American Journal of Biomedical Sciences
ISSN: 1937-9080
nwpii.com/ajbms

C_2-Carbon Isostere of *N*-acetylglucosamine as Substrate for Bacterial Polysaccharide Remodeling

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Received: 17 December 2010; | Revised: 24 February 2011; | Accepted: 1 March 2011

Abstract

Introduction of unique structural modifications into a living cell presents opportunities to investigate and manipulate specific biological pathways and functions. Cell surface oligosaccharides represent one area where structural modifications can easily be made which allow for the investigation and manipulation of such processes. Bacterial cell surface oligosaccharides, while both structurally diverse and biological relevant, have not been extensively probed as a source for introducing chemically modified structures. In this study, we demonstrate the incorporation of an unnatural ketone containing monosaccharide, which heterogeneously is incorporated into the cell surface oligosaccharides of *E. coli*. Through bioorthogonal chemistry we selectively label the cell surface oligosaccharides containing the ketone modified monosaccharide with various probes, which are used to image and identify the cell surface oligosaccharides. In summary, this study presents a facile and effective method for the *in vivo* incorporation of a ketone containing monosaccharide which can be exploited for studying the bacterium.

Keywords: 2-Ketosugars; labeling; metabolic pathways; aminooxybiotin.

Abbreviations: *Escherichia coli*, *E. coli*; Lipopolysaccharide, LPS; *N*-acetylglucosamine, GlcNAc; *N*-acetylgalactosamine, GalNAc; Galactose, Gal; Glucose, Glc; 2-acetonyl-2-deoxy-D-galactose, 2-KetoGal; 2-acetonyl-2-deoxy-D-glucose, 2-KetoGlc; peracetylated 2-KetoGlc, Ac2KetoGlc; *N*-(aminooxyacetyl)-*N*'-(D-biotinoyl) hydrazine, aminooxybiotin; fluorescein isothiocyanate, FITC; LB, Luria-Bertani broth.

1. Introduction

2-acetamido-2-deoxy-D-glycopyranosides (*N*-acetylglucosamine/galactosamine, also called GlcNAc/GalNAc) are widely distributed in living systems as oligosaccharides and glycoconjugates, and play significant roles in a wide range of biological processes [1; 2]. For example, GlcNAc is a major constituent of the backbone of bacterial peptidoglycan, meanwhile both sugars are fundamental components of many important polysaccachrides such as glycosaminoglycans [3-7]. Hence, there is a considerable interest in chemical modification of GlcNAc/GalNAc residues by an unnatural analogue in such a polysaccharide chain for further understanding and modulating the targets of these glycosides [8-13]. Especially, analogues containing bioorthogonal groups (e.g. reactive ketones, azides, alkynes, etc.) are of great interest since they can be further probed by chemoselective reactions [14-16]. Among the various analogues, 2-acetonyl-2-deoxy-D-galactose (2-ketoGal, Figure 1) has attracted much attention because it bears much resemblance to GalNAc, and most significantly, contains a ketone handle. This substrate serves as a carbon isostere of GalNAc and was incorporated into cell surface glycoprotein through metabolic pathways. Then the ketone epitope was chemoselectively reacted with biotin hydrazide, stained with fluorescein isothiocyanate (FITC) labeled avidin, and detected by flow cytometry for quantification [17]. Moreover, 2-ketoGal, which is transferred from the corresponding donor molecule UDP-2-ketoGal by a mutated glycosyltransferase [18] was also widely used as a ketone probe for *in vitro* *O*-GlcNAc glycoprotein detections [14-16; 19; 20].

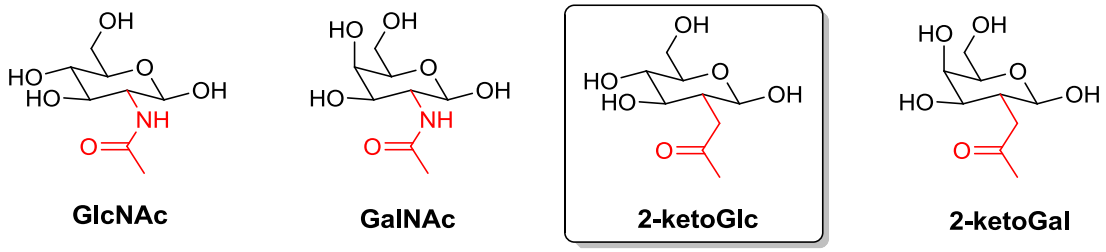

Figure 1. Chemical structure of GlcNAc/GalNAc and their 2-carbon isosteres.

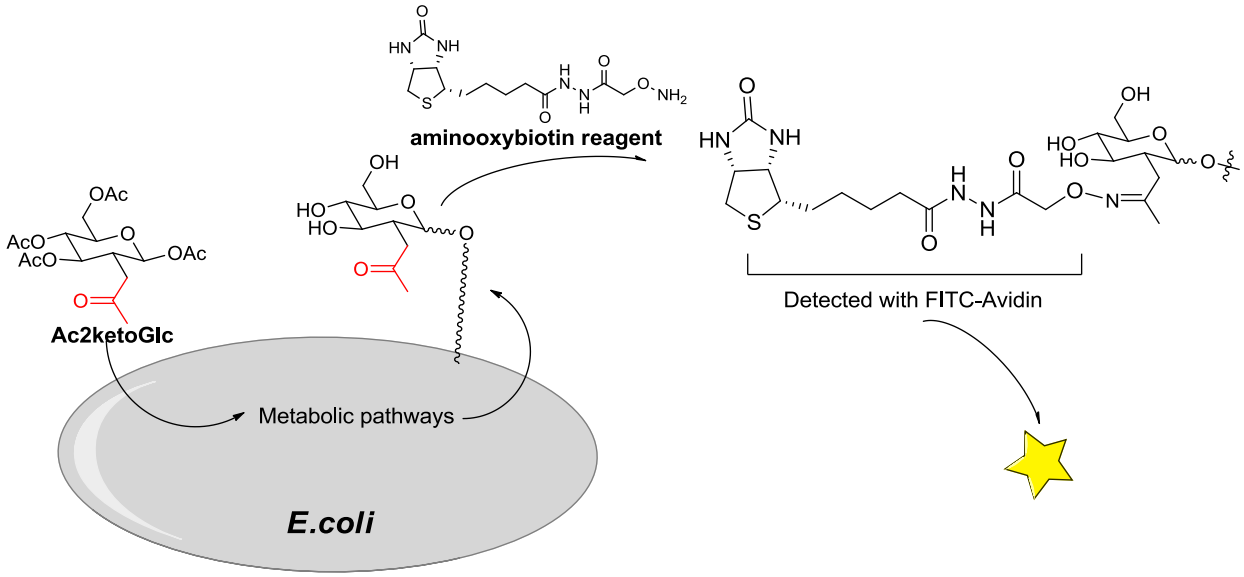

Figure 2. Metabolic incorporation of ketone isotope on *E.coli* surface

However, the corresponding 2-keto isostere of GlcNAc (2-ketoGlc) is missing from current literature. Bertozzi and co-workers demonstrated 2-ketoGal was successfully taken as a substrate for metabolic glycoprotein engineering through the salvage pathway while the GlcNAc counterpart could not be incorporated [17]. One explanation for this observation is because, firstly, GlcNAc is abundant and the 2-ketoGlc lies in strong competition with the endogenous GlcNAc. In addition to this, unlike GalNac, current data suggests that there is a lack of suitable metabolic pathway (salvage pathway) which is critical for the generation of UDP-sugar donor.

In this work, we demonstrate that unnatural 2-ketoGlc can be processed by *E. coli* and displayed on the cell surface (Figure 2). The incorporated ketone epitope allowed further detection by conjugation with commercially available aminooxybiotin reagent. Through western blot analysis, using purified lipopolysaccharide (LPS), we show that the LPS is modified with the ketone epitope.

2. Materials and methods

2.1 Bacterial strains and reagents

E. coli competent cell BL21 (DE3) [F *ompT* $hsdS_B$ (r_Bm_B) *gal dcm* (DE3)] was obtained from Stratagene. All reagents were purchased from Sigma unless otherwise noted.

2.2 Synthesis of ketosugar

Peracetylated 2-ketoGlc was chemically synthesized from glucal as previously described [21].

2.3 Labeling live bacterial cells via aniline-catalyzed oxime ligation

Either *E. coli* BL21 (DE3), and *E. coli* O86:B7 Δ*wecA*/Δ*waaL* was inoculated in 200 µL in LB medium supplemented with the sugar analogue (peracetylated GlcNAc was used as negative control), grown overnight, at 37 °C, and shaking at 250 rpm. Cells were collected by centrifugation at 4 °C, washed 3 times with PBS buffer, and subsequently resuspended in a solution containing 20 mM MOPS, pH 6.7, 1 mM *N*-(aminooxyacetyl)-*N*'-(D-biotinoyl) hydrazine (Invitrogen), and 10 mM aniline. Cells were then incubated at 4 °C 90 minutes (on a rocker), after which the cells were harvested by centrifugation, and subsequently washed 3 times with PBS buffer. Cells were then resuspended in 0.5 mL of PBS, to which 5 µL of FITC conjugated avidin (0.5 mg/mL, Invitrogen) was added. The resuspension was incubated at 4 °C on a rocker for 90 minutes, after which the cells were extensively washed with PBS buffer, and subsequently analyzed by both flow cytometry (BD FACS LSR II) and fluorescence microscopy (Olympus IX81 inverted microscope).

2.4 Functional inactivation of *wecA* and *waaL*

The *wecA* and *waaL* genes were replaced by a kanamycin resistance cassette, using the RED recombination system of phage lambda [22; 23]. For creation of the knock out strain we used the plasmids provided by the Quick and Easy *E. coli* Gene Deletion Kit (Gene Bridges). The *wecA* gene was first replaced from the chromosomal DNA with the kanamycin resistance cassette following the manufacturer's protocol, which was verified by PCR. The kanamycin resistance cassette was then removed by FLP/FLPe expression and the *E. coli* O86:B7 Δ*wecA* was then used as the host to create the *waaL* inactivated strain, which was created following the manufacturer's protocol. The final *E. coli* O86:B7 Δ*wecA*/Δ*waaL* was confirmed by PCR as well as observing the change in LPS phenotype by LPS silver staining.

2.5 LPS isolation and analysis

To determine the relative location of the labeled sugar *E. coli* O86:B7 Δ*wecA*/Δ*waaL* was inoculated with 10 mM of the sugar analogue and subsequently labeled with the aminooxybiotin as mentioned. Then the LPS was extracted according to the published proteinase K digestion protocol, and analyzed by both SDS-PAGE (with silver staining) and western blot [24]. To perform the western blot analysis, the LPS was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with HRP conjugated streptavidin (1:5000, Thermo Scientific). The blot was developed by using the ECL Western Blotting Detection Reagents (GE Healthcare).

3. Results and Discussion

3.1 Incorporation of 2-ketoGlc in cell surface oligosaccharides

In this study we sought to remodel the bacterial cell surface by introduction of an unnatural keto sugar (2-ketoGlc), which can be probed by using bioorthogonal chemistry. Previous work by various groups have demonstrated the utility of modifying cell surface oligosaccharides *in vivo*, typically exploiting very specific biosynthetic mechanisms, primarily in eukaryotic organisms. More specifically, we incubated 2-ketoGlc with various bacterial strains, with the notion that sugar may act as a substrate for various enzymes within the cells' oligosaccharide biosynthetic pathways, which could eventually be incorporated in the cell surface oligosaccharides. For example, there exists multiple salvage pathways, such as the GalNAc salvage pathway, in which a bacterium can obtain a specific sugar from the external medium, and directly convert this sugar to the required sugar nucleotide donor [25].

Previously, we have demonstrated that the key sugar nucleotide donor molecule for 2-ketoGlc incorporation could be synthesized *in vitro* by bacterial kinase (NahK) and pyrophosphorylase (GlmU) [21]. To test whether or not 2-ketoGlc could be incorporated into the cell surface oligosaccharides, we first incubated overnight cultures of *E. coli* BL21 (DE3) with 10 mM of 2-ketoGlc or peracetylated GlcNAc (negative control). Since the ketone is not a common functional group found in living organisms, by virtue of bioorthogonal chemistry, those cells that incorporate the 2-ketoGlc into the cell surface oligosaccharides can be easily identified. Therefore, to the overnight cultures both containing and void of the 2-ketoGlc, *N*-(aminooxyacetyl)-*N*'-(D-biotinoyl) hydrazine (aminooxybiotin) in the presence of the aniline catalyst at neutral pH was added [26]. Ketone groups that had been metabolically engineered into the cell surface polysaccharides can react with the aminooxybiotin reagent resulting in biotin-coated bacterial cells. The biotinylated bacterial cells can be probed by staining with FITC conjugated avidin, and the resulting fluorescence signal can be analyzed by flow cytometry. As shown in Figure 3A, *E. coli* BL21 (DE3) cells fed with 2-ketoGlc showed an increase in fluorescence intensity compared with control samples. Two negative controls are shown, with the teal line representing *E. coli* BL21 (DE3) cells that were treated with the aminooxybiotin reagent followed by subsequent coupling with FITC conjugated avidin, and the orange line representing the fluorescence intensity of just the naked *E. coli* BL21 (DE3). Cells incubated with the biotin linker and subsequently incubated with the FITC conjugated but were void of the 2-ketoGlc sugar observed no change in fluorescence (from the negative *E. coli* BL21 (DE3)) suggesting that the biotin hydrazine is reacting specifically with the ketone in 2-ketoGlc. A similar result was observed for an *E. coli* O86:B7 Δ*wecA*/Δ*waaL* strain (Figure 3B), which is used a model strain to identify the location of the modified (Section 3.3). These results are surprising, for the simple fact that there exists no endogenous GlcNAc-1-kinase, such as NahK, which *in vitro* is utilized for the synthesis of the UDP-GlcNAc donor [21]. Fluorescent microscopy imaging also confirmed successful incorporation of ketone group on *E.coli* cell surface (Figure 4). White arrows in Figure 4A point at FITC labeled *E. coli* rods. Therefore, *in vivo*, these results suggest that the 2-ketoGlc may be being processed either as a mock Glc or GlcNAc due to structural similarities, or is possibly being epimerized by GalE to either a mock Gal or GalNAc. Thus, the obtained results are suggestively very complex as it is challenging to identify which biosynthetic pathway(s) the 2-ketoGlc is traveling by.

In order to try and regulate the glycosylation we attempted at knocking out the *de novo* UDP-GlcNAc biosynthesis pathway, more specifically by removing *glmM* from the chromosome. To a strain of *E. coli* containing NahK (GlcNAc kinase) we attempted to knock out *glmM* and drive UDP-GlcNAc biosynthesis through NahK, however, we were unsuccessful. This is likely due in part because of the abundance of UDP-GlcNAc required by the cell, and eliminating this biosynthetic pathway is lethal for the bacterium. Lastly, In addition to the flow cytometry results, incubation with 2-ketoGlc appeared to have a

significant inhibitory effect on the cell growth. No such effects were reported in previous studies in which unnatural sugars were incorporated with either eukaryotic or prokaryotic organisms.

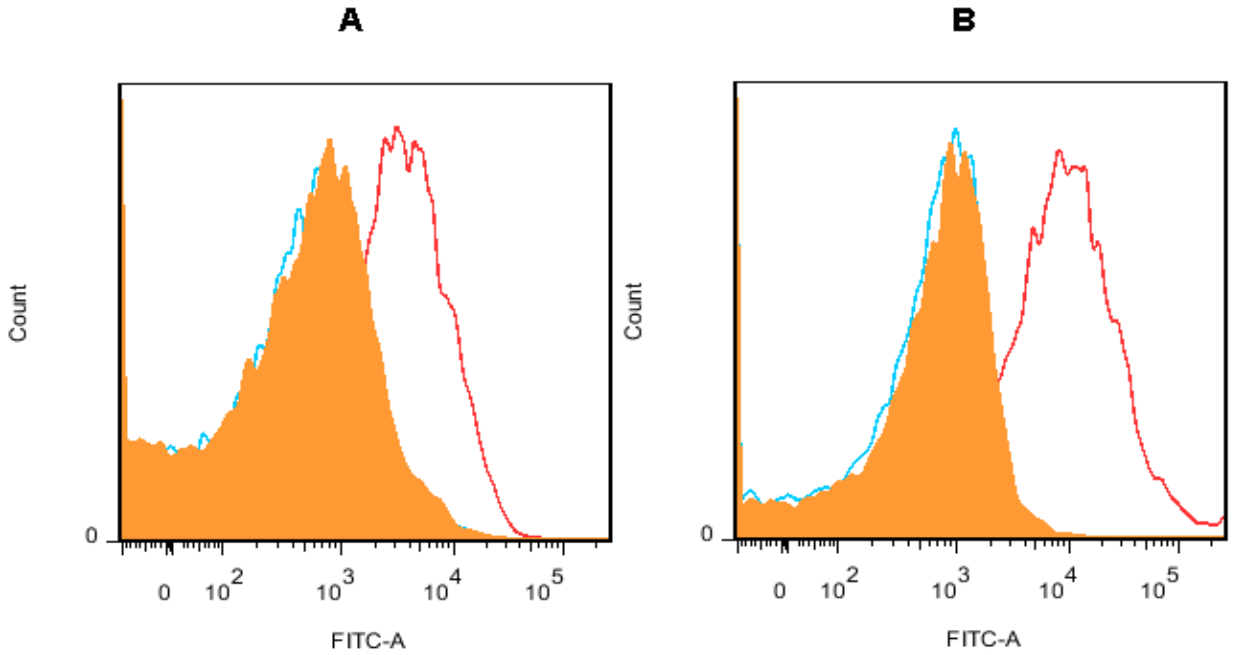

Figure 3. Bacterial cell surface labeling by introduction of 2-ketoGlc, conjugated with aminooxybiotin, and detection by FITC conjugated avidin. The fluorescence signal was analyzed by flow cytometry. A) Fluorescence labeling of 2-ketoGlc fed *E. coli* BL21 (DE3). Red line: Positive reaction; Teal line: Negative control (no 2-ketoGlc but treated with aminooxybiotin and FITC-avidin; Orange fill: Negative control (naked *E. coli* BL21 (DE3)). B) Fluorescence labeling of 2-ketoGlc fed *E. coli* O86:B7 Δ*wecA*/Δ*waaL*. Red line: Positive reaction; Teal line: Negative control (no 2-ketoGlc but treated with aminooxybiotin and FITC-avidin; Orange fill: Negative control (naked *E. coli* O86:B7 Δ*wecA*/Δ*waaL*).

3.2 Labeling LPS

Previous work by our lab has used *E. coli* O86 as a model system for the pin point modification of the fucosylated LPS by using metabolic pathway engineering, and in this work we sought to expand our ability to modify this model strain with 2-ketoGlc [27]. Structurally 2-ketoGlc is very similar to GlcNAc, with the exception of the 2 amide, and thus may be incorporated as GlcNAc in the LPS. In the R3-type core oligosaccharide, there exists multiple GlcNAc residues, several Glc residues, and several other hexoses, thus representing several possible glycosyltransferases that may tolerate and incorporate this unnatural sugar when synthesizing the core oligosaccharide [28].

To examine one possible location where the unnatural sugar is being incorporated we created a double knock out strain of *E. coli* O86:B7, which is functionally missing *wecA* and *waaL*. These enzymes are involved in the biosynthetic pathway of the LPS, more specifically, the synthesis and ligation of the O-antigen, respectively [29; 30]. By removing the *wecA* gene from the chromosome, we remove the glycosyltransferase which is responsible for the first sugar found in the O-antigen, thus removing the O-antigen from the LPS. Furthermore, by disruption of the *waaL* gene, we ensure that there is no ligation of any oligosaccharide onto the LPS core oligosaccharide. Thus, the resulting LPS displayed on the cell surface will only contain the core oligosaccharides, which can verified by the LPS phenotype observed in Figure 5. Figure 5, lane 1 shows the wild type LPS phenotype for *E. coli* O86:B7, whereas lane 2 is the LPS phenotype for the *E. coli* O86:B7 Δ*wecA*/Δ*waaL* strain. The single band observed in lane 2 is characteristic of the core oligosaccharide, which contains several GlcNAc residues [30]. Thus, to determine one

possible location where the 2-ketoGlc may be incorporated, *E. coli* O86:B7 Δ *wecA/ΔwaaL* was fed 2-ketoGlc and incubated overnight, from which the LPS was isolated in analyzed with western blot. To both the *E. coli* O86:B7 Δ *wecA/ΔwaaL* fed 2-ketoGlc and void of 2-ketoGlc, the cells were incubated with aminooxybiotin, which biotinylates cells that incorporate the 2-ketoGlc into the cell surface oligosaccharides. After excessive washing of the cells to remove unreacted aminooxybiotin, the LPS was isolated and separated electrophorically by SDS-PAGE. Then after transfer of the LPS to a nitrocellulose membrane, the presence of biotinylated LPS was probed by using HRP conjugated to streptavidin. Figure 5 lane 2 shows the single band of core oligosaccharide for the *E. coli* O86:B7 Δ*wecA/*Δ*waaL*, and in Figure 5 lane 4, we observe the same band in the western blot. Furthermore, absence of the band in Figure 5 lane 3, suggests that *E. coli* O86:B7 Δ*wecA/*Δ*waaL* is incorporating the 2-ketoGlc into the core oligosaccharides.

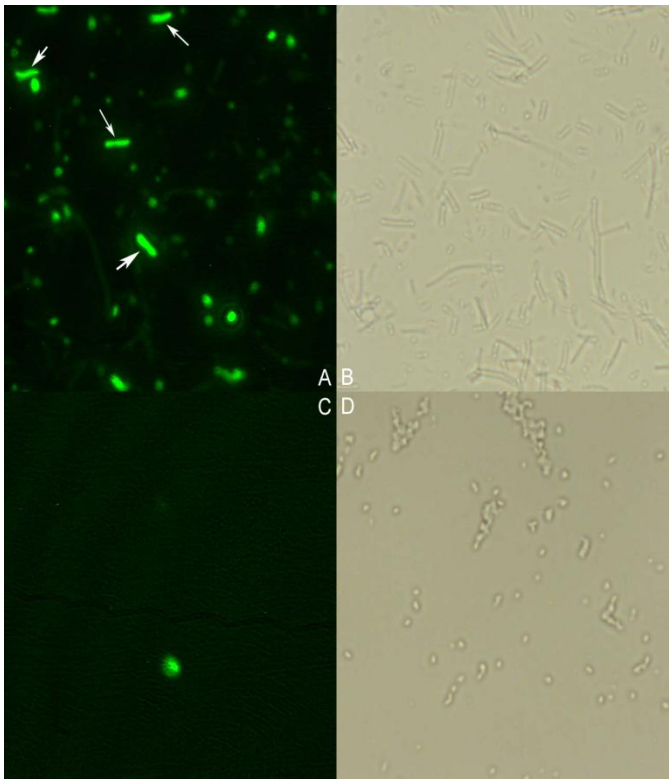

Figure 4. Incorporation of 2-ketoGlc or GlcNAc (negative control) into *E. coli.* A and B: *E. coli* O86:B7 Δ*wecA/*Δ*waaL* incubated with peracetylated 2-ketoGlc, labeled with aminooxybiotin, and probed with FITC-avidin; C and D: *E. coli* O86:B7 Δ*wecA/*Δ*waaL* incubated with peracetylated GlcNAc, labeled with aminooxybiotin, and probed with FITC-avidin; A and C: Fluorescence images taken under the Olympus inverted microscope, white arrows point at FITC labeled *E. coli* rods; B and D: phase contrast images taken at the same fields of A and C respectively.

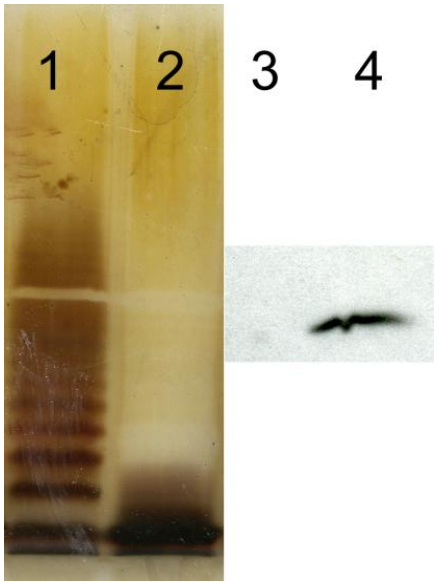

Figure 5. Lane 1: LPS phenotype for *E. coli* O86:B7, visualized by silver staining; lane 2: LPS phenotype for *E. coli* O86:B7 Δ*wecA/*Δ*waaL*, visualized by silver staining; lane 3: LPS phenotype for *E. coli* O86:B7 Δ*wecA/*Δ*waaL* incubated with peracetylated GlcNAc, visualized by HRP conjugate streptavidin; lane 4: LPS phenotype for *E. coli* O86:B7 Δ*wecA/*Δ*waaL* incubated with peracetylated 2-ketoGlc, visualized by HRP conjugate streptavidin.

4. Conclusion

In conclusion, we demonstrate that the 2-ketoGlc is incorporated in some form into the LPS core oligosaccharide, however due to the complexity of the biosynthetic pathways in which this sugar could conceivably interact with, it remains a challenge to identify the exact location that this sugar occupies on the LPS [31]. The key to this success, however, likely resides in the promiscuity of sugar nucleotide biosynthetic pathways, when creating the sugar nucleotide donor UDP-2-ketoGlc. Furthermore, this result may also demonstrate the promiscuity of the glycosyltransferases which are responsible for the

synthesis of the oligosaccharides that are eventually displayed on the cell surface. In conjunction with the incorporation of modified fucose derivatives into the LPS of *E. coli* O86:B7, we further demonstrate proof of principle that other unique monosaccharides can be engineered into the cell surface oligosaccharides in bacteria [27]. Unlike our previous work where we could control the exact location of the modification to the LPS, because of the possible pathways that 2-ketoGlc may take, exact identification of which residue is being modified in the core oligosaccharides is challenging. Also in conjunction with previous observations, we observed a competition effect, whereby it was critical to have an extracellular concentration of 2-ketoGlc at 10 mM. This is likely important for competing with the high intracellular concentrations of UDP-GlcNAc [17]. Further studies are ongoing by incorporating different monosaccharides (GalNAc, GlcNAc) which include different modifications (e.g. azido, alkyne) at different positions which may expand the understanding in which biosynthetic pathways the 2-acetamido-2-deoxy-D-glycopyranosides analogues interact with.

Acknowledgements

P.G.W. acknowledges NIH (R01 AI083754, R01 HD061935 and R01 GM085267) for financial support. We gratefully acknowledge Dr. Xinhe Wang and Dr. Jiyan Ma (Department of Molecular and Cellular Biochemistry, The Ohio State University) for aiding in the fluorescent microscopy.

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