Identification of a Sialate O-Acetyltransferase from Campylobacter jejuni

DEMONSTRATION OF DIRECT TRANSFER TO THE C-9 POSITION OF TERMINAL α-2,8-LINKED SIALIC ACID

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We have identified a sialate O-acetyltransferase in the lipo-oligosaccharide biosynthesis locus of Campylobacter jejuni. Strains possessing this locus are known to produce sialylated outer core structures that mimic host gangliosides, and have been implicated in triggering the onset of Guillain-Barré syndrome. The acetyltransferase, which was cloned and expressed as a fusion construct in Escherichia coli, is soluble and homologous with members of the NodL-LacA-CysE family of O-acetyltransferases. This enzyme catalyzes the transfer of O-acetyl groups onto oligosaccharide-bound sialic acid, with a high specificity for terminal α2,8-linked residues. The modification is directed to C-9 and not C-7 as is believed to occur more commonly in other organisms. Despite their widespread prevalence and importance in both eukaryotes and prokaryotes, this is the first report to describe the characterization of a purified sialate O-acetyltransferase.

As the terminal residue of membrane-associated glycoconjugates in vertebrates and higher invertebrates, sialic acids are important parts of the cellular apparatus devoted to the detection and integration of environmental stimuli. Several pathogenic microorganisms are known to incorporate sialic acids into their surface structures, thereby mimicking an abundant molecular component displayed by host cells and providing a mechanism to evade immune response (1).

O-Acetylation of sialic acids has been implicated in a growing number of physiological and pathological processes. In developing animals, ganglioside O-acetylation is associated with tissue growth and differentiation (4, 5). This process is of clinical importance because different degrees of modification have been observed in human cell lines that have undergone malignant transformation (6, 7). Therefore O-acetylated gangliosides could serve as targets for directed cancer therapies. O-Acetylation has contrasting effects on the process of viral attachment to membrane-bound sialoglycans; this is an obligatory modification for the association of some enteric and respiratory viruses but inhibits binding by others (8, 9). As a final example, the sialylated polysaccharide capsules of group B Streptococcus (10), Escherichia coli K1 (11, 12), and Neisseria meningitidis serogroups C, W-135, and Y (13) have been shown to be O-acetylated in some cases, which results in altered immunogenic properties.

As a result of the biological importance of sialic acid O-acetylation in both eukaryotes and prokaryotes, there has been considerable effort directed toward the identification and characterization of SOATs. Recently, the genes encoding putative SOATs involved in capsule O-acetylation have been identified in E. coli K1 (11) and N. meningitidis (14). As yet, there have been no reports detailing their expression and characterization in vitro. Progress on the eukaryotic front has been slower; there have been no SOATs identified in any organism. A number of obstacles have hindered the ability to clone and purify these enzymes from a eukaryotic source. Two important contributing factors are that they are membrane-associated, and their activity may be dependent on the presence of other proteins (for a more detailed discussion, see Ref. 15).

The mucosal pathogen Campylobacter jejuni is a leading cause of diarrheal disease and of food-borne gastroenteritis worldwide (16). This organism exhibits a highly variable array of cell-surface glycans that are associated with virulence (17, 18). In several strains of C. jejuni, the glycan component of the lipo-oligosaccharide (LOS) is sialylated and structurally similar to gangliosides (19–23). There is a convincing body of evidence to suggest that the LOS from these strains may be responsible for generating antibodies that are cross-reactive with host epitopes found in abundance in nervous tissue, triggering an auto-immune response, which gives rise to Guillain-Barré syndrome (24, 25). In this report, we describe the identification of a SOAT gene (orf1) in the LOS biosynthesis locus of C. jejuni, which was cloned into E. coli and stably expressed as a fusion construct. Our identification of this gene is the first evidence, to our knowledge, to indicate that LOS-bound NeuAc may be O-acetylated in C. jejuni or any other bacterial species. The expression of the enzyme in a heterologous source, and subsequent
purification, enabled precise biochemical characterization, which as noted above, has proven intractable in studies of SOATs found in other organisms to date.

MATERIALS AND METHODS

Cloning of the SOAT from C. jejuni—The orf11 gene was amplified from the various C. jejuni strains using the Pwo polymerase and the following primers: CJ-175 (5′-CTTAAATGC-3′) and CJ-176 (5′-CCTAGGACTTTAAATAGATTAAAATTTTTTGGATTTAG-3′). The PCR products were digested with Ndel and SalI and cloned in pCWori(-lacZ) containing the sequence encoding the E. coli maltose-binding protein (without the leader peptide) and the thrombin cleavage site.

Purification of the Recombinant SOAT—E. coli AD202 containing construct CJL-130 (Orf11 from C. jejuni ATCC 43446 in pCWori+) was grown in 2 YT medium containing 150 μg/ml ampicillin and 2 g/liter glucose. The culture was incubated at 37 °C until A600 was 0.35, induced with 1 mM isopropyl 1-thio-β-d-galactopyranoside, and then incubated overnight at 20 °C. The cells were broken using an Avestin C5 Emulsiflex cell disruptor (Avestin, Ottawa) and the MalE-Orf11 fusion was purified by affinity chromatography on amylose resin following the manufacturer’s instructions (New England Biolabs, Beverly, MA).

In Vitro O-Acetyltransferase Reaction—6-(5-Fluorescein-carboxyamido)-hexanoic acid succimidyl ester (FCHASE)-labeled oligosaccharides were prepared as described previously (26). The acetyltransferase activity was assayed using 0.5 mM NeuAc, 2.8-NeuAc, 2.3-Galβ-1,4-Glc-FCHASE, 1 mM acetyl-CoA, 50 mM Mes (pH 6.5), 10 mM MgCl2, and 1 mM dithiothreitol. The enzymatic reactions were performed at 37 °C for 5 min and were stopped by the addition of acetonitrile (25% final). The samples were analyzed by capillary electrophoresis (CE) as described previously (26). One unit of activity was defined as the amount of enzyme that produces 1 μmol of 9-O-acetylated product in 1 min. Quantitation of the reactions was performed by integration of the CE trace peaks using the MDQ 32 Karat software (Beckman, CA). TLC analysis was performed with aluminum backed silica plates that were developed in ethyl acetate:methanol:water:acetic acid (4:2:1:0.1).

Determination of the O-Acetylation Site by NMR Spectroscopy—Lyoophilized O-acetyl-NeuAc, 2.8-NeuAc, 2.3-Galβ-1,4-Glc-FCHASE was dissolved in D2O at a concentration of ~3 mM. Standard 1H,13C HSQC and HMBC spectra were acquired to assign the position of the O-acetyl group to C-9. The reported 1H chemical shifts are referenced with respect to the methyl group of acetone appearing at 2.225 ppm.

To address whether there was postenzymatic migration of the O-acetyl group on the glycerol side chain, the acetylation of NeuAc, 2.8-NeuAc, 2.3-Galβ-1,4-Glc-FCHASE by MalE-Orf11 was monitored in real time by 1H NMR. This was accomplished by recording successive1H one-dimensional spectra of the reaction at 2-min intervals over a period of 100 min at 37 °C. The reaction mixture contained 2.5 mM of the substrate, 5 mM acetyl-CoA, and 10 mM MgCl2 in deuterated phosphate buffer (pH = 6.5). The reaction was initiated upon addition of an aliquot of enzyme (8 milliunits). Roughly 5 min elapsed between the addition of the enzyme, and the acquisition of the first time point (defined as t = 0 min). The reaction progress was followed and quantified by integrating the O-acetyl and H9'-resonances, which both gave rise to well resolved peaks. The uncertainty associated with integration measurements is ~10%. All NMR data were acquired on Varian instruments operating at 500 and 600 MHz, and processed using the software Topspin (Bruker Biospin).

Whole-cell Neuraminidase Treatment—C. jejuni cells, harvested from Mueller-Hinton plates, were rinsed twice in phosphate buffer (50 mM, pH 5.5) and then resuspended in a buffered solution of neuraminidase (100 milliunits/ml, from Clastidium perfringens, Sigma) and incubated at 37 °C for 3 h. The cells were spun down (9000 × g, 2 min), and the supernatant was lyophilized. The presence of NeuAc and 9-O-acetyl-NeuAc was verified by NMR and capillary electrophoresis-mass spectrometry (CE-MS). For NMR analysis, the cell-free suspension was redissolved in D2O, and standard 1H one-dimensional and carbon-correlated spectra were obtained. The observed chemical shifts correspond with previously published values for these molecules in solution (27). For mass spectrometric analysis, CE was performed using a Prince CE system (Prince Technologies, The Netherlands). The CE system was coupled to an API 3000 mass spectrometer (Applied Biosystems/Sciex, Concord, Canada) via a microspray interface. A sheath solution (isopropanol-methanol, 2:1) was delivered at a flow rate of 1 μl/min. Separations were obtained on ~90-cm length of bare fused-silica capillary with a background electrolyte of 30 mM morpholine in deionized water (pH 9.0). A voltage of 30 kV was typically applied at the injection, and ~5.0 kV was applied at the capillary outlet as electrospray voltage (negative detection mode).

RESULTS AND DISCUSSION

Orf11 of C. jejuni ATCC 43446 Has Sialate O-Acetyltransferase Activity—Based on the gene complement in the LOS biosynthesis locus, C. jejuni strains can be grouped into eight classes (28). Class A, B, and C strains are capable of synthesizing sialylated oligosaccharide cores that mimic ganglioside structures (17) and are thought to be those responsible for triggering the onset of Guillain-Barré syndrome in humans (24, 29). Strains belonging to classes A and B also possess a gene, originally named orf11, that encodes a protein showing homology with various acetyltransferases of the NodL-LacA-CysE family, although it was not possible to determine its acceptor based on the activity of the homologues. This gene is located immediately downstream of the four genes (cst-II, neuB, neuC, and neuA) involved in sialylation of the LOS outer core, which suggested that Orf11 could be a SOAT. In the biosynthetic pathway of sialoglycans, free forms of sialic acids are CMP-activated prior to their transfer, by sialyltransferases, onto nascent oligosaccharides. Sialic acids could conceivably be targeted for O-acetylation at any of the three stages in the pathway, i.e. either as free NeuAc, after activation as CMP-NeuAc, or after transfer to a glycan. In almost all systems studied to date, the substrates for these enzymes are known to be NeuAc-linked oligosaccharides. However, in group B Streptococcus, sialic acid destined for the capsular polysaccharide is O-acetylated prior to activation (10), and in the Golgi apparatus of mammalian cells there is evidence that CMP-NeuAc may be the substrate for certain SOATs (30).

Orf11 from C. jejuni ATCC 43446 was expressed as a MalE fusion construct (MalE-Orf11) in E. coli and was purified by affinity chromatography. There was no evidence of O-acetylated product when purified MalE-Orf11 was incubated with either NeuAc or CMP-NeuAc as acceptor in the presence of acetyl-CoA (data not shown). We have previously used FCHASE glycosides as synthetic acceptors to confirm the biological role of several glycosyltransferases cloned from bacterial sources (31, 32). FCHASE derivatives were employed to establish that Orf11 from C. jejuni ATCC 43446 catalyzes the transfer of an O-acetyl group to oligosaccharide-bound NeuAc and that it has a strong preference for terminal α2,8-linked residues. Following incubation in the presence of acetyl-CoA, NeuAc, NeuAc, 2.8-NeuAc, 2.3-Galβ-1,4-Glc-FCHASE and NeuAc, 2.8-NeuAc, 2.3-Galβ-1,4-Glc-FCHASE glycosides possessing terminal α2,8-linked NeuAc exhibited more rapid migration rates on a TLC plate as a result of O-acetylation
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(Fig. 1). The level of conversion to a higher mobility form was barely detectable when NeuAc-2,3-Galβ-1,4-Glc-FCHASE, possessing a terminal α2,3-linked NeuAc, was used as the acceptor (Fig. 1). The specific activity of the purified MalE-Orf1143446 was 0.4 milliunits/mg with the monosialylated acceptor, 77.8 milliunits/mg with the disialylated acceptor and 17.2 milliunits/mg with the triisialylated acceptor. A recent study with solubilized Golgi membranes from guinea pig liver tissue possessing endogenous SOAT activity reported a similar ability to O-acetate several compounds, at significantly diminished levels, that were not believed to be the natural substrates of the enzyme (33).

Sequence Variation of Orf11 in Various C. jejuni Strains—We previously sequenced the copy of the entire LOS biosynthesis locus from

![FIGURE 1. The SOAT from C. jejuni ATCC 43446 (MalE-Orf114346) O-acetylates terminally α2,8-linked NeuAc. The acceptor specificity of the enzyme was assayed by monitoring the migration of FCHASE glycosides on a TLC plate following incubation in the absence (lanes 1, 3, and 5) and presence (lanes 2, 4, and 6) of acetyl-CoA. The acceptor possessing terminal α2,3-linked NeuAc, NeuAc-2,3-Galβ-1,4-Glc-FCHASE (lanes 1 and 2), shows negligible levels of O-acetylation (barely visible in lane 2 as a faster migrating band). Enhanced migration rates resulting from O-acetylation are observed for the two acceptors possessing terminal α2,8-linked NeuAc, NeuAc-2,8-NeuAc-2,3-Galβ-1,4-Glc-FCHASE (lanes 3 and 4) and (NeuAc-2,8)-, NeuAc-2,3-Galβ-1,4-Glc-FCHASE (lanes 5 and 6). The arrow indicates the origin of migration. The image is shown with reversed gray scale levels.](image1)

FIGURE 2. Sequence alignment of Orf11 from various C. jejuni strains using ClustalW. The alignments are based on the orf11 translation products from various C. jejuni strains with either a class A or B LOS biosynthesis locus. Only variable residues are shown in addition to the consensus sequence. The asterisks (*) indicate conserved residues, the colors (.) indicate strongly similar residues, and the periods (.) indicate weakly similar residues. The C. jejuni strain numbers are indicated to the left of the sequences. The GenBank accession numbers are AF215659 for ATCC 43432, AF400048 for ATCC 43438, AF167344 for ATCC 43446, AF041529 for ATCC 43449, AF041528 for ATCC 43456, and AF130984 for OH4384.

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strains encode truncated forms of Orf11 as a result of single base deletions or insertions, as well as G to A substitutions (see supplemental Table 1). The presence of many inactive and phase-variable orf11 alleles in clinical strains suggests that C. jejuni is undergoing selective pressure to modulate the activity of this gene.

In a broader context, there is a high level of sequence divergence among proteins known to possess O-acetyltransferase activity. Some, however, exhibit sufficient homology to enable grouping into one of two families. The first, known as the NodL-LacA-CysE family, comprises water-soluble cytoplasmic proteins (34), whereas the second comprises a group of integral membrane proteins (35). The enzyme we have identified in C. jejuni shows homology with the NodL-LacA-CysE family. Interestingly, a recently published sequence for a SOAT, which is involved in capsule production in N. meningitidis, also shows homology with the water-soluble family of O-acetyltransferases (14).

The SOAT from C. jejuni HS:19 O-Acetylates Directly at the 9-Position—Sialic acids have been found to be modified with O-acetyl groups at C-4 and at any one of the three hydroxyl groups on the exo...
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FIGURE 4. NeuAc and 9-O-acetyl-NeuAc are released from C. jejuni cells upon treatment with neuraminidase. C. jejuni strains that have class A and B LOS loci are often sialylated. When these cells are incubated in the presence of neuraminidase, sialic acid is released. A, $^1$H NMR spectra of the cell-free suspension of sialidase-treated GB11 cells reveals the presence of NeuAc, with its characteristic N-acetyl peak, $\delta$, in cells from strain GB17, 9-O-acetyl-NeuAc is also liberated following sialidase treatment, as demonstrated by the presence of the modified species. The ratio of 9-O-acetyl-NeuAc to NeuAc, as determined by integration of the well resolved H9- and H11015 resonances from the start of the reaction (Fig. 3). Both the O-acetyl and H9' resonances of the 9-O-acetylated species were well resolved thereby allowing us to unambiguously monitor its formation. Our data clearly establish that MalE-Orf1143446 transfers the O-acetyl group directly to C-9. This is demonstrated by the growth in signal at 4.38 ppm in the earliest time points following the addition of the enzyme (Fig. 3B). This resonance appears as a result of esterification at the 9-position and establishes the creation of the 9-O-acetylated species. In addition, the signal intensity of the nascent O-acetyl group grows at the same rate as H9' (Fig. 3C). This indicates that the formation of the O-acetyl ester bond, and the downfield shift of the 9-protons occurs in parallel. Our results are incompatible with the possibility that O-acetylation is directed originally to C-7, followed by migration, a process that under similar conditions was determined to have a $t_{1/2}$ of $\approx 10$ h (37).

It will be of interest to determine how rare this SOAT is in its ability to O-acetylate the 9-position directly. Characterization of SOATs from other organisms will be required to clarify when and why O-acetylation may be directed to C-7 versus C-9. It seems unlikely that this is a random process, because 7-O-acetyl species exhibit distinct binding and chemical characteristics than the corresponding 9-O-acetylated forms (42, 43). It is possible that in cases where C-7 is the target, this isomer may be required for proper processing and transport of the glycoconjugate or for a time-limited physiological response at the cell surface.

Detection of LOS-bound O-Acetylated NeuAc in C. jejuni—NeuAc has been found to be incorporated into the LOS of Haemophilus influenzae (44), Neisseria spp (45), and C. jejuni (21). However, there have been no reports of O-acetylated NeuAc in the LOS of any of these organisms. Modified NeuAc, if indeed present, would likely have been missed because of the unique challenges associated with the characterization of the glycan component of the LOS. Conventional spectral techniques designed to accomplish this task invariably require the prior removal of the fatty acyl components. Unfortunately, chemical treatments used to strip the fatty acyl chains from the oligosaccharide have the undesired consequence of cleaving NeuAc residues and/or saponification of O-acetyl modifications.

Once the orf11 gene was identified as a SOAT, we sought a method to detect the presence of O-acetyl-NeuAc at the surface of C. jejuni cells. The
presence of this modified residue was eventually found by treating whole cells with neuraminidase and analyzing the cell-free suspensions by both $^1$H NMR and CE-MS. Several strains belonging to type A and B LOS classes were screened (supplemental Table 1). In addition to orf11, class A and B strains possess several genes that are involved in the sialylation of the LOS (17). Neuraminidase treatment of 10 strains resulted in the release of NeuAc (Fig. 4A), whereas in 4 others, we detected both NeuAc and 9-O-acetyl-NeuAc (Fig. 4B). A significant proportion of the released sialic acid was the modified species, as demonstrated by the 1:3 ratio of 9-O-acetyl-NeuAc to NeuAc observed in the $^1$H NMR spectra of the GB17 cell-free suspension (Fig. 4B).

Several C. jejuni strains that possess an active orf11 allele do not display 9-O-acetyl-NeuAc, as indicated by the neuraminidase assay. Two principal explanations can account for this. First, the accompanying sialyltransferase gene in the LOS locus, cst-II, will dictate whether a suitable substrate is available to accept the O-acetyl modification. We have previously demonstrated that this sialyltransferase exists as either a monofunctional version (Thr-51) that can transfer $\alpha$-2,3-linked NeuAc to galactose, or as a bifunctional version (Asn-51) that can transfer both $\alpha$-2,3-NeuAc to galactose and $\alpha$-2,8-NeuAc to NeuAc (17). 9-O-Acetyl-NeuAc was only detected in strains that possess a bifunctional Cst-II, i.e. those capable of synthesizing LOS with terminal $\alpha$2,8-linked sialic acid. This is in full agreement with our biochemical characterization of this SOAT, which demonstrated that the enzyme has a high specificity for $\alpha$2,8-linked residues (Fig. 1). Second, SOAT activity in C. jejuni appears to be modulated by the presence of homopolymeric nucleotide tracts in the strains that have the combination of an active orf11 allele and a bifunctional Cst-II (supplemental Table 1). Polymeric G-tracts have been reported in earlier studies of C. jejuni genes involved in LOS biosynthesis (17), and their presence can lead to premature translation termination, resulting in truncated gene products. Many of the orf11 genes that were sequenced have heterogeneous G-tracts that can abolish SOAT activity in strains where active orf11 alleles are present.

Our emerging understanding of LOS biosynthesis in C. jejuni, and other bacteria, indicates that the genes involved are found in a highly mutable genetic locus, and there are several mechanisms employed to vary the structure of the LOS outer core (17, 46, 47). It was noted earlier that among the 34 orf11 genes that were sequenced (8 sequences from previously sequenced LOS loci (Fig. 2) and 26 additional sequences in the supplemental Table 1), we found several instances of point mutations, nucleotide insertions and deletions, and polymeric G-tracts. These accumulated differences within the nucleotide sequence serve to modulate the expression of the SOAT. Coupled with the modulation of the expression of other genes within the LOS locus, such as cst-II, class A and B strains have the potential to synthesize a considerable variety of sialylated oligosaccharides, some of which will be O-acetylated.

Conclusions—In most organisms that possess sialylated glycoconjugates, O-acetylation has been found to be a frequent modification (2, 3). This is true in some pathogenic bacteria, where NeuAc residues residing on the capsule have been shown to exhibit varying levels of O-acetylation (10, 12, 13). We have now confirmed the existence of an enzyme in C. jejuni, which O-acetylates LOS-bound NeuAc. Future effort will be geared toward establishing whether this modification is a virulence factor.

In this paper, we have described the cloning, purification, and characterization of a soluble SOAT that has high specificity for terminal $\alpha$2,8-linked sialic acid. This enzyme may serve as a valuable tool for the development of therapeutic agents directed against malignant cell lines overexpressing O-acetylated gangliosides.
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