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Article

Group B *Streptococcus cpsE* Is Required for Serotype V Capsule Production and Aids in Biofilm Formation and Ascending Infection of the Reproductive Tract during Pregnancy

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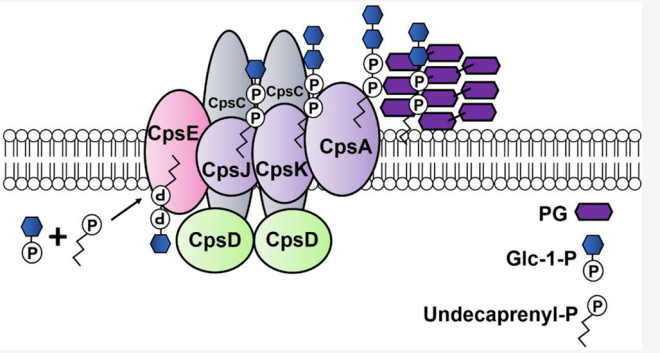
ABSTRACT: Group B *Streptococcus* (GBS) is an encapsulated Gram-positive pathogen that causes ascending infections of the reproductive tract during pregnancy. The capsule of this organism is a critical virulence factor that has been implicated in a variety of cellular processes to promote pathogenesis. Primarily comprised of carbohydrates, the GBS capsule and its synthesis is driven by the capsule polysaccharide synthesis (*cps*) operon. The *cpsE* gene within this operon encodes a putative glycosyltransferase that is responsible for the transfer of a Glc-1-P from UDP-Glc to an undecaprenyl lipid molecule. We hypothesized that the *cpsE* gene product is important for GBS virulence and ascending infection during pregnancy. Our work demonstrates that a GBS *cpsE* mutant secretes fewer carbohydrates, has a reduced capsule, and forms less biofilm than the wild-type parental strain. We show that, compared to the parental strain, the Δ*cpsE* deletion mutant is more readily taken up by human placental macrophages and has a significantly attenuated ability to invade and proliferate in the mouse reproductive tract. Taken together, these results demonstrate that the *cpsE* gene product is an important virulence factor that aids in GBS colonization and invasion of the gravid reproductive tract.



KEYWORDS: *Streptococcus, carbohydrate, capsule, pathogenesis, innate immunity*

Group B *Streptococcus* (GBS) or *Streptococcus agalactiae* is a Gram-positive, encapsulated bacterium that colonizes the gastrointestinal and reproductive tracts of humans.[1,2] GBS colonization of the rectovaginal mucosa during pregnancy is a risk factor for an invasive ascending infection of the gravid reproductive tract.[3] When these tissues are invaded, GBS causes an inflammation of fetal membranes (chorioamnionitis), which can lead to a preterm premature rupture of membranes (PPROM), preterm birth, neonatal sepsis, and maternal-fetal morbidity and mortality.[1] Intrapartum antibiotic use in developed countries has significantly reduced rates of GBS-induced early onset sepsis in full-term neonates.[4] However, this treatment does not prevent a GBS-induced preterm birth with associated long-term neurodevelopmental deficits, nor have there been improvements in rates of late-onset sepsis.[5,6] Additionally, the emergence of antibiotic resistance in perinatal strains foreshadows the need for novel treatment approaches.[7] Understanding mechanisms of GBS virulence is critical to develop effective preventative and therapeutic treatments for pregnant people.

The polysaccharide capsule is a major virulence factor for pathogens in the *Streptococcus* genus. The streptococcal polysaccharide capsule facilitates evasion of the innate immune response by protecting the bacterial cell from deposition of complement, opsonization, and phagocytosis.[8−10] Additionally, the capsule mediates an interaction with viral pathogens that results in superinfection.[11] Capsular synthesis occurs via Wzy- and synthase-dependent mechanisms in Gram-positive bacteria, including streptococci.[12] Wzy-dependent mechanisms involve a cytoplasmic synthesis of polysaccharides anchored to an undecaprenyl-phosphate lipid carrier. A subsequent transmembrane transport to the extracellular peptidoglycan environment occurs via the Wzx flippase.[12] On the cytoplasmic

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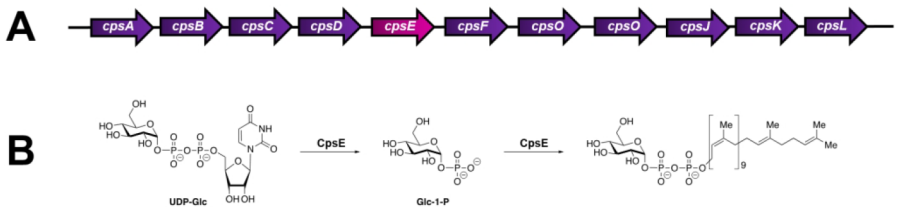

Figure 1. Genetic architecture and putative activity of CpsE. (A) Genetic arrangement of the cps operon in *S. agalactiae* GB37. (B) Putative activity of CpsE (as determined in *S. pneumoniae*). CpsE cleaves a glucose-1-phosphate from uridine diphosphate glucose and transfers this to a polyprenyl phosphate acceptor (such as undecaprenyl phosphate).

face of the bacterial cell wall, Wzy polymerase further elaborates the lipid-polysaccharide capsular component.[13]

The GBS capsular polysaccharide (CPS) forms the outermost layer made of repeating structures of the monosaccharides glucose, galactose, *N*-acetylneuraminic acid, and *N*-acetylglucosamine that define serotypes.[14] There are 10 GBS CPS serotypes (Ia, Ib, and II−IX) that demonstrate varying involvement in specific human disease conditions. For example, CPSIII strains are associated with higher rates of invasive neonatal disease.[15] Despite variable disease outcomes, all of the CPS structures across a serotype share terminal sialic acid (Sia) residues that allow a molecular mimicry of human cell surface sialic acids. This allows an interaction with Sia receptors, Siglecs, on innate immune cells that serve to dampen inflammatory responses.[16] GBS Sia binding to Siglec-9 on platelets inhibits the platelet killing of GBS.[17]

The initiation of GBS CPS synthesis occurs in the bacterial cytoplasm with protein products of the *cps* operon (Figure 1A); however, details of this process are not fully elucidated. The GBS *cps* operon consists of genes that encode proteins involved in bacterial regulation and transport, capsular assembly and polymerization, and sialic acid synthesis and transport.[18] The *cpsE* gene encodes a putative galactosyltransferase involved in capsular assembly and polymerization, which likely occurs at the cytoplasmic face of the bacterial cell wall. *cpsE* adds a monosaccharide to an undecaprenyl phosphate acceptor to initiate the assembly of the oligosaccharide repeating unit as shown in Figure 1B. Deletion of the *cpsE* gene from the type Ia GBS strain 515 (*cpsIaE*) causes a complete loss of reactivity with antiserum to the type Ia GBS polysaccharide.[19] Despite a genetic deletion, the rest of the *cps* operon is still transcribed indirectly indicating that the CpsIaE function is necessary for capsule formation.[19] Interestingly the role of *S. pneumoniae* CpsE in capsule formation is serotype-specific.[12]

Biofilm formation is critical for the colonization of the host and an important primary step in streptococcal pathogenesis. GBS strains vary in their capacity to form biofilms, and these differences are associated with phylogenetic lineage, isolation source, and capsular serotype.[20] Previous work has shown that carbohydrate metabolism influences GBS biofilm formation.[21] Specifically, GBS serotype V strains form strong biofilms in the presence of glucose.[22] In this work, we sought to determine the role of capsular synthesis in a hypervirulent GBS serotype V in biofilm formation, immune evasion, and ascending infection of a pregnant host.

RESULTS

CpsE Is Implicated in Production of Serotype V Capsule. To evaluate the production of the capsule, wild-type (WT) GB37 and the isogenic Δ*cpsE* mutant cells were evaluated by negative stain transmission electron microscopy (TEM) analyses. TEM of negatively stained whole bacterial cells revealed the production of the capsule by GB37 cells (Figure 2A,C). Conversely, the capsule production was

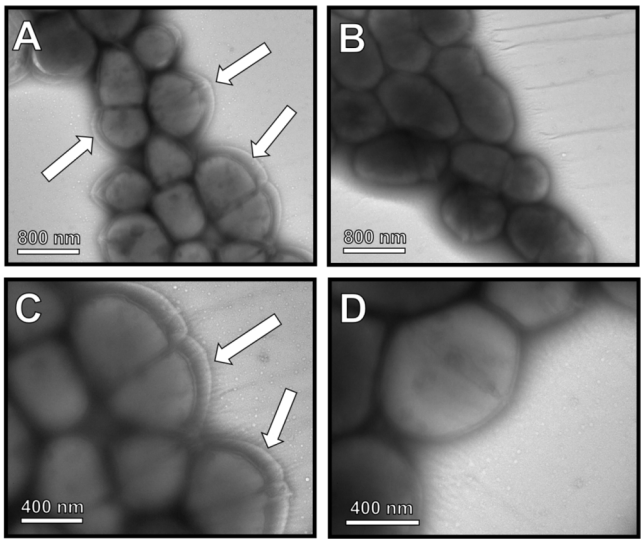

Figure 2. TEM analysis of GBS capsule biosynthesis. Ammonium molybdate staining and TEM of whole bacterial cells reveals (A, C) wild-type GB37 produces a thick capsule visualized by negative stain as a white region surrounding the cell (arrows). (B, D) An isogenic Δ*cpsE* mutant exhibits diminished capsule associated with the cell surface. Magnification is 6500× (A, B) and 11 000× (C, D). Micrographs are representative of three biological replicates.

severely attenuated in the isogenic Δ*cpsE* mutant cells (Figure 2B,D), indicating that the *cpsE* locus is critical for the production of capsule in GBS strain GB37. Prior studies have demonstrated that the CpsE function in serotype Ia is upstream of other tested gene products of the cps operon.[19] Our data demonstrate a similar significant loss of capsule and support the role of CpsE early in the production of the capsule in serotype V as well.

CpsE Aids in GBS Biofilm Formation. Extracellular surface features like a capsule have been implicated in cellular processes, such as biofilm formation, which are critical for colonization and pathogenesis in the vertebrate host. We hypothesized that the isogenic Δ*cpsE* mutant cells, which form less capsule, would have perturbed biofilm formation compared to the parental strain. To test this, bacteria were grown under static conditions to facilitate the biofilm formation. The following day, the biofilms were analyzed by a standard crystal violet spectrophotometric biofilm quantification (Figure 3). This revealed that the isogenic Δ*cpsE* mutant was 60% attenuated in its ability to form biofilms

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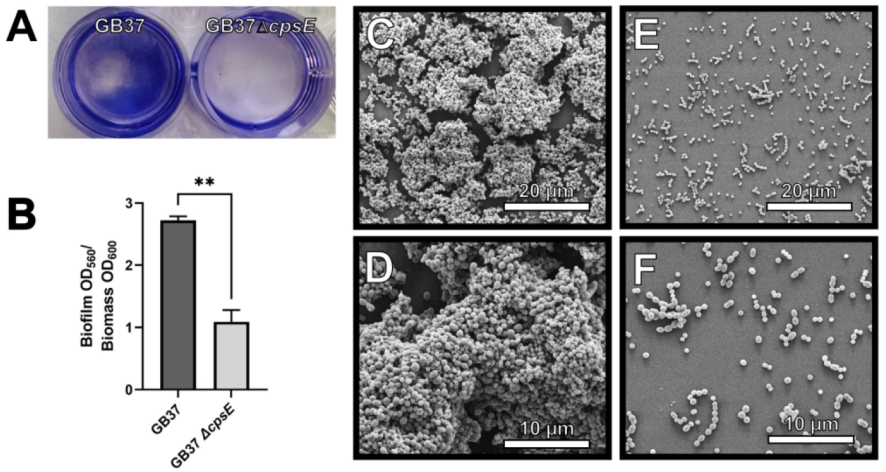

Figure 3. Analysis of the role of *cpsE* in GBS biofilm formation. (A) Bacterial biofilm was visualized with crystal violet stain (purple), which revealed that GB37 forms a robust biofilm when cultured in THB supplemented with glucose. The isogenic Δ*cpsE* mutant has diminished the capacity to form biofilms on polystyrene. (B) Quantification of biofilm by spectrophotometric measurement (bars indicate mean optical density of 560 nm (OD_{560}) of solubilized crystal violet in ratio to cellular density (OD_{600}) prior to staining) indicates the WT GB37 forms a significantly more quantifiable biofilm than the isogenic Δ*cpsE* mutant. High-resolution scanning electron microscopy imaging reveals the WT GB37 forms larger, tertiary architectural structures of cells with well-formed channels between cellular clusters (C, D). Conversely, the isogenic Δ*cpsE* mutant adheres sparsely to the abiotic surface and rarely forms tertiary cellular structures. (C, E) 5000× magnification. (D, F) 10 000× magnification. Bars indicate mean ± SEM $N = 3–6$ biological replicates. $^{**}P < 0.01$, Student's *t*-test with Welch's correction.

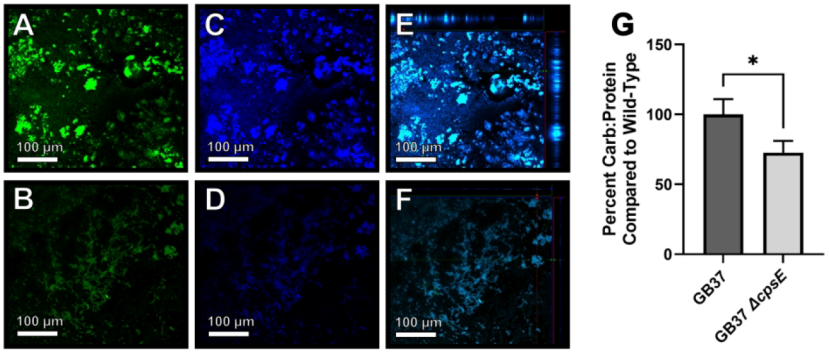

Figure 4. Analyses of carbohydrates within a GBS biofilm. Confocal laser scanning microscopy of (A, B) GBS (stained green with Syto9) and (C, D) cell-associated carbohydrates (stained blue with calcofluor white stain) reveals that GB37 (A, C, E) secretes more carbohydrate matrix in its biofilm than a Δ*cpsE* mutant (B, D, F). Merged images (E, F) include ortho stack analyses, which confirm that GB37 (E) forms thicker biofilms than the Δ*cpsE* mutant (F). (G) Monomeric carbohydrate assay of adherent bacterial cells indicates the Δ*cpsE* mutant secretes fewer carbohydrates compared to the WT strain. $^{*}P < 0.05$, Student's *t*-test.

compared to the parental strain ($P = 0.0039$, paired Student's *t*-test; $P = 0.0019$, unpaired Student's *t*-test with Welch's correction). Interestingly, the Δ*cpsE* and the WT parental strain have no significant difference in growth rate in vitro (Figure S1A). High-resolution scanning electron microscopy also showed that the isogenic Δ*cpsE* mutant had sparse cells adhering to the abiotic substrate (glass coverslip) compared to the parental strain, which formed robust biofilms. Together, these results indicate that the GBS biofilm formation is aided by the *cpsE* locus.

Inactivation of the cpsE Locus Results in Attenuated Carbohydrate Secretion into Biofilms. The *cpsE* gene is predicted to encode a galactosyltransferase that is critical for cell surface polysaccharide transport. Therefore, we hypothesized that cell-associated surface carbohydrates within the biofilm would be attenuated in the isogenic Δ*cpsE* mutant cells compared to the parental strain. To test this, we employed confocal laser scanning microscopy of biofilm samples stained with Syto9 (green fluorescent signal) to visualize cells and calcofluor white stain (blue fluorescent signal) to visualize carbohydrate matrices within the biofilms (Figure 4A–F). Microscopical analyses of the wild-type GB37 strain revealed a robust carbohydrate production within biofilms (Figure 4A–C), which was attenuated in the isogenic Δ*cpsE* mutant (Figure 4D–F). A confirmation of changes in biofilm-associated carbohydrates was done by employing the L-cysteine sulfuric acid monomeric carbohydrate assay to quantify carbohydrates (normalized to total cellular protein content) of bacterial cells adhering to a polystyrene substrate. A quantitation of the total carbohydrate to protein ratio (Figure 4G) revealed that the isogenic Δ*cpsE* mutant cells had diminished carbohydrates associated with adherent cells compared to the parental strain.

Inactivation of the cpsE Locus Results in Enhanced Phagocytosis of GBS by Placental Macrophages. A capsule is an important virulence factor that aids in the bacterial evasion of the innate immune cell phagocytosis. We hypothesized that, due to its defect in capsule production, a

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Δ*cpsE* mutant would exhibit enhanced phagocytosis by innate immune cells in the gravid reproductive tract. To test this, we utilized human placental macrophages in coculture with either a wild-type parental strain of GBS (GB37) or the *cpsE* isogenic mutant strain. Bacterial cells were labeled with a fluorescence marker, and the evaluation of phagocytosis was measured by determining the fluorescence of bacteria associated with macrophages. There were no discernible differences in the fluorescein isothiocyanate (FITC) labeling between the Δ*cpsE* and the WT parental strain as determined by the mean fluorescence of serial dilutions of known cellular densities (Figure S1B). Our results indicate that the Δ*cpsE* mutant had a 42% enhancement of bacteria associated with placental macrophages compared to that of the parental strain (Figure 5). This indicates that GBS CPS V capsule can aid in the evasion of phagocytosis of GBS by human placental macrophages.

A cpsE Mutant Has Diminished Capacity to Invade the Gravid Reproductive Tract of a Mouse. Because the Δ*cpsE* mutant was attenuated in its ability to evade innate immune cells, we hypothesized that this isogenic strain might also be attenuated in virulence in a vertebrate host model of infection and disease. To test this, we employed the mouse model of ascending GBS vaginal infection during pregnancy pioneered by the Randis and Ratner laboratories, which our lab has refined.[23] Pregnant mice were challenged on embryonic day 13.5 with a vaginal inocula of either wild-type GB37 or Δ*cpsE* mutant, and uninfected controls were also maintained. Two days postinfection, mice were sacrificed, and reproductive tissues were collected for an analysis of bacterial burden by quantitative culture techniques (Figure 6). A three-log decrease in bacterial burden was observed in the vaginal and uterine tissues derived from animals infected with a Δ*cpsE* mutant compared to animals infected with the parental strain, a result that was statistically significant ($P < 0.0001$ and $P = 0.0019$, respectively, Student's *t*-test with Welch's correction). A four-log decrease in the bacterial burden was observed in decidua, placenta, fetal membrane, and fetal tissues from animals infected with a Δ*cpsE* mutant compared to animals infected with the parental strain, a result that was statistically

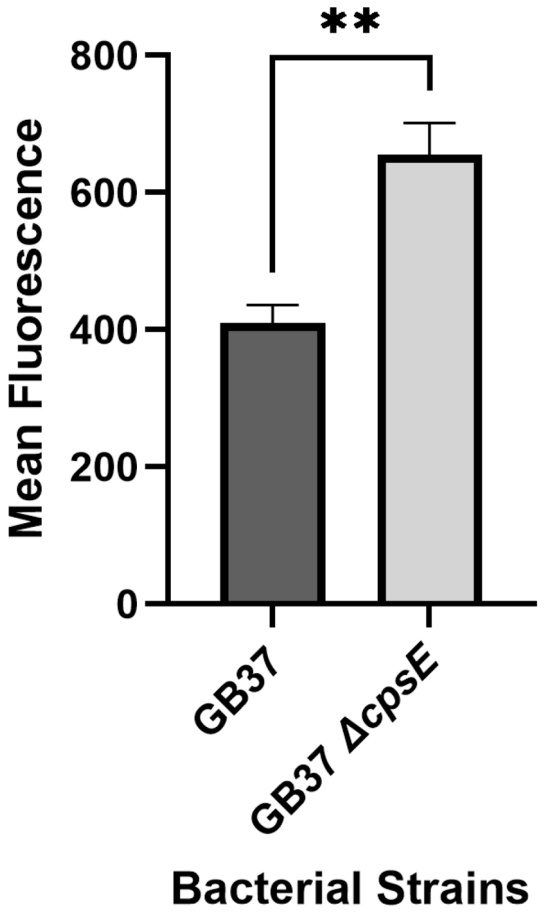

Figure 5. Evaluation of placental macrophage phagocytosis of wild-type vs *cpsE* mutant. Bacterial cells were labeled with a fluorescent dye (FITC) and cocultured with primary human placental macrophages for 3 h before the extracellular bacteria were washed away or quenched with trypan blue stain. Mean fluorescence was measured in placental macrophages cocultured with fluorescently labeled GB37 or isogenic Δ*cpsE* mutant (±SEM). An isogenic isogenic Δ*cpsE* mutant is more readily taken up by placental macrophages than the parental strain, indicating *cpsE* aids in evasion of phagocytosis. $^{**}P < 0.01$, Student's *t*-test with Welch's correction ($N = 4$).

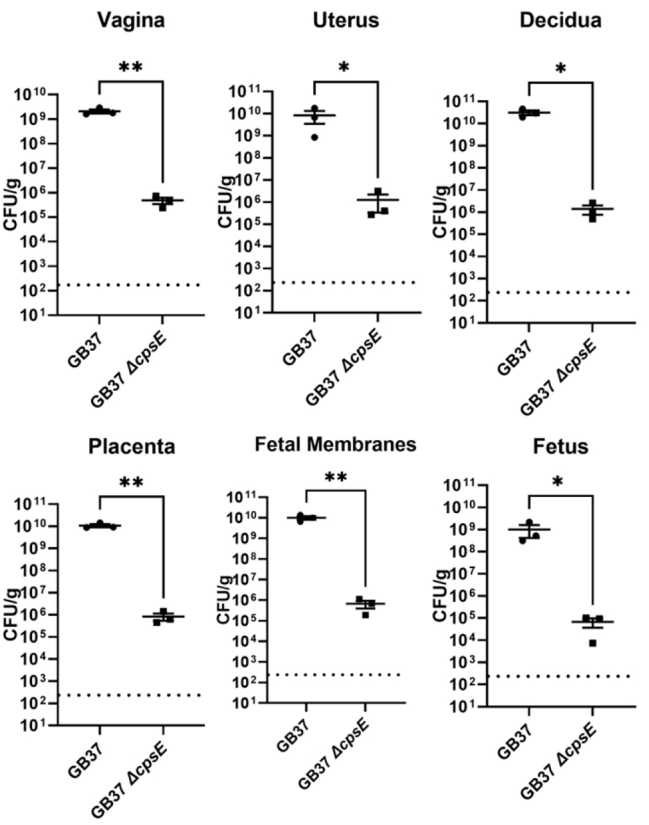

Figure 6. Analysis of bacterial burden within reproductive tissues from the mouse model of ascending GBS infection of the vagina during pregnancy. Pregnant mice received a vaginal inoculation of bacterial cells (either GB37 or isogenic Δ*cpsE* mutant) at embryonic day 13.5. Two days postinfection, mice were sacrificed, and reproductive tissues were collected, homogenized, and analyzed by a quantitative culture to determine the bacterial burden (colony forming units per gram of tissue; CFU/g). GB37 was able to ascend and invade the reproductive tract more effectively and had an enhanced burden in the vagina, uterus, decidua, placenta, and fetal membranes and fetus compared to the isogenic Δ*cpsE* mutant. $N = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, Student's *t*-test with Welch's correction. Dotted line indicates the limit of detection.

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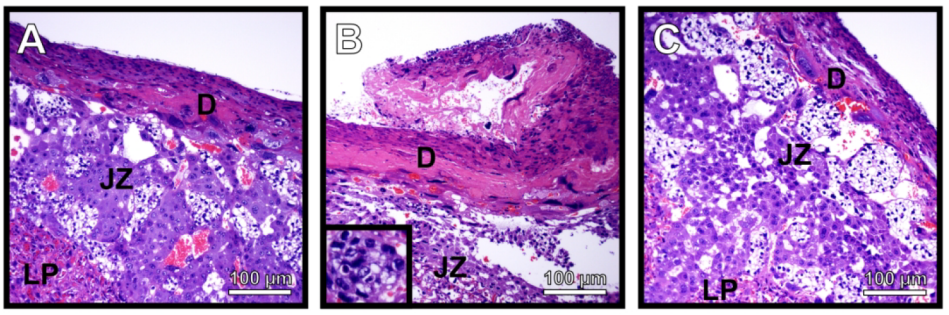

Figure 7. Histopathological evaluation of gravid reproductive tissues in response to GBS infection. Microscopical analysis of hematoxylin and eosin-stained placental tissue sections shows that (A) uninfected tissues have an intact tissue architecture with few detectable polymorphonuclear cells. (B) Infection with wild-type GB37 results in a disruption of tissue integrity and polymorphonuclear cell infiltrates (inset) indicative of inflammation. (C) Infection with the isogenic Δ*cpsE* mutant results in less disruption of tissue integrity, and fewer polymorphonuclear cell infiltrates, indicating less tissue damage in these animals. Micrographs were collected at 400× magnification and are representative of three separate experiments. Tissue compartments are labeled as follows: decidua (D), junctional zone (JZ), labyrinthine placenta (LP).

significant ($P = 0.0002$, $P < 0.0001$, $P = 0.0004$, $P = 0.0006$, respectively, by Student's *t*-test with Welch's correction).

A ΔcpsE Mutant Has Attenuated Ability to Cause Tissue Destruction and Inflammation of the Decidua and Placenta. Our previously published work has shown that GBS can invade and cross the placenta to traverse the maternal−fetal interface and cause an infection of the fetus.[24] This work also showed that, in response to a GBS infection, polymorphonuclear immune cells (neutrophils) were recruited to the decidua and placenta to exert an antimicrobial activity against the bacterial invasion. These neutrophils are a hallmark of chorioamnionitis in human patient samples and are often correlated with active infection and cognate inflammation, which leads to tissue injury and disease progression. We hypothesized that a decreased bacterial burden in these tissues could be correlated with decreased changes in immunopathology associated with a Δ*cpsE* mutant infection compared to animals infected with the parental strain. To test this, we collected fetal-placental units from uninfected, wild-type-infected, and Δ*cpsE* mutant-infected animals and performed a histopathological examination under light microscopy (Figure 7). The results indicate that uninfected animals have a preserved architecture of the decidua (maternal-facing tissues) and placenta with limited presence of polymorphonuclear cells. Conversely, an infection of the host with wild-type GBS results in a disruption of the tissue architecture consistent with a loss of tissue integrity and an enhanced presence of polymorphonuclear cells indicative of acute inflammation. Interestingly, the infection of the host with a Δ*cpsE* mutant resulted in a diminished disruption of tissue architecture and the presence of polymorphonuclear cells compared to animals infected with the parental strain, a result that was similar to uninfected controls.

DISCUSSION

Encapsulated and nonencapsulated strains of *Streptococcus* species are frequently isolated from clinical samples.[25−27] The polysaccharide capsules in *S. pneumoniae* and *S. agalactiae* (GBS) are synthesized by the gene products of the *cps* operon,[15,28,29] Within this operon, the *cpsE* locus encodes an enzyme responsible for the addition of activated sugars to a lipid carrier in the bacterial membrane.[29] Previous work in the human pathogen *S. pneumoniae* has shown that a single nucleotide base substitution (C to G at the 1135 nucleotide position) in the *cpsE* locus results in an amino acid change from arginine to glycine at residue 379 in the CpsE protein, rendering the CpsE protein enzymatically inactive. This single nucleotide polymorphism (SNP) is responsible for the lack of capsule production.[30] Similarly, our work has demonstrated that the loss of the *cpsE* locus results in a significant defect related to serotype V capsule production, indicating that the *cpsE* locus is required for the capsule biogenesis in *S. agalactiae*.

Variations in capsule serotype have been associated with variation in bacterial biofilm formation in *Streptococcus* species, highlighting the important intersection of capsule, biofilm, and pathogenesis.[31] Acidic conditions that mimic the vaginal environment enhance biofilm formation in GBS serotypes III and V.[22,32] In our study, the *cpsE* mutant exhibited an attenuated capacity to form biofilms in vitro in Todd-Hewitt broth (THB) supplemented with glucose. This result demonstrates that proper capsule production is important for a biofilm formation and that CpsE plays an essential role in the GBS serotype V capsule biosynthesis. Similarly, previous work by Xia et al. has shown that a capsule is critical for GBS serotype III biofilm formation in a cell culture medium supplemented with human plasma.[33] Interestingly, several groups have reported that acapsular mutants of *S. pneumoniae* have an enhanced ability to adhere and form biofilms on abiotic and biotic surfaces;[29] this indicates the molecular mechanisms that underpin these processes could vary across strains or species or that other environmental factors could influence a *Streptococcus* biofilm formation independent of bacterial capsule biogenesis.

In *S. pneumoniae*, the inactivation of *galU*, a gene involved in undecaprenyl-glucose metabolism, which influences capsule biosynthesis, results in an enhanced adherence to epithelial cells and phagocytosis by macrophages.[34] Similarly, a capsule has been implicated in resistance to complement activity as well as neutrophil phagocytosis in *S. pneumoniae*[35] and CPS III *S. agalactiae*.[36] A bacterial capsule has been shown to confer resistance to dendritic cell phagocytosis in both *S. agalactiae* and *S. suis*.[37,38] Results from these studies demonstrate that GBS CPS V, which lack *cpsE* and consequently experience defects in capsule biosynthesis, are unable to evade phagocytosis by placental macrophages to the same extent as GBS with a functional *cpsE* gene. Consistent with our results is a similar uptake defect by mouse alveolar macrophages of a type III GBS *cpsE* mutant.[39] Interestingly, the absence of a capsule has also been associated with worse clinical outcomes. For example, nonencapsulated *S. pneumoniae* display multidrug

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resistance,[35] and a lack of capsule in GBS CPS III was isolated from a case of endocarditis.[26] In a GBS CPS III hyperhemolytic strain (A909), a *cpsE* deletion mutant caused an increased phagocytosis in mouse bone marrow derived macrophages.[40] This is consistent with what we see in ex vivo infected human placental macrophages. However, the double mutant in that study, Δ*cpsE*Δ*covr*, caused significantly increased mortality in a mouse model after an intravenous injection of a large CFU burden (1×10^8).[40] Important differences in these two mouse models are the routes of infection (intravenous vs intravaginal), the strain capsular serotypes (III vs V), and the dose of infection. Our mouse model, with an infectious vaginal dose of 1×10^4 CFU, phenotypically mimics human GBS intrauterine infection in that there is very little maternal mortality, the primary sites of infection are the gestational tissues, and the strain was isolated from human neonatal infection. Clearly, capsule-dependent virulence varies among and within bacterial species. Additionally, the pregnant reproductive tract is an immunologically unique environment with a specific susceptibility to certain pathogens, including GBS. Our work is the first to implicate *cpsE*-associated capsule biogenesis as an important immune evasion strategy that GBS CPS V uses to circumnavigate phagocytosis by primary human placental macrophages. This has major implications for understanding the pathogenesis of a devastating infectious disease during pregnancy.

Variations in capsule-associated serotypes have been associated with streptococcal disease outcomes.[31] *S. pneumoniae* rapidly shed their capsule upon interaction with antimicrobial peptides, which allows an enhanced invasion of host epithelial cells.[41] Additional results demonstrate that the CPS type and amount affect transmission dynamics and may contribute to the marked differences in prevalence and shedding phenotypes among streptococcal strains.[42] In *S. pneumoniae*, the *cpsE* expression is linked to a capsule level in serotype 4 strain TIGR4. Mild reductions in *cpsE* transcript levels result in the reduction in capsule and avirulence in murine models of lung and blood infection.[43] Similarly, we show that the *cpsE* mutant bacterial ascension in pregnant mice is significantly lower compared to an infection with the parental wild-type strain. Thus, our study implicates *cpsE* and cognate capsule biosynthesis as an important virulence factor in ascending GBS serotype V infections of the reproductive tract during pregnancy. Additionally, infection with the isogenic *cpsE* mutant yields reduced decidual and placental architectural disruption and inflammation compared to the wild-type strain. This, in combination with decreased bacterial burden, suggests that the *cpsE* mutant does not ascend the reproductive tract as readily as the wild-type strain. It is also possible that the ascension kinetics of the mutant and wild-type strain are similar, but the differences noted in burden and tissue destruction are secondary to the increased killing of the *cpsE* mutant. In either case, this likely results in a diminished capacity to cause tissue damage.

Notably, regional shifts in the relative abundance of circulating GBS,[7] potential capsular switching,[44] and the presence of nontypeable strains[45] highlight the need for vaccine strategies that are independent of a capsular structure. Recently, a live attenuated vaccine strain of *S. pneumoniae* was generated by Amonov and colleagues.[29] This strain features gene deletion of *cpsE* and *endA* (encoding an endonuclease required for neutrophil immune evasion). This *cpsE-endA* double mutant strain has a 23-fold attenuation of virulence in a mouse model of nasopharyngeal infection. Furthermore, the murine immune response to the double mutant vaccine strain results in protection from a high dose mucosal challenge of the D39 wild-type strain of *S. pneumoniae*.[29] This study highlights the importance of understanding the biology of serotype-specific capsular streptococcal mutants for vaccine generation. Our work demonstrates that *cpsE* is a critical virulence factor in serotype V strains of GBS. We have shown that *cpsE* is vital for GBS capsule biosynthesis, biofilm formation, immune evasion, and pathogenicity. Future studies are needed to explore the use of the GBS serotype V *cpsE* mutant as a vaccine candidate to prevent GBS-associated diseases, especially in pregnancy.

CONCLUSIONS: A CONCEPTUAL MODEL OF THE ACTION OF CPSE IN GBS CAPSULE SYNTHESIS, BIOFILM FORMATION, IMMUNE EVASION, AND ASCENDING INFECTION DURING PREGNANCY

In summary, this study revealed that the *cpsE* gene is required for the secretion of polysaccharides and capsule biogenesis by GBS. The acapsular *cpsE* mutant is attenuated in its ability to form biofilms, evade phagocytosis by placental macrophages, and cause an ascending infection of the reproductive tract in a pregnant host (Figure 8).

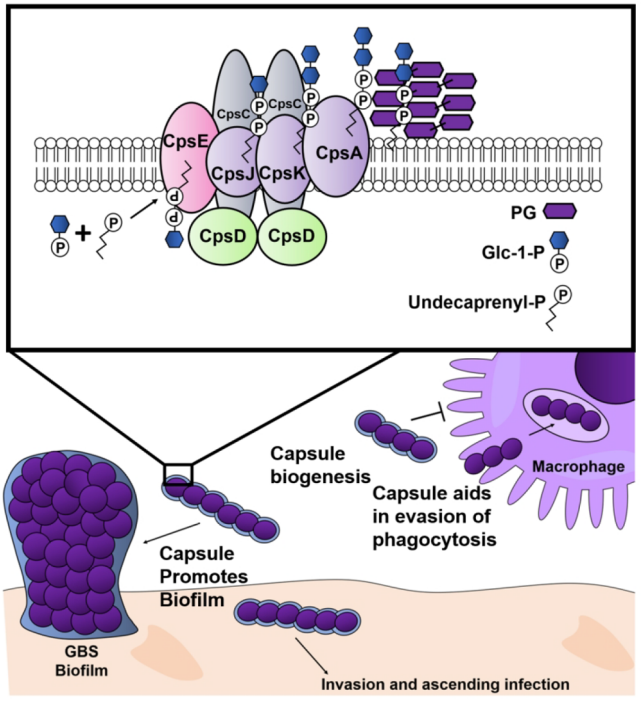

Figure 8. Conceptual model of the role of *cpsE* during a GBS infection of the reproductive tract. GBS CpsE catalyzes the transfer of glucose-1-phosphate (Glc-1-P) to an undecaprenyl phosphate acceptor, initiating capsule synthesis. CpsJ is a predicted glycosyltransferase that transports the molecule to the opposite side of the cellular membrane, where CpsK facilitates sialylation of the capsular polysaccharide. CpsA is an LytR-domain protein that acts as a transcriptional regulator and assists in insertion of the capsular polysaccharide into the peptidoglycan (PG) cell wall. Capsular biogenesis promotes biofilm formation, evasion of phagocytosis by placental macrophages, invasion, and ascending infection during pregnancy.

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METHODS

Bacterial Strains and Culture Conditions. *S. agalactiae* strain GB37 (capsular type V), a highly virulent clinical strain derived from a human case of neonatal sepsis, was used for these studies.[46] This strain is among the most common serotypes found in invasive neonatal disease, is genetically tractable, and is an established strain to study GBS pathogenesis in a pregnant mouse model of ascending vaginal infection. The GB37 *cpsE* isogenic mutant was generated as previously described.[40] Briefly, the *cpsE* mutation was amplified from A909 Δ*DcpsE* mutant chromosomal DNA with primers designed with flanking BamH1 and *Kpn*I ends, which were subsequently digested with BamH1 and Kpn1 and ligated into *Bam*HI/*Kpn*I-digested pJR233, a temperature-sensitive mutagenic plasmid, to create pJR22:Δ*cpsE*. The resulting plasmid was introduced into GB37 by electroporation, and allelic exchange was achieved by a two-step process. First, selection for isogenic derivatives carrying the plasmid was performed on agar plates supplemented with 3 μg/mL erythromycin at 30 °C. Subsequently, cultures were shifted to 37−42 °C to promote an integration into the chromosome by a homologous recombination. Integrant strains were serially passaged on a solid medium at 37 °C, and erythromycin-sensitive strains were screened for the expected deletion mutant by polymerase chain reaction (PCR) and sequencing. All bacterial strains were cultured from freezer stocks onto tryptic soy agar plates supplemented with 5% sheep blood (blood agar plates) at 37 °C in ambient air overnight. Bacteria were subcultured from blood agar plates into THB or THB supplemented with 1% glucose (THB + 1% glucose) and incubated (aerobically, shaking at 200 rpm) at 37 °C in ambient air overnight. To analyze the bacterial growth, the bacterial density was measured spectrophotometrically at an optical density of 600 nm (OD_{600}), and bacterial numbers were determined with a coefficient of 1 OD_{600} = 10^9 colony forming units (CFU) per mL.

Transmission Electron Microscopy Analyses. Bacterial strains were grown in THB + 1% glucose overnight. The following day, bacteria were prepared for a whole cell negative stain and transmission electron microscopy analyses as previously described.[47] Briefly, 10 μL of bacterial culture was spotted onto Formvar-coated copper 100 mesh grids (Electron Microscopy Sciences) and allowed to settle onto the grid for 5−10 min before supernatants were removed. Cells were stained with 1% ammonium molybdate negative stain and imaged with a Philips/FEI T12 transmission electron microscope to visualize cellular features.

Monomeric Carbohydrate Assay. Total monomeric carbohydrates within bacterial biofilms were quantified using the L-cysteine monomeric carbohydrate assay as previously described.[48] Briefly, bacteria were grown in THB overnight and subcultured at a 1:100 dilution into fresh THB + 1% glucose in 3 mL polystyrene tubes. Cultures were incubated statically at 37 °C in ambient air overnight. The following day, cultures were decanted, and adherent bacteria cells were scraped from the sides of the tubes into 0.5 mL of sterile phosphate-buffered saline (PBS). An aliquot (0.2 mL) of culture was removed, and the protein concentration was determined using a standard Bradford reagent assay in conjunction with a known standard curve. A 0.2 mL aliquot of culture was removed and subjected to the colorimetric monomeric carbohydrate assay. 0.8 mL of L-cysteine in sulfuric acid (0.07 g L-cysteine in 86% sulfuric acid solution) was added, and each sample was incubated on ice for 10 min. Samples were gently mixed and heated to 100 °C for 3 min before being plunged into ice again for 5 min. Spectrophotometric readings were taken at OD_{415} nm of both experimental samples, negative control samples, and a standard curve. A ratio of total carbohydrates to total protein was calculated to evaluate total carbohydrates normalized to cellular density.

Quantitative Analysis of Biofilms. The biofilm formation was determined by a crystal violet staining of overnight static cultures as described before.[49,50] Briefly, bacterial cultures were grown overnight in THB and subcultured at a 1:100 dilution into fresh THB + 1% glucose in culture plates (12-well). Cultures were incubated statically at 37 °C in ambient air overnight. The following day, the OD_{600} was measured for each well to ascertain the cell density, and cultures were decanted and washed three times before staining with 0.1% crystal violet. Wells were washed three times with water and allowed to dry before macroscopic imaging. Crystal violet was resolubilized in 80% ethanol/20% acetone solution, and the total biofilm quantification was measured at OD_{560}. The total biofilm to biomass was calculated by the ratio of OD_{560} of resolubilized crystal violet to the OD_{600} measurement of total cell density.

Confocal Laser Scanning Microscopy Analyses. Bacterial biofilms were analyzed by confocal laser scanning microscopy analyses (CLSM) and fluorescent staining. Bacterial cells were stained with 10 μg/mL Syto 9 (ThermoFisher; green fluorescence; excitation wavelength of 486 nm and emission at 501 nm), and extracellular carbohydrates were stained with 10 μg/mL calcofluor white (Sigma-Aldrich; blue fluorescence; excitation wavelength at 380 nm and emission at 475 nm). Samples were mounted with ProLong Gold antifade reagent and imaged with a Zeiss LSM 710 CLSM. Samples were analyzed in both widefield and confocal modalities at 630× magnification, and micrographs were collected with Zen 2010 software. Micrographs shown are representative of three biological replicates.

Scanning Electron Microscopy Analyses. Samples were prepared for scanning electron microscopy analyses as previously described.[49−51] Briefly, samples were subjected to primary fixation with 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.05 M sodium cacodylate buffer at room temperature for 24 h. Subsequently, samples were washed three times with a 0.05 M sodium cacodylate buffer and subjected to a secondary fixation step with 0.1% osmium tetroxide for 15 min. Samples were washed three times with 0.05 M sodium cacodylate buffer before being sequentially dehydrated with increasing concentrations of ethanol. After dehydration, samples were dried with a Tousimis CO_2 critical point dryer, mounted onto aluminum SEM stubs, and painted with a thin stripe of colloidal silver to dissipate excess charging. Samples were imaged with an FEI Quanta 250 field emission gun scanning electron microscope at an accelerating voltage of 5.0 keV.

Isolation of Placental Macrophages. Placental macrophages were isolated from deidentified placenta samples from term, nonlaboring caesarean section deliveries as previously described[30] and in accordance with a protocol approved[52] by the Vanderbilt University Medical Center Institutional Review Board #IRB #181998 and #00005756. Villous core tissue was macerated and enzymatically digested with hyaluronidase, collagenase, and DNase before being strained through a

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stainless-steel filter and suspended in RPMI with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), L-glutamine, and fetal bovine serum supplemented with antibiotic and antifungal factors. The sample was centrifuged at 1500 rpm at room temperature for 10 min, and the supernatant was decanted leaving a cell pellet. The pellet was strained through increasingly fine screens and enriched via a Percoll gradient. Red blood cells (RBC) were lysed with RBC lysis buffer, and macrophages were positively selected with CD14+ beads and a magnet selection. After the purification, placental macrophages were cultured in RPMI overnight at 37 °C in ambient air supplemented with 5% CO_2.

Evaluation of Placental Macrophage Phagocytosis of GBS. Evaluation of the placental macrophage phagocytosis of GBS was performed as previously described.[53] Briefly, placental macrophages were cultured in a fresh medium alone or with a 10:1 inocula of bacterial cells that were pretreated with FITC stain. Bacteria were serially diluted, and the mean fluorescence was measured to ensure FITC labeling was consistent across strains. Cocultures were incubated for 3−4 h before being washed three times with sterile PBS. The extracellular bacterial fluorescence was quenched with trypan blue stain, and the intracellular fluorescence was measured at an excitation wavelength of 495 nm and emission at 519 nm to ascertain the fluorescence intensity as a proxy for the intracellular bacterial presence within placental macrophages.

Ascending Vaginal Infection of Pregnant Mice. The GBS infection of pregnant mice and subsequent analyses were performed as previously described.[23,24,54] Briefly, C57BL6/J mice were purchased from Jackson laboratories and mated in harem breeding strategies overnight. The following day, pregnancy was confirmed by the presence of a vaginal mucus plug, establishing the embryonic date (E0.5). On embryonic day 13.5 (E13.5) pregnant dams were anesthetized via the inhalation of isoflurane and vaginally infected with 10^2−10^3 CFU in 0.05 mL of THB plus 10% gelatin. Uninfected controls were also maintained. On embryonic day 15.5 (E15.5) animals were euthanized, and necropsy was performed to harvest reproductive tissues, including the vagina, uterus, placenta, decidua, and fetal membranes, and the fetus.

Ethics Statement. All animal experiments were performed in accordance with the Animal Welfare Act, U.S. federal law, and NIH guidelines. All experiments were performed under a protocol approved by Vanderbilt University Institutional Animal Care and Use Committee (IACUC: M/14/034 and M/17/012), a body that has been accredited by the Association of Assessment and Accreditation of Laboratory Animal Care Act (AAALAC).

Quantitative Culture to Determine Bacterial Burden in Tissues. To determine the bacterial burden in reproductive tissues quantitative culture methods were employed as previously described.[24] Briefly, reproductive tissues were weighed and homogenized in sterile THB. Homogenates were subjected to a serial dilution and plated onto blood agar to determine the CFU/mg of host tissue.

Histopathological Examination of Reproductive Tissues. Reproductive tissues were subjected to a primary fixation in 10% formalin (neutral buffered) overnight. The following day, tissues were embedded in paraffin and sectioned into 5 m thick sections for staining and microscopical analyses. Sections were stained with hematoxylin and eosin for histopathological examination and imaged with an OMAX M83ES compound light microscope.

Statistical Analyses. A statistical analysis of biofilm, carbohydrate, and fluorescence quantifications was performed using the Student's *t*-test. Bacterial burden assays were analyzed by a log transformation of CFU and a Student's *t*-test with a Welch's correction. $P \leq 0.05$ was considered significant. All data analyzed in this work were derived from at least three separate biological replicates and are reported with the standard error of measure (SEM). Statistical analyses were performed using GraphPad Prism Software (ver. 9.0, GraphPad Software Inc., La Jolla, CA) and Microsoft Excel (ver. 14.6.3, Microsoft Corporation, Redmond, WA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.1c00182.

Bacterial growth and mean fluorescence assays (PDF)

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Notes

The authors declare no competing financial interest.

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