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Genetics

Transposon mutagenesis of type III group B *Streptococcus*: Correlation of capsule expression with virulence

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ABSTRACT **The capsular polysaccharide of type III group B *Streptococcus* (GBS) is thought to be a major factor in the virulence of this organism. Transposon mutagenesis was used to obtain isogenic strains of a GBS serotype III clinical isolate (COH 31r/s) with site-specific mutations in the gene(s) responsible for capsule production. The self-conjugative transposon Tn*916* was transferred to strain COH 31r/s during incubation with *Streptococcus faecalis* strain CG110 on membrane filters. Eleven transconjugant clones did not bind type III GBS antiserum by immunoblot. Immunofluorescence, competitive ELISA, and electron microscopy confirmed the absence of detectable GBS type III capsular polysaccharide in one of the transconjugants, COH 31-15. Southern hybridization analysis with a Tn*916* probe confirmed the presence of the transposon sequence within each mutant. A 3.0-kilobase *Eco*RI fragment that flanked the Tn*916* sequence was subcloned from mutant COH 31-15. This fragment shared homology with DNA from the other GBS serotypes, suggesting a common sequence for capsulation shared by organisms of different capsular types. Loss of capsule expression resulted in loss of virulence in a neonatal rat model. We conclude that a gene common to all capsular types of GBS is required for surface expression of the type III capsule and that inactivation of this gene by Tn*916* results in the loss of virulence.**

Group B *Streptococcus* (GBS) is currently the most common cause of neonatal bacterial sepsis and meningitis in the United States. GBS are classified into serotypes based on specific capsular polysaccharides. Capsular type III GBS are responsible for the majority of neonatal infections (1).

Immunological and epidemiological data suggest that the type III GBS polysaccharide capsule is a major factor contributing to the virulence of this organism. Antibody directed against the type III polysaccharide has been shown to be protective in experimental animals and to promote opsonophagocytosis of GBS *in vitro* (2–4). Serologic studies of human infection have demonstrated a strong correlation between low levels of anti-capsular antibody in neonates and susceptibility to infection (5). The capsular polysaccharide is a high molecular weight polymer containing galactose, glucose, *N*-acetylglucosamine, and *N*-acetylneuraminic acid (sialic acid) arranged in a regular repeating-unit native structure (6). Removal of the terminal side-chain sialic acid residues leaves a core polysaccharide that is identical in structure to the capsular polysaccharide of type XIV *Streptococcus pneumoniae* (7, 8).

To study the role of the type III capsule in virulence directly, isogenic mutants with specific defects in capsule production or structure could serve as powerful tools. Transposon mutagenesis is useful for the derivation of isogenic strains that differ in expression of a specific virulence factor. The genetics of streptolysin (9) and streptokinase (10) production in group A *Streptococcus* have been investigated utilizing transposon insertional mutagenesis and cloning. Hemolysin and bacteriocin genes in *Streptococcus faecalis* also have been analyzed using these techniques (11, 12). To date, GBS virulence factors have not been investigated by genetic analysis. This report describes the derivation and characterization of type III GBS mutants that no longer elaborate capsular polysaccharide on their cell surfaces.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. GBS strains 18RS21 (type II), H36b (type Ib), and 090R (unencapsulated type Ia derivative) were provided by R. Lancefield (Rockefeller University). Strain 515 is a type 1a GBS clinical isolate (13). COH 31r/s is a type III clinical isolate made rifampin and streptomycin resistant as described (14). COH 31-15 is an unencapsulated tetracycline-resistant mutant of COH 31r/s described in this study. *S. faecalis* CG110, a high-frequency donor of Tn*916*, and *Escherichia coli* DH1 have been described (15). PGL101 is a 2.4-kilobase (kb) plasmid coding for ampicillin resistance (16). pAM120 is a 21.2-kb plasmid coding for ampicillin and tetracycline resistance and contains the entire Tn*916* transposon (15). pCER3115 and pCER3115-1 are recombinant pGL101 plasmids described in this study.

Isolation of GBS Mutants. Tn*916* is a 16.4-kb transposon coding for tetracycline resistance. Tn*916* is capable of self-conjugation from *S. faecalis* to other Gram-positive species. Conjugation of Tn*916* from *S. faecalis* CG110 (donor) to COH 31r/s (recipient) was performed after growing each strain in Todd–Hewlitt broth (THB) to an A_{600} of 0.4, then mixing at a donor/recipient ratio of 1:10. After filter mating for 18 hr on sheep-blood agar, the cells were washed off the filter with 5 ml of THB, allowed to grow 4 hr at 37°C, harvested at 19,800 × *g*, and washed twice with isotonic phosphate-buffered saline (PBS, pH 7.3). The cells were resuspended in 5 ml of PBS and rocked for 2 hr at 4°C with 1.4 mg of wheat germ lectin bound to agarose (Sigma). The terminal sialic acid residue on the capsular polysaccharide binds to wheat germ lectin (17). Agglutinated cells—i.e., those that still produced native capsular polysaccharide—were removed by centrifugation at 1500 × *g* for 5 min, and the bacteria remaining in the supernatant (presumably enriched for cells deficient in sialic acid) were plated on Todd–Hewitt agar (THA) containing tetracycline at 10 μg/ml and streptomycin at 750 μg/ml to isolate COH 31r/s GBS transconjugants containing the Tn*916* transposon. Transconjugants were confirmed to be GBS by β-hemolysis and by CAMP factor production on sheep blood agar (18).

Preparation of Antisera. Rabbit anti-GBS type III and anti-group B polysaccharide sera were prepared as described

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Abbreviation: GBS, group B *Streptococcus*.

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(8). Rabbit anti-pneumococcal type XIV serum was purchased from Dan-Serum (Racine, WI)

Immunoblot Analysis. Transconjugant clones were analyzed for expression of capsular polysaccharide by a modification of the Meyer procedure (19).

Immunofluorescence Analysis. Bacterial cells (10^8 colony-forming units/ml) were incubated with either the antiserum to type III GBS or antiserum to type XIV *S. pneumoniae*, washed in PBS, and stained with fluorescein-conjugated goat anti-rabbit immunoglobulin, diluted 1:100 (20). Positive and negative controls included type III GBS COH 31r/s, GBS strain 090R, and *S. faecalis* strain CG110. Mutants were graded on reactivity compared to controls.

Preparation of Capsular Extracts for ELISA Inhibition. Organisms were grown for 4–6 hr to an A_{650} of 0.6 in 500 ml of THB that had been passed through a dialysis membrane with a molecular weight cut-off of 10 kDa (Millipore). Bacterial cells were separated from the broth by centrifugation, washed once with PBS, then incubated with mutanolysin at 0.5 mg/ml (Sigma) in a slurry at 37°C overnight. Bacterial cells and debris were removed by centrifugation, and the mutanolysin supernatant was combined with the original broth culture supernatant. The culture supernatant was concentrated to 100 ml over a 10,000 molecular weight cut-off membrane (Amicon), brought to 80% (vol/vol) ethanol, and allowed to stand at 4°C overnight. The precipitate was recovered by centrifugation, air dried, dissolved in 3 ml of 20 mM sodium phosphate (pH 7.0), and treated with DNase (Sigma) at 0.1 mg/ml and RNase (Sigma) at 0.5 mg/ml at 37°C overnight, then with Pronase (Sigma) at 1 mg/ml at 37°C overnight.

The solution was treated with 1 M NaOH at 4°C overnight to depolymerize contaminating group B polysaccharide, then neutralized by dialysis against 20 mM sodium phosphate (pH 7.0). To assure detection of any type III capsule possibly altered in molecular size or net charge, further purification steps were omitted, although these steps have been used (6).

ELISA Inhibition. Purified GBS type III capsule was coupled to poly(L-lysine) (Sigma) by the method of Gray (21), diluted to 1 μg of capsular polysaccharide per ml in 40 mM sodium phosphate (pH 7.0), then coated onto 96-well flat-bottom plastic plates (Dynatech Laboratories, Alexandria, VA) (100 μl at 37°C for 1 hr). Wells were washed three times with the same buffer containing 0.05% Tween-20 (PBT). ELISA inhibition was done to detect capsular polysaccharide in extracts. One hundred microliters of capsular extract (inhibitor) and 100 μl of type III antiserum diluted 1:1000 were added to each well and incubated at 37°C for 1 hr. After washing three times with PBT, 200 μl of alkaline phosphatase-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) diluted 1:3000 in PBT was added and incubated at 37°C for 1 hr. After washing three times, *p*-nitrophenyl phosphate, disodium salt, at 1 mg/ml (Sigma) was added in 40 mM sodium carbonate (pH 9.6). After incubation at 37°C for 1 hr, A_{405} was determined using an ELISA reader (model EL-307, Bio-Tek, Burlington, VT).

Immune Electron Microscopy. GBS strains were examined by immune electron microscopy as described (22).

Preparation of ^{32}P-Labeled Tn*916* Probe and DNA·DNA Hybridization. The 18.8-kb *Eco*RI fragment of plasmid pAM120 that contains the Tn*916* sequence was isolated from low-temperature agarose by electroelution (23) and labeled with ^{32}P-labeled deoxynucleotides *in vitro* by nick-translation as described (24).

Total cellular DNA from the COH 31r/s type III mutants was isolated utilizing a modification of the Hull procedure (25) in which mutanolysin (50 μg/ml) was used to prepare protoplasts of GBS cells in place of lysozyme. The cells were lysed with 1% $NaDodSO_4$ and proteinase K (50 μg/ml), and DNA was purified from the cell lysate by centrifuging in a buoyant-density cesium chloride gradient (25). Restriction enzyme digestion of DNA with *Eco*RI or *Hin*dIII, agarose gel electrophoresis, Southern transfer of DNA fragments to nitrocellulose, and hybridization with a ^{32}P-labeled Tn*916* DNA probe have been described (23).

Cloning. Isolation and cloning of the *Eco*RI fragment containing the Tn*916* sequence from COH 31-15 was performed by electroelution, ligation to purified *Eco*RI-digested pGL101 vector DNA, and transformation into the *E. coli* recipient DH1 (15). All transformants were selected on L-agar containing ampicillin at 25 μg/ml and tetracycline at 4 μg/ml (15). Transformants resistant to both drugs were maintained on the same medium.

Virulence Assay. A neonatal rat sepsis model described by Zeligs *et al.* (26) was used to determine the LD_{50} of COH 31r/s and COH 31-15.

RESULTS

Isolation and Immunologic Analysis of GBS Mutants Lacking Capsular Polysaccharide. Capsular mutants of type III GBS were derived from strain COH 31r/s by Tn*916* transposon mutagenesis. Tn*916* was transferred from *S. faecalis* CG110 to GBS COH 31r/s at a frequency of 1 per 5×10^{-7} recipient cells. Immunoblot analysis was performed on tetracycline-resistant transconjugant COH 31r/s clones to identify those clones deficient in capsular expression. Transconjugants deficient in type III capsule failed to bind type III antiserum and were identified as nonradioactive colonies by autoradiography (Fig. 1).

Nonreactive transconjugant clones were further characterized by using pneumococcal type XIV antiserum and antiserum to the common group B polysaccharide antigen (anti-090R antiserum) utilizing the immunoblot technique. Eleven clones were isolated that failed to react with type III GBS and type XIV *S. pneumoniae* antisera. All reacted with the group B antiserum. Results from immunoblot analysis of these mutants were confirmed by immunofluorescence analysis utilizing the same antisera.

A single representative mutant, COH 31-15, was chosen for further analysis. As a more sensitive method for detection of capsular polysaccharide, mutanolysin extracts and culture supernatants were used as inhibitors of the binding of type III antiserum to purified type III capsular polysaccharide in a

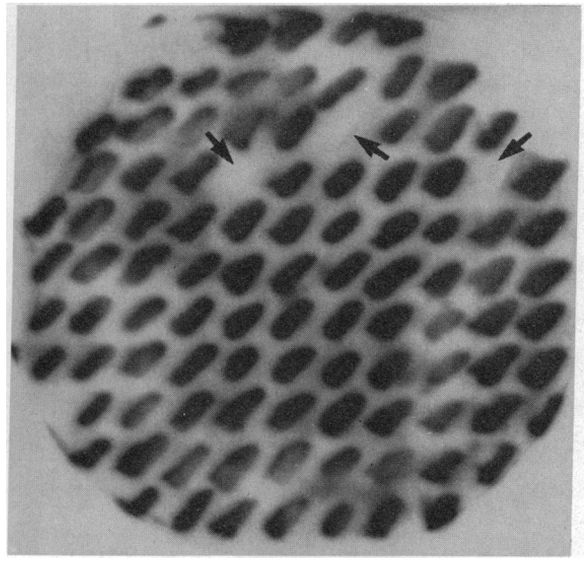

FIG. 1. Immunoblot of COH 31r/s Tn*916* transconjugant clones. Light spots (arrows) are clones that are nonreactive to the primary antibody (type III GBS antiserum, diluted 1:8000).

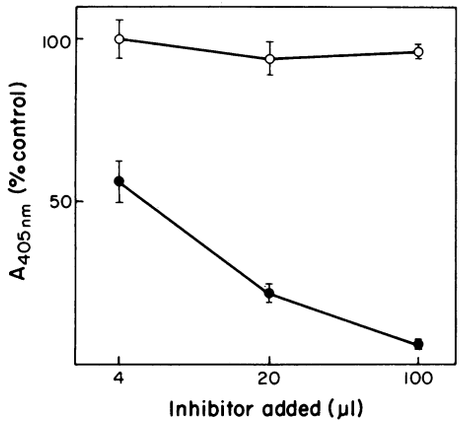

FIG. 2. Inhibition curves of competitive ELISA with increasing concentrations of capsular extracts (inhibitor) from wild-type COH 31r/s (●) and type III mutant COH 31-15 (○).

competitive ELISA. The capsular extract from the parent strain COH 31r/s produced dose-dependent inhibition of the ELISA, whereas the extract from the mutant strain COH 31-15 was not inhibitory (Fig. 2).

Immune Electron Microscopy. Immune electron microscopy was performed on COH 31r/s and COH 31-15 using type III GBS antiserum. After incubation with immune serum, cells were treated with ferritin-conjugated goat anti-rabbit IgG, then processed for electron microscopy. As shown in Fig. 3*A*, the parent strain COH 31r/s was surrounded by a layer of extracellular capsule outlined by ferritin particles. The COH 31-15 mutant strain showed only a small amount of extracellular staining (Fig. 3*B*). Two explanations for the appearance of the mutant strain were possible: (*i*) the antibody bound to the mutant strain could have been directed against a small amount of capsular material not detected by our other immunologic assays, or (*ii*) the antibodies bound to the surface of the mutant strain might be directed against other surface antigens, such as the group B polysaccharide. To distinguish between these possible explanations, immune electron microscopy was done using type III GBS antiserum absorbed with 090R cells to remove antibodies directed against the group B polysaccharide. The parent strain (COH 31r/s) reacted with absorbed serum essentially the same as with unabsorbed serum (Fig. 3*C*); however, the transconjugant COH 31-15 did not bind any antibody (Fig. 3*D*) and had no visible extracellular capsular material.

Comparative Lethality of Parent and Mutant Strains in the Neonatal Rat. The LD_{50} for COH 31r/s was 5×10^5 colony-forming units/g of body weight. No deaths occurred in animals receiving up to 5×10^7 colony-forming units/g of mutant strain COH 31-15. GBS infection was confirmed by culturing the spleens of dead rats.

Molecular Characterization of Type III Mutants. The Tn*916* transposon sequences in several capsule-deficient mutants were confirmed by Southern blot DNA·DNA hybridization analysis using ^{32}P-labeled Tn*916* to probe *Eco*RI digests of whole-cell DNA. *Eco*RI does not cut within the Tn*916* sequence. Autoradiography of the DNA fragments from these mutants showed from 1 to 4 Tn*916* insertions. The size of the DNA fragments ranged from 18 to 25 kb (Fig. 4). No background hybridization was observed between cell-type COH 31r/s total cellular DNA and the probe (Fig. 4, lanes 8).

To confirm the number of Tn*916* insertions in each type III capsule mutant, total cellular DNA was digested with *Hin*dIII. *Hin*dIII cleaves the Tn*916* sequence once internally resulting in two junction fragments (15). Total cellular DNA

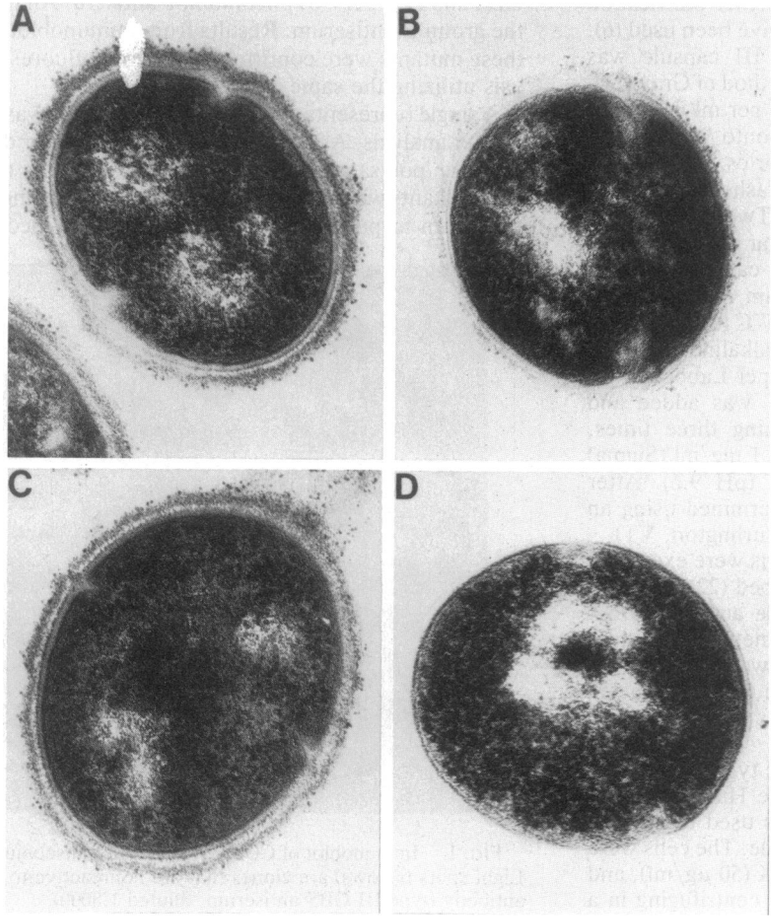

FIG. 3. Immune electron microscopy of COH 31r/s and COH 31-15. (*A* and *B*) COH 31r/s and COH 31-15, respectively, incubated with unabsorbed type III GBS antiserum followed by ferritin-conjugated goat anti-rabbit IgG. (*C* and *D*) COH 31r/s and COH 31-15, respectively, incubated with type III GBS antiserum absorbed with GBS strain 090R cells to remove antibody to the common group B polysaccharide, followed by ferritin-conjugated goat anti-rabbit IgG. (× 49,500.)

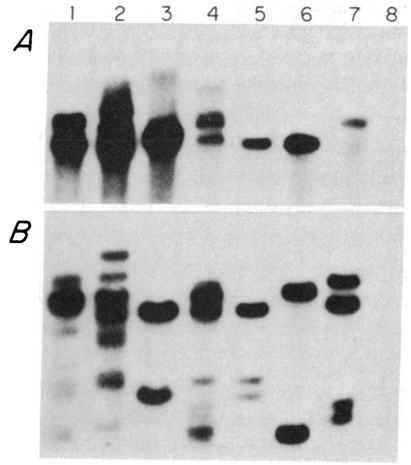

FIG. 4. (*A*) Southern hybridization analysis of whole-cell DNA from mutants of COH 31r/s digested with *Eco*RI and probed with ^{32}P-labeled Tn*916* DNA probe. Lanes: 1, COH 31-9; 2, COH 31-12; 3, COH 31-15; 4, COH 31-17; 5, COH 31-19; 6, COH 31-20; 7, COH 31-22; 8, COH 31r/s (wild-type strain). (*B*) Whole-cell DNA digested with *Hin*dIII from the same mutants, hybridized to same probe, and located in the same lanes as in *A*.

from the type III capsule mutants digested to completion with *Hin*dIII and probed with the same ^{32}P-labeled Tn*916* DNA probe resulted in two fragments for each Tn*916* insertion present. As shown in Fig. 4, although some mutants appeared to have only one Tn*916* insertion after *Eco*RI digestion (Fig. 4*A*, lanes 5 and 7), subsequent blots of the same DNA after *Hin*dIII digestion showed additional fragments indicating more than one insertion of Tn*916* (Fig. 4*B*, lanes 5 and 7). These data indicated that Tn*916* inserted into multiple distinct sites within the GBS chromosome, some of which were on similar-size but distinct *Eco*RI fragments. However, the type III capsule mutants COH 31-15 and COH 31-20 (Fig. 4, lanes 3 and 6) have only a single Tn*916* insertion. The phenotypic effects of multiple Tn*916* copies in chromosomes of the other mutants have not been determined.

Subcloning DNA Flanking Tn*916*. High molecular weight *Eco*RI fragments of COH 31-15 cellular DNA were isolated from agarose by electroelution. A 19.4-kb fragment composed of Tn*916* (16.4 kb) and 3.0 kb of flanking GBS DNA was ligated to the plasmid vector pGL101 (2.4 kb) and transformed into *E. coli* DH1. Selection on plates containing ampicillin and tetracycline gave clones that contained only a pGL101 recombinant plasmid and the 19.4-kb *Eco*RI Tn*916* fragment (designated pCER3115). Tn*916* is unstable and

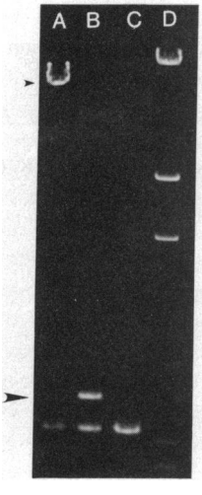

FIG. 5. Agarose gel of *Eco*RI-digested plasmids. Lanes: A, pCER3115; B, pCER3115-1; C, pGL101 vector only; D, the *Hin*dIII fragments of phage λ DNA molecular size standards. pCER3115 and pCER3115-1 were grown with and without tetracycline, respectively, and as a result pCER3115-1 lacks the 16.4-kb Tn*916* insert (small arrowhead). The 3.0-kb fragment from pCER3115-1 is GBS DNA in which Tn*916* was originally inserted (large arrowhead).

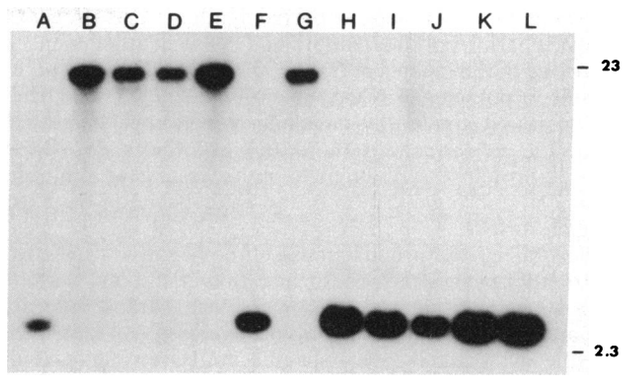

FIG. 6. Southern hybridization of DNA from COH 31r/s mutants (lanes A–G) and from other GBS serotypes (lanes H–L) digested with *Eco*RI and probed with a ^{32}P-labeled 3.0-kb fragment DNA cloned from COH 31-15. Lanes: A, COH 31-9; B, COH 31-12; C, COH 31-15; D, COH 31-17; E, COH 31-19; F, COH 31-20; G, COH 31-22; H, COH 31r/s (wild type); I, H36b (type 1b); J, 515 (type Ia); K, 18RS21 (type II); L, COH 8 (type Ic). Molecular size designations are on the right in kb.

precisely excises and segregates from *E. coli* unless the strain is maintained on tetracycline-containing medium (15). Plasmid DNA from DH1 (pCER3115) grown in tetracycline yielded two fragments of 19.4 and 2.4 kb after *Eco*RI digestion (Fig. 5, lane A). When the same strain was grown without tetracycline, Tn*916* excised from the pCER3115 plasmid. The resultant plasmid, designated pCER3115-1, was 5.4 kb and was composed of the pGL101 vector and the 3.0 kb of COH 31-15 chromosomal DNA that had flanked the Tn*916* sequence as shown by *Eco*RI digestion (Fig. 5, lane B). Since Tn*916* precisely excises, presumably the integrity of the 3.0-kb fragment in pCER3115-1 should be restored and, therefore, should contain a gene sequence involved in type III capsule expression.

The 3.0-kb fragment was used as a probe against whole-cell DNA from COH 31-15 and the six other type III mutants (Fig. 6). The probe hybridized with the 19.4-kb *Eco*RI fragment in COH 31-15 as expected and similarly in four other mutants (Fig. 6, lanes B–E and G). However, in two type III mutants, only a 3.0-kb *Eco*RI fragment hybridized to the probe suggesting that the Tn*916* sequence was inserted elsewhere in the chromosome (Fig. 6, lanes A and F).

The same 3.0-kb probe was used in Southern hybridization against *Eco*RI-digested whole-cell DNA from GBS type Ia, Ib, Ic, II, and III (COH 31r/s) strains. Fig. 6, lanes H–L, shows this sequence is present in all serotypes and is not unique to type III GBS.

DISCUSSION

Polysaccharide capsules of several organisms are thought to play important roles as virulence factors in human infections (27). The primary function of bacterial capsules in pathogenesis is to help the pathogen evade phagocytic host-defense mechanisms (28). The GBS type III polysaccharide antigen has been implicated as a major factor in virulence and immunity: epidemiologic surveys of human infection and animal model studies indicate that anti-capsular antibodies are protective (27).

The type III capsule is a complex carbohydrate composed of four different monosaccharides arranged as a pentasaccharide repeating structure in a polymer of 100 or more repeating units. The terminal side-chain sialic acid on these repeating units appears to confer an antigenically important structure (7). Because of its complexity, the biosynthesis of the GBS extracellular polysaccharide may require several

genes involved in monosaccharide synthesis and transport, oligosaccharide polymerization, and cell wall attachment, as suggested in the studies of *E. coli* K1 capsule (29). *E. coli* K1 capsule, a polymer of *N*-acetylneuraminic acid (sialic acid), has been investigated by molecular genetic analysis to elucidate the genes responsible in its biosynthesis (29, 30). At least 12 proteins are thought to be necessary for synthesis, transport, and organization of the K1 capsule on the extracellular surface.

We utilized the promiscuous self-conjugative transposon Tn*916* to derive site-specific insertion mutations within a GBS type III clinical isolate. We selected transconjugants that are aberrant in their phenotypic expression of the type III capsule as recognized by specific antiserum. Transconjugants identified by immunoblot and immunofluorescence analysis no longer reacted with type III GBS antiserum. These mutants still expressed the common group B polysaccharide. A single representative mutant, COH 31-15, was used for further analysis. By competitive ELISA and immune electron microscopy with type III GBS antiserum, we found that this mutant failed to express the type III immunodeterminant on its surface. If this mutant produces any capsular polysaccharide, it must be structurally altered to such an extent that it is not recognized by type III GBS antiserum. Tn*916* inserted in as many as four distinct sites in the chromosome of some mutants; however, only a single insertion was necessary to completely interrupt the expression of the type III capsule in COH 31-15. This mutation did not simply result in loss of the terminal sialic acid residue of the polysaccharide because pneumococcal type XIV antiserum also failed to react with the mutant.

We have cloned the 3.0-kb *Eco*RI fragment from COH 31-15 containing the DNA sequence interrupted by Tn*916*. This fragment also contained the Tn*916* sequence in four out of six other mutants with the same phenotype. However, two out of six mutants that are phenotypically similar have the transposon inserted elsewhere. It is likely that other genes are involved in capsule biosynthesis, and mutations in different locations may effect capsule expression. The 3.0-kb fragment from COH 31-15 hybridized to a similar size fragment in chromosomal DNA from the other GBS serotypes Ia, Ib, Ic, and II. These data suggest that the gene(s) carried on the 3.0-kb fragment is not unique to the type III serotype. The gene(s) may code for a regulatory protein, for a structural protein, or for a specific glycosyltransferase common to all the serotypes.

Prior studies with chemically altered type III GBS or with spontaneous but genetically uncharacterized mutants (31, 32) have suggested that the loss or alteration of the capsule results in diminished virulence in animal models. We have demonstrated that a specific, single mutation that completely interrupted capsule expression resulted in a GBS isogenic mutant that was avirulent when compared to the wild-type strain. These results provide compelling evidence for the role of type III capsule as a major virulence factor for type III GBS.

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