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The Serotype of Type Ia and III Group B Streptococci Is Determined by the Polymerase Gene within the Polycistronic Capsule Operon

DONALD O. CHAFFIN, STEPHEN B. BERES, HARRY H. YIM, and CRAIG E. RUBENS*

Department of Pediatrics, Division of Infectious Diseases, Children's Hospital Regional Medical Center, Seattle, Washington 98105

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***Streptococcus agalactiae* is a primary cause of neonatal morbidity and mortality. Essential to the virulence of this pathogen is the production of a type-specific capsular polysaccharide (CPS) that enables the bacteria to evade host immune defenses. The identification, cloning, sequencing, and functional characterization of seven genes involved in type III capsule production have been previously reported. Here, we describe the cloning and sequencing of nine additional adjacent genes, $cps_{III}FGHIJKL$, $neu_{III}B$, and $neu_{III}C$. Sequence comparisons suggested that these genes are involved in sialic acid synthesis, pentasaccharide repeating unit formation, and oligosaccharide transport and polymerization. The type III CPS (*cps*III) locus was comprised of 16 genes within 15.5 kb of contiguous chromosomal DNA. Primer extension analysis and investigation of mRNA from mutants with polar insertions in their *cps*III loci supported the hypothesis that the operon is transcribed as a single polycistronic message. The translated *cps*III sequences were compared to those of the *S. agalactiae cps*Ia locus, and the primary difference between the operons was found to reside in $cps_{III}H$, the putative CPS polymerase gene. Expression of $cps_{III}H$ in a type Ia strain resulted in suppression of CPS Ia synthesis and in production of a CPS which reacted with type III-specific polyclonal antibody. Likewise, expression of the putative type Ia polymerase gene in a type III strain reduced synthesis of type III CPS with production of a type Ia immunoreactive capsule. Based on the similar structures of the oligosaccharide repeating units of the type Ia and III capsules, our observations demonstrated that $cps_{Ia}H$ and $cps_{III}H$ encoded the type Ia and III CPS polymerases, respectively. Additionally, these findings suggested that a single gene can confer serotype specificity in organisms that produce complex polysaccharides.**

Group B streptococci (GBS) (*Streptococcus agalactiae*) are the leading cause of serious bacterial infections (bacteremia, pneumonia, and meningitis) in newborns, causing two to three cases per 1,000 live births (47). An indispensable GBS virulence determinant is the production of a type-specific capsular polysaccharide (CPS), which prevents the deposition of host complement factor C3b and inhibits opsonophagocytosis (45). Nine distinct capsular serotypes, Ia, Ib, and II to VIII, have been identified (54), and their chemical compositions and structures have been determined (16–19, 51, 55, 56, 58). Type Ia, Ib, II to V, and VII CPS consist of the monosaccharides glucose, galactose, *N*-acetylglucosamine, and *N*-acetylneuraminic acid. Serotypes VI and VIII lack *N*-acetylglucosamine, and type VIII contains rhamnose (19). Although serotypes Ia, III, and V are currently the most common isolates from the United States associated with early-onset disease (within 1 week of birth), comprising 82% of isolates (27), type III GBS are the most prevalent isolates associated with neonatal disease (5).

We previously identified a region of the GBS chromosome encoding genes involved in type III capsule production (the *cps*III locus) by screening genomic transposon libraries for CPS mutants (45, 59). DNA sequence analysis of the acapsular type III mutants led to the identification of four genes designated *cpsA*, *cpsB*, *cpsC*, and *cpsD* (43). To conform to the emerging consensus nomenclature in the CPS literature, these genes have been designated $cps_{III}B$, $cps_{III}C$, $cps_{III}D$, and $cps_{III}E$, respectively. $Cps_{III}E$ was assigned a function as a galactosyltransferase, but due to potential endogenous C-4 epimerase activity, the possibility that $Cps_{III}E$ may act as a glucosyltransferase could not be ruled out (43). Mutants which produced a capsule lacking sialic acid were also identified (25, 57, 59). The asialo mutants had a common transposon insertion site in a gene approximately 9 kb downstream of $cps_{III}E$, which was designated *cpsF* (now referred to as $neu_{III}A$). The $neu_{III}A$ gene was shown to encode a CMP-*N*-acetylneuraminic acid synthetase (15, 46) which complemented an *Escherichia coli* K1 *neuA* mutant, restoring synthesis of the polysialic acid capsule (15). A gene homologous to *E. coli* K1 *neuD* (formerly designated *cpsE* and now called $neu_{III}D$) was found adjacent to the 5′ end of $neu_{III}A$ (8). Additionally, upstream of $cps_{III}B$, two genes similar to regulatory proteins, designated *cpsY* and *cpsX* (referred to here as $cps_{III}A$ to conform to the usage for the *S. agalactiae* type Ia homologue), have been identified, although their functions have not been confirmed (24). Thus, seven genes of the *cps*III locus have previously been described, along with the divergently transcribed *cpsY*, whose role in CPS synthesis is not known.

The type III CPS produced under the direction of the *cps*III operon possesses a backbone of repeating [→6)-β-D-*N*-acetylglucosamine-(1→3)-β-D-galactose-(1→4)-β-D-glucose-(1→]$_n$ trisaccharide units. Each repeating unit carries a disaccharide side chain of α-D-*N*-acetylneuraminic acid-(2→3)-β-D-galactose, 1→4 linked via the galactose to the backbone *N*-acetylglucosamine. The type Ia CPS is composed of a polymer of β-1,4-linked lactose to which a trisaccharide side chain of α-D-

* Corresponding author. Mailing address: Department of Pediatrics, Division of Infectious Diseases, Children's Hospital Regional Medical Center, Mailstop CH-32, 4800 Sandpoint Way NE, Seattle, WA 98105. Phone: (206) 528-2767. Fax: (206) 527-3890. E-mail: cruben@chmc.org.

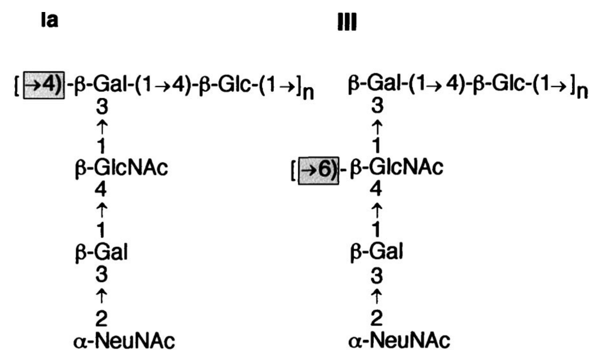

FIG. 1. GBS type Ia and III CPS repeating unit structures. Ia, *S. agalactiae* type Ia CPS subunit structure; III, *S. agalactiae* type III CPS subunit structure; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; NeuNAc, *N*-acetylneuraminic (sialic) acid. The critical linkages differentiating the type Ia and III CPS are shown in the shaded boxes.

N-acetylneuraminic acid-(2→3)-β-D-galactose-(1→4)-β-D-*N*-acetylglucosamine is attached to each repeating unit galactose by a β-D-*N*-acetylglucosaminyl-(1→3) linkage. Traditionally, the GBS CPS repeating unit structures have been depicted to emphasize these differences and to reflect their conformational structure in the native polysaccharide configuration (16, 17). However, if the repeating unit structures are redrawn as in Fig. 1, it can be seen that the type Ia and type III CPS oligosaccharide structures differ only by the linkage between repeating units. Therefore, the essential difference between the two capsules is due to the glycosidic bond formed during CPS polymerization. The structural similarity of these capsule oligosaccharides has led us to hypothesize that there is a high degree of genetic relatedness between their capsule synthesis loci. This hypothesis is supported by two further observations: first, the CPS structures of *S. pneumoniae* type 14 and type III GBS are identical except for the lack of the terminal sialic acid side chain residue of the former (Fig. 1); second, the *cps* loci of *S. pneumoniae* type 14 and GBS type Ia are highly homologous within their regulatory, repeat unit, and oligosaccharide transport genes (60).

In this study, we have completed the molecular characterization of nine additional genes that make up the remainder of the *cps*III operon. We present the organization of the 15.5 kb of DNA containing the 16 genes involved in CPS III synthesis, describe its similarity to other complex polysaccharide synthesis loci, and demonstrate its expression as a polycistronic operon. Last, we provide evidence that a single polymerase gene determines the unique bond formed during polymerization of the type Ia or type III repeating units and thus confers CPS serotype specificity.

MATERIALS AND METHODS

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

Nucleotide sequencing and DNA analysis. Sequencing was performed on plasmid subclones containing COH1 genomic DNA (25), purified PCR products, and a subclone generated by plasmid insertion/rescue (Fig. 2). Fluorescent dye terminator sequencing reactions were performed using a Ready Reaction sequencing kit (Perkin-Elmer Cetus, Foster City, Calif.) according to the manufacturer's specifications and analyzed on an Applied Biosystems 373A automated sequencer. Both strands of each DNA template were sequenced.

$cps_{III}D$-$cps_{III}E$ and IS*861*-$cps_{III}B$ intergenic regions were amplified from the COH1 chromosome by PCR. Primers used for amplifying the IS*861*-$cps_{III}B$ intergenic region hybridized to a region downstream of IS*861* (5′-GAAAGGT TTGCTTTGTCGTGTCGGATATAG-3′) (44) and to the 5′ end of $cps_{III}B$ (5′-CTTCAACGCTTTTGGGCCCATCATCTACATC-3′) (43). The second PCR product was an amplified region of $cps_{III}D$-$cps_{III}E$ that was produced by using primers 5′-GAAGTAAGGGACTCTGGTATTGA-3′, which hybridizes to the 3′ end of $cps_{III}D$, and 5′-GAGCAAACCTATAATAGCACCCGT-3′, which hybridizes to $cps_{III}E$. The PCR products were prepared for sequencing by purification using a QiaQuick PCR purification kit (Qiagen Inc., Valencia, Calif.).

DNA sequence analysis and FASTA homology searches were performed using the Genetics Computing Group (University of Wisconsin) software on a UNIX computer at the University of Washington. ENTREZ and BLAST homology searches were performed using the National Center for Biotechnology Information Internet server.

Plasmid insertion/rescue. To clone and sequence chromosomal DNA 3′ of $neu_{III}A$, an intragenic '$neu_{III}A$' fragment was amplified and cloned into the pT7Blue vector (Novagen Inc., Madison, Wis.) to create pSH101. An *Apa*I/*Eco*RV fragment containing the '$neu_{III}A$' sequence was isolated from pSH101 and ligated into the temperature-sensitive vector pVE6007 (30) previously digested with the same restriction enzymes. This new plasmid was designated pSH102 and transformed into competent COH1 as described elsewhere (13). Transformants were screened for plasmid integration within the chromosomal wild-type (wt) $neu_{III}A$ as described elsewhere (61). Subsequent digestion of integrant chromosomal DNA with *Sac*I, followed by intramolecular ligation, rescued the plasmid and flanking chromosomal sequences. The ligated DNA was transformed into *E. coli* DH5α, and chloramphenicol-resistant clones were isolated by growth at 30°C on L agar containing 10 μg of chloramphenicol per ml.

Generation of *cps* gene allelic exchange plasmids. Intragenic fragments of $cps_{III}A$, $cps_{III}C$, and $cps_{III}D$ were amplified from COH1 chromosomal DNA by PCR and directly ligated into the vector pT7Blue. The intragenic fragments were excised from pT7Blue with *Kpn*I and *Hin*dIII and ligated into *Kpn*I- and *Hin*dIII-digested pVE6007. To clone the intragenic fragment of $cps_{III}B$, pCER107 (43) was digested with *Apa*I and *Hin*dIII, removing 47 nucleotides (nt) from the 5′ end and 425 nt from the 3′ end of $cps_{III}B$. This 616-bp intragenic fragment was separated by agarose gel electrophoresis, extracted (QiaEx gel purification kit; Qiagen), and ligated into *Apa*I- and *Hin*dIII-digested pVE6007.

The ΩKm-2 fragment (38) from pCIV2 was subsequently cloned into sites within each of the intragenic *cps* gene fragments in the pVE6007-based vectors described above. For the $cps_{III}C$ and $cps_{III}D$ constructs, the ΩKm-2 fragment was digested from pCIV2 with *Bam*HI and cloned directly into the *Bgl*II site within each gene fragment. To insert ΩKm-2 within the $cps_{III}B$ construct, an *Fsp*I site in $cps_{III}B$ was ligated to the ΩKm-2 cassette previously prepared by *Bam*HI digestion and end filled with Klenow fragment. The ΩKm-2 fragment was ligated into the *Nsi*I site in the $cps_{III}A$ construct via a *Bam*HI-*Nsi*I adapter, 5′-GATC AGCGGCCGCTTGCA-3′. Plasmid constructs containing ΩKm-2 cassettes in the same orientation relative to the chromosomal *cps* genes were chosen for deriving the allelic exchange mutants in COH1. After transformation of the allelic exchange vectors into GBS, the cells were grown under conditions promoting homologous recombination as described previously (61). Recombination occurring on both sides of the ΩKm-2 cassette resulted in a double-crossover recombination event and an allelic exchange mutation within the recipient gene.

PCR amplification. PCRs were performed using standard conditions (4) and *Taq* polymerase (Promega Corp., Madison, Wis.). To amplify larger (>2.0-kb) PCR products, *Taq* extender (5 U; Stratagene Cloning Systems, La Jolla, Calif.) was added to a standard 100-μl reaction according to the manufacturer's instructions.

RNA analysis. Total bacterial cell RNA was extracted from GBS using a rapid cell disruption method with a dental amalgamator and glass beads as described previously (62). Dot blots were generated as follows. A 2.5-μg aliquot of total cellular RNA was applied to Magnagraph nylon membrane (Micron Separations Inc., Westborough, Mass.) using a Minifold I dot blot vacuum apparatus (Schleicher & Schuell, Keene, N.H.) according to the manufacturer's recommendations and UV cross-linked. Blots were hybridized using standard 50% formamide buffers (31) and ^{32}P-labeled RNA gene probes. After hybridization, the blot was washed twice for 15 min with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at room temperature and twice with 0.1× SSC–0.1% SDS at 65°C, excess buffer was removed, and the filter was subjected to autoradiography.

Primer extension reactions were used to identify the 5′ end of *cps*-specific mRNA. Primer 5a (5′-CACATACACCTCTACGG-3′) was end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase, hybridized to 50 μg of total cellular RNA, and extended, using displayTHERMO-RT RNA polymerase (Display Systems Biotech, Inc., Vista, Calif.). The sample was ethanol precipitated, resuspended in 8 μl of RNase A (200 μg/ml), and incubated for 20 min at 23°C. A reference standard sequencing ladder was generated using [γ-^{32}P]ATP-labeled primer 5a with a cycleSEQ manual cycle sequencing kit (Display Systems Biotech) and loaded next to the primer extension reaction on a 7% acrylamide–8 M urea–1× Tris-borate-EDTA gel. The samples were separated by electrophoresis at 45 W for 2.5 h; the gel was transferred to Whatman 3MM filter paper, dried, and exposed to X-ray film.

Generation of *cps* type Ia and III gene expression constructs. Plasmid constructs were generated to test the effect on capsule production of heterologous *cps* type Ia and III gene expression. The $cps_{Ia}GH$ and $cps_{III}GH$ genes were PCR amplified from strain A909 and COH1 chromosomal DNA, respectively, using primer Pol1f, which contained a 5′ *Apa*I adapter (5′-CCCGGGCCCAGAT GTTATCATATCA-3′), and Pol1r, containing a 5′ *Afl*II adapter (5′-CCAGA TCTTAAGTTTCGTCTTTTCTTC-3′) (adapters are underlined). The 2.35-kb $cps_{Ia}GH$ amplicon was digested with *Apa*I and *Afl*II and ligated to the *Apa*I/

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype/phenotype[a]	Reference or source
S. agalactiae		
A909	Wild type/*cps*Ia	29
COH1	Wild type/*cps*III, Tet^r	32
COH1-13	COH1, Tn*916*ΔE *cpsE*/CPS^- Tet^r Em^r	43
COHY-104	COH1, $cps_{III}B$, ΩKm-2 via pHY108/CPS^- Tet^r Kan^r Cm^s	This study
COHY-105	COH1, $cps_{III}C$, ΩKm-2 via pHY113/CPS^- Tet^r Kan^r Cm^s	This study
COHY-106	COH1, $cps_{III}D$, ΩKm-2 via pHY109/CPS^- Tet^r Kan^r Cm^s	This study
COHY-107	COH1, $cps_{III}A$, ΩKm-2 via pHY120/CPS^- Tet^r Kan^r Cm^s	This study
E. coli		
DH5α	F^- *endA1* (r_K^- m_K^+) *supE44 thi-1 hsdR17 gyrA* (Nal^r) *recA1* Δ(*lacZYA-argF*)*U169 deoR relA1* [ϕ80Δ*lac*(*lacZ*)*M15*]	BRL, Inc.
MC1061	F^- *araD139* Δ(*ara-leu*)*7696* Δ(*lac*)*X74 galU galK hadR2* (r_K^- m_K^+) *mcrB1 rpsL* (Str^r)	53
Plasmids		
pBS	Cloning vector, Ap^r 2.8 kb	Stratagene
pBS9.0	pBS, $cps_{III}IJKLneuBCDA$/Ap^r 11.8 kb	This study
pCER107	pBS, $cps_{III}ABCDE$/Ap^r 5.7 kb	25
pCER108	pBS, $cps_{III}EFGHI$/Ap^r 6.1 kb	25
pDC128	Cm^r, pDC123 with a 7,122-bp *Xba*I fragment containing $cps_{III}KLneuBCDA$; 11.6 kb	This study
pCIV2	Kan^r/ΩKm-2 5.9 kb	38
pHY101	Cm^r, 597 bp '$cps_{III}B$' fragment cloned from pCER107 via *Apa*I/*Hin*dIII ligation into pVE6007	This study
pHY104	Cm^r, 412-bp '$cps_{III}D$' fragment cloned from pHY204 via *Kpn*I/*Hin*dIII ligation into pVE6007	This study
pHY105	Cm^r, 537-bp '$cps_{III}C$' fragment cloned from pHY205 via *Kpn*I/*Hin*dIII ligation into pVE6007	This study
pHY108	pHY101 with ΩKm-2 from pCIV2 cloned into *Fsp*I site of '$cps_{III}B$'/Kan^r	This study
pHY109	pHY104 with ΩKm-2 from pCIV2 cloned into *Bgl*II site of '$cps_{III}D$'/Kan^r	This study
pHY113	pHY105 with ΩKm-2 from pCIV2 cloned into *Bgl*II site of '$cps_{III}C$'/Kan^r	This study
pHY115	Cm^r, 648-bp '$cps_{III}A$' fragment cloned into pVE6007 from pHY208 via *Kpn*I/*Hin*dIII ligation	This study
pHY120	pHY115 with ΩKm-2 from pCIV2 cloned into *Nsi*I site of $cps_{III}A$ using a *Bam*HI-*Nsi*I adapter/Kan^r	This study
pHY204	Ap^r, 345-bp '$cps_{III}D$' amplicon cloned into pT7Blue vector	This study
pHY205	Ap^r, 470-bp '$cps_{III}C$' amplicon cloned into pT7Blue vector	This study
pHY208	Ap^r, 583-bp '$cps_{III}A$' amplicon cloned into pT7Blue vector	This study
pSH101	Ap^r, 541-bp '$neu_{III}A$' amplicon cloned into pT7Blue vector	This study
pSH102	Cm^r, 525-bp '$neu_{III}A$' cloned from pSH101 via *Apa*I/*Eco*RV ligation into pVE6007	This study
pSH104	Cm^r, plasmid rescue construct containing pSH102 and sequences downstream of $neu_{III}A$, 7.3 kb	This study
pT7Blue	Ap^r 2.9-kb T-overhang PCR cloning vector	Novagen
pVE6007	Cm^r temperature-sensitive shuttle vector, 3.4 kb, ori[Ts]	30
pDC123	Cm^r, *phoZ*, gram-positive/negative blue/white screening expression vector	8
pDC123(GH_{Ia})	Cm^r, 2,350-bp PCR product from type Ia GBS strain A909 containing $cps_{Ia}GH$ cloned into *Afl*II/*Apa*I-cut pDC123	This study
pDC123(H_{Ia})	Cm^r, 1,767-bp *Xba*I-cut PCR product containing $cps_{Ia}H$ from type Ia GBS strain A909 cloned into *Sma*I/*Xba*I-cut pDC123	This study
pDC123(GH_{III})	Cm^r, 2,244-bp PCR product containing $cps_{III}GH$ from type III GBS strain COH1 cloned into *Afl*II/*Eco*RV-cut pDC123	This study
pDC123(H_{III})	Cm^r, 209-bp *Bcl*I deletion of pDC123(GH_{III})	This study

[a] Tet^r, tetracycline resistance; Em^r, erythromycin resistance; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Cm^s, chloramphenicol susceptible; Kan^r, kanamycin resistance; Nal^r, nalidixic acid resistance; CPS^-, capsule negative.

*Afl*II-cut pDC123 expression vector, forming pDC123(GH_{Ia}). The 2.24-kb $cps_{III}GH$ amplicon was digested with *Afl*II and ligated with *Eco*RV/*Afl*II-cut pDC123, forming pDC123(GH_{III}). The $cps_{Ia}H$ gene was amplified from the GBS A909 chromosome using primers Pol2f (5′-CTGAGATTGTTATCACAC-3′) and Pol1r. The $cps_{Ia}H$ amplicon (1.95 kb) was digested with *Xba*I and cloned into *Sma*I/*Xba*I-cut pDC123, forming pDC123(H_{Ia}). Constructs for the expression of $cps_{III}H$ were derived from pDC123(HI_{III}) by excision of a 0.21-kb *Bcl*I fragment intragenic to $cps_{III}G$, forming pDC123(I_{III}). The constructs were transferred to the appropriate GBS hosts as described (13), and their identity was subsequently confirmed by PCR and restriction analyses.

Serotype determination of cell-associated CPS. A modification of the colony immunoblot method described by Rubens et al. (45) was used to identify the type of CPS produced on recombinant strains compared to the wt strains. Aliquots (3 μl) of stationary-phase cultures were spotted onto a NitroPlus membrane (Micron Separations), fixed for 5 min with 70% ethanol, air dried, and blocked with BLOTTO (5% nonfat dry milk [NFDM] in phosphate-buffered saline [PBS]) for 30 min. The blots were washed three times for 5 min each with PBS and then incubated for 1 h with either type Ia or III rabbit anti-CPS antiserum (kindly provided by Michael Wessels, Channing Laboratory, Boston, Mass.) diluted 1:20,000 (type Ia) or 1:30,000 (type III) in BLOTTO. The blots were washed three times (5 min each in PBS) and then incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody, diluted 1:20,000 in BLOTTO (Sigma, St. Louis, Mo.). After three 5-min PBS washes, 1 ml of SuperSignal chemiluminescent substrate for HRP (Pierce, Rockford, Ill.) was added to the surface of the filter according to the manufacturer's directions. Chemiluminescence was detected by exposing the blot to X-ray film (Kodak, Rochester, N.Y.).

Quantification of cell-associated CPS. Mutanolysin digests of bacterial cells for extraction of CPS were carried out as described by Paoletti et al. (39), with minor modifications as follows. GBS were grown overnight in 10 ml of Todd-Hewitt broth, with antibiotic selection if necessary, harvested by centrifugation at 10,000 × *g* for 10 min at 4°C, and washed twice with 1.5 ml of 50 mM sodium phosphate buffer (pH 7.0). The cell pellets were resuspended in a final volume of 0.6 ml of 50 mM phosphate buffer (pH 7.0); 200 μl of the cell solution was transferred in duplicate to fresh microcentrifuge tubes; the cells were pelleted at 12,000 × *g* for 10 min at 4°C and then resuspended in 750 μl of 40% sucrose (wt/vol) in 50 mM sodium phosphate buffer (pH 7.0). To each tube, 250 μl of mutanolysin (1 mg/ml in 50 mM sodium phosphate buffer [pH 7.0]) was added. The samples were incubated at 37°C with end-over-end mixing for 1 h. Protoplasts and cell debris were removed by centrifugation at 13,000 × *g* for 4 min at 4°C, and the supernatants were transferred to fresh tubes and stored at −20°C.

Plates for quantitative enzyme-linked immunosorbent assay (ELISA) were

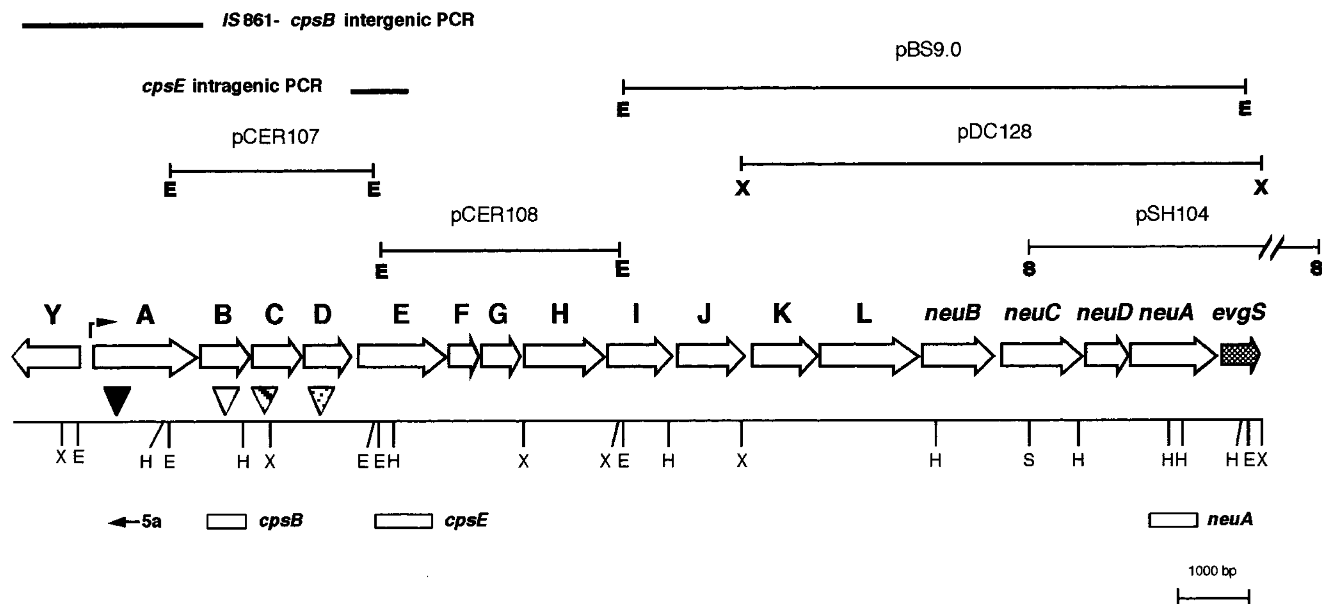

FIG. 2. Organizational map of the type III GBS *cps* operon. ORFs within the operon, with the direction of transcription, are indicated by the open arrows. The corresponding gene designation is shown above each ORF. Restriction site designations: E, *Eco*RI; H, *Hin*dIII; S, *Sac*I; X, *Xba*I. Plasmids used in sequencing the locus are depicted above the operon map. PCR products used to derive additional sequence are shown as solid bars above the operon map. The *cps*III transcriptional start site is indicated with an arrow in the *cpsY-cpsA* intergenic region. The ΩKm-2 insertion sites are indicated with triangles: filled triangle, $cps_{III}A$ insertion; open triangle, $cps_{III}B$ insertion; striped triangle, $cps_{III}C$ insertion; dotted triangle, $cps_{III}D$ insertion. Sequences used in generating RNA probes are marked with open boxes. The location and orientation of the oligonucleotide used to initiate the primer extension reaction (primer 5a) is also shown.

prepared by coupling poly-L-lysine to purified type Ia or III CPS (kindly provided by Lawrence Paoletti, Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School, Boston, Mass.), which was used to coat 96-well microtiter plates as described elsewhere (45). Coating solution (CPS [1.0 μg/ml] coupled to poly-L-lysine in 40 mM sodium phosphate buffer [pH 7.0]; 10 ml/plate) was prepared. To each well of the microtiter plate, 100 μl of the coating solution was dispensed, and the plates were incubated at 37°C for 1 h. The coating solution was discarded, and the plates were washed three times with 40 mM sodium phosphate (pH 7.0)–0.05% Tween 20 (PBT; 200 μl/well) and then blocked with 0.5% (wt/vol) NFDM in 40 mM sodium phosphate buffer (pH 7.0). The blocking solution was decanted, the wells were washed three times with 200 μl of PBT; and the plates were stored with 200 μl of PBT per well for up to 48 h before use at 4°C.

The amount of cell-associated, serotype-specific CPS produced by each strain was determined by competitive inhibition ELISA assay as described elsewhere (39). Assays were performed with duplicate plates in at least three independent experiments for each strain and serotype combination. CPS standards were prepared by dissolving purified type Ia or III CPS in H_2O to a final concentration of 10 ng/μl. Then 160 μl of CPS standard or CPS mutanolysin extract was transferred to the first column of the plate, and serial fourfold dilutions were performed across the plate from left to right into 40 mM sodium phosphate (pH 7.0). Rabbit anti-CPS antiserum (diluted 1:10,000 in 5% NFDM–50 mM sodium phosphate [pH 7.0]) was dispensed into the plate (120 μl/well), which was then allowed to stand at room temperature for 30 min, allowing the antibody to bind. From each well of the dilution series plate, 200 μl was transferred to the corresponding well (i.e., A1 to A1) of a CPS–poly-L-lysine-coated microtiter plate. One well of the plate, chosen as a blank, received 5% NFDM–50 mM Na phosphate (pH 7.0) instead of an antibody-CPS dilution. The ELISA was then developed as described elsewhere (39). The ELISA data were fitted to the four-parameter model of Rodbard (42), and the concentration of type-specific CPS in the undiluted extracts was calculated (50% inhibitory concentration of standard/50% inhibitory concentration of unknown) × concentration of undiluted standard).

Nucleotide sequence accession number. The *cps*III DNA sequence has been deposited in GenBank with accession no. AF163833.

RESULTS

Organization of the type III GBS *cps* locus. We have completed the sequencing of the entire GBS type III capsule synthesis locus by subcloning or amplifying the genomic DNA 5′ and 3′ of the *cps* genes identified previously (Fig. 2). Analysis of the compiled sequence revealed a total of 16 *cps*-related open reading frames (ORFs), of which 9 were previously undescribed. Additionally, a divergently transcribed monocistronic ORF 5′ of the type III locus containing $cps_{III}Y$ was observed. To facilitate comparison with the type Ia *S. agalactiae* and type 14 *S. pneumoniae cps* loci, we have changed the gene designations for *cpsABCDEF*, and thus the gene order for the CPS synthesis locus is $cps_{III}ABCDEFGHIJKL$ $neu_{III}BCDA$ (Fig. 2). All of the ORFs except $cps_{III}Y$ are preceded by recognizable ribosome binding sites. Most of the ORFs are closely linked, with stop codons that overlap the translational initiation sites of the adjacent ORFs. There are significant gaps, however, between $cps_{III}D$ and $cps_{III}E$ (51 bp), $cps_{III}I$ and $cps_{III}J$ (33 bp), $cps_{III}J$ and $cps_{III}K$ (84 bp), and $neu_{III}B$ and $neu_{III}C$ (75 bp). We have examined these intergenic gaps and found that they do not contain potential regions of dyad symmetry, transcriptional terminators, or consensus promoter sequences. If we allow the ORFs to initiate with alternative start codons such as CUG or UUG, the intragenic gaps between $cps_{III}D$ and $cps_{III}E$, $cps_{III}I$ and $cps_{III}J$, and $neu_{III}D$ and $neu_{III}A$ decrease in size from 51 to 7, 33 to 0, and 69 to 21 bp, respectively. The alternative start codons for these putative ORFs also contain upstream ribosome binding sites.

The *cps*III locus is nearly identical to the *cps*Ia operon of *S. agalactiae* type Ia (Fig. 3 and Table 2). Identity between translated ORFs ranges between 94 and 100% with the exception of $Cps_{III}G$ and $Cps_{III}H$, where it falls to 72 and 24%, respectively. A homologue for $Cps_{III}Y$ was not reported for type Ia *S. agalactiae*. The arrangement of genes from $cps_{III}A$ to $cps_{III}L$ of the GBS type III *cps* operon is also strikingly similar to that of the entire capsule locus from *S. pneumoniae* type 14 (Fig. 3). The nucleotide sequence similarity over the length of the common genes is 52% (7,784 identical nt out of 15,133 nt, with 215 gaps) when the type 14 capsule gene sequence is aligned with the GBS type III locus. The resemblance between the genes when translated is also high at approximately 50%

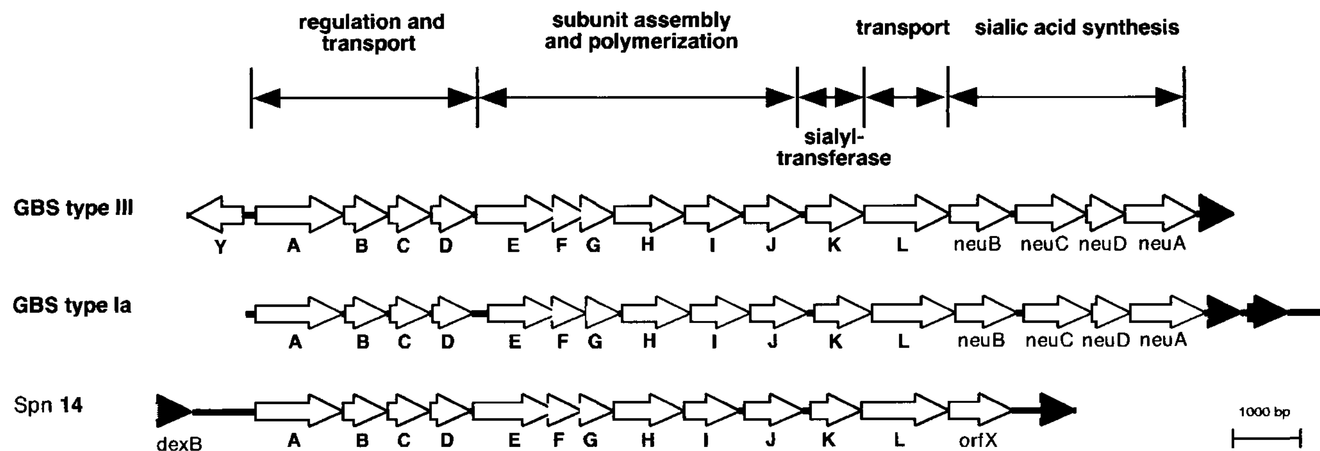

FIG. 3. Schematic representation of the organization of the CPS synthesis loci of *S. agalactiae* type III (GBS type III), *S. agalactiae* type Ia (GBS type Ia) (60), and *S. pneumoniae* type 14 (*Spn* 14) (23). The unfilled arrows indicate ORFs within the operons and the direction of transcription. Filled arrows indicate ORFs not involved in CPS synthesis that flank the *cps* gene regions. Letters below the arrows indicate the gene designations. The organization of the *cps* loci into regions of associated gene function is indicated above the ORF diagrams.

identity and 60% similarity as anticipated, based on their identical capsular structures if the terminal sialic acid residue present on the type III CPS is excluded.

Upstream of the GBS CPS synthesis locus, but in the opposite orientation, is $cps_{III}Y$. It is separated by an intergenic span of 189 nt from the *cpsABCDEFGHIJKL* $neu_{III}BCDA$ (*cps*III) operon. At the 3′ end of the operon, downstream of $neu_{III}A$ is a gap of 170 nt before another ORF is detected. This putative ORF has amino acid sequence homology with the central portion of the *E. coli* gene *evgS*, a putative sensor protein from a two-component regulatory system, and is therefore probably not a component of the capsule synthesis genes.

Sequence similarities of the CPS ORFs to other genes. All of the *cps* gene sequences were translated, and the amino acid sequences were compared to protein sequence databases at the National Center for Biotechnology Information using the gapped BLAST version 2.0 program (1). Similarities to proteins in the sequence databases are presented in Table 2. The translated sequences of $cps_{III}A$ through $cps_{III}J$ are similar to those of a number of genes from gram-positive organisms involved in CPS or exopolysaccharide synthesis.

The $cps_{III}Y$ and $cps_{III}A$ (previously *cpsX*) genes coding for homologues of LysR and LytR, respectively, were previously described (24). The genes $cps_{III}B$, $cps_{III}C$, $cps_{III}D$, and $cps_{III}E$ have also been described (43). $Cps_{III}B$ is homologus to *S. pneumoniae* CpsB, a tyrosine phosphatase involved in CPS regulation (36). Capsule expression is reduced when CpsB dephosphoryates CpsD, an autophosphorylating protein kinase involved in CPS chain length determination and transport. $Cps_{III}D$ is homologous to the *S. pneumoniae* CpsD and also shares amino acid similarity with the carboxy terminus of Wzc, an autophosphorylating tyrosine kinase for lipopolysaccharide synthesis in *E. coli*. $Cps_{III}C$, transcribed from the ORF immediately 5′ of $cps_{III}D$, is homologous to the amino-terminal end of Wzc (40). $Cps_{III}E$ is a glycosyl-1-phosphate transferase (60). The function of $Cps_{III}F$ is unknown, but its *S. pneumoniae* type 14 homologue, Cps14F, is thought to enhance the activity of Cps14G. $Cps_{III}G$ is homologous to Cps14G of *S. pneumoniae* and $Cps_{Ia}G$ from *S. agalactiae* type Ia, β-1,4-galactosyltransferases that catalyze the addition of the second monosaccharide in the formation of the CPS repeating unit.

The $cps_{III}H$ gene is predicted to encode a protein with eight transmembrane domains which is homologous to the putative polysaccharide polymerase encoded by *cps14H* from *S. pneumoniae* type 14 (22). This homology led us to speculate that $Cps_{III}H$ was responsible for polymerization of the oligosaccharide precursors in the type III CPS and thereby introduction of the structural determinant distinguishing type III from type Ia capsule. The aligned sequences of the $cps_{III}H$ gene product, Cps14H from *S. pneumoniae*, and $Cps_{Ia}H$, a GBS type Ia homologue (60), are depicted in Fig. 4. The translated type Ia and type III gene products are strikingly similar outside the $Cps_{III}H$ region (99.3% similarity and 99.2% identity) but diverge markedly from the carboxy-terminal end of $Cps_{III}G$ to the amino-terminal end of $Cps_{III}I$ (41% similarity and 28% identity). Further analysis of this region is discussed below. The $Cps_{III}I$ and $Cps_{III}J$ protein sequences are highly homologous to sequences of several glycosyltransferases, including Cps14I and Cps14J from *S. pneumoniae* type 14 (20, 22, 23).

The $cps_{III}K$ gene shares homology with its type Ia GBS homologue, $cps_{Ia}K$, with the lipooligosaccharide synthetase *orfY* (HI0871) from *Haemophilus influenzae*, and *lst*, which encodes a novel sialyltransferase in *H. ducreyi* (7). Lst, like $Cps_{III}K$, lacks homology to sialyltransferases in *E. coli*, *H. influenzae*, *Neisseria gonorrhoeae*, *N. meningitidis*, and mammals but shows significant homology to the *H. influenzae* OrfY. Interestingly, Lst catalyzes an α(2→3) linkage between the terminal sialic acid and a galactose residue in the lipooligosaccharide of *H. ducreyi*, the same specific glycosidic bond between these residues in the GBS capsular polysaccharides. These observations suggest that $Cps_{III}K$ is the *cps*III sialyltransferase.

The $cps_{III}L$ gene encodes a protein which is weakly similar to several known and presumed oligosaccharide transport proteins. The genes $neu_{III}B$, $neu_{III}C$, $neu_{III}D$, and $neu_{III}A$ encode the final four proteins in the locus. $Neu_{III}D$ (formerly called CpsE) and $Neu_{III}A$ have been previously described (8, 15). These four genes are most similar to the *neuABCD* genes from the gram-negative organism *E. coli* K1 (6) and the *siaCAB* genes of *N. meningitidis* serogroup B (11), which are responsible for the synthesis and activation of sialic acid for capsule production in these species. We previously demonstrated that $neu_{III}A$ encodes a CMP-NeuNAc synthetase, catalyzing the activation of sialic acid with CMP (15). Homologues of $Neu_{III}B$ are NeuNAc synthetases (2). *neuC* of *E. coli* K1 has been characterized as a UDP-*N*-acetylglucosamine→ManNAc epi-

TABLE 2. Sequence comparisons to the NCBI database[a]

Gene product	Proposed function	Similar gene products	% Identity	Range (amino acid)	Accession no.
$Cps_{III}Y$	Transcriptional regulator[b]	*Azorhizobium caulinodans*, NAC[c]	42	1–80	AJ006238
		Klebsiella pneumoniae, NAC[c]	44	1–74	L01114
		Comamonas testosteroni, TsaR[f]	37	1–80	U32622
		H. influenzae Rd KW20, OxyR[c]	27	1–205	U49355
		E. coli, NAC[c]	43	1–74	U56736
$Cps_{III}A$	Transcriptional regulator[b]	*S. agalactiae* type Ia, $Cps_{Ia}A$[f]	95	1–486	AB028896
		S. thermophilus Sfi6, EpsA[f]	47	1–486	U40830
		S. thermophilus, EpsA[f]	52	78–486	AF053346
		S. pneumoniae, Cps14fA[f]	42	5–485	X85787
		S. pneumoniae, Cps23fA[f]	42	5–485	AF030373
		S. pneumoniae, Cps33fA[f]	42	5–485	AJ006986
$Cps_{III}B$	Tyrosine phosphatase[b]	*S. agalactiae* type Ia, $Cps_{Ia}B$[g]	99	1–243	AB028896
		S. thermophilus, EpsB[g]	65	1–243	AF053346
		S. thermophilus Sfi6, EpsB[g]	64	1–243	U40830
		S. salivarius, CpsB[g]	64	1–243	X94980
		S. pneumoniae, Cps19fB[c]	60	1–243	AF030367
$Cps_{III}C$	Chain length regulator[b]	*S. agalactiae* type Ia, $Cps_{Ia}C$[f]	99	1–230	AB028896
		S. thermophilus MR-1C, EpsC[f]	50	9–230	AF053348
		S. salivarius, CpsC[f]	50	1–230	X94980
		S. thermophilus Sfi6, EpsC[f]	49	1–230	U40830
		S. pneumoniae, Cap33fC[f]	45	1–230	AJ006986
		S. pneumoniae, Cps23fC[f]	44	9–230	AF030373
$Cps_{III}D$	Tyrosine kinase, CPS chain length regulator/exporter[b]	*S. agalactiae* type Ia, $Cps_{Ia}C$[f]	99	1–229	AB028896
		S. salivarius, CpsD[f]	57	1–222	X94980
		S. thermophilus Sfi6, EpsD[f]	56	1–222	U40830
		S. pneumoniae, Cps23fD[f]	51	1–229	AF030373
		S. pneumoniae, Cps19fD[c]	53	1–224	U09239
$Cps_{III}E$	Glc-1-P transferase[c]	*S. agalactiae* Ia, $Cps_{Ia}E$[c]	99	1–449	AB028896
		S. salivarius, CpsE[f]	47	1–432	X94980
		S. pneumoniae, Cap33fE[f]	48	33–432	AJ006986
		S. pneumoniae, Cps19fE[f]	44	1–432	U09239
		S. pneumoniae, Cps14fE[c]	44	1–432	X85787
$Cps_{III}F$	β-1,4-Gal transferase enhancer[b]	*S. agalactiae* Ia, $Cps_{Ia}F$[g]	96	1–149	AB028896
		S. pneumoniae, Cps14fF[f]	83	1–149	X85787
		Lactococcus lactis NIZO B40, EpsE[c]	38	2–149	U93364
		Sphingomonas strain s88, SpsK[c]	32	1–142	U51197
		E. coli, N-GlcA[c]	30	3–146	AF013583
$Cps_{III}G$	β-1,4-Gal transferase[d]	*S. agalactiae* Ia, $Cps_{Ia}G$[c]	72	1–147	AB028896
		S. pneumoniae, Cps14fG[c]	63	1–126	X85787
		L. lactis NIZO B40, EpsF[c]	32	1–157	U93364
		Rhizobium leguminosarum, PssE[c]	34	1–111	X99850
		R. leguminosarum, PssE[c]	34	1–111	AF014054
$Cps_{III}H$	CPS polymerase[e]	*S. pneumoniae*, Cps14fH[f]	25	1–375	X85787
		S. agalactiae Ia, $Cps_{Ia}H$[f]	24	137–363	AB028896
		S. suis Cps1H[f]	23	6–361	AF155804
$Cps_{III}I$	β-1,3-GlcNAc transferase[d]	*S. agalactiae* Ia, $Cps_{Ia}I$[c]	98	5–320	AB028896
		S. pneumoniae, Cps23fE[f]	53	1–276	AF030373
		S. thermophilus Sfi6, EpsI[f]	35	1–255	U40830
		S. pneumoniae, Cap33f[f]	36	5–237	AJ006986
		S. pneumoniae, Cps14fE[c]	27	1–290	X85787
$Cps_{III}J$	β-1,4-Gal transferase[d]	*S. agalactiae* Ia, $Cps_{Ia}J$[c]	99	1–315	AB028896
		S. pneumoniae, Cps14fJ[c]	38	7–224	X85787
		S. thermophilus Sfi6, EpsI[f]	37	7–242	U40830
		S. pneumoniae, Cps23fE[f]	37	9–237	AF030373
		S. pneumoniae, Cap33f[f]	27	9–247	AJ006986
$Cps_{III}K$	Sialyltransferase[b]	*S. agalactiae* Ia, CpsIaK[g]	99	1–460	AB028896
		H. ducreyi Lst[c]	27	143–299	AF101047
		H. influenzae Rd KW20, OrfY[g]	26	102–298	HI0871
$Cps_{III}L$	Repeating unit transporter[b]	*S. agalactiae* Ia, CpsIaL	100	1–460	AB028896
		H. influenzae Rd KW20[g]	26	102–298	M94855
		Staphylococcus aureus, Cap1F[f]	24	183–400	U10927
		Streptococcus pneumoniae, Cps23f[f]	19	4–456	AF030373
$Neu_{III}B$	NeuNAc synthetase[c]	*S. agalactiae* Ia, NeuB[f]	99	1–341	AB028896
		E. coli, NeuB[c]	56	2–337	U05248
		Methanococcus jannaschii[f]	47	3–329	H64432
		N. meningitidis, SiaC[c]	38	4–315	M95053
		Bacillus subtilis, SpsE[f]	34	2–334	X73124
$Neu_{III}C$	UDP-GlcNAc 2-epimerase[c]	*S. agalactiae* Ia, NeuC[f]	94	1–320	AB028896
		E. coli, NeuC[c]	39	1–380	M84026
		N. meningitidis, SiaA[f]	38	4–315	M95093
		Rattus norvegicus[c]	29	1–318	Y07744
		Methanococcus thermoautotrophicum[f]	47	3–329	AE000860

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TABLE 2—*Continued.*

Gene product	Proposed function	Similar gene products	% Identity	Range (amino acid)	Accession no.
$Neu_{III}D$	Sialic acid synthesis[c]	*S. agalactiae* type Ia, NeuD[f]	100	1–209	AB028896
		E. coli, NeuD[c]	30	1–209	U05248
		B. subtilis, YvfD[g]	25	3–208	Z71928
		Caulobacter crescentus, LpsB[g]	27	56–206	AF062345
		Campylobacter jejuni, WlaI[g]	27	65–207	Y11648
$Neu_{III}A$	CMP-NeuNAc synthetase[c]	*S. agalactiae* type Ia, NeuA[b]	94	1–370	AB028896
		E. coli, NeuA[c]	37	2–395	J05023
		N. meningitidis, SiaB[c]	35	4–213	U04328
		H. influenzae, NeuA[c]	33	1–213	U32807
		H. ducreyi, NeuA[c]	29	1–209	U54496

[a] Translations of *cps*III ORFs were aligned using BLAST 2.0 with the BLASTP algorithm using the nonredundant database and the BLOSUM62 matrix at the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md. The extent of the alignment returned by BLAST is given as the range.
[b] Predicted by sequence similarity.
[c] Enzymatic activity determined.
[d] Predicted based on similarity to *S. agalactiae* type Ia.
[e] Determined in this study.
[f] Proposed function.
[g] Unknown function.

merase (R. P. Silver et al., submitted for publication). In this study, complementation of an *E. coli* K1 *neuC* mutant by $neu_{III}C$ indicated that $Neu_{III}C$ is functionally equivalent to the *E. coli* epimerase. Although the exact function of $neu_{III}D$ is unknown, a role in sialic acid synthesis has recently been established, since a nonpolar mutation in the *E. coli* K1 homologue *neuD* can be complemented by the addition of exogenous sialic acid or expression of $neu_{III}D$ in *trans* (9a).

Identification of the *cps*III transcriptional start site. We hypothesized that the start of transcription occurred proximal to the $cps_{III}A$ gene. To investigate this possibility, we used COH1 mRNA and a radiolabeled primer complementary to the 5′ end of $cps_{III}A$ to identify the 5′ transcriptional start site of the *cps*III locus by primer extension analysis. The products of the primer extension reaction are shown in Fig. 5A. We mapped the 5′ end of the transcript (indicated by the arrow) to a G nucleotide on the DNA template 38 bp upstream of the $cps_{III}A$ start codon. We examined the sequence directly upstream of the G nucleotide to identify canonical consensus promoter sequences. A sequence (TATAAT) identical to the -10 region of the consensus σ^{70} *E. coli* promoter sequence was detected 7 bp above the 5′ end of the *cps*III transcript. A sequence 17 bp 5′ of the -10 region (TTGAAT) matched the canonical -35 region from *E. coli* at four of six positions. It also matched a consensus sequence for streptococcal promoters which is similar to the *E. coli* promoter, except that the -10 region is extended on the 5′ side to include the sequence TGN (with N being any nucleotide) (12, 52). We concluded this sequence is the *cps*III promoter.

Polar effects of allelic exchange mutagenesis within the *cps*III locus. To determine if there were additional transcriptional start sites within the *cps*III locus, we developed strains incorporating transcriptional terminators within their *cps* loci by allelic exchange mutagenesis (26, 61). To ascertain if these mutations were polar, we tested each clone for type III capsule production by immunoblot analysis as described. The type III antiserum recognizes both sialylated and asialo forms of the type III CPS. The wt strain, COH1, reacted with the antibody as expected; however, each of the CPS mutants failed to react, indicating they were acapsular (data not shown). These data demonstrated that mutations generated by homologous recombination in the 5′ region of the CPS synthesis locus prevented expression of capsule synthesis. These results also suggested that mutations in these upstream genes were polar and altered expression of downstream *cps* genes. This was expected, as a transposon mutant in $cps_{III}B$ was previously shown to be acapsular (43).

Since each of the allelic exchange mutations above abolished capsule production, we sought to determine the effect of the mutations on the expression of downstream *cps* genes. RNA dot blot analysis was performed on total cellular RNA isolated from COH1 and each of the isogenic mutants. Equal amounts of RNA from each strain were hybridized to three different *cps* gene probes independently. The probes were specific for different regions of the CPS synthesis locus: the 5′ end ($cps_{III}B$), the central portion ($cps_{III}D$), and the 3′ end ($neu_{III}A$). As shown in Fig. 5B, each of the probes hybridized to the RNA isolated from COH1 (wt) as expected and demonstrated comparable levels of mRNA transcribed from $cps_{III}B$, $cps_{III}E$, and $neu_{III}A$. In contrast, virtually no hybridization was observed between the three probes and the RNA from the $cps_{III}A$ mutant. Since all of the probes are specific for genes 3′ of the

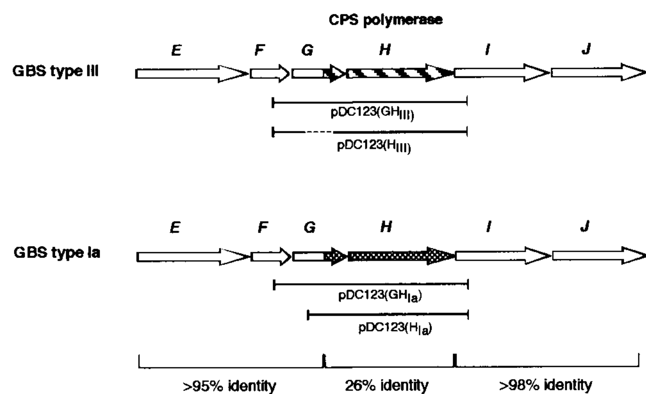

FIG. 4. Capsule subunit assembly locus. Regions of divergent sequence between type Ia and III strains are shown either striped (type III) or checked (type Ia). The degree of homology between the conserved and nonconserved regions is shown at the bottom. Gene designations are shown as single letters above each ORF map, and the CPS polymerase (H) is also indicated. The plasmids used in the polymerase expression constructs are shown beneath each map. Sequences deleted in the construction of pDC123(H_{III}) are shown with dashes.

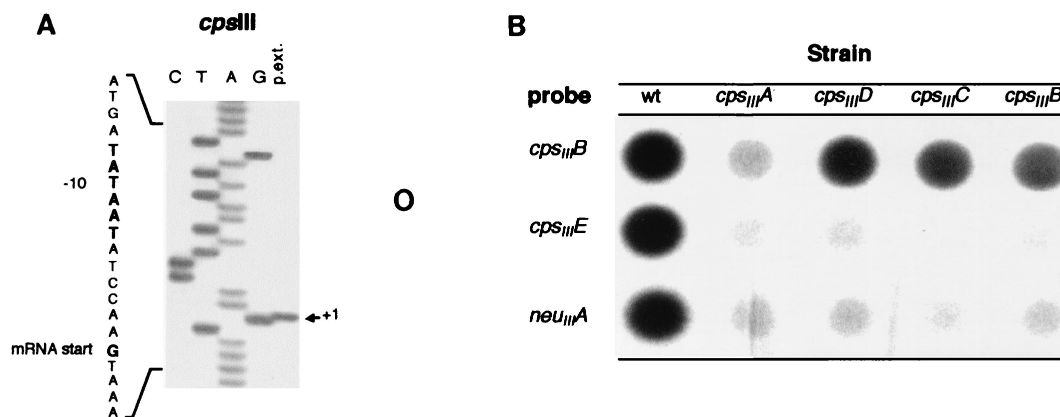

FIG. 5. (A) Primer extension reaction. Primer extension reactions were carried out on total cellular RNA derived from GBS strain COH1, using an antisense primer that hybridized to $cps_{III}A$ mRNA. A sequencing ladder was generated from $cps_{III}A$ double-stranded DNA using the same primer and was separated on a sequencing gel in lanes adjacent (lanes CTAG) to the primer extension products (p.ext.). The position of the apparent first ribonucleotide of the primer extension product (+1) is indicated to the right. The DNA sequence surrounding the start position of the mRNA is depicted vertically on the left, with the consensus −10 promoter sequence and the mRNA start site highlighted in bold. (B) RNA dot blots of total cellular RNA derived from the wt GBS strain COH1 and the ΩKm-2 cassette allelic exchange mutant strains COHY-107 ($cps_{III}A$), COHY-106 ($cps_{III}D$), COHY-105 ($cps_{III}C$), and COHY-104 ($cps_{III}B$). The blots were hybridized to intragenic probes derived from the wt COH1 $cps_{III}B$, $cps_{III}E$, and $neu_{III}A$ genes and exposed to X-ray film.

ΩKm-2 insertion in $cps_{III}A$, these data suggest that this mutation was polar and blocked expression of all downstream genes.

RNA dot blot analysis using $cps_{III}B$-, $cps_{III}C$-, and $cps_{III}D$-derived probes demonstrated hybridization proximal but not distal to each mutation as far downstream as $neu_{III}A$ (data not shown). These results confirmed the observation that the *cps* genes are transcribed up to the site of the ΩKm-2 mutation but not distal to the site of the ΩKm-2 insertion for any of the mutants. Therefore, the ΩKm-2 cassette mutations interrupted transcription of the distal genes, providing further evidence that the CPS synthesis locus is transcribed as a large polycistronic message from $cps_{III}A$ to $neu_{III}A$.

Development of strains expressing heterologous CPS. Based on the observation that the GBS type Ia and type III CPS oligosaccharide repeating units are structurally identical in vivo, the serotype specificity of these two polysaccharides would be determined by the unique linkage introduced during polymerization of the repeating units into high-molecular-weight CPS (Fig. 1). Homology analysis between the CPS type Ia and III loci revealed a striking divergence between $Cps_{Ia}H$ and $Cps_{III}H$ (Fig. 4), both of which share homology with repeating unit polymerase genes in other polysaccharide operons. In addition, we observed that the homology between the *cps*Ia and *cps*III loci diverged at amino acid 110 of the upstream glycosyltransferase gene $cps_{Ia}G$ (Fig. 4). We hypothesized that the putative polymerases are responsible for catalyzing the linkage between the type Ia or III subunits, which confer structural and therefore serotype specificity.

To address this hypothesis, we investigated how expression of the $cps_{Ia}H$ and $cps_{III}H$ genes in the heterologous serotype strain would affect the type of CPS produced. $cps_{Ia}H$ and $cps_{III}H$ were cloned independently into the gram-positive expression vector pDC123 (Fig. 4; Table 1). The $cps_{Ia}H$ construct, pDC123(H_{Ia}), was transformed into the type III capsule-producing strain, COH1. The type Ia-producing strain, A909, was transformed with pDC123(H_{III}) containing $cps_{III}H$. Due to the divergence in their 3′ termini, we also explored the contribution of the glycosyltransferase genes, $cps_{Ia}G$ and $cps_{III}G$, to the specific serotype of CPS synthesized. Therefore, additional constructs containing either $cps_{Ia}GH$ [pDC123 (GH_{Ia})] or $cps_{III}GH$ [pDC123(GH_{III})] were generated and transformed into the respective heterologous wt hosts. Immunoblot analysis was performed using rabbit type Ia or type III CPS polyclonal antisera to determine the nature of the CPS being expressed by the recombinant strains compared to the wt strains as controls. As seen in Fig. 6, cross-reactivity was not observed between A909 and the type III antisera or COH1 and antisera against type Ia CPS. When $cps_{III}GH$ or $cps_{III}H$ was expressed in A909, however, reactivity with the type III antiserum was observed, but reactivity with the type Ia antiserum was absent. Similarly, expression of $cps_{Ia}GH$ or $cps_{Ia}H$ alone in COH1 resulted in reactivity with the type Ia antisera (in addition to reactivity to the type III antisera for $cps_{Ia}GH$). These

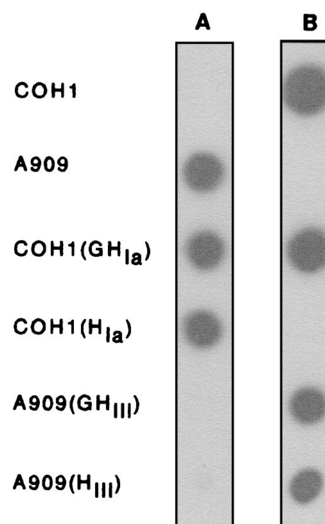

FIG. 6. Immunoblots directed against immobilized whole GBS utilizing rabbit polyclonal anti-CPS antisera, detected with HRP-conjugated, goat anti-rabbit secondary antibody and chemiluminescent HRP substrate. Strains used: COH1, GBS type III strain; A909, GBS type Ia strain; COH1(GH_{Ia}), strain COH1 containing pDC123(GH_{Ia}); COH1(H_{Ia}), strain COH1 containing pDC123(H_{Ia}); A909(GH_{III}), strain A909 containing pDC123(GH_{III}); A909(H_{III}), strain A909 containing pDC123(H_{III}). (A) Blot exposed to type Ia antisera; (B) blot exposed to type III antisera.

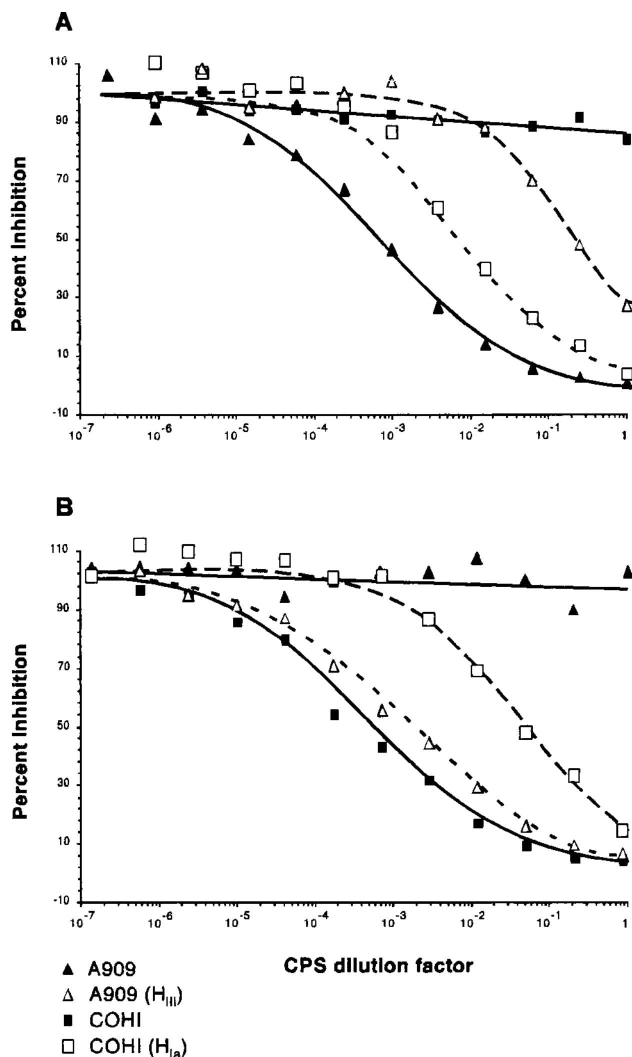

FIG. 7. Competitive ELISA inhibition curves produced from mutanolysin extracts of wt and heterologous *CPS* polymerase-expressing strains. CPS extracts were used to compete with immobilized purified type Ia CPS for anti-type Ia polyclonal antibody (A) or with immobilized purified type III CPS for anti-type III polyclonal antibody (B). Strains A909 and COH1 produce native type Ia and type III CPS, respectively. Strain A909(H$_{III}$) expresses the $cps_{III}H$ allele from COH1, and strain COH1(H$_{Ia}$) expresses the $cps_{Ia}H$ allele from strain A909.

data suggested that expression of $cps_{III}H$ in A909 alone was capable of altering the type of CPS produced from Ia to III. Likewise, expression of $cps_{Ia}H$ in COH1 converted the CPS produced from type III to type Ia. Inclusion of the glycosyltransferase gene(s) ($cps_{Ia}G$ or $cps_{III}G$) just 5′ of the putative polymerase gene did not influence the nature of the CPS produced, as was anticipated from their roles as glycosyltransferases (60).

The amount of CPS produced by the wt strain and the strains carrying the heterologous polymerase genes was quantified by competitive ELISA to determine if any native CPS in the latter strains was produced. Mutanolysin CPS extracts from the wt and recombinant strains were used to competitively inhibit the binding of serotype-specific polyclonal antisera to purified type Ia or type III CPS immobilized on microtiter plates. The degree of inhibition was compared to that of purified CPS similarly preincubated with the antibody, prior to transfer to the coated plates (Fig. 7). The CPS extracts from the wt strains A909 and COH1 inhibited type Ia and III antibody binding, respectively, as expected, and failed to inhibit reactivity of the heterologous CPS serotype antisera. For A909 containing pDC123(GH$_{III}$) or pDC123(H$_{III}$), production of type Ia CPS was reduced (1.7 and 1.2 ng/μl, respectively, versus 63.5 ng/μl for A909 alone). Both of these strains demonstrated a >37-fold decrease in type Ia-reactive CPS material compared to wt but produced a substantial amount of CPS which reacted with the type III antisera (20.8 and 26.1 ng/μl). COH1 containing pDC123(H$_{Ia}$), expressing the type Ia CPS polymerase, produced less type III CPS (10.5 ng/μl) and synthesized a quantity of anti-type Ia-reactive CPS equal to 72% of the wt A909 levels (45.4 ng/μl). A less dramatic effect was seen with COH1 containing pDC123(GH$_{Ia}$), expressing both the $cps_{Ia}G$ and $cps_{Ia}H$ alleles. The reason for this disparity is unclear but could be due to decreased expression of the CPS polymerase in this construct. Nevertheless, an anti-type Ia-reactive CPS was produced (3.2 ng/μl) with a concomitant decrease in type III CPS production (61.9 versus 91.0 ng/μl). These data demonstrated that expression of the heterologous CPS polymerase gene alone, in A909 or COH1, is sufficient to confer binary synthesis of CPS that are immunoreactive to both type Ia and type III antisera. Furthermore, inclusion of $cps_{Ia}G$ or its type III homologue $cps_{III}G$ in the expression constructs was unnecessary for production of the altered CPS phenotype. These data also support the hypothesis that the putative polymerase gene is responsible for the glycosidic linkage between the repeating units of type Ia or III CPS and ultimately confers the serotype specificity of the CPS.

DISCUSSION

The complete DNA sequences of several bacterial CPS and exopolysaccharide synthesis operons have been reported (10, 23, 28, 33, 35, 41, 48, 49). The organization of the regulatory and structural genes within these loci is remarkably well conserved across CPS and exopolysaccharide-producing species. The typical arrangement begins with regulatory determinants, then genes coding for control of polymer chain length and export, followed by structural genes for repeating unit assembly (glycosyltransferases) and polymerization (polymerases), and finally additional gene(s) involved in subunit transport and/or activated monosaccharide biosynthesis. The basic organization of the GBS type III operon reported here is similar to that of polysaccharide operons in other species and parallels that reported for the *S. agalactiae* type Ia operon (60).

Identification and characterization of the glycosyltransferases responsible for the structural organization of the oligosaccharide repeating units of *S. pneumoniae* types 3 (3, 10) and 14 (20, 22, 23) and the *eps* genes of *Lactococcus lactis* (49, 50) have recently been reported (Table 2). In addition, the *S. agalactiae* type Ia glycosyltransferases have recently been characterized and found to be functionally equivalent to their *S. pneumoniae* type 14 homologues Cps14E, Cps14G, Cps14I, and Cps14J (22, 60). In both species, these enzymes are responsible for the assembly of a tetrasaccharide that is chemically identical to the asialo type III GBS CPS repeating unit. We previously reported that $cps_{III}E$ most likely encodes a galactosyltransferase. We could not, however, rule out the possibility that it may encode a glucosyltransferase (43). Given the homology of the type Ia glycosyltransferase genes to those in the *cps*III operon, and the identical structure of the oligosaccharide repeating unit, it is likely that these genes encode the same functions in both serotypes. Subsequent biochemical analysis of Cps$_{Ia}$E and Cps14E in *E. coli* has shown they are glucosyl-1-phosphate transferases (22, 60), which catalyze the

transfer of glucose to a receptor lipid intermediate to begin synthesis of the repeating unit. Based on these observations collectively, $cps_{III}E$ should also code for a glucosyltransferase, and the assignment of $cps_{III}E$ as a probable galactosyltransferase must be reevaluated.

Since the glycosidic linkage between the Ia and III pentasaccharide repeating units is the only apparent difference between the two CPS structures, and the type Ia and type III operons are virtually identical except for the region encoding the CPS polymerase, we hypothesized that the *cpsH* gene in both serotypes encoded the polymerase responsible for the repeating unit linkage. Although the carboxy-terminal domain of the upstream-encoded β-1,4-galactosyltransferases, $Cps_{III}G$ and $Cps_{Ia}G$ diverged, our results confirm that these enzymes are not involved in linking the repeating units. Overexpressing the $cps_{Ia}H$ and $cps_{III}H$ alleles in the heterologous serotype strains resulted in decreased expression of the native CPS and promoted synthesis of the alternative serotype CPS. We postulate that since plasmid pDC123 replicates at ~90 copies/cell (8), the *cps* gene dosage of the recombinant plasmids was significantly higher than that of the chromosomal copy. Hence, depletion of the CPS precursor pool by the episomal polymerase gene product redirected polymerization of the repeating units into the alternative CPS structure. These results demonstrated that $Cps_{Ia}H$ and $Cps_{III}H$ were responsible for the linkage between the repeating units of each CPS polymer.

Despite a number of recent publications characterizing the CPS glycosyltransferases in various gram-positive species, there is a lack of studies describing the CPS polymerases. The identification of genes encoding CPS polymerases within gram-positive *cps* loci has been based on the weak homology of the translated genes to the O-antigen polymerases of *Shigella flexneri* (37) and *Salmonella enterica* serovar Typhimurium (9). Direct evidence of polymerase function has been difficult to obtain presumably because biochemical approaches require significant amounts of the precursor oligosaccharides, which are difficult to isolate or synthesize. The need to reconstitute the polymerase with potential membrane-bound accessory proteins in a host suitable for carrying out the assays, such as *E. coli*, presents another difficulty. As a result, direct in vitro evidence of CPS polymerase activity has not been reported for gram-positive hosts producing complex, branched CPS. Only one other gene product from any gram-positive CPS- or exopolysaccharide-producing species has been unambiguously assigned CPS polymerase function, *cap3B* of type 3 *S. pneumoniae* (14). This enzyme does not share homology with $Cps_{III}H$ and differs in activity, as it processively polymerizes an unbranched β(1→3)-linked glucuronic acid-(1→4)-β-D-glucose heteropolymer (7a). Examining CPS polymerase function by heterologous gene expression, as done here, provides the advantage of using the original host's native machinery in an intact cell for oligosaccharide production and provides a novel approach to assigning CPS gene function.

Divergent CPS polymerase genes have been detected in several pneumococcal serotypes and may be responsible for altered CPS structures in this species. In a study examining the genetic diversity of pneumococcal CPS synthesis loci (21), DNA from 26 different *S. pneumoniae* serotypes was probed with *cps14H*, the gene encoding the putative CPS polymerase. Only serotypes 15b and 15c, which have the same CPS core structure as type 14, hybridized to this probe. The structural differences between *S. pneumoniae* type 19A and type 19F CPS have been attributed to the potential activities of their respective CPS polymerases (34). Transformation of a type 19F strain with DNA containing the *cps19AH* and *cps19AI* genes resulted in a strain that produced a type 19A serotype. These data suggest that although CPS structural diversity within pneumococci is generally due to modification of glycosyltransferase and monosaccharide precursor usage, it can also be achieved through changes in the specificity of the polysaccharide polymerases. The *S. agalactiae* type Ia and type III data presented here provides compelling evidence that the polymerase gene is responsible for generating the linkage which confers the type-specific glycosidic bond between the oligosaccharide subunits and hence the serotype CPS produced.

Mutagenesis of the first four genes in the *cps*III operon and RNA dot blot analysis failed to show transcription starting distal to the promoter upstream of $cps_{III}A$, suggesting the absence of additional active promoters in the rest of the operon. These data indicated that the *cps*III operon is transcribed as a large polycistronic message. In contrast, Yamamoto et al. (60) identified an additional promoter in the type Ia locus between $cps_{Ia}D$ and $cps_{Ia}E$ (homologues of $cps_{III}D$ and $cps_{III}E$, respectively). This promoter may arise from differences between the type Ia and type III nucleotide sequences within the region where the type Ia secondary promoter was located. The most significant of these is the insertion of an additional A at base 3894 in the Ia nucleotide sequence that introduces a frameshift and subsequent stop codon. This results in the truncation of $Cps_{Ia}E$ compared to the type III homologue (the type III $cps_{III}E$ ORF extends an additional 58 codons 5′ of the start site of the homologue type Ia $cps_{Ia}E$ gene). The additional adenine is located within the 17-bp spacer between the −35 and −10 regions of the type Ia promoter sequence. The shorter spacer in the type III locus may account for the lack of secondary promoter activity in our transcriptional analysis.

We are continuing to investigate the *cps* loci of the other GBS serotypes by analyzing the sequence differences of their operons. As might be expected from their CPS structure, we have obtained preliminary evidence, using the same genetic approach, that the CPS of serotype Ib *S. agalactiae* may be converted to type Ia by transferring and overexpressing a single type III galactosyltransferase gene. Similarly, an antigenically indistinguishable type III CPS can be produced from a type VI strain by transfer of a single type III glycosyltransferase gene. This approach promises to enable a rapid means of assigning CPS gene function and investigating polysaccharide biosynthesis in GBS and other gram-positive organisms.

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