Doctoral Thesis

Bacterial N-glycosilation: a model to study lipid-linked oligosaccharide translocation across the membrane

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Bacterial N-glycosylation:
a model to study lipid-linked oligosaccharide
translocation across the membrane

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Summary

The work presented in this thesis concerns the N-linked glycosylation pathway of the bacterium *Campylobacter jejuni* and the process of lipid-linked oligosaccharide (LLO) transfer across the membrane, common to every exopolysaccharide biosynthesis and N-glycosylation process. Lipid-transfer across biological membranes is an essential process for the physiology of bacterial and eukaryotic cells. A special class of proteins, known as translocases or flippases, plays the role of transferring lipids and LLO across membranes, but their features and mechanism are still unknown.

In the first part of this thesis, the membrane protein PglK (formerly WlaB), encoded by the second gene of the recently identified *C. jejuni* pgl-operon, has been shown to be the lipid-linked-oligosaccharide (LLO) translocase of the bacterial N-glycosylation pathway. The requirement of PglK in the N-glycosylation process was investigated first in a *C. jejuni* pglK-knockout and then in *Escherichia coli* by reconstituting a pglK-deficient N-glycosylation pathway.

The similarity between the bacterial N-glycosylation pathway and the lipopolysaccharide biosynthetic pathway was exploited to further characterize the flippase-mediated LLO-translocation. Similarly to the membrane protein Wzx, which has been proposed to mediate the translocation across the plasma membrane of lipopolysaccharide O antigen subunits, PglK has been proposed to mediate the translocation of the undecaprenylpyrophosphate-linked heptasaccharide involved in the *Campylobacter* N-linked protein glycosylation pathway. We provided evidence of the flippase-activity of PglK, showing that it can functionally substitute Wzx in the biosynthesis of lipopolysaccharide. In addition, it has been shown that a Nucleotide Binding Domain (NBD), conserved among ABC-transporters, was required for PglK-activity. Its modification by introduction of specific point mutations was associated with loss of the PglK-dependent glycosylation phenotype. On the basis of these data, we proposed that PglK is an ABC-transporter.
We provided evidence for the existence of two distinct mechanisms for LLO translocation across membranes, catalyzed by distinct proteins, an ABC type and a not-ABC type protein, having a relaxed specificity toward the composition of their substrate and being exchangeable despite the absence of any sequence similarity.

In the second part of this work, the N-glycosylation pathway from Campylobacter was combined with the newly discovered and still not investigated N-glycosylation system of Wolinella succinogenes. We could show that one of the N-glycosylated proteins of Campylobacter can be also glycosylated by the Wolinella pgl operon, suggesting common requirements of the glycosylation substrate among the two systems. Furthermore, the finding that the LLO-flippase of the Wolinella N-glycosylation pathway can functionally substitute the Campylobacter protein provided further evidence for the relaxed substrate-specificity of this class of transporters.

In the final part of this thesis, a method for detection and quantification of glycoproteins in biological samples is presented. The assay is ELISA-based and it was conceived in order to facilitate any kind of studies regarding glycoprotein production or inhibition, with the possibility to be applicable to screening procedures. In particular, such a method may be a convenient tool for the glycoengineering facility offered by the bacterial system.
Oggetto della presente ricerca è il processo di N-glicosilazione del batterio *Campylobacter jejuni* e il trasferimento attraverso la membrana del complesso lipide-oligosaccaride, processo condiviso da biosintesi di esopolisaccaridi e N-glicosilazione.

Il trasferimento di lipidi attraverso le membrane è un processo essenziale per la fisiologia di batteri e di eucarioti. Una specifica classe di proteine, denominate traslocasi o flippasi, ha il ruolo di trasferire lipidi e complessi lipide-oligosaccaridi attraverso le membrane, ma le caratteristiche e il funzionamento di tali proteine sono tuttora oggetto di studio.

Nella prima parte di questa tesi viene dimostrato che la proteina di membrana PglK (originariamente WlaB), codificata dal secondo gene dell’operone pgl, di recente identificato in *C. jejuni*, è la traslocase del complesso lipide-oligosaccaride (LLO) del pathway di N-glicosilazione batterico.

Il coinvolgimento di PglK nel processo di N-glicosilazione è stato inizialmente investigato in un ceppo di *C. jejuni* dove pglK era stato mutato, e successivamente in *E. coli*, dopo avere ricostituito in tale batterio il processo di N-glicosilazione mutante in PglK.

La somiglianza tra i processi batterici di N-glicosilazione e di biosintesi dell’lipopolisaccaride (LPS) è stata utilizzata per caratterizzare ulteriormente il processo di traslocazione del LLO mediato dalla flippase. Infatti così come le proteine di membrana Wzx sono candidate al trasferimento attraverso la membrana delle subunità costituenti l’O-antigen, a PglK viene attribuito il ruolo di traslocare il complesso eptasaccaride-undecapreno-pirofosfato coinvolto nel processo di N-glicosilazione di *Campylobacter*. In questo lavoro vengono fornite prove indirette sull’attività di flippase di PglK attraverso esperimenti di complementazione di Wzx nel processo di sintesi dell’LPS. Inoltre viene dimostrato come il dominio di legame dell’ATP (NBD), conservato tra gli ABC-transporters, sia necessario per l’attività di PglK. Infatti, la sua inattivazione, per introduzione di specifiche mutazioni puntiformi, è stata associata alla perdita di

In conclusione, viene dimostrata l’esistenza di due diversi meccanismi di trasporto dell’LLO attraverso la membrana, svolti da due diverse classi di proteine, ABC-transporter e non, e la loro relativa aspecificità nei confronti del substrato.


Nell’ultima parte di questa tesi, viene presentato un metodo per identificare e quantificare le glicoproteine nel campione biologico. Il metodo esposto si basa sul sistema ELISA ed è stato concepito per essere applicato a studi di screening sulla produzione o l’inibizione di glicoproteine. In particolare, tale sistema risulta un utile strumento nel campo della glico-ingegneria batterica.
CHAPTER 1

Introduction
1 Protein glycosylation

1.1 N-linked protein glycosylation in yeast

N-glycosylation is the most common protein-modification in eukaryotes, and until a few years ago, it was thought to be restricted to this class of organisms. This modification is required for many different functions, including protein stability and solubility, protein turnover, enzymatic activity, antigenicity, intracellular transport and targeting (Helenius, 2004).

Many loci associated with the N-glycosylation process have been identified in yeast. Furthermore, homologues of these genes have been found in other eukaryotes, suggesting that the pathway involved in this protein modification is highly conserved (Burda, 1999).

The lipid-linked-oligosaccharide (LLO) biosynthesis starts on the cytosolic side of the endoplasmic reticulum (ER), where an oligosaccharide consisting of two N-acetylglucosamine and five mannose residues is assembled on a dolichyl pyrophosphate carrier (Fig. 1). In the ER lumen, the oligosaccharide is further modified by the addition of four mannose and three glucose residues. The complete oligosaccharide is then transferred to target proteins (Hirschberg, 1987; Schenk, 2001).

The glycan-moieties are linked to the protein via the amine nitrogen of asparagine residues within the specific consensus sequence, Asn-X-Ser/Thr, where X can be any amino acid except proline (Gavel, 1990). In eukaryotes not every consensus sequence found in a protein is glycosylated, and it was proposed that only those polypeptides able to assume the “asparagine turn” (Impriali, 1995) are glycosylated. This protein conformation is recognized by the key enzyme of the eukaryotic N-glycosylation pathway, the oligosaccharyltransferase (OTase), which transfers the glycan from the lipid anchor to the protein.

The N-glycoproteins produced in the ER then move to the Golgi for further modifications. The nature of these modifications differs among species and may be required for the activity and final targeting of the glycoprotein.
N-linked glycosylation is proposed to proceed through the sequential addition of nucleotide-activated sugars onto a lipid carrier, resulting in the formation of a lipid-linked-oligosaccharide (LLO). This LLO is then ‘flipped’ across the inner membrane into the lumen of the ER. Glycans are further processed to the final polysaccharide that is then transferred to the growing polypeptide by a complex of proteins collectively known as the oligosaccharyltransferase (OTase).

Glc, glucose; GlcNAc, N-acetylglucosamine; Man, mannose

RFT1 has been proposed to be, in the yeast *Saccharomyces cerevisiae*, the “flippase” that transfers the dolichyl-PP-linked Man$_3$GlcNAc$_2$ intermediate across the ER membrane (Helenius, 2002). The importance of RFT1 in the N-glycosylation pathway has been strongly supported by the presence of RFT1-homologues in all eukaryotic genomes sequenced to date, with the notable exception of *Plasmodium falciparum*, a eukaryotic parasite that lacks N-glycoproteins (Davidson, 2001). RFT1-proteins have multiple predicted transmembrane regions and their amino acid sequences do not contain any ATP binding domains. The RFT1-associated flipping-mechanism of the LLO and its energetic requirement are still unknown.
1.2 N-linked protein glycosylation in bacteria

Initially, the N-glycosylation operon of *Campylobacter jejuni*, a human pathogen causing gastroenteritis, was erroneously associated with LPS biosynthesis (Fry, 1998) and accordingly called wla-operon (standard bacterial polysaccharide gene nomenclature). Subsequently, the operon was shown to be responsible for protein glycosylation (Szymanski, 1999) and therefore renamed the pgI-operon. Only recently, the operon was demonstrated to encode for proteins involved in N-glycosylation (Wacker, 2002; Young, 2002). This was the first reported case of bacterial N-glycosylation, breaking the dogma that N-glycosylation was restricted to eukaryotes and archaebacteria.

Bacterial N-glycosylation is remarkably similar to the eukaryotic N-glycosylation pathway. The main obvious difference is the compartmentalization, as bacteria do not have an ER. In bacteria, the biosynthesis of the LLO (GalNAc-α1,4-GalNAc-α1,4-[Glc-β1,3]-GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-Bac) takes place on the cytoplasmic face of the inner membrane and the transfer of the glycan to target proteins occurs in the periplasmic space.

The role of the individual glycosyltransferases in the *Campylobacter* N-glycosylation pathway has been determined by *in vitro* and *in vivo* studies (Glover, 2005; Linton, 2005). As the enzymes of this entire pathway can be functionally expressed in *E. coli* (Feldman, 2005; Wacker, 2002), it is possible to study the function of individual components of the glycosylation machinery in this heterologous system. In the work of Linton and co-workers (Linton, 2005), *E. coli* strain BL21 harbouring a functional pgI locus was used for the structural characterization of the N-glycans synthesized. The glycan structure transferred on the protein was analyzed by mass-spectrometry using knockout mutants of putative pgI-encoded glycosyltransferases to determine the effect of such mutants on the glycan structure and in turn elucidate the function of the deleted glycosyltransferase genes (Fig. 2). A more precise role for the individual pgI-encoded glycosyltransferases was recently determined by *in vitro* studies, using chemically synthesized Und-PP-bacillosamine and different combinations of the glycosyltransferases PglA, PglJ, PglH and PglI (Glover, 2005).
Fig. 2: Structure of the N-linked glycans synthesized in pgl mutant strains (Linton, 2005). Glycosylated peptides produced in E. coli strain BL21 carrying mutant versions of the pgl locus were analysed by MS-MS. Indicated is the N-glycan structure corresponding to each pgl mutant.

From these studies, a model for the biosynthetic pathway of the LLO was proposed (Fig. 3). The first step involves the synthesis of UDP-bacillosamine, from UDP-GlcNAc or UDP-GalNAc, through the action of the dehydratase, PglF, the aminotransferase, PglE and the acetyltransferase, PglD. Subsequently, PglC transfers the UDP-bacillosamine to undecaprenyl-phosphate, on the cytoplasmic side of the plasma membrane, yielding an undecaprenyl-pyrophosphate-linked-bacillosamine. Then, several glycosyltransferases act consecutively on this moiety, transferring hexoses from activated nucleotide precursors, in order to complete the heptasaccharide assembly. Briefly, a GalNAc residue is α1-3 linked to the undecaprenyl-P-P-bacillosamine by PglA, then, a second GalNAc is α1-4 linked to the first one by PglI, and finally, three terminal GalNAc residues are transferred by PglH (α1-4 linkage). Glucose is added by PglI to the third GalNAc to the reducing end.

These data, produced in E. coli, are in agreement with the analysis of pgl knockout mutants of C. jejuni (C. Szymanski, personal communication). An interesting point to note is that when the pgl operon was expressed in the E. coli BL21 strain,
a hybrid glycan with GlcNAc replacing bacillosamine was also observed to be produced. This is thought to result from the activity of the endogenous E. coli glycosyltransferase WecA that competes with PglC and transfers a GlcNAc-phosphate or a GalNAc-phosphate on the undecaprenyl-phosphate (Alexander, 1994; Rick, 1994). Since PglA might be able to transfer a GalNAc residue to either HexNAc or bacillosamine, the final N-glycan differs only at the first sugar (Linton, 2005)(Fig. 2).

Fig. 3: Model for the C. jejuni N-glycosylation pathway. The heptasaccharide is synthesized in the cytoplasm on an undecaprenyl-PP carrier by the action of several pgl-encoded glycosyltransferases acting sequentially. The LLO is then transferred across the plasma membrane by an ABC transporter-like protein, encoded by the pglK (formerly wlaB) gene. In the final step, the heptasaccharide is transferred to Asn residues of target proteins by the oligosaccharyltransferase PglB.
In contrast to the LLO biosynthesis, which is thought to occur in the cytoplasm, several sets of data suggested that the final transfer of the glycan to the acceptor protein, mediated by the oligosaccharyltransferase PglB, occurs in the periplasm. Firstly, only periplasmic, secreted or membrane proteins were found to be N-glycosylated in Campylobacter (Table I). Secondly, in E. coli, the catalytic activity of PglB is localized to the periplasm (Wacker, 2002). Finally, it was recently shown by Feldman and co-workers that PglB can transfer heterologous LLO-substrates known to be periplasmically located to target-proteins (Feldman, 2005).
The recognition sequence of the PglB oligosaccharyltransferase on target proteins contains the eukaryotic consensus Asn-X-Scr/Thr. Additionally, as recently shown by Kowarik and co-workers (Kowarik, 2006), a negatively charged aminoacid is required two positions N-terminal to the glycosylation site. Accordingly, the bacterial N-glycosylation consensus sequence is annotated as Asp/Glu-X-Asn-X-Ser/Thr (Table I).

The second gene in the pgl operon, wlaB, encodes for an ABC transporter-like protein proposed to invert the topology of the LLO, translocating the sugar from the cytoplasmic face to the periplasmic side of the inner membrane (Wacker, 2002). As the function remained hypothetical, the original wla-denomination was retained.

The protein glycosylation system is thought to play a role in Campylobacter virulence, as has been suggested by the observation that genomic mutations in either pglB or pglE resulted in a significant reduction in adherence to and invasion of a human intestinal epithelial cell line, and a reduction in the ability to colonize the intestinal tract of mice (Szymanski, 2002). In addition, assembly of the bacterial type IV secretion system was effected by mutating the N-glycosylation site of one of its component, the VirB10 protein (Larscn, 2004). However, adherence and invasion of C. jejuni are multifactorial, they require motility (Yao, 1994) and various components including the capsular polysaccharide (Bacon, 2001), the outer membrane lipooligosaccharide (Guerry, 2002), a type IV secretion system (Bacon, 2000) and adhesins (Pei, 1998). It remains to be determined, if specific glycoproteins or the surface displayed N-glycans are responsible for C. jejuni virulence.
<table>
<thead>
<tr>
<th>Cj gene</th>
<th>Annotation</th>
</tr>
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<tbody>
<tr>
<td>Cj0081</td>
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</tr>
<tr>
<td>Cj0114</td>
<td>ppp&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<tr>
<td>Cj0289c</td>
<td>Peb3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
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<td>pmp</td>
</tr>
<tr>
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<td>AcrA&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>ppp</td>
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<tr>
<td>Cj1670c</td>
<td>CgpA&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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-16-
Table I: Alignment of glycosylated sequons found in *C. jejuni* (Kowarik, 2006).


Examination of genome sequence data from distant *Campylobacter* species demonstrated that genes from the *pgl* locus are highly conserved, co-linear and clustered (Szymanski, 2005)(Fig. 4). The notable exceptions are *pglG* which is missing in *C. coli* and *C. lari*, and *pglI* which is also missing in *C. lari*. Also interesting is the fact that the *pglEFG* homologues in *C. upsaliensis* are separated from the rest of the cluster by an insertion of 14 genes.

Fig. 4: Gene schematic comparing bacterial N-linked protein glycosylation loci (Szymanski, 2005). Schematic representation of the conserved N-linked protein glycosylation gene clusters in representative δ- and ε-proteobacteria. The gene encoding the essential oligosaccharyltransferase PglB is shown in bold. Slashed lines indicate homologues that are found elsewhere on the chromosome.
Recently, an N-glycosylation pathway, very similar to the *Campylobacter* one, was reported to be present in *Wolinella succinogenes* (Baar, 2003). Furthermore, genes homologous to the *Campylobacter* *pgl* genes can be found in the genome of different bacteria, like *Helicobacter pylori*, where however no PglB homologue is present, and *Desulfovibrio desulfuricans*. But so far no evidences of N-glycosylated proteins have been reported from other bacteria except *Campylobacter*.

1.3 N-linked protein glycosylation in archaea

Prior to the discovery of an N-glycosylation pathway in *C. jejuni*, archaea were the only documented cases of non-eukaryotic N-glycosylation. Unlike most bacteria, archaea have a surface protein layer instead of a rigid sacculus surrounding their plasma membrane. This layer consists of cell surface glycoproteins (CSG), hexagonally arranged into a two dimensional crystal. Interestingly, the extensively studied CSG of *Halobacterium halobium* contains two different types of N-linked glycans (Lechner, 1989). The first type is a glycosaminoglycan chain, made of a repeat of a sulfated pentasaccharide block, whose reducing end GalNAc is N-linked to an Asn residue of the protein. The other N-linked glycan, which has been shown to be also N-linked to halobacterial flagellins (Wieland, 1985), involves sulfated oligosaccharides bound to proteins via an unusual Asn-Glc linkage. In both cases, the glycosylation takes place on the Asn within the consensus sequence Asn-X-Ser/Thr, a recognition sequence for both eukaryotes and cubacteria. However, in archaea, the sequence requirement is not as strict as in eukaryotes or bacteria, as mutation of the serine in one glycosylation site of *H. halobium* CSG did not prevent N-glycosylation of the S-layer (Zeitler, 1998).

Another difference between the glycosaminoglycan and the sulfated oligosaccharides is that they use a different lipid-phosphate anchor during their biosynthesis. The glycosaminoglycan uses a dolichyl-pyrophosphate as is the case for the eukaryotic N-linked glycosylation, whereas the sulfated oligosaccharide uses dolichyl-phosphate. Models propose that both oligosaccharides are synthesized and sulfated while still attached to the lipid on the cytosolic side of the
plasma membrane. They are then translocated to the cell surface and transferred to the proteins. Indeed, the transfer of glycosaminoglycan from the lipid to the protein is inhibited by bacitracin, a compound which does not penetrate the plasma membrane (Mescher, 1978). The localization of the transfer of sulfated oligosaccharides was determined using ionically charged acceptor-peptides, which can not penetrate the plasma membrane. As would be expected if transfer occurred on the cell surface, when added to the medium of a suspension of halobacteria, these peptides were glycosylated with sulfated oligosaccharides (Lechner, 1985). However, it still remains to be determined how the lipid-linked-oligosaccharides are translocated to the cell surface and whether the glycan is completed inside the cell or polymerized after being transported block by block.

Homologues of the bacterial oligosaccharyltransferase PglB and the essential eukaryotic oligosaccharyltransferase subunit STT3, have been found in all of the archaeal genomes sequenced to date. In fact, in the case of Archaeoglobus fulgidus, an operon encoding a putative OTase and several other activities required for the biosynthesis of the lipid-linked-oligosaccharide has been found (Burda, 1999). These results suggest that N-glycosylation is indeed prevalent in archaea.
1.4 Bacterial O-glycosylation

Although the characterized cases of N-glycosylation are fairly limited in archaea and cubacteria, the other class of protein glycosylation, the O-glycosylation, is much more widespread and is found from archaea to eubacteria, including both Gram positive and Gram negative bacteria.

Recent studies suggested that the presence of the glycan on the protein can play a role in adhesion (Benz, 2001; Lindenthal, 1999; Marceau, 1999), protection against degradation (Herrmann, 1996), solubility (Marceau, 1999), antigenic variation (Doig, 1996) and protective immunity (Guerry, 1996; Romain, 1999).

Similar to the eukaryotes, in this modification, the glycan is attached to the peptide backbone via the hydroxyl group of a serine or threonine. In C. jejuni there is an O-linked glycosylation locus which is responsible for the glycosylation of flagellins with pseudaminic acid residues (Doig, 1996; Parkhill et al., 2000; Thibault, 2001). The potential variability of glycoforms encoded by the flagellar glycosylation locus, suggests that this locus provides a mechanism for antigenic diversity and immune evasion for the surface exposed flagellin. Furthermore, it was shown, that O-linked flagella glycosylation is required for the formation of the filament in Campylobacter and in turn, for the motility and infectivity of the organism (Goon, 2003; Logan, 2002). Glycosylation is also required for flagella assembly, and therefore motility, in Helicobacter pylori (Josenhans, 2002). Furthermore, non-motile H. pylori mutants defective in the glycosylation genes were unable to colonize a mouse model (Schirm, 2003). In Neisseria meningitidis and N. gonorrhoe pilin, O-glycosylation was reported (Parge, 1995; Stimson et al., 1995; Virji, 1993), and it was shown that the binding of IgA antibodies to the glycosylated pilin blocks the complement-mediated killing of N. meningitidis strains, suggesting a role of the carbohydrate modification in promoting the disease (Hamadeh, 1995). In Pseudomonas aeruginosa, pili required for adherence and invasiveness were shown to be O-glycosylated with a trisaccharide that is also a component of the bacterial O-antigen (Castric, 2001). A “flagellin glycosylation island” has also been found in P. aeruginosa a-type strains (Arora, 2001; Brimer, 1998), but its function is still not understood, as the assembly and motility of flagella in Pseudomonas is independent of glycosylation (Schirm, 2004).
general, bacterial O-glycosylation has been proposed to occur in the cytoplasm. The notable exception is the pilin glycosylation of *Pseudomonas*. This pathway is particularly interesting as, together with the recently described protein O-mannosylation of *Mycobacterium tuberculosis* (VanderVen, 2005), it is one of the few O-glycosylation processes known to involve a lipid-linked intermediate and taking place in the periplasm (M. Feldman, in preparation).

In contrast to N-glycosylation, the structure of the O-linked glycan is not conserved inside every domain of life.
2 Exopolysaccharides biosynthesis in bacteria

The first glycosylation processes discovered in bacteria were the LPS and capsule biosynthesis pathways. In both cases, but through different paths, glycans are first assembled on a lipid carrier, from nucleotide-sugar donors, on the cytoplasmic side of the plasma membrane, and then transferred across the inner membrane into the periplasm to be further modified and transferred to final acceptor molecules. The role of these glycoconjugates in bacterial virulence has been well documented, and their function in maintaining structural integrity and correct protein localization, as well as inducing host responses, has been elucidated (Beutler and Rietschel, 2003; Hooper and Gordon, 2001). N-glycoproteins, lipooligosaccharide (LOS) and capsule are the main glycoconjugates present on the cell surface of C. jejuni.

2.1 Lipopolysaccharide

LPS, a major building block of the outer membrane in Gram-negative bacteria, consists of an O-specific polysaccharide or O-antigen linked to the lipid A-core oligosaccharide. The pathways for the biosynthesis of the O-antigen and lipid A-core oligosaccharide are independent of each other and converge after both components are translocated to the periplasm (Raetz, 2002). O-antigens can be homo or heteropolymeric oligosaccharides. They are highly variable within a given species, and in the case of E. coli, about 170 different O-serotypes have been identified (Bronner, 1994; Orskov, 1977). Homopolymeric O-antigens are usually synthesized via an “ABC-transporter-dependent” pathway. In this pathway, the polymerization of the glycans onto the undecaprenyl-PP occurs in the cytoplasm. This polysaccharide is then translocated through an ABC transporter to the periplasm, where it is subsequently transferred to the lipid-A core by the ligase WaaL (Bronner, 1994) (Fig. 5B). The ABC transporter involved in this pathway has two subunits (ABC-type 2), a transmembrane domain (TMD) subunit, called Wzm and a nucleotide binding domain (NBD) subunit, called Wzt (Cuthbertson, 2005; Kido, 1995). While the Wzm subunit is functionally interchangeable between the different homopolymeric O-antigen biosynthetic pathways, the Wzt components can not be
exchanged, strongly suggesting that Wzt plays a role in determining substrate specificity (Cuthbertson, 2005).

Only few cases are known where heteropolymeric O-antigens are synthesized via this ABC-transporter pathway. They include the *Klebsiella pneumoniae* O12 antigen (Izquierdo, 2003), the *E. coli* O52 antigen (Feng, 2004) and the *Serratia marcescens* O4 antigen (Saigi, 1999).

The most heteropolymeric O-antigens are synthesized via a “wzy-dependent” biosynthetic pathway. In contrast to the homopolymeric O-antigen, single O-antigen subunits are synthesized in the cytoplasm on an undecaprenyl-PP carrier and then translocated across the plasma membrane by transmembrane proteins called Wzx (Feldman et al., 1999; Liu, 1996; Marolda, 2004; Paulsen, 1997). Once in the periplasm, the O-antigen subunits are polymerized by the O-antigen polymerase, Wzy, and the length of the polymer is regulated by the activity of the chain length regulator, Wzz. Subsequently, the heteropolymeric O-antigen is then transferred to the lipid A core oligosaccharide by the ligase, WaaL (Valvano, 2003) (Fig. 5A).

Interestingly, Wzx proteins from different Wzy-dependent pathways can complement each other in the translocation of different O-antigen sugar precursors (Feldman et al., 1999).

It has recently been suggested that these proteins recognize the oligosaccharide of the reducing end, and the phosphate attached to the undecaprenyl-P, which in many cases is N-acetylglicosamine (GlcNAc) (Marolda, 2004). However, despite these similarities in function and a similar hydropathy profiles (MacPherson, 1995), there is no homology or conserved residues between the different Wzx proteins. Furthermore, their primary sequences do not reveal any ATP binding domains, suggesting a different mechanism from the ABC transporters (Marolda, 1999; Wang, 1998). It is remarkable that there is such diversity in sequence of Wzx proteins considering their proposed similar function.
Fig. 5: Mechanisms of biogenesis of O-specific LPS (Valvano, 2003). The initiation reaction shows conserved features in all mechanisms and involves the formation of an Und-PP-linked sugar. This reaction takes place on the cytosolic face of the plasma membrane, where the nucleotide sugar precursors are available for the initiating enzyme. The three different intermediate steps in the export of O-antigen arc depicted. A, denotes the steps involved in the Wzy-dependent pathway, where the O-repeating subunit is made on the cytosolic side of the plasma membrane and then translocated across the membrane and polymerized at the periplasmic side of the membrane. B, denotes the ABC transporter-dependent pathway. In this case the formation and extension of the O-specific polysaccharide occurs in the cytosol. The polymer is translocated in an ATP-dependent manner by the Wzm/Wzt ABC-2 transporter. The ligation step is common to the two elongation-translocation-polymerization pathways and involves the formation of a glycosidic bond with the terminal residue of the outer core oligosaccharides. This step is mediated by WaaL and results in the release of undecaprenyl-P. The lipid A-core oligosaccharide acceptor molecule is synthesized by an independent
pathway and translocated to the periplasmic side of the plasma membrane by the ABC-transporter MsbA.

In contrast to most of the Gram negative bacteria, Campylobacter species do not have LPS. Instead, they have a lipoooligosaccharide (LOS), made of lipid A-core oligosaccharide (Logan, 1984; St Michael, 2002), missing the long O-antigen repeats typical of the LPS. This LOS has been shown to be involved in Campylobacter adhesion to epithelial cells (McSweegan, 1986). Remarkably, a molecular mimicry mechanism between the bacterial LOS and the GM1 and GM2 carbohydrate epitopes of human gangliosides (Fig. 6) is supposed to be the braking factor for the peripheral nervous system disease Guillain-Barré (Yuki, 1997) and Miller-Fisher syndrome (Salloway, 1996). In fact, in both cases, the disease is frequently preceded by infection with neuropathic Campylobacter strains.

Fig. 6: The complete structure of *C. jejuni* NCTC 11168 LOS (St Michael, 2002). The carbohydrate structures analogous to the GM1 and GM2 carbohydrate epitopes of human gangliosides are indicated in brackets.
2.2 Capsule

The surface of many bacteria is covered by a carbohydrate layer defined as capsule (CPS). CPS plays an important role in bacterial survival and often contributes to pathogenesis (Roberts, 1996). CPS can vary in composition, mimic host antigens, and provide resistance to phagocytosis, helping the bacteria to evade the immune system. CPS consists of polymers of oligosaccharide subunits, whose composition is usually capsule-specific (capsular K-antigen), but in some cases is an extended polymer of the LPS O-antigen (K_{LPS}). While there is antigenic variety of capsules produced by different bacteria in response to their special environment, the mechanisms of capsule biosynthesis can be classified into just four groups based on genetic and biosynthetic criteria (Whitfield, 1999).

Capsules belonging to group 1 and 4 are synthesized by a wzy-dependent pathway, analogous to the wzy-dependent pathway for O-antigen biosynthesis. Individual repeat units are assembled on an undecaprenyl-phosphate anchor, translocated across the inner membrane by Wzx proteins and polymerized from their reducing end by Wzy (Drummelsmith, 1999). For the capsular K antigen of group 1 and 4, the terminal lipid moiety is unknown, and is lipid A for group 1 and 4 K_{LPS}. Group 1 and 4 are distinguished only by the identity of the initiating glycosyltransferase, WbaP (Hex-1-P transferase) or WecA (GlcNAc-1-P or GalNAc-1-P transferase), respectively (Alexander, 1994; Drummelsmith, 2000).

The group 2 and 3 capsules are assembled in a “processive” biosynthetic mechanism, which is wzy-independent. In this case, the K-antigen is polymerized similarly to the homopolymeric O-antigen. In this mechanism, the K-antigen is polymerized in the cytoplasm from its non-reducing end, a process requiring the hetero-oligomeric complex KfiA-D bound to the plasma membrane (Fig. 7). The lipid-linked K-antigen is then translocated across the inner membrane through a two-component ABC transporter (ABC-2), formed by KpsM, the transmembrane component, and KpsT, the ATPase component (Bliss, 1996). This export apparatus is functionally interchangeable between different capsule serotypes and, as with the O-antigen translocation, the reducing terminal lipid modification was proposed to be the moiety recognized (Whitfield, 1999).
Once in the periplasm, the K-antigen is finally transferred to an α-glycerophosphate, which acts as the terminal lipid moiety for such capsules. The mechanism of translocation from the periplasmic side of the inner membrane to the cell surface is still unknown in capsule assembly. "Membrane adhesion sites" have been observed in *E. coli*, with group 1 or 2 capsules, and translocation has been hypothesized to occur in regions where the outer and inner membranes are in close apposition (Whitfield, 1993). For group 1 capsule-assembly, Wza, an outer membrane lipoprotein with a predicted β-barrel structure, and Wzc, an inner-membrane protein with a cytoplasmic ATP-binding motif, are required for this translocation event (Drummelsmith, 1999). The translocation system for group 4 is completely unknown, and no Wza-Wzc homologues have been found in the corresponding gene clusters.

Translocation of group 2 and 3 capsules involves KpsE and D proteins together with porin proteins (Bliss, 1996). KpsE and D, together with the biosynthetic/export complex located on the plasma membrane (KfaA-D, KpsM, T, C and S proteins), seem to form a multiprotein "capsule assembly complex" (Fig. 7). In particular, KpsE could be responsible for the membrane adhesion sites, as it is anchored to the plasma membrane and associated to the outer membrane. KpsD is a periplasmic protein, and it has been suggested to play a role in recruitment of porin proteins to the capsule assembly complex (Roberts, 1996).
Fig. 7: A model for assembly of group 2 capsule (Whitfield, 1999). Proteins involved in polymer synthesis (KfABCD) interact with a "scaffold" comprising KpsCMST to form a biosynthesis-export-translocation complex on the plasma membrane. The polymer is shown growing on an undecaprenyl pyrophosphate carrier before being transferred to a phosphatidyl-Kdo.

From the C. jejuni 81-176 strain, a KpsM-dependent capsule (defined as high molecular weight glycan, HMW glycan) was isolated, which seems to be required for virulence (Bacon, 2001). This HMW glycan structure was determined to contain 6-O-methyl-D-glycero-α-L-gluco-heptose, β-D-glucuronic acid modified with 2-amino-2-deoxyglycerol, β-D-Gal/NAc and β-D-ribose (St Michael, 2002). In addition, Bacon et al (Bacon, 2001) visualized a second glycan ladder-like structure, which was KpsM-independent. Both Campylobacter CPS forms appeared attached to a phospholipid anchor.
3 Protein-mediated translocation of lipid-linked-oligosaccharides

A crucial and common step in exopolysaccharides biosynthesis and N-glycosylation processes is the transbilayer movement of LLOs. Spontaneous occurrence of this event is very rare because of the high energy-barrier to be overcome in taking the charged headgroup of the phospholipid through the hydrophobic core of the membrane. Rather, in biological systems this reaction is catalysed by specific proteins termed flippases or translocases (Bishop, 1985; Doerrler, 2004; Hanover, 1978; McCloskey, 1980; Menon, 1995; Rush, 1998). These transporters have been divided in two broad classes based on the energetic input required for the translocation reaction; ABC-transporters, which require energy, and flippases, which are ATP-independent.

3.1 ABC-type flippases

The name ABC or ATP Binding Cassette transporter was coined in 1990, cementing a general recognition of the importance of this evolutionarily related but functional diverse family of proteins (Higgins, 2001). ATP-binding cassette (ABC) family of transport proteins form one of the largest paralogous families of proteins and are found in various species from microbes to human. Mammalian ABC transporters are often associated with the multidrug-resistance phenotype, which is particularly problematic in cancer and AIDS therapy. In microorganisms, ABC transporters are central to antibiotic and antifungal resistance. Interestingly, and consistent with this hypothesis, it was shown that the number of ABC transporters encoded in bacterial genomes correlates with the physiological severity of the niches in which bacteria live (Davidson, 2004; Garmory, 2004). In general, transporters belonging to the ATP-binding cassette superfamily couple the energy from ATP hydrolysis to the translocation of a wide variety of substances into or out of cells and organelles. ABC transporter proteins are usually composed of complete transporters, expressing two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs). Alternatively, an ABC transporter can be a dimer of two half
transporters, each one expressing only one TMD and one NBD (Higgins, 2001; Venter, 2003).

Recent genetic, biochemical and structural studies have led to the ATP switch model (Higgins, 2004), in which ATP binding and hydrolysis, respectively, induce the formation and dissociation of a NBD dimer. This, in turn, provides an exquisitely regulated switch that induces conformational changes in the TMD to mediate transport across the membrane. This model proposes that substrate binding to the high affinity site of the TMD initiates the transport cycle. This binding of the substrate results in an increase in affinity of the NBD for ATP, as well as the decrease in the activation energy for the closed dimer formation. This decrease favours the closed form of the NBD dimer, which in turn induces a conformational change of the TMD, allowing for the transport and release of the substrate. ATP is then hydrolyzed and finally, with release of the P, and ADP, the transporter restores to its basal open dimer conformation.

The NBD, which is conserved among ABC transporters, contains two highly conserved motifs, the Walker A and Walker B motifs, which together form an ATP binding site. These motifs are also found in ATP-binding proteins not associated with transport. However, the highly conserved consensus signature sequence LSGGQ/X/QR, called ABC signature is specific to the ABC transporter superfamily. This sequence is also known as the linker peptide or C motif, and is located N-terminal with respect to the Walker B motif (Davidson, 2004; Garmory, 2004).

The distribution of ABC transporters and the diversity of pathways they are involved with, suggest that similar translocation mechanisms (ATP-dependent) have been designed to move across membranes different compounds, both soluble in the cytoplasm and lipid-linked.

A well studied example of a prokaryotic ABC transporter is the E. coli MsbA. It is the proposed inner membrane flippase of the lipid A core, and is homologous to the mammalian multi-drug resistance proteins (Doerrler, 2002; Reyes, 2005). The loss of MsbA, or mutations disrupting transport, results in a lethal accumulation of lipid A in the cytoplasmic site of the inner membrane (Chang, 2001).
The *Campylobacter* PglK protein (formerly WlaB), proposed as the flippase of the N-linked glycan, is annotated as a putative ABC transporter and shows 23% identity and 38% similarity with *E. coli* MsbA (Fig. 8).

As previously discussed, type 2 ABC transporters are involved in the translocation of LLO in capsule and homopolymeric O-antigen biosynthetic pathways, and therefore, it is quite possible that the N-glycan LLO is transported in a similar way.

**Fig. 8: Alignment of the NBD sequences of *C. jejuni* WlaB and *E. coli* MsbA.**

Boxed are the three conserved regions with their highly conserved amino acids. WA: Walker A motif, WB: Walker B motif, ABC s: ABC signature or linker region.

Indeed, other ATPases have been described to be involved in translocation of phospholipids at the plasma membrane of eukaryotic cells. Such flipping guarantees the phospholipids-asymmetry required for the functionality of the membrane (Daleke, 1989; Martin, 1987; Seigneuret, 1984).
3.2 Not-ABC type-flippases

This class of membrane proteins facilitates the translocation of charged phospholipids and glycolipids between the leaflets of a bilayer, without any apparent energy input. Despite their crucial role in membrane biology, their mechanism is still unknown. Several examples have been reported of protein-mediated phospholipids or glycolipids flipping activity, including the biosynthesis of bacterial exopolysaccharides, such as the O-antigen LPS; the translocation of glycerophospholipids across the eukaryotic ER or the bacterial inner membrane that is required for membrane growth (Backer, 1987; Bell, 1981; Rothman, 1977); the flipping of the dolichol-PP-GlcNAc₂Man₅ across the ER membrane, required for N-glycosylation; and the flipping across the ER membrane of the precursor of the glycosylphosphatidylinositol (GPI)-anchor, required for GPI-anchoring of secreted/cell-surface proteins (Abeijon, 1990).

Only in few cases the responsible flippase-protein has been identified. Among these are the human P-glycoprotein encoded by *MDR2*, responsible for the transport of phosphatidylcholine in the canalicular membrane of liver (Berr, 1993); the flippase of the dolichol-PP-GlcNAc₂Man₅, RFT1 (Helcnius, 2002); and the Wzx proteins associated with translocation of LLOs in biosynthesis of bacterial exopolysaccharides (Liu, 1996; Paulsen, 1997; Rick, 2003).

The lack of conserved motifs or common requirements among this class of proteins has held back progress of their characterization. Future genetic, biochemical and structural studies are needed to give more insight into the identification and determination of mechanisms for these ATP-independent lipid/glycolipid flippases.
4 Aims of the research

1) To investigate whether PglK/WlaB is required in the N-glycosylation pathway of *C. jejuni*.

2) To analyze the importance of PglK-ABC features, by means of mutagenesis of its NBD.

3) Exploiting the commonalities between bacterial N-glycosylation and O-antigen biosynthesis to investigate the flippase activity of PglK.

4) To analyse if two different translocation systems, an ABC-type (PglK) and a non-ABC transporter (Wzx), can exhibit interchangeable roles.

5) To develop a glycosylation-read out assay
5 References


Reference


CHAPTER 2

Two distinct but interchangeable mechanisms for flipping of lipid-linked oligosaccharides

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(supplementary data pg. 56)

The MS analysis, the ATPase assay and construction of E. coli SCM7 strain were done by coauthors.

Addendum pg 66
Two distinct but interchangeable mechanisms for flipping of lipid-linked oligosaccharides

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Introduction

The biosynthesis of N-linked glycoproteins in the endoplasmic reticulum (ER) of eukaryotic cells and in bacteria, as well as the biosynthesis of O-antigen lipopolysaccharide (LPS), exopolysaccharides and murein in bacteria, share two common principles. First, an oligosaccharide intermediate is assembled on an isoprenoid lipid carrier (dolichylpyrophosphate or undecaprenylpyrophosphate, respectively) at the cytoplasmic side of the ER or plasma membrane (Bugg and Brandish, 1994). Second, the lipid-linked oligosaccharide (LLO) is translocated (flipped) across the membrane into the lumen of the ER, the periplasm in Gram-negative bacteria, or the extracellular space in Gram-positive bacteria. Subsequent reactions in these oligosaccharide-dependent pathways differ significantly. The LLO can be modified further as is the case in eukaryotic protein glycosylation (Burdin and Aebi, 1999). It can be used as a building block in polymerization reactions (O antigen polysaccharide, capsule, and murein biosynthesis), and may be transferred from the isoprenoid lipid anchor to glycolipid (lipid A-core oligosaccharide) or polypeptide (protein glycosylation) acceptor molecules.

LPS, a major building block of the outer membrane in Gram-negative bacteria, consists of a lipid A-core oligosaccharide and, in some bacteria, an O-specific polysaccharide or O antigen. Two independent pathways are involved in the biosynthesis of the O antigen and the lipid A-core oligosaccharide (Raetz and Whitfield, 2002). O antigens can be homopolymeric or heteropolymers made of oligosaccharide repeats, and they are highly variable within a given species (e.g., about 170 different O serotypes have been identified in Escherichia coli) (Orskov et al, 1977; Bronner et al, 1994). Homopolymeric O antigens are usually synthesized via an ‘ABC-transporter-dependent’ pathway, where the undecaprenyl-PP-linked polysaccharide is completely polymerized in the cytoplasm and then translocated through an ABC transporter into the periplasm and subsequently transferred to the lipid-A core oligosaccharide by the ligase WaaL (Bronner et al, 1994). A ‘wzy-dependent’ biosynthetic pathway exists for most heteropolymeric O antigens. In this case, the O antigen subunits are synthesized in the cytoplasm on an undecaprenyl-PP carrier and translocated across the plasma membrane. The O antigen subunits are subsequently polymerized by the concerted action of the Wzy protein and the O antigen chain length regulator Wzz, and finally transferred to the lipid A core oligosaccharide by the WaaL ligase (Valvano, 2003) (Figure 1B). Transmembrane proteins, called Wzx, have been postulated to mediate the translocation of undecaprenyl-PP-linked sugar precursors in the wzy-dependent pathway (Liu et al, 1996; Paulsen et al, 1997; Feldman et al, 1999; Marolda et al, 2004). Wzx proteins can complement each other in the translocation of different O-antigen sugar precursors (Feldman et al, 1999; Marolda et al, 2004) and they have very similar hydropathy profiles (MacPherson et al, 2004).
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1995). It has recently been suggested that these proteins can recognize the first sugar phosphate bound to the undecaprenyl-P, which in many cases is N-acetylglucosamine (GlcNAc) (Marolda et al., 2004). However, no homology or conserved residues are found among Wzx proteins and their primary sequences do not reveal any ATP-binding domains (Wang and Reeves, 1998; Marolda et al., 1999, 2004). Thus, the lack of identifiable regions in the primary amino-acid sequence of Wzx proteins contrasts with their proposed ability to mediate a rather conserved process.

A pathway similar to the O antigen biosynthesis was described for protein N-glycosylation in the bacterium Campylobacter jejuni (Wacker et al., 2002; Young et al., 2002), a human pathogen causing gastroenteritis. In this pathway, the heptasaccharide (GalNAc-Ol,4-GalNAc-3[1,Glc-P3]-GalNAc-Ol,4-GalNAc-3[1,GalNAc-3[1,Bac]) is first synthesized in the cytoplasm on an undecaprenyl-PP carrier by the sequential activity of glycosyltransferases encoded by the N-glycosylation operon pgp (Figure 1A). This LLO is supposedly transferred across the plasma membrane by an ABC-transporter-like protein, encoded by the wlaB gene, and finally N-linked to an asparagine (Asn) residue of target proteins by the oligosaccharyltransferase PglB. The role of the individual glycosyltransferases in this pathway has been defined by in vitro and in vivo studies (Glover et al., 2005; Linton et al., 2005; Weerapana et al., 2005), but the function of WlaB remains hypothetical.

The N-glycosylation pathway of C. jejuni is remarkably similar to the eukaryotic N-glycosylation pathway in the ER. In eukaryotes (Figure 1C), the oligosaccharride biosynthesis starts on the cytosolic leaflet of the ER membrane, but the completion of the oligosaccharide and its transfer to selected asparagine residues in target proteins occur in the ER lumen (Hirschberg and Snider, 1987; Schenk et al., 2001). Genetic experiments in the yeast Saccharomyces cerevisiae (Helenius et al., 2002) identified the RFT1 protein as the putative flippase involved in transferring the dolichyl-PP-linked Man, GlcNAc intermediate across the ER membrane. Similarly to Wzx proteins in bacteria, RFT1 proteins are conserved in eukaryotic organisms. They also have multiple predicted transmembrane regions and lack any characteristic feature in their amino-acid sequences, such as ATP-binding domains.

Therefore, non-ABC- and ABC-type transporters are proposed to catalyze the transbilayer movement of LLOs, a reaction that does not occur spontaneously (Hanover and Lennart, 1978; McCloskey and Troy, 1980; Bishop and Bell, 1985; Rush and Waechter, 1998; Menon, 1995). We have recently discovered that the C. jejuni N-glycosylation system can transfer O polysaccharide from undecaprenyl-PP

![Figure 1 Schematic representation of N-linked protein glycosylation and O-antigen biosynthesis. (A) Bacterial N-glycosylation pathway. The oligosaccharide is assembled on the undecaprenyl-P-P carrier by the activity of PglG and additional glycosyltransferases in reactions that occur on the cytoplasmic side of the plasma membrane. The LLO is translocated into the periplasm by the ABC transporter PglK (NBD = nucleotide-binding domain). The oligosaccharyl transferase (OTase) PglB transfers the oligosaccharide to Asn residues of acceptor proteins. (B) Wzy-dependent pathway for O-antigen synthesis in Gram-negative bacteria. The O-antigen subunit is assembled onto undecaprenyl-P-P by the activity of WecA and additional glycosyltransferases in reactions that occur on the cytoplasmic side of the plasma membrane. Lipid-linked O-antigen subunits are translocated by Wzx, polymerized and transferred to the lipid A-core oligosaccharide, that is assembled by an independent pathway. (C) N-glycosylation in eukaryotes. A dolichylpyrophosphate-linked oligosaccharide is assembled on the cytoplasmic side of the ER membrane and then translocated by RFT1 into the ER lumen. Oligosaccharide synthesis is completed before transfer to Asn residues by the OTase. Symbols have been attributed to the sugar residues according to the CFG nomenclature; the symbols for bacillosamine and rhamnose have been newly introduced.](image-url)
to a periplasmic acceptor protein, a process that requires the activity of the oligosaccharyltransferase PglB (Feldman et al., 2005). In this report, we exploited further the commonalities between bacterial N-glycosylation and O-antigen biosynthesis to demonstrate that WlaB is not only responsible for the translocation of the undecaprenyl-PP-linked oligosaccharide in the C. jejuni N-glycosylation machinery, but also can mediate the translocation of O-antigen subunits. Therefore, we show that two very different proteins, an ABC-type (WlaB) and a non-ABC-type transporter (Wzx), can exhibit interchangeable roles. Given the defined role of WlaB in the transport of LLOs across the plasma membrane, we propose to rename it as PglK.

Results

**N-glycosylation profile of a C. jejuni pglK mutant**

A kanamycin resistance cassette was inserted into the pglK gene of C. jejuni strain 81176 (Korlath et al., 1985) to investigate the involvement of PglK in N-glycosylation. The effect of the mutation on the N-glycosylation system was assessed by the analysis of the AcrA protein. AcrA carries two N-linked glycans (Wacker et al., 2002) that cause a detectable shift in its electrophoretic mobility, and therefore serves as a probe for the functional status of the N-glycosylation system. Figure 2A shows that the AcrA protein in the wild-type (wt) C. jejuni strain has an apparent molecular weight of 47.5 kDa (lane 1), which is consistent with the mass of the diglycosylated form of the protein. In contrast, as reported previously (Wacker et al., 2002), inactivation of the PglB oligosaccharyltransferase resulted in nonglycosylated AcrA that migrates faster in the gel (Figure 2A, lane 2). In the pglK mutant strain, three AcrA-specific bands were detected (Figure 2A, lane 3). These bands correspond to diglycosylated, monoglycosylated, and unglycosylated forms of AcrA.

The hypoglycosylation phenotype of the pglK mutant was confirmed by an experiment using the R12 antiserum. We have shown that this serum reacts with multiple C. jejuni proteins and has a high preference for the glyco-epitope (Wacker et al., 2002). Inactivation of the pgk-dependent general glycosylation system in the pgkB mutant resulted in a different banding pattern as compared with the wt strain, when membrane extracts were reacted with the R12 serum (Figure 2B, lanes 1 and 2). In contrast, the pattern observed in the membrane extract from the pglK mutant corresponded to a mixture of bands found in wt and the oligosaccharyltransferase-deficient cells (Figure 2B, lane 3). In particular, the R12 antiserum detected diglycosylated AcrA in the wt C. jejuni (Figure 2B, lane 1) and, confirming the result obtained with the anti-AcrA antiserum, mono- and diglycosylated AcrA in the pglK mutant (Figure 2B, lane 3). As expected, AcrA was not glycosylated in the pgkB mutant (Figure 2B, lane 2).

We concluded from these experiments that inactivation of the pgkK gene resulted in a hypoglycosylation phenotype, confirming that pgkK encodes a protein involved in the N-glycosylation process. Given the homology of PglK with ABC-type transporters, it is likely that this protein is involved in the membrane translocation of the LLO intermediate. As the N-glycosylation defect in the pgkK mutant is only partial, it is possible that other translocases in C. jejuni partially complemented the pgkK defect.

**PglK with a functional nucleotide-binding domain was necessary for N-glycosylation in E. coli SCM7**

The C. jejuni N-glycosylation pathway can be functionally expressed in E. coli (Feldman et al., 2005; Wacker et al., 2002), making it possible to address the role of individual components of the glycosylation machinery in this heterologous system. To verify the hypothesis that PglK is the LLO translocase of the C. jejuni N-glycosylation pathway, it was necessary to construct first an E. coli strain lacking all flippases of the Wzx family. This provided a genetic background that prevented potential interference of other LLO flippases present in E. coli. For this purpose, we constructed strain SCM7 (see Table I), which has a large deletion eliminating the O antigen and colanic capsule gene clusters and another deletion eliminating the enterobacterial common antigen (ECA) cluster. Therefore, this strain lacks uzxC (colanic acid), uzxO16 (O antigen), and uzxE (ECA) flippase genes.

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In order to determine whether PglK can be regarded as an ABC transporter, we investigated the requirement of the ATP-binding domain (NBD), present in the C-terminal part of the protein, for its activity. Conserved amino-acid residues in the Walker A, Walker B and ABC-signature motifs of PglK, some of which are necessary for the ATPase activity of other ABC transporters (Urbatsch et al., 2000; Venter et al., 2003; Szentpetery et al., 2004), were mutated and the glycosylation profile of AcrA in the presence of the mutated PglK forms was analyzed. We also evaluated the function of a PglK mutant carrying the deletion of the entire NBD (ANBD) (Venter et al. 2003).

Periplasmic protein extracts from E. coli strain SCM7 expressing a soluble version of AcrA (Nita-Lazar et al., 2004; Feldman et al., 2005), the C. jejuni-derived pgl operon with a deletion of the pglK locus, and a wt or mutated version of pglK were analyzed by immunoblot using anti-AcrA and R12 antiserum. As noted above, the R12 serum preferentially reacts with the C. jejuni-derived glycan.

Unglycosylated AcrA was observed in the absence of functional PglK (Figure 3, lane 1), whereas mono- and diglycosylated AcrA proteins were detected in cells where the pglK deficiency was complemented by the pglK expression plasmid pCA1 (Figure 3, lane 2). AcrA protein was fully glycosylated in the presence of the R492C mutant (Figure 3, lane 8), whereas reduced glycosylation efficiency was observed with mutants L506A and K388A (Figure 3, lanes 4 and 5) and loss of glycosylation was detected in the presence of ANBD, S389A, and G488D (Figure 3, lanes 3, 6, and 7). Expression and membrane localization of wt-PglK and the NBD-mutant PglK was confirmed by cell fractionation and subsequent immunodetection with anti-myc antibodies (see Figure S1 in Supplementary data).

The phenotype observed with the pglK mutant in E. coli SCM7 was identical to that observed with the pglB mutant in C. jejuni (Figure 2A, lane 2). Thus, these results established our experimental system where PglK activity could be monitored in vivo by the analysis of AcrA glycosylation, and suggested that PglK required a functional NBD domain for its function, supporting the notion that it is an ABC-type transporter.

**ATPase activity of purified PglK**

To verify the ATPase activity of PglK, the protein tagged with a (His)$_{10}$ sequence at the C-terminus was expressed in E. coli strain C43 and purified by Ni-NTA affinity chromatography (Catrein et al., in preparation). The mutant S389A PglK protein served as a negative control.

Using a colorimetric assay for release of P, (Chifflet et al., 1988), the wt PglK protein showed an ATPase activity of 47.5 nmol/mg/min (Figure 4A), which is in the range of values reported for other ABC transporters (Schneider and Hunke, 1998). Furthermore, the ATPase activity of wt-PglK was strongly inhibited by addition of orthovanadate (Figure 4B), a well-known inhibitor of ABC transporters (Urbatsch et al., 1995a, b). No ATPase activity was detected in the presence of the mutant S389A PglK.

**PglK substituted the function of Wzx in O-antigen LPS biosynthesis**

To obtain direct genetic evidence for a flippase activity of the PglK protein, we took advantage of the similarity between the N-glycosylation pathway of Campylobacter and the LPS biosynthesis pathway of Campylobacter and the LPS.
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Figure 4 PglK-ATPase assay. (A) Purified PglK (●) and mutant PglK-S389A (■) protein (each 10 µg/ml) was incubated in the presence of 4 mM ATP, 100 mM Tris, pH 7.5, 8 mM MgCl₂, 1 mM dithiothreitol, 1% ANAPOL-C₁₂Es (Anatrace), 500 mM NaCl, 25 mM imidazol, and 10% glycerol for various times at 37°C. The generation of P₀ was measured at 850 nm. (B) The purified PglK protein (●) (10 µg/ml) was preincubated on ice for 15 min with increasing amounts of sodium orthovanadate (10-500 µM) in the presence of 4 mM ATP, 100 mM Tris, pH 7.5, 8 mM MgCl₂, 1 mM dithiothreitol, 1% ANAPOL-C₁₂Es (Anatrace), 500 mM NaCl, 25 mM imidazol, and 10% glycerol for 30 min at 37°C, and the generation of P₀ was measured at 850 nm. All values are the averages of triplicate determinations shown with standard deviations.

biosynthesis pathway of E. coli. We investigated whether PglK had the ability to complement O-antigen biosynthesis in the absence of a functional Wzx, the O-antigen translocase. E. coli strain CLM17, which carries a wzzO16 deletion and is defective in O₁₆ LPS synthesis due to wbll1 mutation (Table I), was used to test PglK-dependent O₁₆ LPS production. CLM17 cells were transformed with plasmid pMF19 (expressing the rhamnosyltransferase wbl1 gene required for O₁₆ LPS biosynthesis) and with plasmid pCA1 encoding pglK. Plasmids pCM223 (encoding WzxO₁₆) and pBAD/Myc-His served as positive and negative controls, respectively. Mutant forms of PglK were also included in this experiment. Biosynthesis of O₁₆ LPS was examined by immunodetection with O₁₆-specific antiserum after LPS separation by sodium-dodecylsulfate (SDS)-PAGE. No O₁₆ antigen was detected in strain CLM17 in the presence of the vector control (Figure 5A, lane 1), whereas formation of O₁₆-specific polysaccharide with the typical ladder-like banding pattern was observed in CLM17 cells expressing WzxO₁₆ (Figure 5A, lane 8) or a functional PglK protein (Figure 5A, lane 2). Thus, we concluded that pglK substituted for wzz to mediate the translocation of the undecaprenyl-PP-linked O₁₆ subunits. In addition, complementation of the Wzx function required an intact PglK nucleotide-binding domain, since the NBD deletion mutant did not complement (Figure 5A, lane 3), and also the amino-acid replacement mutants in the conserved Walker A and ABC-signature protein motifs provided various degrees of in vivo complementation of O₁₆ polysaccharide synthesis (Figure 5A, lanes 4-7).

The ability of PglK to functionally substitute Wzx was investigated further in the O₇-LPS biosynthesis pathway. The

Figure 5 PglK-dependent LPS biosynthesis. (A) LPS prepared from E. coli CLM17 cells carrying the rhamnosyltransferase-expressing plasmid pMF19 and a vector control (lane 1), a pglK-expressing plasmid (lane 2) with mutations in the NBD (lanes 3-7), or a wzzO16-expressing plasmid (lane 8) was separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. O₁₆ LPS was detected with O₁₆-specific antiserum. (B) LPS prepared from E. coli S0874 cells carrying cosmid pJHCV32::Tn5HoHol-128, expressing the wzz mutant-O7 cluster, with the vector control (lane 1), a plasmid expressing pglK (lane 2) or wzx-O7 (lane 3) was separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. O₇ LPS was detected with O₇-specific antiserum.

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O7 and O16 oligosaccharide units have only one hexose, GlcNAc, in common, while the remaining sugars in both subunits are different (L’Vov et al., 1984; Stevenson et al., 1994) (see Figure S2 in Supplementary data). We introduced into E. coli S0874 the cosmid pHCV32::Tn3Hol-128 (Marolda et al., 1991, 1999) that carries the O7 antigen synthesis cluster with an inactivated wzxO7 gene. Either PglK (encoded by plasmid pCMW27) or WzxO16 (encoded by plasmid pMF21) complemented O7 polysaccharide synthesis, as demonstrated by immunodetection with O7-specific antisera, which revealed a typical O antigen ladder in both cases (Figure 5B). This experiment demonstrates that PglK can also complement the wzxO7 defect. The weak ladder observed in the absence of PglK or WzxO16 (Figure 5B, lane 1) was attributed to partial complementation by the chromosomally encoded WzxO7 flippase in E. coli S0874.

Altogether, the ability of PglK to complement wzx mutations in two different O antigen systems supported its role as a flippase. Moreover, our results indicated a relaxed specificity of PglK activity with respect to the oligosaccharide structure.

The same conclusion, regarding PglK-relaxed specificity, was supported by the analysis of the AcrA-glycosylation phenotype associated to mutant forms of the pgl operon (see Figures S3 and S4, in Supplementary data).

Our data supported the hypothesis that PglK is an ATP-dependent LLO flippase with a relaxed specificity with respect to the oligosaccharide structure.

**WzxO7 and WzxO16 replaced PglK in the presence of WecA**

The interchangeability of the two LLO translocation systems associated with protein glycosylation and O-antigen LPS biosynthesis made it possible to address the substrate specificity of the Wzx flippases. We introduced plasmids encoding PglK, WzxO16, or WzxO7 into E. coli SC7 strain expressing AcrA and the wt or the pglK-deficient pgl operon. The AcrA glycosylation profile was analyzed from periplasmic extracts as before. AcrA N-glycosylation was only detected when a functional PglK was expressed (Figure 6, lanes 2 and 5). The inability of the Wzx proteins to complement PglK deficiency suggested that the Wzx-dependent flipping mechanism was not compatible with the components of the N-glycosylation process.

Previous work (Feldman et al., 1999; Marolda et al., 2004) suggested that Wzx proteins recognize the proximal sugar bound to undecaprenyl-PP. WecA is the UDP-GlcNAc:Und-P GlcNAc-l-P transferase that initiates the biosynthesis of O7 and O16 antigens (Alexander and Valvano, 1994; Yao and Valvano, 1994). WecA also mediates the first step in the ECA biosynthesis (Barr and Rick, 1987), an outer membrane glycolipid made of trisaccharide repeat units produced in all the Enterobacteriaceae (Meier-Dieter et al., 1992) via a Wzy-dependent mechanism (Rick and Silver, 1996) that requires WzxE, the translocase of the undecaprenyl-PP-GlcNAc-ManNAc-FucNAc ECA precursor (Rick et al., 2003). Since strain SCM7 lacks the ECA cluster, including the wecA gene, it cannot synthesize Und-P--GlcNAc. We reasoned that the lack of complementation of the pglK deficiency by the Wzx flippases could be due to absence of the WecA activity. Therefore, we repeated the PglK-complementation assay described above, but in the presence of a plasmid encoding WecA. The glycosylation profile of AcrA was analyzed in periplasmic extracts of cells expressing AcrA, the pglK-deficient operon, one of the investigated flippases (PglK, WzxO16, WzxO7, or WzxE), and the WecA-expressing plasmid or the vector control. WzxE and WzxO7 complemented the N-glycosylation defect of the pglK-deficient operon only when WecA was expressed (Figure 7, lanes 5–6 and 9–10), while PglK activity was independent of WecA expression (Figure 7, lanes 3–4). No significant WzxO16-mediated complementation was observed (Figure 7, lanes 7–8), independently of WecA expression. We concluded that the WzxE and WzxO7 flippases complemented the pglK deficiency in a WecA-dependent manner. This is consistent with the observation that, in the presence of a functional WecA and the C. jejuni pgl operon, a hybrid C. jejuni oligosaccharide initiating with HexNAc instead of Bacillosamine is formed (Wacker et al., 2002; Linton et al., 2005), and with previous results suggesting that the WzxO7 and WzxE flippases recognize Und-PP-GlcNAc or function in association with WecA (Marolda et al., 2004).

**Discussion**

The translocation of highly hydrophilic LLO across cellular membranes is a key biological reaction in prokaryotes and eukaryotes. However, little is known about the molecular mechanisms of LLO translocation (Menon, 1995). Based on the genetic analysis of LPS biosynthetic pathways in bacteria, it was proposed that translocation can be mediated by...
Our finding that the function of different types of LLO flippases can be analyzed in vivo using a heterologous system offered unique opportunities to study the substrate specificity of the different flippases. We confirmed previously published results that demonstrated a relaxed specificity of the wzx flippases (Feldman et al., 1999; Marolda et al., 2004). The wzxE and the wzxO7 activity for flipping a C. jejuni-transferred LLO was WecA dependent, suggesting that GlcNAc as the reducing-end hexose was of central importance for substrate recognition. In the case of the C. jejuni pglK, oligosaccharide specificity was even more relaxed and our experiments did not reveal a defined requirement. However, we noted that all the LLO substrates tested in our in vivo complementation assay contained a 2-acetamido group in the sugar directly linked to undecaprenylphosphosphate.

The ability to translocate a wide variety of LLOs across the membrane is therefore a common feature of most of the flippases tested in this report. From a biophysical point of view, the major function of these flippases is the translocation of the hydrophilic oligosaccharide across the membrane and aqueous channels to accommodate these structures seem the most likely route to perform this activity. However, the presence of such channels would predict a high specificity to prevent translocation of other hydrophilic components across the membrane. It is evident that a detailed biochemical and structural analysis of LLO flippases, as reported for the highly specific flippase MsAb (Doerrler et al., 2004a; Reyes and Chang, 2005), is required to establish the translocation mechanism mediated by these enzymes. The fact that two very different types of proteins can catalyze this translocation shows that two distinct mechanisms exist for a reaction that interests both biochemists and biophysicists.

Materials and methods

Bacterial strains, growth conditions, and plasmids

C. jejuni strains were grown on Mueller Hinton agar (Difco) at 42°C under microaerophilic conditions (85% N2, 10% CO2, and 5% O2). E. coli strains were grown on Luria Bertani medium at 37°C. Chloramphenicol (20 μg/ml), ampicillin (100 μg/ml), tetracycline (20 μg/ml), spectinomycin (80 μg/ml), and kanamycin (50 μg/ml) were added to the media as needed. Bacterial strains and plasmids used are listed in Tables I and II, respectively.

Construction of a C. jejuni pglK mutant

C. jejuni strain 81-176 (Roklath et al., 1985) was transformed with 10 μg of NcoI-cleaved pACYC184::kan DNA (Liston et al., 2005), carrying the pgl operon with a kanamycin resistance gene cassette inserted in pglK (pglK::kan). For this purpose, five C. jejuni colonies were selected and transferred to a fresh plate. The linearized plasmid (in a 20-μl volume) was added on the top of the bacterial colonies, mixed gently and let dry on the plate before incubating for 8 h under microaerophilic conditions at 42°C. Transformants were repurified on plates containing kanamycin. The integration of the transforming plasmid DNA in pglK by a double crossover event was confirmed by colony-PCR analysis using primers B3 and B4, annealing to pglK, and primers U1 and UL, annealing to g1Acra and the lac-amycin resistance cassette, respectively. The primers were designed according to the sequenced genome data from the Sanger Center (http://www.sanger.ac.uk/Projects/C._jejuni) (see Table I in Supplementary data). PCR reactions (30 μl) were carried out in 200 μl Tris-HCl, pH 8.8, 20 mM MgSO4, 100 mM KCl, 100 mM [NH4]2SO4, 1% Triton X-100, 1 mg/ml BSA with 1 μM concentrations of forward and reverse oligonucleotide primers, and 25 U PfuTurbo DNA polymerase (Stratagene). Amplification was achieved with the following thermal cycling conditions: 1 cycle at 95°C for 5 min, 30 cycles consisting of a 95°C denaturation step for...
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Table II Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACYCpgl::kan</td>
<td>C. jejuni pgl cluster containing a kan cassette in pglC, CmR, KanR</td>
<td>Linton et al (2005)</td>
</tr>
<tr>
<td>pACYCpgl::kan</td>
<td>C. jejuni pgl cluster containing a kan cassette in pglD, CmR, KanR</td>
<td>Linton et al (2005)</td>
</tr>
<tr>
<td>pACYCpgl::kan</td>
<td>C. jejuni pgl cluster containing a kan cassette in pglE, CmR, KanR</td>
<td>Linton et al (2005)</td>
</tr>
<tr>
<td>pACYCpgl::kan</td>
<td>C. jejuni pgl cluster containing a kan cassette in pglF, CmR, KanR</td>
<td>Linton et al (2005)</td>
</tr>
<tr>
<td>pACYCpgl::kan</td>
<td>C. jejuni pgl cluster containing a kan cassette in pglG, CmR, KanR</td>
<td>Linton et al (2005)</td>
</tr>
<tr>
<td>pACYCpgl::kan</td>
<td>C. jejuni pgl cluster containing a kan cassette in pglH, CmR, KanR</td>
<td>Linton et al (2005)</td>
</tr>
<tr>
<td>pACYCpgl::kan</td>
<td>C. jejuni pgl cluster containing a kan cassette in pglI, CmR, KanR</td>
<td>Linton et al (2005)</td>
</tr>
<tr>
<td>pACYCpgl::kan</td>
<td>C. jejuni pgl cluster containing a kan cassette in pglJ, CmR, KanR</td>
<td>Linton et al (2005)</td>
</tr>
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<td>pHAD/Myc-HisA</td>
<td>Cloning vector, AmpR</td>
<td>Invirotrogen</td>
</tr>
<tr>
<td>pHBBRlMCS-3</td>
<td>Cloning vector, SpcR</td>
<td>Lefebvre and Valvano (2002)</td>
</tr>
<tr>
<td>pKD4-M6</td>
<td>χ, β, and exo from λ phage, araC-MaraB, AmpR</td>
<td>Datsenko and Wanner (2000)</td>
</tr>
<tr>
<td>pKVI</td>
<td>wecA&lt;sub&gt;Ac&lt;/sub&gt;, cloned into pBAD24, Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Datsenko and Wanner (2000)</td>
</tr>
<tr>
<td>pCM238</td>
<td>wecE cloned into pBAD24, Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>R Vigeant</td>
</tr>
<tr>
<td>pLMA3</td>
<td>Soluble periplasmic AcrA-(His)&lt;sub&gt;6&lt;/sub&gt;, expression controlled by Tet promoter, in pBBRlMCS-3, TetR</td>
<td>This work</td>
</tr>
<tr>
<td>pLMA4</td>
<td>Soluble periplasmic AcrA-(His)&lt;sub&gt;6&lt;/sub&gt;, expression controlled by Tet promoter, in pBBRlMCS-4, AmpR</td>
<td>This work</td>
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<tr>
<td>pCA1</td>
<td>pgk in pBAD/Myc-HisA, AmpR</td>
<td>This work</td>
</tr>
<tr>
<td>pCV27</td>
<td>pgk in pHBBRlMCS/Myc-His&lt;sub&gt;6&lt;/sub&gt;, TetR</td>
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<tr>
<td>pCA1A</td>
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<td>pLM1</td>
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<td>pLM4</td>
<td>pgk&lt;sup&gt;kan&lt;/sup&gt;DK in pBAD/Myc-HisA, AmpR</td>
<td>This work</td>
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<tr>
<td>pLM5</td>
<td>pgk&lt;sup&gt;kan&lt;/sup&gt;DK in pBAD/Myc-HisA, AmpR</td>
<td>This work</td>
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<tr>
<td>pHCV52::Tn3His Hol-128</td>
<td>O7 LPS biosynthesis gene cluster, wecA::Tn3Hol1-128, TetR, AmpR</td>
<td>Marolda et al (1999)</td>
</tr>
</tbody>
</table>

30℃, 50℃ annealing for 1 min, and 68℃ extension for 2.5 min, and a final extension at 68℃ for 10 min.

Construction of E. coli strain SCM7

Deletion of the ECA cluster was in strain SO874 as described by Datsenko and Wanner (2000). We generated plasmids pweC<sub>Ac</sub> and pweG<sub>Ac</sub> (see Table I in Supplementary data) of 40-45 kbp, corresponding to regions adjacent to the gene targeted for deletion and also containing 20 additional nucleotides that annealed to the template DNA from plasmid pKD4. This plasmid carries a kanamycin-resistance gene flanked by FRT (FLP recognition) sites. Competent cells were prepared by growing E. coli S0874 carrying pKD46 in LB containing 0.5% (w/v) arabinose and a final extension at 68℃ for 10 min.

Construction of recombinant plasmids

E. coli strain Top10 (Invitrogen) was used for DNA cloning experiments and all constructed plasmids were verified by DNA sequencing. All PCR reactions were carried out as described above, using 0.1 µg of template DNA. Plasmid pNA2, expressing a soluble form of ACA that locates in the periplasm (Feldman et al., 2005), was digested with Ethl and EcoRI. The resulting 1.7-kb fragment was ligated into plasmid pBBRlMCS-3 (Kovach et al., 2005) that had been cleaved with SmaI and EcoRI, resulting in plasmid pLMA4. A 1.8-kb fragment containing acaA under the control of a constitutive promoter was excised from pLMA4 with Apel and ScaI and subcloned into Apel-ScaI-cleaved pBBRlMCS-3 (Kovach et al., 1995), resulting in plasmid pLMA3.

This work
protein expression and immunodetection

Arabinose inducible expression (pGk, pGK mutants and uveX) was achieved by adding arabinose at a final concentration of 0.2% (w/v) to E. coli cells grown in Luria Bertani medium up to an OD600 of 0.3. The same amount of arabinose was added again 5 h post-induction, and incubation continued for 15 h. WeCA expression was induced by adding IPTG to a final concentration of 0.5 mM to cultures reached an OD600 of 0.3. Induction was maintained for 20 h. The expression of membrane proteins was monitored by fractionation of cells extracts as described previously (Wacker et al., 2002). Total E. coli cell extracts were prepared for immunodetection analysis using cells at a concentration equivalent to 1 OD600 units from overnight cultures, which were resuspended in 50 μl SDS loading buffer (Lemmli, 1970). Aliquots of 10 μl were loaded on 10% SDS-polyacrylamide gels. Periplasmic extracts of E. coli cells were prepared by lysozyme treatment (Feldman et al., 2003), and 10 μl of the final sample (corresponding to 0.2 OD600 units of cells) was analyzed by SDS-polyacrylamide gel electrophoresis.

After being blotted on nitrocellulose membrane (Protran Bioscience, Dassel, Germany, pore size 0.45 μm), the sample was immobilized with the specific antisem as described (Aebi et al., 1996). Anti-AcrA and R12 sera (Wacker et al., 2002), anti-Flag (Sigma Aldrich, St Louis, MO) antibodies and anti-Myc (Calbiochem, Darmstadt, Germany) antibodies were used. Antiserum against E. coli O7 was obtained from the Statens Serum Institut (Copenhagen). Antiserum against E. coli O16 was obtained from the Laboratorio de Referencia de E. coli (Lugo, Spain). Anti-rabbit IgG-HRP (Santa Cruz) was used as secondary antibody in combination with the anti-O antigen, anti-AcrA, and R12 antisera. Anti-mouse IgG-HRP (Santa Cruz) was used as secondary antibody in combination with anti-Flag and anti-Myc antibodies. Detection was carried out with ECL™ Western Blotting Detection Reagents (Amersham Biosciences, Little Chalfont Buckinghamshire).

ATPase assay

The wt and 5389A mutant Pgk protein in 1% ANAP08-C65E2s were assayed for ATPase activity at a concentration of 10 μg/ml in 350 μl reaction mixture containing 100 mM Tris-HCl, pH 7.5, 4 mM ATP, 8 mM MgCl₂, 25 mM imidazole, 1 mM DTT, 500 mM NaCl, and 10% glycerol. ATPase reactions were incubated for the indicated times at 37°C and 50 μl samples were taken to measure the released P₃ (Chifflet et al., 1988). If sodium orthovanadate (Sigma) was included in the assay, a 15 min preincubation of all components on ice was performed before the incubation at 37°C for 30 min.

LPS analysis

Cells corresponding to 1 OD600 units of an overnight culture were resuspended in 100 μl SDS loading buffer (Lemmli, 1970), heated at 95°C for 5 min, and incubated with 2 μl Protease K (50 μl/ml; Roche) at 60°C for 2 h. Samples (10 μl) were separated by SDS-PAGE on 12% gels, blotted onto nitrocellulose membranes, and the LPS detected with O7 or O16 specific antisem as described above.

Supplementary data

Supplementary data are available at The EMBO Journal Online.

Acknowledgements

We thank Karen Vigrean for providing pKv1. This research was supported by the Swiss National Science Foundation (grants 3001B0-105541 and 3000-057082 to MA), the Gebert-Rüth Foundation, the EU (grant Flippases MRTN-CT-2004-005330), and the Canadian Institutes of Health Research (grant MT-0286 to MAV). MAV holds a Canada Research Chair in Infectious Diseases and Microbial Pathogenesis.

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proteins and primers used are listed in Tables II and I (in Supplementary data).

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Supplementary data
Fig. S1 Expression of PglK and PglK-mutants in *E. coli* SCM7. Membrane proteins-samples were separated by 9% SDS-PAGE transferred to nitrocellulose membrane and PglK detected with αMyc antibody.
Fig. S2. Structures of E. coli O16 and O7 antigen unit
Relaxed substrate specificity of PglK

We wanted to examine whether PglK activity required a complete LLO intermediate. Using *E. coli* BL21 containing a plasmid-encoded *pgl* operon with mutated genes, Linton and coworkers (Linton, 2005) showed that incomplete oligosaccharides are transferred to protein. To investigate whether transfer of biosynthetic intermediates depended on *pglK* activity, mutant forms of the *pgl* operon that altered the structure of the glycan were expressed in *E. coli* SCM7 in combination with the AcrA reporter. Glycosylation was analyzed by SDS-PAGE with anti-AcrA and R12 immunodetection. In *pglll* and *pglJ* mutant strains, incomplete oligosaccharides were transferred to AcrA (Fig. S3A, lanes 3 and 5), as indicated by the altered mobility of glycosylated AcrA. In *pgllJ* mutant strain the electrophoretic mobility of glycosylated AcrA was not affected (Fig. S3A and B, lane 4) because only the branching glucose was missing on the oligosaccharide. The sugar-epimerase mutant, GalE mutant, was previously described to abolish N-glycosylation (Linton, 2005). In our experiment, the R12 antiserum did not recognize AcrA when the protein was expressed in presence of the GalE mutant (Fig. S3 lane 6), meaning that it was not glycosylated.
Fig S3
Fig. S3. AcrA N-glycosylation in presence of pgl mutant plasmids.
Total extracts prepared from E. coli SCM7 cells carrying the AcrA expression plasmid and the pgl operon with the deletions indicated (lanes 1-6) or wild-type pgl operon (lane 7), were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. AcrA and glycosylated proteins were detected with anti-AcrA (A) and the glycoprotein specific R12 (B) antisera, respectively. The position of bands corresponding to unglycosylated (AcrA), monoglycosylated (g1AcrA), and diglycosylated AcrA (g2AcrA) is indicated. The glycan-structure reported by Linton and coworkers (Linton, 2005) for each of the pgl mutant is shown above each lane. Symbols were attributed to the sugar-residues according to the CFG nomenclature, the symbol for bacillosamine was newly introduced.
PglK-dependent transfer is not specific for the lipid-linked-bacillosamine

The N-glycan form transferred to AcrA in presence of pglE and pglF mutants (Fig. S3 A and B, lanes 1 and 2) were also analyzed by MALDI-TOF-MS. As shown in Fig. S4 A and B, N-acetylhexosamine instead of bacillosamine was detected at the reducing end of the oligosaccharide. As N-glycosylation in strain SCM7 is exclusively dependent on PglK for LLO translocation (see main text), these data show that PglK translocates oligosaccharides with relaxed specificity relative to the length of the oligosaccharide and to the nature of the sugar bound to undecaprenyl-PP.

Materials and methods

AcrA purification

Soluble AcrA protein was purified from periplasmic extracts that were brought to 30 mM Tris-HCl-300 mM NaCl (pH8) (buffer A), and loaded on a His Trap HP column (Amersham Pharmacia Biosciences Uppsala Sweden) at a flow rate of 1-5 ml/min. The column was washed with at least 25 column volumes of buffer A containing 20 mM imidazole and bound proteins eluted into 1-3 ml of buffer A containing 250 mM imidazole.

Characterization of AcrA-glycosylated peptides

Tryptic peptides extracted from polyacrylamide gel bands upon in-gel trypsinization were desalted over a NuTip 10 Carbon clean-up tip (Glygen, Columbia, MD) in 0.05% trifluoro-acetic acid (TFA), upon preconditioning the tip with 3 washes in 5 μl of 60% acetonitrile, 0.05% TFA followed by 3 washes in 5 μl of 0.05% TFA. The sample (in 5 μl of 0.05% TFA) was applied by pipetting it up and down 25 times over the tip. Subsequently, the tip was washed 10 times with 10 μl of 0.05% TFA and the peptides were eluted by 5 washes with 1.75 μl of 60% acetonitrile+0.05% TFA (pipetting up and down the eluent 10x for each elution, and pooling the 5 eluates). Then, the pooled eluate was vacuum-evaporated to dryness and reconstituted in 2 μl of "super-DHB" MALDI matrix solution, a 9:1 mixture of 20 mg/ml 2,5-dihydroxybenzoic acid (2,5-DHB) and 20 mg/ml 5-methoxysalicylic acid in 70% MeOH +0.1% TFA (both matrix compounds: Fluka, Buchs, Switzerland). 0.8 μl of this final sample was applied to a stainless steel ABI 192-target MALDI plate (Applied Biosystems, Foster City, CA) and air-dried. MALDI-TOF MS in the positive
ion reflectron mode was performed on an ABI 4700 instrument, using a laser fluency tuned for an optimal balance between sensitivity and spectral resolution for this analyte-matrix combination. External on-plate calibration in the m/z range 750-4250 was performed using a peptide calibration standard (ABI). The spectra were averaged over at least 2,500 laser shots and were baseline-corrected and smoothed through a Gaussian fit. The blank sample was derived from a piece of the same SDS-PAGE gel where no protein was detected by CBB staining. It was treated exactly as the AcrA-containing SDS-PAGE bands.

For TOF/TOF MS/MS, 10,000 laser shots were averaged. Air was used as the collision gas, at a pressure of 5.10E-7 Torr. For comparison, the MS/MS spectrum (Fig. 4S B, lower panel) derived from the same AcrA tryptic peptide as analysed in the top panel of Fig. 4S B is shown (non-glycosylated mass of 551 Da), but this time derived from AcrA which was secreted via the Tat-system of an E. coli strain carrying the wild type pgI locus (Feldman and Kowarik, manuscript in preparation). Consequently, this peptide is glycosylated with the native C. jejuni N-glycan structure, i.e. with baciilosamine as the peptide-proximal residue.
Fig. S4. Mass spectrometrical analysis of the N-glycan structure of AcrA secreted by *E. coli* SCM7 containing *pglE* and *pglF* mutant *pgl* operons.

A. MALDI TOF/TOF spectral regions of the glycosylated derivatives of a tryptic peptide with unglycosylated mass of 551.3 Da. Whereas the bacillosamine-containing *C. jejuni* N-glycan structure on this peptide leads to a mass of 1956.7 Da, we did not observe this predicted analyte in the AcrA tryptic peptide map of *pglE* and *pglF* mutants. Instead, we observed an analyte at 1931.8 in this region for both mutant-derived proteins. This corresponds to the predicted mass of this peptide if it would be glycosylated with the same glycan, but with an N-acetylhexosamine instead of bacillosamine as the peptide-proximal residue.

B. To confirm this, we performed a TOF/TOF MS/MS analysis on this analyte. Despite the low quantities of material available and despite the typical very low ionisation efficiency of glycopeptides (as compared to their non-glycosylated counterparts), we were able to obtain an MS/MS spectrum that was interpretable (average of 10000 laser shots, only possible through the use of the high-frequency laser in the ABI4700 analyzer). A complete y-ion series was discernable, down to the unmodified peptide at m/z 551.3. A spectrum with higher signal/noise than was obtainable from the material under study, derived from the same glycopeptide glycosylated with the bacillosamine-containing N-glycan is shown for comparison in the lower panel. One observes virtually identical fragmentation behaviour for both analytes, with the exception for the difference in mass between the Y₀ and Y₁ ions, which is 203.1 Da for the *pglE* case (top panel), whereas it is 228.1 Da for the known bacillosamine-containing analyte (lower panel). This leaves no doubt that the analyte at 1931.8 in *pglE*-mutant derived AcrA tryptic peptide map has an N-acetylhexosamine residue as peptide-proximal residue, whereas the rest of the glycan behaves identical to the 'wild-type' *C. jejuni* N-glycan structure. It remains to be said that the signal/noise of the other analytes around m/z 1900-2000 was too low to be analyzed via MS/MS.
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**Table 1:** Primers table
Addendum

2.1 Wzx proteins from *E. coli* O157, *S. enterica*, *P. aeruginosa* and *S. flexneri* do not complement *Campylobacter* PglK

The ability of Wzx proteins from *E. coli* O157, *S. enterica*, *P. aeruginosa* and *S. flexneri* to complement *Campylobacter* PglK, was as well investigated. In *E. coli* SCM7 strain expressing the specific Wzx flippase-encoding plasmid (Marolda, 2004), the *pglK*-deficient *pgl* operon and the AcrA protein, N-glycosylation was detected only when *Campylobacter* PglK was expressed (Fig. 1, lane 5). No glycosylation was detected when any of the investigated Wzx proteins were expressed (Fig. 1, lanes 1-4).

Results shown in chapter 2 could explain this finding, suggesting that WecA (or a protein with equivalent function) could be required for the activity of the Wzx proteins here analysed.

![Fig. 1: Complementation of PglK deficiency with Wzx proteins from *Salmonella*, *Pseudomonas*, *Shigella* and *E. coli*. Periplasmic extracts prepared from *E. coli* SCM7 cells carrying the AcrA expression plasmid and the *pglK*-deficient *pgl* operon (lanes 1-5) complemented with a plasmid expressing *pglK* (lane 5), *S. enterica* wzx (lane 1), *P. aeruginosa* wzx (lanes 2), *S. flexneri* wzx (lane 3) or *E. coli* O157 wzx (lane 4) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Glycosylated AcrA was detected with the glycoprotein specific R12 antisera. The position of bands corresponding to monoglycosylated (g1AcrA) and diglycosylated AcrA (g2AcrA) is indicated.](image-url)
2.2 *E. coli* WeeD is the candidate enzyme that complements *Campylobacter* PglD

The MALDI-TOF MS analysis of the N-glycan transferred to AcrA in presence of *pglD* mutant (see Fig. S3A-B lane 3 in chapter 2) gave an unexpected result, since hexosamine instead of a bacillosamine at the reducing end of the oligosaccharide was detected (Fig. 2). Apparently, this result was in contradiction with data obtained by Linton et al (Linton, 2005), who have found a bacillosamine residue at the reducing end of the oligosaccharide when the *Campylobacter* protein Peb3 was glycosylated in presence of the *pglD* mutant *pgl* operon. The reason of our different result is, most probably, due to the different *E. coli* strain we used in our experiment. In fact, the genetic background of *E. coli* SCM7, compared with *E. coli* BL21 used by Linton and coworkers, misses the *wecD* gene together with the rest of the *wec* cluster. In the biosynthetic pathway of the *wec*-encoded ECA, WeeD transfers an acetyl group in position 4 of a 4-amino-4,6-dideoxi-L-galactopyranose residue, while during bacillosamine biosynthesis PglD transfers an N-acetyl group in position 4 of a 4-amino-2-acetamido-2,4,6-trideoxi-D-glucopyranose residue. Because of the equivalent orientation of the aminogroup in the two molecules, we formulated the hypothesis that WeeD was responsible for the PglD-complementation previously observed in *E. coli* BL21.

![Fig. 2: MALDI-TOF MS of glycosylated peptide derived from tryptic cleavage of AcrA in presence of *pglD* deficient *pgl* operon.](image)

The arrow indicates the position of the HexNAc-peptide at 1931.8.
2.3 PglK cannot complement the O9-ABC transporter Wzm-Wzt

The relaxed specificity shown by PglK in the O16 and O7-antigen complementation, together with its ABC-transporter features, suggested that it may also show activity in the biosynthesis of the polymannan O9 antigen.

An ABC-transporter, made of two subunits Wzt-Wzm, is responsible for the transfer of the lipid-linked O9 homopolymer across the bacterial inner membrane. *E. coli* strain CWG638 (Clarke, 2004), a mutant with deletions of both the membrane translocase (Wzm) and the ATPase subunit (Wzt) encoding genes, provided the O9-LPS deficient genetic background. The PglK expressing plasmid (pCA1) or the vector control (pBAD) were introduced in *E. coli* CWG638 and the O9-LPS biosynthesis analysed after mannose and arabinose have been added or not to the culture. The *E. coli* wt E69 strain (Orskov, 1977) and the O9-LPS deficient *E. coli* strain 44 (Jayaratne, 1994) transformed with the empty vector (pBAD), were used as positive and negative control for the O9-LPS production, respectively. No difference was observed between *E. coli* CWG638 containing PglK and *E. coli* CWG638 containing the empty vector, independent of mannose and arabinose addition (Fig. 3, lanes 7-8, and lanes 3-4). As expected, a strong O9-LPS signal corresponded to *E. coli* E69 (Fig. 3, lanes 1 and 5) and no signal was detected in *E. coli* 44 (Fig. 3, lanes 2 and 6).

Since, PglK protein was normally expressed (data not shown) in strain CWG638, we concluded that, in contrast to the heteropolymeric O-antigen pathway, PglK was not active in the homopolymeric polymannan O-antigen pathway.
Fig. 3: PglK expression does not complement the \textit{Wzm-Wzt} deletion of the O9-LPS cluster. LPS prepared from \textit{E. coli} strains: E69 carrying the vector control, arabinose not-treated and treated (lanes 1 and 5, respectively); 44 carrying the vector control, arabinose not-treated and treated (lanes 2 and 6, respectively); CWG638 transformed with PglK plasmid, arabinose not-treated and treated (lanes 3 and 7, respectively), or with the vector control arabinose not-treated and treated (lanes 4 and 8, respectively). O9 LPS was detected using O9 specific antiserum after 12\% SDS-PAGE and transfer to nitrocellulose membranes.
Materials and methods

Bacterial strains and growth conditions
E. coli strains SCM7, E69 (E. coli O9a:K30), E. coli CWG638 (E69 derivative, wzm-wzt::aphA-3) and E. coli 44 (E69 derivative, deficient in O-antigen biosynthesis) were grown in Luria Bertani medium at 37°C. Ampicillin (100μg/ml), tetracycline (20 μg/ml) and kanamycin (50 μg/ml) were added to the media as needed.

Protein expression and immunodetection
Plasmids pJV4, pJV5, pJV6 and pJV7 were used to express Wzx protein from S. enterica, S. flexneri, P. aeruginosa and E. coli O157, respectively (Marolda, 2004). Plasmids pLMA3 and pCA1 used, respectively, to express AcrA and PglK, and plasmids expressing the pglD and pglK-deficient pgl operon, were described in chapter 2.

Arabinose-inducible expression of PglK and Wzx proteins was achieved by adding arabinose at a final concentration of 0.2% (w/v) to E. coli cells grown in Luria Bertani medium up to an OD_{600} of 0.3. The same amount of arabinose was added again 5 h post induction, and incubation continued for 15 h.

Periplasmic extracts were prepared by lysozyme treatment (Feldman, 2005), and 10 μl of the final sample (corresponding to 0.2 OD_{600} units of cells) was analyzed by SDS-polyacrylamide gel electrophoresis.

After being blotted on nitrocellulose membrane (Protran Bioscience, Dassel Germany, pore size 0.45mM), the sample was immunostained with R12 antisera as described (Aebi, 1996).

MALDI-TOF MS
The MALDI-TOF MS analysis of the N-glycan transferred to AcrA in presence of pglD mutant was performed as described in chapter 2.
LPS analysis

The O9 LPS production was achieved by adding mannose at a final concentration of 0.4% (w/v) to *E. coli* cells grown in Luria Bertani medium up to an OD$_{600}$ of 0.3.

The LPS analysis was performed as already described in chapter 2. The antiserum against *E. coli* O9 was a gift from C. Whitfield.
References


CHAPTER 3

The N-glycosylation systems of *C. jejuni* and *W. succinogenes* can be functionally combined
Introduction

Based on genomic sequence data, potential N-glycosylation was reported to take place in *Wolinella succinogenes* (Baar, 2003), an organism belonging to the epsilon (ε) subclass of proteobacteria together with the group of Campylobacteriaceae and Helicobacteriaceae. In the *Wolinella pgl* locus, the order is identical for 10 of the 13 *Campylobacter-pgl* cluster gene homologues. The main differences are the exchanged positions of *pglH* and *pglI*, and the location of the homologues of *pglG* and *galE*, which are found outside of the cluster (Fig. 1). In addition, five genes are found interspersed between *pglA* and *pglB*, as well as *pglJ* and *pglII*. The latter insertion harbors four genes, two of which are also clearly related to protein glycosylation. The presence of these additional genes, a galactosyl transferase (WS0046) and a glycosyl transferase (WS0047), might be indicative for the formation of an oligosaccharide that deviates in its structure from the heptasaccharide described for *C. jejuni* (Wacker, 2002; Young, 2002).

Fig. 1: Gene schematic comparing bacterial N-linked protein glycosylation loci (Szymanski, 2005). Schematic representation of the conserved N-linked protein glycosylation gene clusters in *C. jejuni* and *W. succinogenes*. 
Materials and Methods

Bacterial strains, growth conditions and plasmids

*E. coli* strains were grown on Luria Bertani medium at 37°C. Chloramphenicol (20ug/ml), tetracycline (20ug/ml), kanamycin (50ug/ml) and ampicillin (100ug/ml) were added to the media as needed. Bacterial strains and plasmids used are listed in table I.

**Construction of *Wolinella*-WlaB expressing plasmid, pCAW**

The *wlaB* gene was amplified by PCR with oligonucleotides *W-wlaB*-Neol fw (5’ AACCATGGTGCCAAACTCCGCACTCTTCTCACC 3’) and *W-wlaB*-EcoRI rv (5’ AAGAATTCAACTCTTCATGCACCTCCCTCCCTTTGATGCGG 3’) from a cosmid encoding the entire *Wolinella*-pgl locus (gift from S. Schuster, see table I). The amplicon was digested with *EcoRI* and *Neol* and ligated into *EcoRI*-Neol-cleaved pBAD/Myc-His6 (Invitrogen Carlsbad, California). This resulted in plasmid pCAW encoding the *Wolinella*-WlaB protein with a C-terminal Myc and His6 tag.

**Protein expression and immunodetection**

Arabinose inducible expression of *Wolinella*-WlaB was achieved by adding arabinose at a final concentration of 0.2% (w/v) to *E. coli* cells grown in Luria Bertani medium up to an OD_{600} of 0.3.

Expression of Pgl-proteins encoded by *W. succinogenes* pgl-cosmid was performed by adding IPTG at a 0.5 mM final concentration when the culture reached an OD_{600} of 0.3. Induction was maintained for 6 hours.

Preparation of total *E. coli* cell extracts and immunodetection analysis were performed as described in chapter 2.
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**Table 1:** Strains and plasmids used in this work.
Results

N-glycosylation of a Campylobacter protein mediated by W. succinogenes pgl operon

The similarity between the Campylobacter and Wolinella pgl clusters was exploited by investigating the ability of the Wolinella N-glycosylation pathway to glycosylate the Campylobacter protein AcrA. Total extracts from E.coli BL21 expressing AcrA in presence or absence of a cosmid carrying the Wolinella-pgl operon, were analyzed by immunoblot using anti-AcrA antiserum. Based on the mobility shift, the Wolinella pgl cluster was functional with the heterologous protein AcrA. In fact, unglycosylated, mono and diglycosylated AcrA forms were detected in presence of the Wolinella pgl cluster (Fig. 2, lane 1), whereas only the unglycosylated AcrA was detected in absence of the Wolinella pgl cluster (Fig. 2, lane 2). However, the glycosylated forms of AcrA did not react with the R12 antiserum (data not shown), suggesting the presence of a different glycan structure.

Fig. 2: AcrA N-glycosylation mediated by the W. succinogenes pgl operon.

Total extracts prepared from E. coli BL21 cells carrying the AcrA expression plasmid and the Wolinella pgl cluster (lane 1), or the AcrA expression plasmid alone (lane 2), were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. AcrA and glycosylated proteins were detected with anti-AcrA antiserum. The position of bands corresponding to unglycosylated (AcrA), monoglycosylated (g1AcrA), and diglycosylated AcrA (g2AcrA) is indicated.
Campylobacter PglK complementation with Wolinella-pgl encoded WlaB

The high percentage identity (49.1%) and similarity (73.4%) between C. jejuni PglK and W. succinogenes WlaB was exploited to investigate the ability of Wolinella-WlaB to rescue the glycosylation-deficient phenotype of the Campylobacter pglΔpglK locus. E. coli SCM7 cells were transformed with pgl or pglΔpglK plasmid, the AcrA expressing plasmid (pLMA3) and a plasmid expressing Wolinella WlaB under the control of a promoter inducible with arabinose (pCAW). Periplasmic extracts were analyzed by immunoblot using R12 antiserum. Mono and diglycosylated AcrA protein were detected in presence of the wild-type pgl operon (Fig. 3, lane 1) or in cells where the pglK deficiency was complemented by the Wolinella WlaB expression plasmid pCAW (Fig. 3, lane 2), whereas glycosylated AcrA was not detected in the absence of functional flippase activity (Fig 3, lane 3).

![Fig. 3: C. jejuni PglK complementation with Wolinella WlaB.](image)

Periplasmic extracts prepared from E. coli SCM7 cells carrying the AcrA expression plasmid, the pgl operon (lane 1) or a pglK-deficient pgl operon (lanes 2-3), complemented with the vector control (lanes 1 and 3) or with a Wolinella WlaB expressing plasmid (lane 2), were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Glycosylated AcrA was detected with R12 antiserum. The position of bands corresponding to monoglycosylated (g1AcrA) and diglycosylated AcrA (g2AcrA) is indicated.

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Discussion

A second example of a bacterial N-glycosylation pathway was recently discovered in *W. succinogenes*. Although the *Wolinella pgl* operon has not been investigated in such a detail as the *Campylobacter* operon, we analyzed if besides the genetic similarity between the two *pgl* operons the corresponding bacterial N-glycosylation reactions had similar requirements.

Firstly, the ability of *Wolinella* WlaB to rescue the N-glycosylation deficiency of the *Campylobacter pglApglK* operon (Fig. 3) suggested similar substrate recognition requirements for the two flippases. Secondly, the *Wolinella pgl*-dependent glycosylation of a *Campylobacter* protein (AcrA) (Fig. 2) implied conserved features between the two glycosylation systems in the recognition of the target protein.

The inability of R12 to detect *Wolinella pgl*-dependent glycosylation, suggested that this glycan structure differs from the one produced by the *Campylobacter pgl* locus, as it was predicted from the presence of extra glycosyltransferase-encoding genes in the *Wolinella pgl* locus.

Future studies, addressing the structure of the N-linked oligosaccharide produced by the *Wolinella pgl* pathway and the nature of *Wolinella*-encoded N-glycosylated proteins, will give more insight into the understanding of this new N-glycosylation pathway and its similarity with the *Campylobacter* N-glycosylation pathway.
References


CHAPTER 4

An ELISA-based assay for detection and quantification
of glycoprotein production

Cristina Alaimo, Laura Morf and Markus Aebi

(To be submitted)
Abstract

The discovery of different glycoprotein biosynthetic pathways in bacteria and the important role of the glycan moiety in bacterial pathogenesis make bacterial glycoengineering a promising field for future researches. Studies concerning glycoprotein production or inhibition require the development of new glycoprotein detection techniques. Here we describe the development of an accurate and reproducible ELISA-based method for the detection of His-tagged glycoproteins. Due to the high sensitivity of this method, there is no need for sample purification or concentration. Many samples can be analyzed concurrently, and the method can be applied to automated screening systems. Compared to other techniques, such as immunodetection, the method is faster, has higher throughput, and is more reliable and more sensitive.
Introduction

Detection of glycoproteins is generally performed by immunodetection or band-shift analysis. Both techniques do not allow glycoprotein-quantification and are not suitable to screen glycoprotein production in large scale.

An ELISA-based approach to detect glycosylated proteins in biological samples with high sensitivity and without need to perform additional protein purification steps is the focus of this work. The ELISA system described was applied to detect and discriminate different levels of glycoprotein biosynthesis and was successfully applied to the detection of different protein-linked glycan moieties.

To validate this procedure we took advantage of the bacterial protein-glycosylation system that has been well established (Feldman, 2005; Linton, 2005; Wacker, 2002). In particular, the N-glycosylation system from C. jejuni and the E. coli lipopolysaccharide (LPS) biosynthetic pathway were combined and resulting glycoprotein production analysed.
Materials and Methods

Bacterial strains and plasmids
All the bacterial strains and plasmids used are described in Table I.

AcrA expression
Cloning and expression of the periplasmic soluble and His-tagged version of AcrA protein (plasmid pWA2) was performed as described (Feldman, 2005).

Production of glycosylated protein
Protein glycosylation with the Campylobacter N-glycan was achieved according to the conditions described (Alaimo, 2006). Protein glycosylation with the E. coli O16 antigen was achieved according to the conditions described (Feldman, 2005).

Periplasmic extraction
Periplasmic extracts were prepared as described (Feldman, 2005).

AcrA-purification
Periplasmic extracts from E. coli Top10 cells (Invitrogen) expressing AcrA in presence of the pgl-operon were purified by a HisTrap HP column (Amersham Pharmacia Biosciences) as described (Feldman, 2005). One µg of the purified AcrA proteins was analyzed by 10% SDS-PAGE and visualized by Coomassie blue staining (data not shown). This allowed the relative abundance of the glycosylated protein fraction to be confirmed, since only the glycosylated protein was detectable.
The Ni-NTA coating of HisSorb™ 96 wells microplates (Quiagen) allows selective binding of His-tagged proteins. The binding reaction between the His-tagged protein and the Ni-NTA plate was performed by shaking over night at 4°C. Each sample was analysed in triplicate. Periplasmic protein extracts from *E. coli* SCM7 expressing AcrA, the *pgl*/*apglK* operon and a wild type or mutated version of *pglK* (Alaimo, 2006), were applied to Ni-NTA HisSorb™ Plates in volumes of 150 µl/well. Periplasmic extract from *E. coli* SCM7 expressing the *pgl* operon was used as negative control to eliminate the background due to any non specific binding of *E. coli* proteins to the wells and interaction with the antisera. After four washes with phosphate-buffer-saline containing 0.05% Tween (PBST 0.05%) the His-tagged protein bound to the wells was incubated with three different reagents in a volume of 150 µl/well; 1) rabbit-αAcrA antiserum, recognizing the total AcrA protein; 2) rabbit-R12 antiserum, binding to the glycan moiety; 3) biotinylated SBA lectin, recognizing the terminal GalNAc residue of the glycan moiety. After 2 h incubation at room temperature, the unbound antiserum/lectin was removed by washing four times with PBST 0.05%. Plates were then incubated with 150 µl/well of the secondary antibody: rabbit IgG-HRP for αAcrA or R12 antisera, and streptavidin-conjugated peroxidase for the biotinylated SBA lectin. After 2 h incubation at room temperature, the unbound reagent was removed as described above. Finally, the amount of bound protein was measured by addition of the substrate-chromogen mixture (150 µl/well) made of 0.012% H₂O₂ and 10 mg 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonicacid) (ABTS) in 0.1 M NaH₂PO₄ pH 4. Colour development was measured at 405 nm (Spectra Max plus, Bucher Biotec, Basel CH). The kinetics of the reaction was followed for 60 min, taking measurements every 5 minutes.

The same procedure was followed to detect the protein glycosylated with the O16 antigen of *E. coli*. In this case, a 1:100 dilution of the periplasmic extract, from *E. coli* CLM24 strain expressing AcrA, the wild type or the mutated *Campylobacter* oligosaccharyltransferase PglB, and the rhamnosyltransferase enzyme (pMF19), was applied on the plate and an anti-O16 antiserum was used to detect the glycosylated protein.
**Antisera/lectin**

Anti-AcrA antiserum (Wacker, 2002) was used in the ELISA assay to detect total AcrA protein bound to the wells, and in the western blot analysis to detect non-glycosylated AcrA as well as AcrA glycosylated with the O16-antigen glycan. R12 antiserum (Wacker, 2002) and biotinylated soybean agglutinin (10 µg/ml) (SBA) (Vector Laboratories, Burlingame, CA) were used to detect the fraction of AcrA protein carrying the *Campylobacter* N-glycan. The antiserum against *E. coli* O16 (obtained from Laboratorio de Referencia de *E. coli*, Lugo, Spain) was used in the ELISA to detect the AcrA protein glycosylated with the *E. coli* O16-antigen polymer. Rabbit IgG-HRP (0.2µg/ml) (Santa Cruz) was used as secondary antibody in combination with the anti-AcrA, R12 and *E. coli* O-antigen antisera. Streptavidin-conjugated peroxidase (5µg/ml) (Vector Laboratories, Burlingame, CA) was used as secondary antibody in combination with the biotinylated SBA lectin. All antibodies were diluted in PBS containing 0.2%BSA.

**Protein immunodetection**

Protein immunodetection was performed as described (Aebi, 1996).
Results/Discussion

In order to develop and validate the procedure with different amounts of glycoproteins, the N-glycosylation pathway of the bacterium Campylobacter jejuni (Szymanski, 1999; Wacker, 2002; Young, 2002) was exploited. 

E. coli SCM7 strain was transformed with a plasmid expressing the Campylobacter N-glycosylation pgI-operon and a plasmid expressing the Campylobacter protein AcrA. The glycosylated AcrA was purified from periplasmic samples and different glycoprotein-amounts were applied to ELISA-plates and detected with the SBA lectin (Fig. 1). The kinetics of the colour development was followed taking measures of each sample every 10 minutes for 1 hour. The different kinetics observed for the different samples corresponded to the increase in glycoprotein concentration. In fact, at the same time point increasing OD was measured corresponding to increasing amounts of protein (Fig. 1A). Data from the 10 minutes time point were collected to make a standard curve (Fig. 1B). This shows a concentration-dependent increase in the detected OD (405nm) up to a concentration of 2 µg, suggesting that this method may allow quantification of glycosylated proteins in the biological sample.

We have previously identified PglK as the ABC transporter protein responsible for transferring the lipid-linked-glycan across the inner membrane (Alaimo, 2006) in the Campylobacter N-glycosylation pathway. Mutations in the nucleotide binding domain (NBD) affect to different degrees the activity of PglK and, consequently, the glycosylation process. Production of the N-glycosylated Campylobacter protein AcrA in E. coli cells was analysed and quantified in presence of the pglK-deficient N-glycosylation pgl operon (pglApglK operon), complemented with wild type or mutant forms of PglK. The corresponding periplasmic samples were applied to the ELISA-plate and incubated with the R12 antiserum or the SBA lectin, in order to detect the glycoprotein fraction. At the same time the samples were incubated with the αAcrA antiserum to detect the total amount of AcrA proteins produced. The influence of the different PglK mutations on the level of glycoprotein production, previously observed by immunoblot analysis, was now confirmed by the ELISA results. In particular, we could now quantify the "glycosylation-efficiency" by measuring the ratio between the signal specific for
the glycosylated protein fraction (resulting from R12 or SBA detection) and the signal corresponding to the amount of the total protein bound to the well (given by αAcrA detection). The kinetics of the colour development was followed taking measures of each sample every 5 minutes for 1 hour (Fig. 2C). The 15 minutes time point was chosen to estimate the R12, SBA and αAcrA value of each sample and calculate their ratio (Fig. 2A and 2B). The ratio obtained for the negative control (pBAD, the empty vector), where no PglK was expressed and, consequently, no glycoproteins produced (Alaimo, 2006), was used as blank value and was subtracted from all the other ratios. The R12/αAcrA and the SBA/αAcrA ratios are expressed as percentages of the wt-PglK glycosylation efficiency.

Confirming the previous results of the immunodetection analysis (Alaimo, 2006), the highest glycosylation-signal was detected in presence of the wild type PglK followed by the R/C-PglK and K/A-PglK mutants. No significant glycosylation was detected in presence of the G/D-PglK mutant. Noteworthy, the two different detection systems utilized, the R12 antiserum and the SBA lectin, gave comparable results, although SBA was less sensitive than R12.

To test the efficacy of this technique in detecting glycoproteins with different glycan moieties, production of AcrA glycosylated with the O16 antigen was investigated. Feldman and coworkers have shown that, in the E. coli CLM24 strain, AcrA-glycosylation with the O16 antigen depends on the presence of the functional oligosaccharyltransferase PglB (Feldman, 2005). Periplasmic fractions from E. coli CLM24 cells expressing AcrA protein in presence of a functional or a mutated version of the oligosaccharyltransferase PglB (PglB+ and PglB-, respectively) were analysed by western blot and with the ELISA assay in order to detect the fraction of protein glycosylated with the bacterial O16 antigen. The PglB-dependent O16-glycosylation of AcrA was first analysed by immunodetection with αAcrA antiserum (Fig 3A), in order to confirm that the covalent attachment of the O16-antigen polymer to AcrA (Fig. 3A; ladder-like structure indicated with *) occurred only in presence of PglB. Then, the ELISA was performed using the αO16 antiserum, in order to detect and quantify the AcrA-fraction glycosylated with the O16-antigen. The kinetics of the colour reaction for the sample expressing O16-glycosylated AcrA (PglB+) and non
glycosylated AcrA (PglB-), are shown in Fig. 3B. The non-specific binding of the lipid-linked O16-antigen to the plate could be the reason for the background signal detected in the presence of the non functional PglB (PglB-). In this condition no glycoprotein has been produced, as shown by the anti-AcrA immunodetection (Fig 3A).

Glycoengineering is becoming a subject of great interest, as new studies have proposed that bacterial systems may be useful tools for glycoprotein production (Feldman, 2005; Szymanski, 2005; Wacker, 2002). In particular, development of inhibitors of protein-glycosylation or recombinant glycoprotein-vaccines are among the possible applications of bacterial glycoengineering, due to the significant role of the glycan moiety in several bacterial infections (Bacon, 2001; Hooper and Gordon, 2001; Logan, 2002; Szymanski, 2002; Hamadeh, 1995; Lindenthal, 1999; Schirm, 2003; Benz, 2001). The ELISA-based method described here may be a useful tool for such studies, as it allows detection and quantification of glycoprotein production with the only requirement of a His tagged protein and a primary antiserum or lectin specific for the glycan of interest. This method is more accurate and reproducible than western blot analysis, since the entire kinetics of the detection reaction is visualized. Additionally, 96 microwell plates allow many samples to be processed simultaneously, an advantage required for large-scale glycosylation screens.
References


Fig 1: Quantification of AcrA glycosylation.

Glycosylated His-tagged AcrA was purified from periplasmic samples by a HisTrap HP column and applied to the wells of the ELISA plate in different amounts.

A) The kinetics of colour development of glycosylated-AcrA samples is reported for each amount (in µg) listed in the legend on the right. B) AcrA-Standard curve; X-values are the amounts in µg of AcrA-proteins analysed, and Y-values the corresponding OD405-values collected from the 10 minutes time-point.

Detection of glycosylated AcrA was performed with the SBA lectin.
Fig. 2: ELISA-based quantification of AcrA-glycosylation in presence of PglK-NBD mutants. Periplasmic proteins prepared from *E. coli* SCM7 cells carrying the AcrA expression plasmid and the *pglK*-deficient *pgl* operon, complemented with wild type or mutant forms of PglK, were applied to the ELISA-plate, and the amount of glycoproteins produced was investigated. For each sample the ratio between the signal detected with R12 (A) or SBA (B) and the signal detected with αAcrA antiserum after the ratio-value of the negative control (pBAD vector) was subtracted. The R12/αAcrA and the SBA/αAcrA ratios are expressed as percentages of the wt-PglK glycosylation efficiency (100 %). Bars refer to the standard error. C) Kinetics of the colours development for the different samples during the 1 hour time course.
Fig. 3: ELISA-based detection of AcrA glycosylated with the O16 antigen. A) Periplasmic extracts from *E. coli* CLM24 expressing the O16-antigen and AcrA in presence of functional (+) or mutated PglB (-) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. O16-glycosylated AcrA (lane 1) and non glycosylated AcrA (lane 2) proteins detected with αAcrA antiserum. B) Kinetic-ELISA of the periplasmic extracts containing O16-glycosylated AcrA (PglB+) and non-glycosylated AcrA (PglB-). Colours development was followed for 1 hour after addition of the substrate-chromogen solution. For each sample, the values were determined every 10 minutes.
<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Description</th>
<th>Source</th>
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<tbody>
<tr>
<td>SCM7</td>
<td>SØ874, Δwec</td>
<td>Alaimo et al., 2006</td>
</tr>
<tr>
<td>CLM24</td>
<td>W3110, waaL</td>
<td>Feldman et al., 2005</td>
</tr>
<tr>
<td>pWA2</td>
<td>Soluble periplasmic hexa-His-tagged AcrA under control of Tet promoter, in pBR322, AmpR</td>
<td>Feldman et al., 2005</td>
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<td>pglΔpglK</td>
<td><em>C. jejuni</em> pgl cluster containing a kan cassette in <em>wlaB</em>, CmR, KanR</td>
<td>Linton et al., 2005</td>
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<td>pCA1</td>
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<td>Alaimo et al., 2006</td>
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<td>pglK-G488D in pBAD/Myc-HisA, AmpR</td>
<td>Alaimo et al., 2006</td>
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<td>pMF19</td>
<td>wbbL of <em>E. coli</em> O16 in pEXT21, SpcR</td>
<td>Feldman et al., 1999</td>
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<td>PACYC(pglmut)</td>
<td>Encodes the <em>C. jejuni</em> pgl containing mutations W458A and D459A in PglB, CmR</td>
<td>Feldman et al., 2005</td>
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<td>pMLBAD</td>
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<td>Lefebre &amp; Valvano, 2002</td>
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<td>pMAF10</td>
<td>IIA-tagged PglB cloned in pMLBAD, TmpR</td>
<td>Feldman et al., 2005</td>
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*Table I: E. coli* strains used in this study
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Curriculum Vitae

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PERSONAL DATA

Date of birth: April 01 1973
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EDUCATION

1991-1996 Graduate in Molecular Biology, University of Palermo, Department of Molecular and Developmental Biology.
Honors: Dissertation passed “with Distinction”.

Dissertation: “HCMV-DNA and genotype for diagnosis of Cytomegalovirus Congenital infection”

October 2002 Beginning of the PhD program. Supervisor: Prof. Markus Aebi, Institute of Microbiology, ETH Zürich. Project title: “Oligosaccharyl flipping in the N-glycosylation pathway of Campylobacter jejuni”

FELLOWSHIPS

1996 – 2000 Student Fellowship, Institute of Microbiology of Palermo, from the MURST, Ministero dell’Università per la Ricerca Scientifica e Tecnologica.
1999 Fellowship from the Italian Department for Health to follow and characterize Rotavirus epidemiology in Italy.

PREVIOUS WORKING EXPERIENCE

2001-2002 Chiron Vaccines Research Center

2000 Children’s Hospital of Palermo: research project regarding “Genetic predisposition in Coeliac Disease”. Screening study of a school population, families of celiac patients and celiac patients.

1999 Institute of Microbiology of Palermo: Rotavirus Epidemiology study for the Italian Department for Health.

PUBLICATIONS AND PAPERS

• Interplay of the Wzx translocase and the corresponding polymerase and chain length regulator proteins in the translocation and periplasmic assembly of lipopolysaccharide O antigen. Accepted in J Bac. Marolda CL, Tatar LD, Alaimo C, Aebi M, Valvano MA


LANGUAGES

**Italian** – native language; **English** – speak, read and write with good proficiency; **German** – speak, read, and write with basic competence.