The Novel Fibrinogen-Binding Protein FbsB Promotes *Streptococcus agalactiae* Invasion into Epithelial Cells

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*Streptococcus agalactiae* is a major cause of bacterial sepsis and meningitis in human newborns. The interaction of *S. agalactiae* with host proteins and the entry into host cells thereby represent important virulence traits of these bacteria. The present report describes the identification of the *fbsB* gene, encoding a novel fibrinogen-binding protein that plays a crucial role in the invasion of *S. agalactiae* into human cells. In Western blots and enzyme-linked immunosorbent assay (ELISA) experiments, the FbsB protein was demonstrated to interact with soluble and immobilized fibrinogen. Binding studies showed the N-terminal 388 residues of FbsB and the Aα-subunit of human fibrinogen to recognize each other. By reverse transcription (RT)-PCR, the *fbsB* gene was shown to be cotranscribed with the *gbs0851* gene in *S. agalactiae*. Deletion of the *fbsB* gene in the genome of *S. agalactiae* did not influence the binding of the bacteria to fibrinogen, suggesting that FbsB does not participate in the attachment of *S. agalactiae* to fibrinogen. In tissue culture experiments, however, the *fbsB* deletion mutant was severely impaired in its invasion into lung epithelial cells. Bacterial invasion could be reestablished by introducing the *fbsB* gene on a shuttle plasmid into the *fbsB* deletion mutant. Furthermore, treatment of lung epithelial cells with FbsB fusion protein blocked *S. agalactiae* invasion of epithelial cells in a dose-dependent fashion. These results suggest an important role of the FbsB protein in the overall process of host cell entry by *S. agalactiae*.

*S. agalactiae* is a frequent cause of pneumonia, sepsis, and meningitis in neonates, with a prevalence of 0.2 to 2 cases per 1,000 live births (2, 4, 5, 7, 58). *S. agalactiae* is also the cause of substantial pregnancy-related morbidity and has emerged as an increasingly common cause of invasive disease in the elderly and in immunocompromised persons (77, 83). Besides being the causative agent of many different types of infections, *S. agalactiae* is also part of the normal flora of the human gastrointestinal and genital tract. In the United States and in different European countries, the genital tracts of 15 to 35% of pregnant women are colonized with *S. agalactiae* (58, 61). About 50% of infants born to these women become colonized with *S. agalactiae* during delivery and 1% of the colonized infants develop a severe *S. agalactiae* infection, including pneumonia, sepsis and/or meningitis (61). The main route of neonatal infection is ascending spread of *S. agalactiae* into the amniotic fluid and aspiration of contaminated amniotic fluid by the fetus. After gaining access to the lung, the bacteria can colonize and infect the lung, resulting in pneumonia. Subsequent transmigration of *S. agalactiae* across the epithelial border allows the bacteria to invade the bloodstream and eventually reach the meninges. It is hypothesized that the invasion of host cells represents an important pathogenicity mechanism in invasive *S. agalactiae* disease (53, 54). Numerous studies have demonstrated the capability of *S. agalactiae* to invade human cells (22, 31, 34, 44, 53, 54, 66, 73). However, the underlying events of host cell invasion by *S. agalactiae* are only poorly understood.

In many pathogenic bacteria, the invasion of host cells is mediated by bacterial surface proteins that recognize specific ligands in the extracellular matrix (ECM) or on the surface of host cells. The ECM of mammalian tissues is a stable macromolecular structure underlying epithelial and endothelial cells, and it is composed of structural glycoproteins such as collagen, laminin, fibronectin, and fibrinogen (29).

Several interactions between *S. agalactiae* and ECM components have been reported and are proposed to be of importance for bacterial tissue adhesion and invasion. *S. agalactiae* binds to human laminin via the cell wall protein Lmb (63). However, a role of this interaction for the virulence of *S. agalactiae* has yet to be defined. Fibronectin-binding by *S. agalactiae* is dependent on the glutamine transporter gene *glnQ* (67) and is mediated by the C5a peptidase of the bacteria (3). As described by Cheng et al. (8), C5a peptidase also plays a role in the invasion of host cells by *S. agalactiae*. Recently, we identified in *S. agalactiae* the fibrinogen-binding protein FbsA and demonstrated that FbsA protects the bacteria from opsonophagocytosis in human blood (57).

In the present report we describe the isolation and characterization of a novel *S. agalactiae* fibrinogen-binding protein, termed FbsB. We present data about the ligand-binding domain in the FbsB protein and its binding site within human fibrinogen. A respective *fbsB* deletion mutant was constructed to unravel the role of FbsB in the interaction of *S. agalactiae* with human fibrinogen. Furthermore, tissue culture experiments were performed to elucidate the importance of FbsB for the adherence and the invasion of epithelial cells by *S. agalac-
Our findings indicate that FbsB plays an important role in the entry of *S. agalactiae* into epithelial cells.

**Bacterial strains and culture conditions.** *S. agalactiae* 6313 is a serotype III clinical isolate obtained from an infected neonate (73). *Escherichia coli* dh5α (27) was used for cloning purposes, and *E. coli* BL21 (18) served as host for the production of FbsB, BspA, and Gbs0851 fusion proteins. *S. agalactiae* was cultivated at 37°C in Todd-Hewitt broth (THY) consisting of Todd-Hewitt broth with 1% yeast extract. Recombinant *S. agalactiae* clones, carrying the plasmid pGEM-5Z (Promega), encoding region, was amplified into *E. coli* DH5α (27). Partial digestion of the cosmid with SstI, cloning the BamHI sites used for cloning are underlined. After the C-terminus-encoding region of fbsB was performed with the primers 5′-CTTCATTAACAATATCTGAG-3′ and 5′-GAG, and the region CCAGCTACATCGG and 5′-TTCGCAACTGGTATACTCAGGAC was amplified with the primers fbsB_del5 5′-CCCACTCCTGACTCCTATCAACATCAGGACGTTAAGGCACT-3′ and fbsB_del4 5′-GGGGGATCCCTTCATTAACAATATCTGAG-3′. The PCR product was cut with HindIII and SalI, ligated, and transformed into *E. coli* DH5α. The resulted plasmid, pGEM-5Z, was electroporated into *S. agalactiae* 6313, and transformants were grown at 30°C with erythromycin selection. Cells in which pGEM-5Z had integrated into the chromosome were sequenced at 37°C under erythromycin pressure. Four of such strains were serially passaged for 6 days in liquid medium at 30°C without erythromycin selection to facilitate the excision of plasmid pGEM-5Z, leaving the desired fbsB deletion in the chromosome. Dilutions of the serially passaged cultures were transferred onto agar plates, and single colonies were tested for erythromycin sensitivity to identify pGEM-5Z excisants. Chromosomal DNA of *S. agalactiae* 6313 and of 24 erythromycin-sensitive *S. agalactiae* excisants were HindIII digested and tested by Southern blot, using a digoxigenin-labeled probe, obtained by PCR with the primers fbsB_del1 and fbsB_del4.

**Plasmid-mediated expression of fbsB in *S. agalactiae*.** A DNA fragment containing the lactococcal P23 promoter (76) was isolated from plasmid pMG36e (75) by EcoRI/SmaI digestion and ligated into the EcoRI/SmaI-digested vector pTA26 (71), resulting in the expression vector pAT32. The fbsB structural gene, including its ribosomal binding site, was amplified from *S. agalactiae* 6313 DNA by PCR using the primers 5′-CCAGCTACATCGG and 5′-CCGACAACCTGACCTTTATACCGGATAG-3′. The PCR product was performed with the primers 5′-GTGCTCTGCTCAGGTGTTAAGGCACTCCTTCTTAATACCCGGGATAG-3′ and 5′-CCGACAACCTGACCTTTATACCGGATAG-3′. The N-terminus-amplification region of fbsB was performed with the primers 5′-GTGCTCTGCTCAGGTGTTAAGGCACTCCTTCTTAATACCCGGGATAG-3′ and 5′-CCGACAACCTGACCTTTATACCGGATAG-3′. The NcoI and XhoI restriction sites used for cloning are underlined. After the fbsB-deleted PCR derivative was amplified and of plasmid pET28a with NcoI and XhoI, the fbsB derivatives were ligated into pET28α and transformed into *E. coli* BL21. The full-length FbsB fusion protein was termed FbsB, and the fusion proteins that carried the N or the C terminus of FbsB were accordingly named FbsB-N and FbsB-C, respectively. The gbs0851 gene, devoid of its signal-peptide-encoding sequence was amplified with the primers CCGCGGAATTCATCGG and TGGCACAAGCTTCTTACCTTGAGCGAAGAC. The BamHI and HindIII restriction sites used for cloning are underlined. The obtained PCR product was digested with BamHI and HindIII, and ligated into the BamHI/HindIII-digested vector pET28α. After transformation into *E. coli* BL21, the resultant clone produced the Gbs0851 fusion protein.

**RNA preparation, RT-PCR and quantitative real-time PCR analysis.** *S. agalactiae* 6313 was grown in 50 ml of THY broth to exponential growth phase (optical density at 600 nm = 0.30), and RNA was isolated using the RNeasy kit (Qiagen) as described previously (51). Contaminating DNA was degraded for 1 h in 150 U of RNase-free DNase (Promega). In control experiments, RNA samples were subjected to PCR amplification without prior reverse transcription. As no PCR amplification products were obtained, DNA contamination during RNA preparation could be excluded. Reverse transcription of RNA and subsequent PCR was carried out with 1 μg of RNA, using Ready-To-Go RT-PCR beads (Amersham/Pharmacia) according to the instructions of the manufacturer. The transcriptional organization of the region gbs0851 to gbs0859 to fbsB was analyzed with the primers 5′-GGCGGATCCCTACTCTTTTATACGCGATG-3′ and 5′-CAATCCCAGGGAGCATC-3′.

**Preparation of the fusion proteins FbsB, BspA, Gbs0851, and Bsp,** respectively. Fusion proteins were synthesized in recombinant *E. coli* BL21 by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) after the culture had reached an optical density of 1.0. The cells were disrupted using a French Press cell and purification of the fusion protein was performed according to the instructions of Qiagen using Ni²⁺-affinity chromatography. FbsB fusion proteins were subsequently dialyzed against 20 mM Tris–HCl, pH 8.0, loaded onto a MonoQ column (Amersham/Pharmacia), and eluted by fast-performance liquid chromatography with a linear NaCl gradient in 20 mM Tris–HCl, pH 8.0. The fusion proteins were finally dialyzed against phosphate-buffered saline (PBS). The Bsp protein represents a cell surface protein from *S. agalactiae*, involved in the morphogenesis of the bacteria (52). The synthesis of the fusion proteins FbsA and Bsp has been described previously (52,57).

**Western blot and dot blot analysis.** For Western blot experiments, proteins were size-separated by SDS-PAGE and electroblotted onto nitrocellulose. In dot blot experiments, proteins were directly spotted onto a nitrocellulose membrane. The membrane was subsequently blocked overnight with 1% (w/v) blocking (nonfat dry milk) in PBS and incubated for 1 h at room temperature in a blocking solution. FbsB fusion proteins or human fibronectin at a concentration of 2 μg/ml. To remove unbound proteins, the membrane was washed three times with PBS. The mem-
brane was subsequently incubated for 1 h with either rabbit anti-fibrinogen antibodies (1:1,000 in PBS) or mouse anti-HisTag antibodies (1:500 in PBS). Afterwards, the membrane was washed three times with PBS containing 0.05% Tween 20 (PBST), followed by three washes with PBS. The membrane was subsequently incubated for 1 h with either peroxidase-labeled anti-rabbit IgG (1:1,000 in PBS) or with peroxidase-labeled anti-mouse IgG Fab fragments (1:20,000). To remove unbound antibodies, the membrane was washed again three times with PBST and two times with PBS. Finally, peroxidase-conjugated antibodies were detected by chemiluminescence, using the ECL kit from MoBiTec.

Detection of fibrinogen-binding by ELISA. Microtiter plates were coated with human fibrinogen (21 nM) in 300 μL of PBS at 4°C overnight, and nonspecific binding sites were blocked with 10% bovine serum albumin in PBS for 1 h at room temperature. Different concentrations of the fusion proteins FbsA, FbsB, FbsB-N, or Bsp in 200 μL of PBS were added to the wells, and this was followed by a 1-h incubation at room temperature. Bound fusion proteins were detected by incubating the wells for 1 h with mouse anti-HisTag antibodies (1:500 in PBS), followed by peroxidase-labeled goat anti-mouse IgG Fab fragments (1:10,000 in PBS). The peroxidase reaction was started by the addition of 100 μL 3,3',5,5'-tetramethyl-benzidine (Sigma), and the color reaction was stopped with 100 μL 2 M H2SO4. The absorption of the solution at 450 nm was quantitated in an enzyme-linked immunosorbent assay (ELISA) reader (Anthos hthIII). After every incubation, the plate was washed three times with PBST. Capture ELISA experiments, which tested the influence of FbsB protein on the binding of FbsA protein to immobilized fibrinogen were performed with the following modifications: Each well was incubated for 1 h with a fixed concentration of FbsA protein (0.36 μM) and increasing concentrations of FbsB protein (0.004, 0.084, 0.238, 0.475, 0.699, 1.160, 2.320, or 4.640 μM). Bound FbsA protein was detected with mouse anti-FbsA antibodies (1:1,000 in PBS), followed by peroxidase-labeled goat anti-mouse IgG Fab fragments (1:10,000 in PBS). Dissociation constants (Kd) for the binding of FbsA, FbsB and FbsB-N to human fibrinogen were calculated as described elsewhere (6, 38).

Binding of FITC-labeled S. agalactiae to immobilized fibrinogen and fibronectin. Terasaki plates were coated with human fibrinogen and fibronectin and the binding of fluorescein isothiocyanate (FITC)-labeled bacteria to the immobilized fibrinogen or fibronectin was measured as described previously (26).

Epithelial cell adherence and invasion assay. Adherence of S. agalactiae to epithelial cells and invasion into epithelial cells was assayed as previously described (26). Briefly, A549 cells were transferred to 24-well tissue culture plates at approximately 4 × 10^5 cells per well and cultivated overnight in RPMI tissue culture medium, supplemented with 10% of fetal calf serum. After replacement of the medium with 1 ml of fresh medium, the cells were infected with S. agalactiae at a multiplicity of infection of 10:1, and incubated for 2 h at 37°C. As S. agalactiae reveals growth in tissue culture medium, thereby influencing the number of bacteria that can adhere to and invade the host cells, the number of bacteria after growth for 2 h in tissue culture medium was set as input inoculum as described elsewhere (19). To determine the number of cell-adoherent bacteria, the infected host cells were washed three times with PBS, lysed with distilled water, appropriate dilutions were plated onto agar plates, and the number of CFU was subsequently counted. Intracellular bacteria were determined after a further incubation of the infected cells for 2 h with RPMI medium, containing penicillin G (10 U) and streptomycin (0.01 mg) to kill extracellular bacteria. After three washes with PBS, the A549 cells were lysed in distilled water and the amount of intracellular bacteria was quantitated by plating serial dilutions of the lysate onto agar plates. The number of cell-adoherent and intracellular bacteria was related to the number of bacteria after growth for 2 h in RPMI medium. All assays were performed in triplicate and the experiments were repeated at least three times.

To assess the effect of FbsB fusion proteins on the adherence and invasion of S. agalactiae, the adherence and invasion assays were performed as described above, with the following modifications: Epithelial cells in tissue culture wells were incubated for 15 min in 100 μL of PBS with different amounts of purified proteins as described elsewhere (39). Bacterial cells were then added in tissue culture medium and the wells were incubated at 37°C for 2 h. The remainder of the experiment was carried out as described above.

Nucleotide sequence accession numbers. The genomic DNA sequence of S. agalactiae NEM 316 can be accessed in the EMBL database under accession number AL73256. In the SwissProt TREPML database, the polypeptides described in this report and their accession numbers are as follows: Gbs0849 (Q8E5Y0), FbsB (Q8E5X9), Gbs0851 (Q8E5X8), and Gbs0853 (Q8E5X6).

RESULTS

Isolation of the fbsB gene, encoding a fibrinogen-binding protein. A screen for fibrinogen-binding proteins from S. agalactiae 6313 previously identified three cosmids with genomic S. agalactiae fragments that conferred fibrinogen-binding activity to E. coli DH5α (57). Partial digest of one of the cosmids resulted in the isolation of the fbsA gene, encoding a fibrinogen-binding protein from S. agalactiae (57). However, one of the three cosmids did not hybridize to an fbsA-specific probe in Southern blot experiments (data not shown). We therefore suggested that this cosmids carries a gene, encoding a fibrinogen-binding protein which is distinct from the FbsA protein. This prompted us to initiate studies to isolate and characterize the respective gene from S. agalactiae. After partial digestion of the respective cosmids with Sau3A and subcloning of fragments in the range of 2 to 4 kb in the plasmid pGEM-5Z, we obtained a DNA fragment of 2.9 kb that conferred fibrinogen-binding activity to E. coli DH5α (data not shown). Western blot analysis identified in crude extracts of this strain a single fibrinogen binding protein of 66 kDa (data not shown). Sequencing of the insert in pGEM-5Z identified two complete open reading frames (ORF), exhibiting 100% identity to the ORFs gbs0850 and gbs0851 in the genome of S. agalactiae NEM 316 (23). The localization of the two ORFs in the genomic context of S. agalactiae NEM 316 is depicted in Fig. 1. As subsequent studies demonstrated that gbs0850 encodes a fibrinogen-binding protein, this ORF was termed fbsB. The fbsB gene has a size of 1,905 bp and encodes a protein of 635 amino acids (aa) with a putative signal peptide of 27 aa at its N terminus. The gbs0851 gene has a length of 543 bp and codes for a polypeptide of 181 aa with a putative signal peptide sequence of 23 aa at its N terminus. Neither of the two proteins carries a typical cell wall anchor motif (LPXTG) of gram-positive bacteria at its C terminus. In database analysis, the FbsB protein revealed 22% identity to the fibronectin-binding protein SfbX from S. pyogenes (33). Profile searches did not identify in the FbsB polypeptide motifs with similarity to sequences stored in the PROSITE database. As predicted by the Kyte and Doolittle algorithm (37), the mature FbsB protein is devoid of transmembrane regions. However, the FbsB protein contains two C-terminal repeat regions, from aa 411 to 431 and from aa 456 to 476 (Fig. 1), that exhibit 33% identity to each other. In database analysis, the Gbs0851 protein did not reveal similarity to polypeptides of other organisms.

The FbsB protein binds to human fibrinogen. To unravel the identity of the novel fibrinogen-binding protein from S. agalactiae, the genes fbsB and gbs0851, devoid of their signal peptide-encoding sequences, were cloned in-frame into the expression vector pET28. The vector placed a hexahistidine affinity tag to the C terminus of the resultant FbsB protein, and to the N terminus of the Gbs0851 fusion protein. The fusion proteins FbsB and Gbs0851 were synthesized in E. coli BL21 and subsequently purified by Ni2⁺-affinity chromatography. In Western ligand blots, solely the FbsB fusion protein revealed binding to soluble fibrinogen (Fig. 1B), demonstrating that FbsB represents the novel fibrinogen-binding protein from S. agalactiae. Neither FbsB nor Gbs0851 interacted with human fibronectin, laminin, or collagen I or IV in Western blots (data not shown). To localize the fibrinogen-binding domain in
FbsB, the N-terminal and C-terminal regions of FbsB were synthesized as FbsB-N and FbsB-C fusion proteins which were subsequently tested for fibrinogen binding. As depicted in Fig. 1B, the FbsB-N fusion protein exhibited binding to fibrinogen, indicating that the N-terminal 388 aa of FbsB mediate binding to human fibrinogen.

**FbsB binds to fibrinogen in a dose-dependent and saturable manner.** The ability of the different FbsB proteins to bind to immobilized fibrinogen was quantified in an ELISA. In these studies, the fibrinogen-binding protein FbsA served as positive, and the Bsp protein, involved in the morphogenesis of the bacteria (52), as negative control. Human fibrinogen was immobilized in microtiter wells and incubated with increasing concentrations of the different fusion proteins. Bound fusion proteins were detected with anti-HisTag antibodies, followed by peroxidase-conjugated goat anti-mouse antibodies. As depicted in Fig. 2, the proteins FbsA, FbsB, and FbsB-N bound avidly in a dose-dependent and saturable manner to immobilized fibrinogen. In contrast, the fusion proteins Bsp and FbsB-C failed to bind to human fibrinogen, confirming that the N terminus of FbsB recognizes human fibrinogen. Binding of FbsA, FbsB, and FbsB-N to immobilized fibrinogen could be abrogated with an excess of soluble fibrinogen (data not shown), demonstrating the specificity of the interaction between these proteins and fibrinogen. The FbsA protein interacted with human fibrinogen with an apparent $K_D$ of $1.6 \times 10^{-8}$ M, which corresponds to previous findings (57). The proteins FbsB and FbsB-N revealed apparent $K_D$ values of $2.6 \times 10^{-7}$ M and $1.3 \times 10^{-6}$ M, respectively, indicating a significantly lower affinity of FbsB to fibrinogen. The data also suggest that truncation of the C terminus of FbsB decreases the affinity of the FbsB protein to human fibrinogen.

As *S. agalactiae* possesses the FbsA protein as well as FbsB, we addressed the question if the two proteins compete with each other for the binding to human fibrinogen. For this purpose, fibrinogen was immobilized onto microtiter wells, and purified FbsA was captured to fibrinogen in the presence of increasing concentrations of FbsB protein. Binding of FbsA to fibrinogen was detected by ELISA with mouse anti-FbsA antibodies and peroxidase-conjugated anti-mouse antibodies.
However, fibrinogen-binding of FbsA remained unaffected by an excess (up to 13-fold) of FbsB protein (data not shown), suggesting distinct binding sites of FbsA and FbsB in human fibrinogen.

The FbsB protein binds to the Aα-subunit of human fibrinogen. Fibrinogen is composed of six polypeptide chains—two Aα-, two Bβ-, and two γ-chains—that can be separated from each other on a polyacrylamide gel under reducing conditions. To unravel the binding sites of FbsA and FbsB in human fibrinogen, the Aα-, Bβ-, and γ-chains of fibrinogen were size-separated by SDS-PAGE, transferred onto a nitrocellulose filter, and probed with FbsA or FbsB fusion proteins by Western blot analysis. As control, the binding of the FbsA and FbsB fusion proteins to native human fibrinogen was tested in dot blot experiments. Binding of the fusion proteins was detected with anti-HisTag antibodies and peroxidase-conjugated anti-mouse IgG Fab fragments. As shown in Fig. 3B, both fusion proteins interacted with native fibrinogen in dot blot experiments, confirming that FbsA and FbsB bind to native, immobilized fibrinogen. Western blot analysis with fibrinogen subunits that had been separated from each other by SDS-PAGE under reducing conditions revealed binding of the FbsB fusion protein to the Aα-subunit of human fibrinogen whereas the FbsA protein did not interact with any of the subunits (Fig. 3A). In the absence of fusions proteins, no binding of the used antibodies to human fibrinogen was observed. These findings suggest that the FbsA protein requires for fibrinogen-binding the three-dimensional structure or different subunits of the hexameric protein.

The fbsB gene forms an operon with the gene gbs0851. To study the transcriptional organization of the fbsB gene in S. agalactiae, total RNA from S. agalactiae 6313 was subjected to RT-PCR analysis. Each of the primer pairs amplified from chromosomal S. agalactiae DNA a product of the expected size (Fig. 4). In the absence of reverse transcriptase, no PCR products were obtained from total S. agalactiae RNA (Fig. 4). As shown in Fig. 4, RT-PCR specifically amplified the region fbsB to gbs0851 from S. agalactiae RNA, suggesting that the genes fbsB and gbs0851 comprise an operon in S. agalactiae. No amplification products were obtained by RT-PCR with primer pairs specific to the genes gbs0849 to fbsB, and gbs0851 to gbs0853, respectively, indicating transcriptional initiation upstream of fbsB and transcriptional termination downstream of gbs0851.

The FbsB protein is not required for fibrinogen-binding by S. agalactiae. As a first step to determine the role of FbsB for the interaction of S. agalactiae with fibrinogen, the fbsB gene was deleted in the chromosome of S. agalactiae 6313. Successful deletion of fbsB in the genome of S. agalactiae 6313 was confirmed by Southern blot analysis (data not shown), and the resultant mutant was termed accordingly S. agalactiae ΔfbsB. Quantitative real-time PCR analysis revealed equal expression of the gbs0851 gene in the S. agalactiae strains 6313 and ΔfbsB (data not shown), demonstrating that the deletion of the fbsB gene in mutant ΔfbsB does not influence its gbs0851 expression. For complementation studies, the fbsB gene was cloned into the expression vector pAT32, placing the fbsB gene under the control of the lactococcal P32 promoter. The resultant plasmid, pAT/fbsB, was subsequently introduced into S. agalactiae ΔfbsB. Additionally, the vector pAT32 was transformed into the S. agalactiae strains 6313 and ΔfbsB. The binding of the resulting S. agalactiae strains 6313 pAT32, ΔfbsB pAT32, and ΔfbsB pAT/fbsB to immobilized fibrinogen was subsequently quantitated using FITC-labeled bacteria. Interestingly, all of the tested strains revealed about 45% binding to immobilized fibrinogen (data not shown), suggesting that fibrinogen-binding of S. agalactiae is not directly dependent on the FbsB protein.

FbsB is important for the invasion of S. agalactiae into host cells. To assess the role of FbsB in the interaction of S. agalactiae with host cells, we determined the ability of the S. agalactiae strains 6313 pAT32, ΔfbsB pAT32, and ΔfbsB pAT/fbsB to adhere to and to invade the lung epithelial cell line A549. As depicted in Fig. 5A, the tested strains revealed very
similar adherence to A549 cells, indicating that fbsB is not involved in the adherence of *S. agalactiae* to epithelial cells. However, cells of mutant ΔfbsB pAT32 were approximately 70% reduced in their invasion into A549 cells (Fig. 5B). Plasmid-mediated expression of fbsB restored the invasion capability of strain ΔfbsB pATfbsB to levels comparable to that of the *S. agalactiae* wild type. This result demonstrates that *S. agalactiae* requires the fbsB gene for efficient host cell invasion.

To analyze, whether the FbsB protein is directly involved in the invasion of epithelial cells by *S. agalactiae*, competitive inhibition experiments with purified FbsB protein were performed. As shown in Fig. 6A, the FbsB fusion protein did not interfere with the adherence of *S. agalactiae* 6313 to A549 cells. However, purified FbsB protein competitively inhibited the host cell invasion of the bacteria in a concentration-dependent manner (Fig. 6B). In control experiments with the *S. agalactiae* surface protein Bsp, no effect on the bacterial adherence and invasion was observed (Fig. 6). These findings suggest that the FbsB protein is directly involved in the invasion of epithelial cells by *S. agalactiae*.

As the FbsB protein is a fibrinogen-binding protein in *S. agalactiae*, we tested the effect of fibrinogen on the bacterial adherence and invasion of A549 cells. After preincubating *S. agalactiae* 6313 with fibrinogen in concentrations of 0.3, 0.75, 1.5, and 3.0 nM, the bacterial adherence and invasion of A549 cells was inhibited by 35, 50, 75, and 85%, respectively. However, microscopic inspection revealed a fibrinogen-dependent clumping of the bacteria (data not shown). The observed inhibition of the bacterial adherence and invasion by fibrinogen may therefore be caused either by the blocking of fibrinogen-binding sites on the surface of the bacteria or by the fibrinogen-mediated clumping of the bacteria. The role of fibrinogen in the FbsB-mediated invasion of epithelial cells thus remains to be determined.

**DISCUSSION**

The invasion of host cells and tissues by microorganisms is a critical step in the series of events that lead to a successful infection. In many cases, components of the eukaryotic ECM serve as ligands for the entry of pathogenic bacteria into host cells (16, 47, 65). Fibrinogen, a blood plasma protein, is also found in the ECM and plays an important role in wound healing (21, 29, 42). It is cleaved by thrombin to form fibrin, which is the major component of blood clots. Fibrinogen also interferes with the activation of complement, and protects pathogens that accumulate fibrinogen on their surface from opsonophagocytosis (10, 57, 80). In addition, fibrinogen is used...
by _Staphylococcus aureus_ for the adherence to human cells (59). Also in _S. pyogenes_, fibrinogen-binding M-proteins have been shown to play a role in the bacterial host cell adherence and invasion (12, 17). However, up to now the importance of fibrinogen-binding proteins for the adherence and invasion of human cells by _S. agalactiae_ remained unknown. The present report describes a novel fibrinogen-binding protein from _S. agalactiae_ and its involvement in the overall process of bacterial entry into human cells.

Numerous studies have described the ability of clinical _S. agalactiae_ isolates to interact with human fibrinogen (11, 56, 62, 81). Recently, Schubert et al. (57) identified in _S. agalactiae_ a fibrinogen-binding protein, which was termed FbsA. The FbsA protein is characterized by the presence of repetitive units, each 16 aa in length. Fibrinogen-binding of FbsA is mediated by the repeat region of the protein, and even a single repeat was demonstrated to bind to human fibrinogen (57). In the present study, a second fibrinogen-binding protein was identified in _S. agalactiae_ and was named in analogy FbsB. On the amino acid level, however, the proteins FbsA and FbsB do not exhibit significant similarity to each other. Furthermore, the FbsA protein carries at its C terminus a cell wall anchor motif (LPKTG), whereas the FbsB protein is devoid of such a motif. This indicates that FbsA is covalently attached to the cell wall of _S. agalactiae_ while the FbsB protein appears to be secreted into the culture medium or to be noncovalently attached to the bacterial cell wall. In Western blot analysis with concentrated culture supernatant of _S. agalactiae_ 6313, a fibrinogen-binding protein of the size of FbsB was identified (data not shown), indicating that _S. agalactiae_ indeed secretes the FbsB protein into the medium. However, in a genome-wide screen for surface proteins, the FbsB protein was also identified on the surface of _S. agalactiae_ by fluorescence-activated cell sorting analysis (69), suggesting that at least a fraction of FbsB is anchored to the cell wall of _S. agalactiae_. Also the fibrinogen-binding proteins Eap from _S. aureus_ (20) and the fibronectin- and fibrinogen-binding protein FBP54 from _S. pyogenes_ (13) are attached to the bacterial cell wall although they lack a typical cell wall anchor motif. In recent years, further examples of cell-wall associated adhesins and invasins that are devoid of a cell wall anchor motif have been described in Gram-positive bacteria (9). Therefore, it can be speculated that these proteins, including FbsB, are attached to the bacterial cell wall by hitherto unidentified mechanisms.

Construction of FbsB fusion proteins allowed a tentative localization of the fibrinogen-binding domain within the N-terminal 388 residues of FbsB. An N-terminal fibrinogen-binding domain in FbsB is reminiscent of the reported fibrinogen-binding sites of other streptococcal proteins such as the FbsA protein from _S. agalactiae_ (57), the M protein from _S. pyogenes_ (1) and the proteins FAI and FgBP from group C streptococci (41, 64). However, no apparent homologies were found between the N-terminal part of FbsB and other fibrinogen-binding proteins from streptococci and staphylococci, indicating that the binding of FbsB to human fibrinogen is mediated by a different sequence motif.

The present study demonstrates that _S. agalactiae_ possesses at least two fibrinogen-receptors. Also _S. pyogenes_ and _S. aureus_ have been shown to harbor several fibrinogen-binding proteins (14, 35, 40, 43, 48, 78, 79). However, in _S. pyogenes_, M-proteins are postulated to be the major fibrinogen receptors (15, 32, 36, 49, 50, 70), and in _S. aureus_ fibrinogen-binding is mediated, dependent on the growth-phase, by either ClfA or ClfB (40, 43). In _S. agalactiae_, deletion of the _fbsA_ gene results in a complete loss of fibrinogen-binding activity (57). The FbsA protein is therefore suggested to represent the major fibrinogen-binding protein in _S. agalactiae_. In agreement to this, an _fbsB_ deletion mutant of _S. agalactiae_ retained wild-type binding activity to immobilized fibrinogen. The fact that FbsB does not contribute to the binding of _S. agalactiae_ to immobilized fibrinogen may be caused by a low amount of FbsB in the cell wall of the bacteria. Alternatively, the cell wall-bound FbsB protein may be less accessible to exogenous fibrinogen than FbsA. Finally, a higher fibrinogen-binding affinity of the FbsA protein may explain its greater importance to the fibrinogen-binding of _S. agalactiae_. As shown in the present study, the FbsA protein indeed exhibited a significantly increased affinity to immobilized fibrinogen compared to the FbsB protein. The high affinity of FbsA to immobilized fibrinogen may thus be the basis for its importance to the fibrinogen-binding of _S. agalactiae_. The FbsA possesses similar binding affinities towards immobilized and soluble fibrinogen (57), suggesting that FbsA recognizes these conformational states of fibrinogen with equal affinity. Also the FbsB protein interacts with both immobilized and soluble fibrinogen although the affinity of FbsB to soluble fibrinogen remains to be determined. Interestingly, the deletion of the _fbsA_ gene in _S. agalactiae_ abrogated the binding of the bacteria to both immobilized and soluble fibrinogen (57). It can thus be hypothesized that the low binding affinity of FbsB towards soluble and immobilized fibrinogen does not allow it to take over the function of the FbsA protein in an _fbsA_ deletion mutant of _S. agalactiae_.

A recent report by Harris et al. (28) described a novel surface protease from _S. agalactiae_, termed CspA, which protects the bacteria from opsonophagocytosis and increases their virulence in an animal model. In functional studies, the CspA protease was shown to cleave the Aa subunit of human fibrinogen. It is therefore tempting to speculate that the fibrinogen-binding proteins FbsA and FbsB play a role in the accumulation of fibrinogen on the surface of _S. agalactiae_ for subsequent cleavage by CspA. Alternatively, the CspA protease may interfere with the binding of FbsA or FbsB to human fibrinogen. However, as the cleavage of fibrinogen by CspA was studied over a period of 16 h, it is currently unclear if CspA indeed influences the interaction of FbsA or FbsB with human fibrinogen. Further studies are therefore required to test the influence of CspA on the fibrinogen-binding of _S. agalactiae_ and to analyze the importance of FbsA and FbsB for the bacterial cleavage of fibrinogen by CspA.

Although _S. agalactiae_ has long been considered to be a typical extracellular organism, several reports have documented the capacity of these bacteria to enter human cells (8, 26, 54, 74, 82). Epithelial cell invasion by _S. agalactiae_ represents a putative mechanism in traversing the epithelial cell barrier of the newborn lung in the pathogenesis of pneumonia, which ultimately allows the bacteria to enter the bloodstream and to disseminate systemically (44). The intracellular environment also provides a niche in which the bacteria are protected from host defense mechanisms and antibiotics (54). Binding of _S. agalactiae_ to human fibronectin has been postulated to play...
a role in the bacterial adherence and invasion of host cells (3, 8, 67, 68). Recently, C5a peptidase from *S. agalactiae* was found to interact with human fibronectin (3) and to mediate bacterial invasion into epithelial cells (8). Inactivation of C5a peptidase in *S. agalactiae* reduced the binding of the bacteria to human fibronectin but only partially impaired the bacterial invasion into host cells. Unexpectedly, the C5a peptidase mutant exhibited a significantly increased adherence to host cells (8). Of note, a recent study by Tyrrell et al. (72) revealed no correlation between the amount of fibronectin on the surface of eukaryotic cells and the efficiency of *S. agalactiae* invasion into these cell lines. These data suggest that C5a peptidase is only one of several invasins in *S. agalactiae*, and that besides fibronectin, other host structures are involved in host cell invasion by these bacteria (8, 72).

Here, we demonstrate that the ability of *S. agalactiae* to invade A549 human epithelial cells is markedly dependent on the fibronectin-binding protein FbsB. Plasmid-mediated expression or deletion of *fbsB* in *S. agalactiae* did not influence the bacterial adherence to epithelial cells, indicating that the *fbsB* gene is not involved in the adherence of *S. agalactiae* to host cells. Deletion of the *fbsB* gene, however, significantly reduced the entry of *S. agalactiae* into lung epithelial cells, suggesting an important role of *fbsB* in the bacterial invasion of host cells. Plasmid-mediated expression of *fbsB* restored the capability of the *fbsB* mutant to invade epithelial cells to wild-type levels. This demonstrates that the impaired host cell invasion of the *fbsB* mutant is caused by its *fbsB* deficiency and not by unrelated mutations in its chromosome.

As the FbsB protein has a surface-exposed localization in *S. agalactiae* (69), we tested the effect of externally added FbsB fusion protein on the adherence and invasion of A549 cells by *S. agalactiae*. In agreement to the results obtained with the *fbsB* deletion mutant and the *fbsB*-complemented strain, the addition of FbsB fusion protein did not block the adherence of *S. agalactiae* to host cells. These data confirm that the FbsB protein is apparently not involved in the adherence of *S. agalactiae* to epithelial cells. However, the invasion of *S. agalactiae* into A549 cells was specifically inhibited by increasing concentrations of FbsB fusion protein. This finding indicates that a direct interaction of FbsB with host cell structures is crucial for the efficient entry of *S. agalactiae* into host cells. However, it remains to be determined if FbsB requires further factors for host cell invasion. Interestingly, the *fbsB* gene was shown by RT-PCR analysis to form an operon with the gbs0851 gene in *S. agalactiae*. This indicates that the two gene products may display a similar function during the course of bacterial adherence and invasion. Experiments are currently under way to unravel the function of the gbs0851 gene for the adherence and invasion of host cells by *S. agalactiae*.

Although the present study convincingly demonstrates the binding of FbsB to human fibronogen, the host molecule(s) involved in FbsB-mediated entry of *S. agalactiae* into A549 cells remains to be determined. The lung epithelial cell line A549 is known to synthesize small amounts of fibronogen constitutively and large amounts of this protein upon stimulation with interleukin-6 (25), a proinflammatory mediator of the acute phase response (30). However, only 10 to 20% of the secreted fibronogen is directed to the apical side of A549 cells (24), making a relatively small amount of fibronogen accessible to the FbsB protein. In animal experiments with the pathogenic protozoan *Pneumocystis carinii*, however, the organism was shown to adhere to lung cells by binding to fibronogen, located on the apical face of the epithelium (60). This suggests that the apical side of lung epithelial cells contains sufficient amounts of fibronogen to allow its interaction with pathogenic microorganisms. The mechanisms by which binding of FbsB to fibronogen might trigger the bacterial uptake into host cells remains unknown. However, fibronogen has been shown to bind to the integrin receptors αvβ5 and αvβ1 on A549 cells, resulting in endocytic uptake processes (46). Although highly speculative, fibronogen-binding by the FbsB protein might eventually trigger integrin-mediated uptake processes that allow the invasion of the host cells by *S. agalactiae*. As an alternative to fibronogen-mediated invasion by FbsB, the FbsB protein may recognize a different ligand on the surface of epithelial cells to allow host cell invasion by *S. agalactiae*. Interestingly, the fibronogen-binding protein CIB from *S. aureus* was recently shown to bind to cytokeratin 10 on the surface of eukaryotic cells (45). This finding demonstrates that bacterial fibronogen-binding proteins may interact with distinct ligands on the host cell surface. Currently, studies are under way to identify the nature of ligand(s) to which FbsB binds to during the course of host cell entry by *S. agalactiae*.

Taken together, the results reported here highlight the role of FbsB in the overall process of host cell invasion by *S. agalactiae* and give a first insight into the underlying events required for the successful establishment of an infection. The understanding of virulence mechanisms of *S. agalactiae* on the molecular level may contribute to the development of an efficient vaccine against these bacteria.

**REFERENCES**


