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.

S. 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), CJBl11 (Carol Baker, Baylor College of Medicine, Houston, TX), and the derivative mutants 515ΔcsrR and 2603ΔcsrR (26). Unless otherwise specified, GBS strains were grown in Todd-Hewitt broth (THB; Difco) or on Tryp-
| Bacterial strain or plasmid | Relevant property(ies) | Source or reference |
|-----------------------------|------------------------|---------------------|
| **GBS 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                  |
| 515ΔcsrR                    | 515 with deletion of csrR gene | 26               |
| 515Δsal0824/5              | 515 with deletion of sal0824-sal0825 genes | This study |
| 515Δsal0825                | 515 with deletion of sal0825 gene | This study |
| 515ΔcsrRΔsal0824/5         | 515 with deletion of csrR and sal0824-sal0825 genes | This study |
| 515ΔcsrRΔsal0825            | 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 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                  |
| 2603ΔcsrR                   | 2603V/R with deletion of csrR gene | 26               |
| 2603Δsal0824/5              | 2603V/R with deletion of sal0824-sal0825 genes | This study |
| 2603ΔcsrRΔsal0824/5         | 2603V/R with deletion of csrR and sal0824-sal0825 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**                |                        |                     |
| pRS233                      | Temperature-sensitive E. coli–GBS shuttle vector; Er‘ | 57               |
| pNZ8048                     | E. coli-L. lactis shuttle vector containing nisin-inducible promoter PnisA and start codon in NcoI site; Cm‘ | 27               |
| pNZ8048 sal0825             | pNZ8048 plasmid containing VSV-G-tagged sal0825 gene; Cm‘ | This study |
| pNZ-A                       | pNZ8048 plasmid containing VSV-G-tagged fragment A of sal0825 gene; Cm‘ | This study |
| pNZ-AB                      | pNZ8048 plasmid containing VSV-G-tagged fragment AB of sal0825 gene; Cm‘ | This study |
| pNZ-BC                      | pNZ8048 plasmid containing VSV-G-tagged fragment BC of sal0825 gene; Cm‘ | This study |
| pNZ-C                       | pNZ8048 plasmid containing VSV-G-tagged fragment C of sal0825 gene; Cm‘ | This study |
| pNZ-SEC                     | Modified pNZ8048, containing P44 promoter from plasmid pNZ44 and SEC signal from the L. lactis MG1363 chromosome; Cm‘ | 27               |
| pNZ-SEC-sal0825             | pNZ-SEC containing sal0825; Cm‘ | This study |
| pNZ-SEC-AB                  | pNZ-SEC plasmid containing VSV-G-tagged fragment AB of sal0825 gene; Cm‘ | This study |
| pNZ-SEC-A                   | pNZ-SEC plasmid containing VSV-G-tagged fragment A of sal0825 gene; Cm‘ | This study |
| pNZ-SEC-BC                  | pNZ-SEC plasmid containing VSV-G-tagged fragment BC of sal0825 gene; Cm‘ | This study |
| pNZ-SEC-C                   | pNZ-SEC plasmid containing VSV-G-tagged fragment C of sal0825 gene; Cm‘ | 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 (MI7 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 MgCl₂, 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 saq0824 and 799 bp of adjacent upstream flanking sequence using GBS strain 513 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 1484 and 1490.2 to create a 33-bp vesicular stomatitis virus G protein (VSV-G) epitope tag sequence. Primers 1486 and 1485 included a 33-bp downstream flanking DNA. Primer 1388.4 was designed to ligate fragments A, BC, and C to 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 628 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, Sal0825, and 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, BC-VSV-G, and C-VSV-G, respectively. The overlap PCR product was digested with Ncol and XbaI and ligated into 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 0.05% 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).

Results

Comparison of bacterial adherence to artificial and native ECM proteins. Plates coated with fibronectin or laminin were used to evaluate 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 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 follows: (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 in 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 513, which is present in all
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 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-

### TABLE 2 Oligonucleotide primers used for PCR

| Category and locus | Primer | Direction | Sequence (5’–3’) |
|--------------------|--------|-----------|-----------------|
| **Primers for analysis of regulation by CsrR** | | | |
| sal0824 | 1375 | F | CCATTGTCGAATAGGCAATGTT |
| sal0824 | 1376 | R | TCATTGCAATACCTCCACCT |
| sal0825 | 1377 | F | ACCGTGAACGCTAAACGTC |
| sal0825 | 1378 | R | GGTGACACTCTGTCAACCTCT |
| sal0826 | 1416 | F | AGACGGTGATCAGCTGTTTTG |
| sal0826 | 1417 | R | AAGGGCAGAGCCAAGAATA |
| sal0827 | 1418 | F | TGCTCTCACAATGTGATACCG |
| sal0827 | 1819 | R | GTCACCCCTGTITATCGAC |
| **Primers for analysis of transcriptional linkage** | | | |
| sal0823 | 1304 | F | TCTGTATTAGGCAAGTCTC |
| sal0824 | 1305 | R | ATTAACCAAAGTCAGCCAC |
| sal0824 | 1304 | F | ATTAACCAAAGTCAGCCAC |
| sal0825 | 1306 | R | ACTACACTGTGACAGCAAGTCCAA |
| sal0825 | 1307 | F | TTTACCTAAGAGCATAAGGTAG |
| sal0825 | 1308 | R | AAACGGTGTATTAGCTTAC |
| sal0825 | 1309 | F | AAACGGTGTATTAGCTTAC |
| sal0825 | 1310 | R | AAGGGCAGAGCCAAGAATA |
| sal0826 | 1311 | F | AAGGGCAGAGCCAAGAATA |
| sal0826 | 1312 | R | AAGGGCAGAGCCAAGAATA |
| **Primers for deletion of sal0824/5 or sal0825** | | | |
| sal0823 | 1331 | F | GCGGGATCCATTTAAGCCACCTCAGGA (BamHI site is underlined) |
| sal0824 | 1332 | R | TCTCTTTTAACAAAGTCAG |
| sal0825 | 1333 | F | CTGCATTCTCCTAATTTTACCCAC |
| sal0825 | 1334 | R | TGTGGGTCAACTTCAGTTTGAATG |
| sal0826 | 1335 | F | CACCCAGTAAAGGCACAGGCATAGTTA |
| sal0826 | 1336 | R | TGCATCTAAAACACAGTGAAT |
| **Primers for expression of Sal0825 and truncated peptides in pNZ8048** | | | |
| sal0825 | 1351 | F | CGGCCATGGGTAATAAAAATCTTCAATACCTAATAATTAG (NcoI site is underlined) |
| sal0825 | 1352 | R | TTTACTTTAACAGATTATTGACGTTGCTCATAGCTTCTTGGG (underlined sequence is complementary to VSV-G sequence) |
| sal0825 | 1353 | F | TTATACATCATAATTACATAGTACATAGTTTGGG (underlined sequence is the VSV-G sequence) |
| sal0825 | 1354 | R | GGGCTCTAGAATTTTTGAATGCAAGCTACTAGT |
| Fragment AB | 1390.1 | R | CGATTCTCATTTCATAGCTATAGGATGCTATAGGATG |
| Fragment A | 1390.2 | R | CGATTCTCATTTCATAGCTATAGGATGCTATAGGATG |
| Fragment BC | 1407 | F | GCCCATTCAGGTAAGTGTCATATGCTAGTTG |
| Fragment A | 1408 | R | GCCCATTCAGGTAAGTGTCATATGCTAGTTG |

* F, forward; R, reverse.
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 peptid 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 sal0826 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. 1 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 515ΔcsrR mutant. The result demonstrated a marked increase in the expression of sal0825 in strain 515ΔcsrR 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 515ΔcsrR. 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 515Δsal0824/5 than to those of wild-type strain 515 (Fig. 3). Similarly, adherence of strain 515Δsal0824/5 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, 515ΔcsrRΔsal0824/5, and compared its adherence with that of 515ΔcsrR. 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 515ΔcsrR (Fig. 3).

Bacterium-host tissue interaction often involves the attachment of the bacterium to human ECM components, which can turn bind host cell surface integrins (41). To investigate whether the...
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 $6 \times 10^6$ 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 515ΔcsrR (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 515ΔcsrR 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.

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 in 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 515Δsal0824/5. 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 515ΔcsrR, in which expression of sal0824/5 is upregulated. Our results showed that inactivation of sal0824/5 in 515ΔcsrR 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 2603ΔcsrRΔsal0824/5 relative to that in strain 2603ΔcsrR. As shown in Fig. 5, there is still some production of biofilm in the double mutant 2603ΔcsrRΔsal0824/5, 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 production 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).

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 Blot analysis followed by staining with crystal violet. As shown in Fig. 6B and C, there was no expression of Sal0825 in the double mutant strain 515Δsal0824/5ΔcsrR, indicating that Sal0824/5 is not the only factor contributing to biofilm formation. To address this, we constructed recombinant *pNZ* plasmids for expression of Sal0825 in *L. lactis* to confirm the BID1 domain's role in adherence and biofilm formation. As shown in Fig. 5E and F, a striking increase in biofilm formation was observed in *L. lactis* harboring the pNZ8048 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.*
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 (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. Expressions

**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 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 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 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 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 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 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 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 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).
sion 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.

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