M2Δ*weeH*::kan (*weeH*+)	M2Δ*pilA*::kan with a mini-Tn7 element containing the *pilA* gene fused to a FLAG tag transcribed from its predicted promoter	This study
M2 (A1S_1193-his+)	Strain M2 with a mini-Tn*7* element containing A1S_1193-his transcribed from its predicted promoter	This study
M2Δ*tfpO*::kan (A1S_1193-his+)	M2Δ*tfpO*::kan with a mini-Tn*7* element containing A1S_1193-his transcribed from its predicted promoter	This study
M2Δ*pglL*::kan (A1S_1193-his+)	M2Δ*pglL*::kan with a mini-Tn*7* element containing A1S_1193-his transcribed from its predicted promoter	This study
A. baumannii ATCC 17978	Reference *A. baumannii* strain	ATCC
A. baumannii ATCC 19606	Reference *A. baumannii* strain	ATCC
A. baumannii 27413	*A. baumannii* clinical isolate isolated at Nationwide Children's Hospital (NCH) from body fluid	NCH
A. baylyi ADP1	Environmental isolate	de Berardinis *et al*., (2008)
A. baylyi Δ$pglL_{ComP}$::kan	Strain ADP1 with $pglL_{comP}$ deleted and replaced with a kanamycin resistance casette	de Berardinis *et al.* (2008)
A. baylyi Δ$pglL_{ADP1}$::kan	Strain ADP1 with $pglL_{ADP1}$ deleted and replaced with a kanamycin resistance casette	de Berardinis *et al.* (2008)
E. coli DH5a	General cloning strain	Invitrogen
E. coli EC100D *pir*+	General cloning strain, pir^{+}	Epicentre
E. coli DY380	Recombineering strain	Lee *et al*., (2001)
E. coli DH5a(pFLP2)	Carries FLP recombinase gene under temperature control	Kumar *et al*. (2010)
E. coli HB101(pRK2013)	Conjugation helper strain	Figurski & Helinski, (1979)
E. coli EC100D(pTNS2*)*	Carries transposase genes for mini-Tn*7* transposition	Choi *et al*., (2005)
E. coli Origami 2(DE3)	K-12 derivative containing mutations in *trxB* and *gor* genes and a host lysogen of λDE3	Novagen
E. coli Stellar chemically competent cells	HST08 strain derivative for high transformation efficiencies	Clontech
E. coli CLM24	Constructed from E. *coli* W3110 (IN(rrnD-rrnE)1 rph-1).	Feldman *et*

Table S1.2: Primers included in the study

Primer Set		Sequence
1	F	AGAATACTTGCATAGTGACAGGTTACAG
	R	GTTATGGCGGCGGTGGAGGTC
2	F	CAAAAAGCTTATATAAAAACATACATACAATCTTTGGGGAAAAGGCTATGATTCCGGG GATCCGTCGACC
	R	GGATTGACCTCTCTTTTTTATTTCTAAAATTACGATGCTACAAATGATTGTGTAGGCTG GAGCTGCTTCG
3	F	GCGGGATCCGCAAATTGGTGATGTGATGTCTCG
	R	GCGGGTACCGCTGCGAGGAATAAAAAGAATACT
4	F	GCGGGATCCGCAAATTGGTGATGTGATGTCTCG
	R	GCGGGTACCTCGTATTGTGAACTAGACCATCCT
5	F	GCGGGATCCGCAAATTGGTGATGTGATGTCTCG
	R	GCGGGTACCGCTGCGAGGAATAAAAAGAATACT
6	F	AGAATACTTGCATAGTGACAGGTTACAG
	R	CGCATTTATATTTGGGGATTACTC
7	F	CTTCCATGTATAATTCTTCTCAAGTTTTTGGTCTGTAACCTGTCACTATGATTCCGGGG ATCCGTCGACC
	R	AAAATCCCCTTGAAAACAAGGGGATTTTTTTATTTATCTTTTAATAATTGTGTAGGCTG GAGCTGCTTCG
8	F	CTTCCTCAATCATTTGTAGCAGCGTAATTTTAGAAATAAAAAAG
	R	CTTTTTTATTTCTAAAATTACGCTGCTACAAATGATTGAGGAAG
9	F	ATGAAAAAACTTGAGCACCTTGC
	R	TGTTTGCTCTTATTTCTACTG
10	F	TTGTCATTTATAAAGTTAGTCAC
	R	TGTACACCTGATTTTAATATTCTA
11	F	GAAATAAGAGCAAACAATTCCGGGGATCCGTCGACC
	R	CTTTATAAATGACAATGTAGGCTGGAGCTGCTTCG
12	F	CTCAAGTTTTTTCATCGCCATGGCGGCCGGGAGCATG
	R	AAAATCAGGTGTACAACTAGTGAATTCGCGGCCGCCTGCA
13	F	CGTCCCCAAAAGCGTGAA
	R	TTAGGCAAATTTCGAAGCGTGAT
14	F	GCGCCCGGGATAAGTGCTCAATTGATGG
	R	GGTACCGAGATCCCAAACCAGCAAC
15	F	ACTAGTGAATTCGCGGCCGCCTGCA
	R	CGCCATGGCGGCCGGGAGCATG
16	F	ATTCCGGGGATCCGTCGACC
	R	TGTAGGCTGGAGCTGCTTCG
17	F	CCGGCCGCCATGGCGATGACGATTGGTTTAATTTTTTC
	R	ACGGATCCCCGGAATCATACTTGTAAAAAAAAAAGTATTT
18	F	CAGCTCCAGCCTACAATGGAAGAAAATTCTTTATTAATTT
	R	CGCGAATTCACTAGTTTAAACATATTTTTCCCATTTC
19	F	CCGGCCGCCATGGCGATGACTCCTGCCGGAGG

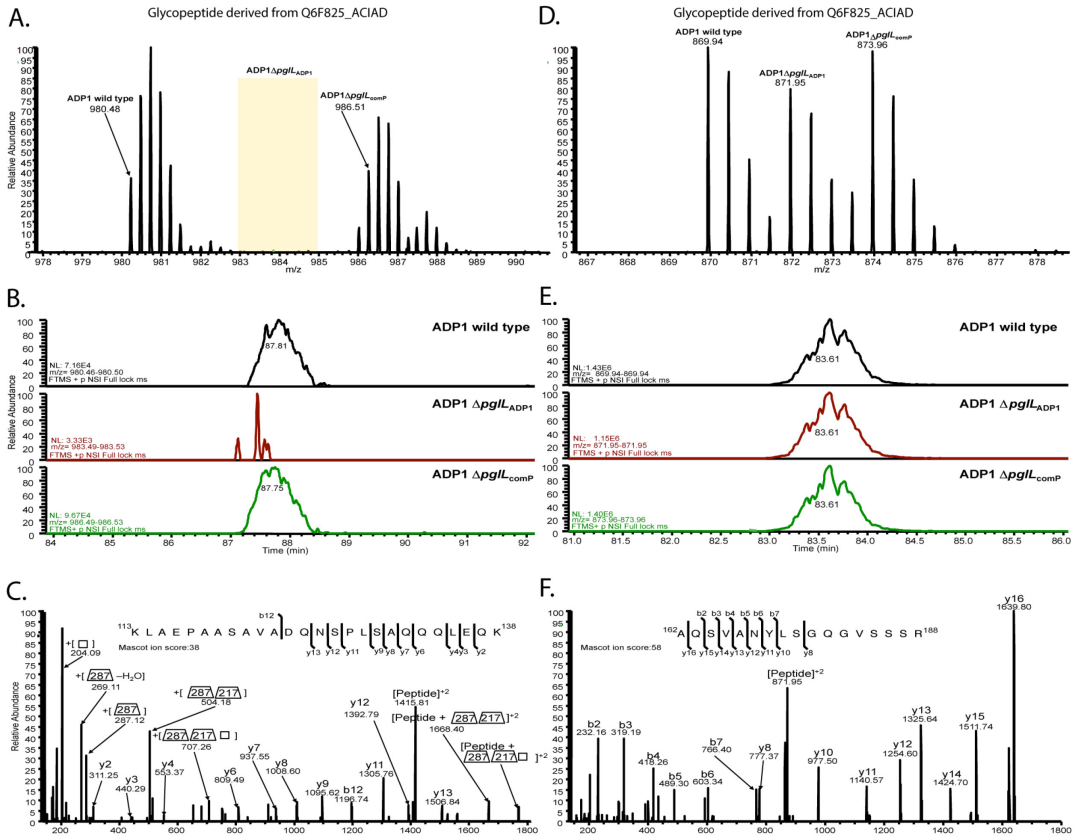
	R	ACGGATCCCCGGAATTTAATAAAGAATTTTCTTCCATTTAC
20	F	CAGCTCCAGCCTACAATAGTAGGACTAAAAAAATGATTTCG
20	R	CGCGAATTCACTAGTTTATTTATATAACCCTTTTTCTTTC
21	F	CGGCCGCCATGGCGATGTTTAAAAATGTATTAATTACTGG
	R	ACGGATCCCCGGAATCATTTATTTATATAACCCTTTTTCT
22	F	CAGCTCCAGCCTACAATAAATTTAAAATATTCATAAATCT
	R	CGCGAATTCACTAGTTTATAATTTAAGTTCTTGAATCAAC
23	F	CTACATTGTTTATTTTTACCAGAA
	R	GAAGCTTGAAGTTATCCACGAA
24	F	CATCAAAAATACCAGCCTAAATTATC
	R	CCATTGTTTGAAATTATTTAGGG
25	F	CATCAAAAATACCAGCCTAAATTATC
	R	GAAGAAAATTCTTTATTAATTTCTG
26	F	CATCAAAAATACCAGCCTAAATTATC
	R	GAAAAAGGGTTATATAAATAAATG
27	F	GCGCCCGGGCTACATTGTTTATTTTTACCAGAA
	R	GCGGGTACCACCATCATTGACTACTAAGACCTC
28	F	GCGCCCGGGCTACATTGTTTATTTTTACCAGAA
	R	GCGGGTACCTTCTACATCCAATACCAGTCGT
29	F	GCGCCCGGGCTACATTGTTTATTTTTACCAGAA
	R	GCGGGTACCGAAGCTTGAAGTTATCCACGAA
30	F	GCGCCCGGGCCGAAGCAGGGTGGGTGTTAGT
	R	GCGGGTACCTTAGTGGTGGTGGTGGTGTTGAGCTACTGAAAACTCAATAC
31	F	GCCATATGGCTTATCAAAACTATATTGCTAAATCTC
	R	GCGGATCCCTCTTTTTTATTTCTAAAATTACGATGCT
32	F	ATAGGATCCATGAATGCACAAAAGGGTTTTACC
	R	TATGTCGACTCAGTGGTGGTGGTGGTGGTGACCACGACATTCTGATGG
33	F	ATATGGATCCGTGGTTGATAGTAGTACTATATGG
	R	ATATGTCGACTCAGTGGTGGTGGTGGTGGTGATATTCTATTGAACAAAATTTTAACTTAGG
34	F	ATATGGATCCGTGGCTGGTTCCCCGCGTGTGTATAATAGC
	R	ATATGTCGACTTAGTGGTGGTGGTGGTGGTGCTTGGATGACTCTACAGCAGAAGC
35	F	ACTGGGATCCATGTTAAAAAAAATTATTCTATTTC
	R	ACTGGTCGACTAAGTGGTGGTGGTGGTGGTGGTGGTGGTGAGGAATTTTTGAGGTTGGTAC
36	F	ACTGGGATCCATGCAAGTATTCTTTCTGTTC
	R	ACTGGTCGACTAAGTGGTGGTGGTGGTGGTGGTGGTGGTGTTTATTTTTTAACAACTCTGCC
37	F	GGTGGACGTGGAGGAG
	R	CTTGCTTGGGTTACATCAGTGCT
38	F	AATTATTGTACAGCCTTTTG
	R	CATCATCATCATCATCACTAATATTAAAAATGTATAAAAAACACC
39	F	CCAGTTGAATTACTTCCTCAAGCATTTGTAGCATCGTAAT
	R	ATTACGATGCTACAAATGCTTGAGGAAGTAATTCAACTGG

Results:

Glycopeptide quantitative labeling

Quantitative dimethylation labeling enabled comparison of all three strains simultaneously providing an internal positive control for glycopeptide enrichment and led to the detection of seven unique glycopeptides (Appendix A Table S1.4). Consistent with the requirement of $PglL_{ADP1}$ for glycosylation, no glycopeptides derived from the $\Delta pglL_{ADP1}$ mutant (Appendix A figure S1.1 A-C) could be detected, while non-glycosylated peptides within the samples were observed at a ~1:1:1 ratio (Appendix A Figure S1.1 D-E, Appendix A table S1.4). Glycosylation was observed at near 1:1 ratio in $\Delta pglL_{ComP}$ compared to wild type (ranging from 46% to 170%) (Appendix A table S1.5) with MS/MS identifications enabling the confirmation of glycopeptides originated from strain $\Delta pglL_{ComP}$ (Appendix A Figure S1.1 C). Taken together these data suggests $PglL_{ADP1}$ is a general *O*-linked OTase while $PglL_{ComP}$ is responsible for glycosylation of a specific subset of the glycoproteome, which was not detectable given the sensitivity of the method employed.

Figure S1.1: Quantitative analysis of glycosylation in *A. baylyi* ADP1 WT, *A. baylyi* ADP1Δ*pglL*$_{ADP1}$, and *A. baylyi* ADP1Δ*pglL*$_{comP}$ using dimethyl labeling.



Using dimethyl labeling and ZIC-HILIC, the *O*-OTase responsible for glycosylation of individual glycopeptides was confirmed. Glycopeptides derived from *A. baylyi* ADP1 WT, labeled with light label and *A. baylyi* ADP1Δ*pglL*$_{comP}$ labeled with heavy label, were observed at near 1:1 levels; whereas, *A. baylyi* ADP1Δ*pglL*$_{ADP1,}$ labeled with medium label, was undetectable within samples. Conversely non-glycosylated peptides were observed at a near 1:1:1 level between all three strains. **A and D)** The MS spectra of the light, medium and heavy isotopologues of the glycopeptide ^{113}KLAEPAASAVADQNSPLSAQQQLEQK^{138} (Q6F825_ACIAD) and non-

glycosylated peptide ^{166}AQSVANYLSGQGVSSSR^{182} (Q6FDR2_ACIAD) enabled the comparison of glycosylation across all three strains. No glycopeptides were observed within ADP1Δ*pglL*$_{ADP1}$ while non-glycosylated peptides were observed a near 1:1:1 ratio. **B and E)** Comparison of the extracted ion chromatograms of the light, medium and heavy isotopologues confirm the absent of ADP1Δ*pglL*$_{ADP1}$ derived glycopeptides and the 1:1:1 ratio of non-glycosylated peptides. **C)** HCD fragmentation confirming the identification of the heavy isotopologues of the glycopeptide ^{113}KLAEPAASAVADQNSPLSAQQQLEQK^{138}, confirming its origins from ADP1Δ*pglL*$_{comP}$. **F)** HCD fragmentation confirming the identification of the medium isotopologues of the non-glycosylated peptide ^{166}AQSVANYLSGQGVSSSR^{182}, confirming its origins from ADP1Δ*pglL*$_{ADP1}$.

Figure S1.2: Genomic organization of OTase(s) in (A) *A. baumannii* ATCC 17978 and (B) *A. baumannii* ATCC 19606.

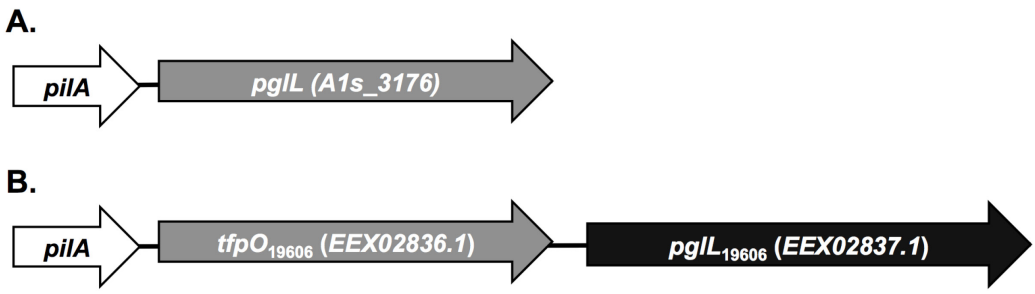


Figure S1.3: Biofilm assays in *A. baylyi*

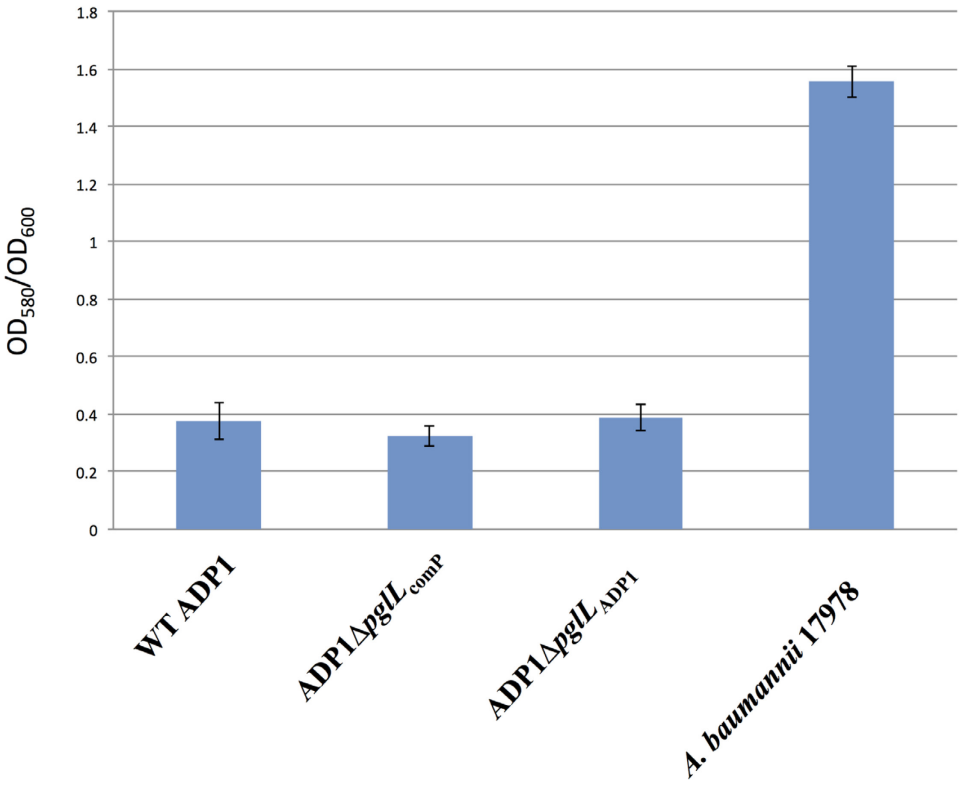


Table S1.3: Glycopeptides identified in *A. baylyi* ADP1

Protein accession number	Protein name	Precursor m/z [Da]	Precursor MH+ [Da]	Precursor Charge	RT [min]	Peptide	Mascot score	Peptide mass	Glycan mass
Q6F875_ACIAD	Uncharacterized protein	703.98	2109.92	3	19.49	AAHAASAAASK	62	955.50	1154.43
Q6F875_ACIAD	Uncharacterized protein	717.98	2151.93	3	20.80	AAHAASAAASK	35	955.50	1196.43
Q6FCV1_ACIAD	Uncharacterized protein	766.33	2296.97	3	29.47	DAAHDAAASVEK	54	1184.56	1112.41
Q6FCV1_ACIAD	Uncharacterized protein;	720.06	2877.23	4	27.44	IDAAADHAAASTEHAADK	40	1764.82	1112.41
Q6FCV1_ACIAD	Uncharacterized protein;	730.57	2919.24	4	27.93	IDAAADHAAASTEHAADK	32	1764.82	1154.42
Q6FCV1_ACIAD	Uncharacterized protein;	741.07	2961.25	4	28.51	IDAAADHAAASTEHAADK	46	1764.82	1196.44
Q6FCV1_ACIAD	Uncharacterized protein;	701.72	3504.57	5	37.64	IDAAADHAAASTEHAADKAEVATR	46	2392.15	1112.42
Q6FCV1_ACIAD	Uncharacterized protein;	710.12	3546.58	5	38.00	IDAAADHAAASTEHAADKAEVATR	38	2392.15	1154.43
Q6FCV1_ACIAD	Uncharacterized protein;	897.90	3588.59	4	39.70	IDAAADHAAASTEHAADKAEVATR	21	2392.15	1196.44
Q6F7K5_ACIAD	Uncharacterized protein	1579.39	4736.14	3	83.54	IYQNTDTSSAASQTSASPTTQGLGDFLHAQEQLR	23	3623.72	1112.42
Q6F7K5_ACIAD	Uncharacterized protein	1593.39	4778.15	3	84.29	IYQNTDTSSAASQTSASPTTQGLGDFLHAQEQLR	47	3623.72	1154.43
Q6F7K5_ACIAD	Uncharacterized protein	1607.39	4820.16	3	84.77	IYQNTDTSSAASQTSASPTTQGLGDFLHAQEQLR	50	3623.72	1196.44
Q6F825_ACIAD	Uncharacterized protein	1278.95	3834.82	3	55.92	KLAEPAASAVADQNSPLSAQQQLEQK	50	2722.40	1112.42
Q6F825_ACIAD	Uncharacterized protein	1292.95	3876.83	3	55.87	KLAEPAASAVADQNSPLSAQQQLEQK	58	2722.40	1154.43
Q6F825_ACIAD	Uncharacterized protein	980.46	3918.84	4	55.74	KLAEPAASAVADQNSPLSAQQQLEQK	48	2722.40	1196.44
Q6F814_ACIAD	Putative secretion protein (HlyD family)	814.04	2440.11	3	20.96	NTAASSVAATHKK	65	1285.69	1154.42
Q6F814_ACIAD	Putative secretion protein (HlyD family)	828.05	2482.12	3	20.59	NTAASSVAATHKK	56	1285.69	1196.44
Q6F8B6_ACIAD	Uncharacterized protein	1283.91	3849.72	3	37.41	SASKPNVEASVSSQNATLSASQPQHQ	57	2653.28	1196.44
Q6F7U4_ACIAD	Uncharacterized protein	1260.88	3780.64	3	66.46	SSELEDLFNSDGGAASEPAASDKTAAK	60	2668.22	1112.41
Q6FAJ2_ACIAD	Uncharacterized protein	989.48	3954.88	4	66.33	VEQIVAQPAPASSVQFKPSNPEIDYK	26	2842.46	1112.42
Q6FAJ2_ACIAD	Uncharacterized protein	999.98	3996.89	4	66.92	VEQIVAQPAPASSVQFKPSNPEIDYK	21	2842.47	1154.43

Glycopeptides identified in *Acinetobacter baylyi* ADP1 wild type. Identifications are grouped according to the corresponding Uniprot number. The protein name, parent m/z, charge state, glycan mass, peptide mass, glycan composition, peptide sequence and mascot ion score are provided for each identified glycopeptide.

Table S1.4: Dimethylated glycopeptides identified in *A. baylyi* ADP1

Protein	Fasta headers	peptide	Charge	Glycan mass	Precursor m/z	Precursor MH+	Mascot ion score	Number of labels
Q6F7U4	>tr|Q6F7U4|Q6F7U4_ACIAD Uncharacterized protein OS=Acinetobacter baylyi (strain ATCC 33305 / BD413 / ADP1) GN=ACIAD3186 PE=4 SV=1	SSELEDLFNSDGGAASEPAASDKTAAK	4	1112.41	966.9369	3864.7238	96	Dimethyl (K); Dimethyl (K); Dimethyl (N-term)
Q6F825	>tr|Q6F825|Q6F825_ACIAD Uncharacterized protein OS=Acinetobacter baylyi (strain ATCC 33305 / BD413 / ADP1) GN=ACIAD3092 PE=4 SV=1	KLAEPAASAVADQNSPLSAQQQLEQK	4	1112.41	980.4813	3918.9013	47	Dimethyl (K); Dimethyl (K); Dimethyl (N-term)
Q6FCV1	>tr|Q6FCV1|Q6FCV1_ACIAD Uncharacterized protein OS=Acinetobacter baylyi (strain ATCC 33305 / BD413 / ADP1) GN=ACIAD1233 PE=4 SV=1	IDAAADHAAASTEHAADKAEVATR	4	1112.41	890.9106	3560.6187	112	Dimethyl (K); Dimethyl (N-term)
Q6F8B6	>tr|Q6F8B6|Q6F8B6_ACIAD Uncharacterized protein OS=Acinetobacter baylyi (strain ATCC 33305 / BD413 / ADP1) GN=ACIAD2990 PE=4 SV=1	SASKPNVEASVSSQNATLSASQPQHQ	4	1154.43	966.6921	3863.7684	52	Dimethyl (K); Dimethyl (N-term)
Q6FCV1	>tr|Q6FCV1|Q6FCV1_ACIAD Uncharacterized protein OS=Acinetobacter baylyi (strain ATCC 33305 / BD413 / ADP1) GN=ACIAD1233 PE=4 SV=1	IDAAADHAAASTEHAADKAEVATR	4	1154.43	901.4081	3602.6325	122	Dimethyl (K); Dimethyl (N-term)
Q6F8B6	>tr|Q6F8B6|Q6F8B6_ACIAD Uncharacterized protein OS=Acinetobacter baylyi (strain ATCC 33305 / BD413 / ADP1) GN=ACIAD2990 PE=4 SV=1	SASKPNVEASVSSQNATLSASQPQHQ	4	1196.43	977.1939	3905.7756	39	Dimethyl (K); Dimethyl (N-term)
Q6FCV1	>tr|Q6FCV1|Q6FCV1_ACIAD Uncharacterized protein OS=Acinetobacter baylyi (strain ATCC 33305 / BD413 / ADP1) GN=ACIAD1233 PE=4 SV=1	IDAAADHAAASTEHAADKAEVATR	4	1196.43	911.9099	3644.6396	118	Dimethyl (K); Dimethyl (N-term)

Dimethylated Glycopeptides identified in *A. baylyi* ADP1 wild type (light) and OTase complement (heavy). Identifications are grouped according to the corresponding Uniprot number. The protein name, parent m/z, charge state, glycan mass, peptide mass, glycan composition, peptide sequence and mascot ion score are provided for each identified glycopeptide. For each identified peptide the dimethylation observed is denoted to aid read distinguish glycopeptide observed from wild type (containing dimethyl N-term and K) and the complement (containing dimethyl N-term 2H(6)13C(2) and K2H(6)13C(2)) .

Table S1.5: Quantitative Glycopeptides identified in *A. baylyi* ADP1

Protein	Fasta header	Best score ra	Best score sc	peptide	Charge	Glycan mass	Mascot ion score	Precursor m/z [Da] light	Precursor MH+ [Da] light	Area under the curve of light replicate 1	Area under the curve of light replicate 2	Precursor m/z [Da] Medium	Precursor MH+ [Da] Medium	Area under the curve of heavy replicate 1	Area under the curve of heavy replicate 2	Precursor m/z [Da] Heavy	Precursor MH+ [Da] Heavy	Area under the curve of heavy replicate 1	Area under the curve of heavy replicate 2	Ratio M/H replicate 1	Ratio M/H replicate 2	Number of labels
Q6F7U4	>tr|Q6F7U4	Nsco_ADPI_(	21586	SSELEDLFNSDGGAASEPAASDKTAAK	4	1112.41	96	966.9369	3864.7238	1537751.0000	1797646.0000	969.9369	3876.7238	-	-	972.96965	3888.8548	1710540.0000	2158696.0000	0.8990	0.8327	Dimethyl (K); Dimethyl (K); Dimethyl (N-term)
Q6F825	>tr|Q6F825	Nsco_ADPI_(	18114	KLAEPAASAVADQNSPLSAQQQLEQK	4	1112.41	47	980.4813	3918.9013	2185053.0000	2710910.0000	983.4813	3930.9013	-	-	986.51384	3943.0315	2905830.0000	3977781.0000	0.7520	0.6815	Dimethyl (K); Dimethyl (K); Dimethyl (N-term)
Q6FCV1	>tr|Q6FCV1	Nsco_ADPI_(	14446	IDAAADHAAASTEHAADKAEVATR	4	1112.41	112	890.9106	3560.6187	19324257.0000	30994030.0000	892.9106	3568.6187	-	-	894.9317	3576.7030	11270073.0000	18267839.0000	1.7147	1.6966	Dimethyl (K); Dimethyl (N-term)
Q6F8B6	>tr|Q6F8B6	Nsco_ADPI_(	14234	SASKPNVEASVSSQNATLSASQPQHQ	4	1154.43	52	966.6921	3863.7684	2243644.0000	1831981.0000	968.6921	3871.7684	-	-	970.71827	3879.8493	1327726.0000	3118958.0000	1.6898	0.5874	Dimethyl (K); Dimethyl (N-term)
Q6FCV1	>tr|Q6FCV1	Nsco_ADPI_(	14609	IDAAADHAAASTEHAADKAEVATR	4	1154.43	122	901.4081	3602.6325	23868968.0000	28896927.0000	903.4081	3610.6325	-	-	905.43505	3618.7164	16076139.0000	19332716.0000	1.4847	1.4947	Dimethyl (K); Dimethyl (N-term)
Q6F8B6	>tr|Q6F8B6	Nsco_ADPI_(	14321	SASKPNVEASVSSQNATLSASQPQHQ	4	1196.43	39	977.1939	3905.7756	690676.0000	879831.0000	979.1939	3913.7756	-	-	981.22031	3921.8574	1480071.0000	1840772.0000	0.4667	0.4780	Dimethyl (K); Dimethyl (N-term)
Q6FCV1	>tr|Q6FCV1	Nsco_ADPI_(	14691	IDAAADHAAASTEHAADKAEVATR	4	1196.43	118	911.9099	3644.6396	8561782.0000	7574176.0000	913.9099	3652.6396	-	-	915.93764	3660.7267	10178794.0000	6814193.0000	0.8411	1.1115	Dimethyl (K); Dimethyl (N-term)

Dimethylated Glycopeptides identified in *Acinetobacter baylyi* ADP1 wild type (light) and OTase complement (heavy). Identifications are grouped according to the corresponding Uniprot number. The protein name, parent m/z, charge state, glycan mass, peptide mass, glycan composition, peptide sequence and mascot ion score are provided for each identified glycopeptide. For each identified peptide the dimethylation observed is denoted to aid read distinguish glycopeptide observed from wild type (containing dimethyl N-term and K) and the complement (containing dimethyl N-term 2H(6)13C(2) and K2H(6)13C(2))

References:

Actis, L.A., Potter, S.A., and Crosa, J.H. (1985) Iron-regulated outer membrane protein OM2 of Vibrio anguillarum is encoded by virulence plasmid pJM1. *J Bacteriol* **161**: 736–42.

Aranda, J., Poza, M., Pardo, B.G., Rumbo, S., Rumbo, C., Parreira, J.R., Rodríguez-Velo, P., and Bou, G. (2010) A rapid and simple method for constructing stable mutants of *Acinetobacter baumannii*. *BMC Microbiol* **10**: 279.

Boersema, P.J., Raijmakers, R., Lemeer, S., Mohammed, S., and Heck, A.J.R. (2009) Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. *Nat Protoc* **4**: 484–94.

Carruthers, M.D., Harding, C.M., Baker, B.D., Bonomo, R.A., Hujer, K.M., Rather, P.N., and Munson, R.S. (2013) Draft Genome Sequence of the Clinical Isolate *Acinetobacter nosocomialis* Strain M2. *Genome Announc* **1**: e00906–13

Carruthers, M.D., Nicholson, P.A., Tracy, E.N., and Munson, R.S. (2013) *Acinetobacter baumannii* utilizes a type VI secretion system for bacterial competition. *PLoS One* **8**: e59388.

Choi, K.-H., Gaynor, J.B., White, K.G., Lopez, C., Bosio, C.M., Karkhoff-Schweizer, R.R., and Schweizer, H.P. (2005) A Tn7-based broad-range bacterial cloning and expression system. *Nat Methods* **2**: 443–8.

Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**: 6640–5.

de Berardinis, V., Vallenet, D., Castelli, V., Besnard, M., Pinet, A., Cruaud, C., Samair, S., Lechaplais, C., Gyapay, G., Richez, C., Durot, M., Kreimeyer, A., Le Fèvre, F., Schächter, V.,Pezo, V., Döring, V., Scarpelli, C., Médigue, C., Cohen, G. N., Marlière, P., Salanoubat, M. and Weissenbach, J.,. (2008) A complete collection of single-gene deletion mutants of *Acinetobacter baylyi* ADP1. *Mol Syst Biol* **4**: 174.

Dykxhoorn, D.M., Pierre, R. St., and Linn, T. (1996) A set of compatible tac promoter expression vectors. *Gene* **177**: 133–136.

Feldman, M.F., Wacker, M., Hernandez, M., Hitchen, P.G., Marolda, C.L., Kowarik, M., Morris, H.R., Dell, A., Valvano, M.A., and Aebi, M. (2005) Engineering N-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli*. *Proc Natl Acad Sci U S A* **102**: 3016–21.

Figurski, D.H., and Helinski, D.R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci* **76**: 1648–1652.

Harding, C.M., Tracy, E.N., Carruthers, M.D., Rather, P.N., Actis, L.A., and Munson, R.S. (2013) *Acinetobacter baumannii* strain M2 produces type IV pili which play a role in natural transformation and twitching motility but not surface-associated motility. *MBio* **4**: e00360–13–.

Hunger, M., Schmucker, R., Kishan, V., and Hillen, W. (1990) Analysis and nucleotide sequence of an origin of an origin of DNA replication in *Acinetobacter calcoaceticus* and its

use for *Escherichia coli* shuttle plasmids. *Gene* **87**: 45–51.

Kumar, A., Dalton, C., Cortez-Cordova, J., and Schweizer, H.P. (2010) Mini-Tn7 vectors as genetic tools for single copy gene cloning in *Acinetobacter baumannii*. *J Microbiol Methods* **82**: 296–300.

Lee, E.C., Yu, D., Martinez de Velasco, J., Tessarollo, L., Swing, D.A., Court, D.L., Jenkins, N.A., and Copeland, N.G. (2001) A highly efficient *Escherichia coli*-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* **73**: 56–65.

Nakar, D., and Gutnick, D.L. (2001) Analysis of the wee gene cluster responsible for the biosynthesis of the polymeric bioemulsifier from the oil-degrading strain *Acinetobacter* lwoffii RAG-1. *Microbiology* **147**: 1937–1946.

Niu, C., Clemmer, K.M., Bonomo, R.A., and Rather, P.N. (2008) Isolation and characterization of an autoinducer synthase from *Acinetobacter baumannii*. *J Bacteriol* **190**: 3386–92.

Scott, N.E., Kinsella, R.L., Edwards, A.V.G., Larsen, M.R., Dutta, S., Saba, J., Foster, L.J., and Feldman, M.F. (2014) Diversity Within the O-linked Protein Glycosylation Systems of *Acinetobacter* Species. *Mol Cell Proteomics* **13**: 2354–70.

Wacker, M., Linton, D., Hitchen, P.G., Nita-Lazar, M., Haslam, S.M., North, S.J., Panico, M., Morris, H.R., Dell, A., Wren, B.W., and Aebi, M. (2002) N-linked glycosylation in *Campylobacter jejuni* and its functional transfer into E. *coli*. *Science* **298**: 1790–3.

Appendix B:

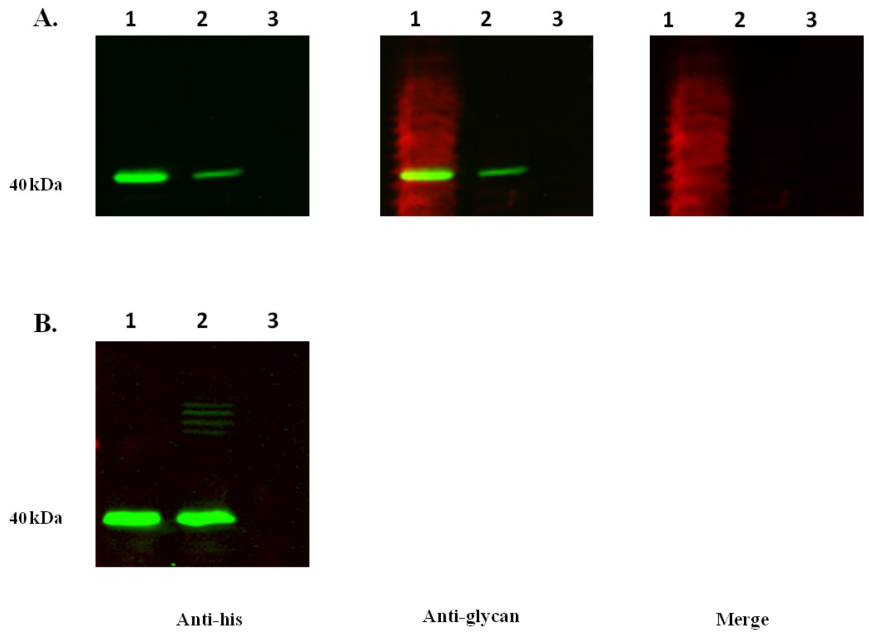


Figure S2.1 Glycosylation of AcrA and DsbA1 by CjPglB and NmPglL respectively.

A. Western blot analyses of whole cell lysates of *E. coli* SDB1 coexpressing AcrA, CjPglB and CPS14. No bands of lower electrophoretic mobility could be detected in lane 2 where PglB is coexpressed with AcrA and CPS14, in comparison with unglycosylated AcrA in lane 1 expressed with CPS14 alone. Proteinase K digests abolished the signal (lane 3). Expression was probed for with a monoclonal anti-his antibody (green) and a polyclonal anti-CPS14 antibody (red).

B. Western blot analyses of whole cell lysates of *E. coli* SDB1 coexpressing AcrA, CjPglB and O7 antigen. Bands with lower electrophoretic mobility could be detected in lane 2 where PglB is coexpressed with AcrA and O7, in comparison with unglycosylated AcrA in lane 1 expressed with O7 alone. Proteinase K digests abolished the signal (lane 3). Expression was probed for with a monoclonal anti-his antibody (green).

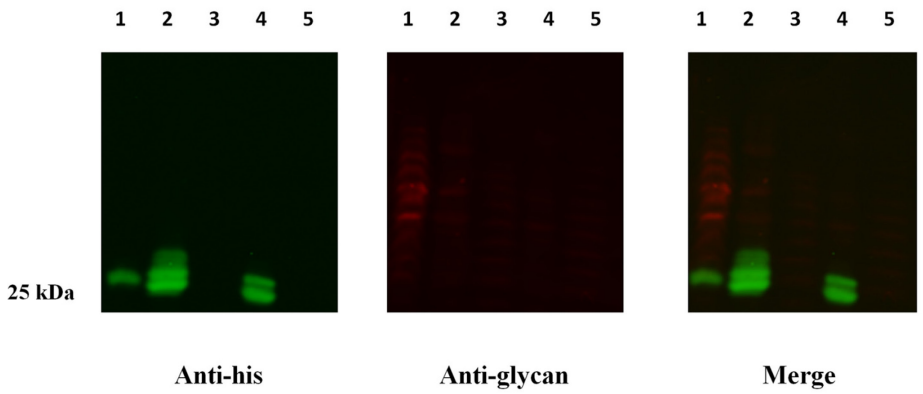


Figure S2.2 Western blot analyses of whole cell lysates of *E. coli* SDB1 probing for DsbA1 and CPS14 expression. No bands of lower electrophoretic mobility that react to both antibodies could be detected in lane 2 and 4, where $PglL_{ADP1}$ and NmPglL are coexpressed respectively. Lower electrophoretic mobility bands detected by anti-his are likely modifications by the *E. coli* unknown glycan moiety. Proteinase K digests abolished the signals (lanes 3 and 5). Expression was probed for with a monoclonal anti-his antibody (green) and a polyclonal anti-CPS14 antibody (red)