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Molecular Analysis of a Region of the Group B Streptococcus Chromosome Involved in Type III Capsule Expression

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Type III group B streptococci (GBS) are the most common cause of neonatal sepsis and meningitis in the United States. The important role of the type III polysaccharide capsule and of the terminal sialic acid moiety of the capsule in the virulence of GBS has been demonstrated by using Tn*916* mutagenesis. Several of the transposon insertion sites that resulted in defective type III capsule synthesis were located in a 30-kilobase (kb) region of the chromosome. Hybridization analysis of two other type III strains that differed in their relative virulence and of GBS serotypes Ia, Ib, Ic, and II showed that this region of the chromosome was highly conserved. A repetitive 1.4-kb sequence was found only in the 30-kb region of the more virulent type III strain, COH 1. The *Escherichia coli* maxicell in vivo expression system and an in vitro coupled transcription-translation system successfully identified the proteins expressed from the 30-kb region. Comparison of the proteins expressed from the same DNA fragments in these two assays indicated that some of these proteins may contain leader sequences that would ultimately result in their secretion to the cell surface. Identification and further characterization of the genes and their products will provide the foundation for understanding the genetic and biochemical events in GBS capsular polysaccharide production.

Group B streptococci (GBS) are a major cause of bacterial sepsis and meningitis in newborn infants (2). Capsular type III GBS is the predominant serotype isolated from infants with early-onset meningitis or with late infections. The type III capsule is a high-molecular-weight polymer containing galactose, glucose, *N*-acetylglucosamine, and *N*-acetylneuraminic acid (sialic acid) moieties. Removal of the terminal side-chain sialic acid from the native form leaves a core polysaccharide that is identical in structure to the capsular polysaccharide of type XIV *Streptococcus pneumoniae* (18).

The role of the type III capsular polysaccharide in the virulence of GBS has been demonstrated by Rubens et al. (13), using Tn*916* insertional mutagenesis. Insertion of Tn*916* into the chromosome of a type III GBS clinical strain, COH 31rs, generated several isogenic mutants lacking capsular polysaccharide. One of these mutants, COH 31-15, was avirulent when compared with the wild-type strain in a neonatal rat sepsis model.

In COH 31-15, a single Tn*916* insertion in a 3.0-kilobase (kb) *Eco*RI chromosomal fragment ablated capsule expression. This fragment was cloned and, when used as a probe in Southern hybridization analysis, was found to be homologous to a similar-sized fragment in all five GBS serotypes. These data suggested that the genes encoded by this fragment may be part of a common pathway for capsular biosynthesis in all the GBS serotypes.

The importance of the terminal sialic acid side-chain moiety of type III capsular polysaccharide to the virulence of GBS has also been demonstrated by Tn*916* mutagenesis of COH 31rs. A single GBS type III mutant, COH 31-21, that contained two Tn*916* inserts in the chromosome was recently derived. This mutant produced a capsule missing the terminal sialic acid side chain and was also avirulent compared with the wild-type strain (M. R. Wessels et al., submitted for publication). These data confirmed previous reports that suggested that sialylation of the capsular polysaccharide of GBS was an important virulence factor (14).

The current report describes the mapping of a region of the GBS chromosome involved in type III capsule expression. The locations of the transposon insertion sites in the chromosomes of the COH 31rs capsule mutants are described. The DNA fragments from the capsule gene region have been cloned from a genomic library of a wild-type strain, and the proteins expressed from these fragments have been identified.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1, and the plasmids used are listed in Table 2.

Cosmid libraries. Genomic cosmid libraries of GBS type III strains were generated by using total cellular DNA from GBS strains isolated by a modification of the procedure of Hull et al. (8) in which 50 μg of mutanolysin per ml was used in place of lysozyme. Chromosomal DNA, partially digested with *Sau*3A, was ligated to *Bam*HI-digested cosmid vector pLH79 (pHC79 cosmid vector [7] with the *tet* gene removed). Ligation mixtures were packaged in lambda bacteriophage heads in vitro by using the GigaPak packaging kit (Stratagene Cloning Systems, La Jolla, Calif.) After transfection of *Escherichia coli* DH1, cosmid clones were selected on medium containing 25 μg of ampicillin per ml for wild-type libraries and 25 μg of ampicillin per ml and 4 μg of tetracycline per ml for mutant libraries to select for clones containing the Tn*916* transposon.

Colony blots and preparation of ^{32}P-labeled probes. After overnight incubation on selective agar plates, *E. coli* transformants were lifted onto nitrocellulose filters. The colonies were lysed and fixed onto the nitrocellulose by sequential placement of the filters for 5 min each onto Whatman no. 1 filter papers soaked with (i) 1.5 M NaCl–0.5 M NaOH, (ii) 1.5 M NaCl–0.5 M Tris (pH 7.5), (iii) 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (9), and (iv) 70% ethanol.

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TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype and phenotype	Source
S. agalactiae		
COH 31rs	Rif^r Str^r type III	Clinical isolate (13)
COH 31-6	31rs Tn*916* Tet^r	Acapsular COH 31rs mutant
COH 31-8	31rs Tn*916* Tet^r	Acapsular COH 31rs mutant
COH 31-9	31rs Tn*916* Tet^r	Acapsular COH 31rs mutant
COH 31-12	31rs Tn*916* Tet^r	Acapsular COH 31rs mutant
COH 31-15	31rs Tn*916* Tet^r	Acapsular COH 31rs mutant (13)
COH 31-17	31rs Tn*916* Tet^r	Acapsular COH 31rs mutant
COH 31-20	31rs Tn*916* Tet^r	Acapsular COH 31rs mutant
COH 31-21	31rs Tn*916* Tet^r	Desialylated COH 31rs mutant
COH 31-22	31rs Tn*916* Tet^r	Acapsular COH 31rs mutant
COH 1	Type III	Clinical isolate
COH 28	Type II	Clinical isolate
COH 46	Type Ia	Clinical isolate
COH 50	Type Ib	Clinical isolate
COH 8	Type Ic	Clinical isolate
M732	Type III	D. Kasper
D136C	Type III	R. Lancefield
E. coli DH1	F$^-$ *recA1 endA1 gyrA96 thi-1 hsdR17 supE44*	D. Clewell

The filters were then baked for 30 min at 80°C in a vacuum-drying oven. Fragments of DNA were isolated from agarose gels with Gene Clean (Bio 101, La Jolla, Calif.) and labeled with ^{32}P-deoxynucleotides in vitro by using a random hexanucleotide labeling kit (Pharmacia-LKB Biotechnology, Uppsala, Sweden). Labeled DNA probes were hybridized to the colonies on the filters as described by Maniatis et al. (9).

Cloning and DNA-DNA hybridization. GBS DNA fragments were isolated and subcloned from *E. coli* cosmid clones by purification of cosmids, electrophoresis of restriction endonuclease-digested cosmids on agarose gels, excision of fragments with Gene Clean, ligation to restriction endonuclease-digested pBS vector DNA (Stratagene Cloning Systems), and transformation into *E. coli* DH1 (9). Southern transfer of DNA fragments to nitrocellulose and DNA-DNA hybridization have also been described (9).

Identification of gene products by maxicell analysis. The *E. coli* maxicell in vivo system (17) was used to analyze protein products encoded by cloned genes. *E. coli* cells harboring plasmids that contained GBS DNA fragments were irradiated with UV light and then incubated in the presence of [^{35}S]methionine to label plasmid-encoded proteins. Radiolabeled protein products were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by autoradiography.

TABLE 2. Plasmids used in this study

Plasmid	Relevant genotype and phenotype	Source or reference
pLH79	Amp^r cosmid (5.0 kb)	C. E. Rubens and L. H. Heggen
pBS	Amp^r *lacZ* (2.7 kb)	Stratagene Cloning Systems
pCER3115-1	Amp^r (5.4 kb)	13
pCER212-1	Amp^r (3.7 kb)	This study
pCER3121-1	Amp^r (10.2 kb)	This study
pCER109	Amp^r (6.0 kb)	This study
pCER108	Amp^r (6.3 kb)	This study
pCER107	Amp^r (5.7 kb)	This study
pCER106	Amp^r (6.2 kb)	This study
pCER101	Amp^r (7.3 kb)	This study
pCER105	Amp^r (5.2 kb)	This study
pCER111	Amp^r (4.7 kb)	This study
pCER112	Amp^r (3.2 kb)	This study

In vitro coupled transcription-translation system. A modified Zubay method (11) for coupled transcription-translation in a procaryotic cell-free system was used to synthesize proteins from recombinant pBS plasmids containing GBS DNA fragments. The plasmids were incubated with a crude extract from *E. coli* supplemented with amino acids, an energy-regenerating system, cofactors, and [^{35}S]methionine. Radiolabeled protein products were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by autoradiography.

RESULTS

Mapping of the type III capsule genes. A genomic cosmid library of COH 1, a virulent type III GBS clinical strain, was generated and used as a source for unmutagenized fragments of GBS chromosomal DNA. COH 1 was chosen for mapping studies because it produces more capsule than COH 31rs and is more virulent in the infant rat model, therefore being more typical of invasive GBS strains isolated from infected infants than is COH 31rs. The 50% lethal dose of COH 1 was 10^2 CFU/g of animal (10), versus 5×10^5 CFU/g of animal for COH 31rs (13).

The 3.0-kb *Eco*RI fragment that flanked the single transposon insertion site in the chromosome of COH 31-15, resulting in an acapsular phenotype, was previously subcloned into *E. coli*, and the recombinant plasmid was designated pCER3115-1 (13). The 3.0-kb fragment was labeled with ^{32}P and hybridized to *E. coli* cosmid clones containing COH 1 DNA. Cosmid DNA from reactive clones was purified and mapped by using restriction endonuclease analysis. GBS DNA fragments from these cosmids were subcloned and used as probes for chromosome-walking studies (1) in Southern hybridizations with restriction endonuclease-digested whole-cell DNA from GBS strains COH 1 and COH 31rs. These studies confirmed the arrangement of the 30-kb putative capsule regions shown in Fig. 1.

These studies also demonstrated two differences in this genomic region between COH 31rs and COH 1. The first difference was that a restriction fragment length polymorphism was observed (Fig. 1, asterisk). An *Eco*RI site that cut

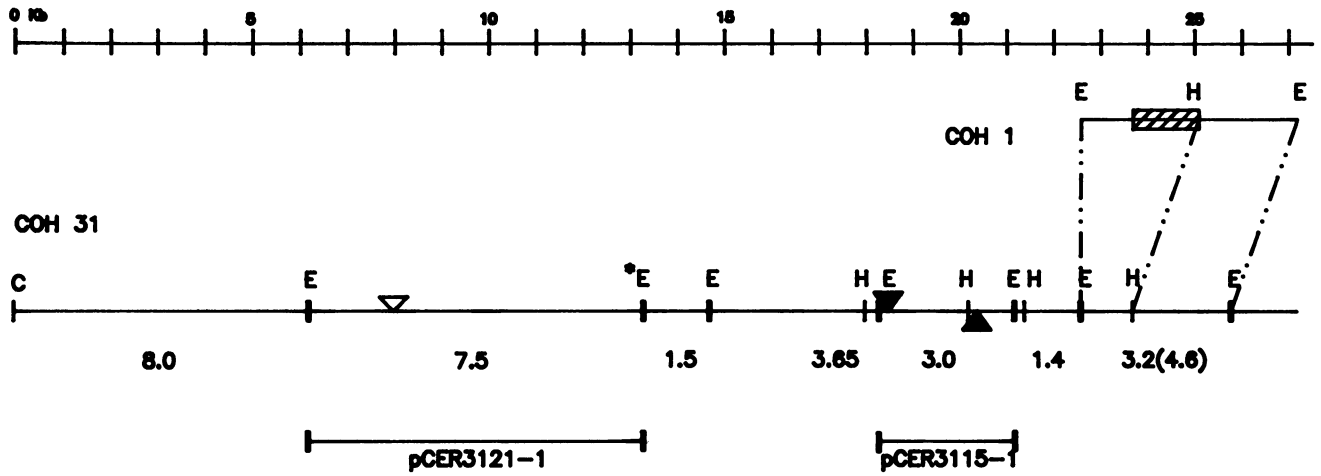

FIG. 1. Chromosomal map of type III capsule gene region. Abbreviations: C, *Cla*I; E, *Eco*RI; H, *Hin*dIII. Not all of the *Hin*dIII sites are shown. Numbers below the map indicate distances in kilobases between *Eco*RI sites in COH 31rs DNA. The number in parentheses is the size of the fragment in COH 1 DNA. Differences in this genomic region between COH 31rs and COH 1 were observed. An *Eco*RI site was not found in COH 1 (*). The 1.4-kb insertion sequence IS*861* was found in COH 1 but not in COH 31rs (▨). One of the two Tn*916* insertion sites in COH 31-21 (▽), the Tn*916* insertion site in COH 31-15 (▼), and the Tn*916* insertion site in COH 31-6 (▲) are indicated. The *Eco*RI fragments that flanked the transposons in COH 31rs that were subcloned into pBS are shown below the map.

the COH 31rs chromosome into adjacent 7.5- and 1.5-kb *Eco*RI fragments was missing in the COH 1 chromosome; only a single 9.0-kb fragment was found in COH 1. The second difference was that a 4.6-kb *Eco*RI fragment in COH 1 shared homology with a smaller (3.2-kb) fragment in COH 31rs. A 1.4-kb sequence within the COH 1 4.6-kb *Eco*RI fragment did not hybridize to the COH 31rs DNA (Fig. 1).

Mapping of the Tn*916* insertion sites in the COH 31rs mutants. The 3.0-kb *Eco*RI fragment from the COH 31-15 acapsular mutant was used as a probe against *Eco*RI-digested whole-cell DNA from nine COH 31rs capsule-negative mutants. The probe hybridized to a 19.4-kb *Eco*RI fragment in COH 31-15 (Fig. 2A, lane 2) and in six other acapsular mutants. In the remaining two mutants (Fig. 2A,

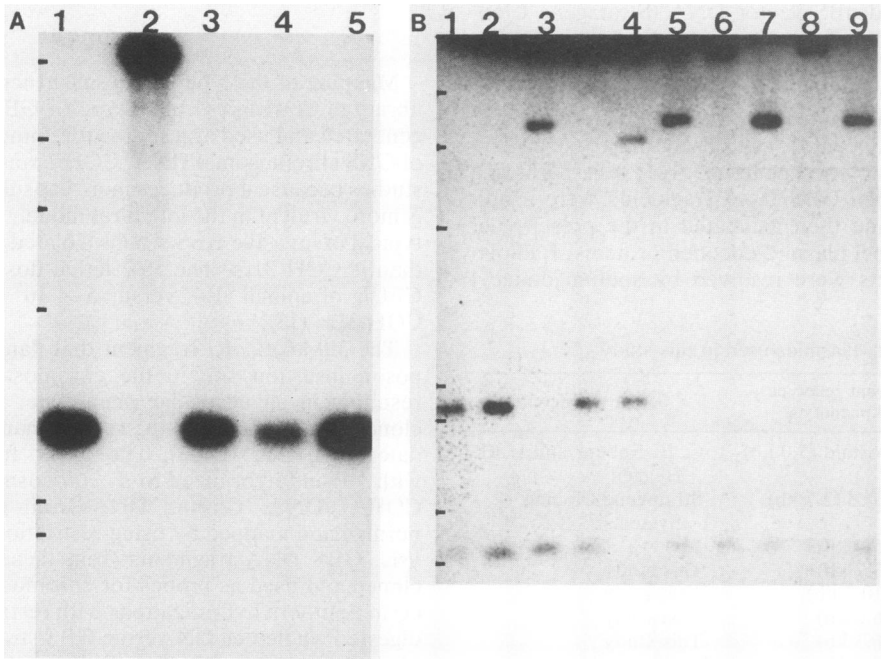

FIG. 2. (A) Southern hybridization analysis of whole-cell DNA from COH 1, COH 31rs, and mutants of COH 31rs digested with *Eco*RI and probed with a ^{32}P-labeled 3.0-kb *Eco*RI DNA fragment from COH 31-15 that flanked Tn*916*. Lanes: 1, COH 31rs; 2, COH 31-15; 3, COH 31-9; 4, COH 31-20; 5, COH 1. The bars to the left of the figure indicate size standards (23, 9.4, 6.7, 4.4, 2.3, and 2.0 kb from the top). (B) Whole-cell DNA digested with *Hin*dIII and probed with the same probe as in panel A. Lanes: 1, COH 1; 2, COH 31rs; 3, COH 31-8; 4, COH 31-6; 5, COH 31-12; 6, COH 31-15; 7, COH 31-17; 8, COH 31-19; 9, COH 31-22. Size standards are 23, 9.4, 6.7, 4.4, 2.3, 2.0, 1.35, and 1.08 kb from the top.

lanes 3 and 4), only a 3.0-kb *Eco*RI fragment hybridized to the probe, indicating that the transposon had not inserted into that fragment. Hybridization studies in which the *Eco*RI fragments adjacent to the 3.0-kb fragment were used as probes showed that the transposon was not inserted within 3.65 kb to the left or 1.4 kb to the right of the 3.0-kb fragment.

Whole-cell DNA from two wild-type strains and from the seven mutants that contained Tn*916* in the 3.0-kb *Eco*RI fragment was digested with *Hin*dIII and analyzed by Southern hybridization analysis, again with the 3.0-kb fragment from COH 31-15 as a probe (Fig. 2B). The 3.0-kb *Eco*RI fragment hybridized to two *Hin*dIII fragments, of 2.2 and 1.2 kb, in COH 31rs (lane 1) and COH 1 (lane 2). In six of the mutants, the 3.0-kb *Eco*RI fragment hybridized to the 1.2-kb *Hin*dIII fragment and to another fragment larger than 2.2 kb (lanes 3 and 5 through 9). Therefore, Tn*916* inserted into the 2.2-kb *Hin*dIII fragment that overlaps the left-hand side of the 3.0-kb *Eco*RI fragment. The transposon was located in a 1.9-kb DNA fragment with an *Eco*RI site on the left and a *Hin*dIII site on the right (Fig. 1). Although Tn*916* is cleaved by *Hin*dIII into two fragments of 5.3 and 10.9 kb, the probe hybridized to only one larger fragment. The transposon inserted very close to the *Eco*RI site on the left end of the 1.9-kb *Eco*RI-*Hin*dIII fragment, and the probe could hybridize to only one of the two fragments containing Tn*916* DNA. The transposon appears to have inserted in the same location but in different orientations in these six mutants. In lanes 3, 5, 7, and 9, the probe hybridized to a 7.2-kb fragment containing the smaller piece of Tn*916*, and in lanes 6 and 8, the probe hybridized to an 18.1-kb fragment containing the larger piece of Tn*916*. In one mutant, COH 31-6, the 3.0-kb *Eco*RI fragment hybridized to the 2.2-kb *Hin*dIII fragment and to a 6.3-kb *Hin*dIII fragment (lane 4). Therefore, Tn*916* inserted into the 1.2-kb *Hin*dIII fragment that overlaps the right-hand side of the 3.0-kb *Eco*RI fragment (Fig. 1).

To determine the sites of the Tn*916* insertions in the desialylated mutant, COH 31-21, we generated a genomic cosmid library. Cosmid clones were selected on medium containing ampicillin and tetracycline to isolate cosmids with GBS DNA containing the transposon. Tn*916* is unstable in *E. coli* and will excise and segregate unless the strain is maintained on tetracycline-containing medium (5). The DNA flanking the transposon in each cosmid containing Tn*916* was obtained by growing the clones with and without tetracycline. Figure 3 shows the *Eco*RI digests of plasmid DNA from a tetracycline-resistant COH 31-21 cosmid clone grown with (lane C) and without (lane D) tetracycline. The arrow designates the 7.5-kb *Eco*RI fragment that flanked Tn*916*. COH 31-21 contained two Tn*916* inserts in the chromosome. A 1.0-kb *Eco*RI fragment flanked the other Tn*916* insertion site (data not shown). The two *Eco*RI fragments that flanked each of the two transposon insertions were purified from agarose and subcloned into the *Eco*RI site of the pBS vector. The recombinant plasmids, designated pCER212-1 (containing the 1.0-kb fragment) and pCER3121-1 (containing the 7.5-kb fragment), were used for restriction endonuclease mapping and DNA probes.

The Tn*916* insertion site in the 7.5-kb *Eco*RI fragment of COH 31-21 mapped only 10.5 kb to the left of the transposon insertion site in COH 31-15 (Fig. 1). The 1.0-kb flanking DNA from the second Tn*916* insertion site in COH 31-21 was not located within the mapped region.

Hybridization of type III GBS chromosomal fragments to DNA from other GBS serotypes. Several *Eco*RI fragments from COH 1 were used as probes in Southern hybridization

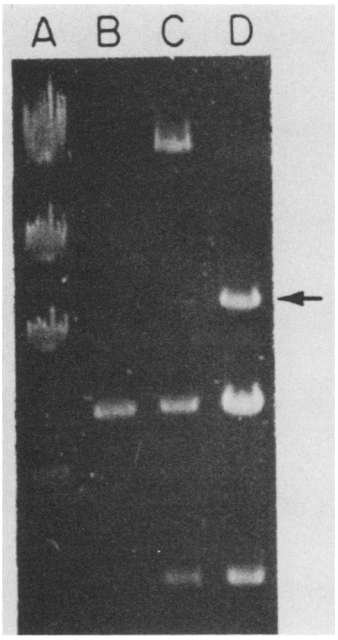

FIG. 3. Agarose gel of *Eco*RI-digested plasmids. Lanes: A, size standards (23, 9.4, 6.7, and 4.4 kb from the top); B, pLH79 vector alone; C, cosmid DNA from tetracycline-resistant COH 31-21 cosmid clone grown with tetracycline; D, cosmid DNA from the same COH 31-21 cosmid clone grown without tetracycline. The arrow designates the 7.5-kb *Eco*RI fragment that flanked one of the Tn*916* insertion sites in COH 31-21.

experiments against *Eco*RI-digested whole-cell DNA from the other GBS types. In a previous study (13), the 3.0-kb *Eco*RI fragment from COH 31-15 hybridized to a similar-sized *Eco*RI fragment in chromosomal DNA from GBS serotypes Ia, Ib, Ic, and II. The 9.0-, 3.65-, and 1.4-kb *Eco*RI fragments and the 2.5-kb *Eco*RI-*Hin*dIII fragment from COH 1 (Fig. 1) were used as probes against the same blot containing *Eco*RI-digested whole-cell DNA from three other types III GBS and from serotypes II, Ia, Ib, and Ic. The results are shown in Fig. 4.

Like the 3.0-kb *Eco*RI fragment, the 3.65- and 1.4-kb *Eco*RI fragments hybridized to similar-sized fragments, respectively, in chromosomal DNA from the eight GBS tested (Fig. 4A and B). However, the 9.0-kb *Eco*RI fragment from COH 1 hybridized to 7.5- and 1.5-kb fragments in COH 31rs (Fig. 4C, lane d). As mentioned above, a restriction fragment length polymorphism was observed between COH 31rs and COH 1 in that region. Hybridization of the 9.0-kb fragment to DNA from the other two type III GBS and to the type II and Ib isolates resulted in a similar-sized fragment as the probe (9.0 kb), whereas serotypes Ic and Ia yielded fragments similar in size to those in COH 31rs (7.5 and 1.5 kb).

The 2.5-kb *Eco*RI-*Hin*dIII fragment from COH 1 was a subclone of the larger (4.6-kb) *Eco*RI fragment. When the 2.5-kb *Eco*RI-*Hin*dIII fragment was used as a probe against *Eco*RI-digested whole-cell DNA from COH 1, it hybridized to the 4.6-kb *Eco*RI fragment and to eight other fragments (Fig. 4D, lane a). The same pattern of nine fragments was observed when the probe was hybrized to *Eco*RI-digested whole-cell DNA from M732, another type III GBS (Fig. 4B, lane b). In DNA from COH 31rs and the type I GBS, only one fragment (3.2 kb) hybridized to the probe (Fig. 4D, lanes d and f through h). A 3.2-kb *Eco*RI fragment and a larger *Eco*RI fragment in the DNA from a type III strain (lane c)

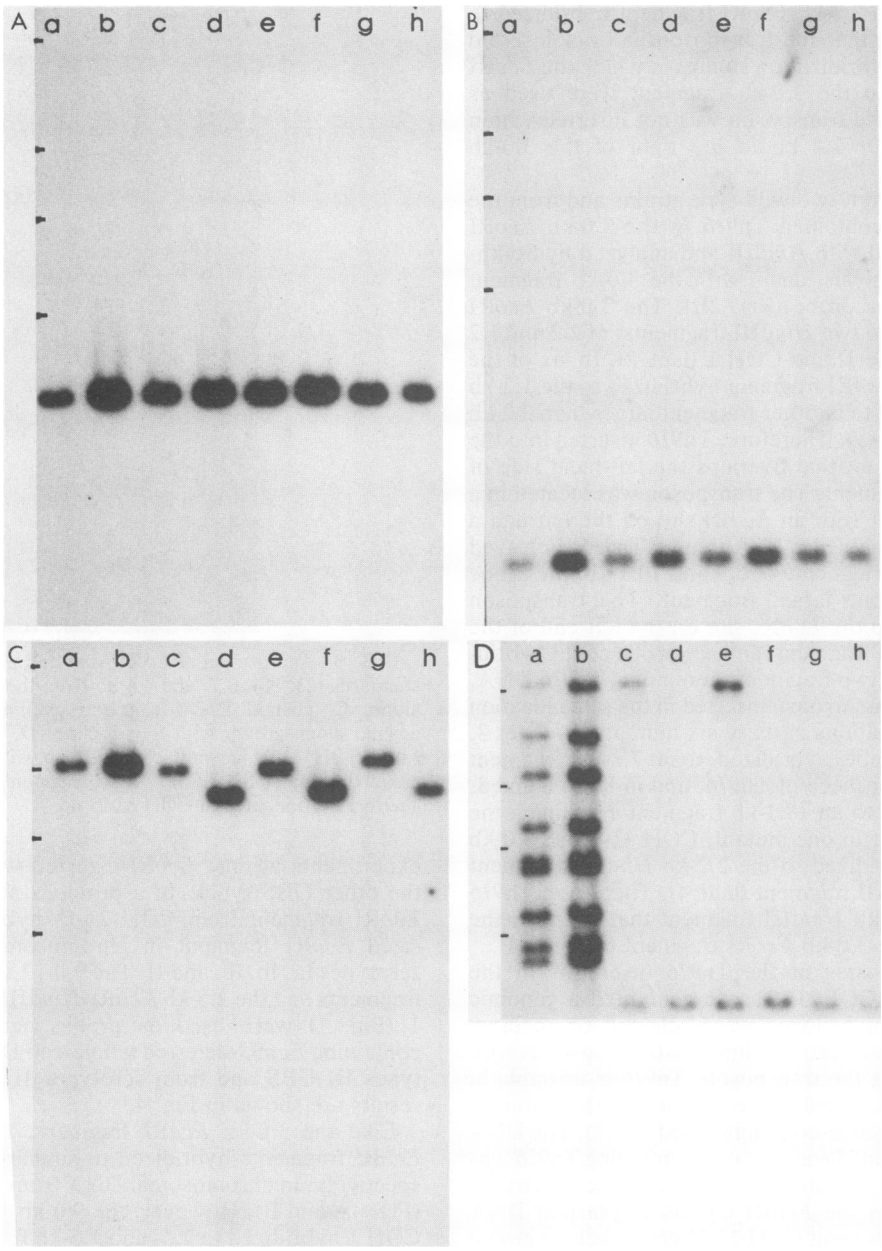

FIG. 4. Southern hybridization analysis of whole-cell DNA from strains of GBS representing all five serotypes digested with *Eco*RI and probed with ^{32}P-labeled DNA fragments from COH 1. Probes are the 3.65-kb *Eco*RI fragment (A), the 1.4-kb *Eco*RI fragment (B), the 9.0-kb *Eco*RI fragment (C), and the 2.5-kb *Eco*RI-*Hin*dIII fragment (D). Lanes: a, COH 1 (type III); b, M732 (type III); c, D136C (type III); d, COH 31rs (type III); e, COH 28 (type II); f, COH 8 (type Ic); g, COH 50 (type Ib); h, COH 46 (type Ia). The bars to the left of the panels indicate size standards (23, 9.4, 6.7, and 4.4 kb from the top).

and a type II isolate (lane e) hybridized to the 2.5-kb *Eco*RI-*Hin*dIII probe.

Identification of type III capsule gene products. The *E. coli* maxicell gene expression system was used to identify gene products of subcloned fragments from the region of the COH 1 chromosome associated with capsule expression. *E. coli* DH1 and clones containing COH 1 chromosomal fragments in the pBS vector were made into maxicells in the presence of [^{35}S]methionine. The proteins made from each set of maxicells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gels were autoradiographed (Fig. 5). DH1 alone did not synthesize any plasmid-encoded proteins, and background expression of chromosomal DNA was not observed (Fig. 5A and B, lanes 1). The vector encoded a β-lactamase of 29 kilodaltons (kDa) and occasionally a small amount of the larger precursor β-lactamase (31 kDa) (Fig. 5A and B, lanes 2). Plasmids containing GBS inserts produced proteins in addition to β-lactamase. Figure 6 shows a map of the capsule gene region of the COH 1 chromosome. The lines and numbers below the map indicate the fragments that were subcloned and the sizes of the proteins expressed by maxicells containing that fragment.

The 9.0-kb *Eco*RI fragment and the smaller (3.3-kb)

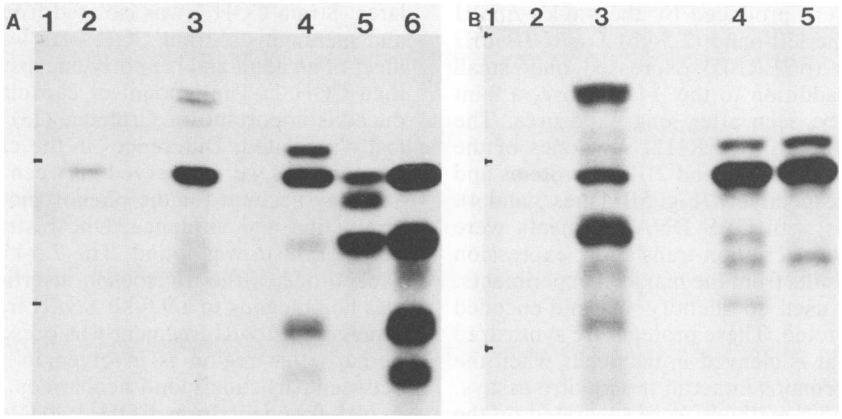

FIG. 5. ^{35}S-labeled proteins from *E. coli* DH1 maxicell clones were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gels were autoradiographed. (A) Lanes: 1, no plasmid; 2, pBS vector; 3, pCER109; 4, pCER108; 5, pCER107; 6, pCER106. (B) Lanes: 1, no plasmid; 2, pBS vector; 3, pCER101; 4, pCER111; 5, pCER112. The bars to the left of the figures indicate the migration distances of standard proteins (43, 29, and 18.4 kDa from the top).

*Eco*RI-*Ava*I fragment within it expressed similar-sized proteins. Figure 5A, lane 3, shows the 40-, 38-, and 34-kDa proteins produced by maxicell clones containing the 3.3-kb *Eco*RI-*Ava*I fragment (pCER109). The 9.0-kb fragment expressed the same 38- and 34-kDa proteins and also a 43-kDa protein instead of the 40-kDa protein produced by the 3.3-kb fragment. This smaller, 40-kDa protein may be a truncated form of the larger, 43-kDa protein whose gene spans the *Ava*I restriction endonuclease site.

The maxicell clone with the 3.65-kb *Eco*RI fragment (pCER108) produced 31-, 23-, 18-, and 16-kDa proteins (Fig. 5A, lane 4). The 3.0-kb insert (pCER107) produced 27-, 24-, and 22-kDa proteins (Fig. 5A, lane 5). The 3.5-kb *Xba*I fragment was a subclone (pCER106) that straddled the center of the adjacent 3.65- and 3.0-kb *Eco*RI fragments (Fig. 5A, lane 6). This fragment produced two proteins (18 and 16 kDa) that comigrated with proteins produced by the 3.65-kb fragment, one protein (22 kDa) that comigrated with a protein produced by the 3.0-kb fragment, and one unique protein (25 kDa) that may be a partial protein corresponding to the 31-kDa protein from the 3.65-kb fragment or the 27-kDa protein from the 3.0-kb fragment. The locations within the cloned fragments of the genes encoding these proteins have yet to be determined.

Several subclones from the 4.6-kb *Eco*RI segment of the chromosome were analyzed (Fig. 5B). Four proteins of 36-,

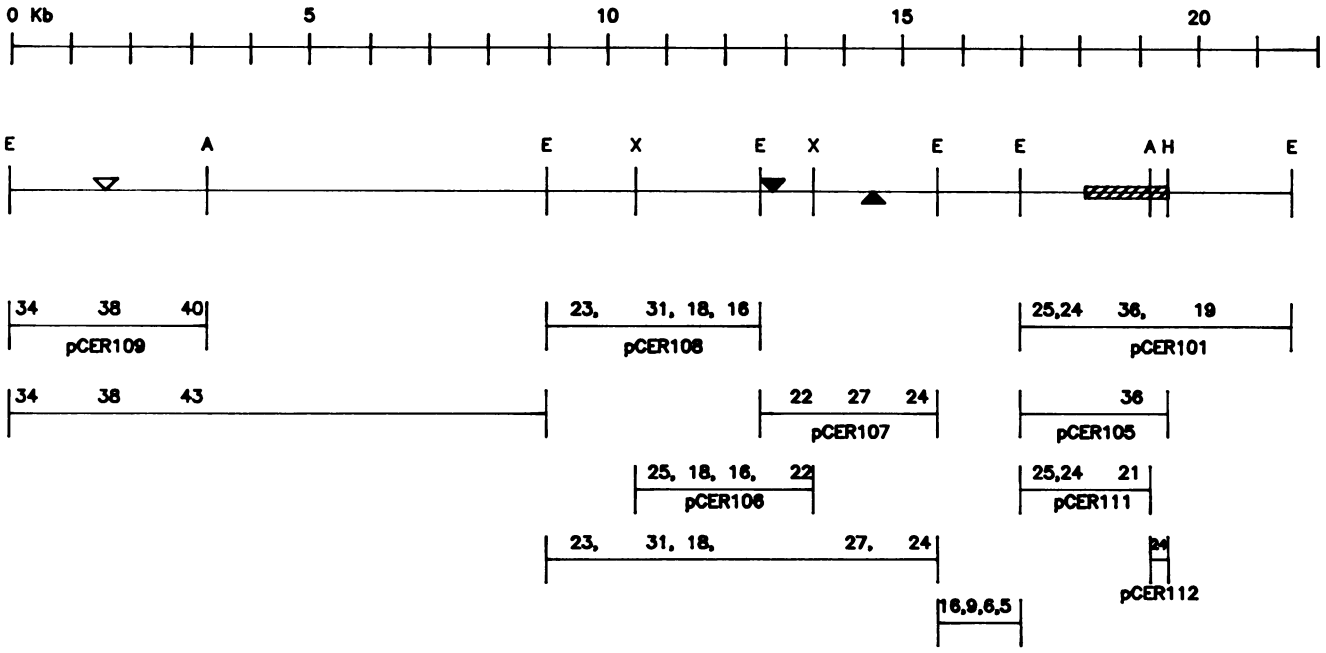

FIG. 6. Chromosomal map of COH 1 type III capsule gene region with approximate sizes and locations of expressed proteins. Abbrevations: A, *Ava*I; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I. Lines below the map represent fragments of COH 1 DNA that were subcloned into the pBS vector and analyzed by a maxicell gene expression method. Numbers above each line are the approximate sizes in kilodaltons of the proteins expressed by maxicells containing that fragment. One of the two Tn*916* insertion sites in COH 31-21 (▽), the Tn*916* insertion sites in COH 31-15 and 31-6 (▼ and ▲, respectively), and the location of IS*861* not found in COH 31rs (▨) are indicated.

25-, 24-, and 17 kDa were produced by the 4.6-kb *Eco*RI fragment (pCER101). The left-hand (2.5-kb) *Eco*RI-*Hin*dIII subclone of that insert (pCER105) expressed only small amounts of protein. In addition to the β-lactamase, a faint band at 36 kDa could be seen after long exposures. The 2.0-kb (pCER111) and 0.5-kb (pCER112) subclones of the 2.5-kb fragment produced 25-, 24-, and 21-kDa proteins and only a 24-kDa protein, respectively (Fig. 5B, lanes 3 and 4).

Some of the plasmids with GBS DNA fragments were tested in an in vitro transcription-translation expression system to confirm the results from the maxicell experiments. This system was also used to identify plasmid-encoded proteins that may be secreted. These proteins are synthsized with a signal peptide that is cleaved in maxicells when the protein is secreted but remains intact in the in vitro assays. Proteins expressed by both methods were separated on the same polyacrylamide gel, and the molecular masses were compared. The apparent mass of a secreted protein will be 1 or 2 kDa larger in the in vitro system than in the maxicell assay. In the in vitro transcription-translation assay, the pBS vector expressed a 31-kDa protein that was the precursor form of the β-lactamase. The 38-kDa protein from the maxicell clone harboring pCER109 was 39 kDa in the in vitro experiments. Two proteins encoded by the maxicells containing pCER106, with apparent molecular masses of 22 and 18 kDa, were 23 and 20 kDa, respectively, when expressed in vitro.

DISCUSSION

Because the type III capsule of GBS is a complex polysaccharide composed of 100 or more repeating units of four different monosaccharides, many genes may be involved in the biosynthesis of this extracellular polymer. In support of the polygenic nature of capsule biosynthesis, the molecular analysis of *E. coli* K1 capsule, a simple sialic acid polymer, has demonstrated that approximately 15 kb of DNA encoding at least 12 proteins is required for the synthesis, transport, and organization of the capsule on the cell surface (4, 15, 16). In addition, some of the K1 capsule genes share significant DNA homology with genes involved in the production of other K-type capsules in *E. coli* (12).

We have identified some of the genes that code for GBS type III capsule biosynthesis by using the technique of Tn*916* mutagenesis. Two distinct phenotypes of capsular mutants were isolated. One class of mutant expressed no capsular material that reacted with native or core antibody (anti-type XIV *S. pneumoniae* (13). The other class of mutant bound antibody to core antigen only (Wessels et al., submitted) and was shown to produce only the desialylated form of the capsule. Therefore, the defect in this mutant occurs in the metabolic pathway for sialic acid or in the process which adds sialic acid to the capsular polysaccharide. We have localized the sites of the Tn*916* insertions that resulted in the altered capsular phenotypes to a 30-kb section of the GBS chromosome. However, for two of the nine acapsular mutants, the transposon site responsible for the mutation did not fall within this 30-kb section. In addition, only one of the two transposon insertion sites for the desialylated mutant was mapped to this region. These data suggest a regional location on the GBS chromsome for some of the genes necessary for type III capsular biosynthesis. They also show that some type III capsule genes may be located elsewhere in the GBS chromosome.

The region of the GBS chromosome responsible for type III capsular biosynthesis was mapped in two type III isolates. Strain COH 1 was isolated from an infant with sepsis and meningitis. Strain COH 31rs was isolated from a leg ulcer of an adult and is poorly encapsulated and less virulent than COH 1. The amount of capsular polysaccharide produced is important for virulence (13) and is probably genetically regulated. Differences in the capsule gene regions of the genomes were observed between COH 31rs and COH 1 and may account for the phenotypic differences in capsule production and virulence. One restriction fragment length polymorphism was found. The 7.5-kb *Eco*RI fragment that flanked one of the transposon insertion sites in COH 31-21 was homologous to a 9.0-kb *Eco*RI fragment in COH 1. The same-sized *Eco*RI fragment was present in Ia and Ic strains. Perhaps this region is involved in specifying the linkage between particular monosaccharides. In addition, the 4.6-kb *Eco*RI fragment from COH 1 located to the right of the transposon insertion site in COH 31-15 contained a 1.4-kb sequence not found in COH 31rs. A probe containing this sequence hybridized to nine *Eco*RI fragments in the COH 1 genome. We have recently shown that the 1.4-kb sequence within the COH 1 1.4-kb *Eco*RI fragment is an insertion sequence that has been designated IS*861* (C. E. Rubens, L. M. Heggen, and J. M. Kuypers, J. Bacteriol., in press). It is intriguing to speculate that the presence of this sequence in the capsule gene region of the chromosome of COH 1 may in some way influence capsule expression.

The genes associated with type III capsular biosynthesis in COH 1 were cloned and used as probes against DNA from other GBS capsular serotypes. Southern hybridization analysis showed that most of this region was highly conserved among all five GBS serotypes. This result suggests that the particular genes identified in this region are involved in capsular biosynthesis in all serotypes. As in *E. coli* (12), the different GBS serotypes may share specific functional gene regions that are responsible for common pathways in all capsular types. An exception to this finding was the 2.5-kb *Eco*RI-*Hin*dIII fragment that contains IS*861*. This probe hybridized to nine *Eco*RI fragments in two serotype III isolates but to only one or two *Eco*RI fragments in two other serotype III isolates and in serotypes II, Ia, Ib, and Ic. The significance of these results is currently being investigated.

Protein products encoded by the cloned type III capsule genes were identified. GBS DNA in an *E. coli* maxicell gene expression system was readily expressed. We have not determined whether the capsule gene products made in *E. coli* are similar to those expressed in GBS or whether transcription was initiated by the GBS promoters. However, other streptococcal genes cloned into *E. coli* were expressed and were functional (6). In addition, pneumococcal DNA fragments cloned into *E. coli* exhibited strong promoter activity (3).

Because the 7.5- and 3.0-kb *Eco*RI fragments were interrupted by Tn*916* in COH 31-21 and 31-15, respectively, the protein products encoded by the genes within those fragments are particularly interesting. Some of the proteins expressed by those regions may be secreted proteins that function outside the cytoplasmic membrane. For example, the desialylated phenotype of COH 31-21 may be due to a mutation of a sialyltransferase that catalyzes the incorporation of sialic acid into the core polysaccharide. Secreted proteins are synthesized as precursor forms with signal peptides that are cleaved when secreted, and maxicells most often demonstrate the cleaved product (16). An in vitro coupled transcription-translation expression system was used to identify plasmid-encoded proteins that retained a signal peptide, inferred by a higher molecular mass in this

system than in the maxicell system (11). Three of the proteins expressed by the capsule genes flanking the transposon insertions in the 7.5- and 3.0-kb *Eco*RI fragments appeared to be larger when synthesized in vitro than when synthesized by maxicells and are possibly secreted proteins. We are currently undertaking further characterization of the proteins expressed by the genes in both of these regions.

We have begun to identify the genes and their products responsible for type III capsular biosynthesis. We believe that other genes that have not yet been identified are also necessary for type III capsule production. Isolation and characterization of the type III capsule genes will enable us to learn more about the molecular and genetic events controlling capsule synthesis, transport, and secretion.

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