Study of the N-linked protein glycosylation pathway in *Campylobacter* species

by

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Abstract

Campylobacter jejuni was the first Gram- negative bacterial species demonstrated to possess an N-linked protein glycosylation pathway (Pgl), however it is now well established that all known *Campylobacter* species and a few other bacterial species possess this pathway. In *Campylobacter*, the pathway is responsible for the synthesis of species specific oligosaccharides that are covalently attached to asparagine residues on multiple proteins to form N-glycosylated proteins, and also releases free oligosaccharides (fOS) into the periplasmic space. The central enzyme, the membrane bound oligosaccharyltransferase (OTase), PglB, is responsible for the formation of both major products and is extensively studied due to its application in the production of glycoconjugate vaccines and therapeutics.

N-glycosylated proteins and fOS were discovered in *C. jejuni* several years ago, however further studies are important to understand their generation, abundance and specific role(s). In addition, the mechanism of protein N-glycosylation and fOS generation by PglB are not well understood. This PhD thesis focuses on developing tools to study the generation, abundance and structural diversity of fOS. In addition, new tools were developed to characterize and understand the fOS generation and protein glycosylation activity of PglB in greater detail.

Efficient and sensitive fOS purification, quantitation and analysis methods were successfully developed in this thesis. These methods are based on thin layer chromatography, porous graphitized carbon purification, high performance anion exchange chromatography, mass spectrometry (MS) and nuclear magnetic resonance. The amount of fOS purified by these methods is 120 times more compared to previously published methods. This method also allows determination of molar quantities of fOS compared to previously published semi-quantitative methods and is applicable to the structurally diverse fOS generated by different *Campylobacter*

species. fOS from selected species were quantitated and found to range from 49.8± 0.5 nMoles in *C. fetus fetus* to 7.8± 0.8 nMoles in *C. lari*. In addition, novel phosphorylated fOS structures were also discovered in *C. lari*, that were not detected by previous methods. The methods established in this project are more sensitive, significantly faster and more efficient compared to previously published methods that were time-consuming and produced lower yields of fOS.

Studies focused on understanding the fOS generation activity of PglB revealed that N-glycosylation of PglB itself may affect both its ability to N-glycosylate other cellular proteins and generate fOS in *C. jejuni*. Bioinformatic analysis showed that the N-glycosylation site is conserved in the majority of the PglB homologues in *Campylobacters*. Significantly lower fOS levels were detected in *C. jejuni* cells expressing unglycosylated PglB compared to wild-type suggesting a role of N-glycosylation in the fOS generation activity of PglB. In addition, overall N-glycoprotein profiles were different between the two strains as determined by Western blot analysis with anti-N-glycan antibodies. This is the first report of the N-glycosylation of an OTase enzyme possibly affecting its own enzymatic activities.

In order to better understand the N-glycosylation activity of PglB, a fluorescence resonance energy transfer (FRET) assay was developed. This method is much faster compared to the other gel electrophoresis or ELISA based assays currently used to assess OTase activity. The FRET assay utilizes a peptide labeled with fluorophore/quencher at each end, along with a glycosylation acceptor sequon and a Factor Xa cleavage site within the peptide sequence. After incubation with the OTase enzyme, the peptide is exposed to the Factor Xa protease. The glycosylated peptide is protected from protease mediated cleavage due to the glycan modification, whereas the unglycosylated peptide gets cleaved resulting in fluorescence that is measured in a plate reader. In addition, MS based methods were successfully established to

identify the peptide composition following glycosylation. This assay can be adapted to a 96-well plate based high-throughput assay that allows quick analysis of OTase activity.

This study has made important contributions to understanding the generation of both major products of the N-linked protein glycosylation pathway in *Campylobacter* species and provided efficient and faster tools to further characterize the pathway in *Campylobacter*s and other bacterial species that possess an N-linked protein glycosylation pathway.

Preface

Chapter 1 is my original work. A version of Chapter 2 was published in October 2013 as "Generation of free oligosaccharides from bacterial protein N-linked glycosylation systems", Biopolymers, Anniversary special issue on Glycosciences, vol.99, issue 10, pg 772-783. I was responsible for designing the method development protocol. In addition, I performed data collection and analysis of experiments involving thin layer chromatography, porous graphite carbon chromatography, silica gel chromatography and high performance anion exchange with pulsed amperometric detection (HPAEC-PAD). Technical training of HPAEC-PAD equipment was provided by Harald Nothaft. All samples for mass spectrometry and NMR were prepared by me. Bela Reiz and Randy Whittal assisted with all mass spectrometry data generation and analysis, as well as, mass spectrometry figures. Portion of NMR figure was re-published from a previously published study by permission from the respective journal. Christine M. Szymanski was responsible for overlooking all experiments performed in this study. I was responsible for manuscript preparation and all co-authors provided their input in the editing process.

Chapter 3 is my original work based on preliminary work performed by Yasmin Barre (MSc, Dr. Szymanski's laboratory) that is not shown in the chapter. Chapter 4 is my original work that was based on preliminary work performed by Dr. Abofu Alemka (Postdoctoral fellow, Dr. Szymanski's laboratory) that is not shown in the chapter. In addition, I was responsible for sample preparation for mass spectrometry analysis, however sample analysis and interpretation of data was performed by Bela Rèiz and Jing Zheng (Chemistry mass spectrometry facility, University of Alberta). Chapter 5 is my original work as well.

A version of the Appendix has been submitted for publication. The data was generated through collaborative work with Dr. Alain Stintzi at the University of Ottawa, Ontario, Canada and Dr.

Arnoud Van Vliet at the Institute of Food Research, Gut Health and Food Safety Programme, Norwich, United Kingdom. I was responsible for performing biofilm assays, scanning electron microscopy, developing the chemotaxis assay and performing chemotaxis assays. I also assisted Jolene Garber in growth assays. Christine M. Szymanski was responsible for overlooking all experiments performed in this study. I was also responsible for manuscript preparation and all co-authors provided their valuable input during the editing process.

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List of abbreviations

Amp	Ampicillin
ATP	Adenosine triphosphate
AtpA	Adenosine triphosphate synthase subunit alpha
BHI	Brain heart infusion
Ca	Calcium
CDG	Congenital disorders of glycosylation
Cm	Chloramphenicol
CPS	Capsular polysaccharide
DDM	n-Dodecyl-ß-D-maltopyranoside
diNAcBac	di-N-acetylated bacillosamine
Dol-P	Dolichol phosphate
EL	External loop
ELISA	Enzyme linked immunosorbent assay
ENGase	Endo-β-N-acetylglucosaminidase
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
ESI	Electrospray ionization
fOS	Free oligosaccharides
Fruc	Fructose
Fuc	Fucose
FRET	Fluorescence resonance energy transfer
HPAEC-PAD	High performance anion exchange chromatography with pulsed amperometric detection

Hex	Hexose
HexA	Hexuronic acid
HexNAc	N-acetylated hexose
HexNAcA	N-acetylamido-deoxy-hexuronic acid
HMW	High molecular weight
Hyl	Hydroxylysine
Hyp	Hydroxyproline
Kan	Kanamycin
FT-ICR	Fourier transform ion cyclotron resonance
Gal	Galactose
GalNAc	N-acetylgalactosamine
GlcNac	N-acetylglucosamine
Glc	Glucose
IL	Interleukin
INT	Intestine
Kan	Kanamycin
kDa	Kilodalton
K-Glu	Potassium glutamate
LC	Liquid chromatography
LLO	Lipid linked oligosaccharide
LB	Luria-Bertani
LOS	Lipooligosaccharide
Man	Mannose

MALDI	Matrix assisted laser desorption ionization
MEM	Minimal essential medium
MGL	Macrophage galactose lectin
MH	Mueller Hinton
mM	Millimolar
MS	Mass spectrometry
N	Asparagine
NaCl	Sodium Chloride
NMR	Nuclear magnetic resonance
nod1	nucleotide binding oligomerization domain 1
OPG	Osmoregulated periplasmic glucan
OTase	Oligosaccharyltransferase
P	Phosphate
PBS	Phosphate buffered saline
PE	Phosphoethanolamine
P-fOS	Mono-phosphorylated free oligosaccharide
PP-fOS	Di-phosphorylated free oligosaccharide
PG	Periplasmic glucan
PGC	Porous graphitized carbon
Pgl	N-linked protein glycosylation
PNGaseF	Peptide:N-glycanase
Ppt	Precipitated
R_f	Distance traveled by compound/ distance traveled by solvent

R_t	Retention time
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SG	Self glycosylation
SGC	Silica gel chromatography
Sq	Semi-quantitative
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TOF	Time of flight
TTC	2,3,5 triphenyltetrazolium chloride
Und-PP	Undecaprenyl pyrophosphate
Und-P	Undecaprenyl phosphate
WT	Wildtype
Xyl	Xylose
QuiS	6-sulfoquinovose

CHAPTER 1

Introduction

1.1 *Campylobacter jejuni* as a model organism for carbohydrate studies

C. jejuni is the leading cause of foodborne bacterial gastroenteritis in the world (Allos, 2001; Silva *et al.,* 2011). In addition, it has also been linked to auto-immune diseases, such as, Guillain Barrè and its clinical variant Miller fisher syndrome (Allos, 2001; Silva *et al.,* 2011; Ansar and Valadi, 2015). In 2009, 1.3 million cases of *Campylobacter* infections were estimated in the United States and 9.2 million cases were estimated in the European Union with *C. jejuni* and *C. coli* attributing for the majority of the infections (Wagenaar *et al.,* 2013). In addition, upto 40% of Guillain Barrè syndrome cases and its variant Miller fisher syndrome cases are associated with prior *C. jejuni* infections (Dingle *et al.,* 2001; Poropatich *et al.,* 2010). Other diseases, such as, reactive arthritis, irritable bowel syndrome, inflammatory bowel disease and haemolytic uraemic syndrome have also been linked to *Campylobacter* infections (Wagenaar *et al.,* 2013; Keithlin *et al.,* 2014; Zautner *et al.,* 2014).

C. jejuni possesses several carbohydrate pathways and these have proved to be major contributors in the infection and pathogenesis lifecycle of this bacterium. This makes *C. jejuni* an excellent model organism for bacterial carbohydrate studies and understanding the specific mechanisms by which these carbohydrate structures provide an advantage to this organism in its physiology. *C. jejuni* encodes O- and N- linked protein glycosylation pathways (Szymanski *et al.,* 1999; Wacker *et al.,* 2002; Ewing *et al.,* 2009), peptidoglycan (Amano and Shibata, 1992), capsular polysaccharide (CPS), as well as, lipooligosaccharide (LOS) structures (St Michael *et al.,* 2002; Kelly *et al.,* 2006).

Peptidoglycan is required for the helical shape of this pathogen (Frirdich *et al.,* 2012). Mutations that affect the peptidoglycan structure reduce the fitness of the organism in the chicken colonization model of *C. jejuni* (Frirdich *et al.,* 2014). In addition, structural mutations in the

peptidoglycan cause reduction in biofilm formation, motility and stimulation of human Nod1 (nucleotide binding oligomerization domain 1) proinflammatory mediator as well (Frirdich *et al.,* 2012).

The CPS and LOS pathways are also important pathogenic traits of *C. jejuni*. The CPS of *C. jejuni* is involved in modulation of host immune responses (Maue *et al.,* 2013). CPS mutants display increased serum sensitivity (Corcionivoschi *et al.,* 2009; Keo *et al.,* 2011), reduced colonization of intestinal tracts in mouse models (Maue *et al.,* 2013), defective invasion of epithelial cells (Bacon *et al.,* 2001; Bachtiar *et al.,* 2007; Corcionivoschi *et al.,* 2009), reduced virulence in the ferret diarrhoeal disease model (Bacon *et al.,* 2001) and increased sensitivity to hyperosmotic stress (Cameron *et al.,* 2012). In *C. jejuni*, LOS is required for conferring resistance to cationic antimicrobials (Keo *et al.,* 2011). LOS also contributes towards the development of Guillain Barrè syndrome through mimicry of host gangliosides (Ellstrom *et al.,* 2013).

C. jejuni has bipolar flagella that are composed of two protein subunits, FlaA and FlaB, that are O-glycosylated with pseudaminic and legionaminic acid at upto 19 serine (S)/ threonine (T) sites per subunit (Thibault *et al.,* 2001; Goon *et al.,* 2003; Logan *et al.,* 2009; Ewing *et al.,* 2009). O-linked glycosylation of the subunits is essential for proper assembly of the flagellar filaments (Goon *et al.,* 2003; Ewing *et al.,* 2009). Flagellar mutants are non- motile and, defective in colonization of the intestinal tracts of chicken (Nachamkin *et al.,* 1993), mouse colonization (Newell *et al.,* 1985), invasion of epithelial cells (Konkel *et al.,* 2004), as well as, biofilm formation (Reeser *et al.,* 2007).

In addition, *C. jejuni* was the first bacterium reported to possess an N-linked protein glycosylation pathway (Szymanski *et al.,* 1999; Wacker *et al.,* 2002). The pathway is responsible

for the modification of >60 proteins (Scott *et al.,* 2011) and mutations in the pathway result in multiple phenotypes ranging from reduced adherence/invasion of human epithelial cells to reduced colonization of the intestinal tracts of chicken and mouse colonization models (Szymanski *et al.,* 2002; Larsen *et al.,* 2004; Karlyshev *et al.,* 2004; Kelly *et al.,* 2006; Hendrixson, 2006; van Sorge *et al.,* 2009; Scott *et al.,* 2011; Kakuda *et al.,* 2012; Alemka *et al.,* 2013).

1.2 Introduction to protein glycosylation

Protein modification occurs across all domains of life, i.e. eukaryotes, archaea and bacteria, and these modifications include lipidation, methylation, acetylation, glycosylation, phosphorylation, ubiquitination and proteolysis (Spiro, 2002; Wang *et al.,* 2014; Cain *et al.,* 2014). Protein modifications add another level of complexity to protein regulation and have multiple affects on proteins, including protein folding, localization, stability and activity (Spiro, 2002; Wang *et al.,* 2014; Cain *et al.,* 2014).

Over 70% of eukaryotic proteome is thought to be glycosylated (Dell *et al.,* 2010), however the abundance of glycosylation in bacteria is unknown due to the relatively recent emergence of the field. Protein glycosylation involves the covalent attachment of carbohydrate units onto side chains of amino acids in proteins and so far glypiation, rhamnosylation, phosphoglycosylation, C-, N- and O- linked glycosylation have been found to exist in nature (Spiro, 2002; Wang *et al.,* 2014; Cain *et al.,* 2014; Lassak *et al.,* 2015). The first evidence of protein glycosylation was found in 1938 in egg albumin (Neuberger, 1938). Since the first discovery of an N-acetylglucosamine (GlcNAc) linked to an asparagine (N) in ovalbumin (Neuberger, 1938; Johansen *et al.,* 1961), several carbohydrate modifications on various functional groups found on proteins have been described across all domains of life.

C- glycosylation linkages involving the C-C bonds between the C-2 of the indole ring of tryptophan and mannose (Man) are rare and have so far been found on a few mammalian proteins, such as, RNase2 (Spiro, 2002) and interleukin-12 (Spiro, 2002). Another distinct type of carbohydrate protein linkage, phosphoglycosylation occurs through phosphodiester linkage to S amino acid residues (Spiro, 2002). So far Man, xylose (Xyl), fucose (Fuc) and GlcNAc linkages have been reported on multiple proteins in certain eukaryotic organisms such as, the soil dwelling amoeba *Dictyostelium discoideum* and the protozoan parasite *Leishmania mexicana* (Haynes, 1998; Spiro, 2002). In addition, glypiation that involves the linkage of Man to proteins through phosphoethanolamine is quite abundant in eukaryotes and has also been reported in archaea (Spiro, 2002).

The N- and O- linked glycoproteins are the most abundant type of post-translational modifications (Haynes, 1998) that are present in all domains of life. N-linked glycosylation involves the attachment of a carbohydrate molecule, usually an oligosaccharide, to the amide nitrogen of the amino acid, asparagine (N). Multiple proteins have been reported to be N-glycosylated in various organisms across all domains of life (Spiro, 2002; Dell *et al.,* 2010). Linkages between a carbohydrate and hydroxyl group of an amino acid are known as O-linked glycosylation (Haynes, 1998; Spiro, 2002). In eukaryotes, proteins are O-glycosylated with a variety of carbohydrates including, N-acetylgalactosamine (GalNAc), GlcNAc, Man, Fuc and Xyl (Dell *et al.,* 2010). Every amino acid with a hydroxyl group, such as, S, T, tyrosine (Y), hydroxyproline (Hyp) and hydroxylysine (Hyl) has been reported in an O-glycosidic linkage (Spiro, 2002; Dell *et al.,* 2010). Multiple reports of O-linked glycosylation have been made in bacteria as well and mostly involve glycosylation of flagellar or pilin subunits with many different glycan structures ranging from derivatives of sialic acid in *C. jejuni* to Gal and GalNAc

containing oligosaccharide in *Acinetobacter baumannii* (Borud *et al.,* 2010; Dell *et al.,* 2010; Iwashkiw *et al.,* 2012). In addition, to the previously mentioned carbohydrate pathways, *C. jejuni* also possesses an N-linked glycosylation pathway that is discussed in more detail below.

1.3 The N-linked protein glycosylation pathway: overview

N-linked protein glycosylation is present in all three domains of life. As mentioned previously, the pathway involves co- or post- translational modification of proteins by the attachment of an oligosaccharide to N residues within specific protein sequences. Despite the structural diversity of the oligosaccharide across all systems, conserved mechanisms exist in the pathway. However, certain exceptions occur, generally the oligosaccharide is assembled from nucleotide activated precursors onto a membrane anchored lipid carrier which is then flipped into the periplasmic space in bacteria (Fig. 1.1), into the endoplasmic reticulum (ER) lumen across the ER membrane in eukaryotes (Fig. 1.3), and across the cell membrane in archaea (Ruiz-Canada *et al.,* 2009; Schwarz and Aebi, 2011; Eichler, 2013). The *en bloc* transfer and covalent attachment of the oligosaccharide to proteins is performed by an oligosaccharyltransferase (OTase) complex consisting of the conserved catalytic Stt3 subunit in eukaryotes (Schwarz and Aebi, 2011; Eichler, 2013) and a single subunit oligosaccharyltransferase, homologous to Stt3, in bacteria and archaea (Nothaft *et al.,* 2010; Eichler, 2013). The N- linked pathway OTase enzymes share the conserved WWDYG motif across all domains of life which is essential for catalysis of the N-protein glycosylation reaction (Szymanski *et al.,* 1999; Wacker *et al.,* 2002; Yan and Lennarz, 2002; Calo *et al.,* 2010). In eukaryotes and *Campylobacter* species, the OTase also releases the glycan structure, as free oligosaccharides (fOS) directly from the lipid linked oligosaccharides (LLOs), into the ER lumen and periplasmic space respectively (Chantret *et al.,* 2003; Liu *et al.,* 2006; Chantret and Moore, 2008; Nothaft *et al.,* 2009; Chantret *et al.,* 2011; Harada *et al.,* 2013).

This phenomenon has not been reported in archaea. In addition, cytoplasmic fOS are also released during protein recycling and degradation in eukaryotic organisms (Chantret *et al.,* 2003; Chantret and Moore, 2008; Chantret *et al.,* 2011).

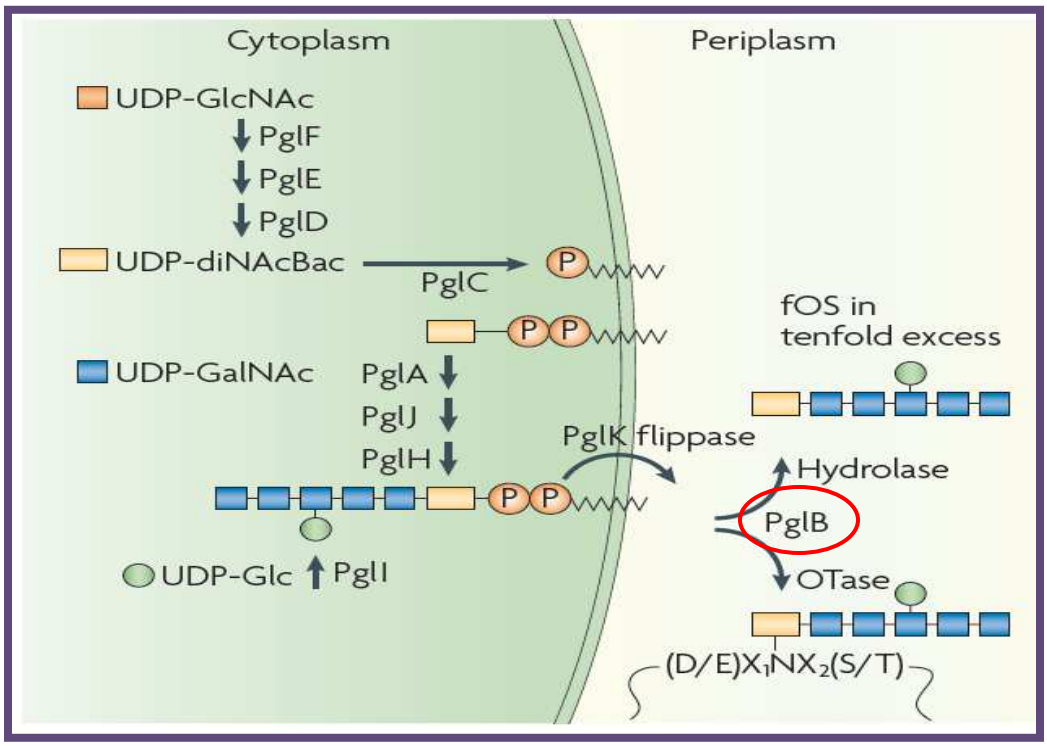

Fig. 1.1 The protein N-linked glycosylation pathway (Pgl) in *Campylobacter jejuni*. A heptasaccharide is synthesized by the sequential action of Pgl glycosyltransferases (PglF, E, D, A, J, H, I) on the lipid carrier, undecaprenyl phosphate, on the cytoplasmic side of the inner membrane. The lipid linked oligosaccharide (LLO) is then flipped into the periplasmic space by a flippase enzyme (PglK). The oligosaccharide is transferred to asparagine residues of proteins within the sequon D/E-X_1-N-X_2-S/T (where X cannot be proline) by the key oligosaccharyltransferase, PglB (circled in red), that also hydrolyzes LLOs and generates free oligosaccharides (fOS) into the periplasmic space in approximately ten times abundance compared to the N-linked counterpart (Nothaft *et al*., 2009; Nothaft and Szymanski, 2010) (glucose, Glc; N-acetylgalactosamine, GalNAc; di-N-acetylbacillosamine, diNAcBac). GalNAc

is shown as blue rectangle, Glc is shown as green circle and diNAcBac is shown as yellow rectangle. Figure modified from Nothaft and Szymanski, 2010.

1.3.1 Diversity in N-glycosylation pathways and structures across all domains of life

N-glycan structures are quite diverse across all three domains of life and variations exist in the oligosaccharide synthesis pathways, the lipid carrier, the characteristics/cellular location of the OTases and the glycosylation sequons as described below.

In eukaryotes, proteins are glycosylated at the conserved sequon, N-X-S/T by the OTase complex, where X cannot be proline (Aebi, 2013). The lipid, dolicholphosphate, serves as a carrier for LLO synthesis. The process of N-glycosylation takes place in the membrane of the ER and the LLO is synthesized by a series of glycosyltransferases encoded by the *alg* (asparagine linked glycosylation) genes (Aebi, 2013) (Fig. 1.3). First, $Man_5GlcNAc_2$ is synthesized on dolichol pyrophosphate on the cytoplasmic side of the ER membrane from nucleotide activated precursors (Aebi, 2013). This is then flipped into the ER lumen, where the LLO is further elongated to a 14-mer oligosaccharide, $Glc_3Man_9GlcNac_2$ (Aebi, 2013). The membrane anchored OTase complex then transfers this oligosaccharide onto proteins (Aebi, 2013). In mammals and other higher eukaryotes, the oligosaccharide is further trimmed by glucosidases, mannosidases and further extended by other glycosyltransferases resulting in complex structures carrying sialic acid, Fuc and galactose (Gal) (Kornfeld and Kornfeld, 1985; Liebminger *et al.,* 2010). Similar to mammals, in plants, the oligosaccharide is further trimmed by glucosidases and mannosidases and modified with Fuc, Xyl, Gal and GlcNAc by different glycosyltransferases in the Golgi (Lerouge *et al.,* 1998; Ruiz-May *et al.,* 2012). In insects, the oligosaccharide is usually only decorated with Fuc residues after trimming (Shi and Jarvis, 2007). However, in lower eukaryotes, such as the yeast, *Saccharomyces cerevisiae*, the N-glycan is not further trimmed and

only elongated by addition of more Man residues (Kornfeld and Kornfeld, 1985). The OTase complex in all cases contains the catalytically active Stt3 subunit that is required for N-glycosylation and the WWDYG motif, in the Stt3 subunit, that has been shown to be essential for catalysis (Aebi, 2013).

In bacteria and eukaryotes, N-glycosylated proteins do not belong to a specific class of proteins or specific functionality, however, most studies in archaea have reported N-glycosylation of S-layer proteins and flagellin subunits. In contrast to eukaryotes, the oligosaccharide has been shown to be synthesized on both dolichol phosphate and dolichol pyrophosphate lipid carriers depending on the species (Jarrell *et al.,* 2010; Calo *et al.,* 2010; Eichler, 2013; Jarrell *et al.,* 2014). In *Haloferax volcanii*, all archaeal glycosylation (*agl*) genes are clustered in the genome (Jarrell *et al.,* 2014), however in other species, such as, *Methanococcus maripaludis* and *Sulfolobus acidocaldarius,* the *agl* genes are found in two or more genetic clusters/locations (Jarrell *et al.,* 2014).

As mentioned earlier, in eukaryotic organisms the OTase is a multimeric complex, while in archaea it consists of a single unit enzyme, AglB, which is homologous to the eukaryotic OTase catalytic subunit Stt3 and also contains the WWDYG motif (Calo *et al.,* 2010). In all archaea, AglB is a membrane bound enzyme with multiple transmembrane domains that glycosylates proteins within the sequon N-X-S/T (X cannot be proline) (Abu-Qarn and Eichler, 2007; Calo *et al.,* 2010). However, recently in *Halobacterium salinarum,* N-glycosylation was reported to occur at N-X-N/Leu/Valine (Kandiba and Eichler, 2015).

Hbt. salinarum was the first non eukaryotic organism to be shown to possess an N-glycosylation pathway (Mescher and Strominger, 1978). Its S-layer proteins and flagellin have been shown to be N-glycosylated with either a tetrasaccharide or a pentasaccharide consisting of glucose (Glc)

and glucuronic acids that are also sulfated (Eichler, 2013; Kandiba and Eichler, 2015). *Hfx. volcanii* is a halophile that N-glycosylates its flagellin and S-layer proteins with a pentasaccharide structure composed of a Hex, two hexuronic acids (HexA), a methyl ester of hexuronic acid and a Man (Mescher and Strominger, 1978; Kaminski *et al.,* 2010; Eichler, 2013). *Haloarcula marismortui* also decorates its S-layer protein shell with the same N-glycan as *H. volcanii,* however the synthesis pathway differs slightly (Calo *et al.,* 2011). *Pyrococcus furiosis*, an extremophile that has an optimum growth of 100°C (Yip *et al.,* 1995), has also been shown to N-glycosylate its proteins with a heptasaccharide composed of two HexNAc, two Hex, one HexA and two pentoses (Igura *et al.,* 2008). The acidophile, *S. acidocaldarius* grows ideally at pH 2-3 and its S-layer protein is highly glycosylated with a hexasaccharide $Glc_1Man_2GlcNAc_2QuiS$ (where QuiS is 6-sulfoquinovose) (Peyfoon *et al.,* 2010). In addition, previously the cytochrome $b_{558/566}$ was also reported to be N-glycosylated in this strain with the same glycan (Eichler, 2013). The N-glycan structure of this strain contains unusual moieties, such as a 6-deoxy-6-sulphoglucose, that is commonly found in photosynthetic membranes of plants and phototrophic bacteria (Eichler, 2013). Interestingly, N-glycan structures with amino acids have been found in the archaeal *Methanococcus* species. Quite unique is the N-glycan structure of *M. maripaludis* that decorates its flagellin with a tetrasaccharide that includes GalNAc and GlcNAc residues and the amino acid T (Eichler, 2013; Ding *et al.,* 2013). In addition, *Methanococcus voltae* strain PS*27* N-glycosylates its flagellin and S-layer proteins with a trisaccharide consisting of N-acetylated Man and Glc residues in addition to amino acid T as well (Voisin *et al.,* 2005; Chaban *et al.,* 2006).

In bacteria, epsilonproteobacteria (*Campylobacter, Helicobacter* and *Wolinella* species) (Szymanski *et al.,* 1999; Wacker *et al.,* 2002; Jervis *et al.,* 2010), deltaproteobacteria

(*Desulfovibrio* species) (Santos-Silva *et al.,* 2007; Ielmini and Feldman, 2011), and gammaproteobacteria (*Haemophilus* and *Actinobacillus* (Grass *et al.,* 2003; Gross *et al.,* 2008; Choi *et al.,* 2010; Schwarz *et al.,* 2011; Naegeli *et al.,* 2014) have been reported to possess N-glycosylation pathways.

So far members of the class epsilonproteobacteria are reported to possess periplasmic glycosylation pathways (Fig. 1.1). All known 29 *Campylobacter* species were analyzed and reported to N-glycosylate their proteins with oligosaccharide structures that were similar at the reducing end but varied at the non-reducing end (Fig. 1.2) (Nothaft *et al.,* 2012). The N-linked protein glycosylation genes (*pgl*) are clustered in all *Campylobacter* species (Nothaft and Szymanski, 2010; Nothaft *et al.,* 2012; Nothaft and Szymanski, 2013). The oligosaccharide is synthesized on undecaprenyl phosphate by enzymes encoded in the *pgl* cluster along with the N-glycosylating OTase enzyme which is homologous to the eukaryotic Stt3 catalytic subunit (Szymanski *et al.,* 1999; Wacker *et al.,* 2002; Reid *et al.,* 2008; Reid *et al.,* 2009; Reid *et al.,* 2010; Nothaft and Szymanski, 2010; Nothaft *et al.,* 2012; Nothaft and Szymanski, 2013). Gammaproteobacteria, such as *Haemophilus influenzae* and *Actinobacillus pleuropneumoniae* have been shown to possess cytoplasmic N-glycosylation pathways that do not synthesize LLOs and instead single glycan moieties are transferred to proteins from nucleotide activated precursors (Grass *et al.,* 2003; Schwarz *et al.,* 2011; Naegeli *et al.,* 2014). The N-glycosylation performing glycosyltransferases in these organisms are also completely unrelated to the conventional N-glycosylation performing Stt3 homologous OTases and belong to a different family of glycosyltransferases, however the N-glycosylation sequon is the same as eukaryotes (Gross *et al.,* 2008; Schwarz *et al.,* 2011; Naegeli *et al.,* 2014).

Bacterial N-glycan structures are best studied in *Campylobacter* species (Nothaft *et al.,* 2012; Jervis *et al.,* 2012). The first bacterial N-linked protein glycosylation system was described in *C. jejuni* over a decade ago (Szymanski *et al.,* 1999; Wacker *et al.,* 2002). In *C. jejuni*, the pathway is responsible for the synthesis and attachment of a heptasaccharide to >60 proteins (Scott *et al.,* 2011). *Helicobacter pullorum* was recently reported to possess a Pgl pathway when N-glycosylated peptides were detected *in vitro* upon incubation with membrane preparations of *H. pullorum* (Jervis *et al.,* 2010). The N-glycan was identified to be a linear pentasaccharide consisting of an N-acetylhexosamine (HexNAc), two 217 Da residues, a 216 Da residue and a reducing end residue of 203 Da or HexNAc (Jervis *et al.,* 2010). *Wolinella succinogenes* also synthesizes a pentasaccharide with unusual moieties of 216 Da, 217 Da and 232 Da similar to *H. pullorum* (Jervis *et al.,* 2012). *H. influenza* N-glycosylates its adhesion, HMW1, with hexoses or dihexoses within N-X-S/T sequon (Gross *et al.,* 2008). So far none of the native proteins from *A. pleuropneumoniae* and *Yersinia enterocolitica* have been identified to be N-glycosylated. However, homologues of the *H. influenza* N-glycosyltransferase from *A. pleuropneumoniae* and *Yersinia enterocolitica* have been shown to N-glycosylate exogenous peptide acceptors *in vitro* (Schwarz and Aebi, 2011). In addition, recently some novel glycosylation sites were identified in N-glycosylated proteins from *C. jejuni* 11168 O strain where the -2 position was identified to have leucine and glutamine residues whereas an alanine was identified in the +2 position of another glycoprotein (Scott *et al.,* 2014) .

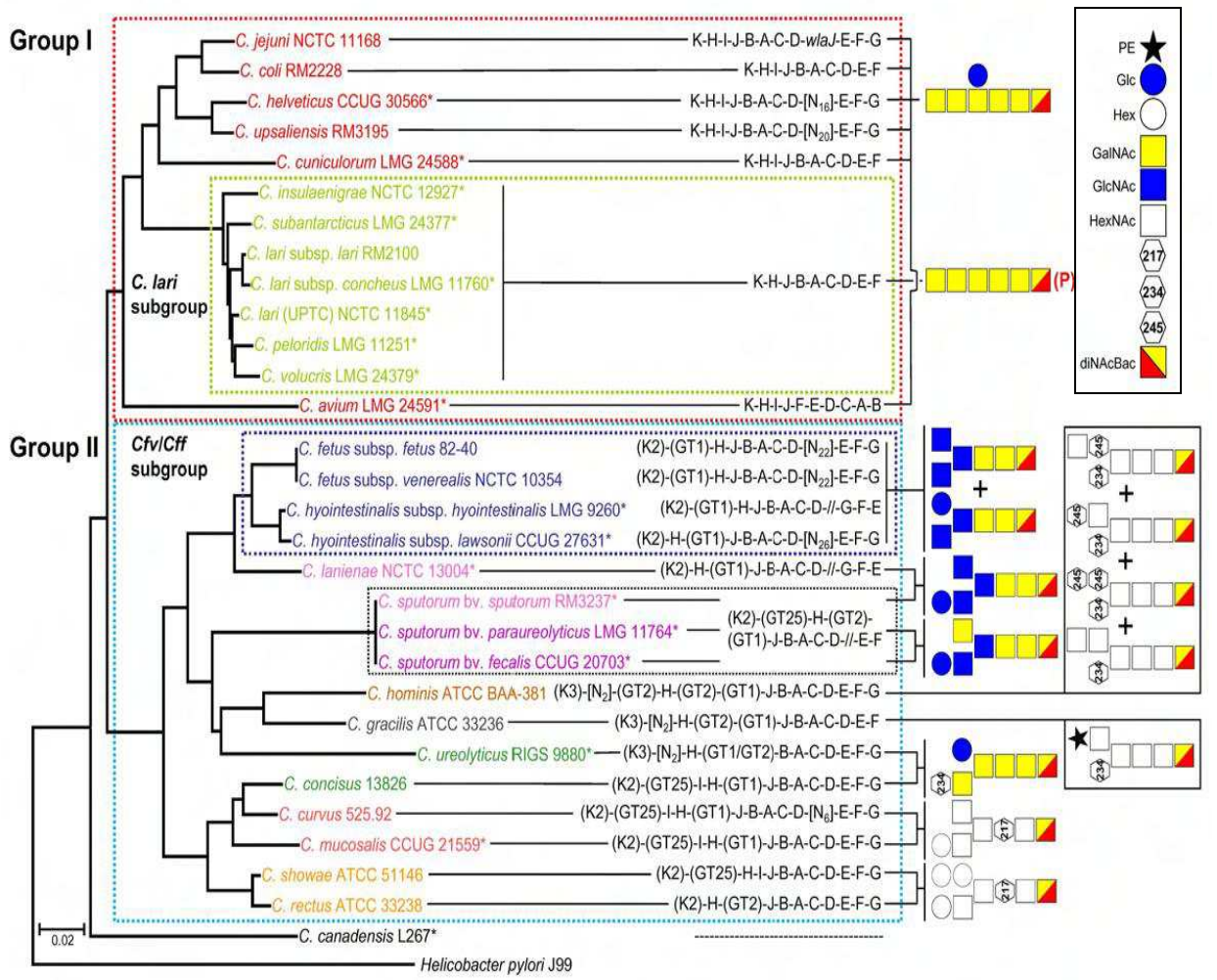

Fig. 1.2 N-glycan and fOS structures in *Campylobacter* species. *Campylobacter* species synthesize a diversity of species specific oligosaccharides for N-glycosylation and fOS generation. A dendogram of *Campylobacter* species based on their AtpA sequences grouped them into two major subgroups (Group I and Group II). Group I species are thermophilic (grow at 42°C), whereas Group II species are non-thermophilic (grow at 37°C). The reducing end, diNAcBac, is conserved in all strains whereas variability exists at the non-reducing end (legend for structures is boxed and shown in upper right corner, phosphoethanolamine, PE; glucose, Glc; hexose, Hex; N-acetylgalactosamine, GalNAc; N-acetylglucosamine, GlcNAc; di-N-acetylbacillosamine, diNAcBac; identities of molecules with

molecular weights of 217, 234 and 245 Da are unknown). Letters representing each *pgl* gene in each glycosylation cluster are indicated by the species name. Figure modified from Nothaft *et al.*, 2012.

1.4 N-linked glycosylation pathway in *C. jejuni*: protein glycosylation and free oligosaccharide generation

As mentioned earlier, in all *Campylobacter* species, the protein glycosylation pathway is encoded by the *pgl* gene cluster (Nothaft and Szymanski, 2010; Nothaft *et al.,* 2012; Jervis *et al.,* 2012) and the N-glycan is synthesized by the sequential transfer of the carbohydrate moieties by the *pgl* gene products. In *C. jejuni*, the N-glycan structure is the heptasaccharide: GalNAc-α1,4-GalNAc-α1,4-[Glcβ1,3]-GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-diNAcBac-β1 (diNAcBac is 2,4-diacetamido-2,4,6-trideoxyglucopyranose) (Wacker *et al.,* 2002; Young *et al.,* 2002; Reid *et al.,* 2010) (Fig. 1.1). First, uridine diphosphate activated GlcNAc is used as a precursor to synthesize diNAcBac by the dehydratase (PglF), aminotransferase (PglE), and the acetyltransferase (PglD) (Fig. 1.1) (Schoenhofen *et al.,* 2006; Olivier *et al.,* 2006). The diNAcBac is then transferred to undecaprenyl phosphate (Und-P) by PglC (Fig. 1.1) (Glover *et al.,* 2006). The glycosyltransferases PglA, PglJ, PglH extend the molecule to an oligosaccharide by adding five GalNAc residues (Fig. 1.1). PglA and PglJ each transfer one GalNAc whereas, PglH transfers the next three GalNAc molecules (Fig. 1.1) (Karlyshev *et al.,* 2004; Glover *et al.,* 2005; Linton *et al.,* 2005; Glover *et al.,* 2006). Then the glucosyltransferase, PglI, transfers the Glc branch to the third GalNAc in the oligosaccharide (Fig. 1.1) (Glover *et al.,* 2005; Kelly *et al.,* 2006) to further extend the lipid-linked oligosaccharide (LLO). This LLO is subsequently flipped into the periplasmic space by the flippase, PglK (Fig. 1.1) (Kelly *et al.,* 2006; Alaimo *et al.,* 2006). The central enzyme, the OTase PglB, then transfers the heptasaccharide onto the D/E-

X_1-N-X_2-S/T sequon of proteins (Fig. 1.1) (Wacker *et al.,* 2002; Nita-Lazar *et al.,* 2005; Kowarik *et al.,* 2006; Alaimo *et al.,* 2006). In addition to OTase activity, PglB possesses hydrolase activity and also releases the oligosaccharide directly from LLOs as free oligosaccharide (fOS) into the periplasmic space (Fig. 1.1) (Liu *et al.,* 2006; Nothaft *et al.,* 2009). In *C. jejuni, pgl* pathway mutants display several important phenotypes including reduction in the attachment and invasion of human epithelial cells (Szymanski *et al.,* 2002) and impaired colonization of the intestinal tracts of mice and chickens (Szymanski *et al.,* 2002; Karlyshev *et al.,* 2004; Hendrixson and DiRita, 2004; Kelly *et al.,* 2006). Moreover, N-glycans attached to *Campylobacter* surface proteins protect them from proteolytic degradation through chicken gut proteases resulting in increased bacterial fitness (Alemka *et al.,* 2013).

1.4.1 Generation of fOS in *Campylobacter* species

The first report of fOS generation from an N-linked protein glycosylation pathway in a bacterial system was made in *C. jejuni* (Liu *et al.,* 2006). In a study by Liu *et al*. (2006), the authors reported a new method to determine N-glycan structure on proteins in eukaryotes and bacteria. The procedure required pronase E digestion of whole cell lysates for 48 hr which was then subjected to porous graphitized carbon purification. The resulting mixture of fOS and glycopeptides was permethylated and analysed by mass spectrometry (MS). The study successfully used the methodology to identify N-glycopeptides on eukaryotic proteins, as well as, on whole cell extracts of *C. jejuni.* However, in addition to observing heptasaccharide attached to asparagine, free heptasaccharide or fOS was also observed. The authors confirmed the fOS was not a breakdown product of LLOs as further analysis revealed that fOS was absent in *pglB* mutant cell extracts indicating that a functional OTase enzyme was required for detection of fOS (Liu *et al.,* 2006).

This observation was further explored in a detailed study by Nothaft *et al.* in *C. jejuni* (2009). In addition to analysis of a *pglB* mutant, fOS and N-glycan structures were analyzed in other selected *pgl* mutants (Nothaft *et al.,* 2009). The authors performed semi-quantitative MS (sqMS) to determine the structures and levels of fOS in the mutant strains relative to the wildtype strain. Whereas, *pglH*, *pglJ*, *pglK* and *pglB* mutants did not release fOS (Nothaft *et al.,* 2009), the *pglD* acetyltransferase mutant released incomplete fOS that lacked the diNAcBac at the reducing end. Interestingly, the N-linked glycan in the *pglD* mutant was determined to be $HexNAc_5Hex$-NAcBac-Asn which was different than the observed fOS structure (Nothaft *et al.,* 2009). In contrast, *pglH* and *pglJ* mutants N-glycosylated their proteins with the truncated N-glycan structures, $HexNac_2$-diNAcbac and HexNac-diNAcBac respectively. Interestingly, whereas *pglE* and *pglF* mutants N-glycosylate proteins at low levels, fOS was not detected in these strains (Nothaft *et al.,* 2009). The *pglI* mutant released wildtype levels of fOS and N-glycosylated proteins with $HexNAc_5Hex$-diNAcBac oligosaccharide (Nothaft *et al.,* 2009). Similar N-glycan structures were found in the mutant strains upon analysis of the reporter CmeA protein in *C. jejuni* (Nothaft *et al.,* 2009; Nothaft *et al.,* 2010).

In addition to *pgl* mutant analysis, the authors also investigated the abundance of fOS under osmotic stress and growth phase by sqMS (Nothaft *et al.,* 2009). The authors found that fOS levels were most abundant during exponential growth phase (Nothaft *et al.,* 2009). Interestingly, the authors found novel effects of osmotic stress on fOS (Nothaft *et al.,* 2009). The wildtype, *pglB* and *pglD* mutants had similar growth rates under standard *C. jejuni* growth conditions in Mueller Hinton broth, however the *pgl* mutant strains had significantly reduced growth rates under hyperosmotic stress exerted by the addition of salt to the growth media (Nothaft *et al.,* 2009). Further analysis revealed that concentrations of fOS are dramatically decreased under

osmotic stress caused by salts (i.e. sodium chloride; NaCl and potassium glutamate; K-Glu) and sucrose (Nothaft *et al.,* 2009).

In addition to *in vivo* analysis, the fOS generation activity of the PglB enzyme was also analysed *in vitro*. Analysis with *pglB* membrane preparations *in vitro* in the presence of the ionic salts, NaCl or K-Glu revealed a dramatic decrease in the fOS release activity of PglB compared to the absence of salts (Nothaft *et al.,* 2009). However, the same phenomenon was not observed in the presence of the neutral osmotic agent, sucrose (Nothaft *et al.,* 2009). This suggested that PglB may not be influenced by the same mechanisms by osmotic agents and perhaps more complex regulatory mechanisms are involved in regulating the fOS generation/ LLO hydrolysis activity of PglB (Nothaft *et al.,* 2009). The conserved WWDYG motif in the PglB enzyme was also found to be essential for fOS release as membrane preparations with the mutant enzyme did not release any fOS *in vitro* (Nothaft *et al.,* 2009).

Recently, Nothaft *et al.* (2012) demonstrated that all known *Campylobacter* species perform N-linked protein glycosylation with species-specific N- glycan structures and also release identical fOS structures into the periplasmic space (Nothaft *et al.,* 2012) (Fig. 1.3). This warrants further studies into the importance of N-glycosylated proteins and fOS in *Campylobacter* species.

1.4.2 Analogy of fOS to periplasmic glucans in proteobacteria

The presence of fOS in the periplasmic space of *Campylobacter* species is similar to the presence of glucose polymers known as osmoregulated periplasmic glucans (OPGs) in other proteobacteria, such as *E. coli* and *Shigella flexneri* (Arellano-Reynoso *et al.,* 2005; Arellano-Reynoso *et al.,* 2005; Lequette *et al.,* 2008; Bhagwat *et al.,* 2012). OPG synthesis enzymes are also inner membrane proteins, similar to Pgl enzymes and both fOS and OPG levels decrease under hyperosmotic stress caused by ionic (e.g. sodium chloride, potassium glutamate) and

neutral (e.g. sucrose) osmotic agents (Kennedy, 1982; Rumley *et al.,* 1992; Nothaft *et al.,* 2009; Lee *et al.,* 2009). Under hypoosmotic conditions, fOS can constitute upto 2.5% of dry cell weight in *C. jejuni* (Dwivedi *et al.,* 2013), similarly OPG have been reported to range from 0.75 to 20% of dry cell weight depending on the species (Breedveld *et al.,* 1994; Bohin and Lacroix, 2006; Lequette *et al.,* 2007). One major difference exists in the generation of these polymers. OPG enzymes are believed to synthesize the glucose polymers in the cytoplasm from nucleotide activated glucose precursors and transport the polymer into the periplasm via a channel (Bohin, 2000; Guidolin *et al.,* 2015), whereas PglB generates fOS by LLO hydrolysis and releases fOS directly into the periplasmic space (Nothaft *et al.,* 2009).

OPG mutants display reduced growth in low osmolarity media however they exhibit optimal growth rates upon increasing extracellular osmolarity suggesting important roles for PG in hypo-osmotic adaptation (Dylan *et al.,* 1990; Cangelosi *et al.,* 1990; Taylor *et al.,* 1993; Lee *et al.,* 2009). In addition to playing crucial roles in hypo-osmotic adaptation, OPGs have been shown to affect multiple phenotypes, such as, motility, antibiotic resistance, intracellular signalling, pathogenesis and plant symbiosis (Bhagwat *et al.,* 2009; Bouchart *et al.,* 2010; Gay-Fraret *et al.,* 2012; Martirosyan *et al.,* 2012). In *C. jejuni, pgl* mutations do not result in reduced growth in hypoosmotic media (Nothaft *et al.,* 2009) suggesting that fOS does not play roles in hypo-osmotic adaptation however, fOS may play other important roles in pathogenesis, similar to OPGs that are mentioned above.

The regulation of OPG synthesis and OPG synthesis enzymes is a relatively well studied area and, in general, the enzymes are reported to be directly inhibited *in vitro* by ionic osmotic agents that cause reduction in OPG concentrations *in vivo* (Zorreguieta *et al.,* 1990; Rumley *et al.,* 1992; Ingram-Smith and Miller, 1998; de Iannino *et al.,* 2000). The activity of OPG synthesis

enzymes from *Agrobacterium tumefaciens, E. coli* and *Rhizobium meliloti* is inhibited *in vitro* upon incubation with salts that cause reduction in cellular OPG concentrations when the bacterial cells are grown in broth cultures (Rumley *et al.,* 1992; de Iannino *et al.,* 2000). Interestingly, neutral osmotic agents, such as sucrose, do not inhibit enzyme activity *in vitro* in these strains however sucrose does cause reduction in cellular OPG concentrations (i.e. *in vivo*) (Rumley *et al.,* 1992; Ingram-Smith and Miller, 1998). These observations are strikingly similar to *in vitro* fOS release activity of PglB in membrane preparations, as fOS release is not affected in the presence of sucrose *in vitro* but is only decreased in the presence of salts (Nothaft *et al.,* 2009).

Genetic regulation of OPG synthesis genes has also been studied in various species. Transcript levels of OPG synthesis genes in *Pseudomonas aeruginosa* are lower when cells are grown in high osmolarity media (Lequette *et al.,* 2007) whereas, transcription of the OPG genes is unaltered by extracellular osmolarity in *A. tumefaciens* and *R. meliloti* indicating that regulation of OPG synthesis is variable depending on the species (Zorreguieta *et al.,* 1990; Rumley *et al.,* 1992; Gay-Fraret *et al.,* 2012). Interestingly, genetic regulation of *pglB* appears to be similar to that in *A. tumefaciens* and *R. meliloti* as the transcript levels of *pglB* remain unaltered in the presence of salts (Nothaft *et al.,* 2009). Experiments involving sudden upshift from hypoosmotic to hyperosmotic conditions suggest that decrease in OPG concentrations under hyperosmotic conditions is achieved by the dilution affect of cellular division and not degradation (Bohin, 2000). This is also observed with fOS in *C. jejuni* and fOS levels directly correlate with the growth rate of the bacteria (Nothaft *et al.,* 2009).

In addition, periplasmic glucans are substituted with non-carbohydrate modifications. For example, OPG are substituted with phosphoglycerol, succinyl and phosphoethanolamine in *E. coli* (Lequette *et al.,* 2008). Further studies are required to determine the roles of these

modifications, although recent studies suggest they play important roles. A recent study in a strain of *S. flexneri* that modifies 99% of its OPG molecules with the anionic succinyl and phosphoglycerol residues revealed that these residues are important for optimal growth in the presence of anionic detergents such as sodium dodecyl sulfate and deoxycholate (Taylor *et al.,* 1993; Bhagwat *et al.,* 2012). *C. lari* fOS is substituted with phosphate residues whereas fOS from *C. gracilis* was found to be substituted with phosphoethanolamine molecules (Nothaft *et al.,* 2012), however, the exact roles of these modifications are yet to be determined (Nothaft *et al.,* 2012; Dwivedi *et al.,* 2013). As *pgl* mutations result in loss of N-glycosylated proteins and fOS, the roles of these Pgl pathway products is difficult to exactly correlate with the phenotypes.

1.4.3 Generation and importance of fOS in eukaryotes

Interestingly, fOS generation by OTases from N-linked protein glycosylation pathways appears to be a conserved mechanism. Soon after a study reported fOS generation in all *Campylobacter* species (Nothaft *et al.,* 2009; Nothaft *et al.,* 2012), another study reported the OTase mediated release of the $Glc_3Man_9GlcNac_2$ fOS structure from LLOs in the yeast, *S. cerevisiae* (Harada *et al.,* 2013). The authors provided genetic and biochemical evidence for this novel phenomenon, and the OTase from the N-linked glycosylation pathway was shown to generate fOS by *in vivo* and *in vitro* methods (Harada *et al.,* 2013). In addition, the Stt3 subunit from the protozoa, *Leishmania major*, was found to possess LLO hydrolytic activity as well when expressed in *S. cerevisiae* in the same study (Harada *et al.,* 2013).

In eukaryotes, the majority of fOS is released by the endoplasmic reticulum associated degradation (ERAD) pathway (Chantret and Moore, 2008; Hirayama *et al.,* 2010; Hirayama and Suzuki, 2011; Chantret *et al.,* 2011). As mentioned earlier, in eukaryotes the OTase transfers $Glc_3Man_9GlcNac_2$ onto nascent polypeptide chains (Roth *et al.,* 2010). Soon after glycosylation,

the covalently attached oligosaccharide is trimmed down to $Glc_1Man_9GlcNAc_2$ by the sequential action of glucosidase I and II enzymes after which the glycoprotein is directed towards the calnexin/calreticulin cycle (Roth *et al.,* 2010). Calnexin and calreticulin are ER membrane bound and luminal proteins, respectively, that are involved in correctly folding proteins (Roth *et al.,* 2010). Correctly folded proteins are de-glucosylated by glucosidase II enzyme and exit the cycle (Roth *et al.,* 2010). Misfolded proteins may go through another calnexin/calreticulin cycle, however, consistently misfolding proteins enter the ERAD pathway (Suzuki, 2015). Such proteins are translocated to the cytosol where they are degraded by the 26S proteosome (Suzuki, 2015). In this process, the cytoplasmic peptide:N-glycanase (PNGaseF) removes the glycan, $Man_9GlcNAc_2$, from the peptide (Suzuki and Harada, 2014) which is then trimmed to $Man_9GlcNAc_1$ by endo-β-N-acetylglucosaminidase (ENGase) in most eukaryotic organisms (Suzuki and Harada, 2014). The latter structure corresponds to the majority of cytosolic fOS in eukaryotic cells (Suzuki and Harada, 2014).

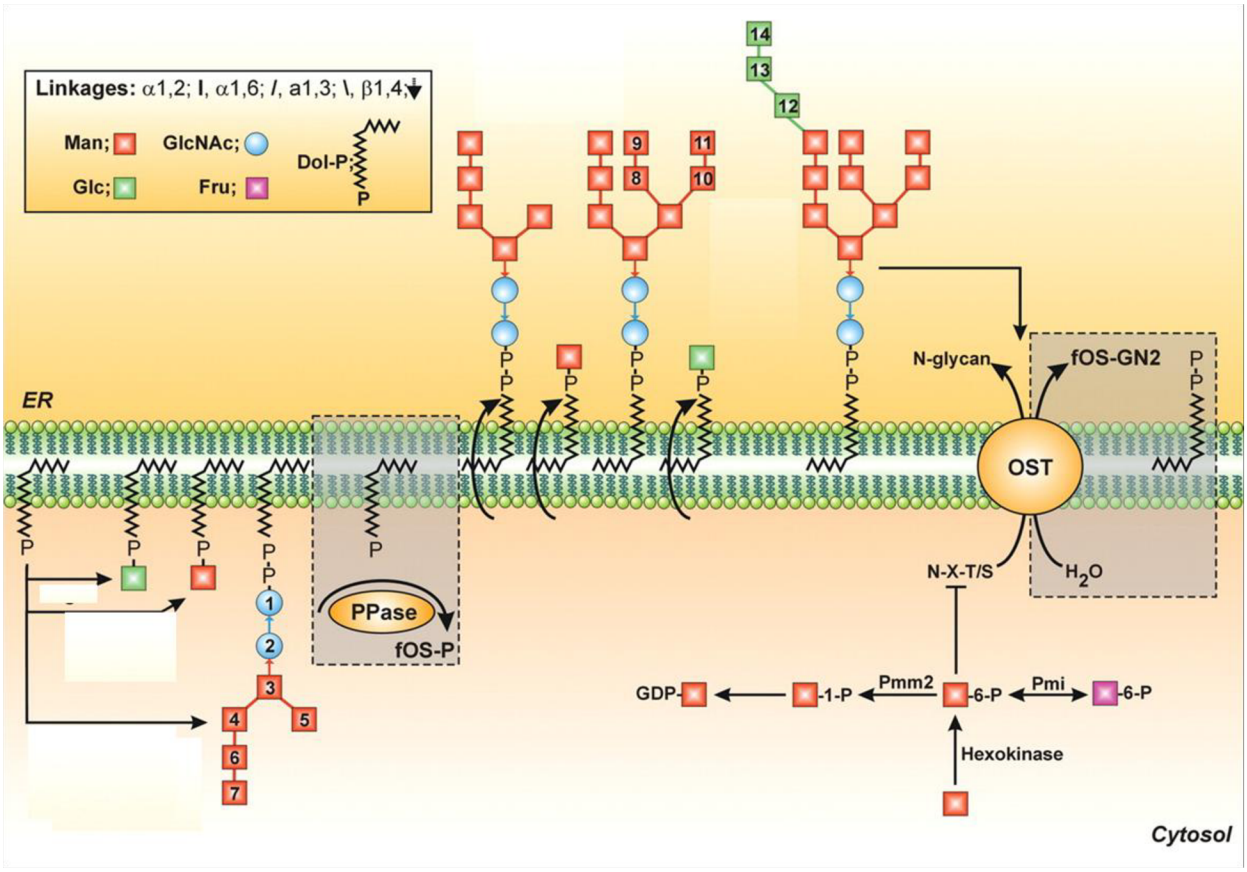

Fig. 1.3 The eukaryotic N-glycosylation pathway. The initial synthesis of the N-glycan oligosaccharide occurs in the cytosol where a $Man_5GlcNAc_2$ is assembled on a dolicholphosphate lipid carrier. The oligosaccharide is then flipped into the lumen of the endoplasmic reticulum (ER) where it is further extended to $Man_9GlcNAc_2$. The oligosaccharyltransferase complex (OTase) N-glycosylates proteins and releases free oligosaccharides (fOS) into the ER lumen (Chantret and Moore, 2008; Harada *et al.*, 2013). Phosphorylated fOS are generated by another mechanism in the cytosol by a yet unidentified pyrophosphatase enzyme on the dolichol pyrophosphate linked $Man_5GlcNAc_2$. fOS are also generated by the degradation of glycoproteins in the cytosol (not shown) (mannose, Man; glucose, Glc; N-acetylglucosamine, GlcNAc; fructose, Fru; dolichol phosphate, Dol-P). Figure modified from Chantret and Moore, 2008.

In addition, to the above mechanisms for fOS generation, phosphorylated fOS (P-fOS) have also been observed in eukaryotes. Cytosolic ($Man_{0-5}GlcNAc_2$-P) and luminal ($Man_{6-7}GlcNAc_2$-P) P-fOS are also generated from LLO pools by an unknown putative pyrophosphatase enzyme(s) (Suzuki and Harada, 2014). It is believed that this mechanism may have evolved to avoid glycosylation of proteins with incomplete oligosaccharides (Suzuki and Harada, 2014). However, although strong evidence for the enzymatic activity associated with this phenomenon has been demonstrated, the location and identity of the enzyme(s) is yet to be determined (Suzuki and Harada, 2014).

The fOS are also further degraded and recycled (Suzuki and Harada, 2014). However, the exact mechanism of how the fOS that are generated in the ER lumen are transported to the cytosol is unknown, evidence suggests the presence of a transport machinery in the ER membrane that is dependent on ATP and Ca^{2+} (Suzuki and Harada, 2014). In mammals, the $Man_9GlcNAc_2$ may be trimmed to $Man_8GlcNAc_2$ in the ER before transport (Chantret and Moore, 2008). This fOS bearing terminal $GlcNAc_2$ are then quickly trimmed to $GlcNAc_1$ by the cytoplasmic ENGase followed by partial demannosylation by a cytosolic mannosidase, M2C1, to $Man_5GlcNAc_1$ (Chantret and Moore, 2008). The process is slightly different in yeast which lacks an ENGase, and $Man_9GlcNAc_2$ are trimmed by a homologous protein, the vacuolar mannosidase, Ams1p, to $Man_8GlcNAc_2$ (Chantret and Moore, 2008). In yeast, it is believed that this $Man_8GlcNAc_2$ structure has a quick turnover time since the pool has a very short half-life and is cleared without evidence of any other intermediate products (Chantret and Moore, 2008). In mammals, the $Man_5GlcNAc_1$ is transported into lysosomes in an ATP dependent process and further degraded into individual Man and GlcNAc residues (Chantret and Moore, 2008; Harada *et al.,* 2015). It is

yet to be determined how exactly P-fOS are degraded, however it is speculated that they are dephosphorylated and then degraded via the same pathways as neutral fOS (Harada *et al.,* 2015). Current evidence suggests that a majority of neutral fOS arise from deglycosylation of proteins by PNGase and not from OTase activity in *S. cerevisiae* (Chantret and Moore, 2008). Approximately, 4% of fOS is generated by the OTase in *S. cerevisiae* (Chantret and Moore, 2008). In contrast, in mammalian cells, a majority of the fOS is attributed to a PNGase independent activity, presumably OTase activity (Harada *et al.,* 2013). Recently, complex type extracellular fOS that are substituted with sialic acid, fucose and galactose residues and bear $GalNAc_2$ at the reducing end were identified in human sera and are thought to originate from novel sources, such as an extracellular PNGase F like enzyme (Iwatsuka *et al.,* 2013).

S. cerevisiae cells deficient in fOS production (Suzuki *et al.,* 2000) and degradation (Kuranda and Robbins, 1987; Cueva *et al.,* 1990) do not exhibit any obvious phenotypes (Chantret and Moore, 2008). However, in higher eukaryotic organisms, such as *Caenorhabditis elegans*, ENGase mutants exhibit a slightly shorter life cycle (Kato *et al.,* 2007). In the fungus, *Trichoderma atroviride,* a homologue of the ENGase enzyme was recently identified and mutants were observed to have significantly reduced growth rates (Dubey *et al.,* 2012). In some human cell lines, downregulation of mannosidases that trim down fOS and N-linked glycan structures, result in changes in cell morphology and adhesion between cells (Yue *et al.,* 2004; Qu *et al.,* 2006; Chantret and Moore, 2008). However, although these studies indicate important roles of fOS in various organisms, the specific roles fOS play are yet to be determined (Chantret and Moore, 2008). In addition, fOS are being extensively studied as biomarkers for various types cancer as well, however it is difficult to determine if they are a cause or an effect of the disease (Ishizuka *et al.,* 2008; Yabu *et al.,* 2013).

Relatively less information on fOS generation and degradation/recycling is available in plants. Similar to mammals, the mannosylated fOS that are generated by the cytosolic PNGase from misfolded proteins and display two terminal GalNAc at the reducing end, are further trimmed by the ENGase enzyme to bearing one GalNAc (Maeda and Kimura, 2014). On the other hand, fOS with Fuc, Xyl and other modifications are known as complex type and are only found to display $GalNAc_2$, and are therefore believed to be devoid of ENGase action (Maeda and Kimura, 2014). These complex type fOS are generated from matured and secreted glycoproteins from the Golgi (i.e. not misfolded proteins) by a putative PNGase enzyme that is genetically different from the cytosolic PNGase enzyme and is optimally functional in an acidic environment, however the exact location of this acidic PNGase enzyme is unknown (Maeda and Kimura, 2014). Similar to the presence of extracellular fOS in human sera, the presence of mannose and complex fOS in the extracellular space of plants suggest that an additional acidic type PNGase may be found in the extracellular space, such as the cell wall or apoplastic area (Maeda *et al.,* 2010; Maeda and Kimura, 2014).

It has been proposed that fOS play signalling roles in plants as the amount of mannosylated fOS displaying GlcNAc1 at the reducing end increase significantly during tomato fruit maturation (Nakamura *et al.,* 2008; Nakamura *et al.,* 2009; Maeda and Kimura, 2014). In *Arabidopsis thaliana*, plants that had both ENGase enzymes knocked out were constructed. No morphological differences were observed between double knockout ENGase mutants and wildtype plants in this study, however in order to properly investigate the roles of fOS in plants, the construction of plants that have the PNGase, as well as, both ENGase enzymes knocked out has been proposed (Maeda *et al.,* 2010; Fischl *et al.,* 2011; Kimura *et al.,* 2011; Maeda and Kimura, 2014). In addition, mannosylated fOS displaying $GlcNAc_2$, as well as, complex type

fOS displaying one or two GlcNAcs at the reducing end have been detected in rice culture medium. However, the exact roles of these extracellular fOS structures is unknown (Maeda *et al.,* 2010).

1.5 The roles and importance of N-glycan protein modification in eukaryotes and archaea

In contrast to fOS, several studies have reported various roles for the N-glycan modification on proteins across all domains of life.

N-glycan modification of proteins has been shown to affect the localization, stability and functional activity of several proteins in humans. For example, the human apical sodium dependent bile acid transporter (ASBT) is mono-glycosylated and, is defective in function and more sensitive to degradation by proteases in the absence of N-glycan modification (Muthusamy *et al.,* 2015). On the other hand, N-glycosylation affects the localization, but not the function of the di-glycosylated human amino acid transporter SLC1A5 (Console *et al.,* 2015). Absence of proper N-glycosylation causes several diseases that are grouped into the category of congenital disorders of glycosylation (CDG) (Parodi, 2000; Jaeken, 2013; Bieberich, 2014; Krasnewich, 2014; Scott *et al.,* 2014; Console *et al.,* 2015; Min *et al.,* 2015). CDG are categorized into two types based on defects in LLO assembly (Type I) or faulty processing of N-glycans on glycoproteins (Type II) (Leroy, 2006). Symptoms are present as early as infancy for CDG disorders. Soon after birth poor suckling, lethargy, hypothermia and dysmaturity are observed (Leroy, 2006). Type I is associated with 20% fatality risk in infancy (Leroy, 2006). Although milder cases have been recorded, the individuals are usually reported to be speechless and to some extent, physically crippled (Leroy, 2006). Type II is associated with characteristics such as poor eye contact, weak neuromotor development and weak learning behaviour (Leroy, 2006). In addition, differential glycosylation of proteins can serve as biomarkers for various types of

cancer, although it is unknown if these are a cause or effect of cancer (Leroy, 2006; Nakata, 2014).

In plants, mutations in the N-glycosylation pathway cause multiple defects in the life cycle and serious growth/developmental defects are observed, such as abnormal cell walls, reduced fertility and defects in cellulose biosynthesis (Lerouxel *et al.,* 2005; Zhang *et al.,* 2009). In *Arabidopsis thaliana*, mutations in subunits of the OTase complex result in activation of the unfolded protein response and this severely increases the sensitivity of the plant to osmotic/salt stress (Koiwa *et al.,* 2003; Farid *et al.,* 2013). N-glycosylation plays important roles in yeast as well. In *S. cerevisiae*, N-glycosylation mutants exhibit cell division defects, slower growth rates and temperature sensitivity (Klebl *et al.,* 1984; Zhou *et al.,* 2007), whereas in *Schizosaccharomyces pombe* N-glycosylation mutants are sensitive to osmotic stress and exhibit cell division defects (Huang and Snider, 1995).

In archaea, mutations in the N-glycosylation pathway cause serious defects in S-layer and flagellar protein function (VanDyke *et al.,* 2009; Jarrell *et al.,* 2010; Tripepi *et al.,* 2012). In *S. acidocaldarius*, however N-glycosylation is not important for proper assembly of the flagellar filament, it is required for proper motility (Meyer *et al.,* 2014). N-glycosylation mutants also have a reduced growth rate of approximately 50% compared to the wildtype under hyperosmotic stress (Meyer *et al.,* 2013). In addition, N-glycosylation is believed to be essential in the species as a chromosomal OTase mutation could not be created unless a second copy was inserted into a non-essential gene in the genome (Meyer and Albers, 2014). Although, N-glycosylation is not essential in other species, such as, *Hfx. volcanii*, *M. maripaludis* and *M. voltae,* studies indicate that the process is definitely advantageous for the organisms (Chaban *et al.,* 2006; Abu-Qarn and Eichler, 2007; VanDyke *et al.,* 2009; Jarrell *et al.,* 2014). In addition, interesting effects of

osmotic stress on N-glycan structure have been reported. In *H. volcanii*, the N-glycan structure on S-layer proteins, as well as, the site of N-glycosylation changes in response to the extracellular salinity (Guan *et al.,* 2012) and this response involves two distinct N-glycosylation pathways (Kaminski *et al.,* 2013). This suggests that N-glycosylation may play roles in osmotic adaptation in this species, however it is unclear how changing the N-glycan structure or sites exactly impart any advantages under the conditions examined (Guan *et al.,* 2012). In addition, mutants lacking the N-glycan modification exhibit 4 fold less stability of the S-layer, as well as, increased sensitivity to proteases, such as proteinase K and trypsin, compared to fully N-glycosylated S-layer (Kaminski *et al.,* 2010; Jarrell *et al.,* 2014). Also, OTase mutants released more S-layer proteins into the growth medium indicating a role of the N-glycan in proper S-layer assembly or stability (Abu-Qarn and Eichler, 2007). OTase mutants also do not express flagella and are non-motile (Tripepi *et al.,* 2012; Jarrell *et al.,* 2014). In the methanogens, *M. voltae* and *M. maripaludis*, absence of a complete N-glycan on flagellar units results in complete absence of flagella and therefore an absence of motility (Chaban *et al.,* 2006; VanDyke *et al.,* 2008; Jarrell *et al.,* 2014). Additionally, in *M. maripaludis*, the type IV pili have been shown to be N-glycosylated (VanDyke *et al.,* 2008). Mutations that result in severely truncated N-glycan do not affect the assembly of pili, however the attachment of the pili to the cell seems to be affected as detached pili are found in the culture medium (VanDyke *et al.,* 2008; Jarrell *et al.,* 2014).

1.5.1 Roles of protein N-glycosylation in bacteria

As mentioned above, N- glycosylation is reported to play many roles in eukaryotes and archaea in terms of protein stability, localization and function. However, only a few examples exist that show specific effects of N-glycan modification on bacterial proteins. For example, in *H. influenza*, N-glycosylation of adhesion HMW1 is essential for its stability (Grass *et al.,* 2003).

Absence of N-glycosylation appears to affect the tethering of the protein to the cell surface (Grass *et al.,* 2003). Wildtype cells express high levels of glycosylated HMW1 on the cell surface compared to cells expressing unglycosylated HMW1 indicating that glycosylation plays important roles in the correct cellular localization of the protein (Grass *et al.,* 2003). Also, smaller amounts of unglycosylated protein is present in whole cell extracts indicating the possibility that the unglycosylated form might be more susceptible to proteolytic degradation in the cell (Grass *et al.,* 2003).

In *C. jejuni,* mutations in the *pgl* pathway result in multiple phenotypes. The growth rate of a *pglB* mutant is reported to be comparable to wildtype in standard Mueller Hinton broth, the mutant adhered at 38% and invaded at 4.4% relative to the wildtype in adherence and invasion assays with the human intestinal epithelial cell line (INT407) (Szymanski *et al.,* 2002). Also, the *pglB* mutant was only able to colonize the intestinal tracts of mice at approximately 30% levels compared to wildtype *C. jejuni* (Szymanski *et al.,* 2002). This study obtained similar results with a *pglE* mutant as well (Szymanski *et al.,* 2002). Another study found that mutations in *pglE, pglF* and *pglH* caused a 100-1000 fold reduction in the ability of the bacterium to colonize gastrointestinal tracts of leghorn chicks (Hendrixson, 2006). Further studies confirmed the colonization defects of *pgl* mutants in chick colonization studies in other strains of *C. jejuni* (Karlyshev *et al.,* 2004; Kelly *et al.,* 2006). Later, Larsen *et al.* (2004) found that the VirB10 protein, which is a component of the Type IV secretion system of *C. jejuni*, is N-glycosylated at two sites (Larsen *et al.,* 2004). The authors found that upon mutating one of the asparagine residues to an alanine residue within one of the N-glycosylation sites in VirB10, the DNA uptake competence of the mutant strain was reduced by approximately 60% compared to wildtype and was similar to the competence of *pglB* and *pglE* mutants that had dropped to 80- 90% compared

to the wildtype strain (Larsen *et al.,* 2004). This was the first report of N-glycosylation affecting the function of a specific protein in *C. jejuni* (Larsen *et al.,* 2004). Another interesting observation the authors made in the study was that whereas glycosylated protein was detectable in *C. jejuni*, unglycosylated VirB10 was not detected in periplasmic extracts of *C. jejuni* cells by Western blot analysis (Larsen *et al.,* 2004). This indicated that unglycosylated VirB10 is either not transported to the periplasmic space and/or is unstable and rapidly degraded due to its inability to properly interact with other components of the Type IV secretion system (Larsen *et al.,* 2004). Binding by the human Macrophage C-type lectin, MGL receptor, is also reduced in *pgl* mutants (van Sorge *et al.,* 2009). The authors demonstrated that the MGL receptor on dendritic cells is able to recognize N-glycan structures via the GalNAc residues. The recognition was abolished upon addition of exogenous free GalNAc and cell extracts from a *pglA* mutant were not recognized in Western blot analysis with the Fc binding region of the MGL receptor (van Sorge *et al.,* 2009). Analysis of IL-6 production by dendritic cells from donors showed that higher IL-6 levels were produced by a *pglA* mutant compared to wildtype suggesting that the presence of the *pgl* locus reduces the production of IL-6 possibly via interactions with the MGL receptor (van Sorge *et al.,* 2009). N-glycans attached to *Campylobacter* surface proteins also protect them from proteolytic degradation by chicken gut proteases resulting in increased bacterial fitness (Alemka *et al.,* 2013). The authors of this study found a significant reduction in the colony forming units of a *pglB* mutant after incubation with chicken caecal contents compared to the wildtype strain of *C. jejuni* (Alemka *et al.,* 2013). Kakuda *et al.* (2012) investigated the function of two N-glycosylated mechanosensitive channels in *C. jejuni*. The authors found that although cells expressing the unglycosylated forms of the channels were able to survive hypososmotic shocks at similar levels compared to the wildtype strain, a *pglB* mutant

is approximately 4 times more sensitive to hypoosmotic shock (Kakuda *et al.,* 2012). This indicated that protein N-glycosylation plays important roles in the ability of *C. jejuni* to survive osmotic shock (Kakuda *et al.,* 2012). As mentioned above, disruption of the Pgl pathway results in several phenotypes in *C. jejuni*, however since pathway mutants lack N-glycans and fOS, these phenotypes cannot be attributed specifically to loss of N-linked protein glycosylation or fOS.

So far studies in *C. jejuni* have revealed that whereas fOS levels change under certain conditions such as osmotic stress, the overall protein N-glycosylation status of the cell is not affected and the N-glycan structure is unaltered (Nothaft *et al.,* 2009). Together with the above mentioned findings, the constant expression of N-glycosylated proteins appears to be an important aspect of *C. jejuni* physiology.

1.6 Oligosaccharyltransferase enzymes: current studies and future applications

It has been a decade since the *C. jejuni pgl* locus was discovered and shown to be functional upon transfer into the glycoengineering host, *E. coli* (Wacker *et al.,* 2002). Since then, several other N-glycosylation systems have been discovered in other bacterial and archaeal organisms (Grass *et al.,* 2003; Chaban *et al.,* 2006; Grass *et al.,* 2010; Choi *et al.,* 2010; Ielmini and Feldman, 2011; Kaminski *et al.,* 2013; Matsumoto *et al.,* 2013; Cohen-Rosenzweig *et al.,* 2014; Meyer and Albers, 2014). The OTase enzymes together with their protein structures, glycan donor and substrate specificities are being studied extensively due to their potential applications in the production of recombinant vaccines and therapeutics (Wacker *et al.,* 2002; Ihssen *et al.,* 2010; Schwarz *et al.,* 2010; Valderrama-Rincon *et al.,* 2012; Wetter *et al.,* 2012; Cuccui *et al.,* 2013).

Previously, glycoconjugate vaccines were generated by chemical conjugation of oligosaccharides to carrier proteins. However, some of the problems with this method are inconsistencies in the structures of the chemically conjugated products and the presence of toxic contaminants generated during the process (Ihssen *et al.,* 2010). Therefore, the production of glycoconjugate vaccines in *E. coli* is being studied extensively since the system is able to generate consistent structures with less toxic contaminants. Ihssen *et al.* (2010) conjugated *Shigella* O-antigen to exotoxin A carrier protein from *Pseudomonas aeruginosa* in *E. coli.* Previously, a chemically synthesized version of the same vaccine was shown to induce strong immune responses in adult volunteers proving to be a promising candidate in the prevention of shigellosis (Taylor *et al.,* 1993). Ihssen *et al.* (2010) also optimized the production of glycoconjugates in *E. coli* by testing different growth conditions such as, culturing conditions, culture supplements, induction times and inducer concentrations in order to determine optimal conditions for increased glycoprotein yields (Ihssen *et al.,* 2010). Generation of eukaryotic N-glycan structures in bacteria is also important to generate therapeutics for treatment of various CDG diseases, such as erythropoietin deficiency, which can be treated by exogenous supplementation with properly N-glycosylated recombinant erythropoietin therapeutics (Jelkmann, 2013). In another recent study, the authors optimized a previously published method by Shwarz *et al.* (2010) to generate recombinant proteins modified with $Man_3GlcNAc_2$ (i.e. $mannose_3$-*N*-$acetylglucosamine_2$) (Valderrama-Rincon *et al.,* 2012). The authors utilized yeast glycosyltransferases enzymes to synthesize and transfer the core structure of eukaryotic N-linked pathways, $Man_3GlcNAc_2$ (i.e. $mannose_3$-*N*-$acetylglucosamine_2$) onto an antibody fragment in *E. coli* (Valderrama-Rincon *et al.,* 2012). Recently, through structure guided mutagenesis, the glycosylation efficiency of PglB for *Salmonella enterica* O-antigen and *S. aureus* capsular polysaccharide was increased (Ihssen *et*

al., 2015). The polysaccharide was conjugated to *P. aeruginosa* exotoxin A and this vaccine candidate has been shown to illicit strong immune responses against the pathogen in clinical trials (Jones, 2005; Wacker *et al.,* 2014). These studies highlight the importance of studying these enzymes in detail in terms of their structure, mechanism, substrate/donor specificities and kinetics.

Since the application of OTases in the biotechnology industry has been demonstrated, several publications have reported protocols for studying the kinetics and acceptor/substrate specificity of OTases (Jervis *et al.,* 2010; Ihssen *et al.,* 2012). So far, OTase studies have relied on semi-quantitative protocols that involve separation of glycosylated and unglycosylated reaction products followed by gel imaging with fluorescent peptides or Western blots with the fluorescent antibodies generated against the products (Jervis *et al.,* 2010; Gerber *et al.,* 2013; Musumeci *et al.,* 2013; Liu *et al.,* 2014; Ishiwata *et al.,* 2015). Other studies have developed ELISA assays in order to determine glycosylation efficiencies using antibodies against the glycan of interest (Ihssen *et al.,* 2012; Ihssen *et al.,* 2015; Kampf *et al.,* 2015). In order to demonstrate the presence of N-linked glycosylation in *Helicobacter pullorum,* Jervis *et al.* (2010) used fluorophore labelled peptides for *in vitro* glycosylation with membrane preparations containing OTase enzymes from either *C. jejuni* or *Helicobacter pullurom*. The reaction products (i.e. glycosylated and unglycosylated peptide) were then separated by gel electrophoresis on tricine gels and were visualized and quantified using a fluorescence imager. Recently, Ihssen *et al.* (2012) tested the efficiency of several mutated PglB enzymes to transfer the *S. aureus* CPS to the *P. aeruginosa* exotoxin A by a new ELISA based assay they developed in the study. The authors generated PglB variants by error prone PCR and quantified glycosylation efficiencies by ELISA using 96-well plates coated with antibodies against the glycan used in the study (i.e. CPS of

Staphylococcus aureus serotype 5). Musumeci *et al.* (2013) characterised the kinetics and substrate specificity of the O-linked OTase, PglL, from *Neisseria meningitides* using fluorescent peptide and tricine gel separation followed by fluorescence imaging as well. The current assays are time consuming since they require electrophoretic separation and required expensive equipment capable of fluorescent image analysis. For ELISA analysis, generation of antibodies against the glycan of interest is necessary. Development of new and faster assays is required to effectively and accurately determine OTase mediated glycosylation rates, and donor/substrate specificities.

1.7 Thesis objectives

The aims of this PhD thesis are to better understand the abundance, generation and roles of both products of the Pgl pathway in *Campylobacter* species: fOS and N-linked glycoproteins.

As described earlier, fOS release is a universal feature coupled with the N-linked protein glycosylation pathway in *Campylobacter* species. Previously, fOS purification and analysis consisted of multiple time- consuming steps (Liu *et al.,* 2006; Nothaft *et al.,* 2012) Analysis using MS required proteolytic digestion of cells up to 72 hr (Liu *et al.,* 2006) or cell disruption by sonication followed by porous graphitized carbon purification (Liu *et al.,* 2006; Nothaft *et al.,* 2012). In some cases, permethylation of the glycans was required to increase the sensitivity of MS analysis and to obtain structural information on the oligosaccharide (Liu *et al.,* 2006). Although amounts that corresponded to 1 mg of whole cell lysates were sufficient for ESI-based sqMS of *C. jejuni* fOS, non-stoichiometric isolation of fOS did not allow accurate quantification (Liu *et al.,* 2006; Nothaft *et al.,* 2012). For structural analysis by nuclear magnetic resonance (NMR) upto 12-20 g of bacterial cell material was required as starting material and several chromatography steps were required to purify the required amount of fOS (Nothaft *et al.,* 2012).

One of the aims of this thesis is to develop highly efficient and faster fOS isolation and analysis methods that can be applied to study the abundance of structurally diverse fOS structures. fOS analysis techniques that will be developed will be based on a combination of thin layer chromatography, high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), MS and NMR. As it is time-consuming to grow a large amount of bacterial cells that is required by the previously published methods, the starting cell material required for fOS isolation and analysis will be downscaled. In addition, it would be useful to develop an HPAEC-PAD based method to accurately quantify fOS in molar concentrations. If this method is established successfully according to the above objectives, the method can be used to study neutral, charged and mixed fOS species, including those with labile substitutions, such as the fOS from *C. lari* that contains phosphate substitutions (Nothaft *et al.,* 2012). We will be able to accurately quantify fOS and determine its abundance in the cell and these methods will in turn help us gain more insights into the generation and roles of fOS in *Campylobacter* species.

In addition, as previously mentioned the OTase activity of PglB is being studied extensively whereas the fOS generation mechanism by the PglB enzyme is not well understood. The *C. lari* PglB OTase was recently crystallized and reported to have a transmembrane domain (residues 1-432) and a periplasmic domain (residues 433-712) that have extensive non-covalent interactions between them (Lizak *et al.,* 2011). The transmembrane domain has 13 transmembrane segments connected by short cytoplasmic and periplasmic loops with the exception of two external loops (EL1 and EL5) (Lizak *et al.,* 2011). The *C. jejuni* PglB N-glycosylates itself at N534 (Scott *et al.,* 2011) and the *C. lari* PglB is also N-glycosylated at N535 and N556 (Lizak *et al.,* 2011). It is unknown whether the N-glycosylation of PglB plays specific roles in stability or glycosylation/fOS generation activities of the enzyme. Preliminary findings made by an MSc

student in the lab (Yasmin Barre) suggest that N- linked glycosylation of PglB influences its fOS generation activity. Removal of the N-glycosylation site in PglB from *C. jejuni* result in approximately 50% reduced fOS levels *in vivo* compared to cells expressing the N-glycosylated form of PglB as determined by sqMS. The aim of this second project is to accurately quantify the levels to which fOS and OTase activity of PglB is affected by its N-glycan modification. Once the previously mentioned fOS analysis and quantitation methods are established, we will apply those methods for absolute quantitation of fOS in the PglB N-glycosylation mutant. In addition, the effect of this mutation on PglB OTase (i.e. glycosylation) activity will be studied by previously well established Western blot analysis methods. Furthermore, the exact mechanism(s) by which these alterations in activity may occur will be investigated.

The final aim of my thesis is to develop faster methods to assess the glycosylation efficiency of the PglB enzyme. I plan to develop a 96-well plate fluorescence resonance energy transfer (FRET) based assay to effectively and accurately determine OTase mediated *N*-glycosylation rates, and donor/substrate specificities using the PglB OTases from *Campylobacter* species. A similar method has previously been applied to study inhibitors of O-linked OTase enzymes (Gross *et al.,* 2008). This study will provide an important tool for quick screening of the N-glycosylation activity of OTases from *Campylobacter* species and can potentially be further extended to study other OTase enzymes as well.

In summary, this thesis will focus on:

1) Developing efficient fOS isolation, analysis and quantitation techniques in order to better understand their abundance, structural features and importance in *Campylobacter* species.
2) Understanding the contribution of self-glycosylation on the enzymatic activity of the PglB enzyme from *C. jejuni.*

3) Developing a 96-well plate FRET based assay for determination of N-glycosylation activity of OTase enzymes from various *Campylobacter* species.

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CHAPTER 2

Generation of free oligosaccharides from bacterial protein N-linked glycosylation systems

A version of this chapter has been published.

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I was responsible for designing the method development protocol. In addition, I performed data collection and analysis of experiments involving thin layer chromatography, porous graphite carbon purification, silica gel chromatography and high performance anion exchange with pulsed amperometric detection (HPAEC-PAD). All samples for mass spectrometry and nuclear magnetic resonance (NMR) were prepared by myself. Technical training of HPAEC-PAD equipment was provided by Harald Nothaft. Bela Reiz and Randy Whittal assisted with all mass spectrometry analysis and mass spectrometry figure generation. Portion of NMR figure was re-published from a previously published study by permission from the respective journal. Christine M. Szymanski was responsible for overlooking all experiments performed in this study. I was responsible for manuscript preparation and all co-authors provided their input in the editing process.

2.1 Introduction

The first bacterial N-linked protein glycosylation system encoded by the *pgl* gene cluster, was described in *Campylobacter jejuni,* a pathogen that is the leading cause of gastroenteritis worldwide (Szymanski *et al.,* 1999; Allos, 2001). The *C. jejuni* pathway involves the synthesis of a heptasaccharide: GalNAc-α1,4-GalNAc-α1,4-[Glcβ1,3]-GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-diNAcBac-β1 (where diNAcBac is 2,4-diacetamido-2,4,6-trideoxyglucopyranose) on the lipid carrier, undecaprenylphosphate (Und-P), on the inner side of the periplasmic membrane (Wacker *et al.,* 2002; Young *et al.,* 2002; Reid *et al.,* 2010). First, diNAcBac is synthesized from UDP-GlcNAc by the consecutive actions of a dehydratase (PglF), an aminotransferase (PglE), and an acetyltransferase (PglD) (Olivier *et al.,* 2006; Schoenhofen *et al.,* 2006) and transferred to Und-P by PglC (Glover *et al.,* 2006). Four glycosyltransferases (GTases), PglA (first GalNAc), PglJ (second GalNAc), PglH (third, fourth and fifth GalNAc), and PglI (Glc branch) (Glover *et al.,* 2006; Kelly *et al.,* 2006) extend the lipid-linked oligosaccharide (LLO) that is subsequently flipped to the periplasmic space by the flippase, PglK (Kelly *et al.,* 2006; Alaimo *et al.,* 2006) and transferred onto asparagine (N) residues in the sequon D/E-X1-N-X2-S/T (X cannot be proline) by the membrane bound oligosaccharyltransferase (OTase), PglB (Wacker *et al.,* 2002; Young *et al.,* 2002; Kowarik *et al.,* 2006). In addition, the Pgl pathway-derived glycans are released from the lipid-linked carrier into the periplasmic space as free oligosaccharides (fOS) by the hydrolytic activity of PglB (Liu *et al.,* 2006; Nothaft *et al.,* 2009). In *C. jejuni*, this hydrolytic PglB-activity is dependent on the extracellular osmolarity, suggesting a role of fOS in the survival of *C. jejuni* under osmotic stress (Nothaft *et al.,* 2009). Recently, we demonstrated that all *Campylobacters* modify their proteins

with species-specific N-linked glycans and also release structurally identical fOS, indicating that this component is ubiquitous among *Campylobacter* species (Nothaft *et al.,* 2012).

The presence of fOS in the *Campylobacter* periplasm is reminiscent of osmoregulated periplasmic glucans (OPGs) in other proteobacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Rhozibium* and *Brucella spp.* Both fOS and OPG concentrations decrease with increasing extracellular osmolarity (Lee *et al.,* 2009; Nothaft *et al.,* 2009). OPGs play crucial roles in osmotic adaptation, motility, antibiotic resistance, intercellular signalling, pathogenesis and plant symbiosis (Mah *et al.,* 2003; Lequette *et al.,* 2007; Bhagwat *et al.,* 2012; Gay-Fraret *et al.,* 2012; Martirosyan *et al.,* 2012; Bontemps-Gallo *et al.,* 2013). In *C. jejuni,* mutants in the *pgl* pathway display several altered phenotypes such as reduced attachment and invasion of human epithelial cells (Szymanski *et al.,* 2002), impaired colonization of the intestinal tracts of mice and chickens (Szymanski *et al.,* 2002; Karlyshev *et al.,* 2004; Kelly *et al.,* 2006; Hendrixson, 2006), reduced natural competence in strains with Type IV secretion pathways (Larsen *et al.,* 2004) and reduced binding by the human macrophage C-type lectin, MGL (van Sorge *et al.,* 2009). Moreover, N-glycans attached to *Campylobacter* surface proteins protect them from proteolytic degradation by chicken gut proteases resulting in increased bacterial fitness (Alemka *et al.,* 2013). However, since *pgl* pathway mutants lack N-glycans and fOS, these phenotypes cannot be attributed specifically to loss of N-linked protein glycosylation or fOS production.

Previously, fOS analysis using mass spectrometry required proteolytic digestion of cells up to 72 hr (Liu *et al.,* 2006) or cell disruption by sonication followed by porous graphitized carbon (PGC) purification. In some cases, permethylation of the glycans was required to increase the sensitivity of mass spectrometry analysis and to obtain structural information on the

oligosaccharide (Liu *et al.,* 2006). Although amounts that corresponded to 1 mg of whole cell lysates were sufficient for electrospray ionization (ESI) based semi-quantitation of *C. jejuni* fOS, non-stoichiometric isolation of fOS did not allow accurate quantification. For structural analysis by NMR, large scale growth, in the range of 12-20 g of bacterial cell pellets, and several chromatography steps were required to purify the required amount of fOS (Nothaft *et al.,* 2012). In this study, we describe a highly efficient fOS isolation method that was applied to the analysis of structurally diverse fOS from *C. fetus fetus, C. jejuni, C. lari* and *C. rectus*. We show that fOS extraction is highly efficient requiring less starting material therefore reducing the presence of potential contaminants and eliminating the need for additional purification steps. Moreover, the purification/enrichment of fOS by thin layer chromatography (TLC) or silica gel chromatography (SGC) in combination with porous graphitized carbon purification does not require additional labelling procedures for downstream analysis by matrix assisted laser desorption ionization (MALDI)-mass spectrometry (MS), nuclear magnetic resonance (NMR) and quantitation by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (scheme summarized in Fig. 2.1). Neutral, charged and mixed fOS species can be efficiently isolated and analyzed using this method, including those with labile substituents. Accurate quantification showed that fOS ranges from 7.80 ± 0.84 to 49.82 ± 0.86 nmoles per gram of wet cell pellet (WCP). In *C. jejuni*, fOS comprises 2.5 % of the dry cell weight. This method is a valuable tool to investigate the structure, biosynthesis, degradation and change in fOS levels under varying biological conditions in order to better understand the role of these products in bacteria.

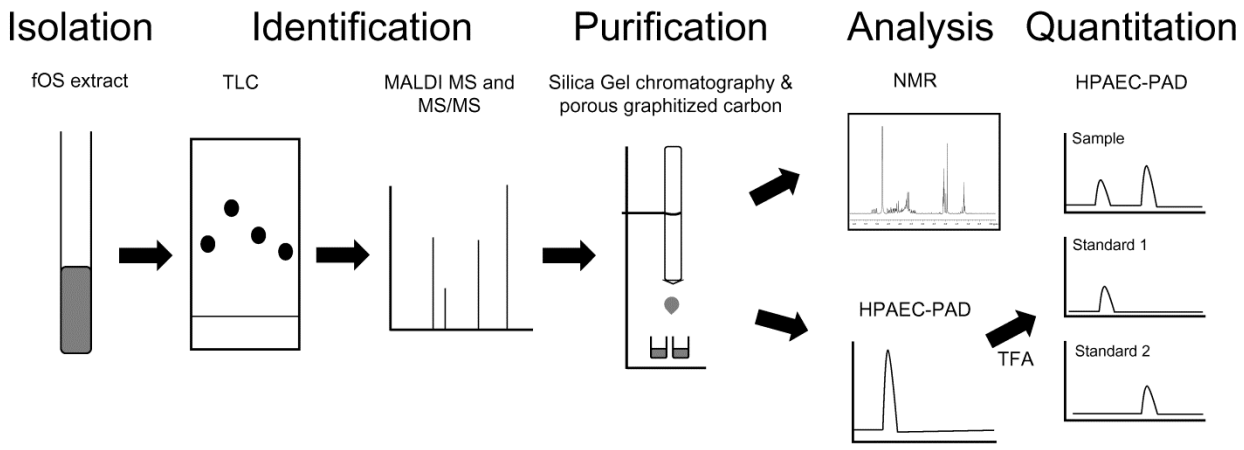

Fig. 2.1 Isolation, purification and analysis of fOS. A flowchart of the methods established in this study for the isolation and analysis of fOS is shown. fOS extracts prepared as described in *Materials and Methods* were analysed by TLC and spots were identified by MALDI MS/MS. Silica gel chromatography followed by porous graphitized carbon chromatography can be carried out for large scale fOS purification. Purified fOS samples can be analysed by HPAEC-PAD or NMR to determine the structural composition. In addition, TFA hydrolyzed fOS can be quantified by HPAEC-PAD using appropriate monosaccharide standards.

2.2 Materials and methods

2.2.1 Bacterial strains and growth conditions

C. jejuni NCTC 11168 (Parkhill *et al.,* 2000), *C. fetus* subspecies *fetus* (Veron and Chatelain, 1973), and *C. lari* subspecies *lari* RM2100 (Fouts *et al.,* 2005) strains were grown on BHI agar or BHI broth for 18 hr under microaerobic conditions. *C. rectus* RM3267 (Tanner *et al.,* 1981) was grown on under anaerobic conditions as described previously (Nothaft *et al.,* 2012). *C. jejuni pglI* and *pglB* mutants (Szymanski *et al.,* 1999; Kelly *et al.,* 2006) were grown in the presence of Kanamycin at a final concentration of 25 µg/mL.

2.2.2 Ethanol extraction and analysis by TLC

A protocol for the isolation of bacterial periplasmic OPGs (Breedveld *et al.,* 1995) was modified to isolate fOS from *Campylobacter* species. Bacterial cells were harvested from overnight cultures by centrifugation at 4300 g for 45 min at 4°C, washed once with 4 mL of deionized water per gram of wet cell pellet (WCP) and centrifuged at 16000 g for 10 min at room temperature (RT). Cells were resuspended in 75% ethanol at 1.5 mL per gram WCP and incubated in a pre-heated 70°C water bath for 30 min. Cell suspensions were centrifuged at 16000 g at RT and the supernatant was diluted with deionized water to 20% ethanol, frozen at -80°C and lyophilized. Alternatively, for small volumes (< 500 µL), the 75% ethanol supernatant was evaporated to dryness in a *fumehood* before further processing. The obtained pellet was resuspended in methanol (600 µL per gram of WCP) and vigorously vortexed for 2 min followed by centrifugation at 16000 g for 5 min at RT. The supernatant was evaporated in a rotatory speed vacuum, centrifuged and resuspended in 120 µL deionized water per gram of WCP. Five µL of sample was spotted onto a TLC plate (Whatman®, aluminum backed, silica coated) and developed until the mobile phase was 3 cm below the upper edge of the plate in a solvent system consisting of a 3:3:2 ratio of acetic acid: n-propanol: water. Carbohydrates were visualized using p-anisaldehyde staining.

To check for residual fOS after 75% ethanol extraction in the pellet, if any, we treated the pellet as follows: the pellet was resuspended in 5 mL of deionized water and sonicated for 2 min in a VWR Scientific, Branson Sonifier 450 at Output Control 1 and a Constant Duty cycle. The suspension was frozen at -80°C and lyophilized. The protocol for fOS extraction was followed as

above, except, 1 mL of methanol was used per gram of WCP and 10 µL of the supernatants were analysed by TLC as described above.

2.2.3 fOS purification by SGC

fOS extracted from 5-10 g cell pellet as described above was purified by SGC using a solvent system consisting of a 3:3:2 ratio of acetic acid: n-propanol: water. An SGC column was prepared in a glass column with a diameter of 2.5 cm by the wet packing method with 54 g of silica gel in the solvent (SiliCycle, 60 Å pore size, 40-63 µM particle size). Lyophilized 75% ethanol extracts (prepared as described above) were resuspended in 60 µL solvent per WCP and centrifuged at 13000 g for 5 min at RT. The supernatant was mixed with 0.5 g-1 g of silica gel, loaded onto the column and topped with 2 g of sand. Then, 62 x 10 mL elution fractions were collected and evaporated to dryness in a fume hood. The resulting pellets were resuspended in 500 µL deionized water and 5 µL of every third fraction was analysed by TLC for the presence of fOS. Fractions that contained fOS were combined and adjusted with deionized water to 120 µL per gram of WCP. fOS samples were stored at -20°C until further use.

2.2.4 NMR analysis of fOS

fOS was isolated from 2 g of WCP as described above and passed through PGC as described previously (Liu *et al.,* 2006). The purified fOS sample was frozen and lyophilized. The one-dimensional solution-state ^{1}H NMR spectrum was obtained for *C. jejuni* fOS in D_2O at 27 °C on a 700 MHz Agilent spectrometer equipped with a cold probe. The spectrum was referenced to an external standard of acetone (2.22 ppm for ^{1}H). A presaturation pulse sequence was used to reduce the intensity of the residual HOD signals (4.78 ppm at 27 °C).

2.2.5 Analysis of fOS by HPAEC-PAD

fOS was extracted from *Campylobacter* pellets as described above. A volume of 100 µL of the fOS sample was TLC extracted using 3 mL of methanol as a solvent and centrifuged at 13000 g for 5 min. The supernatant was evaporated in a fumehood, resuspended in 3 mL of deionized water and passed through a PGC cartridge (Extract Clean TM SPE Carbo 150 mg/4ml, Grace Davison Discovery Sciences) as described previously (Nothaft *et al.,* 2010). The pellet obtained after lyophilization was resuspended in 100 µL of deionized water and 44 µL of this fOS preparation was adjusted to a final volume of 220 µL. For the dephosphorylation of phosphorylated fOS from *C. lari*, 44 µL of the sample was adjusted with concentrated HCl to a final concentration of 20 mM. After incubation at 100°C for 30 min the solution was evaporated in a rotatory speed vacuum to dryness, centrifuged and resuspended in 220 µL of deionized water. Twenty five µL of sample was analysed by HPAEC-PAD on a Dionex ICS3000 system equipped with a CarboPac®PA100 (9 x 250 mm) coupled with a PA100 guard column (3x 50 mm) at a flow rate of 0.400 mL/min. A multistep gradient was used as described (Townsend *et al.,* 1988). Briefly, after an initial isocratic step at 0.1 M NaOH for 5 min a linear gradient from 0.1 M NaOH to 0.1 M NaOH, 0.6 M NaOAc for 68 min was applied. After another isocratic step at 0.1 M NaOH, 0.6 M NaOAc for 5 min, the column was re-equilibrated with 0.1 M NaOH for 10 min.

For quantification of fOS, 44 µL of fOS was hydrolyzed in a final volume of 220 µL in the presence of 4 M trifluoroacetic acid (TFA) for 2 hr at 100°C. The TFA hydrolysed fOS samples were evaporated in a rotatory speed vacuum centrifuge and resuspended in 220 µL of deionized water. GalNAc and glucose standards were prepared to final concentrations of 0, 25, 50, 100 and 150 µM in a final volume of 300 µL in de-ionized water and hydrolyzed in the presence of 4 M

TFA as described above, evaporated and resuspended in 300 µL of de-ionized water. A volume of 25 µL of hydrolyzed fOS and hydrolyzed standards were analysed by HPAEC-PAD on a Dionex ICS3000 system equipped with a CarboPac®PA 1 (4 x 250mm) coupled with PA1 guard column (4 x 50mm) with a flow rate of 1.0 mL/min using isocratic elution of 16 mM NaOH for 25 min. Two technical replicates of monosaccharide standards and fOS were prepared, each replicate was analysed in duplicate.

Amounts of fOS (in nmoles) were determined as follows: for *C. jejuni* and *C. lari* fOS GalNAc peak areas of TFA hydrolysed fOS were divided by 5 and values were plotted against the hydrolyzed GalNAc standard curve. The amount of GalNAc containing fOS per gram WCP was obtained after multiplication of the obtained value with the dilution factors, also taking into account the injection volume for HPAEC-PAD. For *C. jejuni* a hydrolyzed glucose standard was used in addition to hydrolyzed GalNAc. An electrochemical detector in the pulse amperometric mode with a gold working electrode, an Ag/AgCl reference electrode and a quadruple-potential waveform was used for monitoring. The column temperature was maintained at approximately 30°C. Data were processed using the Chromeleon software 7.0 package and standard curves were generated by plotting the peak area of known concentrations of TFA hydrolyzed and unhydrolyzed monosaccharide standards.

2.2.6 Extraction of samples from TLC plates and analysis by MALDI MS and MS/MS

Five-10 µL of TLC extracted fOS was passed through a 0.22 µm filter (Millipore) and lyophilized followed by resuspension in 10 µL of deionized water and analysed by MALDI MS/MS analysis as follows. Samples were spotted on a Bruker Daltonics MTP AC800 Anchorchip^{TM} target plate and air dried. A volume of 0.65 µL of 2,5-dihydroxybenzoic acid (DHB, 10 mg/mL in 80% H_2O and 20% MeOH containing 0.1% trifluoroacetic acid, TFA) was

spotted on top and allowed to dry. Mass spectra were obtained in the positive mode of ionization using a Bruker Daltonics (Bremen, GmbH) UltrafleXtreme MALDI TOF/TOF mass spectrometer. The FlexAnalysis, BioTools and Sequence Editor software packages provided by the manufacturer were used for analysis of the mass spectra. The MS/MS spectra were obtained manually with CID (collisional induced dissociation) set to off. Elemental composition of analytes was determined using a Bruker Daltonics (Billerica, MA) Apex Qe 9.4T FTICR MS instrument using the MALDI source.

2.3 Results

2.3.1 TLC analysis, extraction, purification and identification of fOS by MALDI MSMS

Eukaryotic fOS generation through ERAD processing of N-linked glycosylated proteins and LLO recycling is well characterized (Chantret *et al.,* 2003; Hirayama *et al.,* 2010; Chantret *et al.,* 2011; Hirayama and Suzuki, 2011). In this study, we have developed novel bacterial fOS extraction and analysis methods and also determined whether TLC can be applied to study bacterial fOS similar to eukaryotic fOS studies (Fig. 2.1). To identify potential fOS spots, we first compared the TLC profiles of *C. jejuni* wild-type and the *C. jejuni pglI* mutant (lacking glucosyltransferase activity) with the respective *pglB* OTase mutant profiles.

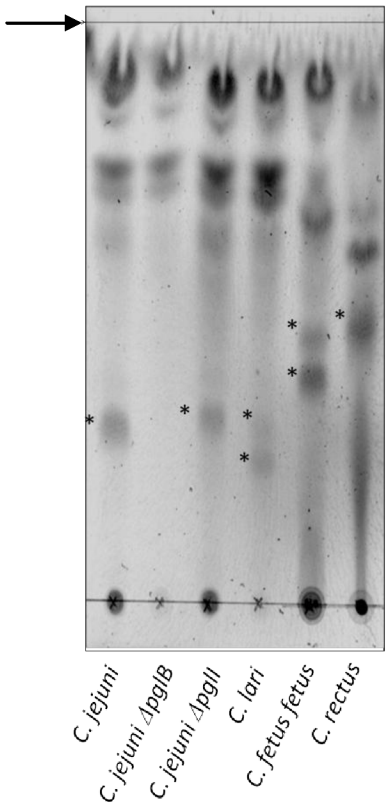

Fig. 2.2 TLC analysis of fOS extracts. Samples obtained after 75% ethanol extraction from the indicated *Campylobacter* species were analysed by TLC and p-anisaldehyde staining. Spots that were identified as fOS by MALDI-MS and MALDI-MS/MS are marked with an *asterisk* (*). The solvent front is marked with an arrow.

Spots that were present in the wild-type and in the *pglI* mutant, but absent in the *pglB* mutant were picked for MALDI-MS and MALDI-MS/MS analysis. For *C. jejuni* wild-type, one spot was found to run at an R_f value of 0.36 (Fig. 2.2). MALDI-MS identified an ion with a m/z of 1446.94 Da indicating the presence of the *C. jejuni* heptasaccharide (data not shown). Subsequent MALDI-MSMS analysis confirmed that this mass corresponded to the $[M+Na]^{1+}$ ion of the branched heptasaccharide HexNAc-HexNAc-[Hex]-$HexNAc_3$-diNAcBac (Fig. 1.3A). In the *C. jejuni pglI* mutant a spot with an R_f of 0.38 (Fig. 2.2) was identified by MALDI-MS to

represent a compound with a mass of m/z of 1284.55 Da (data not shown). MALDI-MS/MS analysis confirmed that this mass corresponds to the $[M+Na]^{1+}$ ion of the linear oligosaccharide $[HexNAc]_5$-diNAcBac (Fig. 2.3B). In *C. fetus fetus,* two spots with R_f values of 0.51 and 0.45 were identified by TLC (Fig. 2.2). MALDI-MS and MALDI-MS/MS analysis of these spots resulted in the identification of compounds with m/z of 1243.60 Da and 1284.83 Da (data not shown) corresponding to the $[M+Na]^{1+}$ ions of HexNAc-[Hex]-$HexNAc_3$-diNAcBac (Fig. 2.3C) and HexNAc-[HexNAc]-$HexNAc_3$-diNAcBac (Fig. 1.3D) oligosaccharides, respectively.

For *C. rectus*, the spot that was identified as fOS had an R_f of 0.52 on the TLC. FT-ICR-MS analysis of this spot showed a peak at m/z 1581.56450 Da resulting in an elemental composition of $C_{60}H_{98}N_6Na_1O_{41}$ with a mass accuracy of 0.96 ppm (data not shown). This elemental composition together with MALDI-TOFTOF-MSMS data (Fig. 2.3E) was determined to correspond to an oligosaccharide sequence of Hex-HexNAc-[Hex_2]-HexNAc-HexNAcA-HexNAc-diNAcBac, where HexNAcA represents N-acetylamido-deoxy-hexuronic acid.

TLC analysis of ethanol extracts from *C. lari* identified two spots with R_f values of 0.31 and 0.38. The spot with the R_f value of 0.38 subsequently analysed by MALDI-MS and MALDI-MSMS identified a peak with m/z 1284.64 Da (Fig. 2.3F) that corresponded to the $[M+Na]^{1+}$ ion of the linear, unphosphorylated hexasaccharide structure $[HexNAc]_5$-diNAcBac similar to *C. jejuni pglI* mutant. This was not surprising since both compounds had similar R_f values. MALDI-MS analysis of the compound with an R_f value of 0.31 revealed the presence of two peaks with m/z 1386.60 Da and with m/z of 1487.76 Da (data not shown). The compound with m/z 1386.60 Da might correspond to $[M+2Na-H]^{1+}$ of the linear, mono-phosphorylated *C. lari* hexasaccharide structure $[HexNAc]_5$-diNAcBac-[P] (P-fOS) that was identified previously (Nothaft *et al.,* 2012), the peak at m/z of 1487.76 Da indicated the presence of a previously unidentified fOS species

that might correspond to $[M+3Na-2H]^{1+}$ of the linear, di-phosphorylated hexasaccharide $[HexNAc]_5$-diNAcBac-$[P]_2$. In order to confirm the sequence of the oligosaccharides and the presence of the phosphate, samples were run by FT-ICR-MS and MALDI-TOFTOF-MS/MS. FT-ICR-MS showed peaks at m/z 1386.45573 Da which corresponds to the $[M+2Na-H]^{1+}$ ion (data not shown) having an elemental composition of $C_{50}H_{83}N_7Na_2O_{33}P_1$ with a mass error of 0.15 ppm. Presence of the phosphate was confirmed by MSMS analysis in which the neutral loss of H_2PO_4Na species can be observed (Fig. 2.3G). Mass spectrometric analysis was not able to confirm the exact location of the phosphate group. Because of the lower sensitivity of FT-ICR-MS, the peak at 1487.76 Da observed in the MALDI-TOFTOF-MS was not observed and therefore no elemental composition was obtained. MS/MS analysis of this peak has shown a loss of 221.9 Da which most likely corresponds to the neutral loss of $H_2P_2O_7Na_2$ (Fig. 2.3H). The TLC and MALDI-MS/MS results are summarized in Table 2.1.

To evaluate whether our extraction method causes dephosphorylation of otherwise fully di-phosphorylated fOS consequently generating un-phosphorylated and mono-phosphorylated fOS species we tested longer incubation times in 75% ethanol at 70°C. TLC profiles were identical independent on the incubation time of the extraction method indicating that the phosphate residue is not affected (data not shown). The possibility that di-phosphorylated fOS species may be released from LLOs during ethanol extraction at 70°C can be eliminated since we did not detect LLO degradation products in the *C. jejuni pglB* mutant. The latter one has been shown to accumulate Und-PP-heptasaccharide on the periplasmic side of the inner membrane (Reid *et al.,* 2010). Hence, it can be concluded that all three fOS i.e. un-phosphorylated, mono- and di-phosphorylated forms of fOS occur naturally in *C. lari* RM2100.

No spots that corresponded to fOS were found in the TLC profiles of extracts prepared from the residual pellet after EtOH extraction (data not shown) showing that all fOS was efficiently extracted from the pellet by our extraction method.

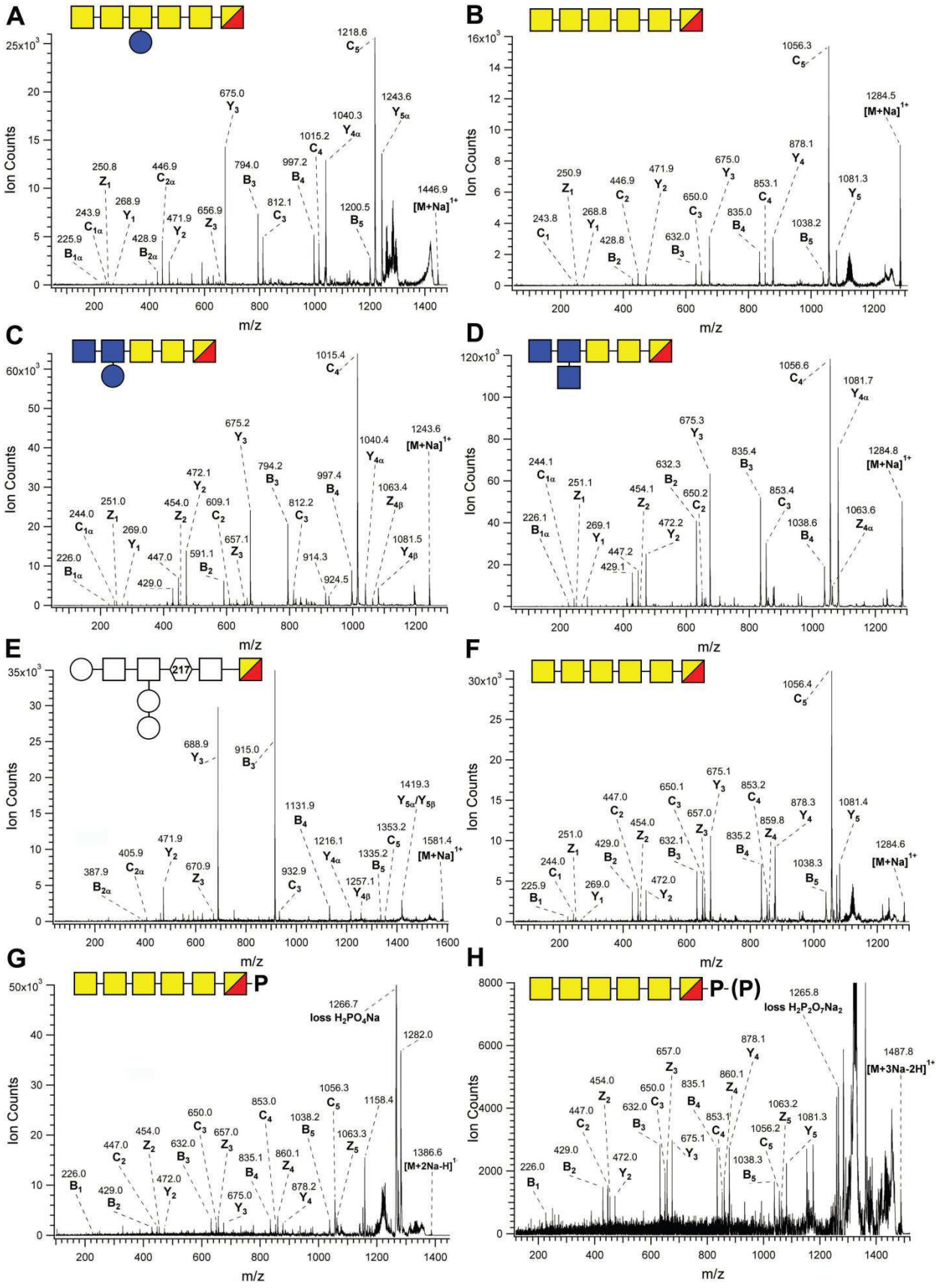
A
B
C
D
E
F
G
H
Ion Counts
m/z
[M+Na]1+
[M+2Na-H]1-
[M+3Na-2H]1+
loss H2PO4Na
loss H2P2O7Na2
217
P
P-(P)

Fig. 2.3 Identification of fOS by mass spectrometry. MALDI-MSMS spectra of: (A) HexNAc-HexNAc-[Hex]-$HexNAc_3$-diNAcBac from *C. jejuni* wild-type, (B) $[HexNAc]_5$-diNAcBac from *C. jejuni pglI*, (C) HexNAc-[Hex]-$HexNAc_3$-diNAcBac from C. *fetus fetus,* (D) HexNAc-[HexNAc]-$HexNAc_3$-diNAcBac from *C. fetus fetus,* (E) Hex-HexNAc-$[Hex_2]$-HexNAc-HexNAcA-HexNAc-diNAcBac from C. *rectus*, (F) $[HexNAc]_5$-diNAcBac from *C. lari,* (G) $[HexNAc]_5$-diNAcBac-[P] from *C. lari,* and (H) $[HexNAc]_5$-diNAcBac-$[P]_2$ from *C. lari*. Precursor ions of non-phosphorylated fOS correspond to $[M+Na]^{1+}$ ions. Precursor ions of mono-phosphorylated fOS correspond to $[M+2Na-H]^{1+}$. Precursor ions of di-phosphorylated fOS correspond to $[M+3Na-2H]^{1+}$. All labelled fragment ions within the mass spectra correspond to sodiated fragment ions. Labels are as follows (if sugar configurations were not known): hexose, open circle; N-acetyl-hexosamine, open square; N-acetylamido-deoxy-hexuronic acid, hexagon (mass 217). The sugar configuration was determined previously by NMR (Nothaft *et al.,* 2012) glucose, blue circle; diNAcBac, red-yellow square; N-acetyl-galactosamine, yellow square; N-acetyl-glucosamine, blue square; P, phosphate; brackets indicate the suggested but not verified position of the P residue.

2.3.2 Structural analysis of fOS by NMR

NMR experiments can be done using fOS or N-glycans to obtain structural information. So far, NMR analysis of fOS required up to 12-20 g of wet cell pellets to purify the amount of fOS needed (Nothaft *et al.,* 2012). Moreover, the purification of N-glycans or glycopeptides is a multi-step and time consuming process (Young *et al.,* 2002; Szymanski *et al.,* 2003). Although HR-MAS has been shown to be a powerful tool to study N-linked glycans *in vivo*, requiring as little as 40 L of cell suspension, such analysis requires the generation of capsular polysaccharide (CPS) mutants to eliminate the stronger and overlapping anomeric resonances resulting from the

CPS on the cell surface (Fig. 2.4B, D, E) (Szymanski *et al.,* 2003). Using our fOS extraction and purification protocol, we were also able to perform one dimensional proton NMR experiments on purified *C. jejuni* fOS extracts prepared from 2 g of WCP (Fig. 2.4G). We observed identical anomeric resonances for *C. jejuni* fOS as published in previous literature (Nothaft *et al.,* 2012) that were in agreement with those obtained from purified N-linked glycans (Fig. 2.4A) (Szymanski *et al.,* 2003). No resonances were observed that correspond to capsular polysaccharides (Fig. 2.4F), indicating that our extraction and purification methods are specific for fOS and suitable for structural determination of these compounds by NMR.

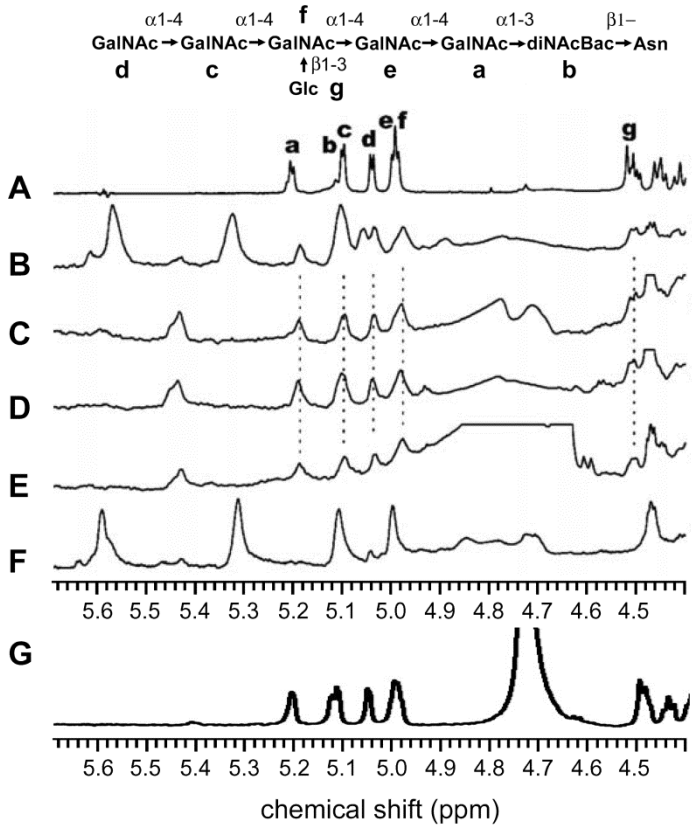

Fig. 2.4 The N-linked glycan in the HR-MAS proton NMR spectra from various *Campylobacter* strains in comparison to purified fOS in a solution-state proton NMR spectrum. The structure of the N-linked glycan is shown above the spectra. (A) Spectrum of the

purified N-linked glycan from *C. jejuni* NCTC11168 showing the anomeric resonances labeled a–g. (B) HR-MAS NMR spectra using a 10-ms CPMG filter of whole cells of *C. jejuni* NCTC11168, (C) *C. jejuni* NCTC11168 *kpsM*-, (D) *C. jejuni* HS:19 serostrain, (E) *C. coli* HS:30 serostrain, and (F) *C. jejuni* NCTC11168 *pglB*-. Common resonances in B-E compared with those in A are indicated by vertical dotted lines. The HOD resonance at 4.8 ppm was saturated and digitally filtered. (G) The one-dimensional solution-state ^{1}H NMR spectrum of purified fOS performed in this study is shown for comparison. Note for Fig. A-F: This work was originally published in The Journal of Biological Chemistry. Christine M. Szymanski, Frank St. Michael, Harold C. Jarrell, Jianjun Li, Michel Gilbert, Suzon Larocque, Evgeny Vinogradov, and Jean-Robert Brisson. Detection of Conserved N-Linked Glycans and Phase Variable Lipooligosaccharides and Capsules from Campylobacter Cells by Mass Spectrometry and High Resolution Magic Angle Spinning NMR Spectroscopy. *J. Biol. Chem.* 2003;278:24509-24520. © the American Society for Biochemistry and Molecular Biology.

2.3.3 Analysis and quantification of fOS by HPAEC-PAD

fOS purified by SGC and PGC was analysed and quantified by HPAEC-PAD (Table 2.1) that separates carbohydrates on the basis of charge. *C. jejuni* fOS had a retention time (R_t) of 15.4 min (Fig. 2.5A) whereas the *C. jejuni pglI* mutant fOS lacking the glucose branch had an R_t of 16.0 (Fig. 2.5B). For *C. lari* we only detected mono-phosphoryated fOS species during HPAEC-PAD analysis with an R_t of 20.0 min although TLC and MALDI-MS, identified un-phosphorylated, mono- and di-phosphorylated fOS. This further confirms that P-fOS is the major form in *C. lari* since the other two forms were below the detection limit for HPAEC-PAD. Chemically de-phosphorylated *C. lari* fOS had a similar retention time compared to the R_t of the *C. jejuni pglI* mutant fOS (data not shown). *C. fetus fetus* fOS that contains either terminal

glucose or GlcNAc ran at an R_t of 22.2 min and 20.0 min respectively (Fig. 2.5D). In all cases, the respective peak fractions were collected to confirm the presence and identity of fOS by MALDI-MSMS analysis (data not shown).

TFA hydrolysis of fOS results in the breakdown into monosaccharide components that can be quantified using appropriate monosaccharide standards. Untreated and TFA hydrolysed monosaccharide standards were used to create standard curves (Fig. 2.5I and J) and to identify and accurately determine the concentrations of fOS per gram of WCP. We found that after TFA treatment, GalNAc had a similar R_t as galactosamine on the PA-1 column (data not shown). This is not a side effect of HPAEC-PAD conditions, as the GalNAc standard performs adequately (data not shown) but is a consequence of the TFA hydrolysis. An increase in GalNAc signal intensity after TFA was likely due to better retention of the hydrolyzed GalNAc form (Fig. 2.5J). The retention time of glucose after TFA treatment was not affected (data not shown), however TFA hydrolysis lead to a slight decrease in signal intensity (compared to untreated glucose) (Fig. 2.5I).

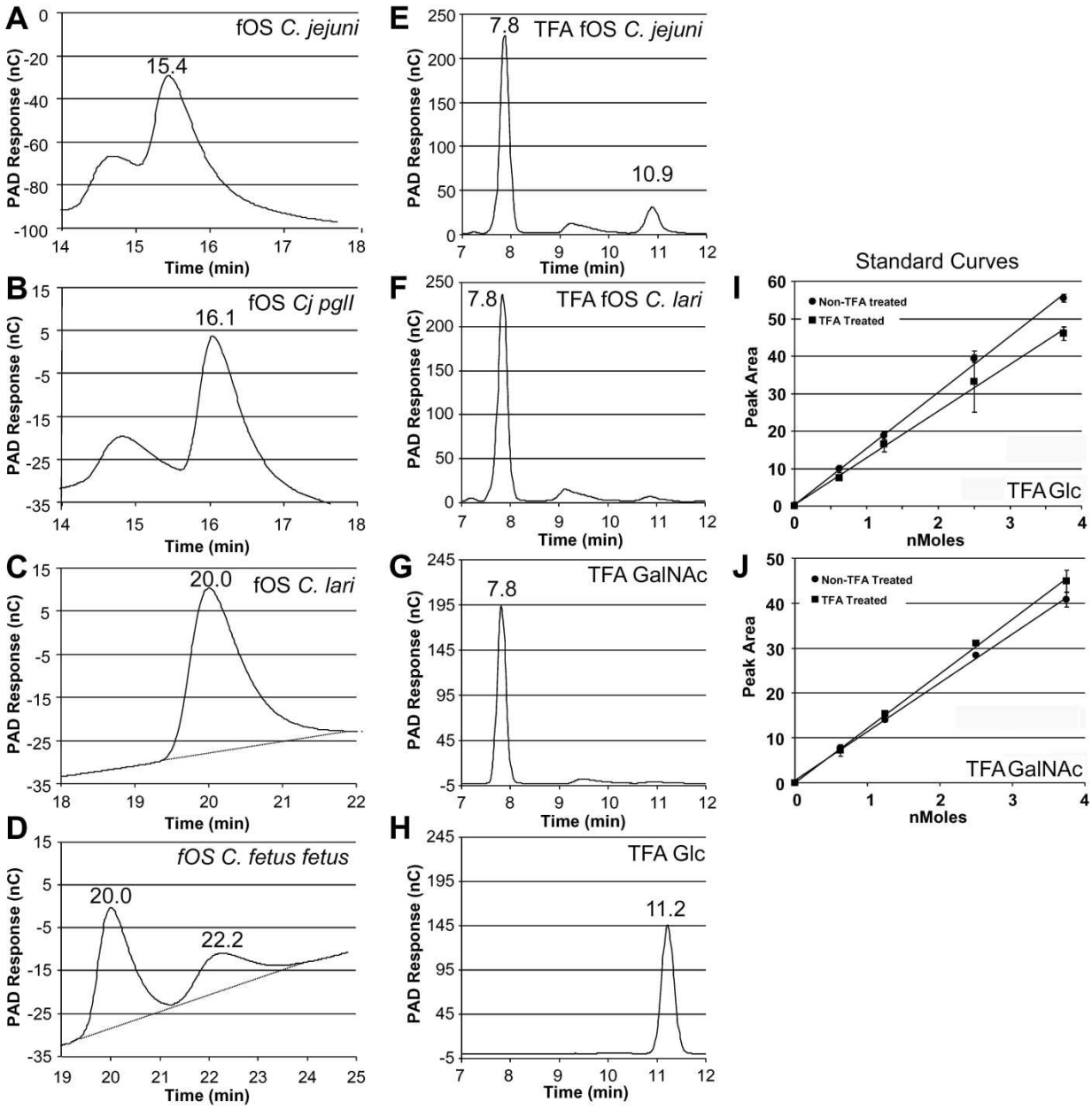

Fig. 2.5 fOS analysis and quantitation by HPAEC-PAD. Profiles of fOS from (A) *C. jejuni,* (B) *C. jejuni* (*pglI*), (C) *C. lari,* and (D) *C. fetus fetus.* TFA treated fOS profiles from (E) *C. jejuni* and (F) *C. lari* are shown in addition to TFA treated monosaccharide standards for (G) GalNAc and (H) glucose. Standard curves generated from the monosaccharides are shown for (I) glucose and (J) GalNAc. The retention times are indicated above the peaks.

For *C. jejuni,* we determined that our method enables us to extract 48.8 ± 6.8 nmoles of fOS from 1 g of WCP which corresponds to approximately 27 mg of fOS per 1.10 g of dry *C. jejuni* pellet and indicates that 2.5 % of the total cell weight is comprised of fOS. Compared to previous methods, this new technique allows us to extract 50-60 times more fOS (Liu *et al.,* 2006; Nothaft *et al.,* 2009). It is worth mentioning that both GalNAc and glucose standards gave similar measurements indicating that both standards are suitable for quantification of fOS. The amount of fOS in the *C. jejuni pglI* mutant was 21.9 ± 5.4 nmoles per gram of WCP (Table 2.1). Although, the N-linked protein glycosylation profile in the *C. jejuni pglI* mutant seems unaffected (Kelly *et al.,* 2006), the amount of fOS is ~2.24 times less compared to the wild-type strain. It can be hypothesized that the absence of the glucose branch may cause leakage of *pglI* mutant fOS into the extracellular environment, however, previous findings did not support this hypothesis (Nothaft *et al.,* 2010). Moreover, we have further confirmed this result using the fOS analysis techniques that were developed in this study (data not shown). This raises more questions about regulation of fOS production and its abundance in N-glycosylation pathway mutants.

Table 2.1 Summary of TLC, MALDI MSMS and HPAEC-PAD analysis and quantitation of fOS from *Campylobacter* species.

***Campylobacter* species**	**fOS MALDI MSMS**	**Rf value TLC**	**HPAEC-PAD Analyses**	
			Retention time (min)	**nMoles per gram wet cell pellet**
C. jejuni	HexNAc-HexNAc-[Hex]-$HexNAc_3$-diNAcBac	0.36	15.40	48.8± 6.8
C. jejuni (*pglI*)	$[HexNAc]_5$-diNAcBac	0.38	16.05	21.8± 5.4
C. lari	$[HexNAc]_5$-diNAcBac	0.38	20.00	7.8± 0.8
	$[HexNAc]_5$-diNAcBac-P $[HexNAc]_5$-diNAcBac-P(P)	0.31	nd	
C. fetus fetus	HexNAc-[HexNAc]-$HexNAc_3$-diNAcBac	0.45	22.16	49.8 ± 0.5
	HexNAc-[Hex]-$HexNAc_3$-diNAcBac	0.51	20.00	
C. rectus	Hex-HexNAc-$[Hex_2]$-HexNAc-HexNAcA-HexNAc-diNAcBac	0.52	nd	nd

nd, not determined

For *C. lari,* we measured 7.8 ± 0.8 nmoles of fOS per gram WCP (Table 2.1). This was ~6.3 times less than *C. jejuni*. The amount of fOS in *C. fetus fetus* was determined to be 49.8 ± 0.5 nmoles (Table 2.1) and this is comparable to the amount of fOS in *C. jejuni*. Our data indicates that fOS amounts vary between the examined species. fOS amounts may vary between species depending on the fOS generation abilities of the PglB enzymes and/or LLO abundance.

2.4 Discussion

All *Campylobacter* species release the same oligosaccharide derived from the N-glycosylation pathway as free oligosaccharide into the periplasmic space (Nothaft *et al.,* 2009; Nothaft *et al.,* 2012). The ratio of *C. jejuni* fOS to N-linked species was determined to be 10:1 under standard laboratory growth conditions (Liu *et al.,* 2006) making fOS an attractive target for structural analysis and glycoconjugate engineering. Moreover, fOS levels can change with mutagenesis of the pathways components as shown in this study with the *pglI* mutant, and in response to the extracellular osmolarity (Nothaft *et al.,* 2009). In order to investigate the variations in the abundance of this product, a robust quantification tool is required. In this study, we have developed efficient analytical methods that will not only help to understand the bacterial N-glycosylation pathways in more detail, but also shed more light on the generation, function and fate of fOS in these organisms.

Previously, fOS analysis techniques required longer processing times, especially for sample preparation, such as extensive proteolytic digests of cell lysates. In addition, in some cases permethylation of the glycans was required to increase sensitivity for ESI-MS and to obtain structural information for the oligosaccharide (Liu *et al.,* 2006). Moreover, the ESI-MS based semi-quantitative method did not allow accurate quantitation of total fOS amounts (Nothaft *et al.,* 2009). Our fOS extraction and purification method by TLC or SGC followed by label-free MALDI-MS, NMR or HPAEC-PAD analysis serves as a convenient way to not only determine the total amount of fOS independent of charge or presence of labile substituents but, to also analyze relative levels of substituted fOS. We determined that fOS amounts range from 7.8 to 49.8 nmoles in the examined *Campylobacter* species. In *C. jejuni,* the fOS amount corresponds to approximately 2.5 % of dry cell weight which is comparable to the abundance of OPGs found

in other proteobacteria that range from 0.75% to 5% of dry cell weight (Lequette *et al.,* 2007; Martirosyan *et al.,* 2012).

Due to the non-destructive nature and high extraction efficiency, higher fOS quantities can be obtained enabling the characterization of potentially unstable fOS species. One example is the *C. rectus* fOS, where mainly degradation products were observed in our earlier studies with only minor amounts of the full length fOS (Nothaft *et al.,* 2012), and here we successfully isolated intact fOS confirming that it is identical to the known N-linked glycan structure. We also proved that the 217 Da sugar, as previously speculated, represents N-acetyl-hexuronic acid (Nothaft *et al.,* 2012). The di-phosphorylated fOS species in *C. lari* has not been observed before likely due to loss of labile phosphate groups as a result of the analysis method or lower amount of starting material (Nothaft *et al.,* 2012). We determined that our extraction method does not result in de-phosphorylation of P-fOS indicating that three forms, mono,-di, -and non-phosphorylated fOS occur naturally in *C. lari*. Although un-phosphorylated *C. lari* fOS was observed before by ESI-MS, the authenticity of this form could not be confirmed by NMR (Nothaft *et al.,* 2012). For separation and exact quantitation of mono-phosphorylated and di-phosphorylated forms by TLC and HPAEC-PAD, the solvent system would require further improvement. Nevertheless, questions about the biological function and genesis of the phosphorylated fOS in *C. lari* require further study.

In eukaryotes, P-fOS is generated by the action of an unknown pyrophosphate phosphatase on LLO pools whereas neutral fOS are released by the OTase complex (Cacan *et al.,* 1992; Chantret and Moore, 2008; Peric *et al.,* 2010; Vleugels *et al.,* 2011). In *C. lari*, un-phosphorylated fOS species may arise from PglB hydrolysis, whereas, P-fOS may be generated from an LLO recycling mechanism. However, the possibility of a periplasmic kinase involved in P-fOS

generation also cannot be eliminated. Periplasmic phosphorylation has been reported previously using Und-PP as a phosphate donor to generate Und-P as an essential bacterial recycled product (Touze *et al.,* 2008). We could not determine the position of the second phosphate on the di-phosphorylated fOS structure, therefore it is possible that the second phosphorylation is linked at another position in the hexasaccharide. Alternatively, it is more likely that *C. lari* fOS is in fact substituted with a pyrophosphate. Although protein pyrophosphorylation has been described before (Azevedo *et al.,* 2009) there is no report of carbohydrate pyrophosphorylation to the best of our knowledge.

Phosphate substitution on the *C. lari* fOS might aid in increasing the net negative charge in the periplasmic space preventing charged antimicrobials from entering the cell or to retain the fOS in the periplasm to fulfill a similar function in osmo-adaptation in *C. lari* as has been shown for *C. jejuni* fOS (Nothaft *et al.,* 2009). In other proteobacteria, OPGs can be substituted with a variety of non-carbohydrate components dependent on the media or growth phase (Geiger *et al.,* 1991; Breedveld *et al.,* 1995; Cho *et al.,* 2009) and have been shown to increase the resistance to anionic detergents in *Shigella flexneri* (Bhagwat *et al.,* 2012). In *Brucella species,* cyclic periplasmic glucans are potent activators of human and mouse dendritic cells and increase IL-6 production (Martirosyan *et al.,* 2012). The *C. jejuni* N-linked glycan (same in structure as the fOS) has been shown to be recognized by the human macrophage galactose lectin and also enhance IL-6 secretion (van Sorge *et al.,* 2009). Interestingly, the fungus *Apergillus fumigatus* secretes GalNAc and Gal containing polysaccharides during host infections and causes immune suppression resulting in enhanced *A. fumigatus* infection (Fontaine *et al.,* 2011). The similarities between *Brucella* cyclic OPGs, the *A. fumigates* polysaccharide and *C. jejuni* fOS in terms of composition, abundance and recognition by host immune-factors suggest that fOS can potentially

have immuno-modulatory functions. Although there is no evidence for fOS secretion from *C. jejuni* (Nothaft *et al.,* 2009), fOS is likely released through complement mediated cell lysis during infection of the host. Other roles of fOS may include intracellular signalling. In *Dickeya dadantii,* a pytopathogen*,* OPG mutant strains have been shown to have a defect in the Rcs phosphorelay system, consequently reducing bacterial pathogenicity in plants (Bouchart *et al.,* 2010; Bontemps-Gallo *et al.,* 2013).

The presented label-free universal fOS isolation and characterization methods do not require the use of specific lectins/antibodies, or multiple chromatography steps for fOS purification. The method established in this study will be helpful to determine, compare and characterize the abundance and diversity of fOS in bacterial glycosylation systems. In addition, the isolated fOS can also be used in chemical conjugation reactions to generate novel glycoconjugate vaccines (Nothaft *et al.,* 2012). The method can also be applied as a quick screening tool when characterizing glycosyltransferases in the glycosylation operon of various bacterial species to directly couple gene function to a mutant phenotype. The tools developed in this study will be useful to determine the diversity, regulation and function of fOS in bacterial N-linked glycosylation systems.

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2.6 References

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CHAPTER 3

N-glycosylation of the *Campylobacter jejuni* PglB oligosaccharyltransferase may influence its enzymatic activities

3.1 Introduction

Campylobacter jejuni was the first bacterium demonstrated to encode an N-linked protein glycosylation pathway (Pgl) (Szymanski *et al.,* 1999; Wacker *et al.,* 2002). The heptasaccharide GalNAc-α1,4-GalNAc-α1,4-[Glcβ1,3]-GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-diNAcBac-β1 (where diNAcBac is 2,4-diacetamido-2,4,6-trideoxyglucopyranose) is synthesized by Pgl enzymes on the lipid, undecaprenylphosphate, resulting in a lipid-linked oligosaccharide (LLO) intermediate (Young *et al.,* 2002; Wacker *et al.,* 2002; Szymanski *et al.,* 2003; Kelly *et al.,* 2006; Reid *et al.,* 2008; Reid *et al.,* 2009; Reid *et al.,* 2010). The oligosaccharide is then transferred onto asparagine residues of proteins by the central oligosaccharyltransferase enzyme (OTase), PglB, within the sequon D/E-X_1-N-X_2-S/T (where X cannot be proline) (Szymanski *et al.,* 1999; Wacker *et al.,* 2002; Szymanski *et al.,* 2002; Nita-Lazar *et al.,* 2005; Linton *et al.,* 2005; Glover *et al.,* 2005; Wacker *et al.,* 2006; Kowarik *et al.,* 2006; Glover *et al.,* 2006; Reid *et al.,* 2010). PglB glycosylates >60 proteins in *C. jejuni* (Scott *et al.,* 2011; Scott *et al.,* 2014). In addition to glycosylation activity, PglB also possesses hydrolase activity and the heptasaccharide is released as free oligosaccharide (fOS) into the periplasmic space by hydrolysis of the LLOs (Nothaft *et al.,* 2009). Recently, all *Campylobacter* species have been shown to perform N-linked protein glycosylation and release fOS with species specific N-glycan structures and, fOS concentrations in select strains of *Campylobacter* have been quantified as well (Nothaft *et al.,* 2012; Dwivedi *et al.,* 2013).

The OTase activity of PglB is being studied extensively due to its applicability in the production of glycoconjugate therapeutics (Feldman *et al.,* 2005; Ihssen *et al.,* 2010; Ihssen *et al.,* 2012; Valderrama-Rincon *et al.,* 2012; Wetter *et al.,* 2012; Cuccui *et al.,* 2013; Ollis *et al.,* 2014; Kampf *et al.,* 2015; Srichaisupakit *et al.,* 2015). Many recent studies have focused on

determining motifs/amino acids that play important roles in catalysis and substrate/acceptor binding specificities (Wacker *et al.,* 2002; Lizak *et al.,* 2011; Ihssen *et al.,* 2012; Gerber *et al.,* 2013; Liu *et al.,* 2014; Lizak *et al.,* 2014; Ihssen *et al.,* 2015). Recently, the crystal structure of the *C. lari* PglB enzyme bound to an acceptor peptide was also determined and this study provided crucial insights into the mechanism of the glycosylation reaction (Lizak *et al.,* 2011). In addition, other studies have focused on the lipid carrier preferences of PglB (Liu *et al.,* 2014; Ishiwata *et al.,* 2015). In contrast, the mechanism of LLO hydrolysis by PglB is not well understood (Nothaft *et al.,* 2009).

In *C. jejuni*, mutations in the *pgl* pathway result in multiple phenotypes including reduced attachment and invasion of epithelial cells and decreased colonization of the intestinal tracts of mice and chickens (Szymanski *et al.,* 2002; Larsen *et al.,* 2004; Karlyshev *et al.,* 2004; Kelly *et al.,* 2006; Hendrixson, 2006; Hall *et al.,* 2014). A *pglB* mutant also exhibits significantly decreased survival under hypoosmotic conditions versus the wildtype (Kakuda *et al.,* 2012). However, it is unknown whether these are direct effects of impaired N-glycosylation of proteins and/or due to losing functionality of proteins and/or losing fOS. Very few bacterial examples exist where the N-glycan modification affects the protein function/stability. For example, the absence of N-glycosylation on the VirB10 protein, a component of the Type IV secretion system in *C. jejuni* 81-176*,* results in decreased DNA uptake (Larsen *et al.,* 2004). On the other hand, N-glycan modification was found to have no influence the functionality of the mechanosensitive channel proteins Cj0263 and Cj1025 (Kakuda *et al.,* 2012) as well as Cj0143 that is a component of a putative zinc transport system (Davis *et al.,* 2009).

Mass spectrometry analysis of the *C. jejuni* 11168 proteome revealed that PglB is N-glycosylated at N534 (Scott *et al.,* 2011) (i.e. self-glycosylated, SG). In addition, the *C. lari* PglB enzyme is

also N-glycosylated at N535 and N556 (Lizak *et al.,* 2011). In this study, we show that unglycosylated PglB exhibits significantly reduced hydrolase activity in *C. jejuni*. In addition, upon Western blot analysis of *C. jejuni* whole cell lysates, we found that unglycosylated PglB displays a different N-glycosylation profile than the wildtype cells expressing glycosylated PglB. Moreover, we found that glycosylation of CmeA, a well studied substrate of PglB (Wacker *et al.,* 2002; Nothaft *et al.,* 2009), was significantly different in cells expressing unglycosylated PglB compared to wildtype. This study suggests important roles of PglB mediated N-glycosylation on its own enzymatic activities and warrants further research into the mechanism of fOS release, as well as, protein N-glycosylation by PglB.

3.2 Materials and methods

3.2.1 Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 4.1. *C. jejuni* 11168 strains were grown on Mueller Hinton (MH, Difco™) agar plates under microaerobic conditions (10% CO_2, 5% O_2, 85% N_2) at 37°C for 18 hrs. Chloramphenicol (25 µg/mL) and kanamycin (25 µg/mL) were added as needed.

Table 3.1 Strains and plasmids used in this study.

Strain	Description	Source
C. jejuni		
11168 NCTC	Clinical isolate used for genome sequencing	Carrillo *et al.*, 2004
11168-*pglB*::kan	*pglB* mutant in *C. jejuni* 11168, Kan^R	Nothaft *et al.*, 2009
11168-*pglB* (pCj-*pglB*$_{WT}$)	*pglB* mutant complemented with pCE111-28 carrying wildtype *pglB*, Kan^R, Cm^R	Nothaft *et al.*, 2009
11168-*pglB* (pCj-*pglB*$_{WWDYG}$)	*pglB* mutant complemented with pCE111-28 carrying ^{457}WAAYG^{461} mutation in *pglB* gene, Kan^R, Cm^R	Nothaft *et al.*, 2009
11168-*pglB* (pCj-*pglB*$_{N534Q}$)	*pglB* mutant complemented with pCE111-28 carrying ^{534}Q (self-glycosylation) mutation in *pglB* gene, Kan^R Cm^R	Received from Yasmin Barre

3.2.2 Alignment of PglB sequences and PglB crystal structure

Amino acid sequences of PglB homologues were downloaded from www.ncbi.nlm.gov in FASTA format and examined by ClustalW (http://www.ch.embnet.org/software/ClustalW.html). The alignment was run with the following settings: scoring matrix, Blosum; opening gap penalty and end gap penalty, 10; extending gap penalty and separation gap penalty, 0.05; Output format, GCG/MSF; output order, Input. The output file data was further processed using BoxShade server (www.ch.embnet.org/software/BOX_form.html) to generate the output in the format shown in Fig. 4.1. Sequences containing the N-glycosylation sequon D/E-X_1-N-X_2-S/T (X cannot be proline) were highlighted in green.

The crystal structure of *C. lari* PglB was downloaded from www.ncbi.nlm.gov as a Cn3D file. The glycosylated residues were highlighted according to the program instructions and used use to create Fig. 4.1B.

3.2.3 Western blot analysis

Whole cell lysates were prepared as follows: cells were harvested from agar plates and resuspended in PBS followed by sonication in a VWR Scientific, Branson Sonifier 450 at output control 1 and a constant duty cycle for 40 sec. After sonication, the sample was centrifuged at 18,000 g for 30 min at 4°C. The supernatant was used in Western blot analysis. The protein concentration in the supernatant was quantified by the BioRad DC Bradford assay kit with bovine serum albumin as a protein standard. An amount of 10 µg of protein was loaded per lane on the gel. Protein expression profiles and N-glycosylation patterns were analyzed by 12.5 % SDS-PAGE followed by Western blot analysis. Equal loading of protein samples was confirmed by Coomassie staining. For Western blot analysis, primary antibodies were used at the following dilutions: 1:6,000 for CmeA specific (Ielmini & Feldman, 2011), and 1:7,500 for *C. jejuni*-*N*-glycan specific hR6 antisera (Wacker *et al.,* 2002). Alkaline phosphatase conjugated anti-rabbit (Santa Cruz) served as the secondary antibody at 1:2,000 dilution. The alkaline phosphatase conjugated rabbit anti-serum was visualized on Western blots using NBT/BCIP (Promega) according to manufacturer's instructions.

Band intensities of glycosylated and unglycosylated forms of CmeA were quantified on Western blots using software ImageJ according to the software instructions (http://rsbweb.nih.gov/ij/).

3.2.4 ELISA assay for quantitation of N-glycosylation in whole cell lysates of *C. jejuni*

Whole cell lysates were prepared as described above with the following changes. Protease inhibitor cocktail (cOmplete EDTA Free, Roche) was added to the cell suspensions before

sonication. Protein concentrations were measured by the DC Bradford assay (BioRad). Protein concentrations were set to 0.015mg/mL in PBS and 200 µL was pipetted into the ELISA plate (Nunc, MaxiSorp® flat-bottom 96 well plate). Following this, a series of 6 dilutions of 1 in 2 were made by adding 100 µL of sample to 100 µL of PBS in the ELISA plate. Wells containing 200 µL of PBS served as negative controls. Dilutions were made in duplicates of 200 µL in each well. The samples were incubated in the ELISA plate overnight at 4°C with shaking. Then the samples were pipetted out and incubated with 200 µL of 4% skim milk (Difco) in PBS-0.05% Tween 20 for 1 hr at room temperature with shaking. The wells were then emptied and a 1:7500 dilution of the anti N-glycan antibody in 4% skim milk employed above in PBS-0.05% Tween 20 (1:3 dilution) was added in a volume of 200 µL and incubated for 1 hr at room temperature with shaking. The wells were then washed 3 times with PBS-0.05% Tween 20. Then, a 1:2000 dilution of the anti-rabbit antibody was added to a 1:3 dilution of 4% skim milk employed above in PBS-0.05% Tween 20 and 200 µL was added to each well and incubated for 1 hr at room temperature with shaking. The wells were washed again as described above and incubated with 200 µL of 5mM para-Nitrophenylphosphate (Sigma) in 100mM NaCl, 100mm Tris-HCl ph 8.5, 1% Tween 20 for 25 mins before reading the absorbance at 405nm. A standard curve was plotted using the Microsoft ® Excel software to determine the linear range of absorbance of absorbance at 405nm on Y- axis and µg of protein the wells on the X-axis. A R^2 was determined to confirm good linearity and a trendline equation was generated. Using the linear range of absorbance values, a X-value was calculated for the other samples and expressed as a % of the *C. jejuni*$_{pglB\text{WT}}$ absorbance that was set to 100%.

3.2.5 fOS quantitation in *C. jejuni* cells and analysis

fOS extraction, quantitation and analysis was performed as described earlier (Dwivedi *et al.,* 2013).

3.3 Results

3.3.1 The N-glycosylation site on PglB is conserved in most *Campylobacter* species

Bioinformatic analysis was performed on PglB homologues from all *Campylobacter* species (Fig. 4.1). We found that the N534 SG site is conserved in 22 out of 29 PglB homologues (i.e. ~76% of *Campylobacter* species) (Fig. 4.1). Amino acid D appears to be preferred for the -2 position, and Y for the -1 position. For the +2 position, both S and T appear to be equally preferred (Fig. 4.1). We found that the -4 and -5 positions mostly consisted of hydrophobic residues, such as M, V and L residues (Fig. 4.1). We did not find any conserved residues in the +1/3/4 sites of the glycosylation site. Upon analysis of the published crystal structure of the PglB enzyme from *C. lari* (Lizak *et al.,* 2014), we found that the published SG sites N535 and N536 are both exposed to the periplasmic space.

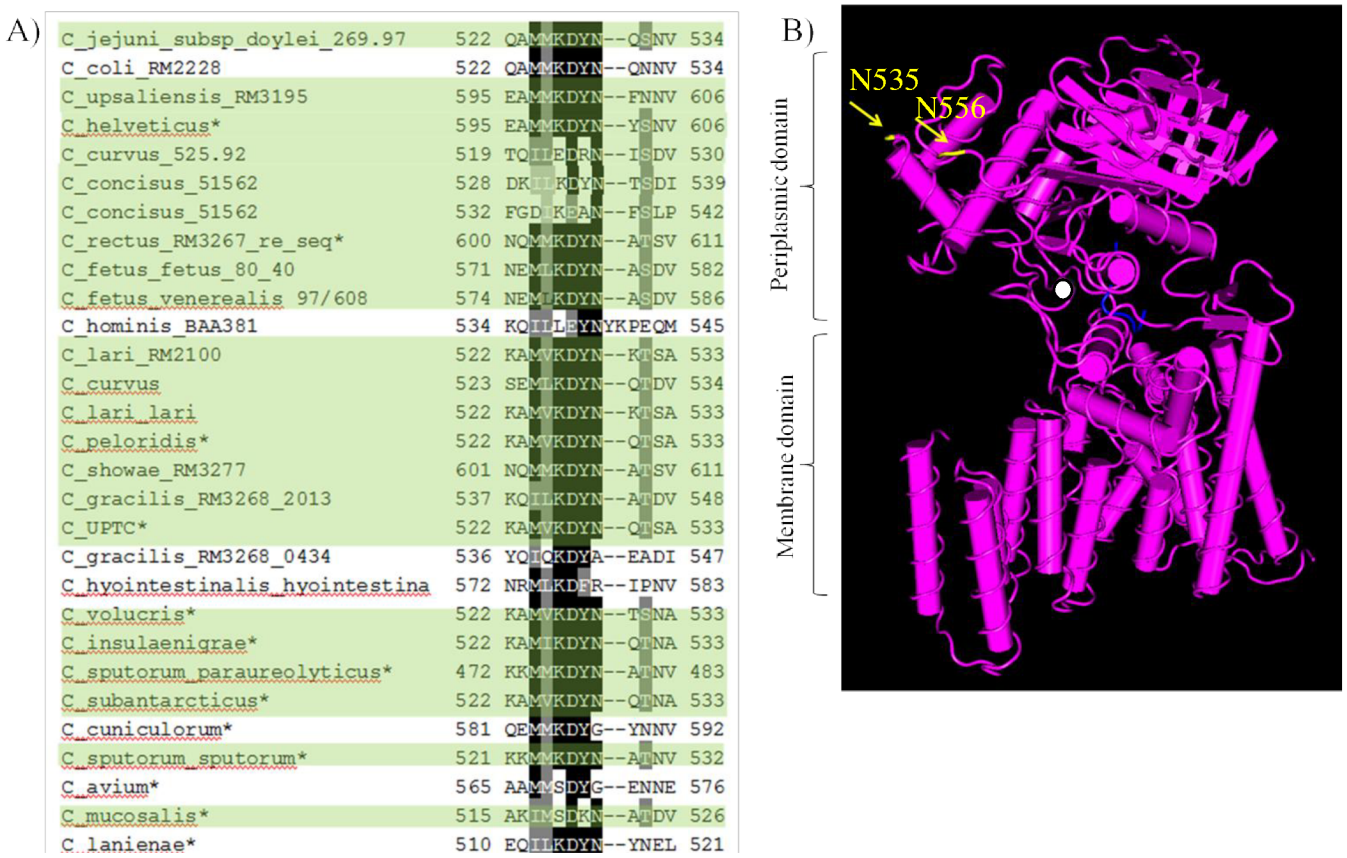


Fig. 3.1 Alignment and modeling of PglB. A) Alignment of the self-glycosylation (SG) site of PglB homologues from all *Campylobacter* species. Species with the SG sequon corresponding to position 534 of *C. jejuni* PglB are highlighted in green. Strongly conserved amino acids are highlighted in black and less conserved residues are in grey. B) X-ray crystal structure of the *C. lari* PglB enzyme with SG sites N535 and N556 highlighted in yellow and indicated with yellow arrows. Acceptor peptide (DQNATF) shown in blue and manganese ion is shown as a white circle. Periplasmic and membrane domains are indicated. Structure was downloaded from www.ncbi.nlm.nih.gov and residues were highlighted using Cn3D software.

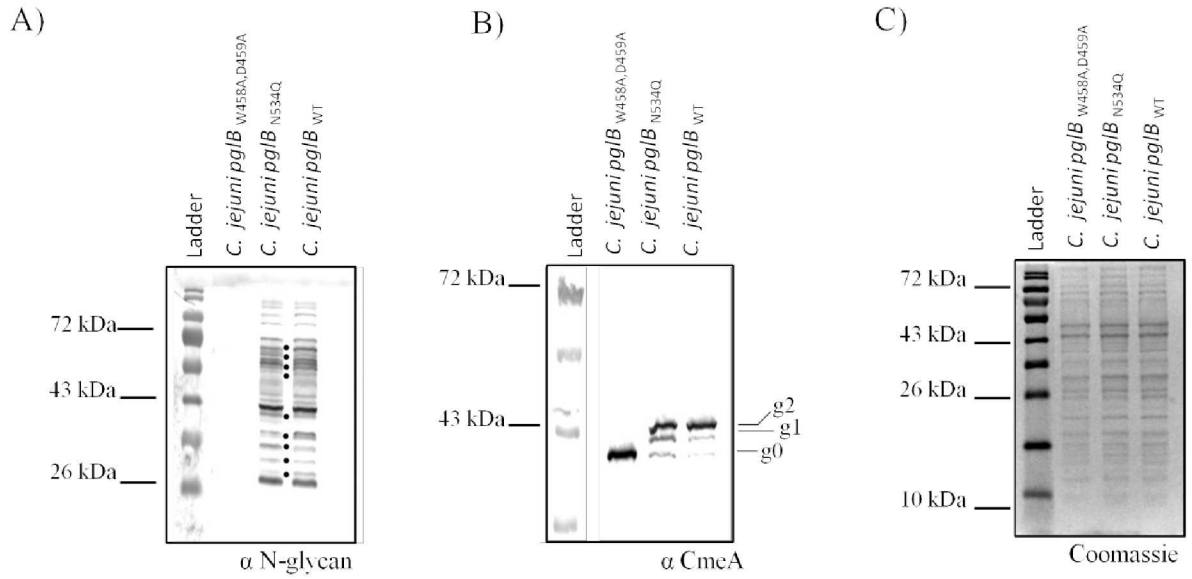
3.3.2 N-glycosylation of PglB may affect its ability to glycosylate other proteins *in vivo*

To investigate whether the N-glycosylation of PglB affects its protein N-glycosylation activity in *C. jejuni*, we generated three strains by complementing the *C. jejuni pglB* mutant with three different *pglB* constructs i.e. wildtype version of PglB ($PglB_{WT}$), self-glycosylation mutant version of PglB where N534 was mutated to a glutamine (Q) ($PglB_{N534Q}$), and inactive version of PglB with a mutation in the essential ^{457}WWDYG^{461} motif, where W458 and D459 are both mutated to an A residue ($PglB_{W458A,D459A}$) (Wacker *et al.,* 2002). Plasmids expressing the three variants of PglB were complemented in the *C. jejuni pglB* mutant background. The complemented strains will be referred to as *C. jejuni* $pglB_{W458A,D459A,}$ *C. jejuni* $pglB_{N534Q,}$ *C. jejuni* $pglB_{WT.}$

We performed Western blot analysis with anti-N-glycan (hR6) and anti-CmeA antibodies on whole cell lysates of the above constructs. Anti- N-glycan antibody allows a snapshot of overall cellular protein N-glycosylation levels, whereas, anti-CmeA allows an insight into the N-glycosylation status of the single CmeA glycoprotein in *C. jejuni*. CmeA is a di-glycosylated protein in *C. jejuni* and is routinely used as a reporter of the N-glycosylation activity of PglB *in vivo* (Wacker *et al.,* 2002; Nothaft *et al.,* 2009; Nothaft *et al.,* 2010; Scott *et al.,* 2011). Western blot analysis with anti-N glycan antibodies revealed that *C. jejuni* $pglB_{N534Q}$ cells were able to perform protein N-glycosylation however the banding pattern was different compared to the *C. jejuni* $pglB_{WT}$ as indicated (Fig. 4.2A and D). In the first set of analysis, the banding pattern for the band at ~70 kDa, two bands at ~65 kDa, two bands at ~55 kDa, 43 kDa, three bands at ~37 kDa and ~26 kDa were different between the two samples (Fig. 4.2 A). In the second Western blot analysis, the band at ~70, two bands at ~65 kDa, band at ~42 kDa, two bands at ~35kDa were different between the two strains (Fig. 4.2D).

Interestingly, further investigation with anti-CmeA antibodies showed that *C. jejuni pglB*$_{N534Q}$ was not able to N-glycosylate CmeA to wildtype levels (i.e. levels of *C. jejuni pglB*$_{WT}$) (Fig. 4.2B) and comparatively, darker bands of unglycosylated and monoglycosylated CmeA were present in this strain (Fig. 4.2B and E). *C. jejuni pglB*$_{WT}$ had high amounts of di-glycosylated CmeA and small amounts of mono- and un-glycosylated CmeA, whereas *C. jejuni pglB*$_{W458A,D459A}$ only had unglycosylated CmeA. In addition, Coomassie staining confirmed equal loading of all samples (Fig. 4.2C and F). To confirm the clearly evident differences in the glycosylation of CmeA in *C. jejuni pglB*$_{N534Q}$ and *C. jejuni pglB*$_{WT}$, we performed densitometry on the CmeA bands (Fig. 4.2G). Densitometry has been used previously to quantitate the relative levels of substrate glycosylation by PglB and other OTases (Yan and Lennarz, 2002; Jaffee and Imperiali, 2011; Ollis *et al.,* 2014; Cohen-Rosenzweig *et al.,* 2014). For our analysis, the levels of total CmeA in each lane was adjusted to 100% and levels of un-, mono and di- glycosylated CmeA were expressed in comparison to total CmeA levels (Fig. 4.2D). Densitometry analysis of the di-glycosylated CmeA in *C. jejuni pglB*$_{WT}$ revealed a percentage of 90.71% compared to total CmeA signal in that lane, whereas in *C. jejuni pglB*$_{N534Q}$ the signal intensity was significantly lower at 61.58% (p-value of 0.02 in a paired t-test analysis) (Fig. 4.2G). The intensity of unglycosylated CmeA in the *C. jejuni pglB*$_{WT}$ was 1.03% whereas in *C .jejuni pglB*$_{N534Q}$ was 11.84% however this difference was not statistically significant ($p>0.05$) in a paired t-test (p-value= 0.13) (Fig. 4.2G). In addition, the density of monoglycosylated CmeA in the *C. jejuni* pglB$_{WT}$ was 8.25% whereas it was 26.58% in the *C. jejuni pglB*$_{N534Q}$ strain however this difference was not statistically significant with a p-value >0.05 (p-value= 0.11) (Fig. 4.2G). The densitometry analysis confirmed the differential glycosylation of CmeA observed in Western blot analysis between *C. jejuni pglB*$_{N534Q}$ and *C. jejuni pglB*$_{WT}$ strains. ELISA analysis of whole

cell lysates of the three strains with the anti- N-glycan antibody in two independent experiments indicated that *C. jejuni* $pglB_{N534Q}$ had ~30% less intensity of absorbance signal (69.30±8.05) compared to *C. jejuni* $pglB_{WT}$ that was set to 100%, whereas the negative control *C. jejuni* $pglB_{W458A,D459A}$ had a background absorbance of 4.29 ±1.04 (Fig. 4.3). However, the signal intensity between the *C. jejuni* $pglB_{N534Q}$ and *C. jejuni* $pglB_{WT}$ strains was not significantly different in a paired t-test analysis (p-value of 0.10) (Fig. 4.3).



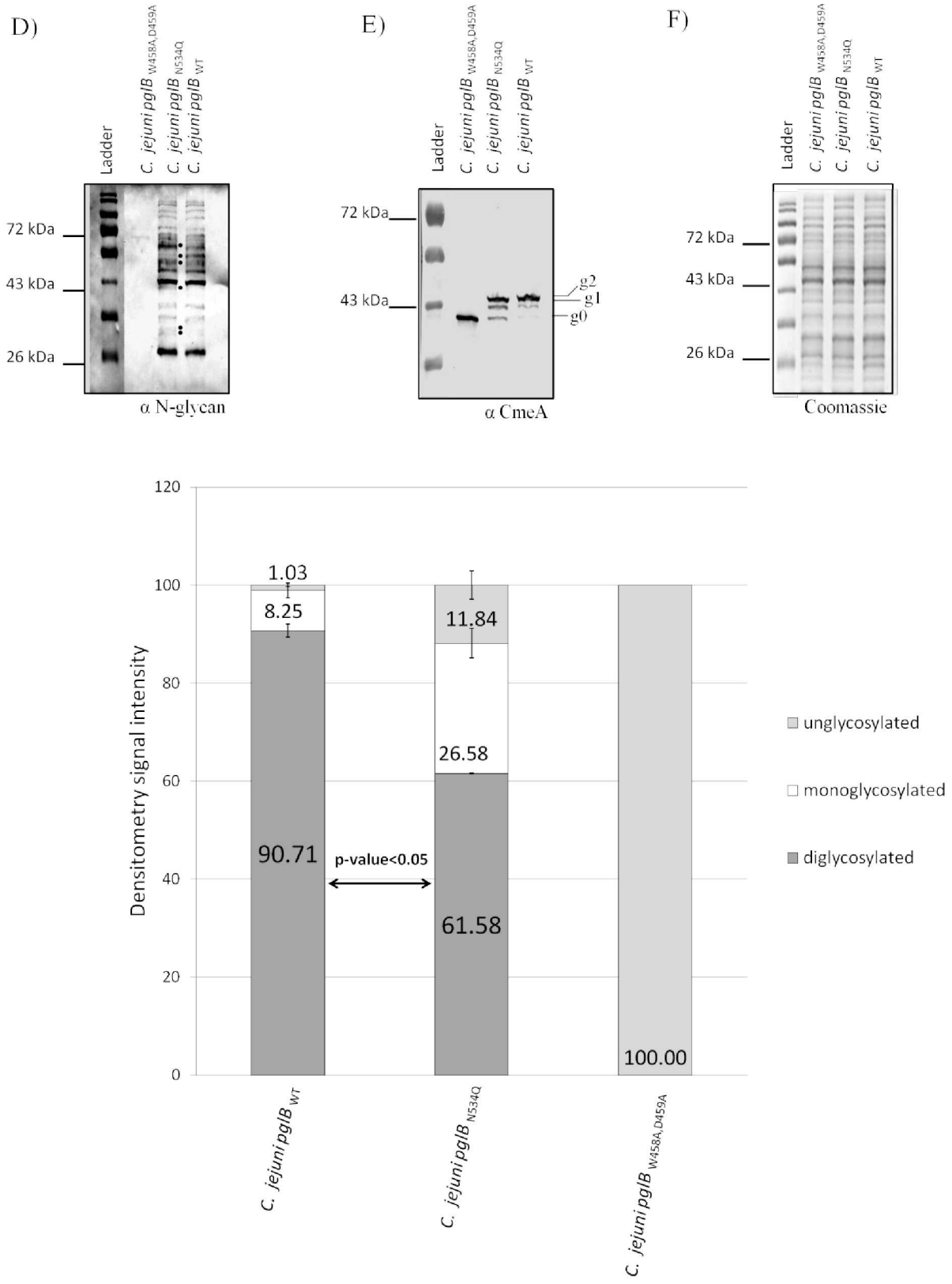

D)
Ladder
C. jejuni pglB W458A,D459A
C. jejuni pglB N534Q
C. jejuni pglB WT
72 kDa
43 kDa
26 kDa
α N-glycan
E)
Ladder
C. jejuni pglB W458A,D459A
C. jejuni pglB N534Q
C. jejuni pglB WT
72 kDa
43 kDa
g2
g1
g0
α CmeA
F)
Ladder
C. jejuni pglB W458A,D459A
C. jejuni pglB N534Q
C. jejuni pglB WT
72 kDa
43 kDa
26 kDa
Coomassie
Densitometry signal intensity
120
100
80
60
40
20
0
1.03
8.25
90.71
p-value<0.05
11.84
26.58
61.58
100.00
unglycosylated
monoglycosylated
diglycosylated
C. jejuni pglB WT
C. jejuni pglB N534Q
C. jejuni pglB W458A,D459A

Fig. 3.2 N-glycosylation of PglB may affect its ability to glycosylate other proteins *in vivo* in *Campylobacter jejuni*. Western blot analysis of whole cell lysates of *C. jejuni.* Ten µg of protein was loaded per lane. A) and D) Analysis with anti- N-glycan antibodies from two independent experiments. Bands that are different in the *C. jejuni* $pglB_{NQ534}$ compared to the *C. jejuni* $pglB_{WT}$ are indicated with black dots on the right side of the lane. B) and E) Analysis with anti- CmeA antibodies from two independent experiments (g0: unglycosylated, g1 : mono-glycosylated, g2: di-glycosylated). C) and F) Coomassie staining of *C. jejuni* whole cell lysates from two independent experiments. G) Densitometry analysis of CmeA bands observed in western blot analysis of *C. jejuni* whole cell lysates with anti CmeA antibodies. Densitometry was performed on two western blots (B) and (E) from independent experiments.

To confirm equal expression of PglB, we performed Western blot analysis of the constructs with anti-HA (hemagglutinin) antibodies as our constructs express HA-tagged PglB. However, we had difficulty detecting PglB reproducibly (data not shown).

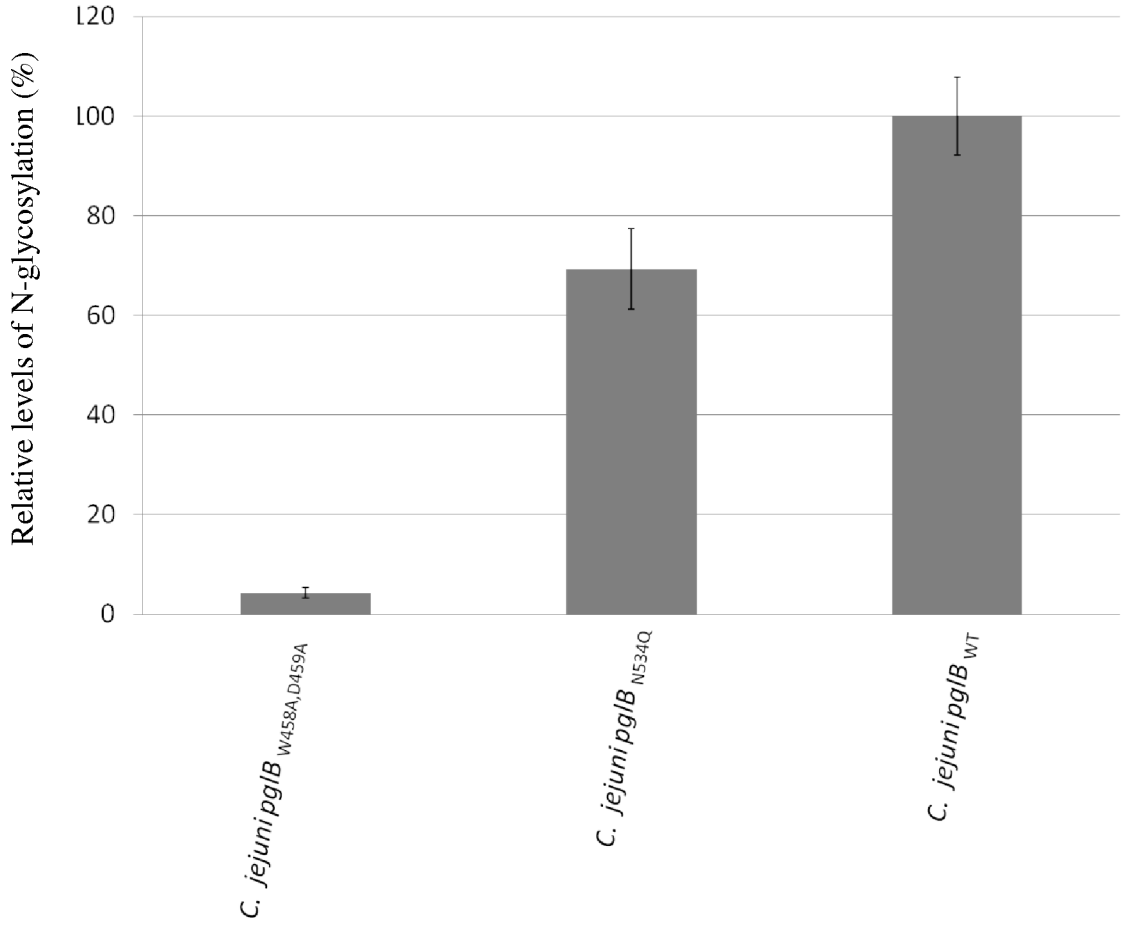


Fig. 3.3 ELISA analysis of N-glycosylation levels in whole cell lysates of the indicated *C. jejuni* strains. The signal intensity of the *C. jejuni* $pglB_{WT}$ was set to 100% and the signals of the other samples were expressed in relative percent levels to this sample. The bars represent the average of atleast two independent experiments.

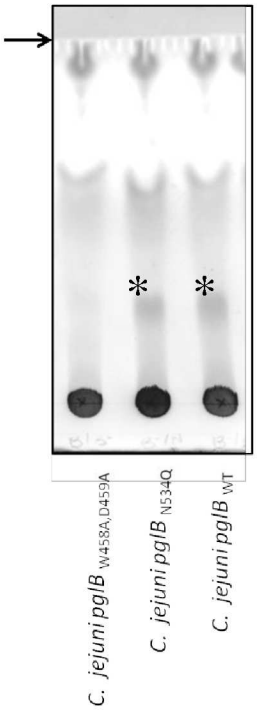
3.3.3 N-glycosylation of PglB may affect its fOS generation activity *in vivo*

We performed fOS analysis by thin layer chromatography (TLC) on our strains in order to detect any differences in the fOS profiles of the three complemented strains i.e. *C. jejuni* $pglB_{W458A,D459A,}$ *C. jejuni* $pglB_{N534Q,}$ *C. jejuni* $pglB_{WT}$. We observed that fOS profiles of *C. jejuni* $pglB_{N534Q,}$ *C. jejuni* $pglB_{WT}$ looked similar with no major changes in the staining pattern (Fig. 4.4A). fOS travelled at the expected R_f value of 0.36 in all lanes where it was detected (Fig.

4.4A). There was no fOS as expected in the *C. jejuni* $pglB_{W458A,D459A}$ due to loss of PglB activity when this motif is mutated (Fig. 4.4A) (Wacker *et al.,* 2002; Nothaft *et al.,* 2009). MALDI-MS was performed on fOS and the *C. jejuni* $pglB_{N534Q}$ mutant was found to produce the same heptasaccharide fOS structure as the wildtype *C. jejuni* and the *C. jejuni* $pglB_{WT}$ strains (Dwivedi *et al.,* 2013).

In order to investigate this in more detail, we used a previously published HPAEC-PAD method for more sensitive quantification of fOS in these strains (Dwivedi *et al.,* 2013). The sensitivity of this method gave us new insights into the results. The *C. jejuni* $pglB_{W458A,D459A}$ had low levels of background noise with signal of 8.8 µM± 4.2 µM (Fig. 4.4B). The *C. jejuni* $pglB_{WT}$ had fOS concentrations of 273.6 µM ± 17.5 µM, whereas the *C. jejuni* $pglB_{N534Q}$ had significantly reduced fOS concentrations at 220.6 µM ± 13.1 µM which is 19.4% less fOS compared to the *C. jejuni* $pglB_{WT}$ strain (Fig. 4.4B) (p- value of 0.03 in a paired t-test analysis).

A)


*
*
C. jejuni pglB W458A,D459A
C. jejuni pglB N534Q
C. jejuni pglB WT

B)

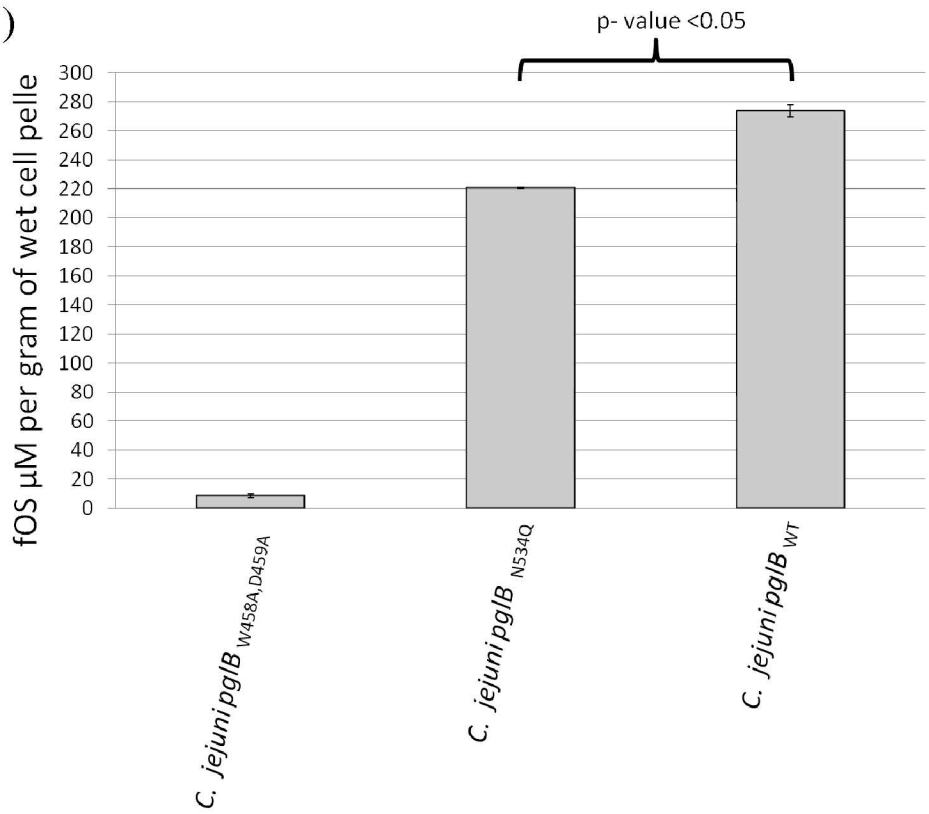

p- value <0.05
fOS µM per gram of wet cell pelle
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
C. jejuni pglB W458A,D459A
C. jejuni pglB N534Q
C. jejuni pglB WT

Fig. 3.4 N-glycosylation of PglB may affect its fOS generation activity *in vivo* in *C. jejuni*. A) Thin layer chromatography analysis of fOS extracts from the indicated *C. jejuni* strains. fOS spots are indicated with an asterisk. B) HPAEC-PAD quantitation of fOS (μM) in the indicated *C. jejuni* strains. The figure represents the average obtained from at least 3 independent experiments (HPAEC-PAD; high performance anion exch ange chromatography with amperometric detection).

3.4 Discussion

The PglB OTase enzymes from the protein N-glycosylation pathways from *Campylobacter* species possess both protein N-glycosylation and LLO hydrolysis activities (Szymanski *et al.,* 1999; Wacker *et al.,* 2002; Young *et al.,* 2002; Nita-Lazar *et al.,* 2005; Nothaft *et al.,* 2009; Nothaft *et al.,* 2010; Reid *et al.,* 2010; Scott *et al.,* 2011; Nothaft *et al.,* 2012; Dwivedi *et al.,* 2013). All *Campylobacter* species have been shown to synthesize species specific N-glycans and perform N-linked protein glycosylation and generate fOS (Nothaft *et al.,* 2012). Interestingly, fOS quantities vary, ranging from 48.75 nmoles in *C. jejuni* to 7.80 nmoles in *C. lari* (Dwivedi *et al.,* 2013). In addition, fOS accounts for up to 2.5% of the cell weight in *C. jejuni* and exists in up to 10 fold higher concentrations compared to its protein-linked counterpart under hypoosmotic conditions (Nothaft *et al.,* 2009; Dwivedi *et al.,* 2013). This suggests that fOS may play important roles in *C. jejuni* under hypoosmotic stress (Nothaft *et al.,* 2009; Dwivedi *et al.,* 2013). Similar to fOS in *Campylobacter* species, many other proteobacteria synthesize periplasmic glucans under hypoosmotic conditions and these play crucial roles in hypoosmotic adaptation and pathogenesis (Lequette *et al.,* 2008; Lee *et al.,* 2009; Bhagwat *et al.,* 2012).

The protein N-glycosylation activity of PglB is being extensively studied due to its application in the production of glycoconjugate therapeutics (Ihssen *et al.,* 2012; Wetter *et al.,* 2012; Cuccui *et*

al., 2013; Ollis *et al.,* 2014; Wacker *et al.,* 2014; Ihssen *et al.,* 2015; Kampf *et al.,* 2015; Srichaisupakit *et al.,* 2015; Cuccui and Wren, 2015). Recently, the crystal structure of the *C. lari* PglB enzyme bound to an acceptor peptide was reported and important amino acids that play roles in the glycosylation reaction mechanism were identified (Lizak *et al.,* 2011). Further studies have focused on determining additional important motifs/amino acids that play roles in catalysis and substrate/acceptor binding specificities, such as the H479 in the *C. jejuni* PglB and the Y293 in the *C. lari* PglB enzyme that are required for optimal glycosylation of substrates (Lizak *et al.,* 2011; Ihssen *et al.,* 2012; Gerber *et al.,* 2013; Liu *et al.,* 2014; Lizak *et al.,* 2014). The *C. jejuni* and *C. lari* PglB enzymes have also been engineered to have relaxed acceptor specificities by mutation of the conserved R331 in the *C. lari* PglB amino acid sequence and the corresponding R328 in the *C. jejuni* PglB enzyme (Ollis *et al.,* 2014). In contrast, the mechanism of LLO hydrolysis (i.e. fOS generation) by PglB is not well understood. The hydrolytic activity of PglB changes in response to the extracellular environment of *C. jejuni*, such as osmolarity and growth phase*,* but the mechanism behind this phenomenon is not understood (Nothaft *et al.,* 2009). The ^{457}WWDYG^{462} motif, however, is essential for both protein glycosylation and LLO hydrolysis/fOS generation in *C. jejuni* (Wacker *et al.,* 2002; Nothaft *et al.,* 2009). In comparison with the bacterial OTase, recently, the eukaryotic OTase complex also generates neutral fOS *in vivo* and *in vitro* (Harada *et al.,* 2013). Analysis of the fOS release activity of the eukaryotic OTase revealed that although the OTase multi-subunit complex releases fOS, the Stt3 catalytic subunit, homologous to the PglB enzyme, is enough for release of fOS from eukaryotic LLOs (Harada *et al.,* 2013). This suggests that LLO hydrolysis might be a conserved activity in all OTases belonging to N-linked protein glycosylation pathways.

Mass spectrometry analysis of the *C. jejuni* proteome revealed that PglB is also N-glycosylated at N534 (Scott *et al.,* 2011). In addition, the *C. lari* PglB enzyme has also been shown to be N-glycosylated at N535 and N556 (Lizak *et al.,* 2011). In this study, we investigated the effects of the N-glycan modification on *C. jejuni* PglB with respect to its N-glycosylation and LLO hydrolysis activity *in vivo*.

We found that the N-glycan modification site is conserved in most *Campylobacter species* and it is exposed to the periplasmic space in the *C. lari* PglB enzyme. The *C. jejuni* PglB enzyme releases lower levels of fOS under hyperosmotic stress and also, during lag and stationary growth phases, however overall cellular protein N-glycosylation levels are unaltered (Nothaft *et al.,* 2009). In this study, we discovered that the N-glycan modification on PglB has significant effects on the LLO hydrolysis (fOS generation) activity of the enzyme in *C. jejuni* and unglycosylated PglB generates lower levels of fOS compared to wildtype. It is possible that when *C. jejuni* is grown under certain conditions, such as osmotic stress, incomplete glycosylation of PglB maybe one mechanism to regulate fOS release. As mentioned previously, fOS are analogous to osmoregulated periplasmic glucans (OPG) found in other proteobacteria (Bohin, 2000; Lequette *et al.,* 2007; Lequette *et al.,* 2008; Lee *et al.,* 2009; Bhagwat *et al.,* 2009). Recently, the phosphoglycerol transferase enzyme involved in decoration of OPG molecules with phosphoglycerol moieties in *E. coli* was also reported to alter its cellular location and activity based on its processing (Lequette *et al.,* 2008). Based on its proteolysis status, the enzyme was reported to either add (membrane bound, pre-proteolysis) or swap phosphoglycerol (periplasmic form, post proteolysis) between OPG molecules (Lequette *et al.,* 2008). Inner membrane proteins in Gram-negative bacteria, such as histidine kinases that from two component signalling systems, undergo changes in phosphorylation status in response to

extracellular stimuli i.e. osmolarity and pH. This allows a connection between the extracellular and intracellular environment ultimately allowing regulation of appropriate intracellular networks for optimal responses to the extracellular conditions (Levit and Stock, 1999; Stock *et al.,* 2000). The methylation status influences the activity of the aspartate chemoreceptor in *E. coli* that is involved in regulation of cellular responses to extracellular aspartate or temperature changes (Borkovich *et al.,* 1992; Nara *et al.,* 1996). Although, the mechanism behind how glycosylation exactly affects PglB activity could not be determined, it is possible that glycosylation affects the structure of PglB and/or its ability to interact with other cellular components, such as periplasmic proteins involved in signaling. The N-glycan of *C. jejuni* is indeed involved in interactions with itself, as well as, amino acid interactions with PglB via the N-acetyl moieties as determined by structural modeling by Ihssen *et al.* (2015).

We also found that N-glycosylation of PglB influences its ability to glycosylate other proteins in *C. jejuni*. We found that the N-glycosylation levels were not significantly different in the cells expressing unglycosylated PglB as determined by ELISA assays, however the overall N-glycosylation protein profile was altered in cells expressing unglycosylated PglB in comparison to the wildtype in Western blot analysis. Several eukaryotic proteins require N-glycan modification for optimal localization/activity and diseases caused by absence of proper protein N-glycosylation are grouped in a category known as congenital disorders of glycosylation (Muthusamy *et al.,* 2015; Console *et al.,* 2015; Min *et al.,* 2015). In addition, in *C. jejuni*, the function/stability of the VirB10 component of the Type IV secretion system is affected upon removal of N-glycosylation (Larsen *et al.,* 2004). Another example is the HMW1 adhesin from *H. influenza* that exhibits increased susceptibility to proteolytic degradation and is mislocalized in the cell in the absence of N-glycosylation (Grass *et al.,* 2003). It is possible that SG of PglB

maybe a novel way to regulate the activity/localization of several proteins in the cell at once by modulating the levels of its own N-glycan modification. In addition, it is possible to repeat the ELISA assay a few more times to confirm that the levels of N-glycoproteins are not significantly altered in the cells expressing unglycosylated PglB compared to wildtype. In addition, ELISA signals from LLOs may misrepresent the differences in overall N-glycosylation levels in SG mutant cells compared to wildtype. Therefore, the ELISA assay can be improved by including whole cell lysates of a *pglA* mutant that does not accumulate LLOs unlike the *pglB* mutant (Glover *et al.,* 2005; Reid *et al.,* 2008) or, purified LLOs. This will aid in determining whether the ELISA signals correspond purely to the N-glycoprotein content of the cells or are affected by LLO content of the cells as well.

In this study, we have reported potentially novel effects of the N-glycan modification on the enzymatic activities of the PglB oligosaccharyltransferase in *C. jejuni*. Our study suggests that the absence of the conserved N-glycan modification may influence both protein N-glycosylation and hydrolase activities of the enzyme. This study warrantees further studies on the mechanism of N-glycosylation and fOS release mechanisms of this enzyme.

3.5 Acknowledgements

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CHAPTER 4

Fluorescence resonance energy transfer (FRET) assay for quantitative analysis of oligosaccharyltransferase enzymatic activity

4.1 Introduction

Campylobacter jejuni was the first bacterium shown to possess an N-linked protein glycosylation system (Pgl) (Szymanski *et al.,* 1999; Wacker *et al.,* 2002; Young *et al.,* 2002). The Pgl pathway encodes enzymes for the synthesis of the heptasaccharide, GalNAc-α1,4-GalNAc-α1,4-[Glcβ1,3]-GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-diNAcBac-β1 (diNAcBac is 2,4-diacetamido-2,4,6-trideoxyglucopyranose) on the lipid carrier, undecaprenylphosphate (i.e. lipid linked oligosaccharide, LLO) (Young *et al.,* 2002; Wacker *et al.,* 2002; Nita-Lazar *et al.,* 2005; Kelly *et al.,* 2006; Reid *et al.,* 2008; Reid *et al.,* 2009). In addition, the central oligosaccharyltransferase (OTase) enzyme, PglB, is also encoded within the *pgl* locus (Wacker *et al.,* 2002). PglB transfers the heptasaccharide from the LLOs to asparagine residues of proteins within the sequon, D/E/X_1-N-X_2-S/T (X cannot be proline) (Szymanski *et al.,* 1999; Wacker *et al.,* 2002; Nita-Lazar *et al.,* 2005). PglB is responsible for glycosylating more than 60 proteins in *C. jejuni* (Scott *et al.,* 2011; Scott *et al.,* 2014) and the WWDYG motif is essential for this activity (Wacker *et al.,* 2002).

New horizons for glycoengineering were unfolded when the *C. jejuni pgl* gene locus was transferred into *Escherichia coli* and N-glycosylation of the efflux pump protein,CmeA, from *C. jejuni* was successfully achieved *in vivo* (Wacker *et al.,* 2002). Since then, PglB and other N-OTases have been extensively studied with a major focus on their structure, glycan donor and substrate specificities (Ielmini and Feldman, 2011; Igura and Kohda, 2011; Ihssen *et al.,* 2012; Gerber *et al.,* 2013; Musumeci *et al.,* 2013; Baker *et al.,* 2013; Cohen-Rosenzweig *et al.,* 2014; Musumeci *et al.,* 2014; Meyer and Albers, 2014). The OTases have also been exploited in the production of recombinant vaccines containing bacterial or eukaryotic glycans (Wacker *et al.,* 2002; Ihssen *et al.,* 2010; Schwarz *et al.,* 2010; Valderrama-Rincon *et al.,* 2012; Wetter *et al.,*

2012; Cuccui *et al.,* 2013; Kampf *et al.,* 2015; Ihssen *et al.,* 2015). So far, studies on substrate glycosylation by OTases have relied on ELISA assays that use antibodies against the glycan of interest following *in vivo* glycosylation or methods that rely on gel electrophoresis, fluorescent gel imaging or Western blot analysis (Jervis *et al.,* 2010; Ihssen *et al.,* 2012; Gerber *et al.,* 2013; Musumeci *et al.,* 2013; Ihssen *et al.,* 2015; Kampf *et al.,* 2015). Although these methods are useful, they involve time-consuming procedures and the development of quicker methods will allow faster data generation and analysis.

In this study, we have developed a Fluorescence Resonance Energy Transfer (FRET) based 96-well plate assay to accurately determine OTase mediated *N*-glycosylation rates using the PglB OTase from *C. jejuni*. The assay is based on a previously published FRET protease protection assay for OTases for O- glycosylation (Gross *et al.,* 2008). This study provides an important tool for quick screening of OTase activity. Our method uses a terminally labelled fluorophore/quencher (FRET) peptide (Dabcyl-DQNATIDGRKQ-Edans, Edans-fluorophore and Dabcyl-quencher) carrying protease cleavage sites (IDGR) close to the N-glycan acceptor sequon (DQNAT) (Fig. 1). The FRET peptide is incubated with the OTase enzyme *in vitro* and precipitated out of the reaction. The peptide is then analyzed by Factor Xa mediated proteolysis. Whereas unglycosylated peptide is cleaved by Factor Xa and results in fluorescence, the glycosylated peptide is protected from proteolysis due to the glycan modification. This sensitive technique allows direct quantification of the levels of glycosylated peptide relative to unglycosylated peptide (Fig. 1). This 96-well plate based assay can be used for quick and accurate analysis of the glycosylation activity of N-OTases.

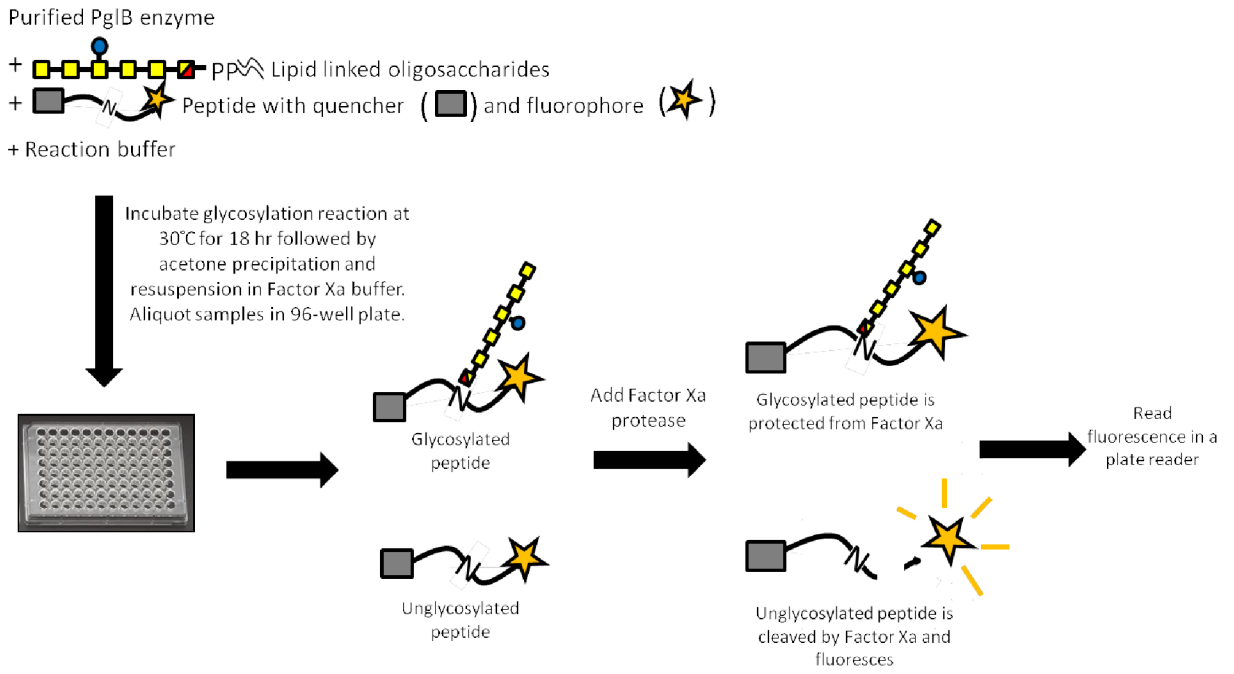

Fig. 4.1 A schematic illustration of the FRET assay. Peptides are incubated *in vitro* with purified PglB enzyme and LLOs for 18 hr at 30°C. The FRET peptide is then precipitated out of the reaction with acetone and aliquoted in 96-well plates for FRET analysis with or without Factor Xa enzyme.

4.2 Materials and methods

4.2.1 Bacterial strains and plasmids

E. coli strains were grown in 2xYT at 37°C with shaking at 220 rpm. Antibiotics were added at the following final concentrations: ampicillin (200µg/mL), kanamycin (25µg/mL) and chloramphenicol (25µg/mL). *E. coli* SCM7 strain expressing the pACYC (pgl_{mut}) was obtained from a previously published study (Kowarik *et al.*, 2006). *E. coli* DE3 strains expressing wildtype PglB enzyme (pET24-$PglB_{WT}$) and PglB enzyme with mutation in the WWDYG motif (pET24b-$PglB_{W458A,D459A}$) were kindly provided by Yasmin Barre (MSc, Szymanski lab, University of Alberta). The $PglB_{WWDYG}$ has been mutated to WAAYG and renders the enzyme inactive (Wacker *et al.*, 2002).

4.2.2 Details of the FRET peptide

Terminally labelled fluorophore/quencher (FRET) peptide (Dabcyl-DQNATIDGRKQ-Edans, Edans-fluorophore and Dabyl- quencher) carrying protease cleavage sites (i.e. IDGR) close to an N-glycan acceptor sequon (i.e. DQNAT) were custom ordered from GenScript, Inc. Peptides were resuspended in 10% isopropanol in deionized water to a final concentration of 573 µM and stored at -20°C until use. Peptides and reactions containing peptides were protected from light and were wrapped in aluminium foil at all times unless stated otherwise.

4.2.3 Purification of PglB for *in vitro* assays and reaction conditions

PglB expression and enrichment was performed as described previously (Li *et al.,* 2010) with the following modifications: the pET24b vector containing His_6-tagged *C. jejuni* full length wildtype PglB and WWDYG mutant expressing genes was transformed into *E. coli* C43 (DE3). Single colonies were grown overnight in 5 ml LB broth. Overnight cultures were grown in 1L of 2XYT medium supplemented with antibiotics at 37°C to an OD_{600} of ~ 0.95 – 1.1. PglB expression was induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After a 5 hour induction period at 30°C under aerobic conditions, cells were harvested by centrifugation at 12,000g for 30 min. All other procedures from this point were carried out on ice or with ice cold buffer. Pellets were resuspended in 25 ml of lysis buffer (50mM Tris-HCL pH 8, 200 mM NaCl, supplemented with 1 mM PMSF, 1 tablet of EDTA free cOmplete™ protease inhibitor cocktail (Roche), according to the manufacturer's protocol. Pellets were disrupted by cell disruption by passing twice at 30 psi and unbroken cells removed by centrifugation at 12,000g for 15 min at 4°C. Membrane fractions were isolated by high speed centrifugation (100,000g, 70 min, 4°C). The supernatant was discarded and PglB containing membranes were solubilized overnight with shaking at 4°C using 15 ml solubilization buffer (50 mM Tris-HCL, pH 8, 200 mM NaCl, 5%

glycerol, 1% DDM (n-Dodecyl-ß-D-maltopyranoside). Resuspension of PglB membranes was done carefully with a plastic spatula to a final volume of 25 ml with wash buffer (50 mM Tris-HCL, pH 8, 200 mM NaCl, 30 mM imidazole, 5% glycerol, 0.1% DDM). Ultracentrifugation (100,000g 70 min, 4°C) was then performed again. Ni-NTA agarose (2 ml) was equilibrated with wash buffer and incubated with solubilized PglB membranes (supernatant after second centrifugation) for 2 hours at 4°C. The resin was washed with 300 ml wash buffer and eluted with 1- 1.5 ml of elution buffer (50 mM Tris-HCL, pH 8, 200 mM NaCl, 300 mM imidazole, 10% glycerol, 0.1% DDM). Alternatively, elution can be carried out with 5X 300 µl elution buffer. Enriched PglB was detected by SDS-PAGE, Coomassie staining, and Western blotting with anti-His antibodies, 1:500 dilution (Santa Cruz). Protein concentration was measured using the Bio-Rad DC Bradford protein assay according to manufacturer's instructions and BSA was used for generating the standard curve. PglB can be stored at -20°C with 15% glycerol in the elution buffer. If desired, PglB was desalted using Millipore Amicon Ultra 10 kDA cut off 0.5mL centrifugal columns with the elution buffer without imidazole. The purified protein sample from the column was loaded into the spin filter and centrifuged at 4°C at 4,000 g for 15 min or until the desired concentrated volume was achieved.

Functionality of PglB was confirmed with CmeA as a substrate as described previously (Glover *et al.,* 2005). The reaction was set-up as follows: 2 µL of 1M $MnCl_2$, 100 µL 2x assay buffer (Glover *et al.,* 2005), 10 µL of LLOs (speedvac dried and resuspended in 10 µL DMSO), 60 µL of protease inhibitor (prepared by resuspending 1 tablet of Roche EDTA free cOmplete protease inhibitor cocktail in 500 µL of water), 2 µg of PglB enzyme, 4µL of FRET peptide (573 µM). Deionized water was added to a final volume of 200 µL. A volume of 100 µL was removed at

time zero and stored at -20°C until further analysis. The reaction was incubated at 30°C for 18 hr with mixing.

4.2.4 Preparation of lipid-linked oligosaccharide samples

LLOs were isolated from *E. coli* SCM7 expressing the plasmid pACYC (pgl_{mut}) as described previously (Kowarik *et al.,* 2006). Only minor changes were made as follows: after the chloroform/methanol/water extraction, the insoluble material was removed by centrifugation at 8,000 g for 30 min at 4°C. Also, the supernatant was evaporated to dryness in a fumehood and resuspended in 1 mL of deionized water per 1 L of starter culture.

4.2.5 Analysis of FRET peptide reactions

The reaction was prepared in a 1 mL Eppendorf tube as follows:

Following incubation, 12.5 µL of 1 mg/mL bovine serum albumin (BSA) was added and mixed into the solution followed by 1 mL of 100% pre-chilled acetone (-80 °C) to the tube and incubated at -80°C for 1 hr. The mixture was then centrifuged at 18,000g for 1 hr at 4°C and the supernatant was discarded. The pellet was dried in the dark at room temperature overnight. A volume of 180 µL of milli-Q water was added to the pellet and vortexed until the pellet completely dissolved. The solution was incubated at 37°C with mixing to ensure proper solubilization of the peptide. Then a volume of 20 µL of 10x Factor Xa buffer (200 mM Tris-HCl pH 8.0, 1 M NaCl, 20 mM $CaCl_2$) was added to the tube and vortexed to obtain a homogeneous solution. The solution was split into two 70 µL aliquots into a 96-well plate for the assay. Fluorescence was read at excitation/emission of 355/530 nm before addition of enzyme. Then, 1 µL of Factor Xa enzyme was added to one aliquot and mixed well by pipetting and the fluorescence was read at time zero and then every 5 min until the fluorescence did not increase for the next three readings. The plate was at room temperature for the duration of the

fluorescence readings. Positive (with 2 µL of stock peptide) and negative (no peptide) controls were setup in the same volumes/aliquots. A precipitated peptide control was also included in which the peptide was acetone precipitated and treated the same as the other reactions.

4.2.6 Mass spectrometry analysis

Following acetone precipitation of the reaction and resuspension in 180 µL as described above, 50 µL was dried in a speedvac and resuspended in 10 µL 2% acetonitrile/1% formic acid. The peptides were cleaned with ZipTip® pipette tips (Millipore) as follows: The tips were pipetted with 3x 10 µL 100% acetonitrile and 3x 10 µL 0.1% formic acid. The samples were pipette up and down the tip 20 times followed by washing with 10 µL of 0.1% formic acid. The samples were then eluted with 60% acetonitrile and 0.1% formic acid and analysed by MALDI-MS and MALDI-MS/MS, as well as, LC-MS and LC-MS/MS at the Chemistry Mass Spectrometry Facility (University of Alberta).

4.3 Results

4.3.1 Preliminary FRET analysis showed the peptide is susceptible to proteases

Preliminary FRET analysis by Dr. Abofu Alemka (Postdoctoral fellow, Dr. Szymanski's lab) revealed peptide degradation following incubation. FRET signals were very low after overnight incubation with $PglB_{W458A,D459A}$ purified or membrane preparations compared to the positive control peptide that was incubated alone (data not shown). This indicated that the peptide was degraded by intrinsic proteases in the preparations of PglB expressing membranes and purified PglB and/or donor LLO preparations. I started troubleshooting the assay by trying different varieties of protease inhibitor cocktails and varying their concentrations. Finally, I was able to achieve comparable levels of fluorescence between the positive control and purified and membrane preparations of $PglB_{W458A,D459A}$ (data not shown).

4.3.2 Troubleshooting incompatibility between the *in vitro* glycosylation assay buffer and Factor Xa enzyme and developing ways to detect peptide glycosylation by mass spectrometry

Previous assays performed by Dr. Abofu Alemka involved directly adding Factor Xa enzyme along with Factor Xa buffer to the glycosylation reaction. Fluorescence was not detected in this reaction mixture indicating that the enzyme was unable to cleave the peptide and, therefore not optimally functional in this mixture (data not shown). It appeared that Factor Xa needed to be resuspended in Factor Xa buffer for optimal activity and the components of the glycosylation reaction mixture interfered with Factor Xa activity.

I tried different methods to remove the peptide from the glycosylation reaction mixture, without major losses of peptide, so that it can be resuspended purely in the Factor Xa reaction buffer to achieve maximum Factor Xa activity. We tried variations of chloroform/methanol protein precipitation and C18 column purifications, however little to no signal was observed after the peptide was precipitated using these techniques suggesting that either these techniques caused loss of the peptide and/or damaged the peptide due to harsh chemical treatment involved in the procedures (data not shown). Finally, acetone precipitation at -80°C proved to work well and comparable levels of fluorescence signals were observed between the positive control and the precipitated peptide (Fig. 4.2).

In addition, Dr. Alemka was able to detect the manufacturer's unprocessed peptide by HPLC (data not shown). This method was also used by the manufacturer to confirm the mass of the peptide, however, this procedure does not accurately identify the peptide and its composition. In addition, this procedure was not tried on the FRET peptide that was mixed in the glycosylation reaction mixture. After troubleshooting the detection procedures, I was able to develop a quick

C18 column based procedure that allowed mass spectrometry of the FRET peptide directly from the glycosylation reaction mixture (Fig. 4.3). The peptide that had been incubated *in vitro* with purified PglB enzymes was successfully detected by MALDI-MS (Fig. 4.3) and MALDI-MS/MS (data not shown), as well as LC-MS and LC- MS/MS (data not shown).

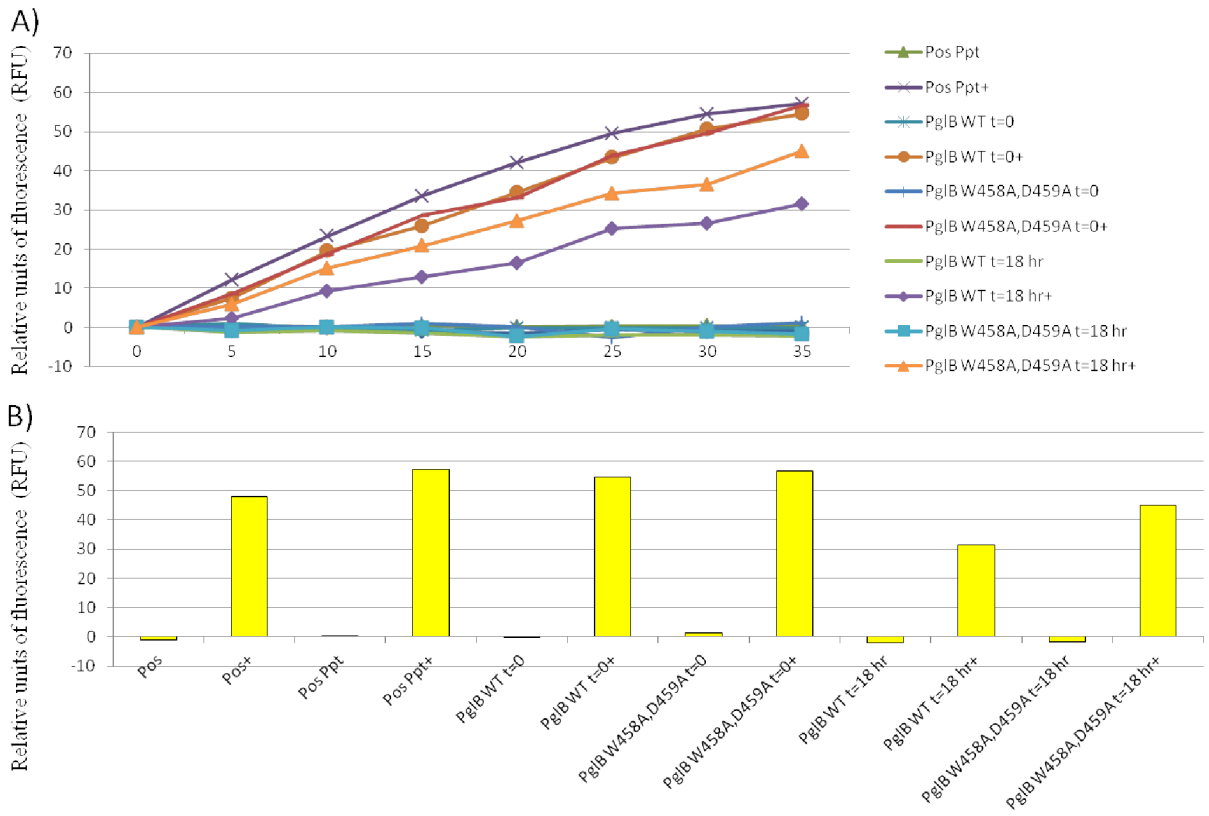

Fig. 4.2 Analysis of *in vitro* reactions containing FRET peptide incubated with purified $PglB_{W458A,D459A}$ and $PglB_{WT}$ enzymes. A) Time course analysis of the relative fluorescence units (RFU) of reactions aliquoted out before and after 18 hr of incubation. B) Final RFU of all reactions after 35 min of incubation with or without Factor Xa. Reactions not containing Factor Xa served as negative controls (Pos, positive control peptide only; Pos Ppt, positive control containing acetone precipitated peptide; Factor Xa addition is indicated with + symbol).

4.3.3 FRET analysis with purified PglB enzymes shows evidence of *in vitro* glycosylation

The FRET peptide was incubated with purified $PglB_{WT}$ and $PglB_{W458A,D459A}$ enzymes and the fluorescence was analyzed before (t=0) and after 18 hr of incubation (Fig. 4.2).

The reactions were split into two aliquots and Factor Xa was added to one aliquot whereas no enzyme was added to the other aliquot and the relative fluorescence units (RFU) were read for 35 min. The fluorescence of the reaction without Factor Xa addition was monitored for background RFU levels at all time points (i.e. noise) (Fig. 4.2A).

All reactions that did not contain Factor Xa maintained basal fluorescence at 1 to 5 RFU at 35 min indicating that increase in RFU in the other reactions was due to Factor Xa mediated cleavage of the peptide (Fig. 4.2B).

Two positive control reactions had been set-up. One from the manufacturer without any further processing (Pos) and the other where the peptide had been acetone precipitated to mimic the processing of the *in vitro* reaction samples (Pos Ppt). This was done in order to test the efficiency of the acetone precipitation method. Both reactions had a final fluorescence of 48.06 and 56.67 RFU at 35 min upon Factor Xa addition showing that acetone precipitation was a reliable method to achieve complete precipitation of the peptide.

The PglB containing reactions were monitored at t=0 min and t=18 hr. The RFU of the reactions at t=0 for $PglB_{WT}$ and $PglB_{W458A,D459A}$ were 54.6 and 48.0 respectively at 35 min after Factor Xa addition (Fig. 4.2A). These RFU were close to the positive control RFU mentioned above and indicated that the peptide was not glycosylated at t=0 min as expected (Fig. 4.2A). As mentioned before, $PglB_{W458A,D459A}$ is an inactivated enzyme that does not perform glycosylation due to the mutation of W458 and Y459 to alanine residues (Wacker *et al.,* 2002). At t=18 hr, the RFU of the $PglB_{W458A,D459A}$ was 45.1 which was similar to the t=0 RFU of the reaction indicating that the

peptide had not degraded after the incubation and was not glycosylated as expected. The reaction containing $PglB_{WT}$ has an RFU of 31.5 after t=18 hr at 35 min which was less than the RFU at t=0 min (Fig. 4.2). This indicated that approximately 25% of the peptide may be glycosylated based on RFU and full glycosylation of the peptide had not been achieved as the RFU had not dropped completely to background levels in this reaction.

In order to detect glycosylation of the peptide, MALDI-MS (Fig. 4.3) and MALDI-MS/MS was performed (data not shown). We observed peaks at 1746 m/z in the sample containing the manufacturer's peptide (i.e. unprocessed) (Fig. 4.3A). This peak corresponded to the correct sequence of the peptide in MALDI-MS/MS (Fig. 4.4). Additional peaks that had high intensity signals were observed in the MALDI-MS (Fig. 4.3A), however, further analysis revealed that these were not FRET peptide related peaks but other contaminants present in the manufacturer's peptide (data not shown). We also observed the same peak at 1746 m/z in the sample containing acetone precipitated peptide (Fig. 4.3B). This confirmed that acetone precipitation worked successfully to remove the peptide from the solutions. Analysis of peptide incubated with $PglB_{\Delta WWDYG}$ enzyme revealed an unglycosylated peptide peak at the expected m/z as well (Fig. 4.3D). Analysis of peptide that had been incubated with $PglB_{WT}$ enzyme showed a peak corresponding to unglycosylated peptide, however a peak corresponding to the glycosylated peptide at an expected 3171 m/z was not observed (Fig. 4.3C).

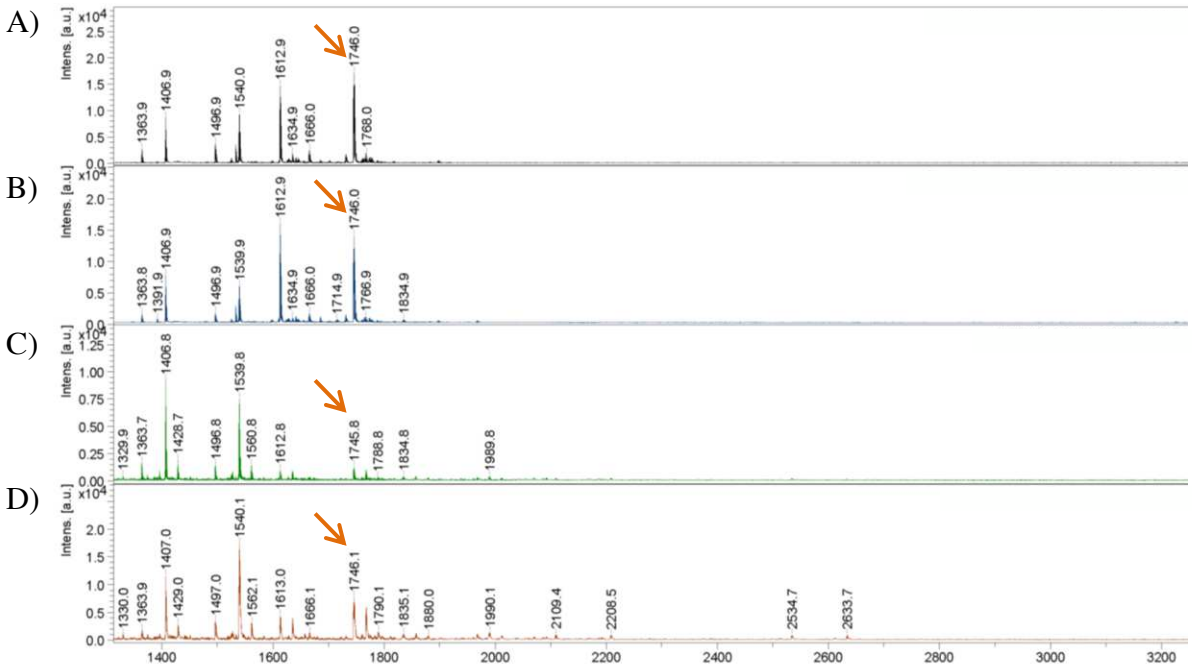

Fig. 4.3 MALDI-MS analysis of control peptides and peptides from *in vitro* reactions with purified $PglB_{W458A,D459A}$ and $PglB_{WT}$ enzymes. A) Peptide from the manufacturer (control). B) Acetone precipitated peptide. C) Acetone precipitated peptide from *in vitro* reactions containing $PglB_{WT}$ enzyme. D) Acetone precipitated peptide from *in vitro* reactions containing $PglB_{W458A,D459A}$ enzyme. The orange arrows point towards the m/z signal of the FRET peptide.

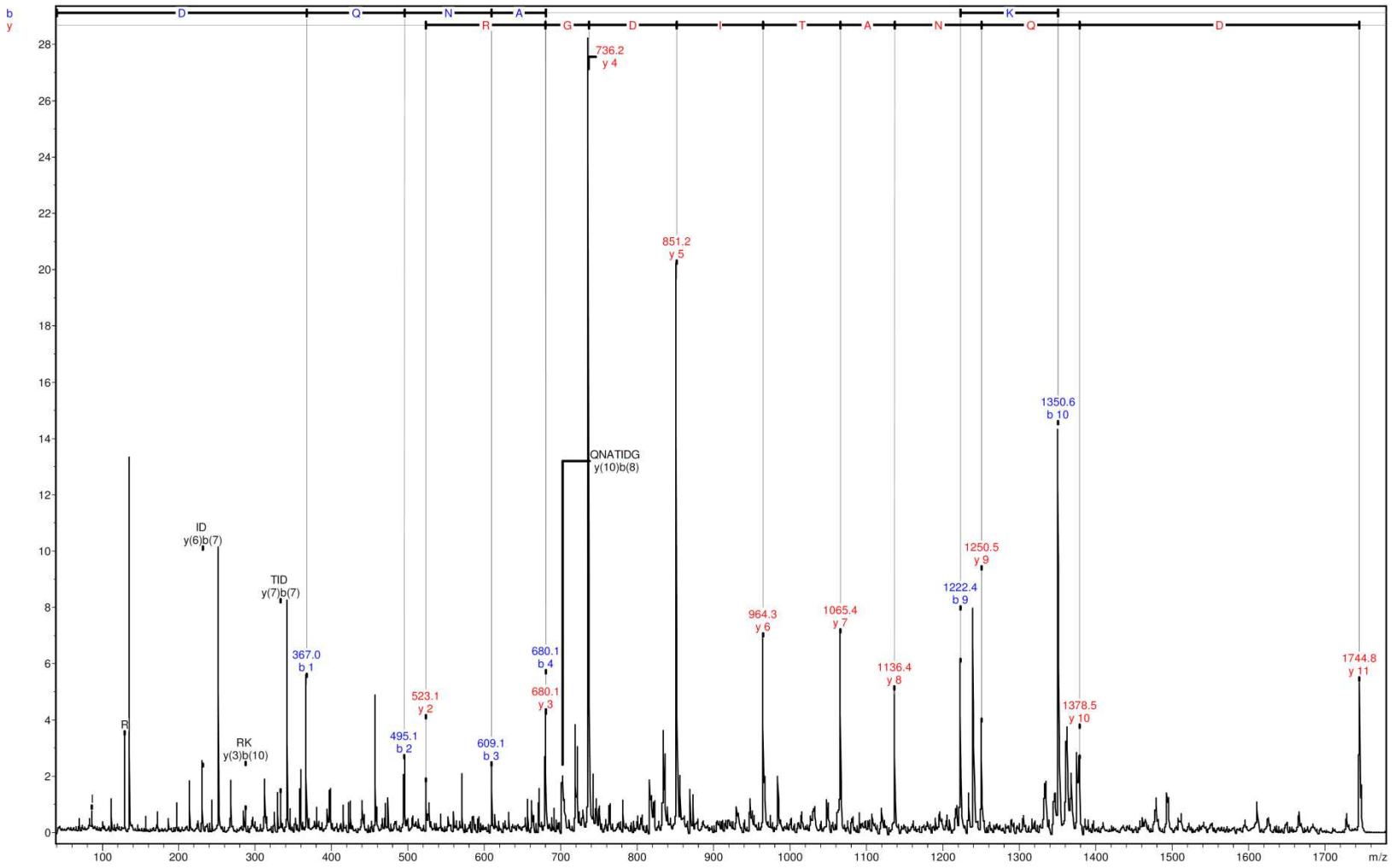

Fig. 4.4 MALDI-MS/MS analysis of control peptide. The Y ions and B ions, as well as internal fragments of the peptide are indicated. The peptide Dabcyl-DQNATIDGRKQ-Edans (Edans-fluorophore and Dabcyl-quencher) was correctly identified.

4.4 Discussion

The first study showing the application of the PglB OTase from *C. jejuni* in glycoengineering was over a decade ago (Wacker *et al.,* 2002). The N-glycan structure from *C. jejuni* was expressed in *E. coli* and successfully transferred onto a model acceptor glycoprotein, CmeA, a component of the major efflux pump in *C. jejuni* (Wacker *et al.,* 2002). Further studies reported the use of PglB in generating many different glycoconjugate vaccines (Feldman *et al.,* 2005; Ihssen *et al.,* 2010; Cuccui *et al.,* 2013; Wetter *et al.,* 2013; Garcia-Quintanilla *et al.,* 2014; Cuccui and Wren, 2015; Srichaisupakit *et al.,* 2015; Kampf *et al.,* 2015). These included the O-antigen polysaccharide from *Francisella* conjugated to detoxified exotoxin A from *P. aeruginosa* that resulted in good immune responses to this human pathogen in mouse models (Cuccui *et al.,* 2013) and, the O-antigen of *Burkholderia pseudomallei* conjugated to the CmeA protein that was partially protective in mouse models (Garcia-Quintanilla *et al.,* 2014). In addition, attempts to generate glycoconjugates with eukaryotic glycan structures have been made with PglB (Schwarz *et al.,* 2010; Hug *et al.,* 2011; Valderrama-Rincon *et al.,* 2012; Srichaisupakit *et al.,* 2015). Also, Srichaisupakit *et al.* (2015) successfully transferred glycosyltransferases from *S. cerevisiae* into *E. coli* and showed that PglB is able to transfer eukaryotic like N-glycan structures to acceptor proteins which would be useful in the production of glycoconjugate therapeutics (Srichaisupakit *et al.,* 2015).

In addition, many researchers have developed ELISA and gel electrophoresis based methods to analyze the activity of OTases, particularly PglB (Ihssen *et al.,* 2010; Schwarz *et al.,* 2010; Cuccui *et al.,* 2013; Ishiwata *et al.,* 2015; Ihssen *et al.,* 2015; Kampf *et al.,* 2015; Cuccui and Wren, 2015). Jervis *et al.* (2010) used gel electrophoretic separation of glycosylated and unglycosylated fluorophore labelled peptides to demonstrate the presence of N-linked

glycosylation in *Helicobacter pullorum* and to characterize the glycan structure. The same principle has been used to study the lipid carrier preferences of PglB as well (Liu *et al.,* 2014). Ihssen *et al*., (2012) generated PglB variants by error prone polymerase chain reaction and quantified glycosylation efficiencies by ELISA using 96-well plates coated with antibodies against the glycan used in the study (i.e. capsular polysaccharide of *Staphylococcus aureus* serotype 5). Ihssen *et al*. (2015) were able to generate PglB mutants that had relaxed oligosaccharide specificity and higher glycosylation efficiencies. The authors of this study were also to compare several mutants to the wildtype enzyme by ELISA assays with antibodies against the glycan of interest (Ihssen *et al.,* 2015). Kampf *et a*l., (2015) also used ELISA assays to determine the efficiency of their improved *in vivo* glycoconjugate production techniques. In this study, the authors were able to increase glycoconjugate yield by 46-fold by determining the optimal times for the induction of PglB and acceptor protein, as well as, optimal concentration of magnesium ions in addition to other parameters (Kampf *et al.,* 2015). Although these methods are useful, they are time-consuming and take several hours to complete and quicker assays are required to make data generation and analysis faster. ELISA assays also require specific antibodies against the glycan of interest. Fluorescence anisotropy has also been used to investigate the binding of acceptor substrates by PglB (Gerber *et al.,* 2013; Lizak *et al.,* 2014), however this technique requires advanced training and specific calculations for data analysis. This technique is also not useful to study the glycosylation efficiency of the OTase enzymes.

A fast and efficient 96 well plate based FRET assay was developed in this study for the analysis of OTase activity. Previous analysis by Dr. Alemka had suggested severe degradation of the peptide during incubation *in vitro*. In addition, Factor Xa enzyme was incompatible with the *in vitro* glycosylation assay buffer and did not cleave the FRET peptide efficiently. I was able to

resolve both difficulties and apply the assay to be used with purified PglB enzymes. Previously, HPLC was used for the detection of peptides however this method does not provide information on the exact composition of the peptide. I was able to develop a quick acetone precipitation and C18 column based method to accurately analyse and detect the peptide following the glycosylation reaction by MALDI-MS, MALDI-MS/MS, LC-MS and LC-MS/MS. Comparable fluorescence values were observed for the peptide in the positive controls, as well as, the $PglB_{\Delta WWDYG}$ reactions before and after overnight incubations. Therefore, earlier difficulties with peptide degradation and incompatibility between Factor Xa enzyme and the *in vitro* glycosylation assay buffer were resolved successfully. A slight drop in the fluorescence of the $PglB_{WT}$ containing reaction was observed, however, I was unable to detect glycosylated peptide in the reaction and only unglycosylated peptide was detected. The slight drop indicated that only a small portion of the peptide is glycosylated and a major portion remained unglycosylated. It is possible that MALDI-MS is not sensitive enough to detect the small portion of glycosylated peptide, especially in the presence of other intense contaminant peaks in the manufacturer's peptide. It may be possible to detect glycosylated peptide upon enrichment with SBA agarose in addition to concentrating the sample. SBA agarose has been previously used to enrich for N-glycosylated proteins from *C. jejuni* since it recognizes terminal GalNAc residues (Young *et al.,* 2002; Larsen *et al.,* 2004; Karlyshev *et al.,* 2004; Linton *et al.,* 2005; Scott *et al.,* 2011). In addition, glycosylation efficiency can be improved by adding higher amounts of PglB enzyme to the reaction mixture and/or alternatively adding smaller amounts of the acceptor peptide. It is also possible to incubate the reaction mixture longer than 18 hr to obtain higher amounts of glycosylated peptide. Previously published studies with PglB suggest that although glycosylation efficiency can be improved, it is difficult to obtain complete glycosylation of the acceptor

substrate *in vitro* (Ihssen *et al.,* 2010; Schwarz *et al.,* 2010; Cuccui *et al.,* 2013; Ishiwata *et al.,* 2015; Ihssen *et al.,* 2015; Kampf *et al.,* 2015; Cuccui and Wren, 2015).

This study provides a quick and efficient high-throughput tool for the analysis of OTase activity. The method can be applied to study the activity of other OTases and can be further extended to study the kinetics and acceptor/substrate preferences of multiple OTases in a 96-well high-throughput format.

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CHAPTER 5

Conclusions and future directions

5.1 Purpose of research

The research conducted in this PhD thesis focused on better characterizing the N-linked protein glycosylation (Pgl) pathway in *Campylobacter* species. The two products of the pathway are generated by the central enzyme, PglB (Szymanski *et al.,* 1999; Wacker *et al.,* 2002; Nothaft *et al.,* 2009; Nothaft *et al.,* 2012). This enzyme possesses oligosaccharyltransferase (OTase) activity and N-glycosylates multiple proteins (Szymanski *et al.,* 1999; Wacker *et al.,* 2002; Nothaft *et al.,* 2012). Secondly, this enzyme also possesses hydrolase activity and generates periplasmic free oligosaccharides (fOS) by hydrolyzing the lipid linked oligosaccharide (LLO) intermediates of the pathway (Nothaft *et al.,* 2009; Nothaft *et al.,* 2010; Nothaft *et al.,* 2012). The purpose of this thesis was to develop efficient methods to understand the generation of these products and to better characterize the OTase and hydrolase activities of the PglB enzyme. Specifically, the objectives were to understand the regulation of the enzymatic activity of PglB and, develop faster and efficient methods for the purification and analysis of fOS that can be applied to the diversity of structures that are generated by the different *Campylobacter* species. Another objective was to develop a 96-well plate based assay for the analysis of PglB OTase activity which would be faster and simpler than the methods currently available.

5.2 Summary and future directions

5.2.1 Generation of fOS in *Campylobacter* species

At the beginning of the project, only semi-quantitative mass spectrometry (sqMS) was available for the quantitation of fOS (Liu *et al.,* 2006; Nothaft *et al.,* 2009). These techniques indicated that fOS is present in ten times abundance compared to the N-linked counterpart, however the exact amounts of the oligosaccharide that is present in *Campylobacter* strains was unknown (Nothaft *et al.,* 2009). In addition, a high amount of starting material was required for isolation

and purification and, analysis was time consuming (Liu *et al.,* 2006; Nothaft *et al.,* 2009). Moreover, some *Campylobacter* species, like *C. lari,* generate fOS with labile phosphate substitutions that get cleaved during harsh analytical procedures, making characterization of such fOS structures difficult (Nothaft *et al.,* 2012). The first objective of this PhD thesis was to develop fast, sensitive and efficient techniques for isolation, purification and quantitation of fOS in molar quantities. These techniques were successfully applied to analyze and quantitate a variety of different fOS structures generated by *C. jejuni*, *C. lari*, *C. fetus fetus* and *C. rectus.* The analysis showed for the first time that neutral, mono- and di- phosphorylated fOS are naturally produced in *C. lari*. In addition, mixed fOS species, such as the two fOS structures generated by *C. fetus fetus* were efficiently isolated and quantified. The fOS structures of some *Campylobacter* species, such as *C. hominis*, *C. showae*, *C. rectus*, *C. curvus* and *C. gracilis* are not completely characterized (Nothaft *et al.,* 2012). Previously, it was not possible to perform NMR due to the small quantities of the fOS obtained from the purification methods, therefore, the identity of certain fOS components is unknown and only the mass has been determined by MS (Nothaft *et al.,* 2012). However, it is possible to now extract high amounts of fOS for NMR analysis in order to determine the identities of the unknown components of *Campylobacter* fOSs. It is also possible to extend these tools to detect the presence of fOS in other bacteria possessing the Pgl pathway. The domain of archaea has not been reported to generate fOS as yet, and it may be possible to determine whether fOS are generated in archaea using the methods established in this study as well. It is also possible to investigate into the generation of fOS in OTase dependent O-linked glycosylation pathways that use LLO intermediates, such as the pathways in *Acinetobacter baumanni* and *Neisseria gonorrhoeae* (Iwashkiw *et al.,* 2013).

For future studies, it is possible that the fOS quantitation method maybe modified to study the protein N-linked counterpart by HPAEC-PAD since the glycan structure is the same. It may be possible to treat the total protein cell extract with proteinase K to digest the proteins and further purify the glycoproteins by porous graphite carbon column before HPAEC-PAD analysis to determine the exact quantities of N-linked glycoproteins in molar quantities. Analysis of exact protein content would give us exact ratios of fOS to N-linked counterpart in the cell. So far analysis of N-glycosylation resorts to Western blot analysis and studies suggest that the relative levels of N-glycosylation in *C. jejuni* seem to be similar under different extracellular conditions such as osmolarity, pH, oxygen and iron availability (Nothaft *et al.,* 2009). However, as Western blot analysis is not a quantitative technique it is possible that some important changes in glycosylation levels are not detected. Therefore, extending fOS quantitation techniques to N-glycoprotein quantitation in *Campylobacter* species would be a useful tool. In addition, *in vitro* fOS generation by PglB has also been studied by sqMS (Nothaft *et al.,* 2009). It may also be possible to extend the HPAEC-PAD method to quantitate fOS release *in vitro* by the PglB enzyme. In combination with the fluorescence energy resonance transfer (FRET) assay that was also developed in this thesis (Chapter 4), this would be a useful tool to study the dynamics of protein glycosylation and LLO hydrolysis by PglB. It may be possible to study and compare the OTase and (LLO) hydrolytic activity of PglB *in vivo* and *in vitro* and, it would be possible to determine if other factors, such as, interactions with other proteins and lipid composition of the membrane affect PglB activity *in vivo*. In addition, many PglB mutations that affect its OTase activity (Ihssen *et al.,* 2012; Lizak *et al.,* 2014; Ihssen *et al.,* 2015; Ollis *et al.,* 2015) have been reported in the recent literature and an *in vitro* fOS analysis assay can be applied to study fOS generation by these PglB mutants. Such studies may help us gain more insights into the

mechanism of LLO hydrolysis by PglB and provide ideas to generate fOS mutants in *C. jejuni*. As *pgl* mutants lack both fOS and protein glycosylation it is difficult to correlate their phenotypes to one or the other product, however fOS mutants may allow us to determine the specific role(s) of fOS in the physiology of *Campylobacter* species.

5.2.2 Understanding the regulation of PglB enzymatic activity

Another project was focused on investigating whether the N-glycan modification of PglB affects its enzymatic activity as some other proteins, such as VirB10 in *C. jejuni* and HMW1 in *H. influenza* have been reported to be influenced by their N-glycan modifications (Grass *et al.,* 2003; Larsen *et al.,* 2004). Preliminary studies by Yasmin Barre (MSc student, Szymanski lab) suggested that fOS levels are reduced by approximately 50% *in vivo* in cells expressing unglycosylated PglB as determined by sqMS. Further studies were conducted based on this observation and the HPAEC-PAD based method, developed in Chapter 2 in this thesis, was used to determine exact quantities of fOS in the *C. jejuni* strain expressing unglycosylated PglB. The amount of fOS in this strain was approximately 20% lower compared to wildtype cells and in addition, these cells had a slightly different N-glycosylation profile than wildtype cells as observed by Western blot analysis. These findings suggest that absence of N-glycosylation may affect the hydrolase (i.e. fOS generation), as well as, the OTase activity of PglB. Upon alignment of PglB sequences, we found that the N534 site is conserved in most PglB homologues in *Campylobacter* species suggesting that the N-glycosylation of PglB may have some important implications in the physiology of *Campylobacters*.

In the future, sensitive techniques should be developed to detect the glycosylation status of the PglB protein within *Campylobacter* cells. These techniques can be used to determine if the N-glycosylation status of PglB changes in *C. jejuni* under certain conditions, such as osmotic stress

and growth phase. If such conditions are identified, the fOS and N-glycosylation levels can be compared as well. In turn, it is possible to make stronger connections between the glycosylation status of PglB and changes in its enzymatic activities. In addition, it can also be determined whether glycosylation has any effect on the stability of PglB. Future studies should also focus on determining whether the expression of unglycosylated PglB has the same effect on the OTase and hydrolase activities of PglB enzymes in other *Campylobacter* species as in *C. jejuni*. It may also be worth investigating whether the expression of unglycosylated PglB affects fOS and glycoprotein profiles in other *Campylobacters*.

In addition, the *C. jejuni* strain expressing unglycosylated PglB should be further characterized. Identification of specific proteins that are glycosylated differently in the mutant strain will offer more insights into phenotypes that can be tested. Based on the proteins that are modified differently and their predicted functions, it is possible to test the phenotypes involving the pathways that the proteins are functional in.

If stronger connections are established, it would be useful to perform further studies to understand the mechanism behind the effect of the N-glycan modification on PglB OTase and LLO hydrolysis activity. It is possible that the N-glycan affects the interaction of PglB with itself or other cellular components, such as proteins, similar to what has been reported earlier (Ihssen *et al.,* 2015). Protein cross-linking studies can be performed and crosslinking patterns can be compared between the strains expressing glycosylated and unglycosylated PglB. These studies will shed more insights into our understanding of PglB activity.

5.2.3 Tools to study the enzymatic activity of PglB

A FRET assay that can be used to study the N-glycosylation activity of the N-OTases was designed and established in this thesis. The assay is efficient and quick, and was shown to work well with purified PglB enzymes. Initial difficulties with peptide degradation, Factor Xa incompatibility and peptide detection following the enzymatic reaction were resolved successfully.

In the future, this assay may be adapted to a high-throughput 96 well plate format and used to study the glycosylation efficiencies of multiple purified N-OTase enzymes. This assay may also be used to study the kinetics of multiple OTase enzymes at once by taking aliquots at different time points. In addition, wildtype or mutated enzymes may be screened for activity at once. It may be possible to further extend the assay to study both glycosylation and fOS generation by OTase enzymes resulting in a huge repertoire of information. In order to study acceptor sequon preferences, it is possible to design multiple peptides with different sequons and fluorophores that can be detected at different wavelengths. This way, the glycosylation of one acceptor sequon can be compared to the other.

Also, if a bacterial species is suspected to encode an N-linked glycosylation system with a membrane bound OTase, the peptide can be incubated with membrane preparation of the species and both the presence of the glycosylation system, as well as, the oligosaccharide can be identified using our assay. This is similar to another study that used gel electrophoresis to demonstrate N-glycosylation in *Helicobacter pullorum* (Jervis *et al.,* 2010), however our assay is quicker compared to the method used in this study since electrophoretic separation of reaction products is not required. For cytoplasmic N-glycosylation, it may be possible to extend the assay

to work with whole cell lysates of bacteria and detect glycosylation of the peptide in addition to the carbohydrate moiety.

Overall, the FRET assay has the potential to be applied to study many different aspects of OTase enzymes and N-linked protein glycosylation.

5.2.4 Concluding remarks

The study of bacterial glycobiology is currently an exciting field with the discovery of new glycosylation systems. Moreover, extensive research is being conducted to apply these systems in glycoengineering as well. Developing new tools to detect and study these systems will enhance our understanding of bacterial glycomics and allow us to explore further beneath the tip of the ice berg.

5.3 References

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APPENDIX A-1

The fucose gene locus mediates fucose dependent chemotaxis and biofilm formation in *Campylobacter jejuni*

A version of this chapter has been submitted for publication.

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The data was generated through collaborative work with Dr. Alain Stintzi at the University of Ottawa, Ontario, Canada and Dr. Arnoud Van Vliet at the Institute of Food Research, Gut Health and Food Safety Programme, Norwich, United Kingdom. I was responsible for performing biofilm assays, scanning electron microscopy, developing the chemotaxis assay and performing chemotaxis assays. I assisted Jolene Garber in the growth assays as well. I was also responsible for manuscript preparation and all co-authors provided their valuable input during the editing process.

A-1.1 Introduction

Campylobacter jejuni is the leading cause of bacterial food borne diarrhoeal disease in the Western world (Allos, 2001; Silva *et al.,* 2011) and is associated with the development of Guillian Barrè syndrome and its variants (Taboada *et al.,* 2007; Keithlin *et al.,* 2014). *C. jejuni* was previously considered to be asaccharolytic and rely on other carbon and nitrogen sources, such as amino acids and intermediates of the citric acid cycle for growth (Velayudhan and Kelly, 2002; Stahl *et al.,* 2012; Szymanski and Gaynor, 2012; Hofreuter, 2014). Recently, this dogma was broken when *C. jejuni* NCTC 11168 was reported to possess a *fuc* locus which encodes an L-fucose permease responsible for fucose transport across the inner membrane (*fucP)* along with other uncharacterized genes required for L-fucose metabolism (Muraoka and Zhang, 2011; Stahl *et al.,* 2011).

Fucose is found in human mucin (Macfarlane *et al.,* 2005), on epithelial cell surfaces (Becker and Lowe, 2003; Pickard *et al.,* 2014; Wacklin *et al.,* 2014) and in our diet (Chaturvedi *et al.,* 2001; Chow and Lee, 2008; Zivkovic and Barile, 2011). *C. jejuni* binds to fucosylated structures (Ruiz-Palacios *et al.,* 2003; Day *et al.,* 2009) and this binding is inhibited by fucose-containing compounds, such as fucosylated human milk oligosaccharides (Cervantes *et al.,* 1996; Ruiz-Palacios *et al.,* 2003; Newburg *et al.,* 2005; Weichert *et al.,* 2013). Indeed, the presence of L-fucose provides an advantage to *C. jejuni* NCTC 11168 and enhances its growth in laboratory media (Muraoka and Zhang, 2011; Stahl *et al.,* 2011) and more importantly, provides the wild-type a competitive advantage in the piglet model of human disease over a *fucP* mutant (Stahl *et al.,* 2011). In addition, the wild-type also outcompetes the *fucP* mutant at low infection doses in chickens fed with a fucose rich diet (Muraoka and Zhang, 2011). These findings suggest an

important role for fucose binding, uptake and metabolism in *Campylobacter* host colonization and pathogenesis.

Chemotaxis also plays an important role in the pathogenicity of *C. jejuni*. Mutants in the chemotaxis signal transduction pathway (*che*) are less virulent and exhibit reduced colonization in chicken and mouse colonization models (Hendrixson and DiRita, 2004; Chang and Miller, 2006), and are attenuated in the ferret disease model (Yao *et al.,* 1997). Interestingly, L-fucose is the only carbohydrate chemoattractant for *C. jejuni* (Hugdahl *et al.,* 1988) and chemotaxis mutants, such as *cheA* (Reuter and van Vliet, 2013), do not swim towards this compound.

Biofilm formation is also associated with persistence and infection in several bacteria, such as *Pseudomonas aeruginosa*, *Streptococcus pneumonia* and enteropathogenic *Escherichia coli* (Costerton *et al.,* 1999; Hall-Stoodley *et al.,* 2004; Aparna and Yadav, 2008; Hall-Stoodley and Stoodley, 2009). *C. jejuni* forms biofilms on epithelial cells (Haddock *et al.,* 2010) and on abiotic surfaces (Trachoo *et al.,* 2002; Kalmokoff *et al.,* 2006; Sanders *et al.,* 2008; Gunther and Chen, 2009; Nguyen *et al.,* 2010; Moe *et al.,* 2010; Maal-Bared *et al.,* 2012) and biofilm formation in *C. jejuni* is affected by multiple environmental factors. For example, in *C. jejuni* M129, biofilm formation is reduced in the presence of NaCl and sucrose, and this is attributed to the osmotic stress induced by these compounds (Reeser *et al.,* 2007). In addition, nutrient rich media, such as Brucella and Bolton broth, inhibit biofilm formation in this strain (Reeser *et al.,* 2007) while the presence of organic materials and bio-fouling can increase biofilm formation (Brown *et al.,* 2014). Temperature and oxygen tension also alter biofilm formation (Reeser *et al.,* 2007; Reuter *et al.,* 2010). *C. jejuni* mutants with defects in biofilm formation show reduced chicken colonization and, adhesion and invasion of epithelial cells, as well as, reduced intracellular survival (Svensson *et al.,* 2009; Theoret *et al.,* 2011; Theoret *et al.,* 2012; Rahman *et al.,* 2014).

In this study, we investigated the influence of the *fuc* locus in biofilm formation and chemotaxis by *C. jejuni* NCTC 11168. We found that biofilm formation by the wild-type is reduced in the presence of L-fucose, but remains unaltered in a *fucP* mutant. Transfer of the *fuc* locus genes (*cj0481-cj0490*) from NCTC 11168, into the *fuc* locus deficient strain 81-176, allowed the recombinant strain to actively transport L-fucose and enhance its growth in the presence of this carbohydrate. Interestingly, we found that both *C. jejuni* NCTC 11168 wild-type and the *fucP* mutant perform chemotaxis towards L-fucose and the transfer of the *fuc* locus into 81-176 also triggers a positive chemotaxis response towards this carbon source. We also discovered a correlation between the presence of the *fuc* locus and chemotaxis towards L-fucose, and identified a *fuc* gene that plays a role in both fucose metabolism and chemotaxis.

A-1.2 Materials and methods

A-1.2.1 Strains, plasmids and growth conditions

C. jejuni strains were grown in MH broth (Difco^{TM}), MEM (Gibco) or on MH agar plates at 37^{o}C under microaerobic conditions (85% N_2, 10% CO_2 and 5% O_2). *E. coli* was grown on LB medium at 37^{o}C under aerobic conditions. If required, antibiotics were added to a final concentration of 25µg/mL for kanamycin and chloramphenicol, 100µg/mL for trimethoprim and ampicillin, and 12.5µg/mL for tetracycline. If not stated otherwise, L-fucose was added to a final concentration of 25 mM. Growth analysis of strains in the presence or absence of L-fucose was performed as described in (Stahl *et al.,* 2011). Plasmids and oligonucleotides used in this study are listed in Table A-1.1.

Table A-1.1 Strains, plasmids and oligonucleotides

Strains	Description/genotype	Source
E. coli DH5α	F⁻ *endA1 hsdR17 supE44 thi-1 recA1* Δ*(argF-lacZYA)U169* (φ80d *lacZ* Δ*M15*) *gyrA96* λ⁻	Invitrogen
E. coli C600 (RK212.2)	*leu thr thi lacY supE44 tonA*; pRK212.2; Amp^R, Tet^R	(Figurski and Helinski, 1979)
C. jejuni 81-176	Clinical isolate	(Korlath *et al.,* 1985)
C. jejuni NCTC 11168	Clinical isolate used for genome sequencing	(Parkhill *et al.,* 2000)
C. jejuni RM1221	Retail chicken skin isolate	(Miller *et al.,* 2000)
C. jejuni 81116	Human isolate	(Manning *et al.,* 2001)
C. jejuni NCTC 11168 *fucP::kan*	*C. jejuni* NCTC 11168 fucose permease, *fucP* (Cj0486) mutant. The *fucP* was disrupted with a kanamycin cassette, Kan^R	(Stahl *et al.,* 2011)
C. jejuni NCTC 11168 *flaA::kan*	*C. jejuni* NCTC 11168 *flaA* (*cj1339*) mutant. The *flaA* gene was disrupted with a kanamycin cassette, Kan^R	(Jones *et al.,* 2004)
C. jejuni NCTC 11168 *cheY::cat*	*C. jejuni* NCTC 11168 chemotaxis (*cheY*) mutant. The *cheY* (*cj1118*) was disrupted with a chloramphenicol cassette, Cm^R	This study
C. jejuni 81-176 (*fuc*)	*C. jejuni* 81-176 with pCE111-28 (*fuc*), Cm^R	This study
C. jejuni NCTC 11168 *cj0481::cm*	*C. jejuni* NCTC 11168 *cj0481* mutant. Gene *cj0481* was disrupted with a chloramphenicol cassette, Cm^R	(Stahl *et al.,* 2011)
C. jejuni NCTC 11168 *cj0483::cm*	*C. jejuni* NCTC 11168 *cj0483* mutant. Gene *cj0483* was disrupted with a chloramphenicol cassette, Cm^R	(Stahl *et al.,* 2011)
C. jejuni NCTC	*C. jejuni* NCTC 11168 *cj0484* mutant. Gene *cj0484* was disrupted with a chloramphenicol cassette, Cm^R	This study

11168 *cj0484::cm*		
C. jejuni NCTC 11168 *cj0485::cm*	*C. jejuni* NCTC 11168 *cj0485* mutant. Gene *cj0485* was disrupted with a chloramphenicol cassette, Cm^R	This study
C. jejuni NCTC 11168 *cj0487::cm*	*C. jejuni* NCTC 11168 *cj0487* mutant. Gene *cj0487* was disrupted with a chloramphenicol cassette, Cm^R	(Stahl *et al.,* 2011)
C. jejuni NCTC 11168 *cj0488::cm*	*C. jejuni* NCTC 11168 *cj0488* mutant. Gene *cj0488* was disrupted with a chloramphenicol cassette, Cm^R	This study
C. jejuni NCTC 11168 *cj0490::cm*	*C. jejuni* NCTC 11168 *cj0490* mutant. Gene *cj0490* was disrupted with a chloramphenicol cassette, Cm^R	(Stahl *et al.,* 2011)
Plasmids		
pBluescriptKS+	*E. coli* cloning vector, Amp^R	Stratagene
pBluescriptKS+ (*fuc*)	pBlueskriptKS+ with genes *cj0481* to *cj0490* from *C. jejuni* NCTC 11168, Amp^R	This study
pCE111-28	*C. jejuni* expression vector; plasmid pRY111 with σ^{28} promoter of *flaA*; Cm^R	(Larsen *et al.,* 2004) and (Yao *et al.,* 1993)
pCE111-28 (*fuc*)	pCE111-28 with genes *cj0481* to *cj0490* from *C. jejuni* NCTC 11168, Cm^R	This study
Oligonucleotides		
CS618	5'TTGGCAGTTAATAAGAATAAAATACGAATTTTTACCAAGTTAACAGC3'	This study
CS619	5'TTTATCGTGCTCTTTAGGCATAGATCTTGAAAAAATTACAGG3'	This study
CS620	5'TTTAGATAAAGGAGAATAAATGGATTTAAAAATTAAAAATAAGG3'	This study
CS621	5'TTTGCGCTCTTTTCTAACCAATTGATTTATGGTAGGC3'	This study
CheY_SE	5'CGGTACCCGGGGATCCGGTGGAGTGAGCTTGC	This study

	TTCT3’	
CheY_AS	5’CGACTCTAGAGGATCCCCAAAGGCTAAGGCTGGATT3’	This study
CheY_SE_inv	5’GAACTAAAGGGCGCAACTCCACAAGTTTTAAAGGAAAAA3’	This study
CheY_AS_inv	5’GAACACCGCCGAGCACCAACTCCAAGCCATTCATT3’	This study

A-1.2.2 Identification of the *fuc* locus in *C. jejuni* and *C. coli* genome sequences

A total of 3,746 *C. jejuni* and 486 *C. coli* genome sequences were obtained from public collections such as the *Campylobacter* pubMLST website (http://pubmlst.org/campylobacter/) (Jolley and Maiden, 2010) and Genbank (http://www.ncbi.nlm.nih.gov/genome/browse/). Genomes were searched using MIST (Kruczkiewicz *et al.,* 2013) and the BLAST+ (v2.28) suite with each individual gene of the *C. jejuni* NCTC 11168 fucose locus (*cj0480c-cj0490*). Genes were considered to be present if matching ≥ 90% with the query sequence. The MLST-clonal complex designation was determined for all genomes using MIST, with the definition file provided by the *Campylobacter* pubMLST website. All genomes were also searched for the presence of the predicted proteins of the *fuc* locus using BLAST (data not shown). A phylogenetic tree of all genomes was constructed using Feature Frequency Profiling of whole genome sequences using a word length of 18 (van Vliet and Kusters, 2015), and the resulting tree was visualised using Figtree using the proportional setting for presentational purposes.

A-1.2.3 Construction of the Δ*cheY, Δcj0484, Δcj0485,* and *Δcj0488* isogenic deletion mutants

Construction of the isogenic deletion mutants was performed using the In-fusion Dry-down PCR cloning kit (Clontech). Briefly, the target gene plus flanking regions were amplified using

Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Scientific) and the corresponding primers (Invitrogen) listed in Table A-1.1. The In-fusion Dry-down cloning kit was used to directionally clone the amplified gene product into BamHI (Invitrogen) digested pUC19. Subsequently, inverse PCR was performed to amplify pUC19 plus the flanking end regions and part of the target gene. A chloramphenicol antibiotic resistance cassette was directionally cloned into the inverse PCR product, disrupting the target gene. The final construct was sequenced to confirm the absence of point mutations and then naturally transformed into *C. jejuni* NCTC 11168. Clones were selected on chloramphenicol supplemented MH agar plates and positive colonies were confirmed by PCR.

A-1.2.4 Biofilm assay

Campylobacter cells were grown in 5 ml of MH broth with required antibiotics for 18 hr at 37^{o}C under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2) with shaking. Cultures were adjusted to an OD_{600} of 0.05 and supplemented with 25 mM L-fucose or 25 mM D-galactose in MH broth and 1 mL of culture was added to borosilicate test tubes (13 x100 mm, Fisher Scientific). Test tubes containing only MH broth or MH broth supplemented with either L-fucose or D-galactose were used as negative controls. The tubes were sealed with parafilm and further incubated at 37^{o}C under microaerobic conditions for five days without shaking. Cultures were removed and the tubes were stained with 100 µl of 1% crystal violet in 95% ethanol for 20 minutes at room temperature. The crystal violet stain was rinsed off thoroughly with distilled water until the wash was clear. Biofilms were dislodged by adding 500 µl of 2% SDS in water and vortexing until a homogenous solution was formed. One hundred microlitres of the solution was transferred into a 96-well plate and the absorbance at 570 nm was measured in a plate reader. The crystal violet

absorbance of the negative control tubes was subtracted from the absorbance readings of the other samples.

A student's paired t-test analysis was performed using Excel software (Microscoft®) and a $p<0.05$ was considered statistically significant.

A-1.2.5 Scanning electron microscopy (SEM) of *C. jejuni* biofilms on glass

Cells were grown as described above (biofilm assay). One milliliter of OD_{600} = 0.05 cells were transferred into 24 well plates containing 10x20 mm glass slides (Thomas Scientific). After 5 days of incubation without shaking, supernatants containing planktonic cells were removed. Biofilms formed on the glass slide were fixed with 2 mL of fixative reagent (2.5 % glutaraldehyde, 2 % paraformaldehyde in 0.1 M phosphate buffer) at 4°C until the samples were processed. The slides were washed three times for 10 min with 0.1 M phosphate buffer (PBS, pH7.5). The biofilm samples were sequentially dehydrated for 10 min in 50% ethanol, 70% ethanol, 90% ethanol, 2 × 100% ethanol, 75:25 ethanol: hexamethyldisilazane (HMDS), 50:50 ethanol: HMDS, 25:75 ethanol: HMDS, 100% HMDS and dried overnight in the fume hood. The slides were mounted on an SEM stub for coating with gold using a Nanotech SEMPrep 2 DC sputter coater. The EOL 6301F field emission scanning electron microscope was used for the SEM with liquid nitrogen cooled lithium drifted silicon energy dispersive x-ray (EDX) detector with a Norvar window manufactured by PGT.

A-1.2.6 Transfer of the *fuc* locus from *C. jejuni* NCTC 11168 into 81-176 and analysis of [^{3}H]-L-fucose uptake

The *fuc* locus genes *cj0481* to *cj0490* (but lacking, *cj0480/fucR*) was amplified from *C. jejuni* 11168 chromosomal DNA as follows: 3883 bp and 4964 bp fragments, both including a native *EcoR*I restriction site, were amplified from chromosomal DNA with primers CS618-CS619 and

CS620-CS621 and Pfx polymerase (Invitrogen). Both fragments were digested with *Eco*RI and inserted into a three arm ligation reaction into plasmid pBluescriptKS+ linearized with *Eco*RV. Positive clones with insertion of the *fuc* operon in the orientation of the *lacZ* gene were confirmed by restriction analyses and named pBluescriptKS+ (*fuc*). Plasmid pBluescriptKS+ (*fuc*) was subsequently digested with *Eco*RV and *Xho*I and the 8633 bp DNA fragment (containing *cj0481* to *cj0490*) was purified and inserted into the *E. coli* to *Campylobacter* shuttle vector pCE111-28 (Larsen *et al.,* 2004) treated with the same enzymes. Formation of the correct ligation product was screened and confirmed by restriction analyses. Plasmid DNA from one positive clone was named pCE111-28 (*fuc*) and used to transform *E. coli* C600 (RK212.2). *E. coli* C600 (RK212.2) (*fuc*) cells were used to conjugate the pCE111-28 (*fuc*) plasmid into *C. jejuni* 81-176 wild-type cells as described (Yao *et al.,* 1997). Chloramphenicol resistant colonies were selected on MH plates supplemented with chloramphenicol and the presence of the plasmid was confirmed after plasmid-DNA isolation from 81-176 (*fuc*) and restriction analyses. [^{3}H]-L-fucose uptake was performed as described previously (Stahl *et al.,* 2011) with strains *C. jejuni* NCTC 11168, *C. jejuni* NCTC 11168 *fucP*, *C. jejuni* 81-176 wt and *C. jejuni* 81-176 (*fuc*).

A-1.2.7 Reverse transcriptase PCR (RT-PCR)

Analysis of *fucP* mRNA transcripts was performed as described earlier (Stahl *et al.,* 2011).

A-1.2.8 Chemotaxis assays

Chemotaxis assays were performed as follows: 500 µL of cells (2.8 mL per gram of cell pellet) in 0.4% PBS-agar were transferred to the bottom of a 2 mL Eppendorf tube and allowed to solidify for 30 min at room temperature. Samples were overlaid first with 100 µL of PBS agar that was allowed to set for 30 min, followed by 900 µL of 0.4% PBS agar and allowed to solidify for an additional 30 min at room temperature. A sterile piece of Whatman paper, soaked with 50

µL of a 1 M solution of L- fucose, L-serine, or 1xPBS was placed on top and samples were incubated under microaerobic conditions for 72 hrs at 37°C. Active bacterial cells that migrated through the upper layer of PBS-agar towards the compound added to the Whatman paper were visualised by adding 500 µL of 0.01% 2,3,5 triphenyltetrazolium chloride (TTC) in PBS. The respiratory dye TTC detects redox activity from active bacterial cells and results in formation of red rings of bacterial cells that are visible after 3-4 hr incubation under microaerobic conditions (Brown *et al.,* 2013; Reuter and van Vliet, 2013). After an incubation of 72 hrs, we added 0.01% TTC which gives an insoluble red precipitate upon reduction by metabolically active *C. jejuni* and this compound has been used previously for the detection of actively respiring cells (Brown *et al.,* 2013; Reuter and van Vliet, 2013). In addition, plating of the accumulated bacteria from the top layer of the agar confirmed the presence/absence of viable cells that migrated from the bottom of the tube towards the substrate on the top.

A-1.3 Results

A-1.3.1 Distribution of the *fuc* locus in *C. jejuni* and *C. coli* genomes

In our previous study, we reported that the *fuc* locus is present in *C. jejuni* strains NCTC 11168, RM1221, CF93-6, 84–25, *C. jejuni* subsp. *doylei* 269.97, and *C. coli* RM2228, but absent in *C. jejuni* strains 81–176, CG8486, HB93-13, 260.94, and 81116 (Stahl *et al.,* 2011). We have determined the prevalence of the *fuc* locus (*cj0480c-cj0490*) in 4,232 *C. jejuni* and *C. coli* genome sequences, which were phylogenetically clustered using feature frequency profiling (van Vliet and Kusters, 2015). The *fuc* locus was present in 2,431 out of 3,746 *C. jejuni* genomes (64.9%) and 354 out of 486 *C. coli* genomes (72.8%) (Fig. A-1.1). The distribution of the *fuc* locus was associated with specific MLST-clonal complexes, such as ST-21, ST-48, ST-206, ST-354 and ST-257 in *C. jejuni*¸ while MLST-clonal complexes such as ST-45, ST-283, ST-42, ST-

353 and ST-464 are mostly lacking the *fuc* locus (Fig. A-1.1). In *C. coli*, the *fuc* locus was primarily found in the MLST-clonal complex ST-828, but not in the riparian isolates (Sheppard *et al.,* 2013). Livestock associated lineages are shown in red whereas, water and wildlife associated lineages are shown in blue in Fig. A-1.1 (Stabler *et al.,* 2013). Interestingly, there appears to be a trend toward agriculture isolates possessing the pathway and environmental isolates lacking this pathway (Fig. A-1.1), although the data set is currently skewed toward agriculture isolates linked with human infections.

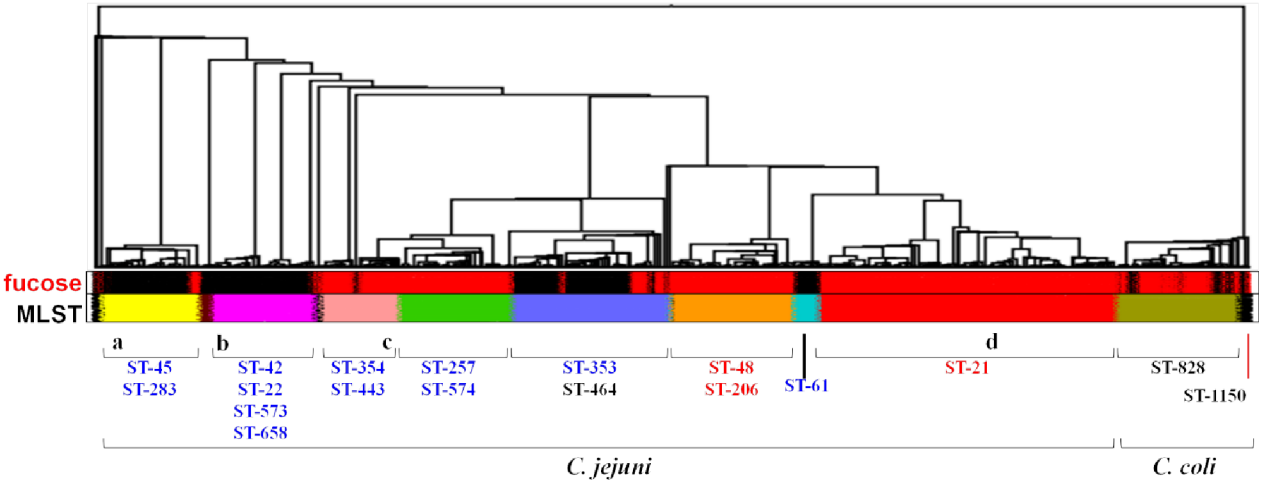


Fig. A-1.1 Prevalence of the *fuc* locus among 3,746 *C. jejuni* and 486 *C. coli* genomes. Genomes were phylogenetically clustered using FFPry feature frequency profiling with L=18 (van Vliet & Kusters, 2015), and the resulting phylogenetic tree has been transformed using the "proportional" setting of Figtree for presentational purposes. The first row labeled 'fucose' indicated genomes possessing the fucose pathway, which are shown in red while those lacking the pathway are shown in black. The second bar shows the primary combinations of MLST clonal complexes for *C. jejuni* and *C. coli*, with red-labeled clonal complexes representing livestock-associated lineages, blue-labeled clonal complexes representing water/wildlife-associated lineages (Stabler *et al.*, 2013). The association of some clonal complexes such as ST-

464 was not reported previously, and these are in black font. The lowercase letters indicate the approximate position of strains 81116 (a), 81-176 (b), RM1221 (c) and NCTC 11168 (d).

A-1.3.2 L-fucose modulates biofilm formation in *C. jejuni* NCTC 11168

We investigated biofilm formation in the presence of 25 mM L-fucose in static glass tube cultures. We found that addition of L-fucose to the MH culture medium caused an approximately 2.7 fold reduction in the amount of biofilm formed by wild-type *C. jejuni* NCTC 11168 as determined by the absorbance at 570 nm via the crystal violet staining method (Fig. A-1.2). The reduction was significantly different in a paired student's t-test (p-value of 0.00056) (Fig. A-1.2). Previously, a mutation in *fucP* in this strain has been shown to inactivate L-fucose uptake from the extracellular environment (Muraoka and Zhang, 2011; Stahl *et al.,* 2011). To confirm the roles of L-fucose in biofilm formation, we examined the ability of the *fucP* mutant to form biofilms in unsupplemented media and in the presence of L-fucose. We found that biofilm formation by the *fucP* mutant was similar regardless of the presence of L-fucose in the media.

The specific effect of L-fucose was shown by the addition of 25 mM D-galactose to the media. Biofilm formation by the wild-type and *fucP* mutant in media supplemented with D-galactose was not significantly different to the amount of biofilm formed by the strain in unsupplemented media (Fig. A-1.2). Our results also demonstrate that the amount of biofilm formation in unsupplemented media by wild-type *C. jejuni* and the *fucP* mutant are similar (Fig. A-1.2).

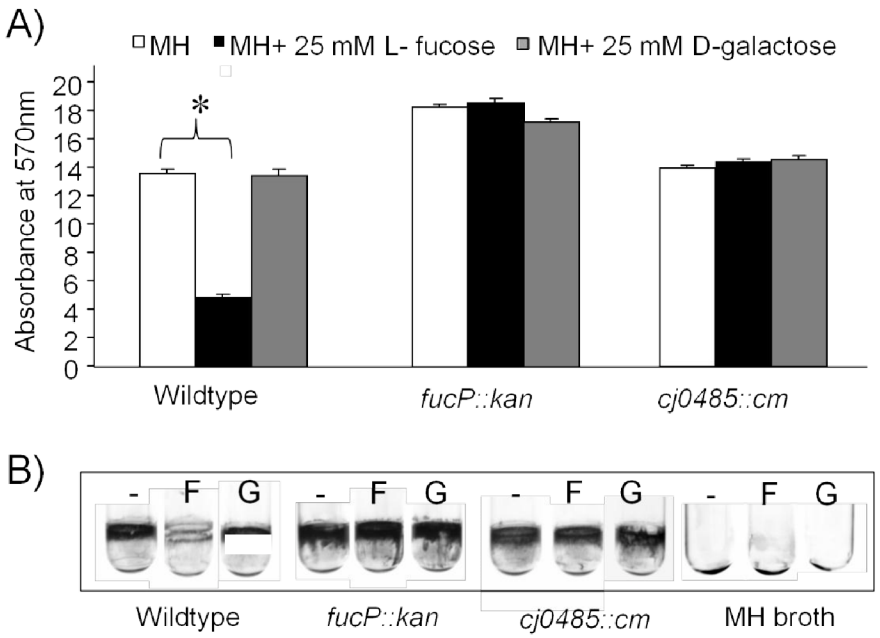


Fig. A-1.2 Effect of L-fucose on biofilm formation by *C. jejuni* NCTC 11168. A) Absorbencies of solubilized biofilm obtained from the crystal violet assay measured at 570 nm. P- value of <0.001 obtained in a paired student's t-test is indicated by an asterisk (*). The bars represent an average of technical triplicates and the data is representative of at least 6 biological replicates. Error bars indicate standard error. B) Test tubes with crystal violet stained biofilms formed under the indicated conditions, (-) indicates growth in MH broth alone, (F) indicates growth in MH broth in the presence of 25 mM L-fucose, (G) indicates growth in MH broth in the presence of 25 mM D-galactose.

We further investigated the effects of L-fucose on appearance and architecture of biofilms by scanning electron microscopy (SEM). We analysed both *C. jejuni* NCTC 11168 wild-type and *fucP* mutant biofilms that had formed on glass slides in MH or MH with 25 mM L-fucose. Wild-type *C. jejuni* formed well established biofilms in the absence of 25 mM L-fucose (Fig. A-1.3). These biofilms had a very dense mass of cells comparable to previous observations of *C. jejuni* biofilms (Joshua *et al.,* 2006; Brown *et al.,* 2014) (Fig. A-1.3). Interestingly, biofilm formation

was severely reduced when cells were grown in the presence of 25 mM L-fucose. The glass slides only had a few *C. jejuni* detectable on the surface (Fig. A-1.3). In contrast, biofilm formation by the *fucP* mutant appeared similar in density and architecture in the presence or absence of fucose. Biofilms formed by the *fucP* mutant were also comparable to wild-type biofilms grown in the absence of L-fucose (Fig. A-1.3). In addition, no biofilms were detected in negative control samples that contained MH broth only and no bacterial cells. Our SEM analysis supported the results obtained from the crystal violet staining assay.

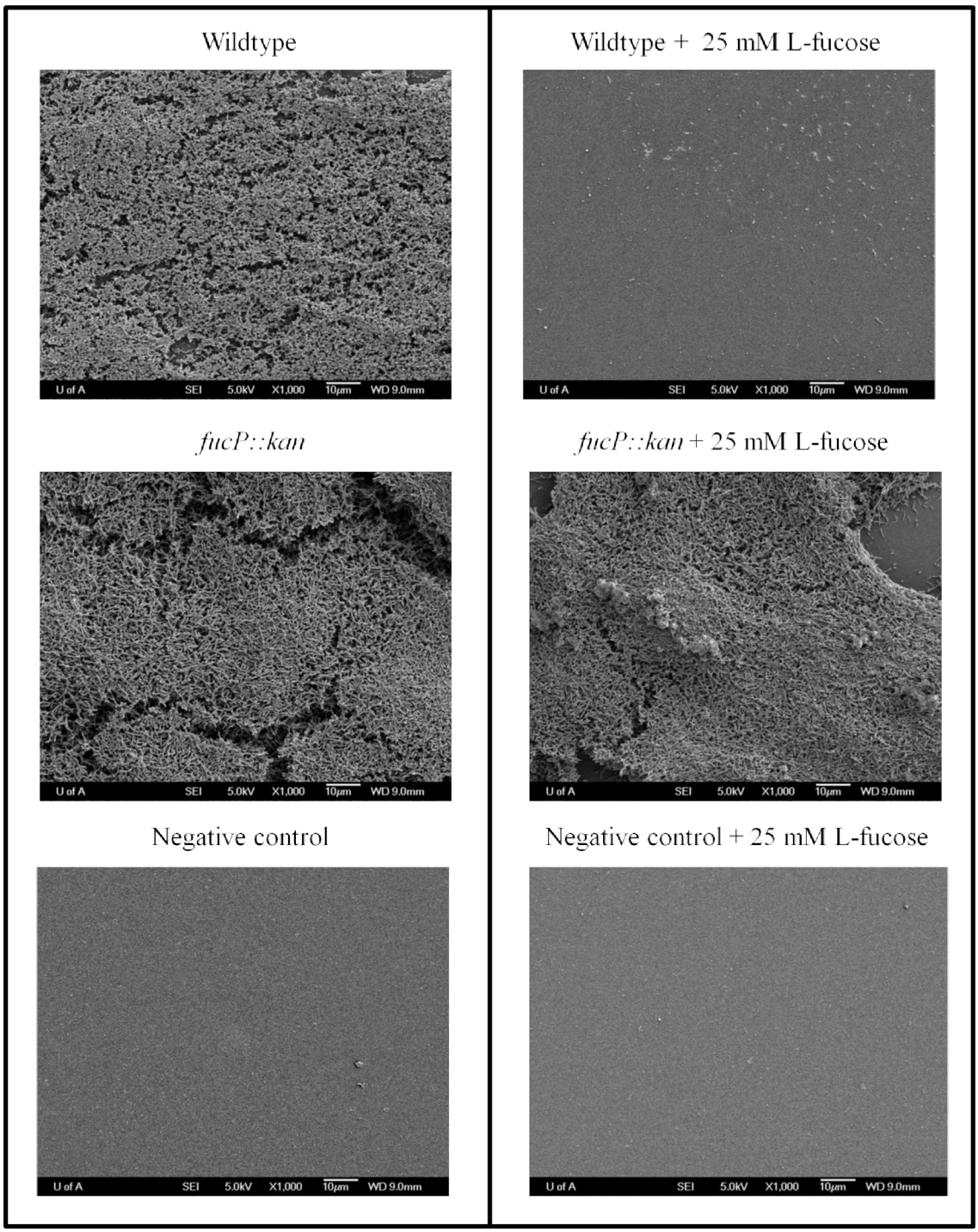


Fig. A-1.3. Scanning electron microscopy of *C. jejuni* NCTC 11168 biofilms on glass slides formed under the indicated conditions in MH broth at 1000x magnification. The images are representative of three independent experiments. Negative control contained no bacterial cells.

A-1.3.3 Transfer of the *fuc* locus from *C. jejuni* NCTC 11168 into *C. jejuni* 81-176

C. jejuni 81-176 naturally lacks the *fuc* locus (Stahl *et al.,* 2011). A plasmid encoding genes *cj0481* to *cj0490* was constructed and transferred into 81-176 resulting in 81-176 (*fuc*). To test the functionality of the *fuc* locus on the introduced plasmid, the uptake of ^{3}H-L-fucose into 81-176 (*fuc*) was measured and compared to 81-176 and NCTC 11168.

Wild-type NCTC 11168 showed basal ^{3}H-L-fucose uptake rates (3.3 pmol ^{3}H-L-fucose/min/10*9 cfu) when grown in MH alone (Fig. A-1.4A). [^{3}H]-L-fucose uptake rates significantly increased (around 4-fold to 12.0 pmol ^{3}H-L-fucose/min/10*9 cfu) when wild-type cells were grown in the presence of fucose demonstrating the induction of the system in the presence of its substrate (Fig. A-1.4A).

In contrast, no ^{3}H-L-fucose transport was observed in 81-176 in the presence of fucose in the growth medium consistent with the absence of the *fuc* locus in this strain (Fig. A-1.4A). Interestingly, 81-176 (*fuc*) exhibited significantly higher uptake rates of ^{3}H-L-fucose independent of the presence or absence of fucose in the growth medium (34.8 pmol 3H-L-fucose/min 10*9 cfu and 36.5 pmol 3H-L-fucose/min/10*9 cfu, respectively), indicating that the pathway is constitutively expressed in this strain (Fig. A-1.4A). Analysis of growth rates further demonstrated that *C. jejuni* 81-176 is not able to utilize L-fucose for enhanced growth. Growth curves and final OD_{600} were similar in the absence or presence of this carbon source (Fig. A-1.4B). In contrast, the *C. jejuni* 81-176 (*fuc*) strain grown in the presence of L-fucose showed significant enhanced growth when compared to growth in MEM alone (Fig.A-1.4B) (p-value <0.05). Similarly, wild-type *C. jejuni* NCTC 11168 showed significant enhanced growth in MEM+L-fucose compared to MEM alone (Fig. A-1.4B). These results were similar to what has been reported previously (Muraoka and Zhang, 2011; Stahl *et al.,* 2011).

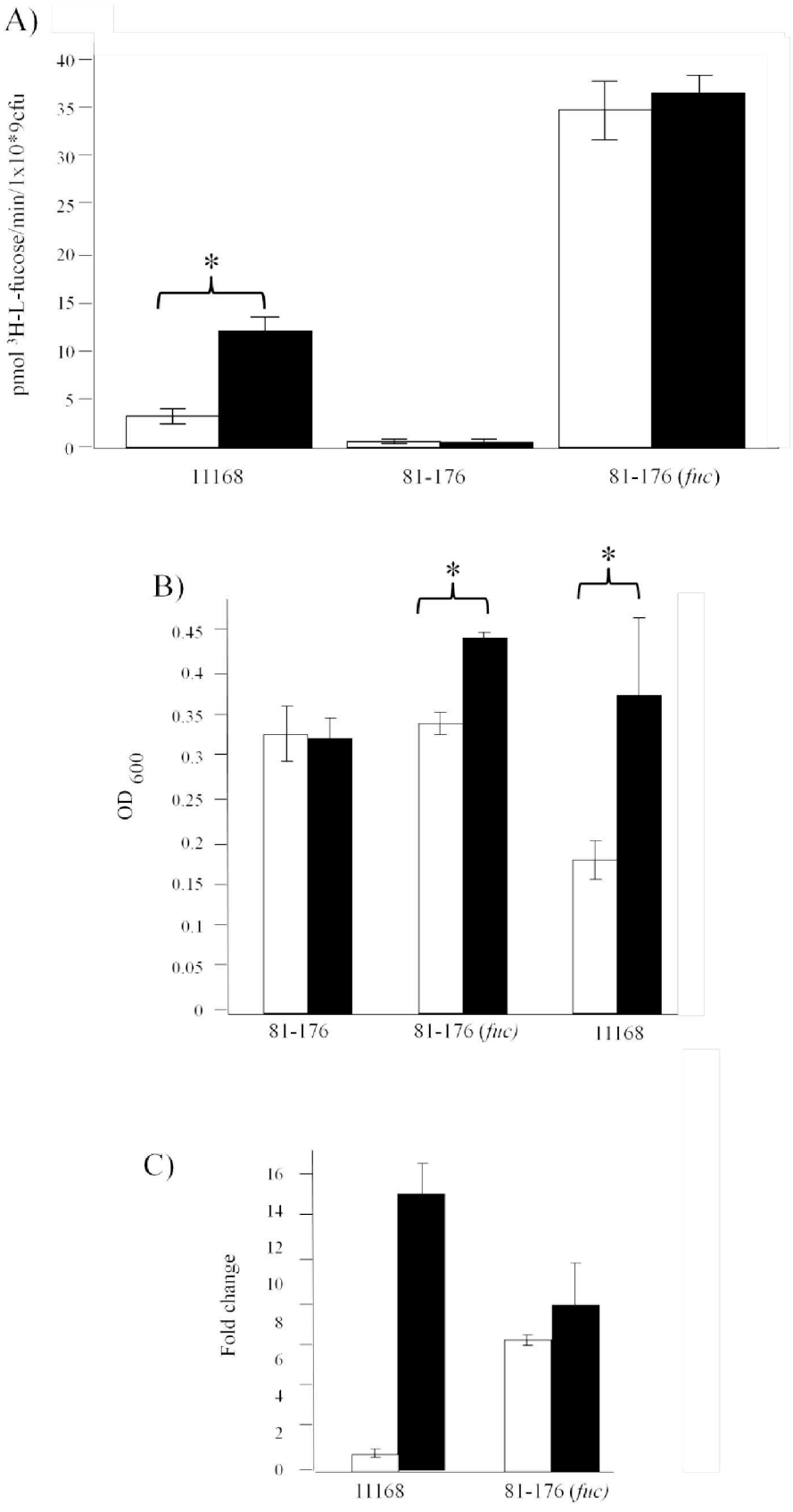

A)
pmol ^{3}H-L-fucose/min/1x10*9cfu
40
35
30
25
20
15
10
5
0
*
11168
81-176
81-176 (*fuc*)
B)
OD $_{600}$
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
*
81-176
81-176 (*fuc*)
11168
C)
Fold change
16
14
12
10
8
6
4
2
0
11168
81-176 (*fuc*)

Fig. A-1.4 Transfer of the *fuc* locus (*cj0481- cj0490*) from *C. jejuni* NCTC 11168 into *C. jejuni* 81-176 and functional analysis. A) Uptake of [^{3}H]- L-fucose in the indicated strains including *C. jejuni* 81-176 and *C. jejun*i 81-176 *(fuc).* B) Growth of indicated strains in minimal essential medium supplemented with (black bars) or without L-fucose (white bars). C) Fold change in the expression of quantitative reverse transcriptase PCR analysis of *fucP* in the indicated strains grown in minimal essential medium supplemented with (black bars) or without L-fucose (white bars). P-value of <0.05 in a student's paired t-test is indicated by an asterisk. All experiments were repeated atleast three times. Error bars indicate standard error.

Quantitative reverse transcriptase PCR (RT-PCR) was used to compare transcript levels of *fucP* (Fig. A-1.4C). The expression levels were significantly higher when NCTC 11168 cells were grown in the presence of fucose compared to cells grown in unsupplemented medium (Fig. A-1.4C). This confirmed previous observations demonstrating induction of the *fuc* pathway in the presence of its substrate (Muraoka and Zhang, 2011; Stahl *et al.,* 2011). Elevated *fucP* transcripts were detected at comparable levels when the *C. jejuni* 81-176 (*fuc*) strain was cultured in MEM or MEM+ 25 mM L-fucose (Fig. A-1.4C). This indicates that the pathway is constitutively expressed in *C. jejuni* 81-176 (*fuc*), thus supporting the observed increased and constitutive ^{3}H-L-fucose uptake rates in this strain are due to the absence of *fucR* (*cj0480*). We conclude that *fuc* locus is fully functional in 81-176 and results in increased uptake of L-fucose and enhanced growth.

We also tested whether mutations in *cj0484*, *cj0485* and *cj0488* in *C. jejuni* NCTC 11168 affected the growth of the strain in MEM supplemented with or without 25 mM L-fucose (Fig. A-1.5). In agreement with previous observations, the wild-type strain showed approximately 2-fold enhanced growth in the presence of L-fucose (p-value <0.05) whereas the *fucP* mutant

showed similar growth in MEM medium regardless of fucose addition (Fig. A-1.5) (Muraoka and Zhang, 2011; Stahl *et al.,* 2011). We found that a mutation in *cj0485* abolished enhanced growth in the presence of L-fucose and the OD_{600} was similar to unsupplemented medium whereas *cj0488* mutant behaved similarly to wildtype (p-value <0.05) (Fig. A-1.5). The *cj0484* mutant also showed enhanced growth in the presence of fucose, however this increase was not significantly different compared to growth in unsupplemented media (Fig. A-1.5).

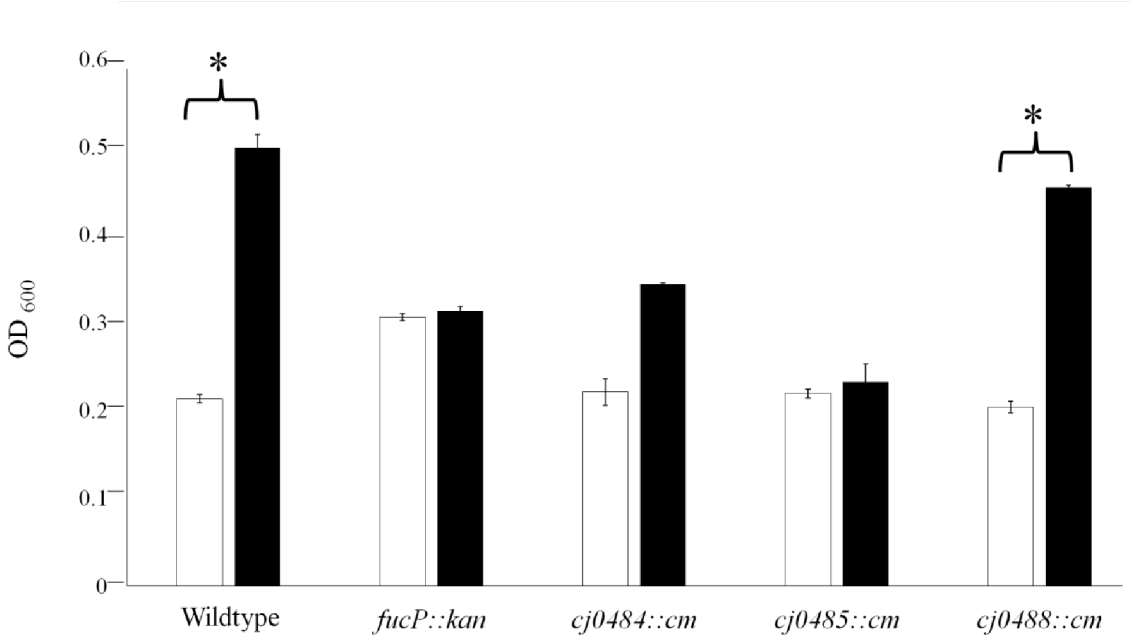
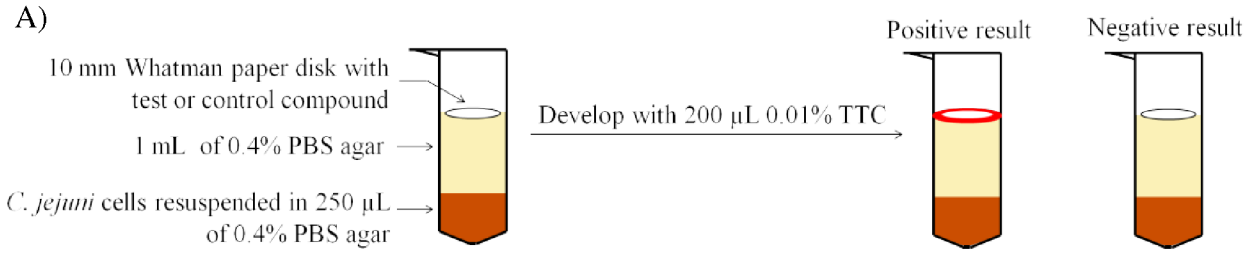


Fig. A-1.5 Growth analysis of indicated *C. jejuni* NCTC 11168 wild-type and specific *fuc* mutant strains in minimal essential medium supplemented with (black bars) or without L-fucose (white bars). The bars represent two biological duplicate experiments. A student's paired t-test was performed and an asterisk represents a p-value <0.05. Error bars indicate standard error.

A-1.3.4 The roles of the *fuc* locus in chemotaxis towards L-fucose

To examine *C. jejuni* chemotaxis towards L-fucose, we attempted to use the PBS agar plate assay that is typically used for the analysis of chemotaxis in this species (Hugdahl *et al.,* 1988; Khanna *et al.,* 2006; Vegge *et al.,* 2009; Baserisalehi and Bahador, 2011), however, this assay resulted in false positives in our hands when we used chemotaxis and flagellar mutants as negative controls (data not shown), similar to another study (Kanungpean *et al.,* 2011).

To circumvent these problems, we established and confirmed the reproducibility of a novel assay with chemotaxis and flagellar mutants (*cheY* and *flaA*). We also established and validated our assay using L-serine and L-fucose as positive and previously described chemoattractants for *C. jejuni* NCTC 11168 (Hugdahl *et al.,* 1988; Vegge *et al.,* 2009; Baserisalehi and Bahador, 2011), as well as, PBS as a negative control. The assays were set-up as described in materials and methods and in Fig. A-1.6. We only found culturable *C. jejuni* in the top layer of the chemotaxis tubes showing positive results indicating that no passive diffusion of cells occurred and that the presence of cells is due to active migration through the agar as a chemotactic response towards the added substrate (data not shown). Most importantly, no rings were observed with PBS, L-fucose or L-serine in tubes containing the *flaA* and *cheY* mutants (Fig. A-1.6). This indicated that our assay does not give false positives and had been established successfully.



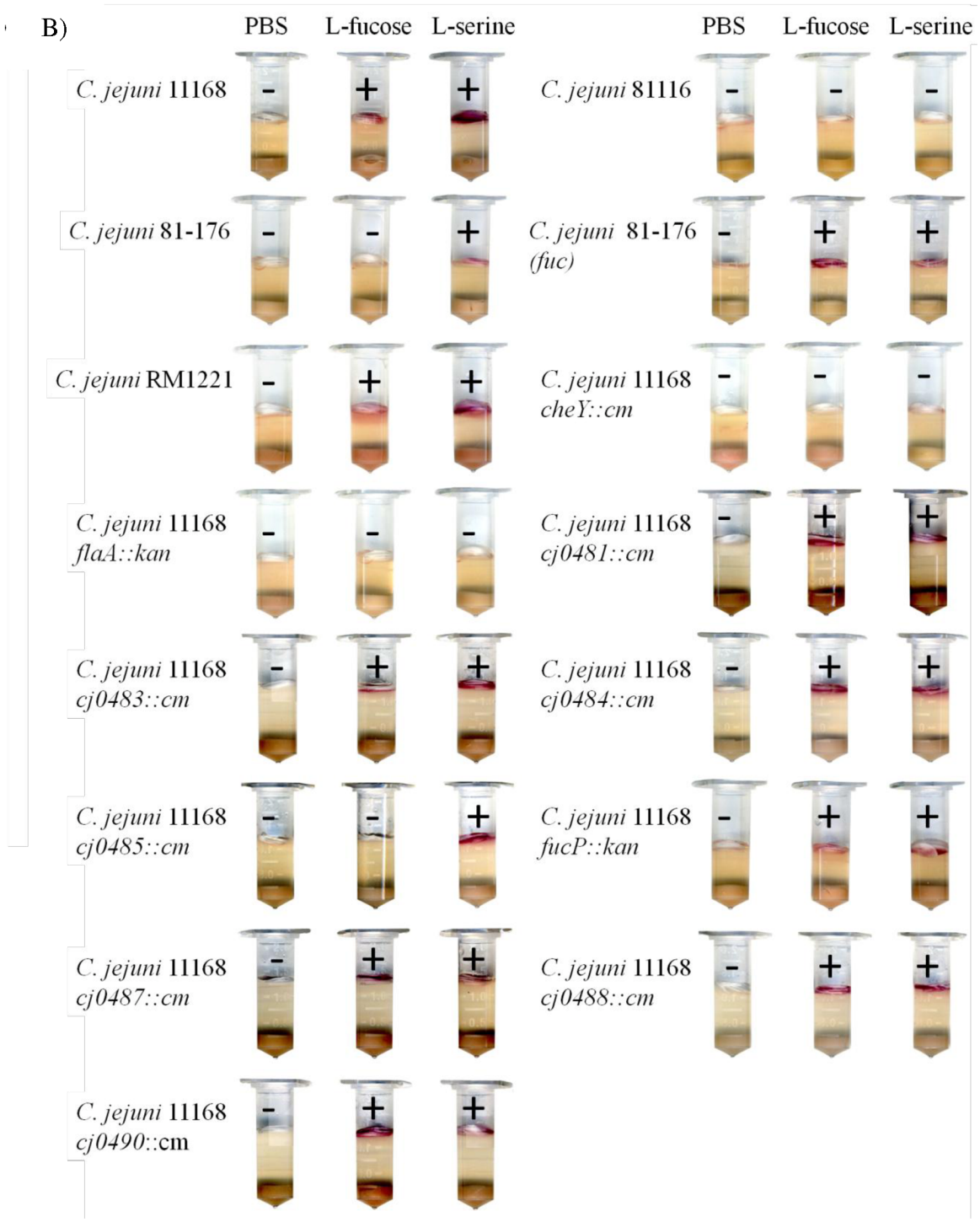


Fig. A-1.6 Analysis of the chemotaxis response of the indicated *C. jejuni* strains. A) A schematic of the chemotaxis assay. B) Images of the chemotaxis assay tubes of the indicated strains after development with 0.01% TTC (2,3,5 triphenyltetrazolium chloride). The + and –

signs indicate the presence or absence of chemotaxis towards the compounds. The images are representative of atleast two independent experiments.

We applied the assay to examine the chemotactic responses of the *C. jejuni fucP* mutant. Red rings were observed around the positive control compound L-serine. Red rings were also observed in the test tube containing L-fucose indicating that this strain is still chemotactic towards this compound (Fig. A-1.6). Next we analysed the chemotactic responses of *C. jejuni* 81-176 and *C. jejuni* 81-176 (*fuc*). Interestingly, 81-176 (*fuc*) was strongly chemotactic towards L-fucose as indicated by dark red rings around L-fucose in the tube whereas no red rings near L-fucose were observed for the parent 81-176 (Fig. A-1.6). Consistent with published reports 81-176 was chemotactic towards L-serine (Hugdahl *et al.,* 1988; Baserisalehi and Bahador, 2011; Reuter and van Vliet, 2013) and the recombinant strain, 81-176 (*fuc*), showed a similar chemotactic behaviour towards this amino acid. To further investigate the correlation between the presence of the *fuc* locus and fucose chemotaxis, we analysed the chemotaxis responses of the *fuc* locus deficient strain *C. jejuni* 81116 and the *fuc* locus positive strain *C. jejuni* RM1221. We showed that strain RM1221 was chemotactic towards L-fucose while strain 81116 was not (Fig. A-1.6), indeed suggesting a correlation between the presence of the *fuc* locus and the ability to swim towards L-fucose. In addition red rings were observed around L-serine with RM1221 cells but not with 81116 cells (Fig. A-1.6) indicating that 81116 is naturally not chemotactic towards L-serine. No red rings were observed around PBS for all the tested strains.

Next, we investigated if the loss of specific *fuc* genes has an impact on the chemotaxis response towards L-fucose. Mutation in either *cj0481, cj0483, cj0484, cj0487, cj0488* and *cj0490* did not affect the ability of *C. jejuni* to swim towards L-fucose or L-serine (Fig. A-1.6). Interestingly, the *cj0485* mutant completely lost the ability to swim towards L-fucose (Fig. A-1.6) but was still

capable of swimming toward the L-serine positive control. This indicates that the *cj0485* gene product is crucial for chemotaxis specifically towards L-fucose in *C. jejuni* NCTC 11168.

We further examined whether a mutation in *cj0485* affected biofilm formation in response to L-fucose and observed that a *cj0485* mutant behaved similar to the *fucP* mutant in the test tube assay. We observed that this mutant formed biofilms regardless of the presence of L-fucose (Fig. A-1.2). As expected biofilm formation by the *cj0485* mutant was also unaffected in the presence of D-galactose (Fig. A-1.2).

A-1.4 Discussion

The L-fucose uptake and utilization locus (*fuc*, *cj0480- cj0490*) in *C. jejuni* NCTC 11168 provides the strain with a competitive advantage in avian and animal colonization models (Stahl *et al.,* 2011; Muraoka and Zhang, 2011).

From recent studies, it is becoming apparent that *C. jejuni* and *C. coli* have lineage-specific patterns of distribution of metabolic markers, such as the vitamin B5 biosynthesis cluster (Sheppard *et al.,* 2013) and the *fuc* locus investigated here. The distribution of the *fuc* locus was previously suggested to be restricted to specific multilocus sequence types of *C. jejuni* and *C. coli* (de Haan *et al.,* 2012), and we have conformed and extended these observations here using a large collection of *C. jejuni* and *C. coli* genome sequences. The *fuc* locus is virtually universally present in the clonal complexes ST-21, ST-48, ST-206, ST-257 and ST-354, which includes the reference isolates NCTC 11168 and RM1221 used in this study, whereas the *fuc* locus is absent in other major lineages such as ST-42, ST-45 and ST-283, which includes other reference isolates such as 81116 and 81-176 used here (Fig. A-1.1). In *C. coli*, the majority of ST-828 isolates is positive for the *fuc* locus, while the riparian *C. coli* isolates lack the locus. It is not completely clear what causes the distribution pattern of the *fuc* locus, as many of the positive and

negative isolates share the agricultural space and hence should have the opportunity for acquisition by horizontal gene transfer or natural transformation. Hence there may be multiple, potentially subtle effects which govern the acquisition, functionality and maintenance of the *fuc* locus, rather than direct exchange by natural transformation. It is also possible that the *fuc* negative strains are naturally weak in DNA uptake and recombination, however this needs to be tested. The successful expression and functionality of the *fuc* locus in strain 81-176 from a plasmid suggests that there are no direct negative effects of expression of the *fuc* locus, and hence other, yet unknown factors may play a role in this phenomenon.

We found that the addition of L-fucose caused at least a 3-fold reduction in wild-type biofilm formation in the standard glass test tube assay, whereas inactivation of *fucP* abolished this phenotype. This indicates that active uptake of L-fucose is necessary to sense this carbon source. Examination of wild-type NCTC 11168 and *fucP* biofilms by SEM analysis showed that the biofilm architecture was similar to previously published reports (Joshua *et al.,* 2006; Kalmokoff *et al.,* 2006; Brown *et al.,* 2014), and was consistent with the crystal violet assay results. In many organisms, such as *Escherichia coli* and *Pseudomonas aeruginosa,* biofilm formation is tied to stress responses that can be induced by DNA damage, the presence of antibiotics at sub-inhibitory or high concentrations and by extracellular metal ions (Landini, 2009). In *C. jejuni*, biofilm formation is also related to extracellular stresses, such as oxidative and osmotic stress (Fields and Thompson, 2008; Svensson *et al.,* 2009). These studies indicate that there exists an intracellular regulatory network between stress responses and biofilm formation in *C. jejuni*. It is likely that the inability to uptake/metabolize L-fucose by the *fucP* mutant and starvation in the absence of L-fucose in the case of the wild-type may trigger a stress response and lead to higher amounts of biofilm formation. Starvation and biofilm formation are linked in *C. jejuni* and

nutrient rich medium, such as Brucella and Bolton broth, inhibit biofilm formation (Reeser *et al.,* 2007). The observed phenomenon may allow for planktonic growth of *C. jejuni* in the stressful and highly competitive environment of the intestinal tract, which may consequently cause enhanced infections and efficient spread during diarrhoeal disease.

To further investigate the importance of the *fuc* pathway in *C. jejuni*, we transferred the *fuc* locus (*cj0481-cj0490*) from NCTC 11168 into 81-176, a strain that is naturally *fuc* deficient. We found that the *fuc* pathway is functional in the recombinant strain and results in active uptake of L-fucose. Since the *fuc* locus was expressed on a plasmid, the copy number of the *fuc* genes in 81-176 is much higher than in NCTC 11168. In addition, the repressor *fucR* (*cj0480*) (Stahl *et al.,* 2011) was not included on the plasmid resulting in constitutive expression in 81-176. This caused overall higher expression levels of *fuc* genes in 81-176 (*fuc*) and consequently ~3.5 fold higher uptake of L-fucose in the recombinant strain compared to NCTC 11168. However, similar to NCTC 11168, L-fucose also enhanced the growth of the 81-176 (*fuc*) strain indicating that the *fuc* locus encodes all the proteins that are required to uptake and metabolize L-fucose.

Chemotaxis plays important roles in the pathogenicity of *C. jejuni*. Chemotaxis mutants are defective in chicken colonization and attenuated in the ferret diarrhoeal disease model (Yao *et al.,* 1997; Hendrixson and DiRita, 2004). Interestingly, *C. jejuni* exhibits chemotaxis towards many amino acids, salts of organic acids and purified mucin (Hugdahl *et al.,* 1988; Vegge *et al.,* 2009). Surprisingly, L-fucose is the only carbohydrate that serves as a chemoattractant for this pathogen (Hugdahl *et al.,* 1988) and *C. jejuni* to bind to structures terminally decorated with L-fucose (Day *et al.,* 2009) which is inhibited by the addition of exogenous terminally fucosylated compounds, such as fucosylated human milk oligosaccharides (Cervantes *et al.,* 1996; Ruiz-Palacios *et al.,* 2003; Newburg *et al.,* 2005; Weichert *et al.,* 2013). This suggests a potential link

between sensing and chemo-attraction towards L-fucose in *C. jejuni*. We investigated the roles of the *fuc* pathway in the chemotaxis response of *C. jejuni* NCTC 11168. We established a new assay that eliminates false positive observations as reported for previous published procedures (Hugdahl *et al.,* 1988; Khanna *et al.,* 2006; Vegge *et al.,* 2009; Baserisalehi and Bahador, 2011). We found that in addition to wild-type NCTC 11168 that has been reported to perform chemotaxis towards L-fucose previously (Hugdahl *et al.,* 1988; Reuter and van Vliet, 2013), the *fucP* mutant is still able to perform chemotaxis towards L-fucose as well. This indicates that intracellular L-fucose is not required for this chemotactic response and extracellular presence of L-fucose is sufficient to trigger chemotaxis. *C. jejuni* 81-176 encodes a functional chemotaxis pathway (Yao *et al.,* 1997), however, it does not swim towards L-fucose due to absence of the *fuc* locus in its genome. We found that the recombinant strain 81-176 (*fuc*) displayed a strong chemotaxis response towards L-fucose. We also discovered that the *fuc* locus positive strain RM1221 was motile towards L-fucose, while the *fuc* locus deficient strain 81116 did not swim towards L-fucose. Our results also indicate that 81-176 is missing the intracellular regulatory network to alter biofilm formation in response to L-fucose since this compound had no influence on biofilm formation in wild-type 81-176 or the 81-176 (*fuc*). We found that *cj0485* that encodes for a protein homologous to short chain dehydrogenases/reductases, had completely lost its ability to swim towards L-fucose. This mutant had also lost the ability to reduce biofilm formation in the presence of L-fucose, similar to a *fucP* mutant that is unable to uptake and metabolize the substrate NCTC 11168 (Stahl *et al.,* 2011). Short chain dehydrogenase/reductase enzymes are generally involved in metabolism of compounds such as carbohydrates, amino acids and lipids (Kavanagh *et al.,* 2008; Bijtenhoorn *et al.,* 2011) but are also implicated in quorum sensing pathways in bacteria such as *E. coli* and *P. aeruginosa* (Bijtenhoorn *et al.,* 2011; Lord *et*

al., 2014). It seems that *cj0485* encodes a protein that is involved in both fucose metabolism and sensing of L-fucose similar to quorum sensing homologues in other bacteria.

Fucose is highly abundant in the intestine and plays an important role in the virulence of intestinal pathogens such as Enterohaemorrhagic *Escherichia coli* (EHEC) and *Salmonella* Typhimurium (Robbe *et al.,* 2004; Pacheco *et al.,* 2012; Weichert *et al.,* 2013; Wang *et al.,* 2015). However, due to lack of secreted or surface exposed fucosidases, EHEC, *S.* Typhimurium, as well as *C. jejuni,* are most likely unable to release fucose from commonly found oligosaccharides (Pacheco *et al.,* 2012). However, other members of the intestinal microbiota, such as *Bacteroides thetaiotaomicron*, possess multiple glycoside hydrolases and have been shown to provide free carbohydrates for EHEC, *S.* Typhimurium and *Clostridium difficile* resulting in increased pathogenicity (Pacheco *et al.,* 2012; Ng *et al.,* 2013; Tailford *et al.,* 2015).

In this study, we report that L-fucose reduces biofilm formation in *C. jejuni* and allows the bacterium to maintain a planktonic state which may be more suitable for maintenance of infectiousness virulence and/or spread during diarrhoeal disease. We also show that the *fuc* locus encodes all proteins required for fucose uptake and metabolism, and also encodes a protein(s) that is involved in chemotaxis towards L-fucose. Chemotaxis may enhance the attachment of *C. jejuni* to fucosylated structures in the intestine, facilitating the persistence of *C. jejuni* during infection. This study contributes to the recent advances in our understanding of the roles of L-fucose in *Campylobacter* pathogenesis and may highlight potential targets for the treatment of *Campylobacter* infections, particularly if this pathway is associated with strains causing human infections.

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