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Selective biochemical labeling of C. jejuni cell-surface glycoconjugates

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Selective biochemical labeling of *C. jejuni* cell-surface glycoconjugates

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Supplementary information: Figure legends for Supplementary Figures 1-3. Figure S1.

LC characterization of PEB3 before and after the GalO reaction. Figure S2.

Deconvoluted MS data for PEB3 before and after the GalO reaction. Figure S3.

Comparison of the GalO-catalyzed conjugation of biotin and Flag-tag to wild-type *C. jejuni* strains 11168 and 81176.

Abstract

The display of cell-surface glycolipids and glycoproteins is essential for the motility, adhesion and colonization of pathogenic bacteria such as *Campylobacter jejuni*. Recently, the cell-surface display of *C. jejuni* glycoconjugates has been the focus of considerable attention, however, our understanding of the roles that glycosylation plays in bacteria still pales in comparison to our understanding of mammalian glycosylation. One of the reasons for this is that carbohydrate metabolic labeling, a powerful tool for studying mammalian glycans, is difficult to establish in bacterial systems and has a significantly more limited scope. Herein, we report the development of an alternative strategy that can be used to study bacterial cell-surface glycoconjugates. Galactose oxidase (GalO) is used to generate an aldehyde at C-6 of terminal GalNAc residues of *C. jejuni* glycans. This newly-generated aldehyde can be conjugated with aminooxy-functionalized purification tags or fluorophores. The label can be targeted towards specific glycoconjugates using *C. jejuni* mutant strains with N-glycan or lipooligosaccharides (LOS) assembly defects. GalO-catalyzed labeling of cell-surface glycoproteins with biotin, allowed for the purification and identification of known extracellular N-linked glycoproteins as well as a recently identified O-linked glycan modifying PorA. To expand the scope of the GalO reaction, live-cell fluorescent labeling of *C. jejuni* was used to compare the levels of surface exposed LOS to the levels of N-glycosylated, cell-surface proteins. While this study focuses on the GalO–catalyzed labeling of *C. jejuni*, it can in principle be used to evaluate glycosylation patterns and identify glycoproteins of interest in any bacteria.

Introduction

Carbohydrate modifications of proteins (N- and O-linked glycosylation) and lipids are known to play important roles in the pathogenicity of *C. jejuni*. In particular, N-linked glycosylation, which was first identified in the epsilon-proteobacteria *C. jejuni,* (Szymanski et al. 1999), is known to be essential for the proper adhesion, invasion and colonization of target hosts (Szymanski et al. 2002, Hendrixson and DiRita 2004, Karlyshev et al. 2004). Additionally, O-linked glycosylation of flagellin proteins with legionaminic acid derivatives impacts autoagglutination and biofilm formation (Howard et al. 2009), while O-linked glycosylation with pseudaminic acid also affects autoagglutination and is required for proper flagellin assembly and motility (Guerry et al. 2006, Schoenhofen et al. 2006). In addition to protein glycosylation, lipooligosaccharides (LOS) are involved in the invasion of host cells (Kanipes et al. 2004). The importance of *C. jejuni* glycosylation has been firmly established, however, the localization and relative abundance of extracellular glycosylation is poorly understood and proteins decorated by N-linked glycans continue to be identified (Scott et al. 2014).

In mammalian systems, metabolic labeling using azide- and alkyne-modified carbohydrates that can be incorporated into select glycans in cell-based systems represents a powerful approach for identifying glycoproteins and determining localization and abundance (Vocadlo et al. 2003, Baskin et al. 2007, Laughlin and Bertozzi 2007). Unfortunately, application of this methodology is more challenging in

bacteria and faces a number of technical hurdles. Typically, in mammalian systems simple acetylated azide- or alkyne-modified monosaccharides can passively diffuse into cells, be deprotected by nonspecific cellular esterases and exploit advantageous salvage pathways to become incorporated into glycans (Laughlin and Bertozzi 2007). The azide/alkyne-modified glycan can then be conjugated with appropriately-activated purification tags or fluorophores for identification and localization studies. In contrast, in bacterial systems growth rates are rapid and nonspecific cellular esterase activity appears to be relatively low (Antonczak et al. 2009, Pons et al. 2014), imposing limitations on the supplies of deprotected azide/alkyne-modified monosaccharides for integration into glycan assembly pathways. To our knowledge, only Dube and coworkers have successfully applied metabolic labeling to bacterial glycoproteins with simple azide-modified, peracetylated carbohydrates in *Helicobacter pylori* (Champasa et al. 2013, Kaewsapsak et al. 2013). In all other examples of metabolic labeling in bacteria, the modified carbohydrate was delivered as a deprotected sugar and prior knowledge of a salvage/metabolic pathway was available (Liu et al. 2009, Dumont et al. 2012, Pons et al. 2014). In the absence of these pathways the information that can be readily generated via metabolic labeling of glycans is limited. Therefore alternative methods of glycan-specific labeling are needed to further our understanding of physiological roles of bacterial protein glycosylation.

Galactose oxidase (GalO) is a promiscuous enzyme that oxidizes the C-6 position of galactose (Gal) or N-acetyl-D-galactosamine (GalNAc) when these carbohydrates are the terminal residues in a linear or branching glycan (Morell et al. 1966, Avigad 1985). The introduced aldehyde can subsequently be reacted with

aminooxy-functionalized probes, including for example fluorophores, generating stable oxime adducts with the targeted glycan. In the case of *C. jejuni*, both the N-glycan and LOS are known to include terminal GalNAc residues, making them potential substrates for GalO. For example, the N-linked glycan that is found modifying proteins with asparagine in the consensus sequence (D/E-X-N-X-T/S) includes di-*N*-acetyl bacillosamine (diNAcBac, Figure 1) followed by a linear chain of five N-acetylgalactosamines (GalNAcs) with a branching Glc modifying the C3-hydroxyl group of the third GalNAc unit. Interestingly, recent protein engineering efforts with GalO, have resulted in enzyme variants that oxidize glucose (Glc), mannose (Man) and N-acetyl-D-glucosamine (GlcNAc), thus expanding the library of glycans that can be labeled by GalO (Sun et al. 2002, Rannes et al. 2011). While further engineering will be required to confer selectivity for alternative carbohydrates, in the current study the wild-type GalO is highly selective and can be used to explore the *C. jejuni* glycoconjugates that are known to include terminal GalNAc residues.

In this study, we establish the utility of GalO-catalyzed labeling of the *C. jejuni* N- and O-linked glycans as well as LOS using aminooxy-functionalized probes. In initial studies, a purified, His-tagged N-linked glycoprotein expressed in *C. jejuni* is used for *in vitro* validation of the two-step labeling approach. Following this, *C. jejuni* (strain 81176) is used to establish whether GalO can be used to modify both, LOS and cell-surface glycoproteins *in vivo*. These studies are complemented by parallel experiments using glycosylation deficient mutant strains of *C. jejuni* that confer selectivity for either LOS or glycoprotein modification. Through these studies it is demonstrated that the application of GalO enables modification of *C. jejuni* glycans that incorporate terminal GalNAc

moieties. These modifications can include purification and identification handles, in the form of aminooxy-functionalized biotin (AO-biotin) and Flag-tag (AO-Flag) derivatives, as well as aminooxy-functionalized fluorophores. Together, these studies demonstrate the versatility of GalO-mediated labeling of *C. jejuni* cell-surface glycoconjugates and provide a flexible experimental platform for future studies focused on the identification of cell-surface glycoproteins from other bacteria that are modified by galactose or GalNAc terminating glycans. In addition, expansion of this method to encompass glycans with different terminal carbohydrates can be envisioned using engineered variants of GalO (Sun et al. 2002, Rannes et al. 2011).

Results

GalO oxidizes C. jejuni N-linked glycoproteins.

To determine conditions for GalO oxidation of the *C. jejuni* N-linked glycan, a His-tagged PEB3 ectopically expressed in *C. jejuni* (strain 11168, Table 1) was purified, incubated overnight with GalO and AO-biotin, and analyzed by western blot (Figure 2). An α-His antibody was used to detect PEB3 (~28 kDa), while visualization using an α-N-glycan antibody (Nothaft et al. 2012) confirmed that PEB3 was glycosylated. In the presence of GalO and AO-biotin a second slower migrating species was seen when probed with the α-His antibody (Figure 2A), and the N-glycan specific antibody (Figure 2B). Given the well-established selectivity of GalO (Mcpherson et al. 1993), it can be surmised that the C6-hydroxyl of the terminal GalNAc residue was oxidized to an aldehyde, followed by nucleophilic attack of the AO-biotin to generate a stable oxime.

When PEB3 was incubated with either GalO or AO-biotin alone, the second protein band was absent. Western blots probed with a streptavidin-alkaline phosphatase (Strept-AP) conjugate confirmed that PEB3 was biotinylated (Figure 2C). MS analysis provided further confirmation that PEB3 was N-glycan modified and that incubation with GalO and AO-biotin resulted in a biotinylated derivative (Supplementary Figures 1 and 2). This confirms that the GalO recognizes the terminal GalNAc residue of the *C. jejuni* N-glycan and that the reactive AO-biotin selectively modifies the newly generated aldehyde.

Biotinylation of N-glycan modified proteins coating the surface of C. jejuni.

The *in vitro* modification of purified PEB3 with AO-biotin indicates that the *C. jejuni* heptasaccharide is a substrate for GalO. The next step was to see if extracellular N-glycan modified proteins were accessible for GalO oxidation. *C. jejuni* strain 11168, was harvested from blood agar plates, resuspended in buffer and incubated with GalO and AO-biotin. To optimize the labeling reaction, samples were taken at regular intervals over a 36 h period for reactions with three different enzyme concentrations (0.2, 1.1, and 6.6 units of GalO per reaction). The cells were harvested, lysed and analyzed by western blot using the α-N-glycan antibody and Strept-AP for detection. Optimal labeling was seen after 10 h with 1.1 units of GalO per reaction (data not shown), these conditions were used for all subsequent cell-surface labeling. It was also observed that while *C. jejuni* did not grow while incubated in PBS for the 36 h time-course experiment, the cells were still viable after the GalO reaction as evidenced by growth on Mueller-Hinton plates and in a microaerophilic environment. This is an ideal

situation because halting *C. jejuni* growth during GalO labeling, limits the loss of labeled

glycans due to the recycling of cell-surface glycoproteins or LOS. Using the optimized

reaction conditions and the α-N-glycan antibody it was observed that the control lanes

with either GalO or AO-biotin and the reaction lane (both GalO and AO-biotin) were

comparable (Figure 3A). However, with Strept-AP detection an increase in signal is

seen in the reaction lane with two significant protein bands at ~35 and 33 kDa (Figure

3B). Prominent, corresponding signals can also be seen in the α-N-glycan blot,

suggesting that these are relatively abundant glycoproteins on the surface of *C. jejuni*.

In place of AO-biotin, aminooxy-Flag (AO-Flag) (Wu et al. 2009) was also used to label

purified PEB3 (data not shown) and *C. jejuni* strain 11168 and 81176 (Supplementary

Figure 3), however detection levels were lower compared to AO-biotin labeling.

Therefore AO-biotin was used in the follow up experiments designed to confirm that the

labeling was occurring on the glycan. To accomplish this *pglE* and *galE* deletion strains

were employed to inhibit biosynthesis of the N-glycan by interrupting either UDP-

diNAcBac or UDP-GalNAc synthesis respectively (Szymanski et al. 1999, Bernatchez et

al. 2005). Detection using the α-N-glycan antibody showed the expected reduction of

glycosylation (Figure 3A), while Strept-AP analysis indicated a similar decrease in

biotinylation (Figure 3B). Notably the prominent protein bands at ~35 and 33 kDa, seen

with the wild type strain, are completely absent in the lanes representing Δ*pglE* and

Δ*galE*, thus establishing that these two bands are N-linked glycoproteins.

Biotinylation of C. jejuni lipo-oligosaccharide (LOS).

The *C. jejuni* LOS, a low molecular weight form of bacterial lipopolysaccharides, are also surface exposed, glycoconjugates (Figure 4). To determine if LOS is also modified by the GalO/AO-biotin procedure, *C. jejuni* mutant strains that are deficient in WaaF and CgtA activity, which would lead to truncated LOS structures (Guerry et al. 2002, Kanipes et al. 2004), were investigated. The *C. jejuni* LOS is known to display a terminal GalNAc, which would be a good substrate for GalO if it is accessible on the cell surface. CgtA transfers the terminal GalNAc to the LOS, while WaaF is responsible for transferring the second heptose to the core oligosaccharide of *C. jejuni* LOS (Figure 4). Therefore, the deletion of *cgtA* and *waaF* will prevent the formation of an LOS structure with a terminal GalNAc, subsequently interfering with the biotinylation of LOS after treatment with GalO. Colonies from wild type strain 81176 and the two mutant strains were harvested and incubated overnight with GalO and AO-biotin. First, Western blots probed with the α-N-glycan antibody (Figure 3C) and Strept-AP (Figure 3D) were analyzed to confirm that N-glycosylation was normal and that the GalO/AO-biotin procedure successfully labels 81176. Interestingly the ~35 kDa protein band is prominent in both the α-N-glycan (Figure 3C) and Strept-AP (Figure 3D) blots, while the ~33 kDa band displayed lower intensity in the α-N-glycan blot (Figure 3C). Importantly, Δ*waaF* and Δ*cgtA* do not significantly affect N-glycosylation.

Previously, Guerry and coworkers used gel electrophoresis to observe differences in LOS electrophoretic mobility patterns between the mutant strains and 81176 (Kanipes et al. 2004). Knowing this, biotinylation of LOS after incubation with GalO for the two wild type strains 81176 and 11168 and the four mutant strains (Δ*pglE*, Δ*galE*, Δ*waaF* and Δ*cgtA*) were investigated. After harvesting and labeling the cells,

proteinase K was used to digest all proteins, leaving the LOS intact. Strept-AP detection indicated that LOS from *C. jejuni* strains 81176 and 11168 as well as Δ*pglE* were biotinylated (Figure 4B) while Δ*galE*, Δ*waaF* and Δ*cgtA* were not biotinylated. This confirms that the LOS from *C. jejuni* extends far enough away from the surface to be accessible to GalO. Presumably Δ*galE*, which blocks UDP-GalNAc and UDP-Gal biosynthesis, generates an intermediate LOS structure between Δ*waaF* and Δ*cgtA* (Figure 4A).

Live-cell labeling of C. jejuni LOS and N-glycan modified extracellular proteins.

The reliable activity of GalO with the series of *C. jejuni* mutants at our disposal now provide tools to selectively label and analyze extracellular, N-glycan modified proteins and LOS. GalO labeling of *waaF* and *cgtA* mutant strains enables selective observation of N-glycans, labeling the *pglE* mutant selectively probes the LOS while Δ*galE* prevents all GalO initiated labeling. A strong fluorescent signal was observed for wild type *C. jejuni* and was in stark contrast to the minimal signal observed in the absence of GalO (Figure 5). In comparison to the wild-type labeling, a modest decrease in fluorescence was observed for Δ*pglE*, while a more noticeable decrease was observed with both of the LOS-deficient strains, Δ*waaF* and Δ*cgtA* (Figure 5). As expected, labeling of Δ*galE* was indistinguishable from the aminooxy-functionalized AlexaFluor 488 (AO-488) control reaction due to the complete lack of a substrate for GalO. The ability to label select glycoconjugates on live *C. jejuni* with a fluorophore further demonstrates the versatility of GalO as a tool to study bacterial glycosylation.

Purification and mass spectrometry analysis of biotinylated, cell-surface glycoproteins.

GalO-mediated oxidization of terminal GalNAc residues followed by biotinylation of extracellular glycoproteins in the presence of an AO-biotin derivative also provides the opportunity to use the technique to selectively purify biotinylated cell-surface glycoproteins for MS identification. Monomeric avidin bound to agarose was used to purify the biotinylated glycoproteins away from other proteins. The purification was analyzed by Western blot using the α-N-glycan antibody (Figure 6A) and Strept-AP (Figure 6B) for detection. The distinct difference between α-N-glycan and Strept-AP blots is the presence of glycoprotein detected with the α-N-glycan antibody in the flow through and early washes, while no signal was detected in the corresponding lanes with Strept-AP. These results indicate that while the GalO-catalyzed biotinylation of extracellular glycoproteins does not go to completion under these prescribed conditions, all biotinylated proteins (within limits of detection) are immobilized on the monomeric avidin resin. Next InstantBlue stain was used to monitor total protein concentration and to prepare a sample for MS analysis (Figure 6C). While the protein-staining pattern is quite different, owing to the fact that all *C. jejuni* proteins are released after cell lysis, unlabeled proteins in the flow-through and early washes can be removed, resulting in a sample eluting from the beads that is enriched with biotinylated glycoproteins.

Finally, soy bean agglutinin-conjugated to alkaline phosphatase (SBA-AP) was used to detect unmodified glycoproteins in the elution fraction (Figure 6D). The α-N-glycan antibody is a polyclonal antibody raised against the entire N-glycan epitope (Nothaft et al. 2012), biotinylation of the terminal GalNAc residue should not have a major effect on α-N-glycan recognition of the biotinylated glycan. However SBA is a

lectin that specifically recognizes the terminal GalNAc residue of glycans, therefore it is assumed that it would not recognize the biotinylated glycan. Probing with SBA-AP supported this assumption; proteins were still detected in the biotinylated sample (Figure 6D, lane 3) because biotinylation does not go to completion, but upon avidin purification no distinct protein bands are visible. This indicated that the purification protocol was sufficient, and therefore a region of the InstantBlue-stained gel that corresponded to the most distinct bands (~32 – 40 kDa based on molecular weight standards) was excised for MS analysis. The gel slice was digested with trypsin and analyzed by MS to examine whether known N-linked glycoproteins on the surface of *C. jejuni* could be identified. Six proteins were identified: Histidine binding protein (HisJ/CjaC), a putative lipoprotein (JlpA), the major outer membrane protein (PorA), the major antigen (PEB4A), a probable thiol peroxidase (tpx), and a DNA protection during starvation protein (dps), (see Table 2). Of the six proteins, HisJ/CjaC, PorA, PEB4A, and JlpA are all known cell surface-exposed proteins (Cordwell et al. 2008), thus providing support for the selectivity of the GalO-catalyzed labeling reaction. HisJ/CjaC and JlpA are both known *C. jejuni* N-linked glycoproteins (Scott et al. 2011, Scott et al. 2012), indicating that the labeling and purification procedure successfully isolated previously reported glycoproteins. With respect to PorA, recent studies by Mahdavi and co-workers reported identification of a glycan modifying *C. jejuni* PorA (Mahdavi et al. 2014). From MS analysis it was proposed that the terminal carbohydrate was galactose and that the glycan was a linear tetrasaccharide with the structure Gal(β1–3)-GalNAc(β1–4)-GalNAc(β1–4)-GalNAcα1-Thr[268]. This glycan would be an excellent substrate for GalO and therefore, this explains its isolation through the GalO-mediated

labeling procedure. The glycosylation state of PEB4A is currently unknown, however, the sequence contains two sites that have recently been reported as non-canonical N-linked glycosylation sites (Scott et al. 2014). The two remaining proteins, tpx and dps, are not glycoproteins and also are of molecular weight significantly lower than that predicted by the approximate range of the excised gel slice, suggesting that these proteins may have co-purified with one of the biotinylated glycoproteins.

Discussion

Glycans that form part of the glycoconjugates decorating the surface of *C. jejuni* play important roles in pathogenicity and are involved in adhesion, invasion, colonization, motility and biofilm formation (Szymanski et al. 2002, Hendrixson and DiRita 2004, Kanipes et al. 2004, Karlyshev et al. 2004, Guerry et al. 2006, Schoenhofen et al. 2006, Howard et al. 2009). Preventing one form of glycosylation is known to impair the ability of *C. jejuni* to invade a host cell (Szymanski et al. 2002, Hendrixson and DiRita 2004, Kanipes et al. 2004, Karlyshev et al. 2004, Guerry et al. 2006, Schoenhofen et al. 2006, Howard et al. 2009), however our understanding of the complex interplay of interactions that occurs between pathogen and host (LOS/glycoprotein and host receptor(s)) remains an important topic for study and the development of synergistic experimental approaches. In mammalian systems metabolic labeling with azide- and alkyne-modified carbohydrates represents a powerful approach for determining the identity and function of specific glycans or glycoproteins, unfortunately this methodology is not readily transferable to similar studies in bacterial systems. To advance the understanding of bacterial glycosylation, versatile methods

that can be applied in bacterial systems and provide comparable information to metabolic labeling are needed. GalO, a well-studied Gal/GalNAc oxidase that generates an aldehyde at the C6-hydroxyl of terminal Gal/GalNAc residues of glycans was used to study *C. jejuni* glycoconjugates. The *C. jejuni* LOS and N-glycan have terminal GalNAc residues that could be modified by GalO. To establish protocols for GalO-mediated labeling, PEB3, a known N-linked glycoprotein from *C. jejuni* was used. After incubation with GalO and AO-biotin a new, slower migrating protein band was observed in gel analysis indicating that the N-glycan-modified PEB3 was oxidized by GalO and that the AO-biotin had reacted with the newly generated aldehyde (Figure 2).

This initial study confirmed that GalO efficiently catalyzed the oxidation of an N-glycan attached to a purified protein. However, to show comparable utility to metabolic labeling, it was critical to demonstrate whether GalO would oxidize LOS or N-glycan modified proteins on the sterically hindered, extracellular surface of the *C. jejuni* outer membrane. GalO was incubated with *C. jejuni* strain 11168 in the presence of AO-biotin and probed with Strept-AP to observe biotinylation (Figure 3B). Two distinct protein bands were seen in the 33 – 35 kDa range, suggesting that the corresponding proteins are abundant and N-glycan modified. Corresponding signals were detected by the α-N-glycan antibody (Figure 3A), supporting the assumption that the proteins are modified by the *C. jejuni* N-glycan. These signals were absent in the Δ*pglE* and Δ*galE* strains, further confirming them as N-glycan modified proteins. Interestingly there are two defined protein bands that are detected by Strept-AP at ~64 kDa in the Δ*pglE* strain but not in the Δ*galE* strain. Corresponding protein bands in either mutant strain were not observed with the α-N-glycan antibody, suggesting that they may represent a

glycoprotein modified by a different Gal/GalNAc terminating glycan (studies to determine the identity of these glycoproteins are ongoing). The GalO-catalyzed labeling of *C. jejuni* glycoproteins was straightforward and distinct differences were observed between the labeled wild type strain, the control reactions and the two mutant strains. As an added benefit, GalO selectively labels extracellular glycoproteins, which represent a sub-population of the most interesting glycoproteins due to their potential involvement in host-pathogen interactions, because it cannot cross the outer membrane to gain access to periplasmically-localized glycoproteins.

Initial studies clearly demonstrate that GalO is labeling the *C. jejuni* N-glycan and possibly a protein(s) modified by an unknown Gal/GalNAc terminating glycan. The next goal was to determine if the LOS was also labeled. The *C. jejuni* LOS is thought to be associated with the development of Guillain-Barré syndrome, a post infectious autoimmune disease, because of the ganglioside mimicry displayed by certain LOS structures (Koga et al. 2006). Multiple LOS structures have terminal Gal/GalNAc moieties (Houliston et al. 2011), therefore, we used the wild type *C. jejuni* strain 81176 and two mutant strains, $\Delta waaF$ and $\Delta cgtA$, that assemble truncated LOS variants (Guerry et al. 2002, Kanipes et al. 2004) to investigate LOS labeling catalyzed by GalO. Comparison of the N-glycan modified proteins revealed no significant differences between 81176 and the genetic knockout strains (Figure 3C). With the genetic knockouts only affecting the LOS and not the N-glycan, similar protein banding patterns, when using Strept-AP for detection, were observed (Figure 3D). Interestingly there was a noticeable difference in the abundance of labeled glycoprotein between wild type 81176 and $\Delta waaF$ and $\Delta cgtA$ for the two prominent bands at ~33 – 35 kDa. Proteinase

K digestion, followed by LOS extraction was used to remove protein contamination, allowing for the comparison of LOS biotinylation between the wild type and mutant strains. Biotinylation was observed for the samples with a complete LOS assembly pathway (*C. jejuni* wild type strains 81176, 11168 and Δ*pglE*), while disruption of this pathway by Δ*waaF* and Δ*cgtA* generated an abbreviated LOS that lacked a terminal Gal/GalNAc residue and was therefore not biotinylated (Figure 4B). The Δ*galE* strain, which prevents *C. jejuni* from synthesizing UDP-Gal and UDP-GalNAc was also devoid of biotinylated LOS.

　　　With the knowledge that GalO could be used to label LOS and/or N-glycans, attention was focused on fluorescence microscopy studies where GalO could be used to probe for localization of N-linked glycoproteins on the surface of *C. jejuni*. Using the same series of genetic mutants, selective labeling of N-glycoproteins (Δ*waaF* and Δ*cgtA*), LOS (Δ*pglE*) or no labeling (Δ*galE*) was observed with an aminooxy-functionalized AlexaFluor 488. Encouragingly, after the GalO-catalyzed labeling reaction was complete, *C. jejuni* observed under the microscope had comparable motility to unreacted cells and a similar growth rate when cultured on Mueller-Hinton (MH) plates (with the suitable antibiotics, data not shown). This suggests that fluorescence labeling of live *C. jejuni* could be applied for monitoring the various stages of infection during a host-pathogen invasion assay (Guerry et al. 2002). The wild type strains 81176 and 11168 displayed robust labeling compared to the control reactions (Figure 5), with minimal background fluorescence in the presence of the fluorophore alone and no detectable fluorescence in the absence of the fluorophore. The *pglE* deletion strain was used to selectively label LOS and displayed similar fluorescence to the wild type

labeling (Figure 5), suggesting that the majority of the fluorophore was attached to the LOS and not the N-glycan. Corroborating this hypothesis was the more modest increase in fluorescence between LOS genetic mutants, Δ*waaF* and Δ*cgtA,* and their respective AlexaFluor 488 control reactions. The fluorescence images from the labeled bacteria have a defined shape, closely mimicking the bright field. While the minimal fluorescence observed in the control reactions is due to non-specific interactions between the fluorophore and *C. jejuni*. Finally, the fluorescence observed from Δ*galE* closely resembles the control reaction, indicating that there is not GalO-catalyzed labeling in the absence of UDP-Gal and UDP-GalNAc. Due to resolution limitations, selective labeling of Δ*waaF* and Δ*cgtA* strains did not reveal cell-surface glycoprotein localization. However future work with super-resolution microscopy and GalO labeling should be able to determine if extracellular localization of glycoproteins occurs.

MS identification of proteins modified by the *C. jejuni* N-glycan has recently been accomplished using zwitterionic-hydrophilic interaction liquid chromatography (ZIC-HILIC) enrichment and LC-MS analysis (Scott et al. 2011, Scott et al. 2012). This is an excellent method that has rapidly expanded the library of known N-glycan modified proteins in *C. jejuni*. Scott and coworkers recently expanded this analysis (Scott et al. 2014) and identified glycoproteins that were missed in the initial studies. To complement this powerful technique, enrichment, through covalent labeling of glycoproteins with a high affinity purification tag such as biotin could lead to the identification of as yet unidentified N-glycan modified proteins. The purification and MS identification of HisJ/CjaC and JlpA, validates our approach, furthermore the identification of PorA due to its modification by a recently identified tetrasaccharide (Mahdavi et al. 2014) suggests

that GalO-catalyzed labeling could also be used to identify other proteins modified by this tetrasaccharide. Finally, PEB4A, a known major antigenic protein, was also identified and will be further explored, to determine conclusively whether it is actually glycosylated.

Together, this work describes a versatile method for labeling extracellular LOS and O- and N-modified glycoproteins for localization and identification studies. It is anticipated that live-cell fluorescence labeling of *C. jejuni* will be useful for studying the interactions that occur during a host-pathogen interactions or as a secondary labeling method when studying specific fluorescently-tagged proteins. Biotinylation of extracellular glycoproteins coupled with monomeric avidin purification and MS analysis successfully identified known N-linked glycoproteins and PorA, a recently identified O-linked glycoprotein (Mahdavi et al. 2014). Labeling select classes of glycoconjugates with purification tags such as biotin is a powerful method of enriching samples and identifying proteins of interest using MS techniques. For this study we focused on exploring *C. jejuni* glycosylation, however, GalO-catalyzed labeling can potentially be used to explore glycosylation in other bacteria. The requirement of a Gal/GalNAc terminating glycan was not a limiting factor when studying *C. jejuni* as Gal/GalNAc is found at the terminus of LOS and N-and O-linked glycans. Finally, the scope of this method could be increased through the use of genetically engineered GalO variants that recognize different terminal carbohydrates.

Experimental

General Western blotting procedure

Samples were loaded onto a 12% SDS-Page gel and run at 180 V for 90 min, followed by transfer to nitrocellulose at 100 V for 90 min. The nitrocellulose was blocked with 10 mL TBS-T (100 mM, pH 7.5, 0.05% Tween-20) + 1% BSA for 30 min. the Primary antibody in fresh TBS-T + 1 % BSA (10 mL) was then added and incubated for 1 h at 21 °C or overnight at 4 °C with gentle rocking. TBS-T + 1 % BSA (10 mL) was used to wash away excess primary antibody before adding the secondary antibody-alkaline phosphatase conjugate in fresh TBS-T + 1 % BSA (10 mL) which was incubate for 1 h at 21 °C (if a secondary antibody was not required this step was omitted). The nitrocellulose was washed with TBS-T for 15 min 2X and TBS for 5 min 2X before adding the developer NBT/BCIP (5 mL, Thermo Scientific), following this the developer was incubated with the nitrocellulose until desired staining intensity occurred.

C. jejuni culture

All bacterial strains used in this study are shown in Table 1. *C. jejuni* strains were cultured on Mueller-Hinton (MH) plates in the presence of trimethoprim (10 µg/mL) alone or with either chloramphenicol (15 µg/mL) or kanamycin (20 µg/mL). Microaerophilic conditions were generated using the BD GasPak™ EZ Campy Gas Generating Pouch System and plates were grown for 24 - 72 h at 37 °C. *C. jejuni* strains were stored at -80 °C in a 12.5% solution of glycerol in MH media.

PEB3 expression and purification

C. jejuni (strain 81176) expressing His_6 tagged PEB3 was grown on MH plates in the presence of trimethoprim and chloramphenicol and in a microaerophilic environment at 37 °C for 72 h. A 1.5 mL aliquot of PBS was used to harvest the cells from the plate.

After thorough resuspension, 750 µL of cells were added to 200 mL of sterile MH media containing trimethoprim (10 µg/mL) and chloramphenicol (15 µg/mL). Cells were grown in a microaerophilic environment at 37 °C for 72 h with shaking. Following this, cells were transferred to centrifuge tubes and spun at 3300 rpm and 4 °C for 30 min. The supernatant was removed and cells were resuspended in 30 mL of PBS (100 mM, pH 7.4) and sonicated on ice for 90 s, three times. Cells were again spun at 3300 rpm, and 4 °C for 30 min and then the supernatant was immediately loaded onto a 5 mL Ni-NTA column. Once the supernatant was passed through the column, the column was washed with 20 mL of buffer 1 (20 mM imidazole, 50 mM Tris, 100 mM NaCl, pH 7.4), 20 mL of buffer 2 (40 mM imidazole, 50 mM Tris, 100 mM NaCl, pH 7.4), and then eluted with 15 mL of buffer 3 (300 mM imidazole, 50 mM Tris, 100 mM NaCl, pH 7.4). The elutions were transferred to a dialysis cassette (Thermo Scientific, 3500 MWCO) and dialyzed three times against PBS (100 mM, pH 7.4) at 4 °C. The final dialysis product was concentrated from 15 mL down to 3 mL using Amicon Ultra centrifugal filters (3000 NMWL).

Chemoenzymatic labeling of PEB3

Purified PEB3 (50 µL, [0.5 mg/mL]) was incubated with catalase (30 µL, 4000 units/mL, Sigma Aldrich (bovine liver)), GalO (2 µL, 65 units/mL, Worthington Biochemical), and AO-biotin (0.5 µL, 250 mM, Thermo Scientific) in a total volume of 100 µL (PBS 100 mM, pH 7.4). The reaction was allowed to mix by gentle rocking for 10 h at 21 °C (At this stage an aliquot was reserved for LC-MS analysis, LC-MS samples were diluted to 5% ACN and 0.1% TFA before injection into LC-MS). To stop the reaction, 30 µL of 6X loading buffer was added and the samples were boiled for 10 min. Samples were

loaded onto a 12% SDS-Page gel and analyzed by Western blot with α-6x-His mouse monoclonal antibody (α-His, 1:2000, Thermo Scientific), α-N-glycan antibody (1:5000, from rabbits (Nothaft et al. 2012)) and streptavidin-conjugated alkaline phosphatase (Strept-AP, 1:5000, Sigma Aldrich). α-Mouse and α-rabbit, alkaline phosphatase conjugated secondary antibodies (1:10000, Thermo Scientific) were used with the α-6x-His mouse monoclonal antibody and the α-N-glycan antibody, respectively.

Cell-surface labeling

C. jejuni (strains 81176 and 11168) was grown on MH plates with trimethoprim (wild type 81176 and 11168), trimethoprim and kanamycin ((Δ*pglE*, and Δ*galE*) or trimethoprim and chloramphenicol (Δ*cgtA*, and Δ*waaF*) for 48 h at 37°C under microaerophilic conditions. Cells were harvested in 1.5 mL phosphate buffered saline (PBS, 100 mM, pH 7.4) and labelled essentially as described by Rannes *et al.*(Rannes et al. 2011) Briefly, cells were pelleted at 12,000 rpm for 2 min, resuspended in 1.2 mL PBS + 50 mM methoxyl amine and incubated for 30 min at room temperature (21°C). Cells were washed with PBS and pelleted at 12,000 rpm for 2 min, 3 times. Cells were then resuspended in 1.2 mL PBS and divided equally into three 1.7 mL Eppendorf tubes. Cells were incubated with 30 µL catalase (Sigma Aldrich, 4000 units/ml), 10 µL GalO (Worthington, 65 units/ml) and 0.5 µL alkoxyamine-PEG4-biotin (Pierce, 250 mM in DMSO) or 2.5 µL aminooxy-Flag (50 mM in water, synthesized according to Wu et al. (Wu et al. 2009)) or 2 µL AlexaFluor 488 Hydroxylamine (Life Technologies, 10 mM in H$_2$O) for 12 h at room temperature. Control reactions for wild type 81176, 11168, Δ*pglE*, Δ*galE*, Δ*cgtA*, and Δ*waaF* were incubated without the aminooxy derivative (alkoxyamine-PEG4-biotin, or aminooxy-Flag or AlexaFluor 488 Hydroxylamine) or

without GalO. After 10 h cells were spun at 12,000 rpm for 2 min and supernatant was removed. The pellet was resuspended in 500 μL PBS and spun at 12,000 rpm for 2 min, three times, to remove any remaining alkoxyamine-PEG4-biotin or aminooxy-Flag or AlexaFluor 488 hydroxylamine.

Western blot analysis

Cells (wild type 81176, 11168, Δ*pglE*, Δ*galE*, Δ*cgtA*, and Δ*waaF*) labeled with alkoxyamine-PEG4-biotin and the control reactions where cells were incubated without alkoxyamine-PEG4-biotin or GalO were redissolved in 200 μL of PBS (100 mM, pH 7.4). A 25 μL aliquot from each sample was removed and used to determine the O.D.$_{600}$. O.D.$_{600}$ values were used to normalize loading samples. The remaining 175 μL of cells suspended in PBS was mixed with 35 μL of 6X loading buffer and heated at 100 °C for 10 min. Samples were centrifuged for 2 min at 10,000 rpm, equal amounts were loaded onto a 12% SDS-Page gel and analysed by Western blotting. The α-N-glycan primary antibody (1:5000, from rabbits) along with a α-Rabbit, alkaline phosphatase conjugated secondary antibody (1:10000) and streptavidin-conjugated alkaline phosphatase (Strept-AP, 1:5000) were used for detection.

Microscopy

C. jejuni (wild type 81176, 11168, Δ*pglE*, Δ*galE*, Δ*cgtA*, and Δ*waaF*), labelled with AlexaFluor 488 hydroxylamine, were used for live-cell microscopy. Agarose pads (1%) were added to 15-well multitest slides (MP biomedicals), followed by 5 μL of bacteria suspended in PBS (100 mM, pH 7.5) per well. Bacteria were allowed to settle for 5 min before a cover glass was added (VWR). A 41012 filter set was used and images were

acquired using a Nikon E800 microscope equipped with a CCD camera (Hamamatsu, model C4742-95) and Improvision Openlabs 4.0 software. All images were colored using OpenLab software, transferred to Adobe Photoshop and assembled in Adobe Illustrator.

Biotinylated protein purification

C. jejuni strain 81176 was grown and surface glycoproteins were labeled as previously described. After washing away excess AO-biotin an aliquot of biotin labelled cells (1/3 of the cells harvested from a MH plate) was resuspended in 200 µL of PBS (100 mM, pH 7.4). 1 µL of protease inhibitors (Calbiochem, Protease inhibitor set 3, EDTA free) and a spatula tip of glass beads (Sigma) were added. The suspension was vortexed for 45 s and then cooled on ice for 90 s. Vortexing and cooling were repeated 3 times. The cell debris and glass beads were pelleted at 10,000 rpm for 5 min. The supernatant was removed and transferred to a 1.7 mL Eppendorf tube with 100 µL of monomeric avidin agarose (Pierce). The supernatant was incubated with the monomeric avidin agarose at 4 °C for 14 h, after which the supernatant was removed and 1 mL of PBS (100 mM, pH 7.4) was added to the beads. The beads were washed on a tube rotator (VWR) at 21 °C for 5 min and then centrifuged at 4,000 rpm for 4 min. The buffer was removed and the washing process was repeated 5 more times to remove any proteins that were binding non-specifically. Samples (200 µL) from the initial supernatant and each subsequent wash (1 mL wash samples were concentrated down to 200 µL using Amicon Ultra Centrifugal Filters (Millipore, 3000 MWCO)) were taken and added to 50 µL of loading buffer. PBS (200 µL, 100 mM, pH 7.4) and 50 µL of loading buffer was added to the beads. All samples were heated at 100 °C for 10 min, centrifuged at 4000 rpm for 4 min

and 6 µL of each sample was loaded onto a 12% SDS-Page gel. The gel was run at 180 V for 90 min and stained with InstantBlue (Expedeon) for 20 – 30 min. A gel slice corresponding to proteins with a molecular weight of ~32 – 40 kDa was excised from the lane containing the monomeric avidin-purified proteins. The excised gel was submitted to the Biopolymers and Proteomics Core Facility at the Koch Institute for trypsin digest and LC-MS analysis.

Acknowledgements

Abbreviations

AO-488, aminooxy-functionalized AlexaFluor 488; AO-biotin, aminooxy-functionalized biotin; AO-Flag, aminooxy-functionalized Flag-tag; Gal, galactose; GalNAc, N-acetyl-D-galactosamine; GalO, galactose oxidase; Glc, glucose; GlcNAc, N-acetyl-D-

glucosamine; LOS, lipooligosaccharide; Man, mannose; MH, Mueller-Hinton; SBA-AP, soy bean agglutinin-conjugated to alkaline phosphatase, SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis; Strept-AP, streptavidin-alkaline phosphatase; UDP, uridine diphosphate; ZIC-HILIC, zwitterionic-hydrophilic interaction liquid chromatography.

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Table and figure captions

Table 1. Bacterial strains used in this paper.

Table 2. MS identification of biotinylated, monomeric avidin purified proteins with an electrophoretic mobility corresponding to ~32 – 40 kDa.

Figure 1. Proposed chemoenzymatic labeling of *C. jejuni* N-glycans. The terminal GalNAc residue of the *C. jejuni* heptasaccharide acts as a substrate for GalO, which generates a reactive C-6 aldehyde. An aminooxy derivative forms a stable oxime with the terminal GalNAc residue allowing for conjugation of affinity tag and fluorophores. *C. jejuni* pgl deficient strains can be used to confirm the glycoprotein as the site of modification.

Figure 2. GalO labeling of PEB3 N-glycan. Purified, N-glycan modified PEB3 was reacted with either AO-biotin or GalO or both GalO and AO-biotin to determine if the N-glycan was a substrate for PEB3. Modification was detected when both GalO and AO-biotin were present using α-His (A) and α-N-glycan (B) antibodies. Biotinylation of PEB3 was confirmed with a Strept-AP conjugate(C).

Figure 3. Biotinylation of *C. jejuni* cell-surface glycoproteins using GalO. Glycoproteins and biotinylated glycoproteins from *C. jejuni* strain 11168, with Δ*pglE* and Δ*galE*, and strain 81176 with Δ*cgtA*, and Δ*waaF*. **A** and **C** show proteins modified by the *C. jejuni* N-glycan while **B** and **D** show proteins modified by the *C. jejuni* N-glycan and biotinylated by GalO. Control lanes have been incubated with either GalO or AO-biotin.

Figure 4. Modification of *C. jejuni* LOS by GalO for wild type and mutant strains. *C. jejuni* LOS is capped with a GalNAc residue (center box) and could be a substrate for GalO. A) Wild type strains 11168 and 81176 as well as the *pglE* mutant have fully formed LOS and act as GalO substrates, leading to modification by aminoxy derivatives designated by "R". The *galE*, *cgtA* and *waaF* mutants have abbreviated LOS structures that do not act as substrates for GalO. B) A Tris-Tricene LOS gel showing biotinylation of LOS after GalO incubation as detected by a Strept-AP conjugate. Lane 1) 11168, Lane 2) ΔpglE, Lane 3) ΔgalE, Lane 4) 81176, Lane 5) ΔcgtA, Lane 6) ΔwaaF.

Figure 5. Purification of biotinylated glycoproteins. Biotinylated *C. jejuni* glycoproteins were purified away from non-biotinylated proteins using monomeric avidin modified agarose beads. Lane 1) Benchmark prestained ladder, Lane 2) control reaction, excludes GalO, Lane 3) reaction before incubation with monomeric avidin, includes GalO and AO-biotin, Lane 4) flow through after incubation with monomeric avidin, Lane 5) wash 1, Lane 6) wash 2, Lane 7) wash 6, Lane 8) elution of proteins bound to monomeric avidin. Proteins were visualized with A) α-N-glycan primary antibody and a α-Rabbit, alkaline phosphatase conjugated secondary antibody, B) Strept-AP, C) InstantBlue, D) SBA-AP.

Figure 6. Live-cell fluorescent labeling of *C. jejuni*. GalO was used to label wild type and mutant strains with AlexaFluor 488 Hydroxylamine (488). Phase-contrast microscopy displays presence of cells (grey panels) while fluorescence microscopy indicates fluorescent labeling catalyzed by GalO (black panels).

Figure 1

Strain or mutation	Genotype or decription	Reference or source
11168	Wild type	(Carrillo et al. 2004)
11168 Δ*pglE*	11168 *pglE::Km*	(Szymanski et al. 1999)
11168 Δ*galE*	11168 *galE::Km*	(Bernatchez et al. 2005)
81176	Wild type	(Black et al. 1988)
81176 Δ*waaF*	81176 *waaF::Cm*	(Kanipes et al. 2004)
81176 Δ*cgtA*	81176 *cgtA::Cm*	(Guerry et al. 2002)
81176 Δ*peb3*	81176 *peb3::Cm*	Julie Silverman (unpublished)

C. jejuni number	Gene name	Protein name	Molecular weight (kDa)	Identified peptides	Known glycoprotein	Glycan
Cj0734c	hisJ	Histidine binding protein HisJ/CjaC	29	6	Yes(Scott et al. 2011)	N-glycan
Cj0983	jlpA	Putative lipoprotein JlpA	42	2	Yes(Scott et al. 2011)	N-glycan
Cj1259	porA	Major outer membrane protein PorA	46	3	Yes(Mahdavi et al. 2014)	Predicted tetrasaccharide: Gal(β1–3)-GalNAc(β1–4)-GalNAc(β1–4)-GalNAcα1-Thr[268]
Cj0596	PEB4A	Major antigen PEB4A	31	5	No	
Cj0779	tpx	Probable thiol peroxidase	18	4	No	
Cj1534c	dps	DNA protection during starvation protein	17	2	No	

R = aminooxy-PEG4-Biotin
aminooxy-Flag
aminooxy-Alexa Fluor 488

GalO   -   +   +
AO-biotin   +   -   +

A

37

26

α-N-glycan

B

37

26

α-6X-His

C

37

26

Strept-AP

A 11168 · PglE · GalE
GalO: − + + · − + + · − + +
AO-biotin: + − + · + − + · + − +
α-N-glycan

B 11168 · PglE · GalE
GalO: − + + · − + + · − + +
AO-biotin: + − + · + − + · + − +
Strept-AP

C 81176 · CgtA · WaaF
GalO: − + + · − + + · + − +
AO-biotin: + − + · + − + · − + +
α-N-glycan

D 81176 · CgtA · WaaF
GalO: − + + · − + + · + − +
AO-biotin: + − + · + − + · − + +
Strept-AP

A

R—GalNAc —β1-4— Gal —β1-4— Glc —β1-2— HepII —α1-3— HepI —α1-3— Kdo —2— Lipid A
                                                              PEtN|6
              α2-3|
              Neu5Ac   β1-4|
                                                              Glc

①②④

GalO + R

PEtN
|6
GalNAc —β1-4— Gal —β1-4— Glc —β1-2— HepII —α1-3— HepI —α1-3— Kdo —2— Lipid A
        α2-3| β1-4|
        Neu5Ac   Glc

ΔwaaF →

PEtN
|6
HepI —α1-3— Kdo —2— Lipid A
β1-4|
Glc

⑥

ΔgalE →

Glc —β1-2— HepII —α1-3— HepI —α1-3— Kdo —2— Lipid A
                         PEtN|6
                         β1-4|
                         Glc

③

ΔcgtA ↓

PEtN
|6
Gal —β1-4— Glc —β1-2— HepII —α1-3— HepI —α1-3— Kdo —2— Lipid A
α2-3| β1-4|
Neu5Ac   Glc

⑤

R = aminooxy-PEG4-biotin
aminooxy-Flag
aminooxy-AlexaFluor 488

B

Strept-AP

Wild type (488)   Wild type (488+GalO)
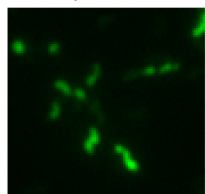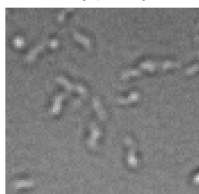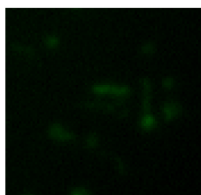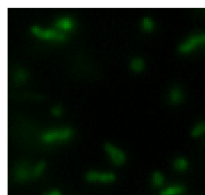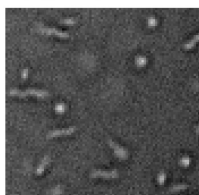
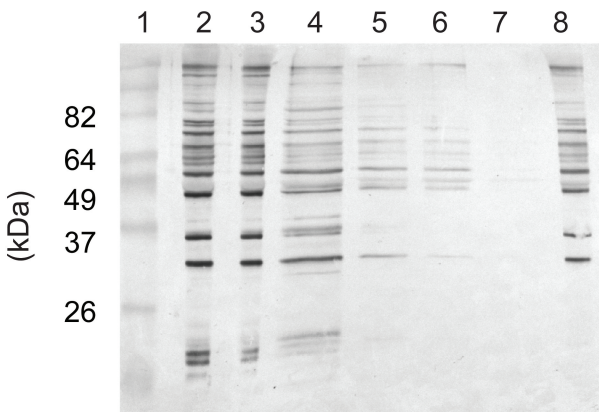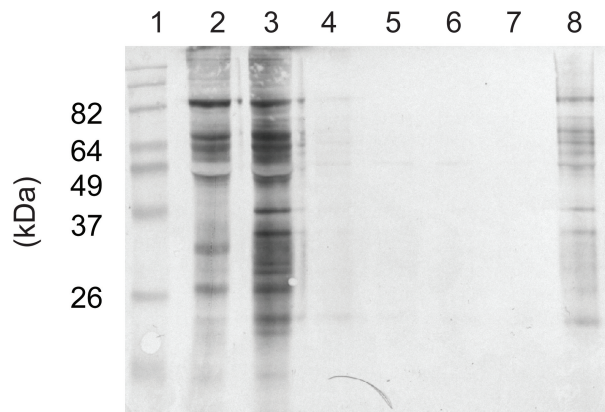Δ*pglE* (488)   Δ*pglE* (488+GalO)

Δ*waaF* (488)   Δ*waaF* (488+GalO)

Δ*cgtA* (488)   Δ*cgtA* (488+GalO)

Δ*galE* (488)   Δ*galE* (488+GalO)

A
1 2 3 4 5 6 7 8

(kDa)
82
64
49
37
26

α-N-glycan

B
1 2 3 4 5 6 7 8

(kDa)
82
64
49
37
26

Strept-AP

C
1 2 3 4 5 6 7 8

(kDa)
82
64
49
37
26

InstantBlue

D
1 2 3 4 5 6 7 8

(kDa)
82
64
49
37
26

SBA-AP
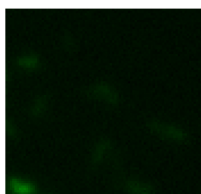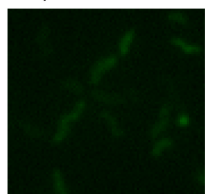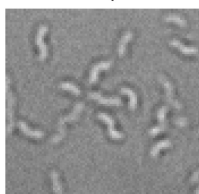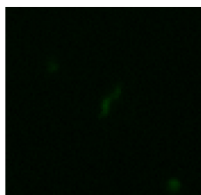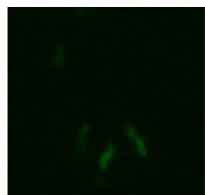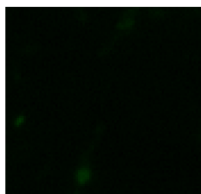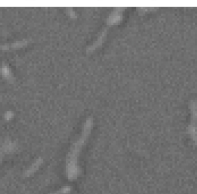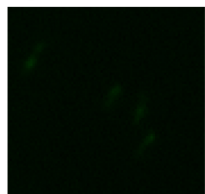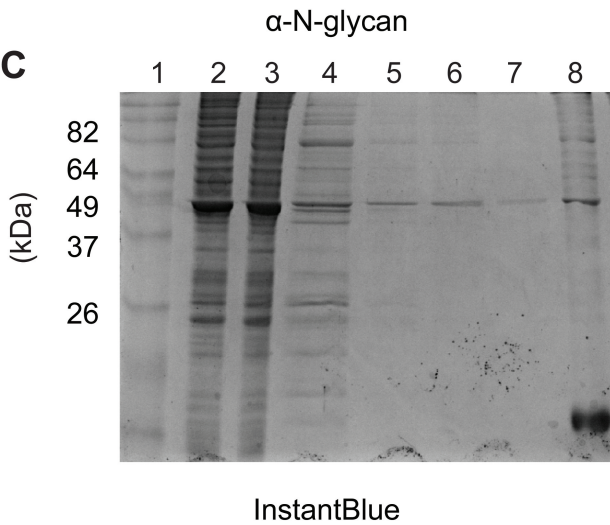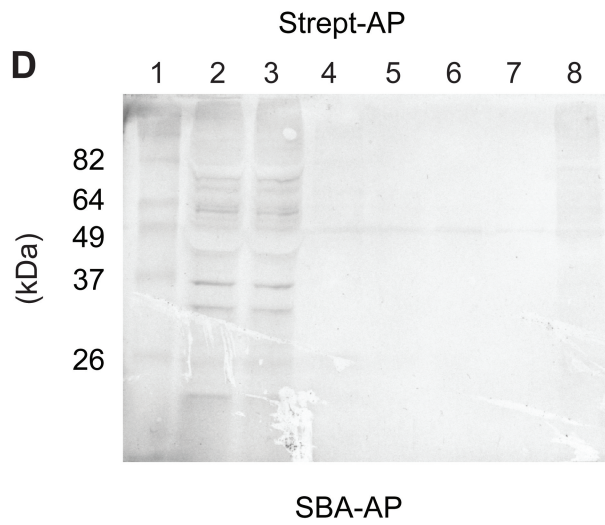
Supplementary Figure 1. LC trace of purified PEB3 before (**A**) and after the GalO reaction (**B**). PEB3 elutes at ~ 8 min in both samples. Additional peaks for the PEB3 sample that underwent the GalO reaction are due to the commercially purchased GalO and catalase.
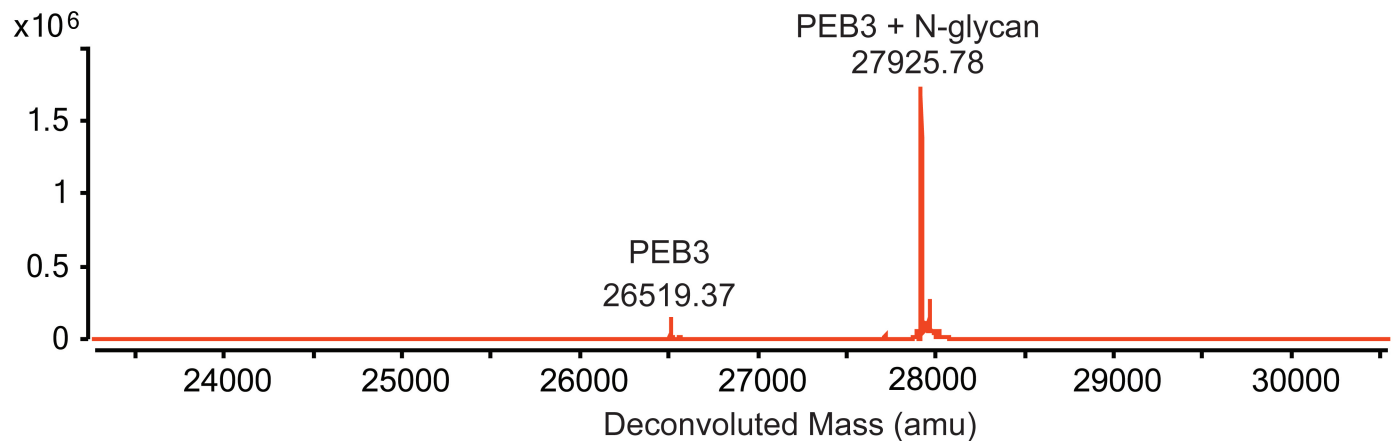
Supplementary Figure 2. Deconvoluted MS spectrum of PEB3 peak at ~8 min as seen in LC trace (Supplementary Figure 1). A) Purified PEB3 before the GalO reaction identifies PEB3 (calculated M+1 = 26519.03) and N-glycan modified PEB3 (calculated M+1 = 27925.53). B) PEB3 after the GalO reaction identifies PEB3 (calculated M+1 = 26519.03), N-glycan modified PEB3 (calculated M+1 = 27925.53) and biotinylated, N-glycan modified PEB3 (calculated M+1 = 28339.80).

Supplementary Figure 3. Comparison of the GalO-catalyzed conjugation of biotin and Flag-tag to cell-surface glycoproteins from wild-type *C. jejuni* strains 11168 and 81176. A) *C. jejuni* strains 11168 and 81176 were incubated with AO-biotin or AO-biotin and GalO, streptavidin-alkaline phosphatase (Strept-AP) was used for detection. B) *C. jejuni* strains 11168 and 81176 were incubated with AO-Flag or AO-Flag and GalO, α-Flag antibody (Monoclonal ANTI-FLAG M2 primary antibody, Sigma Aldrich) followed by an α-Mouse secondary antibody conjugated to alkaline phosphatase were used for detection.
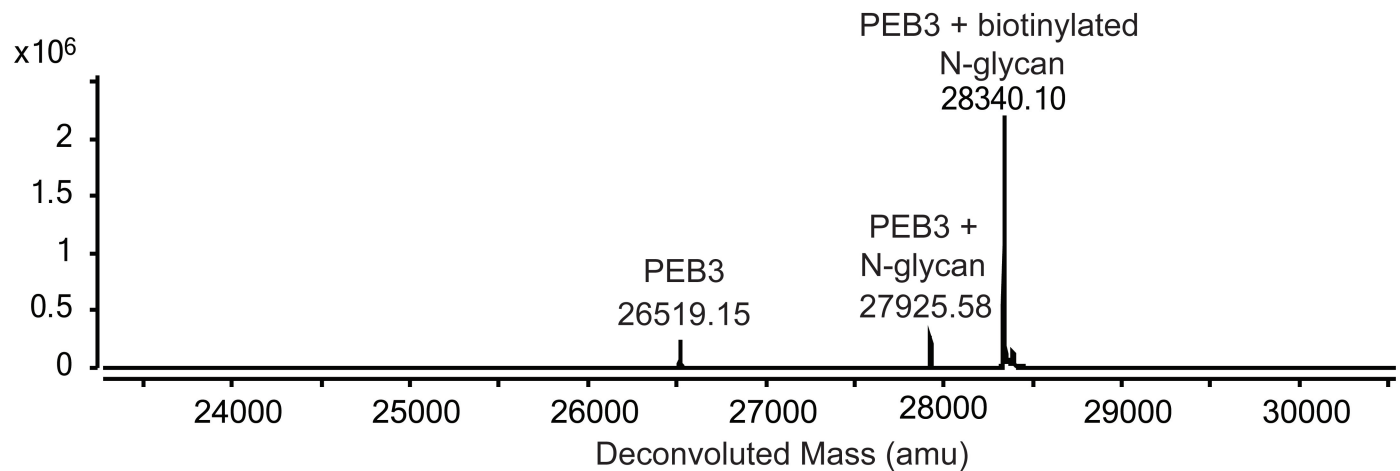
A +ESI TIC Scan Frag=175.0V PEB3

B +ESI TIC Scan Frag=175.0V PEB3 + GalO reaction
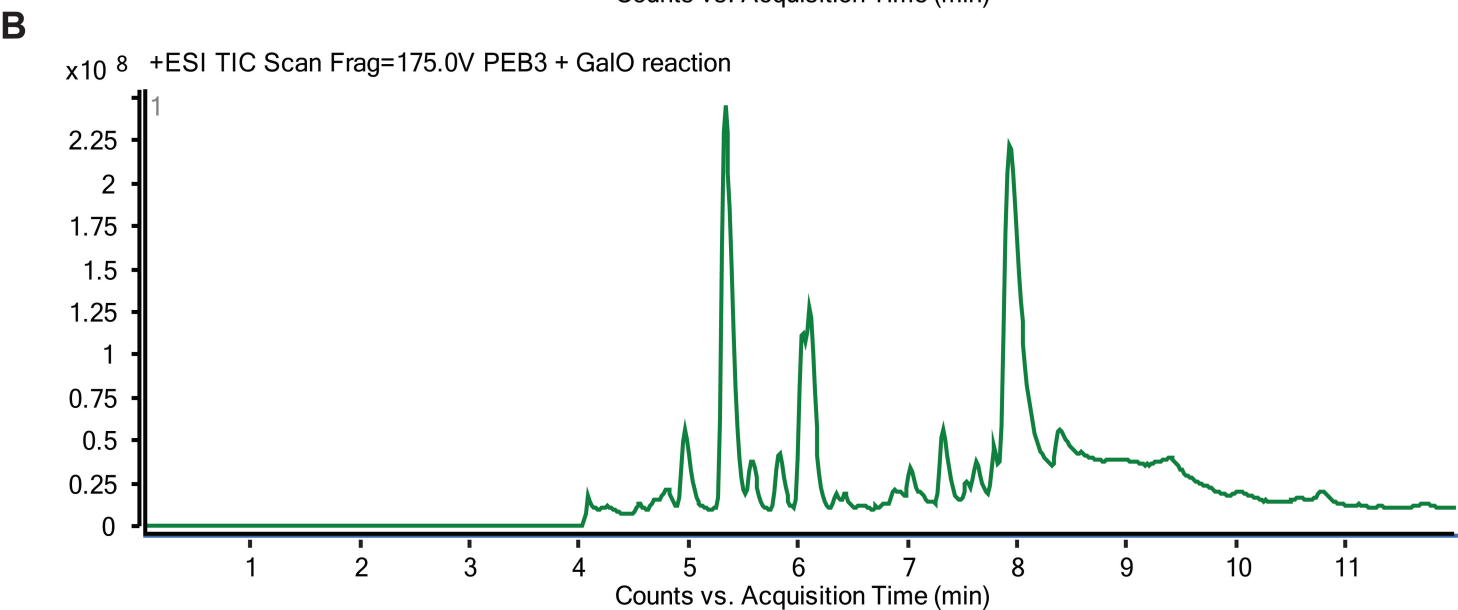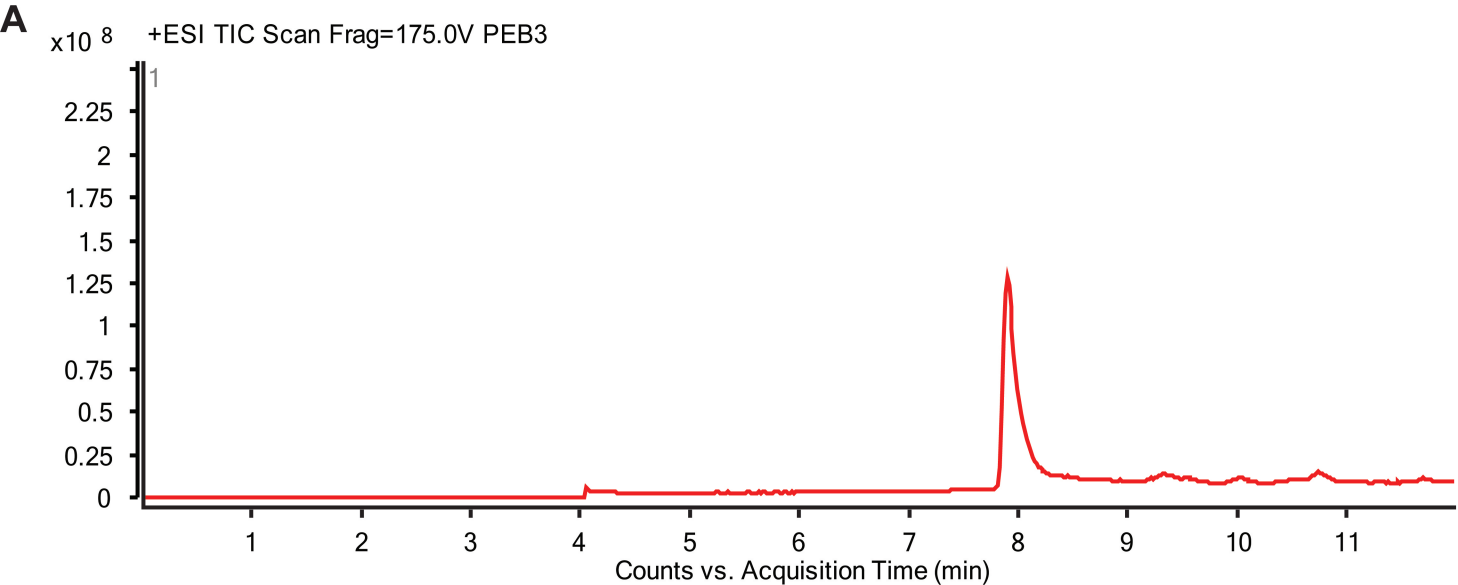
A + Scan (7.81-8.21 min, 25 Scans) PEB3 Deconvoluted (Isotope Width=13.0)

PEB3 + N-glycan
27925.78

PEB3
26519.37

B + Scan (7.76-8.18 min, 26 Scans) PEB3 + GalO reaction Deconvoluted (Isotope Width=13.0)

PEB3 + biotinylated
N-glycan
28340.10

PEB3
26519.15

PEB3 +
N-glycan
27925.58

A

	11168		81176	
GalO	−	+	−	+
AO-biotin	+	+	+	+

82
64
49
37
26
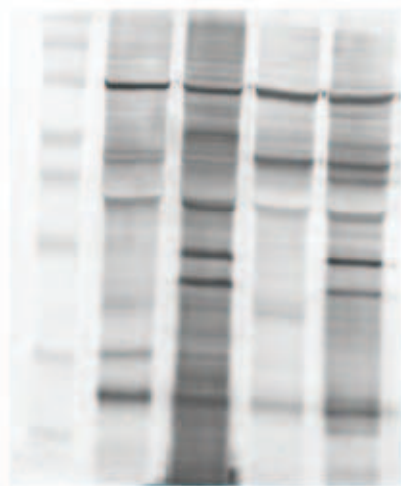
Strept-AP

B

	11168		81176	
GalO	−	+	−	+
AO-Flag	+	+	+	+

82
64
49
37
26

α-Flag antibody