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The characterization ofnovel protein glycosylation in Helicobacter pylori

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ABSTRACT

Protein glycosylation in *Helicobacter pylori* is known to modify the flagellins with pseudaminic acid (pse). This modification is required for proper flagellum production and *H. pylori* motility, which implicates protein glycosylation in *H. pylori's* pathogenesis. We investigated whether protein glycosylation extends beyond pse-specific flagellin glycosylation by the use of *H. pylori pse* biosynthesis mutants and the detection of glycoproteins by glycoprotein-specific chemical labelling. Using these methods, we demonstrated the presence of novel protein glycosylation in *H. pylori,* in terms of both the identification of membrane glycoproteins and at least two alternative glycosylation pathways. In addition, a link between protein glycosylation and lipopolysaccharide (LPS) biosynthesis was elucidated, in which the O-antigen ligase, WaaL, is a candidate glycoprotein and was shown to exhibit general oligosaccharyltransferase activity. Overall, this study highlights that protein glycosylation affects several proteins in *H. pylori* and has a key role in the production of multiple virulence factors.

Keywords: *Helicobacter pylori,* protein glycosylation, flagellins, lipopolysaccharide, virulence factors, glycoproteins

DEDICATION

This is dedicated to my family. Thank you for your constant support and encouragement. I will never be able to thank you enough.

Mom and Dad, I would not have reached the end without you.

Jennifer and Dennis, and Karen and Simon, thank you for always reminding me that life exists outside of the lab and for the motivation to keep going.

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2-DE	2-dimensional gel electrophoresis
6XHis	hexa-Histidine tag
Ala	alanine
Asn	asparagine
Asp	aspartic acid
BHI-YE	brain heart infusion yeast extract
BHz	biotin hydrazide
CAT	chloramphenicol resistance cassette
CE	capillary electrophoresis
CFE	cell-free extract
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CMP	cytidyl monophosphate
CTAB	cetyltrimethylammonium bromide
CV	column volume
DIG	digoxigenin
DMF	dimethylformamide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ESI	electrospray ionization
FlaA	flagellin A
FlaB	flagellin B
Fuc	fucose
FucT	fucosyltransferase
Gal	galactose
GalNAc	N-acetyl-galactosamine
Glc	glucose
GlcNAc	N-acetyl-glucosamine
Glu	glutamic acid
Hep	heptose
HPLC	high performance liquid chromatography
IEF	isoelectric focusing
Ile	isoleucine
IM	inner membrane
LB	Luria Bertani
LC	liquid chromatography
Le^a	Lewis a blood group antigen
Le^b	Lewis b blood group antigen

Leu	leucine
Le^x	Lewis x blood group antigen
Le^y	Lewis y blood group antigen
LOS	lipooligosaccharide
LPS	lipopolysaccharide
m/z	mass to charge ratio
MALDI	matrix assisted laser desorption ionization
MALT	mucosa-associated lymphoid tissue
Man	mannose
Met	methionine
MS	mass spectrometry
Mtz	metronidazole
MWCO	molecular weight cut-off
NeuNAc	N-acetyl-neuraminic acid
OD	optical density
OM	outer membrane
OMP	outer membrane protein
OMV	outer membrane vesicle
OTase	oligosaccharyltransferase
PAS	periodic acid-Schiff staining
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pgl	protein glycosylation (locus)
pI	isoelectric point
pse	pseudaminic acid
PVDF	polyvinylidene fluoride
SabA	sialic acid binding adhesin
SAM	S-adenosyl-methionine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	serine
Thr	*threonine*
TOF	time of flight
UDP	uridine diphosphate
UndP	undecaprenyl phosphate
UreA	urease subunit A
UreB	urease subunit B
Val	valine
xg	gravitational force

CHAPTER 1: Introduction

1.1 *Helicobacter pylori*

Helicobacter pylori is a Gram-negative, spiral-shaped, microaerophilic bacterium with multiple polar sheathed flagella (reviewed in [1]). Presently, *H. pylori* is acknowledged as a cause of gastritis, peptic ulcer disease and gastric cancer [2]. However, at the time of its discovery, the finding of a bacterium in the stomach was controversial, as it was believed that no microorganisms were able to survive in the acidic stomach environment.

Dr. Robin Warren originally observed a bacterium associated with gastric biopsies in 1979, but it was not successfully cultured until 1982 when a plate was left serendipitously incubating over the Easter long weekend [3]. Based on its spiral-shape, the bacterium was named *Campylobacter pyloridis,* but was later found not to be part of the *Campylobacter* genus based on disparity of 16S rRNA sequencing and discrepancies in morphology, cellular fatty acids, and growth characteristics. The bacterium was re-named *Helicobacter pylori* [4, 5]. In 1985, Drs. Barry Marshall and Robin Warren demonstrated the association of *H. pylori* infection with gastritis and peptic ulcer disease when Dr. Marshall drank a culture of *H. pylori* and subsequently developed peptic ulcer disease. He later cleared the infection with antibiotic treatment. Dr. Marshall's experiment was groundbreaking as it fulfilled Koch's postulates and disproved the theory that the stomach was a sterile environment [6]. As a result, Drs. Robin Warren and Barry Marshall won the Nobel Prize in Physiology and Medicine in 2005 for the discovery of *H. pylori* and its involvement in chronic gastrointestinal disease.

1.1.1 General *H. pylori* characteristics

H. pylori range from 2.5μm to 5.0μm in length and are 0.5μm to 1.0μm wide [7]. Genome size varies from 1.6 to 1.73Mb [8]. A high level of genetic diversity has been observed among *H. pylori* isolates, which exhibit silent nucleotide variation within genes and limited gene clustering [9]. Up to 6-7% of encoded genes are predicted to be strain-specific. These strain-specific genes are localized to a hypervariable region that is common amongst *H. pylori* strains, termed the 'plasticity zone' [10]. This genetic diversity of distinct *H. pylori* strains is believed to be associated with the variation in the severity and disease outcome of *H. pylori* infections [10, 11].

Moreover, *H. pylori* is a slow-growing fastidious organism that requires rich, complex media and microaerophilic conditions for growth [1, 12]. When growth conditions are not optimal for the bacterium, such as after several sub-culturing events or during extended growth periods, *H. pylori* is known to change from its spiral shape to a coccoid form. The coccoid form is believed to be viable, but non-culturable *in vitro* [13]. Thus, strict growth conditions in the laboratory need to be maintained to prevent conversion of *H. pylori* to its non-culturable form.

1.1.2 *H. pylori* prevalence and transmission

H. pylori is a ubiquitous microorganism as it is found worldwide. It is estimated that more than half of the world's population is affected by *H. pylori,* making it one of the most common human bacterial infections [14]. Although widespread, *H. pylori* prevalence demonstrates large geographic variation. In general, this variation is associated with socioeconomic status with a higher prevalence of infection occurring in developing countries, estimated at 74%, compared to a lower prevalence in developed

countries, estimated to be 58% [15]. It is proposed that this divergence is due to differential rates of acquisition during the early years of life. In developing countries it is believed that infection occurs most often during childhood years and continues throughout life as a chronic infection. Comparatively, infection in developed countries is thought to occur later in life, likely due to better sanitation and standards of living [16].

Despite the global pattern, variance in the prevalence of *H. pylori* infection within countries is also common [16]. A recent study on *H. pylori* prevalence in Canadian children from three urban cities indicated a low prevalence with an observed infection rate of 7.1% [17]. However, prevalence in Arctic Aboriginal populations can range from 50-90%, indicating large variations in *H. pylori* infection rates within sub-populations of developed countries [18, 19].

In addition to socioeconomic status, other risk factors have been documented for *H. pylori* infection. In particular, age has a positive correlation with *H. pylori* prevalence, with infection being more common among adults [20]. As well, genetic factors are believed to have a role in susceptibility [21]. Overall, many factors contribute to infection and although infection rates are declining in developed countries, due to high seroprevalence of sub-populations and multiple risk factors, *H. pylori* remains a global health burden [22].

Transmission of *H. pylori* remains largely uncharacterized. The only known reservoir of *H. pylori* is the human stomach, despite several studies aimed at identifying other environmental sources, such as water, food, and animal sources [23-28]. As such, person-to-person spread is the most likely mode of transmission, with the route of transfer proposed as either an oral-oral, oral-fecal, or gastro-oral route [16]. A recent

study conducted on the acquisition of *H. pylori* in rhesus macques supports the oral-oral route of transmission as *H. pylori* infection rapidly spread among animals despite no cultivation of *H. pylori* from any environmental source [29]. Conversely, *H. pylori* has been detected in the gastric juice, vomitus, and stool samples of infected individuals, suggesting spread by gastro-oral and oral-fecal routes and the potential for increased transmission by other gastrointestinal diseases [30, 31]. Moreover, interfamilial transmission is considered a predominant method of infection. Mother-to-child spread is believed to be the most common, in which *H. pylori* isolates from children and their mothers are identical [32, 33]. In summary, although *H. pylori* is common around the world, how infection spreads between geographic locations and communities remains largely unknown.

1.1.3 *H. pylori* and disease

The majority of *H. pylori* infections are asymptomatic; however, infection results in chronic inflammation of the stomach mucosa that remains life-long unless eradication therapy is employed. Chronic gastritis ultimately leads to the erosion of the gastric mucosa which can result in the development of severe gastrointestinal diseases including peptic ulcer disease, gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma [34].

Overall, it is estimated that 6-10% of individuals infected with *H. pylori* will develop gastroduodenal disease, up to 3% will develop gastric cancer and less than 0.1% will develop MALT lymphoma [35]. In 1994, *H. pylori* was classified as a class I carcinogen by the International Agency for Research on Cancer (IARC) [36] and is currently recognized as the leading infectious agent known to cause cancer [15].

Importantly, gastric cancer remains the second leading cause of cancer-related deaths [37], of which, 63% of cases is attributable to *H. pylori* infection [15]. *H. pylori* infection is also associated with the development of MALT lymphoma in which 92% of cases are thought to arise from *H. pylori* infection [38]. Eradication of *H. pylori* has been shown to lead to regression of gastric lymphomas, indicating that treatment of *H. pylori* infection is a feasible measure for the prevention of gastrointestinal disease. In fact, due to the success of *H. pylori* eradication, gastric MALT lymphoma is considered the first treatable cancer with antibiotic therapy [39, 40].

Typical treatment for *H. pylori* infection involves the use of triple therapies; a combination of proton pump inhibitors and at least two antibiotics. However, high rates of treatment failure, due to patient non-compliance from various side effects and rising antibiotic resistance worldwide, threatens the efficacy of current treatment regimens, and indicates the need to identify new therapeutic targets [41, 42].

While the association of *H. pylori* with gastrointestinal diseases is well established, more recently, a protective role of *H. pylori* in the progression of esophageal cancer has been described, indicating the potential of the co-adaptation of humans with *H. pylori* [43, 44]. This highlights the complexity of *H. pylori* infection and the host-microbe interaction.

Overall, much remains unknown regarding the progression of *H. pylori* infection and its involvement in disease. The severity and disease outcome of *H. pylori* infection is believed to be due to a combination of environmental, host genetic, and bacterial factors [34]. In fact, many bacterial virulence factors have been characterized and shown to contribute to *H. pylori's* pathogenesis.

1.2 *H. pylori* virulence factors

In addition to host and environmental factors, various bacterial factors aid in *H. pylori's* ability to cause disease. Bacterial virulence factors aid *H. pylori* in all aspects of infection; survival within the hostile gastric environment, colonization, and modulation of host immune responses, all of which contribute to *H. pylori's* ability to cause persistent infection. A brief overview of a few of these factors related to this work is provided below.

1.2.1 Urease

Urease is a multi-subunit enzyme produced by *H. pylori* that aids in its ability to survive within the stomach by converting urea into ammonia and carbon dioxide by-products, thereby neutralizing the local pH of the stomach. The importance of urease in *H. pylori* colonization was first highlighted by urease-negative mutants that were unable to cause infection in gnotobiotic piglets [45]. Urease is highly abundant in the cell and represents 10-15% of total bacterial proteins [46]. The enzyme is composed of two structural subunits, UreA (~29.5 kDa) and UreB (~66 kDa), that form a hexadimer and function in conjunction with UreE-H, which are responsible for transporting nickel for urease activity, and UreI, which encodes a pH-gated urea channel that controls urea concentration [47, 48]. The majority of urease is cytoplasmic, where it acts to maintain the cytoplasmic pH and the proton motive force, and thereby also reduces the acidity of the periplasm [49]. External urease attached to the outer membrane of *H. pylori* has also been observed, and is believed to neutralize the pH of the direct surrounding environment of the bacteria [50].

1.2.2 Adhesins

Initial colonization and persistence of infection is mediated by adherence to host cells. *H. pylori* possesses several adhesins and outer membrane proteins (OMPs) that assist in its adherence to host cells. In fact, 4% of the *H. pylori* genome is predicted to encode OMPs [51]. One of the most characterized adhesins in *H. pylori* is the blood group antigen binding adhesin (BabA). BabA binds fucosylated Lewis b blood group antigens (Le^b) found on the surface of gastric epithelial cells. Le^b-mediated colonization of transgenic mice expressing Le^b by *H. pylori* was shown to result in more severe gastritis, indicating that BabA may increase *H. pylori's* pathogenesis [52]. As well, the presence of BabA in clinical strains is associated with an increased risk of developing duodenal ulcer disease and gastric cancer [53].

Sialic acid-binding adhesin (SabA) is another *H. pylori* adhesin. SabA binds the Lewis x blood group antigen (Le^x) expressed by the gastric epithelium [54]. Le^x expression is induced during chronic gastritis, suggesting that *H. pylori* modulates host cell glycosylation patterns to enhance its own attachment, and therefore may play a role in the persistence of *H. pylori* infection [55].

As well, other less characterized adhesins that likely play a role in colonization are AlpA/AlpB [56], HopZ [57]and OipA [51], however, their host targets have yet to be identified.

1.2.3 Toxins

H. pylori produces a variety of factors that directly target the host and manipulate cell functions. One of the most extensively studied toxins is CagA. CagA is a 145 kDa protein that is translocated into host cells via a type IV secretion system of which CagA is

the only known effector protein [58, 59]. CagA is encoded within the *cag* pathogenicity island (*cag* PAI), a 40kb DNA insertion element encoding 27-31 genes including both CagA and components of the bacterial type IV secretion system [60]. Both CagA and the *cag* PAI are strongly associated with the development of disease. Clinical strains of *H. pylori* are categorized as *cagA*-positive or *cagA*-negative. *cagA*-positive strains are considered to be more virulent and are associated with an increased risk for disease [61]. Upon translocation of CagA into host cells, CagA is believed to interact with an estimated 20 host cellular proteins in either a non-phosphorylated or phosphorylated state and interfere with multiple cellular pathways, including actin-cytoskeletal rearrangement, disruption of tight junctions, induction of pro-inflammatory responses, and apoptosis [62]. Further, translocation of CagA results in a characteristic elongation of gastric cells, termed "the hummingbird phenotype" [58]. Based on its numerous host cell effects and association with disease, CagA is considered a bacterial oncoprotein. In fact, transgenic expression of CagA in mice results in gastric epithelial cell proliferation and the production of carcinomas [63].

Another toxin linked to increased disease risk is the secreted vacuolating cytoxin A (VacA). VacA also has multiple effects on host cells. VacA was first characterized for its ability to induce vacuole formation in mammalian cells [64], but it is now known to alter additional cellular functions including induction of apoptosis, membrane channel formation, suppression of T-cell responses, and induction of pro-inflammatory cytokine production [65].

1.2.4 Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) is a common outer membrane and cell-surface component of Gram-negative bacteria. It is composed of three main components; a lipid A moiety that acts as a hydrophobic anchor in the outer leaflet of the membrane lipid bilayer and has endotoxic activity, a core oligosaccharide with conserved length and sugar composition, and a variable O-antigen chain that provides serospecificity [66, 67]. LPS provides a physical barrier from host defences, including bile salts, detergents, and antimicrobial peptides, and is involved in the maintenance of membrane stability, nutrient transport, and host interactions [68]. The main constituents of *H. pylori* LPS are fucose (Fuc), galactose (Gal) and N-acetyl-glucosamine (GlcNAc), as well as minor amounts of glucose (Glc) and heptose (Hep) [69, 70].

While activation of the innate immune system during bacterial infection by the components of LPS has been well established, the LPS of *H. pylori* exhibits unique characteristics that aid in its ability to evade the host immune response. These modifications are thought to reflect an adaptation of *H. pylori* to its ecological niche in the human stomach, which enables persistent infection.

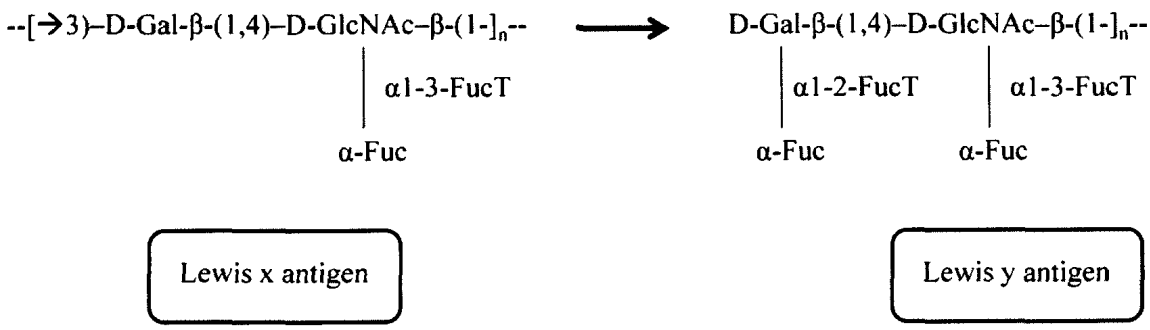
Firstly, the lipid A domain, commonly referred to as endotoxin due to several well-documented immunomodulatory effects on host cells, such as septic shock [66, 67], is underphosphorylated and contains unusual long fatty acid chains in *H. pylori* [71-73]. These modifications result in reduced endotoxicity, including reduced induction of cytokines and chemokines, and lower lethality in mice, when compared to the lipid A of other Gram-negative bacteria [74-76]. In particular, bacterial LPS is known to activate the innate immune response via recognition by Toll-like receptor 4 (TLR-4) [76-78].

However, in the case of *H. pylori* LPS, activation of TLR-4 is reduced due to the atypical acylation and phosphorylation pattern of its lipid A domain [76]. Thus, the unusual modifications of the lipid A moiety result in *H. pylori's* LPS being less immunogenic, in terms of its ability to activate the host immune response, and thereby aids in *H. pylori's* ability to evade the host immune response and generate persistent, chronic infections.

In addition, the O-antigen chain of *H. pylori* contributes to its ability to evade the host immune response as *H. pylori* O-antigen mimics the human Lewis blood group antigens found on gastric epithelial cells [79, 80]. More than 80% of *H. pylori* strains express type 2 Lewis antigens, Le^x and Le^y, whereas less than 5% express type 1 antigen motifs, Le^a and Le^b [81]. Typical *H. pylori* type 2 O-antigen is composed of several internal repeating Le^x units on the precursor O-antigen structure and either Le^x or Le^y at the terminal position [82]. The synthesis of Le^y or Le^x antigens is mediated by the activity of different fucosyltransferases (FucTs) (Figure 1) [82, 83]. A FucT with α1,3-activity glycosylates the backbone O-antigen chain creating the Le^x antigen. A second FucT with α1,2 -activity adds a second fucose to the Le^x structure generating the Le^y antigen. Le^y structures cap the O-antigen and prevent further elongation of the O-antigen chain, thus, they are only found at the terminal position. The relative activities of the FucTs required for Lewis antigen synthesis have been shown to vary amongst *H. pylori* strains, which alters the Lewis antigen production exhibited [83]. For example, the *H. pylori* strain 26695 has relatively lower α1,2-FucT activity, which results in less conversion of Le^x structures to Le^y and thereby produces O-antigen that is decorated with both Le^x and Le^y antigens simultaneously [84, 85]. Conversely, in other *H. pylori* strains,

Figure 1: The synthesis of type 2 Lewis antigens, Le^y and Le^x, of *H. pylori* O-antigen.

Type 2 Lewis antigen synthesis is mediated by fucosyltransferases (FucTs) that modify the O-antigen chain precursor. A FucT with α1,3-activity fucosylates the O-antigen backbone generating the Le^x structure. Le^x antigens are present along the O-antigen chain and in the terminal position. Le^x structures can be further modified by an additional FucT with α1,2-FucT activity to generate the Le^y antigen. Le^y structures terminate the O-antigen chain and prevent further elongation. Gal = galactose, GlcNAc = N-acetyl-glucosamine, Fuc = fucose.


--[→3)–D-Gal-β-(1,4)–D-GlcNAc–β-(1-]n--
α1-3-FucT
α-Fuc
D-Gal-β-(1,4)–D-GlcNAc–β-(1-]n--
α1-2-FucT
α1-3-FucT
α-Fuc
α-Fuc
Lewis x antigen
Lewis y antigen

a high level of α1,2-FucT activity results in the transformation of the majority of terminal Le^x structures to Le^y. Indeed, monoclonal antibodies with specificity for the Lewis antigens found on the O-antigen have been used as a serotyping system for *H. pylori* isolates [86].

In addition to being a form of molecular mimicry, Lewis antigen expression is also phase variable, which further contributes to *H. pylori's* ability to escape detection by the host immune response. Phase variability is due to slipped-strand mispairing during transcription of a polyA-polyC tract in the 5'end and a repeat region in the 3'end of the FucTs genes, which creates an on/off switch for FucT expression and thereby Lewis antigen expression [87]. In fact, *H. pylori* Lewis antigen expression *in vitro* was shown to exhibit phase variation at a rate of 0.2-0.5% in bacterial populations derived from a single cell [88]. As a result, phase variability in the Lewis antigen decoration of *H. pylori* O-antigen allows *H. pylori* isolates to adapt to their host and evade the host immune response.

Lastly, in addition to immune evasion, the O-antigen of *H. pylori* also acts as an adhesin and binds C-type lectin DC-SIGN [89]. This interaction is believed to mediate interactions with immune cells and induce gastric autoimmunity [82].

1.2.4.1 LPS biosynthesis

In general, three O-antigen LPS biosynthesis pathways have been described [67]. In all cases, the O-antigen is assembled on an undecaprenyl phosphate (UndP) lipid carrier by the action of specific glycosyltransferases. Assembly of the O-antigen is typically initiated by WecA, an UDP-GlcNAc:undecaprenyl-phosphate GlcNAc-1-phosphate transferase, that acts to transfer the initiating GlcNAc from the nucleotide

activated sugar donor (UDP-GlcNAc) to UndP [90]. The three pathways diverge in the mechanism by which the O-antigen is assembled and translocated to the periplasm [67]. In the polymerase-dependent pathway, individual O-antigen units are assembled in the cytoplasm and are each translocated to the periplasm by the flippase Wzx, where they are polymerized by Wzy to form the full O-antigen. The length of the final O-antigen chain is regulated by the chain length regulator Wzz. In the ABC transporter-dependent pathway, the entire O-antigen is assembled in the periplasm and flipped by an ATP-dependent transporter composed of the polypeptides Wzm and Wzt that form the membrane channel and produce the energy to drive translocation by ATPase activity, respectively. In the synthase-dependent LPS pathway, the entire O-antigen is also assembled in the cytoplasm and the synthase, WbbF, is required for both glycosyltransferase activity and translocation of the assembled O-antigen to the periplasm. In all three pathways, the fully assembled O-antigen is attached to the lipid A core by WaaL, the O-antigen ligase.

Unlike most bacteria, the genome of *H. pylori* is largely unorganized. As such, genes involved in LPS biosynthesis have been difficult to identify and characterize as they are spread throughout the genome. Indeed, several studies have identified glycosyltransferases involved in O-antigen assembly [82], as well as enzymes involved in the synthesis and modification of the lipid A core [73, 91, 92], however, the enzymes responsible for the translocation and assembly of the completed O-antigen to the lipid A core had remained elusive until recently.

The LPS biosynthesis pathway in *H. pylori* was characterized during the course of this study by Hug *et al.* 2010 [93]. In this study, Hug *et al.* 2010, demonstrated that the *H. pylori* LPS biosynthesis pathway lacks several of the standard LPS enzymes from each

model pathway; no *wzx, wzt, wzm, wbbF, wzy,* or *wzz,* could be identified. However, homologs of WecA and WaaL were identified based on similar membrane topology to the model enzymes from *Escherichia coli.* As such, *H. pylori* possesses a distinct mechanism for the translocation of O-antigen compared to the established pathways. In short, similar to the canonical pathways, O-antigen assembly in *H. pylori* is initiated by a WecA homolog, followed by synthesis of the complete O-antigen by specific glycosyltransferases. The O-antigen is then flipped to the periplasm by the activity of the newly characterized O-antigen translocase, Wzk. Wzk is a homolog of PglK, the enzyme responsible for the translocation of the oligosaccharide intermediate during protein N-glycosylation in *Campylobacter jejuni,* and has not been identified in any other LPS biosynthesis pathways. Wzk exhibits relaxed substrate specificity, which corresponds with the ability of *H. pylori* to express diverse O-antigen chains with varying fucose decoration. After the translocation of the O-antigen to the periplasm, the O-antigen ligase or WaaL, attaches the O-antigen to the lipid A core and the completed unit is transported to the outer membrane. Overall, the elucidation of the *H. pylori* LPS biosynthesis pathway demonstrated a novel translocase involved in LPS biosynthesis. The connection of LPS biosynthesis and protein glycosylation will be discussed further in Section 1.3.4.

1.2.5 Motility and flagella

The ability of *H. pylori* to transverse the viscous gastric mucus to reach the gastric epithelium for colonization is a key component of *H. pylori's* survival in the human stomach. *H. pylori* has 4-6 polar flagella that work in conjunction with its spiral shape to propel the bacterium through the gastric environment [4]. Motility has been shown to be essential for colonization as non-motile variants are unable to establish persistent

infection and are selected against in animal infection models [94-96]. Further, greater motility has been observed in clinical strains isolated from cases of chronic gastritis, peptic ulceration or gastric cancer compared to strains obtained from asymptomatic infections [97], indicating that motility is a significant factor in *H. pylori's* pathogenesis.

Over 40 proteins are involved in flagellum biosynthesis and regulation in *H. pylori* [98]. The overall structure of the *H. pylori* flagellum is similar to the type III flagellar-export apparatus characterized in *E. coli* and *Salmonella* [99, 100]. In general, the flagellar apparatus is composed of three main elements: a basal body, hook, and flagellar filament. The basal body comprises the integral membrane ring structure that forms the foundation of the export channel across the periplasm, as well as the rotor of the flagellum and the motor proteins that mediate the proton-motive force for flagellum rotation [101, 102]. The hook forms a flexible junction between the basal body and the flagellar filament. Mutation of the hook subunit protein in *H. pylori*, FlgE, disrupts motility and production of the flagellar filament [103], which corresponds to the hierarchical flagellum assembly model in most Gram-negative bacteria [104]. Both the hook proteins and the proteins that make up the flagellar filament are secreted through the flagellar apparatus by a type III secretion system and assemble external to the cell. The flagellar filament of *H. pylori* is composed of two structural subunits, flagellin A (FlaA) and flagellin B (FlaB), rather than just a single flagellin species as found in most flagellated bacteria [105]. FlaB is the minor flagellin subunit and assembles adjacent to the flagellar hook. FlaA is the major flagellin subunit and makes up the remainder of the filament structure. The stoichiometric ratio of FlaA and FlaB is approximately ten to one. Inactivation of *flaA* disrupts motility and results in the production of multiple truncated

flagella [106]. Conversely, disruption of *flaB* has no apparent effect on flagellum production but restricts motility by 30-40%. A double knockout mutation of both flagellin genes abolishes *H. pylori* motility and flagellum production. As such, both flagellin structural subunits are required for fully functional flagella and *H. pylori* motility. It is proposed that the complex flagellar filament assembly by two flagellins, rather than one subunit, relates to flagellum strength and may be an adaptation of *H. pylori* to enable its movement through the viscous gastric environment.

In addition, the *H. pylori* flagellar filament is enclosed by a membrane sheath [4]. The sheath forms a terminal bulb at the tip of the flagellum and is an extension of the outer membrane containing both LPS and various outer membrane proteins [107]. The role of the flagellar sheath is incompletely understood, however, it is thought to protect the flagellum from the acidic gastric environment, mask flagellar antigens, or act as an adhesin [108].

The *H. pylori* flagellum is also unique in regards to the genetic organization of its components and transcriptional regulation. Unlike similar flagellar systems, the flagellar genes encoding proteins for flagellum biosynthesis and regulation are primarily unlinked on the chromosome [98, 108-111]. Flagellum biosynthesis is controlled in a hierarchical manner by three RNA polymerase sigma factors, σ^{80}, σ^{54} (RpoN), and σ^{28}. In general, σ^{80} controls housekeeping flagellar genes that encode proteins for chemotaxis and for the basal body and motor components of the flagellum, σ^{54} controls 'middle' flagellar gene expression including the flagellar hook subunits, the minor flagellin FlaB, and the sheath, and σ^{28} controls 'late' flagellar gene expression, including the major flagellin subunit FlaA [111, 112]. The transcriptional regulation of the flagellar genes differs from other

flagellar systems as no master regulator has been identified, however, components of the basal body, FlhA and FlhF, downregulate flagellar gene transcription [112]. As well, the secreted anti- σ^{28} factor, FlgM, well characterized in *Salmonella*, is truncated and lacks a typical N-terminal secretion sequence in *H. pylori*. Recently, it was proposed that FlgM of *H. pylori* exerts its antagonist function on middle flagellar genes in the cytoplasm by interacting with basal body components rather than being secreted [113, 114]. In addition, growth-phase dependent regulation has also been characterized in *H. pylori* for both of the flagellin subunits and the basal body component FlhA [115].

The unique features of the *H. pylori* flagellum also aid in its ability to evade the host immune response. In particular, recognition of FlaA by Toll-like receptor 5 (TLR 5) is dramatically lower than *Salmonella enterica* ser. Typhimurium flagellin, which is demonstrated by approximately 1000-fold less activation of IL-8 production by gastric epithelial cells [116]. The low activation of the innate immune response likely aids in *H. pylori's* ability to cause life-long infection.

Lastly, the flagellin subunits of *H. pylori* are known to be glycosylated with the sugar pseudaminic acid (pse). Glycosylation of both flagellin subunits is required for proper flagellum assembly [117-119]. Disruption of flagellin glycosylation results in *H. pylori* that is non-motile and lack flagella - two components essential to *H. pylori's* colonization and overall pathogenicity. Thus, glycosylation is a key virulence factor of *H. pylori* and is presented in detail in the following section.

1.3 Protein glycosylation

Protein glycosylation is the modification of proteins by the covalent attachment of carbohydrates. It is the most abundant polypeptide chain modification found in nature

[120]. Two dominant forms of protein glycosylation have been characterized - N-linked glycosylation and O-linked glycosylation. N-glycosylation occurs by covalent attachment of the glycan to the amide nitrogen of asparagine residues. Comparatively, O-glycosylation occurs by the attachment of the glycan to the hydroxyl group of serine or threonine residues. Other forms of glycosylation have been reported, including C-C linkage of tryptophan residues, but are rare.

Initially, protein glycosylation was believed to be restricted to eukaryotes, however, the phenomenon is now firmly established among all domains of life and is known to mediate a variety of functions. In general, protein glycosylation is believed to provide the functional diversity required to regulate cellular processes and mediate the interaction of organisms with varying environments [121].

1.3.1 Protein glycosylation in eukaryotes

Protein glycosylation was first reported in eukaryotes [122] and is now predicted to modify two-thirds of eukaryotic proteins [123]. The role of protein glycosylation in eukaryotes spans a variety of functions, which include mediating the immune response, intracellular trafficking, intercellular recognition, protein folding, and protein stability [121].

N-glycosylation in eukaryotes is a co-translational process that occurs at the consensus sequence Asn-X-Ser/Thr (where X represents any amino acid except proline) of newly synthesized polypeptide chains in the lumen of the endoplasmic reticulum (ER) (reviewed in [124]). In general, eukaryotic N-glycosylation occurs "en bloc", in which the glycan is preassembled and attached to the protein target as a unit by a dedicated glycosylation pathway.

The eukaryotic N-glycosylation pathway begins with the assembly of the glycan on the cytoplasmic side of the ER membrane on a dolichol pyrophosphate lipid carrier. The assembled glycan is then translocated to the ER lumen by an ATP-independent "flippase" protein. The remaining biosynthetic steps occur in the ER lumen, producing a conserved tetradecasaccharide ($Glc_3Man_9GlcNAc_2$) core oligosaccharide, which is subsequently transferred to the target protein by a conserved oligosaccharyltransferase (OTase) [125]. The eukaryotic OTase is a multimeric, membrane-associated enzyme, composed of at least eight protein subunits [126]. Of the subunits, Sttp3, is highly conserved, being found in all eukaryotic organisms, and is involved in the catalytic activity of the OTase complex [127]. In fact, the OTase responsible for bacterial N-glycosylation in *C. jejuni*, PglB, is homologous to Sttp3 [128] and suggests that components of protein glycosylation systems are highly conserved. After transfer to the target protein, the oligosaccharide becomes further modified in the ER and during transport through the Golgi apparatus by the action of various glycosidases and glycosyltransferases [129].

In comparison, the mechanism of O-linked glycosylation in eukaryotes is more variable. No dedicated O-linked glycosylation pathway has been identified and no consensus sequence for O-glycosylation sites has been observed, such that O-linked glycosylation may occur on any exposed serine or threonine residue (reviewed in [130]). While O-linked glycosylation in eukaryotes occurs predominantly in the Golgi apparatus during protein maturation, O-glycosylation has also been observed in the ER.

In general, eukaryotic O-glycosylation is initiated by addition of a sugar from a nucleotide-activated sugar, most commonly N-acetyl-galactosamine (GalNAc), by a

specific glycosyltransferase, followed by sequential addition and elongation of the sugar chain by the transfer of additional sugars, such as Gal, GlcNAc, Fuc, and N-acetyl-neuraminic acid (NeuNAc), by dedicated glycosyltransferases [130, 131]. In yeast, O-glycosylation is initiated by addition of a single mannose from a lipid-linked carrier in the ER, followed by further modification by the addition of other sugars in the Golgi apparatus [131]. On the whole, O-linked glycans are smaller than the branched oligosaccharide attached during N-glycosylation, but both forms of modifications are similar in that they contribute to diverse roles on a variety of target proteins.

1.3.2 Protein glycosylation in prokaryotes

Protein glycosylation was originally thought to be absent in prokaryotes as they lack the cellular compartmentalization necessary for the eukaryotic protein glycosylation systems [124]. However, since the initial discovery of prokaryotic protein glycosylation on the surface layers (S-layers) of *Halobacterium salinarium* [132], protein glycosylation has been established as a common phenomenon in both Archaea and Bacteria.

In comparison to eukaryotic protein glycosylation that occurs on unfolded polypeptides, prokaryotic protein glycosylation occurs on flexible surface-exposed regions of folded proteins [133]. As well, the sugars involved in glycosylation are more diverse. In particular, bacterial glycosylation systems make use of amino- and deoxy-sugars that are not found in eukaryotes [134]. As such, prokaryotic sugar biosynthesis pathways may provide unique targets for therapeutic development.

Similar to eukaryotic glycosylation, no general function has been associated with prokaryotic glycosylation, however, unlike eukaryotic glycosylation, where the function of many glycoproteins has been studied in-depth, the roles of prokaryotic glycosylation

remain largely uncharacterized. Regardless, prokaryotic glycosylation has been reported to have a diverse array of functions, including roles in protein stability and resistance to denaturation [135], cell competence and cellular localization [136], host colonization [137, 138], and modulation of the immune response [139]. Most relevant to this study is the involvement of glycoproteins in bacterial virulence, which has been widely observed among several bacterial pathogens [140].

1.3.2.1 Bacterial N-linked glycosylation

Bacterial N-linked glycosylation is exemplified by the system characterized in *C. jejuni.* To date, *C. jejuni* is the only bacterium with both well-characterized N-and O-glycosylation pathways, making it a model organism for the study of bacterial protein glycosylation. Based on the pathway initially described in *C. jejuni,* bacterial *N*-glycosylation is similar to the eukaryotic protein glycosylation pathway, in which both pathways are membrane-mediated and involve synthesis of the glycan on a lipid-linked intermediate prior to transfer to the target protein by an OTase.

In *C. jejuni,* over 53 proteins are now predicted to be N-glycosylated with the majority being localized to the periplasm or surface-associated [141-143]. N-glycosylation occurs within an extended consensus sequence compared to that of eukaryotes and archaea, Asp/Glu-X_1-Asn-X_2-Ser/Thr, where X_1 and X_2 are any amino acid except for proline [144]. This sequence is required, but not sufficient for glycosylation [145, 146]. The genes involved in *C. jejuni* N-glycosylation are organized in an operon termed, *pgl,* for protein glycosylation, and include genes *pglA-G* [142] that synthesize the sugars and assemble them into the final heptasaccharide, $GalNAc_2$-[Glc]$GalNAc_3$-diacetamido bacillosamine [143]. The resulting heptasaccharide is

subsequently flipped to the periplasm by an ATP-dependent flippase, PglK, and transferred to an asparagine residue of a target protein by the OTase PglB [142]. Unlike eukaryotic glycosylation, no further modification of the glycan is performed.

1.3.2.1.1 Additional N-glycosylation systems

In addition to the general N-glycosylation pathway described in *C. jejuni,* an N-glycosylation pathway has recently been identified in *H. pullorum* based on identification of a PglB ortholog, the OTase responsible for mediating N-linked glycosylation in *C. jejuni* [147]. The N-linked glycosylation system differs from *C. jejuni* in that a pentasaccharide glycan is produced rather than the heptasaccharide identified in *C. jejuni.* This indicates significant differences in the glycosylation machineries employed. Interestingly, while PglB orthologs were identified in *H. canadensis* and *H. pullorum,* no PglB ortholog was evident in the genomes of *H. pylori, H. acinonychis, H. mustelae,* and *H. hepaticus.*

Moreover, a distinct N-glycosylation pathway has been described in *Haemophilus influenzae* [148-150]. N-glycosylation of *H. influenzae* occurs by the sequential addition of sugars in the cytoplasm to the target protein rather than "en bloc" transfer of the synthesized glycan in the periplasm.

These large differences in protein glycosylation machineries highlight the diversity and complexity of bacterial protein glycosylation and imply that other protein glycosylation pathways and mechanisms have yet to be explored.

1.3.2.2 Bacterial O-linked glycosylation

O-linked glycosylation has also been described in several bacteria. Most common is the observation of O-glycosylation on surface appendages, such as flagella and pili.

The pilin subunits of *Neisseria spp.* [151, 152] and *Pseudomonas aeruginosa* [153] have been shown to be O-glycosylated. Pilin glycosylation has been implicated in virulence as modification is required for host cell adherence and twitching motility [154, 155]. As well, among other species, the flagellins of *C. jejuni* [156], *H. pylori* [117], and *P. aeruginosa* [157] are also known to be O-glycosylated. Glycosylation of the flagellin structural subunits is known to be required for the proper production of flagella [117, 118, 156], indicating that in addition to virulence characteristics, O-glycosylation is also directly associated with production of bacterial virulence factors.

1.3.2.2.1 The model system: O-glycosylation of the *C. jejuni* flagellins

Flagellin glycosylation was originally identified in *Campylobacter spp.* Glycosylation in *C. jejuni* was demonstrated based on the sensitivity of flagellin sero-specificity to periodate oxidation and their affinity for a sialic acid-specific lectin [158]. A flagellar glycosylation locus was later identified including the genes that encode the flagellins. Structural analysis demonstrated that the flagellins were modified with a derivative of sialic acid, pse, a nine carbon sugar [156]. However, it is now established that the *C. jejuni* flagellins are modified by a variety of sugars, including pse and pse derivatives (PseAcOAc, PseAm, PseAmOGln), as well as an acetamidino derivative of legionaminic acid (LegAm) [156, 159-161]. As well, the extent and variety of flagellin glycosylation appears to vary from strain to strain which demonstrates the heterogeneity in the flagellar glycosylation loci.

The *C. jejuni* flagellins are modified at up to 19 sites, representing up to 10% of protein mass [156, 159, 160]. As such, glycosylation results in an observed ~10 kDa size increase when separated by SDS-PAGE [162]. The glycosylation sites are present along

the central domain of the flagellin subunits and are surface exposed [156], suggesting that glycosylation may be involved in the antigenic variation of the flagellar surface [158].

Although, the mechanism of flagellin O-glycosylation is largely uncharacterized, it is speculated that a glycosyltransferase acts to modify the flagellins at the basal body of the flagellar export apparatus, or during secretion. A recent study indicated that the flagellar glycosylation machinery is localized to the flagellar basal body in the cytoplasm, which supports that glycosylation occurs in the cytoplasm prior to export and that glycosylation is independent of the flagellar regulon as it occurs separate from flagellum assembly [163].

The biological function of flagellin glycosylation remains incompletely understood, such that flagellin glycosylation may be required for flagellin secretion or may mediate protein interactions [163]. Nevertheless, *Campylobacter* flagellin glycosylation is known to be required for proper flagellum assembly in which mutations of pse biosynthetic genes result in *C. jejuni* that is non-motile [162]. As such, glycosylation plays a key role in *C. jejuni* virulence as motility is well characterized as an essential component for *C. jejuni* colonization in human hosts [164].

1.3.2.2.2 General O-glycosylation systems

Until recently, O-glycosylation was thought to be restricted to single protein targets, such as the flagellins. However, general O-glycosylation systems have now been identified. These pathways are similar to the N-glycosylation pathway in which various proteins are modified with a specific glycan after sugar assembly on lipid carriers by the action of an OTase.

The general O-glycosylation system of *Neisseria spp.* is a prime example [165, 166]. Originally, the pilin protein subunit, PilE, of the Type IV pilus of *N. gonorrhoeae* was shown to undergo O-glycosylation at a single serine residue [167]. This glycosylation system was demonstrated to be similar to the N-glycosylation system of *C. jejuni* by interspecies complementation. The two glycosylation systems differed only by the oligosaccharide transfer in which the OTase, PglO, rather than a PglB homolog of the *C. jejuni* system, was required for the transfer of the assembled glycan to the protein target [168]. The presence of a general glycosylation pathway was initially observed by the immunoreactivity of multiple proteins with an antibody raised against glycosylated PilE, which indicated the presence of a shared epitope [166]. All proteins of the *N. gonorrhoeae* O-glycosylation system are believed to be periplasmic or cell surface associated. The identification of periplasmic glycoproteins indicates that O-glycosylation is not limited to surface exposed proteins [166].

Moreover, the O-glycosylation system of *Bacteroides fragilis* has also been implicated in the glycosylation of several extracytoplasmic proteins, including some proteins that are localized to the inner membrane [169, 170]. As well, O-glycosylation in *B. fragilis* has been demonstrated to modify essential proteins, such as those involved in cell division and chromosomal segregation. The *B. fragilis* O-glycosylation system is unique in that a consensus sequence has been identified; Ser/Thr-Ala/Ile/Leu/Met/Thr/Val. It is anticipated that over half of the extracytoplasmic proteins of *B. fragilis* are glycosylated [170].

Overall, the diversity of the O-glycosylation systems identified thus far, including the variety of protein targets based on cellular function and localization, indicate that

glycosylation is much more widespread than previously believed and that protein glycosylation may have significant implications for both essential cellular functions for viability as well as in the production of bacterial virulence factors.

1.3.3 *H. pylori* and protein glycosylation

The flagellins, FlaA and FlaB, are the only glycoproteins fully characterized in *H. pylori.* The first evidence of flagellin glycosylation in *H. pylori* arose from characterization of the *neuA/flmD* gene cluster hypothesized to be involved in protein glycosylation [119]. In this study, the flagellins were chemically labelled with digoxigenin (DIG)-hydrazide, indicating that they were modified via glycosylation. Interestingly, glycosylated forms of the flagellins in the cytoplasm could not be detected, which may indicate that glycosylation of the flagellins is likely coupled to their secretion from the flagellar apparatus [119]. This finding remains to be further characterized.

Purified flagellins were subsequently analyzed by mass spectrometry and the sugar substituent identified as pse, similar to *C. jejuni* [117]. Unlike the flagellins of *C. jejuni,* the *H. pylori* flagellins demonstrated little heterogeneity in sugar content or sugar distribution. This likely reflects the presence of the membrane sheath around the flagellar filament that limits interaction with the surrounding environment. This is in contrast to the *C. jejuni* flagellins, which are decorated by a variety of carbohydrates and are believed to be involved in antigenic recognition [160, 161]. As well, the *H. pylori* flagellins were both shown to be less heavily glycosylated than the *C. jejuni* flagellins. *H. pylori* FlaA was observed to be glycosylated at up to 7 sites and FlaB to be glycosylated at up to 10 sites [117]. Comparatively, the *C. jejuni* flagellins are glycosylated at up to 19 sites, which is reflected in a noticeable molecular mass increase by SDS-PAGE. A similar

molecular mass increase by SDS-PAGE is not observed with the *H. pylori* flagellins [118]. Regardless, similar to *C. jejuni,* the glycosylation sites of the *H. pylori* flagellins are located in the central core of the flagellin subunits. This suggests that flagellin glycosylation in both species may share a similar biological role. Furthermore, a similar set of enzymes for pse biosynthesis in *C. jejuni* has been described for *H. pylori* [171].

1.3.3.1 The requirement for pse biosynthesis in *H. pylori*

Our laboratory and others have demonstrated that mutants of *H. pylori* defective in the enzymes required for pse biosynthesis are non-motile and lack production of proper flagella [117-119, 162]. As motility is a requirement for *H. pylori's* colonization, protein glycosylation can be considered a key virulence factor in *H. pylori's* pathogenesis. Despite the well-established role of flagellin glycosylation in *H. pylori* flagellum production, the exact biological role of glycosylation remains uncharacterized.

The biosynthesis pathway for pse was initially investigated based on mutations of sugar-nucleotide modifying enzymes [117, 119]. In particular, our laboratory demonstrated that FlaA1 exhibits UDP-GlcNAc C_6 dehydratase/C_4 reductase activity [172, 173]. As well, HP0366 was shown to be an aminotransferase and to convert the product of FlaA1 [174]. Knockout mutants of *flaA1* (*hp0840*) and *hp0366* resulted in *H. pylori* that lacked proper flagella, were non-motile, and exhibited altered LPS profiles [118, 174]. Both mutants were successfully complemented *in trans* with restoration of motility and LPS production [118, 174]. The entire pse biosynthesis pathway has since been solved and is known to start with UDP-GlcNAc and result in the generation of an activated form of pse, CMP-pse. The entire pathway has been observed in a "single pot" reaction [171]. FlaA1 and HP0366 perform the first two steps of the pse pathway as

depicted in Figure 2. While the pse biosynthesis machinery has been fully elucidated in *H. pylori,* the mechanism of O-glycosylation remains uncharacterized. However, it is thought to require an unidentified glycosyltransferase which adds pse directly to the flagellins, similar to the eukaryotic O-glycosylation system.

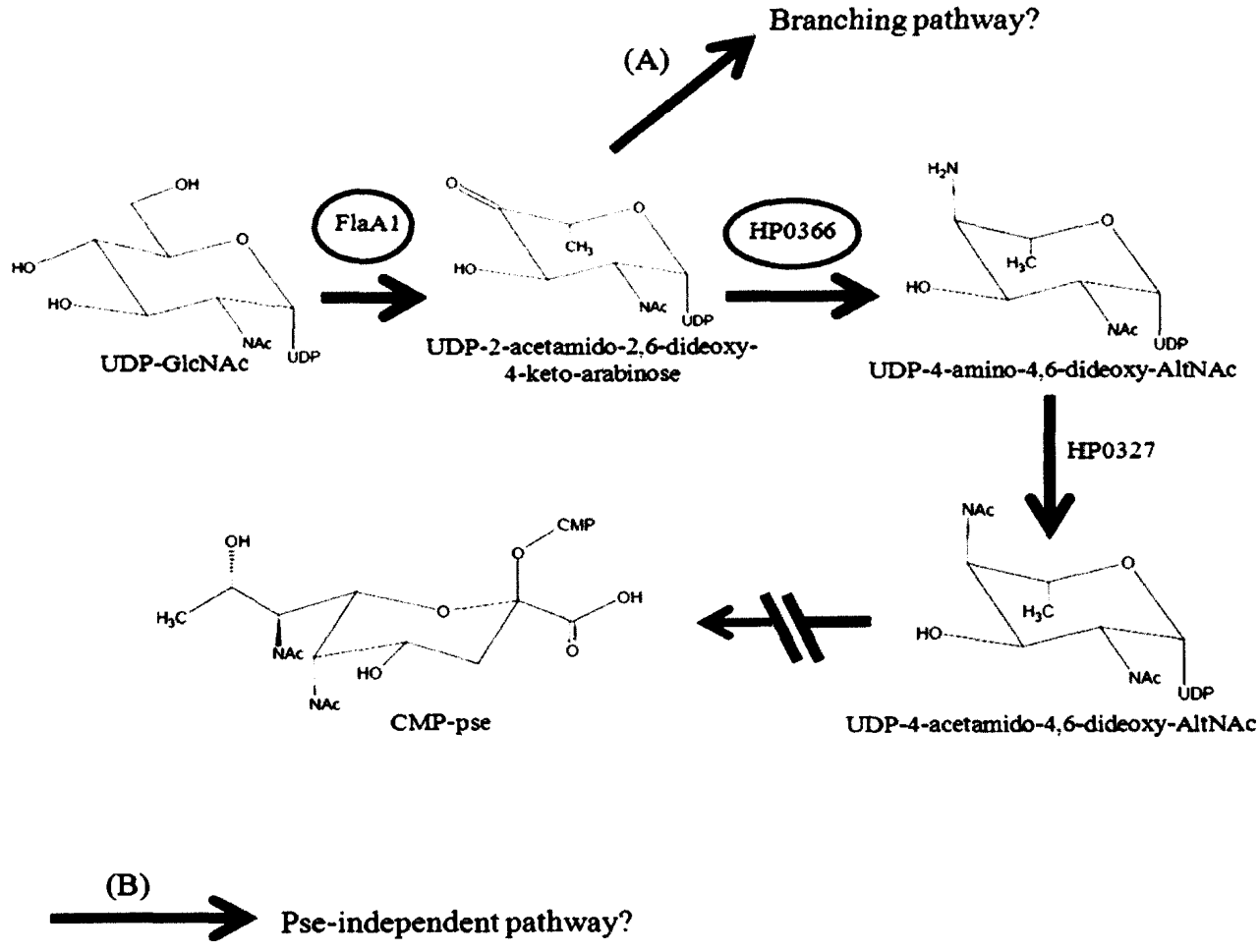
1.3.3.2 Evidence of additional protein glycosylation in *H. pylori*

In *H. pylori,* the only accepted glycoproteins are the flagellins. However, the observation of general glycosylation systems in related species, *C. jejuni* and *H. pullorum* [142, 147], suggest that *H. pylori* may also possess additional glycoproteins.

Upon investigation of the pse biosynthesis pathway, our laboratory has observed the presence of several anomalies that indicate that both additional glycoproteins and additional glycosylation pathways are likely present in *H. pylori.*

Characterization of isogenic mutants of the first two steps in the pse biosynthesis pathway, *flaA1* and *hp0366,* first indicated that glycosylation extends beyond the flagellins and pse glycosylation in *H. pylori.* Both *pse* mutants, *flaA1* and *hp0366,* were shown to be non-motile and lack flagella, as expected since pse biosynthesis is disrupted and pse is known to be required for flagellum production [118, 174]. The flagellins from total cell lysates of both of the *pse* mutants were assessed for glycosylation by chemical labelling with DIG-hydrazide, as used commonly in the field of glycoproteomics [175]. As anticipated, the flagellins of the *flaA1* mutant were non-glycosylated. Comparatively, the flagellins of the *hp0366* mutant appeared glycosylated. This was surprising as pse biosynthesis is disrupted in the mutant [174]. The observation of glycosylated flagellins in the *hp0366* mutant suggests that there exists an alternative glycosylation pathway in *H. pylori,* such that there may be a branch in the pse biosynthesis pathway (Figure 2: A).

Figure 2: Initial steps for pse biosynthesis and the proposed alternative glycosylation pathways in *H. pylori*. The pse biosynthesis pathway in *H. pylori* has been fully elucidated. The first two steps in the pse biosynthesis pathway are mediated by FlaA1, a dehydratase, and HP0366, an aminotransferase, respectively. The HP0366 product is subsequently converted to CMP-pse by the remaining enzymes in the pathway for incorporation on the flagellins. In addition to pse-glycosylation, several lines of evidence indicate the presence of additional glycosylation pathways in *H. pylori*. The demonstration of glycosylated flagellins in the *hp0366* mutant, but not the *flaA1* mutant, suggests that there may be an alternative glycosylation pathway that branches from the pse pathway (A). As well, based on glycosylation of the *hp0366* flagellins and the lack of flagellum production in the *waaL* mutant, a completely pse-independent glycosylation pathway is hypothesized (B).


Branching pathway?
(A)
FlaA1
HP0366
HP0327
UDP-GlcNAc
UDP-2-acetamido-2,6-dideoxy-4-keto-arabinose
UDP-4-amino-4,6-dideoxy-AltNAc
UDP-4-acetamido-4,6-dideoxy-AltNAc
CMP-pse
(B)
Pse-independent pathway?

Despite glycosylation of the *hp0366* flagellins, the mutant was non-motile indicating that the modification of the *hp0366* flagellins is not due to the presence of an additional aminotransferase that can complement the defect in *hp0366* and that the *hp0366* flagellin glycan is distinct from pse [118]. Alternatively, the glycosylation of the *hp0366* flagellins may be due to a separate pse-independent glycosylation pathway in *H. pylori* (Figure 2: B).

In addition to the surprising flagellin glycosylation of *hp0366,* both *pse* mutants displayed altered LPS profiles [118, 174]. In comparison to WT LPS, the LPS of *flaA1* exhibited a reduced O-antigen with an altered serotype, while the LPS of *hp0366* was completely O-antigen deficient. This effect on LPS production by the *pse* mutants was unexpected as pse is not involved in the production of *H. pylori* LPS [69, 70]. As well, this effect on LPS production was not due to joint transcriptional regulation as quantitative real-time PCR (qRT-PCR) data on the *pse* mutants indicated no significant changes in the gene expression of LPS biosynthetic enzymes that would result in decreased production of O-antigen (Creuzenet Lab, unpublished). The observation of a defect in LPS production in the *pse* mutants suggests that other proteins are affected by pse glycosylation. In particular, this finding indicated that LPS biosynthetic enzymes may require pse glycosylation for function [175].

Moreover, the *hp0366* O-antigen deficient LPS profile is reminiscent of a defect in the WaaL, or O-antigen ligase, required for O-antigen biosynthesis. As such, the *H. pylori* WaaL could require glycosylation for function. A *waaL* knockout mutant in *H. pylori* was constructed and characterized [175]. As expected, the *waaL* LPS profile was O-antigen deficient, similar to *hp0366.* Upon characterization of the *waaL* mutant, it was

observed that the mutant was non-motile and lacked production of flagella. This was not anticipated as *waaL* is not involved in flagellar assembly [104]. As such, it was proposed that *waaL* may be required for protein glycosylation [175]. Interestingly, the flagellins of the *waaL* mutant appeared glycosylated by chemical labelling. This suggests that if *waaL* is required for protein glycosylation, it acts separately from the pse glycosylation pathway and modifies other proteins besides the flagellins (Figure 2: B). These proteins may include other components of the flagellar apparatus, which prevent flagellum assembly in the *waaL* mutant.

Furthermore, a recent study conducted by our laboratory examined the presence of additional glycoproteins in the soluble fraction of *H. pylori* and their dependence on pse biosynthesis [176]. In this study, glycoproteins were detected by chemical DIG-labelling. By this method, additional glycoproteins besides the flagellins were observed in the soluble protein fraction of *H. pylori*, indicating additional pse-dependent glycoproteins. Moreover, glycoproteins were also observed in the soluble protein fraction of *flaA1* and *hp0366,* suggesting the presence of pse-independent glycoproteins. This is consistent with a study that utilized a metabolic labelling approach to detect glycoproteins, which also observed the presence of additional glycoproteins in *H. pylori* [177].

In addition to the detection of both pse-dependent and pse-independent soluble glycoproteins in *H. pylori,* sugars were extracted from the protein fractions containing glycoproteins by acid hydrolysis and identified by mass spectrometry (MS). The sugars identified from the glycoprotein fractions included pse, previously identified on the *H.*

pylori flagellins, as well as uncharacterized sugars in *H. pylori* that include pse derivatives, bacillosamine derivatives and a legionaminic acid derivative [176].

Together, these findings indicate that protein glycosylation in *H. pylori* affects proteins other than the flagellins and that multiple glycosylation pathways are likely present. The study presented here, aims to further characterize protein glycosylation in *H. pylori.* In particular, the possibility of glycoproteins in the membrane fraction of *H. pylori* is investigated. As well, the link between protein glycosylation and LPS biosynthesis is further explored.

1.3.4 Similarities between protein glycosylation and LPS biosynthesis

Based on comparison of the biosynthetic pathways leading to the production of glycolipids and glycoproteins, striking similarities can be observed. In many cases, the biosynthesis of LPS and protein glycosylation pathways involve homologous enzymes, which suggest a possible evolutionary connection [178].

The similarity of the two pathways can be appreciated by the number of reports which inaccurately identify components of LPS biosynthesis for protein glycosylation and vice versa. For instance, the *pgl* locus for N-glycosylation in *C. jejuni* was originally identified and proposed to be required for the biosynthesis of lipooligosaccharide (LOS), a derivative of LPS which lacks the repeating O-antigen units [179].

In particular, both LPS biosynthesis and "en bloc" N- or O- glycosylation are membrane-mediated events which are preceded by assembly of the oligosaccharide units on an undecaprenyl lipid carrier. This is similar to the N-glycosylation pathway in eukaryotes in which the glycan is assembled on a dolichol lipid carrier in the ER membrane. As well, assembly of the oligosaccharide is initiated by similar

glycosyltransferases and in some cases the same initiating enzyme is utilized for both pathways [178]. For example, *P. aeruginosa* employs the WecA homolog, WbpL, for both LPS synthesis and pilin glycosylation [180] and the glycosyltransferase, WbpO, for both LPS synthesis and flagellin glycosylation [181]. Moreover, some *P. aeruginosa* pilin glycans have been shown to share structural similarity to that of its O-antigen [153]. As well, the UDP-GlcNAc/Glc-4 epimerase of *C. jejuni,* Gne, is involved in the production of both the N-linked heptasaccharide for protein glycosylation and LOS [182].

In addition, both "en bloc" bacterial glycosylation and O-antigen biosynthesis pathways require the translocation of the lipid-linked intermediate oligosaccharides to the periplasm. Interestingly, as described, the flippase responsible for the translocation of the O-antigen chain in *H. pylori,* Wzk, is a homolog of the translocase involved in *C. jejuni* N-glycosylation, PglK [93]. Furthermore, the *H. influenzae* N-glycosylation pathway was recently shown to involve the transfer of sugar precursors previously implicated in LOS biosynthesis [149].

Lastly, the assembled glycans are attached to their respective cell structures in the periplasm. O-antigen is attached to lipid A by the O-antigen ligase, or WaaL, whereas the substrate for protein glycosylation is attached to serine or threonine residues for O-glycosylation or asparagine residues for N-glycosylation by an OTase. It is believed that WaaL ligases and O-linked OTases are evolutionarily related, whereas the origin of N-linked OTases is likely distinct [178]. However, in all cases, OTase activity is believed to be similar to that of WaaL ligases. In fact, the OTase responsible for N-linked protein glycosylation in *C. jejuni,* PglB, was shown to be able to use O-antigen as a substrate [183]. Interestingly, a Wzy_C motif characteristic of WaaL ligases is also present in O-

linked OTases, including PglL of *N. meningitidis* and PilO of *P. aeruginosa,* that mediate O-linked pilin glycosylation [153, 184]. Indeed, the WaaL of *H. pylori* exhibits the same degree of sequence similarity to PglL as it does with known WaaLs from other species [175]. Further, WaaL ligase activity appears to be independent of the substrate glycan structure, suggesting the potential for WaaL to act as a ligase for a variety of substrates [67]. In fact, it has been suggested that glycosylation of the adhesin EmaA in *Aggregatibacter actinomycetemcomitans* is mediated by WaaL [185]. This may be similar to the phenomenon observed by our laboratory, in which is it hypothesized that the *H. pylori* WaaL may act as a general glycosyltransferase and modify proteins involved in flagellar biosynthesis [175].

In general, the similarity of protein glycosylation pathways and LPS biosynthesis in bacteria suggest that further interconnections are likely present. As such, it is anticipated that with the increasing reports of bacterial protein glycosylation, further involvement and overlap with the LPS biosynthesis machinery will be observed.

1.3.5 Methods to detect glycoproteins

Several methods have been developed for the study of glycoproteins. However, the heterogeneity of glycan composition and linkage sites poses many challenges. Furthermore, the strategy employed for glycoprotein characterization depends on the quantity and purity of the sample. In many cases, complementary approaches are required in order to fully characterize both the sugar composition of the glycan and the linkage site [186]. As well, many of these methods originated from characterization of eukaryotic glycoproteins in which the core structure and sugar components are fairly conserved. In

the case of prokaryotic glycosylation, many glycans are composed of unique sugars, which cannot be identified via the same methods [187].

Initial steps in identifying and characterizing glycoproteins typically begins with separation of samples by 1D SDS-PAGE or 2-dimensional gel electrophoresis (2-DE) [186]. By 1D SDS-PAGE separation, glycoprotein bands are often diffuse due to heterogeneous glycosylation and the presence of multiple glycoforms or exhibit aberrant migration. Similarly, by 2-DE, multiple protein spots are evident in a diagonal pattern representing various glycoforms of differing molecular mass and isoelectric point [188]. However, in many cases, glycoproteins, especially those that are membrane associated, are poorly represented by 1D SDS-PAGE or 2-DE analysis due to poor solubility or, in the case of the latter, difficulties with isoelectric focusing [186]. Thus, several additional methods are utilized to enrich samples in glycoproteins and aid in their detection and characterization.

1.3.5.1 Lectins

Lectins are non-enzymatic proteins that bind specifically to mono- or oligo-saccharides. In many cases their binding specificity has been defined. Thus, lectins serve as a valuable tool to detect, purify, and assess the presence and structure of glycoproteins [189]. As such, panels of lectins with known specificities have been used to detect the presence of specific sugar epitopes [190]. Furthermore, the development of lectin affinity chromatography matrices has allowed purification of glycoprotein pools by specific lectin binding [186, 191]. As well, the availability of lectin arrays allows for qualitative and quantitative glycoprotein glycan profiling [192]. Nevertheless, this form of analysis typically requires an *a priori* knowledge of the type of glycan expected. This information

is not always readily available or predictable, especially in the case of bacterial glycoproteins in which unusual sugars are the norm.

1.3.5.2 Enzymatic and chemical release of glycans

Removal of intact glycans for MS analysis can be facilitated by enzymatic cleavage by glycosidases. The effectiveness of this approach is based on the substrate specificity of the enzyme. As such, a prior knowledge of the type of sugar linkage is required, as not all glycans can be released [186]. Enzymatic release of N-glycans is most common as a greater variety of enzymes have been identified with varying substrate specificity. The most commonly used enzyme is the amidase peptide N-glycosidase F, PNGase F, which cleaves the linkage between GlcNAc and asparagine and allows for the broad removal of N-linked sugars [193, 194]. Conversely, the enzymes available to cleave O-linked glycans are much more limited in substrate specificity. Thus, alternative methods are generally used to investigate O-glycosylation. Enzymatic release can be used in conjunction with reverse phase (RP) high performance liquid chromatography (HPLC) [186]. This strategy allows the carbohydrate content to be estimated based on differences in the elution profiles before and after enzymatic treatment.

Alternatively, chemical release of glycans can be employed. Hydrazinolysis preferentially releases O-linked glycans but conditions can be adjusted for the release of N-linked glycans as well [186, 194]. However, in the case of large O-linked glycan structures, the oligosaccharide can be sequentially degraded resulting in the release of monosaccharide units, which complicates analysis. O-linked glycans are also commonly released by β-elimination in alkaline conditions [194, 195]. However, chemical removal of glycans can result in degradation.

As well, acid hydrolysis can be employed to non-specifically cleave monosaccharides for compositional analysis of the glycan of interest [196]. Conversely, treatment of glycoproteins with trifluoromethanesulfonic (TFMS) acid can be used to degrade the attached glycan, leaving the protein component intact for MS analysis [197].

1.3.5.3 Chemical labelling strategies

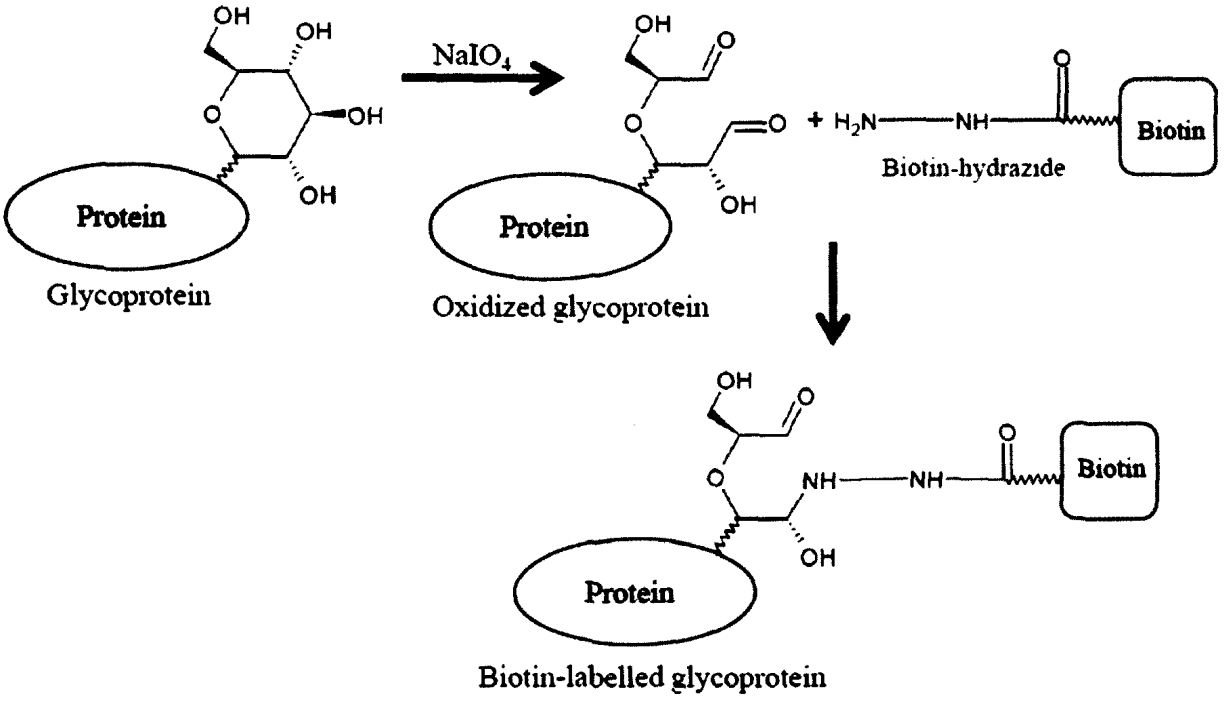
In order to enhance detection and subsequent analysis of glycoproteins, several glycoprotein-specific chemical labelling strategies have been developed based on periodic acid-Schiff staining (PAS) [198, 199]. This strategy enables the introduction of a detectable label after mild oxidation of the glycan and has been employed for glycoprotein samples in-solution or after immobilization on a membrane support [200]. Briefly, samples containing glycoproteins are treated by mild oxidation with sodium periodate or periodic acid. Mild oxidation converts vicinal diols present on carbohydrates to reactive aldehyde groups that specifically react with hydrazide groups at basic pH to form a hydrazone linkage and allow incorporation of a specific tag. A variety of tags have been developed for this purpose, including hydrazide-linked digoxigenin (DIG) that can be detected by an anti-DIG Western blot, chromophores and fluorescent probes [201-204]. For the purpose of glycoprotein detection in this study, a hydrazide-linked derivative of biotin was utilized that can be detected by streptavidin (Figure 3).

1.3.5.4 Metabolic labelling

Metabolic labelling strategies have also been developed to detect cellular glycoproteins. Compared to chemical labelling strategies that are conducted after cell lysis, metabolic labelling incorporates the label during growth via supplementation of growth media with unnatural sugars [205]. Unnatural sugar monosaccharides have been

Figure 3: Glycoprotein-specific chemical labelling with biotin-hydrazide.

Glycoproteins can be specifically labelled in-solution or immobilized on a nitrocellulose support by a modification of periodic acid Schiff (PAS)-staining. By this method, glycoproteins are chemically labelled and detected by the incorporation of a specific tag, such as biotin. Samples containing glycoproteins are treated by mild oxidation with sodium periodate. Mild oxidation converts vicinal diols present on sugar glycans to reactive aldehyde groups that specifically react with hydrazide groups at basic pH to form a hydrazone linkage. This allows for specific incorporation of the biotin tag. Biotin-labelled glycoproteins can subsequently be detected by interaction with a fluorescent conjugate of streptavidin.


OH
OH
OH
OH
O
Protein
Glycoprotein
NaIO4
OH
O
O
OH
Protein
Oxidized glycoprotein
+ H2N
NH
Biotin
Biotin-hydrazide
OH
O
NH
NH
Biotin
OH
Protein
Biotin-labelled glycoprotein

developed containing ketone, azide or thiol groups. During growth, these unnatural sugars are converted to nucleotide-activated sugar analogs that enter normal biosynthetic pathways, which ultimately lead to their incorporation in glycoproteins [206]. The tag can then be selectively detected by Western blot or other methods. This technique provides the advantage of being able to track glycosylation over time in live cells. However, the unnatural sugar derivatives can also be incorporated into other cell structures [206], which can interfere with the interpretation of results. This system was originally optimized for eukaryote cells, however, metabolic labelling with azido-GlcNAc has since been used to assess the glycoprotein content in *H. pylori* [177]. While the results of this study indicate that several additional glycoproteins besides the flagellins are present in *H. pylori*, the validity of the technique in bacteria and the specific labelling of glycoproteins remains to be established.

1.3.5.5 Mass spectrometry analyses of glycopeptides and sugars

Most often enzymatic analysis and labelling strategies only provide preliminary evidence of the presence of glycoproteins, the linkage site, and the glycan structure [186]. For complete characterization of the glycosylation site and the identification of the glycan, analysis by mass spectrometry (MS) is required. In short, MS is an analytical technique that separates sample components after ionization based on their mass to charge ratio (m/z). MS has been particular useful in the characterization of bacterial glycosylation in which the sugars are often unknown [187]. However, in general, MS analysis of glycoproteins poses many more challenges than non-glycosylated proteins [186]. Firstly, glycopeptides are poorly abundant relative to non-glycosylated peptides. This is further compounded by the existence of multiple glycoforms which results in

lower signal intensities and less efficient ionization of glycosylated peptides. As well, MS analysis relies on the digestion of proteins into peptides. Glycosylation can hinder protein digestion by masking accessibility to proteinase cleavage sites, thereby limiting peptide coverage of glycoproteins and preventing the detection of glycosylated peptides.

In order to enhance the detection of glycoproteins, previous enrichment strategies are often required. If the carbohydrate moiety is known, purification methods such as lectin affinity chromatography can be used. As well, for oligosaccharide analysis, initial separation of glycans by capillary electrophoresis (CE) or HPLC is beneficial to enhance MS sensitivity [207]. However, in the case of novel bacterial glycosylation in which the glycan is typically unknown, crude separation techniques can often be used to enrich samples.

Furthermore, recent improvements in MS instrumentation have allowed for analysis of complex glycoprotein samples. Briefly, target glycoproteins are separated by 1D SDS-PAGE or 2-DE and the respective protein band or spot is excised. Glycoproteins can subsequently be analyzed by "top down" or "bottom up" MS strategies [208]. In a top down approach to analysis, intact glycoproteins are assessed by electrospray ionization (ESI)-MS. Low range masses often correspond to glycan ions that fragment from the glycoprotein backbone. Comparatively, a bottom up MS approach requires glycoproteins to be enzymatically digested into peptides and analyzed by liquid chromatography-matrix assisted laser desorption ionization with time of flight analysis, LC-MALDI-TOF MS, and/or by ESI-MS. By this approach, masses are initially separated by LC. The peptide masses can be identified to determine the glycoprotein candidate. Further, masses can be selected for collision-induced dissociation (CID) and MS/MS analysis, which provides a

further fragmentation pattern. In general, glycosidic bonds are weaker than peptide bonds and fragment more easily allowing glycan sequence information to be obtained by MS/MS analysis [187]. The MS/MS fragmentation pattern can also decipher the glycan attachment site. In many cases, final sugar composition and structure is often obtained by nuclear magnetic resonance (NMR) due to the unusual nature of bacterial glycans [208]. More recently, alternative MS techniques by electron capture dissociation (ECD) and electron transfer dissociation (ETD) have been applied to glycopeptide analysis [207]. Altogether, MS analysis is typically the ultimate proof of glycosylation and can be used to analyze the glycan, the attachment site, and the candidate glycopeptides, if the correct form of analysis is employed.

1.4 Hypothesis and objectives

At the onset of this research, the only glycoproteins fully characterized in *H. pylori* were the flagellins, flagellin A (FlaA) and flagellin B (FlaB). Both flagellins are modified with the sugar pseudaminic acid (pse) and flagellin glycosylation is known to be required for proper flagellum assembly [117-119]. Disruption of pse biosynthesis by knockout mutations of the first two enzymes in the pse biosynthesis pathway, *flaA1* and *hp0366*, resulted in *H. pylori* that lack flagellum and are non-motile, as expected since pse glycosylation is interrupted. While the flagellins of *flaA1* were non-glycosylated, the flagellins of *hp0366* still appeared glycosylated by glycoprotein-specific chemical labelling, despite disruption of pse biosynthesis. This finding suggests that there is an alternative protein glycosylation pathway in *H. pylori*, which may be completely independent of the pse biosynthesis pathway or branch from the pse biosynthesis pathway (Figure 2). Furthermore, this finding indicates a complex biological role for flagellin

glycosylation in which there is a strict requirement for pse for proper flagellum production. The exact role of flagellin glycosylation is unknown, however, the alternatively glycosylated flagellins of the *pse* mutants will provide insight to the role of pse-specific flagellin glycosylation.

In addition, both *pse* mutants displayed altered LPS profiles. This finding was surprising as the sugar products from both FlaA1 and HP0366 are not precursors for LPS biosynthesis. As such, additional glycoproteins besides the flagellins are predicted to be present in *H. pylori.* In relation to LPS, the products of FlaA1 and HP0366 may be precursors for sugars that glycosylate membrane proteins involved in LPS biosynthesis. Moreover, the LPS phenotype of *hp0366* is O-antigen deficient, which is reminiscent of a defect in the O-antigen ligase, WaaL, required for LPS biosynthesis. This suggests that the WaaL of *H. pylori* may require glycosylation by pse or a pse-derivative for proper function.

Furthermore, an apparent link was observed between protein glycosylation and LPS biosynthesis in *H. pylori.* While both FlaA1 and HP0366 are dedicated to the pse biosynthesis pathway and are required for flagellin glycosylation and flagellum assembly, both *pse* mutants displayed unexpected altered LPS profiles, as described above. Similarly, while WaaL, the O-antigen ligase, is conserved in LPS biosynthesis, a knockout mutation of *waaL* in *H. pylori* prevented flagellum assembly. Interestingly, flagellin glycosylation was not altered in the *waaL* mutant suggesting that other components of the flagellar assembly are modified by WaaL. This finding suggests that WaaL may act as a general glycosyltransferase and may modify both lipid A and

proteins. Overall, these discrepancies in established pathways suggest an intimate link between LPS biosynthesis and protein glycosylation in *H. pylori.*

Based on the combined observations of the *pse* and *waaL* mutants, the following hypothesis was proposed: Protein glycosylation in *H. pylori* affects several proteins in addition to the flagellins involving both pse-dependent and pse-independent glycosylation and plays a key role in virulence factor production.

To address this hypothesis, three main objectives were investigated:

(i) To elucidate the novel flagellin glycosylation pathway observed in the *hp0366* mutant.

(ii) To demonstrate the presence of novel membrane-associated glycoproteins in *H. pylori,* including proteins other than the flagellins modified by pse or by other sugars.

(iii) To characterize the link between protein glycosylation and LPS biosynthesis in *H. pylori.*

CHAPTER 2: Materials and Methods

2.1 Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Unless indicated, all *H. pylori* strains were grown on brain heart infusion yeast extract (BHI-YE) agar plates (EMD, BioShop) supplemented with 7.5% horse serum (Gibco), 4.55mM sodium pyruvate (Alfa Aesear), 4μg/mL amphotericin B (Calbiochem), 5μg/mL trimethoprim (Sigma), and 10μg/mL vancomycin (BioBasic). When necessary, selection with 5μg/mL kanamycin (BioBasic), 12μg/mL chloramphenicol (BioBasic) and/or 18μg/mL metronidazole (Biobasic) was applied. All strains were grown under microaerophilic conditions at 37°C with 5% oxygen, 10% carbon dioxide and 90% humidity in a tri-gas incubator (NuAire) or in gas jars with Campygen gas packs (Oxoid) for 36 hour periods until confluent.

All *H. pylori* strains were maintained as freezer stocks at -80°C containing 25% glycerol. Freezer stocks were revived on BHI-YE agar plates and cells were expanded in three stages. After initial revival from freezer stock, bacteria were grown for 36-48 hours and then harvested with a sterile cotton swab, spread to a new plate and grown for an additional 36 hours. The confluent lawn was resuspended in 1mL of BHI broth containing no supplements or antibiotics with a sterile cotton swab and distributed to 5-8 BHI-YE agar plates and grown for 36 hours. These plates were then harvested in 10mL of BHI broth and with sterile cotton swabs were transferred to up to 50 BHI-YE agar plates and grown for a final 36 hour period. The final 50 plates of growth were harvested in 3 separate volumes of 45mL of 0.85% saline in sequence to prevent oversaturation of each resulting suspension and to ensure recovery of the maximum yield of cells. Cells were pelleted by centrifugation at 3,220*xg* for 30 minutes at 4°C in a clinical

Table 1: Description of bacterial strains used in this study

Strain	Description	Reference
Helicobacter pylori		
26695	Sequenced wild-type strain	[209] Lab stock provided by Dr. D.E. Taylor
SS1	Mouse colonization strain	[210]
NCTC 11637	Wild-type strain	[211] Lab stock provided by Dr. S. Logan
flaA1::kan	*flaA1 (hp0840)* gene disrupted in strain NCTC 11637 by a kanamycin resistance cassette	[118]
hp0366::kan	*hp0366* gene disrupted in strain NCTC 11637 by a kanamycin resistance cassette	[174]
waaL::kan	*waaL (hp1039)* gene disrupted in strain NCTC 11637 by a kanamycin resistance cassette	[175]
Escherichia coli		
DH5α	F'Φ80dlacZΔM15 recA1 endA1 gyrA26 thi-1 hsdR17 supE44 relA1 deoRΔ (lacZYA-argF)U169	[212]
JM109	F′ *traD36 proA^{+}B^{+} lacI^{q} Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14^{-} gyrA96 recA1 relA1 endA1 thi hsdR17*	[213]

centrifuge (Eppendorf). The supernatant was discarded and the cell pellets were resuspended in 15mL of 0.85% saline, combined, and spun again at 3,220*xg* for 30 minutes at 4°C. The supernatant was removed and the cell pellet was frozen at -20°C for future protein analysis.

E. coli strains were grown at 37°C in Luria Bertani (LB) Miller broth with agitation for up to 16 hours. Selection with 100μg/mL of ampicillin and 34μg/mL of chloramphenicol was applied when necessary.

2.2 Growth of *H. pylori* in liquid culture and analysis of the secretome

Wild-type and *pse* mutant *H. pylori* strains were grown on BHI-YE agar plates with background and selective antibiotics for 36 hours. After initial growth on solid medium, the cells were harvested in brucella broth (BD). Brucella broth for liquid cultures was prepared with 1% β-cyclodextrin (EMD Millipore) as a protein-free nutrient source [214, 215] with the background antibiotics and supplements used for standard *H. pylori* growth conditions (see Section 2.1). Prepared broth was inoculated with each *H. pylori* suspension to a final OD_{600nm} of 0.2, where OD_{600nm} of 1.0 is equal to approximately $3X10^8$ cells. Broth cultures were grown with gentle agitation at 37°C in microaerophilic conditions supplied by growth in anaerobic jars with Campygen gas packs (Oxoid) for 40 hours. After growth in broth, cells were pelleted by centrifugation at 3,220*xg* for 30 minutes at 4°C. The supernatant was removed and ultracentrifuged at 100,000*xg* for 1 hour at 4°C to pellet membrane blebs. The ultracentrifuged supernatant was removed and concentrated by ammonium sulphate precipitation and membrane bleb pellets were resuspended in 1mL of 50mM ammonium bicarbonate, pH 7.5. Samples were further concentrated by lyophilization.

2.3 Phenol red urease activity assay to monitor cell lysis

In order to assess the degree of cell lysis of *H. pylori* grown in broth, total urease activity and urease activity in cell pellets and cell supernatants were compared. A 2mL sample from each *H. pylori* liquid culture was recovered and normalized to an OD_{600} of 0.2. Two 1mL aliquots were used for analysis; 1mL was centrifuged at 2057*xg* for 10 minutes to pellet cells and separate the supernatant and the other 1mL was used to measure urease activity from total cells. After centrifugation, the supernatants were removed to a new eppendorf tube and cell pellets were resuspended in 1mL of brucella broth, the original sample volume. Thirty microlitres of each sample were added to the wells of a 96-well plate in duplicate. To each sample, 200µL of urease reagent (5mM sodium phosphate pH 7.5, 0.15M NaCl, 0.33M Urea, 0.001% phenol red) were added. Urease activity was detected by measuring the OD_{600nm} over time for 1.5 hours at 120 second intervals at 37°C using a Microplate Reader (BioRad). The pH indicator, phenol red, reflected urease activity as the colour change of phenol red from yellow to red signified conversion of urea to ammonia and carbon dioxide.

2.4 Supernatant concentration by step-wise ammonium sulphate precipitation

The supernatant of each *H. pylori* strain was recovered from ultracentrifugation and split into three 50mL centrifuge tubes in even volumes for concentration. Ammonium sulphate precipitation was performed at 4°C. Ammonium sulphate (J.T Baker) was added to the samples to 40% (w/v) gradually over the course of 30 minutes while being mixed on a nutating platform. After all ammonium sulphate was dissolved, the samples were mixed for an additional 30 minutes and then centrifuged at 18,514*xg* in a clinical centrifuge for 20 minutes. The supernatants were removed to new centrifuge tubes and

ammonium sulphate was gradually added to 60% (w/v) from 40% with continuous mixing on a nutator. The samples were mixed for an additional 30 minutes and then spun at 18,514*xg* for 20 minutes to pellet protein. The supernatants were removed to new centrifuge tubes and ammonium sulphate was gradually added to 80% (w/v) from 60% and mixed overnight. The samples were then centrifuged at 18,514*xg* for 20 minutes to pellet remaining protein. The final supernatants were removed to a new centrifuge tubes and frozen at -20°C. All ammonium sulphate pellets were resuspended in 1mL of 20mM Tris-HCl pH 7.5, lyophilized for further concentration and analyzed by SDS-PAGE (see Section 2.9). Alternatively, samples were dialyzed overnight in binding buffer, 20mM Tris pH 7.5, 50mM NaCl, at 4°C prior to further separation by anion exchange chromatography.

2.5 Supernatant concentration and buffer exchange by ultrafiltration

To limit protein loss by ammonium sulphate precipitation and/or dialysis, ultrafiltration was used for concentration and buffer exchange of cell supernatants prior to anion exchange chromatography into binding buffer (20mM Tris pH 7.5, 50mM NaCl). After ultracentrifugation of cell supernatants, supernatants were processed individually through a pressurized ultrafiltration stirred cell with a polyethersulfone porous membrane disc with a 10 kDa molecular weight cut off (MWCO) (Millipore). Operating pressure was maintained below 70 psi. Diafiltration was performed 5X to remove pepsin and other contaminating proteins present in the broth culture media. The retentate from each sample was subsequently separated by anion exchange chromatography.

2.6 Anion exchange chromatography of cellular supernatants

Supernatants were dialyzed into anion exchange binding buffer by either dialysis or ultrafilatration (see Section 2.4 or Section 2.5), as described. Anion exchange chromatography was performed using the AKTA Explorer fast protein liquid purification system (Amersham) with a 1.04mL AcroSep™ Q Ceramic Hyper D F column (PALL Life Sciences) according to the manufacturer's instructions. The system was run with a constant flow rate of 1mL/min with backpressure no greater than 0.3 mPa. The column was equilibrated with 10 CV (column volumes) of binding buffer before sample injection. Samples were filtered through a 0.45 μm PVDF membrane filter (Millipore) prior to sample loading. The protein samples were injected individually with a syringe into a 10mL super-loop (GE Healthcare). After sample loading, the column was washed with 10 CV of binding buffer to remove all unbound proteins. Bound proteins were eluted over 40 CV of a linear gradient to 100% elution buffer (20mM Tris pH 7.5, 1M NaCl). One millilitre fractions were collected and directly concentrated by 80% ammonium sulphate precipitation. Pellets were resuspended in sodium dodecyl sulphate (SDS) loading buffer and analyzed by 12% SDS-PAGE and anti-FlaA/B Western blot (see Section 2.9 and refer to Table 2). Following purification and between samples, the column was stripped with 10 CV of elution buffer and re-equilibrated in 5 CV of binding buffer. The column was stored in 20% (v/v) ethanol until further use.

2.7 Cell fractionation by differential centrifugation

H. pylori strains were grown on 50 BHI-YE plates, as described (see Section 2.1). After 36 hours of growth, plates were harvested in 0.85% saline and whole cells were pelleted by centrifugation at 4,000*xg* for 30 minutes at 4°C. Pellets were resuspended and

washed in 25mL of 0.85% saline and pelleted at 4,000*xg* for 30 minutes at 4°C again. The final pellet was resuspended in 30-40mL of 0.85% saline to a final OD_{600} of 6-10. The cells were subsequently lysed by passage through a French press pressure cell (GlenMills) four times at 15,000 psi on ice. All centrifugation steps were performed at 4°C. Cellular debris and unlysed cells were pelleted by centrifugation at 5,000*xg* for 30 minutes. Insoluble proteins were removed from the supernantant by centrifugation at 13,000*xg* for 1 hour. The insoluble protein pellet was washed in 50mM sodium phosphate pH 7.2, 0.3M NaCl and pelleted again by centrifugation in a table top microcentrifuge (Eppendorf). Membrane proteins were pelleted by ultracentrifugation (Optima-XL 100K ultracentrifuge, Beckman Coulter, 70-Ti rotor) at 100,000*xg* for 1 hour at 4°C. The supernatant recovered containing soluble protein underwent a second ultracentrifugation spin at 100,000*xg* for 1 hour. The initial membrane pellet was washed in 500µL 50mM sodium phosphate pH 7.2, 0.3M NaCl and pelleted by ultracentrifugation in a micro-ultracentrifuge (Optimax ultracentrifuge, Beckman Coulter, TLA-110 rotor). The supernatant was discarded and the total membrane pellet was used for separation of inner and outer membrane proteins.

2.8 Separation of inner and outer membrane proteins

To solubilize inner membrane proteins, the washed total membrane pellet was resuspended in 500µL of solubilization buffer (50mM sodium phosphate pH 7.2, 0.3M NaCl, with 1% N-lauroylsarcosine (w/v) (Sigma-Aldrich)) and mixed on nutator for 1 hour at room temperature followed by incubation overnight at 4°C on the nutator. Outer membrane proteins were pelleted by ultracentrifugation at 100,000*xg* (Optimax ultracentrifuge, Beckman Coulter, TLA-110 rotor) for 1 hour at 4°C. To further enhance

the separation, the supernatant containing inner membrane proteins was ultracentrifuged at 100,000*xg* for 1 hour at 4°C for an additional three times until no pellet was visible. Similarly, the original outer membrane pellet was resusupended and washed in solubilization buffer, mixed for 1 hour at room temperature, and pelleted again by ultracentrifugation at 100,000*xg* for 1 hour at 4°C. The final outer membrane pellet was resuspended in 50mM sodium phosphate pH 7.2, 0.3M NaCl for protein analysis by SDS-PAGE.

2.9 SDS-PAGE and Western blot analysis

Bacterial protein fractions or protein purification samples were denatured in SDS loading buffer (0.625M Tris pH 6.8, 2% SDS, 2% β-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue) and incubated for 5 minutes at 100°C, unless otherwise indicated. Proteins were separated on 12% SDS polyacrylamide gels by the Laemmli system [216] (Bio-Rad, mini-gel system). Electrophoresis was performed at 12mA. Proteins were visualized by Coomassie blue staining (10% acetic acid, 25% ethanol, 0.001% (w/v) Brilliant Blue R-250), silver nitrate staining (see Section 2.21) or Western blotting (see below).

LPS preparations (see Section 2.20) were loaded directly on 12% SDS-polyacrylamide gels for separation. Carbohydrates were visualized by silver nitrate staining (see Section 2.21) or Western blotting.

The antibodies and Western blot conditions used in this study are summarized in Table 2. Western blotting was performed after electrophoretic transfer of proteins or LPS to nitrocellulose (Bio-Rad). Wet transfer was performed for 90 minutes for membrane protein samples and 60 minutes for all other protein and LPS samples in Tris-Glycine

Table 2: Antibodies and Western blot conditions used in this study

Epitope detected	Blocking buffer	Primary antibody/label and dilution used	Secondary antibody and dilution used	Wavelength detected (nm)
H. pylori flagellins (FlaA and FlaB)	2.5% skim milk	Anti-FlaA/B (rabbit) [118], 1/2500	Anti-rabbit (goat) conjugated to IRDye 800 (Rockland Immunochemicals), 1/5000	800
Biotin	Odyssey blocking buffer (Licor) or 10% Horse serum	BHz-labelled glycoproteins (see Section 2.10)	Streptavidin conjugated to AlexaFluor 680 (Invitrogen), 1µg/mL	700
6XHis-tag	2.5% skim milk	Anti-polyHistidine (mouse) (Sigma), 1/3000	Anti-mouse (goat) conjugated to IRDye 680 (Licor), 1/10000	700
Lewis y	2.5% skim milk	Anti-Lewis y, clone F3 (mouse) (Calbiochem), 1/100	Anti-mouse (goat) conjugated to IRDye 680 (Licor), 1/10000	700
Lewis x	2.5% skim milk	Anti-Lewis x, clone P12 (mouse) (Calbiochem), 1/100	Anti-mouse (goat) conjugated to IRDye 680 (Licor), 1/10000	700

Transfer buffer (192mM glycine, 25mM Tris, 20% methanol, 0.01% SDS) with a constant current of 180mA (Bio-Rad transblot system). After transfer, the membrane was rinsed in distilled water and stained with Ponceau S (0.1% (w/v) Ponceau S (Sigma-Aldrich) in 1% (v/v) acetic acid) to visualize the transfer of proteins to the membrane. The Ponceau S stain was removed from the membrane by two 20mL washes with phosphate buffered saline (PBS) buffer (137mM NaCl, 2.7mM KC1, 8mM Na_2HPO_4, 1.46mM KH_2PO_4, pH 7.2). The membrane was blocked overnight at 4°C followed by an hour at room temperature with gentle shaking on a gel surfer (Dia-med) in 2.5% skim milk, Odyssey Blocking Buffer (Licor), or 10% Horse Serum (Gibco), as appropriate for the antibodies to be used (refer to Table 2). After blocking, all steps were performed at room temperature and with gentle shaking on the gel surfer. The membrane was washed twice in 20mL PBS-Tween-20 (PBS with 0.1% Tween-20) and once in PBS buffer for 5 minutes each and then incubated with primary antibody for 1 hour at room temperature. After incubation, the membrane was washed four times in PBS-Tween-20 and once in PBS for 5 minutes each and then incubated with secondary antibody for 30 minutes in the dark. The membrane was washed in the dark with PBS-Tween-20 and PBS as above. All antibodies were centrifuged at 6,300*xg* for 10minutes prior to use. Proteins or LPS were detected by the Licor Infrared Imaging system at wavelengths of 700nm or 800nm.

2.10 Glycoprotein-specific chemical labelling

2.10.1 In-solution glycoprotein labelling with biotin-hydrazide

Protein samples of approximately 0.1-0.5 μg/μL, as estimated by Coomassie staining of membrane protein fractions, were diluted 1:1 in 200mM sodium acetate pH 5.5, for a total starting volume of 20μL. Carbohydrates were mildly oxidized by

incubation with 10mM sodium periodate (Sigma-Aldrich) for 20 minutes in the dark. The reaction was quenched by addition of 20mM meta-bisulphite and incubation for 5 minutes. One micromole of biotin-hydrazide (BHz) from a 5mM BHz stock solution made in DMF (Sigma-Aldrich) was added to label glycoproteins and incubated for 1 hour. All steps were performed at room temperature with gentle shaking on a nutator. Labelled samples were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane for detection with fluorescent streptavidin. Mock samples were treated in the same fashion with the exception of incubation with DMF rather than with the BHz label.

2.10.2 Labelling of immobilized glycoproteins with biotin-hydrazide

Immediately after transfer of protein samples onto a nitrocellulose membrane, the membrane was washed two times in 20mL of PBS (9mM sodium phosphate pH 7.2, 27mM NaCl) buffer. The membrane was subsequently incubated in the dark with 10mM sodium periodate in 100mM sodium acetate pH5.5, 5mM EDTA, for 20 minutes for mild oxidation of carbohydrates and washed for 5 minutes in 20mL of PBS. The reaction was stopped by incubation with 20mM meta-bisulphite in 100mM sodium acetate pH 5.5, 5mM EDTA for 10 minutes and washed 3 times for 10 minutes each with 20mL of PBS. Glycoproteins were labelled by incubation with 1μM BHz, prepared fresh from a 5mM BHz stock made in DMF, in 100mM sodium acetate pH 5.5, 5mM EDTA for 1 hour at room temperature. The membrane was washed three times for 10 minutes each in 20mL of PBS and rinsed in distilled water. Total protein was stained with Ponceau S. Ponceau S stain was removed from the membrane by a wash in 20mL PBS. All incubation steps for the membrane were conducted with gentle shaking on a nutator. Mock labelling was

performed on a separate membrane in the same manner with the exception of incubation with DMF rather than the BHz label.

2.10.3 Detection of biotin-hydrazide labelled glycoproteins with fluorescent streptavidin

Glycoproteins immobilized on a nitrocellulose membrane, either previously labelled with BHz in-solution or labelled with BHz after transfer to nitrocellulose, were detected by incubation with 1μg/mL streptavidin-AlexaFluor-680 (Molecular Probes), a fluorescent conjugate of streptavidin. The nitrocellulose membrane was blocked overnight at 4°C and for 1 hour at room temperature with gentle shaking in 10% horse serum or Odyssey blocking buffer (Licor). The membrane was subsequently washed two times in 20mL PBS-Tween for 10 minutes each and then incubated for 30 minutes with 1μg/mL streptavidin-AlexaFluor-680 in the dark. After incubation with the fluorescent probe, the membrane was washed 3 times in PBS-Tween for 10 minutes each and once in PBS for 10 minutes. The membrane was scanned on a Licor Odyssey Infra-red scanner at 700nm to detect the fluorescent streptavidin conjugate bound to BHz-labelled glycoproteins.

2.11 In-gel trypsin digestion of proteins for mass spectrometry analysis

Samples for mass spectrometry were prepared according to the procedure outlined by the University of Western Ontario Biological Mass Spectrometry Laboratory (BMSL). Briefly, after separation of proteins by SDS-PAGE and protein staining with Coomassie Blue stain, the protein band of interest was excised from the polyacrylamide gel and cut into $1mm^3$ pieces and combined in a 1.5mL eppendorf tube. The gel pieces were washed with 1 volume of autoclaved and filtered distilled water with occasional vortexing

throughout. The distilled water was removed and the gel pieces were treated three times with 1 volume of 1:1 distilled water and acetonitrile for 15 minutes. The liquid was removed and the gel pieces were incubated with enough acetonitrile to just cover the gel pieces until the gel pieces visibly shrunk in size, appeared white in colour, and stuck together (approximately 5-10 minutes). The gel pieces were subsequently spun down in a microcentrifuge and the acetonitrile was removed. The gel pieces were rehydrated in 1 volume of 0.1M ammonium bicarbonate for 5 minutes. One volume of acetonitrile was added, resulting in a 1:1 mixture of acetonitrile and 0.1M ammonium bicarbonate, and the gel pieces were incubated for an additional 15 minutes. The liquid was removed and the gel pieces were dried in a vacuum centrifuge (Eppendorf) on the organic phase setting. Once completely dry, the gel pieces were rehydrated in 1 volume of 10mM DTT, 0.1M ammonium bicarbonate, and incubated for 45 minutes at 56°C to reduce the protein. The remaining liquid was removed and 1 volume of 55mM iodoacetamide, 0.1M ammonium bicarbonate was added. The gel pieces were incubated in the dark for 30 minutes and then washed with 1 volume of 0.1M ammonium bicarbonate for 5 minutes and 1 volume of 50% acetonitrile in distilled water for 15 minutes. The wash solution was removed and 1 volume of acetonitrile was added and incubated until the gel pieces became white and sticky (approximately 5-10 minutes). The liquid was removed and an additional 0.1M ammonium bicarbonate and acetonitrile washing cycle was performed to remove residual Coomassie stain. The samples were completely dried in a vacuum centrifuge. The gel pieces were rehydrated in 1 volume of trypsin digestion buffer (50mM ammonium bicarbonate, 5mM $CaCl_2$, 10ng/μL trypsin) and incubated for 45 minutes on ice. The gel pieces were spun down in a microcentrifuge and the supernatant

removed. The gel pieces were covered with digestion buffer minus trypsin (50mM ammonium bicarbonate, 5mM $CaCl_2$) and incubated overnight at 37°C. To extract peptides, the gel pieces were spun down in a microcentrifuge and the supernatant removed to a clean 1.5mL eppendorf tube. One volume of 25mM ammonium bicarbonate was added to the gel pieces until they were completely covered. The gel pieces were incubated for 15 minutes with vortexing every 2-3 minutes. One volume of acetonitrile was added to make a 1:1 solution of 0.1M ammonium bicarbonate and acetonitrile and the gel pieces were incubated for an additional 15 minutes. The supernatant was removed and combined with the initial supernatant following overnight digestion. To the gel pieces, 1 volume of 5% formic acid was added and incubated for 15 minutes. The same volume of acteonitrile was added and incubated for an additional 15 minutes. The gel pieces were spun down and the supernatant was combined with the earlier steps. An additional extraction cycle with 5% formic acid and acetonitrile was performed and the supernatant combined. To the pooled supernatant, DTT was added to a final concentration of 1mM. Lastly, the combined supernatant was completely dried in a vacuum centrifuge and submitted to the BMSL facility for mass spectrometry analysis. All steps were performed at room temperature unless otherwise stated.

2.12 Construction of pBKS-UreB::RdxA-CAT for chromosomal integration of *rdxA*

The plasmids and primers used in this study are listed in Table 3 and Table 4, respectively. The strategy used to construct pBKS-UreB::RdxA-CAT is depicted in Figure 4. *H. pylori ureB (hp0072)* was amplified from chromosomal DNA of strain 26695 using primers HP0072P1 and HP0072P2. The resulting PCR product was cloned into pBlueScriptKS by Xuan Bui Thanh using *NotI* and *KpnI*, generating the initial

Table 3: Description of plasmids used in this study

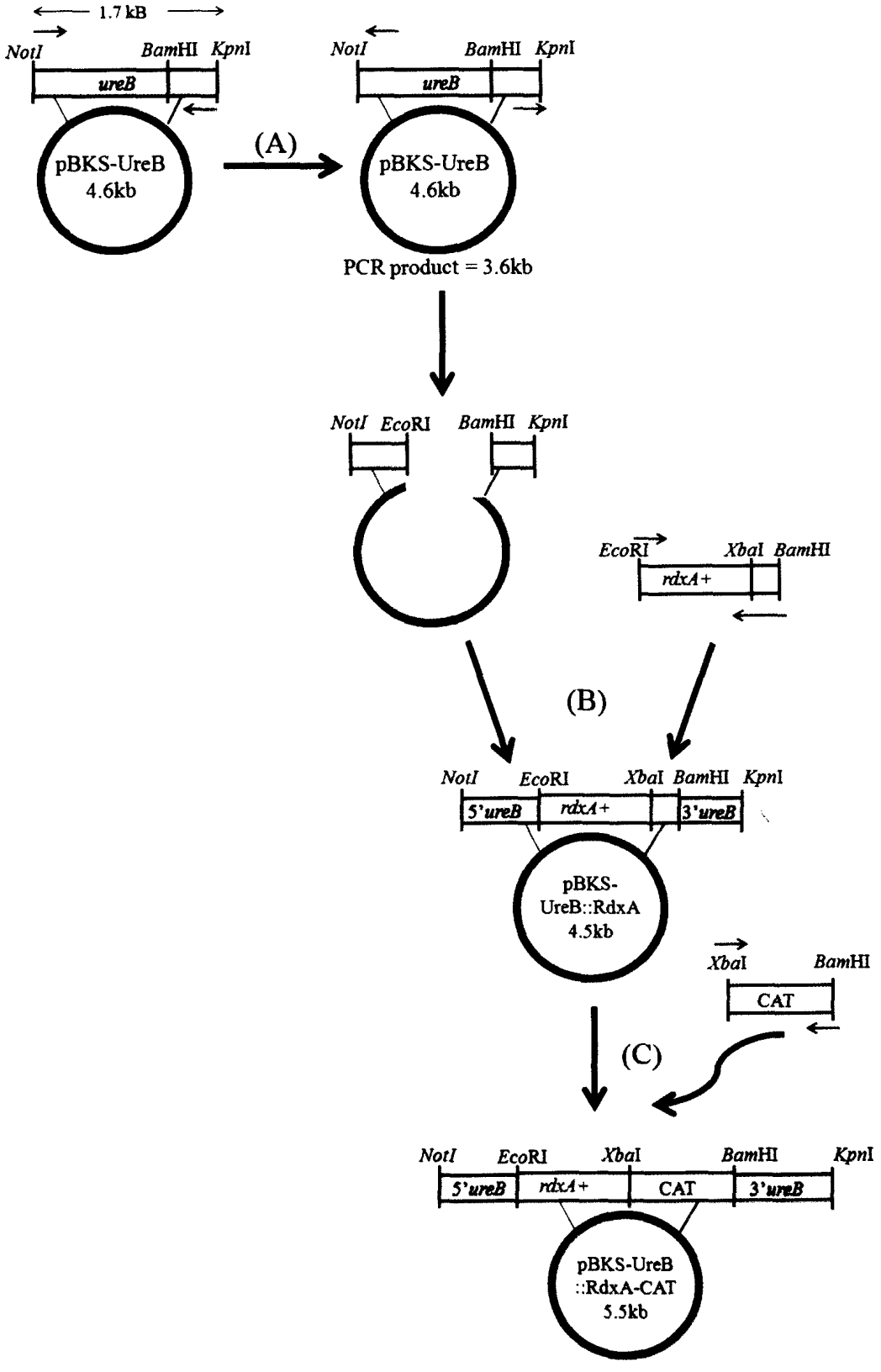
Plasmid	Description	Reference
pProCompWaaL	2.3kb PCR fragment containing *waaL (hp1039)* and upstream promoter region was digested with *Bam*HI and *Kpn*I and cloned into similarly cut pHel2. Cat^R	This Study. Constructed by Dr. Alexandra Merkx-Jacques
pHel2	Shuttle vector containing chloramphenicol resistance cassette. Cat^R	[217]
pET634comp	Derivative of pET21a containing *jhp0634* as an insert with flanking regions of *rdxA*. Used for construction of pRF-1 and pRF-2. Amp^R, confers Mtz^R in *H. pylori.*	Provided by Dr. Trent (University of Texas) [218]
pRF-1	1.75kb PCR fragment containing *flaB (hp0115)* and 166bp upstream of the gene was digested with *Bam*HI and *Eco*RI and cloned into similarly cut pET634comp. Amp^R, confers Mtz^R in *H. pylori.*	This Study
pRF-2	1.66kb PCR fragment containing *flaA (hp0601)* and 164bp upstream of the gene was digested with *Bam*HI and *Nde*I and cloned into similarly cut pRF-1. Amp^R, confers Mtz^R in *H. pylori.*	This Study
pBlueScriptKS	Suicide vector used for construction of pBKS-UreB::RdxA-CAT. Amp^R	Stratagene
pBKS-UreB	1.72kb PCR fragment encoding *ureB (hp0072)* was digested with *Not*I and *Kpn*I and cloned into similarly cut pBlueScriptKS. Amp^R	This Study. Constructed by Xuan Bui Thanh.
pBKS-UreB::RdxA	950bp PCR fragment encoding *rdxA (hp0954)* and 300bp upstream was digested with *Eco*RI and *Bam*HI and cloned into similarly cut 3.55 kb inverse PCR product of pBKS-UreB. Amp^R	This Study
pBKS-UreB::RdxA-CAT	1.07kb PCR fragment containing the chloramphenicol resistance cassette from pHel2 was digested with *Bam*HI and *Xba*I and cloned into similarly cut pBKS-UreB::RdxA. Amp^R, Cat^R	This Study

Table 4: Primers used for this study

Primer Name	Sequence (5' to 3')
HP0072P1	GGAATTC**CATATG**AAAAAGATTAGCAGAAAAGAA
HP0072P2	GG**GGTACC**TAGAAAATGCTAAAGAGTTGC
HP0072P3	CG**GAATTC**AGTAGCAGGACCTACGC
HP0072P4	CGC**GGATCC**CTACCCCACAACCAG
HP0954P1	CG**GAATTC**ATGAGTTGCGTTATCCCAGC
HP0954P2	GC**GGATCC**AG**TCTAGA**CTACAACCAAGTAATCGCATCAAC
CatHelP1	GC**TCTAGA**CTCAAAACGATGAAATAGGGC
CatHelP2	GA**GGATCC**CCGGGTACCG
CatHelP3	CCTTAGCTCCTGAAAATCTCGG
HPFlagBP5	CAG**GGATCC**ATGGAACGCCTTTAATAAATG
HPFlagBP7	CAC**GAATTC**TCAGTGGTGGTGGTGGTGACTTCCGGA**CATAT G**TTGTAAAAGCCTTAAGACATTTTG
HPFlagBP8	GACGGGTGTGGAAGCGAGC
HP0115P1	GCTCTCCATGGGCATGAGTTTTAGGATAAATAC
HPFlagAP5	CAC**GGATCC**TTATAGCCCATTTTCATGCTCC
HPFlagAP6	GGAATTC**CATATG**AGTTAAAAGCCTTAAGATATTTTG
HP0954P3seq	CCCATTAAGCTCACGCCCATG
HP1039P1	CAGGATCCGTTATGGCTTTGAATCTGGAGAAA
HP1039P7	GATTGCAAAAAGCGTTTAAAG
Ctermhis	CGGGATCCTCAGTGGTGGTGGTGGTGG
pHel2CAT1	CATATTGTGTGGAAACACCGC
pHel2CAT2	CACTCATCGCAGTACTGTTG

*Restriction sites are **bolded**

Figure 4: Strategy for construction of pBKS-UreB::RdxA-CAT. Inverse PCR was performed on pBKS-UreB containing the full-length *ureB* gene (A). *rdxA* and 300bp upstream of the gene (*rdxA*+) was amplified from chromosomal DNA of *H. pylori* strain 26695. The PCR products of pBKS-UreB (from inverse PCR) and *rdxA*+ were cut with *Eco*RI and *Bam*HI to generate pBKS-UreB::RdxA (B). pBKS-UreB::RdxA and the CAT cassette amplified from the shuttle vector pHel2 were subsequently cut with *Xba*I and *Bam*HI resulting in the final construct pBKS-UreB::RdxA-CAT (C). Arrows indicate the location of the primers used for each stage of cloning.


1.7 kB
NotI
BamHI
KpnI
ureB
pBKS-UreB
4.6kb
(A)
NotI
BamHI
KpnI
ureB
pBKS-UreB
4.6kb
PCR product = 3.6kb
NotI
EcoRI
BamHI
KpnI
EcoRI
XbaI
BamHI
rdxA+
(B)
NotI
EcoRI
XbaI
BamHI
KpnI
5'ureB
rdxA+
3'ureB
pBKS-
UreB::RdxA
4.5kb
XbaI
BamHI
CAT
(C)
NotI
EcoRI
XbaI
BamHI
KpnI
5'ureB
rdxA+
CAT
3'ureB
pBKS-UreB
::RdxA-CAT
5.5kb

construct, pBKS-UreB, containing the full-length *ureB* gene. pBKS-UreB was verified by PCR amplification of the *ureB* fragment using primers HP0072P1 and HP0072P2 and by restriction digest with *Kpn*I and *Bam*HI (restriction site within *ureB),* which generated an expected fragment of 600bp.

Similarly, *rdxA (hp0954)* and the sequence 300bp upstream, were amplified from chromosomal DNA of strain 26695 using primers HP0954P1 and HP0954P2. HP0954P2 contained a linker sequence with an *Xba*I site to allow insertion of the chloramphenicol resistance cassette (CAT) from the shuttle vector pHel2 [217] at a later stage of cloning.

Inverse PCR amplification of pBKS-UreB was performed with the primers HP0072P3 and HP0072P4 to remove the middle portion (1043bp) of UreB containing the *Bam*HI restriction site. Both *rdxA* and pBKS-UreB (from inverse PCR) PCR products were cut with *Eco*RI and *Bam*HI and allowed to ligate with T4 ligase (NEB) overnight at room temperature to generate the intermediate construct, pBKS-UreB::RdxA.

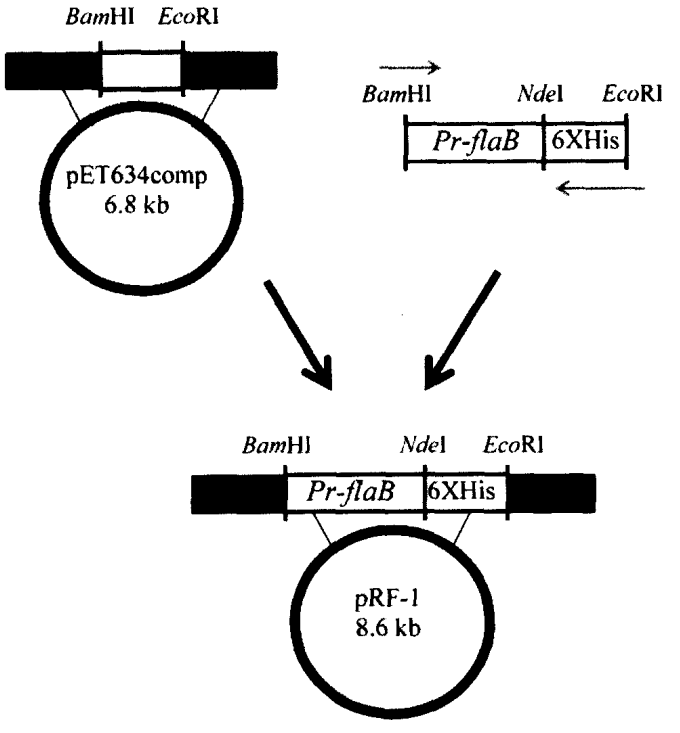
The CAT cassette from the shuttle vector pHel2 [217] was amplified with primers CATHelP1 and CATHelP2. The PCR product was cloned into pBKS-UreB::RdxA via restriction digest with *Xba*I and *Bam*HI resulting in the final construct, pBKS::UreB-RdxA-CAT. All PCR reactions were performed with Expand Long-Range Template polymerase (Roche) with an annealing temperature of 56°C. Sequencing of *rdxA* within pBKS-UreB::RdxA-CAT was performed with primers CatHel3 and HP0954P1.

2.13 Construction of Histidine-tagged flagellin constructs, pRF-1 (FlaB-His) and pRF-2 (FlaA-His), for expression in *H. pylori*

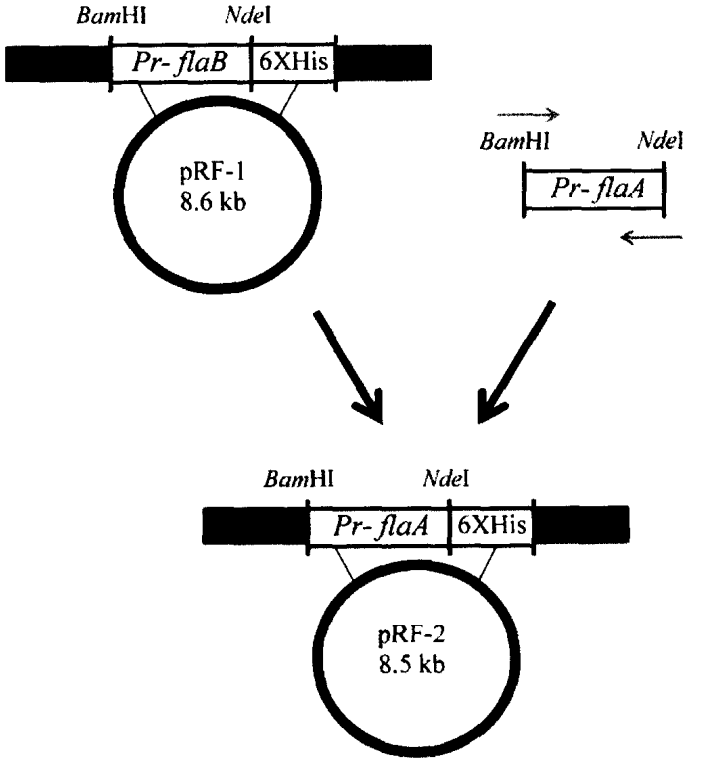
The strategy used to construct pRF-1 and pRF-2 is depicted in Figure 5. Refer to Table 3 and Table 4 for the plasmids and primers utilized. *H. pylori* flagellin genes, *flaA*

Figure 5: Strategy used to construct pRF-1 and pRF-2 for recombinant expression of the *H. pylori* flagellins. The vector pET634comp containing flanking regions of *rdxA* was cut with *Bam*HI and *Eco*RI to remove the previous insert. *flaB* and its promoter (*Pr-flaB)* were PCR amplified from chromosomal DNA of strain 26695 and similarly cut with *Bam*HI and *Eco*RI to generate pRF-1, encoding *Pr-flaB* with a C-terminal hexa-Histidine tag (6XHis) (A). To construct pRF-2, *flaA* and its promoter (*Pr-flaA)* were PCR amplified from chromosomal DNA of strain 26695. Both the *Pr-flaA* PCR product and the previously constructed pRF-1 vector were cut with *Bam*HI and *Nde*I to produce pRF-2 by replacing the *Pr-flaB* insert with *Pr-flaA* (B). Arrows indicate the positions of the primers used.

(A)


BamHI EcoRI
pET634comp
6.8 kb
BamHI NdeI EcoRI
Pr-flaB 6XHis
BamHI NdeI EcoRI
Pr-flaB 6XHis
pRF-1
8.6 kb

(B)


BamHI NdeI
Pr-flaB 6XHis
pRF-1
8.6 kb
BamHI NdeI
Pr-flaA
BamHI NdeI
Pr-flaA 6XHis
pRF-2
8.5 kb

(hp0601) and *flaB (hp0115),* and their respective promoters were amplified from chromosomal DNA of *H. pylori* strain 26695 using primers HPFlagBP5 and HPFlagBP7 for *Pr-flaB,* and HPFlagAP5 and HPFlagAP6 for *Pr-flaA.* A hexa-Histidine tag (6XHis) was encoded within HPFlagBP7 behind an *Nde*I site that enabled sequential cloning of the flagellin constructs and addition of the C-terminal Histidine-tag to both flagellins. The *Pr-flaB* PCR product was cloned into the suicide vector pET634comp [218], provided by Dr. S. Trent (University of Texas), by restriction digest with *Bam*HI and *Eco*RI and overnight ligation, generating pRF-1 *(flaB-His).* pRF-1 was subsequently digested with *Bam*HI and *Nde*I to remove *Pr-flaB*, but leaving the hexa-Histidine tag intact. The *Pr-flaA* PCR product was similarly cut with restriction enzymes *Bam*HI and *Nde*I and cloned into pRF-1 in place of *Pr-flaB*, generating pRF-2 *(flaA-His).* The flagellin constructs were sequenced with HP0115P1 and HPFlagBP8 for pRF-1, and HPFlagAP5 and HPFlagAP6 for pRF-2. Both constructs were also sequenced with HP0954P3 to ensure that the Histidine-tag was in-frame with the gene coding region.

2.14 Transformation of *E. coli* competent cells

Standard transformation by heat shock was used to transform all plasmid DNA into DH5α or JM109 $CaCl_2$-competent *E. coli* cells. Briefly, $CaCl_2$-competent *E. coli* cells were thawed on ice and divided into 150μL aliquots. One microlitre of purified plasmid DNA (~25ng/μL) or 15μL of a ligation mixture was added and mixed gently into the competent cells. The mixture of competent cells and plasmid DNA was incubated on ice for 30 minutes. The cells were heat shocked for 90 seconds at 42°C and then placed on ice for 1 minute. Six-hundred microlitres of LB broth were added to the cells, followed by recovery for 1.5 hours at 37°C in a shaking incubator. After recovery, 50μL

of the culture were plated on LB agar plates supplemented with the appropriate antibiotic selection using glass beads. The remaining cells were pelleted by centrifugation at 7,320*xg* for 2 minutes and the majority of the supernatant removed. The pellet was resuspended in approximately 70-80µL of the remaining supernatant and 50µL of concentrated cells were plated on LB agar plates as described. All plates were incubated at 37°C up to 16 hours. Six-hundred microlitres of LB broth were added to the remaining cells and incubated at 37°C in a shaking incubator to allow recovery overnight. After overnight recovery, the cells were plated on LB agar plates with selection as described and incubated at 37°C for up to 16 hours.

2.15 *In vitro* site-specific methylation of plasmid DNA with *H. pylori* cell-free extract

In order to overcome the restriction-modification barrier of *H. pylori* to transformation, all plasmid DNA was treated with WT *H. pylori* cell-free extract (CFE) as described by Donahue *et al.* 2000 [219] prior to transformation.

2.15.1 *H. pylori* cell-free extract (CFE) preparation

WT *H. pylori* was harvested from 2-5 plates after 36 hours of growth on BHI-YE agar plates as described. Bacterial pellets were resuspended in up to 5mL of extraction buffer (20mM Tris-acetate pH7.9, 50mM potassium acetate, 5mM Na_2EDTA, 1mM DTT) and supplemented with protease inhibitor cocktail (Roche) according to the manufacturer's instructions. For cell suspensions greater than 3mL, cells were lysed by passage through a Mini-French Pressure Cell (GlenMills) at 15,000 psi three or four times until visible clearing of the suspension was observed. For smaller volumes, cells were lysed with acid-washed glass beads; an approximate 50µL volume of neutralized acid-washed glass beads was added to the cell suspension. The mixture was vortexed for

30 seconds, followed by incubation on ice for 1 minute and repeated six times until clearing of the cell lysate was visible. Cellular debris were removed by centrifugation at 13,000*xg* for 15 minutes at 4°C. The supernatant was removed to a new tube. Protein content was assessed by the Bradford protein assay (see Section 2.15.2) and used to treat plasmid DNA.

2.15.2 Determination of protein concentration by Bradford protein assay

Protein concentration of CFE was determined by the Bradford protein assay [220]. A standard curve was generated using serial dilutions of 1.36g/L BSA in extraction buffer. The dilutions were added to a 96-well plate in triplicate. CFE was prepared at three dilutions, 1/25, 1/5, and undiluted, by serial diluting and added to the 96-well plate in triplicate. Protein reagent (Bio-Rad) was added to the samples and OD_{600nm} was read by a microplate reader (Bio-Rad). Based on the standard curve, the protein concentration of the prepared CFE was determined.

2.15.3 Treatment of plasmid DNA with WT *H. pylori* cell-free extract

Eight to twelve micrograms of plasmid DNA was combined with 300-400μg of WT *H. pylori* CFE in a 150μL reaction containing 20mM Tris-acetate pH 7.9, 50mM potassium acetate, 5mM Na_2EDTA, and 1mM DTT. As the methyl donor for the reaction, 200μM of S-adenosyl-methionine (SAM) (BetaPharma Inc.) were added. The reaction was incubated at 37°C for 1 hour. Plasmid DNA was extracted by phenol/chloroform/isoamyl alcohol (25:24:1) extraction. Briefly, 1 volume of phenol/choloroform/isoamyl alchohol (25:24:1) was added to the reaction tube and vortexed until an emulsion formed. The mixture was centrifuged at 13,400*xg* for 1 minute at room temperature and the aqueous phase was removed to a new tube. Extraction was

repeated three times until no protein interface was visible. To remove residual phenol, 1 volume of chloroform was added to the final aqueous phase, vortexed, and the aqueous phase removed to a new tube. Plasmid DNA was recovered by ethanol precipitation; 1/10 volume of 3M sodium acetate pH 7 and 2.5 volumes of anhydrous ethanol were added to the extracted DNA. The sample was mixed 6-10 times by inversion. DNA was allowed to precipitate for 2 hours or overnight at -20°C. After incubation, DNA was pelleted by centrifugation at 13,400*xg* for 30 minutes at 4°C. The supernatant was removed and the pellet was washed with 750µL of 70% ethanol. The final pellet was air dried and then resuspended in 15µL-30µL of autoclaved distilled water.

2.16 Electroporation of *H. pylori*

One plate of actively growing *H. pylori* was resuspended in a 15% glycerol, 9% sucrose solution and pelleted at 5,900*xg* for 2 minutes. The pellet was resuspended and washed three times in 750µL of the 15% glycerol, 9% sucrose solution. The final pellet was resuspended in 200µL of the 15% glycerol, 9% sucrose solution and the volume of the cell suspension was adjusted to a final OD_{600nm} of 2.5. The cell suspension was divided into 70µL aliquots. CFE-treated plasmid DNA (1µg) was mixed gently into the cells. The mixture was transferred to ice-cold 0.2 cm electroporation cuvettes (Bio-Rad). The cells were electroporated at 25 µF, 800 Ω, and 2.5 kV. After electroporation, 100µL of recovery solution (5% Horse serum, 20mM glucose in BHI-YE broth) were added to the cells. The cell suspension (~160-180µL) was divided into three and spotted on BHI-YE agar plates with no antibiotic selection. Cells were allowed to recover for 16-24 hours in standard conditions. After recovery, cells were resuspended in 1mL of BHI-YE broth and plated on BHI-YE agar plates containing appropriate antibiotic selection.

Transformants were screened for the presence of plasmid or chromosomal integration by conventional PCR of the gene of interest.

2.17 Extraction of chromosomal DNA by CTAB

Chromosomal DNA was extracted from total cells using cetyltrimethylammonium bromide (CTAB) buffer. One millilitre of cell suspension at an OD_{600nm} of 4 was centrifuged at 2,000*xg* for 5 minutes to pellet cells. The cell pellet was resuspended in 0.5mL of CTAB buffer and vortexed. To the cell suspension, 0.5mL of phenol/chloroform/isoamyl alcohol (25:24:1) were added and vortexed until an emulsion formed. The lysate was centrifuged at 13,000*xg* for 5 minutes. The aqueous phase was removed and treated with 0.5mL of chloroform-isoamyl alcohol (24:1) to remove residual phenol. The mixture was centrifuged at 13,000*xg* for 5 minutes. The aqueous phase was removed and 0.08 volumes of cold 7.5M ammonium acetate and 0.54 volumes of chilled isopropanol were added to precipitate the DNA. The contents were mixed by inversion 20-30 times and incubated on ice for 30-40 minutes. The precipitated DNA was collected by centrifugation at 13,000*xg* for 10 minutes at 4°C. The DNA pellet was washed once with cold 70% ethanol and air dried. The final DNA pellet was resuspended in 50μL of autoclaved distilled water and stored at 4°C.

2.18 Screening of *H. pylori* transformants by colony PCR

H. pylori transformants were screened by PCR amplification of the gene of interest. Genomic DNA was isolated using the InstaGene Matrix (Bio-Rad) according to the manufacturer's instructions. In brief, the equivalent of the size of a small single colony of each clone, previously patched on BHI-YE agar plates, was suspended in 1mL of autoclaved distilled water and pelleted by centrifugation at 13,400*xg*. The supernatant

was removed and 200μL of InstaGene Matrix was added. The suspension was incubated at 56°C for 15 minutes, vortexed, and boiled at 100°C for 8 minutes. The suspension was vortexed again and the InstaGene Matrix pelleted by centrifugation at 13,400*xg* for 2 minutes. The supernatant, containing genomic DNA, was used as the template for PCR with Expand Long-Range template polymerase (Roche) and the PCR products visualized by separation on a 0.7% agarose gel and imaging using the GelDoc (Bio-Rad). Positive clones were further expanded on BHI-YE agar plates for protein analysis.

2.19 Protein analysis of *H. pylori* clones to verify chromosomal integration

Integration of *rdxA* and disruption of *ureB* in WT and *hp0366 H. pylori* strains was monitored by analyzing the total protein profile of the *H. pylori* clones for the lack of UreB production. Total cell pellets of the *H. pylori* clones were resuspended in SDS-PAGE loading buffer and separated on 12% SDS-PAGE. Total protein was stained with Coomassie Blue.

2.20 LPS sample preparation

LPS samples were prepared via the method described by Marolda *et al.* 2006 [221] with minor modifications for the preparation of *H. pylori* LPS. This protocol is modified from the original LPS sample preparation method [222] as an extra step is incorporated to remove residual protein content from samples. Approximately 1-2 plates of actively growing *H. pylori* were harvested in 1.5mL of 1X PBS. The optical density of the suspension was adjusted to an OD_{600nm} of 4.0. Cells were pelleted by centrifugation at 5,000*xg* for 2 minutes and the supernatant removed. The cell pellet was resuspended in lysis buffer (2% (w/v) SDS, 4% β-mercaptoethanol, 0.5M Tris-HCl pH 6.8) and boiled for 10 minutes. Ten microlitres of 20mg/mL proteinase K was added to the cell lysate and

incubated overnight at 60°C. To remove residual protein and/or peptides, 1 volume of prewarmed (70°C) phenol solution was added to the cell lysate and incubated at 70°C for 15 minutes with vortexing every 5 minutes. The suspension was incubated on ice for 10 minutes and subsequently centrifuged at 10,000*xg* for 10 minutes to separate the aqueous phase. The aqueous phase was transferred to a new tube and 10 volumes of ethyl ether saturated with Tris-EDTA were added and mixed by inversion 10-15 times. The samples were centrifuged at 10,000*xg* for 1 minute and the ether phase (top phase) was discarded by aspiration. Sample loading buffer (4X) for SDS-PAGE was added and the samples were stored at -20°C until needed.

2.21 Detection of LPS and protein by silver nitrate staining

After separation of LPS or protein samples by 12% SDS-PAGE, polyacrylamide gels were stained via a modified ultra-fast silver staining procedure by Fomsgaard *et al.* 1990 [223]. Samples were oxidized in-gel by incubation for 20 minutes in the oxidation solution (0.7% periodic acid, 40% ethanol, 5% acetic acid) followed by 4-5 washes with distilled water for a total of 15 minutes. The gel was subsequently incubated for 10 minutes with freshly prepared staining solution (10% (w/v) silver nitrate, 0.002% NaOH, 0.4% (v/v) NH_4OH). The staining solution was removed and the gel was washed 4-5 times in distilled water for 15 minutes. Developing solution (0.00005% (w/v) citric acid, 0.00054% (v/v) formaldehyde) was added allowing visualization of LPS and protein. The reaction was stopped by removal of the developing solution, addition of 10% acetic acid for 5 minutes, and several washes in distilled water. All staining steps were performed at room temperature with gentle shaking on the GelSurfer. All solutions were made in

freshly cleaned glassware to limit contaminants and solutions were removed by vacuum aspiration.

2.22 Enrichment of WaaL-His by nickel affinity chromatography

H. pylori strains carrying the plasmid pProCompWaaL for WaaL-His expression or pHel2 as a control were grown on 100 BHI-YE agar plates for 36 hours until confluent. To confirm the maintenance of each plasmid throughout each stage of growth (see Section 2.1), 500μL of the cell suspension used to expand cells or from the final cell suspension, were removed and pelleted by centrifugation at 3,220*xg* for 5 minutes. The supernatant was discarded and the resulting pellet was used for plasmid purification. Due to low plasmid yield, plasmid was verified by PCR. Primers specific for WaaL-His, HP1039P1 and Ctermhis, were used to detect pProCompWaaL and primers specific for the CAT cassette, pHel2CAT1 and pHel2CAT2, were used to verify the presence of both pHel2 and pProCompWaaL.

Once plasmid maintenance was verified, cells were harvested in 0.85% saline, lysed in the presence of protease inhibitor cocktail (Roche) according to the manufacturer's instructions, and separated by differential centrifugation as described in Section 2.7. Total membrane samples were further fractionated into inner and outer membrane samples by selective solubilization of inner membrane proteins in 1% N-lauroylsarcosine as outlined in Section 2.8. Inner membrane samples containing WaaL-His were diluted 1:1 in 50mM sodium phosphate pH 7.2, 0.3M NaCl, 5mM imidazole, and 2% Triton, for a final Triton X-100 concentration of 1%. Samples were mixed gently on a nutator at 4^{0}C to allow further solubilization of proteins in the detergent for 1 hour. Gravity flow columns (ThermoFisher) were prepared with 1mL of nickel chelation resin

(Chelating Fast Flow Sepharose; Pharmacia). Columns were loaded with 5 column volumes (CV) of 0.1M nickel sulphate, followed by rinsing of the columns with 10 CV of distilled water, 5 CV of 50mM sodium phosphate pH 7.2, 0.3M NaCl, 5mM imidazole, and 15 CV of binding buffer (50mM sodium phosphate pH 7.2, 0.3M NaCl, 5mM imidazole, 1% Triton). Inner membrane samples were loaded on the columns and the flowthrough loaded two times. Columns loaded with inner membrane samples were incubated at 4^{0}C for 1 hour to allow proteins to bind to the resin. Unbound proteins were removed by sequential washes in 10 CV of binding buffer, 20 CV of wash buffer 1 (50mM sodium phosphate pH 7.2, 0.3M NaCl, 5mM imidazole, 0.2% Triton X-100), 12 CV of wash buffer 2 (50mM sodium phosphate pH 7.2, 0.3M NaCl, 50mM imidazole, 0.2% Triton X-100), and 12 CV of wash buffer 3 (50mM sodium phosphate pH 7.2, 0.3M NaCl, 75mM imidazole, 0.2% Triton X-100). Bound proteins were removed by step-wise elution with increasing concentrations of imidazole; 2 CV each of 50mM sodium phosphate pH 7.2, 0.3M NaCl, 0.2% Triton X-100 with 250mM imidazole, 375mM imidazole, 500mM imidazole, and 1M imidazole. The fractions were screened for the presence of WaaL-His by SDS-PAGE and detection via anti-Histidine Western blot (refer to Table 2).

2.23 Treatment of inner membrane protein samples to disrupt protein aggregates

Aggregate removal was performed by modification of the protocol described by Marani *et al.* 2006 [224]. Inner membrane samples were diluted 1:1 in PBS containing 10M urea. Samples were mixed for 1 hour on a nutator at 4^{0}C. Insoluble protein was removed by ultracentrifugation at 100,000*xg* for 1 hour. Urea treated inner membrane samples were assessed for the presence of WaaL-His by anti-Histidine Western blot

(refer to Table 2). No improvement in WaaL-His migration within SDS-PAGE gels was observed.

2.24 Proteinase K digestion of protein fractions

Samples were digested by sequential treatment with proteinase K to ensure degradation of the majority of the protein present in the samples. Three-hundred microlitres of approximately 0.125μg/μL of each cellular protein fraction, as estimated by Coomassie staining, was split into two equal volumes. The first aliquot was treated with proteinase K to degrade all protein by addition of 5μL of 20mg/mL proteinase K. The same volume of autoclaved distilled water was added to the second aliquot as untreated samples. All samples were incubated overnight (approximately 16 hours) at 60°C for maximum enzymatic activity. To degrade remaining proteins, an additional 2μL of 20mg/mL proteinase K or autoclaved distilled water were added to treated and untreated samples respectively. The samples were incubated for an additional 4 hours at 60°C and stored at -20°C until needed.

2.25 Separation of outer membrane and insoluble protein fractions by 2-DE

Outer membrane or insoluble protein fractions containing 100-250μg of protein, as estimated by Coomassie blue staining, were pelleted by ultracentrifugation at 100,000*xg* or centrifugation at 13,000*xg* for 1 hour at 4°C, respectively. Samples were resuspended in the appropriate volume of rehydration sample buffer (7M urea, 2M thiourea, 4% CHAPS, 30mM Tris-HCl pH 8.8, 0.2% Bio-Lyte 3/10 ampholytes, trace bromophenol blue) for either 7cm or 11cm isoelectric focusing (IEF) strips. Sample was dispensed along the length of the rehydration/equilibration tray and the desired ReadyStrip IPG strip (Bio-Rad) was placed gel-side down on the sample. Initial

rehydration was allowed to proceed for 30 minutes and then 2-3mL of mineral oil was overlayed along the strip. The strip was incubated overnight at room temperature to allow rehydration of the IEF strip and sample loading. After rehydration, the strip was loaded onto the Protean IEF cell (Bio-Rad). IEF focusing was performed for 11-cm ReadyStrip IPG strips with a gradient of pH 4-7 as follows: 250V for 20 minutes, 8,000V for 2.5 hours, and 8,000V until 30,000V-hours were reached. For 7-cm ReadyStrip IPG strips with a non-linear gradient of pH 3-10, IEF focusing was performed as follows: 250V for 20 minutes, 4,000V for 2 hours, and 4,000V until 10,000V-hours were reached. All IEF steps were conducted at 20^0C. After IEF, strips were subjected to disulfide reduction with DTT by incubation for 10 minutes in equilibration buffer I (6M urea, 2%SDS, 0.375M Tris-HCl pH 8.8, 20% glycerol, 2% DTT), followed by cysteine alkylation with iodoacetamide by incubation for 10 minutes in equilibration buffer II (6M urea, 2% SDS, 0.375M Tris-HCl pH 8.8, 20% glycerol, 0.025g/mL iodoacetamide). Proteins were further separated in the second dimension by standard electrophoresis using 12% SDS-PAGE gels. After 2-DE, total protein was visualized by Coomassie Blue staining or samples were transferred to nitrocellulose for detection of proteins by Western blot.

CHAPTER 3: Results

3.1 Elucidation of an alternative pse-independent glycosylation pathway in *H. pylori*

To demonstrate the presence of a novel glycan on the flagellins of the *hp0366* mutant, purification of the flagellins from both WT and *hp0366 H. pylori* strains was required to allow for the analysis of the glycan present on the *hp0366* flagellins and compare it to pse from WT flagellins. Conversely, the flagellins of the *flaA1* mutant were non-glycosylated, therefore, further investigation of their glycosylation was not warranted. However, *flaA1* may provide insight into the biological requirement for flagellin glycosylation as the *flaA1* flagellins represent non-glycosylated flagellin species. Previous studies have described flagellin purification from WT strains of *H. pylori* by mechanical shearing of flagella from cells and acid dissociation of the flagellin subunits [105, 107]. However, both *pse* mutants lack proper flagellum assembly despite flagellin production. As such, various new strategies were explored in order to purify flagellins from different cellular fractions as outlined below.

3.1.1 Disruption of pse glycosylation prevents *H. pylori* flagellin secretion

Both *pse* mutants, *flaA1* and *hp0366,* produce flagellins but are non-motile and lack production of proper flagella. This indicates that disruption of pse-glycosylation interrupts proper flagellum assembly. In general, flagellum assembly in *H. pylori* is thought to occur via the prototypical bacterial type III flagellar protein-export system, in which the structural subunits of the flagellar filament are secreted by an export system homologous to the type III secretion system identified in several Gram-negative bacteria [99, 100]. Briefly, in the cytoplasm, the flagellin subunits associate with specific chaperone proteins that maintain them in their unfolded state and target them to the flagellar basal body at the cytoplasmic membrane. The flagellin subunits are

subsequently secreted via the type III export apparatus, composed of the flagellar basal body and ring structure. This allows the flagellins to transverse the periplasmic space and travel through the hollow core of the growing flagellar filament to the tip, where they polymerize and are incorporated in the flagellar filament [100].

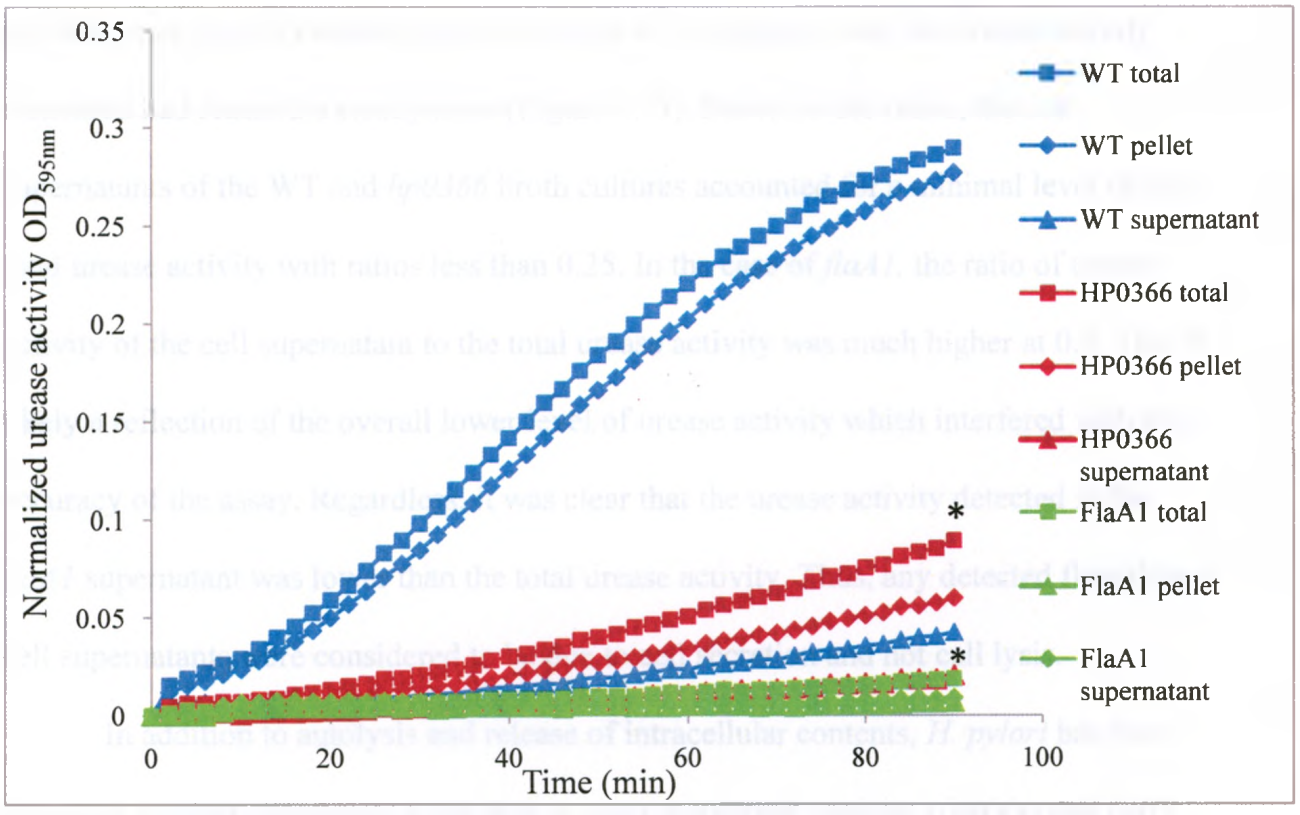
Based on this model of flagellum assembly, it is reasoned that flagellin glycosylation must occur in the cytoplasm, as the flagellins are not exposed to the periplasm during export and glycosylation is unlikely to occur at the tip of the flagellar filament [225]. Furthermore, as the *H. pylori* flagellum is covered by the membranous sheath, it is unlikely that the flagellin subunits interact with extracellular components. Therefore, it was proposed that disruption of flagellin glycosylation by pse could alter production of the flagellar filament in two ways. Firstly, a glycosylation defect could inhibit protein interactions between the flagellin subunits and their cognate chaperones or prevent interaction with the flagellar basal body and export apparatus, which would result in a secretion defect of the flagellins and an accumulation of the flagellins in the cytoplasm. Secondly, a glycosylation defect could alter protein interactions between flagellin subunits and prevent polymerization of the flagellins into the flagellar filament, which would result in the loss of flagellins to the cell supernatant by secretion from the flagellar export apparatus. Since the biological role of flagellin glycosylation is unknown, both scenarios are possible. Nevertheless, a defect in flagellin polymerization would be advantageous for the purification of the *hp0366* flagellins for future glycan analysis since secreted flagellins are predicted to be modified as glycosylation is believed to occur in the cytoplasm prior to secretion. Both the flagellins of *flaA1* and *hp0366* were

investigated to assess the effect of non-glycosylated flagellins and alternatively glycosylated flagellins on flagellum assembly.

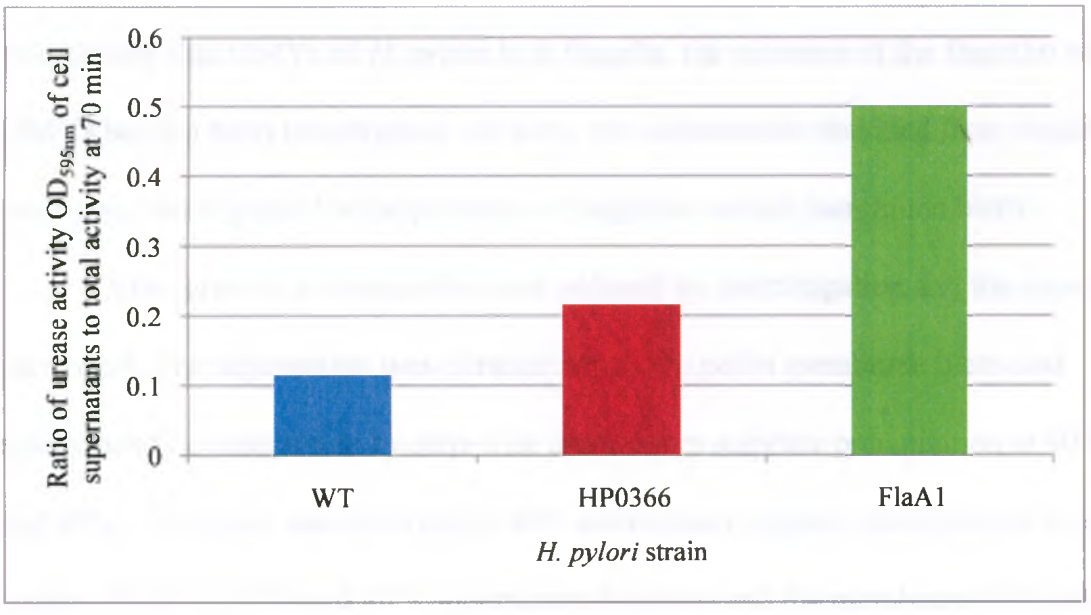
To investigate whether pse-glycosylation affects flagellin polymerization or secretion, WT, *flaA1,* and *hp0366 H. pylori* strains were grown in broth and the supernatants were assessed for the presence of flagellins via anti-FlaA/B Western blots. Protein-free media was used for this purpose as standard growth medium is supplemented with serum, which contains various proteins that are difficult to remove from the extracellular proteins of *H. pylori* [226]. As well, growth of *H. pylori* in broth required specific conditions, as when growth conditions are not optimal, *H. pylori* is known to turn into a coccoid viable, non-culturable form, or undergo autolysis. This would result in the release of intracellular content [50, 215, 227, 228]. To ensure that any detection of the flagellins in the supernatant was due to flagellar secretion and not cell lysis, urease activity was measured to monitor cell lysis. Urease converts urea to ammonia and carbon dioxide and is known to largely reside in the cytoplasm [45, 229, 230]. Therefore, by measuring urease activity, the degree of cell lysis could be inferred. It should be noted that the total urease activity of *flaA1* and *hp0366* is statistically different from WT as characterized previously [118, 174, 175]. However, for the purpose of assessing cell lysis within a particular *H. pylori* strain, the total urease activity of each strain can be compared to the urease activity detected in the cell pellets versus the supernatant. By this method, the majority of urease activity detected over time from total cell suspensions was found in the cell pellets, which suggests a low degree of cell lysis (Figure 6: A). As urease activity increases over time due to the conversion of urea to ammonia and carbon

Figure 6: Measurement of urease activity in total cultures, cell pellets, and cell supernatants to assess the degree of cell lysis after growth of WT, *flaA1*, and *hp0366* *H. pylori* in broth. Cell lysis was monitored by detection of urease, primarily a cytoplasmic protein, in the total cultures, and separated cell supernatants and cell pellets of WT, *flaA1*, and *hp0366 H. pylori* strains grown in broth. Urease activity was assessed by the phenol red assay which detects changes in pH by OD_{595nm} over time (A). Change in pH reflects the conversion of urea to ammonia and carbon dioxide by urease. Values represent the average urease activity from samples evalulated in duplicate for a single growth period. The total urease activity of WT is statistically different from *flaA1* and *hp0366* (* indicates $p < 0.05$). As the urease activity detected by this method increases over time, a ratio of the urease activity from cell supernatants compared to total urease activity was determined at 70 minutes once a steady-state had been reached (B). The minimal level of urease activity detected in culture supernatants compared to the total urease activity for all *H. pylori* strains indicates low levels of cell lysis.

(A)


Normalized urease activity OD_{595nm}
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
20
40
60
80
100
Time (min)
WT total
WT pellet
WT supernatant
HP0366 total
HP0366 pellet
HP0366 supernatant
FlaA1 total
FlaA1 pellet
FlaA1 supernatant
*
*

(B)


Ratio of urease activity OD_{595nm} of cell supernatants to total activity at 70 min
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
HP0366
FlaA1
H. pylori strain

dioxide, the ratio of urease activity from cell supernatants compared to total urease activity from the cell cultures was determined at 70 minutes, once the urease activity measured had reached a steady-state (Figure 6: B). Based on the ratios, the cell supernatants of the WT and *hp0366* broth cultures accounted for a minimal level of the total urease activity with ratios less than 0.25. In the case of *flaA1,* the ratio of urease activity of the cell supernatant to the total urease activity was much higher at 0.5. This is likely a reflection of the overall lower level of urease activity which interfered with the accuracy of the assay. Regardless, it was clear that the urease activity detected in the *flaA1* supernatant was lower than the total urease activity. Thus, any detected flagellins in cell supernatants were considered to be due to cell secretion and not cell lysis.

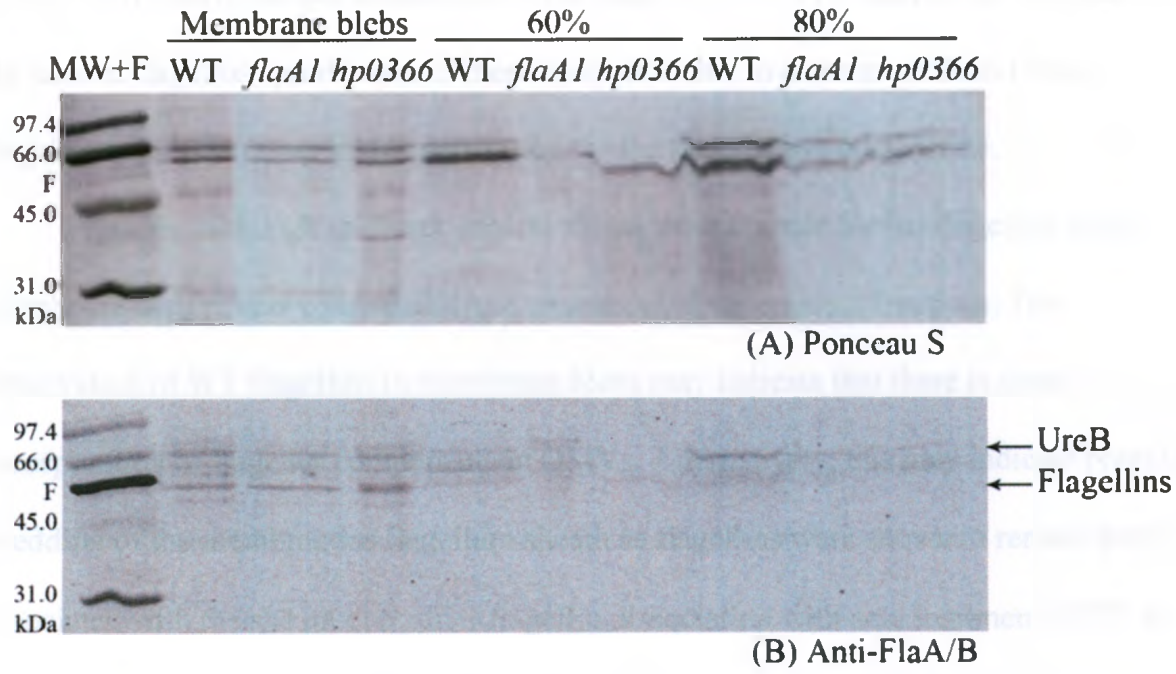
In addition to autolysis and release of intracellular contents, *H. pylori* has been shown to produce membrane blebs and/or outer membrane vesicles (OMVs) that carry outer membrane proteins [231-233]. While Keenan *et al.* 2000 demonstrated by electron microscopy that OMVs of *H. pylori* lack flagella, the presence of the flagellin subunits in OMVs has not been investigated. As such, the supernatants obtained from liquid culture were also investigated for the presence of flagellins within membrane blebs.

After growth in broth, cells were pelleted by centrifugation and the supernatant recovered. The supernatant was ultracentrifuged to pellet membrane blebs and subsequently concentrated by step-wise ammonium sulphate precipitation at 40%, 60%, and 80%. No pellet was observed at 40% ammonium sulphate precipitation in any of the strains. Both the 60% and 80% supernatant fractions and the membrane bleb pellets were assessed for the presence of flagellins by Western blot. Immunoblotting was performed using a polyclonal antiserum raised in rabbits against WT *H. pylori* FlaA, the major

flagellin subunit (distinct from FlaA1 required for pse biosynthesis), which was overexpressed in *E. coli* and purified by nickel affinity chromatography via a Histidine-tag. This antiserum is known to cross-react with FlaB, the minor flagellin subunit, due to the high sequence identity between FlaA and FlaB (57.2%) and therefore detects both flagellin subunits [118]. As a positive control, *H. pylori* flagellins cloned with a Histidine-tag that were overexpressed in *E. coli* and purified by nickel affinity chromatography were utilized and loaded with the molecular weight standards.

Based on Ponceau S staining of total proteins in each cell fraction examined, it is apparent that protein loading of the *pse* mutants was slightly lower than WT (Figure 7: A). Nevertheless, the overall protein profile of the WT fractions compared to the fractions of the *pse* mutants is similar, which indicates that there is no defect in the general secretion machinery of the *pse* mutants. By anti-FlaA/B Western blot, only slight signal was observed in the membrane bleb and supernatant fractions (Figure 7: B). Two bands were predominant. The higher migrating band of approximately 66 kDa was previously identified as the large subunit of urease, UreB. UreB is 54% homologous to FlaA and contains a 113 amino acid sequence identical to FlaA, which may explain the cross-reactivity observed by the anti-FlaA/B antibody [118]. The lower migrating band of approximately 54 kDa corresponds with the flagellins. The flagellin band was poorly abundant compared to other proteins in the *H. pylori* secretome as the flagellins were not visible by Ponceau S stain (Figure 7: A) and only a limited signal was observed by anti-FlaA/B Western blot (Figure 7: B). This finding was expected of the WT secretome as the flagellins polymerize into the flagellar filament and are not aberrantly secreted into the cell supernatant, and is consistent with previous studies of the *H. pylori* secretome

Figure 7: Screening of *H. pylori* broth culture supernatants for flagellins by anti-FlaA/B Western blot. WT, *flaA1*, and *hp0366 H. pylori* strains were grown in broth to detect a defect in the secretion of flagellins from the flagellar export apparatus or a defect in the polymerization of the flagellin subunits. Membrane bleb proteins and supernatant proteins concentrated by 60% and 80% ammonium sulphate precipitation were separated by 12% SDS-PAGE for each WT, *flaA1* and *hp0366*. Total protein content in the supernatant fractions was detected by Ponceau S stain (A). Flagellins were detected by anti-FlaA/B Western blot (B). Reactive bands correspond to UreB and the flagellin band, as described in the results. MW+F, molecular weight standard spiked with purified recombinant *H. pylori* flagellins.


Membrane blebs
60%
80%
MW+F WT flaA1 hp0366 WT flaA1 hp0366 WT flaA1 hp0366
97.4
66.0
F
45.0
31.0
kDa
(A) Ponceau S
97.4
66.0
F
45.0
31.0
kDa
UreB
Flagellins
(B) Anti-FlaA/B

[226]. Conversely, the low abundance of the flagellins in the *pse* secretome indicates that the lack of flagellum production in these mutants is due to a secretion defect from flagellar apparatus rather than a deficiency for the flagellins to polymerize.

Further, although minimal, greater signal was obtained for the flagellins in the membrane bleb pellets compared to the concentrated supernatant fractions. The observation of WT flagellins in membrane blebs may indicate that there is some incorporation of flagellar components in OMVs. Alternatively, this may indicate potential shedding of the membranous flagellum sheath as flagellins were shown to remain partly associated with the sheath even after flagellin dissociation with acid treatment [107]. In the case of the *pse* mutants, flagellins detected in the membrane bleb fractions may indicate an association of the *pse* flagellins with other flagellar components that are ultimately incorporated into OMVs.

An additional attempt to purify the flagellins in the supernatant by anion exchange chromatography was conducted. Ammonium sulphate precipitation fractions were combined and dialyzed into anion exchange binding buffer prior to loading on the anion exchange column. No flagellins were detected in the fractions collected or in the flowthrough, further suggesting low recovery of the flagellins from culture supernatants (data not shown). Ultrafiltration, rather than ammonium sulphate precipitation, was also used to concentrate the cell supernatants in order to enhance the recovery of the flagellins prior to anion exchange chromatography, however low detection of the flagellins was also observed by anti-FlaA/B Western blot, similar to the results observed by direct ammonium sulphate precipitation (data not shown).

Overall, minimal levels of flagellins were detected in the supernatant and membrane blebs of liquid cultures of both WT and the *pse* mutants. The level of flagellins observed from the *pse* mutants was similar to WT. This indicates that a defect in pse flagellin glycosylation, either by the absence of glycosylation or alternative glycosylation, as demonstrated by the flagellins of *flaA1* and *hp0366,* respectively, likely results in a secretion defect of the flagellins from the flagellar basal body. This finding suggests that the flagellins of the *hp0366* mutant likely reside in the cytoplasm. Therefore, in order to further assess the observation of alternative glycosylation in the *hp0366* mutant, intracellular fractions were investigated for the presence of flagellins.

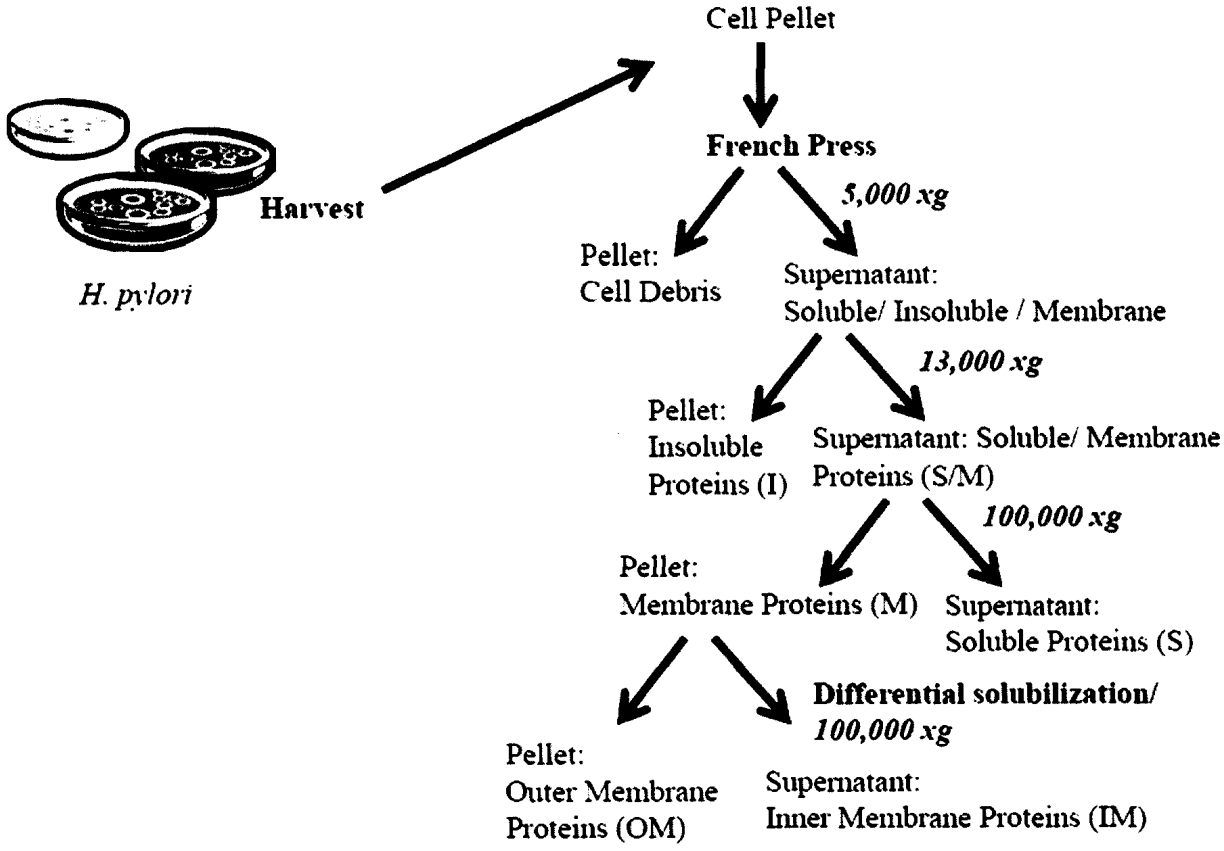
3.1.2 *H. pylori* flagellins are identified intracellularly

As altered glycosylation in the *pse* mutants resulted in an apparent secretion defect from the flagellar apparatus, it was necessary to determine the intracellular localization of the flagellins. In particular, comparing the localization of pse glycosylated flagellins from WT to the alternatively glycosylated flagellins of the *hp0366* mutant would aid in characterizing the role that pse-specific glycosylation plays in flagellum assembly. As such, in order to investigate the effect of alternative glycosylation, the localization of the flagellins in WT and *hp0366* was examined.

H. pylori cells were fractionated by differential centrifugation into insoluble, soluble, and membrane fractions. The total membrane fraction was further separated into inner membrane (IM) and outer membrane (OM) components by selective solubilization of the IM by N-lauroylsarcosine [234] and separation by ultracentrifugation (Figure 8). Each cell fraction was extensively washed to limit protein contaminants between protein fractions. The cell fractions were screened for the presence of flagellins by anti-FlaA/B

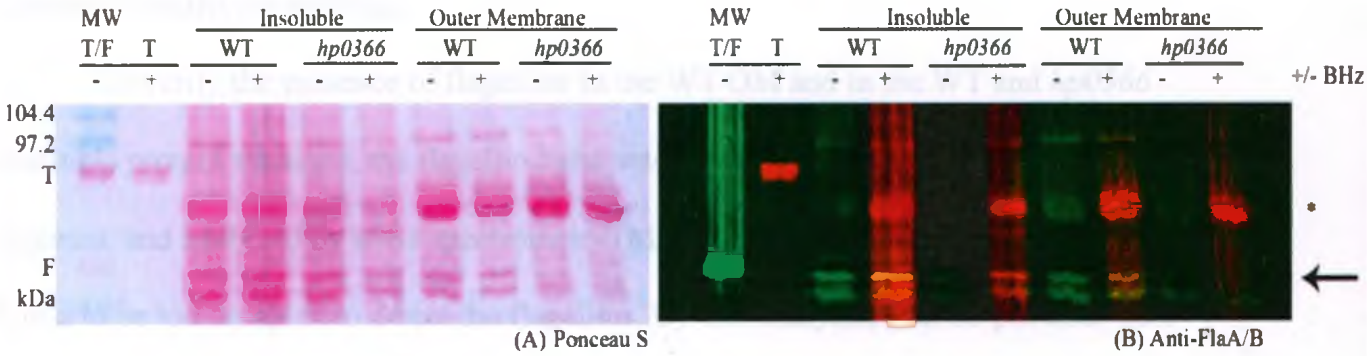
Figure 8: Fractionation of *H. pylori* cellular proteins by differential centrifugation.

In order to facilitate the analysis and localization of the flagellins and other glycoproteins in *H. pylori,* total *H. pylori* cells were separated into cellular protein fractions by differential centrifugation. This allowed for enrichment of specific pools of glycoproteins. *H. pylori* was grown on agar plates and harvested. Total cells were lysed by French press. Unlysed cells and cellular debris were pelleted by centrifugation at 5000*xg*. The supernatant was removed and insoluble proteins (I) were separated from soluble proteins (S) by centrifugation at 13,000*xg*. Membrane proteins (M) were pelleted by ultracentrifugation at 100,000*xg*. Inner membrane proteins (IM) were further separated from outer membrane proteins (OM) by the differential solubilization of IM proteins in N-lauroylsarcosine and separation by ultracentrifugation at 100,000*xg*. Separated cellular protein fractions were subsequently assessed for the presence of flagellins or glycoproteins and subjected to further downstream separation to simplify the cell fractions for MS analysis.


Cell Pellet
French Press
5,000 xg
Harvest
H. pylori
Pellet:
Cell Debris
Supernatant:
Soluble/ Insoluble / Membrane
13,000 xg
Pellet:
Insoluble
Proteins (I)
Supernatant: Soluble/ Membrane
Proteins (S/M)
100,000 xg
Pellet:
Membrane Proteins (M)
Supernatant:
Soluble Proteins (S)
Differential solubilization/
100,000 xg
Pellet:
Outer Membrane
Proteins (OM)
Supernatant:
Inner Membrane Proteins (IM)

Western blot. Glycosylated flagellin species were detected by glycoprotein-specific labelling in-solution with biotin-hydrazide (BHz) and detection of the label with a fluorescent conjugate of streptavidin after transfer to nitrocellulose. Transferrin, a known glycoprotein, was used as a positive control for glycoprotein-specific labelling, and the non-glycosylated recombinant *H. pylori* flagellins purified from *E. coli* were run with the molecular weight standards as a control for the anti-FlaA/B Western blot. No flagellins were identified by anti-FlaA/B Western blot in the soluble or inner membrane cell fractions of WT or *pse H. pylori* strains (data not shown). However, flagellins were detected in the OM fraction of WT *H. pylori* (Figure 9). This finding was expected as flagellins assemble into a proper flagellum in WT and the *H. pylori* flagellum is known to be enclosed with a membranous sheath continuous with the outer membrane [107]. Thus, without further purification, the WT flagellins should remain associated with the flagellar sheath as part of the OM fraction. Surprisingly, flagellins were also detected in the insoluble protein fraction of WT. Moreover, in *hp0366,* flagellins were also identified in the insoluble protein fraction (Figure 9). No flagellins were identified in the OM of *hp0366,* as expected as *hp0366* is unable to form proper flagella. By glycoprotein-specific labelling, the WT flagellins in both the OM and insoluble protein fractions appeared to be glycosylated. As well, the flagellins in the insoluble fraction of *hp0366* also appeared glycosylated (Figure 9). Interestingly, by glycoprotein specific-labelling, a protein band was strongly detected in both the insoluble and OM fractions of WT and *hp0366*. As glycosylation is not dependent on *hp0366,* this glycoprotein candidate may represent an additional glycoprotein that is glycosylated by a sugar other than pse in *H. pylori.* The respective protein band was cut from a Coomassie-stained polyacrylamide gel

Figure 9: Detection of WT and *hp0366* glycosylated flagellins in insoluble and outer membrane protein fractions by anti-FlaA/B Western blot and glycoprotein-specific chemical labelling. WT and *hp0366* insoluble and outer membrane (OM) cell fractions obtained by differential centrifugation were labelled in-solution for glycoproteins with (+) or without (-) BHz and analyzed by SDS-PAGE. Total protein was stained with Ponceau S (A). Glycoproteins were identified by detection of the BHz label with a fluorescent conjugate of streptavidin (Red) and flagellins were detected by anti-FlaA/B Western blot (Green). The arrow indicates the flagellin band. Overlap of the signals obtained by anti-FlaA/B Western blot and by detection of glycoproteins with streptavidin indicate the presence of glycosylated flagellin species. The asterisk indicates the glycoprotein band sent for analysis. MW = molecular weight standards, T = transferrin, F = purified recombinant *H. pylori* flagellins (non-glycosylated).



(A) Ponceau S

(B) Anti-FlaA/B Streptavidin 680

and sent for glycopeptide analysis by our collaborator, Dr. Anne Dell (Imperial College, London). Results are pending.

To verify the presence of flagellins in the WT OM and in the WT and *hp0366* insoluble protein fractions, the flagellin band was cut from 1D SDS-PAGE gels, trypsin digested, and analyzed by mass spectrometry (MS). Unfortunately, the samples were found to be too complex to detect the flagellins. By MS analysis, over 30 proteins were identified in the single cut band with high confidence (data not shown). As such, it is likely that the flagellins were recovered in low amounts by differential centrifugation, which prevents further glycopeptide analysis. Regardless, the additional evidence of glycosylated flagellins in *hp0366* further suggests that there is a pse-independent glycosylation pathway in *H. pylori*. This observation warranted the development of new strategies to assess the insoluble protein fraction of WT and *hp0366*. Therefore, new approaches were utilized in order to simplify the protein fractions containing glycosylated flagellins for further purification and glycan analysis.

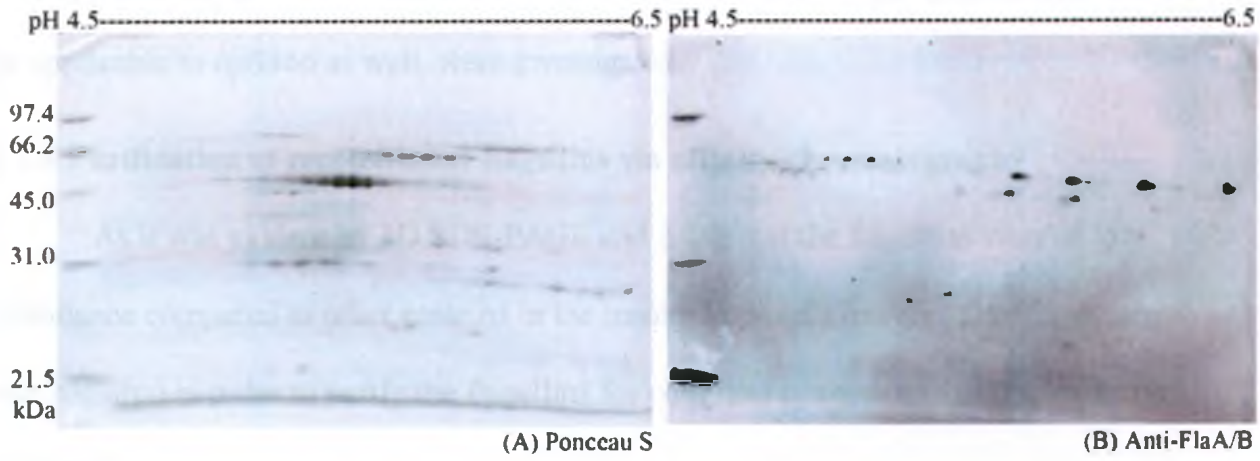
3.1.3 Separation of WT *H. pylori* insoluble flagellins by 2-D gel electrophoresis

In order to identify the flagellins in the insoluble protein fraction of both WT and *hp0366* strains of *H. pylori*, it was necessary to separate the flagellins from other protein content present in the samples. For this purpose, 2-dimensional gel electrophoresis (2-DE) was investigated as a potential preparative method. 2-DE separates proteins based on charge in one dimension and based on molecular mass in the second dimension, resulting in distinct protein spots. 2-DE has previously been used to resolve different glycoforms of glycoproteins [188].

To determine if 2-DE would provide enough resolution to obtain purified flagellins from the insoluble protein fractions, the WT insoluble sample was assessed by 2-DE. Previous characterization of the *H. pylori* flagellins indicated that WT flagellins exhibited a pI of 5.2 [105]. As such, the pH range of 4.5 to 6.5 was chosen for analysis. Total protein was visualized by Ponceau S stain and the flagellins were detected by anti-FlaA/B Western blot (Figure 10). By anti-FlaA/B Western blot, flagellins were detected as a series of several protein spots rather than a single protein spot. While the detection of multiple protein spots may be due to flagellin degradation or non-specificity of the anti-FlaA/B antibody, horizontal streaking by 2-DE is typically indicative of post-translational modifications, such as glycosylation [188]. Thus, the detection of multiple protein spots by anti-FlaA/B Western blot could reflect the presence of several flagellin glycoforms. This was anticipated as previous analysis of the WT flagellins has indicated that they are heterogeneously glycosylated [117]. In the present analysis, the flagellin spots of the WT insoluble protein fraction appear as two populations. This likely represents the separation of FlaA and FlaB and/or the separation of the different glycoforms of each flagellin. As evident by 2-DE, the WT flagellins are poorly abundant compared to other proteins in the insoluble protein fraction and are not visible by Ponceau S. The low abundance of the flagellins in the insoluble fraction may explain why the flagellins could not be conclusively identified by MS analysis from 1D SDS-PAGE.

Overall, 2-DE of the WT insoluble fraction allowed for the separation of the WT flagellins from more abundant proteins that would otherwise contaminate MS analysis. As such, the protein spots corresponding to the flagellins could be excised and used for MS analysis. However, the flagellin spots are not detectable by Ponceau S. Thus, it is

Figure 10: Analysis of WT insoluble flagellins separated by 2-DE and detected via anti-FlaA/B Western blot. Total protein in the WT insoluble protein fraction was separated by 2-DE and stained with Ponceau S (A). Flagellins were detected by anti-FlaA/B Western blot (B). By 2-DE, several flagellin protein spots were observed. These likely represent the various glycoforms of FlaA and FlaB. All flagellin spots were poorly abundant compared to other proteins in the WT insoluble protein fraction. Thus, a different method to obtain purified flagellins for glycoprotein analysis was investigated.



(A) Ponceau S (B) Anti-FlaA/B

unlikely that enough flagellins could be recovered for complete glycoprotein analysis by 2-DE preparative gels. Therefore, methods to recover more of the flagellins, which would be applicable to *hp0366* as well, were investigated.

3.1.4 Purification of recombinant flagellins via affinity chromatography

As it was evident by 1D SDS-PAGE and 2-DE that the flagellins were of low abundance compared to other proteins in the insoluble protein fraction, another strategy was required in order to purify the flagellins for complete analysis of the glycosylation observed.

In order to enable purification of the flagellins, both of the flagellin subunits, FlaA and FlaB, were tagged with a C-terminal hexa-Histidine tag to allow purification from *H. pylori* by affinity chromatography. Expression of the flagellins from *H. pylori,* rather than a different bacterial host, was required in order to assess the effect of pse glycosylation versus alternative glycosylation. However, genetic manipulation of *H. pylori* is hindered by the availability of minimal gene transfer systems. Although some shuttle vectors have been designed, problems are encountered with plasmid stability and the occurrence of recombination events [235]. In addition, limited strategies for chromosomal integration have been described in *H. pylori.* A shuttle vector based strategy was employed for expression of the glycoprotein candidate WaaL in *H. pylori,* as described in Section 3.3.2, however, problems with plasmid stability were encountered. Therefore, to express tagged versions of the flagellins in *H. pylori*, a chromosomal integration strategy was utilized.

The flagellins were integrated into the chromosome in the *rdxA* gene as previously reported [218, 236]. Few other sites for chromosomal integration have been

reported for *H. pylori.* RdxA *(hp0954)* is a nitroreductase that converts the antibiotic metronidazole (Mtz) from its inactive pro-drug form to its active form [237], to which most laboratory strains of *H. pylori* are susceptible. This site of integration is advantageous as *rdxA* is dispensable for *H. pylori* viability; Mtz-resistant strains exhibit no metabolic or growth differences from isogenic Mtz-susceptible strains [237]. As well, inactivation of *rdxA* allows for positive selection without incorporation of additional resistance markers as disruption of *rdxA* prevents conversion of Mtz to its active form and results in *H. pylori* that is resistant to Mtz [236]. The vector used for chromosomal integration in *rdxA* is a pET21a (Novagen) derivative that contains flanking regions of *rdxA* around a gene of interest for chromosomal integration [218]. This vector, pET634comp, was obtained from Dr. S. Trent (University of Texas) and was modified to contain either flagellin gene with their native promoter, *hp0601* (*Pr-flaA*) and *hp0115* (*Pr-flaB*), and a C-terminal hexa-Histidine tag, to be used for chromosomal integration of the flagellins in *rdxA* and subsequent expression of the tagged flagellins in *H. pylori.*

However, our laboratory WT *H. pylori* strain is naturally resistant to Mtz, due to insertion of the mini-IS605 transposon and deletion of the flanking regions of *rdxA* [238]. The truncated and non-functional copy of *rdxA* in the WT chromosome prevented the use of the flagellin pET634comp derivatives for chromosomal integration into *rdxA.* Therefore, in order to utilize this system for flagellin expression in WT and *hp0366* strains, an additional integration step was required to introduce a full-length functional copy of *rdxA* into WT and the isogenic mutant *hp0366.*

In order to introduce a functional copy of *rdxA*, a new site of integration within the WT chromosome was required. For this purpose, *ureB (hp0072),* which encodes for

urease subunit B (UreB), was chosen. Disruption of *ureB* was chosen as deletion of *ureB* in *H. pylori* has been widely characterized with no observed defect in growth and motility or effect on glycosylation [118, 239]. In addition, disruption of *ureB* is advantageous for the purification of the flagellins as UreB has been found by several different methods to cross-react with the anti-FlaA/B antibody, including during attempts to purify the flagellins from cell fractions as described in Section 3.1.2. This co-migration has hindered past MS analysis of samples as UreB is highly abundant in the cell and likely masks detection of the flagellins, which are present at lower levels. Upon incorporation of *rdxA* within *ureB, rdxA* could be subsequently inactivated with the tagged flagellin genes for the expression of tagged flagellins in WT and *hp0366* and the purification of the flagellins by affinity chromatography. This strategy is outlined below.

3.1.4.1 Integration of *rdxA* into WT and *hp0366 H. pylori*

Incorporation of *rdxA* into WT and *hp0366 H. pylori* strains will convey Mtz susceptibility and allow for the use of the pET634comp derivatives for chromosomal integration of the cloned flagellin genes for flagellin expression in *H. pylori*. *ureB* was chosen as the site of integration for a full-length copy of *rdxA*. A suicide vector, pBKS-UreB::RdxA-CAT, containing *rdxA* in-frame with a chloramphenicol antibiotic resistance cassette (CAT) with flanking regions of *ureB* was constructed (refer to Figure 4). For transformation into *H. pylori,* the vector was treated with WT *H. pylori* cell-free extract (CFE). CFE treatment of foreign DNA is advantageous for transformation into *H. pylori* as the presence of multiple restriction-modification systems act as a barrier to transformation [219]. Treatment with CFE results in specific *in vitro* site-specific methylation of foreign DNA with the "signature" of *H. pylori* DNA by endogenous

methyltransferases. This allows for the restriction-modification barrier to be overcome [219, 240]. Without CFE treatment of foreign DNA, *H. pylori* strains are often resistant to transformation or exhibit extremely low transformation efficiencies. After CFE treatment, pBKS-UreB::RdxA-CAT was transformed into WT and *hp0366* by electroporation. Clones were selected on BHI-YE agar plates supplemented with 12μg/mL chloramphenicol and were apparent after 3-5 days of incubation in microaerophilic conditions.

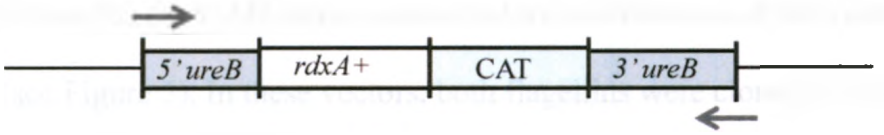
Successful integration of *rdxA-CAT* was screened by conventional PCR of chromosomal DNA for the upstream and downstream regions of *ureB*. PCR amplification of a 2.7kb band indicated that *rdxA-CAT* had properly integrated within *ureB* and not elsewhere in the genome (Figure 11: A). Furthermore, the disruption of *ureB* was confirmed by analysis of the total protein profiles of select clones (Figure 11: B). Compared to WT and *hp0366* strains, clones with successful integration of *rdxA* into *ureB*, lacked the abundant UreB band of approximately 66 kDa.

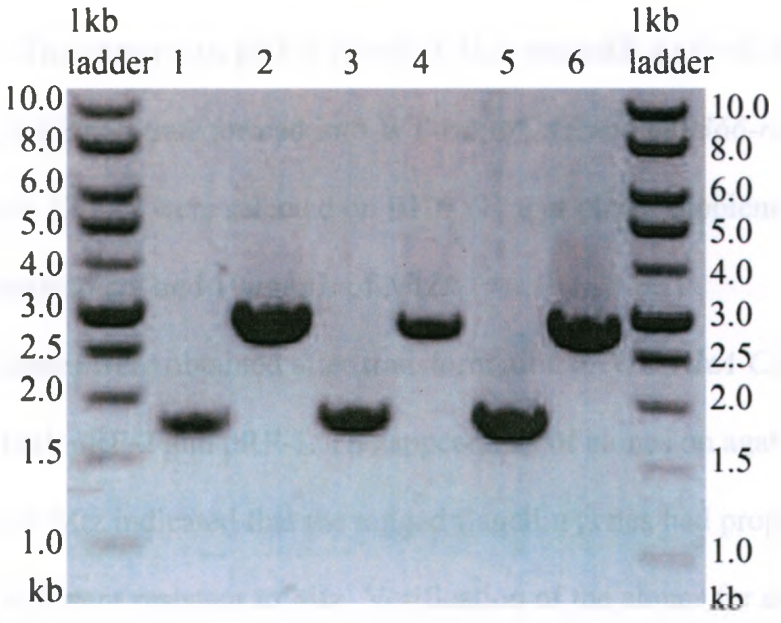
To verify the integration of a functional copy of *rdxA*, clones were patched onto BHI-YE agar plates supplemented with 6μg/mL, 12μg/mL, and 18μg/mL of Mtz. In all cases, WT and *hp0366* clones containing *rdxA* were unable to grow in the presence of Mtz (data not shown). Therefore, a full-length functional copy of *rdxA* was successfully integrated into both WT and *hp0366 H. pylori* strains.

3.1.4.2 Inactivation of *rdxA* via chromosomal integration of the tagged flagellins

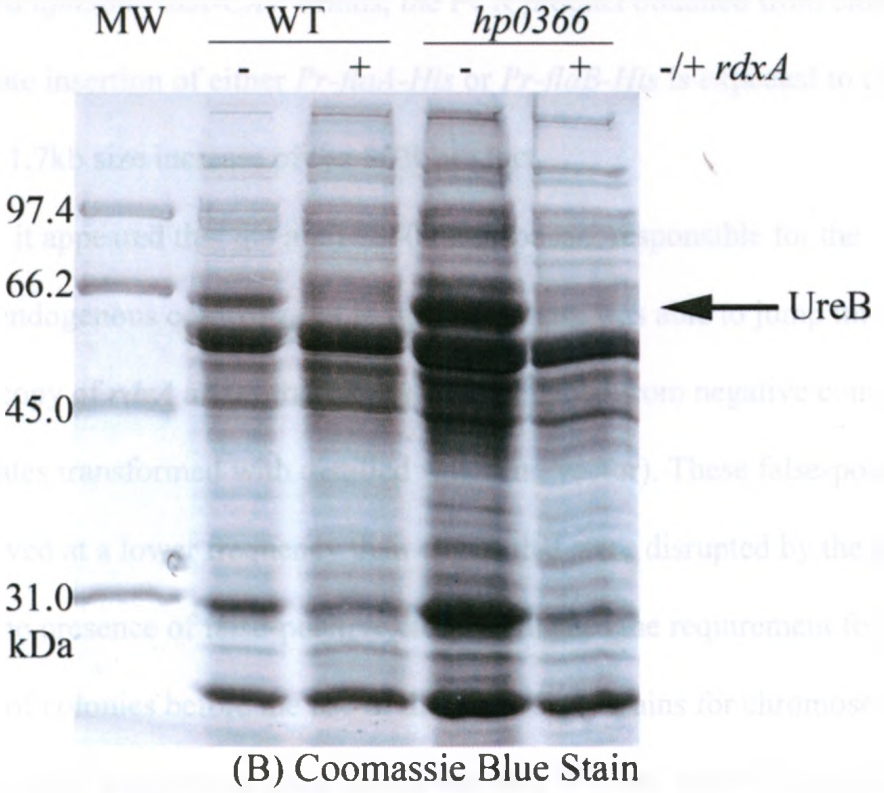
Representative clones of WT and *hp0366* with verified integration of *rdxA-CAT* were used for subsequent inactivation of *rdxA* by tagged versions of the *H. pylori* flagellin genes. As described previously, suicide vectors containing either *Pr-flaA-His*

Figure 11: Verification of chromosomal integration of a functional copy of *rdxA* in WT and *hp0366 H. pylori.* *rdxA-CAT* was integrated into the chromosome of both WT and *hp0366 H. pylori* strains by electroporation with the suicide vector pBKS-UreB::RdxA-CAT. Successful integration of *rdxA-CAT* was confirmed by PCR amplification with primers specific for *ureB* from chromosomal DNA of WT, *hp0366,* and representative clones containing *rdxA-CAT* from each strain. Clones of both WT and *hp0366* that had undergone successful chromosomal integration of *rdxA-CAT* demonstrated a size increase of ~1.0kb. 1 = pBKS-UreB (plasmid control), 2 = pBKS-UreB::RdxA-CAT (plasmid control), 3 = WT, 4 = WT-*rdxA-CAT,* 5 = *hp0366,* 6 = *hp0366-rdxA-CAT* (A). The disruption of *ureB* was verified by the loss of UreB production as seen by analysis of the total protein content of the *rdxA-CAT* clones. Total protein from WT, *hp0366,* and representative *rdxA-CAT* clones from each strain were separated by 12% SDS-PAGE and visualized by Coomassie Blue staining (B). A noticeable loss of UreB (~66 kDa) in the clones containing *rdxA-CAT* indicated the successful disruption of *ureB.* MW = molecular weight standards.





(A) 0.7% agarose gel



(B) Coomassie Blue Stain

(hp0601) or *Pr-flaB-His (hp0115)* were constructed by modification of the vector pET634comp (see Figure 5). In these vectors, both flagellins were cloned with their native promoter as the flagellins are known to be differentially regulated by alternative promoters [112]. The constructs, pRF-2 *(Pr-flaA-His)* and pRF-1 *(Pr-flaB-His),* were treated with WT CFE and transformed into WT-*rdxA-CAT* and *hp0366-rdxA-CAT* strains by electroporation. Clones were selected on BHI-YE agar plates supplemented with 12μg/mL chloramphenicol and 18μg/mL of Mtz.

Several clones were obtained after transformation of WT-*rdxA-CAT* and *hp0366-rdxA-CAT* with both pRF-2 and pRF-1. The appearance of clones on agar plates supplemented with Mtz indicated that the tagged flagellin genes had properly integrated within *rdxA* as they were resistant to Mtz. Verification of the clones for each strain is underway via PCR amplification using primers that flank the *ureB* gene. Compared to the WT-*rdxA-CAT* and *hp0366-rdxA-CAT* strains, the PCR product obtained from clones with the appropriate insertion of either *Pr-flaA-His* or *Pr-flaB-His* is expected to exhibit an approximately 1.7kb size increase of the PCR product.

Moreover, it appeared that the mini-IS605 transposon, responsible for the disruption of the endogenous copy of *rdxA* in our WT strain, was able to jump into the newly integrated copy of *rdxA* as several clones were obtained from negative control transformation plates transformed with distilled water (no vector). These false-positive clones were observed at a lower frequency than clones that were disrupted by the tagged flagellin genes. The presence of false-positive clones indicates the requirement for routine screening of colonies before the use of the *rdxA-CAT* strains for chromosomal integration in the future. Regardless, once clones for each WT and *hp0366* expressing *Pr-*

flaA-His and *Pr-flaB-His* are confirmed, the expression of the tagged flagellins will be verified by an anti-Histidine Western blot from total cell lysates.

Overall, once fully characterized, the generation of WT and *hp0366* strains that express the flagellins with a Histidine-tag will enable purification of large amounts of the flagellins from both *H. pylori* strains by affinity chromatography. Purified flagellins can then be analyzed by MS. The ultimate goal is to identify the glycan present on the *hp0366* flagellins and compare it to that of pse on the WT flagellins. The demonstration that a glycan distinct from pse is present on the *hp0366* flagellins will definitively reveal the presence of an alternative glycosylation pathway in *H. pylori.*

3.2 Detection of additional glycoproteins besides the flagellins in *H. pylori*

Pse is known to only modify the flagellins of *H. pylori*. However, both *pse* mutants, *flaA1* and *hp0366,* displayed unexpected altered LPS profiles in addition to a defect in flagellum production. *flaA1* exhibited an altered O-antigen compared to WT [118, 175], while *hp0366* was completely O-antigen deficient [174]. This observation was surprising as pse is not involved in LPS biosynthesis [69-71]. Further, the effect observed on LPS production was not due to joint transcriptional regulation of LPS and flagella biosynthesis as quantitative real-time PCR demonstrated no significant changes in the expression of LPS biosynthetic genes in the *hp0366* mutant (Creuzenet Lab, unpublished). In *flaA1,* a slight upregulation of the O-antigen ligase, WaaL, involved in LPS biosynthesis was observed, however, this would not contribute to the altered O-antigen profile observed.

Overall, the unexpected effect by the disruption of *flaA1* and *hp0366* on LPS production indicated that other proteins, such as those involved in LPS biosynthesis, may

require glycosylation for proper function. The proposed presence of additional glycoproteins in *H. pylori* is supported by recent data from our laboratory in which soluble glycoprotein candidates in *H. pylori* were identified [176]. The following section describes detection of additional glycoproteins in the membrane protein fraction of *H. pylori.* The identification of glycoproteins in the membrane protein fraction of *H. pylori* may exemplify a link between protein glycosylation and LPS biosynthesis as LPS biosynthetic enzymes are known to be integral IM proteins. As well, surface-associated glycoproteins in the OM of *H. pylori* could represent a pool of novel virulence factors.

3.2.1 Pleiotropic effects on membrane stability do not account for the LPS defects observed in *flaA1* and *hp0366*

Pse glycosylation of the flagellin subunits is well-established as a component required for flagellum production [117, 225]. Moreover, the flagellar apparatus is a prime component of the bacterial membrane that transverses both the IM and OM [104]. Thus, interruption of flagellum production by disruption of pse biosynthesis may have pleiotropic effects on the stability of the cell membrane, which may result in the LPS defects observed in the *flaA1* and *hp0366* mutants. To investigate if the LPS profiles observed in *flaA1* and *hp0366* could be due to a general effect on membrane stability, rather than a link between protein glycosylation and LPS biosynthesis, the cell membranes of WT and the *pse* mutants were analyzed.

For this purpose, total membrane fractions of WT, *flaA1,* and *hp0366* were separated into IM and OM components by differential solubilization of the IM proteins by 1% N-lauroylsarcosine and the protein profiles were analyzed by 12% SDS-PAGE. In

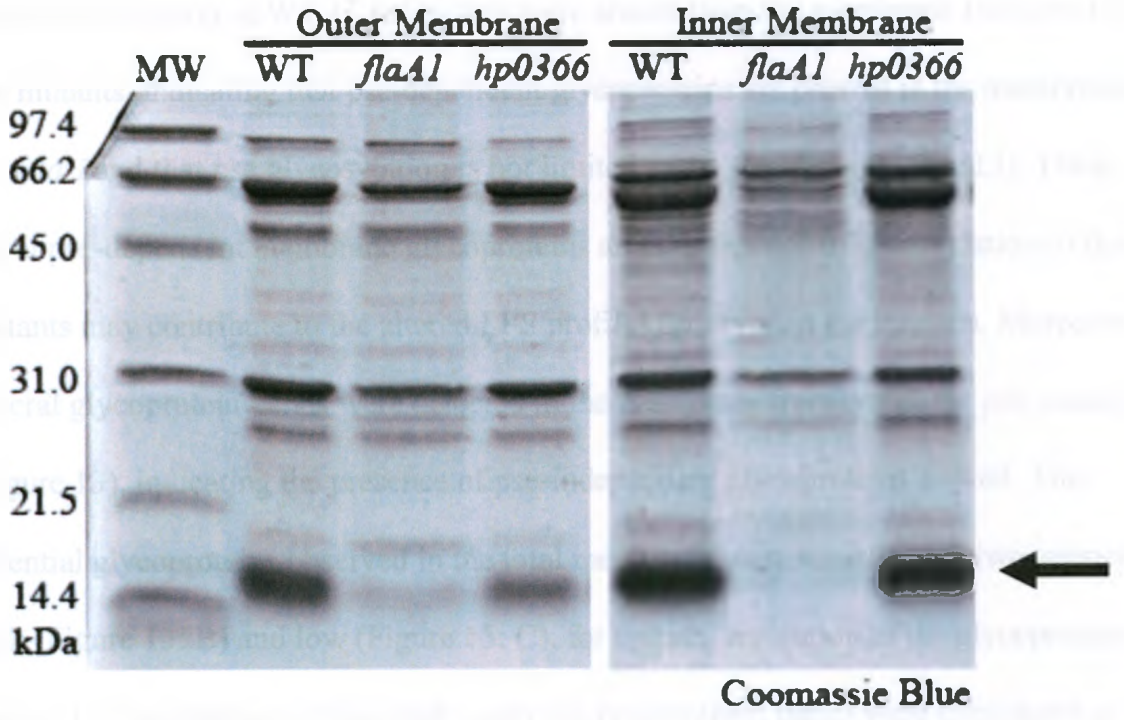
general, by Coomassie stain, the IM and OM protein profiles of WT, *flaA1,* and *hp0366* were similar (Figure 12). The similar protein pattern observed indicates that the LPS defects observed in *flaA1* and *hp0366* are gene-specific and not the result of pleiotropic effects on membrane stability. Of interest are the reduced levels of a ~15 kDa band in the membrane fractions of *flaA1.* This protein could represent a potential pse-dependent glycoprotein in the membrane of *H. pylori* that follows a similar pattern to that observed for the flagellins; when *flaA1* is disrupted, the flagellins are non-glycosylated and expressed at reduced levels [175]. Comparatively, the flagellins of *hp0366* are alternatively glycosylated and are expressed at levels close to WT [174]. The corresponding ~15 kDa protein band observed by Coomassie staining was cut from the polyacrylamide gel and sent for protein identification by MS analysis. Results are pending.

3.2.2 Demonstration of novel membrane glycoproteins in *H. pylori*

The alteration of LPS production in *flaA1* and *hp0366* was unexplained by transcriptional regulation and potential pleiotropic effects on membrane stability, indicating that the effect on LPS production was gene-specific. Thus, the defect observed in LPS production in the *pse* mutants suggests that LPS biosynthetic enzymes in *H. pylori* may require glycosylation for function. As such, glycoproteins are likely present in the membranes of *H. pylori.*

To investigate whether the LPS defect observed in the *pse* mutants was due to the presence of membrane glycoproteins in *H. pylori*, total membrane samples of WT, *flaA1,* and *hp0366* were chemically labelled with BHz to specifically label glycoproteins, and separated by 12% SDS-PAGE. Glycoproteins were detected with a fluorescent conjugate

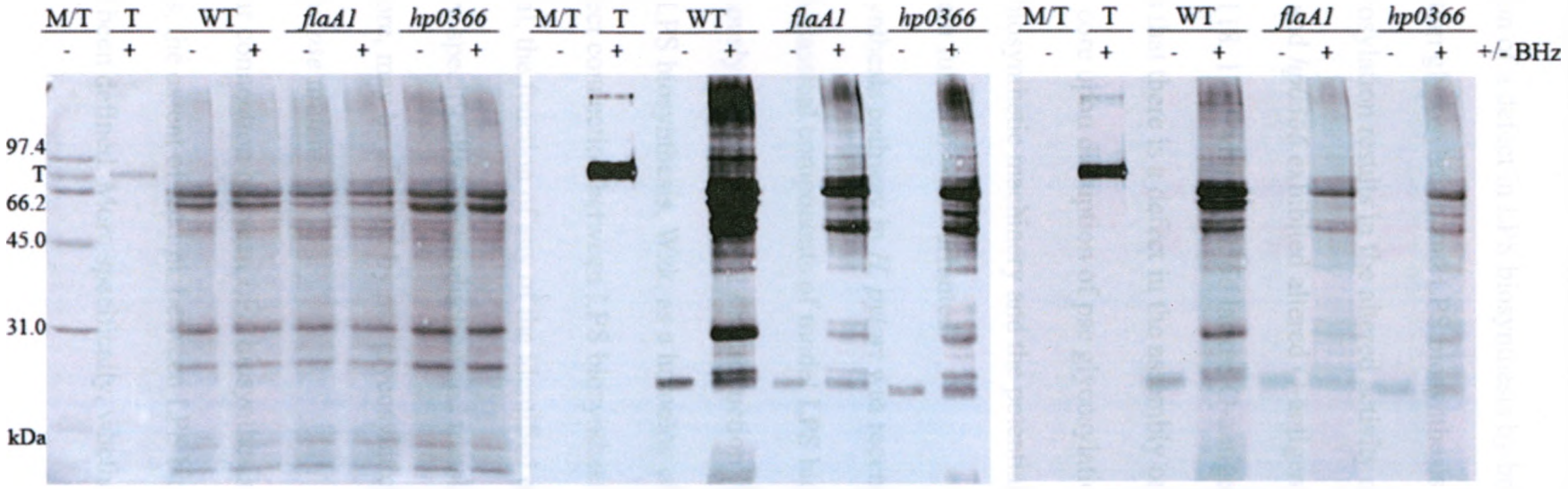
Figure 12: Inner membrane and outer membrane protein profiles of WT, *flaA1*, and *hp0366*. WT, *flaA1*, and *hp0366* total membrane samples were separated into inner and outer membrane components by differential solubilization of inner membrane proteins in 1% N-lauroylsarcosine. The protein profiles of the inner membrane and outer membrane fractions were analyzed by 12% SDS-PAGE and stained with Coomassie Blue. The protein profiles of WT, *flaA1*, and *hp0366* are similar, indicating no pleiotropic effects on membrane stability caused by the disruption of flagellin glycosylation and flagellum production in *flaA1* and *hp0366*. Interestingly, a ~15 kDa protein appeared dependent on *flaA1* but not *hp0366*. This protein band of interest is indicated by the arrow. MW = molecular weight standards.


Outer Membrane
Inner Membrane
MW
WT
flaA1
hp0366
WT
flaA1
hp0366
97.4
66.2
45.0
31.0
21.5
14.4
kDa
Coomassie Blue

of streptavidin. By this method, several glycoprotein bands were observed in the total membrane fraction of WT *H. pylori* that were absent from the membrane fractions of the *pse* mutants, indicating that pse-dependent glycoproteins are present in the membranes of *H. pylori* and that pse glycosylation is not limited to the flagellins (Figure 13). These novel pse-dependent membrane glycoproteins and the absence of glycosylation in the *pse* mutants may contribute to the altered LPS profiles observed in the mutants. Moreover, several glycoprotein bands were detected in the membrane fractions of the *pse* mutants (Figure 13), indicating the presence of pse-independent glycoproteins as well. The potential glycoproteins observed in the total membrane were visualized at two intensities, high (Figure 13: B) and low (Figure 13: C), for optimal resolution of the glycoprotein bands. For the purpose of this study, only the predominant bands were considered as candidates for further investigation. This likely underestimates the number of glycoproteins found in the membranes of *H. pylori* as several additional bands are observed upon visualization at a higher intensity.

Overall, the observation of pse-independent glycoproteins further demonstrates the presence of a novel glycosylation pathway in *H. pylori,* which corresponds with the alternative glycosylation of the *hp0366* flagellins. As well, the detection of several pse-dependent glycoproteins demonstrates that additional proteins besides the flagellins are glycosylated with pse. Together, these findings go against the current dogma of protein glycosylation in *H. pylori* in which only the flagellins are believed to be glycosylated by pse.

Figure 13: Detection of membrane glycoproteins in WT, *flaA1*, and *hp0366 H. pylori.* WT, *flaA1,* and *hp0366* total membrane samples were subjected to chemical labelling in-solution, which specifically labels glycoproteins with BHz. Samples were treated with (+) or without (-) BHz to demonstrate specific detection of labelled proteins, separated on 12% SDS-PAGE gels, and transferred to a nitrocellulose support. Total protein on the nitrocellulose membrane was stained with Ponceau S (A). BHz-labelled glycoproteins were detected with a fluorescent conjugate of streptavidin. Detection of glycoproteins was performed at high intensity (B) and at a low intensity (C) to resolve the distinct glycoprotein bands. It is evident that several pse-dependent glycoprotein bands are present in the WT membrane. These distinct bands are clearly evident by detection of glycoproteins at low intensity. Moreover, several pse-independent glycoprotein bands are also evident in the membrane fractions of *flaA1* and *hp0366.* M = molecular weight standards, T = transferrin (glycoprotein control).



(A) Ponceau S (B) Streptavidin 680 - High Intensity (C) Streptavidin 680 - Low Intensity

3.3 Investigation of *H. pylori* LPS biosynthetic enzymes as novel glycoproteins

The observation of a defect in LPS biosynthesis by both *pse* mutants is suggestive of a link between protein glycosylation and LPS biosynthesis in *H. pylori,* such that disruption of pse glycosylation results in the altered activity of LPS biosynthetic enzymes. Both *flaA1* and *hp0366* exhibited altered O-antigen profiles; *flaA1* displayed a modified O-antigen [118, 175] while *hp0366* lacked O-antigen production completely [174], which suggests that there is a defect in the assembly or translocation of the O-antigen to the lipid A core upon disruption of pse glycosylation. As a result, investigation of the *H. pylori* LPS biosynthetic machinery and the potential role of protein glycosylation on protein function was warranted.

The LPS biosynthesis pathway in *H. pylori* was recently characterized and was shown to exhibit both classical components of model LPS biosynthesis and novel components not previously associated with LPS production [93]. The identification of the flippase required for LPS biosynthesis, Wzk, as a homolog of protein glycosylation [93] further supports a direct connection between LPS biosynthesis and protein glycosylation in *H. pylori.* In general, the function of any of the identified enzymes involved in LPS biosynthesis, and more specifically, the production and translocation of the O-antigen chain to the lipid A core, may be affected by pse glycosylation and result in the LPS defects observed in the *pse* mutants.

Overall, a clear connection between LPS biosynthesis and protein glycosylation is evident. Nevertheless, the extent of the link between LPS biosynthesis and protein glycosylation has not been defined. More specifically, whether the LPS biosynthetic

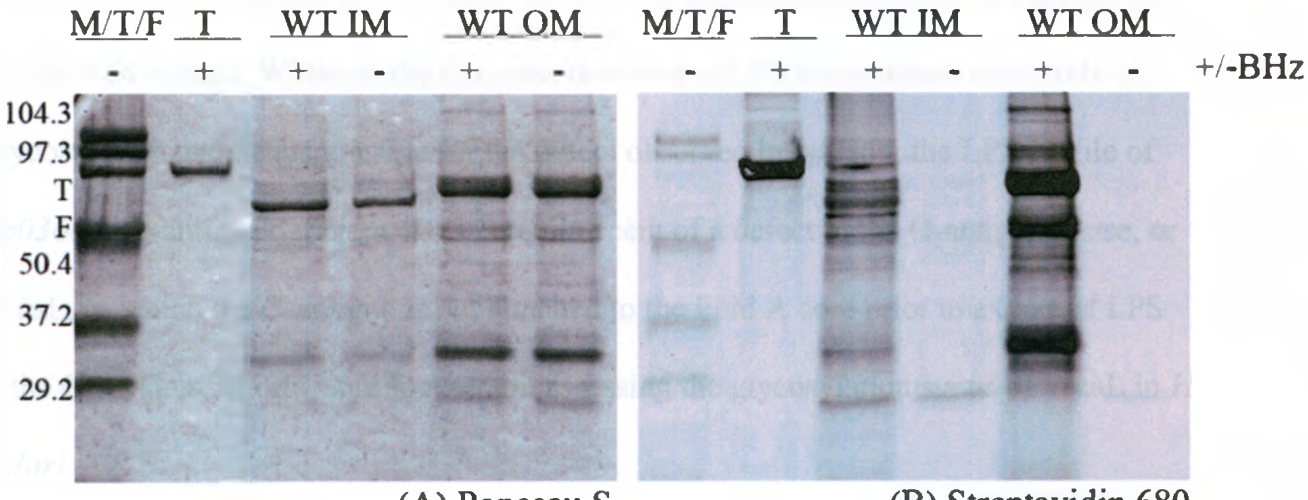
enzymes are glycosylated themselves and if they require glycosylation for function has not been previously investigated. This is examined in the following sections.

3.3.1 Glycoprotein candidates are present in the inner membrane of *H. pylori*

The LPS biosynthetic machinery responsible for the assembly and attachment of the O-antigen to the lipid A core is localized to the IM. As such, in order to explore whether LPS biosynthetic enzymes are glycosylated, it was first necessary to determine whether glycoproteins are present in the IM of *H. pylori.* As described in Section 3.2.2, several membrane glycoprotein candidates were detected via glycoprotein-specific labelling in total membrane samples (Figure 13). This represents potential glycoproteins in both the IM and OM. To determine if any of the labelled glycoproteins could be LPS biosynthetic enzymes, WT total membrane samples were separated into IM and OM components by differential solubilization of IM proteins in 1% N-lauroylsarcosine. The IM and OM fractions were subsequently labelled for glycoproteins in-solution with BHz and glycoproteins were detected with a fluorescent conjugate of streptavidin.

Several glycoprotein bands were detected in both the IM and OM protein fractions (Figure 14). The observation of glycoprotein bands in the IM fraction indicates that LPS biosynthetic enzymes may be glycosylated as all enzymes involved in LPS biosynthesis for the attachment and translocation of the lipid A core are integral IM proteins. Nevertheless, it is difficult to ascertain if any of the detected glycoprotein bands correspond to LPS biosynthetic enzymes based on molecular mass alone, as anomalous migration within SDS-PAGE gels has been reported for several integral IM proteins [241-243].

Figure 14: Detection of *H. pylori* inner membrane glycoproteins. WT total membrane samples were separated into IM and OM components by selective solubilization of IM proteins in 1% N-lauroylsarcosine. IM and OM proteins were labelled in-solution for glycoproteins with BHz (+) or without (-), separated by 12% SDS-PAGE, and transferred to nitrocellulose. Total proteins were stained with Ponceau S (A). Glycoproteins were detected by affinity of the BHz-label with a fluorescent conjugate of streptavidin (B). Several glycoprotein bands are evident in both the IM and OM fractions. The presence of IM glycoproteins corresponds with the hypothesis that LPS biosynthetic IM enzymes may be glycosylated and require glycosylation for function. M = molecular weight standards, T = transferrin (glycoprotein control), F = purified recombinant flagellins.



(A) Ponceau S (B) Streptavidin 680

In order to further investigate whether LPS biosynthetic enzymes are glycosylated, a candidate glycoprotein, WaaL, was chosen based on the LPS profile of the *hp0366* mutant. While all the enzymes involved in LPS biosynthesis could rely on glycosylation and be responsible for the defect observed in *hp0366*, the LPS profile of *hp0366* is O-antigen deficient. This is reminiscent of a defect in the O-antigen ligase, or WaaL, in which the O-antigen is not attached to the lipid A core prior to export of LPS tothe OM. Thus, efforts were focused on assessing the glycosylation status of WaaL in *H. pylori.*

3.3.2 Characterization of the candidate glycoprotein WaaL

Previous work in the Creuzenet Laboratory identified the WaaL in the sequenced *H. pylori* strain 26695 as *hp1039* based on a homology search to known WaaLs and comparison of hydrophobicity profiles [175]. In general, known WaaLs lack sequence similarity, but demonstrate similar secondary structures and membrane topology [67]. Comparison of the *H. pylori* WaaL, *hp1039,* to characterized WaaLs in *E. coli* K12 and *Salmonella enterica* indicated that they are similar in both the distribution and size of transmembrane domains [175]. As well, *hp1039,* possesses a Wzy_C domain (Pfam domain PF04932), which is annotated as an O-antigen polyermerase motif, but has in practice been found among O-antigen ligases. The hydrophobicity profile of *hp1039* indicated that the *H. pylori* WaaL contains 12 transmembrane domains with a large periplasmic loop of approximately 90 amino acids. This secondary structure is consistent with known WaaLs in which the large periplasmic loop has been shown to possess the catalytic site for ligase activity [244]. As such, if glycosylation does affect the activity of WaaL, it is likely to occur within the periplasmic loop. In fact, based on the sequence and

membrane topology of *hp1039,* several serine and threonine amino acid residues for O-linked glycosylation as well as one putative eukaryotic N-linked glycosylation sequence are present within the periplasmic loop [175].

The identification of *hp1039* as a true WaaL was further demonstrated by construction of a knockout mutant of *hp1039* [175]. The mutant displayed an O-antigen deficient LPS profile as expected of an O-antigen deficient strain and will be referred to as *waaL*. As anticipated, the O-antigen deficient profile matched that of *hp0366*, which supports the proposed theory that WaaL is non-functional in *hp0366* due to a glycosylation defect.

In order to investigate WaaL as a candidate glycoprotein, several obstacles needed to be overcome. Firstly, as an integral inner membrane protein, WaaL is expressed at low levels. To date, all WaaL proteins that have been characterized have been studied after overexpression [244, 245]. No WaaL has been purified from endogenous levels. However, in order to study glycosylation of WaaL in *H. pylori,* expression in another host was not possible. As well, overexpression of WaaL in *H. pylori* was undesired as the effects on glycosylation are unknown, such that overexpression may congest the glycosylation machinery resulting in detection of non-glycosylated forms of the protein. As well, the LPS machinery acts as a complex and may require strict stoichiometric interactions between complex components for proper function. Furthermore, likely due to difficulties in purification, no WaaL-specific antibody is available to detect WaaL. To accommodate these barriers, WaaL, under its native promoter, was tagged with a C-terminal hexa-Histidine tag on the shuttle vector pHel2 by Dr. Alexandra Merkx-Jacques. The Histidine-tag was incorporated to serve a dual purpose; to allow enrichment of WaaL

by nickel affinity chromatography and for its subsequent detection by anti-Histidine Western blot. Using this strategy, attempts to purify WaaL-His from *H. pylori* in order to assess its glycosylation status were performed.

3.3.2.1 Expression and localization of WaaL-His to the WT inner membrane

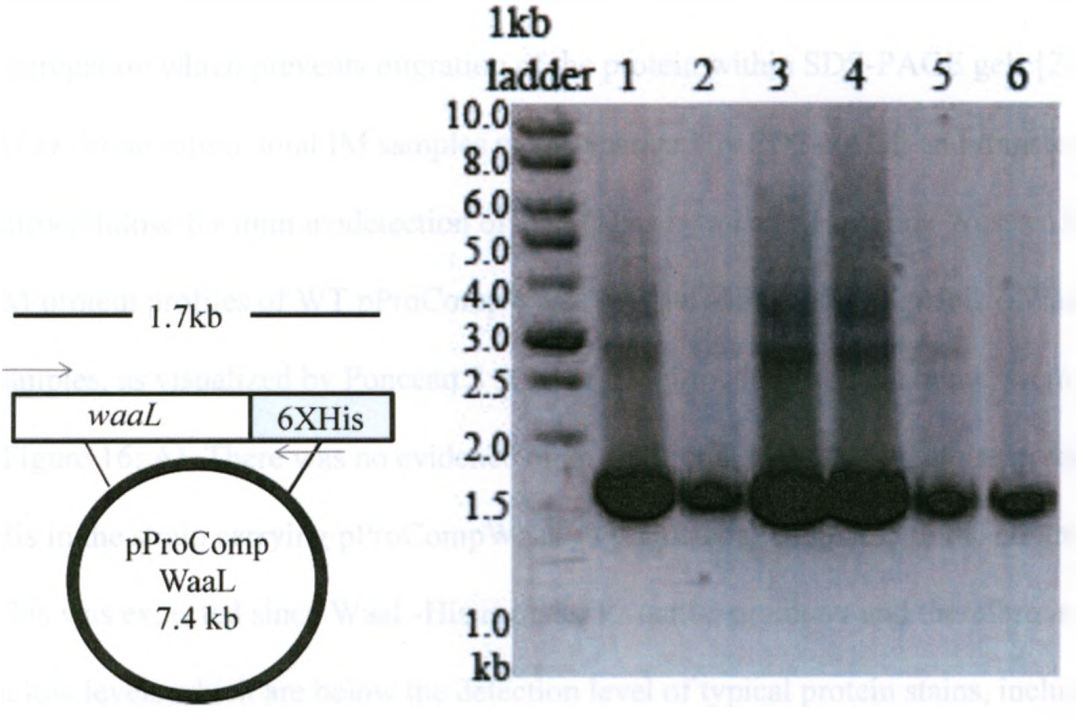
The shuttle plasmid encoding *H. pylori* WaaL *(hp1039)* under its native promoter with a C-terminal Histidine-tag, pProCompWaaL, was previously constructed by Dr. Alexandra Merkx-Jacques. The shuttle plasmid was treated with WT cell-free extract (CFE) for *in vitro* site-specific methylation of plasmid DNA in order to overcome the restriction-modification barrier present in *H. pylori,* as described in Section 3.1.4. The CFE-treated plasmid was transformed into WT *H. pylori* by electroporation. In tandem, WT *H. pylori* was also transformed with the backbone vector, pHel2, treated with WT CFE. This WT strain, carrying pHel2, was used as a negative control for WaaL-His expression in all experiments to verify that the signal detected by anti-Histidine Western blot was specific to WaaL-His expression and not from the plasmid backbone. Transformants were screened by colony PCR using primers specific for the promoter region of WaaL (HP1039P1) and for the C-terminal Histidine-tag (Ctermhis) (data not shown - see Figure 15). The primer specific for the C-terminal Histidine-tag was used to distinguish the foreign copy of WaaL-His from the endogenous *waaL.* To screen for pHel2 transformants, colony PCR for amplification of the CAT cassette from the vector was performed using primers pHel2CAT1 and pHel2CAT2.

As discussed in Section 3.1.4, limited gene transfer systems are available in *H. pylori.* While shuttle plasmids have been used with success, problems are encountered with plasmid stability in which plasmid loss can occur over time. To limit the occurrence

of plasmid loss, *H. pylori* strains carrying each plasmid were grown in a strict timeline with two cell expansions. Each growth period did not exceed 36 hours. As well, to further verify plasmid maintenance in each strain, after each expansion of WT strains carrying pProCompWaaL (+WaaL-His) or pHel2 (-WaaL-His), an aliquot of cells was used for plasmid purification and PCR amplification of WaaL-His or of the CAT cassette to ensure that each plasmid was retained during growth. PCR amplification from purified plasmid was necessary to assess the presence of the plasmid as pHel2 is a low copy vector [217] and very limited plasmid recovery is obtained from *H. pylori.* The presence of a 1.7kb band from PCR amplification of WaaL-His from pProCompWaaL extracted from *H. pylori* at each stage of growth verified that the plasmid was not lost (Figure 15). Similarly, a 750bp band from PCR amplification of the CAT cassette from pHel2 or pProCompWaaL extracted from *H. pylori* after each stage of growth indicated that both plasmids were not lost during growth (data not shown).

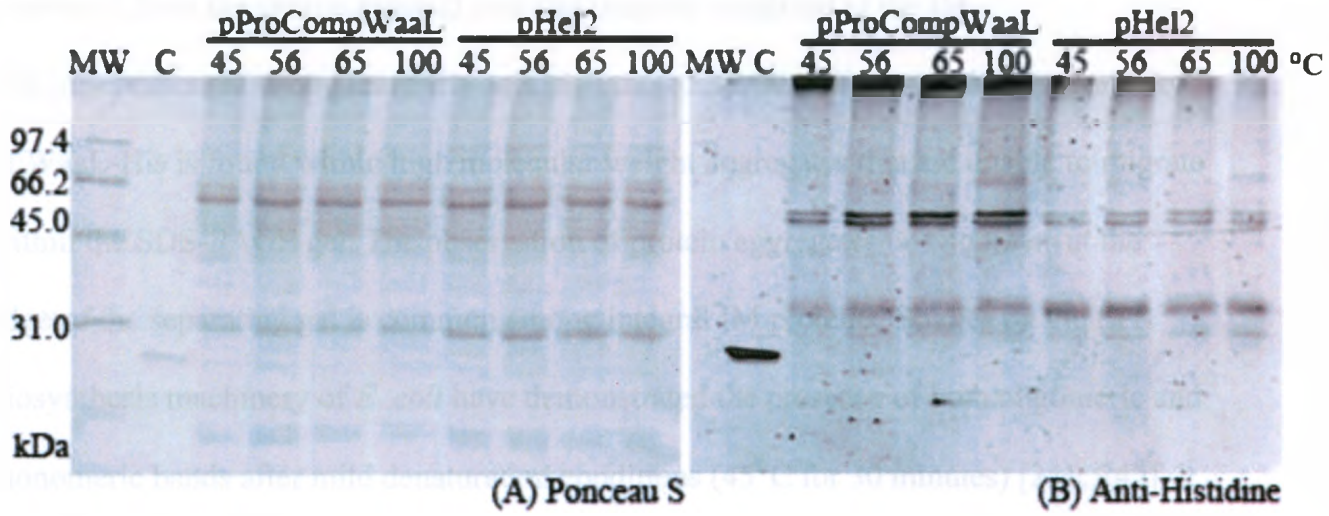
After validation of the presence of plasmid by PCR amplification, cell pellets of WT *H. pylori* carrying pProCompWaaL and WT pHel2 were lysed and the cell lysate separated by differential centrifugation. The total membrane pellet was further separated into IM and OM fractions. The initial cell separation acted to enrich samples in WaaL-His prior to purification by affinity chromatography as WaaL was expected to be localized to the IM. After separation, expression of WaaL-His was assessed in the IM fractions. Total IM samples of both WT pProCompWaaL (+WaaL-His) and WT pHel2 (-WaaL-His) were denatured by various conditions before separation by SDS-PAGE; incubation at 45^{0}C for 30 minutes, 56^{0}C for 10 minutes, 65^{0}C for 10 minutes or 100^{0}C for 5 minutes. Several denaturing conditions were tested as membrane proteins have

Figure 15: Verification of pProCompWaaL maintenance in WT *H. pylori* by PCR analysis of purified plasmid. To verify the presence of pProCompWaaL in WT *H. pylori* after each stage and round of growth for 36 hours on BHI-YE agar plates, plasmid was extracted from *H. pylori* by plasmid purification. Recovery of plasmid DNA from *H. pylori* is too low to ascertain the presence of the plasmid after plasmid purification alone. Therefore, purified plasmid DNA was used for PCR amplification of WaaL-His to detect pProCompWaaL. The 3'primer was specific for the C-terminal Histidine-tag to ensure amplification of WaaL-His from the vector rather than the endogenous copy of WaaL. Detection of a 1.7kb band from *H. pylori* at each stage of growth after each cell harvest indicated that pProCompWaaL was retained throughout each growth period. PCR amplification of WaaL-His was also conducted on the original pProCompWaaL vector extracted from *E. coli* as a PCR control at two dilutions (undiluted and 1/10). Lane 1: pProCompWaaL from *E. coli* undiluted, Lane 2: pProCompWaaL from *E. coli* 1/10 dilution, Lane 3: pProCompWaaL from *H. pylori,* final harvest, round 1, Lane 4: pProCompWaaL from *H. pylori,* final harvest, round 2, Lane 5: pProCompWaaL from *H. pylori,* first expansion, round 1, Lane 6: pProCompWaaL from *H. pylori,* first expansion, round 2.


1kb
ladder
1
2
3
4
5
6
10.0
8.0
6.0
5.0
4.0
3.0
2.5
2.0
1.5
1.0
kb
1.7kb
waaL
6XHis
pProComp
WaaL
7.4 kb

previously been reported to aggregate upon standard boiling for protein denaturation due to their high hydrophobic nature [243]. In fact, other integral IM proteins involved in LPS biosynthesis have been undetectable if boiled before SDS-PAGE due to protein aggregation which prevents migration of the protein within SDS-PAGE gels [241, 242]. After denaturation, total IM samples were separated by SDS-PAGE and transferred to nitrocellulose for immunodetection of WaaL-His by an anti-Histidine Western blot. Total IM protein profiles of WT pProCompWaaL (+WaaL-His) and WT pHel2 (-WaaL-His) samples, as visualized by Ponceau S stain of the nitrocellulose membrane, were similar (Figure 16: A). There was no evidence of an additional band that would represent WaaL-His in the strain carrying pProCompWaaL (+WaaL-His) compared to the control strain. This was expected since WaaL-His is under its native promoter and therefore is expressed at low levels which are below the detection level of typical protein stains, including Ponceau S. As well, no apparent difference in protein profile was observed from the various denaturing conditions. In order to detect expression of WaaL-His, an anti-Histidine Western blot was conducted (Figure 16: B). Reactivity of a ~50.0 kDa doublet was observed to be more intense in the samples containing pProCompWaaL (+WaaL-His) when denatured at 56^{0}C, 65^{0}C, or 100^{0}C, compared with the control strain. This doublet was later found to be due to non-specific signal from the secondary antibody alone and was not investigated further. However, a highly reactive, high molecular weight band was also detected in the IM samples from the strain with pProCompWaaL (+WaaL-His), but not from the control strain. This high molecular weight band was thought to be WaaL-His as it was specific to the WT strain carrying pProCompWaaL by

Figure 16: WaaL-His is expressed and localized to the inner membrane of WT *H. pylori* after expression *in trans*. IM samples of WT *H. pylori* carrying pProCompWaaL (+WaaL-His) or pHel2 (-WaaL-His) were denatured at various conditions; 45^{0}C for 30 minutes, 56^{0}C for 10 minutes, 65^{0}C for 10 minutes, and 100^{0}C for 5 minutes. Denatured samples were separated by 12% SDS-PAGE. Total protein was stained with Ponceau S (A). WaaL-His was detected by anti-Histidine Western blot (B). WaaL-His was apparent as a high molecular weight aggregate in samples containing pProCompWaaL (+WaaL-His). MW = molecular weight standards, C= control Histidine-tagged protein.


pProCompWaaL
pHel2
pProCompWaaL
pHel2
MW C 45 56 65 100 45 56 65 100 MW C 45 56 65 100 45 56 65 100 °C
97.4
66.2
45.0
31.0
kDa
(A) Ponceau S
(B) Anti-Histidine

anti-Histidine Western blot. The detection of WaaL-His indicated that WaaL-His was expressed from the shuttle plasmid and was properly localized to the IM.

The observation of strong reactivity as a high molecular band indicates that the majority of WaaL-His is found within high molecular weight aggregates that are unable to migrate within the SDS-PAGE gel. The observation of protein aggregates or oligomers at the edge of the separating gel is common among integral IM proteins. Studies on the LPS biosynthesis machinery of *E. coli* have demonstrated the presence of both oligomeric and monomeric bands after mild denaturation conditions (45^{0}C for 30 minutes) [241, 242]. It has been proposed that the mild denaturation conditions used to detect membrane proteins are insufficient to fully denature all protein aggregates resulting in the presence of high molecular weight oligomers [90, 242]. On the contrary, upon harsher denaturation by boiling, no protein monomers can be detected. In the case of WaaL-His, no monomers were observed, which suggests that the denaturing conditions tested were unable to fully denature protein aggregates containing WaaL-His.

Various strategies were employed in order to disrupt the aggregates. Firstly, IM samples were treated with 5M urea to remove aggregates as described previously for OM proteins by Marani *et al.* 2006 [224], however, no difference in the migration of WaaL-His was observed (data not shown). As well, IM samples were also denatured in a modified sample buffer containing urea as previously described for the detection of Wzx in *E. coli* [242], however, no difference in the detection of WaaL-His as a high molecular weight aggregate was observed (data not shown). In all cases, WaaL-His was detected as a high molecular weight aggregate, which suggests that the denaturation conditions tested for WaaL-His were insufficient to disperse protein aggregates from the IM.

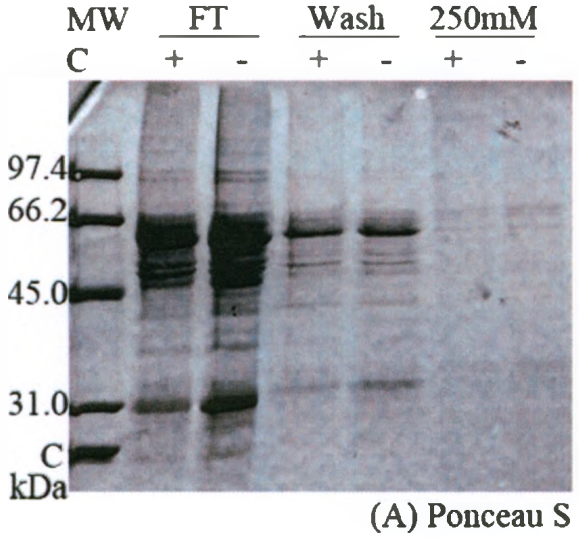
3.3.2.2 Enrichment of WaaL-His by nickel affinity chromatography

Although WaaL-His could not be resolved by SDS-PAGE, the presence of WaaL-His in the IM was observed as high molecular weight aggregates that were present in *H. pylori* containing pProCompWaaL (+WaaL-His) and not the control strain. In order to assess whether WaaL was glycosylated, nickel affinity chromatography was employed to further purify WaaL-His from the total IM samples.

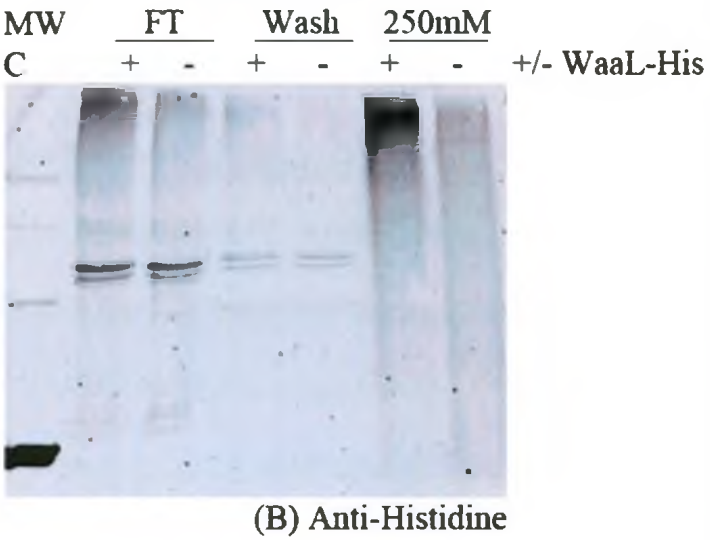
IM samples containing WaaL-His from *H. pylori* carrying pProCompWaaL (+WaaL-His), as well as the control samples from *H. pylori* carrying pHel2 (-WaaL-His) were processed through a gravity flow column of chelating sepharose resin loaded with nickel sulphate. Both samples were subjected to step-wise elution with increasing concentrations of imidazole. Fractions were analyzed by SDS-PAGE and detection of WaaL-His was performed by immunoblot for the Histidine-tag. WaaL-His was detected in the flowthrough and appeared to primarily elute with 250mM imidazole as a high molecular weight aggregate as observed by anti-Histidine Western blot (Figure 17). The high molecular weight band was not detected in the fractions collected from the control strain.

To determine the purity of WaaL-His, the 250mM elution fraction from WT pProCompWaaL (+WaaL-His) and the 250mM elution fraction from the control strain, were separated by SDS-PAGE and were stained with silver nitrate (Figure 18). Multiple protein bands were detected in samples with pProCompWaaL (+WaaL-His) indicating that WaaL-His was not purified by nickel affinity chromatography. However, the 250mM elutions containing WaaL-His from WT pProCompWaaL (+WaaL-His) was much simpler in protein content compared to the total IM fraction. Thus, although WaaL-His

Figure 17: Detection of WaaL-His in nickel affinity chromatography fractions. Total IM samples with pProCompWaaL (+WaaL-His) or pHel2 (-WaaL-His) were processed through gravity flow columns containing chelating sepharose resin charged with nickel sulphate. The flowthrough (FT), wash, and elution at 250mM fractions were analyzed on 12% SDS-PAGE gels. Total protein was stained with Ponceau S (A). WaaL-His was detected by anti-Histidine Western blot (B). WaaL-His was detected specifically in the flowthrough and 250mM imidazole elution from the WT pProCompWaaL (+WaaL-His) strain as a high molecular weight aggregate. MW = molecular weight standards, C = control Histidine-tagged protein.

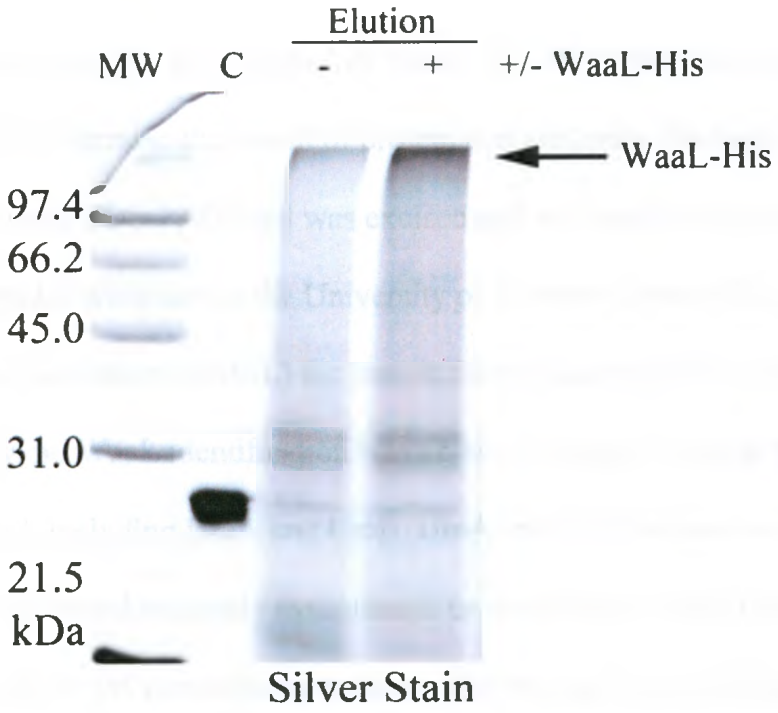


(A) Ponceau S



(B) Anti-Histidine

Figure 18: Analysis of WaaL-His purified by nickel affinity chromatography. To assess the purity of WaaL-His after nickel affinity chromatography, the 250mM imidazole elution fractions from WT pProCompWaaL (+WaaL-His) and WT pHel2 (-WaaL-His) were separated by 12% SDS-PAGE and total protein was stained with silver nitrate. Several protein bands were evident in both the elution from WT pProCompWaaL (+WaaL-His) and WT pHel2 (-WaaL-His) indicating that WaaL-His was only enriched by nickel affinity chromatography. MW = molecular weight standards, C = control Histidine-tagged protein.


Elution
MW
C
-
+
+/- WaaL-His
WaaL-His
97.4
66.2
45.0
31.0
21.5
kDa
Silver Stain

could not be fully purified by nickel affinity chromatography, this method allowed for enrichment of WaaL-His.

In order to verify the presence of WaaL-His within the high molecular weight aggregates and determine the extent of protein contaminants, the high molecular weight region on a 1-well SDS-PAGE gel was excised and subjected to trypsin digestion. The extracted peptides were sent to the University of Western Ontario Biological Mass Spectrometry Laboratory (BMSL) for protein identification by MS analysis. Unfortunately, no WaaL identification was obtained. Instead, several protein complexes were identified, including UreA and UreB. UreA and UreB are not known to be present in the IM and are predominantly cytoplasmic or surface associated [230]. Thus, their identification from IM preparations suggests that the region containing WaaL-His does consist of protein aggregates that were not solubilized by treatment with detergent, in addition to the insufficient protein denaturation of samples prior to SDS-PAGE. As well, it is possible that WaaL-His was expressed at levels too low for detection by MS. Furthermore, as WaaL-His was only enriched by affinity chromatography, the protein contaminants present may have a greater ability to be ionized. In particular, it is known that glycosylated peptides are much harder to detect because they are more difficult to ionize [187]. Thus, if WaaL-His was poorly expressed and our hypothesis is correct that WaaL is glycosylated, WaaL peptides would be masked by more abundant, well-ionized peptides, such as those of UreA and UreB. As well, peptides were extracted from SDS-PAGE gels by trypsin digestion. Trypsin is commonly used to prepare samples for MS analysis and cleaves the C-terminus of lysine and arginine residues [246]. Proximity to

post-translational modifications has been shown to inhibit protease activity [247]. Thus, if WaaL is glycosylated, trypsin may have been inadequate for peptide digestion.

Overall, it is evident that expression by this system was not sufficient to purify WaaL-His from WT *H. pylori.* It is likely that expression from the shuttle plasmid was poor. Moreover, problems with plasmid stability remain a problem. Although the presence of the shuttle plasmid was verified by PCR amplification for WaaL-His at each stage of growth, it is possible that the plasmid was highly unstable. Since purified plasmid is difficult to recover from *H. pylori*, PCR amplification was used to ascertain its presence. However, this does not provide quantitative information regarding the percentage of cells that still harbour the plasmid in the whole cell population and therefore, this may over-represent the number of cells that are carrying the plasmid. Thus, in order to investigate the potential glycosylation of WaaL, it will be beneficial to use a chromosomal integration system. As such, the expression system derived for expression of the recombinant flagellins via integration in *rdxA* in *H. pylori* may be used for the expression of WaaL-His.

3.4 Investigation of WaaL as a general glycosyltransferase

Both *pse* mutants demonstrated an altered LPS profile in which the O-antigen of the *flaA1* mutant was altered compared to WT and the LPS of the *hp0366* mutant was O-antigen deficient. The disruption of LPS biosynthesis in the *pse* mutants indicated that a connection exists between protein glycosylation and LPS biosynthesis in *H. pylori.* This prompted investigation of the direct glycosylation of LPS biosynthetic enzymes in *H. pylori* as described in Section 3.3. In particular, WaaL, the O-antigen ligase, was chosen as a candidate glycoprotein based on the O-antigen deficient profile of *hp0366.* For this

study, a *waaL (hp1039)* knockout mutant that was constructed previously was used [175]. Upon characterization of the *waaL* mutant, it was observed that in addition to an O-antigen deficient LPS profile, as expected of a *waaL* mutant, the mutant was also non-motile and lacked production of a proper flagellum [175]. This finding was surprising as WaaL is considered to function exclusively in LPS biosynthesis in *H. pylori*. No additional role of WaaL in *H. pylori* has been described previously. As such, the observation that inactivating WaaL abrogated flagellum production suggested that WaaL function is necessary for proper flagellum production.

Given the known function of WaaL as the O-antigen ligase, which amounts to saccharyltransferase activity similar to that of the oligosaccharyltransferases (OTases) found to be involved in protein glycosylation, it was theorized that WaaL could act as an OTase for glycosylation of the flagellins. However, a prior study demonstrated that while WaaL affects flagellum production, it is not involved in the glycosylation of the flagellins since the *waaL* flagellins were demonstrated to be glycosylated by glycoprotein-specific labelling [175]. Thus, this suggested that WaaL may be necessary for the glycosylation of other flagellar components required for proper flagellum assembly, and that WaaL may serve as a general OTase for these components.

The proposal of WaaL as a general OTase in *H. pylori* is further supported by the finding that WaaL is similar to the pilin glycosyltransferase of *Neisseria meningitidis*, PglL. PglL and WaaL exhibit similar hydrophobicity profiles in which both exhibit several transmembrane domains and a large periplasmic loop. Furthermore, WaaL is 46% similar to PglL, which is the same degree of similarity exhibited between the *H. pylori* WaaL and known WaaLs from other species [175]. As well, PglL was originally

identified as a ligase by homology with the Wzy_C domain (PFAM04932) [184]. Wzy_C domains are common among O-antigen ligases, including the *H. pylori* WaaL [175], however they have also been identified in transferases involved in protein glycosylation pathways including PilO, which is required for the O-glycosylation of pilin subunits in *P. aeruginosa* [153]. PilO and PglL have been classified as a new family of OTases, O-OTases, which mediate O-linked protein glycosylation [248]. A search conducted by Dr. Alexandra Merkx-Jacques for other proteins besides WaaL in *H. pylori* that encode a Wzy_C domain was unsuccessful, indicating that if an OTase for protein glycosylation was present in *H. pylori* the most likely candidate is WaaL. Interestingly, both PilO and PglL exhibit relaxed glycan specificity [249, 250]. In particular, PilO was demonstrated to transfer different O-antigens to the pilin subunits [249] while PglL has been shown to have extreme substrate promiscuity in which potentially any glycan can be transferred to the pilin subunits [250]. As such, if the *H. pylori* WaaL acts similarly as a general OTase, it may have relaxed glycan specificity for both the O-antigen chain and other glycans for protein glycosylation.

Nevertheless, if WaaL functions as an OTase in addition to the O-antigen ligase, the structure of the glycan transferred to potential glycoproteins is unknown. Therefore, to investigate whether WaaL could act as an OTase, it was examined whether the *H. pylori* WaaL could transfer its natural glycan, the Le^y O-antigen chain, to proteins in addition to lipid A.

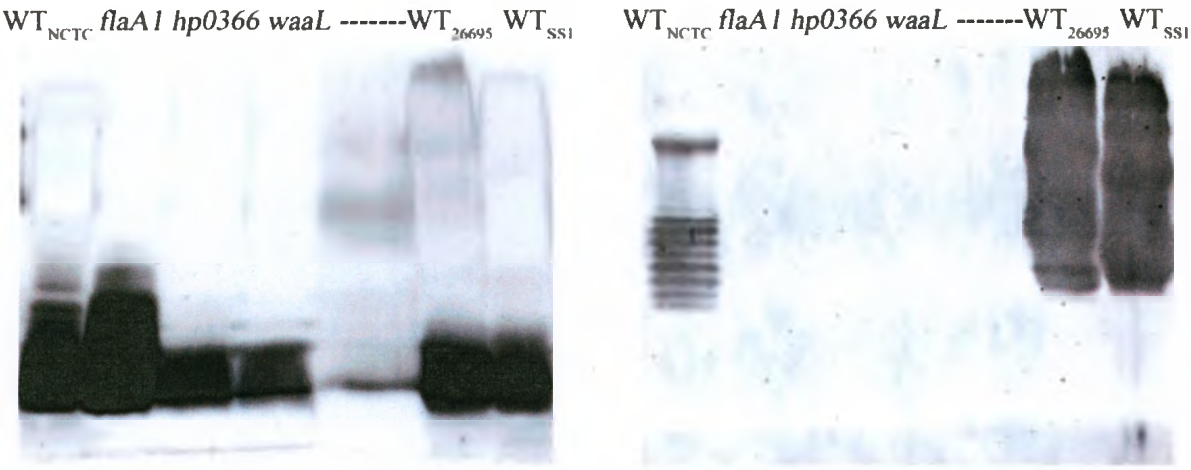
3.4.1 Detection of *H. pylori* O-antigen

In order to deduce whether O-antigen was present on *H. pylori* proteins it was necessary to develop a reliable method to detect *H. pylori* O-antigen. The O-antigen of *H.*

pylori strains are known to be decorated by sugars that mimic the Lewis blood group antigens found on human epithelial cells. This enables detection of *H. pylori* LPS by Western blot with antibodies specific for the Lewis antigens [86]. Previously, our laboratory WT strain was found to be Le^y-positive [118]. However, Lewis antigen expression is known to be phase variable in *H. pylori* and expression of Lewis antigens can be switched on or off within populations derived from a single cell [251]. Therefore, the expression of Lewis antigens needs to be validated consistently after *H. pylori* growth to account for potential phase variation. Furthermore, LPS samples with known Lewis antigen expression were required as positive controls for anti-Lewis Western blots to ensure that the antibodies were specific for O-antigen chains decorated by the different Lewis antigens.

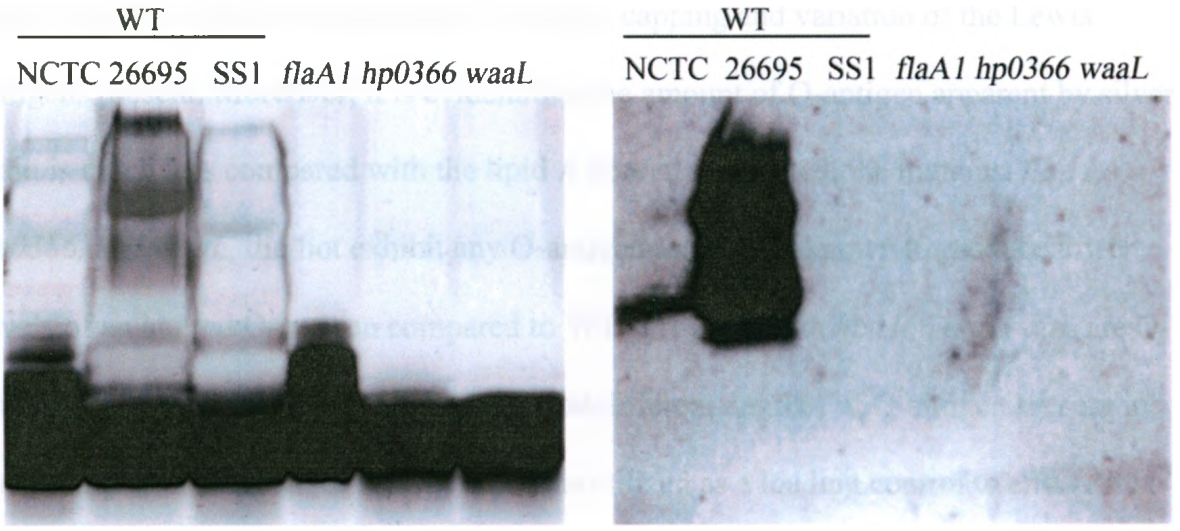
As such, in order to confirm the Lewis expression of our strains and obtain reliable LPS controls for Lewis Western blots, LPS samples were prepared from a panel of *H. pylori* strains expressing the Lewis antigens, Le^y and Le^x, relevant to our study and assessed for the presence of the Lewis antigens by Western blot. The *H. pylori* strains examined included the WT laboratory strain, NCTC 11637, and the isogenic mutants *flaA1, hp0366,* and *waaL,* as well as WT *H. pylori* strains 26695 [209] and SS1 [210]. LPS was extracted from total cell pellets by solubilization of total cell lysates with SDS, removal of proteins by proteinase K treatment, and the subsequent removal of residual proteins and peptides by phenol/chloroform extraction [221]. Each LPS sample was separated by 12% SDS-PAGE and visualized by silver nitrate staining (Figure 19: A, Figure 20: A). By silver stain, it was evident that the different WT strains of *H. pylori,* NCTC 11637, 26695, and SS1, exhibited different O-antigen patterns, which is a

Figure 19: SDS-PAGE analysis of *H. pylori* O-antigen with detection by anti-Le^y Western blot. LPS samples from the WT laboratory strain NCTC 11637 and the isogenic mutants, *flaA1, hp0366,* and *waaL,* as well as other WT strains of *H. pylori,* 26695 and SS1, were separated by SDS-PAGE. LPS was visualized by silver nitrate staining (A). The LPS of strain 26695 was highly reactive by silver nitrate staining and residual amounts from sample spill-over are apparent in the empty lane to the left side of the 26695 sample. The production of Le^y O-antigen was examined by anti-Le^y Western blot (B). All of the WT *H. pylori* strains were detected, indicating that the O-antigen of all of the WT strains tested were decorated with Le^y motifs. The isogenic mutants of the WT laboratory strain were not detected by anti-Le^y Western blot, as expected, since they are either O-antigen deficient or produce an altered O-antigen. The detection of only the LPS of the WT strains is consistent with known results indicating that the anti-Le^y Western blot was successful in detecting *H. pylori* Le^y O-antigen.



(A) Silver stain (B) Anti-Le^y

Figure 20: SDS-PAGE analysis of *H. pylori* O-antigen with detection by anti-Le^x Western blot. LPS samples from the WT laboratory strain NCTC 11637 and the isogenic mutants, *flaA1, hp0366,* and *waaL,* as well as other WT strains of *H. pylori,* 26695 and SS1, were separated by SDS-PAGE. LPS was visualized by silver nitrate staining (A). The production of Le^x O-antigen was examined by anti-Le^x Western blot (B). Only the strain 26695 was detected, indicating that the O-antigen of this strain carries Le^x motifs. It is apparent that some of the signal from strain 26695 spilled over into the WT NCTC 11637 lane due to the high level of reactivity. The lack of reactivity of the LPS of the other *H. pylori* strains is consistent with previous observations indicating that the anti-Le^x Western blot was successful in specifically detecting *H. pylori* Le^x O-antigen.



(A) Silver stain (B) Anti-Le^x

reflection of the different degrees of O-antigen capping and variation of the Lewis antigens present. Moreover, it is evident that the amount of O-antigen apparent by silver stain is much less compared with the lipid A core. As expected, the mutants, *flaA1, hp0366,* and *waaL,* did not exhibit any O-antigen as *flaA1* is known to produce lower levels of an altered O-antigen compared to WT [118] and both *hp0366* and *waaL* are O-antigen deficient [174, 175]. Thus, due to the different degrees of O-antigen present in each sample, the amount of lipid A core was utilized as a loading control to ensure that approximately the same amount of LPS was examined for each strain.

The presence of Le^y and Le^x Lewis antigens was assessed by anti-Le^y and anti-Le^x Western blots. By anti-Le^y Western blot, all WT strains were reactive, indicating the presence of Le^y O-antigen in each strain (Figure 19: B). The reactivity of the WT strains by anti-Le^y Western blot is consistent with previous observations [69, 252]. Conversely, despite similar LPS loading for the mutants, *flaA1, hp0366,* and *waaL,* no Le^y O-antigen was detected by anti-Le^y Western blot. This was expected as *hp0366* and *waaL* lack O-antigen and *flaA1* produces an altered O-antigen that was previously shown to exhibit limited reactivity by anti-Le^y Western blot [118]. The ability to detect Le^x O-antigen by anti-Le^x Western blot was also examined by testing the reactivity of the LPS from each strain via an anti-Le^x Western blot. By anti-Le^x Western blot, only the WT strain 26695 was reactive (Figure 20: B). This is consistent with previous observations for each of the *H. pylori* strains [69, 174, 175, 252]. Indeed, the presence of both Le^y and Le^x is well established in strain 26695 due to the lower relative activities of the FucTs that mediate Lewis antigen expression that limit the conversion of Le^x structures to Le^y [84, 85]. The apparent reactivity observed for the LPS of the WT NCTC strain is due to sample spill-

over from the 26695 sample as the LPS of 26695 reacts very strongly by anti-Le^x Western blot. For the NCTC signal to be real, a ladder-like pattern would be evident as observed by anti-Le^y Western blot for the same strain. The lack of reactivity by anti-Le^x Western blot from our WT strain is consistent with previous observations in the laboratory [118].

Overall, the consistency of Lewis antigen detection on the LPS of the various *H. pylori* strains screened with known or previously observed Lewis antigen expression, demonstrates that immunoblotting is a reliable method to detect O-antigen. As well, screening of the panel of laboratory *H. pylori* strains for Lewis antigen reactivity by Western blot verified that the O-antigen of our laboratory WT strain, NCTC 11637, is Le^y-positive, while strain 26695 exhibits both Le^y and Le^x antigens, making it an adequate control for anti-Le^y and anti-Le^x Western blots. Thus, anti-Le^y and anti-Le^x Western blots provide a means to examine the presence of O-antigen on proteins.

3.4.2 Demonstration of glycoproteins modified with O-antigen in *H. pylori* cell fractions

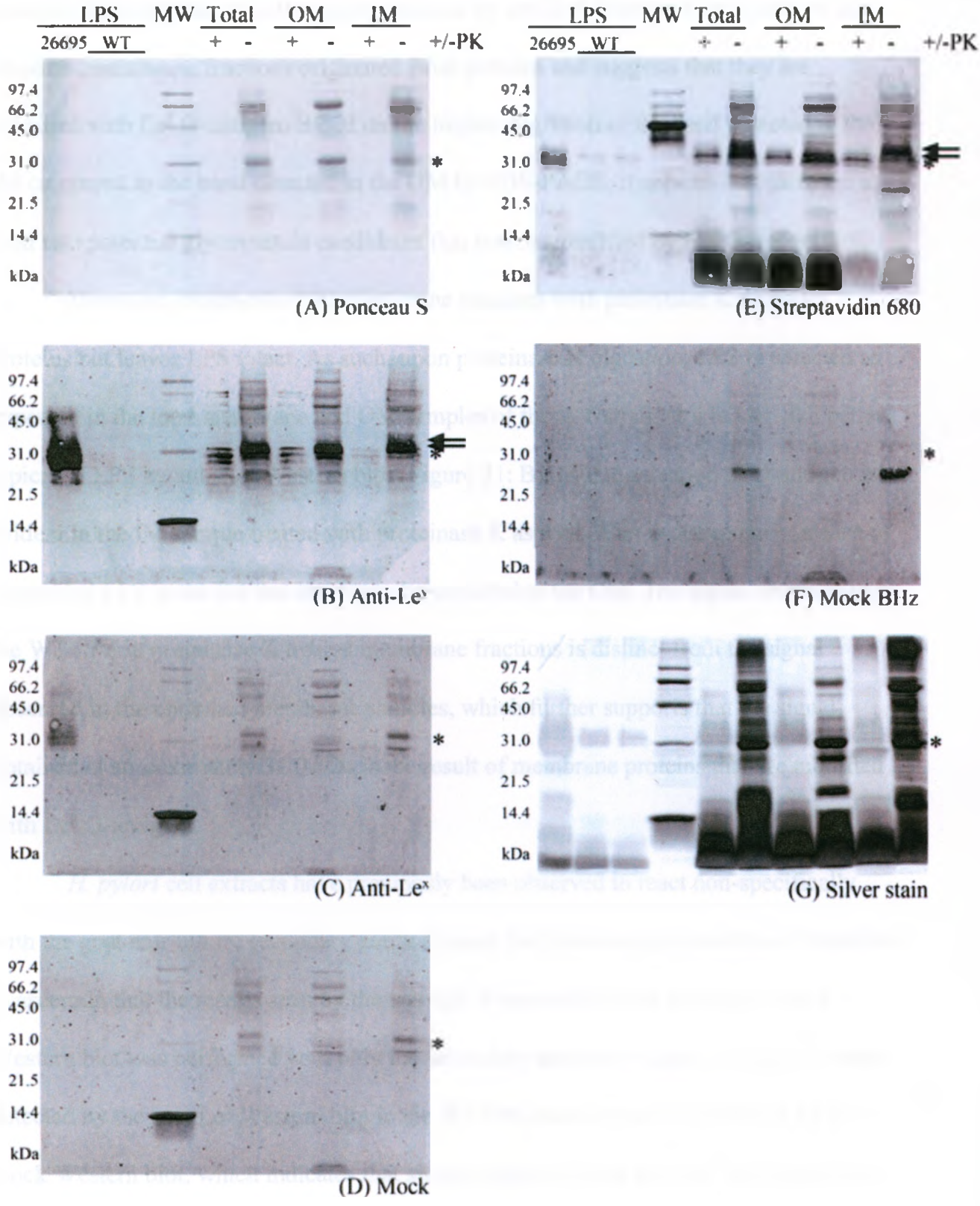
Once detection of the Lewis antigens on the O-antigen of *H. pylori* LPS by Western blot was optimized, it was possible to assess whether *H. pylori* proteins were modified with the O-antigen chain by WaaL. It is predicted that WaaL only has periplasmic activity since its catalytic site is present within a large periplasmic loop [244], therefore, if WaaL acts as a general OTase, only periplasmic and membrane proteins would be accessible to WaaL for modification. Thus, to investigate if periplasmic or membrane proteins were modified with O-antigen, the soluble cell fraction, which contains both cytoplasmic and periplasmic proteins, and the membrane

cell fractions of *H. pylori*, were examined for the presence of proteins that react by anti-Le^y and anti-Le^x Western blots.

WT *H. pylori* cellular proteins were separated by differential centrifugation into soluble, insoluble, total membrane, IM, and OM fractions, as outlined previously (refer to Figure 8). The membrane protein fraction was fractionated into IM and OM components to allow detection of modified proteins in each membrane. The total membrane fraction, and separated IM and OM protein fractions were separated by 12% SDS-PAGE and analyzed by immunoblotting. Total proteins transferred to the nitrocellulose membrane were stained with Ponceau S (Figure 21: A). O-antigen was detected by anti-Le^y (Figure 21: B) and anti-Le^x (Figure 21: C) Western blots. LPS from strain 26695 was used as a control for the anti-Le^y and anti-Le^x Western blots and was detected as expected.

By anti-Le^y Western blot, several bands were detected in the total membrane fraction and in the IM and OM fractions (Figure 21: B). The bands detected by anti-Le^y Western blot in the total membrane fraction appear to be separated between the IM and OM fractions. This is most evident with the principal reactive band of approximately 31.0 kDa in the total membrane fraction that appears to represent at least two proteins, one in the OM and one in the IM, that migrate slightly differently by SDS-PAGE. The IM reactive protein appears to migrate slightly slower than that of the band present in the OM. To determine if the signal observed by anti-Le^y Western blot in each membrane fraction was due to the presence of LPS in the samples or from proteins modified with O-antigen, each membrane fraction was treated with proteinase K to degrade all of the proteins present in the samples. Significantly, the predominant band of approximately 31.0 kDa detected in each membrane fraction was absent upon proteinase K digestion of

Figure 21: Demonstration of WT *H. pylori* membrane proteins modified with Le^y O-antigen by anti-Le^y and anti-Le^x Western blots and by glycoprotein-specific labelling. WT cellular fractions of total membrane and separated IM and OM components were treated with (+) and without (-) proteinase K (+/- PK) to specifically degrade all proteins in the samples. The treated and untreated samples with proteinase K were separated by 12% SDS-PAGE. Total proteins transferred to nitrocellulose were stained with Ponceau S (A). A representative membrane stained with Ponceau S is shown for all Western blots. O-antigen was detected by anti-Le^y (B) and anti-Le^x (C) Western blots. To ensure that detection was specific to O-antigen, a mock secondary only Western blot was performed (D). Glycoproteins were detected by glycoprotein-specific labelling with BHz (E). The specificity of glycoprotein labelling was determined by performing a mock labelling without BHz (F). Total proteins and LPS in each of the fractions were detected by silver stain (G). It is evident that proteinase K treatment degraded all of the proteins from each sample, revealing a distinct LPS pattern in treated samples, detected by both anti-Le^y Western blot and silver nitrate staining. Two independent glycoprotein candidates were observed by anti-Le^y Western blot and glycoprotein labelling, one each in the IM and OM that migrate slightly differently by SDS-PAGE at approximately 31.0 kDa (indicated by arrows). The glycoprotein candidates migrate slightly higher than the abundant 31.0 kDa protein denoted with an asterisk for reference to compare Western blots and total protein detected by Ponceau S and silver stain. No specific bands were detected by anti-Le^x Western blot, indicating that no WT membrane proteins were modified with Le^x O-antigen. A sample of strain 26695 LPS was run as a control for all Western blots.


LPS
26695 WT
MW
Total
OM
IM
+ - + - + -
+/-PK
97.4
66.2
45.0
31.0
21.5
14.4
kDa
*
(A) Ponceau S
(E) Streptavidin 680
(B) Anti-Le
(F) Mock BHz
(C) Anti-Le
(G) Silver stain
(D) Mock

proteins. This indicates that the signal obtained by anti-Le^y Western blot in each of the untreated membrane fractions originated from proteins and suggests that they are modified with Le^y O-antigen. Based on the higher migration of the band detected in the IM compared to the band detected in the OM by SDS-PAGE, it appears that there are at least two potential glycoprotein candidates that may be modified by Le^y O-antigen.

Moreover, treatment of the membrane fractions with proteinase K degrades proteins but leaves LPS intact. As such, upon proteinase K digestion, LPS is detected as expected in the total membrane and OM samples of the WT strain as a ladder-like pattern typical of LPS by anti-Le^y Western blot (Figure 21: B). Minor detection of O-antigen is evident in the IM sample treated with proteinase K as well. This is due to the presence of assembled LPS in the IM that has yet to be exported to the OM. The signal obtained from the WT LPS in proteinase K treated membrane fractions is distinct from the signal observed in the untreated membrane samples, which further supports that the signal obtained at approximately 31.0 kDa is the result of membrane proteins that are modified with Le^y O-antigen.

H. pylori cell extracts have previously been observed to react non-specifically with the goat anti-mouse secondary antibody used for Lewis antigen detection. Therefore, to ascertain that the results seen by the anti-Le^y Western blot were genuine, a mock Western blot was performed with only the secondary antibody (Figure 21: D). The band detected by the anti-Le^y Western blot in the WT OM fraction was not observed by the mock Western blot, which indicates that signal obtained by the anti-Le^y Western blot is real and that the detected protein is modified with O-antigen. Conversely, a band was detected at approximately 31.0 kDa in the WT total membrane fraction and IM fraction in

the mock Western blot. However, the band was detected at a lower intensity compared to the bands observed in these fractions by anti-Le^y Western blot. This non-specific band appears to migrate similarly to the band reactive in the total membrane fraction, but slightly lower than the band detected in the IM fraction by anti-Le^y Western blot. Together, the lower reactivity in the mock Western blot and the slightly lower migration of the non-specific band suggests that the protein band detected in the IM by anti-Le^y is distinct from the non-specific signal observed by the mock Western blot and also represents a glycoprotein modified with Le^y O-antigen. Comparatively, the non-specific band detected by the mock Western blot in the total membrane fraction likely results from non-specific binding of the secondary antibody to an unknown IM protein as the same signal is observed in the IM fraction but not the OM fraction.

The membrane fractions were also assessed for the presence of O-antigen decorated with Le^x antigens. This was conducted to determine whether the same Le^y O-antigen chain was attached to both lipid A and proteins or if a modified O-antigen, such as Le^x, could be attached to glycoproteins even if it is not present on the WT LPS. Interestingly, the ability to attach different O-antigen chains to proteins has been observed for the pilin OTase PilO of *P. aeruginosa* [249]. By anti-Le^x Western blot, a number of faint bands were detected in the membrane fractions in addition to the WT 26695 LPS control (Figure 21: C). However, it is apparent that the bands detected by anti-Le^x Western blot are the same bands that are detected by the mock secondary only Western blot (Figure 21: D) and therefore are due to non-specific binding of the secondary antibody to *H. pylori* membrane proteins. Thus, this data establishes that no glycoproteins in the WT strain are modified by Le^x O-antigen.

To further demonstrate the presence of carbohydrates on the Le^y-reactive glycoproteins, the WT membrane fractions were assessed for glycosylation by glycoprotein-specific labelling of carbohydrates with BHz. Labelling was performed on the nitrocellulose membrane and labelled glycoproteins were detected with a fluorescent conjugate of streptavidin (Figure 21: E). LPS was detected by BHz labelling, which is expected given that this method of chemical labelling relies on the oxidation of carbohydrates. Despite detection of LPS, a distinct signal was observed at approximately 31.0 kDa in each of the membrane fractions that was absent upon proteinase K digestion. The bands detected correspond to the migration of the protein bands detected by anti-Le^y Western blot (Figure 21: B). Further, the detected bands were demonstrated to be specific to BHz labelling as a nitrocellulose membrane that was mock labelled in the same manner, but without BHz, did not show the same protein bands (Figure 21: F).

Lastly, total proteins and LPS were stained by silver nitrate (Figure 21: G). Again, it is evident that upon proteinase K treatment of each membrane fraction, the distinct ladder-like pattern of LPS can be observed, similar to the control LPS preparations of WT and strain 26695.

Together, the demonstration of a specific band reactive by both anti-Le^y Western blot (Figure 21: B) and by glycoprotein-specific labelling (Figure 21: E) in each of the IM and OM fractions indicates that at least two membrane glycoproteins are present in *H. pylori* that are modified with Le^y O-antigen. As such, this suggests that WaaL may be acting as a general OTase that transfers its natural substrate, Le^y O-antigen, onto these proteins.

To investigate whether WaaL can also modify periplasmic proteins in addition to membrane proteins, the soluble protein fraction was separated by 12% SDS-PAGE and immunoblotting was performed with anti-Le^y and anti-Le^x antibodies and a mock Western blot with the secondary antibody only. However, by both anti-Le^y and anti-Le^x Western blots no proteins were specifically detected compared to the mock Western blot (data not shown). This indicates that no periplasmic or soluble proteins are modified by Le^y O-antigen and that only membrane proteins appear to be targeted by WaaL.

3.4.3 Examination of the dependence of the O-antigen-modified membrane glycoprotein candidates on WaaL and pse biosynthesis

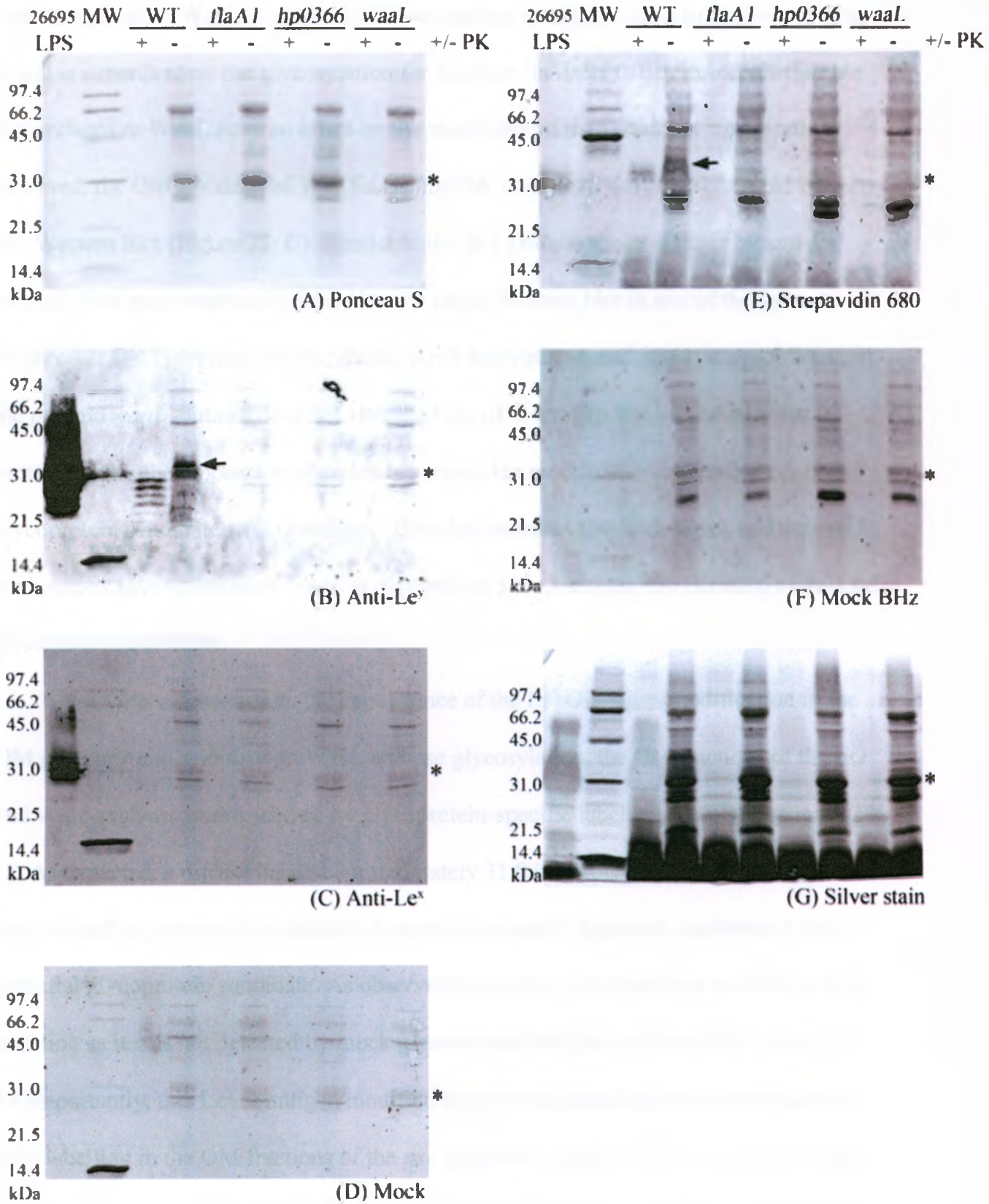
Several findings indicate that LPS biosynthesis and protein glycosylation in *H. pylori* are interconnected. As well, it is proposed that the WaaL ligase of *H. pylori* functions as both the O-antigen ligase and as a general OTase that is able to modify proteins. Firstly, the *pse* mutant, *hp0366,* is O-antigen deficient, suggesting that WaaL activity is abrogated upon disruption of pse. As well, the *H. pylori waaL* mutant lacks proper flagellum production in addition to being O-antigen deficient. This unexpected role of WaaL on flagellum production is suspected to be due to the modification of flagellar components by WaaL. The presence of *H. pylori* membrane proteins modified with Le^y O-antigen was demonstrated by the specific detection of Le^y O-antigen on proteins by anti-Le^y Western blot. However, the dependence of the glycosylation of these proteins on WaaL and pse biosynthesis could not be established from the WT alone. Therefore, based on the above hypotheses and findings, it is proposed that if WaaL acts as a general OTase and is responsible for the modification of the WT membrane proteins with Le^y O-antigen, the Le^y O-antigen should be absent from the membrane protein

fractions of the *waaL* mutant. Similarly, if WaaL function is dependent on pse-specific glycosylation, the modification of proteins with O-antigen should not occur in the *pse* mutants.

To investigate the reliance of the Le^y O-antigen modified membrane glycoprotein candidates on WaaL and pse glycosylation, the total membrane fractions of WT, *flaA1, hp0366,* and *waaL H. pylori* strains were separated into OM and IM components. While both OM and IM will need to be analyzed to assess the glycoprotein candidate in each membrane fraction, initial analyses were performed on the OM. The OM fraction of each strain was treated with and without proteinase K, separated by 12% SDS-PAGE, and assessed for the presence of O-antigen as described above for the WT membrane fractions.

The presence of the OM glycoprotein candidate modified with the Le^y O-antigen in the OM of WT, *flaA1, hp0366,* and *waaL* was assessed by anti-Le^y Western blot (Figure 22: B). The same approximately 31.0 kDa protein-dependent band was observed in the WT OM fraction as observed previously. By the separation obtained by this Western blot, two potential reactive bands are evident in the WT OM. One of the bands may correspond to the protein detected by Ponceau S stain at approximately 31.0 kDa and the other migrates slightly higher which is not detected by Ponceau S (Figure 22: A). Alternatively, the glycoprotein of interest may appear as a doublet to reflect the modification with O-antigen in which O-antigen is typically visualized as a ladder-like pattern. The same doublet was not detected in the OM fractions of the *pse* mutants and *waaL.* This indicates that the modification of the OM glycoprotein candidate with O-antigen is dependent on both WaaL and pse glycosylation. This observation matches the

Figure 22: Demonstration of the dependence and specificity of the Le^y O-antigen modification of the WT OM glycoprotein candidate on WaaL and pse biosynthesis. The OM fractions of WT, *flaA1, hp0366,* and *waaL* were treated with (+) and without (-) proteinase K (+/- PK) to degrade all proteins in the samples. The treated and untreated samples with proteinase K were separated by 12% SDS-PAGE. Total proteins transferred to nitrocellulose were stained with Ponceau S (A). A representative membrane stained with Ponceau S is shown for all Western blots. O-antigen was detected by anti-Le^y (B) and anti-Le^x (C) Western blots. To ensure that detection was specific to O-antigen, a mock secondary only Western blot was performed (D). Glycoproteins were verified by glycoprotein-specific labelling with BHz (E). Specificity of glycoprotein detection by glycoprotein labelling was controlled for by performing a mock labelling without BHz (F). Total proteins and LPS in each of the fractions were detected by silver stain (G). It is evident that proteinase K treatment was incomplete as low molecular weight proteins are evident in treated samples by silver stain. Regardless, a distinct protein-dependent band of approximately 31.0 kDa was detected by anti-Le^y Western blot and glycoprotein labelling as expected in the WT OM (indicated by an arrowhead). The OM glycoprotein candidate migrates slightly higher than the abundant 31.0 kDa protein denoted with an asterisk for reference. A similar band was not detected in the *pse* and *waaL* mutants, indicating that Le^y O-antigen modification of the OM glycoprotein candidate is dependent on pse biosynthesis and WaaL. A sample of strain 26695 LPS was run as a control for all Western blots.


26695 MW WT flaA1 hp0366 waaL
LPS + - + - + - + - +/- PK
97.4
66.2
45.0
31.0
21.5
14.4
kDa
*
(A) Ponceau S
(B) Anti-Le^Y
(C) Anti-Le^X
(D) Mock
(E) Strepavidin 680
(F) Mock BHz
(G) Silver stain

results expected if WaaL is a general OTase that can modify proteins in addition to lipid A and is dependent on pse glycosylation for function. In order to determine whether pse biosynthesis or WaaL have an effect on the specificity of the O-antigen modification observed, the OM fractions of WT, *flaA1, hp0366,* and *waaL* were also analyzed by anti-Le^x Western blot (Figure 22: C). Similar to the WT OM, no signal distinct by anti-Le^x Western blot was observed compared to the mock Western blot in any of the mutants (Figure 22: D). Therefore, the disruption of pse biosynthesis and inactivation of WaaL in the *pse* and *waaL* mutants does not alter the type of O-antigen that can be attached to proteins, but rather appears to completely prevent the modification of the OM glycoprotein candidate with O-antigen. This also indicates that both WaaL and potential pse-specific glycosylation of WaaL is required for Le^y O-antigen modification of the OM glycoprotein candidate.

To further demonstrate the dependence of the Le^y O-antigen modification of the OM glycoprotein candidate on WaaL and pse glycosylation, the OM fractions of the *pse* and *waaL* mutants were analyzed by glycoprotein-specific labelling with BHz (Figure 22: E). As expected, a diffuse band at approximately 31.0 kDa was detected in the WT OM fraction and its presence was abolished upon proteinase K digestion, indicating it was a potential glycoprotein candidate. As observed previously, this band was specific to BHz labelling as it was not detected by mock glycoprotein labelling without BHz (Figure 22: F). Importantly, this Le^y O-antigen modified glycoprotein candidate was not evident by BHz labelling in the OM fractions of the *pse* and *waaL* mutants. This also suggests that the glycoprotein candidate observed to be modified with Le^y O-antigen in the WT OM is dependent on both WaaL and pse glycosylation.

Total proteins and LPS from the OM fraction of WT and the *pse* and *waaL* mutants were stained by silver nitrate (Figure 22: G). It is evident that proteinase K digestion of all proteins in the samples was not complete as similar low molecular weight bands are present in the proteinase K treated fractions of all *H. pylori* strains. However, the majority of proteins in the samples have been degraded, especially in the range of molecular weight greater than 31.0 kDa around the glycoprotein candidate of interest. WT LPS was detected in the WT OM sample treated with proteinase K by both silver staining (Figure 22: G) and anti-Le^y Western blot (Figure 22: B). No LPS was detected in the OM fractions of *flaA1, hp0366,* and *waaL* as expected since *hp0366* and *waaL* are O-antigen deficient and *flaA1* produces an altered O-antigen that is only visible upon much higher loading than present on this gel. By silver stain, it was apparent that the approximately 31.0 kDa region where the Le^y O-antigen modified OM glycoprotein candidate is observed is quite complex and therefore will require further separation for MS analysis and its identification.

In addition, the dependence of the Le^y O-antigen modification of the IM glycoprotein candidate on WaaL and pse glycosylation was assessed in the same manner (data not shown). Interestingly, this glycoprotein candidate appears to be dependent on strict growth conditions and further optimization of the conditions required for its production is required before its production in the *pse* and *waaL* mutants can be determined.

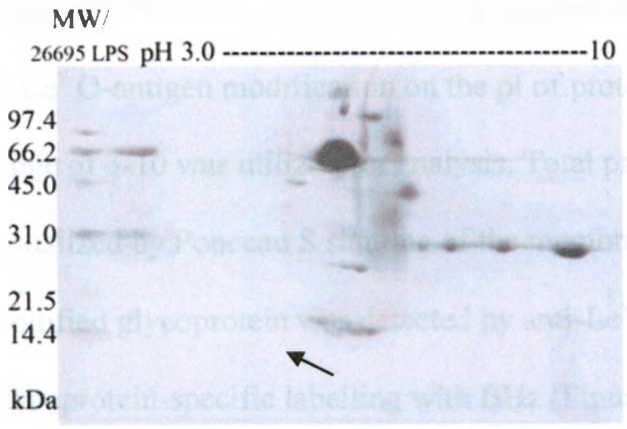
3.4.4 Analysis of the Le^y O-antigen modified membrane glycoprotein candidates by 2-dimensional gel electrophoresis (2-DE)

Two membrane glycoprotein candidates modified with Le^y O-antigen were observed in the membrane fraction of WT *H. pylori:* one in the IM and one in the OM. As the *waaL* mutant was previously shown to exhibit a defect in flagellum production, it was proposed that WaaL may act as a general OTase to modify components of the flagellar apparatus required for flagellum assembly. To determine if the membrane glycoprotein candidates modified with Le^y O-antigen are part of the flagellar apparatus, identification of the glycoprotein candidates is required. Since the *H. pylori* flagellar apparatus is similar to the prototypical type III secretion flagellar machinery described among Gram-negative bacteria and transverses both the cytoplasmic and outer membranes [99], it is possible that both glycoprotein candidates may be flagellum components.

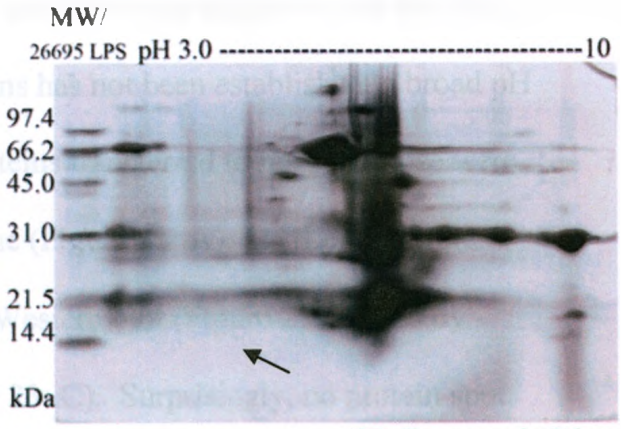
Unfortunately, the IM and OM fractions of *H. pylori* appear complex by 1D SDS-PAGE with several proteins visible that migrate similar to the glycoprotein candidates. Therefore, before the glycoprotein candidates can be identified by MS analysis, the IM and OM fractions need be simplified in order to enhance detection of the glycoprotein candidates over non-glycosylated proteins. Thus, 2-DE was employed to separate the glycoprotein candidates from non-glycosylated proteins.

Efforts were first focused on identifying the OM glycoprotein candidate as no growth requirements were observed for its production and OM proteins are believed to be more compatible with the conditions used for isoelectric focusing for the first dimension of 2-DE compared to IM proteins [253, 254]. As such, the WT OM fraction was

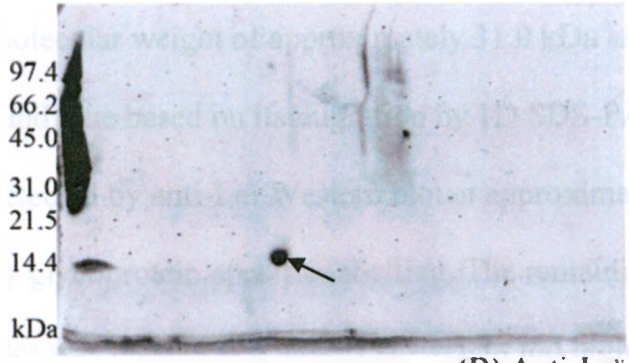
Figure 23: Detection of the OM glycoprotein candidate modified with Le^y O-antigen by anti-Le^y Western blot and glycoprotein labelling after separation by 2-DE. The WT OM fraction containing the OM glycoprotein candidate modified with Le^y O-antigen was separated by 2-DE with a broad pH range of 3-10. Total proteins transferred to nitrocellulose were detected by Ponceau S (A). A representative membrane stained with Ponceau S is shown for all Western blots. The Le^y O-antigen modified OM glycoprotein candidate was detected by anti-Le^y Western blot (B) and by glycoprotein-specific labelling with BHz (C). A distinct protein spot with a molecular weight of approximately 14.5 kDa was detected by both methods. The same spot was not evident by Coomassie Blue staining of total protein despite high protein loading (D). This indicates that the glycoprotein candidate of interest modified with Le^y O-antigen is of too low abundance to be detected by MS analysis. The location of the protein spot detected is indicated by an arrow.



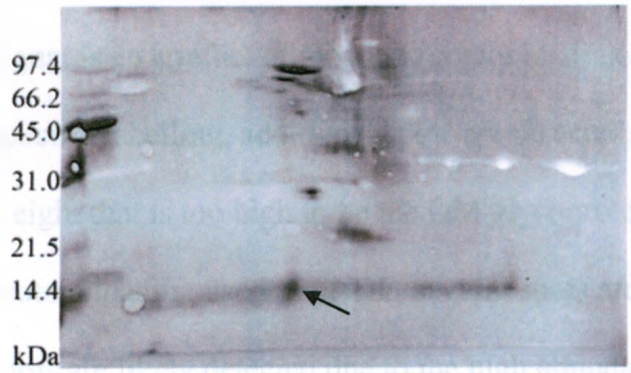
(A) Ponceau S



(D) Coomassie Blue



(B) Anti-Le^y



(C) Streptavidin 680

separated by 2-DE. As the pI of the glycoprotein candidate was unknown and the effect of Le^y O-antigen modification on the pI of proteins has not been established, a broad pH range of 3-10 was utilized for analysis. Total proteins transferred to nitrocellulose were visualized by Ponceau S staining of the membrane (Figure 23: A). The Le^y O-antigen modified glycoprotein was detected by anti-Le^y Western blot (Figure 23: B) and by glycoprotein-specific labelling with BHz (Figure 23: C). Surprisingly, no protein spot was detected by either anti-Le^y Western blot or by glycoprotein-specific labelling with a molecular weight of approximately 31.0 kDa as anticipated for the OM glycoprotein candidate based on its migration by 1D SDS-PAGE. However, a predominant spot was detected by anti-Le^y Western blot at approximately 14.5 kDa. A similar spot was detected by glycoprotein-specific labelling. The remaining signal observed by anti-Le^y Western blot was present at a molecular weight too high to be the OM glycoprotein candidate of interest and appears as a smear in the second dimension, which suggests that this residual signal is an artefact of the high protein loading used for 2-DE. Similarly, by glycoprotein-specific labelling, additional spots are detected, however, they also exhibit a molecular weight that is too high to be the OM glycoprotein candidate. As well, due to the high protein loading used for 2-DE, several spots are detected by negative staining. These spots are likely detected due to the high abundance of these proteins. Therefore, as no other common signal between the anti-Le^y Western blot and glycoprotein-specific labelling was observed, the detected protein spot of approximately 14.5 kDa is likely the OM glycoprotein candidate of interest that is modified with Le^y O-antigen.

The identification of a 14.5 kDa protein spot rather than the expected molecular weight of approximately 31.0 kDa suggests that the original 31.0 kDa protein detected by

1D SDS-PAGE in the OM fraction may have been a dimer of the 14.5 kDa protein detected by 2-DE. It is possible that the denaturing conditions used for 1D SDS-PAGE were insufficient to disrupt oligomers. This is consistent with the fact that OM proteins are known to be resistant to SDS solubilization [255]. Comparatively, the conditions used for 2-DE involve additional chaotropes and detergents which may be more capable of disrupting protein oligomers and results in the detection of the OM glycoprotein candidate at the lower molecular weight of approximately 14.5 kDa.

The WT OM fraction separated by 2-DE was also stained by Coomassie Blue for total proteins (Figure 23: D). Unfortunately, the protein spot detected by anti-Le^y Western blot and glycoprotein-specific labelling was not apparent by Coomassie Blue staining, even upon the high protein loading utilized. This indicates that the Le^y O-antigen modified glycoprotein candidate is of low abundance. The inability to detect the glycoprotein candidate by Coomassie Blue staining prevents the protein spot from being excised and sent for MS analysis for protein identification. Although the protein spot may be visualized by other staining methods that are more sensitive, such as by silver nitrate staining, to obtain the recommended amount of protein for MS analysis, multiple 2-DE spots would likely be required, which is impractical for both protein identification and further glycopeptide analysis. Therefore, due to the low abundance of the glycoprotein candidate, additional enrichment and purification strategies need to be performed before identification of the OM glycoprotein candidate can be obtained.

Although the Le^y O-antigen modified OM glycoprotein candidate was found to be poorly abundant, the same is not necessarily true for the IM glycoprotein candidate modified with Le^y O-antigen. Thus, once growth conditions for the production of the IM

glycoprotein candidate are optimized, the same methodology will be applied to separate the IM glycoprotein candidate from non-glycosylated proteins to allow for eventual identification of the glycoprotein candidate by MS analysis.

In general, once MS identification of the Le^y O-antigen modified glycoprotein candidates can be obtained and glycopeptide analysis performed, this data will provide conclusive evidence of the existence of both additional glycoproteins in *H. pylori* and verification that a second protein glycosylation pathway exists in *H. pylori.* Furthermore, confirmation of the modification of the glycoprotein candidates with Le^y O-antigen by glycopeptide analysis will implicate WaaL in both LPS biosynthesis and protein glycosylation pathways. These findings will undoubtedly change the current dogma of protein glycosylation in *H. pylori* in which only the flagellins are believed to be glycosylated and only the pse glycosylation pathway is thought to be present.

CHAPTER 4: Discussion

4.1 Deciphering the biological role of pse-glycosylation of the flagellins in *H. pylori*

At the onset of this research, the flagellins of *H. pylori* were known to be glycosylated with pse [117]. Glycosylation of the flagellins with pse is required for production of proper flagella as mutations in the genes responsible for pse biosynthesis results in *H. pylori* that are non-motile and lack formation of flagella [117, 118, 174, 175]. Thus, protein glycosylation in *H. pylori* is considered a virulence factor as it is required for motility - a key factor in *H. pylori's* colonization and overall pathogenesis [111, 256]. However, the biological role of flagellin glycosylation in *H. pylori* and other bacteria has yet to be elucidated.

Previous studies by the Creuzenet Laboratory characterized the first two enzymes required for pse biosynthesis in *H. pylori,* FlaA1[118, 175] and HP0366 [174]. Knockout mutants for each enzyme lacked flagella and were non-motile, as expected, since pse biosynthesis is inactivated. However, preliminary evidence obtained by glycoprotein-specific labelling suggested that the flagellins of the *hp0366* mutant were glycosylated, despite disruption of pse biosynthesis. This alluded to the presence of an alternative glycosylation pathway in *H. pylori* in addition to the pse glycosylation pathway and suggests that the *hp0366* flagellins are modified with a sugar other than pse. Comparatively, the flagellins of the *flaA1* mutant were non-glycosylated as expected. As such, the flagellins of the *flaA1* and *hp0366* mutants represent variations of flagellin glycosylation compared to the flagellins from WT *H. pylori* and provide a useful tool to investigate the biological requirement for the flagellins to be glycosylated specifically with pse.

Based on the requirement for flagellin glycosylation on flagellum assembly [117, 118, 174, 175], it was proposed that glycosylation could be required for the interaction or polymerization of the flagellin subunits to form the flagellar filament, or that glycosylation could be required for the secretion of the flagellins from the flagellar type III secretion apparatus. Both defects were investigated by analyzing the supernatants of *H. pylori* liquid cultures of WT and the *pse* mutants for flagellins. No evidence of the flagellins was found in the supernatants of the *pse* mutants, whereas the secreted flagellins of WT *H. pylori* are known to assemble into the flagellar filament (Figure 7). This indicates that the flagellins of the *flaA1* and *hp0366* mutants, which do not form proper flagella and are not glycosylated by pse, were unable to be secreted from the cell.

This finding demonstrates that the defect in flagellum production, due to the inactivation of pse-specific glycosylation in the *flaA1* and *hp0366* mutants, is a result of a defect in the secretion of the flagellins from the flagellar apparatus. Further, a defect in secretion implies that flagellin glycosylation likely occurs at the base of the flagellar basal body as opposed to within the flagellar ring structure or filament during flagellum assembly. This is consistent with the recent observation that components of the flagellin glycosylation machinery of *C. jejuni* are localized to the flagellar basal body [163]. Interestingly, the same secretion defect was observed in both *flaA1* and *hp0366.* This implies that specific glycosylation of the flagellins by pse is required for their secretion as the flagellins of *hp0366* are believed to be alternatively glycosylated.

Overall, this study provides the first known evidence that flagellin glycosylation is required for the secretion of the flagellin subunits by the flagellar export system and that the *H. pylori* glycosylation machinery is likely present at the flagellar basal body.

4.2 The implication of alternative flagellin glycosylation in *H. pylori*

As described, the flagellins of the *hp0366* mutant appeared unexpectedly glycosylated by glycoprotein-specific labelling of total cell lysates, despite the lack of pse biosynthesis [175]. Moreover, the *hp0366* mutant was unable to form proper flagella, indicating that the *hp0366* flagellins are modified with a sugar distinct from pse. As such, a novel alternative glycosylation pathway for the glycosylation of the *hp0366* flagellins was predicted.

As the *hp0366* flagellins are unable to assemble into proper flagella, the glycosylated flagellins could not be present in the OM cell fraction as in WT, where the flagellins associate with the OM cell fraction due to the flagellar membrane sheath. Instead, glycosylated flagellins were observed in the insoluble protein fraction (Figure 9). This further indicates that the flagellin glycosylation machinery is likely present at the flagellar basal body as opposed to within the flagellar filament during flagellum assembly since the glycosylated flagellins were identified intracellularly in a mutant that does not produce flagella.

Moreover, the *hp0366* glycosylated flagellins were identified in the insoluble protein fraction rather than the cytoplasmic fraction. The absence of cytoplasmic glycosylated flagellins is consistent with the first report of glycosylated flagellins in *H. pylori* in which the *neuA/flmD* gene cluster was investigated for its involvement in flagellar biosynthesis or flagellin glycosylation [119]. In this study, it was suggested that the lack of cytoplasmic glycosylated flagellins implied that glycosylated flagellin species were only present in their polymerized state as a part of the assembled flagellar filament and that glycosylation must be coupled to flagellin secretion. As such, the observation of

glycosylated flagellins in the *hp0366* mutant and their localization to the insoluble protein fraction demonstrates that contrary to the speculation from this initial study, glycosylated flagellins are present intracellularly.

The demonstration of glycosylated flagellins of the *hp0366* mutant as insoluble proteins rather than soluble proteins suggests that when the flagellins are alternatively glycosylated with a sugar other than pse, the flagellins are unstable and may aggregate. Furthermore, this indicates that pse-specific glycosylation of the flagellins may be required for proper protein folding, such that when the flagellins are alternatively glycosylated with a sugar different from pse, as in the case of the *hp0366* flagellins, the flagellins are mis-folded, aggregate, and pellet as insoluble proteins. The role of protein glycosylation on protein folding and stability has been characterized in-depth, in which protein glycosylation has been shown to mediate final protein conformation [257].

Interestingly, glycosylated flagellins were also observed in the WT insoluble protein fraction (Figure 9). This suggests that some WT flagellins may also be alternatively glycosylated by the alternative glycosylation pathway proposed for the *hp0366* flagellins. This may indicate that glycosylation could act as a quality control or feedback mechanism for flagellin secretion, such that when they are improperly folded or not required by the cell, they are alternatively glycosylated and cannot be secreted from the flagellar basal body. This is reminiscent of the well-characterized role of protein glycosylation on eukaryotic proteins in which proper glycosylation is required for protein folding and stability [121]. Conversely, the production of WT insoluble flagellins could be due to an interaction between non-glycosylated and pse-glycosylated flagellins. In this case, it is hypothesized that non-glycosylated flagellins act similarly to the alternatively

glycosylated flagellins of *hp0366* and are unstable, resulting in their aggregation and insolubility. It could be reasoned that the presence of WT insoluble glycosylated flagellins is the result of pse-glycosylated flagellins aggregating with non-glycosylated flagellins. Thus, glycosylation could be acting as a feedback mechanism to control the secretion of the flagellins and potentially the length of the flagellum. In fact, although the overall structure of the *H. pylori* flagellar apparatus is believed to be similar to the general model of the type III flagellar export system of the *Enterobacteriaceae*, notable differences have been observed in regards to the regulation of the flagellar assembly. In particular, no master control regulator has been identified [112] and posttranscriptional regulation of the major flagellin subunit, *flaA,* has been reported [258], both of which indicate the presence of unique regulatory mechanisms in *H. pylori* for flagellum assembly. The current study may indicate that flagellin glycosylation also acts as a regulatory mechanism for flagellar assembly. Overall, the impact of the proposed alternative flagellin glycosylation pathway in *hp0366* highlights the need to fully characterize this novel glycan to further elucidate the function of flagellin glycosylation.

4.3 Difficulties associated with the purification of the insoluble glycosylated flagellins

In order to definitively demonstrate the presence of an alternative flagellin glycosylation pathway in *H. pylori*, identification of the glycan present on the glycosylated *hp0366* flagellins was necessary. Therefore, purification of the *hp0366* flagellins was required. However, in order to purify the flagellins from the insoluble protein fraction, several difficulties were encountered.

Firstly, the insoluble protein fraction of *H. pylori* is very complex. The single flagellin protein band cut from a 1D SDS-PAGE gel contained over 30 positive protein identifications by MS (refer to Section 3.1.2). The inherent complexity of the insoluble protein fractions thereby hindered sample preparation for glycopeptide analysis and sugar identification of the glycan present on the *hp0366* flagellins. In general, glycopeptide and sugar analyses by MS are difficult due to the poor abundance of glycosylated peptides compared to non-glycosylated peptides, and the existence of multiple glycoforms which dilutes the pool of glycopeptides available for analysis [187]. As well, glycosylated peptides are less efficiently ionized compared to non-glycosylated peptides [186]. Therefore, the low level of the flagellins in the insoluble protein fractions and the co-migration of the flagellins with unglycosylated proteins by 1D SDS-PAGE, prevented the detection of the flagellins by MS and subsequent glycopeptide and sugar analyses.

Thus, 2-DE was utilized to further separate and simplify the insoluble protein fractions to obtain purified WT and *hp0366* glycosylated flagellins. 2-DE is often used to separate complex protein samples and has been used to analyze glycoproteins, making it a suitable technique to examine the flagellins [188]. By 2-DE, the WT insoluble flagellins did separate into distinct protein spots, however, the flagellins were found to be poorly abundant in the insoluble protein fraction (Figure 10). In addition, several glycoforms were evident. As such, the availability of glycopeptides for MS analysis from 2-DE was anticipated to be too low for complete glycopeptide analysis.

Overall, the complexity of the insoluble protein fractions and poor abundance of the flagellins prevented the purification of the *hp0366* flagellins and identification of the *hp0366* flagellin glycan by standard protein preparation methods from cell lysates for MS

analysis. To overcome this limitation of the sample preparation, a strategy was designed to express recombinant *H. pylori* flagellins cloned with a C-terminal Histidine-tag in WT and *hp0366 H. pylori* strains to enable purification of the flagellins from the insoluble protein fraction. This strategy is advantageous as it overcomes both barriers to the analysis of the glycosylated flagellin species: it allows the flagellins to be purified away from other contaminating proteins, which would otherwise hinder glycopeptide analysis, and it enables samples to be enriched with high levels of the flagellins. In general, analysis on purified flagellin samples is expected to allow identification of the glycosylated flagellin peptides, the attachment site, and most importantly, the identification of the alternative glycan present on the *hp0366* flagellins.

4.4 The impact of the presence of an alternative glycan on the *hp0366* flagellins

Glycosylated flagellins were identified in the insoluble protein fraction of *hp0366* by glycoprotein-specific labelling with BHz (Figure 9). The demonstration of glycosylated flagellins in the *hp0366* mutant is surprising as pse biosynthesis has been inactivated, suggesting that an alternative glycosylation pathway is present in *H. pylori.* The strategy developed to express recombinant *H. pylori* flagellins with a C-terminal Histidine-tag to enable their purification by affinity chromatography will allow complete characterization of the glycan present on the *hp0366* flagellins as indicated above. Ongoing characterization of these recombinant *H. pylori* strains, of both WT and *hp0366* expressing tagged versions of the flagellins, both FlaA and FlaB, will allow a direct comparison between the flagellin glycan on the WT flagellins to that identified on the *hp0366* flagellins. Importantly, the verification of an alternative sugar on the *hp0366* flagellins will demonstrate the presence of a novel glycosylation pathway in *H. pylori.*

The occurrence of multiple glycan structures on the *H. pylori* flagellin subunits would not be unique as the flagellins of *C. jejuni* are known to be modified with a variety of carbohydrates including pse, pse derivatives, and legionaminic acid [156, 158].

Moreover, the identification of the alternative glycan on the *hp0366* flagellins may provide insight into the biological role of flagellin glycosylation in *H. pylori.* It can be reasoned that the size or charge of the alternative sugar identified compared to that of pse may allow inference regarding the potential role for flagellin glycosylation, such that pse-specific glycosylation of the flagellins may be required for proper flagellin folding or be more amenable to flagellin secretion from the export flagellar basal body.

Furthermore, the identification of the alternative glycan present on the *hp0366* flagellins will lead to additional studies regarding the elucidation of the sugar biosynthesis pathway and the sugar nucleotide modifying enzymes involved in its production.

Lastly, the identification of an alternative glycosylation pathway, based on the novel glycan on the *hp0366* flagellins, would be consistent with the observation of pse-independent glycoproteins by glycoprotein-specific labelling detected in the membrane fractions of *H. pylori* in this study (Figure 13) and previously in the soluble protein fraction [176]. Thus, identification of the alternative glycan on the *hp0366* flagellins will also reveal new information that can be used to design further approaches to characterize the glycoprotein candidates detected by glycoprotein-specific labelling. For example, when the glycan is identified, lectin affinity may be utilized, if an appropriate lectin is available, to determine if any of the detected glycoproteins are modified by the same

sugar. Thus, identification of the *hp0366* flagellin glycan is crucial to assess whether this alternative glycosylation pathway also targets other proteins in *H. pylori.*

4.5 The significance of membrane glycoproteins in *H. pylori*

The accepted view regarding protein glycosylation in *H. pylori* is that only the flagellins are modified with pse. Nevertheless, increasing evidence suggests that additional glycoproteins are present in *H. pylori.* A recent study demonstrated the presence of additional glycoproteins in *H. pylori* by metabolic labelling, however, no analysis of the sugars involved or identification of the glycosylated proteins was performed [177]. Moreover, the metabolic labelling technique used in this study has only been previously applied to detect glycoproteins in eukaryotic systems, thus, optimization of this labelling technique in prokaryotes is required before direct conclusions regarding the presence of protein glycosylation can be made. Comparatively, a more comprehensive study conducted by our laboratory, demonstrated the presence of additional glycoproteins besides the flagellins in the soluble protein fraction of *H. pylori* by chemical glycoprotein-specific labelling [176]. In addition to the detection of multiple soluble glycoproteins by glycoprotein labelling, this study identified and verified the presence of sugars other than pse from the soluble protein fraction and identified potential glycoprotein candidates. Thus, this study provided the first clear evidence that both additional glycoproteins and alternative glycosylation pathways are present in *H. pylori.*

The present study investigated whether membrane glycoproteins are present in *H. pylori* in addition to the soluble glycoprotein candidates observed in the previous study [176]. Indeed, several membrane glycoprotein candidates were detected by glycoprotein-specific labelling with BHz in both the WT and *pse* mutants (Figure 13) and by anti-Le^y

Western blot in WT *H. pylori* (Figure 21). In general, the identification of additional glycoproteins in the membranes of *H. pylori* goes against the current dogma of protein glycosylation in *H. pylori* and indicates that protein glycosylation likely plays a variety of functions in this organism. Furthermore, the observation of pse-dependent and pse-independent glycoproteins suggests that there are both additional glycoproteins besides the flagellins that are modified with pse as well as one or several alternative pse-independent glycosylation pathways that act to modify proteins with sugars distinct from pse. In particular, the clear loss of the approximately 15-17 kDa protein in the membranes of *flaA1,* but not *hp0366,* is consistent with a requirement for the alternative glycosylation pathway proposed for the *hp0366* flagellins for the glycosylation of other protein targets (Figure 12). This protein appears to be dependent on *flaA1* and thereby pse biosynthesis, but can overcome a defect in *hp0366,* which is similar to the pattern observed for the glycosylation of the *hp0366* flagellins. Identification of this protein by MS analysis may uncover a glycoprotein modified with a glycan similar to that of the *hp0366* flagellins. Moreover, the presence of several membrane glycoproteins suggests the presence of a potential general glycosylation pathway in *H. pylori* similar to *H. pullorum, N. gonorrhoeae,* or *B. fragilis,* as these systems are mediated by the cytoplasmic membrane [147, 166, 169].

More specificially, glycoproteins were evident in both the IM and OM by glycoprotein-specific labelling with BHz (Figure 14). The presence of IM glycoproteins corresponds with the hypothesis that LPS biosynthetic enzymes may be glycosylated, as investigated in Section 3.3, and suggests that protein glycosylation has important intracellular functions. The observation of IM glycoproteins in *H. pylori* is compatible

with the identification of IM glycoproteins in *B. fragilis* [169]. To date, the IM glycoproteins characterized in *B. fragilis* include multiple proteins involved in cell division and a sensor histidine kinase. Thus, protein glycosylation may affect similar proteins in *H. pylori.*

Comparatively, the presence of glycoproteins in the OM suggests that protein glycosylation may have important implications regarding host-pathogen interactions, such that OM glycoproteins may be involved in *H. pylori* colonization or mediating the host immune response. Thus, OM glycoproteins may represent a novel virulence factor in *H. pylori's* pathogenesis that has yet to be explored.

While the glycan present on the majority of the membrane glycoproteins visualized by glycoprotein-specific labelling is unknown, two candidates were found to be modified with the Le^y O-antigen chain typically characteristic of LPS; one glycoprotein candidate in the IM and one glycoprotein candidate in the OM of WT *H. pylori* (Figure 21). The modification of these glycoprotein candidates with Le^y O-antigen represents a second alternative glycosylation pathway in *H. pylori,* distinct from the protein glycosylation pathway proposed for the *hp0366* flagellins. Interestingly, glycosylation of the OM glycoprotein candidate by this alternative glycosylation pathway was still dependent on pse biosynthesis as it was not observed in the *pse* mutants (Figure 22). Thus, the requirement for pse biosynthesis suggests that the two glycosylation pathways are intertwined, such that modification of proteins with Le^y O-antigen is dependent on the previous pse-specific glycosylation of proteins which mediate this alternative glycosylation pathway. In general, the identification of membrane glycoproteins in both WT and the *pse* mutants demonstrates that alternative glycosylation

pathways are indeed present in *H. pylori* and shows that other proteins besides the flagellins are glycosylated.

4.6 Difficulties associated with the purification of the membrane glycoprotein candidates

Several membrane glycoproteins were observed by glycoprotein-specific labelling with BHz (Figure 13 and Figure 14) and by detection of proteins modified with Le^y O-antigen by anti-Le^y Western blot (Figure 21). Thus, the ongoing priority of this study is to identify the membrane glycoproteins observed and characterize their glycosylation by glycopeptide and sugar analyses to definitively demonstrate the presence of novel protein glycosylation in *H. pylori*. However, many difficulties are associated with the purification of these candidates for verification of their glycosylation by MS analysis.

In particular, the membrane fractions containing the glycoprotein candidates are quite complex, such that excision of the glycoprotein bands for trypsin digestion and subsequent MS analysis would likely result in the identification of a large array of proteins. It is predicted that the majority of the identified proteins by 1D SDS-PAGE would originate from contaminating non-glycosylated peptides that are more efficiently ionized compared to glycosylated peptides, as observed for the 1D SDS-PAGE protein band containing the insoluble flagellins (Section 3.1.2).

As such, 2-DE is commonly employed to separate membrane proteins [188]. However, membrane proteins are more difficult to solubilize compared to soluble or insoluble proteins due to their highly hydrophobic nature. In general, the use of the chaotropes, urea and thiourea, and the zwitterionic detergent CHAPS is required [253]. However, it cannot be determined if the solubilization and rehydration buffer will be

adequate to solubilize the membrane protein of interest. IM proteins are particularly notorious as being difficult to solubilize due to the number of transmembrane spanning domains mediated by alpha helices [254]. This may hinder analysis of the IM glycoprotein candidate modified with Le^y O-antigen. Comparatively, OM proteins are believed to be more soluble in the conditions used for isoelectric focusing, as the transmembrane domains of OM proteins are generally composed of beta-barrel sheets that can be fully denatured in the conditions typically used for 2-DE [253]. However, optimization of 2-DE conditions for OM proteins is still required. In fact, the poor abundance of the OM glycoprotein candidate modified by Le^y O-antigen, as seen by Coomassie staining (Figure 23: D), may have partly been due to the limited solubilization of the glycoprotein candidate by the conditions used for 2-DE. The poor abundance of glycoproteins after 2-DE separation for glycopeptide analysis was also reported for the identification of the glycoprotein candidates targeted by the general glycosylation system of *N. gonorrhoeae*. In this prior study, not enough protein was recovered by 2-DE from endogenous protein levels for complete analysis of the glycoproteins detected [166]. Thus, although the complexity of the membrane protein fractions can often be remedied by separation of the glycoprotein candidates from other non-glycosylated proteins by 2-DE, the poor abundance of the glycoprotein candidates has prevented the analysis of their glycosylation directly from cell fractions thus far.

In addition to difficulties associated with the characterization of the unidentified glycoprotein candidates, obstacles were also encountered with investigation of the known glycoprotein candidate WaaL. WaaL was selected as a glycoprotein candidate based on the O-antigen deficient profile of the *hp0366* LPS. As well, the hypothesis that WaaL is a

glycoprotein is consistent with the observation that the modification of the Le^y O-antigen glycoprotein candidates are dependent on both WaaL and pse biosynthesis since disruption of pse biosynthesis appears to prevent the modification of proteins with O-antigen, which is believed to be mediated by WaaL. Problems associated with analysis of the glycosylation of WaaL highlight potential downstream barriers to the detection of other membrane glycoproteins. In particular, WaaL could not be visualized accurately by SDS-PAGE despite the examination of several denaturing conditions. In all cases, WaaL was observed as a high molecular aggregate in total IM samples and after enrichment of WaaL by nickel affinity chromatography, indicating that the denaturing conditions used for SDS-PAGE were unable to disperse oligomer formation. The inability of WaaL to migrate within SDS-PAGE gels prevented WaaL from being separated from other high molecular weight or aggregated proteins. This further prevented subsequent MS analysis of WaaL for glycosylation as purified WaaL could not be obtained. Therefore, to overcome this barrier for the detection of WaaL and for the detection of other membrane glycoprotein candidates, additional electrophoresis techniques should be investigated to improve the separation and visualization of membrane proteins. As such, the use of deoxycholate gels [259] or SDS-PAGE gels supplemented with urea [243] may be advantageous for the denaturation of membrane proteins and their separation.

Furthermore, even after enrichment of WaaL by affinity chromatography, WaaL was of too low abundance to be detected by MS analysis (Section 3.3.2.2). Apart from the low expression of WaaL from its native promoter, the poor abundance of WaaL is likely a reflection of the instability of the shuttle vector used for WaaL expression. However, expression of membrane glycoprotein candidates, including WaaL, from their native

promoters is desired, instead of an overexpression strategy, as the effect of overexpression on the glycosylation machinery is unknown. Thus, future studies to investigate other membrane glycoprotein candidates as well as to further examine the glycosylation of WaaL will require the development of a stable expression system in *H. pylori.*

In general, these obstacles in membrane protein analysis have hindered the identification and characterization of the glycoprotein candidates revealed in this study. Thus, optimization of standard purification and protein analysis techniques for membrane proteins will be advantageous for the future directions of this study. Nevertheless, the eventual identification and purification of the membrane glycoprotein candidates will pave the way for additional studies to investigate the role of glycosylation on these proteins. It is possible, that similar to the general glycosylation system of *B. fragilis*, identification of several of the proposed glycoproteins and analysis of their glycopeptides and glycan attachment sites by MS may reveal a common consensus sequence for protein glycosylation in *H. pylori* and thereby may expose the presence of a general glycosylation system in *H. pylori.* This could lead to the identification of even more glycoproteins in *H. pylori.*

4.7 The demonstration of a connection between protein glycosylation and LPS biosynthesis in *H. pylori*

An obvious link between protein glycosylation and LPS biosynthesis was observed in *H. pylori,* such that disruption of pse-specific glycosylation in the *pse* mutants altered LPS production and mutation of the O-antigen ligase, *waaL,* required for

LPS biosynthesis, prevented flagellum production. This study is the first to characterize the interconnection between the two distinct glycosylation pathways in *H. pylori*.

This link was first examined by investigating the possibility that LPS biosynthetic enzymes may require pse glycosylation for function (Section 3.3). WaaL was chosen as a glycoprotein candidate to explore this connection based on the O-antigen deficient LPS profile of *hp0366,* however, due to difficulties in its purification, it remains to be determined whether WaaL is glycosylated. It is possible that other LPS biosynthetic enzymes are glycosylated instead of, or in addition to WaaL, to account for the LPS defect observed in the *pse* mutants.

As described in Section 4.6, the expression system used for WaaL expression in this study was likely not stable and thereby hindered purification of WaaL due to its poor abundance. The development of a stable expression system will undoubtedly improve the ability to purify larger amounts of WaaL for analysis of its glycosylation. Once WaaL is purified, the glycosylation status of WaaL can be determined via glycoprotein-specific labelling with BHz and further characterization by MS analysis.

In addition, if WaaL is shown to be glycosylated after a stable expression system is achieved, the same system can be used to determine the dependence of WaaL glycosylation on pse biosynthesis. It is predicted that if WaaL is glycosylated with pse, glycosylation will not occur in the *pse* mutants. Moreover, if WaaL is glycosylated with pse, the biological role of WaaL glycosylation can be investigated by examination of WaaL purified from the *pse* mutants. It is theorized that pse glycosylation may directly affect the ligase activity of WaaL. As such, the ability to purify WaaL from both WT and *pse* mutant strains of *H. pylori* will allow differences in the O-antigen ligase activity to be

investigated by the *in vitro* assay previously used to assess the WaaL O-antigen ligase activity of *H. pylori* [93] and other species [244]. Alternatively, it is possible that pse glycosylation of WaaL could affect its cellular localization, similar to pse glycosylation of the flagellins. In this sense, WaaL would not be properly targeted to the IM in the *pse* mutants and would likely be observed in the soluble or insoluble protein fractions. Thus, analysis of the cellular protein fractions obtained by differential centrifugation from WT and *pse* strains expressing WaaL can be examined for the cellular localization of WaaL.

Further, by comparing the glycosylation status of WaaL from WT to that of the *pse* mutants it can be determined whether the alternative glycosylation pathway observed for the *hp0366* flagellins is common among other pse-dependent glycoproteins, such as WaaL. In this scenario, WaaL would be glycosylated with pse in WT *H. pylori,* non-glycosylated in *flaA1,* and alternatively glycosylated in *hp0366.* As such, demonstration of the differential glycosylation of WaaL in the *pse* mutants may explain the variation in the LPS profiles observed between *flaA1* and *hp0366,* such that complete abrogation of WaaL glycosylation results in reduced O-antigen ligase activity in the *flaA1* mutant, while when WaaL is alternatively glycosylated in *hp0366,* ligase activity is completely inactivated. Thus, establishment of a stable expression system for WaaL and analysis of its glycosylation could initiate an array of studies to characterize the requirement of pse glycosylation on WaaL and LPS biosynthesis.

In addition, generation of a stable expression system in *H. pylori* for membrane glycoprotein expression and glycosylation analysis can also be used to examine the other glycoprotein candidates once they are identified. It is possible that the IM glycoprotein candidates observed by glycoprotein-specific labelling with BHz (Figure 14) include one

or several proteins that are involved in LPS biosynthesis which may or may not be WaaL. Thus, identification of the observed IM glycoprotein candidates by glycoprotein labelling will contribute to the exploration of the functional requirement for pse glycosylation for the activity of LPS biosynthetic enzymes.

Nevertheless, the system used in the present study was unable to indicate whether WaaL was glycosylated by pse and if its glycosylation was required for function. It remains possible that WaaL is not glycosylated and that a different component of the LPS machinery may be affected in the *pse* mutants. Despite this caveat, another connection between LPS biosynthesis and protein glycosylation via WaaL was observed, which highly supports that WaaL has a role in both glycosylation pathways and may be glycosylated by pse.

A second connection between LPS biosynthesis and protein glycosylation was evident due to a defect in flagellum production upon *waaL* inactivation. This suggested that WaaL could be acting as a general OTase in addition to its known role as the O-antigen ligase, and may modify proteins required for proper flagellum assembly. Flagellin glycosylation was not affected in the *waaL* mutant [175] suggesting that other flagellar components may be glycosylated by WaaL. Thus, this observation is consistent with the proposal that additional glycoproteins besides the flagellins are glycosylated in *H. pylori*. As such, it was theorized that in addition to the direct glycosylation of WaaL for LPS biosynthesis, WaaL may mediate an alternative glycosylation pathway in *H. pylori*. Together, the dual role of WaaL in protein glycosylation and its direct glycosylation resolve the obvious link between LPS biosynthesis and protein glycosylation in *H. pylori*.

The general OTase activity of WaaL was explored through the detection of O-antigen on proteins by immunblotting. By this method, two membrane glycoprotein candidates modified with Le^y O-antigen were observed; one OM protein and one IM protein (Figure 21). Importantly, the OM glycoprotein candidate was absent in the *waaL* mutant (Figure 22). This supports the hypothesis that WaaL is a general OTase and can modify proteins. This is similar to the protein glycosylation pathway observed in *A. actinomycetemcomitans* in which the glycosylation of the collagen adhesin EmaA is dependent on WaaL [185]. To further demonstrate that the modification of the glycoprotein candidates with Le^y O-antigen are dependent on WaaL, complementation of the *waaL* mutant and re-appearance of the modified glycoprotein bands by immunoblotting would be ideal. However, all previous attempts to complement the *waaL* mutant with the shuttle vector designed for WaaL expression have been unsuccessful (data not shown). The inability to complement a *waaL* defect has been reported by other groups as well [93]. This suggests that there is an inherent property of WaaL that interferes with its complementation. It is proposed that WaaL may function under strict stoichiometric ratios with other components of the LPS biosynthesis or protein glycosylation machineries that may be refractory to its complementation *in trans*. Nevertheless, upon establishment of a stable expression system for *waaL*, complementation can be attempted in the *waaL* mutant again, which may be more successful. Alternatively, if complementation of *waaL* remains unattainable, but purification of WT WaaL can be achieved, the *in vitro* assay previously proposed to monitor the O-antigen ligase activity of WaaL can be adapted to monitor the ability of purified WaaL to attach O-antigen to the membrane glycoprotein candidates. Thus, using

these methods, the dependence of the modification of membrane proteins with Le^y O-antigen can be firmly established.

Significantly, the Le^y O-antigen-modified OM glycoprotein candidate was also absent in the membrane fractions of the *pse* mutants (Figure 22), indicating that the Le^y O-antigen modification observed is dependent on pse biosynthesis despite pse not being involved in O-antigen synthesis. In order to demonstrate that the Le^y O-antigen modification of the membrane glycoprotein candidates is dependent on pse biosynthesis, analysis of the membrane fractions of the complemented *flaA1* and *hp0366 H. pylori* strains previously constructed in the laboratory [174, 175] is underway. The production of the Le^y O-antigen-modified glycoprotein candidates in the complemented strains, but not in the mutant strains, will directly demonstrate that the modification of these glycoproteins with Le^y O-antigen is dependent on pse biosynthesis. It is proposed that the dependence of the Le^y O-antigen modification of proteins on pse biosynthesis indirectly demonstrates that WaaL is glycosylated with pse and requires pse-glycosylation for function, as speculated. To definitively demonstrate this connection, purification of WaaL remains a priority in order to assess its glycosylation directly. Overall, based on the results presented thus far, it is observed that inactivation of pse biosynthesis appears to affect both the O-antigen ligase activity of WaaL, as seen by the defect of LPS production in the *pse* mutants, and its potential OTase activity, as observed by the absence of the Le^y O-antigen-modified glycoproteins in the *pse* mutants. Therefore, the data presented in this study establishes a clear link between protein glycosylation and LPS biosynthesis in *H. pylori.* It is evident that at least two membrane proteins are

modified with Le^y O-antigen, indicating that WaaL has general OTase activity and can modify both lipid A and proteins and that WaaL is likely glycosylated by pse.

4.8 Future directions

The present study demonstrated the presence of novel protein glycosylation in *H. pylori,* in terms of both the detection of membrane glycoproteins and the elucidation of uncharacterized glycosylation pathways. As a result, the future direction of this study is to further characterize the novel membrane glycoproteins identified for the eventual characterization of the protein glycosylation pathways present in *H. pylori.*

As described, the major obstacle that prevents identification of the unknown membrane glycoprotein candidates observed in this study is their poor abundance. As such, the next step in the characterization of these glycoprotein candidates is to develop strategies to enrich samples in the glycoprotein candidates for MS analysis and identification.

It is proposed that enrichment of all membrane glycoprotein candidates can be performed using a similar mechanism to that used for glycoprotein-specific labelling. For enrichment of glycoproteins, the membrane fractions containing the glycoprotein candidates of interest can be chemically labelled with BHz, as used for glycoprotein detection. The BHz-labelled glycoproteins can then be isolated from other membrane proteins by selective affinity for streptavidin-coated beads. The use of streptavidin-coated beads to purify biotinylated glycoproteins has previously been utilized to purify both N- and O-linked glycoproteins [260]. Thus, this provides a suitable means to purify the membrane glycoproteins without any prior knowledge of the type of linkage or the identity of the glycan. In addition, since chemical labelling with BHz was previously

employed to detect the glycoprotein candidates in the membranes of *H. pylori,* the use of streptavidin-coated beads is a suitable method to enrich these candidates. Moreover, the BHz-tag incorporated on the modified carbohydrates can further be used as a signature to identify glycopeptides apart from non-glycosylated peptides, which will be useful in identifying the glycoproteins, glycosylation attachment sites, and the glycans involved, by MS analysis.

In the case of the Le^y O-antigen-modified glycoprotein candidates, affinity for the anti-Le^y antibody can be utilized to purify the glycoprotein candidates from unmodified membrane proteins. For this method, the anti-Le^y IgM antibody could be coupled to a matrix containing Protein L, which has affinity for the Ig light chain and has previously been shown to bind IgM antibodies [261]. Subsequently, the anti-Le^y antibody, which has been coupled to a support via Protein L, can be used to purify the Le^y O-antigen-modified glycoproteins via affinity chromatography. Once purified by this method, the Le^y O-antigen-modified glycoprotein candidates can be sent for MS analysis for identification. Alternatively, a fucose-specific lectin that can detect the fucose of the Le^y O-antigen can be used to similarly capture the Le^y O-antigen-modified glycoprotein candidates. These approaches target identification of the membrane glycoproteins modified by the Le^y O-antigen only.

Moreover, the present study focused on the identification of glycoproteins modified with the known substrate of WaaL, the Le^y O-antigen. However, several WaaL homologues, such as the pilin glycosyltransferase PglL of *N. meningitidis,* exhibit substrate promiscuity [250]. Thus, additional investigations are required in order to study the substrate specificity of the *H. pylori* WaaL. It is possible that WaaL may modify

proteins with carbohydrates other than O-antigen and that the protein glycosylation pathway mediated by WaaL represents a major source of protein glycosylation uncharacterized in *H. pylori*.

It is anticipated that enrichment of the unknown membrane glycoprotein candidates by the methods described above will allow for their identification by MS. However, in order to completely characterize their glycosylation, glycopeptides and sugar analyses are required. These forms of analyses are most accurate with large amounts of pure glycoproteins. Thus, as many of the membrane glycoprotein candidates observed were of low abundance, a further strategy may be required to purify the candidates prior to complete characterization of their glycosylation.

The strategy most commonly utilized to purify glycoprotein candidates is to express recombinant versions of the glycoprotein candidates with a tag to enable the specific purification of each candidate. This strategy was most recently used to confirm the glycosylation of glycoprotein candidates from the general protein glycosylation system of *B. fragilis* [169]. Moreover, the use of this strategy was also applied for the purification of two glycoprotein candidates in this study: the *hp0366* flagellins and WaaL, in which each candidate was tagged with a C-terminal Histidine-tag to allow their purification by affinity chromatography. Purification of the flagellins from WT and *hp0366* is underway, but no problems are anticipated as the flagellins are present in the insoluble protein fraction and a stable expression system was used for expression of the tagged flagellin genes via chromosomal integration. Comparatively, purification of WaaL was met with several obstacles due to the use of a shuttle vector for expression that was unstable. Therefore, in order to allow complete characterization of the glycoprotein

candidates, including WaaL, a stable expression system needs to be designed. In fact, the strategy utilized for the chromosomal integration of the tagged flagellins into *rdxA* can be employed for expression of the membrane glycoprotein candidates and will allow for the stable expression of each of the glycoprotein candidates. Thus, by this method, each glycoprotein candidate can be purified, which will allow for the glycosylation of these proteins to be fully characterized by both glycopeptide analysis and identification of the glycan by MS.

4.9 General significance and applications

In general, the study of bacterial protein glycosylation has received a lot of attention in recent years for both therapeutic development and biotechnological applications. In particular, bacterial protein glycosylation has been demonstrated to have significant applications in glycoengineering. In particular, the ability to transfer the *C. jejuni* N-glycosylation system to *E. coli* [128] has opened the doors for the production of glycoproteins for medical and industrial use. It has been proposed that sugar biosynthetic enzymes from additional glycosylation pathways may be used for glycoengineering purposes, which can be used for the glycosylation of antibiotics or anti-cancer drugs [262] and the production of recombinant peptide antigens [263]. The elucidation of additional glycosylation pathways in *H. pylori* will add to the available sugar biosynthetic enzymes for these purposes.

Moreover, protein glycosylation pathways in bacteria are considered innovative targets for therapeutic development as the sugars typically incorporated in bacterial glycoproteins are distinct from those of their host. Thus, small-molecule inhibitors of the glycosyltransferases or other biosynthetic enzymes involved in sugar biosynthesis and

protein glycosylation provide a new pool of therapeutic targets. In the case of *H. pylori* and the data presented in this study, the inhibition of pse biosynthesis could potentially inhibit the production of several virulence factors - flagella, LPS, and IM and OM glycoproteins - which implicate pse glycosylation as a prime target for therapeutic development. In fact, a previous investigation has identified CMP-pse as a natural inhibitor of pse biosynthesis for both *H. pylori* and *C. jejuni* [264]. The need to develop new therapeutics to treat *H. pylori* infections is evident given the rise of antibiotic resistance in *H. pylori* strains [41].

4.10 Summary

Overall, this study explored the presence of novel protein glycosylation in *H. pylori*, including both novel glycoproteins and glycosylation pathways and demonstrated their existence. The results of this study challenge the current dogma regarding protein glycosylation in *H. pylori* on several accounts. In particular, only the pse glycosylation pathway is believed to modify the flagellins of *H. pylori.* However, this study demonstrates that there is an alternative glycosylation pathway that branches from the pse biosynthesis pathway that can also modify the flagellins. Further, the observation of the flagellins as intracellular insoluble proteins provides the first evidence that the biological role of flagellin glycosylation may be related to the stability, proper folding, or regulation of the flagellin subunits. In addition, this study revealed the presence of several membrane glycoproteins in *H. pylori.* The glycosylation of several of these glycoproteins appears independent of pse biosynthesis, indicating that a pse-independent glycosylation pathway is also present in *H. pylori.* Moreover, it was demonstrated that at least two of these membrane glycoproteins are modified with the Le^y O-antigen typically found on *H.*

pylori LPS. This form of glycosylation involving the O-antigen ligase, WaaL, directly demonstrates the presence of another glycosylation pathway in *H. pylori* and a novel glycan involved in *H. pylori* protein glycosylation.

Furthermore, this study is the first to characterize a link between protein glycosylation and LPS biosynthesis in *H. pylori.* It was demonstrated that WaaL functions as a general OTase and can modify both lipid A and proteins. Moreover, the evidence presented in this study suggests that WaaL is glycosylated by pse and requires pse-glycosylation for function.

Overall, the data presented here implicates protein glycosylation in the production of multiple virulence factors. It was shown that pse-specific glycosylation is required for flagellum assembly, LPS production, and the modification of both IM and OM glycoproteins. As well, the OM glycoproteins detected may have direct roles in virulence, such as in *H. pylori* colonization and protection from host defences, while the IM glycoproteins detected may play pivotal roles in virulence factor production, such as that predicted for the production of LPS via pse-glycosylation of WaaL.

In general, this study has contributed to the further understanding of bacterial protein glycosylation. Overall, much less is known regarding bacterial protein glycosylation compared to eukaryotic protein glycosylation. However, given the important cellular roles of protein glycosylation in eukaryotes, and the increasing number of reports of bacterial protein glycosylation, it is anticipated that bacterial protein glycosylation has many more implications than previously believed. Therefore, continued investigation of bacterial protein glycosylation is warranted in order to fully understand the extent and importance of this phenomenon in bacteria.

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