

Biosynthesis of the N-Linked Glycan in *Campylobacter jejuni* and Addition onto Protein through Block Transfer

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In eukaryotes, N-linked protein glycosylation is a universal modification involving addition of preformed oligosaccharides to select Asn-Xaa-Ser/Thr motifs and influencing multiple biological events. We recently demonstrated that *Campylobacter jejuni* is the first member of the *Bacteria* to possess an N-linked glycan pathway. In this study, high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR) was applied to probe and quantitate *C. jejuni* N-glycan biosynthesis in vivo. To confirm HR-MAS NMR findings, glycosylation mutants were screened for chicken colonization potential, and glycoproteins were examined by mass spectrometry and lectin blotting. Consistent with the mechanism in eukaryotes, the combined data indicate that bacterial glycans are assembled en bloc, emphasizing the evolutionary conservation of protein N glycosylation. We also show that under the conditions examined, PglG plays no role in glycan biosynthesis, PglI is the glucosyltransferase and the putative ABC transporter, and WlaB (renamed PglK) is required for glycan assembly. These studies underpin the mechanism of N-linked protein glycosylation in *Bacteria* and provide a simple model system for investigating protein glycosylation and for exploitation in glycoengineering.

N-linked protein glycosylation is essential in eukaryotes and influences multiple protein functions including sorting, targeting, localization, stability, and quality control of protein synthesis and turnover (9). Although widespread in eukaryotes and archaea, bacteria had been considered unable to synthesize N-glycosylated proteins. However, the prevalent human food-borne pathogen *Campylobacter jejuni* was the first bacterium shown to have a general protein glycosylation pathway (14, 25) that was later identified to be N linked (29, 33).

C. jejuni synthesizes a conserved heptasaccharide with the structure GalNAc- α 1,4-GalNAc- α 1,4-[Glc β 1,3]-GalNAc- α 1,4-GalNAc- α 1,4-GalNAc- α 1,3-Bac- β 1, where Bac is bacillosamine (2,4-diacetamido-2,4,6-trideoxyglucopyranose) (33). Similar to eukaryotes, the glycan is attached to multiple proteins via an N linkage to the Asn residue of the Asn-Xaa-Ser/Thr motif (29, 33), where Xaa can be any amino acid except proline (18). Unlike eukaryotes however, the precise function(s) of the glycan modification in *C. jejuni* is unknown, but loss of the heptasaccharide leads to changes in protein antigenicity, loss of type IV protein complex assembly and DNA uptake, reduction in adherence and invasion in vitro, and loss of colonization in mice and chickens in vivo (13, 20, 21, 25).

The genes involved in the synthesis and transfer of the heptasaccharide are found clustered together in the *pgl* (for protein glycosylation) locus (Fig. 1) and can be functionally transferred into *Escherichia coli* (29). The locus is conserved in gene content and organization throughout the *Campylobacter* genus (24), with the exception of *wlaJ*, which is present in approxi-

mately half of all *C. jejuni* strains. Recent analyses of the gene content from the genome-sequenced strains of *Wolinella succinogenes* and *Desulfovibrio desulfuricans* have identified genes with significant identity to those in the *Campylobacter pgl* locus, including the gene encoding the oligosaccharide transferase, PglB, required for N-linked protein glycosylation. PglB, the homolog of the eukaryotic Stt3p, contains the WWDYG catalytic domain that is conserved in all three domains of life and is essential for N-linked protein glycosylation (29, 31). Thus, although it has not yet been confirmed structurally, it is likely these organisms also synthesize N-linked glycans.

Based on similarity to other bacterial enzymes, knowledge of the N-linked glycosylation pathway in eukaryotes, and published data, a putative model for N-glycan biosynthesis in *C. jejuni* has been proposed where the periplasm mimics the endoplasmic reticulum lumen (Fig. 1) (1, 6, 8, 15, 18). The sugars are sequentially assembled on an inner-membrane-bound lipid carrier (undecaprenyl pyrophosphate) on the cytoplasmic face, before they are presumably transported across the membrane and attached to specific proteins in the periplasm. As shown in Fig. 1, the first sugar Bac is synthesized by the actions of PglF (dehydratase), PglE (aminotransferase), and PglD (acetyltransferase) on a HexNAc sugar and then transferred onto the lipid carrier by PglC. The first GalNAc residue is added to the lipid-linked Bac by PglA, with the four subsequent GalNAc residues being transferred by PglH and PglJ. The bifunctional Glc/GlcNAc epimerase, Gne, is responsible for the biosynthesis of the GalNAc residues. The addition of the Glc branch by PglI completes the heptasaccharide structure, and WlaB (renamed PglK) presumably transports the glycan block across the inner membrane. Finally, the oligosaccharide transferase PglB attaches the glycan to proteins with the appropriate target sequon.

In this study, we examine the biosynthesis of *C. jejuni* N-

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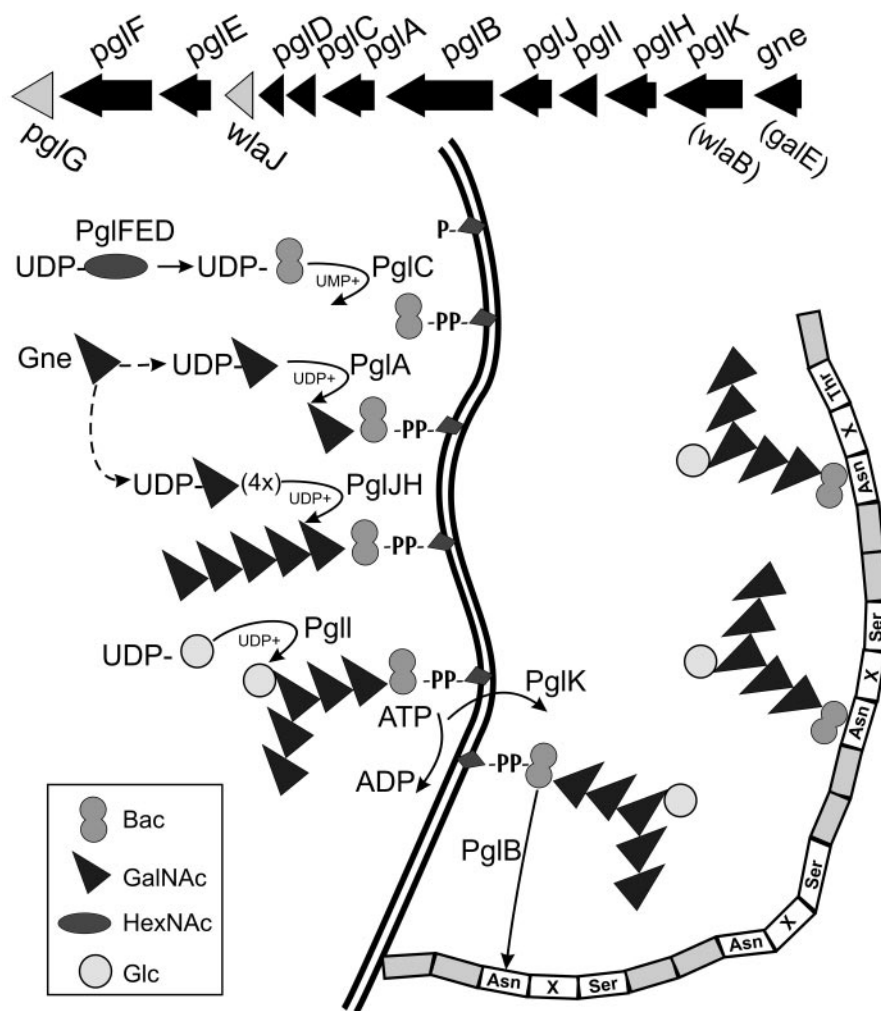


FIG. 1. Schematic of the *pgl* locus and proposed *N*-glycan pathway. (Top) *C. jejuni* NCTC 11168 gene cluster, *cj1119c-cj1131c*, examined in this study. Gray arrowheads indicate genes that do not appear to be involved in *N*-glycan biosynthesis. Note that *glaE* (1) and *wlaB* (this work) have been renamed *gne* and *pglK*, respectively. (Bottom) *N*-glycan biosynthesis in *C. jejuni* proceeds through assembly of glycan on the lipid anchor, undecaprenyl pyrophosphate (6). The glycan is synthesized as a block before being added to proteins at select Asn-X-Ser/Thr motifs.

linked glycans in vivo using high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR) (23) and have applied this method to quantitate the glycosylation efficiency within the cells. To confirm the NMR findings, protein extracts from the *C. jejuni* glycosylation mutants were tested for their reactivity with the lectin soybean agglutinin (SBA), recognizing terminal GalNAc residues; specific glycoproteins were examined by mass spectrometry (MS). Furthermore, based on previous observations that disruption of the *C. jejuni* N-linked pathway results in loss of chicken colonization, we screened our mutants with the chicken model to validate our findings and determined the minimum glycan structure required for *C. jejuni* colonization of its natural commensal host.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Campylobacter jejuni* NCTC 11168 was grown on Mueller-Hinton agar (Oxoid) at 37°C under microaerophilic conditions (85% N₂, 10% CO₂, and 5% O₂). *Escherichia coli* DH10B (Invitrogen) was used as the host strain for cloning experiments, and plasmid pPCR-Script

Amp (Stratagene) was used as the cloning vector. Clones were grown on S-Gal/Luria-Bertani agar (Sigma) at 37°C. When required, kanamycin (Km), chloramphenicol (Cm), and ampicillin were added to the medium at a final concentration of 30 μg ml⁻¹, 20 μg ml⁻¹, and 150 μg ml⁻¹, respectively.

Construction and characterization of *C. jejuni* site-specific mutations. We have previously described the construction of the *pglB* (33), *pglH* (21), and *gne* (1) mutants, while *pglA*, *pglD*, *pglE*, and *pglJ* mutants were kindly provided by B. Wren (14). For construction of the *kpsM*::Cm mutant, a 2,420-bp fragment (*kpsE-Cj1450*) containing the *kpsM* gene was amplified using *kpsM*-F2 and *kpsM*-R2 (see Table 1 for primer sequences) and constructed as previously described (33); the Cm resistance cassette from pRY109 was inserted (32) into the unique BseRI restriction site of *kpsM*. For the *pglF*::Km mutant, a 5,151-bp fragment (*prmA-pglE*) was amplified using *prmA*F and 1121R. A Km cassette from pILL600 (12) was cloned into the unique XbaI restriction site of *pglF*. For the *pglG*::Km mutant, a 1,825-bp fragment (*cheY-pglF*) was amplified using *pglG*-F2 and *pglG*-R2, and the Km cassette was cloned into the unique NdeI site of *pglG*. For the *pglI*::Km mutant, a 3,026-bp fragment (*pglJ-pglH*) was amplified using 1127F and 1129R, and the Km cassette was cloned into the unique PflMI site of *pglI*. For the *wlaB*::Km mutant, a 4,408-bp fragment (*pglH-wlaA*) was amplified using *wlaB*GalE-F1 and *wlaB*GalE-R1, and the Km cassette was cloned into the unique AfeI site of *wlaB*. All the *C. jejuni* antibiotic-resistant transformants were characterized by PCR to confirm that the incoming plasmid DNA

TABLE 1. Primer sequences used for mutant construction and verification

Primer name	Primer sequence (5'-3')
kpsM-F2.....	CATCGTGCCTTACATATCTTGGTGC
kpsM-R2.....	TTCTTTTGGCTTTAACTCTCCGAC
prmAF.....	AAAACATACACCCTCATTGATTTGC
1121R.....	CGCATATGGGTGGTAATGAATTTAA
pglG-F2.....	GCTTTTCCACCCTCAGTTGTAAC
pglG-R2.....	GCACTAAGAGAGTTTGGCGAGC
1127F.....	TATCAAAGGCTTTAGCCCTAGTTTT
1129R.....	TTATTATCGCAACTTTAAATTCAGG
wlaBgalE-F1.....	GCTATTTCATCATCACAACTACC
wlaBgalE-R1.....	GCCAGATGTTGAGCTTATCCG
ckanB.....	CCTGGGTTTCAAGCATTAG

had integrated by a double-crossover event. The orientation of the Km cassettes in the glycan mutants was confirmed to be nonpolar by sequencing with the ckanB primer; microarray analyses also demonstrated that expression of the downstream genes was not affected by mutagenesis (results not shown). To clearly observe the N-linked glycan resonances by HR-MAS NMR, all Km-containing mutants were introduced into the *kpsM::Cm* background to eliminate resonances due to capsular polysaccharide (23). All double mutants were confirmed and characterized by NMR.

HR-MAS NMR spectroscopy. Cells were prepared and analyzed by HR-MAS NMR spectroscopy as previously described (23). Semiquantitative estimates of *N*-glycan content were obtained as described below. HR-MAS samples were prepared by suspending equal amounts of cells, adjusted to the same optical density at 600 nm in 40 μ l of D₂O containing 0.75% (wt/vol) trimethylsilylpropionic acid d₄ sodium salt (TSP), and loaded into the rotor. The samples were shimmed to give the same line width ($\pm 10\%$) based on TSP ¹H resonance. NMR spectra were collected in triplicate for each sample under identical conditions. Resonances were integrated using standard Varian VNMR 6.1C software. Integrals of the *N*-glycan anomeric resonances are expressed as percentages of the TSP integral. Since the spectra were acquired under identical conditions, the signal/noise (S/N) ratio of the TSP is expected to be the same for each sample. Any differences should reflect variations in the concentration of TSP in the sample. Thus, *N*-glycan integrals were normalized to the same TSP S/N ratio. Since the α -1,3-GalNAc peak at 5.24 ppm often had a broad underlying peak for the *pgl*⁺/*kpsM*-negative spectra, the anomeric peaks at 5.14 to 5.04 ppm (i.e., five anomeric protons: b, c, d, e, and f of the *N*-glycan) (Fig. 2 inset) were integrated. For the *pgl*-negative/*kpsM*-negative mutant, the peak at ~ 5.14 ppm was broad and of poor S/N; as a result, integration was based on resonances at 5.08 ppm and 5.04 ppm (i.e., four anomeric protons: c, d, e, and f of the *N*-glycan). For comparison of the *pgl*⁺/*kpsM*-negative and *pgl*-negative/*kpsM*-negative results, the *pgl*-negative integrals were normalized to five protons by scaling the integrals by 5/4. In addition to the use of the TSP external reference, the *N*-glycan integrals were referenced against two *C. jejuni* peaks at 8.28 ppm and 8.62 ppm, which are invariant cell-related resonances (the identity of these peaks has not been determined).

Analysis of total *C. jejuni* glycoproteins by SDS-PAGE and immunoblotting. Total protein extracts were obtained from overnight *C. jejuni* cultures by resuspending 1 mg (wet weight) per 100 μ l of loading buffer (100 mM Tris-Cl [pH 8.0], 2% β -mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 0.2% xylene cyanol, and 20% glycerol) and boiling for 10 min. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% Laemmli gels using Mini Protean II slab gels (Bio-Rad Laboratories) and then transferred onto polyvinylidene difluoride membranes (Millipore). The membrane was blocked with 1% bovine serum albumin (Sigma) in TBS (20 mM Tris base, 500 mM NaCl [pH 7.5]) for 1 h prior to incubation and for another hour with alkaline phosphatase-labeled SBA lectin (EY Laboratories) diluted to 20 mg ml⁻¹ in TBS with 0.05% Tween 20. After being washed three times in TBS-Tween, the blot was developed with nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (Roche).

Purification and analysis of Peb3. The Peb3 glycoprotein was isolated from *C. jejuni* by cation exchange chromatography as described previously (33) with the following modifications. A 1-liter overnight culture was harvested and resuspended in 50 mM HEPES, pH 9.0, and disrupted using the EmulsiFlex (Avestin, Inc.). The lysate was applied to a HiPrep 16/10 SP FF column on an ÄKTA FPLC system (Amersham Biosciences). The column eluate was monitored for UV absorbance at 280 nm, and fractions were examined by SDS-PAGE using

Mini Protean II slab gels (Bio-Rad Laboratories). Fractions containing Peb3 were combined, dialyzed, and freeze-dried before further purification using a Mono S HR 5/5 column as previously described (33). The intact mass analysis of Peb3 was determined by electrospray ionization mass spectrometry (ESI-MS) using a Q-TOF2 hybrid quadrupole time of flight mass spectrometer (Waters). The protein solutions were adjusted to 25% methanol-0.5% acetic acid prior to infusion at a flow rate of 1 μ l/min. Mass spectra were acquired for approximately 1 min over the range *m/z* 600 to 2,000.

Analysis of eluted glycoproteins from SDS-PAGE by mass spectrometry. Glycopeptide analysis was done as previously described (33). Briefly, overnight cultures were disrupted with the EmulsiFlex (Avestin). Glycoproteins were then enriched by affinity chromatography with SBA lectin agarose (Sigma). The appropriate fractions were pooled and separated by SDS-PAGE and stained with colloidal Coomassie blue. Glycoprotein bands were excised, destained, and in-gel digested with modified trypsin (Promega) according to standard protocols (33). The peptide extracts were then analyzed by nanoHPLC-electrospray tandem mass spectrometry (nanoLC-MS/MS) using the Q-TOF2 spectrometer. Approximately one-third of each extract was injected onto a 0.3- by 5-mm C₁₈ Micro-Precolumn cartridge (Dionex/LC-Packings), which was then brought online with a 75- μ m by 50-mm Picofrit nanocolumn (New Objective) packed with BioBasic C₁₈ reversed-phase medium. The peptides were separated using a gradient supplied by a Waters CapLC pump (5 to 80% acetonitrile-0.2% formic acid in 50 min, ~ 300 -nl/min flow rate). The mass spectrometer was set to automatically acquire MS/MS spectra on double-, triple-, and quadruple-charged ions. Database searching was carried out in batch mode using Mascot Daemon (Matrix Science) against the NCBI protein sequence database, as well as the *C. jejuni* NCTC 11168 genome sequence database. Glycopeptide MS/MS spectra were identified manually by the presence of the strong oxonium ions at *m/z* 204.1 and 407.2, corresponding to [HexNAc]⁺ and [HexNAc₂]⁺, respectively.

Colonization of 1-day-old chicks. One-day-old chicks, in groups of 20, were challenged with *C. jejuni* wild-type and select mutants as described previously (4). Motility was measured for all strains in duplicate using 0.4% Mueller-Hinton agar plates to ensure that changes in colonization were not due to alterations in flagellar expression (4). Inocula for the challenge experiments were produced by harvesting cells grown for 18 h into cold 0.85% (wt/vol) NaCl, diluted to the indicated concentration in saline, and maintained on ice until used. Birds were maintained for 7 days after challenge and then euthanized by cervical dislocation according to the approved guidelines of the Canadian Council for Animal Care. Ceca were collected, and the contents were removed and weighed. To determine the level of colonization, serial dilutions of the cecal contents were made using normal saline and were plated on selective Karmali agar (Bacto). For statistical analysis of the data, which were not normally distributed, the levels of colonization were transformed by ranking. A one-way analysis of variance test for significance of differences among the groups was done. If there was a significant difference among the groups, the means of the ranked data were compared using Tukey's multiple-comparison test.

RESULTS

Construction of *C. jejuni pgl* mutants and characterization of N-linked protein glycosylation by HR-MAS NMR. The purified *N*-glycan of *C. jejuni* NCTC 11168 exhibits a specific set of ¹H resonances when examined by HR-MAS NMR (Fig. 2) (23). We recently provided the first demonstration that this method can also be used to detect *N*-glycan residues attached to proteins of intact *C. jejuni* cells (23). In this study, HR-MAS NMR was applied to examine the biosynthesis of the *C. jejuni* *N*-glycan using site-specific insertional *pgl* gene mutants constructed in a capsule minus background. The gene *wlaJ* was not included in this study, as it is not present in all *C. jejuni* strains and as the *pgl* locus can be functionally transferred into *E. coli* without *wlaJ* (29). Despite several attempts, we were unable to mutate *pglC*; however, other groups have also been unable to make mutations in this gene in NCTC 11168 (30), suggesting that in this strain, mutation may be toxic to the cell. The *pglB* (oligosaccharide transferase) (23) and *gne* (Glc/GlcNAc epimerase) (1) mutants have been previously analyzed by HR-MAS NMR and were concluded to be deficient in *N*-glycan biosynthesis, due to

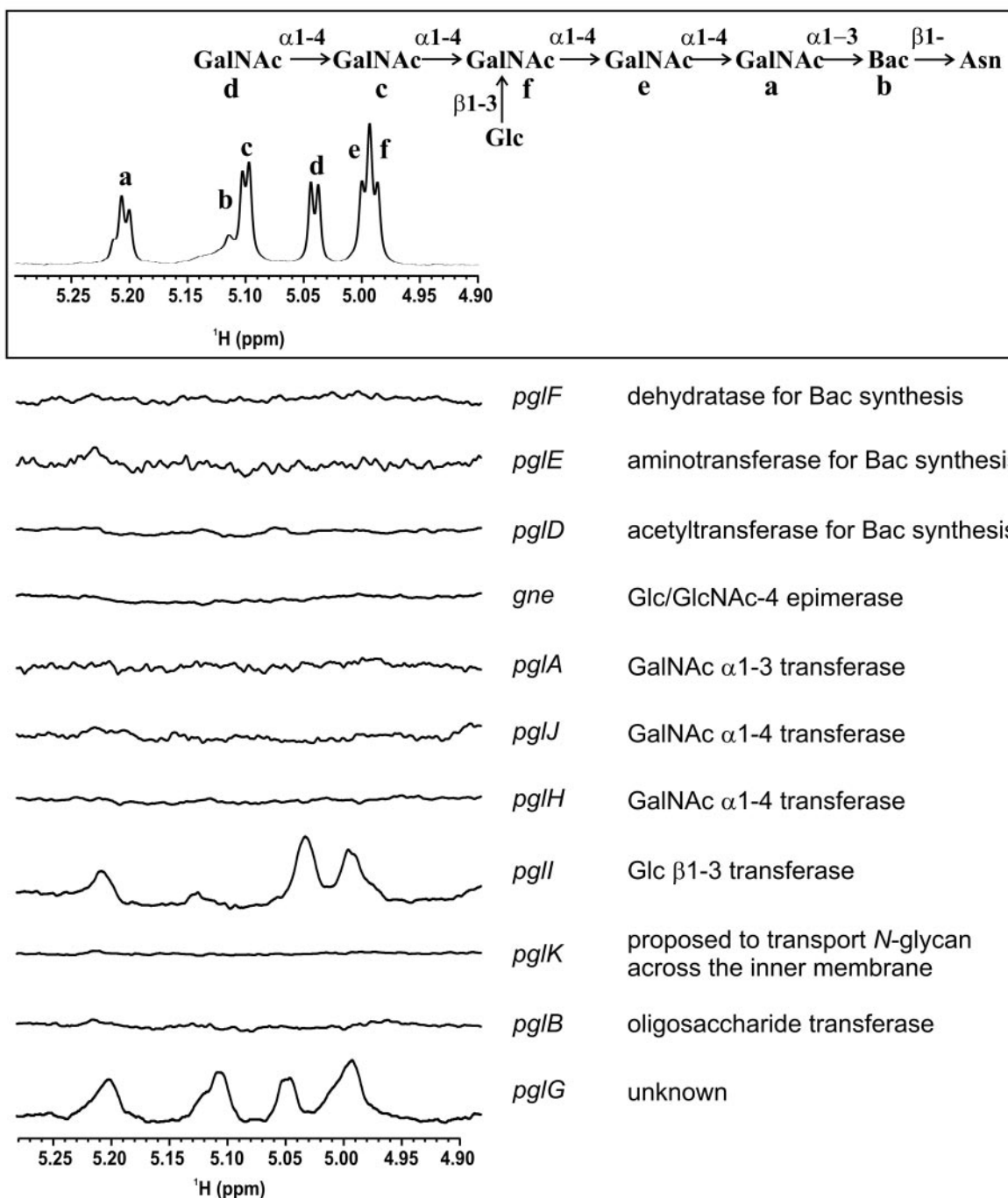


FIG. 2. ^1H HR-MAS NMR of *C. jejuni* NCTC 11168 double mutants. The anomeric region of each indicated mutant in a *kpsM*-negative background is shown, along with the proposed protein function in sequential biosynthetic order. (Inset) Structure of the *N*-linked heptasaccharide along with the anomeric ^1H NMR spectrum of purified glycan from the wild type for comparison (33). This spectrum is similar to those observed for the wild type and *kpsM*-negative whole cells (23). Note that the Glc anomeric region is not shown for the mutants because this resonance is difficult to distinguish in whole-cell spectra.

the loss of α -1,3-GalNAc resonance from the spectra. However, as several anomeric resonances from the capsular polysaccharide overlap with the *N*-glycan resonances in the HR-MAS NMR spectra from NCTC 11168, we constructed all 12 mutants (including *pglB* and *gne*) in a capsule minus (*kpsM* mutant) background to enable unambiguous interpretation.

When examined by HR-MAS NMR, the majority of double *pgl/kpsM* *C. jejuni* mutants were missing *N*-glycan resonances (Fig. 2). These strains included *pglB/kpsM* and *gne/kpsM*, as was predicted from previous experiments; *pglD/kpsM*, *pglE/kpsM*, and *pglF/kpsM* (Bac biosynthesis mutants); *pglA/kpsM*, *pglH/kpsM*, and *pglJ/kpsM* (GalNAc transferase mutants); and

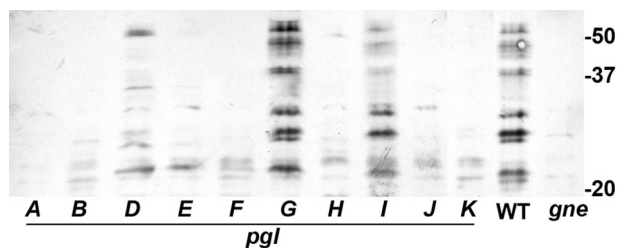


FIG. 3. *C. jejuni* NCTC 11168 whole-cell lysate reactivity with the SBA lectin. The masses (in kilodaltons) are indicated on the right.

pglK/kpsM (putative inner-membrane transport mutant). In contrast, the glycan produced by *pglG/kpsM* appeared to be identical to the wild type. This gene flanks the *pgl* locus (Fig. 1), but its function is currently unknown.

Interestingly, the Glc transferase mutant *pglI/kpsM* was still able to synthesize a glycan. The HR-MAS NMR spectra from this strain exhibited chemical shifts similar to those of the wild-type resonances (Fig. 2, inset) (23) but with altered stoichiometry, which integration has confirmed reflects a shift in the position of resonance c to overlap with resonance d. Integration of the *N*-glycan anomeric resonances relative to that of the external TSP standard allowed estimation of the relative amount of glycan expressed in the *pgl⁺/kpsM*-negative and *pglI*-negative/*kpsM*-negative *C. jejuni* mutants. Based upon triplicate analyses of each mutant, the amount of glycan present was found to be $2.33\% \pm 0.42\%$ and $2.47\% \pm 0.31\%$ (expressed as a percentage of the TSP resonance integral) for the *pgl⁺/kpsM*-negative and *pglI*-negative/*kpsM*-negative mutants, respectively. Comparison of the *N*-glycan integrals with two invariant *C. jejuni* resonances as internal standards also yielded similar results (not shown). The errors in our analyses *in vivo* were consistent with results reported for quantitative solution ^1H NMR and with the difficulty in obtaining accurate measurements with dilute solutions or impure compounds (3). Thus, *in vivo* quantitative HR-MAS NMR demonstrates that deletion of Glc transferase activity encoded by *pglI* does not significantly alter glycan expression.

Lectin reactivity of the *pgl* mutants. The terminal GalNAc residue of the *N*-linked glycan binds to the SBA lectin (14). To corroborate the HR-MAS NMR results, proteins from whole-cell lysates of all the mutants were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with SBA (Fig. 3). Consistent with the HR-MAS NMR data, the *pglG*-negative and *pglI*-negative protein extracts reacted with SBA at a level similar to that of the wild type, indicating the proteins were glycosylated in these mutants. Similarly, the other *pgl* mutants showed very little reactivity with SBA, confirming that the glycosylation pathway had been disrupted. In contrast, the *pglD* mutant showed an intermediate level of reactivity with SBA.

Purification and characterization of Peb3 from select *pgl* mutants. To complement the HR-MAS NMR results, a previously characterized *C. jejuni* glycoprotein, Peb3, was extracted from several *pgl* mutants (14, 33). Strains were chosen to represent each stage of the pathway: *pglD*-negative and *pglE*-negative strains for Bac biosynthesis, *pglH*-negative strain for the GalNAc transferases, *pglI*-negative strain for the Glc

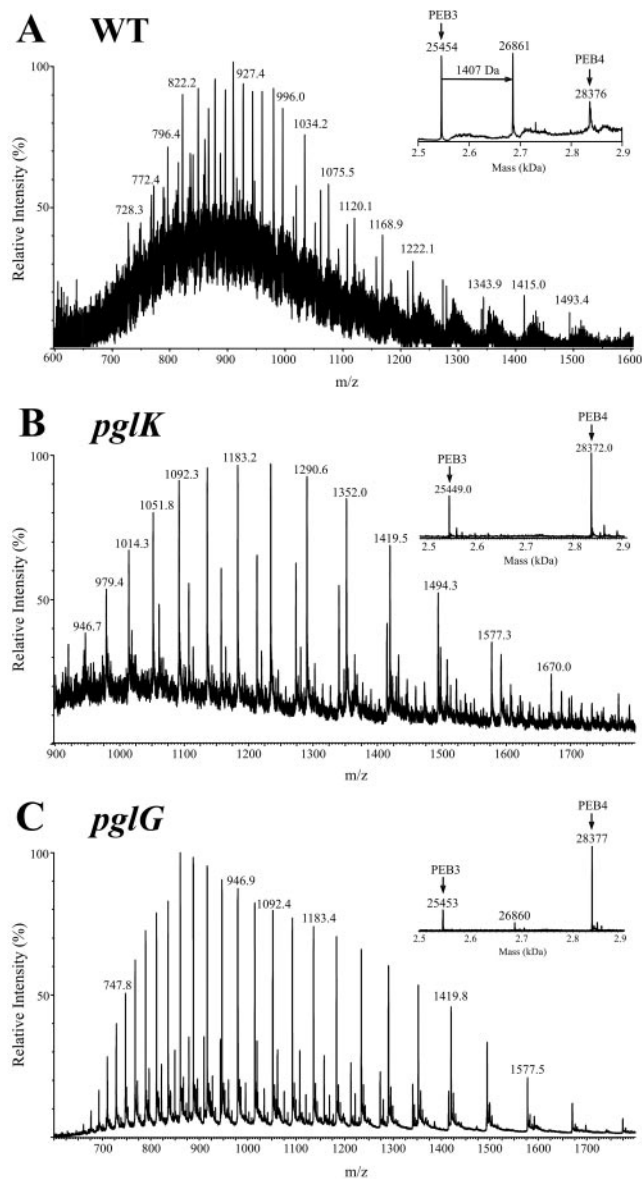


FIG. 4. ESI-MS analysis of combined Peb3-containing fractions from cation-exchange chromatography: wild-type *C. jejuni* NCTC 11168 (A), the *pglK* mutant (B), and *pglG* mutant (C). The reconstructed molecular mass profiles are presented in the insets. The protein peak at 26,861 Da is glycosylated Peb3.

transferase, *pglK*-negative and *pglB*-negative strains involved in transport and transfer of the oligosaccharide, and the mutant of unknown function, a *pglG*-negative strain. Peb3 was isolated by cation exchange chromatography from whole-cell protein extracts. Fractions containing Peb3 were identified by SDS-PAGE and analyzed by electrospray ionization-mass spectrometry for the presence of glycosylated Peb3.

The mass spectra from the wild-type, *pglK*-negative, and *pglG*-negative strains are shown in Fig. 4. The reconstructed mass profiles derived from these spectra are provided in the insets. All the examined strains contained unmodified Peb3 (25,454 Da) and Peb4 (28,376 Da). However, glycosylated

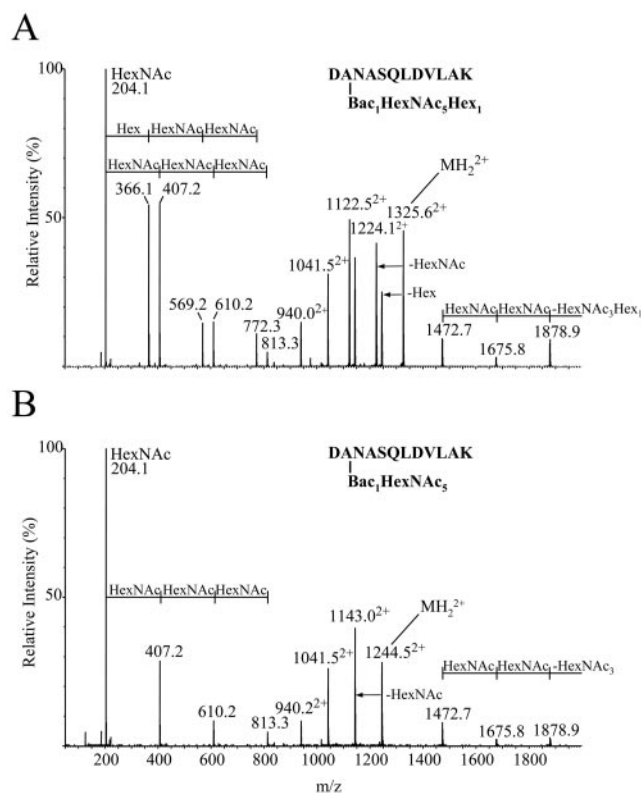


FIG. 5. NanoLC-MS/MS analysis of the tryptic glycopeptide ($T_{327-388}$) from the secreted transglycosylase (Cj0843c) isolated from wild-type *C. jejuni* NCTC 11168 (A) and the *pglI* mutant (B). Presented here are the product ion spectra of the doubly protonated wild-type glycopeptide ion at m/z 1325.6 (A) and the corresponding *pglI*-negative glycopeptide ion at m/z 1244.5 (B). The peptide sequence and the glycan composition are displayed in the insets. The major fragment ions observed in both spectra are either glycan oxonium ions (low m/z region) or are due to sequential loss of the oligosaccharide residues (upper m/z region). Only the glycopeptide containing the complete heptasaccharide structure was observed in the digest of wild-type transglycosylase, and it was never detected in the corresponding *pglI* mutant digest. The reverse was true with respect to the glycopeptide lacking the hexose moiety.

Peb3 (26,861 Da) was only observed in the mass profiles from the wild type and the *pglG* mutant and not from the *pglB*-, *pglD*-, *pglE*-, *pglH*-, and *pglI*-negative strain Peb3 fractions (data not shown).

Structure of the N-linked glycan from the *pglI* mutant. Although the HR-MAS NMR spectrum of the *pglI* mutant showed anomeric resonances that corresponded to those of the N-linked glycan, they exhibited altered stoichiometry compared to the wild type. MS analysis of Peb3 fractions from this mutant did not detect glycosylation, yet *pglI*-negative protein extracts reacted with SBA at a level similar to that of the wild type. To resolve these observations and to determine whether Peb3 was inefficiently glycosylated in this mutant, total glycoproteins were extracted from the *pglI* mutant by SBA affinity chromatography and separated by SDS-PAGE, and the glycoprotein bands were analyzed by in-gel tryptic digestion and nanoLC-MS/MS as described. The MS/MS spectra of glycopeptides derived from one of these glycoproteins, the secreted transglycosylase (Cj0843c) from wild-type *C. jejuni* NCTC 11168

and the *pglI* mutant are presented in Fig. 5A and B. The identity of Cj0843c was confirmed by the observation of weak peptide fragment ions in these spectra (not highlighted in Fig. 5) and by the presence of other peptides derived from this protein in the gel band digest extracts. The MS/MS spectrum of the doubly charged wild-type glycopeptide ion at m/z 1325.6 was dominated by the glycan oxonium ions in the low-mass region of the spectrum (m/z 204.1, HexNAc; m/z 366.1, HexNAc plus Hex; m/z 407.2, $2 \times$ HexNAc) and by the sequential loss of monosaccharides in the upper regions and was entirely consistent with the structure described previously for this glycan (33). On the other hand, the MS/MS spectrum of the corresponding doubly protonated *pglI*-negative glycopeptide ion at m/z 1244.5 lacked the HexNAc plus Hex oxonium ion at m/z 366.1 and any fragment ions in the higher m/z regions indicating the presence of a hexose. This evidence, plus the difference in mass between the two glycopeptides, indicates that the Cj0843c glycan from the *pglI* mutant lacked the hexose moiety representative of Glc. Similar analyses were carried out with the *pglD*, *pglH*, and *pglK* mutants (not shown). As expected, there were no detectable glycopeptides for *pglH* and *pglK* mutants; however, there was evidence of very low levels of glycosylation for the *pglD* mutant. Unfortunately, the levels were too low to analyze further.

Chick colonization studies. We inoculated 1-day-old chicks with several *pgl* mutants to determine colonization potential and to identify the minimum N-linked glycan structure required for survival in vivo (Fig. 6). The *pglG* mutant, which expresses glycoproteins with wild-type N-glycan structures, colonized to wild-type levels. However, where glycan biosynthesis was abolished or severely impaired, i.e., with *pglB*, *pglD*, *pglE*, and *pglK* mutants, there was no colonization detected. Interestingly, the *pglI* mutant, which synthesized glycoproteins lacking the Glc branch, showed levels of colonization statistically similar to those of the wild type. Due to the variability in *pglI* mutant colonization levels observed in Fig. 6, the experiment was repeated a second time with two separate doses of the wild type and the *pglI* mutant (5×10^9 and 5×10^{10}) and again showed no differences in colonization (results not shown).

DISCUSSION

Earlier colonization studies with mice (20) and later with chicks (10, 11, 21) demonstrated that the *C. jejuni* N-linked glycan was required for colonization in vivo. Our current studies indicate that the minimal structural requirement for efficient protein glycosylation and chick colonization is the hexasaccharide backbone with or without the Glc branch. Mutation of the gene bordering the *pgl* locus, *pglG*, was shown to have no effect on N-linked protein glycosylation by HR-MAS NMR, mass spectrometry, and chicken colonization studies, suggesting that *pglG* is not involved in the pathway under the conditions examined. Lectin blotting and mass spectrometry of in-gel digests indicated that another acetyltransferase homolog, such as those identified in the pseudaminic acid pathway of flagellar O glycosylation (22), can partially complement the activity of PglD in vivo. However, complementation occurred at very low levels, since the mutant was unable to colonize chicks and N-linked glycans were not detected by HR-MAS or on Peb3 by MS. In contrast, the intact glycan was observed on Peb3 by Linton et al. when the *C. jejuni* glycosylation pathway

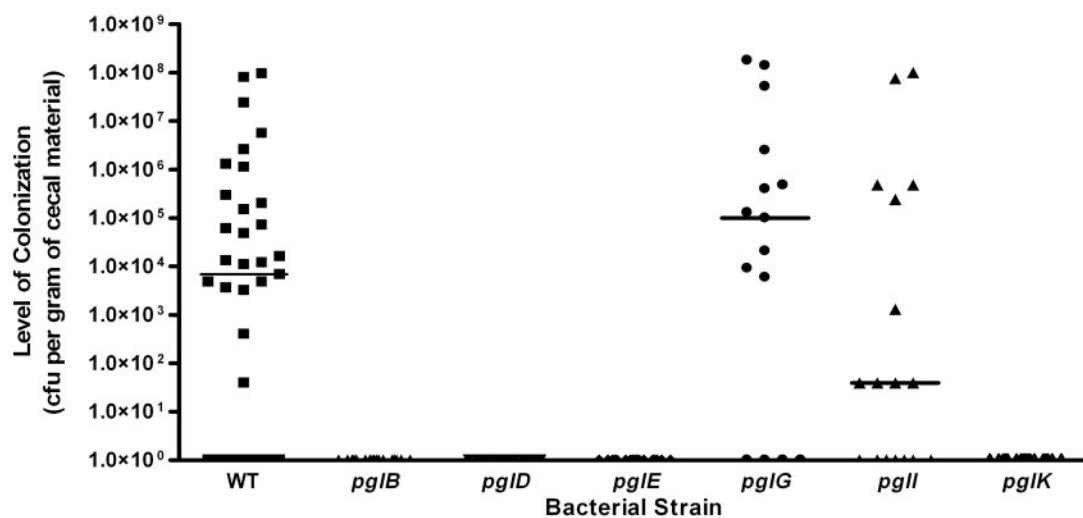


FIG. 6. Colonization of 1-day-old chicks by *C. jejuni* NCTC 11168 and indicated mutants. The bar represents the median level of colonization for each group. The sensitivity level of the assay is as low as 40 CFU of *C. jejuni* per gram of cecal material.

was functionally transferred into *E. coli* and *pglD* was inactivated (15). This may be due to differences in the enzyme repertoire, protein expression, metabolite concentrations, or pathway compartmentalization in an alternate host.

pglI mutation causes loss of the glucose branch, confirming that this gene encodes the glucosyltransferase (8, 15). Interestingly, the oligosaccharide transferred in the *pglI* mutant contains the entire 6-mer, demonstrating that Glc plays no role in determining the length of the GalNAc backbone. HR-MAS NMR measurement of protein glycosylation efficiency demonstrated that rates of protein modification were not altered significantly in this mutant, and this was supported by observations of similar levels of chick colonization *in vivo* and protein reactivity with SBA. However, the observation that Peb3 was not modified in the *pglI* mutant suggests that there are minor differences which need further investigation. For example, we previously demonstrated that only 50% of Peb3 was glycosylated with a single heptasaccharide in the wild-type background, so N-linked modification of this protein may be inefficient (33). In addition, the SBA protein reactivity pattern in the *pglI* mutant differs slightly from the wild type, with one band missing in the same mass range (27 kDa) as glycosylated Peb3 (Fig. 3). In yeast and higher eukaryotes, loss of glucose results in reduced rates of glycan transfer to protein both *in vitro* and *in vivo* (2, 26, 27). Following transfer, removal of Glc is critical for protein quality control in the endoplasmic reticulum to mediate correct protein folding and glycan processing to the complex structures found in these organisms (19). Since there is no further processing of the N-linked glycan in *C. jejuni*, it is not surprising that the Glc is not essential in the bacterial pathway; recently, genome sequencing demonstrated that another *Campylobacter* species was missing the Glc transferase homolog in its *pgl* gene cluster (7, 24).

Loss of N-linked glycan detection by NMR, mass spectrometry, and SBA reactivity, in combination with loss of chick colonization, demonstrated for the first time that the putative ABC transporter, WlaB, is involved in N-linked protein glycosylation in *C. jejuni*. We propose to renaming WlaB as PglK to

reflect these observations. Future studies will determine whether PglK indeed provides the energy to transfer the glycan across the inner membrane. It will also be interesting to establish whether recognition of the glycan block exists at this stage of the pathway, since reports have demonstrated that PglB has relaxed specificity and is capable of adding incomplete glycans, and even foreign O antigens, to protein when expressed in *E. coli* (6, 15). Our results describing protein modification with the complete N-linked heptasaccharide in its native host are in contrast to the results described by Linton et al., where incompletely assembled oligosaccharides were added to protein when the system was transferred into *E. coli* (15). Since inactivation of *C. jejuni* WlaB (PglK) in *E. coli* did not affect protein glycosylation, it is possible that a promiscuous *E. coli* enzyme (such as Wzx) (5, 17) is compensating and providing incomplete glycans to be added through the action of PglB. To support this argument, examples for cross talk between the *E. coli* system and the *C. jejuni* glycosylation machinery have already been demonstrated, i.e., *E. coli* WecA compensation for *C. jejuni* PglC (15) and HexNAc occasionally replacing Bac (29) in recombinant glycoproteins expressed in *E. coli*.

During the process of assembling preformed glycans for block transfer, interference at any stage of glycan synthesis will result in the loss of glycoconjugate formation. In this study, we demonstrate that disruption of the enzymes involved in biosynthesis, transport, and transfer of the hexasaccharide backbone was essential for N-glycan addition and for chick colonization, while removal of the Glc branch showed very little effect. Thus, the initial steps for N-linked protein glycosylation involving the synthesis, flipping, and addition of the preformed oligosaccharides en bloc to select motifs on the nascent protein have been evolutionarily conserved between bacteria and eukaryotes (16). Similarly, it was recently demonstrated that the process of protein O-mannosylation is also conserved between bacteria and eukaryotes (28). Furthermore, we show that under the conditions examined, PglG plays no role in glycan biosynthesis, PglI is the glucosyltransferase, and the putative ABC transporter, WlaB (renamed PglK) is required for N-

glycan formation. HR-MAS NMR is a nondestructive technique that for the first time allowed efficient tracking and quantitation of the N-linked protein glycosylation pathway in vivo. Further characterization of this pathway in the native host will not only provide details describing the coordinated reactions and regulation of the pathway in bacteria and higher organisms but will also provide a wealth of information on optimizing the system for efficient glycoengineering in foreign hosts such as *E. coli*.

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