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Affinity-capture tandem mass spectrometric characterization of polyprenyl-linked oligosaccharides: a novel tool to study protein *N*-glycosylation pathways

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Abstract

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 until the discovery of the general protein glycosylation pathway (Pgl) in *Campylobacter jejuni*. To date most techniques to analyze lipid-linked oligosaccharides (LLOs) of these pathways involves 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 an accurate, rapid, 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 with 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.

Keywords

Campylobacter; *N*-glycan; metabolomics; lipid-linked oligosaccharide; mass spectrometry; affinity-capture; glycoengineering

Introduction

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 (Glc₃Man₉GlcNAc₂) is transferred from a polyisoprenyl

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carrier, dolichol-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- α 1,4-GalNAc- α 1,4-[Glc- β 1,3]-GalNAc- α 1,4-GalNAc- α 1,4-GalNAc- α 1,3-Bac2,4diNAc- β 1, 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 polyisoprenyl 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 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 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 (*i.e.* *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 ¹²⁵I-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 semi-quantitative, non-radioactive assay for LLOs. Its major drawback is the lack of information regarding the heterogeneous make-up 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 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 analyses²⁶. The coupling of CE with mass spectrometry provides a powerful approach for rapid identification of target analytes present at trace levels in biological matrices, 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 *E. coli* “glyco-factory”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-glycan³⁸. 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 Mateo, 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* DH5 α and SCM7 harbouring pACYCp*gl* and pACYCp*glmut* were previously described^{31, 40}. *E. coli* cells were grown in 2 $\times$ 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 described⁴², 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* DH5 α harbouring pACYCp*glmut* 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 $\times$ YT broth supplemented with chloramphenicol to 30 μ g/mL at mixing rate of 200 to 400 r.p.m. Cells were harvested at an O.D₆₀₀ = 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 CHCl₃:MeOH:H₂O (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 NH₄Ac. Fractions containing LLOs were characterized by CE-MS and purity determined by TLC using solvent A as mobile phase with detection with I₂ (vapour) and 20% H₂SO₄ in ethanol.

Isolation of lipid-linked oligosaccharides for analysis

Cells from overnight cultures of either 1 L *E. coli* DH5 α and SCM7 harbouring the functional or inactive *C. jejuni* N-glycan operon (pACYCpgl, pACYCpglmut) or 7.5 L *C. jejuni* 11168 wild type and *pglB*, *pglD*, *pglH*, *pglI*, *pglJ*, *pglK* isogenic mutants were harvested (7 500 $\times$ g, 15 min, 4°C). Cell pellets, which corresponded to 10¹² – 10¹³ bacterial cells, were extracted with 2 $\times$ 20 mL of 10:20:3 (CHCl₃:MeOH:H₂O) and 2 $\times$ 20 mL 2:1 (CHCl₃:MeOH) and the combined extracts 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 μ L SBA-agarose for 1 hr. The resin was collected by centrifugation (2 200 $\times$ 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 NaH₂PO₄, pH 7.4 as per the manufacturer's instructions and used without further purification.

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 μ g depending on the sugar size) was resuspended in 40 μ L of 9:1 EtOH/MeOH with vortexing and sonication, diluted with 360 μ L of Buffer A. Twenty μ L of SBA resin, that was pre-equilibrated with Buffer A, was added and the mixture incubated at 4 °C for 2h. The supernatant was removed, and the resin washed with another 400 μ L of Buffer A. The bound radiolabelled LLOs were eluted with 0.2 M Gal (1 $\times$ 400 μ L, 2 $\times$ 200 μ L) with 15 min incubations. The eluted material was separated by HPLC and radioactivity measured by scintillation counting as previously described⁴⁴. 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, Netherlands). The CE separation was obtained on a 90 cm length of bare fused-silica capillary (365 μ m OD $\times$ 50 μ m ID) 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 ammonium acetate 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 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 NH₄HCO₃ pH 8.0. Cell debris was removed by centrifugation and the protein content precipitated with the addition of ice-cold ethanol to a final concentration of 60%. The precipitated proteins were removed by centrifugation and the supernatant evaporated to dryness. The dried sample was re-dissolved in H₂O and filtered through a Millipore Amicon Ultra (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 supplementary Figure S1). Due to 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 occur⁴⁷.

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 (supplementary 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 (CHCl₃:MeOH:H₂O). 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 (BacdiNAc 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 sub-picomole instrumental detection limit. Additionally, LLOs of the *Pgl* pathway could be isolated from biologically relevant samples containing 10¹²–10¹³ 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 five-fold greater cell 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[–H₂O] 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 due to partial complementation by an alternate acetyltransferase⁶, 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 backgrounds⁶. 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-diacetamido-trideoxyhexose in the first of the α 1,4-GalNAc transferase mutants, *pglJ*42. This conclusion is reinforced by the observed allosteric regulation in the yeast OST complex⁴⁸. 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* DH5 α harbouring pACYCpglmut, which encodes the *C. jejuni* *N*-glycosylation pathway with a nonfunctional PglB³¹, 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 structure⁴. 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 DH5 α 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 removed⁴⁰. Previous analysis of glycoproteins from this *E. coli* strain suggested that only oligosaccharides with Bac2,4diNAc at the reducing end were being produced⁴⁰. 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 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 DH5 α 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 eukaryotes⁵⁰. Metabolic radiolabeling has been an essential technique for analysis of LLOs in intact cells and is a very sensitive technique for the detection of Glc₃Man₉GlcNAc₂-PP-Dol, with detectable levels of LLOs from only 10⁶ 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 [¹²⁵I]-acceptor peptide or solid-state ConA binding with chemiluminescence²³. Neither of these techniques readily provides structural information on the LLO components in the mixture. In the case of the OST endpoint 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 glycoconjugates⁵². While the humanization of bacterial glycoprotein biosynthesis is not yet feasible, glycoengineering in bacteria currently has great potential for the production of glycoconjugate vaccines for 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 endpoint assays using ¹²⁵I-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. Based on the combination of lectin affinity-capture and mass spectrometry, we have developed a non-radioisotope 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 auxillary proteins and nucleotide precursor levels in the control of the pathway. The proposed method is rapid, reliable, and is 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Weerapana E, Imperiali B. *Glycobiology* 2006;16:91R–101.
2. Helenius A, Aebi M. *Ann Rev Biochem* 2004;73:1019–1049. [PubMed: 15189166]
3. Szymanski CM, Yao R, Ewing CP, Trust TJ, Guerry P. *Mol Microbiol* 1999;32:1022–1030. [PubMed: 10361304]
4. Young NM, Brisson J-R, Kelly J, Watson DC, Tessier L, Lanthier PH, Jarrell HC, Cadotte N, St Michael F, Aberg E, Szymanski CM. *J Biol Chem* 2002;277:42530–42539. [PubMed: 12186869]
5. Kowarik M, Young NM, Numao S, Schulz BL, Hug I, Callewaert N, Mills DC, Watson DC, Hernandez M, Kelly JF, Wacker M, Aebi M. *EMBO J* 2006;25:1957–1966. [PubMed: 16619027]
6. Kelly J, Jarrell H, Millar L, Tessier L, Fiori LM, Lau PC, Allan B, Szymanski CM. *J Bacteriol* 2006;188:2427–2434. [PubMed: 16547029]
7. Chavan M, Rekowicz M, Lennarz W. *J Biol Chem* 2003;278:51441–51447. [PubMed: 14530272]
8. Katja Dettmer PAA, Hammock Bruce D. *Mass Spectrom Rev* 2007;26:51–78. [PubMed: 16921475]
9. Bino RJ, Hall RD, Fiehn O, Kopka J, Saito K, Draper J, Nikolau BJ, Mendes P, Roessner-Tunali U, Beale MH, Trethewey RN, Lange BM, Wurtele ES, Sumner LW. *Trends Plant Sci* 2004;9:418–425. [PubMed: 15337491]
10. Mashego M, Rumbold K, De Mey M, Vandamme E, Soetaert W, Heijnen J. *Biotechnol Lett* 2007;29:1–16. [PubMed: 17091378]
11. Wang, Q-z; Wu, C-y; Chen, T.; Chen, X.; Zhao, X-M. *Appl Microbiol Biotechnol* 2006;70:151–161. [PubMed: 16395543]
12. Kell DB. *FEBS J* 2006;273:873–894. [PubMed: 16478464]
13. Hermansson M, Uphoff A, Kakela R, Somerharju P. *Anal Chem* 2005;77:2166–2175. [PubMed: 15801751]
14. Houjou T, Yamatani K, Imagawa M, Shimizu T, Taguchi R. *Rapid Commun Mass Spectrom* 2005;19:654–666. [PubMed: 15700236]
15. Guan XL, He X, Ong WY, Yeo WK, Shui G, Wenk MR. *FASEB J* 2006;20:1152–1161. [PubMed: 16770014]
16. Bijlsma S, Bobeldijk I, Verheij ER, Ramaker R, Kochhar S, Macdonald IA, vanOmmen B, Smilde AK. *Anal Chem* 2006;78:567–574. [PubMed: 16408941]
17. Lavery, SB. *Mass Spectrometry: Modified Proteins and Glycoconjugates*. Burlingame, AL., editor. Vol. 405. Academic Press; San Diego, CA: 2005. p. 300-369.
18. Aas FE, Vik A, Vedde J, Koomey M, Egge-Jacobsen W. *Mol Microbiol*. 2007in Press

19. DiGiandomenico A, Matewish MJ, Bisaillon A, Stehle JR, Lam JS, Castric P. *Mol Microbiol* 2002;46:519–530. [PubMed: 12406226]
20. Horzempa J, Comer JE, Davis SA, Castric P. *J Biol Chem* 2006;281:1128–1136. [PubMed: 16286455]
21. Power PM, Seib KL, Jennings MP. *Biochem Biophys Res Commun* 2006;347:904–908. [PubMed: 16870136]
22. Spiro RG, Spiro MJ, Bhoyroo VD. *J Biol Chem* 1976;251:6409–6419. [PubMed: 977579]
23. Kelleher DJ, Karaoglu D, Gilmore R. *Glycobiology* 2001;11:321–333. [PubMed: 11358881]
24. Gao N, Lehrman MA. *Glycobiology* 2002;12:353–360. [PubMed: 12070078]
25. Kelleher DJ, Banerjee S, Cura AJ, Samuelson J, Gilmore R. *J Cell Biol* 2007;177:29–37. [PubMed: 17403929]
26. Moini M. *Anal Bioanal Chem* 2002;373:466–480. [PubMed: 12172682]
27. Li J, Richards JC. *Mass Spectrometry Reviews* 2007;26:35–50. [PubMed: 16967446]
28. Li YL, Su X, Stahl PD, Gross ML. *Anal Chem* 2007;79:1569–1574. [PubMed: 17297957]
29. Li J, Wang Z, Altman E. *Rapid Commun Mass Spectrom* 2005;19:1305–1314. [PubMed: 15838845]
30. Lacaze JPCL, Stobo LA, Turrell EA, Quilliam MA. *J Chromatogr A* 2007;1145:51–57. [PubMed: 17270196]
31. Wacker M, Linton D, Hitchen PG, Nita-Lazar M, Haslam SM, North SJ, Panico M, Morris HR, Dell A, Wren BW, Aebi M. *Science* 2002;298:1790–1793. [PubMed: 12459590]
32. Smith DF. *Biochem Biophys Res Commun* 1983;115:360–367. [PubMed: 6615536]
33. Curatolo W, Yau AO, Small DM, Sears B. *Biochemistry* 1978;17:5740–5744. [PubMed: 31910]
34. Barua TK, Raychowdhury MK, Chakrabarti P. *Indian J Biochem Biophys* 1984;21:73–75. [PubMed: 6490064]
35. Torres BV, McCrumb DK, Smith DF. *Arch Biochem Biophys* 1988;262:1–11. [PubMed: 3355161]
36. Alroy J, Ucci AA, Goyal V, Woods W. *J Histochem Cytochem* 1986;34:501–505. [PubMed: 3081625]
37. Smith DF, Torres BV. *Methods Enzymol* 1989;179:30–45. [PubMed: 2622354]
38. Linton D, Allan E, Karlyshev AV, Cronshaw AD, Wren BW. *Mol Microbiol* 2002;43:497–508. [PubMed: 11985725]
39. Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, Basham D, Chillingworth T, Davies RM, Feltwell T, Holroyd S, Jagels K, Karlyshev AV, Moule S, Pallen MJ, Penn CW, Quail MA, Rajandream MA, Rutherford KM, van Vliet AH, Whitehead S, Barrell BG. *Nature* 2000;403:665–668. [PubMed: 10688204]
40. Alaimo C, Catrein I, Morf L, Marolda CL, Callewaert N, Valvano MA, Feldman MF, Aebi M. *EMBO J* 2006;25:967–976. [PubMed: 16498400]
41. Gross M, Marianovski I, Glaser G. *Mol Microbiol* 2006;59:590–601. [PubMed: 16390452]
42. Glover KJ, Weerapana E, Imperiali B. *Proc Natl Acad Sci USA* 2005;102:14255–14259. [PubMed: 16186480]
43. Nakamura K, Suzuki M, Taya C, Inagaki F, Yamakawa T, Suzuki A. *J Biochem* 1991;110:832–841. [PubMed: 1783616]
44. Chen MM, Weerapana E, Ciepihal E, Stupak J, Reid CW, Swiezewska E, Imperiali B. *Biochemistry* 2007;46:14342–14348. [PubMed: 18034500]
45. Soo EC, Aubry AJ, Logan SM, Guerry P, Kelly JF, Young NM, Thibault P. *Anal Chem* 2004;76:619–626. [PubMed: 14750855]
46. Weerapana E, Glover KJ, Chen MM, Imperiali B. *J Am Chem Soc* 2005;127:13766–13767. [PubMed: 16201778]
47. Liu HA, Li YM, Du JT, Hu J, Zhao YF. *J Mass Spectrom* 2006;41:208–215. [PubMed: 16382487]
48. Karaoglu D, Kelleher DJ, Gilmore R. *Biochemistry* 2001;40:12193–12206. [PubMed: 11580295]
49. Feldman MF, Wacker M, Hernandez M, Hitchen PG, Marolda CL, Kowarik M, Morris HR, Dell A, Valvano MA, Aebi M. *Proc Natl Acad Sci U S A* 2005;102:3016–3021. [PubMed: 15703289]
50. Lehrman MA. *Glycobiology* 2007;17:75R–85R.
51. Rosner MR, Hubbard SC, Ivatt RJ, Robbins PW. *Methods Enzymol* 1982;83:399–408. [PubMed: 7098942]

52. Hamilton SR, Bobrowicz P, Bobrowicz B, Davidson RC, Li H, Mitchell T, Nett JH, Rausch S, Stadheim TA, Wischnewski H, Wildt S, Gerngross TU. Science 2003;301:1244–1246. [PubMed: 12947202]
53. Linton D, Dorrell N, Hitchen PG, Amber S, Karlyshev AV, Morris HR, Dell A, Valvano MA, Aeby M, Wren BW. Mol Microbiol 2005;55:1695–1703. [PubMed: 15752194]

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

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

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 *pglB* (A) and *pglK* (C) mutants showing accumulation of Und-PP linked heptasaccharide (m/z 1166.0); negative ion mode MS/MS spectrum of *pglB* showing loss of Und-PP (B). 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 (D).

Figure 4.

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

Figure 5.

CE-MS precursor ion scans in the negative ion mode for Und-PP (m/z 907.6) of *E. coli* pACYCpglmut (A) and pACYCpgl+ (B), and *E. coli* SCM7 pACYCpglmut (C) enriched LLO extracts. 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 (D). 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.

Table 1Summary of results of *N*-glycan intermediate analyses in *C. jejuni*

Strain	Function	Glycoprotein phenotype ^a	LLOs	UDP-BacdiNAc ^b
11168	-	heptasaccharide	none	none
<i>pglB</i>	oligosaccharyl transferase	unmodified	yes	none
<i>pglD</i>	acetyltransferase	minor heptasaccharide ^c	none	none
<i>pglH</i>	processive GalNAc transferase	unmodified	none	none
<i>pglI</i>	Glc transferase	hexasaccharide	none	none
<i>pglJ</i>	second GalNAc transferase	unmodified	none	yes
<i>pglK</i>	flippase	unmodified	yes	none

^aReference 6;^bBased on precursor ion scans for nucleotide linked sugars (UDP) from cell lysates;^cMinor levels of *N*-glycosylation observed in this mutant.

Table 2
Results of lipid-linked oligosaccharide analyses of the *E. coli* “glyco-factory” strains

Strain	LLOs observed	Relative Intensity ^a (%)	Glycoprotein phenotype ^b
<i>E. coli</i> DH5a			
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	
<i>E. coli</i> SCM7			
pACYCpglmut			unmodified
	1Bac2,4diNAc + 5HexNAc + 1Hex	100	
pACYCpgl+			heptasaccharide
	1Bac2,4diNAc + 5HexNAc + 1Hex	100	

^aMost abundant ion in spectrum set to 100%;

^bReferences 31, 53