Metabolic Labeling of Legionaminic Acid in Flagellin Glycosylation of Campylobacter jejuni Identifies Maf4 as a Putative Legionaminyl Transferase

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Abstract: Campylobacter jejuni is the major human foodborne pathogen. Its bipolar flagella are heavily O-glycosylated with microbial sialic acids and essential for its motility and pathogenicity. However, both the glycosylation of flagella and the exact contribution of legionaminic acid (Leg) to flagellar activity is poorly understood. Herein, we report the development of a metabolic labeling method for Leg glycosylation on bacterial flagella with probes based on azide-modified Leg precursors. The hereby azido-Leg labeled flagelin could be detected by Western blot analysis and imaged on intact bacteria. Using the probes on C. jejuni and its isogenic maf4 mutant we also further substantiated the identification of Maf4 as a putative Leg glycosyltransferase. Further evidence was provided by UPLC–MS detection of labeled CMP-Leg and an in silico model of Maf4. This method and the developed probes will facilitate the study of Leg glycosylation and the functional role of this modification in C. jejuni motility and invasiveness.

Campylobacter jejuni is the leading cause of bacterial gastroenteritis worldwide. It is a highly motile bacterium due to its bipolar flagella that play an important role in host colonization and infections of humans. The flagellar filament of C. jejuni is composed of two highly homologous flagelin proteins, FlaA and FlaB. The flagellins of C. jejuni are extensively O-glycosylated with microbial nonulosonic acids such as pseudaminic acid (Pse) and legionaminic acid (Leg) (Figure 1). It has been shown that O-glycosylation with Pse is required for proper filament formation in C. jejuni and thus for a functional flagella. Glycosylation of flagellin proteins with Leg derivatives correlates with an ability to form biofilms and auto-agglutinate, the latter activity is often a marker for virulence and interaction with host cells in Gram-negative bacterial pathogens. However, the functional role Leg plays in these processes and in flagella functioning is largely unknown.

In 1994, legionaminic acid was identified by Knirel and coworkers as a component of the lipopolysaccharide (LPS) of Legionella pneumophila, the causative agent of Legionnaires’ disease. Subsequently, legionaminic acid and derivatives thereof have been found in numerous bacterial species, including, Campylobacter, Pseudomonas, Vibrio, Acinetobacter, Escherichia and Salmonella.

The biosynthetic pathway of CMP-legionaminic acid (CMP-Leg) has been elucidated in C. jejuni. It involves six enzymes and starts with the conversion of GDP-GlcNAc into by LegB, a NAD^+ dependent dehydratase (Scheme 1). The second enzyme is a PLP-dependent aminotransferase, LegC, that installs an amino group at C4 to produce 9. The latter intermediate is transformed into 10 by the action of the acetyltransferase LegH. A hydroxylase and 2-epimerase, LegG, converts 10 into the key non-phosphorylated hexose precursor 11 that is condensed with phosphoenolpyruvate (PEP) by legionaminic acid synthase, LegI, to produce Leg. Finally, Leg is activated by the cytidyltransferase LegF as CMP-Leg for glycoconjugate synthesis mediated by a glycosyltransferase that has yet to be identified.
The development of a versatile molecular toolbox of Leg-based probes represents a key approach to illuminate the presence and functional role of Leg glycosylation in bacteria and identify the involved enzymatic activities. We herein report the design and synthesis of six probes (2–7) with azide reporters based on Leg hexose precursor (1). We evaluated their ability for metabolic oligosaccharide engineering (MOE) of Leg glycosylation in C. jejuni (Figure 1) and establish that three of these probes (2, 4 and 6) could be incorporated into the flagella of C. jejuni strains through their legaminic acid biosynthetic pathway. Furthermore, by using probe 6 we identified that the Mafa4 protein of C. jejuni may be a putative Leg glycosyltransferase, as this probe could successfully be converted into its CMP-Leg analogue, but its incorporation into the flagellins of the mafa4 mutant was strongly diminished.

MOE is a powerful approach to explore and visualize cell-surface glycoconjugates, also in bacteria. For legaminic acid it is known that a C6 azide modification of hexose precursor 1 allows for species-specific labeling by MOE of Legionella pneumophila. For this study, we also selected precursor 1 as a suitable target for MOE as we judged that it could be readily synthesized with bioorthogonal chemical reporters and its lack of a charge would hopefully allow for sufficient uptake by C. jejuni. As it was not yet known if one or both of the N-acyl positions of 1 could be used for modification towards a MOE probe, we planned to install the azidoacetaldehyde group on either C2 and C4 or both positions of 1. Additionally, the hydroxyls on these modified precursors could be acetylated to potentially facilitate the passive diffusion into bacterial cells. In situ deacetylation and further processing by the downstream Leg biosynthesis enzymes would then also lead to labeling.

Commercially available D-fucose 12 was chosen as the starting material based on the ability to simultaneously substitute with inversion at the C2 and C4 position, as reported by Chen (Scheme 2). After full acetylation of 12, the p-methoxyphenyl group was installed onto the anomic center in the presence of BF3·Et2O to afford 13. Deacetylation of 13 using sodium methoxide and aminoethyl diphenylborinate-mediated regioselective O3-benzoylation afforded 14 in 73% yield in two steps. Conversion of 14 into the corresponding 2,4-bistriflate, followed by bis-azidation with tetraethylammonium azide through SN2 substitution, resulted in diazide 17 using the N-9-manno configuration in 84% yield over two steps. The benzoate group was removed using sodium methoxide in methanol to obtain 16 in 95% yield. This step proved necessary as migration of the benzoate group was otherwise observed during reduction of the azide in its presence. Reduction of the 2,4-azido groups was performed by hydrogenation over Pd(OH)2/C and followed by N-acylation. For the synthesis of 19 with di-N-azidoacetyl moieties, the amines that formed after reduction of azido groups, were directly coupled with diazide 15 with the N-9-manno configuration in 84% yield over two steps. The benzoate group was removed using sodium methoxide in methanol to obtain 16 in 95% yield. This step proved necessary as migration of the benzoate group was otherwise observed during reduction of the azide in its presence. Reduction of the 2,4-azido groups was performed by hydrogenation over Pd(OH)2/C and followed by N-acylation to give 20 in 80% yield over 2 steps. To synthesize 19 with di-N-azidoacetyl product 19 another more polar product S1 also formed and was identified as having only a N2-azidoacetyl modification (Scheme S1). This reactivity difference between the amine at C2 and C4 could be used to our advantage by subsequently converting S1 into 17 by N4-acylation. For the synthesis of 18, the reactivity difference was again used to install an acetyl group onto N2 selectively. Subsequent N4-azidoacetyl modification and treatment with sodium methoxide provided 18. Removal of the methoxyphenyl group of intermediates 17–20 by ceric ammonium nitrate gave the target chemical probes 2, 4, 6 and native precursor 1 respectively. Finally, acetylation of

Scheme 1. Legaminic acid biosynthetic pathway in C. jejuni.

Scheme 2. Synthesis of Leg precursor 1 and its azido analogues 2–7.
We first set out to screen the set of developed probes 2–7 for their ability to be salvaged and incorporated by \textit{C. jejuni} as legionaminic acid into its glycans. \textit{C. jejuni} 11 168 was cultured for 4 h in the presence of 1 mM of probes 2–7 and afterwards any incorporated Leg probe was labeled through a strain-promoted azide–alkyne cycloaddition (SPAAC) reaction with DBCO (dibenzocyclooctyne)-PEG4-biotin. The resulting biotinylated glycoconjugates in whole cell lysates were detected on Western blot using Streptavidin-HRP. The only biotinylated proteins from the bacterial cells that were detected originated from experiments with the non-acetylated probes (2, 4, 6). Treatment with these probes only produced a single labeled band at approximately 70 kDa (Figure 2a), which is the approximate size of \textit{C. jejuni} flagellins. To confirm that these streptavidin-reactive bands indeed belonged to flagellin, the whole-cell lysates were treated with an anti-flagellin antibody and the hereby labeled bands all proved to have an identical migration patterns to the streptavidin-reactive bands (Figure 2a). Further inspection indicated that probe 6, containing two azido groups, shows the best labeling result compared to probe 2 or 4 (relative band intensity for 2 and 4 was, respectively 36% and 30% lower compared to 6), which only contain a single azido group at C2 or C4 position. With the acetylated versions of these probes (3, 5, 7) we wanted to test whether labeling could also be achieved by entry into the bacteria by passive transport, and intracellular conversion into probes 2, 4 and 6 by non-specific esterase esterases. No labeling was however observed with these three probes, suggesting that there is insufficient esterase activity inside \textit{C. jejuni} to remove the acetyl group. 

Next, a concentration-dependent labeling experiment was performed with probe 6, ranging from 0 to 1.0 mM (Figure 2b). The use of probe 6 as low as 16 μM still resulted in detectable labeling of the flagellar proteins and there was a positive correlation between the signal intensity and the probe concentration. The optimal incorporation of probe 6 was however observed when bacteria were grown with 1 mM of 6 for 4 h at 42°C. To verify whether probe 6 was being incorporated through the native Leg biosynthetic pathway, we conducted a competition experiment with native N-acetylated Leg precursor 1. First, bacteria cells were cultured with 1 and Western blot analysis indicated as expected that the natural precursor 1 did not result in detectable labeling of the flagellin (Figure 2c). Then a competition assay between 1 and 6 was conducted by culturing the bacteria with both substrates simultaneously in different ratios (Figure 2d). The detectable signal from incorporated azido-labeled Leg decreased when 1 was present in twofold excess. This result indicates that probe 6 is competing with native precursor 1 and thus plausibly being incorporated through the same metabolic pathway. Interestingly, the signal originating from incorporation of 6 could still be detected after adding fivefold excess of 1, which indicates that 6 might even be a better substrate for one or several of the enzymes involved. To further confirm that probe 6 is converted into the nucleotide–sugar by the bacterial biosynthetic machinery, we sought to detect the existence of cellular CMP-LegdiNAz. Bacterial cells treated with 1 mM probe 6 for 4 h were lysed, extracted and analyzed by UPLC–MS. For CMP-LegdiNAz, \texttt{[M–H]} is calculated as \textit{m/z} 720.1744, and it was indeed detected as \textit{m/z} 720.1728 (2.2 ppm) in the sample treated with 6 while such signal was not detected in the sample untreated with 6, suggesting that the unnatural nucleotide sugar was indeed produced (Figure 3, S2).

Encouraged by these results, we applied the developed labeling strategy with probe 6 on two other \textit{C. jejuni} strains, 129108 (abbreviated as 108) and 81116. Genomic analysis shows that \textit{C. jejuni} 108 has the genes that encode for the enzymes responsible for the Leg biosynthetic pathway, while \textit{C. jejuni} 81116 lacks this pathway. Both strains were grown in HI media supplemented with probe 6 for 7 h and as expected Western blot analysis revealed that the flagella of \textit{C. jejuni} 108 could indeed be labeled, while those of strain 81116 were not labeled. \textit{C. jejuni} 81116 ΔflaAB, a non-flagellated mutant, was used as an additional negative control that as expected did not show any labeling with 6 (Figure S1). To gain insight into the degree of labeling, a band shift assay was performed with DBCO-PEG5K on \textit{C. jejuni} 11 168 and 108 treated with 6. This showed at least about 25% of FlaA/B subunits were each labeled with a multiple copies of LegdiNAz (Figure S7). Finally, an autoagglutination analysis of both probe 6 treated and untreated \textit{C. jejuni} 108 samples showed no difference in their rate of autoagglutination that tentatively indicates the azido modifications do not impact the functional role of Leg on the bacteria (Figure S5).

We next aimed to visualize incorporated azido-labeled Leg on the flagella of intact bacterial cells. To achieve this, \textit{C. jejuni} 11 168 was grown in the presence of probe 6, and the incorporation of the probe into flagella was detected through a SPAAC reaction with DBCO-PEG4-biotin. Any biotin labeling on the flagella was then visualized with Streptavidin-
Unclear. A previous study by us proved that assembly and motility, but their exact functions remain unclear. The hydroxyl of Ser/Thr residues in the flagellin of C. jejuni has not been annotated yet so we next set out to use our optimal Leg MOE probe (6) to identify the involved bacterial glycosylation gene. Potential candidate genes encoding the glycosyltransferase that uses CMP-Leg to glycosylate the bacterial glycosyltransferase that was assigned as the Leg-glycosylated flagellin. This result suggested Maf4 could be a putative legionaminic acid glycosyltransferase. We next used UPLC–MS again to determine the biosynthesis of the azido-labeled Legionaminic acid glycosylation on the flagellin by the Leg glycosyltransferase, due to the knockout of the maf4 gene.

To further substantiate the role of Maf4 as a glycosyltransferase of microbial sialic acids, we used in silico analysis to build a model of the Maf4 protein (Figure 6) that was compared with the recently published crystal structure of the Maf enzyme of M. magneticum AMB-1, a putative Pse glycosyltransferase. The hereby generated Maf4 model shares high similarity with Maf in the central α/β domain of the bacterial glycosyltransferase that was assigned as the "Communications"
substrate-binding and catalysis region (Figure 6). A sequence alignment of this central α/β domain amino acid stretch for both Maf4 and Maf, together with other selected homologous bacterial proteins (Figure S6) that have been implicated with microbial sialic acid metabolism, showed many fully and highly conserved residues among which previously identified potential catalytic sites in Maf. Taken together, these results provide compelling evidence that Maf4 is a putative glycosyltransferase responsible for the transfer of Leg onto the flagellin in C. jejuni.

In summary, we have designed and synthesized probes based on the Leg hexose precursor (1) with azide reporters that were successfully used to study flagella glycosylation in C. jejuni strains using a MOE approach. This study shows that probes (2, 4, 6) based on 1 with non-natural modifications on either N2 or N4 could be metabolically incorporated into bacterial flagella in specific strains, but not their O-acetylated versions (3, 5, 7). With the azide reporters, the bacterial flagella could be selectively visualized by attaching a biotin and a fluorophore, respectively, thus providing the opportunity to study the location and abundance of Leg glycosylation. Moreover, the identity of Maf4, a putative Leg glycosyltransferase, was further substantiated using the developed probes and in silico modeling. We are currently further developing this approach as an efficient method for labeling C. jejuni flagella and to investigate the functional role of legionaminic acid in the dynamics of flagellin glycosylation and the filament assembly process in C. jejuni, also in relation to pseudaminic acid. An increased understanding of the role these microbial sialic acids have in bacterial motility and invasiveness of C. jejuni will aid in our ability to deal with this common human pathogen.

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Conflict of Interest

The authors declare no conflict of interest.

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