Development and immunogenicity of a prototype multivalent Group B Streptococcus bioconjugate vaccine

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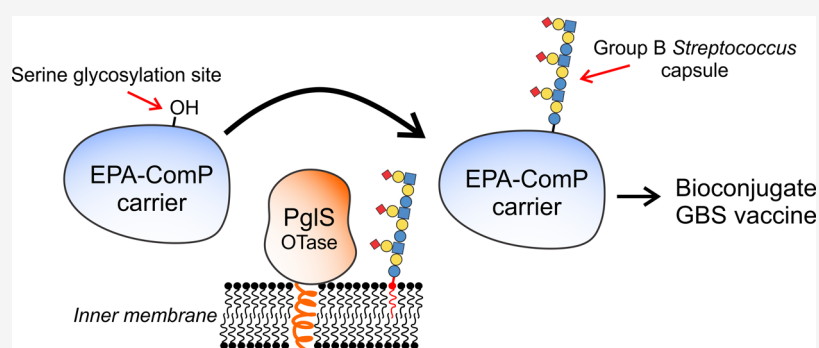Nichollas E. Scott, Mario F. Feldman, Fikri Y. Avci,* and Christian M. Harding*

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ABSTRACT: Group B *Streptococcus* (GBS) is a leading cause of neonatal infections and invasive diseases in nonpregnant adults worldwide. Developing a protective conjugate vaccine targeting the capsule of GBS has been pursued for more than 30 years; however, it has yet to yield a licensed product. In this study, we present a novel bioconjugation platform for producing a prototype multivalent GBS conjugate vaccine and its subsequent analytical and immunological characterizations. Using a glycoengineering strategy, we generated strains of *Escherichia coli* that recombinantly express the type Ia, type Ib, and type III GBS capsular polysaccharides. We then combined the type Ia-, Ib-, and III-capsule-expressing *E. coli* strains with an engineered *Pseudomonas aeruginosa* exotoxin A (EPA) carrier protein and the PglS oligosaccharyltransferase. Coexpression of a GBS capsule, the engineered EPA protein, and PglS enabled the covalent attachment of the target GBS capsule to an engineered serine residue on EPA, all within the periplasm of *E. coli*. GBS bioconjugates were purified, analytically characterized, and evaluated for immunogenicity and functional antibody responses. This proof-of-concept study signifies the first step in the development of a next-generation multivalent GBS bioconjugate vaccine, which was validated by the production of conjugates that are able to elicit functional antibodies directed against the GBS capsule.

KEYWORDS: bioconjugation, bioconjugate, conjugate vaccine, group B *Streptococcus*, multivalent, capsule

roup B *Streptococcus* (GBS) is a Gram-positive, opportunistic bacterium that most often colonizes the lower gastrointestinal and genitourinary tracts. An estimated 10−35% of women are colonized by GBS, resulting in a variety of acute illnesses in pregnant or puerperal women, as well as stillbirth if the infection reaches the growing fetus.[1] GBS can also be transferred to the newborn, where it typically manifests as early-onset disease (EOD, first week of life) or late-onset disease (LOD, first 7−90 days of life) and can cause meningitis, sepsis, and pneumonia. It is estimated that there are >200 000 and >100 000 annual cases of EOD and LOD, respectively.[2−5] In the United States, GBS is the most common cause of infant morbidity and mortality, with a mortality rate of 7%. In Africa, where antibiotic treatments are less readily available, mortality rates can reach as high as 19%.[2] In high-income countries, EOD has been significantly reduced with the implementation of intrapartum antibiotic prophylaxis (IAP), a

standard clinical practice of administering intravenous antibiotics to pregnant women who tested positive for GBS colonization or whose status is unknown. Unfortunately, IAP has failed to lower rates of LOD,[6] necessitating the development of new types of treatment. Moreover, although historically associated with pregnant women and neonates, recent studies have revealed that GBS also causes significant morbidity in nonpregnant adults over the age of 18 and particularly those over 65. In U.S. adults, incidence rates of GBS diseases in these populations were found to be

comparable to *Streptococcus pneumoniae* (pneumococcal) disease,[7] for which routine vaccination is recommended.

Earlier studies with GBS showed that immunoglobulin G (IgG) antibodies could be placentally transferred,[8] making vaccines a promising method to protect pregnant mothers, fetuses, and neonates. GBS vaccines have been in varying stages of development for more than 30 years, and two formulations have recently entered clinical testing: a serotype-independent protein-based vaccine (ClinicalTrials.gov identifier: NCT04596878) and a multivalent capsular polysaccharide (CPS) conjugate vaccine.[9] The GBS conjugate vaccine is produced by purifying the capsule from GBS cells and chemically cross-linking their CPS to purified carrier proteins, resulting in polysaccharide–protein conjugates.[10] Over the last three decades, conjugate vaccines against *S. pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* have been licensed and are in widespread use.[11] While conjugate vaccines have been instrumental in lowering disease burden, they are among the most technically challenging and costly vaccines to manufacture.[12] Furthermore, the chemical approaches used to cross-link capsular polysaccharides to carrier proteins result in significant conjugate heterogeneity with potential destruction of critical polysaccharide and/or carrier protein epitopes.[13] This increases batch-to-batch variability, complicating chemistry, manufacture, and control (CMC) activities and necessitating intense regulatory scrutiny and quality control processes. Complex regulatory activities, in turn, result in high costs and slow developmental timelines for many conjugate vaccines.

A more recently developed, alternative method to produce polysaccharide–protein conjugates, termed bioconjugation, has been shown to simplify conjugate vaccine production[14] and has been adopted by major pharmaceutical companies. Production of bioconjugate vaccines relies on a bacterial enzyme called an oligosaccharyltransferase (OTase) that transfers fully assembled polysaccharides from lipid-linked precursors to engineered carrier proteins in the periplasm of *Escherichia coli*.[15,16] OTases transfer target polysaccharides to specific amino acid side chains in conserved protein sequences called sequons.[17−19] As such, conventional vaccine carrier proteins can be engineered to contain OTase sequons, resulting in site-specific bioconjugation with minimal alteration to the carrier and no alterations to the target polysaccharide. Typically, bioconjugates are produced in engineered *E. coli* strains expressing an OTase, an engineered carrier protein, and the targeted vaccine-specific polysaccharide. Coexpression of these three components results in the production of bioconjugate vaccines in a one-pot system that can be industrially scaled using conventional infrastructures for large-scale microbial fermentation. In addition, bioconjugation offers several advantages over chemically produced conjugate vaccines. For one, the ability to site-specifically glycosylate carrier proteins at sequons significantly reduces the heterogeneity of the product and allows known T-cell epitopes to be preserved. Bioconjugates also obviate the need for separate culturing and purification of CPS and carrier proteins, thereby reducing the number of release controls, which ultimately lowers production costs.[14] Indeed, the reduced manufacturing cost of bioconjugates may open the doors to new vaccine markets including those in high-need, low-income countries that have been neglected due to the high cost of chemical conjugates.[14] Finally, bioconjugation does not rely on harsh oxidative activation of CPS to cross-link to protein, thereby preserving labile epitopes on the polysaccharide that may otherwise be lost. Bioconjugate vaccines have been developed for a variety of bacterial pathogens led by a multivalent *Shigella* bioconjugate vaccine,[20,21] as well as multivalent *E. coli* bioconjugate vaccine[22] currently in advanced stages of clinical trials.

There are 10 known GBS capsular serotypes;[23,24] however, six serotypes (type Ia, type Ib, type II, type III, type IV, and type V) account for nearly all GBS infections across all age groups.[25] Serotype III alone accounts for ∼60% of all invasive neonatal infections and ∼40% of all stillbirths.[25] Global GBS seroepidemiology data indicate that the type Ia, Ib, and III serotypes combined account for 66% of all maternal GBS disease cases, 78% of all EOD cases, and 94% of all LOD cases in developed countries.[2] While the seroepidemiology of invasive and noninvasive GBS diseases in nonpregnant adults is not as well characterized, serotypes Ia and III have previously been shown to account for a large portion of disease isolates. The type Ia, Ib, and III CPSs are structural isomers containing glucose, galactose, and *N*-acetylneuraminic acid (sialic acid) in different arrangements.[23] The sialic acid residue serves as a terminal cap within the repeat unit and plays an important role in protecting the pathogen from the host immune system by blocking the binding of complement.[26] All GBS capsules have glucose as the reducing end sugar (the monosaccharide directly linked to the lipid carrier). Although bioconjugation systems have been investigated for almost two decades, there have been no reports on the development of a GBS bioconjugate vaccine. This is directly attributable to the fact that two OTases employed to generate bioconjugate vaccines (PglB and PglL) do not naturally transfer polysaccharides with glucose at their reducing end.[27] Recently, we discovered a new family of OTases (PglS) that has the broadest polysaccharide substrate versatility of all known OTases, including the ability to transfer glycans with glucose at the reducing end.[27,28]

Here, we report on the production of a trivalent bioconjugate vaccine targeting the type Ia, Ib, and III GBS serotypes. Using the PglS system, we generated fully sialylated bioconjugates of the three CPS serotypes linked to a genetically deactivated *Pseudomonas aeruginosa* exotoxin A (EPA) containing a large fragment of the ComP protein, the native substrate of PglS. Using a variety of analytic techniques, including NMR spectroscopy on glycoengineered GBS polysaccharides and mass spectrometry on intact bioconjugates, we validated the structural composition of each vaccine drug substance. Immunogenicity and opsonophagocytic killing assay (OPKA) studies subsequently demonstrated that the trivalent GBS bioconjugate vaccine immunizations elicited a robust IgG antibody production and a functional antibody response.

■ RESULTS

Glycoengineering *E. coli* for Heterologous Expression of GBS Type Ia, Ib, and III Capsular Polysaccharides. Bioconjugation relies on *E. coli* assembling non-native polysaccharide precursors prior to their transfer by an OTase to engineered carrier proteins. As such, the first step in bioconjugation is cloning and introducing genes encoding for proteins required to produce the targeted non-native polysaccharide and subsequently validating efficient expression and correct assembly of the glycan. All 10 GBS capsular polysaccharide loci contain a conserved architecture, with the first four genes (*cpsABCD*) encoding proteins involved in
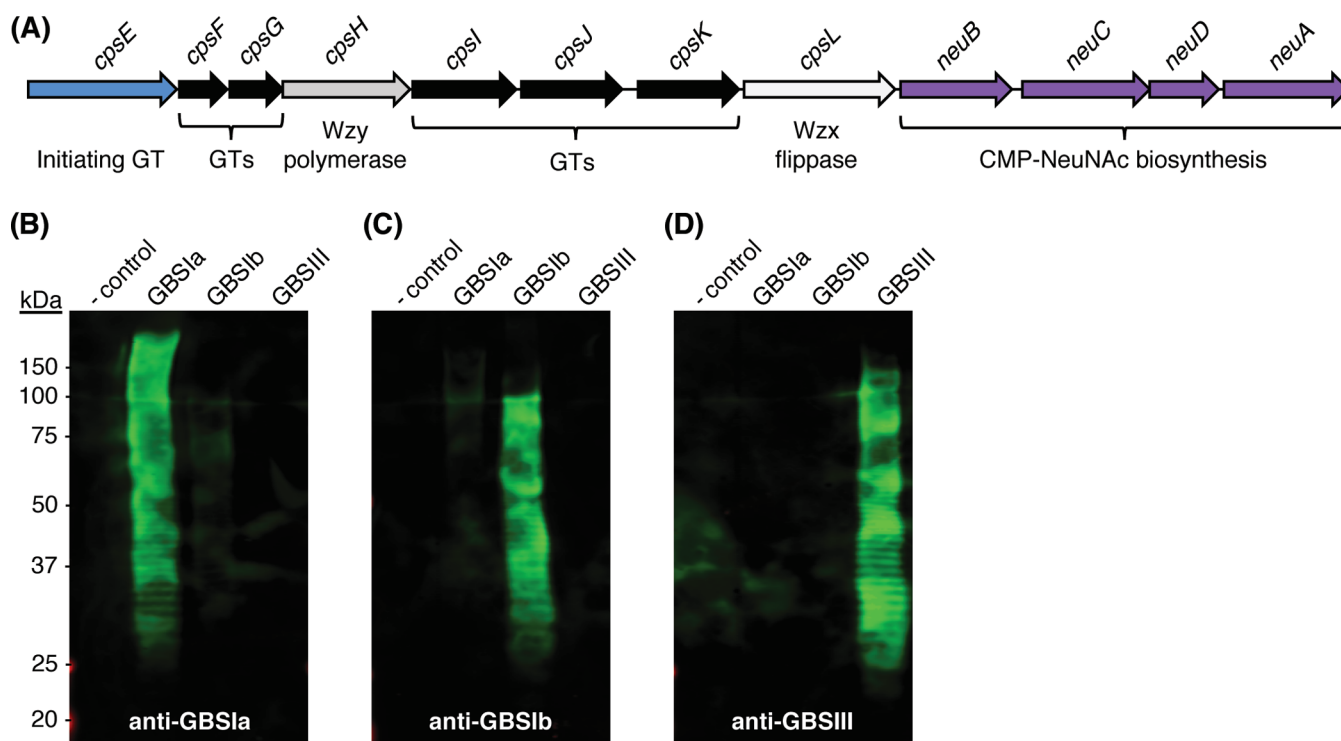
Figure 1. Heterologous expression of GBS capsular polysaccharides type Ia, Ib, and III in *E. coli*. (A) Schematic of the type Ia, Ib, and III genetic loci cloned in this study. The gene architectures for types Ia, Ib, and III are identical, with variations in gene sequence contributing to genetic diversity and serology. (B–D) Western blot analysis of LPS extracted from CLM37 carrying and expressing genes from type Ia, Ib, or III gene loci.

(A)

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                  A       B
          -4-β-Gal-4-β-Glc-
                  |3
α-NeuAc-3-β-Gal-3-β-GlcNAc
          N       C       D
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(C)

Unit		H/C-1	H/C-2	H/C-3	H/C-4	H/C-5	H/C-6a;b	H/C-7	H/C-8	H/C-9
β-Gal A	H	4.47	3.74	3.85	4.40	3.74	3.77; 3.77			
	C	104.1	71.4	82.8	76.1	75.5	62.0			
β-Glc B	H	4.92	3.33	3.69	3.64	3.58	3.77; 3.77			
	C	103.0	74.3	75.5	79.7	75.7	62.0			
β-Gal C	H	4.51	3.55	4.09	3.95	3.68	3.77; 3.77			
	C	104.5	70.1	76.7	68.4	76.2	62.0			
β-Gal C'	H	4.48	3.54	3.65	3.93	3.71	3.77; 3.77			
	C	104.5	71.8	73.7	69.7	76.3	62.0			
β-GlcNAc D	H	4.78	3.89	3.84	3.57	3.51	3.79; 3.94			
	C	103.8	55.9	83.3	69.9	76.5	62.0			
α-NeuNAc N	H			1.79; 2.77	3.70	3.84	3.65	3.60	3.88	3.66; 3.85
	C			40.8	69.4	52.8	73.9	69.2	72.9	63.7

Figure 2. Two-dimensional NMR spectroscopy of the GBS type Ib-containing LPS extracted from *E. coli*. (A) Core structure of the type Ib repeating unit. (B) ^{1}H–^{13}C HSQC spectrum of the GBS type Ib polysaccharide produced in *E. coli* CLM37 strain. (C) ^{1}H and ^{13}C NMR chemical shifts for the GBS type Ib polysaccharide repeating unit sugars.

export of the CPS for surface presentation and the last four genes encoding the machinery to synthesize cytidine-5′-monophospho-*N*-acetylneuraminic acid (CMP-NeuNAc), the

nucleotide-activated sialic acid precursor. Bioconjugation is most efficient when the polysaccharide precursor accumulates in the inner membrane and is not transported to the surface;

therefore, genes encoding for proteins involved in the export of the polysaccharide, like the *cpsABCD* genes, are not necessary. To this end, we cloned the CPS locus from *cpsE* to *neuA* from GBSIa, Ib, or III strain into the IPTG-inducible, low-copy expression vector pBBR1MCS2[29] (Figure 1A). This genetic region for each serotype encodes for all of the necessary glycosyltransferases, the Wzx flippase, the Wzy polymerase, as well as the proteins required to synthesize CMP-NeuNAc.[23] *E. coli* naturally carries genes encoding for proteins required to synthesize uridine diphosphate (UDP)-glucose, UDP-galactose, and UDP-*N*-acetylglucosamine, all of which are also required for type Ia, Ib, and III polysaccharide assemblies. The type Ia, Ib, or III CPS-expressing plasmids were then introduced into *E. coli* CLM37,[30] a W3110 derivative lacking the *wecA* gene that encodes for the phosphoglycosyltransferase required to initiate the synthesis of both O-antigen and enterobacterial common antigen.[31] The CLM37 strain allows for expression and accumulation of non-native polysaccharide precursors that can subsequently be transferred by the O-antigen ligase, WaaL ligase, to the outer core saccharide of lipopolysaccharide (LPS), which can readily be detected via Western blot. CLM37 strains carrying either the GBSIa, Ib, or III CPS-expressing plasmid were grown to mid-logarithmic phase, induced and allowed to grow overnight. LPS was then extracted and analyzed via Western blotting using commercial antisera specific to each of the three GBS CPSs from Statens Serum Insitut. As seen in Figure 1B–D, CLM37 strains carrying either the Ia, Ib, or III CPS-expressing plasmids produced LPS that reacted specifically with the anti-Ia, anti-Ib, or anti-III antisera, respectively.

NMR Analysis of Glycoengineered GBSIa-, Ib-, and III-Containing LPS. Next, we characterized the Ia-, Ib-, and III-containing LPS purified from glycoengineered CLM37 by NMR spectroscopy. The purified polysaccharides demonstrated reasonably clean spectra as determined by ^{1}H NMR spectra overlay of the Ia, Ib, and type III extracted polysaccharides (Figure S1). Two-dimensional (2D) NMR analysis [gradient correlation spectroscopy (gCOSY), total correlated spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY), and gradient heteronuclear single quantum coherence (gHSQC) spectra] subsequently confirmed the structures as shown by Pinto and Berti,[32] with the full 2D NMR spectra and NMR data shown in Figure 2 for the glycoengineered GBSIb-containing LPS O-antigen structure. The 2D NMR spectra and NMR data for the partially sialylated GBSIa are shown in Figure S2, and the fully sialylated GBSIII-containing LPS O-antigen structures are shown in Figure S3. Due to the high viscosity of two of the GBS polysaccharides, spectra were recorded at 60 °C, while the Ib spectrum was resolved at a lower temperature. Even with a modified sodium acetate–sodium dodecasulfate (SDS) buffer extraction protocol, unfortunately, only low-quality 2D spectra were obtained for sialylated Ia GBS polysaccharide. However, given the immunoreactivity of the type Ia-containing LPS with the GBS antitype Ia antisera, and mass spectrometry data presented below of the type Ia bioconjugates, it is highly likely that the sialic acid residue is fully present and was lost during sample preparation for the type Ia LPS.

Glycoengineering Trivalent GBSIa, Ib, and III Bioconjugate Vaccines in *E. coli*. While multiple bioconjugation systems and iterations thereof have been described, we have developed the only bioconjugation platform capable of naturally transferring polysaccharides containing glucose at

their reducing end to engineered carrier proteins.[27] This is essential for developing bioconjugate vaccines targeting GBS as all 10 GBS capsular polysaccharide serotypes contain glucose at the reducing end.[23] For these bioconjugation experiments, the OTase PglS, first characterized in *Acinetobacter baylyi* strain ADP1,[33] was designed to transfer a lipid-linked polysaccharide precursor to a genetically deactivated exotoxin A protein from *P. aeruginosa* (EPA) fused with a fragment of ComP, the natural protein substrate of PglS. ComP is a type IV major pilin subunit involved in natural competence in some *Acinetobacter* environmental isolates.[33] ComP contains an N-terminal membrane-associated domain (first ∼ 28 amino acids) and is natively glycosylated by PglS at an internal serine residue corresponding to serine 84 of the ComP orthologue from *A. baylyi* ADP1.[27] The engineered EPA carrier protein fusion with ComP has been termed EPA$_{ComPΔ28}$ as it is a chimera of the ComP protein missing its first 28 amino acids translationally fused to the C-terminus of EPA (Figure 3A). We introduced the GBSIa-, Ib-, or III-expressing plasmids into the SDB1 strain of *E. coli* that also carries IPTG-inducible plasmids expressing PglS and EPA$_{ComPΔ28}$. The SDB1 strain has two specific mutations that make it ideal for generating bioconjugates consisting of polysaccharides that have glucose at their reducing end.[34] First, it has the same *wecA* mutation as CLM37 that blocks the synthesis of O-antigen and enterobacterial common antigen, both of which in theory can be transferred by PglS to the designed carrier protein. Second, SDB1 has a mutation in *waaL*. By deleting the WaaL O-antigen ligase, the GBSIa, Ib, or III lipid-linked polysaccharide precursors accumulate in the outer leaflet of the inner membrane ensuring an adequate pool of polysaccharide precursor for PglS. SDB1 cells carrying GBS glycan-expressing plasmids, PglS and EPA$_{ComPΔ28}$, were grown to mid-logarithmic phase, induced with IPTG and grown overnight. GBSIa, Ib, and III bioconjugates were subsequently purified by three rounds of chromatography. First, nickel-affinity chromatography was applied as the EPA$_{ComPΔ28}$ carrier protein contains a C-terminal hexahistidine tag. To enrich for high-molecular-weight glycoforms of the bioconjugates and to reduce the amount of unglycosylated EPA$_{ComPΔ28}$ carrier protein in the final preparation, Ni-purified bioconjugates were further purified using anion-exchange chromatography. As a final polishing step, high-molecular-weight glycoforms of each GBS bioconjugate were purified by size-exclusion chromatography. The highly pure GBS bioconjugates were then analyzed by Coomassie staining and Western blotting. As seen in Figure 3B, the unglycosylated EPA$_{ComPΔ28}$ carrier protein runs exclusively near the 75 kDa marker (theoretical mass of 79 kDa). In contrast, the GBSIa-, Ib-, and III-EPA$_{ComPΔ28}$ bioconjugates migrated with electrophoretic mobilities ranging between 100 and 250 kDa with minimal free, unglycosylated protein around 75 kDa. Western blot analysis of the same samples demonstrated that each GBS bioconjugate was reactive with its cognate antisera, indicating that the correct glycan sequences were transferred to the EPA$_{ComPΔ28}$ carrier protein (Figure 3C–E).

To quantify the degree of glycosylation for each GBS bioconjugate, we next performed quadrupole time-of-flight mass spectrometry (Q-TOF-MS) on the intact glycoproteins. This technique enables accurate mass determination, within 50 ppm, of the proteoforms contained within protein preparations. From a chemistry, manufacturing, and controls (CMC) perspective, this is a highly valuable analytical tool allowing for
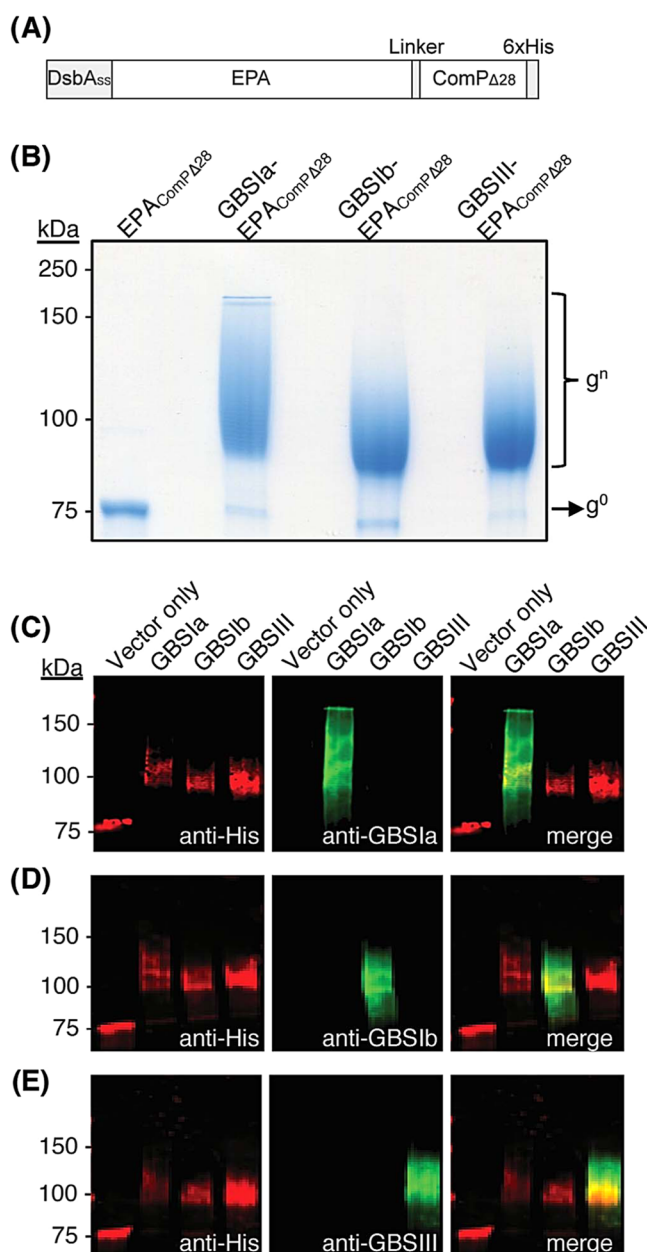
Figure 3. GBSIa-, Ib-, and III-EPA$_{ComPΔ28}$ bioconjugate vaccines. (A) Diagram of the EPA$_{ComPΔ28}$ carrier protein construct used for this study. "DsbA$_{SS}$" corresponds to the DsbA secretion signal required for export of the protein to the periplasm. A single serine residue in ComPΔ28 is the site of glycosylation. (B) Coomassie blue-stained image of purified EPA$_{ComPΔ28}$, GBSIa-EPA$_{ComPΔ28}$, GBSIb-EPA$_{ComPΔ28}$, and GBSIII-EPA$_{ComPΔ28}$. Each lane was loaded with ~5 μg of material based on total protein. (C−E) Western blots were run in triplicate and probed with both the anti-His antibody and either the type Ia-, Ib-, or III-specific antisera from Statens Serum Institut. (C) Anti-GBSIa, (D) anti-GBSIb, and (E) anti-GBSIII Western blots. Each lane was loaded with ~0.5 μg of glycoconjugate based on the total protein.

highly accurate quantification of the number of GBS repeat units covalently attached to the EPA$_{ComPΔ28}$ carrier protein. As seen in Figure 4A−C, the MS1 spectra of the GBSIa-, Ib-, and III-EPA$_{ComPΔ28}$ bioconjugates showed a modal, ladder distribution, with each peak separated by ~980 Da. The Ia, Ib, and III repeat units are structural isomers and, as such, have the same molecular weight and appear nearly identical on the

MS1 spectra for the three different GBS bioconjugates. For all three GBS bioconjugates (Ia, Ib, and III), the glycoform population displayed a bell-shaped distribution, with EPA$_{ComPΔ28}$ containing 10−20 repeat units depending on the bioconjugate. Polysaccharide-to-protein ratios were estimated by first calculating the mass percentage of each glycan (number of repeat units) contributed to the overall mass for each glycoform (glycan mass + carrier protein mass). The summed ion intensities for each glycoform were then multiplied by their respective glycan mass contribution to calculate the ion intensity associated with the polysaccharide portion for each glycoform. The polysaccharide ion intensities were finally averaged and normalized to account for the minor population of unglycosylated EPA$_{ComPΔ28}$ present and are displayed as the polysaccharide-to-protein ratio in Table 1.

Correlates of Protection against GBS Human-Isolated Strains. To begin establishing the efficacy of the trivalent GBS bioconjugate vaccine, immunization groups of four female BALB/c mice were vaccinated intraperitoneally at 2 week intervals in a three-dose regimen using either the trivalent bioconjugate (2 μg polysaccharide per bioconjugate) mixed with alum adjuvant or adjuvant only as control. The postimmunization murine sera were collected on days 14, 28, and 42 for analysis of humoral responses to human clinical isolates of GBS strains expressing the GBS type Ia, Ib, and III CPS. Analysis of the immune response was performed using whole-bacteria-coated enzyme-linked immunosorbent assay (ELISA) to detect the presence of serotype-specific immunoglobulin M (IgM) and IgG antibodies. All mice in the trivalent experimental group generated a significant IgM antibody response to all three strains of GBS compared to the adjuvant immunization control groups (Figure 5A−C). After administration of the second dose on day 14, a booster response indicated by large IgG titers to the individual serotypes was detected starting on day 28 (Figure 5D−F). In addition, all mice in the trivalent experimental group generated a robust anti-EPA$_{ComPΔ28}$ IgG response as compared to adjuvant-only vaccinated mice by day 42 (Figure S4).

To assess the protective capacity of the GBS-specific antibodies, an *in vitro* bacterial killing assay via opsonophagocytic killing assay (OPKA) was performed against the three GBS serotypes. Individual cultures of GBS types Ia, Ib, and III were incubated, with sera obtained from immunized mice before being treated with complement and differentiated HL-60 phagocytes. We observed that serum from mice immunized with the trivalent conjugate vaccine demonstrated significant killing of the GBSIa, GBSIb, and GBSII strains compared to mice injected with the adjuvant only. The results from this assay confirm that the trivalent bioconjugate vaccine can elicit a complement-mediated opsonophagocytic activity against each individual serotype in a manner correlative to the anti-CPS antibody titers generated (Figure 6). Overall, these experiments illustrate the biologically significant response produced by a novel trivalent bioconjugate against GBS through the two hallmark correlates of protection.

■ DISCUSSION

Licensed conjugate vaccines targeting encapsulated bacterial pathogens have had tremendous societal and economical success, significantly reducing the disease burden associated with *H. influenzae* type B, *S. pneumoniae*, and *N. meningitidis*. As such, the development of multivalent conjugate vaccines targeting the capsular polysaccharide of GBS has become a
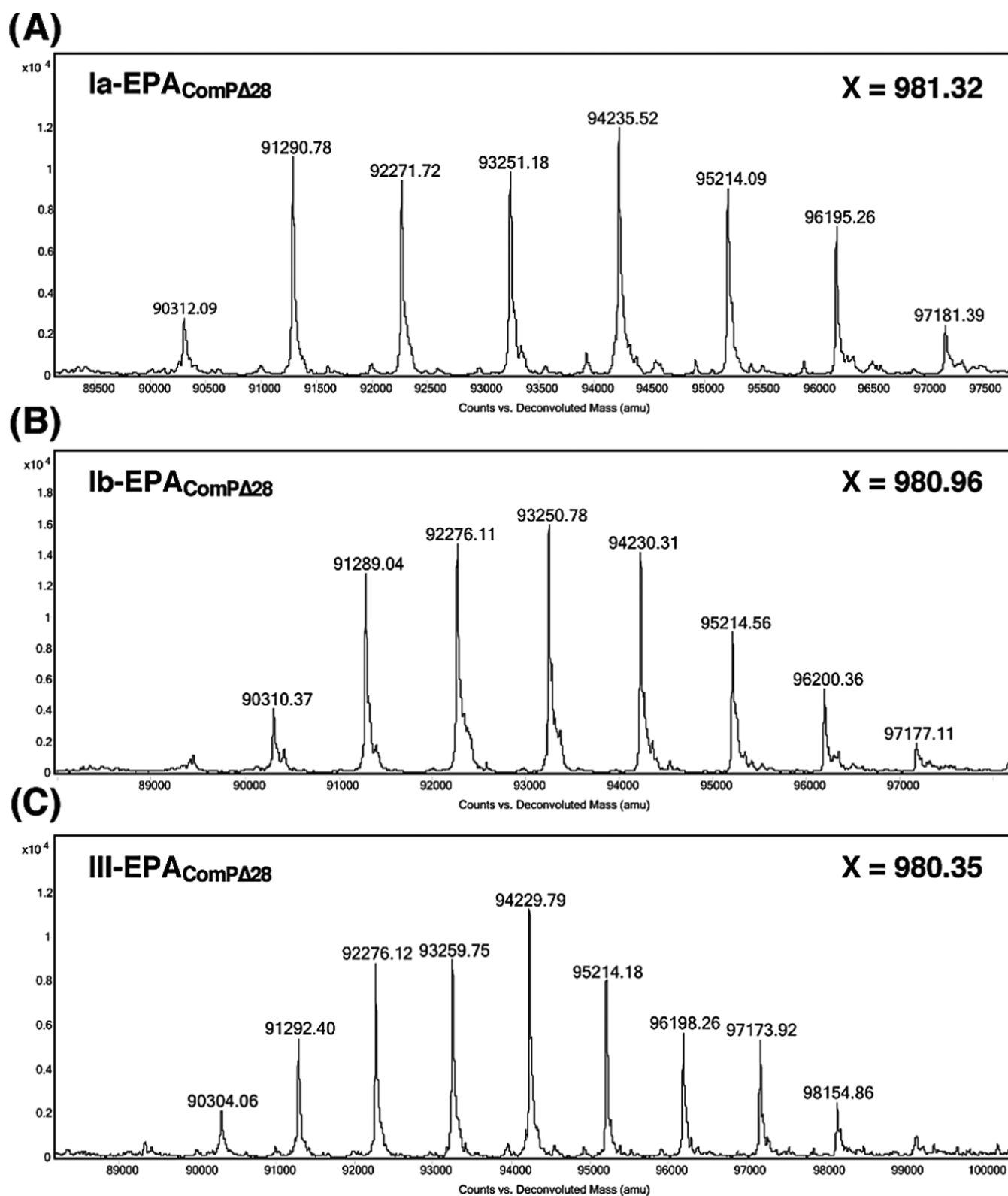
Figure 4. Q-TOF-MS of intact GBS bioconjugates. Intact protein Q-TOF-MS analysis showing the MS1 spectra for (A) Ia-EPA$_{ComP\Delta28}$, (B) Ib-EPA$_{ComP\Delta28}$, and (C) III-EPA$_{ComP\Delta28}$ bioconjugates. Multiple glycoforms for each bioconjugate were observed, with increasing masses corresponding to the type Ia, Ib, or III repeat unit, all three of which have a mass of ~980 ± 1 Da (corresponding to a mass accuracy of < 50 ppm). X equals the mean of the distance between each peak for each bioconjugate.

top priority given the high burden of maternal and neonatal GBS diseases,[1,2] as well as increasing rates of invasive GBS diseases in adults.[7] In this study, we present the early development and characterization of a trivalent bioconjugate

vaccine targeting three of the most clinically prevalent GBS serotypes: type Ia, type Ib, and type III. Using a glycoengineering strategy, we show that an engineered *E. coli* strain expressing a GBS capsule locus is able to correctly

Table 1. GBS Bioconjugate Glycoform Characterizations Based on Intact Mass Spectrometry

| bioconjugate	repeat unit (RU) range	predominant glycoform	polysaccharide:protein ratio
GBSIa-EPA$_{ComP\Delta28}$	11−18	14 RU	0.134
GBSIb-EPA$_{ComP\Delta28}$	10−18	13 RU	0.123
GBSIII-EPA$_{ComP\Delta28}$	10−20	14 RU	0.146

assemble the type Ia, type Ib, or type III polysaccharide as confirmed by Western blotting and NMR spectroscopy. We next combined GBS capsule-expressing *E. coli* strains with our bioconjugation platform, which enables seamless covalent attachment of the target polysaccharide to an engineered carrier protein and successfully demonstrated bioconjugation of the GBS polysaccharides to the EPA fusion protein. Finally, mouse immunizations revealed that the trivalent GBS bioconjugate vaccine elicited GBS serotype-specific serum IgG antibodies that were also functionally protective, as determined by the opsonophagocytic killing assay.

As shown in this and other studies, bioconjugation is mainly highlighted for streamlining the manufacture of conjugate vaccines as compared to conventional chemical procedures,[14] particularly by reducing the number of good manufacturing practice (GMP) production processes from three [(1) polysaccharide extraction, (2) carrier protein production, (3) polysaccharide−protein conjugation] to one. Specifically, in a bioconjugation process, the biosynthesis of the vaccine-targeted polysaccharide and the carrier protein, as well as their subsequent enzymatic covalent linkage, are performed simultaneously within *E. coli*[35] or other Gram-negative bacterial hosts.[18] Less frequently discussed, but equally advantageous, is the fact that the OTase transfers native,
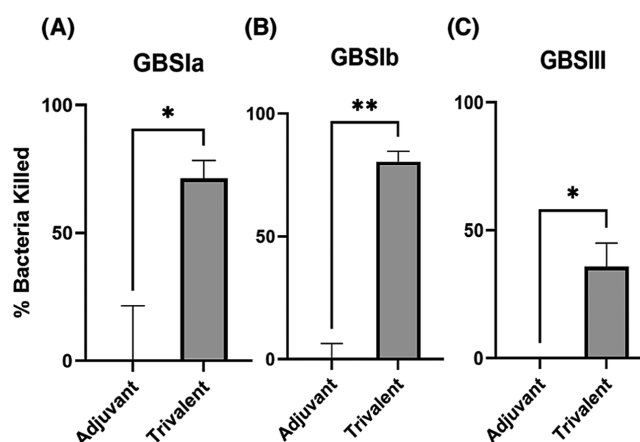
Figure 6. *In vitro* opsonophagocytic killing assay of immunized mouse serum against clinical isolate GBS strains. Pooled mouse sera collected at day 42 of the immunization schedule were used to evaluate the opsonic capacity of antibodies generated against live GBSIa (A), GBSIb (B), and GBSIII strains (C), displaying percent bacteria killed after incubation with HL-60 cells in the presence of serum and complement. Values represent the mean ± SD of the CFU killed. $**p < 0.01$ and $*p < 0.05$ represent significant differences between groups determined using two-tailed Student's *t*-test.

nonderivatized polysaccharides to the carrier protein. This salient feature of bioconjugation ensures that each repeat unit retains critical polysaccharide epitopes important for immunogenicity and eliciting protective, functional antibody responses. In conventional conjugate vaccine preparations, periodate oxidization coupled with reductive amination chemistries is usually required to activate the capsular polysaccharide.[12] In the case of GBS conjugate vaccine production, oxidation occurs through the sialic acid residue within the repeat unit.[36] This can negatively affect the
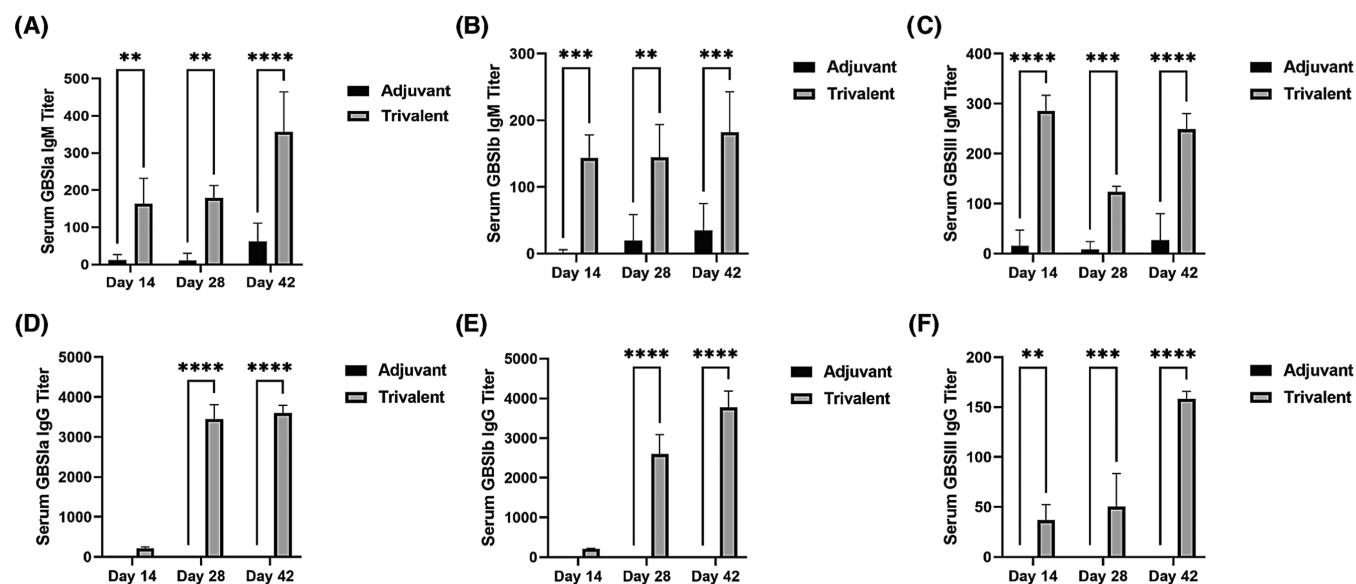
Figure 5. Whole-bacteria ELISA of bioconjugate immunized mice. Groups of four female BALB/cJ mice were vaccinated, with either the trivalent GBS bioconjugate formulation or adjuvant alone. Sera from individual mice at all three time points were subsequently assessed via ELISA for anti-GBS capsular polysaccharide responses. Using fixed GBS bacteria, antigen-specific antibody titers of bioconjugate immunized mice were determined for IgM (A−C) and IgG (D−F). Antibody titers were calculated using the reciprocal dilution that gave an optical density of 0.5 at absorbance 405 nm in the ELISA assay. Values represent the mean ± standard deviation (SD) of the IgM or IgG titers. $****p < 0.0001$, $***p < 0.001$, and $**p < 0.01$ represent significant differences between groups determined using two-way analysis of variance (ANOVA) with Šidák's multiple comparisons test.

immunogenicity of the final vaccine product and must be carefully controlled as modest degrees of oxidation (>20%) can lead to significant reductions in immunogenicity due to epitope destruction.[29] Furthermore, the polysaccharide structure of a protective epitope for the GBS type III CPS was recently solved. These studies demonstrated that the sialic acid residue of an identified six-sugar epitope established direct binding interactions with a monoclonal antibody previously shown to mediate opsonophagocytic killing of the type III GBS strain, providing a mechanistic explanation for the importance of nonoxidized sialic acid residues within GBS conjugate vaccine preparations.[37]

The sialic acid residues of the type Ia, Ib, and III repeat units have also been shown to be nonstoichiometrically O-acetylated at the C_7, C_8, or C_9 position.[38] In both our NMR characterizations of the type Ia, Ib, and III polysaccharides extracted from glycoengineered E. coli, as well as intact mass spectrometric analysis of the type Ia, Ib, and III bioconjugate vaccines, we did not observe O-acetylation of the sialic acid residue. From a vaccine design perspective, the lack of sialic acid O-acetylation in the GBS bioconjugate is not critical owing to the fact that it is not required for immunogenicity and protective immune responses. In particular, de-O-acetylated GBS conjugate vaccines were found to elicit opsonophagocytic killing responses to a panel of GBS isolates with varying degrees of O-acetylated sialic acid residues.[39] Moreover, the structural characterization of the non-O-acetylated protective polysaccharide type III GBS epitope demonstrated that the functional monoclonal antibody studied interacts with the O_7 of sialic acid via a water molecule, potentially allowing for the accommodation of an acetyl group as well.[37]

While the trivalent GBS vaccine in this study was shown to elicit statistically significant increases in serotype-specific IgG responses to all three capsule types, the immune response to the type III antigen was the weakest. We further demonstrated that the antibodies elicited from the GBS bioconjugate vaccine were functional as assessed via the opsonophagocytosis killing assay, a gold-standard correlate of immunity when assessing efficacy for pneumococcal conjugate vaccines[40] and a frequent assay used for assessing the functional activity of vaccine-induced antibodies elicited from GBS conjugate vaccines in clinical trials.[41] The opsonophagocytic killing activity was highest against the type Ib and Ia GBS strains, which is not unexpected given that the IgG titers to type Ia and Ib were also highest. A comparatively moderate opsonophagocytic killing response was observed from sera of mice vaccinated with the trivalent GBS bioconjugate toward the GBSIII strain. Nevertheless, the bioconjugate vaccine was able to induce promising functional antibody responses as measured by an opsonophagocytic killing assay that likely can be improved by increasing the dose of the type III GBS component. This precedent has previously been established for pneumococcal conjugate vaccines, where certain pneumococcal polysaccharide conjugates for a subset of the serotypes are formulated at twice the concentration as others to induce comparable functional antibody responses.[42] As part of our future studies, we will increase GBS polysaccharide concentrations in the bioconjugation product formulation and explore the relationship between polysaccharide concentrations and functional antibody responses.

In an era of accelerated vaccine development, driven by the SARS-CoV-2 pandemic, new technologies and strategies to advance next-generation vaccines to prevent infectious diseases have garnered renewed interest. While mRNA vaccine technologies are the main beneficiary, it is unlikely that mRNA vaccine technologies will be applied as vaccine candidates targeting encapsulated bacterial pathogens. This is due to the fact that polysaccharide synthesis is nontemplate-driven, unlike protein synthesis, and requires a complex biosynthetic pathway of many enzymes to sequentially assemble the target bacterial polysaccharide correctly. As such, bioconjugation technologies hold much promise for the development of next-generation conjugate vaccines. Currently, the trivalent type Ia, Ib, and III bioconjugate vaccines presented in this study would target 65−95% of all neonatal GBS isolates (EOD and LOD events) depending on the geographic region.[2,43] It is anticipated that the GBS bioconjugate vaccine development would benefit from an expanded serotype coverage to include serotype II and serotype V bioconjugates, both of which are associated with maternal GBS disease events, as well as EOD and LOD neonatal GBS diseases.[2,43] Like serotypes Ia, Ib, and III, serotype II and serotype V also contain glucose as the reducing end sugar and are therefore naturally incompatible with other bioconjugation technologies (PglB and PglL) pharmaceutically employed for conjugate vaccine development.[27] As such, the continued development effort to incorporate additional GBS serotypes leveraging the PglS system is a logical and natural next step. In addition, the minimum sequon required for PglS-dependent glycosylation was recently characterized and is expected to further improve conjugate characteristics, including stability and immunogenicity given that the sequon has been reduced from 117 to 11 amino acids and is able to be integrated internally of the target carrier protein.[19] As such, increasing the valency of the vaccine and utilizing an improved sequon are expected to further increase the commercial potential and social impact of the multivalent GBS bioconjugate that covers greater than >90% of all GBS disease isolates. Collectively, this study presents a promising initial characterization of the first multivalent GBS bioconjugate.

■ METHODS

Bacterial Strains, Plasmids, and Growth Conditions. Strains and plasmids used in this study are listed in Table S1. E. coli strains were grown in superoptimal broth (SOB) at 30 °C overnight for LPS and bioconjugate vaccine production. For plasmid selection, the antibiotics were used at the following concentrations: ampicillin (100 μg/mL), chloramphenicol (12.5 μg/mL), and kanamycin (20 μg/mL). GBS strains used for ELISA coating and opsonophagocytic killing assays were obtained from ATCC and correspond to the serotype Ia (Strain 515, BAA-1177), Ib (Strain H36B, 12401), and III (M781, BAA-22). GBS was grown aerobically at 37 °C in brain-heart infusion (BHI) or on tryptic soy agar (TSA) plates supplemented with 5% (v/v) sheep blood.

Construction of the GBSIa, GBSIb, and GBSIII Capsule Locus-Containing Vectors. The GBSIa, GBSIb, and GBSIII capsule loci were individually cloned into the vector pBBR1MCS2 using a Gibson assembly strategy (New England Biolabs) using reference serotype Ia, Ib, and III GBS genomic DNAs as templates. Briefly, each CPS cluster was polymerase chain reaction (PCR)-amplified from wchE to neuA using primers 5′-taccgggcccccccctcgaggATGATTCAAA-CAGTTGTGGTTTAT and 5′-atcaagcttatcgataccggTTA-TAAGGTTTTAACTTCGTCTACAAATAATTG for the type Ia cluster, primers 5′-taccgggcccccccctcgaggATGATT-

CAAACCGTTGTGG and 5′- atcaagcttatcgataccggTTA-TAAGGTTTTAACTTCGTCTACAAATAATTG for the type Ib cluster, and primers 5′- taccgggcccccctcgaggAT-GATTCAAACAGTTGTAGTTTATT and 5′-atcaagcttatcga-taccggTTATAAGGTTTTAACTTCGTCTACAAATAATTG for the type III GBS cluster. Each primer contained a 20 bp homology arm to the multiple cloning site of the pBBR1MCS2 cluster (lower case font). The pBBR1MCS2 vector was linearized via PCR using the primers 5′-taccgggcccccctcgagg and 5′-atcaagcttatcgataccgg. PCR products were gel-purified and then used in a Gibson assembly reaction and transformed into DH10b cells. Transformants were selected on L-agar supplemented with kanamycin. Plasmid DNA was extracted from positive clones and tested for GBSIa, Ib, or III glycan expression.

LPS Extraction for Western Blot Analysis. LPS was extracted following the methods of Marolda et al.[44] Briefly, LPS was extracted using a hot phenol method from 2.0 OD units of stationary phase-grown *E. coli* cells. The precipitated LPS was resolved in 50 μL of 1× Laemmli buffer. A 10 μL aliquot of the GBSIa-, GBSIb-, and GBSIII-*O*-antigen-containing LPS was separated on a 4−20% TGX sodium dodecasulfate (SDS) polyacrylamide gel (Bio-Rad). LPS was also extracted from *E. coli* containing the empty pBBR1MCS2 vector as a negative control.

Western Blotting. The purified LPS or bioconjugate vaccines were separated using 4−20 or 7.5% precast TGX sodium dodecasulfate polyacrylamide gels, respectively (Bio-Rad). The samples were subsequently transferred to nitro-cellulose membranes with a 0.2 μm pore size (Bio-Rad). Nitrocellulose membranes were blocked with a Licor TBS blocking buffer, incubated with primary antibodies for 1 h, washed three times in TBS supplemented with Tween-20, incubated with secondary antibodies for 30 min, washed three times with TBS supplemented with Tween-20, and then visualized using an Odyssey Infrared Imaging System (LiCor Biosciences). Primary antibodies included the reference GBS type Ia antisera (Statens Serum Institut Article Number 22455), the reference GBS type Ib antisera (Statens Serum Institut Article Number 22456), the reference GBS type III antisera (Statens Serum Institut Article Number 22459), and the anti-*Pseudomonas* exotoxin A antibody (P2318-1ML), all used at 1:1000 dilutions. Secondary antibodies included Licor IRDye 680RD goat antimouse (925-68070) and goat antirabbit 800CW (926-32211) used at 1:10 000 dilutions.

O-Antigen Polysaccharide Extraction and Purification. O-Antigen polysaccharides were extracted from *E. coli* CLM37 expressing either the GBS type Ia-, Ib-, or III-containing LPS by heating whole-cell bacteria in 2% acetic acid at 100 °C for 1.5 h. The insoluble material was removed by centrifugation, and the supernatant was separated on a Biogel P6 column (2.5 × 60 cm) in 1% acetic acid. Fractions were monitored for polysaccharides with a refractive index detector (Gilson). The polymeric fraction containing the desired GBS polysaccharides was collected; however, the terminal sialic acid residues for each repeat unit were lost during the acetic acid hydrolysis procedure required to cleave the O-antigen polysaccharide (in this case, the GBS polysaccharide) from the core saccharide. Asialyated GBS polysaccharides were then purified by anion-exchange chromatography. Briefly, up to 50 mg of sample was injected into a HiTrap Q column (Amersham, two columns by 5 mL each connected in series) equilibrated in water at 3 mL/min, washed with water for 5

min, and then eluted with a linear gradient from water to 1 M NaCl over 1 h with UV detection at 220 nm and spot test on silica TLC plate with development by dipping in 5% H_2SO_4 in ethanol and heating with a heat gun until brown spots become visible. Samples were desalted on a Sephadex G-15 column (1.6 × 60 cm) in 1% acetic acid and collected in a refractive index detector. GBS type Ia-, Ib-, or III with the sialic acid residues still intact was obtained by hydrolysis of the phenol-water-extracted LPS with the sodium acetate−SDS buffer.[45] LPS was dispersed in 20 mM sodium acetate−acetic acid buffer (pH 4.5) containing 1% SDS and hydrolyzed at 100 °C for 2 h; the precipitate was centrifuged and removed. The supernatant was dialyzed and then separated on Biogel P6 and subsequent Hitrap Q columns as described above. The degree of sialylation was <100%, most likely due to the hydrolysis procedure.

NMR Spectroscopy Analysis. NMR experiments were carried out on a Bruker AVANCE III 600 MHz (¹H) spectrometer with a 5 mm Z-gradient probe with acetone internal reference (2.225 ppm for ¹H and 31.45 ppm for ¹³C) using standard pulse sequences cosygpprqf (gCOSY), mlevphpr (TOCSY, mixing time 120 ms), roesyphpr (ROESY, mixing time 500 ms), hsqcedetgp (HSQC), hsqcetgpml (HSQC−TOCSY, 80 ms TOCSY delay), and hmbcgplpndqf (HMBC, 100 ms long-range transfer delay). The resolution was kept < 3 Hz/pt in F2 in proton−proton correlations and <5 Hz/pt in F2 of H−C correlations. The spectra were processed and analyzed using the Bruker Topspin 2.1 program. Monosaccharides were identified by COSY, TOCSY, and NOESY cross peak patterns and ¹³C NMR chemical shifts. Aminogroup location was concluded from a high-field signal position of aminated carbons (CH at 45−60 ppm). Connections between monosaccharides were determined from transglycosidic nuclear Overhauser effect (NOE) and heteronuclear multiple bond correlation (HMBC) correlations.

Bioconjugate Vaccine Expression in *E. coli*. *E. coli* SDB1 cells were made electrocompetent by growing cells to the mid-logarithmic stage followed by two rounds of washing in 10% glycerol and a final resuspension in 1/250th of the original culture volume. Cells were electroporated with GBSIa, GBSIb, or GBSIII CPS-expressing plasmids and selected on L-agar supplemented with kanamycin. Colonies were picked, made electrocompetent as described above, and then electroporated with the pACT3-*pglS* and transformants selected on L-agar supplemented with kanamycin and chloramphenicol. Colonies were picked, made electrocompetent again, and then electroporated with pCH4 and transformants selected on L-agar supplemented with kanamycin, chlorophenol, and ampicillin. Five to 10 colonies were swabbed and used to inoculate 250 mL of starter cultures in SOB supplemented with antibiotics for overnight growth at 30 °C with 225 rpm. Starter cultures were then used to inoculate multiple batches of 2 L Erlenmeyer flasks containing 1 L of SOB media supplemented with antibiotics and 0.2 mM of IPTG. Cultures were grown for 24 h at 30 °C with 225 rpm. Cells were pelleted by centrifugation and resuspended in 20 mM Tris, 500 mM NaCl, pH 8.0 and stored at −80 °C.

Bioconjugate Vaccine Purifications. *E. coli* SDB1 cells expressing the GBSIa-EPA$_{ComPΔ28}$, GBSIb-EPA$_{ComPΔ28}$, or the GBSIII-EPA$_{ComPΔ28}$ bioconjugate vaccines were lysed by sonication, and lysates were clarified by centrifugation. The clarified lysates were passed over the Nickel NTA resin,

washed with 20 mM Tris, 500 mM NaCl, 10 mM imidazole, pH 8.0, and eluted with 20 mM Tris, 500 mM NaCl, 300 mM imidazole, pH 8.0. The eluted proteins were diafiltrated using Amicon Ultra Centrifugal Filters with a 50 kDa cutoff into 20 mM Tris, pH 8.0. Buffer-exchanged bioconjugates were subsequently loaded onto a Mono Q 5/50 GL column (Cytiva). Proteins were eluted using a linear gradient of buffer containing 20 mM Tris, 1 M NaCl, pH 8.0. Fractions containing the glycosylated GBSIa-EPA$_{ComP\Delta28}$, GBSIb-EPA$_{ComP\Delta28}$, or the GBSIII-EPA$_{ComP\Delta28}$ bioconjugate vaccines were pooled, concentrated, and separated on a Superdex 200 10/300 GL column equilibrated with a 1× phosphate-buffered saline (Corning 46013CM). GBSIa-EPA$_{ComP\Delta28}$, GBSIb-EPA$_{ComP\Delta28}$, or the GBSIII-EPA$_{ComP\Delta28}$ bioconjugate vaccines were analyzed for the total protein content using a DC Protein Assay (Bio-Rad).

Intact Protein Analysis. Intact mass analysis was performed as described previously.[27,28] Briefly, samples were resuspended in 2% acetonitrile and 0.1% trifluoroacetic acid and loaded onto a Jupiter 300 C5 column (Phenomenex) using an Agilent 1200 high-performance liquid chromatography (HPLC). Five micrograms of the GBSIa-EPA$_{ComP\Delta28}$, GBSIb-EPA$_{ComP\Delta28}$, and GBSIII-EPA$_{ComP\Delta28}$ bioconjugate samples were desalted by washing with 2% acetonitrile ad 0.1% formic acid for 2 min at a flow rate of 0.25 mL/min and then separated using a linear gradient of 80% acetonitrile and 0.1% formic acid (2−80% acetonitrile over 12 min using 0.25 mL/min). Samples were infused into a 6520 Accurate mass Q-TOF mass spectrometer (Agilent), and MS1 mass spectra were acquired at 1 Hz between a mass range of 300 and 3000 m/z. Intact mass analysis and deconvolution were performed using MassHunter B.06.00 (Agilent).

Murine Immunizations. Immunizations consisted of a trivalent formulation, using a mixture of GBSIa, Ib, and II bioconjugates dissolved in phosphate-buffered saline (PBS) and mixed with Alum (Alhydrogel, Invivogen) (1:1, v/v, 1 mL) to form an emulsion equating to 2 μg of the capsular polysaccharide content of each individual bioconjugate serotype per 200 μL of dose, compared to a control of adjuvant immunization alone. Each group of four female BALB/cJ mice (Stock #000651, The Jackson Laboratory) was immunized intraperitoneally on day 0 with two boosts administered on days 14 and 28 with the same 200 μL of adjuvant or trivalent emulsion. Mouse blood samples were collected via tail vein on days 14, 28, and 42 prior to administration of a boost. Mouse serum was prepared by centrifuging clotted blood samples at 1000g for 5 min in a tabletop centrifuge and pipetting serum into sterile tubes for storage at −80 °C until used for experimentation.

ELISA. ELISA plates were prepared by first heat-killing GBS cultures that were in the late-log phase of growth and washing with sterile PBS, before diluting to a concentration of ~2 × 10^8 CFU/mL in PBS. To each well of a 384-well plate (Nunc Maxisorp), 20 μL of heat-killed bacterial suspension was added (~4 × 10^6 CFU) and incubated overnight at 4 °C. The plates were washed with phosphate-buffered saline containing 0.05% Tween-20 (PBST) and incubated with 50 μL of a blocking solution of 3% w/v bovine serum albumin (BSA) in PBST for 2 h with rocking at room temperature. After washing three times with PBST, 20 μL of a 2-fold dilution series of the collected mouse serum (from 1:200 to 1:12 800) diluted in 1% w/v BSA in PBST was added per well and incubated for 2 h at room temperature. The plates were again washed three times

with PBST, and 20 μL per well of a 1:2000 dilution of alkaline phosphatase-linked human-adsorbed goat antimouse IgM or IgG antibody in PBST was introduced and the plates were allowed to incubate for 1 h at room temperature. After a final wash, plates were developed using 20 μL of p-nitro-phenylphosphate (PNPP) (2 mg/mL) dissolved in 100 mM Tris−HCl, 100 mM NaCl, 5 mM MgCl$_2$, pH 9.5 at 37 °C and analyzed at a 405 nm wavelength.

Opsonophagocytosis Killing Assay. An opsonophago-cytic killing assay was performed as described previously,[46,47] as adapted from an earlier protocol with modifications.[48] GBS type Ia, Ib, and III stocks were incubated in triplicate wells in a 96-well round-bottom plate for 1 h at 37 °C with the indicated sera samples (5 μL serum/50 μL total reaction volume/well) in opsonization buffer B (OBB: sterile 1× PBS with Ca2 + /Mg2 +, 0.1% gelatin, and 5% heat-inactivated Hyclone FetalClone I Serum). Cells of the human promyelocytic leukemia cell line HL-60 (ATCC) were cultured in RPMI with 10% heat-inactivated Hyclone FetalClone I Serum and 1% L-glutamine. HL-60 cells were differentiated using 0.6% N,N-dimethylformamide (DMF) for 3 days before performing the OPKA, harvested, and resuspended in OBB. Baby rabbit complement (Pel-Freez) was added to HL-60 cells at a 1:5 final volume. The HL-60−complement mixture was added to the bacteria at 1 × 10^5 cells/well. The final reaction mixtures were incubated at 37 °C for 1 h with shaking. The reactions were stopped by incubating the samples on ice for approximately 20 min. Then, 10 μL of each reaction mixture (triplicate) was diluted to a final volume of 50 μL and plated onto blood agar plates. Plates were incubated overnight at 30 °C and counted the next day. The percentage of bacterial killing was calculated as each sample replicate normalized to the mean value obtained for the control samples, subtracted from 100 (with PBS-treated control sera samples representing 0% survival).

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.1c00415.

One-dimensional (1D) and 2D NMR spectra of GBS polysaccharides and tables of chemical shifts, an anti_EPA$_{ComP\Delta28}$ IgG ELISA, and a table of the plasmids and bacterial strains used in this study (PDF)

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Notes

The authors declare the following competing financial interest(s): Lloyd Robinson, Cory Knoot, Mario Feldman, and Christian Harding each have a financial stake in VaxNewMo, LLC, a for profit entity developing vaccines using the technology described in this and other published manuscripts and patents.

■ ABBREVIATIONS

GBS, group B *Streptococcus*; CPS, capsular polysaccharide; EPA, exotoxin A from *P. aeruginosa*; EOD, early-onset GBS disease; LOD, late-onset GBS disease; OTase, oligosaccharyltransferase

■ REFERENCES

(1) Russell, N. J.; Seale, A. C.; O'Driscoll, M.; O'Sullivan, C.; Bianchi-Jassir, F.; Gonzalez-Guarin, J.; Lawn, J. E.; Baker, C. J.; Bartlett, L.; Cutland, C.; Gravett, M. G.; Heath, P. T.; Le Doare, K.; Madhi, S. A.; Rubens, C. E.; Schrag, S.; Sobanjo-Ter Meulen, A.; Vekemans, J.; Saha, S. K.; Ip, M.; Group, G. B. S. M. C. I. Maternal Colonization With Group B *Streptococcus* and Serotype Distribution Worldwide: Systematic Review and Meta-analyses. *Clin. Infect. Dis.* **2017**, *65*, S100−S111.

(2) Seale, A. C.; Bianchi-Jassir, F.; Russell, N. J.; Kohli-Lynch, M.; Tann, C. J.; Hall, J.; Madrid, L.; Blencowe, H.; Cousens, S.; Baker, C. J.; Bartlett, L.; Cutland, C.; Gravett, M. G.; Heath, P. T.; Ip, M.; Le Doare, K.; Madhi, S. A.; Rubens, C. E.; Saha, S. K.; Schrag, S. J.; Sobanjo-Ter Meulen, A.; Vekemans, J.; Lawn, J. E. Estimates of the Burden of Group B Streptococcal Disease Worldwide for Pregnant Women, Stillbirths, and Children. *Clin. Infect. Dis.* **2017**, *65*, S200−S219.

(3) Seale, A. C.; Blencowe, H.; Bianchi-Jassir, F.; Embleton, N.; Bassat, Q.; Ordi, J.; Menendez, C.; Cutland, C.; Briner, C.; Berkley, J. A.; Lawn, J. E.; Baker, C. J.; Bartlett, L.; Gravett, M. G.; Heath, P. T.; Ip, M.; Le Doare, K.; Rubens, C. E.; Saha, S. K.; Schrag, S.; Meulen, A. S.; Vekemans, J.; Madhi, S. A. Stillbirth With Group B *Streptococcus* Disease Worldwide: Systematic Review and Meta-analyses. *Clin. Infect. Dis.* **2017**, *65*, S125−S132.

(4) Hall, J.; Adams, N. H.; Bartlett, L.; Seale, A. C.; Lamagni, T.; Bianchi-Jassir, F.; Lawn, J. E.; Baker, C. J.; Cutland, C.; Heath, P. T.; Ip, M.; Le Doare, K.; Madhi, S. A.; Rubens, C. E.; Saha, S. K.; Schrag, S.; Sobanjo-Ter Meulen, A.; Vekemans, J.; Gravett, M. G. Maternal Disease With Group B *Streptococcus* and Serotype Distribution Worldwide: Systematic Review and Meta-analyses. *Clin. Infect. Dis.* **2017**, *65*, S112−S124.

(5) Madrid, L.; Seale, A. C.; Kohli-Lynch, M.; Edmond, K. M.; Lawn, J. E.; Heath, P. T.; Madhi, S. A.; Baker, C. J.; Bartlett, L.; Cutland, C.; Gravett, M. G.; Ip, M.; Le Doare, K.; Rubens, C. E.; Saha, S. K.; Sobanjo-Ter Meulen, A.; Vekemans, J.; Schrag, S.; Infant, G. B. S. D. I. G. Infant Group B Streptococcal Disease Incidence and Serotypes Worldwide: Systematic Review and Meta-analyses. *Clin. Infect. Dis.* **2017**, *65*, S160−S172.

(6) Nanduri, S. A.; Petit, S.; Smelser, C.; Apostol, M.; Alden, N. B.; Harrison, L. H.; Lynfield, R.; Vagnone, P. S.; Burzlaff, K.; Spina, N. L.; Dufort, E. M.; Schaffner, W.; Thomas, A. R.; Farley, M. M.; Jain, J. H.; Pondo, T.; McGee, L.; Beall, B. W.; Schrag, S. J. Epidemiology of Invasive Early-Onset and Late-Onset Group B Streptococcal Disease in the United States, 2006 to 2015: Multistate Laboratory and Population-Based Surveillance. *JAMA Pediatr.* **2019**, *173*, 224−233.

(7) McLaughlin, J. M.; Peyrani, P.; Furmanek, S.; Khan, F. L.; Quinn, A.; Jodar, L.; Ramirez, J.; Swerdlow, D. L. Burden of Adults Hospitalized with Group B Streptococcal Infection. *J. Infect. Dis.* **2020**, No. jiaa110.

(8) Baker, C. J.; Rench, M. A.; McInnes, P. Immunization of pregnant women with group B streptococcal type III capsular polysaccharide-tetanus toxoid conjugate vaccine. *Vaccine* **2003**, *21*, 3468−3472.

(9) Absalon, J.; Segall, N.; Block, S. L.; Center, K. J.; Scully, I. L.; Giardina, P. C.; Peterson, J.; Watson, W. J.; Gruber, W. C.; Jansen, K. U.; Peng, Y.; Munson, S.; Pavliakova, D.; Scott, D. A.; Anderson, A. S. Safety and immunogenicity of a novel hexavalent group B *Streptococcus* conjugate vaccine in healthy, non-pregnant adults: a phase 1/2, randomised, placebo-controlled, observer-blinded, dose-escalation trial. *Lancet Infect. Dis.* **2021**, *21*, 263−274.

(10) Anderson, A. S.; Bhalla, A. S. B.; Donald, R. G. K.; Gu, J.; Jansen, K. U.; Kainthan, R. K.; Khandke, L.; Kim, J.; Liberator, P.; Prasad, A. K.; Ruppen, M. E.; Scully, I. L.; Singh, S.; Yang, C. X. Group B *Streptococcus* Polysaccharide−Protein Conjugates, Methods for Producing Conjugates, Immunogenic Compositions Comprising Conjugates, and Uses Thereof. US Patent US2021/0145957 A12009.

(11) Berti, F.; Adamo, R. Antimicrobial glycoconjugate vaccines: an overview of classic and modern approaches for protein modification. *Chem. Soc. Rev.* **2018**, *47*, 9015−9025.

(12) Frasch, C. E. Preparation of bacterial polysaccharide-protein conjugates: analytical and manufacturing challenges. *Vaccine* **2009**, *27*, 6468−6470.

(13) Poolman, J.; Frasch, C.; Nurkka, A.; Kayhty, H.; Biemans, R.; Schuerman, L. Impact of the conjugation method on the immunogenicity of *Streptococcus pneumoniae* serotype 19F polysaccharide in conjugate vaccines. *Clin. Vaccine Immunol.* **2011**, *18*, 327−336.

(14) Rappuoli, R.; De Gregorio, E.; Costantino, P. On the mechanisms of conjugate vaccines. *Proc. Natl. Acad. Sci. U.S.A.* **2019**, *116*, 14−16.

(15) Feldman, M. F.; Wacker, M.; Hernandez, M.; Hitchen, P. G.; Marolda, C. L.; Kowarik, M.; Morris, H. R.; Dell, A.; Valvano, M. A.; Aebi, M. Engineering *N*-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 3016−3021.

(16) Ihssen, J.; Kowarik, M.; Dilettoso, S.; Tanner, C.; Wacker, M.; Thony-Meyer, L. Production of glycoprotein vaccines in *Escherichia coli*. *Microb. Cell Fact.* **2010**, *9*, No. 61.

(17) Kowarik, M.; Young, N. M.; Numao, S.; Schulz, B. L.; Hug, I.; Callewaert, N.; Mills, D. C.; Watson, D. C.; Hernandez, M.; Kelly, J. F.; Wacker, M.; Aebi, M. Definition of the bacterial *N*-glycosylation site consensus sequence. *EMBO J.* **2006**, *25*, 1957−1966.

(18) Pan, C.; Sun, P.; Liu, B.; Liang, H.; Peng, Z.; Dong, Y.; Wang, D.; Liu, X.; Wang, B.; Zeng, M.; Wu, J.; Zhu, L.; Wang, H. Biosynthesis of Conjugate Vaccines Using an O-Linked Glycosylation System. *mBio* 2016, 7, No. e00443-16.

(19) Knoot, C. J.; Robinson, L. S.; Harding, C. M. A minimal sequon sufficient for O-linked glycosylation by the versatile oligosaccharyl-transferase PglS. *Glycobiology* 2021, 31, 1192−1203.

(20) Hatz, C. F.; Bally, B.; Rohrer, S.; Steffen, R.; Kramme, S.; Siegrist, C. A.; Wacker, M.; Alaimo, C.; Fonck, V. G. Safety and immunogenicity of a candidate bioconjugate vaccine against *Shigella dysenteriae* type 1 administered to healthy adults: A single blind, partially randomized Phase I study. *Vaccine* 2015, 33, 4594−4601.

(21) Ravenscroft, N.; Braun, M.; Schneider, J.; Dreyer, A. M.; Wetter, M.; Haeuptle, M. A.; Kemmler, S.; Steffen, M.; Sirena, D.; Herwig, S.; Carranza, P.; Jones, C.; Pollard, A. J.; Wacker, M.; Kowarik, M. Characterization and immunogenicity of a *Shigella flexneri* 2a O-antigen bioconjugate vaccine candidate. *Glycobiology* 2019, 29, 669−680.

(22) van den Dobbelsteen, G.; Fae, K. C.; Serroyen, J.; van den Nieuwenhof, I. M.; Braun, M.; Haeuptle, M. A.; Sirena, D.; Schneider, J.; Alaimo, C.; Lipowsky, G.; Gambillara-Fonck, V.; Wacker, M.; Poolman, J. T. Immunogenicity and safety of a tetravalent *E. coli* O-antigen bioconjugate vaccine in animal models. *Vaccine* 2016, 34, 4152−4160.

(23) Cieslewicz, M. J.; Chaffin, D.; Glusman, G.; Kasper, D.; Madan, A.; Rodrigues, S.; Fahey, J.; Wessels, M. R.; Rubens, C. E. Structural and genetic diversity of group B streptococcus capsular polysaccharides. *Infect. Immun.* 2005, 73, 3096−3103.

(24) Berti, F.; Campisi, E.; Toniolo, C.; Morelli, L.; Crotti, S.; Rosini, R.; Romano, M. R.; Pinto, V.; Brogioni, B.; Torricelli, G.; Janulczyk, R.; Grandi, G.; Margarit, I. Structure of the type IX group B *Streptococcus* capsular polysaccharide and its evolutionary relationship with types V and VII. *J. Biol. Chem.* 2014, 289, 23437−23448.

(25) Bianchi-Jassir, F.; Paul, P.; To, K. N.; Carreras-Abad, C.; Seale, A. C.; Jauneikaite, E.; Madhi, S. A.; Russell, N. J.; Hall, J.; Madrid, L.; Bassat, Q.; Kwatra, G.; Le Doare, K.; Lawn, J. E. Systematic review of Group B Streptococcal capsular types, sequence types and surface proteins as potential vaccine candidates. *Vaccine* 2020, 38, 6682−6694.

(26) Marques, M. B.; Kasper, D. L.; Pangburn, M. K.; Wessels, M. R. Prevention of C3 deposition by capsular polysaccharide is a virulence mechanism of type III group B streptococci. *Infect. Immun.* 1992, 60, 3986−3993.

(27) Harding, C. M.; Nasr, M. A.; Scott, N. E.; Goyette-Desjardins, G.; Nothaft, H.; Mayer, A. E.; Chavez, S. M.; Huynh, J. P.; Kinsella, R. L.; Szymanski, C. M.; Stallings, C. L.; Segura, M.; Feldman, M. F. A platform for glycoengineering a polyvalent pneumococcal bioconjugate vaccine using *E. coli* as a host. *Nat. Commun.* 2019, 10, No. 891.

(28) Feldman, M. F.; Mayer Bridwell, A. E.; Scott, N. E.; Vinogradov, E.; McKee, S. R.; Chavez, S. M.; Twentyman, J.; Stallings, C. L.; Rosen, D. A.; Harding, C. M. A promising bioconjugate vaccine against hypervirulent *Klebsiella pneumoniae*. *Proc. Natl. Acad. Sci. U.S.A.* 2019, 116, 18655−18663.

(29) Kovach, M. E.; Elzer, P. H.; Hill, D. S.; Robertson, G. T.; Farris, M. A.; Roop, R. M., 2nd; Peterson, K. M. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 1995, 166, 175−176.

(30) Linton, D.; Dorrell, N.; Hitchen, P. G.; Amber, S.; Karlyshev, A. V.; Morris, H. R.; Dell, A.; Valvano, M. A.; Aebi, M.; Wren, B. W. Functional analysis of the *Campylobacter* jejuni *N*-linked protein glycosylation pathway. *Mol. Microbiol.* 2005, 55, 1695−1703.

(31) Meier-Dieter, U.; Barr, K.; Starman, R.; Hatch, L.; Rick, P. D. Nucleotide sequence of the *Escherichia coli rfe* gene involved in the synthesis of enterobacterial common antigen. Molecular cloning of the rfe-rff gene cluster. *J. Biol. Chem.* 1992, 267, 746−753.

(32) Pinto, V.; Berti, F. Exploring the Group B *Streptococcus* capsular polysaccharides: the structural diversity provides the basis for development of NMR-based identity assays. *J. Pharm. Biomed. Anal.* 2014, 98, 9−15.

(33) Harding, C. M.; Nasr, M. A.; Kinsella, R. L.; Scott, N. E.; Foster, L. J.; Weber, B. S.; Fiester, S. E.; Actis, L. A.; Tracy, E. N.; Munson, R. S., Jr; Feldman, M. F. *Acinetobacter* strains carry two functional oligosaccharyltransferases, one devoted exclusively to type IV pilin, and the other one dedicated to *O*-glycosylation of multiple proteins. *Mol. Microbiol.* 2015, 96, 1023−1041.

(34) Garcia-Quintanilla, F.; Iwashkiw, J. A.; Price, N. L.; Stratilo, C.; Feldman, M. F. Production of a recombinant vaccine candidate against *Burkholderia pseudomallei* exploiting the bacterial *N*-glycosylation machinery. *Front. Microbiol.* 2014, 5, No. 381.

(35) Harding, C. M.; Feldman, M. F. Glycoengineering bioconjugate vaccines, therapeutics, and diagnostics in *E. coli*. *Glycobiology* 2019, 29, 519−529.

(36) Wessels, M. R.; Paoletti, L. C.; Kasper, D. L.; DiFabio, J. L.; Michon, F.; Holme, K.; Jennings, H. J. Immunogenicity in animals of a polysaccharide-protein conjugate vaccine against type III group B *Streptococcus*. *J. Clin. Invest.* 1990, 86, 1428−1433.

(37) Carboni, F.; Adamo, R.; Fabbrini, M.; De Ricco, R.; Cattaneo, V.; Brogioni, B.; Veggi, D.; Pinto, V.; Passalacqua, I.; Oldrini, D.; Rappuoli, R.; Malito, E.; Margarit, I. Y. R.; Berti, F. Structure of a protective epitope of group B *Streptococcus* type III capsular polysaccharide. *Proc. Natl. Acad. Sci. U.S.A.* 2017, 114, 5017−5022.

(38) Lewis, A. L.; Nizet, V.; Varki, A. Discovery and characterization of sialic acid *O*-acetylation in group B *Streptococcus*. *Proc. Natl. Acad. Sci. U.S.A.* 2004, 101, 11123−11128.

(39) Pannaraj, P. S.; Edwards, M. S.; Ewing, K. T.; Lewis, A. L.; Rench, M. A.; Baker, C. J. Group B streptococcal conjugate vaccines elicit functional antibodies independent of strain *O*-acetylation. *Vaccine* 2009, 27, 4452−4456.

(40) Romero-Steiner, S.; Frasch, C. E.; Carlone, G.; Fleck, R. A.; Goldblatt, D.; Nahm, M. H. Use of opsonophagocytosis for serological evaluation of pneumococcal vaccines. *Clin. Vaccine Immunol.* 2006, 13, 165−169.

(41) Baker, C. J.; Rench, M. A.; Fernandez, M.; Paoletti, L. C.; Kasper, D. L.; Edwards, M. S. Safety and immunogenicity of a bivalent group B streptococcal conjugate vaccine for serotypes II and III. *J. Infect. Dis.* 2003, 188, 66−73.

(42) Juergens, C.; de Villiers, P. J.; Moodley, K.; Jayawardene, D.; Jansen, K. U.; Scott, D. A.; Emini, E. A.; Gruber, W. C.; Schmoele-Thoma, B. Safety and immunogenicity of 13-valent pneumococcal conjugate vaccine formulations with and without aluminum phosphate and comparison of the formulation of choice with 23-valent pneumococcal polysaccharide vaccine in elderly adults: a randomized open-label trial. *Hum. Vaccines Immunother.* 2014, 10, 1343−1353.

(43) Buurman, E. T.; Timofeyeva, Y.; Gu, J.; Kim, J. H.; Kodali, S.; Liu, Y.; Mininni, T.; Moghazeh, S.; Pavliakova, D.; Singer, C.; Singh, S.; Handke, L. D.; Lotvin, J.; Prasad, A. K.; Scully, I. L.; Donald, R. G. K.; Jansen, K. U.; Anderson, A. S.; Novel, A. Hexavalent Capsular Polysaccharide Conjugate Vaccine (GBS6) for the Prevention of Neonatal Group B Streptococcal Infections by Maternal Immunization. *J. Infect. Dis.* 2019, 220, 105−115.

(44) Marolda, C. L.; Lahiry, P.; Vines, E.; Saldias, S.; Valvano, M. A. Micromethods for the characterization of lipid A-core and O-antigen lipopolysaccharide. *Methods Mol. Biol.* 2006, 347, 237−252.

(45) Tirsoaga, A.; El Hamidi, A.; Perry, M. B.; Caroff, M.; Novikov, A. A rapid, small-scale procedure for the structural characterization of lipid A applied to Citrobacter and *Bordetella* strains: discovery of a new structural element. *J. Lipid Res.* 2007, 48, 2419−2427.

(46) Paschall, A. V.; Middleton, D. R.; Avci, F. Y. Opsonophagocytic Killing Assay to Assess Immunological Responses Against Bacterial Pathogens. *J. Visualized Exp.* 2019, No. e59400.

(47) Middleton, D. R.; Paschall, A. V.; Duke, J. A.; Avci, F. Y. Enzymatic Hydrolysis of Pneumococcal Capsular Polysaccharide Renders the Bacterium Vulnerable to Host Defense. *Infect. Immun.* 2018, 86, No. e00316-18.

(48) Burton, R. L.; Nahm, M. H. Development of a fourfold multiplexed opsonophagocytosis assay for pneumococcal antibodies against additional serotypes and discovery of serological subtypes in

Streptococcus pneumoniae serotype 20. *Clin. Vaccine Immunol.* **2012**, *19*, 835−841.