THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 17, pp. 11480–11486, April 28, 2006

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*[S]

Received for publication, November 14, 2005, and in revised form, January 17, 2006 Published, JBC Papers in Press, February 14, 2006, DOI 10.1074/jbc.M512183200

R. Scott Houliston[‡], Hubert P. Endtz[§], Nobuhiro Yuki[¶], Jianjun Li[‡], Harold C. Jarrell[‡], Michiaki Koga[¶], Alex van Belkum[§], Marie-France Karwaski[‡], Warren W. Wakarchuk[‡], and Michel Gilbert[‡1]

From the [‡]Institute for Biological Sciences, National Research Council Canada, Ottawa, Ontario K1A 0R6, Canada, the [§]Department of Medical Microbiology and Infectious Diseases, Erasmus MC, 3015 GD Rotterdam, The Netherlands and the [¶]Department of Neurology, Dokkyo University School of Medicine, Tochigi 321-0293, Japan

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 wide 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).

Sialic acids are a family of carboxylated monosaccharides that possess a common backbone structure of 9 carbon atoms. The structural diversity in the family arises as a result of various modifications to the biochemical precursor and most naturally abundant member, 5-*N*-acetylneuraminic acid (NeuAc)[2] (2, 3). One commonly observed modification is *O*-acetylation at one or more of the hydroxyl groups at positions 4, 7, 8 or 9. This process, which is catalyzed enzymatically by sialate *O*-acetyltransferases (SOATs), changes the binding and recognition characteristics of the underlying molecule and, as a result, diversifies the interaction potential for a given sialoglycan.

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 codependent 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 (*orf11*) 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

* This work was supported by Grant RGP 38/2003 from the Human Frontier Science Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

[1] To whom correspondence should be addressed. Tel.: 613-991-9956; Fax: 613-952-9092; E-mail: Michel.Gilbert@nrc-cnrc.gc.ca.

[2] The abbreviations used are: NeuAc, 5-*N*-acetylneuraminic acid; CE-MS, capillary electrophoresis-mass spectrometry; FCHASE, 6-(5-fluorescein-carboxamido)-hexanoic acid succimidyl ester; LOS, lipo-oligosaccharide; SOAT, sialate *O*-acetyltransferase; Mes, 4-morpholineethanesulfonic acid; HMBC, heteronuclear multiple bond coherence.

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′-CTTAGGAGGT*CATATG*GAAAAAATAAC-CTTAAAATGC-3′ 37 mer, NdeI site in italics) and CJ-176 (5′-CCTAG-*GTCGAC*TTAAAATAGATTAAAAATTTTTTTTGATTTTAG-3′ 44 mer, SalI site in italics). The PCR products were digested with NdeI 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 A_{600} = 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-carboxamido)-hexanoic acid succimidyl ester (FCHASE)-labeled oligosaccharides were prepared as described previously (26). The acetyltransferase activity was assayed using 0.5 mM NeuAcα-2,8-NeuAcα-2,3-Galβ-1,4-Glc-FCHASE, 1 mM acetyl-CoA, 50 mM Mes (pH 6.5), 10 mM $MgCl_2$, 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—Lyophilized *O*-acetyl-NeuAcα-2,8-NeuAcα-2,3-Galβ-1,4-Glc-FCHASE was dissolved in D_2O at a concentration of ~3 mM. Standard ^1H-^{13}C 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 NeuAcα-2,8-NeuAcα-2,3-Galβ-1,4-Glc-FCHASE by MalE-Orf11^{43446} from *C. jejuni* was monitored in real time by ^1H NMR. This was accomplished by recording successive ^1H 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 $MgCl_2$ 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 *Clostridium 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 D_2O, 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 are *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^{43446}) in *E. coli* and was purified by affinity chromatography. There was no evidence of *O*-acetylated product when purified MalE-Orf11^{43446} 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, NeuAcα-2,8-NeuAcα-2,3-Galβ-1,4-Glc-FCHASE and $(NeuAc\alpha\text{-}2,8)_2$-NeuAcα-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

(Fig. 1). The level of conversion to a higher mobility form was barely detectable when NeuAcα-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-Orf11^{43446} was 0.4 milliunits/mg with the monosialylated acceptor, 77.8 milliunits/mg with the disialylated acceptor and 17.2 milliunits/mg with the trisialylated acceptor. A recent study with solubilized Golgi membranes from guinea pig liver tissue possessing endogenous SOAT activity reported a similar ability to *O*-acetylate 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 deposited the sequence of the entire LOS biosynthesis locus from 8 class A and B strains of *C. jejuni* (see legend of Fig. 2 for accession numbers), which all contain a copy of Orf11. The *orf11* gene from ATCC 43460 encodes a truncated protein (63 amino acids) because of a frameshift mutation and is thus inactive. Because the translation products from *C. jejuni* OH4382 and OH4384 are identical, there are six distinct full-length Orf11 variants among the eight class A and B loci that we sequenced. The level of protein sequence identity is high, with 94% of the residues being conserved among the six variants (Fig. 2). To assess how specific amino acid differences affected *O*-acetyltransferase activity, we cloned and expressed the six Orf11 variants as MalE fusion constructs. We compared the activity of the six variants using NeuAcα-2,3-Galβ-1,4-Glc-FCHASE and NeuAcα-2,8-NeuAcα-2,3-Galβ-1,4-Glc-FCHASE as acceptors, and we analyzed the products by TLC (data not shown). As described earlier, MalE-Orf11^{43446} had high activity on terminal α-2,8-linked NeuAc and significantly lower activity on α-2,3-linked NeuAc. MalE-Orf11^{43438} and MalE-Orf11^{43449} also had high activity on terminal α-2,8-linked NeuAc and significantly lower activity on α-2,3-linked NeuAc. MalE-Orf11^{43432} had low activity on α-2,8-linked NeuAc and no activity on α-2,3-linked NeuAc. MalE-Orf11^{OH4382} and MalE-Orf11^{43456} are both inactive. Single mutations are responsible for the inactivation of these variants because Orf11^{OH4382} has only one amino acid difference (D75G) with Orf11^{43446}, as does MalE-Orf11^{43456} with Orf11^{43449} (E91G) (Fig. 2).

We sequenced the *orf11* gene from 26 additional clinical strains (see supplemental Table 1) to study the sequence variation in a set larger than the eight strains discussed above. Eight of the clinical strains had *orf11* genes that encoded full-length versions, whereas 13 contained the presence of homopolymeric G-tracts, which can lead to the expression of a mixture of truncated and full-length gene products in a culture. For instance the *orf11* gene from MF6 has a mixture of 10 and 11-G homopolymeric tracts, which results in the expression of either a full-length protein (276 amino acids) or a truncated one (64 amino acids), respectively. The remaining five *C. jejuni*

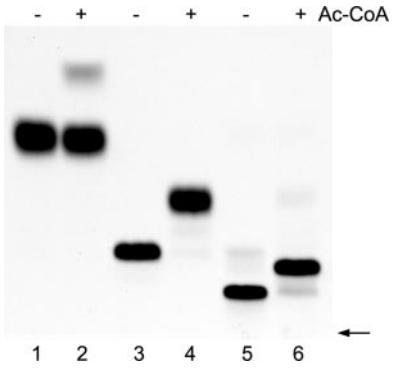

FIGURE 1. **The SOAT from *C. jejuni* ATCC 43446 (MalE-Orf11^{43446}) *O*-acetylates terminally α2,8-linked NeuAc.** The acceptor specificity of the enzyme was assayed by monitoring the migration of FCHASE glycosides on a TLC plate following incubation in the absence (*lanes 1*, *3*, and *5*) and presence (*lanes 2*, *4*, and *6*) of acetyl-CoA. The acceptor possessing terminal α2,3-linked NeuAc, NeuAcα-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, NeuAcα-2,8-NeuAcα-2,3-Galβ-1,4-Glc-FCHASE (*lanes 3* and *4*) and (NeuAcα-2,8)$_2$-NeuAcα-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.

```
                    10        20        30        40        50        60        70        80        90       100
                     |         |         |         |         |         |         |         |         |         |
43449     ..........................................I..K..Q...AA.L.....C...........G............A..G.....A...
43456     ..........................................I..K..Q...AA.L.....C...........G............A..E.....A...
43438     ..........................................I..E..R...AG.-.....C...........G............A..G.....A...
43432     ..........................................I..E..Q...VA.L.....Y...........G............A..G.....A...
43446     ..........................................F..E..Q...VA.L.....C...........G............T..G.....S...
OH4382/4384 ..........................................F..E..Q...VA.L.....C...........D............T..G.....S...
          ******************************************:**:**:***..* ***** ***********.************:** *****:***
Prim.cons. MEKITLKCNKNILNLLKQYNIYTKTYIENPRRFSRLKTKDFITIPLENNQLESAAGLGIEEYCAFKFSNILHEMGSFSFSGSFLPHYAKVGRYCSIADGV

                   110       120       130       140       150       160       170       180       190       200
                     |         |         |         |         |         |         |         |         |         |
43449     ........I.................D.....................................................................
43456     ........I.................D.....................................................................
43438     ........I.................D.....................................................................
43432     ........M.................N.....................................................................
43446     ........M.................D.....................................................................
OH4382/4384 ........M.................D.....................................................................
          ********:******************:*****************************************************************
Prim.cons. SMFNFQHPMDRISTASFTYETNHSFINDACQNHINKTFPIVNHNPSSSITHLIIQDDVWIGKDVLLKQGITLGTGCVIGQRAVVTKDVPPYAIVAGIPAK

                   210       220       230       240       250       260       270
                     |         |         |         |         |         |         |
43449     ......DE............R.....................................................  277
43456     ......DE............R.....................................................  277
43438     ......NE............R.....................................................  276
43432     ......NK............K.....................................................  277
43446     ......DE............K.....................................................  277
OH4382/4384 ......DE............K.....................................................  277
          ******::************:*************************************************************
Prim.cons. IIKYRFDEKTIERLLKIQWWKYHFADFYDIDLNLKINQYLDLLEEKIIKKSISYYNPNKLYFRDILELKSKKIFNLF
```

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 *colons* (:) 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, AF401529 for ATCC 43449, AF401528 for ATCC 43456, and AF130984 for OH4384.

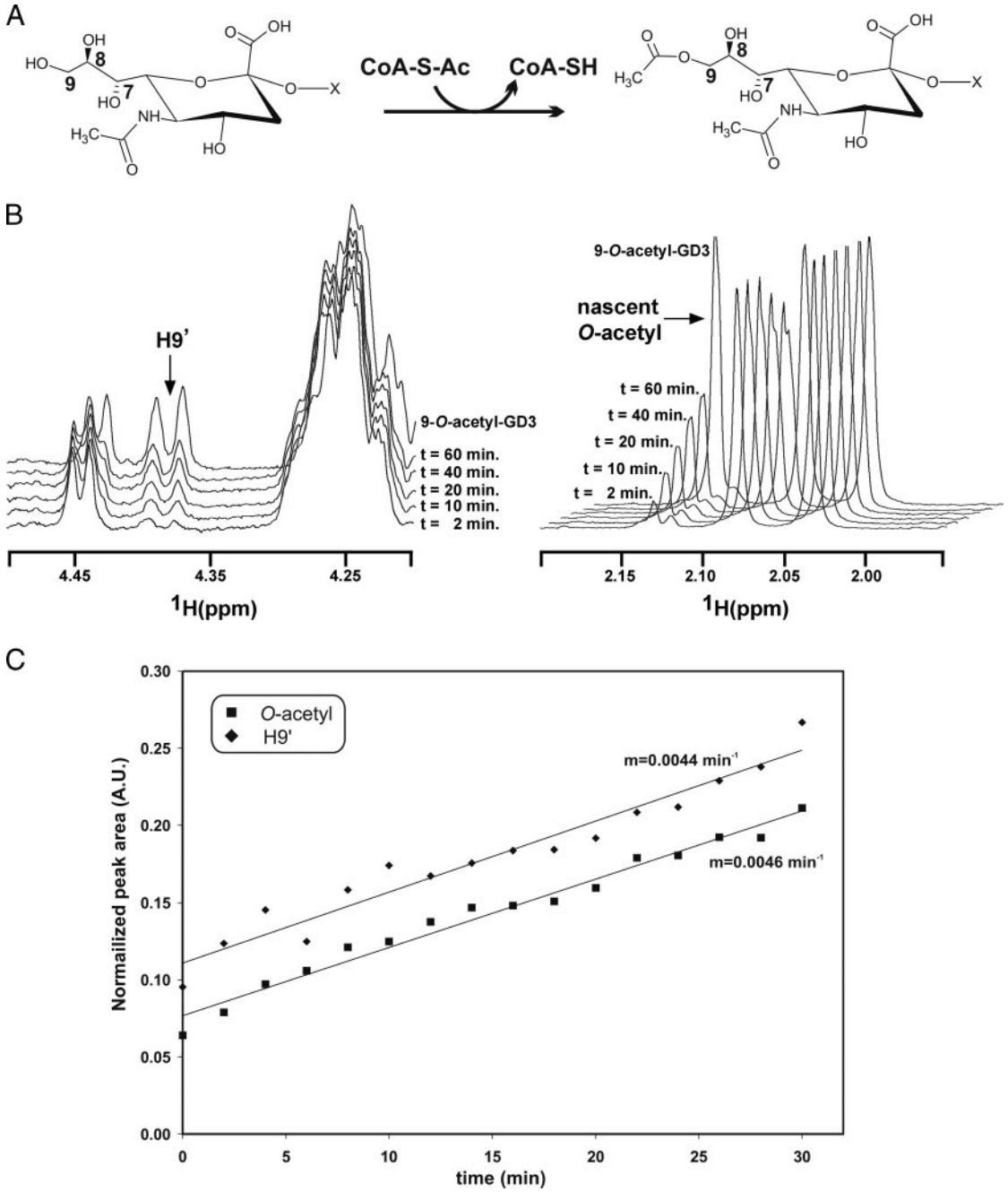

FIGURE 3. **MalE-Orf11^{43446} transfers the *O*-acetyl group directly to C-9 of NeuAc.** *A*, schematic representation of *O*-acetylation of NeuAc. *O*-Acetylation of the terminal NeuAc residue at the 9-position results in the downfield shift of the non-equivalent protons from 3.64 (H9) and 3.88 (H9′) to 4.21 and 4.38 ppm, respectively. *B*, stacked ^{1}H NMR spectra of the acetylation of NeuAcα-2,8-NeuAcα-2,3-Galβ-1,4-Glc-FCHASE by the enzyme shows the buildup in signal of the H9′ and *O*-acetyl resonances at various time points following the initiation of the reaction. The signal from H9′ at 4.38 ppm is evident in the earliest spectra, indicating rapid formation of the 9-*O*-acetylated species. *O*-Acetylation at the 7-position would result in the downfield shift of the H7 resonance from 3.58 to ~5.05 ppm (27). There is no evidence of a downfield shift of the H7 resonance at any point during the in-tube reaction. *C*, the signal intensity for the H9′ (*diamonds*) and the *O*-acetyl (*squares*) resonances grow at almost identical rates, demonstrating direct transfer of the *O*-acetyl group to C-9. The growth rates (slopes or *m* values on the graph) were obtained by fitting the data to a straight line using non-linear least squares regression, with error estimates of 5 and 8% for the H9′ and *O*-acetyl resonances, respectively.

strains encode truncated forms of Orf11 as a result of single base deletions or insertions, as well as a 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-

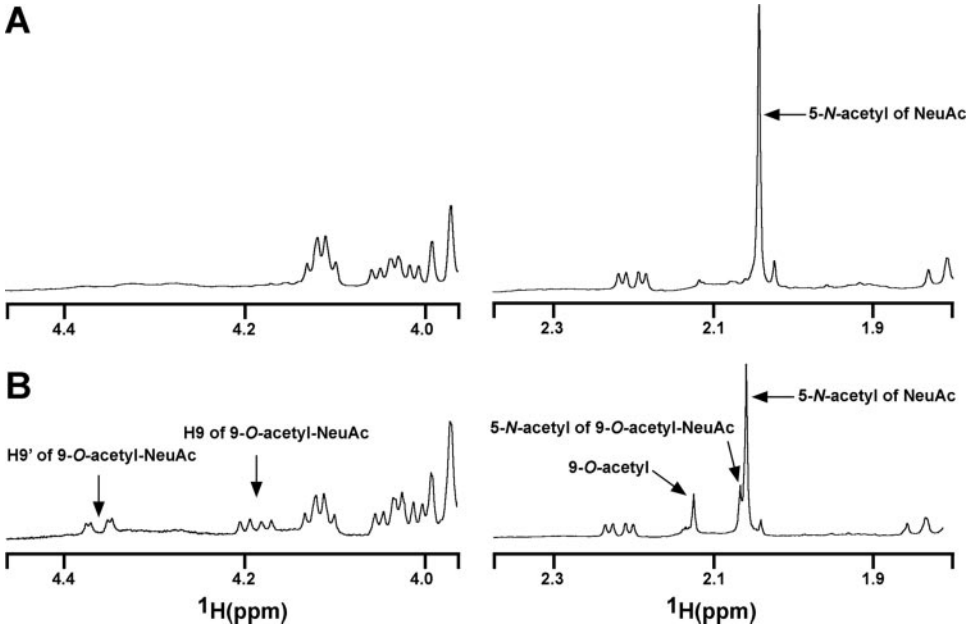

FIGURE 4. **NeuAc and 9-*O*-acetyl-NeuAc are released from *C. jejuni* cells upon treatment with neuraminidase.** *C. jejuni* strains that have class A and B LOS loci are often sialylated. When these cells are incubated in the presence of neuraminidase, sialic acid is released. *A*, ^{1}H NMR spectra of the cell-free suspension of sialidase-treated GB11 cells reveals the presence of NeuAc, with its characteristic *N*-acetyl peak. *B*, in cells from strain GB17, 9-*O*-acetyl-NeuAc is also liberated following sialidase treatment, as demonstrated by the presence of the well resolved H9- and *O*-acetyl peaks from the modified species. The ratio of 9-*O*-acetyl-NeuAc to NeuAc, as determined by integration of the *N*- and *O*-acetyl peaks, is ~1:3. 9-*O*-Acetyl-NeuAc was also detected in strains GB16, GB19, and GC068 (data not shown).

cyclic side chain (*i.e.* C-7, C-8, and C-9) (2, 3). One of the peculiarities associated with *O*-acetylation to the exocyclic side chain is that this group will migrate spontaneously from the 7- to the 9-position, via the 8-*O*-acetyl species (36). 8-*O*-Acetylated sialic acids are very unstable and exist only transiently, unless the hydroxyl group at C-9 has already been modified or is the linkage site. *O*-Acetyl migration from the 7- to the 9-position, which has been observed directly *in vitro* using free forms of modified NeuAc in solution, occurs on a relatively slow time scale under mild solution conditions ($t_{1/2} \approx 10$ h at pH 7, 37 °C) but is accelerated by raising the solution pH (37). There is evidence that some organisms may possess specific enzymes that facilitate *O*-acetyl migration (38). In almost all organisms where 9-*O*-acetylated sialoglycans have been detected, the *de novo* enzymatic product has been postulated to be the 7-*O*-acetylated species, which was converted to the 9-*O*-acetyl isomer following migration (10, 14, 30, 38–40). Varki and colleagues (41, 42) have demonstrated, however, that in the Golgi apparatus of mammalian systems, sialylated glycoconjugates are likely *O*-acetylated at both C-7 and C-9 directly, possibly by different SOATs. The precise acceptor position has been difficult to determine in these studies because of the inability to purify these enzymes.

Using the SOAT from *C. jejuni* ATCC 43446 (MalE-Orf11^{43446}), we synthesized preparative quantities of 9-*O*-acetyl-NeuAcα-2,8-NeuAcα-2,3-Galβ-1,4-Glc-FCHASE for analysis by NMR spectroscopy. We were able to assign the *O*-acetyl group to the C-9 position of the terminal NeuAc. This is demonstrated by the downfield shift of the non-equivalent 9-proton resonances on this residue (H9 = 4.21 and H9′ = 4.38 ppm) relative to the analogous pair in the non-acetylated FCHASE glycoside (H9 = 3.64 and H9′ = 3.88 ppm, from (32)). The esterification position was confirmed through the use of HMBC carbon-correlated proton spectra, where we observed a connectivity between the H9 protons and the carbonyl carbon of the acetyl group.

Our assignment of the *O*-acetyl group to the 9-position in the enzymatic product did not rule out the possibility that this isomer was formed postenzymatically, as a result of migration from C-7. To confirm the acceptor position on NeuAcα-2,8-NeuAcα-2,3-Galβ-1,4-Glc-FCHASE, *O*-acetylation was followed in real time, by acquiring ^{1}H spectra of the reaction mixture continuously at 2-min intervals 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-Orf11^{43446} 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. 3*B*). 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. 3*C*). 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 ~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 different binding and chemical characteristics than the corresponding 9-*O*-acetyl 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. 4*A*), whereas in 4 others, we detected both NeuAc and 9-*O*-acetyl-NeuAc (Fig. 4*B*). 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. 4*B*).

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 α-2,3-linked NeuAc to galactose, or as a bifunctional version (Asn-51) that can transfer both α-2,3-NeuAc to galactose and α-2,8-NeuAc to NeuAc (17). 9-*O*-Acetyl-NeuAc was only detected in strains that possess a bifunctional Cst-II, *i.e.* in those capable of synthesizing LOS with terminal α2,8-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 α2,8-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 α2,8-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.

Acknowledgments—We thank Anna Cunningham, Cathy Lorbetskie, and Denis Brochu for technical help, Michael Masotti for DNA sequencing and Tom Devecseri for figure preparation. We also thank Peggy C. R. Godschalk and Astrid P. Heikema for helpful discussion.

REFERENCES

1. Vimr, E., and Lichtensteiger, C. (2002) *Trends Microbiol.* **10,** 254–257
2. Schauer, R. (1991) *Glycobiology* **1,** 449–452
3. Varki, A. (1992) *Glycobiology* **2,** 25–40
4. Blum, A. S., and Barnstable, C. J. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 8716–8720
5. Constantine-Paton, M., Blum, A. S., Mendez-Otero, R., and Barnstable, C. J. (1986) *Nature* **324,** 459–462
6. Cheresh, D. A., Reisfeld, R. A., and Varki, A. P. (1984) *Science* **225,** 844–846
7. Fahr, C., and Schauer, R. (2001) *J. Investig. Dermatol.* **116,** 254–260
8. Herrler, G., Rott, R., Klenk, H. D., Muller, H. P., Shukla, A. K., and Schauer, R. (1985) *EMBO J.* **4,** 1503–1506
9. Smits, S. L., Gerwig, G. J., van Vliet, A. L., Lissenberg, A., Briza, P., Kamerling, J. P., Vlasak, R., and de Groot, R. J. (2005) *J. Biol. Chem.* **280,** 6933–6941
10. Lewis, A. L., Nizet, V., and Varki, A. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 11123–11128
11. Deszo, E. L., Steenbergen, S. M., Freedberg, D. I., and Vimr, E. R. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 5564–5569
12. Orskov, F., Orskov, I., Sutton, A., Schneerson, R., Lin, W., Egan, W., Hoff, G. E., and Robbins, J. B. (1979) *J. Exp. Med.* **149,** 669–685
13. Bhattacharjee, A. K., Jennings, H. J., Kenny, C. P., Martin, A., and Smith, I. C. (1976) *Can. J. Biochem.* **54,** 1–8
14. Claus, H., Borrow, R., Achtman, M., Morelli, G., Kantelberg, C., Longworth, E., Frosch, M., and Vogel, U. (2004) *Mol. Microbiol.* **51,** 227–239
15. Shi, W. X., Chammas, R., and Varki, A. (1998) *Glycobiology* **8,** 199–205
16. Nachamkin, I., Allos, B. M., and Ho, T. (1998) *Clin. Microbiol. Rev.* **11,** 555–567
17. Gilbert, M., Karwaski, M. F., Bernatchez, S., Young, N. M., Taboada, E., Michniewicz, J., Cunningham, A. M., and Wakarchuk, W. W. (2002) *J. Biol. Chem.* **277,** 327–337
18. Szymanski, C. M., Michael, F. S., Jarrell, H. C., Li, J., Gilbert, M., Larocque, S., Vinogradov, E., and Brisson, J. R. (2003) *J. Biol. Chem.* **278,** 24509–24520
19. Aspinall, G. O., McDonald, A. G., Raju, T. S., Pang, H., Moran, A. P., and Penner, J. L. (1993) *Eur. J. Biochem.* **213,** 1017–1027
20. Aspinall, G. O., McDonald, A. G., Raju, T. S., Pang, H., Kurjanczyk, L. A., Penner, J. L., and Moran, A. P. (1993) *Eur. J. Biochem.* **213,** 1029–1037
21. Aspinall, G. O., McDonald, A. G., Pang, H., Kurjanczyk, L. A., and Penner, J. L. (1994) *Biochemistry* **33,** 241–249
22. St Michael, F., Szymanski, C. M., Li, J., Chan, K. H., Khieu, N. H., Larocque, S., Wakarchuk, W. W., Brisson, J. R., and Monteiro, M. A. (2002) *Eur. J. Biochem.* **269,** 5119–5136
23. Yuki, N., Taki, T., Inagaki, F., Kasama, T., Takahashi, M., Saito, K., Handa, S., and Miyatake, T. (1993) *J. Exp. Med.* **178,** 1771–1775
24. Godschalk, P. C., Heikema, A. P., Gilbert, M., Komagamine, T., Ang, C. W., Glerum, J., Brochu, D., Li, J., Yuki, N., Jacobs, B. C., van Belkum, A., and Endtz, H. P. (2004) *J. Clin. Investig.* **114,** 1659–1665
25. Yuki, N., Susuki, K., Koga, M., Nishimoto, Y., Odaka, M., Hirata, K., Taguchi, K., Miyatake, T., Furukawa, K., Kobata, T., and Yamada, M. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 11404–11409
26. Wakarchuk, W. W., and Cunningham, A. M. (2003) *Methods Mol. Biol.* **213,** 263–274
27. Haverkamp, J., van Halbeek, H., Dorland, L., Vliegenthart, J. F., Pfeil, R., and Schauer, R. (1982) *Eur. J. Biochem.* **122,** 305–311
28. Parker, C. T., Horn, S. T., Gilbert, M., Miller, W. G., Woodward, D. L., and Mandrell, R. E. (2005) *J. Clin. Microbiol.* **43,** 2771–2781
29. Koga, M., Gilbert, M., Takahashi, M., Li, J., Koike, S., Hirata, K., and Yuki, N. (2006) *J. Infect. Dis.* **193,** 547–555
30. Shen, Y., Tiralongo, J., Iwersen, M., Sipos, B., Kalthoff, H., and Schauer, R. (2002) *Biol. Chem.* **383,** 307–317
31. Gilbert, M., Watson, D. C., Cunningham, A. M., Jennings, M. P., Young, N. M., and Wakarchuk, W. W. (1996) *J. Biol. Chem.* **271,** 28271–28276
32. Gilbert, M., Brisson, J. R., Karwaski, M. F., Michniewicz, J., Cunningham, A. M., Wu, Y., Young, N. M., and Wakarchuk, W. W. (2000) *J. Biol. Chem.* **275,** 3896–3906
33. Iwersen, M., Dora, H., Kohla, G., Gasa, S., and Schauer, R. (2003) *Biol. Chem.* **384,** 1035–1047
34. Downie, J. A. (1989) *Mol. Microbiol.* **3,** 1649–1651
35. Hara, O., and Hutchinson, C. R. (1992) *J. Bacteriol.* **174,** 5141–5144
36. Varki, A., and Diaz, S. (1984) *Anal. Biochem.* **137,** 236–247
37. Kamerling, J. P., Schauer, R., Shukla, A. K., Stoll, S., van Halbeek, H., and Vliegenthart, J. F. (1987) *Eur. J. Biochem.* **162,** 601–607

38. Vandamme-Feldhaus, V., and Schauer, R. (1998) *J. Biochem.* (*Tokyo*) **124,** 111–121
39. Higa, H. H., Butor, C., Diaz, S., and Varki, A. (1989) *J. Biol. Chem.* **264,** 19427–19434
40. Lemercinier, X., and Jones, C. (1996) *Carbohydr. Res.* **296,** 83–96
41. Diaz, S., Higa, H. H., Hayes, B. K., and Varki, A. (1989) *J. Biol. Chem.* **264,** 19416–19426
42. Manzi, A. E., Sjoberg, E. R., Diaz, S., and Varki, A. (1990) *J. Biol. Chem.* **265,** 13091–13103
43. Kniep, B., Claus, C., Peter-Katalinic, J., Monner, D. A., Dippold, W., and Nimtz, M. (1995) *J. Biol. Chem.* **270,** 30173–30180
44. Hood, D. W., Makepeace, K., Deadman, M. E., Rest, R. F., Thibault, P., Martin, A., Richards, J. C., and Moxon, E. R. (1999) *Mol. Microbiol.* **33,** 679–692
45. Smith, H., Parsons, N. J., and Cole, J. A. (1995) *Microb. Pathog.* **19,** 365–377
46. Moxon, E. R., Rainey, P. B., Nowak, M. A., and Lenski, R. E. (1994) *Curr. Biol.* **4,** 24–33
47. Gilbert, M., Godschalk, P. C., Parker, C. T., Endtz, H. P., and Wakarchuk, W. W. (2005) in *Campylobacter: Molecular and Cellular Biology* (Ketley, J. M., and Konkel, M. E., eds) pp. 219–248, Horizon Bioscience Norfolk, UK

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

R. Scott Houliston, Hubert P. Endtz, Nobuhiro Yuki, Jianjun Li, Harold C. Jarrell, Michiaki Koga, Alex van Belkum, Marie-France Karwaski, Warren W. Wakarchuk and Michel Gilbert

J. Biol. Chem. 2006, 281:11480-11486.
doi: 10.1074/jbc.M512183200 originally published online February 14, 2006
