Separate Pathways for O Acetylation of Polymeric and Monomeric Sialic Acids and Identification of Sialyl O-Acetyl Esterase in Escherichia coli K1

Susan M. Steenbergen,1 Young-Choon Lee,1,2 Willie F. Vann,3 Justine Vionnet,3 Lori F. Wright,4 and Eric R. Vimr1*

Laboratory of Sialobiology and Comparative Metabolomics, Department of Pathobiology, University of Illinois at Urbana-Champaign, Urbana, Illinois1; Department of Biotechnology, Dong-A University, Busan, South Korea2; Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Bethesda, Maryland3; and Department of Microbiology and Immunology, University of Rochester, Rochester, New York4

Received 4 April 2006/Accepted 15 June 2006

O acetylation at carbon positions 7 or 9 of the sialic acid residues in the polysialic acid capsule of Escherichia coli K1 is catalyzed by a phase-variable contingency locus, neuO, carried by the K1-specific prophage, CUS-3. Here we describe a novel method for analyzing polymeric sialic acid O acetylation that involves the release of surface sialic acids by endo-N-acetylneuraminidase digestion, followed by fluorescent labeling and detection of quinoxalinone derivatives by chromatography. The results indicated that NeuO is responsible for the majority of capsule modification that takes place in vivo. However, a minor neuO-independent O acetylation pathway was detected that is dependent on the bifunctional polypeptide encoded by neuD. This pathway involves O acetylation of monomeric sialic acid and is regulated by another bifunctional enzyme, NeuA, which includes N-terminal synthetase and C-terminal sialyl O-esterase domains. A homologue of the NeuA C-terminal domain (Pm1710) in Pasteurella multocida was also shown to be an esterase, suggesting that it functions in the catabolism of acetylated environmental sialic acids. Our combined results indicate a previously unexpected complexity in the synthesis and catabolism of microbial sialic and polysialic acids. These findings are key to understanding the biological functions of modified sialic acids in E. coli K1 and other species and may provide new targets for drug or vaccine development.

Escherichia coli K1 is a versatile human and animal facultative pathogen that causes a variety of extraintestinal diseases including sepsis, meningitis, cystitis, pylonephritis, cellulitis, pneumonia, and postoperative infections. The ability of E. coli K1 to invade and traverse the mammalian epithelial cell barrier also may contribute to inflammatory bowel syndromes such as Crohn’s disease. A primary virulence determinant in these diseases is the polysialic acid capsule or K1 antigen, a homopolymer of 2-keto-3-deoxy-5-hexosamidino-7,8,9-O-glycerod-galacto-nonulosonic, or N-acetylenuraminic acid (Neu5Ac, the most common sialic acid), residues connected by α2,8-glycoketosidic linkages (36). The kps and neu genes needed for polysialic acid synthesis and export map to a 17-kb accretion domain inserted near pheV (7). Mutation of neu (biosynthetic) genes generally results in no capsule polysaccharide produced while kps mutations usually result in intracellular accumulation of unexported polysaccharides (46, 47). Polysialic acid is also found on the mammalian neural cell adhesion molecule and comprises the group B meningococcal, Pasteurella haemolytica A2, and Moraxella nonliquefaciens capsular polysaccharides (41). Mammalian polysialic acid regulates cell migration, axon pathfinding and targeting, and plasticity in the embryonic and adult nervous system (6). Molecular mimicry of this antigen by the bacterial capsules is thought to account for the relatively low immunogenicity of microbial polysialic acid, which has limited the attempts to produce safe and effective capsulabased vaccines (41). Known functions of the capsule include inhibition of phagocytosis and other innate immune responses to microbial infection, but despite our understanding of capsule function during extraintestinal disease, we know little about its role in colonization of the mammalian large intestine. Increased understanding of the colonization process may suggest new targets for therapeutic development.

Unlike the neural cell adhesion molecule or group B meningococcal polysialic acid, the E. coli K1 capsule may exist in an alternate form in which the individual Neu5Ac residues are variably modified with O-acetyl esters at carbon positions 7 or 9. The O-acetyltransferase gene, neuO, responsible for these modifications is carried on a K1-specific prophage designated CUS-3 (13). In addition to lysogeny, neuO expression is controlled by a translational switch involving slipped-strand DNA mispairing of heptanucleotide repeats located in the 5′ coding region. This switch is designated the polyΨ domain, where loss or gain of heptad repeats in any number other than a multiple of three results in frameshift mutation and synthesis of truncated (inactive) neuO gene products. The neuO contingency locus and its mobile phase delivery vehicle account for at least five capsule forms: (i) permanently acetylation “off” because the cell is not a CUS-3 lysogen, (ii) stochastic variation in the proportion of “on” and “off” forms caused by neuO frameshifting, (iii) variation in the degree of acetylation, which may depend on the length of the polyΨ domain, (iv) variation in the...
positions of acetyl esters on individual Neu5Ac residues of the polysialic acid chains (carbon positions 7 or 9), resulting from nonenzymatic transesterification and (v) variation in the positioning of sialyl O-acetyl esters along the chains resulting from incomplete acetylation. Variation in neuO and its metabolic products thus has the capacity to alter capsule antigenicity and physiochemical properties of the K1 cell surface, with one locus accounting for potentially thousands of different capsule phenotypes (48).

In addition to neuO, the K1 neuD gene product annotates as an acetyltransferase (4), and the group B Streptococcus (GBS) NeuD orthologue has been shown to be a monomeric sialic acid O-acetyltransferase responsible for modification of the streptococcal capsular polysaccharide (23, 24). Complementation of a GBS neuD mutant with K1 neuD+ restores sialyl O acetylation, indicating that K1 NeuD is also a monomeric O-acetyltransferase (23). In GBS, where O-acetyl esters are found at carbon positions 7, 8, or 9, the acetylated monomers are activated and transferred as terminal nonreducing residues of the capsule main chain. Therefore, neuD in E. coli K1 suggests there may be two acetylation pathways in this species, one involving neuO for modification of polysialic acid and the other, involving neuD, for acetylation of monomeric sialic acid. Furthermore, neuA encodes a bifunctional enzyme including N-terminal cytidine 5′-monophospho-N-acetylneuraminic acid (CMP-Neu5Ac synthetase) and C-terminal esterase in both the K1 and GBS systems (26, 52), suggesting a mechanism for converting acetylated monomeric sialic acid to the de-O-acetylated forms. In this communication we describe separate pathways for the O acetylation of polymeric and monomeric sialic acids and provide the first demonstration of a new class of esterase with activity against O-acetylated sialic acids.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. Plasmid pWV200b used for overproduction of E. coli K1 NeuA was derived from pWAI (53, 54) as a template using PCR primers containing EcoRI and HindIII sites. Double-digested PCR product was cloned into similarly digested pKK223-3, placing K1 neuD+ under control of the tac promoter and the ribosome binding site of pKK223-3. A truncated version of K1 neuD4 containing the N-terminal 254 amino acid residues (neuD4) was derived by PCR from pWS313 by mutagenesis methods described earlier (42) with the reverse primer Del 254R (5′-CGGCGTCCA GCCGGATATCAGATATGAGGTCCG) to yield pWS319. This plasmid retains the synthetase domain but lacks the C-terminal esterase domain defined previously by Liu et al. (26). LB (Lennox formulation) was purchased from Fisher Scientific and used as the rich medium in all experiments. Minimal M63 salts medium (35) containing 0.4% glycerol as a carbon source was used as the defined medium where indicated. Ampicillin or chloramphenicol was used at 100 or 20 µg/ml, respectively, for plasmid maintenance. Cultures were grown at 37°C with vigorous aeration in a water bath equipped with a rotary shaker. Unless indicated otherwise, all cultures were supplemented with 20 µg/ml of Neu5Ac and grown to early stationary phase prior to cell harvesting by low-speed centrifugation.

Chemical reagents. Purified bovine submaxillary gland mucin (BSM) was kindly provided by Tony Corfield (Bristol, United Kingdom). Neu5Ac was purchased from ICN (Aurora, OH). N-acetyl-9-O-acetylneuraminic acid (Neu5,9Ac) was a gift from Roland Schauer (Kiel, Germany), while samples of N-acetyl-7-O-acetylneuraminic acid (Neu5,7Ac), N-acetyl-9-O-acetylneuraminic acid (Neu5,9Ac), N-acetyl-7,8 or 9-O-acetylneuraminic acid (Neu5,7,8Ac,9Ac), and N-glycolyl-9-O-acetylneuraminic acid (NeuGc9Ac) were purified from BSM as previously described (43), were provided by Tom Warner (San Carlos, CA). N-glycolylneuraminic acid (Neu5Gc), 3-deoxy-D-manno-octulosonic acid (KDO), para-nitrophenyl-acetate (pNP-Ac), and 1,2-diamino-4,5-methyleneedioxybenzene (DMB) were purchased from Sigma (St. Louis, MO). All other chemicals were purchased from Sigma or Fisher Scientific and were of the highest technical grade available.

Enzymes. Recombinant, histidine-tagged endo-N-acetylneuraminidase (endo-N), from phage PKIE, was purified from a culture of IPTG (isopropyl-β-D-thiogalactoside)-induced E. coli BL21(DE3) essentially as described previously (30). CMP-Neu5Ac synthetase was purified as previously described (53, 54) from a culture of induced cells expressing pWV200b and had a specific activity of 610.3 units/mg protein, where 1 unit activates 1 µmol of Neu5Ac in 1 min at 37°C. Other enzymes were used as induced soluble extracts from cells (BL21 or DH5a) grown in overexpression broth (Zymo Research, Orange, CA) with 1 mM IPTG. Esterase activity was assayed as described by Yu et al. (52), with the absorbance for pNP-Ac at 405 nm after 6 min of incubation at room temperature using a Beckman DU-640 spectrophotometer. Complete hydrolysis of pNP-Ac was accomplished by the addition of NaOH to a final concentration of 0.1 M. Data are expressed as activity relative to complete base hydrolysis, normalized for protein concentration as indicated.

Isolation and characterization of neuO form variants. Strains EV717 (neuO “off”) and EV718 (neuO “on”) were isolated from EV291 essentially as described previously (13). Briefly, EV291 was grown overnight in LB and plated for single colonies on agar medium containing 10% (vol/vol) horse-46-polysialic acid as antiserum. Colonies were examined for surrounding halo representing precipitation responses to polysialic acid, where acetylated capsular polysaccharide gives no or only a weak halo response. The lengths of the polysialic acid bands are indicated. The two variants were determined by PCR analysis using flanking forward and flanking reverse primers (13) and were found to contain 20 and 21 heptad repeats in EV717 and EV718, respectively.

DBM analyses and TLC of α-keto compounds. For the analysis of capsule polysialic acid by thin-layer chromatography (TLC), cells from 10- to 30-ml cultures were harvested by centrifugation. The pellets were resuspended in 1/10 volume of 10 mM phosphate buffer (pH 7.0) and repelleted by centrifugation. Polysialic acid in the supernatants was either applied directly to silica gel thin-layer plates or treated with endo-N (50 µg) at 37°C for 2 h to release sialic acids prior to the application. Plates were developed with n-propanol:water (7:3, vol/vol) solvent, and sialic acids were visualized by orcinol spray as previously described (39). For DMB analysis, oligosialic acids released by endo-N digestion were purchased from Sigma or Fisher Scientific and were of the highest technical grade available.

Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EV1</td>
<td>K-12/K1 hybrid (CUS-3+)</td>
<td>51</td>
</tr>
<tr>
<td>EV5</td>
<td>EV1 neuD</td>
<td>51</td>
</tr>
<tr>
<td>EV36</td>
<td>K-12/K1 hybrid (CUS-3+)</td>
<td>50</td>
</tr>
<tr>
<td>EV50</td>
<td>EV36 nanaA</td>
<td>50, 51</td>
</tr>
<tr>
<td>EV78</td>
<td>MC4100 nanaA</td>
<td>50</td>
</tr>
<tr>
<td>EV136</td>
<td>EV36 neuD</td>
<td></td>
</tr>
<tr>
<td>EV239</td>
<td>EV50 neuB neuD</td>
<td>40</td>
</tr>
<tr>
<td>EV291</td>
<td>Derivative of RS218 (CUS-3+)</td>
<td>16, 17</td>
</tr>
<tr>
<td>EV708</td>
<td>EV291 ΔneuO</td>
<td>13</td>
</tr>
<tr>
<td>EV715</td>
<td>EV5 neuD</td>
<td>29</td>
</tr>
<tr>
<td>EV716</td>
<td>EV5 neuD+ (isogenic with EV715)</td>
<td>29</td>
</tr>
<tr>
<td>EV717</td>
<td>EV291 neuD+ “off”</td>
<td>This study</td>
</tr>
<tr>
<td>EV718</td>
<td>EV291 neuD+ “on”</td>
<td>This study</td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td>T7-polymerase expression</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>DH5a</td>
<td>Cloning, expression</td>
<td>Laboratory</td>
</tr>
<tr>
<td></td>
<td>strain</td>
<td></td>
</tr>
<tr>
<td>MC4100</td>
<td>K-12 (K1 null)</td>
<td>Laboratory</td>
</tr>
<tr>
<td>RS218</td>
<td>Clinical K1 isolate (CUS-3+)</td>
<td>1</td>
</tr>
<tr>
<td>RS2887</td>
<td>EV36 nanaA neuD</td>
<td>12</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>PCR cloning vector</td>
<td>Promega</td>
</tr>
<tr>
<td>pSX785</td>
<td>pGEM-T Easy neuO+</td>
<td>13</td>
</tr>
<tr>
<td>pSX1000</td>
<td>pm1710 expression</td>
<td>37</td>
</tr>
<tr>
<td>pSX1001</td>
<td>pm0187 expression</td>
<td>37</td>
</tr>
<tr>
<td>pWV200b</td>
<td>K1 neuD+ expression</td>
<td>This study</td>
</tr>
<tr>
<td>pWG153</td>
<td>GpB neuD+ expression</td>
<td>This study</td>
</tr>
<tr>
<td>pWS319+</td>
<td>K1 neuD+ expression</td>
<td>This study</td>
</tr>
<tr>
<td>pEndo-N</td>
<td>Endo-N expression</td>
<td>U. Ruthsager</td>
</tr>
<tr>
<td>pRS361</td>
<td>neuD+ expression</td>
<td>12</td>
</tr>
</tbody>
</table>

* Expresses first 254 amino acid residues of NeuA.
of whole cells were hydrolyzed completely to monomers by incubation at 80°C with an equal volume of 4 M acetic acid for 3 h. Any precipitate was removed by centrifugation in a microcentrifuge at 16,100 × g for 10 min at 4°C. Samples were concentrated at least 10-fold in a Savant SpeedVac system and recentlyrifuged, and 15-μl samples were subjected to DMB-labeling exactly as described in the original procedure (18). After being labeled in the dark for 2.5 h at 50°C, samples were centrifuged through 0.22-μm-pore-size nylon filters, and 6-μl aliquots were immediately analyzed fluorescently (373 nm excitation and 448 nm excitation) using a reverse phase (RP) high-performance liquid chromatography (HPLC) system equipped with Dionex AS50 Autosampler, GP50 Gradient Pump, RF2000 Fluorescence Detector, and Chromelone version 6.50 software for data management. The solvent, acetonitrile-methanol-water (8:7:84, vol/vol/vol), was slightly modified from the 9:7:84 mixture originally described (18). One major modification was the use of a 4.6-mm by 10-cm TSKgel Super-ODS column (2 µm, 110 Å; Tosoh Bioscience, Montgomeryville, PA), which resulted in elimination of the reagent peak or DMB-breakdown product that elutes just after Neu5,9Ac2 (18). After the completion of any given set of experiments, the column was washed with 50% methanol before the next use. For the analysis of intracellular sialic acids, soluble cell extracts were prepared by sonication disruption and centrifugation to remove debris and then incubated with an equal volume of 4 M acetic acid for 30 min at 80°C before precipitated material was removed by centrifugation and RP-HPLC analysis as described above. Data were expressed as relative fluorescence versus time as previously described (23, 24), where elution of Neu5Ac quinaxime derivatives (25) varied between 9.5 and 13.6 min. However, on a given day’s experiments the relative elution of all peaks was consistent. Where quantitative comparisons were made, the supporting data are posted on the Laboratory of Sialobiology website (www.cvm.uic.edu/path/sialobiology). Confirmation of O acetylation was obtained by mild-base hydrolysis prior to DMB labeling (24).

**RESULTS**

**Two molecular forms of CMP-sialic acid synthetase.** Activation of sialic acid for transfer to nascent polysialic acid by NeuS (polymerase) is catalyzed by CMP-Neu5Ac synthetase (NeuA). Liu et al. (26) showed that the first 229 amino acid residues of NeuA comprise the synthetase, whereas residues 228 to 419 function as an acetylhydrolase (acylesterase) with predicted tertiary structural similarity to the α1-subunit of bovine brain platelet-activating factor acetylhydrolase (PAF-AH) isoform I. Similarly, the GBS NeuA orthologue C terminus was shown to be an acetyl esterase, and the authors (52) speculated that this activity might regulate accumulation of acetylated Neu5Ac derivatives synthesized by NeuD (23, 24). These observations suggest two molecular forms of NeuA: the long or bifunctional form, and a short form composed of just the synthetase domain. This hypothesis predicts that organisms with neuD might acetylate monomeric sialic acids and use a long form of NeuA to regulate intracellular O-acetylated monomeric sialic acid concentrations, while organisms that synthesize or activate sialic acids but that lack neuD might express only the NeuA short form that lacks esterase activity. We designated the putative sialyl O-acetyl esterase domain of the synthetase NeuA-star (NeuA*).

To investigate the correlation between the NeuA form and sialic acid acetylation, we carried out a BLAST (3) search for K1 NeuA and NeuD orthologues. As shown in Table 2, there was general concordance between acetylation/NeuD and NeuA*. Notable exceptions were the neuA gene products of *Pseudomonas aeruginosa* and *Legionella pneumophila*, which are known to acetylate the sialic acid-like molecules pseudaminic and legionaminic acid, respectively, and *Neisseria meningitidis* groups C, Y, and W-135, which are known to acetylate sialic acid but express the NeuA short form. *Campylobacter jejuni* was unusual because it contains both short and long synthetase forms. *L. pneumophila* expresses the NeuA short form while the *P. aeruginosa* long-form C-terminal domain was more similar to nucleotidyl transferase than esterase. When taken together, the results shown in Table 2 suggest that there may be two acetylation pathways in *E. coli* K1, one for polysialic acid catalyzed by the neuO-encoded O-acetyltransferase and another for acetylation of monomeric sialic acids (Fig. 1).

**Detection of O-acetylated cell surface sialic acids.** Note that the salient features of proposed sialic and polysialic acid synthesis in Fig. 1 show two O acetylation pathways interconnected at the substrate level by specific (NeuA*) or nonspecific O-acetyl esterases and the possibility that NeuO could act on the putative polysialic acid products of the NeuD-catalyzed acetylation pathway. Testing these hypotheses required a simple method of monitoring capsule and intracellular O-acetylated sialic acids. Unfortunately, published methods of polysialic acid isolation and characterization are laborious and require high polysaccharide concentrations, while chemical methods provide no information about the site(s) of O acetylation (28). To facilitate analytical investigation of acetylated polymeric or monomeric sialic acids from multiple strains or relatively small amounts of material, we took advantage of the specificity of K1-lytic phage endo-N. Figure 2 (lane 2) shows that digestion of polysialic acid from strain EV36 with a high concentration of endo-N results in a mixture of monomeric and oligomeric sialic acids. Note that the plate was overloaded so that minor O-acetylated sialic acid could be detected if present. A similar digestion pattern was observed when commercially available polysialic acid (colominic acid), also unacetylated (13), was treated with endo-N (Fig. 2, lane 4). When polysialic acid from EV36 harboring pSX785 (neuO*) was treated with endo-N, a sialic acid species migrating faster than Neu5Ac was detected (Fig. 2, lane 3). The relative mobility of this species was consistent with it being Neu5,7Ac2 or Neu5,9Ac2 (32). Sensitivity of this faster-migrating species to mild-base hydrolysis confirmed its identification as acetylated

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain or ORFb</th>
<th>NeuA accession no.</th>
<th>Acc</th>
<th>NeuD</th>
<th>NeuA*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>K1</td>
<td>P13266</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>O104</td>
<td>AAK64369</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O145</td>
<td>AAXS7593</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
<td>GpB</td>
<td>P0A0Z27</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GpC</td>
<td>P0A0Z27</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GpsY and W-135</td>
<td>P0A0Z28</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus agalactiae</em></td>
<td>Group B</td>
<td>AADS3077</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td><em>Legionella pneumophila</em></td>
<td>Philadelphia 1</td>
<td>1YP_094787</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td><em>Pasteurella multocida</em></td>
<td>Pm70</td>
<td>NP_254124</td>
<td>–</td>
<td>–d</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>O12</td>
<td>AAM27591</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>Campylobacter jejuni</em></td>
<td>Cj1331</td>
<td>NP_282477</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cj1143</td>
<td>NP_282291</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>Haemophilus influenzae</em></td>
<td>Rd KW20</td>
<td>NP_439432</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td><em>Homo sapiens</em></td>
<td>CMAS</td>
<td>Q8NFW8</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

a Wellcome Trust Sanger Institute.

b ORF, open reading frame.

c Sialic acids modified with O-acetyl esterases. Ac, acetate.

d Open reading frame pm1710, the first gene of the *P. multocida nan* operon (37).

e C-terminal domain is similar to nucleotidyl transferase instead of esterase.

**TABLE 2. Distribution of NeuA* and NeuD in humans and in bacteria that are known to synthesize or activate sialic, pseudaminic, or legionaminic acid.**
sialic acid (not shown). The colorimetric TLC method shown in Fig. 2 demonstrates that O acetylation neither prevents polysialic acid hydrolysis by endo-N nor affects the composition of the limit digestion products. This conclusion suggested a simple method for rapid and specific analysis of O-acetylated polysialic acids.

Figure 3 shows the details of our analytical method, indicating release of oligosialic acids by endo-N treatment and subsequent removal of intact cells by centrifugation. The released material is converted to a mixture of free Neu5Ac and O-acetylated monosaccharides (if present) by hydrolysis in dilute acetic acid under conditions that do not destroy sialyl O-acetyl esters. Labeling with DMB converts the sialic acids in the hydrolysate to their quinoxalinone derivatives that have characteristic retention times during RP-HPLC (Fig. 4A). Although all of our analyses were carried out with pmol amounts of sialic acids, DMB labeling is reported to detect as little as 50 to 100 fmol (18). Base treatment of BSM-derived sialic acids resulted in loss of peaks representing Neu5,7Ac2, Neu5Ac9Gc, Neu5,9Ac2, and Neu5,7 (8,9)Ac3 and the expected increased ratio of Neu5Ac to Neu5Gc, which are both base resistant (Fig. 4B). Relative retention times of acetylated sialic acid derivatives were consistent with the originally described method (18), company literature (ProZyme Signal Product Code GKK-407), and tandem electron spray mass spectrometry (24).
any CUS-3 open reading frames other than neuO, strain EV36 transformed with vector or vector expressing neuO/H11001 (pSX785) was subjected to DMB analysis. As shown in Fig. 5A, over 98% of the sialic acid from EV36 was unacetylated, indicating that if NeuD or any other O-acetyltransferase is active in this strain, it can account for only about 2% of the total capsular sialic acid residues. In contrast, polysialic acid derived from EV36 expressing pSX785 produced free Neu5Ac as well as detectable Neu5,7Ac2 and Neu5,9Ac2, which are the two known acetylated forms present in *E. coli* K1 strains lysogenized by CUS-3 (13, 18). Note the absence of triacetylated [Neu5,7(8)9Ac3] derivatives, which would elute after Neu5,9Ac2 (Fig. 4A), indicating that NeuO most likely acetylates either the carbon-7 or carbon-9 position followed by nonenzymatic transesterification between positions. The results of DMB capsule analysis indicate that most of the acetylated Neu5Ac in polysialic acid is synthesized by the NeuO pathway (Fig. 1). Nonetheless, about 2% of the sialic acids appeared to be O acetylated by a NeuO-independent pathway that is likely to depend on NeuD (23).

To provide further evidence for the quantitative importance of the NeuO-catalyzed pathway, we carried out DMB analysis of strain EV291, a derivative of the prototypic K1 clinical isolate RS218 known to carry the CUS-3 prophage and undergo capsule form variation (13). Figure 5C shows the expected sialic acid profile of the isogenic *neuO* deletion mutant EV708. This profile is nearly identical to that of EV36 shown in Fig. 5A. Similarly, an “off” derivative (EV717) of EV291 produced mostly unacetylated polysialic acid (Fig. 5D). In contrast, an “on” form variant of EV291 (EV718) yielded a profile resembling that of EV36 harboring pSX785 (Fig. 5B), except that about 35% instead of 16% of the total sialic acid was O acetylated (Fig. 5E). Variation in the degree of acetylation is consistent with previously reported strain differences (19, 21, 28, 31). Note especially that in the “off” profile a small but detectable amount of material, consistent with Neu5,7Ac2 and Neu5,9Ac2, as well as a peak representing Neu5,8Ac2, was observed (Fig. 5D). These small amounts of NeuO-independent acetylated sialic acids may have resulted from leakage of intracellular monomers produced by a second O acetylation pathway in *E. coli* K1 (Fig. 1). However, this hypothesis is unlikely because, as shown below, neuA/H11001 strains do not accumulate O-acetylated sialic acids. Therefore, they most likely arose from activation and incorporation of a small proportion of O-acetylated monomeric sialic acid that escaped NeuA* recycling (Fig. 1). While some of the O-acetylated forms in strain EV717 may have arisen from the stochastic proportion of “on” forms expected to result during outgrowth of the predominant “off” form (13), this amount can be no more than 1 or 2% (compare O acetylation in Fig. 5D with that of Fig. 5C). These considerations suggest that there is a minor or secondary pathway for the O acetylation of monomeric sialic acid and that about 2% of these forms are ultimately incorporated into polysialic acid independently of NeuO.

A second O acetylation pathway for monomeric sialic acid. Lewis et al. (24) reported that a mutation in the GBS *neuA* orthologue resulted in accumulation of Neu5,7Ac2, Neu5,8Ac2, and Neu5,9Ac2, suggesting that NeuA normally out-competes NeuD or other O-acetyltransferases for Neu5Ac substrate. Alternatively, loss of the NeuA* esterase activity in this mutant might have accounted for the increase in acetylated forms (52).
If a similar pathway is operable in *E. coli* K1, loss of NeuA in a mutant also lacking sialic acid aldolase (NanA) should result in accumulation of acetylated sialic acids (Fig. 1). Elimination of NanA would be necessary to detect this phenotype because in a *neuA* mutant the increased pool of Neu5Ac binds NanR repressor, leading to induction of the *nanATEKycH* catabolic operon and destruction of intracellular sialic acids (22, 29, 30).

When a whole-cell extract (sonicate) of the *nanA* *neuA* double mutant EV715 was subjected to DMB analysis, four peaks other than free Neu5Ac were observed (Fig. 6A). On the basis of BSM-derived standards (Fig. 4A) and analogy to the results of tandem electrospray mass spectrometry (24), the three peaks eluting with retention times greater than the Neu5Ac peak represent Neu5,7Ac₂, Neu5,8Ac₂, and Neu5,9Ac₂, respectively. Confirmation that these peaks were derived from O-acetylated sialic acids was shown by sensitivity to mild-base treatment carried out prior to DMB-labeling (Fig. 6B). Note the increase in the ratio of peak c (Neu5Ac) to peak a in Fig. 6B, indicating conversion of O-acetylated sialic acids to Neu5Ac. Peak a, with the earliest retention time, eluted even before Neu5Gc (Fig. 4A), suggesting that it might be the 8-carbon α-keto sugar acid KDO (34). Spiking a sample of the EV715 extract with Neu5Gc prior to DMB analysis resulted in the addition of one new peak to the profile (Fig. 6C), showing that peak a was not Neu5Gc. Chromatography of derivatized KDO, Neu5Gc, or KDO plus Neu5Gc unambiguously confirmed that peak a was derived from KDO (not shown). We assume that KDO in the intracellular extracts results from partial release of free KDO during acid treatment from lipopolysaccharide present as contaminating membrane material. However, some *kps* genes (*kpsF* and *kpsU*) have been shown to...
function in the KDO biosynthetic pathway, warranting future studies of KDO metabolism in *E. coli* K1 strains. Because free sialic acid is not detectable by colorimetric or amperometric methods in wild-type *E. coli* K1 (29), we concluded that loss of NeuA allows accumulation of sufficient free Neu5Ac in a *nanA* mutant background to produce a detectable pool of acetylated forms, which by analogy to GBS may result from the action of NeuD (Fig. 1). Note that the combined amount of diacetylated forms is at least twice that of the intracellular Neu5Ac concentration (Fig. 6A). Although the concentration of all sialic acid derivatives was reduced in the *nanA*/*neuA* mutant, EV716, acetylated forms were still detectable (Fig. 6D). We assume that the known relative resistance of acetylated sialic acids to sialate aldolase encoded by *nanA* accounts for this observation (32).

Although LB contains a low concentration of free Neu5Ac (45), all of the acetylated forms synthesized by EV715 and EV716 (Fig. 6) were dependent on the mutant background. This conclusion was substantiated by analysis of an extract from EV78, an *E. coli* K-12 derivative of strain MC4100 that lacks the *kps/neu* accretion domain. Note the accumulation of Neu5Ac by EV78 but the absence of diacetylated forms (Fig. 7A). As expected, when EV78 was grown in nonsupplemented minimal medium, no free Neu5Ac peak was observed (Fig. 7B). However, when the medium was supplemented with exogenous Neu5Ac, accumulation of intracellular sialic acid was detected (Fig. 7C). Note that the failure of supplemented EV78 to produce diacetylated sialic acids means that *E. coli* lacking the *kps/neu* genes does not express Neu5Ac O-acetyltransferase. Furthermore, the failure to accumulate diacetylated forms in the *nanA*/*neuB*/*neuS* triple mutant, EV239 (compare Fig. 7D and E) suggests efficient de-O acetylation by NeuA*. Although as expected EV36 (wild type) does not accumulate detectable free sialic acid, the peak eluting with the longest retention time represents DMB-labeled pyruvate, another \(\alpha\)-keto acid that most likely results from sialic acid accumulation and subsequent NanA cleavage during polysialic acid biosynthesis (Fig. 7F). Finally, to determine if activation of sialic acids protects acetylated forms from NeuA*, we analyzed an extract from strain EV136 that accumulates CMP-Neu5Ac due to a NeuS polymerase defect (29, 38, 51). Although the free Neu5Ac peak resulting from acid hydrolysis of CMP-Neu5Ac in this extract was apparent, acetylated forms were not detected, indicating that if acetylated sialic acids are activated by the synthetase, NeuA* deacetylates them prior to DMB labeling (Fig. 7G). We conclude from the results shown in Fig. 5 to 7 that the monomeric O-acetyltransferase, probably NeuD (23), produces diacetylated sialic acids that are normally deacetylated by NeuA* or nonspecific esterase (Fig. 1). As shown in Fig. 5, only a small percentage of the acetylated monomers are ever incorporated into capsular polysialic acid (Fig. 5). This is a major distinction with the minimal medium showing uptake and accumulation of Neu5Ac. (D) Growth of the triple mutant in nonsupplemented minimal medium. (E) Growth of the triple mutant in supplemented minimal medium. (F) Growth of the wild type in nonsupplemented LB. (G) Growth of the polymerase mutant in nonsupplemented LB.
incubation with purified NeuA for 30 min (Fig. 8A).

8D), while all three acetylated forms were eliminated after coccal polysaccharide (23, 24).

acetylated monomeric sialic acids are added to the streptococcal polysaccharide (23, 24).

NeuD catalyzed pathway in GBS, where 50 to 60% of O-acetylated monomeric sialic acids are added to the streptococcal polysaccharide (23, 24).

O-acetyl esterase (NeuA*) activity of NeuA. To determine if NeuA has sialyl O-acetyl esterase activity, recombinant synthetase was added to an extract of EV715. As shown in Fig. 8A, all three diacetylated peaks were sensitive to the NeuA* activity of the purified enzyme. Heating enzyme at 90°C for 5 min prior to addition to the EV715 extract eliminated esterase activity, indicating the absence of nonspecific de-O acetylation (Fig. 8B). Using half the amount of enzyme as the experiment shown in Fig. 8A resulted in incomplete deacetylation (Fig. 8C). That contamination of the enzyme preparation by nonspecific esterase was an unlikely explanation for these results was apparent from the extended (5 h) incubation of the EV715 extract at 37°C. Though this treatment resulted in some loss of Neu5,7Ac2, it had only a minor effect on Neu5,8(9)Ac2 (Fig. 8D), while all three acetylated forms were eliminated after incubation with purified NeuA for 30 min (Fig. 8A).

NeuA* has been shown to hydrolyze pNP-Ac between pH 7.5 and 9.0 (26, 52). As shown in Table 3, the normalized recombinant NeuA activity against this model substrate was as great as the hydrolysis produced by alkali treatment, indicating that NeuA* rapidly and completely deacetylated pNP-Ac. Similarly, an extract containing overproduced K1 NeuA, but not one containing the overproduced NeuA short form from group B meningococci, expressed NeuA* activity that was at least nine times higher than background (Table 3). This background activity was likely due to nonspecific esterase because the same amount of background was observed in an extract prepared from EV715, which lacks NeuA because of mutation (Table 3). In contrast, an extract containing overproduced Pm1710 from Pasteurella multocida (37) had elevated esterase activity while an extract containing the overproduced short form of P. multocida NeuA (Pm0187) did not (Table 3). When taken together, the results shown in Fig. 8 and Table 3 indicate that NeuA*, in addition to its expected activity against pNP-Ac (26, 52), is a sialyl O-acetyl esterase. This conclusion is supported by the identity of conserved blocks I, II, III, and V found in GDSL esterases of the SGNH-hydrolase family (2) whose members include conserved catalytic SGNH residues at the indicated positions of each polypeptide shown in Fig. 9, strongly suggesting that Pm1710 also is a sialyl O-acetyl esterase (37). Further confirmation that NeuA* is an esterase was obtained by showing that an extract containing overproduced, truncated K1 NeuA composed of just the first 254 residues (synthetase domain) lacked elevated activity against pNP-Ac (Table 3).

**Contribution of NeuD to O acetylation of polysialic acid.** To determine if the small amount of NeuO-independent O-acetylated sialic acids represents monomers that escaped NeuA* recycling and were subsequently incorporated into polysialic acid, we used exogenous Neu5Ac to rescue the nanA neuD double mutant RS2887 (12). In addition to its activity as a monomeric sialic acid O-acetyltransferase (23), NeuD is required for sialic acid synthesis (4, 12). The synthetic defect of a neuD mutant can be rescued by adding Neu5Ac to the growth medium as long as the cell lacks NanA to prevent the lyase from destroying transported monosaccharide. When polysialic acid from sialic acid-rescued RS2887 was analyzed by DMB labeling, there were no detectable O-acetylated forms (not shown), indirectly suggesting that NeuD is necessary for the

![Table 3](https://example.com/table3.png)

**Table 3. Relative esterase activities of various NeuA forms and Pm1710 against pNP-Ac**

<table>
<thead>
<tr>
<th>Plasmid (gene)</th>
<th>NeuA form</th>
<th>Sourcea</th>
<th>Relative activityb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSX1000 (pm1710) C-terminus</td>
<td>P. multocida</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>pSX1001 (pm0107) Short</td>
<td>P. multocida</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>pVV200b (neuA) Long</td>
<td>E. coli K1</td>
<td>18.7</td>
<td></td>
</tr>
<tr>
<td>pWG153 (neuA) Short</td>
<td>N. meningitidis group B</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>EV715</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>pWS319 (neuA) Short</td>
<td>E. coli K1</td>
<td>2.1</td>
<td></td>
</tr>
</tbody>
</table>

a Species from which the gene was cloned or strain used as source of extract.
b Normalized to 50 µg of protein unless indicated otherwise.
c First gene of the P. multocida nan operon; lacks synthetase domain (37).
d Truncated K1 neuA lacking NeuA* (Table 1).
e Purified K1 NeuA from overexpressing pWV200b (Table 1).
f Relative activity produced by 10 µg of purified K1 NeuA.
minor O acetylation detected in wild-type strains lacking neuO (Fig. 5). We conclude that NeuD is a functional monomeric O-acetyltransferase in *E. coli* but that NeuA* recycles most monomers to Neu5Ac before incorporation into polymer (Fig. 1). The exact function of this minor pathway is unknown, although we assume that NeuD is responsible for the O acetylation of monomeric sialic acids in species such as GBS, *E. coli* O104, and others (23). However, our results unambiguously demonstrate the quantitative importance of the NeuO-catalyzed pathway, suggesting that NeuD, at least in *E. coli* K1, plays only a minor role in capsule modification.

### DISCUSSION

**Identification and function of NeuA*.** Our results indicate that sialyl O-acetyl esterase activity is catalyzed by the long form of NeuA, a bifunctional CMP-Neu5Ac synthetase in *E. coli* K1 and other bacteria that decorates their surfaces with sialic acids (Table 2). On the basis of three-dimensional molecular modeling and biochemical activity, Liu et al. (26) concluded that the K1 NeuA C terminus (residues 229 to 419) was a PAF-AH. These authors speculated that PAF cleavage may function in *E. coli* K1 invasion of the blood-brain barrier after proteolytic release of PAF-AH from the N-terminal synthetase domain (26). We think this suggestion is unlikely for at least six reasons. First, NeuA is a cytoplasmic enzyme, and even if it were proteolytically processed in vivo, how it gains access to PAF would still remain unclear. Even if PAF were somehow accessible as a substrate, it is unclear how hydrolysis would affect endothelial invasion. *E. coli* K1 invasion of the blood-brain barrier has been studied extensively in tissue culture, and the model seems unconvincingly complicated since mutants with in vitro invasion defects still cause meningitis in the rodent disease model and have only modest invasion defects in vivo (20). Second, our demonstration that O-acetylated sialic acids are NeuA* substrates provides the first direct evidence for this activity, suggesting that PAF may not be a physiological substrate. Third, the failure to accumulate O-acetylated sialic acids in a *neuA*/H11001 strain points to a function of NeuA* in regulating the intracellular monomeric O-acetylated sialic acid concentration. Fourth, the obligate animal commensal and facultative pathogen *P. multocida* expresses CMP-sialic acid synthetase encoded by the short form of *neuA* (pm0187). *P. multocida* also expresses a homologue of *neuA* (pm1710), which maps as the first gene of a functional *nan* operon for catabolism of environmental sialic acids (37). The concentration of O-acetylated sialic acids in nonhuman mammalian serum is more than twice that of Neu5Ac (18), suggesting that the pm1710 gene product may function to remove O-acetyl esters after the cell transports acetylated sialic acids from the host. O acetylation is known to inhibit sialate aldolase (NanA), further suggesting that the NeuA* physiological substrates, independent of species, are O-acetylated sialic acids. Fifth, the active residues in blocks I to III and V of NeuA* are more similar to esterases such as Pm1710 and TesA than to PAF-AH (Fig. 9). Finally, other neuroinvasive strains like *N. meningitidis* and *Haemophilus influenzae* express the short form of neuA (pm0187). *P. multocida* also expresses a homologue of neuA* (pm1710), which maps as the first gene of a functional *nan* operon for catabolism of environmental sialic acids (37). The concentration of O-acetylated sialic acids in nonhuman mammalian serum is more than twice that of Neu5Ac (18), suggesting that the pm1710 gene product may function to remove O-acetyl esters after the cell transports acetylated sialic acids from the host. O acetylation is known to inhibit sialate aldolase (NanA), further suggesting that the NeuA* physiological substrates, independent of species, are O-acetylated sialic acids. Fifth, the active residues in blocks I to III and V of NeuA* are more similar to esterases such as Pm1710 and TesA than to PAF-AH (Fig. 9). Finally, other neuroinvasive strains like *N. meningitidis* and *Haemophilus influenzae* express the short form of neuA, indicating that NeuA* is not essential for cell invasion. Evidence for the in vivo function of NeuA* in vivo may be obtained by constructing an *E. coli* K1 mutant that lacks star (O-acetyl esterase) activity while retaining synthetase.

Human serum contains over 30 times less O-acetylated sialic acid than Neu5Ac (18), which may explain why an obligate human commensal like *H. influenzae* lacks a pm1710 homologue despite the functional expression of other *nan* catabolic genes (44). Note that *H. influenzae* neuA encodes the short

<table>
<thead>
<tr>
<th>Block I</th>
<th>Block II</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBS NeuA (249)</td>
<td>(263) LENYH</td>
</tr>
<tr>
<td>K1 NeuA (251)</td>
<td>(270) DIEVN</td>
</tr>
<tr>
<td>Pm1710 (23)</td>
<td>(270) DQVTA</td>
</tr>
<tr>
<td>TesA (29)</td>
<td>(60) KTSVV</td>
</tr>
<tr>
<td>humanPAF-AH (40)</td>
<td>(64) PLHAL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Block III</th>
<th>Block V</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBS NeuA (295)</td>
<td>(383) HLTS</td>
</tr>
<tr>
<td>K1 NeuA (304)</td>
<td>(394) MVTY</td>
</tr>
<tr>
<td>Pm1710 (81)</td>
<td>(173) RLCT</td>
</tr>
<tr>
<td>TesA (92)</td>
<td>(175) WMQD</td>
</tr>
<tr>
<td>humanPAF-AH (97)</td>
<td>(187) HDMI</td>
</tr>
</tbody>
</table>

FIG. 9. Sequence alignment of four conserved blocks in the SGNH-hydrolase family. Blocks I, II, III, and V found in enzymes of the SGNH-hydrolase family (2) are shown boxed, including the NeuA* domains of GBS and K1 NeuA; the esterase encoded by *P. multocida* open reading frame pm1710: *E. coli* thioresterase I (TesA), accession no. AAC37396; and human PAF-AH, accession no. AAH07863. Other accession numbers are given in Table 2. Single-letter amino acid designations are used, and the numbering in parentheses indicates the relative positions of each block to the N terminus. Note that block I is always found at the N terminus of esterases, consistent with its relative positions in Pm1710, TesA, and PAF-AH. Note that in the two CMP-sialic acid synthetases, the esterase domains are located at the C termini of these bifunctional enzymes. Conserved residues are in boldface, where asterisks indicate catalytic residues.
form of the synthetase (Table 2), suggesting that it does not modify its surface with O-acetylated sialic acids. Because most *E. coli* strains do not synthesize or activate sialic acids, and therefore lack *neuA*, the NeuA* physiological substrates in *E. coli* K1 may be the monomeric O-acetylated sialic acids produced during sialic acid synthesis, which requires NeuC (epimerase), NeuB (synthase), and NeuD (Fig. 1), instead of environmentally derived sialic acids. The niche of the human and animal large intestine occupied by *E. coli* K1 and other *E. coli* is a complex and poorly understood microbial environment that includes a diverse set of organisms expressing sialidases, sialyl O-acetyl esterases, and other catabolic enzymes directed against mucins and mucin-derived monosaccharides (8–10), suggesting that the *E. coli nan* system may primarily scavenge Neu5Ac instead of O-acetylated forms. However, over half of all mucin sialic acid residues are O acetylated (8–10), suggesting that *E. coli* K1 NeuA* may confer a selective advantage over other *E. coli* lacking the esterase. For example, in a study of healthy pregnant females, *E. coli* K1 was the most prevalent aerobic species in 38% of the participants (31), indicating that it may express specialized colonization factors such as efficient deacetylation of environmental sialyl O-acetyl monomers. The temperature dependence of capsule synthesis and nicotineamide auxotrophy of many K1 strains also may indicate ongoing adaptation to an animal environment (48), in which case NeuA* could play an important role in colonization and persistence.

**Contribution of the monomeric O acetylation pathway to capsule modification.** Although NeuA*-sensitive O-acetylated sialic acids accumulate in a *neuA* mutant, most of the acetylated sialic acids in the capsule are derived from the NeuO-catalyzed pathway. However, 2 to 4% of residues in polysialic acid are O acetylated by the NeuO-independent pathway, indicating that all *E. coli* K1 stains, regardless of CUS-3 status, contain a small amount of modified residues. The apparent synthesis and reversion of most O-acetylated monomeric sialic acids to Neu5Ac would seem to be a wasteful or nonsensical process (Fig. 1). However, *E. coli* serotype O104 (Table 2) synthesizes O-acetylated monosialic acid as a structural component of lipopolysaccharide (15). NeuA* offers a mechanism to regulate the degree of acetylation by controlling the concentration of O-acetylated sialic acids available for surface modification. In contrast, no O acetylation of sialic acid was reported for the *E. coli* O145 serotype (14), suggesting that there may be differences in the relative activity of NeuA* or activity of NeuD in some strains. In GBS the relative concentration of O-acetylated monomeric sialic acids is low unless the *neuA* orthologue is inactivated (24). Although Lewis et al. (24) ascribed this phenotype to a competition between the synthetase and O-acetyltransferase encoded by *neud* (23), our results support NeuA* as the molecular explanation for the accumulation of O-acetyl forms in an *E. coli* K1 *neuA* mutant. Indeed, it is unclear how the levels of GBS polysaccharide O acetylation reported could occur in the presence of NeuA*, unless the activity of this enzyme in GBS is less than in *E. coli* K1. In vitro comparisons of NeuA* proteins from *E. coli* K1 and GBS against the model esterase substrate pNP-Ac indicate that the *E. coli* esterase is relatively more active (32).

Similarly, the groups C, Y, and W-135 meningococcus *neuA* homologues lack the genetic information to code for NeuA*, and the degree of O acetylation in these strains is 90%, 47%, and 66%, respectively (21), which is much higher than in *E. coli* K1 strains lacking *neuO*. Meningococcal strains also lack NeuD (Table 2), supporting the negative correlation between the degree of acetylation and NeuA*. In contrast to these NeuA*-negative meningococcal strains, the degree of capsule acetylation in *E. coli* K1 CUS-3 strains may approach 100% (28) despite the simultaneous occurrence of sialyl O-acetyl esterase. Our current results show that capsule O acetylation is largely dependent on *neuO*. That NeuO acetylates polymeric instead of monomeric sialic acid (19) presumably accounts for the lack of an effect of NeuA* on the degree of polysialic acid O acetylation, since it is as yet unclear whether NeuA* recognizes O-acetylated polysialic acid. However, the degree of *E. coli* K1 polysialic acid acetylation is strain specific (13, 19, 28). Sialic acids from stains lacking *neuO* either due to mutation or naturally because they are not CUS-3 lysogens contain few O-acetyl esters. In contrast, CUS-3 lysogens expressing *neuO* in the “on” form vary over a wide range in the degree of acetylation (28), which may not be solely dependent on the stochastic proportion of cells in the “off” form (13). How or even whether the degree of acetylation is affected during host colonization relative to that observed in cells grown in vitro, as in the current study, provides fertile new ground for investigating the biological functions of variable capsule modification in the context of the host-microbe interaction.

**Function of the NeuD-catalyzed pathway.** Our results indicate that the NeuD-catalyzed addition of O-acetyl esters to Neu5Ac is a continuous process in *E. coli* K1 but that NeuA* limits accumulation of O-acetylated forms or their incorporation into capsule. However, while *neuD*” (pRS361) complemented a *Δneud* mutant (RS2887) for sialic acid synthesis, the plasmid did not result in detectable O-acetylated sialic acids when expressed in strain EV78 grown in sialic acid-supplemented medium (not shown). Thus, while NeuD is the most likely candidate for the *E. coli* K1 sialyl O-acetyltransferase, our results do not provide direct evidence for this conclusion despite the recent observation that K1 NeuD appears to acetylate Neu5Ac in GBS (23). Note that Lewis et al. (23) did not identify acetyl-coenzyme A as the presumed two-carbon donor nor demonstrate biochemically that either K1 or GBS NeuD functions as an acetylate in vitro. Both of these biochemical conditions have been demonstrated for NeuO (13). It may be that NeuD requires interaction with other *kps* or *neu* gene products in order to be an active O-acetyltransferase, and a direct interaction between NeuD and NeuB has been demonstrated in vivo (11). However, in unpublished experiments, plasmids expressing *neuBAC* or *neuDRAC* in EV78 did not produce detectable intracellular Neu5Ac, suggesting that stoichiometry or interactions with still other gene products not present in the K-12 background may be critical. Evidence that O-acetylated monomers are not necessarily protected from NeuA* upon activation by the synthetase domain was evident from the phenotype of EV136 shown in Fig. 7G. This mutant has a defect in *neuS* and accumulates high intracellular concentrations of CMP-Neu5Ac (29, 38) but no detectable O-acetylated forms, suggesting that K1 NeuA either does not activate acetylated monomers or, if so, then they, too, must be NeuA* substrates. However, because GBS NeuA clearly activates O-acetylated Neu5Ac, the low incorporation of NeuO-
independent acetylated sialic acids in polysialic acid presumably reflects the efficiency of NeuA* and, perhaps, the efficiency of the polymerization process (39). It will be interesting to determine whether NeuA* is active against O-acetylated polysialic acid. Our current results suggest that the small but constant amount of NeuO-independent acetylation of polysialic acid may be a target for potential vaccination against all K1 strains.

Finally, the NeuD-catalyzed O acetylation pathway may be irrelevant to the biology of E. coli K1. This possibility follows from the bifunctional nature of neuD, where in addition to its acetylase activity, NeuD is required for sialic acid synthesis (12). Dissection of these two functions has been accomplished for GBS NeuD by site-directed mutagenesis, resulting in loss of the acetylase activity but retention of the sialic acid biosynthetic phenotype (23). Therefore, it may be that only the bio-
synthetic function of NeuD is important in E. coli K1. The situation where only one of two biochemical functions is important to polysialic acid biosynthesis might be analogous to KpsF, which is enzymatically active in KDO-precursor biosynthesis (27) but also includes a cystathionine-β-synthase or Bateman domain (5) that is now understood to bind adenosine metabolites (27) but also includes a cystathionine-β-synthase or Bateman domain (5) that is now understood to bind adenosine metabolites (27). This indicates that the metabolism of microbal monocyclic and polymeric sialic acids is far more complex than previously thought. The absence of at least some of these metabolic processes in mammals suggests new targets for potential drug or vaccine development (47–49).

ACKNOWLEDGMENTS

This research was supported by NIH grant R01 AI042015 (E.R.V.). Y.-C. Lee was supported in part by the LG Yeonam Foundation, South Korea, during his sabbatical with the Laboratory of Sialobiology. We thank Kerry Helms for expert photographic assistance. We are grateful to Mark and Theresa Kuhlenschmidt for technical training in enzymatic assay development of sialic acid with fluorogenic reagent, 1,2-diamino-4,5-methylenedioxybenzene. Anal. Biochem. 176:162–166.


Rangenberg, M. A., S. M. Steenbergen, and E. R. Vimr. 2003. The first committed step in the biosynthesis of sialic acid by Escherichia coli K1 does not involve a phosphorylated N-acetylmannosamine intermediate. Mol. Mi-

Downloaded from http://jb.asm.org/ on October 23, 2017 by guest