Several species of streptococci produce extracellular polysaccharides in the form of secreted exopolysaccharides or cell-associated capsules. Although the biological properties and repeating unit structures of these polysaccharides are diverse, sequence analysis of the genes required for their production has revealed a surprising degree of conservation among five genes found in the capsule gene cluster of each of several polysaccharide-producing streptococci. To determine the function of these conserved genes, we characterized a series of isogenic mutants derived from a wild-type strain of type Ia group B Streptococcus by selectively inactivating each gene. Inactivation of cpsIaE resulted in an acapsular phenotype, consistent with previous work that identified the cpsIaE product as the glycosyltransferase that initiates synthesis of the polysaccharide repeating unit. Mutants in cpsIaA, cpsIaB, cpsIaC, or cpsIaD produced type Ia capsular polysaccharide, but in reduced amounts compared with the wild type. Analysis of the mutant polysaccharides and of capsule gene transcription in the mutant strains provided evidence that cpsIaA encodes a transcriptional activator that regulates expression of the capsule gene operon. Mutants in cpsIaC or cpsIaD produced polysaccharide of reduced molecular size but with an identical repeating unit structure as the wild-type strain. We conclude that CpsA to -D are not required for polysaccharide repeating unit biosynthesis but rather that they direct the coordinated polymerization and export of high molecular weight polysaccharide.

Therefore, considerable attention has focused on characterization of the molecular basis for polysaccharide production. Beginning with the identification of the GBS type III capsule locus over a decade ago (1), rapid progress has been made in uncovering the genetic and biochemical basis for capsular polysaccharide expression in the streptococci.

A common theme that has emerged among encapsulated Gram-negative bacteria is that the capsule locus consists of a region encoding polysaccharide-specific genes required for polysaccharide repeating unit biosynthesis flanked by groups of genes involved in broadly conserved functions such as transport and regulation (2). Similarly, the capsule gene region of several polysaccharide-producing streptococci consists of a group of polysaccharide-specific genes encoding glycosyltransferases and polymerases as well as an adjacent group of genes that is conserved among several species (3–6). The functions of these conserved genes are unknown, but they are thought to direct such common processes as repeating unit polymerization, transport, and regulation.

For GBS, nine different capsule serotypes have been described, and the repeating unit structure of each has been determined (7–15). The type Ia polysaccharide has a linear backbone made up of disaccharide repeat units of \(\alpha-D-(1\rightarrow4)-\beta-D-(1\rightarrow4)-\beta-D-Galp(1\rightarrow4)-\beta-D-Galp(1\rightarrow3)-\beta-D-Galp(1\rightarrow4)-\beta-D-Galp(1\rightarrow3)-\beta-D-Galp(1\rightarrow4)-\beta-D-Galp(1\rightarrow3)-\beta-D-Galp(1\rightarrow4)-\beta-D-Galp(1\rightarrow3)-\beta-D-Galp(1\rightarrow4)-\beta-D-Galp(1\rightarrow3)-\beta-D-Galp(1\rightarrow4)-\beta-D-Galp(1\rightarrow3)-\beta-D-Galp(1\rightarrow4)-\beta-D-Galp(1\rightarrow3)-\beta-D-Galp(1\rightarrow4)-\beta-D-Galp(1\rightarrow3)-\beta-D-Galp(1\rightarrow4)-\beta-D-Galp(1\rightarrow3)-\beta-D-Galp(1\rightarrow4)-\beta-D-Galp(1\rightarrow3)-\beta-D-Galp(1\rightarrow4)-\beta-D-Galp(1\right)

These disaccharide repeat units are linked to C3 of each \(\beta\)-d-galactose residue of the backbone (8). The genetic locus for the synthesis of the type Ia GBS capsule includes at least 16 open reading frames and is predicted to contain two promoters (6). One promoter region is upstream of the first gene, \(\text{cpsIaA} (\text{Fig. 1}) (6)\). RT-PCR and primer extension analysis suggested that a second promoter lies between \(\text{cpsIaD}\) and \(\text{cpsIaE} (\text{Fig. 1}) (6)\). On the basis of DNA sequence analysis and enzymatic assays of gene products in \(E.\ coli\), functions have been assigned to many of the genes in the cluster. \(\text{cpsIaE}, \text{cpsIaG}, \text{cpsIaI}\), and \(\text{cpsIad}\) appear to encode all of the specific glycosyltransferases required for biosynthesis of the type Ia polysaccharide repeating unit with the exception of the sialyltransferase, which has not yet been identified (6). \(\text{cpsIaE}\) encodes the glycosyltransferase thought to initiate biosynthesis of the polysaccharide repeating unit. \(\text{neuA to -D}\) encode enzymes involved in sialic acid synthesis and activation (6).

Upstream of \(\text{cpsIaE}\) are \(\text{cpsIaA}\) to \(\text{cpsIaD}\), genes that, together with \(\text{cpsE}\), are well conserved among the GBS capsule serotypes and among other polysaccharide-producing streptococci such as \(S.\ thermophilus\), \(S.\ pneumoniae\), and \(S.\ suis\) (Fig. 1), (4, 6, 16, 17). Homologs of some of these genes are found also in the polysaccharide synthesis gene clusters of other Gram-positive bacteria including \(Lactococcus lactis\) and \(Staphylococcus aureus\) (18, 19). Tentative functions have been suggested for \(\text{cpsA}\) to -D on the basis of sequence similarity to genes in
other species. A homolog of the gene product encoded by cpsA, LytR, appears to regulate transcription of the structural genes of the N-acetylmuramoyl-l-alanine amidase operon of Bacillus subtilis (20). Proteins encoded by homologs of cpsC and cpsD have been implicated in chain length regulation of exopolysaccharide production in R. meliloti and lipopolysaccharide biosynthesis in Escherichia coli (21, 22). A recent report by Morona et al. (23) provided the first experimental evidence for the function of these four conserved genes in polysaccharide-producing streptococci. Nonpolar deletion mutations in any of the four genes reduced capsule production by S. pneumoniae type 19F (23). CpsD was shown to be an autophosphorylating protein-tyrosine kinase, similar to Wzc in E. coli, whose phosphorylation state influences polysaccharide production (23). CpsC and -B appeared to influence phosphorylation and dephosphorylation, respectively, of CpsD (23).

In this report, we describe the construction and characterization of nonpolar deletion mutants in cpsIaA, cpsIaB, cpsIaC, cpsIaD, and cpsIaE in type Ia GBS. Analysis of the mutants and their purified capsular polysaccharides provided experimental evidence for the involvement of each gene in polysaccharide elongation, export, or regulation. In addition, our data specifically identify CpsIaA as an activator of capsule gene transcription and demonstrate that CpsIaC and CpsIaD both control polysaccharide chain length.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—Type Ia GBS strain 515 was used as the parent strain for all mutants constructed in this study. GBS was grown in liquid culture in Todd-Hewitt broth (Difco) supplemented with yeast extract to 0.5% (w/v) (Todd-Hewitt yeast), on trypticasoy agar supplemented with 5% sheep blood (Becton-Dickinson, Cockeysville, MD), or on Todd-Hewitt-yeast agar plates supplemented with antibiotics and 5% defibrinated sheep blood (M PMicrobiologicals, Tulatin, OR). For continuous culture experiments, GBS was grown in a chemically defined medium as described (25) except that glycine was not added, NaOH was added at 0.4 g/liter, and 7 g/liter of Casitone (Difco) was substituted for the amino acid mix. E. coli strain DH5α MCR (Life Technologies, Inc.) was used for cloning and was grown inuria-Bertani medium or on Luria-Bertani agar. When appropriate, the medium was supplemented with ampicillin at 100 μg/ml or with erythromycin (erm) at 1 μg/ml for GBS or 250 μg/ml for E. coli. GBS was grown either without shaking in liquid culture or in continuous culture as described previously (25). E. coli was grown with shaking at 37 °C. Plasmid pGEM-T (Promega, Madison, WI) is an E. coli vector used for the direct cloning of PCR products; pJRS233 is a temperature-sensitive E. coli/Gram-positive shuttle vector (26). pWKS30 is a low copy number cloning vector encoding ampicillin resistance (27).

DNA Isolation and Manipulations—Plasmid DNA was isolated using either the Qiagen midiprep or miniprep kit (Qiagen, Valencia, CA) according to the manufacturer’s recommendations. GBS chromosomal DNA was prepared as described (28). Restriction endonuclease digests, DNA ligations, transformation of CaCl2-competent E. coli, agarose gel electrophoresis, and Southern hybridizations (ECL; Amersham Pharmacia Biotech) were performed using standard techniques (29). GBS electrocompetent cells were prepared as described (30) and transformed by electroporation using a Bio-Rad Gene Pulser II (Bio-Rad) as described (31).

Construction of cps Nonpolar Deletion Mutants—For cpsIaA deletion construction, primers 275 and 276 (Table I) were used to amplify by PCR the first 126 bp of cpsIaA and 736 bp of adjacent upstream flanking sequence using GBS strain 515 chromosomal DNA as template. Primers 267 and 277 (Table I) were used to amplify the last 104 bp of cpsIaA and 1535 bp of downstream flanking DNA. Primer 277 contains 18 bp of DNA that is complementary to the sequence in primer 276. The two gel-purified PCR products containing complementary ends were mixed and amplified with primers 275 and 276 to create a 1106-bp internal deletion of cpsIaA by overlap PCR (32, 33). The resultant 2500-bp PCR fragment was digested with HindIII and ligated into HindIII-digested pJRS233.

A deletion mutant in cpsIaB was constructed as above, except that primer pairs 280 and 314 (Table I) were used to amplify the 5′-terminal 100 and 900 bp of adjacent upstream flanking DNA. Primers 284–315 (Table I) were used to amplify the 3′-terminal 100 and 900 bp of downstream flanking DNA, with primer 284 containing 26 bp of DNA that is complementary to primer 280. The cpsIaB amplification products were used in an overlap PCR with primers 314 and 315 to introduce a 500-bp deletion of cpsIaB. The 2200-bp overlap PCR product was digested with BamHI and PstI and ligated into BamHI/PstI-digested pJRS233.

To construct a cpsIaC deletion, primers 292 and 293 (Table I) were used to amplify a 1900-bp fragment containing the coding sequences for cpsIaC and 600 bp of upstream and downstream flanking DNA. The cpsIaC amplification product was ligated into pGEM-T, and an internal 200-bp fragment of cpsIaC was released by digesting with XbaI and EcoRV. The 5′ overhangs were filled in using the large fragment of DNA polymerase 1 (Klenow) to create blunt ends. The plasmid was then religated to create an internal cpsIaC deletion. The 1600-bp fragment containing the cpsIaC deletion and flanking sequences was released from pGEM-T by digestion with BamHI and KpnI and ligated into BamHI/KpnI-digested pJRS233.

An internal deletion of cpsIaD was constructed as described for cpsIaA and cpsIaB except that primer pairs 254 and 273 and 257 and 274 (Table I) were used to amplify the flanking PCR products, and primers 254 and 257 were used for the overlap extension reaction. The overlap amplicon was digested with SalI and BamHI and ligated into BamHI/KpnI-digested pJRS233.

For construction of an internal deletion of cpsIaE, primers 119 and 121 (Table I) were used to amplify a 1000-bp fragment containing the complete coding sequence of cpsIaE. The amplification product was digested with BgIII and EcoRI to remove all but the first 100 bp of cpsIaE coding sequence. This fragment was digested with BamHI and ligated into BamHI/EcoRI-digested pWKS30 to produce pWKS30-cpsIaE containing a BamHI site at the 5′-end of the insert and an EcoRI site at the 3′-end of the insert. Primers 190 and 191 (Table I) were used to amplify a 670-bp fragment containing the last 570 bp of cpsIaE and 100 bp of downstream flanking DNA. The amplification product of the 3′-end of cpsIaE was digested with EcoRI and HindIII and ligated into EcoRI/HindIII-digested pWKS30-cpsIaE to create a 360-bp internal deletion of cpsIaE. The fragment including the cpsIaE deletion and flanking DNA was released from pWKS30-cpsIaEΔ by digestion with HindIII and XbaI and was ligated into HindIII/XbaI-digested pJRS233.

Gene Replacement Mutagenesis in GBS Strain 515—Each of the cps deletion constructs in the temperature-sensitive shuttle vector pJRS233 was introduced into GBS strain 515 by electroporation, and transformants were selected by growth at 30 °C in the presence of erm. A single erm-resistant colony was used to inoculate a liquid culture supplemented with erm. After overnight incubation at 30 °C, the culture was diluted 10-fold with fresh broth containing erm and incubated at 37 °C to select organisms in which the recombinant plasmid had integrated in the cps locus of the GBS chromosome by homologous recombination (Fig. 2). Dilutions of each culture were plated on medium containing erm and incubated overnight; erm-resistant colonies representing plasmid integrants were serially passaged twice on solid medium at 37 °C. Intact strains were serially passaged at least five times in broth at 30 °C in the absence of erm; excision of the plasmid from the chromosome via a second recombination event either completed the allelic exchange or reconstituted the wild-type genotype (Fig. 2). erm-sensitive colonies were screened for the expected deletion mu-
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**TABLE I**

| Name | Sequence a | Description b/Orientation b | 5'-Terminal restriction endonuclease site |
|------|------------|----------------------------|----------------------------------------|
| 119  | TTGATGCCGATACTAGG | 708 bp upstream of cpsE start/F | None |
| 121  | AAGTGTCGATAGACC | 1245 bp downstream of cpsE start/R | None |
| 190  | CCCGAATTCAATGTAACTGAGGCCAC | 28 bp downstream of cpsE start/F | EcoRI |
| 191  | CCCCAAGATTACCTTTACTGCTGTCG | 1182 bp downstream of cpsE start/R | HindIII |
| 252  | GGAAGGATCCGACATCATAGAAGAAAGGTATG | 625 bp upstream of cpsC start/F | BamHI |
| 253  | GATGTTACCTTCTTTGTCATCCTGTCTTTTG | 1267 bp downstream of cpsC start/R | KpnI |
| 254  | GGAAGGATCCACATTGTTGACATCGTATGTCG | 622 bp upstream of cpsD start/R | BamHI |
| 257  | GATGTTACCTTAGCTGTAAGATATTTTCG | 1072 bp downstream of cpsD start/F | KpnI |
| 267  | GGCCT TGAGAGTTTTCCCTGTCCTCC | 3000 bp downstream of cpsA start/R | PstI |
| 273  | ACTAATCCAACAAGTTTTAGCGCAAGAAAGCATGGGAACAAAG | 736 bp upstream of cpsA start/R | HindIII |
| 275  | CTTAAGGCTTTGAGGATTGTTGCTG | 111 bp downstream of cpsA start/R | None |
| 277  | ATGTTGACGATACAAG | 1300 bp downstream of cpsA start/F | None |
| 278  | CTTATGTATCGTCAAGTGGATTTCTTCCCCT | 115 bp downstream of cpsB start/R | None |
| 280  | GAAACATTACGTAACACCATGTGTCG | 544 bp downstream of cpsB start/F | None |
| 284  | GCAAGGGTTGATGAAATATACATTGTTGATGATCTGATGATCTTCCGG | 997 bp upstream of cpsB start/F | BamHI |
| 314  | GGGCGCCGACGGAAAGATTCCTTATTAC | 1734 bp downstream of cpsB start/F | PstI |
| 315  | GGCCTGCAGGGGTTGATCCTGCAATTACC | 147 bp downstream of recA start/F | None |
| 333  | GCCCTCTAGCCTAGCTCCAA | 617 bp downstream of recA start/R | None |
| 334  | ACCACTCCCTGGATTGTTGATGATCTGATGATCTTCCGG | 2 bp downstream of cpsD start/F | None |
| 335  | GACTCGTTTAGAATAG | 841 bp downstream of cpsD start/R | None |
| 336  | CAATTTAGAGAACACATTAC | 5'-Terminal restriction endonuclease site | None |

*DNA sequences were derived from type Ia GBS capsule gene cluster (accession number AB028896). Where indicated, additional nucleotides were added to the 5'-terminus to create restriction endonuclease sites. Underlined sequences are complementary to noncontiguous sequences and were used to introduce internal deletions by overlap PCR.*

*a* F, forward (coding strand); R, reverse (noncoding strand).

Preparation of Mutanolysin Extracts for Measurement of Surface-expressed Capsular Polysaccharide—For measurement of cell-associated capsular polysaccharide produced by mutant strains, cells from a 100-ml overnight culture were washed in PBS and resuspended in 1 ml of protoplast buffer (30 mM NaHPO<sub>4</sub>, 40% sucrose (w/v), 10 mM MgCl<sub>2</sub>). Capsular polysaccharide was released from the cells by the addition of 1000 units of mutanolysin (Sigma). After 6 h of incubation at 37 °C, the protoplasts were removed by centrifugation at 3800 × g for 15 min. The amount of type Ia polysaccharide in the extract was determined using serial dilutions of the extract in a competition ELISA by comparison with a standard curve generated with purified type Ia capsular polysaccharide as described above (35). The ELISA procedure was performed as described (34).

Type Ia polysaccharide and group B carbohydrate were detected in chromatography column fractions by double diffusion in agarose using Ia-TT antiserum and anti-group B carbohydrate, respectively (35).
ment in the chromosome (from the chromosome of the integrant strain via duplicated GBS segregated strains at the permissive temperature. Excision of the plasmid incubating cells at the nonpermissive temperature under antibiotic was introduced into GBS type Ia strain 515 by electroporation. Inte-

allelic exchange. A recombinant plasmid of pJR8233 taining an internal deletion of the target cps gene and flanking DNA was introduced into GBS type Ia strain 515 by electroporation. Integration of the plasmid into the GBS chromosome was achieved by incubating cells at the nonpermissive temperature under antibiotic selection. Excisants were isolated by multiple passages of the integrated strains at the permissive temperature. Excision of the plasmid from the chromosome of the integrant strain via duplicated GBS sequence on one side of the deletion generated the desired allelic replace-

ment in the chromosome (Deletion Mutant); excision via duplicated sequence on the other side of the deletion regenerated the wild-type allele in the chromosome (Wild-type).

min. After dialysis, extracts were filtered sterile and lyophilized. Each sample was resuspended in 30 ml of 69 mM sodium borate, and the pH was raised to greater than 10 by the addition of 10 N NaOH. The polysaccharide was reacylated by the addition of 1.8 ml of acetic anhydride at room temperature; pH was maintained at 10 or higher by the addition of 1 N NaOH. The mixture was stirred for 2 h, neutralized with HCl, dialyzed, filtered sterile, and lyophilized. Samples were dissolved in 20 ml of enzyme treatment buffer (10 mM Tris, pH 7.4, 10 mM CaCl2, 10 mM MgCl2) and incubated with DNase (1 mg) and RNase (5 mg) overnight at 37 °C. Samples were incubated with Pronase (2 mg) at 37 °C overnight, dialyzed, and lyophilized. Polysaccharide samples were dissolved in water at a concentration of 25 mg/ml and purified by gel filtration chromatography on a 5 × 100-cm column of Sephacryl S-200 (Amersham Pharmacia Biotech) with PBS as eluant. Column fractions were screened for the presence of type Ia capsular polysaccharide and for group B carbohydrate by Ouchterlony immunodiffusion as described above. If necessary, capsular polysaccharides were further purified by anion exchange chromatography on a column of DEAE-Sepharose (Amersham Pharmacia Biotech) equilibrated with 10 mM Tris, pH 7.5, and eluted with a 0–0.4 M NaCl gradient. Fractions were assayed as above.

Carbohydrate Analysis—Samples were analyzed by high performance anion exchange chromatography using a gradient system (Dionex, Sunnyvale, CA) equipped with a pulsed amperometric detector (model PAD 2) and a pellicular anion exchange column (PA-1; 4 × 250 mm). The detector sensitivity was set at 300 nA with a 0.05 V applied pulse potential. Samples were hydrolyzed with 0.5 N trifluoroacetic acid at 100 °C for 18 h for analysis of neutral and amino sugars. After the removal of trifluoroacetic acid by repeated evaporation in water, hydrolyzed samples were applied through a microinjection valve with a 50-μl loop. Neutral sugars were eluted with 20 mM sodium hydroxide at a flow rate of 1 ml/min. For detection of sialic acid, samples were treated with 6% acetic acid at 80 °C for 2 h. After removal of acetic acid by repeated evaporation in water, samples were dissolved in water, applied to the column as above, and eluted with 90 mM sodium acetate and 100 mM sodium hydroxide.

NMR Analysis—Purified polysaccharide samples were dissolved in deuterium oxide at a concentration of 3 mg/ml and subjected to 1H NMR analysis on a Varian Unity 500 spectrometer (Varian Medical Systems, Palo Alto, CA) with a frequency of 500 MHz. Spectra generated by each sample at 60 °C were recorded with chemical shifts referenced relative to water resonance at 4.42 ppm. Water resonance was calibrated externally using diocetyl sodium sulfosuccinate as the standard.

Molecular Size Analysis of Capsular Polysaccharides—Purified capsular polysaccharide was analyzed by gel filtration FPLC on a column of Superose 12 HR 10/30 (Amersham Pharmacia Biotech) equilibrated in PBS. Samples were eluted in the same buffer at a flow rate of 0.5 ml/min with continuous monitoring of refractive index. Elution of capsular polysaccharide in fractions corresponding to the refractive index peaks was confirmed by competition ELISA. Molecular size of each polysaccharide was calculated on the basis of Kav by comparison with a calibration curve generated using dextran standards.

RNA Isolation and Analysis—Total cellular RNA was isolated from GBS grown in liquid culture to A540 = 0.4 using the RNeasy RNA isolation kit (Qiagen) according to the manufacturer’s recommendations except that GBS cells were lysed by shearing with glass beads on a shaker oscillator (36). Total RNA was eluted in 40 μl of RNase-free water and treated with Dnase I (Life Technologies) as recommended by the manufacturer. For RT-PCR, 20 ng of RNA was used for cDNA synthesis and subsequent PCR amplification using the Access RT-PCR kit (Promega) according to the manufacturer’s recommendations. The amplification product was fractioned on 0.7% agarose and stained with ethidium bromide, and the image was digitally captured using the Foto/Analyst Archiver (Fotodyne, Hartland, WI). RT-PCR amplification products were quantified by densitometric analysis using NIH Image version 1.62. Values were normalized as a ratio of target gene RNA to GBS recA RNA.

RESULTS

Construction and Characterization of GBS Mutant Strains—The first five genes of the GBS type Ia capsule synthesis locus (cpsIaABCDE) are proposed to be expressed as part of a single transcriptional unit (6). Accordingly, to investigate the function of these genes, we introduced a nonpolar deletion mutation in each open reading frame. This strategy permitted the assessment of each mutant phenotype independent of potential polar effects on expression of downstream genes. DNA sequences representing the 5′- and 3′-ends of the target gene plus flanking sequences were amplified by PCR and then fused to create an internal deletion encompassing most of the coding sequence of each gene (32, 33). Each internally deleted gene was cloned into the temperature-sensitive shuttle vector pJR8233 (26), and the recombinant plasmid was introduced into the GBS type Ia strain 515 by electroporation. Allelic exchange of the internally deleted gene for the chromosomal wild-type copy was achieved by a two-step process in which the plasmid carrying the mutant allele was first integrated into the cps operon by homologous recombination (Fig. 2). Plasmid excision from the chromosome via a second recombination event at the permissive temperature (30 °C) either completed the allelic exchange or reconstituted the wild-type genotype (Fig. 2). Erythromycin-sensitive colonies were screened by PCR with primers specific to the target gene to confirm the expected internal deletion. The genotype of each mutant was further confirmed by Southern hybridization analysis of HindIII-digested chromosomal DNA probed with the target gene. In addition, the digested chromosomal DNA was probed with pJR8233 to confirm that the plasmid had been lost from each mutant strain. The capsule phenotype that resulted from inactivation of each gene was determined by colony immunoblot analysis. Of the five mutant strains (designated 515-cpsIaΔΔ, 515-cpsIaΔB, etc.), only 515-cpsIaΔΔ was completely devoid of type Ia capsular polysaccharide by immunoblot analysis (Fig. 3). Previous studies have indicated that cpsIaΔE encodes a glycosyltransferase responsible for the addition of a monosaccharide to a lipid intermediate to initiate assembly of the oligosaccharide repeating unit (6, 37, 38). In type III GBS, inactivation of cpsD, a cpsIaΔE homolog, by a transposon insertion resulted in reduced galactosyltransferase activity, but no other apparent polar effects on downstream genes could have contributed to this phenotype (37).
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investigation by inactivation of cpsIAE is consistent with the proposed role of CpsIAE as the initiating enzyme in polysaccharide repeating unit biosynthesis. Unlike the cpsIAE deletion mutant, mutants harboring deletions in cpsIAA, cpsIAB, cpsIAC, or cpsIAD were all positive for cell-associated type Ia capsular polysaccharide by colony immunoblot analysis (Fig. 3). These results suggested that CpsA -D are not absolutely required for repeating unit synthesis but rather that they direct polymerization, export, and/or regulation. Therefore, a detailed examination was undertaken of the effects of each mutation on polysaccharide structure, quantity, cellular location, and chain length.

Analysis of 515-cpsIAAD—Colony immunoblot screening of 515-cpsIAAD was positive, a result that indicated production by the mutant strain of cell-associated polysaccharide recognized by type-specific antiserum (Fig. 3). The amount of polysaccharide produced by the mutant strain relative to that produced by the wild-type parent strain 515 was quantified using an ELISA inhibition assay; strain 515-cpsIAAD produced 24% as much cell-associated polysaccharide as wild-type strain 515 (Fig. 4). Assays of culture supernatants and of cell lysates showed no increase in polysaccharide released into the growth medium nor in intracellular polysaccharide in the mutant relative to the parent strain (data not shown). These results indicated that inactivation of CpsIA decreased cell surface expression of type Ia capsular polysaccharide but was not associated either with intracellular accumulation of polysaccharide or with increased release of polysaccharide from the cell surface.

The apparent reduction in cell-associated polysaccharide observed in 515-cpsIAAD might have been due to one or more different effects on polysaccharide expression such as an altered polysaccharide structure resulting in reduced reactivity with type-specific antiserum, production of fewer polysaccharide molecules at the cell surface, or reduced polysaccharide chain length. To distinguish among these possibilities, capsular polysaccharide was purified from a large scale culture of 515-cpsIAAD grown under controlled conditions. Polysaccharide was extracted from 515-cpsIAAD (or from wild-type strain 515 for comparison) and purified by sequential enzymatic treatment followed by gel filtration chromatography. Component sugar analysis of the purified polysaccharide by high performance anion exchange chromatography with a pulsed amperometric detector demonstrated the presence of N-acetylglucosamine, galactose, glucose, and N-acetylmuramic acid (sialic acid) in a molar ratio of 0.9:2.0:1.4:0.7, an analysis nearly identical to that of polysaccharide purified from parent strain 515 and consistent with the previously reported structure of the type Ia GBS polysaccharide (Fig. 4) (8).

The 1H NMR spectra of the purified 515-cpsIAAD polysaccharide and the parent strain 515 polysaccharide exhibited resonance peaks with identical chemical shifts for each of four anomic protons between 4.5 and 5.1 ppm, evidence that the composition and linkage pattern of the hexoses are the same (Fig. 4). Sialic acid was similarly detected in both samples as evidenced by its characteristic H-3 signals at 1.78 (due to the axial proton) and 2.75 ppm (due to the equatorial proton). Both the 515-cpsIAAD polysaccharide and the parent strain 515 polysaccharide were fully N-acetylated, as indicated by the relative intensity of the signal at 2.04 ppm. The cluster of signals between 3.3 and 4.2 ppm arising from the nonameric sugar ring protons showed a virtually identical pattern in the two spectra except for minor discrepancies attributable to small amounts of impurities in the samples. Thus, results of both component sugar analysis and 1H NMR spectroscopy provided strong evidence that the repeating unit structure of the 515-cpsIAAD polysaccharide is identical to that of parent strain 515. Comparison of NMR spectra of these samples with that of polysaccharide from strain 090, the prototype strain from which the type Ia polysaccharide structure was determined, indicated identical repeating unit structures for the three polysaccharides (8).

To determine whether the apparent reduction in capsule production by 515-cpsIAAD might be due to altered polysaccharide chain length, the molecular size of the purified mutant polysaccharide was assessed by gel filtration FPLC. The 515-cpsIAAD polysaccharide eluted with a $K_v$ of 0.20 compared with a $K_v$ of 0.23 for polysaccharide purified from parent strain 515 (Fig. 5). These $K_v$ values correspond to $M_v$ values of 100,000 and 90,000, respectively, using a calibration curve constructed with dextran standards. Results of this analysis indicated that inactivation of CpsIAA had no significant effect on polysaccharide chain length. Since repeating unit structure and chain length of the 515-cpsIAAD polysaccharide were both essentially identical to the wild-type polysaccharide, it appeared that inactivation of CpsIAA resulted in expression of fewer molecules of type Ia polysaccharide at the cell surface relative to the wild-type strain.

CpsIAA has 44% similarity to LytR, a protein in Bacillus subtilis that is thought to act as a transcriptional regulator and has been suggested to perform the same role in GBS capsule expression (40). Disruption of lytR resulted in a 4-fold increase in transcription of lytABC, supporting a role for LytR as an attenuator of lyt operon transcription (20). A possible function of CpsIAA as a regulator of GBS capsule gene transcription was investigated by measuring transcription of the cps operon in 515-cpsIAAD and in wild-type strain 515. RT-PCR using primers for cpsIAD revealed 2-3-fold more transcripts in strain 515 than in 515-cpsIAAD (Fig. 6). Reduced transcription of the cps operon in 515-cpsIAAD suggests that CpsIAA regulates cps gene expression, but, in contrast to LytR, CpsIAA appears to function as an activator of target gene transcription. The reduced amount of capsular polysaccharide produced by 515-cpsIAAD is an expected consequence of reduced cps operon expression.

Analysis of 515-cpsIBAD—By immunoblot analysis, 515-cpsIBAD appeared to be positive for production of immunoreactive type Ia polysaccharide by comparison with a control type III strain or with the capsule-negative mutant 515-cpsIAE (Fig. 3). However, the signal from 515-cpsIBAD was weaker than that from wild-type strain 515 or from 515-cpsIAAD, 515-cpsICD, or 515-cpsIAAD. Quantitative ELISA inhibition analysis on capsular polysaccharide extracts demonstrated that 515-cpsIBAD produced ~3% of the quantity of cell-associated type Ia polysaccharide produced by parent strain 515 (Fig. 4). No intracellular accumulation of polysaccharide was detected in 515-cpsIBAD; nor was polysaccharide detected in culture supernatants (data not shown). The very small quantity of polysaccharide produced by 515-cpsIBAD precluded further analysis of the mutant polysaccharide.
In experiments like those described above for \(515\)-cpsIaA\(\Delta\), we assessed whether inactivation of CpsIaB affected transcription of the \(cps\) operon. Using \(cpsIaD\) as a target, we found no reduction in \(cps\) gene transcripts by RT-PCR (Fig. 6B). This result provides evidence that the reduced production of capsular polysaccharide in \(515\)-cpsIaB\(\Delta\) was not attributable to an effect on \(cps\) gene expression.

**Fig. 4.** Analysis of capsular polysaccharide produced by \(cps\) deletion mutants. Surface capsular polysaccharide expressed in each of the mutants was determined by ELISA competition and expressed as a percentage of the amount of capsular polysaccharide produced by wild-type strain 515. Purified capsular polysaccharide was analyzed by both Dionex high performance anion exchange chromatography and \(^1\)H NMR. *, insufficient material for analysis.

| Strain       | Polysaccharide Quantity | Sugar Analysis [GlcNAc:Gal:Glc:NeuNAc] | \(^1\)H-NMR Spectrum |
|--------------|-------------------------|----------------------------------------|----------------------|
| 515 (wild-type) | 100%                    | 0.9:2.0:0.9:0.6                        |                     |
| 515-cpsIaA\(\Delta\) | 24%                     | 0.9:2.0:1.4:0.7                        |                     |
| 515-cpsIaB\(\Delta\) | 3%                      | *                                      | *                   |
| 515-cpsIaC\(\Delta\) | 6%                      | 0.9:2.0:0.8:0.6                        |                     |
| 515-cpsIaD\(\Delta\) | 9%                      | 1.0:2.0:1.0:0.6                        |                     |
| 515-cpsIaE\(\Delta\) | None detected           | *                                      | *                   |

In experiments like those described above for \(515\)-cpsIaA\(\Delta\), we assessed whether inactivation of CpsIaB affected transcription of the \(cps\) operon. Using \(cpsIaD\) as a target, we found no reduction in \(cps\) gene transcripts by RT-PCR (Fig. 6B). This result provides evidence that the reduced production of capsular polysaccharide in \(515\)-cpsIaB\(\Delta\) was not attributable to an effect on \(cps\) gene expression.

Analysis of \(515\)-cpsIaC\(\Delta\) and \(515\)-cpsIaD\(\Delta\)—Immunoblot analysis indicated that both \(515\)-cpsIaC\(\Delta\) and \(515\)-cpsIaD\(\Delta\) produced immunoreactive capsular polysaccharide (Fig. 3). Quantitative analysis of cell-associated capsule by ELISA inhibition indicated that \(515\)-cpsIaC\(\Delta\) and \(515\)-cpsIaD\(\Delta\) produced 6 and 9%, respectively, of the amount produced by parent strain 515. Capsular polysaccharide was purified from large scale cultures of both \(515\)-cpsIaC\(\Delta\) and \(515\)-cpsIaD\(\Delta\). Component sugar analysis and \(^1\)H-NMR of the purified polysaccharides indicated that the repeating unit structure of each was identical to that of the type Ia parent strain 515 (Fig. 4).

Several reports have noted the similarity of CpsC and CpsD in GBS and in other streptococci to proteins implicated in the polymerization and export of polysaccharides in Gram-negative bacteria, including ExoP of \(R.\ meliloti\) and the Wza and Wzc proteins of enterobacteriaecae (4, 6, 16, 23). Since these proteins in Gram-negative bacteria appear to affect polysaccharide chain length, we investigated whether inactivation of CpsIaC or CpsIaD affected the molecular size of the GBS type Ia polysaccharide. Analysis of the polysaccharides purified from \(515\)-cpsIaC\(\Delta\) and \(515\)-cpsIaD\(\Delta\) by gel filtration FPLC revealed that both were reduced in size compared with polysaccharide from parent strain 515. The \(515\)-cpsIaC\(\Delta\) polysaccharide had a \(K_\text{av}\) of 0.33 (\(M_r\) of 41,000), and the \(515\)-cpsIaD\(\Delta\) polysaccharide had a \(K_\text{av}\) of 0.32 (\(M_r\) of 45,000), compared with a \(K_\text{av}\) of 0.23 (\(M_r\) of 90,000) for 515 polysaccharide (Fig. 5). In addition, each of the two mutant polysaccharides eluted in a narrower peak than did the wild-type, a result that implies a more restricted range of molecular sizes among the polysaccharide chains produced by the mutants compared with polysaccharide from parent strain 515. The \(515\)-cpsIaC\(\Delta\) polysaccharide had a \(K_\text{av}\) of 0.33 (\(M_r\) of 41,000), and the \(515\)-cpsIaD\(\Delta\) polysaccharide had a \(K_\text{av}\) of 0.32 (\(M_r\) of 45,000), compared with a \(K_\text{av}\) of 0.23 (\(M_r\) of 90,000) for 515 polysaccharide (Fig. 5). In addition, each of the two mutant polysaccharides eluted in a narrower peak than did the wild-type, a result that implies a more restricted range of molecular sizes among the polysaccharide chains produced by the mutants compared with those of the wild-type or \(515\)-cpsIaA\(\Delta\). Since the average chain length of polysaccharide produced by \(515\)-cpsIaC\(\Delta\) and \(515\)-cpsIaD\(\Delta\) is about one-half of that produced by the wild-type, it seems unlikely that reduced chain length alone accounts for the >90% reduction in cell-associated polysaccharide measured by immunoassay. Thus, inactivation of either CpsIaC or CpsIaD results in reduced polysaccharide chain length and, probably, a reduction in the number of polysaccharide molecules expressed on the bacterial surface.
capsIaC gene, a ratio to the amount of product amplified using primers for the control gene, recA. B, ratio of cpsIaD RT-PCR product to recA product of each cps deletion mutant is expressed as a percentage of the ratio for wild-type strain 515. Results shown represent mean values of three experiments; error bars denote S.D.

**Fig. 6. Effect of deletion mutations in cps genes on cps operon gene expression.** PCR was performed using primers for cpsIaD after reverse transcription of cellular RNA prepared from wild-type strain 515 or isogenic mutant strains. A, the amount of product amplified from strain 515 or from 515-cpsIaΔ using primers for cpsIaD is expressed as a ratio to the amount of product amplified using primers for the control gene, recA. B, ratio of cpsIaD RT-PCR product to recA product of each cps deletion mutant is expressed as a percentage of the ratio for wild-type strain 515. Results shown represent mean values of three experiments; error bars denote S.D.

**Lack of Effect of CpsIaC or CpsIaD on cps Gene Transcription**—To investigate whether the decrease in capsular polysaccharide production by 515-cpsIaCΔ and 515-cpsIaDΔ was due to an effect on cps gene transcription, we assessed the abundance of cps gene transcripts by RT-PCR in the two mutant strains using primers specific for cpsIaD. In contrast to the decrease in cps transcripts detected in 515-cpsIaΔ, in 515-cpsIaCΔ and 515-cpsIaDΔ we observed similar levels of cps gene transcription as in wild-type strain 515 (Fig. 6B). Therefore, the reduced production of capsular polysaccharide in 515-cpsIaCΔ and 515-cpsIaDΔ was not attributable to effects of either mutation on cps gene expression.

**DISCUSSION**

In this paper, we describe the effects in type Ia GBS of nonpolar, inactivating mutations in each of five genes that are highly conserved among polysaccharide-producing streptococci. Of the five genes examined, only a disruption of cpsIaE resulted in an acapsular phenotype. The cpsIaEΔ phenotype is consistent with the proposed role of CpsE as a glycosyltransferase responsible for attachment of the first sugar to a lipid carrier to initiate repeating unit synthesis (6, 37, 38). The striking conservation of cpsIa-E among GBS of different serotypes and among other species of streptococci has suggested that the products of these genes have functions that are independent of the repeating unit structure of the associated polysaccharide. Southern analysis using gene-specific probes from type Ia GBS confirmed the presence of all five genes in GBS strains of types Ia, Ib, II, III, IV, V, VI, and VII. cpa to -D, but not cpsE, were detected also in GBS type VIII (data not shown). Like the other known GBS capsule types, the repeating unit structure of the type VIII polysaccharide includes glucose, galactose, and sialic acid; however, type VIII differs in the presence of rhamnose as part the repeating unit (11). The apparent absence of a gene closely related to cpaIaE in type VIII GBS suggests the possibility that a unique enzyme, perhaps with rhamnose as a substrate, initiates repeating unit synthesis in this serotype.

CpsA is similar to LytR, which is suggested to down-regulate transcription of the lyt operon. On the basis of evidence from the present study, CpsIA regulates cpaIa gene expression, but in contrast to LytR, CpsIA acts as an activator of capsule gene transcription. A mutant in cpaIaA produced type Ia capsular polysaccharide of similar M₈ as the wild-type, but in reduced amounts, and inactivation of CpsIAA was associated with reduced expression of the type Ia capsule gene cluster. Several features differentiate LytR from CpsIAA and its homologs. Unlike LytR, the N-terminal portion of CpsIAA includes predicted membrane-spanning domains. Unlike lytR, cpaIaA is transcribed as part of a polycistronic message along with other genes of the type Ia capsule gene cluster, whereas transcription of lytR in B. subtilis is divergent from that of the operon it regulates. In addition, phosphohydrophobic regions found in CpsIAA and its homologs in other streptococci do not correspond to homologous regions in LytR (20). These or other structural differences between the two proteins may account for the observation that CpsIAA appears to act as an activator while LytR attenuates gene expression.

A definitive role of CpsIB in capsule expression has yet to be determined. In S. pneumoniae, inactivation of Cps19fB was associated with increased tyrosine phosphorylation of a 25-kDa protein tentatively identified as Cps19fD. This result suggested the possibility that Cps19fB functions as a phosphotyrosine-protein phosphatase that regulates capsule expression through its effect on the degree of phosphorylation of Cps19fD. In the present study, a GBS mutant in cpaIaB expressed low levels of cell-associated capsular polysaccharide. Whether this decrease in capsule expression is due to a reduction in number of polysaccharide molecules on the cell surface or reduced polysaccharide chain length or both was not determined because of the very small amount of capsular polysaccharide produced by the mutant strain. Inactivation of CpsIB had no effect on expression of the cps gene cluster, so it seems unlikely that CpsIB acts as a transcriptional regulator of capsule gene expression. The presence of CpsB in capsule biosynthetic loci of other species and the fact that inactivation of CpsB has a profound effect on capsule production suggest that it serves a
critical function in transport or polymerization, but the exact nature of that function remains uncertain. It seems unlikely that the sole function of CpsB in GBS or *S. pneumoniae* is to regulate phosphorylation of CpsD, since the effect on capsule production associated with inactivation of CpsIA is more pronounced than that of inactivating CpsIA.

CpsIA and CpsID are similar to amino- and C-terminal regions, respectively, of ExoP, a protein involved in *R. meliloti* exopolysaccharide biosynthesis (22). The amino-terminal portion of ExoP contains predicted membrane-spanning domains and is similar to proteins involved in polysaccharide polymerization or export (22). The C-terminal portion of the protein includes a putative ATP-binding domain (22). Although the similarity between CpsIA and the N-terminal region of ExoP is quite low (<20%), CpsIA does contain predicted membrane-spanning domains similar to ExoP. Disruption of *cpsB* in *S. suis* serotype 2 (73% similarity to *cpsIA*) produced a capsule-negative phenotype by slide agglutination assay (5); however, the acapsular phenotype in the *S. suis* *cpsB* mutant may have been due to polar effects on downstream transcription caused by the insertion mutation. Morona *et al.* (23) showed that deletion mutants in *S. pneumoniae* *cps19C* and *cps19D* had a similar phenotype to the GBS *cpsIaC* and *cpsIaD* mutants characterized in our study insofar as the *S. pneumoniae* mutants also were positive for immunoreactive, cell-associated *S. pneumoniae* characterized in our study insofar as the absence of intracellular polysaccharide. The precise roles of ATP and is similar to proteins involved in polysaccharide polymerization and is similar to proteins involved in polysaccharide polymerization or export (22). 

Results of the present investigation provide evidence that each of the proteins encoded by the conserved genes of the streptococcal polysaccharide gene cluster plays a critical role in the regulation, processing, and/or export of extracellular polysaccharide. Further characterization of the molecular details of the polysaccharide polymerization and export complex will provide a better understanding of an important aspect of the physiology of polysaccharide-producing streptococci and may define novel targets for directed drug design against encapsulated Gram-positive pathogens.

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