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Identification of a Genetic Locus Essential for Capsule Sialylation in Type III Group B Streptococci

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The type III capsular polysaccharide of group B streptococci (GBS) consists of a linear backbone with short side chains ending in residues of *N*-acetylneuraminic acid, or sialic acid. The presence of sialic acid on the surface of the organism inhibits activation of the alternative pathway of complement and is thought to be an important element in the virulence function of the capsule. We showed previously that a mutant strain of GBS that expressed a sialic acid-deficient, or asialo, form of the type III polysaccharide was avirulent, supporting a virulence function for capsular sialic acid. We now report the derivation of an asialo capsule mutant from a highly encapsulated wild-type strain of type III GBS, strain COH1, by insertional mutagenesis with transposon Tn*916ΔE*. In contrast to the wild-type strain, the asialo mutant strain COH1-11 was sensitive to phagocytic killing by human leukocytes in vitro and was relatively avirulent in a neonatal rat model of GBS infection. The asialo mutant accumulated free intracellular sialic acid, suggesting a defect subsequent to sialic acid synthesis in the biosynthetic pathway leading to capsule sialylation. The specific biosynthetic defect in mutant strain COH1-11 was found to be in the activation of free sialic acid to CMP-sialic acid: CMP-sialic acid synthetase activity was present in the wild-type strain COH1 but was not detected in the asialo mutant strain COH1-11. One of the two transposon insertions in the asialo mutant COH1-11 mapped to the same chromosomal location as one of the two Tn*916* insertions in the previously reported asialo mutant COH31-21, identifying this site as a genetic locus necessary for expression of CMP-sialic acid synthetase activity. These studies demonstrate that the enzymatic synthesis of CMP-sialic acid by GBS is an essential step in sialylation of the type III capsular polysaccharide.

Group B streptococci (GBS) are the leading cause of neonatal sepsis and meningitis in the United States (4, 13). Several lines of evidence indicate that the GBS capsular polysaccharide is a critical factor in the ability of the organism to produce invasive infection. While the four major capsular types are found with similar frequencies among isolates cultured as commensals from the genital tracts of colonized women, type III strains predominate among isolates from the blood or cerebrospinal fluid of infants with GBS sepsis. Organisms of capsular type III account for approximately 60% of GBS isolated from infants with neonatal sepsis and 80 to 90% of those with meningitis (1, 6). That type III strains are disproportionately prevalent among GBS associated with invasive disease suggested that the type III capsule serves as a virulence factor. This hypothesis was supported by the observation that type III isolates from the blood or cerebrospinal fluid of ill infants were more highly encapsulated than were colonizing strains (16). More direct evidence for the role of the type III capsule in virulence came from our studies of an unencapsulated mutant strain of type III GBS: loss of capsule expression was associated with loss of virulence in a neonatal rat model of GBS infection (26).

A structural feature of the type III capsule that appears to be important in virulence is the presence of *N*-acetylneuraminic acid, or sialic acid. The type III capsular polysaccharide is a high-molecular-weight polymer with a trisaccharide backbone repeating unit. Attached to each backbone repeating unit is a disaccharide side chain which terminates with a sialic acid residue (38). The presence of these externally located sialic acid residues on the surface of the GBS cell has important implications for interaction of the organism with the host immune system. Sialic acid plays a major role in determining the conformation of the epitope recognized by protective anticapsular antibodies (14). In addition, like other sialic acid-coated particles, fully encapsulated type III GBS inhibit activation of the alternative pathway of complement. Several investigators have shown that surface-located sialic acid residues increase the affinity of surface-bound C3b for the complement regulatory protein factor H, resulting in the inactivation of C3b by factor I, rather than amplification of alternative pathway activity (10, 21). The sialic acid-containing capsular polysaccharides of both *Escherichia coli* K1 and GBS type III have been shown to inhibit complement activation by this mechanism, accounting, at least in part, for the resistance of these organisms to phagocytic killing in the absence of specific antibody (7, 8, 31).

Chemical or enzymatic removal of the sialic acid residues from the GBS type III polysaccharide produces the desialylated or core type III polysaccharide, which has a repeating unit structure and immunoreactivity identical to those of the capsular polysaccharide of type 14 *Streptococcus pneumoniae* (14, 15, 38). We recently reported the derivation and characterization of a transposon mutant strain of type III GBS that expressed the core, or asialo, form of the GBS type III capsular polysaccharide (39). Loss of capsular sialic acid was associated with a similar reduction in virulence as complete loss of capsule, supporting a critical role of sialic acid in the virulence function of the type III capsule (39).

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The wild-type GBS strain COH31r/s from which we derived the asialo mutant reported previously was relatively poorly encapsulated. Since most type III GBS strains isolated from ill infants express larger amounts of capsular polysaccharide than strain COH31r/s, mutants derived from strain COH31r/s provided a useful, but limited, experimental model. To investigate more thoroughly the effects of altered capsule expression on virulence, and also to begin to dissect the molecular mechanisms of capsule biosynthesis and regulation, we developed a system for derivation of capsule mutants in the background of the well-encapsulated type III GBS strain COH1, a wild-type isolate typical of those associated with neonatal sepsis. In contrast to strain COH31r/s, COH1 is relatively resistant to phagocytic killing in the absence of specific antibody and is more virulent in animals than strain COH31r/s.

We now report the derivation of an asialo capsule mutant from the well-encapsulated wild-type strain of type III GBS, strain COH1, by insertional mutagenesis with transposon Tn*916ΔE*. Comparison of the asialo mutant with the wild-type strain in assays of virulence, both in vitro and in vivo, supported the hypothesis that the sialylated type III capsule is a virulence factor for GBS. These investigations defined an essential enzymatic step in sialylation of the type III GBS polysaccharide and identified a genetic locus required for expression of this enzymatic activity in a region of the GBS chromosome previously implicated in capsule biosynthesis.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype[a]	Source
GBS		
COH31r/s	Rf^r Sm^r, type III	26
COH31-21	Tc^r Rf^r Sm^r Tn*916*, asialo type III	39
COH31-15	Tc^r Rf^r Sm^r Tn*916* cap^-, type III	26
COH1	Tc^r, type III	19
COH1-11	Em^r Tc^r Tn*916ΔE*, asialo type III	This study
Escherichia coli		
DH1	F^- *recA1 endA1 gyrA96 thi-1 hsdR17 supE44*	D. Clewell
O18	K1 capsule positive	D. Goldmann
Enterococcus faecalis RH110	Rf^r Fs^r Em^r Tn*916ΔE*	25
Plasmids		
pCER110	Ap^r Em^r Tn*916ΔE* (21.8 kb)	25
pCER3121-1	Ap^r (10.2 kb)	18
pCER212-1	Ap^r (3.7 kb)	18

[a] Rf^r, rifampin resistant; Sm^r, streptomycin resistant; Tc^r, tetracycline resistant; Em^r, erythromycin resistant; cap^-, capsule negative; Fs^r, fusidic acid resistant; Ap^r, ampicillin resistant.

MATERIALS AND METHODS

Antigens and antisera. Purified type III GBS capsular polysaccharide was prepared from broth cultures of type III GBS strain M781 as previously described (37). Type 14 pneumococcal (Pn14) polysaccharide was prepared by Merck Sharpe & Dohme, Inc., and obtained through the American Type Culture Collection, Rockville, Md. Type III GBS antiserum was prepared by immunizing New Zealand White rabbits with formalin-fixed type III GBS organisms (8). Pn14 antiserum was purchased from Dako Corporation, Santa Barbara, Calif. A murine monoclonal antibody (UU218) specific for the native type III GBS polysaccharide (27) was used to identify capsule-deficient mutants in immunoblot assays. This reagent was kindly provided by Harry Hill, University of Utah, Salt Lake City.

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are described in Table 1.

Isolation of mutants. Tn*916ΔE* is a derivative of transposon Tn*916* in which the tetracycline resistance gene has been replaced with a constitutive erythromycin resistance gene (25). Conjugation of Tn*916ΔE* from *Enterococcus faecalis* RH110 to type III GBS strain COH1 was performed as described previously (25). Transconjugants obtained after filter mating were enriched for mutants deficient in expression of capsule or capsular sialic acid by their failure to agglutinate in the presence of wheat germ lectin (26) and were selected by growth on Todd-Hewitt agar containing tetracycline (10 μg/ml) (to select for GBS) and erythromycin (10 μg/ml) (to select for Tn*916ΔE*).

Immunoblot analysis. Immunoblot analysis of transconjugants was performed as described previously (26). Briefly, colonies were transferred from agar plates to nitrocellulose filters, and the bacterial cells were lysed and precipitated. Filters were treated with diluted antiserum or with monoclonal antibody UU218 and then with the appropriate alkaline phosphatase-conjugated second antibody, and color development was achieved with 5-bromo-4-chloro-indolyl-phosphate/Nitro Blue Tetrazolium (Bio-Rad Laboratories, Richmond, Calif.).

DNA techniques. Restriction enzyme digestion of DNA with *Eco*RI and *Hin*dIII, agarose gel electrophoresis, Southern transfer of DNA fragments to nitrocellulose, and hybridization with a ^{32}P-Tn*916ΔE* DNA probe have been described previously (26). The ^{32}P-Tn*916ΔE* probe was prepared from the 20.1-kb *Eco*RI fragment of plasmid pCER110. This fragment was electroeluted from low-melting-temperature agarose and labeled with [^{32}P]deoxynucleotides in vitro by random priming hexamer labeling (Pharmacia-LKB Biotechnology, Uppsala, Sweden). The Tn*916ΔE* insertion sites in COH1-11 were mapped as described previously (18). The *Eco*RI fragments flanking each of the two Tn*916* insertion sites in the genome of COH31-21 have been cloned previously and designated pCER3121-1 and pCER212-1, respectively (Table 1) (18). Plasmid pCER3121-1 contains a 7.5-kb *Eco*RI fragment that was mapped to the 30-kb region of the GBS chromosome associated with type III capsule production. Plasmid pCER212-1 contains the 1.0-kb fragment that flanked the second Tn*916* insertion site in COH31-21, a site remote from the 30-kb capsule region. DNA from each of these two cloned fragments was used to prepare probes to determine whether the two Tn*916ΔE* sequences in COH1-11 were in the same chromosomal locations as the Tn*916* insertions in COH31-21.

Purification of capsular polysaccharide from strain COH1-11. Strain COH1-11 was grown in 80 liters of Todd-Hewitt broth containing 8% glucose with continuous titration to pH 7.0. Capsule was released from the bacterial cells by using mutanolysin as described previously (39). The crude capsular polysaccharide was purified by chromatography on DEAE-Sephacel as described previously (39). The polysaccharide was detected in the eluate which failed to bind to the DEAE column by reactivity in Ouchterlony immunodiffusion gels with Pn14 antiserum (20). The fractions containing

polysaccharide were dialyzed extensively against distilled water and lyophilized. Contaminating group B carbohydrate was depolymerized by treatment with 0.5 M NaOH at 50°C for 48 h; because base treatment may result in partial deacetylation of *N*-acetylglucosamine residues, the NaOH-treated capsular polysaccharide was re-N-acetylated with acetic anhydride (4% by volume) in 5% sodium bicarbonate at room temperature for 2 h. After dialysis and lyophilization, the final product was separated from low-molecular-weight contaminants by chromatography on a column (1.6 by 82 cm) of Sepharose 4B-CL with 20 mM Tris buffer (pH 7.2) as the eluant. The final product was shown to contain less than 1% protein by the Bradford method (5), with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as a standard. Sialic acid content was estimated by the thiobarbituric acid method (34), using sialic acid (Sigma) as a standard.

Determination of molecular size of COH1-11 and COH1 polysaccharides by fast protein liquid chromatography. Each strain was grown to the logarithmic phase in 2 liters of Todd-Hewitt broth (A_{650} = 0.5). The bacterial cells were collected by centrifugation and washed once with 50 mM Tris-HCl buffer (pH 7.6). The cell pellet was resuspended in 100 ml of the same buffer containing 40% (wt/vol) sucrose and treated with mutanolysin (4 mg; Sigma) for 12 h at 37°C to release capsular polysaccharide from the cells. Protoplasts and cell debris were removed by centrifugation at 12,000 × *g* for 20 min, and then the cell capsule extract was treated with DNase (5 μg/ml; Sigma) and RNase (20 μg/ml; Sigma) for 8 h at 37°C and then with pronase (40 μg/ml; Sigma) for 16 h at 37°C. After dialysis against water, the polysaccharide was lyophilized, dissolved in 50 mM Tris-HCl buffer (pH 7.6), and loaded onto a column (2.6 by 85 cm) of Sephacryl S300 (Pharmacia). Fractions containing capsular polysaccharide were identified by testing aliquots in enzyme-linked immunosorbent assay (ELISA) inhibition as described previously (35). Type III GBS ELISA was used to detect COH1 polysaccharide, and Pn14 ELISA was used to detect COH1-11 polysaccharide. Fractions containing polysaccharide by ELISA were pooled, dialyzed against water, and lyophilized. The polysaccharide was dissolved in phosphate-buffered saline and loaded onto a Superose 6 HR 10/30 column (Pharmacia) with phosphate-buffered saline as the running buffer at a flow rate of 0.5 ml/min. Elution of the polysaccharide was monitored by measuring the refractive index of the eluate (model R401 Refractometer; Waters Associates, Bedford, Mass.), and the elution volume of each polysaccharide was confirmed by ELISA inhibition as described above. The molecular size of each polysaccharide was estimated by comparison of its K_{av} with a calibration curve generated by using dextran standards.

ELISA. GBS III or Pn14 capsular polysaccharide was coupled to poly-L-lysine (Sigma) and coated onto 96-well plates as previously described at a concentration of 1 μg of polysaccharide per ml (11, 26). After washing three times with 40 mM sodium phosphate (pH 7.0) containing 0.05% Tween 20 (Sigma), nonspecific protein-binding sites were blocked by incubating wells with 200 μl of 5% skim milk powder (Difco) in the same buffer at 37°C for 2 h. After washing three times with the same buffer, inhibitor and antiserum were added at an appropriate dilution in the same buffer and incubated at 37°C for 1 h. Subsequent steps of addition of alkaline phosphatase-conjugated secondary antibody, addition of substrate, and measurement of color development were done as described previously (26).

In vitro opsonophagocytic assay. Opsonophagocytic assays were performed essentially as described by Baltimore et al. (2). Early log-phase organisms (4 × 10^6 CFU) were incubated with 3 × 10^6 human peripheral blood leukocytes in 0.5 ml of Eagle's minimal essential medium. Each assay contained, in addition, 10% human serum (previously adsorbed with GBS type III organisms and stored at −80°C to preserve complement activity). Aliquots were removed, diluted in sterile water, and spread on blood agar plates for quantitative culture at time zero and after 1 h of end-over-end rotation at 37°C.

Virulence assays. The virulence of mutant strain COH1-11 was compared with that of the wild-type strain COH1 in a neonatal rat model of lethal GBS infection as described previously (26). The 50% lethal dose (LD_{50}) was calculated by the method of Reed and Meunch (23) based on data from two experiments utilizing a total of 80 animals for each strain.

Assay for intracellular sialic acid. GBS strains to be tested for the presence of free intracellular sialic acid were grown in 500 ml of Todd-Hewitt broth to the mid-log phase (A_{650} = 0.5 to 0.8). Bacterial cells were collected by centrifugation, suspended in 5 ml of 10 mM Tris (pH 7.0) containing mutanolysin (3,000 U/ml; Sigma), and incubated at 37°C for 1 h. The cells were ruptured by shaking with glass beads for 20 min (Vibrogen cell mill; Edmund Bühler, Tübingen, Germany). The lysate was clarified by centrifugation at 13,600 × *g* for 5 min (Micro-centrifuge; Fisher Scientific Co., Pittsburgh, Pa.). Nucleic acids were removed by precipitation with 60% ethanol, and the supernatant was analyzed by thin-layer chromatography (TLC) on plastic-backed plates of Silica Gel 60 (E. Merck AG, Darmstadt, Germany). Plates were developed in a solvent system of butanol-acetic acid-water (2:1:1, vol/vol), dried, and stained with diphenylamine spray. Sialic acid (*N*-acetylneuraminic acid) was identified by R_f and characteristic pink staining by comparison with an authentic sialic acid standard run simultaneously.

Purification of sialic acid from strain COH1-11. Asialo mutant strain COH1-11 was grown in broth culture as described above. Lyophilized bacterial cells (10 g) were stirred at 37°C overnight in 250 ml of water containing 1 mM $MgCl_2$ and 50,000 U of mutanolysin. The protoplasts were recovered by centrifugation and lysed by sonication in water for 15 min (Sonifier; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). An equal volume of 95% ethanol was added to the lysate and allowed to stand at 4°C overnight. Precipitate and cell debris were removed by centrifugation, and the supernatant was dried on a rotary evaporator (Rotavapor; Büchl, Flawil, Switzerland). Sialic acid was purified from this material by a modification of the method described by Pozsgay et al. (22) for purification of sialic acid from edible bird's nest. Briefly, the residue was dissolved in 3 ml of water and loaded onto a column (2.6 by 18 cm) containing Dowex AG-50W-X8 cation-exchange resin, equilibrated with water. Fractions corresponding to the void volume were lyophilized, redissolved in 3 ml of water, and loaded onto a column (2.6 by 16 cm) containing Dowex AG 2-X8 anion-exchange resin, 100/200 mesh, chloride form. The sample was eluted with a 500-ml linear gradient of 0.2 to 0.5 M formic acid in water. Fractions were analyzed by TLC for the presence of substances resembling sialic acid by R_f and staining characteristics. Fractions in which sialic acid was detected by TLC were pooled, and formic acid was removed by repeated evaporation on a rotary evaporator. The final product was desalted on a column (1.6 by 80 cm) of P2 Bio-Gel, using water as the running buffer.

CMP-sialic acid synthetase assay. GBS cells from a mid-

log-phase broth culture were collected by centrifugation, yielding 5.0 ± 0.5 g (wet weight) of bacteria per liter of culture. The bacterial pellet was washed in 50 mM Tris-HCl buffer (pH 7.6), resuspended in the same buffer to approximately 0.5% of the original broth culture volume, and lysed by shaking with glass beads (Vibrogen cell mill) for 20 min with ice water cooling. The lysate was clarified by centrifugation at 10,000 × *g* for 20 min. A similar lysate was prepared from *E. coli* O18 (K1 capsule positive), kindly provided by Donald Goldmann, Boston Children's Hospital, except that the cells were grown in Trypticase soy broth and lysed by passage through a French pressure cell (Aminco, Urbana, Ill.) at 12,000 lb/in^2. The assay for CMP-sialic acid synthetase was similar to that utilized by Vann et al. (32), except that ^{32}P-labeled CTP was used as a substrate and the product was detected by TLC and autoradiography instead of by thiobarbituric acid assay. The assay mixture contained 10 μl of cell lysate, 10 μl of 25 mM sialic acid, 5 μl of 50 mM CTP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 1 μCi of [α-^{32}P]CTP (800 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), 15 μl of water, and 10 μl of assay buffer consisting of 1 M Tris-HCl, 1 mM dithiothreitol, and 0.1 M $MgCl_2$ (pH 8.8). After incubation at 37°C for 60 min, a 2.5-μl aliquot of the assay mixture was applied to a TLC plate coated with Silica Gel 60. The plate was developed in a solvent system of 2-propanol–ammonium hydroxide–water (6:1:2, vol/vol). After drying, the plate was cut 1 cm above the origin and the top portion was exposed to X-ray film (Eastman Kodak Co., Rochester, N.Y.). This procedure was used to prevent overexposure of the film from radioactivity remaining at the origin. The band corresponding to CMP-sialic acid was identified by its comigration with a CMP-sialic acid standard (Sigma).

RESULTS

Derivation of a Tn*916ΔE* mutant expressing an asialo capsular polysaccharide. We have reported previously the derivation and characterization of an asialo capsular mutant of type III GBS. That mutant, COH31-21, was shown to produce a capsular polysaccharide with a repeating unit structure identical to that of the wild type, except for the absence of sialic acid residues (39). While animal studies showed a clear reduction in virulence of the asialo mutant strain COH31-21, the wild-type strain COH31r/s was itself not highly virulent and produced a relatively small amount of capsular polysaccharide compared with typical type III GBS strains isolated from neonates with sepsis. The small capsule of COH31r/s (and COH31-21) limited our ability to characterize the biochemical defect in the mutant and also limited the generality of inferences we could make about effects of the mutation on virulence, since the wild-type parent strain COH31r/s was not typical of disease isolates. For these reasons, a new asialo mutant was derived for the purpose of the current studies in the background of a well-encapsulated strain of type III GBS, strain COH1, which was isolated from an infant with GBS sepsis and meningitis. Tn*916ΔE* was transferred from *E. faecalis* RH110 to COH1 by filter mating on blood agar. Several erythromycin-resistant, tetracycline-resistant colonies were identified as deficient in capsule expression by their failure to react with a GBS type III-specific monoclonal antibody in immunoblot assays. Subsequent characterization of these transconjugants identified a single mutant strain, designated COH1-11, that failed to react with the GBS III-specific monoclonal antibody but did react with antiserum against Pn14. The repeating unit structure of the Pn14 capsule is identical to that of the GBS III polysaccharide, except that the Pn14 polysaccharide lacks sialic acid. Therefore, strain COH1-11 was tentatively identified as expressing an asialo, or sialic acid-deficient, type III polysaccharide capsule.

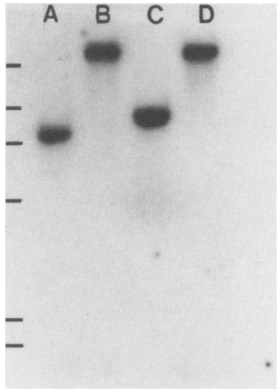

FIG. 1. Southern hybridization analysis of *Eco*RI-digested genomic DNA from COH31r/s (lane A), COH31-21 (lane B), COH1 (lane C), and COH1-11 (lane D) probed with a ^{32}P-labeled 1.3-kb *Hin*dIII fragment flanking the Tn*916* insertion site within a 7.5-kb *Eco*RI fragment from the capsule gene region of COH31-21 (18). The 7.5-kb *Eco*RI fragment corresponds to a 9.0-kb *Eco*RI fragment in COH1 because the COH31r/s chromosome contains an additional *Eco*RI site in this region (Fig. 3). As expected, the probe hybridized to a 7.5-kb fragment in COH31r/s (lane A) and to a 9.0-kb fragment in COH1 (lane C). The probe hybridized to single larger fragments, each containing one transposon insertion, in both mutants COH31-21 (lane B) and COH1-11 (lane D), demonstrating a common location of transposon insertion in the two mutant strains. Markers on the left indicate the position of molecular size standards (*Hin*dIII digest of lambda bacteriophage DNA: 23, 9.3, 6.6, 4.4, 2.3, and 2.0 kb in descending order).

Southern hybridization analysis. Genomic DNA of mutant strain COH1-11 was digested with restriction endonuclease *Eco*RI or *Hin*dIII and examined by Southern analysis by using a ^{32}P-labeled fragment of plasmid pCER110 that contained the Tn*916ΔE* sequence. Two copies of Tn*916ΔE* were identified in COH1-11 (data not shown). This observation was similar to that made previously in an asialo mutant (COH31-21) derived from the type III GBS strain COH31r/s (39). The previously identified asialo mutant COH31-21 contained two Tn*916* insertions, one of which was mapped to a 7.5-kb *Eco*RI fragment located in a region of the chromosome involved in capsular polysaccharide expression (18). A 1.3-kb *Hin*dIII fragment flanking this Tn*916* insertion site in COH31-21 was used as a probe in Southern hybridization analysis against *Eco*RI-digested genomic DNA from COH1 and COH1-11 (Fig. 1). The 1.3-kb probe from COH31-21 hybridized to a 7.5-kb *Eco*RI fragment in COH31r/s and to a corresponding 9.0-kb fragment in COH1 (Fig. 1). These results were anticipated because COH1 was shown previously to lack an *Eco*RI site present in COH31r/s in this region (Fig. 3) (18). The probe hybridized to a fragment larger than 23 kb both in COH31-21 and in COH1-11, indicating transposon insertion in the same restriction fragment in the two mutant strains. The results suggest that the locus of the mutation responsible for the sialylation defect observed in mutants derived both from COH31r/s and from COH1 lies within this 1.3-kb chromosomal segment.

The second Tn*916ΔE* insertion site in strain COH1-11 was

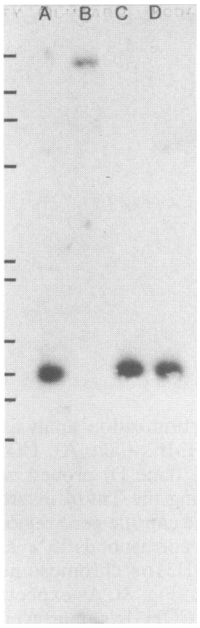

FIG. 2. Southern blot shown in Fig. 1 was stripped of the prior probe and hybridized with a ^{32}P-labeled 1.0-kb *Eco*RI fragment flanking the second Tn*916* insertion site in COH31-21. The probe hybridized to a 1.0-kb fragment in COH31r/s (lane A), COH1 (lane C), and COH1-11 (lane D) but to a 17.4-kb fragment in COH31-21 (lane B), indicating that the site of the second transposon insertion in COH1-11 is in a separate chromosomal location from the second insertion site in COH31-21. Molecular size standards are indicated by markers on the left and include lambda *Hin*dIII digests as in Fig. 1 and φX174 *Hae*III digests (1.3, 1.0, 0.63, and 0.3 kb in descending order).

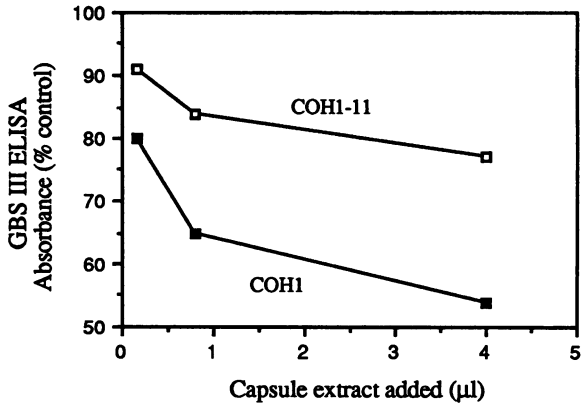

FIG. 4. Inhibition of native type III GBS ELISA by capsular extracts of wild-type GBS strain COH1 or of asialo mutant GBS strain COH1-11. Each datum point represents the mean absorbance of duplicate determinations, expressed as a percentage of the absorbance of control wells to which no capsular extract was added.

not within the previously identified capsule gene region and was in a different chromosomal location from the second Tn*916* insertion observed previously in COH31-21. This conclusion was reached by using the cloned flanking sequence from the second insertion site of COH31-21 as a probe in Southern hybridization analysis with the *Eco*RI-digested genomic DNA of COH1-11. As shown in Fig. 2, the 1-kb probe hybridized to a 17.5-kb fragment in COH31-21 but to a 1-kb fragment in COH1-11. This result indicates that the second Tn*916ΔE* insertion in COH1-11 was in a different site from the second Tn*916* insertion in COH31-21. That the second transposon insertions were in different sites in COH31-21 and in COH1-11 suggests that these insertions were unrelated to the sialylation defect common to the two mutant strains.

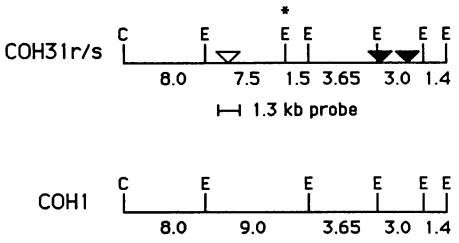

FIG. 3. Chromosomal map of a portion of the type III capsule gene region (adapted from reference 18). A *Cla*I site is indicated by C, and *Eco*RI sites are indicated by E. One *Eco*RI site (*) was present in COH31r/s but absent in COH1. Numbers below the map indicate distances in kilobases between *Eco*RI sites. The sites of transposon insertion in two unencapsulated Tn*916* mutants of COH31r/s are indicated by solid arrowheads. The site of one of the two Tn*916* insertions in asialo mutant COH31-21 is indicated by the open arrowhead. The chromosomal location of the 1.3-kb *Hin*dIII fragment that flanked this transposon insertion site is indicated by the bar labeled 1.3 kb probe. This fragment was used as the probe in the Southern hybridization analysis shown in Fig. 1.

Characterization of capsular polysaccharide of strain COH1-11. To confirm that strain COH1-11 expressed the asialo or core type III polysaccharide, capsule was released from cells of strain COH1-11 by treatment with mutanolysin and then assayed by competitive ELISA for the presence of immunoreactive capsular material. Although there is a small degree of cross-reactivity between the two antigens, the ELISAs for GBS III and for Pn14 are relatively specific for the sialylated and asialo type III GBS polysaccharides, respectively (39). The mutant COH1-11 extract inhibited the reaction of type III GBS antiserum with native type III polysaccharide only slightly compared with the native GBS III capsule of wild-type strain COH1, a result compatible with the absence of sialylated type III antigen on the mutant strain (Fig. 4). The same COH1-11 extract produced dose-dependent inhibition of the reaction between Pn14 antiserum and Pn14 polysaccharide, consistent with the identification of the mutant capsule as the core or asialo GBS type III polysaccharide (Fig. 5).

This conclusion was further supported by studies characterizing the mutant capsular polysaccharide purified from strain COH1-11. The COH1-11 polysaccharide was isolated from a mutanolysin extract of the bacterial cells and purified by ion-exchange and gel filtration chromatography. The purified COH1-11 polysaccharide contained no detectable sialic acid by thiobarbituric acid assay and formed a line of identity with Pn14 polysaccharide in Ouchterlony immunodiffusion gels against Pn14 antiserum. Native GBS type III capsular polysaccharide purified from wild-type strain COH1 produced no reaction with Pn14 antiserum in this immunoassay system. The molecular size of the COH1-11 polysaccharide relative to that of the native COH1 polysaccharide was estimated by fast protein liquid chromatography on a Superose 6 column calibrated with dextran standards. K_{av} of the asialo COH1-11 polysaccharide was 0.50, corre-

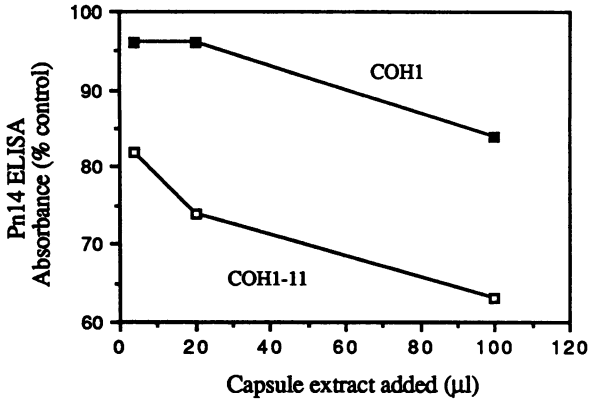

FIG. 5. Inhibition of Pn14 ELISA by capsular extracts of wild-type GBS strain COH1 or of asialo GBS mutant strain COH1-11. Each datum point represents the mean absorbance of duplicate determinations, expressed as a percentage of the absorbance of control wells to which no capsular extract was added.

sponding to an M_r of ~100,000, somewhat smaller than the apparent molecular size of the native polysaccharide purified from COH1 (K_{av} = 0.38, M_r of ~250,000), although both polysaccharides eluted as broad peaks, indicating substantial variation in chain length.

We also attempted to quantify the amount of capsular polysaccharide produced by the asialo mutant strain COH1-11 compared with the wild-type strain COH1, reasoning that a defect in sialylation of the polysaccharide might affect the efficiency of assembly or export of the polysaccharide, either directly or because of inhibitory effects of intracellular accumulation of one or more biosynthetic intermediates. To quantify capsule production by mutant and parent strains in the same immunoassay system, we treated cells of the wild-type strain COH1 with neuraminidase to remove sialic acid from the capsule and then compared capsule extracts of the asialo mutant strain COH1-11 and neuraminidase-treated COH1 for inhibitory capacity in the Pn14 ELISA. The amount of polysaccharide extracted from each strain was quantified by comparison with a standard curve generated with authentic Pn14 polysaccharide. We detected the equivalent of 1.1 μg of Pn14 polysaccharide per 10^9 CFU of the parent strain COH1 and 0.041 μg/10^9 CFU of the asialo mutant strain COH1-11. We have shown previously that the affinity of antibody binding to the Pn14 polysaccharide increases with the chain length of the polysaccharide (36). However, the relatively small difference in molecular size of the COH1-11 versus COH1 polysaccharides is unlikely to account for a difference of 25-fold in the ELISA measurement of COH1-11 versus desialylated COH1 polysaccharide. The 25-fold difference in immunoreactivity probably reflects some diminution in affinity of antibody binding to the shorter-chain-length polysaccharide of COH1-11 as well as a reduction in the number of polysaccharide molecules produced. These results indicate that the capsular polysaccharide produced by the asialo mutant COH1-11 not only lacked sialic acid but also was reduced in amount compared with the wild-type strain.

Effect of altered capsule expression on virulence of mutant strain COH1-11. We reported previously that loss of capsular sialic acid in mutant strain COH31-21 was associated with diminished virulence in a neonatal rat model of GBS infection, comparable to the reduction in virulence associated with complete loss of capsule (26, 39). We observed a similar loss of virulence in the asialo mutant strain COH1-11 compared with wild-type strain COH1: the LD_{50} for mutant strain COH1-11 was greater than 1×10^7 CFU/g of body weight compared with 1.1×10^2 CFU/g for wild-type strain COH1.

TABLE 2. Opsonophagocytic killing of GBS strains by human peripheral blood leukocytes in the presence of normal human serum[a]

Strain	$t = 0$ (10^6 CFU)[b]	$t = 1$ h (CFU)[b]	Log kill	% Kill
COH1	3.8	3.8×10^6	0	0
COH1 + antibody	4.6	3.9×10^4	2.1	99.2
COH1-11	3.4	6.2×10^4	1.7	98.2

[a] Wild-type strain COH1 was assayed in the absence (COH1) or presence (COH1 + antibody) of 1% rabbit antiserum raised against type III GBS organisms.

[b] Data are from a representative experiment. Each value represents the mean of duplicate quantitative cultures taken at time zero (t = 0) or after 1 h of incubation (t = 1 h).

The ability of the sialic acid-rich type III capsule to inhibit complement-mediated opsonophagocytic killing is thought to be an important virulence mechanism for GBS (7). Therefore, we examined resistance to phagocytic killing in vitro of the asialo mutant strain COH1-11. The parent strain COH1 is relatively resistant to phagocytosis in the absence of specific antibody. We observed no change in CFU of strain COH1 after 1 h of incubation with 10% normal human serum (as a complement source) and human peripheral blood leukocytes. In contrast, incubation of the mutant strain COH1-11 under the same conditions resulted in a >95% decrease in CFU (Table 2). The reduced virulence of strain COH1-11, both in neonatal rats and in the opsonophagocytic assay, may be attributable not only to the absence of capsular sialic acid but also to the diminished level of capsule production in this strain.

Characterization of biosynthetic defect in asialo mutant strain COH1-11. The absence of sialic acid in the capsular polysaccharide of COH1-11 could be due to a defect in any of several steps in the biosynthetic pathway of the native sialylated type III polysaccharide, including synthesis of sialic acid, formation of an activated precursor, or transfer of sialic acid from activated precursor to the polysaccharide or to an intermediate acceptor molecule. While the details of this pathway are unknown in GBS, these general steps are likely to be involved, based on analogous biosynthetic schemes in other bacteria (30). As a first step in identifying the specific defect in COH1-11, we examined the asialo mutant COH1-11 and wild-type strain COH1 for the presence of free intracellular sialic acid. While the presence of interfering substances did not permit accurate quantitation by colorimetric methods, sialic acid was readily identified by TLC analysis of lysates of mutant strain COH1-11 and was not seen in lysates of COH1. The sialic acid band was identified by R_f value and characteristic bright pink staining with diphenylamine spray (Fig. 6). A second band with similar staining characteristics but a slightly faster migration on TLC was also present, in smaller amounts. The material tentatively identified as sialic acid by TLC was purified from lysates of mutant strain COH1-11 by combined cation- and anion-exchange chromatography. Analysis of this material by ^{13}C nuclear magnetic resonance (courtesy of Harold J.

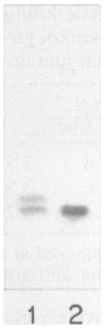

FIG. 6. TLC of intracellular sialic acid purified from a lysate of asialo mutant GBS strain COH1-11, stained with diphenylamine spray. The origin is at the bottom of the figure. The material purified from strain COH1-11 is in lane 1 and consists of two components: the slower-migrating band corresponds to native sialic acid, while the faster-migrating band represents a lactone derivative. Lane 2 contains a sialic acid standard.

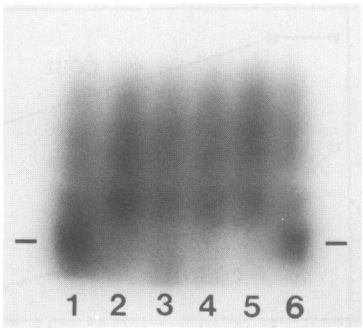

FIG. 7. Autoradiogram of TLC plate showing CMP-sialic acid synthetase assay. Bacterial lysates were incubated with ^{32}P-labeled CTP and sialic acid and then fractionated by TLC. After drying, the plate was cut 1 cm above the origin and the top portion was exposed to X-ray film. The standard assay mixture was incubated with a lysate of COH1 (lane 1); with COH1 lysate previously placed in a boiling water bath for 90 s (lane 2); with COH1-11 lysate (lane 4); with water substituted for bacterial lysate (lane 5); with a lysate of a K1 strain of *E. coli* (lane 6); lane 3 shows the standard assay mixture, with sialic acid omitted, incubated with COH1 lysate. The horizontal bars on either side of the figure indicate the migration of a CMP-sialic acid standard run on the same plate. Results shown are representative of those obtained by using lysates prepared from two separate cultures of COH1 and COH1-11.

Jennings, National Research Council of Canada, Ottawa, Ontario) confirmed it to be authentic sialic acid; the minor product corresponding to the faster-migrating band visualized by TLC was identified as a lactone form, i.e., an internal ester, of sialic acid. This latter species probably represents a degradation product which arose either in the cells as a result of the abnormal accumulation of sialic acid or during the extraction and purification procedures.

The asialo mutant strain accumulated free sialic acid, suggesting that synthesis of sialic acid was intact but that a subsequent step in the biosynthetic pathway was blocked. Synthesis by *E. coli* of the poly-sialic acid K1 capsular polysaccharide proceeds from synthesis of free sialic acid to activation of sialic acid by the action of CMP-sialic acid synthetase. The activated precursor molecule, CMP-sialic acid, then serves as the substrate for the transfer of sialic acid to an acceptor molecule, from which it is incorporated into the growing sialic acid polymer (24, 30). On the basis of the *E. coli* K1 system, we reasoned that the likely defect in mutant strain COH1-11 was in the activation of sialic acid to a nucleotide precursor molecule. To test this hypothesis, we developed an assay for CMP-sialic acid synthetase activity in GBS. The use of ^{32}P-labeled CTP as the substrate in this assay increased the sensitivity over that obtained by using the thiobarbituric acid assay for detection of CMP-sialic acid and allowed us to specifically identify the enzymatic product as CMP-sialic acid, rather than another sialic acid-containing compound. After incubation, the assay mixture was analyzed by TLC and autoradiography: [^{32}P]CMP-sialic acid was identified on the autoradiogram by its comigration with a CMP-sialic acid standard. Incubation of lysates of the wild-type strain COH1 with sialic acid and [^{32}P]CTP resulted in incorporation of the label into the band corresponding to CMP-sialic acid (Fig. 7). No incorporation was observed when sialic acid was omitted or when the cell lysate was boiled, indicating that the reaction required both sialic acid as a substrate and a heat-labile factor in the cell lysate, presumably CMP-sialic acid synthetase. CMP-sialic acid synthetase activity was not detected in lysates of the asialo mutant COH1-11 (Fig. 7). The absence of CMP-sialic acid synthetase activity in the asialo mutant strain COH1-11, together with evidence of intracellular accumulation of the enzyme's substrate, free sialic acid, implicates this enzymatic step as the site of the biosynthetic defect in sialylation of the COH1-11 polysaccharide. These experiments identify the activation of sialic acid to CMP-sialic acid as a required step in synthesis of the native type III polysaccharide.

DISCUSSION

The type III capsular polysaccharide of GBS has been shown to be an important virulence determinant (1, 6, 16, 26). The presence of sialic acid residues on the surface of the organisms inhibits activation of the alternative pathway of complement. Type III GBS from which sialic acid was removed by treatment with neuraminidase activated the alternative pathway, leading to efficient phagocytosis of the desialylated bacteria (7). The importance of the sialic acid-rich type III capsule in virulence was supported by our previous studies of capsule-deficient mutants derived from the type III GBS strain COH31r/s. In those studies, loss of encapsulation or loss of capsule sialylation was associated with reduced virulence of the organisms in animals (26, 39).

We now report the derivation and characterization of a mutant strain with an asialo capsular phenotype, as we described previously (39); the new mutant, however, was derived from the well-encapsulated type III GBS strain COH1, typical of strains isolated from neonates with GBS sepsis. In contrast to poorly encapsulated type III strains, strains such as COH1 are relatively resistant to phagocytic killing in vitro in the absence of type-specific antibody (2, 8). Loss of capsular sialic acid in the mutant strain COH1-11 was associated with loss of resistance to phagocytic killing in vitro and with decreased virulence in vivo. However, these changes in the virulence characteristics of the asialo mutant COH1-11 may reflect not only loss of capsular sialic acid but also a quantitative reduction in the capsule expression. Since poorly encapsulated wild-type strains of type III GBS also have diminished resistance to phagocytosis, at least part of the reduced virulence of strain COH1-11 may be a result of its smaller amount of capsule compared with the parent strain.

While the importance of the sialylated type III capsule in

virulence has been established, little is known at the genetic or biochemical level about the synthesis or regulation of the GBS capsular polysaccharide. On the basis of the biosynthetic pathways of extracellular polysaccharides produced by other bacteria, we presume that synthesis of the GBS capsule must be complex, involving transport into the cell and/or synthesis of the four component sugars, activation of each to a nucleotide precursor, the coordinated transfer of each sugar, in sequence, to the repeating unit oligosaccharide, polymerization, export, and attachment of the polysaccharide to the cell surface (24, 30). The identification of a mutant strain of type III GBS deficient specifically in capsular sialic acid has allowed us to begin to dissect the portion of the capsule biosynthesis pathway involved in sialylation of the type III polysaccharide. In *E. coli*, the following basic scheme comprises the major biosynthetic steps for synthesis and polymerization of sialic acid to form the K1 capsular polysaccharide (24, 30):

$$\text{(i) } N\text{-acetyl-mannosamine} + \text{phosphoenolpyruvate} \xrightarrow{\text{(sialic acid synthase)}} \text{sialic acid} + P_i$$

$$\text{(ii) CTP} + \text{sialic acid} \xrightarrow{\text{(CMP-sialic acid synthetase)}} \text{CMP-sialic acid} + PP_i$$

$$\text{(iii) CMP-sialic acid} + \text{acceptor} \xrightarrow{\text{(sialyltransferase)}} \text{sialic acid-acceptor} + \text{CMP}$$

Our finding of increased amounts of free intracellular sialic acid in mutant strain COH1-11, which expressed the asialo capsular polysaccharide, suggested that sialic acid synthesis was intact and that the block was at a later step in the pathway, e.g., in the activation of sialic acid to a nucleotide-sugar precursor. CMP-sialic acid synthetase activity was not detected in the asialo mutant strain COH1-11 but was readily demonstrated in the wild-type strain. Thus, the formation of CMP-sialic acid by CMP-sialic acid synthetase appears to be an essential step in sialylation of the type III GBS capsular polysaccharide. How the reduction in total capsule expression in COH1-11 is related to the specific defect in sialylation is not yet known; the mechanisms for polymerization or export of the polysaccharide may be less efficient for the asialo form, as suggested by the shorter chain length of the COH1-11 polysaccharide; accumulation of free intracellular sialic acid may down-regulate other steps in capsule biosynthesis by feedback inhibition or as a nonspecific toxic effect; or the mutation(s) in COH1-11 may have more general effects in regulating capsule expression.

The asialo mutants COH31-21 and COH1-11, derived from different type III wild-type strains, both had two transposon insertions, one of which, in each case, was located at the same chromosomal location in a region involved in capsule biosynthesis (18). That two independently derived transposon mutants with the asialo capsular phenotype share a site of transposon insertion implicates this site as a genetic locus necessary for capsule sialylation, although the precise function of the locus is not yet known; it may represent the gene encoding CMP-sialic acid synthetase or a regulatory element that controls expression of this, and perhaps other, biosynthetic genes involved in capsular polysaccharide production. It is possible that two separate mutations are necessary to prevent expression of CMP-sialic acid synthetase activity; however, since the second transposon insertion was at a different site in the two mutants, we think it is more likely that the common insertion site represents the essential gene and that the second insertion in the two mutants is unrelated to the sialylation defect. We observed two or more transposon insertions more commonly than single insertions among transconjugants of both COH31r/s and COH1, so the presence of an irrelevant transposon insertion in COH31-21 and COH1-11 would not be unexpected. The location of the site associated with CMP-sialic acid synthetase expression in the same chromosomal region as transposon insertion sites associated with complete loss of capsule expression suggests that genes involved in encapsulation are clustered in this area of the GBS chromosome, as has been demonstrated for the capsule genes of *Haemophilus influenzae* type b and *E. coli* K1 (3, 9, 12, 17, 28, 29, 33). Further studies are needed to define more precisely the location and function of the genes controlling GBS capsule synthesis. These investigations represent a first step in understanding the biosynthesis and regulation of capsular polysaccharide production by this important human pathogen.

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