Sialylation of Group B Streptococcal Capsular Polysaccharide Is Mediated by \textit{cpsK} and Is Required for Optimal Capsule Polymerization and Expression

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Several bacterial pathogens have evolved the means to escape immune detection by mimicking host cell surface carbohydrates that are crucial for self/non-self recognition. Sialic acid, a terminal residue on these carbohydrates, inhibits activation of the alternate pathway of complement by recruiting the immune modulating molecule factors H, I, and iC3b. Sialylation of capsular polysaccharide (CPS) is important for virulence of group B streptococci (GBS), a significant human pathogen. We previously reported that \textit{cpsK}, a gene within the \textit{cps} locus of type III GBS, could complement a sialyltransferase deficient \textit{lst} mutant of \textit{Haemophilus ducreyi}, implicating its role in sialylation of the GBS capsule. To explore the function of \textit{cpsK} in GBS capsule production, we created a mutant in \textit{cpsK}. Immunoblot analysis and enzyme-linked immunosorbent assay using anti-type III CPS antiserum demonstrated that the mutant CPS did not contain sialic acid. This was confirmed by high-performance liquid chromatography after mild acid hydrolysis of the CPS. Although increased CPS chain length was seen for this strain, CPS production was <20% of the parental isolate. An episomal \textit{cpsK} copy restored synthesis of sialo-CPS to wild-type levels. These data support our hypothesis that \textit{cpsK} encodes the GBS CPS sialyltransferase and provide further evidence that lack of CPS oligosaccharide sialylation reduces the amount of CPS expressed on the cell surface. These observations also imply that one or more of the components involved in synthesis or transport of oligosaccharide repeating units requires a sialo-oligosaccharide for complete activity.

\textit{Streptococcus agalactiae} (group B streptococci or GBS) remains a major cause of serious neonatal bacterial infection in the developed world despite a >65% reduction in early-onset GBS disease (infection within the first week of life) due to the advent of chemoprophylactic prevention measures (37). The GBS capsular polysaccharide (CPS) is well established as a primary virulence determinant in GBS pathogenesis (16). Nine serotypes of GBS have been identified based on their unique CPS antigens (serotypes Ia and Ib and types II through VIII). The individual serotypes arise from the synthesis of distinct CPS precursor oligosaccharide repeating units (RPUs) and/or differences in the way the CPS RPUs are polymerized. The \textit{cps} loci in each serotype are organized similarly with genes in the 5’ region involved in regulation and chain length, a 3’ region encoding sialic acid synthesis, and a central region containing the oligosaccharide RPU structural and polymerization genes (Fig. 1). Despite significant heterogeneity in the central structural region of their \textit{cps} loci, all GBS serotypes produce RPUs with side chains terminated by N-acetylneuraminic acid (sialic acid; Neu5Ac) α2,3 linked to galactose (Gal).

Several bacterial species produce sialylated glycoconjugates on their surfaces. A number of gram-negative species, including \textit{Salmonella enterica}, \textit{Neisseria meningitidis}, \textit{Neisseria gonorrhoeae}, \textit{Haemophilus influenzae}, \textit{Haemophilus ducreyi}, and \textit{Escherichia coli}, are capable of producing sialylated forms of lipopolysaccharides (LPS) or lipooligosaccharides (LOS) (42). Certain serotypes of \textit{E. coli} and \textit{N. meningitidis} also produce capsules of homopolymeric polysialic acid (39). For gram-positive bacteria, only GBS and \textit{Streptococcus suis} produce sialylated polysaccharides (in the form of CPS).

The genes responsible for the addition of sialic acid to procaryotic glycoconjugates can be assigned to three separate families (39). The first group consists of the processive polymerases that produce sialic acid homopolymers on \textit{E. coli} K1 and K92 and \textit{N. meningitidis} serotypes B and C. These sialyltransferases are unrelated to those responsible for sialylation of LOS, LPS, and CPS synthesized by the block-type (Wzy-dependent) processes. The transferases of the latter type have been separated into two unrelated families, one composed of sialyltransferases from \textit{H. ducreyi}, \textit{H. influenzae}, \textit{E. coli} O104, GBS, and \textit{S. suis} (pfam05855.3 and \textit{Lst}), and the other family which contains the \textit{Neisseria} LOS sialyltransferases.

Variants of the above species lacking sialylated LPS, LOS, or CPS are generally less virulent, and a role for sialo-LOS and LPS in serum resistance and antiphagocytic activity has been demonstrated for \textit{N. gonorrhoeae} and \textit{N. meningitidis} (30, 44). Loss of CPS sialylation or capsule production by GBS with animal models has been correlated with decreased virulence (47, 36, 35).

It has been proposed that the presentation on the GBS cell surface of the sialylated terminal CPS RPU disaccharide, α-D-Neu5Ac(2→3)β-D-Galp, enables immune evasion through molecular mimicry (5, 24). This speculation is supported by the fact that a number of host glycoconjugates mediating self/non-self recognition, such as the blood group antigen sialyl-Lewis*
and glycosphingolipids, are terminated with sia1olactosamine \([\alpha-d-Neu5Ac(2\rightarrow3)\beta-d-Galp(1\rightarrow4)\beta-d-GlcpNAC] \). Sialylation of GBS CPS has been shown to be critical for prevention of opsononphagocytosis (36, 49) through inhibition of alternative complement pathway activation (29). Since CPS plays an essential role in GBS virulence, an understanding of the mechanism of CPS sialylation is important for understanding GBS pathogenesis. We have shown that cpsK of serotype III GBS exhibited sialyltransferase activity when expressed in \( E. coli \) in a sialyltransferase mutant of \( H. ducreyi \) (8). This was not confirmed, however, for GBS. In this report, we explore the role of the \( \text{cpsK} \) in GBS CPS sialylation, polymerization, and production through creation of a nonpolar GBS \( \text{cpsK} \) allelic exchange mutant and complementation of the mutation in \( \text{trans} \). Our results confirm that \( \text{cpsK} \) is the GBS sialyltransferase and provide additional insights into the effect of sialylation on CPS synthesis.

MATERIALS AND METHODS

**Bacterial strains and media.** Bacterial strains and plasmids used in this work are listed in Table 1. \( E. coli \) strains were grown in Luria-Bertani broth (LB; Sigma-Aldrich, St. Louis, MO) and either 100 \( \mu \text{g/ml} \) ampicillin (Amp), 5 \( \mu \text{g/ml} \) chloramphenicol (Cm), 50 \( \mu \text{g/ml} \) kanamycin (Km), 100 \( \mu \text{g/ml} \) spectinomycin (Sp), or 400 \( \mu \text{g/ml} \) erythromycin (Erm) (Sigma). Strains were grown at 37°C or at 30°C for the temperature-sensitive plasmid pHY304. GBS strains were grown in Todd-Hewitt broth (THB; Difco, Becton Dickinson and Co., Sparks, MD) at 37°C or 30°C with antibiotic selection of 5 \( \mu \text{g/ml} \) (Cm), 1,000 \( \mu \text{g/ml} \) (Km), 100 \( \mu \text{g/ml} \) (Sp), or 5 \( \mu \text{g/ml} \) (Erm) as required.

**Recombinant DNA procedures.** Restriction enzymes were from New England Biolabs (Beverly, MA). Taq DNA polymerase was obtained from Bioline (Bio-lase; Bioline USA, Randolph, MA). For high-fidelity DNA amplification, Expand Taq polymerase was used (Roche Applied Science, Indianapolis, IN). Other DNA modifying enzymes were from Promega (Madison, WI).

To derive a chromosomal chloramphenicol acetyltransferase gene \((\text{cat})\) replacement of \( \text{cpsK} \) within GBS strain COH1, pbS90, a genomic clone containing 9.0 kb of the COH1 \( \text{cps} \) locus, was digested with EcoRI and NsiI, and the 5.4-kb fragment containing \( \text{cpsK} \) was ligated to EcoRI/PstI restricted pBlueScript II KS(+) (Stratagene, La Jolla, CA) to form pDC143. After electroporation of the ligated mixture into \( E. coli \) DH5\( \alpha \), plasmid pDC143 was used to transform competent \( \text{COH1-350} \); clones carrying pDC143 were selected on LA containing 100 \( \mu \text{g/ml} \) Cm. The identity of the resultant plasmid (pDC147) was confirmed by PCR and restriction digestion. The insert DNA containing the \( \text{cat} \) replacement and flanking \( \text{cps} \) sequences was amplified from pDC147 by PCR using primers \( \text{cps}44 \) and \( \text{cnt}14 \) and high-fidelity Taq polymerase. The \( \text{cat} \) containing PCR fragment from pDC147 was ligated to a pHY304 T-vector created by EcoRV digestion, followed by addition of deoxycytidylymyhlin 3' overhangs using terminal deoxynucleotidyl transferase and dTTP (Promega). The ligation reaction mixture was introduced into DH5\( \alpha \) by electroporation. After growth at 30°C on LA with 5 \( \mu \text{g/ml} \) Cm for 48 h, colonies were screened for the resultant plasmid, pLM103; by PCR and restriction digestion. GBS strain COH1 was made competent as previously described (19) and transformed with plLM103, and \( \text{cat}\) allelic replacement mutants were selected as previously described (51) to produce COH1-350. The chromosomal replacement of \( \text{cpsK} \) by \( \text{cat} \) was confirmed by PCR, restriction digestion, and Southern and Northern blot analyses.

To complement the COH1-350 strain in \( \text{trans} \), a COH1 \( \text{cps} \) promoter plasmid, pBL26, was first constructed. Plasmid pZ12spec was digested with EcoRI and Spl and then ligated to an EcoRI/Splh fragment from pMut3 containing a promoterless \( \text{gfp} \) (mut3) to form pBL2-gfp. The \( \text{cpn} \) promoter region from COH1 was amplified using primers PCPSF2 and PCPSR1, followed by digestion with EcoRI and SmaI; ligation to EcoRI/SmaI-cut pBL2-gfp to form pBL26. Promoter activity was confirmed by fluorescence of DH5\( \alpha \) carrying pBL26 compared to the promoterless pBL2-gfp. To create a \( \text{cpn} \) expression vector, pBL26 was digested with NcoI and HindIII. The cut vector was ligated to a similarly digested PCR fragment containing \( \text{cpsK} \) and amplified using COH1 chromosomal DNA, primers \( \text{cpnK-NcoI} \) and \( \text{cpnK-HindIII} \), and high-fidelity Taq polymerase. \( E. coli \) DH5\( \alpha \) was transformed with the ligation reaction, and colonies expressing the resulting plasmid pLM104 were selected on LA containing 100 \( \mu \text{g/ml} \) Sp. Plasmid DNA isolated from DH5\( \alpha \) (pLM104) was used to transform competent COH1-350; colonies harboring pLM104, designated COH1-350(pLM104), were screened on THB with 100 \( \mu \text{g/ml} \) Sp.

A \( km-\Omega-2 \) insertion mutant of \( \text{cpsL} \) was created by amplification of COH1 chromosomal DNA with high-fidelity Taq polymerase using primers DC88 and DC89, followed by ligation into pGEM-T Easy (Promega), transfer to DH5\( \alpha \) by electroporation, and selection on LA containing 100 \( \mu \text{g/ml} \) Amp to produce pDC132. Small-digested pDC132 was ligated to BamHI linkers (Promega), cut with BamHI, and ligated to introduce an internal BamHI site in \( \text{cpsL} \) to create pDC150. After electroporation of the ligated DNA into \( E. coli \) XL1-Blue (Stratagene) and selection on LA containing 100 \( \mu \text{g/ml} \) Amp, the presence of the inserted site was confirmed by restriction digestion with BamHI. The \( km-\Omega-2 \) fragment of pO12 was amplified using primers PVC1V-1 and PVC2V-2, and the resulting PCR product was cut with BamHI and ligated to BamHI digested pDC150. The ligation reaction was used to transform \( E. coli \) XL1-Blue with selection on LA containing 50 \( \mu \text{g/ml} \) Km to form pDC151. The proper orientation of the \( km-\Omega-2 \) insertion in pDC151 was confirmed by double digestion with BglII and PstI. The \( km-\Omega-2 \) insertion fragment with the flanking \( \text{cpsL} \) DNA was

![FIG. 1. Organization of the Streptococcus agalactiae serotype III CPS synthesis operon. Proposed functions of the \( \text{cps} \) gene products are indicated at the top of the diagram. Locations of the Tn916\( \Delta E \) transposon and \( km-\Omega-2 \) cassette insertions in strains COH1-11 (black triangle), COH1-13 (open triangle), and COH1-355 (shaded triangle) are indicated. The site of the chloramphenicol acetyltransferase allelic exchange replacement of \( \text{cpsK} \) in COH1-350 and the \( \text{cps} \) locus expressed in pLM104 are also indicated. The \( \text{cps} \) gene designations are indicated below the arrows representing each open reading frame. The locations of the \( \text{cpsK} \) and \( \text{cpsL} \) probes for Northern dot blot analysis are shown as grey or open bars, respectively, above the schematic of the \( \text{cps} \) operon.](http://jb.asm.org/Downloaded/from/pa.jpg)
transferred to pH304 after PCR amplification from pDC151 with high-fidelity Taq polymerase. The amplicon from serotype III GBS strain COH1 cloned into pGEM T-Easy; pDC152, and double-crossover allelic replacement mutants were selected as above to produce pDC152. After electroporation into E. coli DH5α, and transferred to pHY304 after PCR amplification from pDC151 with high-fidelity Taq polymerase. The amplicon from serotype III GBS strain COH1 cloned into pGEM T-Easy; pDC152, and double-crossover allelic replacement mutants were selected as above to produce pDC152. After electroporation into E. coli DH5α, and transferred to pHY304 after PCR amplification from pDC151 with high-fidelity Taq polymerase. The amplicon from serotype III GBS strain COH1 cloned into pGEM T-Easy; pDC152, and double-crossover allelic replacement mutants were selected as above to produce pDC152. 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20 mM CaCl₂), along with Na azide to 0.05% final concentration, was added to Na phosphate buffer, pH 7.0, 10 mM MgCl₂, 40% [wt/vol] sucrose, 13.3 U/ml twice with ice-cold PBS. The cells were suspended in 200 ml lysis buffer (25 mM of 0.7. The culture was chilled on ice, and the cells were pelleted and washed

Isolation of capsular polysaccharides. Capsular polysaccharides from the COH1 and COH1-350(pLM104) strains were isolated as previously described (6, 12, 46) with modification. Bacteria were grown overnight in 400 ml of THB with 5 μg/ml Cm, if necessary, diluted to 4 liters in fresh THB, and grown to an OD₆₀₀ of 0.7. The culture was chilled on ice, and the cells were pelleted and washed twice with ice-cold PBS. The cells were suspended in 200 ml lysis buffer (25 mM Na phosphate buffer, pH 7.0, 10 mM MgCl₂, 0.05% Triton X-100, 20 mM Tris, pH 7.0). From this, two 200-
l aliquots were removed to new tubes and centrifuged, the supernatants were carefully

Quantification of cell surface-associated CPS. Mutanolysin extracts were prepared using a procedure modified from Pauletii et al. (33). GBS were grown overnight in THB with antibiotic selection if necessary, then diluted 1:10 in 120-mI fresh medium, and grown to an OD₆₀₀ of 0.8. The cells were harvested, washed once with ice-cold PBS, washed once in 50 mM Na phosphate, pH 7.0, and suspended in 5 ml 50 mM Na phosphate, pH 7.0. Four 1-ml aliquots of cells were transferred to prewetted 1.5 ml microcentrifuge tubes, pelleted by centrifugation, washed once with dH₂O, lyophilized, and weighed. A 300-μl aliquot from the remaining 1.0 ml of cells was transferred to a microcentrifuge tube and diluted 1:2 with 50 mM Na phosphate, pH 7.0. From this, two 200-μl aliquots were removed to new tubes and centrifuged, the supernatants were carefully removed, and the cells were suspended in 875 μl protoplast buffer each (20% [wt/vol] sucrose, 10 mm MgCl₂, 0.05% Triton X-100, 20 mM Tris, pH 7.0). Mutanolysin (125 μl, 10 U/ml) was added, and the samples were incubated at 37°C with rocking for 1 h. After centrifugation to remove protoplasts, 850 μl of the supernatant from each sample was removed to a fresh tube and frozen at −20°C. Competitive inhibition ELISAs were performed as previously described (10) using 96-well microtiter plates coated with poly-C-lysine-coupled purified GBS type III CPS or with CPS-coated plates and type III CPS standards. The assay was done using fourfold serial dilutions of CPS standards (10 μg/ml type III GBS or Spr14 CPS) or CPS extracts. For quantification of CPS from the COH1 and COH1-350(pLM104) strain extracts, anti-type III GBS GBS polyclonal Ab (1:20,000) was used with type III CPS-coated plates and type III CPS standards.
Anti-Spr14 CPS monoclonal Ab (1:100) was used with Spr14 CPS-coated plates and Spr14 CPS standards to determine the amount of CPS in extracts from the COH1-350 and COH1-11 strains. At least five independent assays were performed in duplicate for each strain and the values reported as milligrams of CPS per milligrams (dry weight) of cells.

Cell surface localization of CPS. CPS was determined to be associated with either the peptidoglycan or the cell membrane by the method of Bender et al. (2). GBS were grown to an OD560 of 0.8 in 10 ml THB, rapidly chilled to 4°C, harvested by centrifugation, washed once in PBS at 4°C, resuspended in 100 μl protoplast buffer containing 5 U/ml mutanolysin, and incubated overnight at 22°C. The mutanolysin extracts were centrifuged at 8,000 × g at 4°C for 5 min. The supernatants (cell wall fraction) were removed to a fresh tube, and the pellets (protoplasts) were washed once in 500 μl protoplast buffer and resuspended in 100 μl protoplast buffer. Proteolysis and cell wall fractions (2 μl [each] of strains COH1 and COH1-350 [LM104], 20 μl [each] of the COH1-13, COH1-11, and COH1-350 strains) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) with 4 to 20% gradient gels and transferred to nitrocellulose membranes. The membranes were blocked, incubated with anti-type III CPS polyclonal antiserum at a 1:30,000 dilution in LI-COR (Lincoln, NE) blocking buffer, and the immunobLOTS were developed with Alexa-680-conjugated goat anti-rabbit fluorescent antibody. CPS was detected with a LI-COR Odyssey IR scanner.

TLC of CPS hydrolysates. NeuAc was released from CPS by mild acid hydrolysis of 10 μg purified CPS in 100-μl 2 M acetic acid at 80°C for 1 h. The hydrolysates were dried in a SpeedVac centrifugal evaporator (Thermo Savant, Milford, MA), suspended in 2 μl 60% ethanol, and spotted on a silica gel 60 high-performance thin-layer chromatography (HPTLC) plate (Merck, Darmstadt, DE), along with Neu5Ac standards (5 μg/μl in 60% ethanol). Plates were developed twice in 1-butanol-acetic acid:H2O, 2:1.1 (47), and Neu5Ac was identified by its mobility and characteristic lavender color after being sprayed with diaphenylamine-aniline-phosphoric acid detection reagent (11).

For total hydrolysis of polysaccharides, 20 μg purified CPS was incubated in 200 μl 2 M trifluoroacetic acid at 108°C for 2 h under N2 in sealed glass ampoules. After hydrolysis, the samples were evaporated under a stream of N2 at 60°C, the residue was extracted twice with 20 μl methanol, and the extracts centrifuged briefly and dried in a SpeedVac concentrator. The samples were dissolved in 10 μl methanol, and 1 μl of each hydrolysate was separated by HPTLC on silica gel 60. The plates were developed twice in ethyl acetate:pyridine:H2O, 8:3:1, and the sugars were detected with diphenylamine-aniline-phosphoric acid detection reagent and compared to authentic glucose (Glc), galactose (Gal), glucosamine (GlcNH), and N-acetylgalactosamine (GlcNAc) standards.

Quantitation of total hydrolyzable NeuAc by HPLC. Quantitation of the amount of Neu5Ac in each CPS was achieved by high-performance liquid chromatography (HPLC) with fluorometric detection, after mild acid hydrolysis in 2 M acetic acid and derivitization with 1,2-diamino-4,5-methylendioxobenzene (DMB; Sigma-Aldrich) (1, 8). Assays were performed in triplicate with 2-keto-3-deoxyoctulosonic acid (KDO) added to the samples prior to hydrolysis as an internal standard. 12.5 μg Neu5Ac spiked with 12.5 μg KDO (final concentration) was hydrolyzed in 100 μl 2 M acetic acid as above. The samples were dried, dissolved in 5 μl dH2O, and then reacted with 30 μl 7 mM DMB in 1.4 M acetic acid, 88 mM Na2SO3, and 0.75 mM 8-mercaptoethanol for 2 h at 50°C in the dark. After derivatization, the samples were diluted 1:25 in HPLC mobile-phase buffer (4:25:92 acetonitrile:methanol:H2O [vol/vol]) and briefly centrifuged, and 12.5 μl was injected into the HPLC. Samples were separated on an Alltech Prevail 5 μm C18, column (4.6 by 150 mm) and eluted at a rate of 0.9 ml/min with fluorescence detection at 373 nm excitation and 448 nm emission.

Quantitation of intracellular NeuAc. To determine if intracellular NeuAc accumulated in COH1-350, 10-ml GBS cultures were grown to an OD560 of 0.8 and snap chilled in a dry-ice–ethanol bath; the cells were harvested, washed once in ice-cold PBS, and then suspended in 1 ml ice-cold 20 mM NH4HCO3, pH 7.8. The cells were disrupted at 4°C with glass beads (FP120; ThermoSavant); the lysates were removed; the beads washed once with 400 μl 20 mM NH4HCO3, pH 7.8; and the washed and lysates were combined. Unbroken cells and debris were removed by centrifugation at 8,000 × g and 4°C, and the supernatants were subjected to ultracentrifugation at 100,000 × g for 30 min at 4°C to pellet the cell membranes. The supernatant fraction was lyophilized a resuspended in 200 μl dH2O. The KDO internal standard was added (20 μl 20 mM KDO), and the samples were placed at −20°C for 20 min. The samples were centrifuged at 15,000 × g and 4°C for 20 min; the supernatants were removed, dried in a SpeedVac (ThermoSavant), and resuspended in 80 μl dH2O. The KDO internal standard was added (20 μl 200 μM), and the Neu5Ac extracts were passed over 0.3-ml Dowex 50W-X8 minicolumns. The columns were washed three times with 100 μl of dH2O, and the flowthrough and washes were combined and passed over 0.3-ml columns of Bio-Rad AG1-X8 (formate form). The columns were washed three times with dH2O, and the Neu5Ac was eluted with three 100-μl washes (each) of 2 M formic acid. The column eluates were combined, dried in a SpeedVac, and resuspended in 20 μl dH2O. A total of 5 μl of each extract was derivatized with DMB, and the amount of Neu5Ac present was determined by HPLC as above.

RESULTS

The cpsK gene is located in the cps operon of GBS (Fig. 1) with the other structural genes necessary for RPU synthesis. Because of the similar structure of the type III GBS RPU and the sialylated LOS of H. ducreyi, we hypothesized and subsequently demonstrated that the GBS cpsK could complement a mutation in the H. ducreyi sialyltransferase gene lst (8), restoring the terminal sialic acid to its LOS. This observation, as well as the homology between cpsK and the other bacterial sialyltransferases, suggested a role for cpsK in the sialylation of the RPU5s of GBS CPS.

Construction of a nonpolar cpsK deletion in COH1. To explore the role of cpsK in GBS CPS sialylation, a cpsK allelic exchange mutant was created by exact allelic replacement with a cat gene via homologous recombination. To deliver cat to the cps locus, the cat allele was flanked with cps sequences immediately 5' and 3' of cpsK, the chimeric fragment was ligated into the temperature-sensitive vector pHY304, and the recombinant plasmid was transformed into the type III strain COH1. Under nonpermissive conditions, the cat allele replaced cpsK via homologous recombination, producing strain COH1-350. The replacement was confirmed by Southern blotting and PCR analysis using COH1-350 chromosomal DNA (data not shown).

To determine if insertion of cat into the cps operon caused distal effects on downstream gene expression, RNA dot blot analysis was performed. Probes for cpsK, cpsL, and rpsL, a ribosomal structural gene, were generated and hybridized to RNA isolated from the wild-type (wt) and COH1-350 strains. Total RNA was hybridized to antisense RNA probes that were generated from in vitro transcription of cpsK, cpsL, or rpsL (a ribosomal subunit housekeeping gene). Either 4 or 2 μg of cellular RNA was transferred to a nitrocellulose membrane and hybridized with the probes. Strain designations: COH1, wt; COH1-350, ΔcpsK.
amounts of hybridization with the \textit{cpsL} probe were observed for both strains. These results demonstrated that insertion of the \textit{cat} gene did not exert polar effects on distal genes.

\textbf{Complementation of \textit{cpsK} deletion.} A derivative of COH1-350 containing an episomal copy of \textit{cpsK} was constructed to confirm that the results of the phenotype analysis performed below were a result of the deletion of \textit{cpsK}. A wild-type copy of \textit{cpsK} from COH1 was ligated into the low-copy-number vector pBL26 under control of the COH1 \textit{cps} promoter. The recombinant plasmid, designated pLM104, was transformed into COH1-350 to produce COH1-350(pLM104). This strain was used along with the wild-type and mutant strains to characterize the impact of the \textit{cpsK} mutation on CPS composition and expression.

\textbf{Cell surface CPS expression.} The CPS produced by \textit{Spn14} is structurally and immunologically identical to that of type III GBS asialo-CPS (Fig. 3) (5, 20, 25, 27, 43). MAb HASP-14 raised against \textit{Spn} 14 CPS also recognized GBS serotype III asialo-CPS. To test for cell surface expression of either sialo- or asialo-CPS, HASP-14 Ab (which recognizes type III asialo-CPS), in conjunction with anti-serotype III GBS polyclonal antibody (*) and anti-type III GBS polyclonal antibody (**) are as indicated.

\textbf{FIG. 3.} GBS serotype III and \textit{Spn14} CPS RPU structures, adapted from reference 27. Galp, galactose; Glcp, glucose; GlcpNAc, N-acetylgalactosamine; NeuNAc, N-acetylneuraminic (sialic) acid. The brackets to the right delineate the portions of the structure, corresponding to the CPS RPs produced by \textit{S. pneumoniae} serotype 14 and serotype III GBS, respectively. Structural motifs recognized by anti-Spn14 monoclonal antibody HASP-15 (†), anti-type III GBS sialo-CPS monoclonal antibody S9 (§), and anti-type III GBS polyclonal antibody (*) are as indicated.

\textbf{Biochemical analysis of mutant CPS.} CPS from COH1-350 was isolated by DEAE chromatography to determine if sialic acid was missing. Loss of sialic acid causes serotype III CPS to become neutrally charged, and thus it was not retained on DEAE. Column fractions were assayed for anti-type III CPS immunoreactive material by immuno-dot blot analysis. The immunoreactive material was observed in the void volume of the DEAE column, consistent with a sialic acid content of the mutant CPS.

\textbf{FIG. 4.} Whole cell immuno-dot blots using either anti-serotype III polyclonal (A), anti-serotype III sialo-CPS monoclonal (B), or anti-\textit{Spn14} CPS monoclonal (C) antibodies. Dilutions of cells were made in PBS and spotted on nitrocellulose membranes (1:1 = 10^6 CFU), and the cells were fixed and then probed with the various anti-CPS antibodies. Strains shown are COH1 (wt), COH1-350 (Δ\textit{cpsK}), COH1-350(pLM104) (Δ\textit{cpsK/ cpsK}), COH1-11 (Δ\textit{neuA}), and \textit{S. pneumoniae} NCTC11902 (\textit{Spn14}).
eluate, consistent with the loss of Neu5Ac from the CPS. The purified COH1-350 CPS was subjected to mild and strong acid hydrolysis, and the hydrolysates were analyzed by HPTLC. Mild acid hydrolysis removes sialic acid from the CPS without hydrolyzing other sugar linkages. Strong acid hydrolysis destroys sialic acid but otherwise reduces the CPS to its constituent monosaccharides. After mild acid hydrolysis, separation by HPTLC, and visualization with diphenylamine-aniline-phosphoric acid reagent, CPS purified from the COH1 and COH1-350(pLM104) strains produced a prominent band having the same characteristic lavender color and Rf seen for the Neu5Ac standard (Fig. 5, left). Some lactonization of the Neu5Ac was seen, as was previously reported with this method (47). Neu5Ac was not detected in the hydrolysates of COH1-350 or COH1-11, nor was it seen in the \( \text{Spn}_{14} \) CPS control (not shown). These findings are consistent with the absence of Neu5Ac. Total hydrolysis of CPS from the COH1, COH1-350, COH1-350(pLM104), and \( \text{Spn}_{14} \) strains, followed by HPTLC separation of the hydrolysates, revealed the presence of Glc, Gal, and GlcN (GlcNAc is converted to GlcNH under strong hydrolysis conditions), but no other sugars (Fig. 5, right). These data are consistent with the known composition of the type III GBS and \( \text{Spn}_{14} \) CPSs (48, 43).

To quantitate the amount of Neu5Ac associated with the CPS of the strains, purified CPS from each strain was hydrolyzed under mild acid conditions, and the amount of Neu5Ac released was quantitatively determined by HPLC (Table 3). Sialic acid was not detected in hydrolyzed CPS from COH1-350 or CPS purified from \( \text{Spn}_{14} \) but was present in equal amounts of CPS hydrolyzed from COH1 and COH1-350 (pLM104) (Table 3). The average percent composition of sialic acid in the RPU was 39% for COH1 and COH1-350(pLM104) and correlated well with the predicted 34% for type III CPS in which each repeating unit carries a single molecule of sialic acid. These observations provide compelling evidence for the loss of CPS sialylation in COH1-350 and restoration to parental levels in the \( \text{cpsK} \) mutant complemented by \( \text{cpsK} \) in trans.

**Accumulation of intracellular sialic acid in COH1-350.** To explore the possibility that the asialo phenotype might be due to defects in sialic acid synthesis in COH1-350, intracellular sialic acid concentrations were measured by HPLC. Under the conditions of the assay, CMP-Neu5Ac was hydrolyzed to Neu5Ac, so total intracellular sialic pools were measured. COH1-350 contained threefold more intracellular sialic acid than strain COH1. Intracellular sialic acid was not detected in COH1-355, a strain containing a polar insertion in \( \text{cpsL} \) that abolishes transcription of the downstream sialic acid synthesis genes \( \text{neuBCDA} \) (Fig. 1). The accumulation of sialic acid in COH1-350 confirmed that its asialo phenotype was not due to the lack of endogenous sialic acid synthesis but was due to a block in utilization of sialic acid by the sialyltransferase.

**Quantification of CPS production by COH1-350.** The amount of CPS produced by the COH1, COH1-350, COH1-11, and COH1-350(pLM104) strains was quantitated by competitive inhibition ELISA analysis of cell wall-associated CPS using anti-type III CPS polyclonal Ab (Fig. 6). ELISAs with type III CPS immobilized on microtiter plates and anti-type III CPS

### Table 3. Amount of CPS and degree of CPS sialylation for COH1, COH1-350, COH1-350(pLM104), and COH1-11 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amt of CPS (µg/mg cell dry wt)</th>
<th>% Sialic acid in CPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>COH1</td>
<td>8.7 ± 2.6</td>
<td>40.7 ± 2.9</td>
</tr>
<tr>
<td>COH1-350(pLM104)</td>
<td>6.8 ± 3.9</td>
<td>38.4 ± 3.0</td>
</tr>
<tr>
<td>COH1-350</td>
<td>1.8 ± 1.1</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>COH1-11</td>
<td>1.9 ± 1.2</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

FIG. 5. Thin-layer chromatography of purified CPS acid hydrolysates. (Left) Mild acid hydrolysis of CPS from strain COH1 (wt), COH1-350 (\( \Delta \text{cpsK} \)), and COH1-350(pLM104) (\( \Delta \text{cpsK} \)). The migration of pure sialic acid (Neu5Ac) is seen in the first lane (5 µg Neu5Ac). The Neu5Ac lactone is an artifact of the hydrolysis. (Right) Strong acid hydrolysis of CPS from strain COH1 (wt), COH1-350 (\( \Delta \text{cpsK} \)), and COH1-350(pLM104) (\( \Delta \text{cpsK} \)), serotype 14 \( \text{S. pneumoniae} \) NCTC11902 (\( \text{Spn}_{14} \)), or N-acetylglucosamine hydrolyzed under identical conditions. The migration distances of authentic glucosamine (GlcNH), galactose (Gal), glucose (Glc), and N-acetylglucosamine (GlcNAc) are indicated.
polyclonal antibody showed identical inhibition curves for the COH1 and COH1-350(pLM104) strains (Fig. 6A). This indicated equivalent CPS synthesis levels and identical Ab binding kinetics for both strains and suggested the CPS was immunologically indistinguishable. The COH1-350 and COH1-11 mutant strains, although having identical inhibition kinetics, showed markedly reduced competition for the antibody under these conditions when compared to the results with COH1 and COH1-350(pLM104) (Fig. 6A).

The decreased competition for anti-type III Ab by the CPS from COH1-350 and COH1-11 compared to COH1 and COH1-350(pLM104) is consistent with an asialo phenotype for the former two strains. However, another explanation for these results could be the potential decreased levels of CPS expression. To determine the absolute levels of CPS expression in these strains, ELISAs using Spn14 CPS immobilized on microtiter plates and anti-Spn14 CPS MAb were performed (Fig. 6B). Since Spn14 CPS is structurally identical to the GBS type III asialo-CPS, it was used as the standard in the ELISA to determine the concentration of CPS in the COH1-350 and COH1-11 extracts. The concentration of Spn14 control CPS used in this experiment (10 μg/ml) was equivalent to that of the control wild-type type III CPS used in the anti-Spn14 CPS ELISA above. COH1-350 and COH1-11 extracts demonstrated identical inhibition kinetics to each other, but they competed less effectively for the anti-Spn14 CPS antibody than the control Spn14 CPS, indicating reduced CPS production by the COH1-350 and COH1-11 strains. Quantitatively, the mutant strains produce only 20% of the CPS of the COH1 strain on the basis of milligrams of CPS per milligrams (dry weight) of cells (Table 3).

Localization of CPS in COH1-350. We questioned if increased secretion of CPS into the media by COH1-350 might be responsible for the decrease in anti-type III polyclonal Ab binding to cell extracts observed above. To investigate this possibility, strains COH1, COH1-350, COH1-350(pLM104) and COH1-11, and the acapsular transposon mutant strain COH1-13 were grown in chemically defined medium. After clarification by centrifugation, the spent culture medium was spotted onto nitrocellulose membrane as a twofold dilution series in PBS, either directly [strains COH1 and COH1-350(pLM104)] or after 100-fold concentration over a 10-kDa NMWCO filter (strains COH1-350, COH1-11 and COH1-13). Likewise, a dilution series of washed cells harvested from the cultures was spotted on the membrane along with purified type III GBS and Spn14 CPS standards. After incubation with anti-type III CPS polyclonal antisera and visualization of Ab binding (Fig. 7) it was apparent that the 100-fold concentrated supernatants from strains COH1-350 and COH1-11 had reduced Ab binding compared to strains COH1 and COH1-350(pLM104) (panel A). The binding was only slightly more than that seen for the concentrated supernatant from the acapsular COH1-13 strain. Based on the binding observed for the purified Spn14 and type III GBS CPS standards (Fig. 7C), strains COH1-350 and COH1-11 secreted 50-fold-less CPS into the medium than the parental strain COH1. Ab binding to PBS-washed whole cells of strains COH1-350 and COH1-11 was markedly reduced compared to that seen for strains COH1 and COH1-350.
These results demonstrated that the decrease in Ab binding seen in the COH1-350 and COH1-11 mutant strains in the previous experiments was not due to increased secretion into the bacterial culture supernatant. Further experiments were performed to determine the localization of the cell-associated CPS in COH1-350 compared to that of the control strains. Mutanolysin extracts of whole cells were separated by centrifugation into cell wall and protoplast fractions. Each fraction was separated by SDS-PAGE and transferred to a nitrocellulose membrane, and CPS on the membrane was detected after incubation with polyclonal anti-type III CPS Ab (Fig. 8). For all strains tested, the majority of the CPS was detected in the cell wall fraction, indicating that it had been associated with the cell wall prior to mutanolysin digestion. Only trace amounts of CPS were detected in the protoplast fractions, indicating efficient transfer of polymerized CPS to the cell wall.

**Effect of cpsK mutation on CPS chain length.** SDS-PAGE analysis of CPS from cell wall extracts (Fig. 8), indicated a lower electrophoretic mobility for CPS from strains COH1-350 and COH1-11 than the wild-type and the complemented COH1-350(pLM104) strains. These data suggested an increase in CPS chain length for the asialo mutants. To determine the effect of cpsK on CPS polymerization, CPS from strains COH1, COH1-350, and COH1-11 was characterized by Sephacryl S-400 size exclusion chromatography (Fig. 9). Based on dextran calibration standards, the average CPS molecular mass for COH1 was 118 ± 11 kDa (four independent determinations) which corresponded to ~115 pentasaccharide RPUs. The COH1-350 CPS was found to be significantly larger (410 ± 107 kDa; three independent determinations) corresponding to ~600 tetrasaccharide RPUs. The CPS from strain COH1-11 eluted at the same position as that of COH1-350, and CPS isolated using NaOH or enzymatic digestion gave similar values. Thus, the decrease in total CPS could not be attributed to decreased CPS chain length but rather suggests that the reduc-
tion in quantity is due to fewer (but longer) CPS chains on the COH1-350 strain. Similar analysis by size exclusion chromatography of the CPS from COH1-350(pLM104) revealed it to be indistinguishable in size from the CPS of COH1, demonstrating restoration of the wt phenotype when cpsK was expressed in trans.

**DISCUSSION**

GBS is among a select number of significant human and animal pathogenic bacteria expressing sialic acid on their cell surfaces. For these organisms, sialic acid enhances their virulence by interfering with the mechanisms of host innate immune recognition, specifically with opsonic components of the complement system. We previously demonstrated that GBS cpsK encodes sialyltransferase activity when expressed in *H. ducreyi*. In this report, we used genetic and biochemical approaches to conclusively demonstrate that cpsK is responsible for the addition of sialic acid to the GBS CPS. Additionally, we show that loss of CPS sialylation results in qualitative and quantitative differences in the nature of the CPS produced.

Creation of a cpsK frame deletion mutant was accomplished by allelic replacement of cpsK with a cat allele. Genes distal to cpsK, including the genes responsible for sialic acid synthesis (neuBCDA), were transcribed normally in this strain, and determination of intracellular sialic acid levels confirmed sialic acid synthesis was retained. CPS on the surface of the cpsK deletion strain failed to bind an anti-sialo-CPS MAb. However, it did bind to an anti-type III CPS polyclonal antibody, which recognizes both asialo-CPS and native serotype III CPS, as well as to an anti-Spn14 monoclonal antibody, which only binds the asialo form of type III GBS CPS. Episomal expression of cpsK in the cpsK deletion mutant restored its antibody binding phenotype to that of the wild-type COH1 strain. These data were consistent with the absence of sialic acid on the CPS of the cpsK mutant and indicated that CPS sialylation could be restored by expression of cpsK in trans. We confirmed the above results using mild acid hydrolyses of purified CPS from the cpsK mutant, which did not contain detectable sialic acid. Glucose, galactose, and N-acetylgalactosamine were observed in the expected molar ratio after strong acid hydrolysis of cpsK mutant CPS. Sialic acid was recovered from mild acid hydrolyses of CPS from the COH1-350 mutant expressing cpsK in trans, demonstrating that the asialo phenotype of COH1-350 was due to the absence of cpsK activity. These data provide compelling evidence that cpsK encodes the CPS sialyltransferase.

Of interest was the observation that loss of sialylation had a dramatic impact on the amount of CPS produced. The 80% reduction in surface-associated CPS produced by both asialo mutant strains compared to the parental strain was consistent with our previously reported results for the neuA mutation (47) and suggested that sialylation was important for full synthesis of CPS by GBS. The ELISA data were corroborated by data demonstrating proportionately less CPS isolated per gram (dry weight) of cells from the cpsK mutant than from the COH1 strain. We confirmed that the decrease in cell surface-associated CPS in the cpsK and neuA mutant strains was not due to loss of surface attachment and increased secretion of CPS into the culture supernatants. Our results demonstrated that most of the CPS for the mutant and wild-type strains was located in the cell wall fraction, suggesting that it is efficiently transferred from the lipid carrier and attached to the peptidoglycan regardless of the degree of sialylation. This observation implies that the CPS muro-phosphodiester activity in GBS is insensitive to the presence of sialic acid on the CPS and therefore unrelated to the reduced polysaccharide production seen in the asialo COH1-350 and COH1-11 mutant strains. That these two strains exhibit indistinguishable CPS phenotypes established that these effects are due to the loss of CPS sialylation independent of the specific lesion within the sialic acid biosynthesis and transfer pathway. This observation appears to be in contrast to that found with the lst mutations in *H. ducreyi* and *N. meningitidis*, whereby loss of sialyltransferase activity did not reduce production of the asialo-LPS backbone structure (4).

Differences in the apparent size of CPS produced by the COH1-350 strain were also evident by size exclusion chromatography. A possible explanation is that there was a significant increase in the degree of CPS polymerization in COH1-350 compared to the COH1 strain. If so, the CPS from the cpsK mutant, on average, contained five times more RPUs than CPS from COH1: 410 kDa for COH1-350 or approximately 600 RPUs, compared to 118 kDa and 120 RPUs for the COH1 CPS. In our experiments, the molecular mass of the COH1 CPS differed somewhat from that previously reported (~250 kDa), and the CPS produced by the asialo neuA strain COH1-11 was found to be smaller than that of the parental isolate (~100 kDa) (47). The discrepancy in size could be due to differences in CPS preparation, since group antigen and cell wall fragments were not decoupled from CPS alkaline hydrolysis in our previous report. However, when we extracted the CPS from the COH1-11 strain by only enzymatic means, we observed the same size as that seen by alkaline extraction. The greater degree of CPS polymerization in the COH1-350 strain suggested that regulation of CPS chain length is relaxed for the asialo-CPS and indicated that a decrease in overall CPS expression was not due to reduced chain length in the COH1-350 strain.

Possible reasons for the decrease in CPS production for the asialo mutant strains could include diminished production of CPS oligosaccharide precursors and/or reduced transfer of CPS precursors across the cytoplasmic membrane. If the sialyltransferase is an integral part of a membrane-associated complex of glycosyltransferases, its loss may also disrupt the functional integrity of the CPS synthesis complex. In addition, these data may indicate the role of a feedback mechanism on the functions of cpsBCD, which have been previously shown to control chain length and transport of the polysaccharide (31, 12, 3, 2). We postulate that the asialo RPUs as they are polymerized may fail to provide the maturation signal that leads to control of the overall chain length observed with the wild-type CPS. We are currently investigating the potential mechanisms behind these observations.

Despite significant differences in overall CPS structure, all known serotypes of GBS produce a sialylated CPS. Since our last report, the DNA sequence of cpsK from the remaining serotypes II, VII, and VIII have been determined. Comparison of the capsule genes from each serotype suggests that the cpsK locus is a common crossover point for insertion of DNA sequences encoding novel glycosyltransferases (12a). With the
exception of types Ia and III, which differ only in their RPU polymerase alleles, the region of variable glycosyltransferases ends within cpsK. Thus, the 5’ region of cpsK differs between serotypes, whereas the 3’ end is conserved between serotypes. The variable portions of the alleles are distantly related but are phylogenetically closer to each other than to the sialyltransferases of other species. The one exception is cpsK of serotype VIII, which is unrelated to the other GBS sialyltransferases and is instead homologous to the N. meningitidis and N. gonorrhoeae lipooligosaccharide sialyltransferase family. It remains to be determined if the heterogeneity between cpsK alleles reflects evolutionary drift alone or also functional differences in sialyltransferase specificity to accommodate the structural differences between the nine CPS serotype RPUs. The data above suggest that conservation of the sialic acid decoration among the GBS CPS structures is under strong evolutionary pressure, potentially to retain CPS epitopes crucial to immune evasion and survival in the host.

Sialylation is critical to the function of CPS (18, 38, 41) and, as shown here, is also critical to the biosynthetic process. The various GBS serotypes share virtual identity between the genes that are responsible for regulation and export of CPS production. Their capsule operons differ only in the central glycosyltransferase region responsible for synthesis of their CPS RPU oligosaccharides. The data presented here suggest that the synthesis, export, and regulatory mechanisms are highly integrated with the incorporation of sialic acid on CPS. Indeed, strains that lose the ability to synthesize sialic acid or transfer it to their CPS suffer a significant loss of surface polysaccharide, resulting in an increased sensitivity to opsonization and phagocytic killing (47). This may explain the preservation of a sialylated CPS by the different GBS serotypes in contrast to the inability to sialylate the capsule is understood on the molecular level, it may be possible to construct strains that produce wt levels of asialo-CPS, which will allow direct evaluation of sialic acid’s contribution to the ability of GBS to resist complement opsonization.

Further investigation into the mechanisms by which each GBS serotype adds sialic acid to its CPS should lead to novel insights into the poorly understood areas of CPS export and polymerization by gram-positive organisms and could help to further characterize its role in GBS pathogenesis.

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