INFECTION AND IMMUNITY, June 2004, p. 3495–3504 Vol. 72, No. 6
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.6.3495–3504.2004

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

Heike Gutekunst, Bernhard J. Eikmanns, and Dieter J. Reinscheid*

Department of Microbiology and Biotechnology, University of Ulm, D-89069 Ulm, Germany

Received 2 October 2003/Returned for modification 17 December 2003/Accepted 21 February 2004

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*.**

Streptococcus 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-*

* Corresponding author. Mailing address: Department of Microbiology and Biotechnology, University of Ulm, Albert-Einstein-Allee 11, D-89069 Ulm, Germany. Phone: 49-731-5024853. Fax: 49-731-5022719. E-mail: dieter.reinscheid@biologie.uni-ulm.de.

tiae. Our findings indicate that FbsB plays an important role in the entry of *S. agalactiae* into epithelial cells.

MATERIALS AND METHODS

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, Bsp, and Gbs0851 fusion proteins.

S. agalactiae was cultivated at 37°C in Todd-Hewitt yeast broth (THY) consisting of Todd-Hewitt broth with 1% yeast extract. Recombinant *S. agalactiae* clones, carrying the plasmid pG$^+$host6 or pAT32, were selected with erythromycin (5 μg/ml) or spectinomycin (200 μg/ml). *E. coli* was grown at 37°C in Luria broth (LB) and clones carrying the plasmids pG$^+$host6, pGEM-5Z or pET28a were selected in the presence of erythromycin (300 μg/ml), ampicillin (100 μg/ml), or kanamycin (50 μg/ml).

Antibodies and human proteins. Affinity-purified rabbit anti-fibrinogen antibodies and peroxidase-labeled goat anti-rabbit antibodies were obtained from Dako-Biochemicals. Peroxidase-labeled goat anti-mouse immunoglobulin G (IgG) Fab fragments, and monoclonal anti-HisTag antibodies were purchased from Dianova and Roche Diagnostics, respectively. Mouse anti-FbsA antibodies were kindly provided by Intercell (Vienna, Austria). Human fibrinogen, fibronectin, collagen I, and collagen IV were obtained from Sigma. Human fibrinogen was passed through a gelatin-Sepharose column to remove residual contaminating fibronectin in the preparation. The purity of the fibrinogen preparation was confirmed by Western blotting using anti-fibronectin antibodies.

Isolation and sequencing of the *fbsB*-encoding region from *S. agalactiae* 6313. The *fbsB* gene was isolated from a cosmid that conferred fibrinogen-binding activity to *E. coli* DH5α (57). Partial digestion of the cosmid with Sau3A, cloning of fragments in the range of 2 to 4 kb in the vector pGEM-5Z (Promega), screening for fibrinogen-binding *E. coli* clones, and sequencing of the obtained plasmid was performed as described previously (57).

Construction of plasmids for synthesis of FbsB or Gbs0851 fusion proteins. The vector pET28a (Novagen) was used for the synthesis of hexahistidyl-tagged FbsB or Gbs0851 fusion proteins. The *fbsB* gene, devoid of its signal peptide-encoding region, was amplified from chromosomal DNA of *S. agalactiae* 6313 by PCR with the primers 5′ GTGCCTTGCCATGGCCGGGATAACTAAAG and 5′ GCGGACAGCTCGAGCTCTTTTATACGCGATGAG. The N-terminus-encoding region of *fbsB* was amplified using the primers 5′ GTGCCTTGCCATGGCCGGGATAACTAAAG and 5′ GCGGACAGCTCGAGTTGTTTTTGGGAGCCATTCAACC. Amplification of the C-terminus-encoding region of *fbsB* was performed with the primers 5′ GTGCCTTGCCATGGGTTGAATGGCTCCCAAAAAC and 5′ GCGGACAGCTCGAGCTCTTTTATACGCGATGAG. The NcoI and XhoI restriction sites used for cloning are underlined. After digestion of the *fbsB*-derived PCR products and of plasmid pET28a with NcoI and XhoI, the *fbsB* derivates were ligated into pET28a 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 by PCR with the primers CCGCGGATCCGATGATAACTTTGAAATGCC and TGGCACAAGCTTACATTCTGAGCAGAAAGC. 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 pET28a. 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 with 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 *gbs0849* to *fbsB* was analyzed with the primers 5′GGGCATATGGGACGTACTG and 5′ CAATCCAAAACGCAATAGG. The region *fbsB* to *gbs0851* was investigated by RT-PCR with the primers 5′ CCGGTTCAATCAGTTGTTG and 5′CTTCATTAACAATATCTGAG, and the region *gbs0851* to *gbs0853* was tested with the primers 5′CTAGTTGCAACGACATCGG and 5′GATGCTACTCCTGCTTGAG.

For comparative expression analysis of the *gbs0851* gene in the *S. agalactiae* strains 6313 and Δ*fbsB*, 5 μg of RNA of each strain was reverse transcribed with 200 U of RevertAid M-MuLV reverse transcriptase (MBI Fermentas) using the *gyrA*- and *gbs0851*-specific reverse primers 5′TCAAACTGAGGTACGACG and 5′GTTCAATGGGTATAATCTC. Quantitative real-time PCR was performed with the *gyrA*-specific primers 5′GACGTTCAGGTATTCAC and 5′TCAAACTGAGGTACGACG, and the *gbs0851*-specific primers 5′GACACTGCTATCAAGGCG and 5′GTTCAATGGGTATAATCTC, respectively. LightCycler analysis was performed as described previously (26), and the quantity of *gbs0851*-specific cDNA was normalized to the quantity of *gyrA* cDNA in the two samples.

Construction of an *fbsB* deletion mutant. The *fbsB* gene was deleted in the chromosome of *S. agalactiae* 6313 by a method previously described by Schubert et al. (57). Briefly, two DNA fragments, flanking the *fbsB* gene were amplified by PCR from the genome of *S. agalactiae* 6313 with the primer pair *fbsB*_del1 5′CCGCGGATCCGTCATGTTACTAATCTTATGC and *fbsB*_del2 5′*CCCATCCACTAAACTTAACA*CAATCCAAAACGCAATAGG and with the primer pair *fbsB*_del3 5′*TGTTTAAGTTTAGTGGATGGG*GATCAAGCTTTTGTAGCTAG and *fbsB*_del4 5′GGGGGTACCCTTCATTAACAATATCTGAG. cDNA sequences in the primers *fbsB*_del2 and *fbsB*_del3 are marked in italics and the BamHI and HindIII restriction sites in the primers *fbsB*_del1 and *fbsB*_del4 are underlined. The *fbsB*-flanking PCR products were mixed in equal amounts with each other and subjected to a crossover PCR with the primers *fbsB*_del1 and *fbsB*_del4, resulting in one PCR product that carried the two *fbsB*-flanking regions. The crossover PCR product and the thermosensitive vector pG$^+$host6 were digested with BamHI and HindIII, ligated, and transformed into *E. coli* DH5α. The resulting plasmid, pG$^+$Δ*fbsB*, was electroporated into *S. agalactiae* 6313, and transformants were grown at 30°C with erythromycin selection. Cells in which pG$^+$Δ*fbsB* had integrated into the chromosome were selected 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 pG$^+$Δ*fbsB*, 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 pG$^+$Δ*fbsB* 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*_del3 and *fbsB*_del4.

Plasmid-mediated expression of *fbsB* in *S. agalactiae*. A DNA fragment containing the lactococcal P32 promoter (76) was isolated from plasmid pMG36e (75) by EcoRI/SmaI digestion and ligated into the EcoRI/SmaI-digested vector pAT28 (71), resulting in the expression vector pAT32. The *fbsB* structural gene, including its ribosomal binding site, was amplified from chromosomal *S. agalactiae* 6313 DNA by PCR using the primers 5′ CCGCGGATCCGTCATGTTACTAATCTTATGC and 5′ CCGCGGATCCCTACTCTTTTATACGCGATG. The BamHI sites used for cloning are underlined. The PCR product was cut with BamHI and ligated into the BamHI-digested vector pAT32. The correct orientation of the *fbsB* gene in pAT32 was determined by EcoRI digest, and the resultant plasmid was termed pAT*fbsB*. The plasmids pAT32 and pAT*fbsA* were transformed by electroporation into *S. agalactiae* with subsequent spectinomycin selection.

General DNA techniques. Conventional techniques for DNA manipulation and protein analysis, such as restriction enzyme digests, PCR, ligation, transformation by electroporation, Southern blotting, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting were performed as described by Sambrook et al. (55).

Preparation of the fusion proteins FbsA, FbsB, 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 Ni^{2+} 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% (wt/vol) blocking reagent (Roche) in PBS and incubated for 1 h at room temperature with purified fusion proteins or human fibrinogen 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), following 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 H_2SO_4. The absorption of the solution at 450 nm was quantitated in an enzyme-linked immunosorbent assay (ELISA) reader (Anthos htIII). 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 (K_D) 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 isothiocyante (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-adherent 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-adherent 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 AL732656. In the SwissProt TREMBL 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 cosmid 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 cosmid 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 Gbs0581 fusion protein. The fusion proteins FbsB and Gbs0851 were synthesized in *E. coli* BL21 and subsequently purified by Ni^{2+} 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

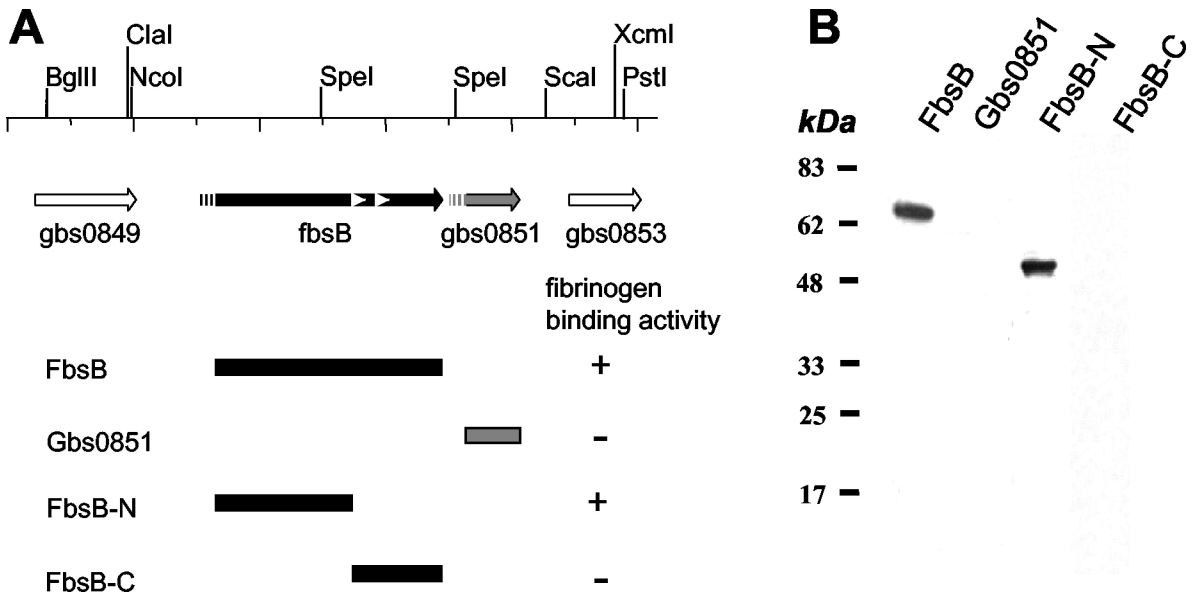

FIG. 1. Restriction map of the *fbsB*-encoding region in *S. agalactiae* (A) and Western blot analysis of FbsB and Gbs0851 fusion proteins for fibrinogen-binding (B). The fusion proteins were size separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and tested for fibrinogen binding by Western blotting. Bound fibrinogen was detected with rabbit anti-fibrinogen antibodies followed by peroxidase-labeled goat anti-rabbit antibodies and was visualized by chemiluminescence. FbsB and Gbs0851 are full-length fusion proteins. Abbreviations: FbsB-N, N-terminal 388 aa of FbsB, FbsB-C: C-terminal 222 aa of FbsB. The dashed lines in the genes *fbsB* and *gbs0851* show the location of their signal peptide encoding regions, and the white arrows in the *fbsB* gene depict the regions that code for the repeats in the FbsB polypeptide.

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 1.6×10^{-8} M, which corresponds to previous findings (57). The proteins FbsB and FbsB-N revealed apparent K_D values of 2.6×10^{-7} M and 1.3×10^{-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.

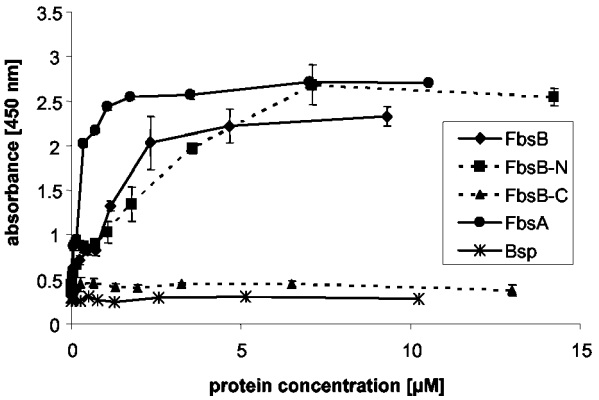

FIG. 2. Binding of the proteins FbsA and Bsp and the FbsB-derived proteins to immobilized fibrinogen in a capture ELISA. Microtiter wells were coated with a fixed amount of human fibrinogen, followed by the addition of increasing concentrations of the different fusion proteins. Bound fusion protein was detected with mouse anti-HisTag antibodies and peroxidase-conjugated goat anti-mouse IgG Fab fragments. Color development was initiated by the addition of tetramethyl-benzidine substrate and stopped with H_2SO_4. The absorbance of the microtiter wells was read at 450 nm. Values represent the means of three independent experiments, each performed in triplicate.

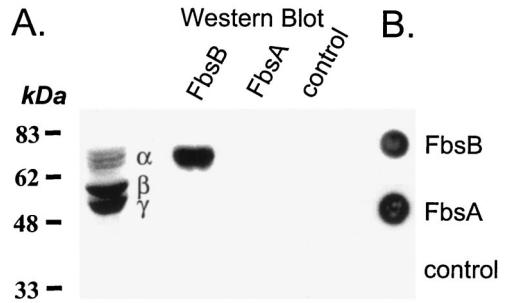

FIG. 3. Western blot experiments to identify the FbsA- and FbsB-binding sites within human fibrinogen (A) and dot blot experiments to detect binding of the FbsA and FbsB fusion proteins to native fibrinogen (B). Human fibrinogen was size-separated by SDS-PAGE and either Coomassie stained (left lane) or transferred onto a nitrocellulose membrane and tested for FbsA- or FbsB-binding by Western blot experiments (A). Native human fibrinogen was spotted onto nitrocellulose and tested for binding of the fusions proteins FbsA or FbsB (B). Bound fusion proteins were detected with mouse anti-HisTag antibodies, followed by peroxidase-conjugated goat anti-mouse IgG Fab fragments and visualized by chemiluminescence. In control experiments (control), no fusion proteins were added and the binding of the used antibodies to human fibrinogen was investigated.

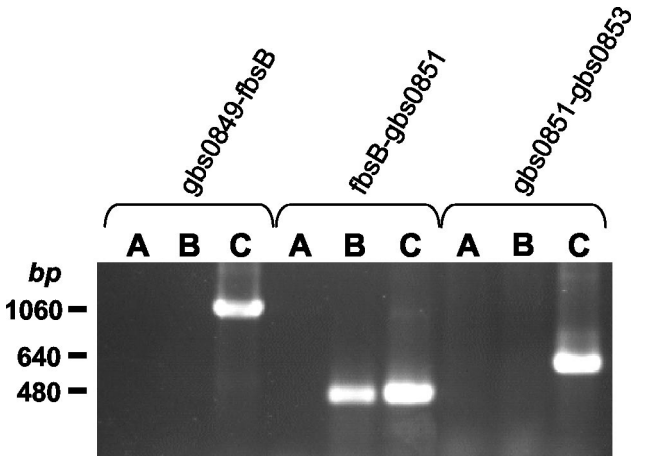

FIG. 4. Transcriptional organization of the *fbsB*-encoding region in *S. agalactiae*. The names on top of the figure indicate the genes to which the primer pairs annealed during PCR with total RNA (A), RT-PCR with total RNA (B) or PCR with chromosomal DNA (C) from *S. agalactiae* 6313.

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* Δ*fbsB*. Quantitative real-time PCR analysis revealed equal expression of the *gbs0851* gene in the *S. agalactiae* strains 6313 and Δ*fbsB* (data not shown), demonstrating that the deletion of the *fbsB* gene in mutant Δ*fbsB* 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* Δ*fbsB*. Additionally, the vector pAT32 was transformed into the *S. agalactiae* strains 6313 and Δ*fbsB*. The binding of the resulting *S. agalactiae* strains 6313 pAT32, Δ*fbsB* pAT32 and Δ*fbsB* 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, Δ*fbsB* pAT32, and Δ*fbsB* pAT*fbsB* to adhere to and to invade the lung epithelial cell line A549. As depicted in Fig. 5A, the tested strains revealed very

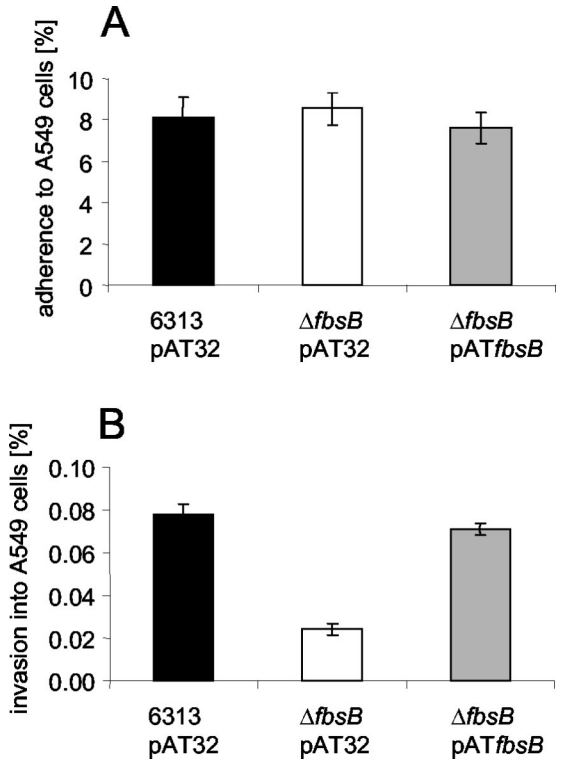

FIG. 5. Adherence (A) and invasion (B) of the lung epithelial cell line A549 by the *S. agalactiae* strains 6313 pAT32, Δ*fbsB* pAT32, and Δ*fbsB* pAT*fbsB*, respectively. Bacterial adherence and invasion were calculated as follows: adherence = number of adherent bacteria/total number of bacteria in the assay × 100. Invasion = number of internalized bacteria/total number of bacteria in the assay × 100. Each experiment was performed at least three times in triplicate.

similar adherence to A549 cells, indicating that *fbsB* is not involved in the adherence of *S. agalactiae* to epithelial cells. However, cells of mutant Δ*fbsB* pAT32 were approximately 70% reduced in their invasion into A549 cells (Fig. 5B). Plasmid-mediated expression of *fbsB* restored the invasion capability of strain Δ*fbsB* pAT*fbsB* 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

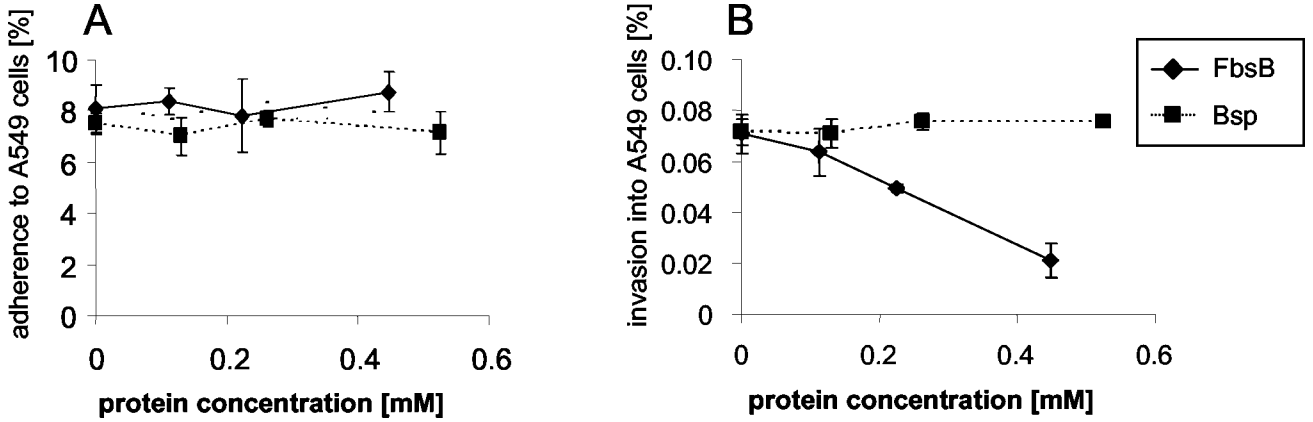

FIG. 6. Eukaryotic cell adherence (A) and invasion (B) of *S. agalactiae* 6313 in the presence of different concentrations of FbsB and Bsp fusion proteins. Bacterial adherence and invasion were calculated as described in the legend of Fig. 5. Each experiment was performed at least three times in triplicate.

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 Aα 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 fibrinogen-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 fibrinogen, 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 fibrinogen 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 fibrinogen is directed to the apical side of A549 cells (24), making a relatively small amount of fibrinogen 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 host fibrinogen, located on the apical face of the epithelium (60). This suggests that the apical side of lung epithelial cells contains sufficient amounts of fibrinogen to allow its interaction with pathogenic microorganisms. The mechanisms by which binding of FbsB to fibrinogen might trigger the bacterial uptake into host cells remains unknown. However, fibrinogen has been shown to bind to the integrin receptors $\alpha_v\beta_3$ and $\alpha_5\beta_1$ on A549 cells, resulting in endocytotic uptake processes (46). Although highly speculative, fibrinogen-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 fibrinogen-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 fibrinogen-binding protein ClfB from *S. aureus* was recently shown to bind to cytokeratin 10 on the surface of eukaryotic cells (45). This finding demonstrates that bacterial fibrinogen-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

1. **Akesson, P., K. H. Schmidt, J. Cooney, and L. Björck.** 1994. M1 protein and protein H: IgGFc- and albumin-binding streptococcal surface proteins encoded by adjacent genes. Biochem. J. **300:**877–886.
2. **Beardsall, K., M. H. Thompson, and R. J. Mulla.** 2000. Neonatal group B streptococcal infection in South Bedfordshire, 1993–1998. Arch. Dis. Child Fetal Neonatal Ed. **82:**F205–F207.
3. **Beckmann, C., J. D. Waggoner, T. O. Harris, G. S. Tamura, and C. E. Rubens.** 2002. Identification of novel adhesins from group B streptococci by use of phage display reveals that C5a peptidase mediates fibronectin binding. Infect. Immun. **70:**2869–2876.
4. **Berner, R.** 2002. Group B streptococci during pregnancy and infancy. Curr. Opin. Infect. Dis. **15:**307–313.
5. **Bignardi, G. E.** 1999. Surveillance of neonatal group B streptococcal infection in Sunderland. Commun. Dis. Public Health. **2:**64–65.
6. **Bozzini, S., L. Visai, P. Pignatti, T. E. Petersen, and P. Speziale.** 1992. Multiple binding sites in fibronectin and the staphylococcal fibronectin receptor. Eur. J. Biochem. **207:**327–333.
7. **Campbell, J. R., S. L. Hillier, M. A. Krohn, P. Ferrieri, D. F. Zaleznik, and C. J. Baker.** 2000. Group B streptococcal colonization and serotype-specific immunity in pregnant women at delivery. Obstet. Gynecol. **96:**498–503.
8. **Cheng, Q., D. Stafslien, S. S. Purushothaman, and P. Cleary.** 2002. The group B streptococcal C5a peptidase is both a specific protease and an invasin. Infect. Immun. **70:**2408–2413.
9. **Chhatwal, G. S.** 2002. Anchorless adhesins and invasins of Gram-positive bacteria: a new class of virulence factors. Trends Microbiol. **10:**205–208.
10. **Chhatwal, G. S., I. S. Dutra, and H. Blöbel.** 1985. Fibrinogen binding inhibits the fixation of the third component of human complement on surface of groups A, B, C, and G streptococci. Microbiol. Immunol. **29:**973–980.
11. **Chhatwal, G. S., C. Lämmler, and H. Blöbel.** 1984. Guanidine extraction enhances the binding of human fibrinogen to group-B streptococci. Med. Microbiol. Immunol. **173:**19–27.
12. **Courtney, H. S., M. S. Bronze, J. B. Dale, and D. L. Hasty.** 1994. Analysis of the role of M24 protein in group A streptococcal adhesion and colonization by use of omega-interposon mutagenesis. Infect. Immun. **62:**4868–4873.
13. **Courtney, H. S., J. B. Dale, and D. I. Hasty.** 1996. Differential effects of the streptococcal fibronectin-binding protein, FBP54, on adhesion of group A

streptococci to human buccal cells and HEp-2 tissue culture cells. Infect. Immun. **64:**2415–2419.
14. **Courtney, H. S., Y. Li, J. B. Dale, and D. L. Hasty.** 1994. Cloning, sequencing, and expression of a fibronectin/fibrinogen-binding protein from group A streptococci. Infect. Immun. **62:**3937–3946.
15. **Courtney, H. S., S. Liu, J. B. Dale, and D. L. Hasty.** 1997. Conversion of M serotype 24 of *Streptococcus pyogenes* to M serotypes 5 and 18: effect on resistance to phagocytosis and adhesion to host cells. Infect. Immun. **65:** 2472–2474.
16. **Cue, D., P. E. Dombek, H. Lam, and P. P. Cleary.** 1998. Streptococcus pyogenes serotype M1 encodes multiple pathways for entry into human epithelial cells. Infect. Immun. **66:**4593–4601.
17. **Dombek, P. E., D. Cue, J. Sedgewick, H. Lam, S. Ruschkowski, B. B. Finlay, and P. P. Cleary.** 1999. High-frequency intracellular invasion of epithelial cells by serotype M1 group A streptococci: M1 protein-mediated invasion and cytoskeletal rearrangements. Mol. Microbiol. **31:**859–870.
18. **Dubendorff, J. W., and F. W. Studier.** 1991. Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with *lac* repressor. J. Mol. Biol. **219:**45–59.
19. **Elsinghorst, E. A.** 1994. Measurement of invasion by gentamycin resistance, p. 405–419. *In* J. N. Abelson and M. I. Simon (ed.), Bacterial pathogenesis, part B. Academic Press, Inc., New York, N.Y.
20. **Flock, M., and J. I. Flock.** 2001. Rebinding of extracellular adherence protein Eap to *Staphylococcus aureus* can occur through a surface-bound neutral phosphatase. J. Bacteriol. **183:**3999–4003.
21. **Fuss, C., J. C. Palmaz, and E. A. Sprague.** 2001. Fibrinogen: structure, function, and surface interactions. J. Vasc. Interv. Radiol. **12:**677–682.
22. **Gibson, R. L., M. K. Lee, C. Soderland, E. Y. Chi, and C. E. Rubens.** 1993. Group B streptococci invade endothelial cells: type III capsular polysaccharide attenuates invasion. Infect. Immun. **61:**478–485.
23. **Glaser, P., C. Rusniok, C. Buchrieser, F. Chevalier, L. Frangeul, T. Msadek, M. Zouine, E. Couve, L. Lalioui, C. Poyart, P. Trieu-Cuot, and F. Kunst.** 2002. Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. Mol. Microbiol. **45:**1499–1513.
24. **Guadiz, G., L. A. Sporn, R. A. Goss, S. O. Lawrence, V. J. Marder, and P. J. Simpson-Haidaris.** 1997. Polarized secretion of fibrinogen by lung epithelial cells. Am. J. Respir. Cell Mol. Biol. **17:**60–69.
25. **Guadiz, G., L. A. Sporn, and P. J. Simpson-Haidaris.** 1997. Thrombin cleavage-independent deposition of fibrinogen in extracellular matrices. Blood **90:**2644–2653.
26. **Gutekunst, H., B. E. Eikmanns, and D. J. Reinscheid.** 2003. Analysis of RogB-controlled virulence mechanisms and gene expression in *Streptococcus agalactiae*. Infect. Immun. **71:**5056–5064.
27. **Hanahan, D.** 1985. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
28. **Harris, T. O., D. W. Shelver, J. F. Bohnsack, and C. E. Rubens.** 2003. A novel streptococcal surface protease promotes virulence, resistance to opsonophagocytosis, and cleavage of human fibrinogen. J. Clin. Investig. **111:** 61–70.
29. **Hay, E. D.** 1991. Cell biology of extracellular matrix. Plenum Press, New York, N.Y.
30. **Heinrich, P. C., J. V. Castell, and T. Andus.** 1990. Interleukin-6 and the acute phase response. Biochem. J. **265:**621–636.
31. **Hulse, M. L., S. Smith, E. Y. Chi, A. Pham, and C. E. Rubens.** 1993. Effect of type III group B streptococcal capsular polysaccharide on invasion of respiratory epithelial cells. Infect. Immun. **61:**4835–4841.
32. **Husmann, L. K., J. R. Scott, G. Lindahl, and L. Stenberg.** 1995. Expression of the Arp protein, a member of the M protein family, is not sufficient to inhibit phagocytosis of *Streptococcus pyogenes*. Infect. Immun. **63:**345–348.
33. **Jeng, A., V. Sakota, Z. Li, V. Datta, B. Beall, and V. Nizet.** 2003. Molecular genetic analysis of a group A streptococcus operon encoding serum opacity factor and a novel fibronectin-binding protein, SfbX. J. Bacteriol. **185:**1208–1217.
34. **Källman, J., and E. Kihlström.** 1997. Penetration of group B streptococci through polarized Madin-Darby canine kidney cells. Pediatr. Res. **42:**799–804.
35. **Katerov, V., A. Andreev, C. Schalen, and A. A. Totolian.** 1998. Protein F, a fibronectin-binding protein of *Streptococcus pyogenes*, also binds human fibrinogen: isolation of the protein and mapping of the binding region. Microbiology **144:**119–126.
36. **Kotarsky, H., A. Thern, G. Lindahl, and U. Sjobring.** 2000. Strain-specific restriction of the antiphagocytic property of group A streptococcal M proteins. Infect. Immun. **68:**107–112.
37. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
38. **Lodish, H., A. Berk, S. L. Zipursky, P. Matsudaira, D. Baltimore, and J. E. Darnell.** 1999. Molecular cell biology, p. 858. W. H. Freeman and Company, New York, N.Y.
39. **Massey, R. C., M. N. Kantzanou, T. Fowler, N. P. Day, K. Schofield, E. R. Wann, A. R. Berendt, M. Höök, and S. J. Peacock.** 2001. Fibronectin-binding protein A of *Staphylococcus aureus* has multiple, substituting, binding regions that mediate adherence to fibronectin and invasion of endothelial cells. Cell Microbiol. **3:**839–851.
40. **McDevitt, D., P. Francois, P. Vaudaux, and T. J. Foster.** 1994. Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*. Mol. Microbiol. **11:**237–248.
41. **Meehan, M., D. A. Muldowney, N. J. Watkins, and P. Owen.** 2000. Localization and characterization of the ligand-binding domain of the fibrinogen-binding protein (FgBP) of *Streptococcus equi subsp. equi.* Microbiology **146:** 1187–1194.
42. **Mosesson, M. W., K. R. Siebenlist, and D. A. Meh.** 2001. The structure and biological features of fibrinogen and fibrin. Ann. N. Y. Acad. Sci. **936:**11–30.
43. **Ni Eidhin, D., S. Perkins, P. Francois, P. Vaudaux, M. Höök, and T. J. Foster.** 1998. Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of *Staphylococcus aureus*. Mol. Microbiol. **30:**245–257.
44. **Nizet, V., K. S. Kim, M. Stins, M. Jonas, E. Y. Chi, D. Nguyen, and C. E. Rubens.** 1997. Invasion of brain microvascular endothelial cells by group B streptococci. Infect. Immun. **65:**5074–5081.
45. **O'Brien, L. M., E. J. Walsh, R. C. Massey, S. J. Peacock, and T. J. Foster.** 2002. Staphylococcus aureus clumping factor B (ClfB) promotes adherence to human type I cytokeratin 10: implications for nasal colonization. Cell Microbiol. **4:**759–770.
46. **Odrljin, T. M., C. G. Haidaris, N. B. Lerner, and P. J. Simpson-Haidaris.** 2001. Integrin $\alpha_v\beta_3$-mediated endocytosis of immobilized fibrinogen by A549 lung alveolar epithelial cells. Am. J. Respir. Cell Mol. Biol. **24:**12–21.
47. **Ozeri, V., I. Rosenshine, D. F. Mosher, R. Fassler, and E. Hanski.** 1998. Roles of integrins and fibronectin in the entry of *Streptococcus pyogenes* into cells via protein F1. Mol. Microbiol. **30:**625–637.
48. **Palma, M., S. Nozohoor, T. Schennings, A. Heimdahl, and J. I. Flock.** 1996. Lack of the extracellular 19-kilodalton fibrinogen-binding protein from Staphylococcus aureus decreases virulence in experimental wound infection. Infect. Immun. **64:**5284–5289.
49. **Perez-Casal, J., M. G. Caparon, and J. R. Scott.** 1992. Introduction of the *emm6* gene into an *emm*-deleted strain of *Streptococcus pyogenes* restores its ability to resist phagocytosis. Res. Microbiol. **143:**549–558.
50. **Podbielski, A., N. Schnitzler, P. Beyhs, and M. D. Boyle.** 1996. M-related protein (Mrp) contributes to group A streptococcal resistance to phagocytosis by human granulocytes. Mol. Microbiol. **19:**429–441.
51. **Reinscheid, D. J., B. Gottschalk, A. Schubert, B. J. Eikmanns, and G. S. Chhatwal.** 2001. Identification and molecular analysis of PcsB, a protein required for cell wall separation of group B streptococcus. J. Bacteriol. **183:**1175–1183.
52. **Reinscheid, D. J., C. Stoesser, K. Moeller, K. Ehlert, R. W. Jack, B. E. Eikmanns, and G. S. Chhatwal.** 2002. Influence of proteins Bsp and FemH on cell shape and peptidoglycan composition in group B streptococcus. Microbiol. **148:**3245–3254.
53. **Rubens, C. E., H. V. Raff, J. C. Jackson, E. Y. Chi, J. T. Bielitzki, and S. L. Hillier.** 1991. Pathophysiology and histopathology of group B streptococcal sepsis in *Macaca nemestrina* primates induced after intraamniotic inoculation: evidence for bacterial cellular invasion. J. Infect. Dis. **164:**320–330.
54. **Rubens, C. E., S. Smith, M. Hulse, E. Y. Chi, and G. van Belle.** 1992. Respiratory epithelial cell invasion by group B streptococci. Infect. Immun. **60:**5157–5163.
55. **Sambrook, J., E. F. Fritsch, and J. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor, N.Y.
56. **Schönbeck, C., L. Björck, and G. Kronvall.** 1981. Receptors for fibrinogen and aggregated beta 2-microglobulin detected in strains of group B streptococci. Infect. Immun. **31:**856–861.
57. **Schubert, A., K. Zakikhany, M. Schreiner, R. Frank, B. Spellerberg, B. E. Eikmanns, and D. J. Reinscheid.** 2002. A fibrinogen receptor from group B streptococcus interacts with fibrinogen by repetitive units with novel ligand binding sites. Mol. Microbiol. **46:**557–569.
58. **Schuchat, A.** 1998. Epidemiology of group B streptococcal disease in the United States: shifting paradigms. Clin. Microbiol. Rev. **11:**497–513.
59. **Shenkman, B., E. Rubinstein, I. Tamarin, R. Dardik, N. Savion, and D. Varon.** 2000. Staphylococcus aureus adherence to thrombin-treated endothelial cells is mediated by fibrinogen but not by platelets. J. Lab. Clin. Med. **135:**43–51.
60. **Simpson-Haidaris, P. J., M. A. Courtney, T. W. Wright, R. Goss, A. Harmsen, and F. Gigliotti.** 1998. Induction of fibrinogen expression in the lung epithelium during *Pneumocystis carinii* pneumonia. Infect. Immun. **66:**4431–4439.
61. **Spellerberg, B.** 2000. Pathogenesis of neonatal *Streptococcus agalactiae* infections. Microbes Infect. **2:**1733–1742.
62. **Spellerberg, B., E. Rozdzinski, S. Martin, J. Weber-Heynemann, and R. Lütticken.** 2002. *rgf* encodes a novel two-component signal transduction system of *Streptococcus agalactiae*. Infect. Immun. **70:**2434–2440.
63. **Spellerberg, B., E. Rozdzinski, S. Martin, J. Weber-Heynemann, N. Schnitzler, R. Lütticken, and A. Podbielski.** 1999. Lmb, a protein with similarities to the LraI adhesin family, mediates attachment of *Streptococcus agalactiae* to human laminin. Infect. Immun. **67:**871–878.
64. **Talay, S. R., M. P. Grammel, and G. S. Chhatwal.** 1996. Structure of a group

C streptococcal protein that binds to fibrinogen, albumin and immunoglobulin G via overlapping modules. Biochem. J. **315:**577–582.

65. **Talay, S. R., A. Zock, M. Rohde, G. Molinari, M. Oggioni, G. Pozzi, C. A. Guzman, and G. S. Chhatwal.** 2000. Co-operative binding of human fibronectin to Sfbl protein triggers streptococcal invasion into respiratory epithelial cells. Cell Microbiol. **2:**521–535.
66. **Tamura, G. S., J. M. Kuypers, S. Smith, H. Raff, and C. E. Rubens.** 1994. Adherence of group B streptococci to cultured epithelial cells: roles of environmental factors and bacterial surface components. Infect. Immun. **62:**2450–2458.
67. **Tamura, G. S., A. Nittayajarn, and D. L. Schoentag.** 2002. A glutamine transport gene, glnQ, is required for fibronectin adherence and virulence of group B streptococci. Infect. Immun. **70:**2877–2885.
68. **Tamura, G. S., and C. E. Rubens.** 1995. Group B streptococci adhere to a variant of fibronectin attached to a solid phase. Mol. Microbiol. **15:**581–589.
69. **Tettelin, H., V. Masignani, M. J. Cieslewicz, J. A. Eisen, S. Peterson, M. R. Wessels, I. T. Paulsen, K. E. Nelson, I. Margarit, T. D. Read, L. C. Madoff, A. M. Wolf, M. J. Beanan, L. M. Brinkac, S. C. Daugherty, R. T. DeBoy, A. S. Durkin, J. F. Kolonay, R. Madupu, M. R. Lewis, D. Radune, N. B. Fedorova, D. Scanlan, H. Khouri, S. Mulligan, H. A. Carty, R. T. Cline, S. E. Van Aken, J. Gill, M. Scarselli, M. Mora, E. T. Iacobini, C. Brettoni, G. Galli, M. Mariani, F. Vegni, D. Maione, D. Rinaudo, R. Rappuoli, J. L. Telford, D. L. Kasper, G. Grandi, and C. M. Fraser.** 2002. Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V *Streptococcus agalactiae*. Proc. Natl. Acad. Sci. USA **99:**12391–12396.
70. **Thern, A., M. Wästfelt, and G. Lindahl.** 1998. Expression of two different antiphagocytic M proteins by *Streptococcus pyogenes* of the OF+ lineage. J. Immunol. **160:**860–869.
71. **Trieu-Cuot, P., C. C. Poyart-Salmeron, and P. Courvalin.** 1990. A pair of mobilizable shuttle vectors conferring resistance to spectinomycin for molecular cloning in *Escherichia coli* and in gram-positive bacteria. Nucleic Acids Res. **18:**4296.
72. **Tyrrell, G. J., A. Kennedy, S. E. Shokoples, and R. K. Sherburne.** 2002. Binding and invasion of HeLa and MRC-5 cells by *Streptococcus agalactiae*. Microbiology **148:**3921–3931.
73. **Valentin-Weigand, P., P. Benkel, M. Rohde, and G. S. Chhatwal.** 1996. Entry and intracellular survival of group B streptococci in J774 macrophages. Infect. Immun. **64:**2467–2473.
74. **Valentin-Weigand, P., and G. S. Chhatwal.** 1995. Correlation of epithelial cell invasiveness of group B streptococci with clinical source of isolation. Microb. Pathog. **19:**83–91.
75. **van deGruchte, M., F. J. van der Wal, J. Kok, and G. Venema.** 1992. Lysozyme expression in *Lactococcus lactis*. Appl. Microbiol. Biotechnol. **37:**216–224.
76. **van der Vossen, J. M., D. van der Lelie, and G. Venema.** 1987. Isolation and characterization of *Streptococcus cremoris* Wg2-specific promoters. Appl. Environ. Microbiol. **53:**2452–2457.
77. **Waite, D. C., E. J. Alper, and B. J. Mady.** 1996. Adult group B streptococcal disease. Ann. Intern. Med. **125:**152–153.
78. **Wann, E. R., S. Gurusiddappa, and M. Höök.** 2000. The fibronectin-binding MSCRAMM FnbpA of *Staphylococcus aureus* is a bifunctional protein that also binds to fibrinogen. J. Biol. Chem. **275:**13863–13871.
79. **Whitnack, E., and E. H. Beachey.** 1982. Antiopsonic activity of fibrinogen bound to M protein on the surface of group A streptococci. J. Clin. Investig. **69:**1042–1045.
80. **Whitnack, E., and E. H. Beachey.** 1985. Degradation products of fibrinogen and fibrin prevent opsonization of group A streptococci. Trans. Assoc. Am. Physicians **98:**392–398.
81. **Wibawan, I. W., and C. Lämmler.** 1992. Relationship between group B streptococcal serotypes and cell surface hydrophobicity. Zentralbl. Veterinarmed. **39:**376–382.
82. **Winram, S. B., M. Jonas, E. Chi, and C. E. Rubens.** 1998. Characterization of group B streptococcal invasion of human chorion and amnion epithelial cells In vitro. Infect. Immun. **66:**4932–4941.
83. **Zangwill, K. M., A. Schuchat, and J. D. Wenger.** 1992. Group B streptococcal disease in the United States, 1990: report from a multistate active surveillance system. Morbid. Mortal. Wkly. Rep. CDC Surveill. Summ. **41:**25–32.

Editor: V. J. DiRita