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Affinity-Capture Tandem Mass Spectrometric Characterization of Polyprenyl-Linked Oligosaccharides: Tool to Study Protein N-Glycosylation Pathways

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N-Glycosylation of proteins is recognized as one of the most common post-translational modifications. Until recently it was believed that N-glycosylation occurred exclusively in eukaryotes before the discovery of the general *p*rotein *gly*cosylation pathway (Pgl) in *Campylobacter jejuni*. To date, most techniques to analyze lipid-linked oligosaccharides (LLOs) of these pathways involve the use of radiolabels and chromatographic separation. Technologies capable of characterizing eukaryotic and the newly described bacterial N-glycosylation systems from biologically relevant samples in a quick, accurate, and cost-effective manner are needed. In this paper a new glycomics strategy based on lectin-affinity capture was devised and validated on the *C. jejuni* N-glycan pathway and the engineered *Escherichia coli* strains expressing the functional *C. jejuni* pathway. The lipid-linked oligosaccharide intermediates of the Pgl pathway were then enriched using SBA-agarose affinity-capture and examined by capillary electrophoresis−mass spectrometry (CE−MS). We demonstrate that this method is capable of detecting low levels of LLOs, the sugars are indeed assembled on undecaprenylpyrophosphate, and structural information for expected and unexpected LLOs can be obtained without further sample manipulation. Furthermore, CE−MS analyses of *C. jejuni* and the *E. coli* "glyco-factories" showed striking differences in the assembly and control of N-glycan biosynthesis.

N-linked glycosylation is an essential protein modification in eukaryotes. The central step of the process takes place on the luminal side of the endoplasmic reticulum (ER) membrane where a preassembled oligosaccharide ($Glc_3Man_9GlcNAc_2$) is transferred from a polyisoprenol carrier, dolichyl-pyrophosphate (Dol-PP), to the asparagine side chain of nascent proteins by the oligosaccharyltransferase (OST) complex (Figure 1A).[1,2]

N-glycosylation systems have traditionally not been observed in bacteria, with the recent exception of the enteric pathogen *Campylobacter jejuni*, where the *pgl* operon encodes a general N-linked protein glycosylation pathway (Figure 1B).[3] The glycan in the *C. jejuni* system is a conserved heptasaccharide with the structure GalNAc-α1,4-GalNAc-α1,4-[Glc-β1,3]-GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-Bac2,4diNAc-β1, where Bac2,4diNAc is 2,4-diacetamido-2,4,6-trideoxyglucopyranose.[4] The similarities between the eukaryotic and bacterial N-glycan pathways include the assembly of an oligosaccharide on a polyisoprenol carrier anchored in the membrane and transfer of the oligosaccharide en bloc to asparagine residues of proteins with specific sequons.[5,6] Furthermore, the *C. jejuni* oligosaccharyltransferase PglB shows modest homology to Stt3p, which is an essential subunit in the eukaryotic OST complex.[3,7] Since the N-glycosylation pathway in *C. jejuni* is not required for bacterial viability and possesses many similarities with the eukaryotic counterpart, it provides an ideal system to study protein N-glycosylation.

Although the understanding of living organisms at the molecular level is still in its infancy, it is evident that comprehensive investigations of the "omics cascade" with genomics, transcriptomics, proteomics, glycomics, and metabolomics will provide important information for advancing our knowledge of these systems.[8] The integrative analysis of biological systems will lead to a better understanding of the biochemical and biological mechanisms at work in an organism. While genomics, transcriptomics, and proteomics have made significant strides in technology development, the tools for the examination of the metabolome

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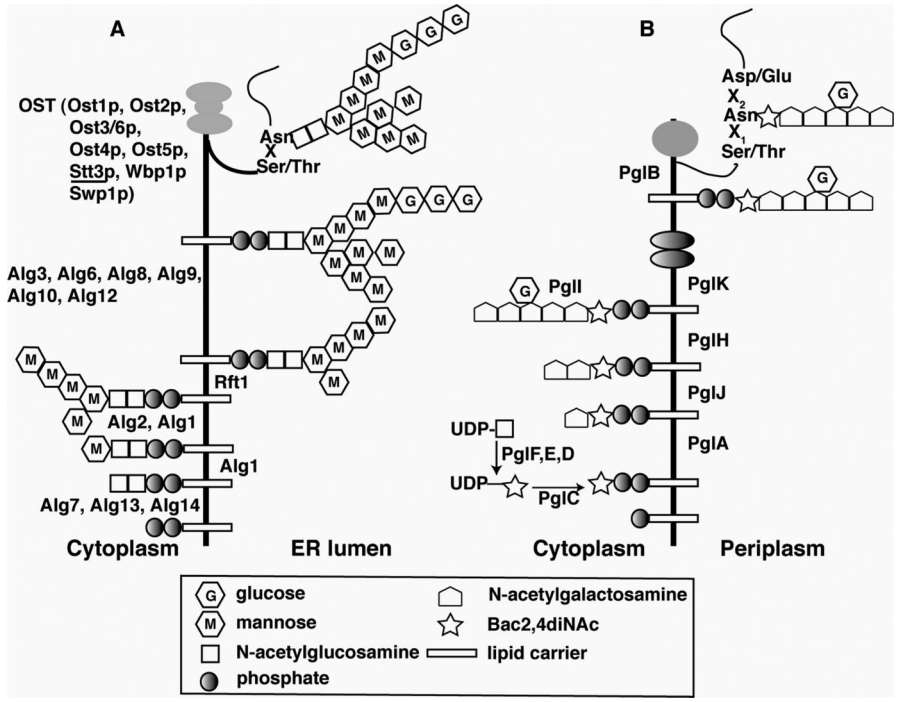

Figure 1. Comparison of N-linked protein glycosylation in (A) eukaryotes and (B) the bacterium, *Campylobacter jejuni*. The underlined OST subunit possesses homology to PglB.

are still emerging.[9–12] The burgeoning interest in lipids, and by extension lipidomics, has been facilitated by the enhanced capabilities of modern mass spectrometry (MS) instruments and interfaces and an increase in the development of global lipid analytical methods.[13–17] This now opens up new opportunities to examine in greater detail bacterial glycan pathways that proceed through lipid-intermediates, such as N-glycosylation, capsular polysaccharides, lipopolysaccharides, and peptidoglycan. Furthermore, recent reports have demonstrated that the pilin O-glycosylation pathways in the important human pathogens, *Neisseria meningitidis* and *Pseudomonas aeruginosa*, also proceed through lipid-linked intermediates.[18–21]

Traditionally, the detection of lipid-linked oligosaccharides (LLO) have been performed using a variety of techniques including metabolic radiolabeling,[22] solid-state analysis of LLOs with conjugated lectins,[23] an OST assay with ^{125}I-labeled acceptor peptides, or fluorophore assisted carbohydrate electrophoresis (FACE).[24] Metabolic radiolabeling of LLOs is hampered by the variable rates of LLO turnover, isotope dilution effects, and difficulties associated with certain cells or tissues not being amenable to metabolic labeling. The solid-state assay employing horseradish peroxidase conjugated Concanavalin A (ConA) lectin provides a semiquantitative, nonradioactive assay for LLOs. Its major drawback is the lack of information regarding the heterogeneous makeup of the LLO pool under investigation. Both the OST assay and FACE analysis of LLOs rely on detection of the LLO associated glycans. In the OST assay, detection of all LLOs in the pool is based on their efficient transfer to a radioactively labeled acceptor peptide and assumes that all LLOs are transferred with equal efficiency to the acceptor peptide. A recent study by Kelleher et al. of several eukaryotic OSTs suggests that equal transfer efficiency is a false assumption.[25] FACE analysis of LLO associated glycans are labeled with a fluorophore, typically 8-amino-1,3,6-naphthalenetrisulfonate (ANTS) or 7-amino-1,3-naphthalenedisulfonate (ANDS), and separated by electrophoresis. With the exception of the solid-state assay, all current methods

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require chromatographic or electrophoretic separation and are not readily amenable to structural characterization.

Capillary electrophoresis (CE) is a high-resolution technique for the separation of complex biological mixtures and has been widely applied to biological analyses.[26] The coupling of CE with mass spectrometry provides a powerful approach for rapid identification of target analytes present at trace levels in biological matrixes and for structural characterization of complex biomolecules.[27–30] Here we report a method for the detection of LLOs from bacterial samples. Affinity capture-mass spectrometry has been applied to the analysis of LLOs from the *C. jejuni* N-glycan pathway and the recently developed *Escherichia coli* "glycofactory".[31] Affinity-capture with agarose immobilized soybean agglutinin (SBA) with affinity for GalNAc was chosen for selective enrichment of LLOs. Lectins have been used successfully to characterize glycolipids from a variety of biological sources,[32–37] and SBA has previously been shown to effectively bind the *C. jejuni* N-glycan.[38] Results from this study provide new insight into the function and control of the *C. jejuni* N-glycan pathway, which will aid in future glycoengineering efforts. The methodology can also be applied to improving our understanding of glycoconjugate biosynthesis pathways in other bacteria, and there is potential for expanding this method to the investigation of eukaryotic systems.

METHODS

Chemicals and Materials. All chemicals were of analytical reagent grade and used as received. SBA-agarose was obtained from EY Laboratories (San Mataeo, CA). Sodium chloride, sodium phosphate, ethylenediamine tetraacetic acid disodium salt (EDTA), and galactose were purchased from Sigma-Aldrich (St. Louis, MO), while *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic sodium salt (HEPES) was acquired from Bioshop Ltd. (Burlington, ON). Chloroform and methanol were purchased from EMD Chemicals (Gibbstown, NJ) and were of analytical grade. Distilled water was deionized on a Millipore Milli-Q water reagent system (Billerica, MA).

Bacteria. Wild-type *C. jejuni* 11168 and *pgl* mutants (*pglB*, *pglD*, *pglH*, *pglI*, *pglJ*, *pglK*) were previously described.[6,39] *E. coli* DH5α and SCM7 harboring pACYCpgl and pACYCpglmut were previously described.[31,40] *E. coli* cells were grown in 2× YT overnight at 37 °C with shaking, while *C. jejuni* was grown in brain-heart infusion (BHI) broth at 37 °C under microaerobic conditions. Bacteria were enumerated using the technique described by Gross et al.[41]

Lipid-Linked Oligosaccharide Standards. Chemo-enzymatically synthesized glycans conjugated to undecaprenyl-pyrophosphate (Und-PP) as previously described,[42] were used to optimize CE−MS conditions. In order to optimize enrichment conditions for LLO extraction from biological samples, bacterial cells were spiked with purified LLOs. Therefore a large scale LLO purification from *E. coli* DH5α harboring pACYCpglmut was performed using a modified protocol adapted from Nakamura et al.[43] Briefly, cells were grown in a 28 L New Brunswick Scientific fermentor in 2× YT broth supplemented with chloramphenicol to 30 μg/mL at mixing rate of 200−400 rpm. Cells were harvested at an OD_{600} = 4.3 by tangential flow filtration to yield a cell pellet of 234 g (wet weight). The cell pellet was extracted with 1 L of $CHCl_3$/MeOH/H_2O (10:20:3) (solvent A) using a Sorval Omni-mixer for 1 h at room temperature. The cell debris was removed by filtration and the combined organic filtrate was concentrated in vacuo. The concentrated sample was dialyzed overnight against water and lyophilized. A 0.85 g sample of the lyophilized crude LLO extract was dissolved in a minimum of solvent A and purified by anion exchange chromatography on DEAE Sephadex A-50. Bound LLOs were eluted with a gradient of solvent A containing increasing amounts of NH_4Ac. Fractions containing LLOs were characterized by CE−MS and purity determined by TLC using solvent A as the mobile phase with detection with I_2 (vapor) and 20% H_2SO_4 in ethanol.

Isolation of Lipid-Linked Oligosaccharides for Analysis. Cells from overnight cultures of either 1 L of *E. coli* DH5α and SCM7 harboring the functional (pACYCpgl) or inactive (pACYCpglmut) *C. jejuni* N-glycan operon or 7.5 L of *C. jejuni* 11168 wild type and *pglB*, *pglD*, *pglH*, *pglI*, *pglJ*, *pglK* isogenic mutants were harvested (7 500*g*, 15 min, 4 °C). Cell pellets, which corresponded to 10^{12}−10^{13} bacterial cells, were extracted with 2 × 20 mL of 10:20:3 ($CHCl_3$/MeOH/H_2O) and 2 × 20 mL 2:1 ($CHCl_3$/MeOH), and the combined extracts were evaporated to dryness. The concentrated lipid extract was dissolved in a minimum of 9:1 EtOH/MeOH, diluted 10-fold with buffer A (10 mM Hepes pH 7.4, 3.4 mM EDTA, 150 mM NaCl) and incubated with 100 μL of SBA-agarose for 1 h. The resin was collected by centrifugation (2 200*g*, 5 min, 4 °C), and washed three times with buffer A. The bound LLOs were eluted from the SBA-agarose with 0.2 M Gal in 25 mM NaH_2PO_4, pH 7.4 as per the manufacturer's instructions and used without further purification.

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Quantitative Assessment of SBA Affinity for Und-PP-Oligosaccharides. Radioactively labeled Und-PP-di-, tri-, hexa-, and heptasaccharides were prepared as previously described.[42,44] A tube containing 0.13 nmol of Und-PP-sugar (0.15−0.20 μg depending on the sugar size) was resuspended in 40 μL of 9:1 EtOH/MeOH with vortexing and sonication, diluted with 360 μL of buffer A. An amount of 20 μL of SBA-agarose, that was pre-equilibrated with buffer A, was added, and the mixture incubated at 4 °C for 2 h. The supernatant was removed, and the resin washed with another 400 μL of buffer A. The bound radiolabeled LLOs were eluted with 0.2 M Gal (1 × 400 μL, 2 × 200 μL) with 15 min incubations. The eluted radioactive material was measured by scintillation counting as previously described.[44] All reactions were done in duplicate and with an associated error of 1−2% for each of the values.

CE−MS Analysis of Lipid-Linked Oligosaccharides. Mass spectra were acquired using a 4000 QTrap mass spectrometer (Applied Biosystems/Sciex, Concord, ON, Canada). CE was performed using a Prince CE system (Prince Technologies, The Netherlands). The CE separation was obtained on a 90 cm length of bare fused-silica capillary (365 μm o.d. × 50 μm i.d.) with CE−MS coupling using a liquid sheath-flow interface and isopropanol/methanol (2:1) as the sheath liquid. An aqueous buffer consisting of 10 mM NH_4Ac was used for all experiments. Precursor ion scanning for Und-PP (*m/z* 907.6) was performed in negative-ion mode with a collision energy of 70 V. For MS/MS experiments in the positive ion mode, the declustering potential on the instrument was set at 400 V in order to produce oligosaccharide ions without the Und-PP linker, which limited detailed structural characterization. Sodiated molecular ions with masses corresponding to the sugar portion of the original Und-PP-linked oligosaccharides, observed previously in the negative ion mode, were then selected for collision-induced dissociation in the positive ion mode to obtain detailed and complete sequence information.

CE−MS Analysis of Nucleotide-Linked Sugars. Nucleotide-sugar pools were analyzed in *C. jejuni* using the protocol developed by Soo et al.[45] Briefly, 1.5 L of cultures of *C. jejuni* 11168, *pglB*, *pglD*, *pglH*, *pglI*, *pglJ*, and *pglK* were harvested and lysed using BugBuster (Novagen, San Diego CA) in 50 mM NH_4HCO_3 pH 8.0. Cell debris was removed by centrifugation, and the protein content was precipitated with the addition of ice-cold ethanol to a final concentration of 60%. The precipitated proteins were removed by centrifugation, and the supernatant was evaporated to dryness. The dried sample was redissolved in H_2O and filtered through a Millipore Amicon Ultra (molecular weight cut off (MWCO) 10 000) spin column. The filtrate was evaporated to dryness and analyzed by CE−MS without further purification.

RESULTS AND DISCUSSION

Development of CE−MS and LLO Enrichment Conditions. Solubilization and ionization conditions were first established using chemo-enzymatically synthesized glycans conjugated to Und-PP resulting in LLOs of varying oligosaccharide size representative of the intermediates in the *C. jejuni* N-glycan pathway.[42,46] Analyses of these Und-PP-linked oligosaccharide standards were performed using precursor ion scan in the negative ion mode for Und-PP (see Supporting Information Figure S1). Because of the limited structural characterization of Und-PP-linked oligosaccharides in the negative ion mode, MS/MS spectra were acquired in the positive ion mode upon orifice fragmentation resulting in the cleavage of the undecaprenyl-pyrophosphate linker to produce free oligosaccharides. Care must be taken when analyzing these samples since a migrating rearrangement of the Und-PP-linked oligosaccharides can occur.[47]

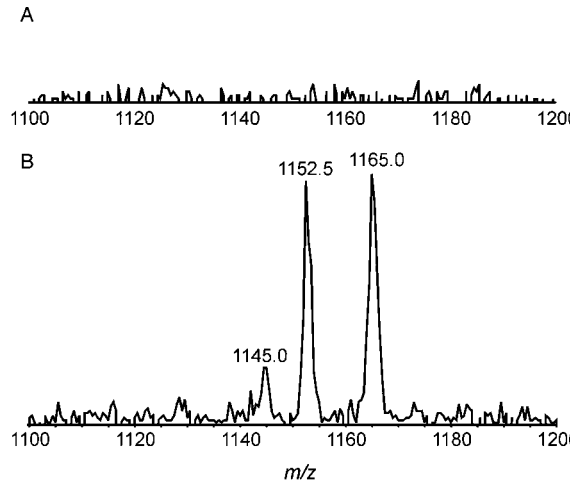

Figure 2. Recovery of LLOs from *E. coli* extracts, (A) unspiked and (B) spiked with 250 μmol of purified Und-PP-heptasaccharide. Precursor ion scan experiments were performed at an ion of *m/z* 907.6 with a collision energy of 70 V.

The ability of immobilized SBA lectin to effectively bind Und-PP-linked oligosaccharides of varying lengths was assessed using radioactively labeled Und-PP-di-, tri-, hexa-, and heptasaccharides. The results indicated that the immobilized SBA lectin could bind LLOs as small as the disaccharide containing only one GalNAc residue (Supprting Information Table S1). In order to demonstrate that the immobilized SBA lectin can selectively enrich LLOs from biological samples, *E. coli* cells lacking the engineered N-glycan pathway were spiked with purified Und-PP-heptasaccharide prior to extraction with 10:20:3 ($CHCl_3$/MeOH/H_2O). The samples were subjected to SBA-agarose enrichment and analysis by CE−MS (Figure 2). The Und-PP-heptasaccharide contained relatively equal amounts of *m/z* 1153.0 (HexNAc at the reducing end) and *m/z* 1165.5 (Bac2,4diNAc at the reducing end). The minor peak observed at *m/z* 1145.0 potentially corresponds to Und-PP-HexNAc alone. Analysis with purified Und-PP-heptasaccharide demonstrated a subpicomole instrumental detection limit. Additionally, LLOs of the Pgl pathway could be isolated from biologically relevant samples containing 10^{12}−10^{13} cells (see below).

LLOs from *C. jejuni* and the *pgl* Mutants. LLO profiling of the *C. jejuni* N-glycan pathway and its isogenic *pgl* mutants has provided unique insight into the synthesis of this oligosaccharide. Attempts to detect LLOs of the N-glycan pathway in wild-type *C. jejuni* 11168 proved unsuccessful even when a 5-fold greater cell

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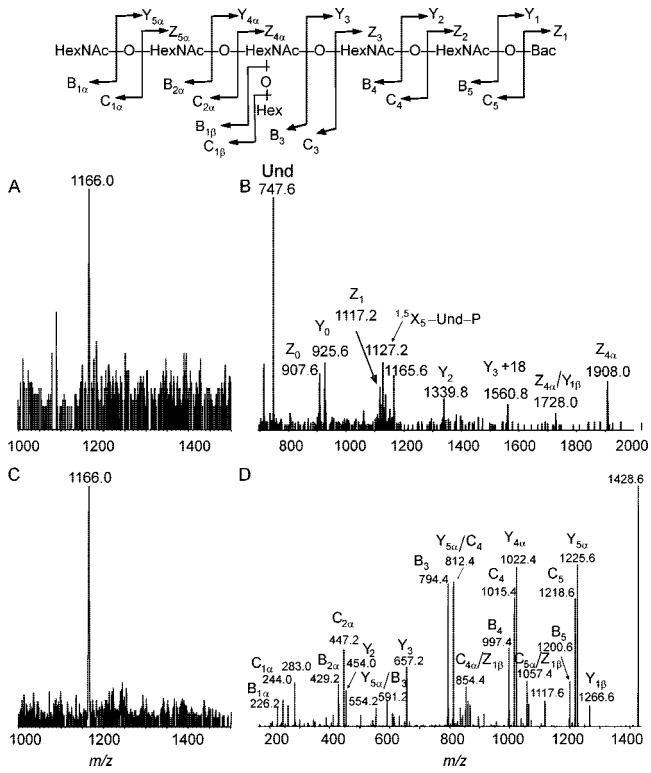

Figure 3. CE−MS negative ion mode precursor ion scans for Und-PP (*m*/*z* 907.6) in glycolipid enriched extracts of *C. jejuni pgl* mutants. Analysis of (A) *pglB* and (C) *pglK* mutants showing accumulation of Und-PP linked heptasaccharide (*m*/*z* 1166.0); (B) negative ion mode MS/MS spectrum of *pglB* showing loss of Und-PP. (D) MS/MS analysis of *m*/*z* 1166.0 in the *pglK* mutant in the positive ion mode with high orifice voltage to cleave off the lipid portion of the molecule, confirming the structure of the N-glycan.

density was used as starting material. The lack of accumulating LLOs in wild-type *C. jejuni* suggests that, in the presence of polypeptide acceptors, LLOs are used immediately as they are formed. However, when the oligosaccharyltransferase (*pglB*) and flippase (*pglK*) mutants were analyzed, LLOs corresponding to the Und-PP-heptasaccharide (*m*/*z* 1166.0) were detected in both samples (Figure 3 and Table 1). Fragmentation of the LLOs in the negative ion mode confirmed that the oligosaccharide is assembled on Und-PP based on fragment ions of *m*/*z* 925.6 and 907.6 corresponding to the masses of Und-PP and Und-PP[$-H_2O$], respectively (Figure 3B). The identity of the oligosaccharide was confirmed by MS/MS analysis in the positive ion mode (Figure 3D). In contrast, analysis of the GalNAc transferase mutants (*pglH* and *pglJ*), the glucosyltransferase mutant (*pglI*) and the Bac2,4diNAc acetyltransferase mutant (*pglD*) showed no measurable accumulation of LLO intermediates. In order to verify that the negative result was due to a low abundance of truncated LLOs and not to a recognition problem by the lectin, a crude lipid extract from the *pglH* mutant was enriched using anion exchange resin (similar to the method described above to isolate LLOs from *E. coli* for use as standards) and analyzed by CE−MS. Analysis of DEAE-Sephadex enriched *pglH* sample confirmed initial results with the immobilized SBA lectin. This suggested that LLOs in these mutants were below the level of detection for this method. Analysis of nucleotide sugar pools in these *pgl* mutants showed an accumulation of UDP-diacetamido-trideoxy-Hex in only the *pglJ* mutant and not in *pglB*, *pglD*, *pglH*, *pglI*, and *pglK*, Figure 4 and Table 1. For the *pglD* mutant, minor levels of protein glycosylation are still observed,[6] so one would expect to see less or no accumulation of intermediates in this mutant background. However, the lack of accumulating LLOs in the *C. jejuni pglH* and *pglJ* mutants was unexpected considering that PglB does not transfer truncated N-glycans to glycoproteins in these backgrounds.[6] Therefore, the lack of detectable truncated LLOs in the *C. jejuni pglH* and *pglJ* mutants must be due to feedback inhibition at preceding steps, which is supported by the detection of UDP-Bac2,4diNAc in the first of the α1,4-GalNAc transferase mutants, *pglJ*.[42] This conclusion is reinforced by the observed allosteric regulation in the yeast OST complex.[48] Since undecaprenol is in limited abundance in the bacterial cell and is involved in a plethora of cellular activities, some of which are vital for cell survival, it is not surprising that a feed-back regulation of the N-glycan pathway would exist to prevent the accumulation of LLOs and sequestering of undecaprenol from other biological processes.

Analysis of LLOs from the *E. coli* "Glyco-Factory". The results from the native *C. jejuni* host were compared to the *E. coli* glyco-factory in an attempt to better understand the system being used for glycoengineering efforts.[31,49] Analysis of LLOs from *E. coli* DH5α harboring pACYCpglmut, which encodes the *C. jejuni* N-glycosylation pathway with a nonfunctional PglB,[31] showed accumulation of multiple LLOs (Figure 5A). A large percentage of these intermediates possess a HexNAc at the reducing end instead of Bac2,4diNAc (Table 2), with the native N-glycan structure constituting less than 10% of the total LLO pool detected. In addition, some intermediates displayed two Hex side-branches on the oligosaccharide in contrast to what was previously reported for the native structure.[4] Similar results were obtained with *E. coli* pACYCpgl with a fully functional PglB under limiting polypeptide acceptor conditions (note that PglB itself is N-glycosylated). The LLOs detected in this background were in significantly reduced amounts with only a few of the LLOs observed (Figure 5B). When the pACYCpglmut and pACYCpgl+ constructs were inserted into the *E. coli* SCM7 background, a more efficient system is observed (Figure 5C). Only LLOs containing Bac2,4diNAc-heptasaccharide were detected in the SCM7 strain (Figure 5C) but at significantly lower amounts compared to the DH5α background. In *E. coli* SCM7, all of the flippases of the lipopolysaccharides (LPS) Wzx family are lacking. In addition, the LPS and enterobacterial common antigen (ECA) initiating GlcNAc transferase WecA are removed.[40] Previous analysis of glycoproteins from this *E. coli* strain suggested that only oligosaccharides with Bac2,4diNAc at the reducing end were being produced.[40] Under conditions where other flippases are not active, the broad distribution of LLOs should not be observed and only those possessing Bac2,4diNAc would be transported to the periplasm. This is probably due in part, to the lack of interference

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Table 1. Summary of Results of N-Glycan Intermediate Analyses in *C. jejuni*

strain	function	glycoprotein phenotype[a]	LLOs	UDP-Bac2,4diNAc[b]
11168		heptasaccharide	none	none
pglB	oligosaccharyl transferase	unmodified	yes	none
pglD	acetyltransferase	minor heptasaccharide[c]	none	none
pglH	processive GalNAc transferase	unmodified	none	none
pglI	Glc transferase	hexasaccharide	none	none
pglJ	second GalNAc transferase	unmodified	none	yes
pglK	flippase	unmodified	yes	none

[a] Reference 6. [b] Based on precursor ion scans for nucleotide linked sugars (UDP) from cell lysates. [c] Minor levels of N-glycosylation observed in this mutant.

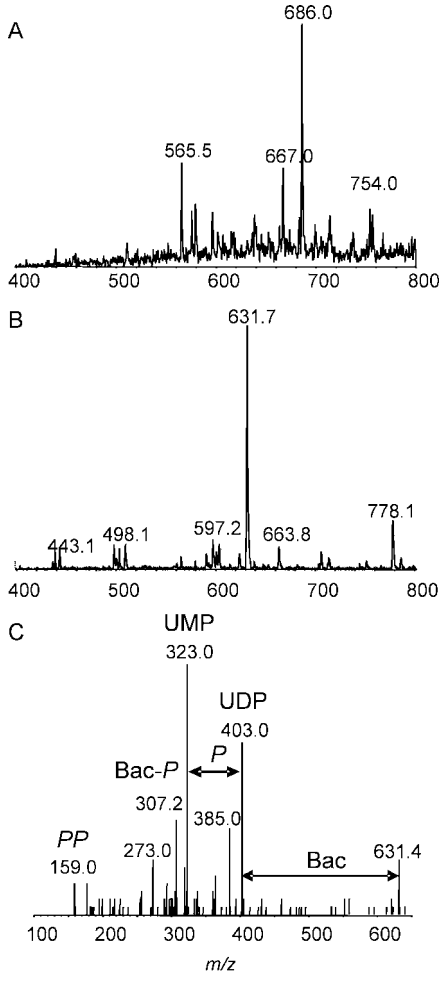

Figure 4. CE−MS precursor ion scans for nucleotide-linked sugars (UMP, *m*/*z* 323.2) in cell lysates of (A) *C. jejuni* 11168 and (B) the *pglJ* mutant. (C) MS/MS spectrum of *m*/*z* 631.5 confirming the presence of UDP-Bac2,4diNAc in the *pglJ* sample.

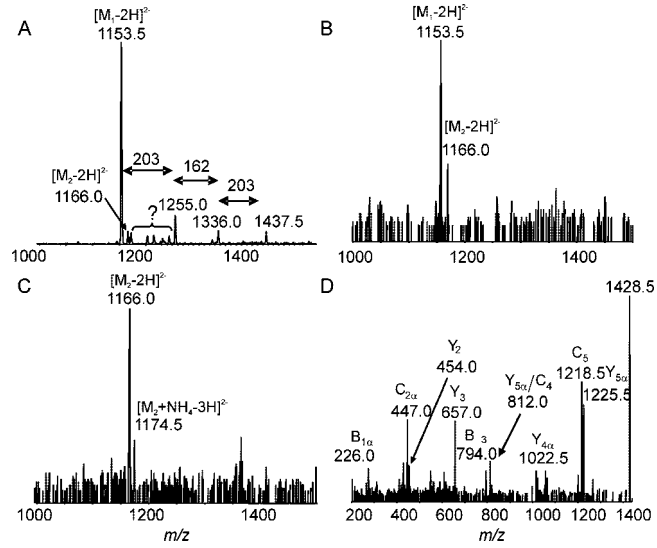

Figure 5. CE−MS precursor ion scans in the negative ion mode for Und-PP (*m*/*z* 907.6) of *E. coli* (A) pACYCpglmut and (B) pACYCpgl+, and (C) *E. coli* SCM7 pACYCpglmut enriched LLO extracts. (D) MS/MS spectra of *m*/*z* 1166.0 in the positive ion mode with high orifice voltage to cleave off the lipid portion of the molecule, confirming the presence of Bac2,4diNAc in the expected N-glycan structure. M_1 refers to an Und-PP-heptasaccharide possessing a HexNAc at the reducing end while M_2 corresponds to the Und-PP-heptasaccharide with Bac2,4diNAc at the reducing end.

from other *E. coli* flippases and WecA on the synthesis of the *C. jejuni* N-glycan.

When comparing the N-glycan pathway in *C. jejuni* and *E. coli*, several striking differences are noted. In *C. jejuni*, the system is tightly controlled, and completely assembled LLOs are efficiently used. Disruptions in steps upstream of *pglK* do not accumulate LLO intermediates. In the case of the *pglI* mutant, PglB is able to recognize and transfer the hexasaccharide as efficiently as the native heptasaccharide with the Glc, thereby preventing accumulation of intermediates in the pathway. With the GalNAc transferase mutants, *pglH* and *pglJ*, the lack of truncated LLO accumulation is a result of negative feedback at the level of PglJ, causing the accumulation of nucleotide-linked sugar precursors. In the case of the *E. coli* glyco-factory, a loosely regulated system is observed in the DH5α background in which a broad distribution of LLOs is detected including LLOs greater in length and with additional Glc branches in contrast with the native heptasaccharide structure. This unregulated synthesis suggests the role for auxiliary proteins in *C. jejuni* that aid in regulating and coordinating glycosylation events which are not present in the *E. coli* system. In addition, endogenous *E. coli* proteins, different *pgl* gene expression levels, and available nucleotide-linked intermediates could interfere and cause this aberrant glycosylation profile. The influence of endogenous *E. coli* proteins in the synthesis of LLOs is supported by the results obtained in the SCM7 background.

There are currently several methods employed to study lipid-linked steps in oligosaccharide biosynthesis in eukaryotes.[50] Metabolic radiolabeling has been an essential technique for

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Table 2. Results of Lipid-Linked Oligosaccharide Analyses of the *E. coli* "Glyco-Factory" Strains

strain	LLOs observed	relative intensity[a] (%)	glycoprotein phenotype[b]
		E. coli DH5α	
pACYCpglmut			unmodified
	5HexNAc + 1Hex	6.2	
	6HexNAc + 1Hex	100	
	1Bac2,4diNAc + 5HexNAc + 1Hex	6.5	
	7HexNAc + 1Hex	14.5	
	7HexNAc + 2Hex	6.8	
	8HexNAc + 2Hex	2.8	
pACYCpgl+			heptasaccharides with HexNAc or Bac2,4diNAc
	1Bac2,4diNAc + 5HexNAc +1Hex	50	
	7HexNAc + 1Hex	100	
		E. coli SCM7	
pACYCpglmut			
	1Bac2,4diNAc + 5HexNAc + 1Hex	100	unmodified
pACYCpgl+			
	1Bac2,4diNAc + 5HexNAc + 1Hex	100	heptasaccharide

[a] Most abundant ion in spectrum set to 100%. [b] References 31, 53.

analysis of LLOs in intact cells and is a very sensitive technique for the detection of $Glc_3Man_9GlcNAc_2$-PP-Dol, with detectable levels of LLOs from only 10^6 CHO-K1 cells.[50,51] Despite this high sensitivity, metabolic radiolabeling techniques have limitations such as the challenge of exposing tissue to sufficiently high concentrations of radioactive precursor and limitations in the incorporation of the radioactive precursor. Alternatives to metabolic radiolabeling include the analysis of LLOs based upon transfer of the glycan to [^{125}I]-acceptor peptide or solid-state ConA binding with chemiluminescence.[23] Neither of these techniques readily provide structural information on the LLO components in the mixture. In the case of the OST end point assay and metabolic radiolabeling experiments, tedious chromatographic steps are required. While the affinity-capture technique described here does not approach the sensitivity currently available from the use of radioisotopes, its major advantage over conventional techniques is the ability to provide structural information on the pool of LLO intermediates, and the method does not depend on the accessibility to an OST preparation. Additionally, by varying the lectin used for affinity capture or developing a general protocol for LLO extraction as we are currently exploring, this method can be applied to a wide range of biological systems. Analysis of bacterial complex carbohydrate biosynthetic pathways such as pilin O-glycosylation and peptidoglycan may provide new insights into the synthesis and control of these virulence factors. This method can also be expanded to eukaryotic systems and analyses of type I congenital disorders of glycosylation that result from disruption of proper LLO biosynthesis.

In light of the information obtained with this method, new directions can be envisaged for glycoengineering efforts in bacteria. While the continued use of *E. coli* as a platform has the advantage of a wide variety of genetic tools to engineer an improved system, alternatively, the native *C. jejuni* host may also provide an alternate vehicle for the production of certain glycoproteins. A truly viable glycoengineering platform in bacteria should efficiently produce only the desired oligosaccharides. Advances in the production of humanized glycoproteins in the yeast *Pichia pastoris* has allowed for the production of human therapeutic glycoconjugates.[52] While the humanization of bacterial glycoprotein biosynthesis is not yet feasible, glycoengineering in bacteria currently has great potential for the production of glycoconjugate vaccines against infectious diseases.

CONCLUSIONS

Currently, the analysis of LLOs of complex carbohydrate biosynthetic pathways has been restricted to examining intermediates through the use of metabolic radiolabeling or OST end point assays using ^{125}I-labeled acceptor peptides followed by chromatographic separation and scintillation counting. While these methods have been immensely useful in the characterization of N-glycosylation pathways in eukaryotes, they are time-consuming and require the handling of radioactive material and its associated waste. To date, there is no reported method for the direct detection and structural characterization of intact lipid-linked oligosaccharides. On the basis of the combination of lectin affinity-capture and mass spectrometry, we have developed a nonradioisotope method for the detection of intact lipid-linked oligosaccharides from biological samples. The advantage of this technique over traditional methods is the ability to obtain structural information, assess the heterogeneity of a lipid population, and avoid labeling steps (metabolic radiolabel, fluorophore). By altering the lectin used (i.e., wheat germ agglutinin, ConA) in the affinity-capture of LLOs, a broad range of pathways can be investigated. LLO analysis of the *C. jejuni* 11168 N-glycan pathway demonstrated a tightly regulated system which is controlled by feedback inhibition when biosynthesis is interrupted. Conversely, when the *E. coli* glyco-factory was analyzed and compared to *C. jejuni*, a loosely regulated synthesis was observed suggesting a potential role for auxiliary proteins and nucleotide precursor levels in the control of the pathway. The proposed method is rapid, reliable, and currently being extended toward the development of a general trapping method for isoprenoid-linked intermediates that is independent of the attached oligosaccharide structures offering broader applications to examining glycoconjugate biosynthesis in bacterial, archaeal, and eukaryotic systems.

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SUPPORTING INFORMATION AVAILABLE

CE−MS/MS analysis of Und-PP-Bac2,4diNAc, Und-PP-Bac2,4diNAc-GalNAc, and Und-PP-Bac2,4diNAc(GalNAc)$_6$ standards, CE−MS spectra of standards in the negative ion mode, and assesment of binding affinity of SBA-agarose with radiolabeled Und-PP-linked sugars. This material is available free of charge via the Internet at http://pubs.acs.org.

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