

Genetic and biochemical modulation of sialic acid *O*-acetylation on group B *Streptococcus*: Phenotypic and functional impact

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Group B *Streptococcus* (GBS) is an important human pathogen and a model system for studying the roles of bacterial glycosylation in host–microbe interactions. Sialic acid (Sia), expressed prominently in the GBS capsular polysaccharide (CPS), mimics mammalian cell surface Sia and can interact with host Sia-binding proteins to subvert immune clearance mechanisms. Our earlier work has shown that GBS partially *O*-acetylates CPS Sia residues and employs an intracellular *O*-acetylation/de-*O*-acetylation cycle to control the final level of this surface Sia modification. Here, we examine the effects of point mutations in the NeuD *O*-acetyltransferase and NeuA *O*-acetyl esterase on specific glycosylation phenotypes of GBS, pinpointing an isogenic strain pair that differs dramatically in the degree of the *O*-acetyl modification (80% versus 5%) while still expressing comparable levels of overall sialylation. Using these strains, higher levels of *O*-acetylation were found to protect GBS CPS Sia against enzymatic removal by microbial sialidases and to impede engagement of human Siglec-9, but not to significantly alter the ability of GBS to restrict complement C3b deposition on its surface. Additional experiments demonstrated that pH-induced migration of the *O*-acetyl modification from the 7- to 9-carbon position had a substantial impact on GBS–Siglec-9 interactions, with 7-*O*-acetylation exhibiting the strongest interference. These studies show that both the degree and position of the GBS *O*-acetyl modification influence Sia-specific interactions relevant to the host–pathogen relationship. We conclude that native GBS likely expresses a phenotype of intermediate Sia *O*-acetylation to strike a balance between competing selective pressures present in the host environment.

Keywords: capsular polysaccharide/*N*-acetylneuraminic acid/*sialidase*/*Siglec*/*Streptococcus agalactiae*

Introduction

Group B *Streptococcus* (GBS) is a Gram-positive bacterial pathogen that colonizes the lower digestive and vaginal tract in 25–35% of healthy women (Campbell et al. 2000). GBS is the leading agent of bacterial sepsis and meningitis in newborns (CDC, C.f.D.C.a.P. 2005), acquired by the fetus through invasion of placental membranes or upon aspiration of contaminated vaginal fluids during passage through the birth canal. GBS is increasingly recognized as a cause of serious infections in elderly, diabetic, and immune-compromised individuals (Edwards and Baker 2005; Maisey et al. 2008).

The GBS capsular polysaccharide (CPS) is a critical virulence factor in animal models, a function largely attributable to the presence of terminal α 2-3-linked sialic acids (Sias) on each of the branched repeating units of the polymer (Wessels et al. 1989). GBS Sia expression represents a form of molecular mimicry, wherein the bacteria decorate its own surface with a sugar moiety expressed in abundance on the surface of all mammalian cells. While there are nine antigenically and structurally distinct GBS capsular serotypes (Baker and Kasper 1985), the repeat unit of the polymer invariably contains this hallmark terminal Sia motif. GBS belonging to serotype III are disproportionately associated with late-onset bacteremia and meningitis, highlighting a particular predilection of these serotype strains to resist host innate immune clearance mechanisms. Sia expression is also an important characteristic of the human bacterial pathogens *Neisseria meningitidis*, *N. gonorrhoea*, *Haemophilus influenzae*, *Campylobacter jejuni*, and *Escherichia coli* K1 (Vimr et al. 2004). *N*-acetylneuraminic acid (Neu5Ac) is the most common Sia found in nature, and the predominant Sia expressed by these microbes and their human host.

Classically, GBS capsular Sia is recognized to promote resistance to immunologic clearance by interfering with alternative complement pathway activation and accumulation of opsonic C3b on the bacterial cell surface (Edwards et al. 1982). In recent studies, an additional mechanism by which bacterial Sia expression can influence host–pathogen dynamics has been uncovered. The Sia-binding immunoglobulin superfamily lectins (Siglecs) are expressed on leukocytes and function to regulate their activation and differentiation (Crocker et al. 2007). GBS of various serotypes bind human Siglecs in a Sia- and Siglec-specific fashion (Carlin et al. 2007), and GBS serotype III engagement of human Siglec-9 (hSiglec-9) was demonstrated to reduce neutrophil activation and bactericidal capacities (Carlin et al. 2009). GBS neutrophil suppression via Siglecs involves recruitment of SHP protein tyrosine phosphatases to immunoreceptor tyrosine-based inhibitory motifs (ITIM) on the receptor's intracellular domain (unpublished observations), a process known to antagonize immune signaling cascades (Avril et al. 2006).

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A further level of complexity in understanding GBS Sia function emerged when it was discovered that GBS of all serotypes partially *O*-acetylate the Neu5Ac residues on their CPS (Lewis et al. 2004). Subsequent biochemical and mutagenesis studies identified two enzymes encoded in the GBS capsule biosynthetic operon as responsible for controlling GBS Sia *O*-acetylation levels. The addition of an *O*-acetyl group is carried out by the *O*-acetyltransferase NeuD, which also plays an essential role in GBS Sia biosynthesis (Lewis et al. 2006). NeuD-catalyzed *O*-acetylation first appears on the GBS surface at the 7-carbon position of the Neu5Ac exocyclic side chain (Lewis et al. 2004), but spontaneous migration of the *O*-acetyl ester from the 7- to the 9-carbon position occurs at pH above 7.0 or below 3.0 (Varki and Diaz 1984; Kamerling et al. 1987; Lewis et al. 2004). Modulation of Neu5Ac *O*-acetylation levels is achieved by a Sia *O*-acetyl esterase activity present in the C-terminal domain of NeuA, itself a bifunctional enzyme that serves as the GBS CMP-Sia synthetase (Lewis et al. 2007). The NeuA *O*-acetyl esterase acts intracellularly to remove a subset of the Sia modifications prior to polymerization of the CPS (Lewis et al. 2007).

The *O*-acetyl modification of Sia residues in mammalian cell surface glycoconjugates is known to influence their recognition by specific lectins and their susceptibility to sialidase enzymes (Shi et al. 1996; Powell and Varki 2001; Chokhawala et al. 2007; Cariappa et al. 2009). Evaluating the potential influence of variable *O*-acetylation upon Sia-dependent molecular interactions is thus important for understanding GBS pathogen–host dynamics. However, potential pitfalls abound in the genetic manipulation of these enzymes since both the NeuD *O*-acetyltransferase and NeuA *O*-acetyl esterase play roles in both Sia biosynthesis and regulation of the *O*-acetyl modification (Lewis et al. 2006; Lewis et al. 2007). Here, we perform thorough biochemical and phenotypic analyses of GBS strains with targeted gene deletion or active site mutation of NeuD and NeuA to successfully isolate Sia *O*-acetylation as a single chemical variable. An isogenic strain pair engineered to express either high or low *O*-acetylation, but similar overall Sia is then utilized, to determine the effect of this modification on GBS complement activation, human Siglec-9 binding, and susceptibility to sialidases. The results suggest that GBS may have evolved a phenotype of intermediate Sia *O*-acetylation in response to competing selective pressures present in the host environment.

Results

Deletion of the gene encoding either the NeuD Sia *O*-acetyltransferase or the NeuA Sia *O*-acetyl esterase/CMP-Sia synthetase leads to complete loss of Sia expression in the GBS CPS (Lewis et al. 2004, 2006). As summarized schematically in Figure 1, single amino acid substitutions to alanine in the enzyme active sites of NeuD (K123A) or NeuA (N301A) can ablate the *O*-acetyltransferase and *O*-acetyl esterase activities, respectively (Lewis et al. 2006, 2007), allowing a more precise level of control of Sia modification. In addition, a naturally occurring polymorphism in NeuD (88F versus 88C) is functionally associated through an unknown mechanism with high or low levels of acetylation, respectively (Lewis et al. 2006). We used these available genetic tools to generate a panel of nine strains in the serotype III GBS background (Figure 1): WT par-

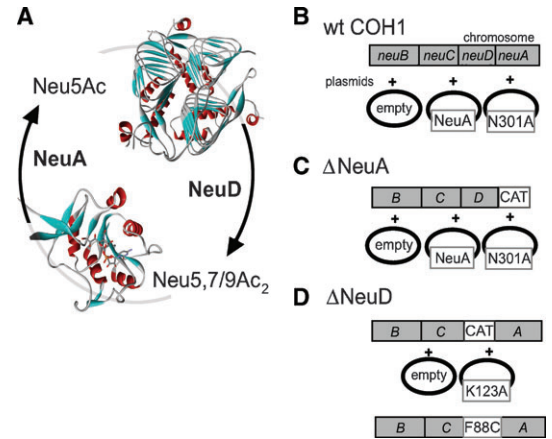


Fig. 1. Genetic engineering of sialic acid metabolism. Schematic depictions of the intracellular cycle of Sia *O*-acetylation in GBS and the isogenic strain sets used in this study. (A) Homology models of NeuD, the GBS intracellular *O*-acetyltransferase and NeuA, the GBS *O*-acetyl esterase, which participate in a cycle of Sia *O*-acetylation and de-*O*-acetylation resulting in the final level of surface Sia modification on GBS. Both enzymes have bifunctional roles in Sia synthesis that are not fully depicted in the schematic. Site-directed mutagenesis of transferase and esterase active sites allows specific control of the *O*-acetyl modification. Polymorphism at amino acid position 88 of NeuD can also lead to natural variations in *O*-acetylation between GBS strains (Lewis et al. 2006). The *O*-acetyl modification is originally located at the 7-carbon position followed by a unidirectional migration along the exocyclic side chain to the 9-carbon position (Lewis et al. 2004). (B–D) Sia biosynthetic genes *neuA* and *neuD* were deleted, mutated, and/or overexpressed in the GBS parent strain COH1 as described in the *Material and methods*. CAT refers to the chloramphenicol acetyltransferase gene cassette used to create in-frame replacements.

Table I. Summary of isogenic-type III GBS strains and their phenotypes

Strain		<i>O</i> Ac	Sia	Gal	CPS
Chromosomal	Plasmid (pDCerm)				
WT (COH1)	Empty	++	+++	–	++
	WT <i>neuA</i> ^a	–	++++	–	++
	N301A <i>neuA</i>	++	++++	–	++
Δ <i>neuA</i>	Empty	–	–	++++	+
	WT <i>neuA</i> ^a	–	++++	–	++
	N301A <i>neuA</i> ^b	++++	++++	–	++
Δ <i>neuD</i>	Empty	–	–	++++	+
	K123A <i>neuD</i>	–	++	+	++
NeuD F88C	None	–	++	+	++

Summary of bacterial glycosylation phenotypes as detailed in Figures 2–5. Sia and *O*-acetylation levels were determined by DMB-HPLC, exposed galactose was quantitated by flow cytometry using the lectin ECA, and CPS was evaluated by relative buoyancy as described in the *Material and methods*.

^aIndicates *O*Ac^{low}

^bIndicates *O*Ac^{high} strains used in Figures 6–8.

ent, WT with 88C NeuD polymorphism introduced, WT overexpressing NeuA or enzyme dead NeuA (N301A), Δ*neuD* mutant, Δ*neuD* mutant + enzyme dead NeuD (K123A), Δ*neuA* mutant, Δ*neuA* mutant + WT NeuA or enzyme dead NeuA (N301A). These strains were then analyzed for total Sia expression, degree of *O*-acetylation, level of underlying galactose exposure, hydrophobicity, and buoyancy (summarized in Table I)—with the aim of pinpointing a strain pair that differed only in Sia *O*-acetylation, thus allowing further examination of the functional consequences of this specific modification on the GBS surface.

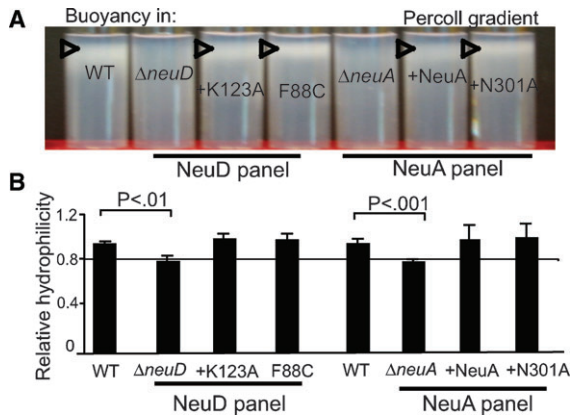


Fig. 2. GBS strains produced by isogenic manipulation of Sia metabolic genes were evaluated for (A) their overall buoyancy after slow-speed centrifugation through a layered Percoll gradient as described in the Materials and Methods. Arrow heads emphasize a layer of buoyant bacteria that do not enter the Percoll gradient. (B) GBS strains were evaluated for their overall hydrophilicity using a standard optical density-based assay to quantitate cells that remain in an aqueous phase when given the opportunity to adhere to a hydrophobic *n*-hexadecane phase.

GBS buoyancy and hydrophilicity are influenced by overall CPS Sia but not by variation in Sia O-acetylation-specific enzyme activities

Increased buoyancy of GBS is a strong surrogate measure for overall encapsulation (Hakansson et al. 1987, 1988). Buoyancy phenotypes were evaluated in undisturbed, nonshaking overnight cultures and after slow speed centrifugation through a Percoll density gradient. While overall growth of the Sia-deficient ($\Delta neuD$ or $\Delta neuA$) strains by colony forming unit enumeration did not differ significantly (data not shown), these strains accumulated in a flocculant pellet at the bottom of the culture tube, leaving the media transparent (data not shown). In contrast, the WT parent and other strains in the panel remained in suspension. Similar findings were observed in the Percoll gradient analysis, where only the Sia-deficient $\Delta neuD$ and $\Delta neuA$ strains penetrated deeper based on reduced buoyancy/increased density (Figure 2A). These findings are consistent with published observations that loss of CPS Sia due to deletion of sialyltransferase *cpsK* reduces overall GBS encapsulation (Chaffin et al. 2005). These data further suggest that complementation of the $\Delta neuD$ and $\Delta neuA$ strains with plasmids bearing either WT or mutated versions of the deleted genes restores GBS encapsulation to WT or near-WT levels. Bacterial CPS expression also provides an enveloping hydrophilic barrier. Overall bacterial hydrophobicity was assessed using an *n*-hexadecane partition assay (Buchanan et al. 2005). Once again, only the Sia-negative strains ($\Delta neuD$ and $\Delta neuA$ mutants) were found to exhibit significantly more association with the hydrophobic phase when compared to WT (Figure 2B).

Manipulations of Sia O-acetylation via the NeuD acetyltransferase also perturb overall Sia expression on the GBS surface

NeuD was examined as a candidate for targeted *O*-acetyl manipulation since a naturally occurring polymorphism was correlated with high (88F, 30–50% *O*-acetylation) versus low (88C, 5–10% *O*-acetylation) levels of this Sia modification in the CPS

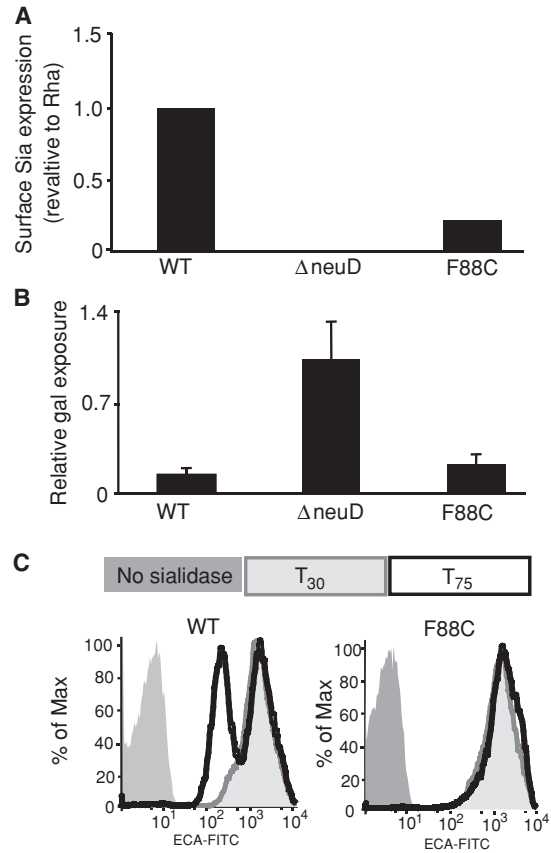


Fig. 3. Genetic manipulation of NeuD by introduction of the natural “low-*O*-acetylation” polymorphism has unintended consequences for Sia expression. (A) Relative Sia expression was measured as described in the *Material and methods* in overnight cultures of WT GBS and compared to isogenic strains in which NeuD was either deleted or replaced with the “low-*O*-acetyl” polymorphic allele of NeuD (88C, Lewis et al. 2006). (B) The extent of Galactose exposure (underlying Sia) was evaluated by flow cytometry using a FITC-conjugated ECA lectin. Data from three independent experiments were normalized based on the signal from the $\Delta neuD$ strain and presented as average relative fluorescence intensity \pm standard deviation. (C) Resialylation assays using WT and F88C GBS were carried out by desialylating GBS with *Arthrobacter urafaciens* sialidase (AUS) and allowing the bacteria to resialylate over the designated time interval. Flow cytometry-based lectin (ECA) recognition of galactose residues (the structure underlying GBS capsule Sias) was used to monitor the kinetics of CPS resialylation as evidenced by the decreased lectin interaction.

of clinical strains (Lewis et al. 2006). As previously published, changing the *neuD* gene sequence in GBS WT strain COH1 to encode 88C instead of 88F markedly reduced *O*-acetylation levels (Lewis et al. 2006). However, in the present more thorough analysis of the NeuD F88C strain (Lewis et al. 2006), it was apparent that decreased surface sialylation accompanies decreases in surface Sia *O*-acetylation (Figure 3A). In contrast to the Sia-negative $\Delta neuD$ mutant, the F88C mutant strain showed only a modest increase (compared to WT) in the extent of surface-exposed galactose, the sugar residue that immediately underlies Sia in the GBS CPS repeating units (Figure 3B). To further probe this difference, we performed an assay measuring the kinetics of Sia addition after complete enzymatic surface desialylation of the GBS WT versus F88C mutant CPS. This assay showed that sialidase-exposed galactose residues were resialylated more quickly in the WT strain than the F88C mutant (Figure 3C).

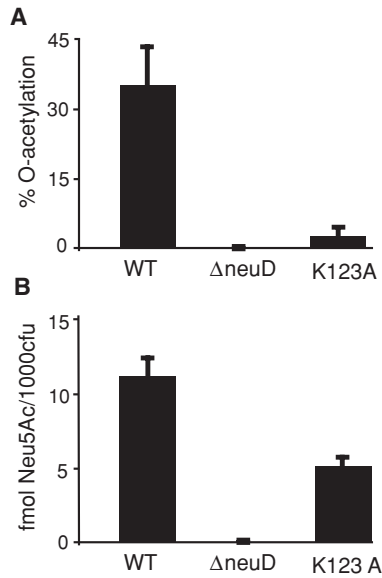


Fig. 4. *O*-Acetyltransferase activity is required for optimal surface sialylation. Isogenic strains varying in the expression or absence of the GBS *O*-acetyltransferase NeuD or the active-site mutated NeuD (K123A) were evaluated for relative *O*-acetylation (as published previously) and surface sialylation relative to colony forming units (cfu).

These data suggest that the F88C polymorphism of NeuD plays a complex role in the surface glycosylation of serotype III GBS, and its impact on overall Sia content makes it an unsuitable candidate for independent manipulation of *O*-acetylation levels.

The K123A active-site mutation of NeuD was created in order to more precisely target the *O*-acetyltransferase activity of the enzyme, apart from its apparent dual role in Sia biosynthesis (Lewis et al. 2006). Complementation of the $\Delta neuD$ mutant with a plasmid expressing the mutated active site K123A NeuD enzyme markedly reduced overall *O*-acetylation (Figure 4A), but failed to restore the fully sialylated CPS of the WT strain (Figure 4B). Thus, engineering of *neuD* activity, even by active site mutation, does not provide specific variation of *O*-acetylation as an independent chemical phenotype.

GBS Sia O-acetylation can be manipulated via the NeuA acylesterase activity while maintaining consistent overall surface Sia expression

The NeuA *O*-acylesterase activity offered a second potential target toward a goal of manipulating GBS surface *O*-acetylation in a background of consistent total CPS sialylation. We had previously shown that a N301A mutation in the C-terminal NeuA *O*-acylesterase active site increases GBS surface Sia *O*-acetylation by eliminating this counter-regulatory enzymatic activity (Lewis et al. 2007). Here, we expressed either WT NeuA or the esterase-deficient N301A NeuA on plasmids in the WT GBS parent strain COH1 or its isogenic $\Delta NeuA$ mutant. In these cases, the level of surface Sia *O*-acetylation was significantly higher in the strains expressing the esterase-deficient N301A NeuA compared to those expressing the WT NeuA enzyme (Figure 5A); however, the total amount of CPS Sia in each pair was nearly identical (Figure 5B). A slight increase in total surface Sia expression of all NeuA (WT or N301A) over-

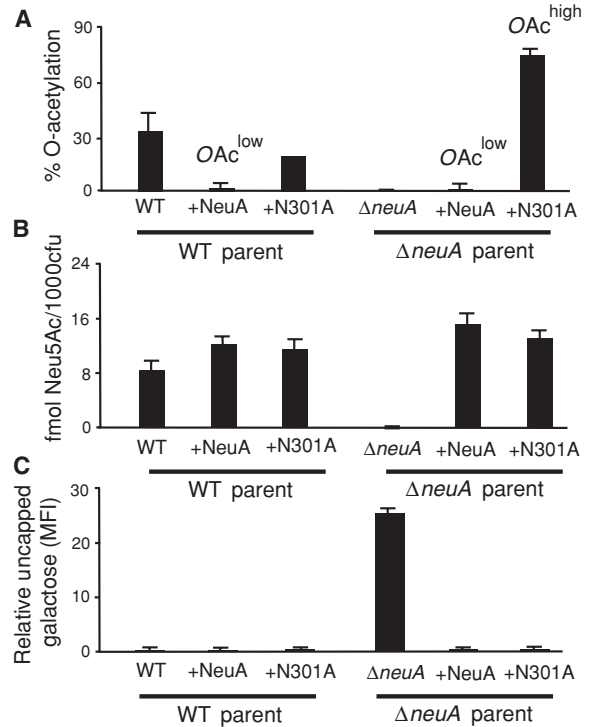


Fig. 5. Modulation of sialic acid *O*-acylesterase activity allows specific alteration of sialic acid *O*-acetylation without loss of sialylation. GBS strains with alterations of NeuA esterase function by chromosomal deletion, overexpression, or site-specific inactivation, as described in the *Material and methods* and depicted in Figure 1, were subjected to chemical analyses of (A) the extent of *O*-acetylation, (B) total surface Sia expression, and (C) Exposure of galactose residues, which normally underlie Sias on the CPS. Data are presented as the average median fluorescence intensity of three independent experiments \pm standard deviation. Strains hereafter referred to in the text as “OAc^{high}” and “OAc^{low}” are depicted.

expressing strains (Figure 5B) likely reflects increased NeuA N-terminal CMP-synthetase function, which activates free intracellular Sia acid residues for incorporation into the CPS. For further analysis of the specific effect of *O*-acetylation on GBS Sia-dependent functions, we compared the very low *O*-acetylation (<5%) strains WT + NeuA or $\Delta neuA$ + NeuA, hereafter designated “OAc^{low},” to the high *O*-acetylation (~80%) strain $\Delta neuA$ + N301A NeuA, hereafter designated “OAc^{high}.” As summarized in Table I, these strains did not differ significantly in overall buoyancy (Figure 2A), hydrophobicity (Figure 2B), exposure of underlying galactose residues (Figure 5C), or CPS Sia content (Figure 5B).

Sia O-acetylation level does not significantly affect complement deposition on the GBS surface

An important GBS virulence phenotype associated with CPS Sia expression is inhibition of alternative complement activation. The complement regulatory protein factor H can recognize host and bacterial Sia, inhibit C3 convertase activity, and facilitate C3b degradation (Pangburn et al. 2009), thereby reducing the amount of opsonic C3b on the cell surface. In murine erythrocytes and cancer cell lines, *O*-acetylation of Sia appears to impair human factor H binding, resulting in increased complement-mediated lysis of these cells (Shi et al. 1996; Varki

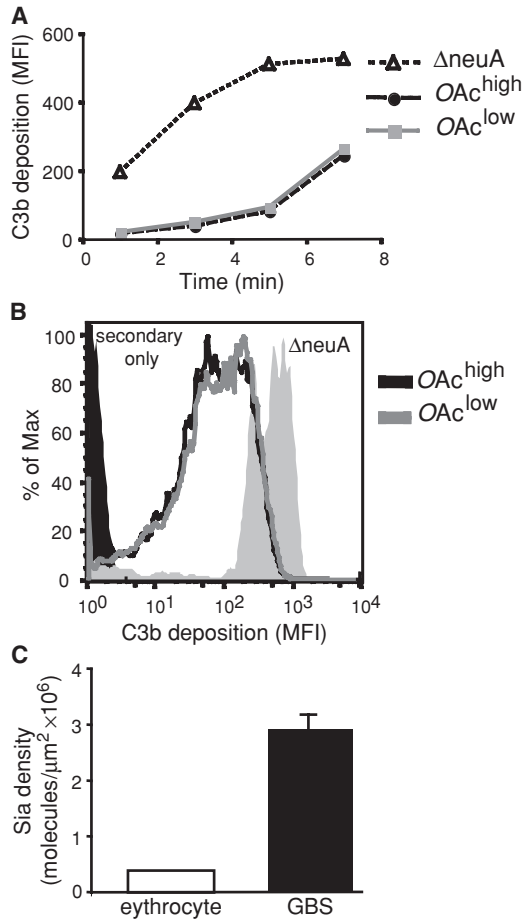


Fig. 6. GBS *O*-acetylation does not impact resistance of the organism to complement opsonization. (A) Isogenic OAc^{high} and OAc^{low} GBS strains were incubated in 50% human serum and the extent of C3b deposition was analyzed by flow cytometry following staining with FITC-labeled anti-human C3b and expressed as median fluorescence intensity. (B) A representative histogram of the 5-min timepoint. (C) Sia density reveals a possible explanation for why *O*-acetylation increases susceptibility of mammalian erythrocytes, but not GBS to the effects of complement.

and Kornfeld 1980), but the effect of bacterial Sia *O*-acetylation on complement deposition has not been studied.

We incubated the isogenic OAc^{low} and OAc^{high} GBS strains in 50% serum for up to 10 min, washed in PBS, incubated with the FITC-conjugated anti-human C3b antibody, and quantified C3b deposition by flow cytometry (Figure 6A, B). The $\Delta neuA$ Sia-negative strain was included in these experiments as a control. As expected from previous studies (Edwards et al. 1982), the $\Delta neuA$ strain showed ~200-fold higher C3b deposition than the OAc^{low} and OAc^{high} strains, even within 1 min (Figure 6A). However, there was no difference in C3b deposition between the OAc^{low} and OAc^{high} strains under these conditions. Thus, in contrast to the finding with murine cells, *O*-acetylation of up to 80% of the Sia in the GBS CPS still did not reduce the ability of the bacterium to inhibit the complement deposition. A possible explanation for this difference is found in the extremely high density of Sia expression on the GBS surface compared to mammalian erythrocytes (Figure 6C). The consistent display of some non-*O*-acetylated Sia on the CPS, as ensured by the

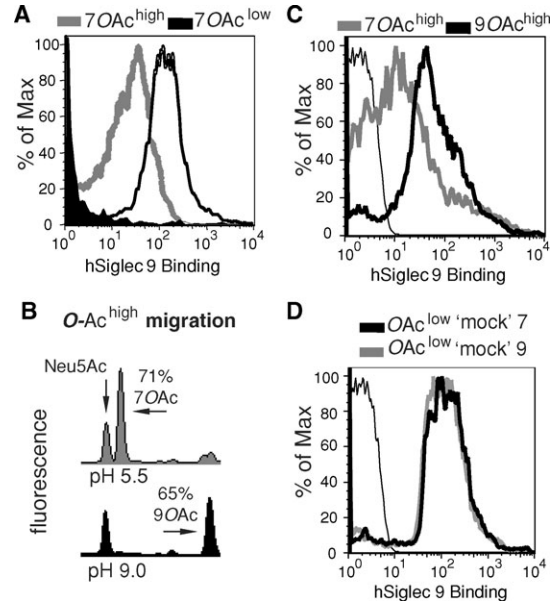


Fig. 7. The amount and placement of CPS Sia *O*-acetylation influences GBS binding to human Siglec-9. (A) Isogenic OAc^{high} and OAc^{low} GBS strains were incubated with hSiglec9-Fc chimeric proteins and analyzed for binding by flow cytometry using a goat anti-human (IgG-Fc)-PE conjugated secondary antibody. (B) The OAc^{high} strain was subjected to pH-dependent *O*-acetyl migration as described in the *Material and methods* and analyzed by DMB-HPLC to examine the amount and position of the *O*-acetyl ester on GBS surface Sias. Percent of total Sias *O*-acetylated at each position is shown. Retention times of Neu5Ac, Neu5,7Ac₂, and Neu5,9Ac₂ were 11.8, 12.9, and 19.5 min, respectively. (C) GBS displaying the OAc^{high} phenotype at positions 7 or 9 or (D) OAc^{low} GBS incubated under identical conditions were analyzed for interactions with hSiglec9-Fc by flow cytometry. Parallel control experiments included the secondary antibody alone (A) and Sia-deficient $\Delta NeuA$ GBS (C and D).

O-acetyltransferase activity of NeuA, may preserve a sufficient reservoir on the GBS surface for factor H binding and subsequent reduction of bacterial C3b opsonization, despite high levels of Sia *O*-acetylation.

O-Acetylation of GBS capsular Sia reduces human Siglec-9 binding

Human Siglec-9 is expressed prominently on the surfaces of neutrophils and macrophages (Angata and Varki 2000; Zhang et al. 2000) and possesses intracellular inhibitory motifs (ITIMs) that send negative signals thought to dampen immune responses upon engagement of sialylated ligands (Crocker et al. 2007). Recent studies have shown that GBS CPS engagement of hSiglec-9 downregulates neutrophil responses to GBS, subverting neutrophil killing of the bacteria (Carlin et al. 2009). To determine whether the *defined* variable of *O*-acetylation impacts GBS interactions with hSiglec-9, we compared the isogenic OAc^{high} and OAc^{low} strains in a flow cytometry-based hSiglec-Fc binding assay. We found an approximately 5-fold reduction of Siglec-9 binding to the OAc^{high} compared to the OAc^{low} strain (Figure 7A). In this light, we can conclude that a previous measurement of decreased Siglec-9 binding following the K123A mutation of NeuD (Carlin et al. 2007) does not reflect the low *O*-acetylation status of the strain, but rather the decreased

overall CPS Sia expression in the strain, compared to WT (Figure 4A, B)

We have previously shown that GBS *O*-acetylates Sia at the 7-carbon position (Lewis et al. 2004), and under the acidic conditions of urogenital tract colonization (pH 4–5), 7-*O*-acetylation would likely be retained at this position. However, upon entry into the lungs or bloodstream of the fetus or infant, neutral pH will likely induce nonenzymatic *O*-acetyl migration to the 9-position, a process observed for Sia *O*-acetyl esters in mammalian glycoconjugates (Vandamme-Feldhaus and Schauer 1998; Varki and Diaz 1984) with a calculated half life of ~5 h (Kamerling et al. 1987). To probe the effect of 7- versus 9-*O*-acetylation of GBS Sia on Siglec-9 binding, we took freshly grown isogenic *OAc*^{high} and *OAc*^{low} strains and either (a) induced complete 7- to 9-*O*-acetyl migration by incubation in the pH 9 buffer at 37°C for 30 min, or (b) retained 7-*O*-acetylation in another aliquot by incubation in the pH 5.5 buffer. For these treatments, surface Sia was analyzed by DMB-HPLC, confirming the expected *O*-acetyl migration (or lack thereof) while retaining equivalent levels of overall Sia and *O*-acetylation (Figure 7B). When these GBS were then incubated with the hSiglec9-Fc chimeric protein and binding analyzed by flow cytometry, 7-*OAc* was found to block Siglec 9 binding to a much greater extent than 9-*OAc* (Figure 7D). This effect was specific to the migration of the *O*-acetyl ester and not related to other theoretical effects of differential pH exposure as demonstrated by the equal binding of *OAc*^{low} strains incubated under the identical pH conditions in parallel (Figure 7C). These data are the first to demonstrate a specific influence of the position of Sia *O*-acetylation upon Siglec binding and suggest that the impairment of GBS hSiglec-9 engagement by *O*-acetylation would be mitigated in the bloodstream by migration from the 7- to the 9-position.

O-Acetylation blocks removal of GBS CPS Sia by bacterial sialidases

When colonizing the host gastrointestinal and vaginal mucosa, GBS coexists with an abundance of other microbes, including both normal flora and transient colonizers. Many of these co-inhabiting microbes express sialidases, which remove terminal Sia residues from the mucosa of the host for use as a carbon source or to adhere to underlying structures (Corfield 1992; Vimr et al. 2004). Microbial sialidases vary in their substrate specificity for different linkages and types of Sias, but most have the capacity to cleave α 2-3-linked Neu5Ac (Powell and Varki 2001). In biochemical assays with purified enzymes, Sia 9-*O*-acetylation has been shown to interfere with substrate recognition and block the activity of sialidases (Varki and Diaz 1983; Varki and Diaz 1984; Chokhawala et al. 2007); however, these observations have not been considered in the context of the intact bacterium and mucosal niche competition.

We hypothesized that GBS might use *O*-acetylation to protect its critical virulence determinant from removal by microbial sialidases. The isogenic GBS *OAc*^{high} and *OAc*^{low} strains were incubated with purified sialidases from *Clostridium perfringens*, *Salmonella typhimurium*, and *Vibrio cholerae*, organisms that can form transient or stable associations with the human gastrointestinal tract. *Arthrobacter urafaciens* sialidase (AUS) was used as a control in each experiment since it displays relaxed substrate flexibility and releases *O*-acetylated Sias (Chokhawala

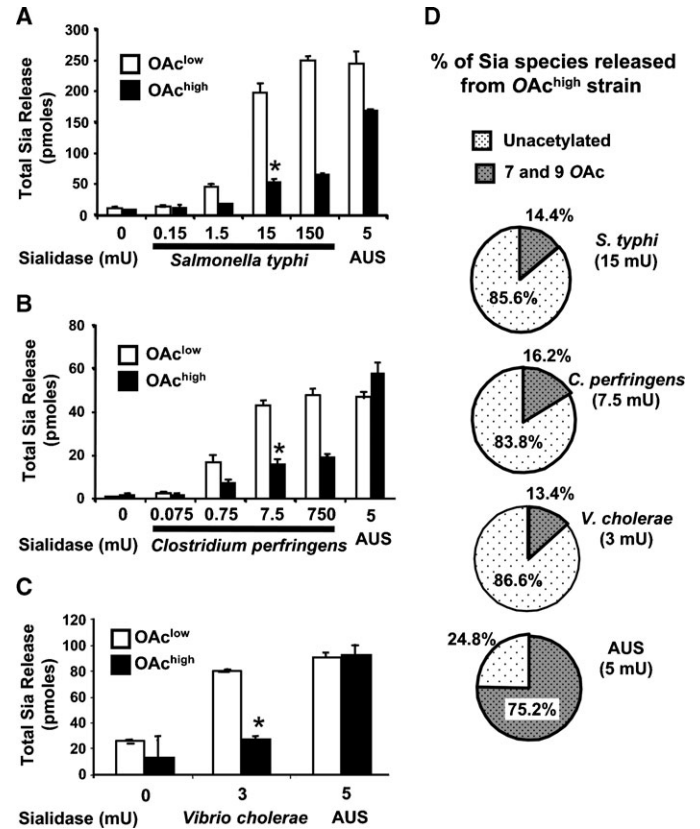


Fig. 8. *O*-Acetylation blocks GBS sialic acid removal by the sialidases of some gastrointestinal bacteria. Isogenic *OAc*^{high} and *OAc*^{low} GBS were treated with varying amounts of purified sialidases from the gastrointestinal pathogens (A) *Salmonella typhimurium* (B) *Clostridium perfringens* or (C) *Vibrio cholerae* and the total Sia released was monitored after NaOH treatment by DMB-HPLC as described in the *Material and methods*. Data are presented as mean \pm standard deviation from analyses performed in triplicate. *Arthrobacter urafaciens* sialidase was used as a control in all experiments as it is known to have a relaxed substrate specificity and to indiscriminately release *O*-acetylated Sias from GBS. (D) Sialidases of *S. typhimurium*, *C. perfringens* and *V. cholerae* were incubated with GBS that express high levels of *O*-acetylation, followed by DMB-HPLC analysis without prior NaOH treatment and quantitation of *O*-acetylated and unmodified Sias. Asterisks highlight experimental conditions in A–C used for the analysis in D. In contrast to the control *A. urafaciens* sialidase, which releases Sias in a more representative fashion, mostly unmodified Sias were released by the tested gastrointestinal pathogens.

et al. 2007). Bacteria were incubated with a range of enzyme concentrations at 37°C for 20 min, supernatants collected and subjected directly to DMB derivatization, allowing analysis of all released Sias including modified and unmodified forms (Figure 8D), or base treated to hydrolyze *O*-acetyl esters, followed by DMB derivatization for a more accurate total Sia quantification (Figure 8A–C).

At all concentrations of each enzyme, total Sia release from the *OAc*^{high} strain was markedly reduced in comparison with the *OAc*^{low} strain (Figure 8A–C). Under saturating enzyme concentrations, the *S. typhimurium*, *C. perfringens*, and *V. cholerae* sialidases released approximately 20%, 50%, or 33% as much Neu5Ac from the *OAc*^{high} strain compared to the *OAc*^{low} strain, respectively (Figure 8A–C). In contrast, Sia release by AUS, the broad-spectrum sialidase encoded by a soil saprophyte, was

unaffected by GBS *O*-acetylation (Figure 8A–C). Additional experiments evaluated the distribution of Sia types (unmodified or *O*-acetylated) that were being released from the *OAc*^{high} strain (Figure 8D). The proportion of Sia types released by AUS (Figure 8D) mirrored the proportion of Sia types displayed by the *OAc*^{high} strain on its surface (Figure 5A). In striking contrast, the vast majority of Sias released by *S. typhimurium*, *C. perfringens*, and *V. cholerae* sialidases were unmodified (Figure 8D). The overall level of non-*O*-acetylated Sia released by each of the sialidases is comparable to that released by AUS, indicating that all non-*O*-acetylated residues available for cleavage are preferentially removed from the GBS surface under these conditions (data not shown). These data directly demonstrate that *O*-acetylation of Sias on intact GBS protects CPS Sias from cleavage by a variety of microbial sialidases. This is the first demonstration that Neu5Ac residues modified with *O*-acetyl esters at the 7-carbon position (as opposed to the 9-carbon position) are effective at blocking sialidase action.

Discussion

Partial *O*-acetyl modification is a newly appreciated biochemical feature of GBS capsular Sia, which is known to be an important virulence feature of the pathogen, and the basis for molecular mimicry of the human host. The enzymatic basis of GBS Sia *O*-acetylation by the *O*-acetyltransferase NeuD and its modulation by the *O*-acetyl esterase activity of NeuA are established. However, the required function of each enzyme in overall CPS Sia biosynthesis posed a challenge in evaluating effects of *O*-acetylation on GBS Sia-mediated phenotypes in isolation. We used overexpression of WT or esterase-deficient NeuA in GBS to generate isogenic strains that differ markedly in overall *O*-acetylation (5% versus 80%) but express similar overall CPS Sia. These studies reveal that higher levels of *O*-acetylation of the GBS capsular Sia reduce its susceptibility to enzymatic removal by different microbial sialidases, do not appreciably influence complement C3b accumulation on the GBS surface, but impair the ability of GBS to engage hSiglec-9. While previous studies have examined effects of *O*-acetylation on Sia-dependent processes, ours is the first to report such influences in the context of intact bacterial cells expressing *O*-acetylation as an isolated chemical variable.

We hypothesize that a phenotype of partial Sia *O*-acetylation in GBS has evolved under competing selective pressures that benefit the organism over strategies of expressing unmodified Sia or 100% *O*-acetylated Sia. Inhibition of Sia removal by microbial or host sialidases may be one major advantage of Sia *O*-acetylation, protecting at least a proportion of this critical virulence factor from enzymatic elimination in the host environment. Increased susceptibility to the alternative complement pathway would be a theoretical disadvantage of GBS Sia *O*-acetylation (Varki and Kornfeld 1980; Shi et al. 1996), but these studies show that even when 80% of GBS Sia residues are *O*-acetylated, the density of GBS surface Sia expression and the preservation of a subset of non-*O*-acetylated Sia residues evidently provides a sufficient reservoir for this complement inhibitory function. Finally, *O*-acetylation reduces binding of hSiglec-9, a newly identified mechanism of GBS immune evasion. However, migration of the *O*-acetyl ester from the 7- to the 9-position under physiologic pH partially restores this binding potential.

The preservation of the NeuD/NeuA system for *O*-acetylation and de-*O*-acetylation of Sia prior to capsule assembly (Figure 1) allows GBS to generate partial Sia *O*-acetylation phenotypes. These phenotypes are likely to exhibit different optimization points based on (a) the underlying biochemical structure of each GBS serotype repeat unit that displays the terminal Sia residue and/or (b) strain variation expression of surface proteins that influence interactions with Sia binding lectins. The approach we have outlined will facilitate future studies to understand how GBS uses *O*-acetylation to strike a balance between sialidase protection, complement resistance, and Siglec engagement to maximize its success in the host environment. *O*-acetyl modifications have also been described on Sia residues in surface expressed glycoconjugates of other bacterial pathogens (Bhattacharjee et al. 1976; Orskov et al. 1979; Gamian et al. 1992, 2000; Gamian and Kenne 1993) and similar analyses of their influence on Sia preservation and interaction with host Sia binding lectins should prove fruitful.

Material and methods

Strains and culture conditions

All strains used in this study were derived in an isogenic manner from the well-characterized wild-type strain COH1. *neuD* and *neuA* mutants were constructed as previously described (Lewis et al. 2004, 2006) by allelic replacement with a chloramphenicol acetyltransferase cassette (Figure 1). Complementation of these mutants was achieved by transformation of the pDCerm plasmid bearing either wild-type or mutated alleles of *neuD* and *neuA* as previously described (Lewis et al. 2004, 2006, 2007). Strains new to this study include WT and mutant GBS bearing empty control pDCerm vectors and the WT strain transformed with the NeuA N301A vector. Although all graphs do not contain “+ empty vector” alongside the strain names, studies of strains containing overexpression constructs were always compared to WT or mutated strains containing the empty pDCerm vector and grown under identical conditions. In the case of the F88C polymorphism of NeuD, allelic replacement by homologous recombination was employed to replace the polymorphic allele into its native position on the GBS chromosome (Lewis et al. 2006). GBS were grown in Todd-Hewitt broth at 37°C without shaking. Strains bearing the pDCerm plasmid were grown in Todd-Hewitt broth containing 5–10 µg/mL erythromycin.

Buoyancy assays

GBS were grown overnight (without shaking). After initial inspection of buoyancy in undisturbed cultures, tubes were vortexed briefly to homogenize the bacterial suspension and 1 mL of culture was pelleted, washed in PBS, and resuspended in 100 µL PBS. GBS were overlaid onto a Percoll gradient in glass tubes consisting of 1 mL each of 60%, 50%, and 40% Percoll diluted in PBS. A qualitative evaluation of buoyancy was made following 500 rpm centrifugation for 10–15 min. Similar results were obtained using log phase cultures.

Hydrophobicity assays

Overall hydrophobicity of the isogenic panel of GBS strains was tested in triplicate using a variation of the method of Rosenberg (2006). Briefly, cells were grown to OD₆₀₀ 0.4, washed in PBS, and resuspended in 3 mL of Milli-Q water to an OD₆₆₀ of

approximately 0.1. To this, 0.6 mL of *n*-hexadecane was added, and the suspension was vortexed three times for 15 s each. The initial and final OD₆₆₀ values of the lower aqueous phase were collected and relative retention of bacteria within the aqueous layer was calculated by dividing the final/initial OD values. Data are represented as mean \pm standard deviation and Student's *t*-test was used for statistical analysis.

Sialic acid quantitation

The quantity of sialic acid residues was measured after hydrolysis of cells or cell fractions in 2N acetic acid, or treatment with various sialidases as described in *Material and methods* and *Results*. Released Sias were filtered over a Centricon-10 cassette, lyophilized, and treated with 0.1 N sodium hydroxide followed by neutralization as previously described (Higa et al. 1989). Sialic acids were then derivatized with 1,2-diamino-4,5-methylene dioxybenzene (DMB) and quantitated by reverse-phase HPLC with fluorescent detection as previously described (Lewis et al. 2004) using commercially available *N*-acetylneuraminic acid as a standard (Inalco, San Luis Obispo, CA). Analyses were performed in triplicate and expressed as mean \pm standard deviation.

Quantitation of O-acetylation

The levels and placement of *O*-acetyl groups on GBS Sias was determined by DMB-HPLC analysis without prior NaOH-treatment of Sias as previously described (Lewis et al. 2007) using peak areas integrated by the Rainin R software package.

Fractionation of GBS for quantitation of surface expressed Sia

For analysis of Sias relative to rhamnose, cell wall extracts were prepared from overnight cultures of GBS strains. One milliliter of culture was washed in PBS and resuspended in 100 μ L Tris-HCl, pH 7, containing 10 mM MgCl₂ and 250 U mutanolysin from *Streptomyces globisporus* (Sigma). After incubation for 3 h at 37°C, protoplasts were pelleted, and the supernatant containing extracted cell wall material was split into two equal parts used for quantitation of sialic acid and rhamnose residues (of CPS or Group B carbohydrate, respectively), both of which are covalently attached to peptidoglycan (Deng et al. 2000).

Quantitation of rhamnose residues present in the Group B carbohydrate

Rhamnose was quantitated by the UCSD glycotecology core resource. Briefly, cell wall extracts were hydrolyzed with 2M TFA, lyophilized, and analyzed by HPAEC-PAD using a CarboPac PA-1 column with parallel preparation and quantitation of commercially available rhamnose standard. Relative sialylation was calculated by comparing the absolute quantity of sialic acids to that of rhamnose (pmol Sia/pmol Rha) in the same volume of sample and expressed relative to the sialylation value found in the WT COH1 parent strain.

Bacterial re-sialylation assay

CPS Sias on GBS were removed by incubating bacteria in sterile PBS with 100 mU/mL *A. ureafaciens* sialidase (AUS) for 30 min at 37°C and then washed five times with PBS. GBS were either analyzed immediately by labeling with ECA-FITC at 4°C fol-

lowed by flow cytometry (time 0) or incubated for indicated time points at 37°C prior to analysis.

Sia quantitation relative to colony forming units

GBS were grown to mid-log phase and resuspended to OD₆₀₀ 0.4 ($\sim 10^8$ cfu/mL) in PBS. One milliliter of each strain was pelleted in triplicate and resuspended in 200 μ L water. Bacteria were lysed using three rounds of freeze-thawing in a dry ice ethanol bath alternating with a 100°C heat block. Cell debris was collected by centrifugation at maximum speed on a tabletop centrifuge for 10 min and any soluble intracellular materials in the supernatant, including intracellular Sias, were discarded. The pellet was washed with PBS, resuspended in 50 mM sodium acetate, pH 5.5, containing 7.5 mU of *A. ureafaciens* sialidase (EY labs). After a 2-h incubation at 37°C, sialic acid residues released into the supernatant were filtered, lyophilized, NaOH-treated, derivatized, and quantitated as described above. In parallel, bacteria were serially diluted and plated on Todd-Hewitt Agar for evaluation of cfu, which were similar between strains. All experiments included parallel Neu5Ac standards during DMB derivatization for construction of standard curves used to estimate Sia expression. After normalization of absolute Sia levels for cfu, which did not vary considerably between samples, relative Sia levels were expressed as fmol Neu5Ac/1000cfu. Similar relative results were obtained when whole bacteria were subjected to 2N acetic acid hydrolysis rather than sialidase-treatment of cell-wall extracts.

ECA-binding assays for estimation of relative uncapped galactose

GBS were grown to mid-log phase and resuspended to an OD₆₀₀ of 0.4. 10^8 bacteria were washed and diluted in PBS. Then 10^6 cells were incubated in a total volume of 100 μ L on ice in the dark with FITC-conjugated *Erythrina cristagalli* agglutinin (ECA) (EY Labs, San Mateo, CA) at a final dilution of 1:100. After a 30-min incubation, cells were pelleted, washed in PBS, and resuspended for analysis using a BD FacsCaliber flow cytometer.

Complement deposition assays

GBS were grown to mid-log phase and resuspended to an OD₆₀₀ of 0.4 in PBS. Approximately, 10^7 cells were resuspended in 50 μ L of 50% serum diluted in the HEPES++ buffer (20 mM Hepes, 5 mM CaCl₂, 2.5 mM MgCl₂, 140 mM NaCl, 0.05% BSA, pH 7.3) and incubated at 37°C with shaking. Eight microliters of aliquots were removed at 1, 3, 5, 7, and 10 min intervals and diluted into 500 μ L ice-cold PBS containing 1% BSA and 5 mM EDTA. Samples were then washed with 1 mL PBS with 1% BSA and resuspended in 50 μ L of 1:50 FITC-conjugated goat anti-human-C3 F(ab)₂ antibody (Protos Immunoresearch) in PBS with 1% BSA. Samples were stained for 30 min on ice, washed once with 1 mL of PBS with 1% BSA, resuspended in 300 μ L of PBS, and analyzed using a BD FacsCaliber flow cytometer.

Calculation of Sia density

The density of Sia expression was compared between GBS and mammalian erythrocytes by calculating the number of Sia molecules expressed per cell and dividing by the estimated surface area of each cell type. For GBS, the data collected for

Figure 5 were used to calculate Sia molecules per cell and estimating GBS diameter at approximately 1 μm to calculate the surface area. For the erythrocyte calculations, we used estimates of Sia molecules per cell published by Powell and Hart (1986) for human erythrocytes and average measurements of erythrocyte diameter in normal individuals (Matsumoto et al. 2003) for calculation of the cell surface area. Sia density was calculated as Sia molecules per cell divided by the (spherical) surface area of each cell type in μm^2 .

Siglec-Fc binding assays

Siglec-Fc chimeric protein (0.4 μg) was pre-complexed with 0.4 μg of goat-anti-human phycoerythrin-conjugated secondary antibody in 50 μL of PBS for 1 h on ice in the dark. Bacterial strains were grown to mid-log phase and resuspended to OD_{600} 0.4 in PBS. 2×10^6 bacteria in 50 μL of PBS were incubated with precomplexed mixture for 40 min on ice in the dark. Samples were then washed with 1 mL of PBS, resuspended in 500 μL of PBS, and analyzed by flow cytometry.

O-Acetyl migration

OAc^{high} and OAc^{low} GBS were grown to mid-log phase and resuspended to an OD_{600} of 0.4 in PBS. 10^8 cells were pelleted and resuspended in 1 mL of either 50 mM sodium acetate pH 5.5 or Tris-HCl pH 9.0 and incubated for 30 min at 37°C to either maintain the *O*-acetyl ester at carbon position 7 (pH 5.5) or induce *O*-acetyl migration to position 9 (pH 9.0). Aliquots of 100 μL were removed and treated as described above for Siglec binding (using PBS at pH 6.5), using the OAc^{low} strain as a positive control for binding and simultaneously as a negative control for the pH incubation. Additional 100 μL aliquots of pH-treated bacteria were incubated with 15 mU *A. ureafaciens* sialidase (AUS) at 37°C for 2 h to remove surface sialic acids, which were analyzed by DMB-HPLC to determine the extent and position of the *O*-acetyl modification as described above. Remaining cells were incubated in parallel with the Siglec incubation on ice for 40 min, during which time we observed little additional movement or loss of the *O*-acetyl ester.

Treatment with sialidases of gastrointestinal bacteria

Bacterial strains were grown to mid-log phase and washed in PBS, and the 10^{-4} – 10^{-6} dilutions were plated for cfu enumeration. 1×10^8 bacteria were resuspended in 20 μL of 100 mM sodium acetate buffer, pH 5.5 (with 1 mM Ca for *V. cholerae*), and sialidases from *S. typhimurium* (Glyko), *Chlostridium perfringens* (Sigma), *V. cholerae* (Sigma), or *A. ureafaciens* (EY Labs) were added over a 4 log range as indicated. After 37°C incubation for 20 min, bacterial cells were pelleted and supernatant was removed for analysis by DMB-HPLC with or without prior NaOH treatment to visualize *O*-acetylated species or condense all Sias into a single peak for injections of 3–4 samples representing independent experiments in the same 50 min elution of the reverse-phase column. Peak areas were integrated and these values were corrected for original cfu, which were always very similar, to compare between OAc^{high} and OAc^{low} strains.

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Conflict of interest statement

None declared.

Abbreviations

CPS, capsular polysaccharide; DMB, 1,2-diamino-4,5-methylene dioxybenzene; ECA, *Erythrina cristagalli* agglutinin; Gal, galactose; GBS, group B *Streptococcus*; ITIM, immunoreceptor tyrosine-based inhibitory motifs; Neu5Ac, *N*-acetylneuraminic acid; *OAc*, *O*-acetylation; Sia, sialic acid; AUS, *Arthrobacter urafaceans* sialidase.

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