

NeuA Sialic Acid O-Acetyltransferase Activity Modulates O-Acetylation of Capsular Polysaccharide in Group B *Streptococcus**

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Group B *Streptococcus* (GBS) is a common cause of neonatal sepsis and meningitis. A major GBS virulence determinant is its sialic acid (Sia)-capped capsular polysaccharide. Recently, we discovered the presence and genetic basis of capsular Sia O-acetylation in GBS. We now characterize a GBS Sia O-acetyltransferase that modulates the degree of GBS surface O-acetylation. The GBS Sia O-acetyltransferase operates cooperatively with the GBS CMP-Sia synthetase, both part of a single polypeptide encoded by the *neuA* gene. NeuA de-O-acetylation of free 9-O-acetyl-N-acetylneuraminic acid (Neu5,9Ac₂) was enhanced by CTP and Mg²⁺, the substrate and co-factor, respectively, of the N-terminal GBS CMP-Sia synthetase domain. In contrast, the homologous bifunctional NeuA esterase from *Escherichia coli* K1 did not display cofactor dependence. Further analyses showed that *in vitro*, GBS NeuA can operate via two alternate enzymatic pathways: de-O-acetylation of Neu5,9Ac₂ followed by CMP activation of Neu5Ac or activation of Neu5,9Ac₂ followed by de-O-acetylation of CMP-Neu5,9Ac₂. Consistent with *in vitro* esterase assays, genetic deletion of GBS *neuA* led to accumulation of intracellular O-acetylated Sias, and overexpression of GBS NeuA reduced O-acetylation of Sias on the bacterial surface. Site-directed mutagenesis of conserved asparagine residue 301 abolished esterase activity but preserved CMP-Sia synthetase activity, as evidenced by hyper-O-acetylation of capsular polysaccharide Sias on GBS expressing only the N301A NeuA allele. These studies demonstrate a novel mechanism regulating the extent of capsular Sia O-acetylation in intact bacteria and provide a genetic strategy for manipulating GBS O-acetylation in order to explore the role of this modification in GBS pathogenesis and immunogenicity.

N-Acetylneuraminic acid (Neu5Ac)² is the most common type of sialic acid (Sia) found in nature and is present as an outer modification on the surfaces of all vertebrate cells, where it plays important roles in biology, evolution, and disease (1, 2). A subset of pathogenic bacteria also express terminal Neu5Ac residues, using a biosynthetic pathway evolutionarily related to that of the vertebrate host. Sia biosynthesis in bacteria appears ancient (1), yet only some bacteria endogenously express this carbohydrate structure (3). Bacterial Sia expression is related to several distinct virulence phenotypes, including complement evasion (4–7), intracellular survival (8), and biofilm formation (9). However, the precise mechanistic underpinnings of Sia-dependent bacterial virulence phenotypes remain largely uncharacterized.

Group B *Streptococcus* (GBS), or *Streptococcus agalactiae*, is the leading cause of neonatal sepsis and has become increasingly associated with invasive infections in elderly and immunocompromised individuals (10). The most extensively studied and widely appreciated virulence factor of GBS is its Sia-capped capsular polysaccharide (CPS). To date, the structures of nine antigenically distinct GBS CPS serotypes have been described, all of which express terminal α 2–3-linked Neu5Ac (11). Animal models of infection confirm that GBS mutants in the Sia biosynthetic pathway are severely attenuated for virulence (12).

In addition to being a virulence determinant, the CPS is also an important target of both innate (lectin-mediated complement) and adaptive (IgG) host immune responses against the pathogen (13, 14). Recently, it was shown that bacterial Sias can facilitate interactions with certain host Sia-binding proteins called Siglecs (15, 16), a phenomenon that could potentially favor pathogen or host, depending on several variables. In short, the sialylated CPS of GBS represents both an important host-pathogen interface and a relevant model for studying Sia-dependent bacterial interactions with the immune system.

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² The abbreviations used are: Neu5Ac, N-acetylneuraminic acid; GBS, group B *Streptococcus*; Sia, sialic acid; Sia-O-Ac, O-acetylated; Neu5,9Ac₂, 9-O-acetylated N-acetylneuraminic acid; DMB, 1,2-diamino-4,5-methylene dioxybenzene; CPS, capsular polysaccharide; WT, wild type; ManNAc, N-acetylmannosamine; HPLC, high pressure liquid chromatography; Erm, erythromycin; Cm, chloramphenicol.

Recently, we described the biochemical and genetic basis of capsular Sia O-acetylation in GBS (17, 18), a modification unrecognized during earlier GBS capsule studies. The O-acetyl modification of Sias and related molecules is not unique to GBS; rather, it is characteristic of many bacterial pathogens (17, 19–26) as well as many vertebrate cell types (27–32). Sia O-acetylation influences several Sia-dependent functions of vertebrate cells, including apoptotic regulation (33), susceptibility to the alternative pathway of complement, and binding to leukocyte-expressed Sia-binding proteins (34). Given the effects of this modification on vertebrate cell-cell interactions, we hypothesize that Sia O-acetylation on the bacterial surface is also likely to modulate interactions between GBS and host. A combined biochemical and genetic approach for manipulating Sia-O-acetyl regulation in GBS is necessary to address these and other pertinent questions about Sia-dependent virulence at the host pathogen interface.

Biosynthesis of sialylated structures requires activation of the Sia monomer by transfer of the α -phosphate of CTP to the anomeric oxygen of Neu5Ac (35). Synthesis of CMP-Neu5Ac is catalyzed by a cytidyltransferase (CMP-Sia synthetase). Some bacterial CMP-Sia synthetases have an additional C-terminal domain related to the GSDL family of hydrolases. In GBS and *Escherichia coli* K1, these two-domain CMP-Sia synthetases are encoded by the *neuA* gene and were recently shown to possess esterase functionality *in vitro* (36–38). Initial experiments on the bifunctional enzymes employed 4-nitrophenyl-acetate and (acetylated) platelet-activating factor as substrates of the esterase reaction (36, 37). More recently, work by Vimr and co-workers (38) suggest that in *E. coli*, the native substrate of the NeuA esterase domain is O-acetylated Sia. Based on these studies and our earlier observations, we hypothesized that GBS NeuA acts natively as a Sia-O-acetyltransferase, an intracellular process that could mitigate overall O-acetylation of the sialylated capsule *in vivo*.

To test this hypothesis, we used a defined *in vitro* system to explore mechanistic aspects of Sia-O-acetyl removal, coupled with live bacterial models to study the biochemical phenotypes resulting from genetic deletion, overexpression, and site-directed enzymatic inactivation of the NeuA esterase in GBS. Taken together, these studies demonstrate that the bifunctional GBS NeuA can act through two different enzymatic pathways and show for the first time that O-acetyl removal prior to capsular assembly is an important regulatory mechanism controlling the extent of capsular O-acetylation in intact bacteria.

EXPERIMENTAL PROCEDURES

Purification of Recombinant NeuA Enzymes—Full-length *neuA* homologs were cloned into pET15b or pET22b(+) vectors from GBS serotype V strain 2603V/R, *Neisseria meningitidis* group B strain MC58, and *E. coli* K1 strain ATCC13027 as previously described. C-terminal hexahistidine-tagged proteins were expressed in isopropyl 1-thio- β -D-galactopyranoside-induced *E. coli* BL21 (DE3) cells and purified by nickel-nitrilotriacetic acid-agarose chromatography (39). Enzymes were stored at 4 °C in Tris-HCl buffer (20 mM, pH 7.5) containing 10% glycerol. We observed some variability in overall GBS enzyme activity between enzyme batches over time. Findings

were confirmed using at least two different batches of GBS enzyme.

Chemoenzymatic Synthesis of Neu5,9Ac₂ and CMP-Neu5,9Ac₂—6-O-Acetyl ManNAc was prepared as previously described (40). Briefly, 200 mg of N-acetylmannosamine (ManNAc) was dissolved in a mixture of CH₃CN and Me₂SO (3:1, v/v, 6 ml). Trifluoroethyl acetate (1 ml) and Protease N (100 mg; Amano Enzyme Inc.) were added, and the suspension was stirred vigorously at 45 °C for 2 days. Protein was then removed by filtration, and the filtrate was concentrated on a rotary evaporator. The resulting residue was purified by flash column chromatography (EtOAc/MeOH, 5:1 v/v) using Silica Gel 60 (40–63 μ m; Sorbent Technologies) to yield 6-O-acetyl ManNAc (121 mg, 51% yield).

9-O-Acetylated N-acetylneuraminic acid (Neu5,9Ac₂) was subsequently prepared in a Tris-HCl (100 mM, pH 7.5) buffer containing MgCl₂ (20 mM), 6-O-acetyl ManNAc (10 mM), sodium pyruvate (50 mM), and the recombinant Sia aldolase from *E. coli* K12 ATCC47076 (0.2 mg/ml) (39). The reaction mixture was incubated in an isotherm incubator at 37 °C with 140 rpm agitation for 2 h. The reaction was stopped by adding EtOH when TLC (EtOAc/MeOH/H₂O/HOAc = 4:2:2:0.2, v/v/v/v) analysis indicated the completion of the reaction. The precipitates in the reaction mixture were removed by centrifugation. The supernatant was concentrated and purified by BioGel P-2 gel filtration chromatography to yield Neu5,9Ac₂ (60%) as a white solid after lyophilization. ¹H NMR (300 MHz, D₂O) δ 4.31 (dd, 1 H, *J* = 3.0, 12.0 Hz), 4.14 (dd, 1 H, *J* = 5.7, 11.4 Hz), 3.86–4.01 (m, 2 H), 3.67–3.70 (m, 1 H), 3.57 (d, 1 H, *J* = 6.6 Hz), 2.20 (dd, 1 H, *J* = 4.5, 12.9 Hz), 2.08 (s, 3 H), 2.02 (s, 3 H), 1.79 (t, 1 H, *J* = 12.9 Hz); ¹³C NMR (75 MHz, D₂O) δ 176.62, 174.83, 174.53, 96.60, 70.21, 68.27, 67.91, 67.26, 66.34, 52.26, 39.40, 22.19, 20.35. N-Acetylmannosamine, EtOAc, and trifluoroethyl acetate were purchased from Sigma. CH₃CN, Me₂SO, MeOH, HOAc, Tris, and MgCl₂ were from Fisher. The presence of small quantities of Neu5,7Ac₂ and Neu5,8Ac₂ seen by HPLC (Fig. 1A) and probably by NMR (see *left shoulder* of the 9-OAc peak in Fig. 3A) probably reflect an equilibrium of O-acetyl migration along the exocyclic 3-carbon side chain of N-acetylneuraminic acid (41).

CMP-Neu5,9Ac₂ was synthesized from 6-O-acetyl ManNAc using the Sia aldolase and NeuA of *N. meningitidis* (the latter does not possess a C-terminal esterase domain) as reported (39), except that a Tris-HCl buffer of pH 7.5 was used instead of pH 8.5. The product CMP-Neu5,9Ac₂ was stored as a lyophilized powder at –80 °C. This species was not completely stable and was broken down spontaneously during the lyophilization and storage to form some CMP and Neu5,9Ac₂ (42). 1,2-Diamino-4,5-methylene dioxybenzene (DMB) derivatization with or without NaBH₄ treatment (as described below) showed that the CMP-Neu5,9Ac₂ used in our assays contained ~50% Neu5,9Ac₂.

Preparation of [9-O-acetyl-³H]Neu5,9Ac₂—Radioactive substrate for NeuA esterase assays was prepared as previously described (43). Briefly, rat liver Golgi vesicles were prepared by the method of Leeleavathi (44) and subsequently labeled with [acetyl-³H]acetyl-coenzyme A, of which 75–85% was incorporated into O-acetylated Neu5Ac (45). Sias were released by

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treatment with *Arthrobacter urafaciens* sialidase and further purified using Dowex-1 and Dowex 3×4A ion exchange resins as previously described. Finally, any O-acetylation existing at the 7-carbon position of Sia was induced to migrate to the 9-carbon position by incubation of the purified Sia pool in 20 mM ammonium hydroxide at 37 °C for 30 min followed by rapid evaporation.

DMB Derivatization and HPLC Analysis of Sias—Sias were derivatized in 7 mM DMB, 18 mM sodium hydrosulfite, 1.4 M acetic acid, and 0.7 M 2-mercaptoethanol for 2 h at 50 °C in the dark. Typically, fresh 2 × DMB reagent was added to an equal volume of released Sias or reaction mixture. Derivatized Sias were resolved on a reverse phase C18 Microsorb-MV column (Varian; 4.6-mm internal diameter, 25 cm, 5 μm) using a Rainin Dynamax SD-200 high pressure liquid chromatograph and a 50-min isocratic elution in 8% acetonitrile and 7% methanol in water at a flow rate of 0.9 ml/min (45). C18 separation of Sias was followed by fluorescence detection using a Jasco FP-2020 Plus intelligent fluorescence detector. Standard Sias prepared from bovine submaxillary mucin as previously described (46) were derivatized in parallel for peak assignment. Integration of HPLC peaks was performed using Rainin R software, allowing calculation of O-acetylation as a percentage of total Sias.

NeuA Esterase Assays—Purified NeuA enzyme was incubated at a concentration of 1–4 μM with Neu5,9Ac₂ or [9-O-acetyl-³H]Neu5,9Ac₂ in 100 mM Tris-HCl, pH 7.5, in the presence or absence of 5 mM CTP and 20 mM MgCl₂ at 37 °C. Control reactions in the absence of enzyme or in the presence of an equimolar concentration of bovine serum albumin showed little background hydrolysis. When (nonradioactive) Neu5,9Ac₂ was used, reactions were quenched at the designated time interval by the addition of 2 × DMB derivatization reagent (see below), followed by incubation at 50 °C for 2 h and then HPLC separation and quantitation of starting material (Neu5,9Ac₂) and reaction product (Neu5Ac). When [9-O-acetyl-³H]Neu5,9Ac₂ was used, reactions were performed with 7,000–12,000 cpm of substrate (3–4 μM final concentration) and were quenched with an equal volume of 125 mM chloroacetic acid dissolved in a 1:1 mixture of 1 M NaOH and 4 M NaCl. Reactions to assess linearity of hydrolysis with varying enzyme concentration were performed in separate tubes; however, a single large reaction was set up to measure hydrolysis over time, with removal of reaction aliquots at various time intervals. Quenched reactions were allowed to sit on ice for 15 min, followed by a 5-min high speed centrifugation step to remove any precipitated protein. Typically, 80 μl of quenched reaction were added to 10 ml of scintillation mixture consisting of 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis(5-phenyloxazol-2-1)benzene (both from U. S. Biochemical Corp.) dissolved in toluene with the subsequent addition of 20% isoamyl alcohol. Released (protonated) free acetate is taken into the organic phase scintillation mixture, whereas the nonhydrolyzed starting material remains in the aqueous phase of the reaction mixture and is not counted. This is based on an assay originally reported for analysis of acetylcholinesterase activity (47).

Reduction of Free Sias in Reaction Products with Sodium Borohydride (NaBH₄)—CMP-Sias are cleaved under the acidic conditions of DMB derivatization. For this reason, the DMB-

HPLC method alone cannot distinguish between free and CMP-activated Sias produced during the NeuA reaction. We modified a previously described method (48) to detect CMP-activated Sias. Products of the NeuA reaction (~400 pmol) were treated with 80 mM sodium borohydride at room temperature for 20 min and then quenched with the addition of 5 M acetic acid (1:7 by volume). The DMB reagent was added directly to NaBH₄-treated or untreated samples. Parallel controls included commercially produced Neu5Ac (Pfanstiehl Laboratories, Waukegan, IL) and CMP-Neu5Ac (Sigma). In control reactions, Neu5Ac was destroyed by NaBH₄, and CMP-Neu5Ac remained >90% intact. Untreated Neu5Ac was used as a standard for quantitation of CMP activation and de-O-acetylation reactions.

¹H NMR Assays—Experiments were carried out in a total volume of 0.7 ml using 2.5 μM GBS NeuA and 2.5 mM substrate in the presence of cofactors (5 mM CTP, 20 mM MgCl₂) in 20 mM deuterated phosphate buffer, pH 7.5. Spectra were acquired every 2 min using 600 MHz ¹H NMR. Peak assignments were made by comparison with the ¹H NMR spectra of standards.

Strains and Culture Conditions—All GBS strains are isolates from human neonates with invasive infection. GBS were propagated on Todd-Hewitt agar plates or liquid broth at 37 °C without shaking, with 5–10 μg/ml erythromycin (Erm) or 2 μg/ml chloramphenicol (Cm) selection when indicated. Allelic exchange mutagenesis of *neuA* was performed in WT serotype III strains COH1 and D136 and serotype Ia strain A909. In addition, NeuA overexpression was performed in serotype Ib (H36B), V (NCTC), and Ia (KL90) strains.

Release of GBS Sias—Total bacterial Sias were released using 2 M acetic acid for 3 h at 80 °C (45). Alternatively, surface Sias were released using *A. urafaciens* sialidase (EY Laboratories) in 50 mM sodium acetate for 3 h at 37 °C.

Deletion, Complementation, and Overexpression of NeuA in GBS—The GBS *neuA* knock-out vector (pNeuA-KO) was constructed as described previously (17) and used for precise, in frame replacement of GBS *neuA* for a Cm acetyltransferase gene (*cat*), which confers resistance to Cm. Briefly, WT GBS strains were transformed by electroporation with pNeuA-KO with selection on Erm at 30 °C. Chromosomal integration events were identified by a shift to the nonpermissive temperature (37 °C) under Erm selection, followed by relaxation in the absence of antibiotics to identify double cross-over mutants with Cm resistance but Erm sensitivity. Precise in-frame allelic replacement of *neuA* by *cat* in the GBS chromosome was confirmed by PCR. A plasmid bearing GBS NeuA under a constitutive promoter (pGBS-NeuA) was generated as described previously (17) and introduced by electroporation to transform WT or NeuA mutant strains.

Site-directed Mutagenesis of *neuA*—Site-directed mutagenesis of NeuA asparagine 301 to alanine in pGBS-NeuA was accomplished with PAGE-purified primer 5'-ctatagggtagctgatctcattacagg-3' and its reverse complement in the QuikChange reaction (Stratagene) followed by DpnI digestion to remove residual template. DNA isolated by ethanol precipitation was used to transform electrocompetent *E. coli* MC1061, and plasmid pGBS-NeuA-N301A was confirmed by sequenc-

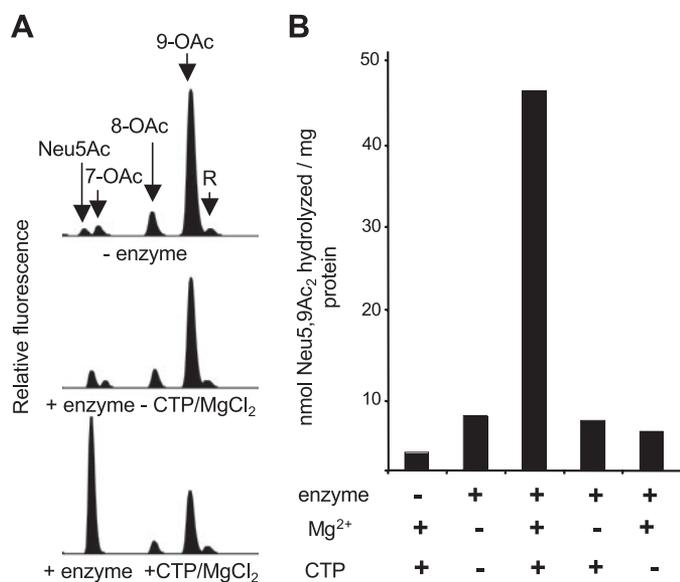


FIGURE 1. Purified GBS NeuA hydrolyzes 9-O-acetylated Neu5Ac in a CTP- and Mg²⁺-dependent manner. His-tagged GBS NeuA was purified, and 9-O-acetylated Neu5Ac (Neu5,9Ac₂ or 9-OAc) was synthesized using a chemoenzymatic approach as described under "Experimental Procedures." Enzyme assays were performed in 100 mM Tris, pH 7.5, with 1 μM enzyme and 8 μM substrate and allowed to proceed for 40 min followed by DMB derivatization and HPLC resolution of the 9-O-acetylated substrate (9-OAc) and non-O-acetylated product (Neu5Ac). R, a reagent peak of unknown identity. A, HPLC profiles of reactions performed in the presence or absence of enzyme or CMP-Sia synthetase co-factors (CTP and Mg²⁺). B, quantitation of Sia-O-acetyl hydrolysis by integration of Neu5Ac peak. Optimal NeuA esterase activity requires the presence of both CTP (5 mM) and MgCl₂ (20 mM).

ing to harbor only the single residue change in the *neuA* coding region.

RESULTS

GBS NeuA De-O-acetylates Free Neu5,9Ac₂ under Conditions That Maintain the NeuA CMP-Sia Synthetase Activity—The GBS O-acetyltransferase, NeuD, acts at the level of free intracellular Sia (17, 18). We hypothesized that the NeuA C-terminal esterase domain could act on free intracellular O-acetylated Sias to regulate capsular O-acetylation on the bacterial surface. Assays were designed to test this hypothesis *in vitro*, using purified recombinant GBS NeuA and synthetic Neu5,9Ac₂. Reaction products were monitored by reverse phase HPLC after fluorescent derivatization of Sias using DMB.

Initial experiments used reaction conditions identical to those previously published for the (presumably nonnative) substrates 4-nitrophenyl-acetate and platelet-activating factor (40). However, little difference was observed between HPLC profiles of the Neu5,9Ac₂ substrate in the presence or absence of purified NeuA enzyme (Fig. 1A). Given the putative bifunctional nature of this enzyme, co-factors of the CMP-Sia synthetase domain (CTP and Mg²⁺) were added in an attempt to boost the proposed secondary activity. The new conditions did, in fact, stimulate de-O-acetylation of Neu5,9Ac₂ as evidenced by a NeuA-dependent increase in the Neu5Ac reaction product by DMB-HPLC (Fig. 1A). Upon removal of either CTP or MgCl₂ from the reaction, esterase activity returned to near base line levels (Fig. 1B).

Spontaneous migration and hydrolysis of Sia-O-acetyl esters has been reported in several contexts, particularly under basic

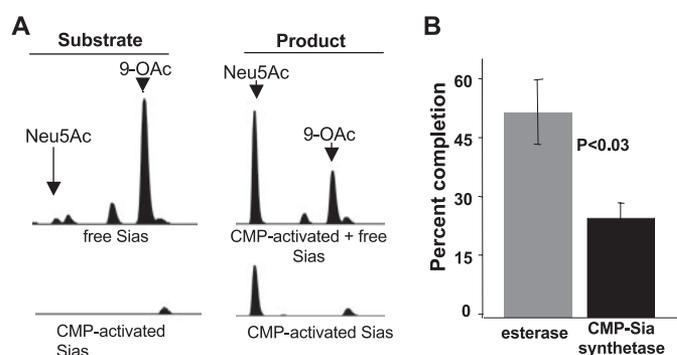


FIGURE 2. GBS NeuA can directly de-O-acetylate Neu5,9Ac₂. NeuA esterase and CMP-Sia synthetase activities were monitored to test the hypothesis that CTP/Mg²⁺ dependence is due to a processive mechanism. A, NeuA enzyme assays were performed in 100 mM Tris, pH 7.5, with (right) or without (left) 2 μM enzyme in the presence of 8 μM substrate and allowed to proceed for 30 min. The CMP-Sia bond is labile under the acidic conditions of DMB derivatization, thereby resulting in combined detection of free and CMP-bound Sias (top). Half of each reaction was treated with 80 mM freshly prepared NaBH₄ to destroy free Sias and detect only CMP-activated Sias (bottom). See the legend to Fig. 1 for complete description of peaks. B, percentage completion of CMP-synthetase and esterase reactions in A. Sia-O-acetyl hydrolysis was measured as a percentage of total Sia substrate in the reaction, using integration of Neu5Ac peaks in samples with or without enzyme without NaBH₄ treatment. CMP-Sia activation was likewise measured by integration of peaks in the NaBH₄-treated samples. Although 52% of the Neu5,9Ac₂ substrate was de-O-acetylated, only 25% of the free Sias were CMP-activated. These data are representative of two independent experiments.

conditions (17, 41). The current experiments were carried out at pH 7.5 with little background hydrolysis. De-O-acetylation did not occur in the presence of an equivalent concentration of a control protein, bovine serum albumin (data not shown). Additionally, hydrolysis of [9-O-acetyl-³H]Neu5,9Ac₂ was monitored over time and with different NeuA concentrations for further verification that the observed reaction had typical enzymatic properties, with product formation being approximately linear over time and with added enzyme (data not shown).

Taken together, these data suggested that the CMP-synthetase activity of NeuA might be required for *in vitro* esterase-mediated de-O-acetylation of free Neu5,9Ac₂. To explore the reaction mechanism further, we monitored CMP activation and de-O-acetylation reactions using a modified DMB derivatization procedure as well as by 600 MHz ¹H NMR.

CMP Activation of Sia Is Not a Prerequisite of the Sia-O-acetyl Esterase Reaction—Conditions of DMB derivatization result in the combined analysis of both free and CMP-activated Sias (since the CMP-Sia bond is hydrolyzed during derivatization). CMP-Sias can be distinguished from free Sias in a mixture, by selectively reducing the anomeric C-2 position of the free molecules using sodium borohydride (NaBH₄) (48). NaBH₄ reduction of free Sias renders them invisible in the DMB derivatization, since this reaction requires the intact α-keto (C-1/C-2) moiety of Sias and related sugars. Unfortunately, sodium borohydride can also cleave O-acetyl groups on Sia. Therefore, NeuA reactions were analyzed by DMB-HPLC with or without prior borohydride treatment to compare overall levels of O-acetyl hydrolysis and CMP activation during *in vitro* NeuA assays (Fig. 2).

Reaction products were incubated with or without NaBH₄ to reveal the degree of enzymatic de-O-acetylation (–NaBH₄) and

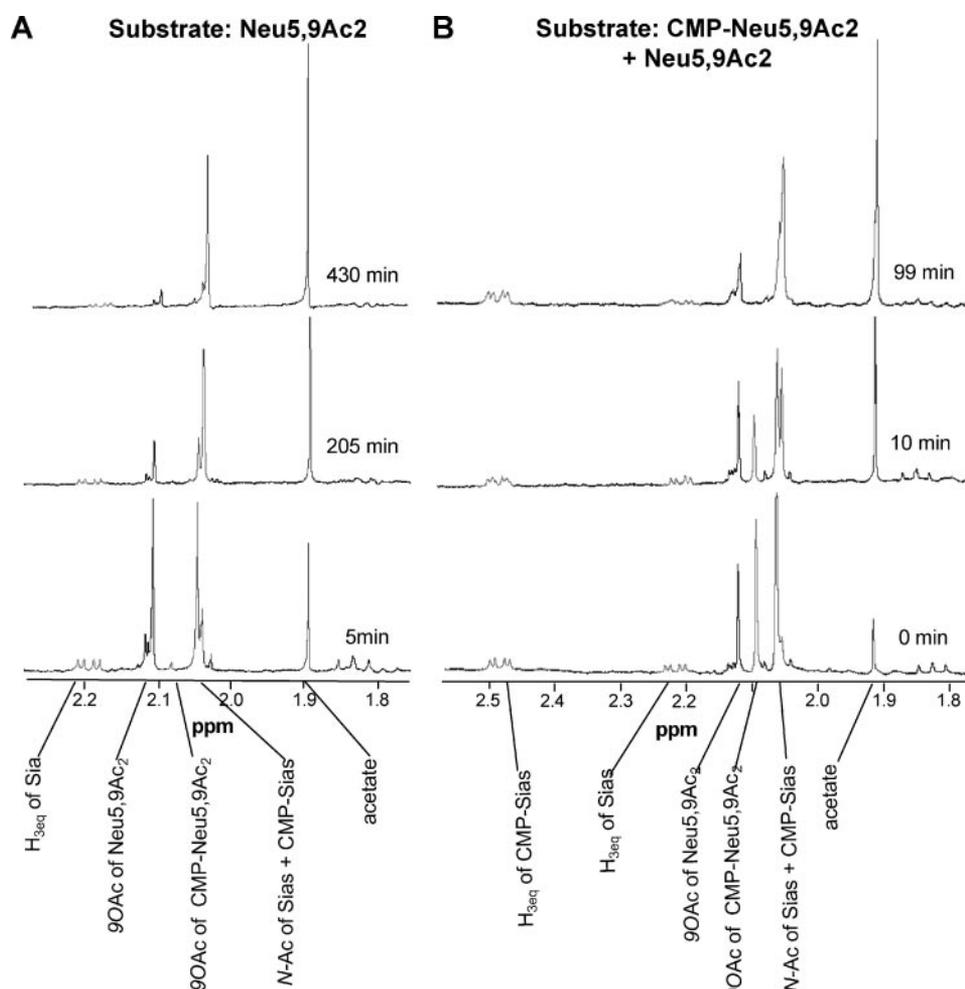


FIGURE 3. CMP-Neu5,9Ac₂ is a direct substrate of the NeuA esterase and is de-O-acetylated more rapidly than free Neu5,9Ac₂. NeuA reactions were monitored by 600 MHz ¹H NMR. Experiments were carried out at 37 °C in a total volume of 0.7 ml. Assays included 2.5 μM GBS NeuA with 2.5 mM substrate, CTP, and MgCl₂ in 20 mM deuterated phosphate buffer, pH 7.5. About 5 min elapsed between the addition of the enzyme and the acquisition of the first time point (defined as *t* = 0 min). NMR spectra were acquired every 2 min. Data shown are representative of the trends observed throughout the reaction. *A*, Neu5,9Ac₂ alone was used as substrate. *B*, enzymatically synthesized CMP-Neu5,9Ac₂ was used as substrate but contained a significant amount of free Neu5,9Ac₂, allowing internal comparison.

CMP activation (+NaBH₄) (Fig. 2A). Quantitation of each reaction showed that when Neu5,9Ac₂ is used as substrate, de-O-acetylation occurs more readily than CMP activation (Fig. 2B). If CMP-Neu5,9Ac₂ were the only substrate of the esterase, the extent of CMP-Sia formation should be greater than or equal to the extent of de-O-acetylation. The dominance of de-O-acetylation over CMP activation suggests that NeuA-mediated hydrolysis of Neu5,9Ac₂ does not require CMP activation and that free Neu5,9Ac₂ can be a direct substrate of the esterase reaction.

GBS NeuA Can Activate Neu5,9Ac₂, Followed by De-O-acetylation of CMP-Neu5,9Ac₂—Next, 600 MHz proton NMR was used to monitor the progress of NeuA reactions over time. Assays employed purified GBS NeuA with cofactors in deuterated phosphate buffer. Spectra were acquired every 2 min using 600 MHz ¹H NMR to monitor reaction progress. Assays using Neu5,9Ac₂ as the substrate showed immediate formation of CMP-Neu5,9Ac₂ (Fig. 3A). Although CMP-Neu5,9Ac₂ was observed at early time points, levels of the activated, O-acety-

lated molecule remained low and disappeared by 205 min (Fig. 3A). It was apparent that the level of substrate Neu5,9Ac₂ decreased over time; however, NMR was unable to determine whether this happened directly or via a CMP-activated O-acetylated intermediate. As expected, the de-O-acetylation reaction was mirrored by corresponding increases in the level of free acetate. The formation of CMP-Neu5Ac was also observed over time by increasing signal associated with protons at the C-3 position (not shown). These results showed that NeuA can activate Neu5,9Ac₂ to CMP-Neu5,9Ac₂ prior to de-O-acetylation. To determine whether this pathway (*i.e.* activation then de-O-acetylation) is the predominant NeuA mechanism, ¹H NMR monitoring was also performed on reactions containing both Neu5,9Ac₂ and CMP-Neu5,9Ac₂.

CMP-Neu5,9Ac₂ Is De-O-acetylated Rapidly by GBS NeuA—CMP-Neu5,9Ac₂ was generated enzymatically from Neu5,9Ac₂ using the *N. meningitidis* NeuA enzyme, which does not possess esterase activity (Fig. 4B). This product was then used as a tool to study the GBS NeuA esterase reaction. Protons of the 9-O-acetyl substituent of CMP-Neu5,9Ac₂ clearly decreased over time, with about half of the CMP-Neu5,9Ac₂ hydrolyzed by 10 min and the remaining substrate hydro-

lyzed by 99 min (Fig. 3B). Notably, some Neu5,9Ac₂ was also present in the starting material, allowing internal comparison of the two potential pathways. The signal arising from the 9-O-acetyl group of Neu5,9Ac₂ also decreased with time, yet much of this substrate remained at 99 min (Fig. 3B). Again, de-O-acetylation was accompanied by an increasing acetate signal. Importantly, CMP hydrolysis was not observed in this reaction, as evidenced by the equatorial hydrogen on C-3 that remained on both CMP-Neu5Ac and CMP-Neu5,9Ac₂ (Fig. 3B). These data showed that CMP-Neu5,9Ac₂ serves as a direct substrate of the NeuA O-acetyl esterase *in vitro*, a reaction that appears to occur more rapidly than de-O-acetylation of free Neu5,9Ac₂. Taken together, these results suggest that whereas there are two possible pathways *in vitro*, the activated molecule may be the preferred substrate *in vivo*.

Structure-activity Comparison of NeuA Homologs—Some sialylated bacterial pathogens like *E. coli* K1 have a NeuA enzyme composed of two domains homologous to those of GBS. Others, like serogroup C *N. meningitidis*, maintain only

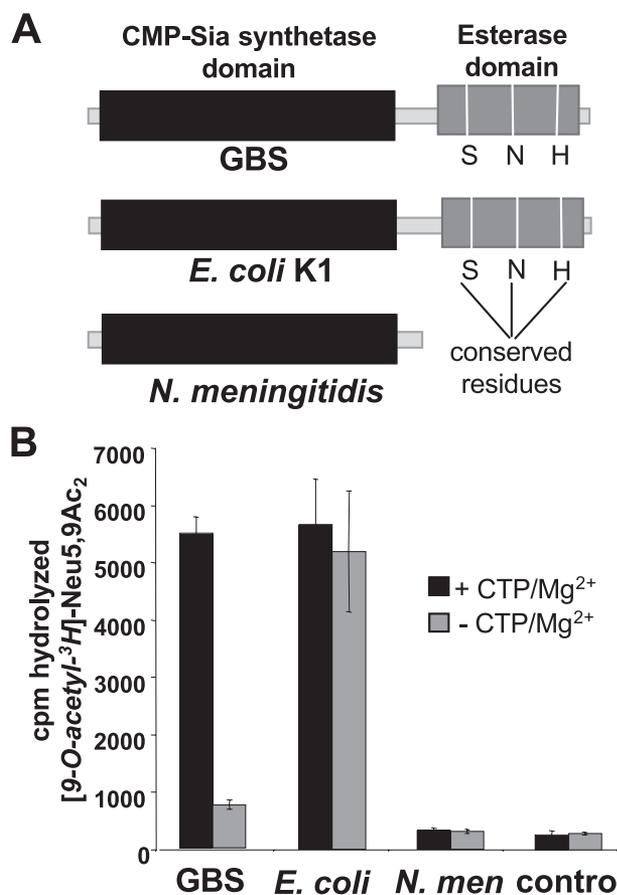


FIGURE 4. Divergence of co-factor dependence between the homologous bifunctional NeuA enzymes of GBS and *E. coli*. A, schematic representation of NeuA homologs in GBS, *E. coli* K1, and *N. meningitidis*. B, radioligand hydrolysis assay comparing requirements of NeuA esterase activity between the three homologs. Reactions were performed as described in the legend to Fig. 2 with (or without) 4 μ M enzyme in the presence or absence of 5 mM CTP and 20 mM MgCl₂. Bars, S.D. of two experiments performed on separate days.

the CMP-Sia synthetase domain and are thus not expected to possess Sia-O-acetyltransferase activity (Fig. 4A). Despite their overall structural similarity, we noted a striking difference between the activities of the GBS and *E. coli* Sia O-acetyltransferases. Whereas the GBS enzyme strongly favored conditions that maintain the CMP-Neu5Ac synthetase function, the *E. coli* enzyme hydrolyzed [9-O-acetyl-³H]-Neu5,9Ac₂ equally well in the presence or absence of CTP and Mg²⁺. This observation suggests that the bifunctional nature of NeuA may have diverged mechanistically along the two bacterial lineages.

Analysis of GBS Sias from Mutant Bacteria That Overexpress or Lack NeuA—To determine whether GBS NeuA esterase activity is important for O-acetyl modification *in vivo*, the focus of our studies turned toward characterization of NeuA in intact bacteria. First, a plasmid construct was used to deliver and overexpress GBS NeuA in five different WT GBS strains. Consistent with the observed *in vitro* esterase activity, fluorescent derivatization and HPLC analysis of acid-released Sias shows that overexpression of GBS NeuA resulted in decreased Sia O-acetylation in each of the GBS strains (Fig. 5).

In addition to NeuA Overexpression, chromosomal deletion of *neuA* was accomplished in three different WT GBS strains, through precise, in-frame allelic replacement of *neuA* with an

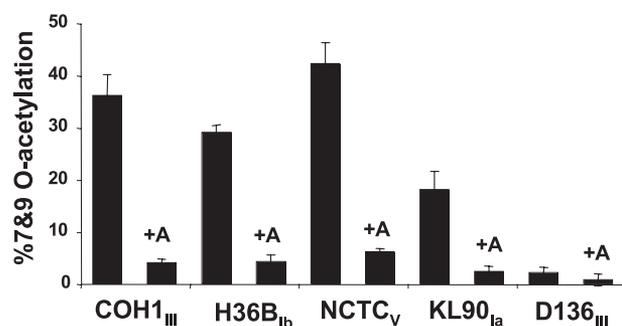


FIGURE 5. Overexpression of GBS NeuA decreases Sia-O-acetylation on GBS strains of multiple serotypes. WT GBS strains were transformed with an expression construct encoding the full-length NeuA enzyme (+A), and O-acetylation was compared with the parent strain. Total cellular Sias were isolated by mild acid hydrolysis and fluorescently derivatized with DMB as described under "Experimental Procedures." Derivatized Sias were resolved by reverse phase HPLC, and the percentage of O-acetylation was determined by software-assisted integration of HPLC peaks corresponding to O-acetylated and non-O-acetylated Neu5Ac peaks. HPLC profiles of the COH1 strain and corresponding NeuA-overexpressing strain were published previously (17).

antibiotic resistance cassette. Following DMB-HPLC analysis of total bacterial Sias, we found that deletion of *neuA* resulted in a significant increase in overall levels of Sia O-acetylation in all three strains. Consistent with the concomitant inability of GBS to activate Sias (*i.e.* loss of CMP-Sia synthetase activity), all Sias in the Δ NeuA strains were found in the intracellular compartment (Fig. 6), as we have previously described (17). Complementation of the mutant strains with plasmid-expressed NeuA reversed the intracellular hyper-O-acetylated phenotype into a capsular hypo-O-acetylated phenotype (Fig. 6).

Asn³⁰¹ Is Important for NeuA Esterase Activity, Which Limits Capsular O-Acetylation in Intact GBS—Because of the dual domains and activities of NeuA, the above experiments cannot definitively exclude the possibility that effects of (full-length) NeuA deletion and overexpression represent a pathway artifact introduced by alteration of the CMP-Neu5Ac synthetase activity. Whereas complete *neuA* deletion resulted in intracellular hyper-O-acetylation with no capsular Sia, we predicted that a specific disruption of NeuA esterase functionality, while preserving CMP-Neu5Ac synthetase activity, would result in a hyper-O-acetylated surface capsule.

Initial attempts to truncate the NeuA C-terminal esterase domain also affected CMP-Sia synthetase activity, as evidenced by the lack of capsular Sia upon introduction of the truncated NeuA enzymes into the GBS *neuA* mutant strains (data not shown). Thus, site-directed mutagenesis was employed to target NeuA esterase activity while avoiding (or minimizing) effects on the N-terminal CMP-Sia synthetase. Others have identified conserved residues within the GDSL family of esterases that are required for esterase/lipase activity (49, 50). In order to verify an essential role for one of these residues in GBS NeuA esterase activity, the asparagine at amino acid position 301 was mutated to alanine using site-directed mutagenesis. Following direct sequence confirmation of the mutated expression vector, NeuA N301A was introduced by transformation into the GBS NeuA mutant strains. Capsular Sias were released using *A. urafaciens* sialidase for analysis by DMB-HPLC.

As predicted, capsular O-acetylation was significantly increased in the N301A-complemented strains (Fig. 7). These

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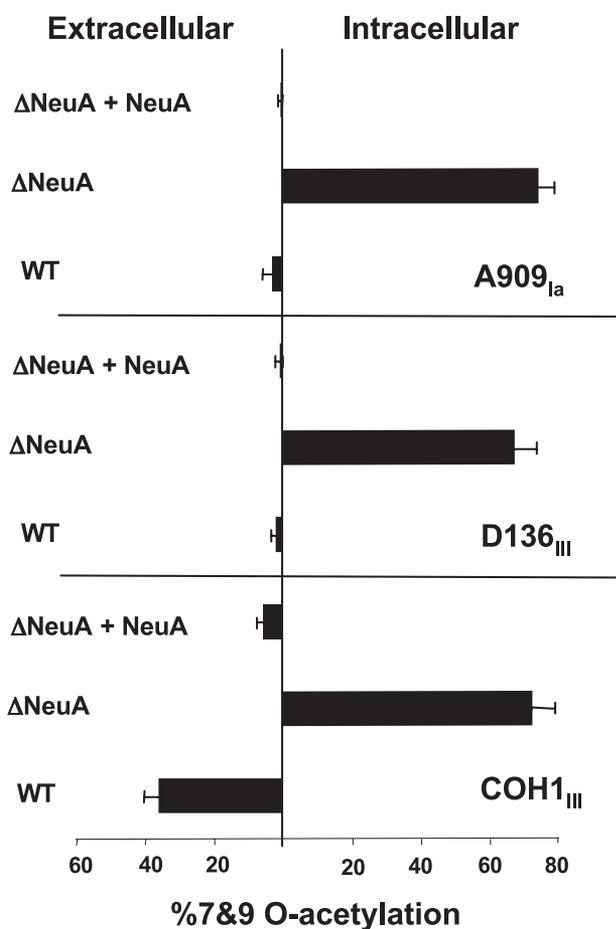


FIGURE 6. Deletion of GBS NeuA increases intracellular Sia-O-acetylation in both high O-Ac and low O-Ac strains. Elimination of NeuA (both CMP-Sia synthetase and esterase domains) in different GBS strains was accomplished by precise allelic replacement of the *neuA* gene with chloramphenicol acetyltransferase to produce the ΔNeuA or “ΔA” strains as described under “Experimental Procedures.” Mutant strains were complemented by plasmid-based expression of NeuA (+A) as in Fig. 4. Intracellular and extracellular Sias were separated as previously described (17), and the percentage of O-acetylation was determined by DMB-HPLC analysis. HPLC profiles of the wild-type COH1 and corresponding ΔNeuA strain were published previously (17). Bars, S.D. of two or more independent experiments.

data clearly demonstrate an important role for conserved residue Asn³⁰¹ in NeuA esterase activity, consistent with structural observations of *E. coli* thioesterase I, where the analogous residue participates in stabilization of the oxyanion generated during nucleophilic attack of the substrate (50). These data also confirm the essential role of NeuA esterase activity in limiting GBS capsular Sia-O-acetylation. Separation of intracellular (free) from extracellular (capsular) Sias as previously described (17) revealed no evidence of intracellular Sia accumulation, confirming preservation of CMP-Neu5Ac synthetase activity in the N301A-complemented strains (data not shown).

DISCUSSION

These studies define a role for NeuA Sia O-acetyltransferase activity in controlling the level of capsular Sia O-acetylation in GBS. *In vitro*, purified NeuA can act through two distinct pathways: de-O-acetylation of free Neu5,9Ac₂, followed by activation of Neu5Ac, or activation of free Neu5,9Ac₂, followed by de-O-acetylation of CMP-Neu5,9Ac₂. Taken together, our data

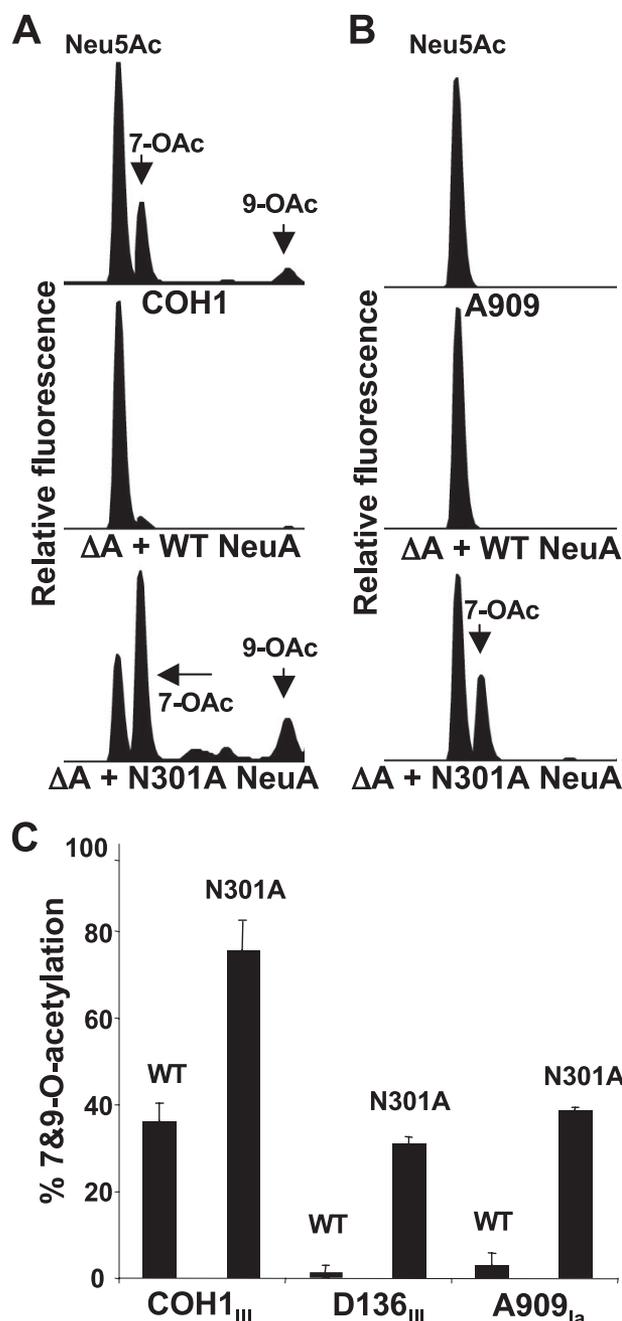


FIGURE 7. Active site mutagenesis of the NeuA esterase increases capsular O-acetylation. Site-directed mutagenesis of putative esterase active site residue Asn³⁰¹ was performed using the WT NeuA expression plasmid as template. WT NeuA and mutated N301A NeuA constructs were then used to complement all three GBS NeuA-deficient ΔA strains, followed by DMB-HPLC analysis of sialidase-released (capsular) Sias. Shown are raw HPLC data for WT, ΔA + WT NeuA, and ΔA + N301A NeuA strains in the COH1 (high OAc) (A) and A909 (low OAc) GBS (B) backgrounds. C, the percentage of O-acetylation of N301A-complemented NeuA-deficient ΔA + N301A NeuA strains is substantially higher than the respective isogenic WT NeuA-complemented strains. Further experiments employing separation of intracellular and extracellular Sias from each strain validated the lack of intracellular Sia accumulation and capsular hyper-O-acetylation of the ΔA + N301A NeuA strains (data not shown). Bars, S.D. of two or more independent experiments.

suggest that CMP-Neu5,9Ac₂ is the preferred substrate for the acetyltransferase activity of GBS NeuA *in vivo*. Overexpression and deletion of *neuA* in GBS have the effect of decreasing and increasing total Sia-O-acetylation, respectively, findings con-

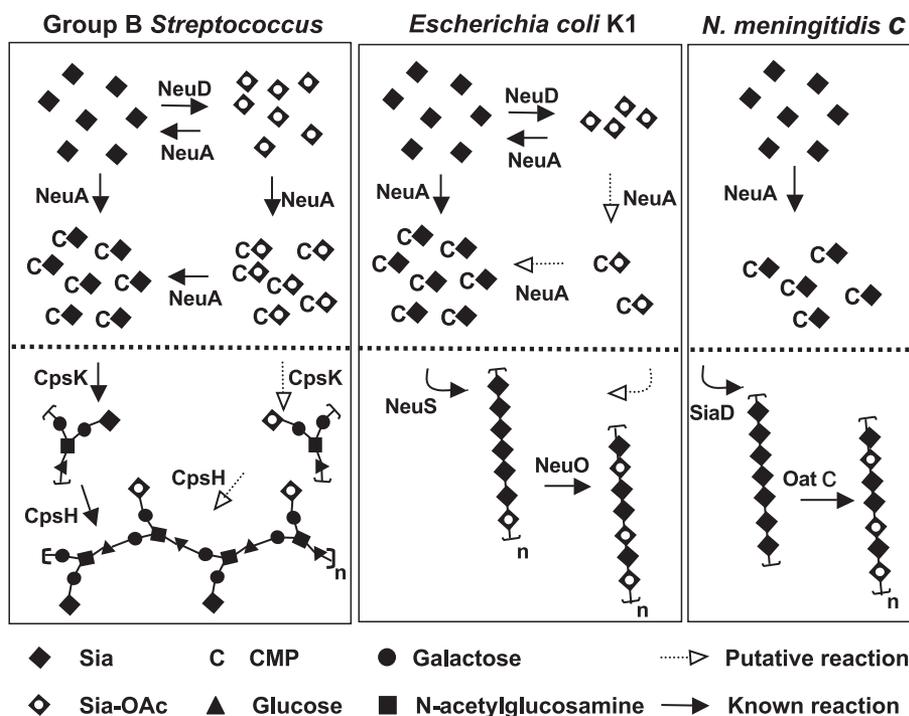


FIGURE 8. NeuA esterase activity in the context of capsule biosynthetic evolution in three prominent Sia-decorated pathogens GBS, *E. coli* K1, and serogroup C *N. meningitidis*. Bacterial Sia biosynthesis can be divided into three main parts: 1) Sia biosynthesis and degradation, 2) intracellular Sia *O*-acetylation and de-*O*-acetylation, and 3) polysaccharide biosynthesis, polymerization, and *O*-acetylation. For simplicity, only the second and third of these are shown (above and below the dotted line, respectively). With respect to the first, these Sia-expressing pathogens use a homologous three-enzyme pathway to generate ManNAc by epimerization of UDP-*N*-acetylglucosamine, condense phosphoenolpyruvate with ManNAc to generate Neu5Ac, and then activate Neu5Ac by transfer of the anomeric oxygen to the α -phosphate of CTP. Only *E. coli* possesses Sia degradation machinery. In GBS, *cpsK* and *cpsH* encode the sialyltransferase and repeating unit polymerase, respectively. Intracellular Sia *O*-acetylation and de-*O*-acetylation is limited to GBS and *E. coli*, both of which possess the NeuD Sia *O*-acetyltransferase and NeuA Sia *O*-acetyl esterase. The *N. meningitidis* pathway is more streamlined in appearance due to the lack of an endogenous *O*-acetylation/de-*O*-acetylation cycle. Despite the pronounced difference between *N. meningitidis* (serogroup C) and *E. coli* K1 in intracellular *O*-acetylation cycling, these pathogens share a poly-Sia antigen made up of Neu5Ac residues (polymerized by SiaD and NeuS, respectively) that can be *O*-acetylated after capsule polymerization (by OatC/NeuO, respectively). See "Discussion." The model is based on Refs. 17, 18, 38, and 51–61.

sistent with those observed *in vitro* esterase activity. Furthermore, site-directed mutagenesis of a conserved active site residue within the NeuA esterase domain (N301A) abolishes esterase activity while retaining CMP-Sia synthetase activity *in vivo*, resulting in GBS that expresses a hyper-*O*-acetylated CPS. Taken together, these data conclusively demonstrate that *O*-acetylated Sias are native substrates of the NeuA *O*-acetyltransferase, which acts prior to CPS assembly, thereby modulating the degree of GBS CPS Sia-*O*-acetylation.

Two Distinct Strategies of Sia-*O*-acetyl Regulation in GBS—WT GBS strains characterized to date modify between 5 and 55% of total Sias by *O*-acetylation (17). A polymorphism within the GBS *O*-acetyltransferase encoded by *neuD* is at least partially responsible for controlling the degree of *O*-acetylation in GBS, where 88C corresponds to the "low-*O*-Ac" phenotype (<5% *O*-Ac) and 88F corresponds to the "high-*O*-Ac" phenotype (>30% Sia-*O*-Ac) (18). Here we show that in high *O*-Ac GBS strains, NeuA prevents even greater levels *O*-acetylation (>70%) as observed in the NeuA esterase-inactive mutant (Fig. 5). In the low *O*-Ac strains, NeuA also prevents further *O*-acetylation, maintaining capsular Sia-*O*-Ac at a low level. These data indicate that the two phenotypes of capsular *O*-acetylation in

GBS are governed by the combined action of two molecular strategies: 1) the genetic polymorphism in *neuD* and 2) NeuA esterase activity. It remains a possibility that polymorphisms within the coding sequence of *neuA* also contribute to natural differences in *O*-acetylation between GBS strains. The two apparently distinct phenotypes of CPS *O*-acetyl modification suggest that GBS strains may have differentially optimized this biochemical parameter based on varied (and perhaps opposing) selective pressures exerted within the animal host.

New Understanding Enriches Model of GBS Sia *O*-Acetylation—In a previous publication, we suggested that NeuA overexpression may decrease Sia *O*-acetylation by altering pathway flux away from the *O*-acetyltransferase (NeuD), which competes for the same intracellular substrate, Neu5Ac (17). Taken together, our current analyses of the NeuA deletion and overexpression are unlikely to result simply from the architecture of the Sia biosynthetic pathway but instead may largely result from the active removal of some Sia-*O*-acetyl groups prior to capsular assembly. Our current studies now allow us to further enrich the model (Fig. 8) by

introduction of a new reaction (de-*O*-acetylation), which may occur at the level of free or CMP-activated intracellular Sias.

Interestingly, when Sias are released from the GBS surface using a sialidase at physiological pH, *O*-acetylation is found to occur predominantly at the 7-carbon position (Fig. 7). 7-*O*-acetyl groups are prone to migration along the exocyclic side chain of sialic acid (C-7 to C-9) over time under physiological conditions (41). This behavior has also been noted for Sias released from the GBS surface by mild acid hydrolysis (17, 18). Although spontaneous migration of the *O*-acetyl position makes the hypothesis difficult to test, we speculate that de-*O*-acetylation of 9-*O*-acetylated free and CMP-Sias intracellularly may allow the dominant expression of 7-*O*-acetylated capsular polysaccharide.

Evolution of Sia-*O*-acetyl Regulation: Comparison of Three Sialylated Pathogens—From an evolutionary perspective, the NeuA de-*O*-acetylation reaction provides an interesting vantage point for observing biochemical pathway optimization among different bacterial species. GBS, *E. coli* K1, and serogroup C *N. meningitidis* are all known to carry the *O*-acetyl modification on their sialylated capsular polysaccharides (17, 19, 21). However, these bacteria employ different combinations

Sia O-Acetyltransferase Activity of NeuA in GBS

of O-acetyltransferase- and O-acetyltransferase-encoding genes (18, 38), which may themselves produce enzymes with varied mechanisms and/or activity levels (Fig. 4) (51). We can compare and contrast the known parameters of O-acetyl regulation in these three pathogens, with reference to a simplified model of capsule biosynthesis (Fig. 8).

GBS, *E. coli*, and *N. meningitidis* generate the O-acetyl modification by different mechanisms involving O-acetyltransferases that act at the level of free intracellular Sia and/or the fully assembled CPS (17, 51). Whereas GBS uses *only* an intracellular O-acetylation mechanism and *N. meningitidis* probably uses only a capsular O-acetylation mechanism, *E. coli* can employ both intracellular and capsular O-acetylation mechanisms to arrive at the final level of surface modification (38, 51). All three bacteria can modulate O-acetylation levels by varying the amino acid sequence of their O-acetyltransferases. However, whereas *E. coli* K1 and *N. meningitidis* accomplish this by slipped strand mispairing of homopolymeric DNA tracts within their poly-Sia O-acetyltransferase genes (17, 51, 52), GBS strains appear to encode a single specific residue polymorphism in the free-Sia O-acetyltransferase gene that results in differential O-acetylation (18).

Despite the similarity of intracellular Sia anabolic pathways in GBS and *E. coli*, these pathogens appear to differentially regulate the O-acetylation/de-O-acetylation cycle catalyzed by NeuD and NeuA, respectively. This differential regulation is evidenced by the wide divergence of capsular O-acetylation specifically attributable to the intracellular process in GBS and *E. coli*. In GBS, NeuD-dependent O-acetylation typically results in >30% capsular Sia O-acetylation (18), indicating that the NeuA esterase does not hydrolyze O-acetylated Sias to completion *in vivo*. In *E. coli*, the intracellular O-acetylation mechanism results in only 2–4% O-acetylation of capsular Sias (38). As reviewed by Steenbergen *et al.* (38), intracellular O-acetylation in *E. coli* may be further regulated by several *E. coli*-specific parameters (38). Overall differences in intracellular Sia O-acetylation between GBS and *E. coli* may also be related to our observation that unlike GBS NeuA, *E. coli* NeuA operates independently of the co-factors necessary to maintain CMP-Sia synthetase activity (Fig. 4B).

In conclusion, our studies show that GBS NeuA displays native Sia-de-O-acetylation activity and demonstrate for the first time that O-acetyl removal prior to capsular assembly is a mechanism controlling the extent of capsular O-acetylation in living bacteria. Identification and site-directed mutation of the Sia-O-acetyl transferase (NeuD K123A) (18) and Sia O-acetyltransferase (NeuA N301A) have yielded inactive enzymes that produce a full-range of capsular Sia O-acetylation when introduced into GBS mutants where the corresponding WT genes have been deleted. This panel of isogenic GBS strains represents a key step toward understanding the role(s) of Sia O-acetylation and the mechanisms of Sia-dependent virulence at the host-pathogen interface.

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