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Group B Streptococcal Pilus Proteins Contribute to Adherence to and Invasion of Brain Microvascular Endothelial Cells[▽]

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Surface filamentous structures known as pili have been discovered recently in the gram-positive streptococcal pathogens that cause invasive disease in humans, including group B *Streptococcus* (GBS). We show that two GBS proteins involved in pilus formation, encoded by *pilA* and *pilB*, also facilitate the interaction of this important agent of central nervous system infection with endothelial cells of the human blood-brain barrier.

Group B *Streptococcus* (GBS), a gram-positive bacterial pathogen, is a major cause of meningitis in newborns. In order to cause central nervous system infection, blood-borne GBS must interact with and breech the blood-brain barrier (BBB), comprised primarily of a single layer of brain microvascular endothelial cells. While GBS adheres to and invades human brain microvascular endothelial cells (hBMEC) (9), the specific GBS factors that contribute to this process are only beginning to be elucidated. Proper anchoring of lipoteichoic acid on the GBS surface facilitates hBMEC invasion, while the pore-forming β-hemolysin/cytolysin is cytolytic for hBMEC; each factor promotes BBB penetration and lethality in a mouse model of hematogenous meningitis (1, 2). A recent study has also demonstrated that GBS fibrinogen-binding protein FbsA contributes to hBMEC adherence and invasion in vitro (14).

Proteins targeted for cell surface expression in GBS are predicted to share a C-terminal sequence (L/IPXTG) for sortase recognition and anchoring to the gram-positive cell wall, and we have examined candidate genes encoding this motif for roles in the cellular pathogenesis of GBS meningitis. Serotype V GBS clinical isolate NCTC10/84 is highly virulent in the mouse model of hematogenous meningitis (2), and our PCR and sequence analysis of this strain revealed a locus encoding the transcriptional regulator RogB (5), three genes encoding proteins with C-terminal L/IPXTG anchor motifs, and two candidate sortase enzyme genes (Fig. 1A). Compared to loci present in the sequenced GBS strains NEM316 (4) and 2603V/R (15), in which the corresponding L/IPXTG-anchored proteins have been demonstrated to be components of pilus appendages (3, 10), this locus exhibited a high level of sequence identity and had a similar position in the chromosome. Comparison of our locus with the previous reports suggested that the second L/IPXTG-anchored protein (encoded by a gene designated *pilB*) could represent the major pilus subunit. Homologues of other pilus genes at a second pilus locus found in several other GBS strains (10) were not detected in the NCTC10/84 strain.

GBS pilus components, including the PilB homologue GBS59, were shown to induce protective immunity in mouse models of GBS disease and thus show promise for use in the development of novel vaccines (8, 10). Given the exposure of these components on the bacterial surface, we hypothesized that pilus expression by GBS could also play a role in the bacterium's initial interactions with hBMEC. Utilizing a molecular genetic approach that combined targeted allelic replacement and heterologous gene expression, we examined the role of two candidate surface-expressed pilin proteins in GBS adherence to and invasion of hBMEC.

To verify the presence of pili on the surface of GBS test strain NCTC10/84, we performed negative staining transmission electron microscopy of intact bacterial cells. Briefly, bacterial suspensions were deposited on Formvar-carbon copper grids precoated with 1% fibronectin. Adherent bacteria were subsequently fixed in 2% paraformaldehyde, washed three times for 5 min in water, and stained with 2% acidic uranyl acetate. As shown in Fig. 1B, characteristic fibrous organelles, or pili, extending from the bacterial surface were visualized. To demonstrate the association of PilB with these structures, we performed immunogold labeling essentially as described previously (10) using mouse antisera raised against recombinant GBS59 (PilB) protein (a generous gift from Guido Grandi, Immaculada Margarit, and colleagues, Novartis/Chiron Vaccines, Siena, Italy). Gold particles were localized on the GBS surface in pilus-like appendages (Fig. 1C and D). No immunogold labeling was observed using preimmune antisera (data not shown).

To probe the functional role of PilA and PilB, we generated knockout mutants of GBS strain NCTC10/84 with mutations in each gene by precise, in-frame allelic replacement with a chloramphenicol resistance cassette using our established methods (6). These mutant strains exhibited growth kinetics equivalent to those of the wild-type (WT) GBS parent strain in Todd-Hewitt broth and the RPMI-based culture medium used in our in vitro assays. Additionally, no differences were observed between the WT and mutant strains with respect to β-hemolysin/cytolysin activity or sensitivity to the penicillin and gentamicin concentrations used in our invasion assays (data not shown).

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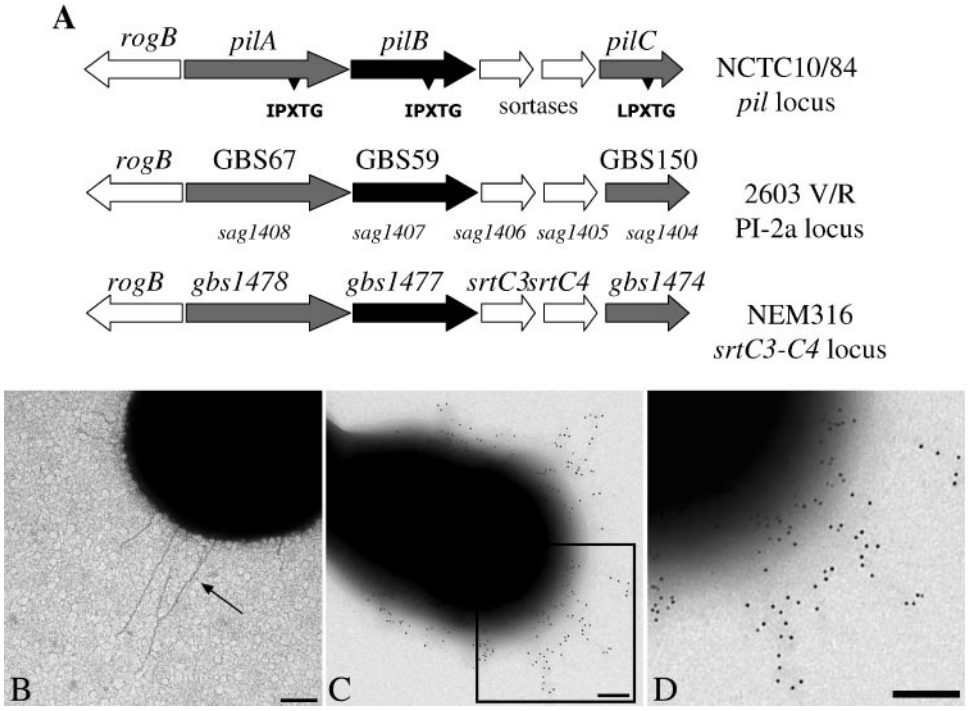

FIG. 1. (A) Schematic diagram of the NCTC10/84 GBS *pil* locus containing *pilA*, *pilB*, and *pilC* compared to previously characterized pilus loci in strains NEM316 (3) and 2603R/V (10). (B) Transmission electron microscopy and negative staining of pilus-like structures (indicated by an arrow) on the cell surface of GBS strain NCTC10/84. (C) Immunogold labeling and transmission electron microscopy with mouse sera against GBS59 (PilB). NCTC10/84 was immobilized on Formvar-carbon copper grids precoated with fibronectin (1%) and stained with specific antisera and 6-nm colloidal gold-conjugated anti-mouse immunoglobulin G antibody. (D) Higher magnification of the area indicated in panel C. Scale bars = 100 nm.

The GBSΔ*pilA* and GBSΔ*pilB* mutants were analyzed using our well-characterized in vitro model of the BBB (2, 9), consisting of hBMEC that maintains the morphological and functional characteristics of primary brain endothelial cells (7, 12). Cell maintenance and quantitative assays for GBS hBMEC adherence and invasion were performed as described previously (9). Briefly, invasion of hBMEC by GBS was quantified using a standard antibiotic protection assay in which intracellular organisms were recovered and enumerated after selective killing of extracellular bacteria by antibiotic treatment. To assess the level of surface-adherent (total cell-associated) bacteria for the mutant and WT strains, bacteria were quantified from hBMEC monolayers prior to the addition of extracellular antibiotics. The data were expressed as percentages of adherence or invasion based on the original inoculum (10^5 CFU; multiplicity of infection, 1 to 3 bacteria/cell) and then were normalized to levels calculated for the WT GBS strain. As shown in Fig. 2A, the GBSΔ*pilA* mutant was 60% less adherent to hBMEC than the WT strain, while for the GBSΔ*pilB* mutant there was not a significant change in adherence. Conversely, when strains were analyzed to determine their abilities to invade hBMEC, the GBSΔ*pilB* mutant exhibited a significant reduction in invasion (40%) compared to the invasion by the WT and GBSΔ*pilA* strains. Thus, it appears that the PilA protein contributes to the initial attachment of GBS to brain endothelium, while the PilB protein contributes to the process of bacterial internalization.

In order to further establish that the GBS *pilA* and *pilB* genes specifically contribute to hBMEC adherence and invasion, respectively, we performed single-gene complementation and heterologous expression analyses. Both *pilA* and *pilB* genes were amplified from GBS genomic DNA, cloned into the *Escherichia coli*-streptococcal shuttle expression vector pDCerm (6) to obtain pDC*pilA* and pDC*pilB*, and used to transform the corresponding GBSΔ*pilA* and GBSΔ*pilB* mutants. Complementation of the Δ*pilA* and Δ*pilB* mutant strains with the corresponding genes on plasmid vectors reversed the observed adherence (PilA) and invasion (PilB) defects (Fig. 2A and B). The presence of the vector-only control in the Δ*pilA* and Δ*pilB* mutant strains did not affect the recovery of adherent or intracellular bacteria (data not shown). To determine if the *pilA* and *pilB* genes are sufficient for hBMEC adherence and invasion, the pDC*pilA* and pDC*pilB* constructs were used to transform the nonpathogenic, noninvasive, gram-positive bacterium *Lactococcus lactis*. The empty pDCerm expression vector in *L. lactis* served as a control. Heterologous expression of the *pilA* gene in *L. lactis* resulted in a dramatic increase (~20-fold) in adherence to hBMEC (Fig. 2C), while similar expression of *pilB* increased the ability of this nonpathogen to invade hBMEC ~55-fold (Fig. 2D). Together, our results demonstrate that *pilA* is necessary and sufficient for hBMEC adherence, while *pilB* is necessary and sufficient for hBMEC invasion.

In summary, here we provide evidence that genes encoding surface-associated pili play a role in the ability of GBS to adhere to and invade brain endothelium. Pili and pilus assembly have been best described in gram-negative bacteria, where

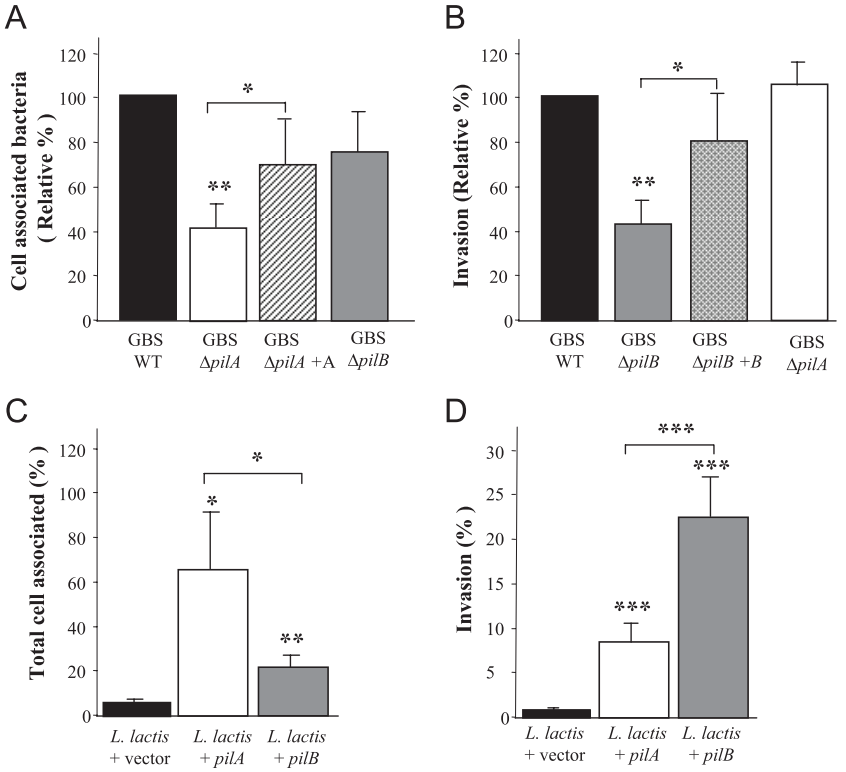

FIG. 2. GBS PilA and PilB contribute to hBMEC adherence and invasion. (A) GBS WT, mutant, and complemented strains were incubated with hBMEC at a multiplicity of infection of 1 to 3 for 30 min, after which adherent or total cell-associated bacteria were isolated and enumerated. (B) Intracellular organisms were quantified following 2 h of incubation with hBMEC and 2 h of incubation with penicillin (5 μg/ml) and gentamicin (100 μg/ml) to kill extracellular bacteria. The percentages of total cell-associated and invasive bacteria were calculated on the basis of the initial inoculum and are expressed relative to the value for the WT GBS strain. (C and D) Adherence (C) and invasion (D) assays were performed using *L. lactis* expressing vector alone or the cloned GBS *pilA* and *pilB* genes. All data are data from a representative experiment, and at least three experiments were performed. The error bars indicate the 95% confidence intervals of the means of three wells. The differences in the values for recovered bacteria were analyzed by Student's *t* test, and the data were compared to data for the parent strain or for other strains as indicated by brackets (one asterisk, $P < 0.05$; two asterisks, $P < 0.005$; three asterisks, $P < 0.0005$).

pili are known to mediate host-pathogen interactions important in colonization or the development of disease (11), and it is likely that they have a similar function in gram-positive pathogens (13). Our results suggest that the GBS PilA protein contributes to initial attachment to hBMEC as the GBSΔ*pilA* mutant was less adherent to host cells. These results are consistent with recent work demonstrating that the PilA homologue GBS 1478, but not the PilB homologue GBS 1477 (Fig. 1A), promoted adhesion to human pulmonary epithelial cells (3). The GBSΔ*pilA* mutant did not exhibit a decrease in invasive ability compared to the WT strain. It is possible that the absence of PilA from the pilus structure results in increased interactions between PilB and hBMEC. Our results also demonstrate that PilB, a major pilus component, mediates GBS intracellular invasion of brain endothelium, a critical step in BBB penetration during GBS meningitis. We speculate that bacterial pilus components, including PilA and potentially PilC, promote the initial contact and attachment of GBS to host cell surfaces, which is followed by PilB-mediated initiation of bacterial internalization. The mechanism by which pili and pilus proteins facilitate bacterial invasion of host cells remains to be determined. Future examinations of the role of these specialized surface appendages in the pathogenesis of central nervous system infection by GBS and other pilus-expressing meningeal pathogens (e.g., *Streptococcus pneumoniae*) are merited.

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