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BsaB, a Novel Adherence Factor of Group B *Streptococcus*

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Streptococcus agalactiae **(group B** ***Streptococcus*** **[GBS]) is a leading cause of neonatal sepsis and meningitis, peripartum infections in women, and invasive infections in chronically ill or elderly individuals. GBS can be isolated from the gastrointestinal or genital tracts of up to 30% of healthy adults, and infection is thought to arise from invasion from a colonized mucosal site. Accordingly, bacterial surface components that mediate attachment of GBS to host cells or the extracellular matrix represent key factors in the colonization and infection of the human host. We identified a conserved GBS gene of unknown function that was predicted to encode a cell wall-anchored surface protein. Deletion of the gene and a cotranscribed upstream open reading frame (ORF) in GBS strain 515 reduced bacterial adherence to VK2 vaginal epithelial cells** ***in vitro*** **and reduced GBS binding to fibronectin-coated microtiter wells. Expression of the gene product in** ***Lactococcus lactis*** **conferred the ability to adhere to VK2 cells, to fibronectin and laminin, and to fibronectin-coated ME-180 cervical epithelial cells. Expression of the recombinant protein in** ***L. lactis*** **also markedly increased biofilm formation. The adherence function of the protein, named bacterial surface adhesin of GBS (BsaB), depended both on a central BID1 domain found in bacterial intimin-like proteins and on the C-terminal portion of the BsaB protein. Expression of BsaB in GBS, like that of several other adhesins, was regulated by the CsrRS two-component system. We conclude that BsaB represents a newly identified adhesin that participates in GBS attachment to epithelial cells and the extracellular matrix.**

Streptococcus agalactiae (group B *Streptococcus* [GBS]) can be considered part of the normal human microbiota, as it colonizes the gastrointestinal and/or genital tracts of 15 to 30% of healthy adults (1). However, in certain circumstances, GBS can behave as a life-threatening pathogen that causes infection in neonates, pregnant women, and elderly or immunocompromised persons. Despite the success of prenatal screening and maternal antibiotic prophylaxis in the United States and other countries, GBS remains the leading cause of early-onset neonatal sepsis (2). Neonates acquire GBS from a colonized mother shortly before or during birth by aspiration of infected amniotic fluid or vaginal secretions or by bacterial contamination of the skin or mucosal surfaces (1, 3). Approximately 50% of infants born to a vaginally colonized mother become colonized as a result of peripartum exposure. The ability of GBS to adhere to mucosal and epithelial surfaces is thought to be an essential early step for GBS colonization and for subsequent development of disseminated infection (4).

GBS, like other Gram-positive pathogens, produces surface proteins that mediate bacterium-host receptor interactions (5). Several surface proteins have been characterized as adhesins that are involved in bacterial attachment to host cells and/or the extracellular matrix (ECM) (6–16). Members of one family of surface proteins contain the LPXTG anchor motif at the C terminus followed by a hydrophobic domain and a positively charged tail (17). These proteins, after being synthesized in the cytosol, are exported via the Sec pathway by means of a cleavable N-terminal signal peptide and then anchored to cell wall peptidoglycan through sortase-mediated cleavage within the LPXTG motif and subsequent linkage to a peptidoglycan cross-bridge (18). Analysis of genome sequences of GBS strain 2603V/R (referred to here as 2603) and strain NEM316 revealed 24 or 21 surface proteins, respectively, bearing the LPXTG motif (19, 20). These LPXTG-containing proteins are often involved in (i) bacterial attachment to human cells, such as, for example, the serine-rich repeat family protein Srr-1 and BibA, and/or (ii) binding to ECM components, such as, for example, fibrinogen-binding protein FbsA and fibronectin-binding protein ScpB (6, 10, 11, 13–16).

Searching the genome sequence of GBS strain 515, a clinical isolate from an infected neonate, we identified a predicted cell wall-anchored protein encoded by *sal0825* (20, 21). The protein harbors a typical N-terminal signal peptide and C-terminal LPXTG sorting signal, consistent with its being anchored to the GBS cell wall. Sal0825 is one of seven surface proteins that are conserved across GBS strains (22). On the basis of these features and results of the functional studies reported here, we named the protein BsaB (bacterial surface adhesin of GBS). *In vitro* functional analysis of BsaB revealed that the protein participates in GBS binding to human fibronectin and laminin, in the adhesion of GBS to human epithelial cells, and in biofilm formation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. GBS strains were 515 (21), 2603 (2603V/R) (23), NEM316 (19), A909, H36B, 18RS21 (24), COH1 (25), CJB111 (Carol Baker, Baylor College of Medicine, Houston, TX), and the derivative mutants 515Δ*csrR* and 2603Δ*csrR* (26). Unless otherwise specified, GBS strains were grown in Todd-Hewitt broth (THB; Difco) or on Tryp-

Received 16 August 2013 Returned for modification 14 September 2013
Accepted 7 December 2013
Published ahead of print 16 December 2013
Editor: A. Camilli
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.01014-13.

doi:10.1128/IAI.01014-13

TABLE 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant property(ies)	Source or reference
Strains		
GBS strains		
NEM316	Wild-type strain	19
2603V/R	Wild-type strain	23
A909	Wild-type strain	24
H36B	Wild-type strain	24
CJB111	Wild-type strain	Carol Baker
COH1	Wild-type strain	25
18RS21	Wild-type strain	24
515	Wild-type strain	21
515Δ*csrR*	515 with deletion of *csrR* gene	26
515Δ*sal0824/5*	515 with deletion of *sal0824-sal0825* genes	This study
515Δ*sal0825*	515 with deletion of *sal0825* gene	This study
515Δ*csrR*Δ*sal0824/5*	515 with deletion of *csrR* and *sal0824-sal0825* genes	This study
515Δ*csrR*Δ*sal0825*	515 with deletion of *csrR* and *sal0825* genes	This study
515pNZ8048	515 containing pNZ8048	This study
515pNZ-*sal0825*	515 expressing Sal0825 from pNZ-*sal0825*	This study
515pNZ-AB	515 expressing peptide AB of Sal0825 from pNZ-AB	This study
515pNZ-A	515 expressing peptide A of Sal0825 from pNZ-A	This study
515pNZ-BC	515 expressing peptide BC of Sal0825 from pNZ-BC	This study
515pNZ-C	515 expressing peptide C of Sal0825 from pNZ-C	This study
515pNZ-SEC-*sal0825*	515 expressing Sal0825 from pNZ-SEC-*sal0825*	This study
515pNZ-SEC-AB	515 expressing peptide AB of Sal0825 from pNZ-SEC-AB	This study
515pNZ-SEC-A	515 expressing peptide A of Sal0825 from pNZ-SEC-A	This study
515pNZ-SEC-BC	515 expressing peptide BC of Sal0825 from pNZ-SEC-BC	This study
515pNZ-SEC-C	515 expressing peptide C of Sal0825 from pNZ-SEC-C	This study
2603V/R	Wild-type strain	23
2603Δ*csrR*	2603V/R with deletion of *csrR* gene	26
2603Δ*sal0824/5*	2603V/R with deletion of *sal0824-0825* genes	This study
2603ΔcsrRΔ*sal0824/5*	2603V/R with deletion of *csrR* and *sal0824-0825* genes	This study
L. lactis strains		
NZ9000	*L. lactis* susp. *cremoris* MG1363	27
NZ9700	Nisin-secreting *L. lactis* strain	27
NZ9000::*sal0825*	NZ9000 expressing Sal0825 from pNZ-*sal0825*	This study
NZ9000::AB	NZ9000 expressing peptide AB of Sal0825 from pNZ-AB	This study
NZ9000::A	NZ9000 expressing peptide A of Sal0825 from pNZ-A	This study
NZ9000::BC	NZ9000 expressing peptide BC of Sal0825 from pNZ-BC	This study
NZ9000::C	NZ9000 expressing peptide C of Sal0825 from pNZ-C	This study
E. coli DH5α	Chemically competent intermediate host; plasmid free	Zymo Research Corp.
Plasmids		
pJRS233	Temperature-sensitive *E. coli*-GBS shuttle vector; Er^r	57
pNZ8048	*E. coli-L. lactis* shuttle vector containing nisin-inducible promoter PnisA and start codon in NcoI site; Cm^r	27
pNZ-*sal0825*	pNZ8048 plasmid containing VSV-G-tagged *sal0825* gene; Cm^r	This study
pNZ-A	pNZ8048 plasmid containing VSV-G-tagged fragment A of *sal0825* gene; Cm^r	This study
pNZ-AB	pNZ8048 plasmid containing VSV-G-tagged fragment AB of *sal0825* gene; Cm^r	This study
pNZ-BC	pNZ8048 plasmid containing VSV-G-tagged fragment BC of *sal0825* gene; Cm^r	This study
pNZ-C	pNZ8048 plasmid containing VSV-G-tagged fragment C of *sal0825* gene; Cm^r	This study
pNZ-SEC	Modified pNZ8048, containing P44 promoter from plasmid pNZ44 and SEC signal from the *L. lactis* MG1363 chromosome; Cm^r	27
pNZ-SEC-*sal0825*	pNZ-SEC containing *sal0825*, Cm^r	This study
pNZ-SEC-AB	pNZ-SEC plasmid containing VSV-G-tagged fragment AB of *sal0825* gene; Cm^r	This study
pNZ-SEC-A	pNZ-SEC plasmid containing VSV-G-tagged fragment A of *sal0825* gene; Cm^r	This study
pNZ-SEC-BC	pNZ-SEC plasmid containing VSV-G-tagged fragment BC of *sal0825* gene; Cm^r	This study
pNZ-SEC-C	pNZ-SEC plasmid containing VSV-G-tagged fragment C of *sal0825* gene; Cm^r	This study

ticase soy agar supplemented with 5% defibrinated sheep blood (PML Microbiologicals). *Escherichia coli* was grown in Luria-Bertani broth, and *Lactococcus lactis* was grown in GM17 broth (M17 broth [Oxoid] supplemented with 0.5% glucose) (27). When appropriate, antibiotics were used at the following concentrations: for *E. coli*, erythromycin (ERM) at 200 μg/ml and ampicillin at 100 μg/ml; for *L. lactis*, chloramphenicol at 10 μg/ml; and for GBS, ERM at 1 μg/ml and chloramphenicol at 10 μg/ml.

RT-PCR. GBS RNA was isolated as described previously (28). For reverse transcription-PCR (RT-PCR), 50 ng of total RNA was used with the Invitrogen kit according to the manufacturer's recommendations. The reaction sequence included reverse transcription (30 min at 45°C), denaturation (2 min at 94°C), and 40 cycles of PCR with the following parameters: 94°C for 15 s, 55°C for 30 s, and 68°C for 60 s. PCR primers used in this study are listed in Table 2.

qRT-PCR. GBS strains were grown to mid-exponential phase, and RNA isolation and quantitative RT-PCR (qRT-PCR) were performed as described previously (28).

Cell culture. VK2, a human vaginal epithelial cell line, and ME-180, a human cervical carcinoma cell line, were cultured as described previously (28).

Cell wall protein extracts. For identification of recombinant protein on the bacterial cell surface, cell wall-anchored proteins were released by the following procedure. Bacterial cells were collected from 20 ml overnight culture, washed twice with phosphate-buffered saline (PBS), pH 7.4, resuspended in 0.5 ml of protoplast buffer (50 mM HEPES, 0.01 M $MgCl_2$, 0.5 M sucrose, pH 7.0), and then treated with lysozyme (2 mg/ml) and mutanolysin (60 U/ml) at 37°C for 1 h. The supernatant was collected after centrifugation of protoplasts at 5,000 × *g* for 45 min at 4°C, and supernatant proteins were analyzed by SDS-PAGE and Western immunoblotting.

Construction of mutagenesis plasmid for deletion of the *sal0824/5* locus. For construction of a plasmid to delete the *sal0824-sal0825* (*sal0824/5*) locus, primers 1381 and 1383 were used to PCR amplify the first 114 bp of *sag0824* and 799 bp of adjacent upstream flanking sequence using GBS strain 515 chromosomal DNA as the template. Primers 1388.4 and 1386.3 were used to amplify the last 99 bp of *sal0825* and 806 bp of downstream flanking DNA. Primer 1388.4 contains 20 bp of DNA that is complementary to primer 1383. The two gel-purified PCR products containing complementary ends were mixed and amplified with primers 1381 and 1386.3 to create a 2,190-bp internal deletion of the *sal0824/5* gene locus by overlap PCR. The 1,817-bp overlap PCR product was digested with BamHI and KpnI and ligated into BamHI/KpnI-digested pJRS233. We used a similar strategy to construct a plasmid to introduce a large internal deletion in *sal0825* using primer pairs 1500/1501 and 1502/1503 to amplify the *sal0825* 5′ and 3′ termini and flanking regions, followed by overlap PCR with primer pair 1500/1503 to fuse the 5′ and 3′ amplicons.

Construction of GBS mutants 515Δ*sal0824/5*, 2603Δ*sal0824/5*, 515Δ*csrR*Δ*sal0824/5*, 2603Δ*csrR*Δ*sal0824/5*, 515Δ*sal0825*, and 515Δ*csrR*Δ*sal0825*. The deletion construct in plasmid pJRS233 was introduced into GBS candidate strains 515, 515Δ*csrR*, 2603, and 2603Δ*csrR* by electroporation. Exchange of the internally deleted *sal0824/5* or *sal0825* locus for the native alleles on the GBS chromosome and identification of mutants was accomplished as described previously (28).

Expression of Sal0825 and truncated peptides in *L. lactis* NZ9000 or GBS 515. To express Sal0825 in *L. lactis* NZ9000 or GBS 515, primers 1484 and 1485 were used to PCR amplify the first 1,419 bp of *sal0825*. Primers 1486 and 1483.2 were used to amplify the last 120 bp of *sal0825*, which encodes the C terminus sorting signal. Primers 1486 and 1485 included a 33-bp vesicular stomatitis virus G protein (VSV-G) epitope tag sequence. The two gel-purified PCR products containing complementary ends were mixed and amplified with primers 1484 and 1383.2 to create a *sal0825*--VSV-G fusion by overlap PCR. The overlap PCR product was digested with NcoI and XbaI and ligated into a similarly digested pNZ8048 vector.

To express the truncated Sal0825 peptides, four primer pairs were used to amplify the specific regions AB, A, BC, and C (see Fig. 6). Primers 1484 and 1490.1 were used to PCR amplify the first 873 bp of *sal0825* (fragment AB), primers 1484 and 1490.2 were used to PCR amplify the first 627 bp of *sal0825* (fragment A), primers 1507 and 1485 were used to PCR amplify the region from bp 628 to 1419 of *sal0825* (fragment BC), and primers 1508 and 1485 were used to PCR amplify the region from bp 874 to 1419 (fragment C) of *sal0825*. As described for the construction of pNZ-*sal0825*, the primers 1486 and 1483.2 were used to amplify the last 120 bp of *sal0825*. The resultant amplicon was fused with fragment AB, A, BC, or C by overlap PCR using primer pairs 1484/1490.1, 1484/1490.2, 1507/1483.2, and 1508/1483.2 to create fusion fragments AB–VSV-G, A–VSV-G, BC–VSV-G, and C–VSV-G, respectively. The overlap PCR product was digested with NcoI and XbaI and ligated onto the pNZ8048 vector digested with the same enzymes. To make pNZ-SEC constructs, the digested PCR product was ligated to the pNZ-SEC vector, which harbors the P44 promoter from plasmid pNZ44 and the SEC signal sequence from the *L. lactis* MG1363 chromosome (27).

The recombinant plasmids were transformed into *E. coli* DH5α chemically competent cells (Zymo Research). After verification of DNA sequences, the plasmid construct was subsequently transformed into electrocompetent *L. lactis* NZ9000 or GBS cells. Colonies were screened by PCR after 24 h of incubation.

Western immunoblotting. For immunoblotting, protein preparations were fractionated by SDS-PAGE under reducing conditions using a NuPAGE 12% bis-Tris gel (Invitrogen) and transferred onto a nitrocellulose membrane. The membrane was incubated in TBS (PBS with 0.005% Tween 20) containing 5% milk to block nonspecific binding, followed by washing three times in TBS. Primary antibody (anti-VSV-G; Sigma) was added at 1:5,000 in TBS for 1 h at room temperature. After being washed three times in TBS, membranes were incubated for 1 h with goat anti-rabbit IgG conjugated to horseradish peroxidase, diluted 1:10,000. Membranes were washed three times in substrate buffer. Positive bands were visualized with the addition of peroxidase substrate (Pierce).

GBS adherence to human epithelial cells. Adherence assays were performed as described previously using cell lines VK2 and ME-180 (28). Assays were repeated at least three times in triplicate. The percentage of adherent GBS was calculated as follows: (number of CFU of adherent GBS/number of CFU in initial inoculum) × 100%.

GBS adherence to human extracellular matrix proteins. To investigate the adhesion of GBS to immobilized ECM components, adherence assays were performed in 24-well polystyrene plates coated with individual ECM proteins. Plates coated with fibronectin or laminin were purchased from BD Biosciences. Adherence assays were performed as described previously (8). Assays were repeated at least three times in triplicate. The percentage of adherent GBS was calculated as follow: (number of CFU of adherent GBS/number of CFU in initial inoculum) × 100%.

Biofilm formation assay. Biofilm assays were performed in 96-well polystyrene flat-bottom microtiter plates (Costar) as described previously (8, 29, 30). Each assay was performed in triplicate and repeated at least three times.

Statistical analysis. Data are reported as means ± standard deviations (SD) unless otherwise stated. Statistical analysis was performed using Prism 5.0 (Graphpad Software Inc.). Differences between groups were analyzed using a two-tailed *t* test. Differences with *P* values of <0.05 were considered statistically significant. Asterisks in the figures represent ranges of *P* values for differences between groups (not significantly different [NS], $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$).

RESULTS

Identification of a GBS gene predicted to encode a surface protein of unknown function. Surface components play an important role for tissue colonization and infection by mediating interactions between the pathogen and the host cells and in evasion of immune defense. Analysis of the completed GBS genomes identified a gene, designated *sal0825* in strain 515, which is present in all

TABLE 2 Oligonucleotide primers used for PCR

Category and locus	Primer	Direction[a]	Sequence (5′–3′)
Primers for analysis of regulation by CsrR			
sal0824	1375	F	CCATTGTCGAATAGGCATGT
	1376	R	TCATTGCCAATACTCCACCT
sal0825	1377	F	ACCTGTGAACGCTAAAGCTG
	1378	R	GCTGACCACTTGTCACCTCT
sal0826	1416	F	AGACGGTGATCAGCTGTTTG
	1417	R	AAGGGCAGAGCCAAAGAATA
sal0827	1418	F	TGCTTCACAAATGGATACCG
	1819	R	GTCACCCCCTGTTTATCGAC
Primers for analysis of transcriptional linkage			
sal0823	1404	F	TCTGTTATGGGCAAGTCTCTCT
sal0824	1405	R	ATTAACCAAAGTCAGCCACATC
sal0824	1443	F	ACTACATCTGATGACACAGTCCAA
sal0825	1444	R	TTGTCACCTCTGACAAAGTTACCA
sal0825	1406	F	TACCACCTACTTCGAAACCAAC
sal0826	1407	R	CCTGCTTCTCTAATTTCACCAC
sal0826	1428	F	TGTGGGTCAACTTCAGTTTGAAGT
sal0827	1429	R	CGTGATATAGAAGCATAAGGTATAG
sal0827	1420	F	AACAGGTTGATTTAGCTTATACCT
sal0828	1421	R	CACTTTCTCCTGCTAACAAATTAT
sal0828	1422	F	AACAGGTGAAATGCCATAGTTTGA
sal0829	1423	R	TCGATCTACAACAACTTGAGGTAT
Primers for deletion of *sal0824/5* or *sal0825*			
sal0823	1381	F	GCGGGATCCATTAAACAACCACCTCAGGA (BamHI site is underlined)
sal0824	1383	R	TCTCTATTAACCAAAGTCAG
sal0825	1388.4	F	CTGACTTTGGTTAATAGAGACTGGTGATCAAGCCATTAG (underlined sequence is complementary to that of primer 1383)
sal0826	1386.3	R	GCGCGGTACCTGGATTATTGGCAAACAGCT (KpnI site is underlined)
sal0824	1500	F	GCGGATCCCAGAGATTCCGCAGATGCCT (BamHI site is underlined)
sal0825	1501	R	CTTGCTGCCATTACTGGTG
sal0825	1502	F	CACCAGTAATGGCAGCAAGGCTATGACAGCCTTAGCTA (the underlined sequence is complementary to that of primer 1501)
sal0826	1503	R	CGGGTACCATTGGCAAACAGCTGATCA (KpnI site is underlined)
Primers for expression of Sal0825 and truncated peptides in pNZ8048			
sal0825	1484	F	CGGCCATGGGTAATAAATCATTCAATACCAAATTAG (NcoI site is underlined)
	1485	R	TTTACCTAAACGATTCATTTCAATATCAGTATAGGCTGTCATAGCTTTTGG (underlined sequence is complementary to VSV-G sequence)
	1486	F	TATACTGATATTGAAATGAATCGTTTAGGTAAATTAGCTAAAAAATTGCCTAAAACTG (underlined sequence is the VSV-G sequence)
	1483.2	R	GGCTCTAGATTATTTGATCGTGATTTTTAAGGAAGCCTAAC (XbaI site is underlined)
Fragment AB	1490.1	R	CGATTCATTTCAATATCAGTATATCGAATAGTATAGCCCTTTGA (underlined sequence is complementary to the VSV-G sequence)
Fragment A	1490.2	R	CGATTCATTTCAATATCAGTATATGATTCTCCAGGTACAATATC (underlined sequence is complementary to the VSV-G sequence)
Fragment BC	1507	F	GCCCATGGGTAGAAGTGCATATGTTAATGTTG (NcoI site is underlined)
Fragment C	1508	F	GCCCATGGGTACTGATGTAGCAGGCTCT (NcoI site is underlined)

[a] F, forward; R, reverse.

reported sequenced strains. Because the genome sequences of several strains indicated disruption of the homologous sequence by frameshift mutations, we amplified by PCR the chromosomal region in strains 2603, NEM316, CJB111, H36B, A909, 18RS21, and COH1 and sequenced the amplicons. The results showed an uninterrupted open reading frame (ORF) for all strains except COH1, with 98 to 99% identity in its predicted amino acid (aa) sequence to that of *sal0825* in strain 515. Strain COH1 appears to have a single nucleotide deletion that results in a frameshift and premature termination at aa 229. Sal0825 harbors a bacterial im-

***sal0824/0825* locus in GBS strain 515**

Gene	Gene Length* (bp)	Protein Length* (aa)	Protein Mass* (kDa)	Proposed Function*
sal0823	1221	406	44.1	Oxalate:formate antiporter
sal0824	**687**	**228**	**26.4**	**Putative membrane protein**
sal0825	**1539**	**512**	**54.3**	**Cell wall surface anchor family protein**
sal0826	1545	514	58.3	peptide chain release factor 3
sal0827	381	126	14.0	conserved hypothetical protein
sal0828	735	244	27.7	ABC transporter, ATP-binding protein
sal0829	663	220	24.9	ABC transporter, permease protein

**sal0824* and *sal0825* of strain 515 were resequenced in this study. Features of the flanking genes and predicted proteins are based on the genome sequence of GBS 2603V/R (20).

FIG 1 Schematic of the chromosomal region of GBS strain 515 that contains the *sal0824/5* locus and flanking genes.

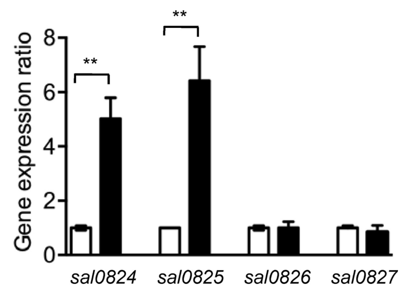

FIG 2 CsrR regulation of expression of *sal0824/5* in GBS strain 515. Relative transcript abundances for the indicated genes were compared by RT-PCR between strains 515 (open bars) and 515Δ*csrR* (filled bars). Values represent gene expression relative to that in 515 (mean ± SD). Each assay was performed at least three times in triplicate. **, $P < 0.01$.

munoglobulin-like domain (BID1) spanning aa 210 to 291. BID1 domains are found in bacterial surface proteins such as intimin-like proteins and cell adhesion molecules of Gram-negative pathogens that mediate bacterial adhesion and/or invasion into host cells (31, 32).

The upstream ORF, *sal0824*, is transcribed in the same orientation as *sal0825* (Fig. 1). Sal0824 contains 6 membrane-spanning domains, which suggests that it is located on the cell surface. Homologs of *sal0824* and *sal0825* were found in GBS strains 2603, NEM316, CJB111, H3bB, A909, 18RS21, and COH1. BLAST analysis performed with *sal0824* did not reveal any homologous proteins of known function in the database. The downstream ORF, *sal0826*, was predicted to encode a protein with homology to peptide chain release factor 3 of *E. coli*, which is involved in the release of newly synthesized polypeptide chains from the ribosome (33, 34). The function of the hypothetical protein encoded by *sal0827* is unknown. We performed RT-PCR to define the transcriptional linkage between *sal0825* and flanking ORFs. The results demonstrated cotranscription of four consecutive ORFs, *sal0824*, *sal0825*, *sal0826*, and *sal0827* (see Fig. S1 in the supplemental material). The functional relationship of proteins encoded by the four cotranscribed genes remains to be investigated.

Expression of Sal0824/5 is repressed by CsrR. The CsrRS (or CovRS) two-component regulatory system controls the expression of multiple virulence factors in GBS (26, 35–40). Inactivation of CsrRS in GBS strains was associated with increased adherence to epithelial cells and increased expression of several adhesins (8, 38). To test whether CsrR regulates the expression of *sal0825*, we compared the expression of *sal0825* in the 515 wild type to that in the 515Δ*csrR* mutant. The result demonstrated a marked increase in the expression of *sal0825* in strain 515Δ*csrR* relative to that in wild-type strain 515 (Fig. 2). As discussed above, three other genes, *sal0824*, *sal0826*, and *sal0827*, were cotranscribed with *sal0825*, so we also tested whether their expression was regulated by CsrR. We found that the expression of *sal0824* was increased to an extent similar to that of *sal0825* in strain 515Δ*csrR*. In contrast, we found no increase in the expression of *sal0826* and *sal0827* (Fig. 2). In summary, the data suggested that the expression of *sal0824* and *sal0825* is under the negative control of CsrR. Despite the apparent transcriptional linkage of the two downstream ORFs to *sal0824* and *sal0825*, their expression may be controlled predominantly by a separate, CsrR-independent, promoter.

Effect of Sal0824/5 inactivation on GBS adherence. We hypothesized that Sal0825 participates in bacterium-host cell interactions based on its surface localization and the presence of a BID1 domain. To investigate the biological function of the protein, we first tested whether it contributes to the adherence of GBS to human epithelial cells. A deletion mutant was developed by allelic exchange in which both *sal0824* and *sal0825* were deleted from GBS strain 515. We chose to include *sal0824* in this deletion with the consideration that it may form a functional unit with *sal0825* based on their transcriptional linkage, similar regulation by CsrR, and surface localization. We then examined the relative association of the GBS wild-type and mutant strains with two types of human epithelial cells, VK2 (vaginal epithelial cell line) and ME-180 (cervical carcinoma cell line). After 1 h of exposure to GBS, cell monolayers were washed to remove the unbound bacteria, detached from wells, and lysed, and quantitative cultures of the lysates were performed to enumerate the cell-bound bacteria. We observed 31% less adherence to VK2 cells of 515Δ*sal0824/5* than to those of wild-type strain 515 (Fig. 3). Similarly, adherence of strain 515Δ*sal0824/5* to ME-180 cells was reduced by 29% compared to that of the wild type, although overall adherence to this cell line was quite low. Since expression of *sal0824/5* is repressed by CsrR in strain 515 (Fig. 2), the relatively modest effect on adherence from inactivation of this locus might reflect the low level of *sal0824/5* expression. To explore this possibility, we constructed a double mutant, 515Δ*csrR*Δ*sal0824/5*, and compared its adherence with that of 515Δ*csrR*. While inactivation of *csrR* resulted in increased adherence to VK2 cells, adherence of the double mutant strain to both cell lines was not significantly different from that of 515Δ*csrR* (Fig. 3).

Bacterium-host tissue interaction often involves the attachment of the bacterium to human ECM components, which in turn bind host cell surface integrins (41). To investigate whether the

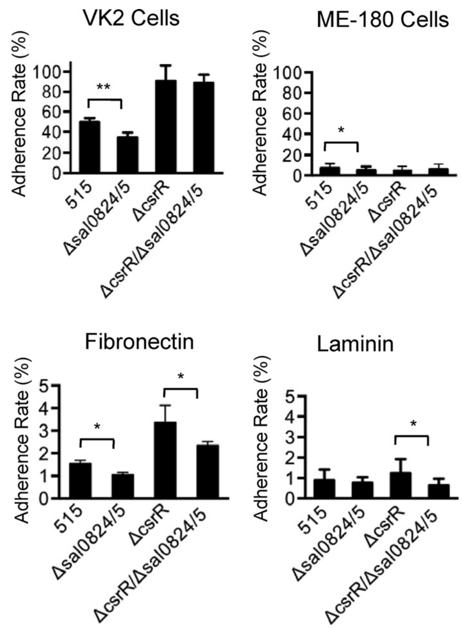

FIG 3 Effect of inactivation of the *sal0824/5* locus on GBS adherence to human epithelial cells or immobilized ECM proteins. Levels of adherence were compared among GBS strains 515, 515Δ*sal0824/5* (Δ*sal0824/5*), 515Δ*csrR* (Δ*csrR*), 515Δ*csrR*/Δ*sal0824/5* (Δ*csrR*/Δ*sal0824/5*). Adherence is shown as a percentage of the initial inoculum (mean ± SD). Each assay was performed at least three times in triplicate. **, $P < 0.01$; *, $P < 0.05$.

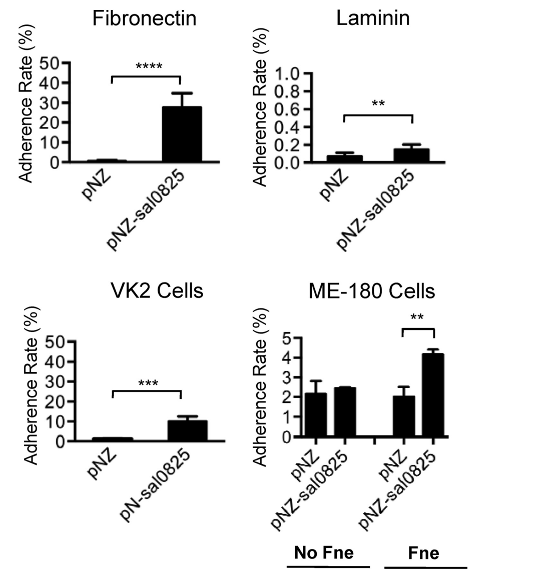

FIG 4 The expression of Sal0825 protein increases the adherence of *L. lactis* to human epithelial cells or immobilized ECM proteins. Levels of adherence were compared among strains *L. lactis*(pNZ) and *L. lactis*(pNZ-*sal0825*). ME-180 cells were exposed to *L. lactis* strains before or after the cell monolayer was coated with human fibronectin (Fne). Adherence is shown as a percentage of the initial inoculum (mean ± SD). Each assay was performed at least three times in triplicate. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$.

inactivation of *sal0824/5* affects GBS adherence to ECM proteins, we compared the levels of adherence of GBS strains using 24-well plates coated with either fibronectin or laminin. Coated wells were each inoculated with approximately 6×10^6 CFU of GBS, and the adherence rate was calculated as the ratio of the number of CFU of GBS bound to the number of CFU in the initial inoculum. We found 32% and 29% decreases, respectively, in adherence to fibronectin when *sal0824/5* was deleted from strain 515 and 515Δ*csrR* (Fig. 3). Deletion of *sal0824/5* resulted in a more modest reduction in binding to laminin, a difference that reached statistical significance only in the 515Δ*csrR* background.

In summary, inactivation of *sal0824/5* led to a moderate reduction in adherence of GBS 515 to human epithelial VK2 cells and immobilized fibronectin. This limited effect on adherence may be explained by the presence of other adhesins on the surface of GBS. Indeed, several surface components of GBS are involved in the adherence to host cells and ECM and may be responsible for the residual adherence of the *sal0824/5* deletion mutant (6–16).

sal0824 is predicted to encode a protein closely associated with the cell membrane; therefore, we thought it unlikely that the reduced-adherence phenotype observed in the *sal0824/5* mutant was attributable to loss of Sal0824. Rather, we focused our subsequent studies on *sal0825*, which is predicted to encode a cell wall-anchored protein with an exposed extracellular domain that is more likely to mediate adherence. To test the role of Sal0825, we constructed a mutant in which only *sal0825* was deleted. We found that loss of Sal0825 had an impact on adherence similar to that of deletion of both Sal0824 and Sal0825 (see Fig. S2 in the supplemental material). Thus, it appears that Sal0825, and not Sal0824, is the major component that contributes to the adherence phenotype.

Effect of Sal0825 expression on the adherence of *L. lactis*. Because of overlapping functions of multiple GBS adhesins, deletion of one or two proteins may not have a major impact on overall bacterial adhesion. To further investigate the potential role of Sal0825, we expressed the protein on the surface of *L. lactis*. Nonpathogenic *L. lactis* has been a useful tool to express and decipher the function of heterologous proteins. It supports expression of surface proteins from Gram-positive bacteria and has the further advantage of relatively low intrinsic adherence to cells and ECM proteins (42, 43). The *sal0825* sequence was fused with a VSV-G epitope tag and cloned into pNZ8048. The recombinant plasmid was introduced into *L. lactis* (see Materials and Methods). The NICE (nisin-induced controlled expression) system of plasmid pNZ8048 enabled expression of Sal0825 protein in *L. lactis* after a 1-h induction with nisin (27). As detected by Western blotting, Sal0825 is anchored to the lactococcal cell wall (see Fig. S3 in the supplemental material). The adherence of *L. lactis* pNZ-*sal0825* to fibronectin was markedly increased (46-fold) relative to that of *L. lactis* pNZ, and its adherence to laminin was modestly increased (2-fold) (Fig. 4). The Sal0825-expressing strain also showed 8-fold-greater adherence to VK2 cells. All these data suggested that Sal0825 functions as an adhesin in bacterial interactions with host cells and/or ECM. The expression of Sal0825 did not change lactococcal adherence to ME-180 cells in initial experiments (Fig. 4). Since ECM proteins can act as bridging molecules between bacterial adhesins and cell surface receptors, such as integrins, we tested whether fibronectin might function in this role for Sal0825-mediated adherence. When ME-180 monolayers were coated with fibronectin (10 μg/ml), we observed a 2-fold increase in the binding of *L. lactis* pNZ-*sal0825* compared to that on the uncoated cell monolayer (Fig. 4). In summary, using *L. lactis* as a heterologous expression host, we identified Sal0825 as a novel surface adhesin. The expression of Sal0825 significantly increased the association of *L. lactis* with the human ECM components fibronectin and laminin as well as human epithelial VK2 cells and ME-180 cells. The data suggest that Sal0825 adherence to epithelial cells is mediated by binding to fibronectin (and perhaps other ECM proteins), which acts as a bridging molecule linking the bacteria to the host cell surface.

Effect of Sal0825 expression on biofilm formation in GBS and *L. lactis*. Biofilm formation may have an important role in the pathogenesis of GBS infection (29, 30, 44). GBS has been isolated from the population of biofilm-forming bacteria on intrauterine devices and has been shown to form biofilm on abiotic and cell surfaces *in vitro*. Two research groups found that pilus PI-2a participates in biofilm formation (29, 30). However, it was also noted that GBS strains that do not produce pilus PI-2a can form biofilms, indicating that additional factors may contribute (30). To investigate whether Sal0825 is involved in biofilm formation, GBS strains were grown in LB medium supplemented with 1% glucose in polystyrene plates, and biofilm formation was detected by crystal violet staining followed by dye solubilization with acetic acid and measurement of absorbance at 540 nm. As shown in Fig. 5A and B, strain 515 produced very little biofilm. Similar results were observed in the mutant strain 515Δ*sal0824/5*. As biofilm formation is relatively low in wild-type strain 515, we also assessed the importance of *sal0824/5* in biofilm formation in the background of 515Δ*csrR*, in which expression of *sal0824/5* is upregulated. Our results showed that inactivation of *sal0824/*5 in 515Δ*csrR* resulted in a 47% reduction in biofilm formation (Fig. 5A and B). We also investigated the role of *sal0824/5* in biofilm formation by GBS strain 2603, as this strain exhibits a greater increase in biofilm formation upon deletion of *csrR* than does strain 515. Biofilm formation was decreased 34% in strain 2603Δ*csrR*Δ*sal0824/5* relative to that in strain 2603Δ*csrR*. As shown in Fig. 5, there is still some production of biofilm in the double mutant 2603Δ*csrR*Δ*sal0824/5*, indicating that Sal0824/5 is not the only factor contributing to biofilm formation. Together, these data suggested that Sal0824/5 is one of the factors involved in biofilm formation in GBS 515 and 2603 and that CsrR regulates biofilm formation, at least partially, through regulation of Sal0824/5 expression.

To test whether Sal0825 alone is sufficient to confer the ability to form biofilm, we compared the biofilm formation of *L. lactis* pNZ-*sal0825* with that of *L. lactis* harboring the pNZ8048 vector alone. As shown in Fig. 5E and F, a striking increase in biofilm production was observed when Sal0825 was expressed in *L. lactis*. Together, these data provide evidence that Sal0825 contributes to GBS biofilm formation.

Analysis of the function of the BID1 domain of Sal0825. As mentioned above, Sal0825 harbors a BID1 domain at aa 210 to 291, which is also present in other adhesion proteins (31, 32). To understand the role of the BID1 domain and other regions of the protein in adherence, we constructed recombinant pNZ plasmids corresponding to various regions of Sal0825 and expressed the recombinant peptides in *L. lactis* (Fig. 6A). Peptide AB corresponds to the first 291 aa, including the amino-terminal portion of the protein and the BID1 domain. Peptide BC corresponds to aa 210 to 512 and includes the BID1 domain and the adjacent C-terminal region. As indicated in Fig. 6A, we also expressed peptides A and C individually, which correspond, respectively, to the N-terminal region and C-terminal region, excluding the central BID1 domain. The N-terminal signal peptide and C-terminal sorting signal were included in each construct to allow correct localization and display of peptides on the bacterial surface. Each recombinant plasmid was introduced into *L. lactis*, and protein expression was induced with nisin. However, while trying to compare the levels of adherence to fibronectin of *L. lactis* strains, we found that our work was hampered by the poor growth of *L. lactis* pNZ-BC and *L. lactis* pNZ-C. Although strains *L. lactis* pNZ-AB and pNZ-A grew normally, neither of them showed any difference in adherence to fibronectin from that of *L. lactis* with the pNZ8048 vector alone (data not shown).

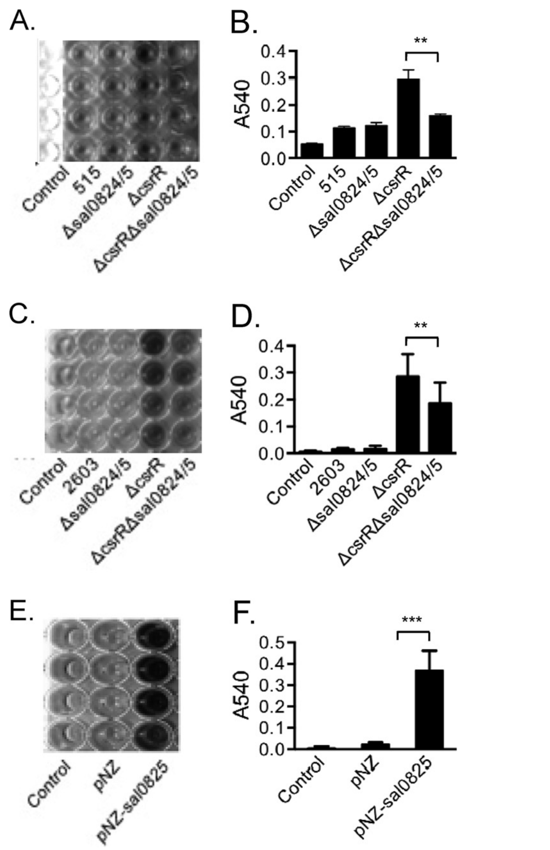

FIG 5 Role of Sal0825 in biofilm formation. Biofilms were compared among GBS strain 515 and isogenic mutants strains 515Δ*sal0824/5* (Δ*sal0824/5*), 515Δ*csrR* (Δ*csrR*), and 515Δ*csrR*/*sal0824/5* (Δ*csrR*/*sal0824/5*) (A and B), among GBS strain 2603 and isogenic mutant strains 2603Δ*sal0824/5* (Δ*sal0824/5*), 2603Δ*csrR* (Δ*csrR*), and 2603Δ*csrR*/*sal0824/5* (Δ*csrR*/*sal0824/5*) (C and D), and between *L. lactis* strains containing the plasmid construct pNZ-*sal0825* and the pNZ vector alone (E and F). (A, C, E) Adherent bacteria stained with crystal violet; (B, D, F) quantification of biofilm by measurement of absorbance at 540 nm after the release of bound dye from each well using glacial acetic acid. ***, $P < 0.001$; **, $P < 0.01$.

In order to further characterize the functional domain, we also expressed the same fragments of Sal0825 in GBS strain 515. Because nisin induction slowed GBS growth, we cloned and expressed full-length Sal0825 and derivative fragments under the control of the constitutive P44 promoter in pNZ-SEC in GBS strain 515 with the following plasmids: pNZ-SEC-*sal0825*, pNZ-SEC-A, pNZ-SEC-AB, pNZ-SEC-BC, pNZ-SEC-C, and the pNZ-SEC vector alone. The expression and localization of the entire or partial Sal0825 protein was confirmed by SDS-PAGE and Western

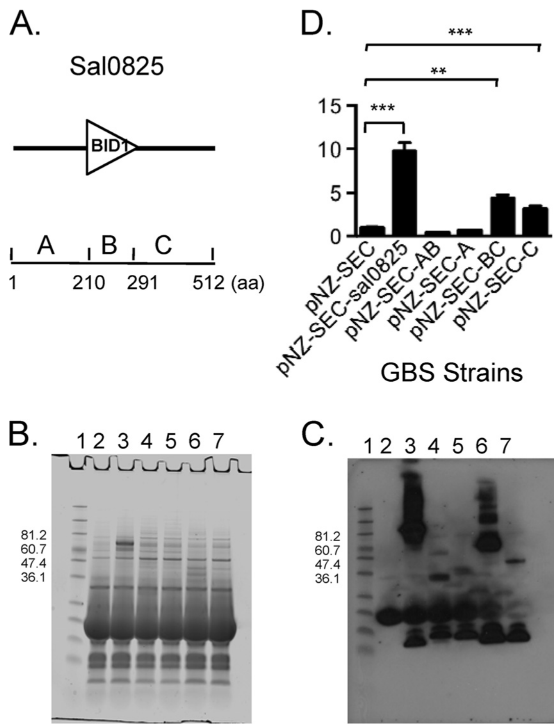

FIG 6 Analysis of the functional domains of Sal0825 in GBS. (A) Diagram of the fragments of Sal0825 expressed from the pNZ8048 vector in GBS 515. (B) Proteins anchored to the bacterial cell wall were isolated and then separated by SDS-PAGE. (C) Western blot analysis with specific antiserum against the epitope tag VSV-G. (D) Relative levels of adherence to human fibronectin among different GBS strains. (B and C) Lanes 1, molecular weight standards (numbers beside the lanes are in thousands); lanes 2, proteins isolated from GBS 515 containing the vector alone (pNZ-SEC); lanes 3 to 7, proteins isolated from 515pNZ-SEC-*sal0825* (pNZ-SEC-*sal0825*), 515pNZ-SEC-AB (pNZ-SEC-AB), 515pNZ-SEC-A (pNZ-SEC-A), 515pNZ-SEC-BC (pNZ-SEC-BC), and 515pNZ-SEC-C (pNZ-SEC-C), respectively. ***, $P < 0.001$; **, $P < 0.01$.

blotting (Fig. 6B and C). We evaluated the relative adherence of each GBS strain to fibronectin-coated plates. We found that overexpression of full-length Sal0825 increased the binding to fibronectin of GBS 515pNZ-SEC-*sal0825* by approximately 10-fold compared to that of 515pNZ-SEC (Fig. 6D), which is in agreement with the result in *L. lactis* (Fig. 4). We then compared the adherence capacities of GBS strains expressing peptides corresponding to various regions of Sal0825. We found that the overexpression of peptide BC (strain 515pNZ-SEC-BC) increased binding by 4.5-fold relative to that of 515pNZ-SEC. For peptide C, we detected only a very small amount on the GBS surface by Western blotting. However, even the small amount of peptide enhanced binding of 515pNZ-SEC-C by 3.2-fold relative to that of 515pNZ-SEC. On the other hand, the surface expression of peptides A and AB in strains 515pNZ-SEC-A and 515pNZ-SEC-AB was very low (Fig. 6B and C), and we did not observe any increase in GBS adherence related to the expression of these two peptides (Fig. 6D). In summary, our data suggest that the C-terminal region of Sal0825 corresponding to peptide C as well as the BID1 domain itself contributes to the adherence function of Sal0825. In addition, region C appears to play an important role in the expression, surface display, or stability of Sal0825, since constructs lacking this domain were poorly expressed on the bacterial surface in both *L. lactis* and GBS.

DISCUSSION

The present study investigated the importance of a previously uncharacterized surface protein encoded by *sal0825* in GBS adherence and biofilm formation. Based on the results of these experiments, we propose to name the protein bacterial surface adhesin of GBS (BsaB). Our data demonstrated that BsaB is localized on the surface of GBS and is able to interact directly with immobilized fibronectin and laminin. BsaB promoted the adherence of GBS to VK2 cells and ME-180 cells, representing human vaginal and cervical epithelial cells. In addition, BsaB enhanced GBS adherence to abiotic surfaces in strains 515 and 2603. In an attempt to identify the functional adherence domain of the protein, we found that binding to immobilized fibronectin involved both a central BID1 domain and the C-terminal region from aa 292 to 512. The latter domain is critical not only for binding but also for expression and proper display of BsaB on the bacterial surface.

Deletion of *sal0824/5* resulted in a moderate decrease in GBS adherence to human vaginal epithelial VK2 cells and immobilized human fibronectin. However, a loss-of-function approach is likely limited by the presence of multiple adherence factors in GBS, so the effect of *sal0824/5* inactivation might be largely compensated for by the function of other adhesins (6–16). A complementary gain-of-function approach provided evidence that overexpression of BsaB resulted in a substantial increase in the adherence of both *L. lactis* and GBS.

The most prominent function of BsaB is fibronectin binding (Fig. 4). Fibronectin is a large glycoprotein constituent of the ECM and blood plasma that has been shown to be a binding substrate for a variety of pathogenic bacteria, such as *Staphylococcus aureus* and *Streptococcus pyogenes* (41, 45–54). At least one of two genes coding for closely related fibronectin-binding proteins is found in almost all clinical isolates of *S. aureus* (55). *Streptococcus pyogenes* can express at least five different cell wall-anchored proteins with fibronectin-binding activity (52). In GBS, ScpB (C5a-peptidase) was identified as a bifunctional protein, working as a peptidase that inactivates human C5a and also mediating bacterial binding to fibronectin (6, 56). Fibronectin forms a molecular bridge between the bacterial surface and host cell integrins and is important for bacterium-host interactions (52). In this study, we found that fibronectin enhanced the attachment of GBS to ME-180 epithelial cells, presumably by a bridging mechanism (Fig. 4).

Our previous study showed that CsrR negatively regulates the expression of multiple GBS adhesins (8). The current work demonstrates that CsrR modulates the expression of BsaB in the same manner, consistent with the previously described central regulatory role of CsrR in GBS adherence (8, 38). *sal0825*, encoding BsaB, is cotranscribed with the upstream ORF *sal0824*, and expression of both is regulated by CsrR, but BsaB alone is sufficient to confer increased adherence on GBS and *L. lactis*.

Previous studies have shown that pilus type 2a is involved in biofilm formation by GBS. In this report, BsaB was found to contribute to biofilm formation in both strain 515, which produces type 2a pili, and strain 2603, which does not. In both strain backgrounds, BsaB-dependent biofilm formation was strongly regulated by CsrR.

Adherence of GBS to epithelial cells and/or ECM is a key step in bacterial colonization of host mucosal surfaces and in subsequent infection. The current work adds BsaB to a growing number of adhesins reported to be involved in GBS-host interaction. Expression of multiple surface proteins with adherence functions enables efficient interactions of GBS with different host components and likely enhances the organism's adaptability in occupying different niches of the host. While redundancy in function makes it difficult to establish a definitive role in pathogenesis for any single GBS adhesin, the multiplicity of adhesins produced by GBS underlines the importance of bacterial attachment in GBS colonization and invasion.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grants AI59502 and AI29952 from the National Institute of Allergy and Infectious Diseases.

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