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Functional analysis of the Helicobacter pullorum N-linked protein glycosylation system

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Abstract

N-linked protein glycosylation systems operate in species from all three domains of life. The model bacterial N-linked glycosylation system from Campylobacter jejuni is encoded by pgl genes present at a single chromosomal locus. This gene cluster includes the pglB oligosaccharyltransferase responsible for transfer of glycans from lipid carrier to protein. Although all genomes from species of the Campylobacter genus contain a pgl locus, among the related Helicobacter genus only three evolutionarily related species (H. pullorum, H. canadensis and H. winghamensis) potentially encode N-linked protein glycosylation systems. Helicobacter putative pgl genes are scattered in five chromosomal loci and include two putative oligosaccharyltransferase-encoding pglB genes per genome. We have previously demonstrated the in vitro N-linked glycosylation activity of H. pullorum resulting in transfer of a pentasaccharide to a peptide at asparagine within the sequon (D/E)XNXS/T. In this study, we identified the first H. pullorum N-linked glycoprotein, termed HgpA. Production of histidine-tagged HgpA in the background of insertional knockout mutants of H. pullorum pgl/wbp genes followed by analysis of HgpA glycan structures demonstrated the role of individual gene products in the PglB1-dependent N-linked protein glycosylation pathway. Glycopeptide purification by zwitterionic-hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry identified six glycosites from five H. pullorum proteins, which was consistent with proteins reactive with a polyclonal antiserum generated against glycosylated HgpA. This study demonstrates functioning of a H. pullorum N-linked general protein glycosylation system.

Key words: bacteria, glycoprotein, glycosylation, Helicobacter, N-linked

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Introduction

In all three domains of life subsets of proteins are modified by the covalent attachment of sugars to asparagine residues within a conserved consensus sequon of N-X-S/T (Calo et al. 2010; Nothaft and Szymanski 2010; Larkin and Imperiali 2011; Schwarz and Aebi 2011; Eichler 2013). Among Bacterial species, two such distinct N-linked glycosylation systems have been identified. The first involves attachment of monosaccharide to asparagine by a cytoplasmic N-glycosyltransferase and has been characterized in Haemophilus influenzae (Grass et al. 2003, 2008, 2010) and Actinobacillus pleuropneumoniae (Choi et al. 2010; Kawai et al. 2011; Schwarz et al. 2011a; Naegeli et al. 2014; Cacciu et al. 2017), with further N-glycosyltransferase orthologues identified in a number of other species including pathogenic Yersinia spp., and enterotoxigenic Escherichia coli (Grass et al. 2010). The second type of bacterial N-linked protein glycosylation system was discovered in Campylobacter jejuni (Szymanski et al. 1999) and subsequently other related species from the Epsilon subdivision of the Proteobacteria (Nothaft and Szymanski 2010). In this system cytoplasmic assembly of an oligosaccharide on an isoprenoid lipid, is followed by transfer across the inner membrane and attachment onto proteins in the periplasm mediated by an integral membrane oligosaccharidyltransferase (OTase). The prototypical OTase-dependent N-linked protein glycosylation system of C. jejuni has been extensively studied. More than 60 extracytoplasmic proteins are known to be glycosylated within an extended N-X-S/T sequon containing an acidic residue (D/E) at the −2 position (Kowarik et al. 2006; Wacker et al. 2006; Chen et al. 2007; Gerber et al. 2013) although examples of non-classical occupied sequons (without the D/E at the −2 position or S/T at the +2 position) have also been demonstrated (Scott et al. 2014). A single locus contains genes required for biosynthesis, transport and linkage of a conserved heptasaccharide to protein. Five cytoplasmic glycosyltransferases (PglA, PglC, PglJ, PglH and PglI) assemble the heptasaccharide on the lipid carrier (Glover et al. 2005a; Linton et al. 2005; Glover et al. 2006). This is transported across the inner membrane and into the periplasm by the “flipase” PglK (Alaimo et al. 2006) and transferred onto protein by the OTase, PglB (Wacker et al. 2002; Glover et al. 2005b; Lizak et al. 2011). Three further proteins (PglD, PglE and PglF) are required for biosynthesis of the reducing end sugar, a diacetamidotrideoxyhexose known as di-acetyl bacillosamine (diNACb), from N-acetyl glucosamine (Olivier et al. 2006). The C. jejuni pgl gene locus when expressed in E. coli results in N-linked protein glycosylation (Wacker et al. 2002) and can be used to glycosylate an array of target proteins with diverse glycan (Feldman et al. 2005; Iwashiki et al. 2012; Jervis et al. 2012). The specificity of the C. jejuni PglB for lipid-linked oligosaccharides (LLOs) with an acetamido group on the C-2 carbon of the reducing end sugar and accessibility of the target sequon on the surface of the folded target protein, are the major limitations of this approach to generating N-linked glycoproteins of choice.

Characterization of further bacterial PglBs has led to identification of OTases from many Campylobacter species (Jervis et al. 2012; Nothaft et al. 2012), Desulfovibrio desulfuricans (Kelm insi and Feldman 2011) and deep sea vent dwelling organisms Nitratireductor tergaricus, Sulfitobacter lithotrophicus and Deferribacter desulfuricans with some displaying differing sequon recognition and glycan promiscuity (Mills et al. 2016). In addition to PglB genes, pgl gene-containing genetic loci are present in the Campylobacter species genomes sequenced to data (Nothaft and Szymanski 2010), and the structures of many of the corresponding N-linked glycans were characterized (Jervis et al. 2012; Nothaft et al. 2012). Although the genera Campylobacter and Helicobacter are closely related, orthologues of the Campylobacter pgl genes are absent in genomes of most Helicobacter species, including Helicobacter pylori. However, pgl gene orthologues are present in a single evolutionarily related group of three Helicobacter species: Helicobacter pullorum, Helicobacter canadensis and Helicobacter winogradensis (Jervis et al. 2010). In contrast to Campylobacter species, the Helicobacter pgl genes are scattered around five loci (Figure 1). A further significant deviation from the C. jejuni model is the presence in these Helicobacter species of not one but two pglB genes potentially encoding distinct putative N-linked OTases. In our previous work we have demonstrated that a H. pullorum membrane extract is capable of in vitro N-linked peptide glycosylation with a linear pentasaccharide glycan consisting of HexNAc-216-217-217-HexNAc where 216 and 217 represent the mass differences between species generated by glycan fragmentation and correspond to residues with MH+ values of 217 and 218 Daltons, respectively. Peptide N-glycosylation was H. pullorum pglB1 dependent and required an acidic residue at the −2 position of the sequon as for C. jejuni (Jervis et al. 2010). In this more in-depth study, we have demonstrated the in vivo functioning of a H. pullorum PglB1-dependent N-linked general protein glycosylation pathway.

Results

H. pullorum N-linked protein glycosylation loci
Orthologues of C. jejuni pglABCDEFHIJK genes that encode the well-characterized N-linked protein glycosylation system are present
in *H. pullorum* (Figure 1) as well as the closely related species *H. canadensis* and *H. umbilicalis* but not in other *Helicobacter* species. These *Helicobacter* species also possess a putative glycosyltransferase-encoding gene located between *pglI* and *pglJ* that is absent in *C. jejuni* and is here designated *pggL* (Figure 1). Two further genes co-located with *Helicobacter* *pgl* genes also lack *C. jejuni* orthologues. Their predicted products have significant levels of sequence similarity to WbpOS enzymes involved in sugar biosynthesis (King et al. 2010) and we have thus named them *wbpO* and *wbpS* (Figure 1). In contrast to *C. jejuni* and the majority of *Campylobacter* species where *pgl* genes are located in a single locus, in *Helicobacter* species these genes are present in five distinct loci. A notable feature of the *Helicobacter* *pgl* gene loci is the presence of two orthologues (*pglB1* and *pglB2*) of the single *Campylobacter* *pglB* gene encoding the *OTase* (Figure 1). Amino acid sequence alignment of the *Helicobacter* PglBs with the structurally and mechanistically characterized PglB of *Campylobacter lari* and the well-characterized *C. jejuni* PglB showed a high degree of conservation of the known essential residues for oligosaccharyltransferase activity. Catalytically active residues D$_{54}$, R$_{145}$, D$_{152}$, D$_{154}$, E$_{316}$ and R$_{372}$ and the 455WWD457 motif required for peptide binding (Lizal et al. 2011, 2013; Gerber et al. 2013) are all absolutely conserved in the *Helicobacter* PglB1 and PglB2 enzymes (Figure S1). This strongly indicates both PglB1 and PglB2 possess oligosaccharyltransferase or related activity with potentially two distinct N-linked protein glycosylation systems operating in *H. pullorum*. To investigate N-linked protein glycosylation in this species we first determined whether both *pglB1* and *pglB2* genes were expressed. Specific intragenic primers were designed for both genes (Table S1) and RT-PCR used to detect corresponding transcripts. The generation of RT-PCR products of the predicted sizes (Figure S2) indicates both genes were transcribed during in vitro growth. To demonstrate N-linked OTase activity of *H. pullorum* PglBs we expressed both genes in the background of a *C. jejuni* *pglB* insertional knockout mutant (*pglB::aphA*). In this N-linked, mutant glycoproteins are not produced and we propose that the presence of lipid-linked heptasaccharide and numerous sequon-containing target proteins provides a sensitive and convenient assay for detecting related N-linked OTase activities. The *pglB* genes were recombined onto the *C. jejuni* *pglB::aphA* chromosome within pseudogene Cj0223 (see Methods), and complementation in this way with the *C. jejuni* *pglB* gene fully restored glycosylation as detected by reactivity of numerous proteins with the N-linked heptasaccharide specific antisera hR6 (Figure 2). Complementation with the *H. pullorum* *pglB1* gene also restored hR6 immunoreactivity though relatively few proteins were glycosylated (Figure 2). In contrast, the *pglB2* gene did not restore detectable levels of hR6 immunoreactivity (Figure 2). These data confirm that *pglB1* encodes an N-linked OTase able to transfer the *C. jejuni* heptasaccharide glycan onto protein whilst the activity of PglB2 remains elusive.

**Identification of *H. pullorum* N-linked glycoproteins**

To directly demonstrate activity of the *H. pullorum* N-linked protein glycosylation system we sought to identify corresponding N-linked glycoproteins. Initial efforts to identify a lectin that interacts with such glycoproteins were unsuccessful (data not shown). We therefore undertook an unbiased approach based on glycopeptide enrichment and site-specific identification using MS/MS. Whole cell protein lysates were digested with trypsin and glycopeptides enriched using ZIC-HILIC prior to identification by CID MS/MS to provide N-glycan structural information and HCD MS/MS to identify the peptide backbone (Scott et al. 2011). We identified 62 glycopeptides modified with the HexNAc-216-217-217-HexNAc pentasaccharide (Figure S3, Table SIV), which represent six confirmed sites of N-glycosylation from five *H. pullorum* proteins (Table I). These proteins are predominantly of unknown function, however all are predicted periplasmic or membrane-associated proteins. Given that many *C. jejuni* N-linked glycoproteins have now been identified (Scott et al. 2011), we searched the five confirmed *H. pullorum* glycoprotein sequences against the *C. jejuni* NCTC11168 genome. *C. jejuni* contained an orthologue for all five *H. pullorum* glycoproteins (Table I), with sequence identities of between 24.0% and 38.0% (data not shown). Examination of the literature confirmed that four of the five orthologues are known *C. jejuni* glycoproteins, with only Cj1259 as an unknown N-glycoprotein. Cj1259 is the major outer membrane protein (PorA or MOMP) in *C. jejuni* and the NCTC11168 sequence contains no N-linked sequons. MOMP has however, recently been identified as a unique O-glycoprotein modified with a four residue glycan at a single threonine residue (Mahdavi et al. 2014). We additionally noted that *H. pullorum* glycoprotein Hp00510 is an orthologue of the *C. jejuni* N-linked glycoprotein A or Cgpa (Wacker et al. 2002). We therefore named this protein HpA (Helicobacter glycoprotein A). HpA is a predicted periplasmic protein with a single N-linked glycosylation sequon of ENNDT and is annotated as HPMG_01281 in the *H. pullorum* MIT 98-5489 genome sequence. To further investigate N-linked glycosylation of HpA, the corresponding gene was cloned and expressed in *E. coli* from plasmid pQEhgpA (Table SII and Methods) along with the *C. jejuni* *pgl* locus on a second plasmid (Wacker et al. 2002). Western blotting with *C. jejuni* N-linked heptasaccharide glycan-specific antisera hK6 demonstrated that HpA was glycosylated in a *C. jejuni* PglB-dependent manner (Figure 3). The HpA protein was similarly glycosylated in *E. coli* by *H. pullorum* PglB1, but not PglB2 (Figure 3). Glycosylation of HpA by both *C. jejuni* PglB and *H. pullorum* PglB1 was dependent on presence of
Identification of *H. pullorum* genes involved in HgpA N-linked glycosylation

To identify genes involved in HgpA N-linked glycosylation, we constructed insertional knockout mutants in seven *H. pullorum* *pgl* genes from five loci (Figure 1). These mutants were constructed in the *H. pullorum* Hp47 genetic background that produces HgpAhis<sub>10</sub> (see above and Table SIII) and the relative electrophoretic mobility of the N-linked glycoprotein investigated by SDS-PAGE of whole-cell lysates followed by Western blotting with an anti-His antisera. HgpAhis<sub>10</sub> electrophoretic mobility increased when derived from a *pglB1* insertional knockout mutant compared to that derived from the wild-type strain indicating modification via a *pglB1* dependent pathway (Figure 4). Similar analyses of *H. pullorum* strains with disrupted glycosyltransferase-encoding *pglC*, *pglA*, *pglH* and *pglJ* genes resulted in increased but varying mobility of the corresponding HgpAhis<sub>10</sub> proteins in SDS-PAGE (Figure 4) indicating their involvement in assembly of the N-linked pentasaccharide. We also investigated *wbpO* and *wbpS* (Figure 1), products of which have significant levels of amino acid sequence identity (32% and 63% respectively) to *Pseudomonas aeruginosa* WbpO and WbpS involved in biosynthesis of 2-acetamido-2-deoxy-<i>D</i>-galacturonamide (GalNAcAN). In *P. aeruginosa*, WbpO converts UDP-GlcNAc to UDP-2-acetamido-2-deoxy-<i>D</i>-glucurionate (UDP-GlcNAcA), and the activated form of this sugar is then converted to UDP-GalNAcA by the isomerase WbpP (<i>P. aeruginosa</i> wbpS involved in biosynthesis of 2-acetamido-2-deoxy-<i>D</i>-galacturonamide (GalNAcAN)).

*Further characterization of HgpA N-linked glycosylation*

A C-terminal deca-histidine-tagged version of hgpA (HgpAhis) was recombined onto the *H. pullorum* NCTC 12824 chromosome via plasmid pHPC2hgpAhis (see Methods) to produce strain Hp47 (Table SIII). In order to verify that HgpAhis<sub>10</sub> was glycosylated, it was purified by nickel affinity chromatography (see Methods). ZIC-HILIC enrichment and MS/MS of tryptic peptides from HgpAhis<sub>10</sub> was performed in this peptide and the underlined Asn is predicted only.

**Table I. Identification of glycopeptides from *H. pullorum* using ZIC-HILIC and LC-MS/MS**

<table>
<thead>
<tr>
<th>Gene number</th>
<th>Protein identification</th>
<th>Protein mass (Da)</th>
<th>Precursor mass (Charge)</th>
<th>MASCOT score&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Peptide sequence&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>H00296c (Cj0114)</td>
<td>Tetratricopeptide Repeat Protein</td>
<td>35,661</td>
<td>1888.8647 (3+)</td>
<td>69.87</td>
<td>160KDTIKEDSVENNGSAPNA&lt;sup&gt;177&lt;/sup&gt;</td>
</tr>
<tr>
<td>H00314c (Cj1565c)</td>
<td>Flagellar Functional Protein</td>
<td>93,678</td>
<td>2371.1319 (3+)</td>
<td>27.25</td>
<td>175PNANANATIESAENNNQKESKQ&lt;sup&gt;196c&lt;/sup&gt;</td>
</tr>
<tr>
<td>H00510 (Cj1670c)</td>
<td>Hypothetical Protein (HpgA)</td>
<td>28,021</td>
<td>1896.9534 (3+)</td>
<td>56.58</td>
<td>42AHHYQQMILQKDPDEAEKKEIQALDDTLINYYEDDNATK&lt;sup&gt;665&lt;/sup&gt;</td>
</tr>
<tr>
<td>H00561c (Cj0633)</td>
<td>Hypothetical Protein</td>
<td>38,588</td>
<td>4941.5575 (6+)</td>
<td>37.78</td>
<td>44KEIPTPNENNDTKEIR&lt;sup&gt;59&lt;/sup&gt;</td>
</tr>
<tr>
<td>H01062c (Cj1259)</td>
<td>Putative Membrane Protein</td>
<td>56,457</td>
<td>1447.6162 (3+)</td>
<td>63.45</td>
<td>68TLQQENQTSQSTIPQIPTPSIQESKPTKPQIPSKPKPQCQK&lt;sup&gt;111&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>87DRVDNGNGDYNGSK&lt;sup&gt;100&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>MASCOT score represents the best score provided for the identified glycosylation sequon.

<sup>b</sup>Sequon is shown in italics, with glycosylation site in bold and underlined.

<sup>c</sup>Full sequon was not identified, with glycosylation site in underlined and italicised.
a presumed N-linked glycan of 1056 Da, consistent with previous data (Jervis et al. 2010). The intact mass value of 26,069 Da for HgpAhis, derived from the H. pullorum pglC insertional knockout mutant was also consistent with production of unmodified protein. Insertional knockout mutagenesis of four further H. pullorum genes (pglA, pglJ, pglH and wbpS) produced HgpAhis, proteins of masses 26,297, 26,488, 26,923 and 26,490 Da, respectively (Table II). These values were intermediate between those obtained for HgpAhis, from wild type and pgb1::aphA genetic backgrounds indicating modification with truncated glycans. The HgpAhis protein from the wbpO knockout mutant did not give consistent values in intact mass analysis experiments likely due to sample heterogeneity. The masses obtained for HgpAhis, proteins derived from mutant strains were consistent with their varying electrophoretic mobility, and combined intact mass and western blotting data demonstrate that H. pullorum pgl and wbp gene products are involved in HgpAhis, modification.

Structural characterization of N-linked glycans produced in mutant backgrounds

To further characterize the variety of HgpA modifications produced in pgl/wbp mutant backgrounds, purified HgpAhis6 proteins were digested with trypsin and peptides analyzed by MALDI-TOF MS. A number of peaks were observed corresponding to the predicted masses of HgpAhis-derived tryptic peptides confirming protein identity (data not shown). However, a peak at m/z 2429 was present in the spectrum for HgpAhis derived from the wild type H. pullorum but not from the pgb1 knockout. This m/z value corresponds to the mass of the predicted tryptic peptide containing the N-linked glycosylation sequon (EIPTPENNNDTK – 1371.4 Da) plus the mass of the H. pullorum pentasaccharide N-linked glycan (1056 Da). This peak was selected for fragmentation by MALDI-LIFT-TOF/TOF MS and the resultant spectrum (Figure 5) is consistent with the previously observed HexNAc-216-217-217-HexNAc pentasaccharide observed on an in vitro generated glycopeptide (Jervis et al. 2010) and present on glycopeptides identified above. Insertional knockout mutagenesis of the H. pullorum pglH gene results in production of an HgpAhis-derived glycopeptide with a tetrasaccharide structure, lacking the nonreducing end residue (Figure 5). Similarly, inactivation of the pglL gene resulted in production of an N-linked disaccharide glycan consisting of the first two sugars of the pentasaccharide (Figure 5). Inactivation of the pglA gene resulted in production of a monosaccharide N-linked glycan, although the spectrum produced indicated presence of a diNACBac rather than a HexNAc residue (Figure 5). In a pglC insertional knockout mutant there was no detectable N-linked glycan suggesting its role as the initiating transferase as seen in C. jejuni (Linton et al. 2005; Glover et al. 2006). These data indicate that biosynthesis of the H. pullorum N-linked pentasaccharide glycan involves sequential action of the PglC, PglA, PglJ and PglH glycosyltransferases and are entirely consistent with the intact mass analysis for each of the mutants described above. The MALDI-LIFT-TOF/TOF MS spectra generated from HgpAhis produced in both the wbpO and wbpS mutants indicated presence of a disaccharide N-linked glycan. In the wbpO mutant the disaccharide consisted of a diNACBac reducing end sugar and a HexNAc residue, whilst in the wbpS mutant the disaccharide was composed of a reducing end HexNAc and a HexNACAN residue, again consistent with intact mass data presented above (Figure 5).

**Demonstration of further H. pullorum N-linked glycoproteins**

To identify further N-linked glycoproteins, a polyclonal antiserum was raised against purified HgpAhis10 from H. pullorum (see Methods). When a wild type H. pullorum whole-cell lysate was probed with this antiserum a number of immunoreactive bands were observed (Figure 6, arrowheads). Major bands at 35–40 kDa are consistent with the predicted masses of proteins Hp00296c and Hp00561c identified by MS/MS (Table I), with additional minor bands above 50 kDa and approximately 90 kDa consistent with the identification of Hp01062c and Hp00314c, respectively. The majority of these bands (Figure 6; shaded arrowheads) were no longer detected in a pgb1 insertional knockout mutant indicating these represented glycoproteins glycosylated in a pgb1-dependent manner. Their immunoreactivity was also dependent on pglABC and wbpOS genes demonstrating their role in this general protein glycosylation pathway. In pglL and particularly pglH knockout backgrounds, bands retained some immunoreactivity suggesting that the antiserum recognizes the shortened glycans likely present on proteins in these backgrounds. Immunoreactivity of an approximately 28 kDa band was unaffected by pgl and wbp gene mutations, however the mobility of this band was increased in these mutants (Figure 6). This band likely represents HgpA detected by antibodies against both the N-linked glycan and the protein itself and this is
consistent with a predicted size of 26 kDa for glycosylated HgpA. The increased HgpA mobility in these backgrounds is presumably due to reduction in size of glycan structures, an interpretation consistent with data obtained above. These and previous data demonstrate the functioning of a H. pullorum PglB1-dependent general protein glycosylation system.

**Discussion**

It is well established that C. jejuni encodes a general N-linked protein glycosylation pathway and similar N-linked glycan structures have been identified in other Campylobacter species (Jervis et al. 2012; Nothaft et al. 2012). Less well characterized are the putative N-linked protein glycosylation systems present in a small number of species.

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**Fig. 5.** Tandem MALDI mass spectrometry of the HgpA tryptic (glyco)peptide derived from wild type H. pullorum and insertional knockout mutants of pglHJA and wbpO genes. Spectra produced from fragmentation of the tryptic glycopeptide (EIPTPNENDT – 1371.4 Da) containing the N-glycosylation site of the glycoprotein HgpAhis with inferred structures of the corresponding N-linked glycans below. The genetic backgrounds from which HgpA protein was purified are indicated top right of each spectrum.
from the related Helicobacter genus. The Helicobacter N-linked protein glycosylation systems are notable for the presence of two distinct OTase encoding pglB genes (Figure 1), with the H. pullorum PglB1 protein more similar to the C. jejuni PglB (31% amino acid sequence identity) than to H. pullorum PglB2 (23% identity). Through a variety of approaches in E. coli (Jervis et al. 2010), C. jejuni and H. pullorum we have demonstrated N-linked oligosaccharyltransferase activity of H. pullorum PglB1 but not PglB2. Furthermore evidence was provided for a H. pullorum PglB1 directed general protein glycosylation pathway (Figure 6). MS/MS analysis of enriched glycopeptides confirmed the H. pullorum N-glycan structure and identified six sites of glycosylation within five proteins. Intriguingly, only five of six glycopeptides contained the anticipated N-linked bacterial sequon D/E-X-N-X-S/T, with a second glycopeptide in Hp00296c containing a nonclassical sequon with a lysine at the +2 position (ENNQK; Table I). Recent work demonstrated that C. jejuni PglB is able to glycosylate nonclassical sequons with three examples identified in the NCTC 11168O strain (Scott et al. 2014). These included sequons lacking the D/E at position −2, or S/T at position +2, but never both. A similar phenomenon has been described for Campylobacter lari PglB (Schwarz et al. 2011b). For Hp00296c, we noted that the occupied nonclassical sequon is close to the identified “classical” sequon (N171 versus N190; Table I), suggesting that hierarchical site occupancy as observed for the C. jejuni lipoprotein JlpA (Scott et al. 2009) may occur, however this is yet to be determined. Knockout of several genes from the H. pullorum pgl gene loci that encode putative glycosyltransferases and enzymes involved in sugar biosynthesis altered the structure of the PglB1-dependent N-linked glycan (Figures 4 and 5). Based on these data we propose a model for H. pullorum N-linked protein glycosylation (Figure 7). The proposed activities of H. pullorum PglCAJHB1 and WbpOS are broadly consistent with those of their similarly named counterparts in C. jejuni (Glover et al. 2005a; Linton et al. 2005). The H. pullorum wbpS mutant produced an HgpA-linked disaccharide of HexNAc–HexNAcA (Figure 5). Again this is consistent with the predicted role for WbpS in the biosynthesis of UDP-HexNAcAN from UDP-HexNAcA in P. aeruginosa (King et al. 2010). Thus if the H. pullorum wbpS knockout mutant is

![Fig. 6. Immunoreactivity of whole-cell lysates from H. pullorum pgl and wbp gene mutants. Whole cell lysates from H. pullorum NCTC 12824 and corresponding pgIBC1AJH and wbpOS insertional knockout mutants were separated by SDS-PAGE, Western blotted and probed with a polyclonal antibody raised against purified HgpA glycoprotein. Shaded arrowheads indicate position of bands corresponding to putative glycoproteins whilst the unshaded arrowhead indicates the band corresponding to HgpA itself.](https://academic.oup.com/glycob/advance-article-abstract/doi/10.1093/glycob/cwx110/4798970)

![Fig. 7. Model of H. pullorum N-linked protein glycosylation pathways. Direct evidence for role of PglCAJHB1 and WbpOS is provided in this study. The proposed roles of PglDEFK and PglB2 are based on established function of C. jejuni orthologues. The H. pullorum PglB1-dependent N-linked pentasaccharide glycan is assembled through sequential action of glycosyltransferases PglCAJH, flipped into the periplasm by PglK and transferred to proteins as indicated in the periplasm by PglB1. A second proposed glycan, with a reducing end 228Da residue synthesized by PglDEF activity, is similarly assembled and flipped into the periplasm where it is transferred to as yet unidentified protein(s) via PglB2 activity.](https://academic.oup.com/glycob/advance-article-abstract/doi/10.1093/glycob/cwx110/4798970)
unable to synthesize HexNAcAN, the structurally related and bio-
synthetic precursor HexNAcA is transferred to the reducing end
HexNAc in its place with the PgB1 transferase presumably unable to
further extend the glycans. In the background of pglA::aphA
and wbpO::aphA mutants, HgpA was modified with glycans includ-
ing a reducing end diNAcBac residue absent in the pentasaccharide
N-glycan produced in the wild type background (Figure 5), but con-
sistent with the presence in H. pullorum of orthologues of the C. jejuni
pglDEF genes encoding enzymes required for diNAcBac
biosynthesis (Figure 1). Surprisingly the H. pullorum pglDEF genes
are not required for biosynthesis of the PglB1-dependent H. pullorum
N-linked pentasaccharide (Jervis et al. 2010) and we hypothesize
that they are involved in biosynthesis of an as yet uncharacterized
glycan potentially transferred by the H. pullorum PglB2 dependent
pathway. Disruption of the PglB1 pathway at the earlier stages of
pentasaccharide assembly may result in cross-talk between the two
systems. Due to its similarity to P. aeruginosa WbpO it is plausible that
H. pullorum WbpO converts UDP-HexNAc to UDP-HexNAcA.
Indeed disruption of wbpO resulted in substitution of the
HexNAcAN of the N-linked glycan with a HexNAc residue albeit
with a diNAcBac reducing end. Despite this, we found no evidence of
enriched glycopeptides with Asn-linked glycans containing a
diNAcBac reducing end sugar in the H. pullorum wild-type strain.
Glycopeptide data analysis was performed by manual interpretation
of all MS/MS scans containing the HexNAc oxonium ion
204.086 m/z, which would identify glycopeptides with truncated
HexNAc-containing glycans or those containing di-NACBac–HexNAc
disaccharides. Searches were also performed to extract MS/MS scans containing di-NACBac-associated ions, however bacillosa-
mime does not produce intense oxonium ions during vibrational frag-
mentation approaches. Database searches using diNAcBac, diNACBac + HexNAc and other combinations of possible glycans
masses also failed to identify additional glycopeptides (data not
shown). This suggests that, in accordance with the production of
diNACBac glycopeptides in pglA::wbpO mutants, such glycans are
only produced under specific conditions that influence pglA::wbpO
expression and these are yet to be determined. We have shown the
involvement of a number of pgl genes in the HpPglB1-dependent gly-
cosylation system but further genes associated with these pgl loci
remain uncharacterized. These include pgll and pgl encoding putative
glycosyltransferases, which we were unable to disrupt and the
putative di-NACBac synthesis genes pglDEF that are not involved in
the HpPglB1-dependent system (Jervis et al. 2010). This suggests the
N-linked protein glycosylation system in H. pullorum may be more
complex than in the C. jejuni model. The parasites Leishmania major
and Trypanosoma brucei possess four and three single subunit N-linked
OTases, respectively, with different glycan and acceptor protein specific-
ities and varying growth phase dependent expression patterns resulting in
production of distinct subsets of the N-linked glycoproteome (Nasab
OTases have not yet been studied experimentally though Haloferax vol-
cantii has been shown to produce two structurally distinct S-layer protein
N-linked glycans (Kaminski et al. 2013a) and Archaeoglobus fulgidus
encodes three OTases and produces two structurally distinct LLOs (Tagachi et al. 2016). Genetic analysis shows that most OTase
gene duplication events are relatively ancient (Kaminski et al. 2013b).
Our combined data enable proposal of a model for N-linked protein
glycosylation in H. pullorum (Figure 7) with experimental evidence for
biosynthesis of pentasaccharide transferred onto proteins in the per-
plasm by PglB1 and a more speculative proposal for PglB2 function
that will require experimental verification.

Materials and methods

Bacterial strains

All E. coli strains were grown in Luria–Bertani (LB) broth or on LB
agar plates. C. jejuni NCTC 11168 and H. pullorum NCTC 12824
strains were grown on Columbia agar containing 5% defibrinated
horse blood (TCS Biosciences) at 42°C in a modified atmosphere
(85% N2, 10% CO2 and 5% O2) generated with a VA5000 workstation
(Don Whitley Ltd.). Antibiotics were used at the following concen-
trations: kanamycin 50 μg/mL, chloramphenicol 17 μg/mL, tetracycline
10 μg/mL, ampicillin 100 μg/mL and erythromycin 300 μg/mL. Primers,
plasmids and strains are described in Tables S1, S2 and S3, respectively.

Reverse transcriptase PCR

Total RNA was extracted from H. pullorum harvested from 48 h
blood agar plates using the Qiagen RNaseasy kit with an additional
in-solution DNase I digestion step. Reverse transcriptase PCR was
performed using the Qiagen OneStep RT PCR kit according to man-
ufacturer’s instructions.

Integration of pglB genes onto the C. jejuni
chromosome

Various pglB genes were integrated onto the C. jejuni 11168 pglB::
aphA chromosome using a modification of a previous method
(Gerber et al. 2013). A chloramphenicol resistance cassette (van
Vliet et al. 1998) was PCR amplified using primers Cm-F (restriction
sites Spel, BgiII and Xhol) and Cm-R (Ncol, Nhel and Spel),
digested with Spel and cloned into the SpeI site of a previously con-
structed vector consisting of pUC18 backbone with a region of the
C. jejuni 11168 pseudogene Cj0223 cloned into the Smal site
(Hitchens et al. 2010). The resulting plasmid was named pCJC1.

Complete C. jejuni pglB, H. pullorum pglB1 and H. pullorum
pglB2 genes were PCR amplified with primer pairs PgB1comp-F/PgB1comp-R, HppgL1comp-F/HppgL1comp-R and HppgL2comp-
F/HppgL2comp-R, respectively, to include approximately 70 bp
upstream of the each start codon. Primers PgB1comp-F, HppgL1comp-
F and HppgL2comp-F included an Ncol site at the 5’ end and the corre-
sponding reverse primers encoded a deca-histidine tag at the 3’ end along
with either an Nhel (PgB1comp-R) or Spel (HppgL1comp-R and
HppgL2comp-R) site. Products were digested with Ncol and either
Nhel or Spel as appropriate and cloned immediately downstream of,
and in the same transcriptional orientation as, the chloramphenicol
resistance cassette of pCJC1. The resulting plasmids pCJC1pglB1,
pCJC1pglB2 and pCJC1HppgL2 (Table SII) were electroporated into
C. jejuni 11168 pgB::aphA cells, chloramphenicol-resistant colonies
selected and the anticipated double crossover integration events
verified by PCR.

Expression of hgpA in E. coli

The predicted H. pullorum NCTC 12824 hgpA coding sequence was
PCR amplified with primers hgpA-F and hgpA-R to introduce
SpI and BgiII restriction sites at the 5’ and 3’ end, respectively.
Following digestion with SpI and BgiII, PCR products were ligated
into similarly digested vector pQE70 to generate pQEhgpA encoding
a C-terminal hexa-his tagged protein. A variant, pQEhgpAN46Q,
was created by site directed mutagenesis using primers hgpAN46SDM-
::aphA chromosome using a modifi-
fication of a previous method
(Gerber et al. 2013). A chloramphenicol resistance cassette (van
Vliet et al. 1998) was PCR amplified using primers Cm-F (restriction
sites Spel, BgiII and Xhol) and Cm-R (Ncol, Nhel and Spel),
digested with Spel and cloned into the SpeI site of a previously con-
structed vector consisting of pUC18 backbone with a region of the
C. jejuni 11168 pseudogene Cj0223 cloned into the Smal site
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Complete C. jejuni pglB, H. pullorum pglB1 and H. pullorum
pglB2 genes were PCR amplified with primer pairs PgB1comp-F/PgB1comp-R, HppgL1comp-F/HppgL1comp-R and HppgL2comp-
F/HppgL2comp-R, respectively, to include approximately 70 bp
upstream of the each start codon. Primers PgB1comp-F, HppgL1comp-
F and HppgL2comp-F included an Ncol site at the 5’ end and the corre-
sponding reverse primers encoded a deca-histidine tag at the 3’ end along
with either an Nhel (PgB1comp-R) or Spel (HppgL1comp-R and
HppgL2comp-R) site. Products were digested with Ncol and either
Nhel or Spel as appropriate and cloned immediately downstream of,
and in the same transcriptional orientation as, the chloramphenicol
resistance cassette of pCJC1. The resulting plasmids pCJC1pglB1,
pCJC1pglB2 and pCJC1HppgL2 (Table SII) were electroporated into
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Following digestion with SpI and BgiII, PCR products were ligated
into similarly digested vector pQE70 to generate pQEhgpA encoding
a C-terminal hexa-his tagged protein. A variant, pQEhgpAN46Q,
was created by site directed mutagenesis using primers hgpAN46SDM-
F and hgpAN46SDM-R. Plasmids pQEhgpA and pQEhgpAN46Q
were transformed into E. coli Novablaue (Stratagene) cells harboring
Introduction of oligo-histidine tagged hgpA onto the *H. pullorum* chromosome

A 1.8 kb internal fragment of the *H. pullorum* 23S rRNA gene (*rrl*) was amplified using primers Hp23s-F and Hp23s-R, ligated into pGEM T-easy to create plasmid pHPC and digested with HindIII to excise 276 bp of *rrl*. An erythromycin resistance cassette (*ermC*) lacking a transcriptional terminator was amplified using primers Ery-F (containing a HindIII site) and Ery-R (HindIII and BamHI sites), and ligated into HindIII digested pHPC in the same transcriptional orientation as flanking *rrl* fragments to create (pHPC1). A derivative of this plasmid, termed pHPC2, was constructed by cloning the promoter region of the *C. jejuni* porA gene into the pHPC1 BamHI site located immediately downstream of the erythromycin resistance cassette with the promoter in the same transcriptional orientation as *rrl* gene and erythromycin cassette. The promoter region was PCR amplified using primers porAP-F (containing XhoI site) and porAP-R (Ndel and Xhol sites). Two *H. pullorum* hgpA expression systems were constructed based on either pHPC1 or pHPC2. In the first the hgpA ORF was PCR amplified using primers hgpABamHI-F and hgpABamHI-R to incorporate a C-terminal hexa-his tag and ligated into the BamHI site at the 3’ end of the erythromycin resistance cassette in pHPC1 to create pHPC1hgpAhis. In the second, the hgpA ORF was PCR amplified with primers hgpANdel-F and hgpANdel-R to include a C-terminal deca-his tag and cloned into the Ndel site of the plasmid pHPC2 downstream of the porA promoter region to create plasmid pHPC2hgpAhis.

Plasmids pHPC1hgpAhis and pHPC2hgpAhis were electroporated into *H. pullorum* cells (van Vliet et al. 1998; Jervis et al. 2010) and erythromycin resistant colonies screened by PCR for the predicted double crossover events within the chromosomal *rrl* gene.

Purification of HgpAHis from *H. pullorum*

Approximately 1 g of *H. pullorum* NCTC 12824 treated with 25 mM Tris-HCl, 25 mM NaCl (pH 8.0) and analyzed by LC-ESI–MS using a Dionex PepSwift RP column (200 μm x 50 mm) connected to a Micromass LCT Q-TOF/TOF mass spectrometer. Data were acquired as.mgf files using the MaxEnt I software (Micromass).

Glycan analysis by MALDI-MS

Coomassie stained SDS-PAGE bands were excised, lyophilized and digested with trypsin (E.C.3.4.21.4, Promega) overnight. Peptides were extracted from gel pieces using a C18 ZipTip (Millipore, UK) according to the manufacturer’s protocol and eluted in 10 μL of 50% acetonitrile, 0.1% formic acid. MALDI-TOF MS and MALDI-LIFT-TOF/TOF MS spectra were acquired by laser-induced dissociation (LID) using a Bruker Ultraflex II mass spectrometer in the positive-ion reflection mode with a matrix of 20 mg/mL 2,5-dihydroxybenzoic acid (DHB) (30% acetonitrile, 0.1% TFA). Data were analysed with FlexAnalysis 3.0 software (Bruker Daltonics).

Enrichment of glycopeptides using zwitterionic-hydrophilic interaction liquid chromatography (ZIC-HILIC) and identification by reversed phase LC–MS/MS

Identification of glycopeptides from *H. pullorum* NCTC 12824 WT was conducted as previously described (17, 33). Lysates were suspended in 6 M urea, 2 M thiourea, 40 mM NH₄HCO₃, and reduced and alkylated with 20 mM dithiotheritol and 40 mM iodoacetamide, respectively, each for 1 h at room temperature. Samples were diluted 1:10 with 40 mM NH₄HCO₃ and digested with porcine sequencing grade trypsin (Promega, Madison WI; 1:100) overnight at 37°C. Peptides were acidified with 2% (v/v) formic acid and 0.1% (v/v) TFA, then desalted by hydrophilic lipidic-balance solid phase extraction (HLB-SPE) (Waters, Milford MA). ZIC-HILIC enrichments were carried out according to Scott et al. (2011). Fractions were resuspended in 0.1% formic acid and loaded directly onto a 20 cm, 75 μm inner diameter, 360 μm outer diameter Reprosil Gold C18 AQ 1.9 μm (Dr. Maisch, Ammerbuch-Entringen, Germany) reversed phase (RP) column using a trapless EASY-nLC II system (Proxeon, Odense Denmark) coupled to an LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Scientific, San Jose, CA). Peptides were loaded in 95% buffer A (0.1% FA) and eluted at 250 μL/min using a linear gradient of buffer B (80% ACN, 0.1% FA) from 5% to 40% over 120 min. The column was washed with 90% buffer B for 10 min before being returned to 95% buffer A. The LTQ-Orbitrap Velos Pro was operated using Xcalibur v2.2 (Thermo Scientific) with a capillary temperature of 200°C in a data-dependent mode automatically switching between MS and higher energy collisional dissociation (HCD)/collision-induced dissociation (CID) MS/MS. For each MS scan, the three most abundant precursor ions were selected for HCD (normalised collision energy 45) and CID (normalised collision energy 35). Data processing was carried out as previously described (Scott et al. 2014). Briefly, HCD scans from *.mgf* files were processed in Proteome Discoverer v1.4.1.14 (Thermo Scientific) and searched using SEQUEST against an in-house, translated *H. pullorum* NCTC 12824 database. MS/MS scans that did not result in identifications were exported as*.mgf* files. The “*.mgf*” feature within the MSMS module of GPMAW 10.0 (Lighthouse Data, Odense, Denmark) was used to highlight all scan events containing the diagnostic HexNAc oxonium ion 204.0866 m/z, in addition to the oxonium ions for the HexNAcA (218.0665 m/z).
and HexNAcAN (217.0824 m/z) species that are constituents of the
H. pullorum N-glycan. MASCOT v2.2 searches were conducted
against the H. pullorum NCTC 12824 database with parent ion mass
accuracy of 20 ppm and product ion accuracy of 0.02 Da, no protease
specificity, instrument set to MALDI-QIT-TOF, as well as the fixed
modification carbamidomethyl (C) and variable modifications oxidation
(M) and deamidation (N). All spectra were searched with the
decoy option enabled, and no matches were detected (FDR 0%).
HCD and CID scans from matched spectra (MASCOT scores >20)
were manually inspected to ensure all major peaks were matched, and
to validate alignment and composition of the N-glycan. Isotopic dis-
tribution analysis was performed with the MS Isotope module of
Protein Prospector (http://prospector.ucsf.edu/prospector).

Construction of H. pullorum insertional knockout mutants
H. pullorum genes were inactivated by insertion of the aphA gene
via double crossover recombination events with appropriately con-
structed suicide vectors introduced into cells by electroporation (van
Vliet et al. 1998). To create suicide vectors, PCR products of
approximately 2 kbp were generated that incorporated regions of
target genes. These were ligated into pGEM-T Easy, and the
aphA cassette lacking a transcriptional terminator cloned into BamHI or
HindIII sites within the central region of cloned PCR products. If
these restriction sites were not present they were introduced by site
directed mutagenesis or overlap PCR as described previously (Jervis
et al. 2010). The individual mutations were made as described
below.

pglA: Primers pglA-F and pglA-R were used to amplify a 1.6 kb
fragment encompassing the complete pglA gene with a natu-
really occurring central HindIII restriction site.

pglC: A 729 bp fragment consisting of the first 111 bp at the 5’ end
of pglC plus upstream region was amplified using primers
pglC-UF and pglC-UR to include a 3’ HindIII site. A 689 bp
fragment consisting of 340 bp of the 3’ end of pglC and
downstream region was amplified using primers pglC-DF and
pglC-DR to include a 5’ HindIII site. Overlap PCR was per-
formed using primers pglC-UF and pglC-DR.

pglH: A 912 bp fragment consisting of the first 561 bp at the 5’ end
of pglH plus upstream region was amplified using primers
pglH-UF and pglH-UR to include a 3’ HindIII site. A 929 bp
fragment consisting of 527 bp of the 3’ end of pglH and
downstream region was amplified using primers pglH-DF and
pglH-DR to include a 5’ HindIII site. Overlap PCR was per-
formed using primers pglH-UF and pglH-DR.

pglI: An 823 bp fragment consisting of the first 236 bp at the 5’ end
of pglI plus upstream region was amplified using primers
pglI-UF and pglI-UR to include a 3’ HindIII site. An 857 bp frag-
ment consisting of 551 bp of the 3’ end of pglI and downstream
region was amplified using primers pglI-DF and pglI-DR to
include a 5’ HindIII site. Overlap PCR was performed using prim-
ers pglI-UF and pglI-DR.

pglJ: An 879 bp fragment consisting of the first 204 bp at the 5’ end
of pglJ plus upstream region was amplified using primers
pglJ-UF and pglJ-UR to include a 3’ HindIII site. An 808 bp fragment
consisting of 196 bp of the 3’ end of pglJ and downstream
region was amplified using primers pglJ-DF and pglJ-DR to
include a 5’ HindIII site. Overlap PCR was performed using primers
pglJ-UF and pglJ-DR.

pglL: An 857 bp fragment consisting of the first 180 bp at the 5’ end
of pglL plus upstream region was amplified using primers
pglL-UF and pglL-UR to include a 3’ HindIII site. An 885 bp
fragment at the 3’ end of pglL, was amplified using primers
pglLD-F and pglLD-R to include a 5’ HindIII site. Overlap
PCR was performed using primers pglL-UF and pglL-DR.

wbpO: Primers wbpO-F and wbpO-R were used to amplify a
1.9 kb fragment including the complete wbpO CDS with a
naturally occurring central HindIII restriction site.

wbpS: Primers wbpS-F and wbpS-R were used to amplify a 1.2 kb
internal fragment of wbpS. A BamHI restriction site was cre-
ated using site directed mutagenesis with primers wbpSBamHI-
F and wbpSBamHI-R.

Supplementary data
Supplementary data is available at GLYCObIOLOGY online.

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Conflict of interest statement
None declared.

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