The SrtA Sortase of *Streptococcus agalactiae* Is Required for Cell Wall Anchoring of Proteins Containing the LPXTG Motif, for Adhesion to Epithelial Cells, and for Colonization of the Mouse Intestine

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*Streptococcus agalactiae* (group B streptococcus [GBS]) is the leading cause of neonatal pneumonia, sepsis, and meningitis. An in silico genome analysis indicated that GBS strain NEM316 encodes 35 proteins containing an LPXTG motif which are thought to be covalently linked to the peptidoglycan by an enzyme called sortase. The role of these cell wall-anchored proteins in GBS pathogenesis was evaluated on a global level by inactivating the *srtA* gene. This gene encodes the major sortase SrtA that anchors most of the LPXTG-containing proteins. We chose the C5a peptidase (ScpB) and Alp2, an abundant immunogenic protein, as prototypical LPXTG-containing proteins. As expected, the SrtA knockout mutant was unable to anchor the C5a peptidase (ScpB) and Alp2 to the cell wall. Complementation with plasmid-borne *srtA* inserted into the chromosome restored the correct surface localization of both ScpB and Alp2. Interestingly, the SrtA mutant was impaired for binding to the major extracellular matrix components fibronectin and fibrinogen and displayed a significant reduction in adherence to human (A549, HeLa, and Caco-2) and murine (L2) epithelial cells compared to the parental wild-type strain. Surprisingly, the inactivation of *srtA* had no effect on the virulence of the type III strain of GBS in a neonatal rat model (measured by the 50% lethal dose and lung colonization) but strongly impaired the capacity of the strain to colonize the intestines of gnotobiotic mice in a competition assay. These results demonstrate that LPXTG-containing proteins are involved in cell adhesion and GBS persistence in vivo.

Gram-positive pathogens express specific surface proteins which may mediate interactions with the components of the host extracellular matrix, adherence to, colonization, and invasion of host cells and tissues, and evasion from the host defenses (39). These bacteria have evolved a variety of different anchoring mechanisms to display proteins to the cell surface, one of which, referred to as “sorting,” results in the covalent attachment of the protein to the peptidoglycan (15, 39). Cell wall-anchored surface proteins contain a characteristic carboxy-terminal sorting signal made of a conserved LPXTG motif followed by a hydrophobic domain and a positively charged tail (19). Following secretion, the sorting signal is cleaved between the threonyl and glycyl residues of the LPXTG motif and the carboxyl group of the threonine is linked to the peptidoglycan by an amide linkage (58). The enzyme that catalyses the protease and transpeptidase activities is a membrane-associated protein called sortase (Srt) (15, 39, 42, 43).

Sortases can be divided into four (classes A, B, C, and D) (17) or five (classes A and B, subfamilies 3, 4, and 5) (13) structural classes depending upon the approach utilized. The class A enzymes, the prototype of which is SrtA of *Staphylococcus aureus*, anchor most of the LPXTG-containing proteins. They contain a hydrophobic NH2-terminal segment functioning both as a signal peptide and a membrane anchor sequence and a carboxylic catalytic signature sequence (TLXTC) containing an essential cysteyl residue (38). SrtA mutants in *S. aureus* (37), *Listeria monocytogenes* (5, 22), *Streptococcus gordonii* (9), *Streptococcus mutans* (36), *Streptococcus pneumoniae* (31), *Streptococcus pyogenes* (3), and *Streptococcus suis* (41) are unable to anchor surface proteins and have significantly reduced adherence to epithelial cells (5, 22, 31) and virulence in animal models (5, 9, 22, 30). Genes encoding class A sortases are ubiquitous among gram-positive bacteria, whereas those encoding class B or C enzymes are not present in all sequenced genomes (13, 17). SrtC is a narrow-range enzyme required for anchoring few substrates (13, 17). In *Streptococcus agalactiae* NEM316, the four class C sortases are clustered by pair, with each pair associated with three LPXTG-containing proteins. It is possible that these accessory C sortases, which are not present in all group B streptococcus (GBS) isolates (data not shown), might specifically anchor the flanking LPXTG-containing proteins.

Lancefield’s GBS (33), also referred to as *Streptococcus agalactiae*, is the leading cause of septicemia, meningitis, and pneumonia in neonates. It is also a serious cause of mortality or morbidity in nonpregnant adults, particularly in elderly per-
sions and those with underlying diseases (18, 40, 51). Colonization of the rectum and vagina of pregnant women with GBS, which causes infection of the amniotic cavity, is correlated with GBS sepsis in newborn infants with early-onset disease. In this case, newborns are colonized intrapartum by the aspiration of contaminated amniotic fluid. The lung is a likely portal entry for GBS into the bloodstream since the bacterium can adhere to and invade alveolar epithelial (47) and endothelial cells (24). The adherence of GBS to the mother’s (intestinal and vaginal) and infant’s (lung) epithelial cells might therefore be essential for virulence.

A genome analysis of S. agalactiae NEM316 revealed the presence of one class A and four class C sortases (17) and 35 surface proteins bearing a cell wall-sorting signal motif (26). Proteins had an LPXTG motif, 4 had an LPXTG motif, 2 had an LPXTS motif, 2 had an LPXTN motif, and 1 had an FPXTG motif (25). For this work, the role of the cell wall anchoring of surface proteins in the virulence of S. agalactiae was envisaged at a global level by inactivating the srtA gene coding for the class A sortase SrtA. The SrtA+ mutant of S. agalactiae was unable to properly anchor two prototypes of LPXTG-containing proteins, Alp2 and ScpB, to the cell surface, was impaired for binding to fibronectin, and displayed a significant reduction in adherence to epithelial cell lines compared to the isogenic wild-type strain. Most importantly, the SrtA+ mutant displayed a 6-log reduction in its capacity to colonize the intestines of gnotobiotic mice in a competition assay, suggesting a key role for LPXTG-containing proteins in bacterial persistence in vivo.

MATERIALS AND METHODS

Bacterial strains, growth, and media. S. agalactiae NEM316 was responsible for a fatal septicemia and belongs to capsular serotype III (21). The complete genome sequence of this strain has been determined (25). Escherichia coli DH5α (Gibco-BRL) was used for cloning experiments. S. agalactiae was cultured in Todd-Hewitt (TH) broth or agar (Difco Laboratories, Detroit, Mich.), and E. coli was cultured in Trypticase soy medium. RPMI 1640 (MerckEurkolab, Fontenay-sous-Bois, France) was also used as a synthetic medium to study the growth of S. agalactiae strains. Unless otherwise specified, antibiotics were used at the following concentrations: for E. coli, ampicillin was used at 100 μg/ml, erythromycin was used at 150 μg/ml, kanamycin was used at 50 μg/ml, and spectinomycin was used at 60 μg/ml; for S. agalactiae, erythromycin was used at 10 μg/ml, kanamycin was used at 1,000 μg/ml, and spectinomycin was used at 250 μg/ml. S. agalactiae liquid cultures were grown in standing filled flasks. All incubations were performed at 37°C.

General DNA techniques. Genomic streptococcal DNAs were isolated as previously described (46). Standard recombinant DNA techniques were used for nucleic acid preparation and analysis (48). Plasmid DNA preparations were isolated with a Nucleospin plasmid (Macherey Nagel, Düren, Germany). PCRs were carried out with AmpliTaq Gold polymerase as recommended by the manufacturer (Applied Biosystems, Roissy, France). Amplification products were purified on Sephadex S-400 columns (Pharmacia, Uppsala, Sweden) and sequenced with an ABI 310 automated DNA sequencer by use of an ABI PRISM manufacturer (Applied Biosystems, Roissy, France). Amplification products were purified on Sephadex S-400 columns (Pharmacia, Uppsala, Sweden) and sequenced with an ABI 310 automated DNA sequencer by use of an ABI PRISM polymerase as recommended by the manufacturer (Applied Biosystems, Roissy, France). Amplification products were purified on Sephadex S-400 columns (Pharmacia, Uppsala, Sweden) and sequenced with an ABI 310 automated DNA sequencer by use of an ABI PRISM manufacturer (Applied Biosystems, Roissy, France). Amplification products were purified on Sephadex S-400 columns (Pharmacia, Uppsala, Sweden) and sequenced with an ABI 310 automated DNA sequencer by use of an ABI PRISM manufacturer (Applied Biosystems, Roissy, France). Amplification products were purified on Sephadex S-400 columns (Pharmacia, Uppsala, Sweden) and sequenced with an ABI 310 automated DNA sequencer by use of an ABI PRISM manufacturer (Applied Biosystems, Roissy, France).

Construction of bacterial strains. To construct an S. agalactiae srtA mutant (NEM2135), we inserted the promoterless and terminatorless kanamycin resistance cassette aphA-3 (59) into srtA in the same direction of transcription. This was done by ligating, after digestion with the appropriate enzymes, the following amplicons: O1-O2 (SrtA end of srtA), KanK-KanB (aphA-3 gene), and O3-O4 (3' end of srtA). The corresponding EcoRI-PstI fragment was cloned into the thermosensitive shuttle plasmid pG7 host5, and the resulting recombinant vector, pG7 host5 srtA, was introduced by electroporation into NEM316. Transformants were selected by growth on erythromycin agar plates at 30°C. Cells in which pG7 host5 srtA had integrated into the chromosome were selected by growth of the transformants at 40°C in the presence of erythromycin. Integrant cells were purified on Sephadex S-400 columns (Pharmacia, Uppsala, Sweden) and sequenced with an ABI 310 automated DNA sequencer by use of an ABI PRISM manufacturer (Applied Biosystems, Roissy, France). Amplification products were purified on Sephadex S-400 columns (Pharmacia, Uppsala, Sweden) and sequenced with an ABI 310 automated DNA sequencer by use of an ABI PRISM manufacturer (Applied Biosystems, Roissy, France). Amplification products were purified on Sephadex S-400 columns (Pharmacia, Uppsala, Sweden) and sequenced with an ABI 310 automated DNA sequencer by use of an ABI PRISM manufacturer (Applied Biosystems, Roissy, France). Amplification products were purified on Sephadex S-400 columns (Pharmacia, Uppsala, Sweden) and sequenced with an ABI 310 automated DNA sequencer by use of an ABI PRISM manufacturer (Applied Biosystems, Roissy, France). Amplification products were purified on Sephadex S-400 columns (Pharmacia, Uppsala, Sweden) and sequenced with an ABI 310 automated DNA sequencer by use of an ABI PRISM manufacturer (Applied Biosystems, Roissy, France). Amplification products were purified on Sephadex S-400 columns (Pharmacia, Uppsala, Sweden) and sequenced with an ABI 310 automated DNA sequencer by use of an ABI PRISM manufacturer (Applied Biosystems, Roissy, France). Amplification products were purified on Sephadex S-400 columns (Pharmacia, Uppsala, Sweden) and sequenced with an ABI 310 automated DNA sequencer by use of an ABI PRISM manufacturer (Applied Biosystems, Roissy, France). Amplification products were purified on Sephadex S-400 columns (Pharmacia, Uppsala, Sweden) and sequenced with an ABI 310 automated DNA sequencer by use of an ABI PRISM manufacturer (Applied Biosystems, Roissy, France).

Protein purification. Outer surface proteins of S. agalactiae grown overnight in TH broth at 37°C were prepared essentially as described previously (54). The bacteria in a 200-ml overnight culture were spun down, washed twice with 10 ml Tris-HCl buffer (50 mM, pH 7.3), and resuspended in 1.5 ml of osmotic digestion buffer (20% sucrose-2.5 μM phenylmethylsulfonyl fluoride in 50 mM Tris-HCl, pH 7.3). Mutanolysin (Sigma Chemical Co., St. Louis, Mo.), dissolved to 5,000 U/ml in potassium phosphate buffer (10 mM, pH 6.2), was then added to the bacterial suspension to give a final concentration of 350 U/ml. The digestion reaction was allowed to proceed for 18 h at 37°C with gentle shaking. After the mutanolysin treatment, the reaction mixture was centrifuged twice (15,000 rpm, 15°C, 15 min; Sigma 2K15) to remove cell debris and remaining protoplasts, and the supernatant containing the proteins released from the cell wall was kept frozen at −20°C for subsequent analysis.

Protein solubility in hot SDS. Cell wall-anchored proteins are insoluble in hot sodium dodecyl sulfate (SDS) unless the peptidoglycan has first been digested enzymatically with mutanolysin. In contrast, membrane-anchored proteins are generally extractable in hot SDS without any prior treatment. An assay described by Garandeau et al. (22) was used to study the solubility of ScpB and Alp2 in NEM316 and its derivatives. The bacteria in a 2-ml overnight culture were collected by centrifugation (6,000 rpm, 4°C, 10 min). The pellet was washed with phosphate-buffered saline (PBS), centrifuged, and resuspended in 100 μl of 4% SDS−0.5 M Tris-HCl, pH 8. The bacterial suspension was boiled for 10 min and then centrifuged at 10,000 rpm for 5 min. The supernatant fractions were further purified by immunoblotting.

Western blot analysis of proteins and immunofluorescence staining. A 594-bp DNA fragment encoding the NH2 moiety of ScpB (CSa peptidase) was amplified by using the primers O7 and O8 and was cloned into pET28a (Novagen, Madison, Wis.) that had been digested with NdeI and BamHI. The corresponding recombinant protein, which was devoid of a signal peptide but contained an NH2-terminal histidyl tag, was expressed in E. coli BL21(DE3) and purified by affinity chromatography on Ni-ntritiociatric acid columns according to the manufacturer’s recommendations (Novagen). This purified truncated ScpB protein was injected into a rabbit to produce polyclonal anti-ScpB antibodies. A
rabbit antisera raised to the R28/Alp2 was kindly provided by G. Lindahl (University of Lund, Lund, Sweden) and was described previously (53). Following electrophoresis under denaturing conditions, the proteins of \textit{S. agalactiae} were transferred onto a nylon membrane and revealed as described previously (23) by using a rabbit anti-ScpB or anti-R28 antisera (diluted 1:1,000 in PBS). Immunofluorescence staining of R28/Alp2 was performed as described previously (34), using specific rabbit polyclonal antibodies, and was revealed with anti-immunoglobulin G (anti-IgG) coupled to Alexa 488 (Molecular Probes, Eugene, Ore.). Images were scanned on a Zeiss LSM 510 microscope.

Cell culture techniques and adherence assays. The human cell lines A549 (ATCC CCL-185; from an alveolar epithelial carcinoma), HeLa (ATCC CCL-2; from a cervical carcinoma), and Caco-2 (ATCC HTB-37; from a colorectal adenocarcinoma) and the rat epithelial cell line L2 (ATCC CCL-149; from a lung) were cultured in Dulbecco's modified Eagle medium containing Glutamax (Biochrom AG, Berlin, Germany) and supplemented with 10% fetal calf serum (Biochrom AG). Cells were incubated in 10% CO2 at 37°C and were seeded at a density of 0.5 \times 10^5 to 1 \times 10^6 cells per well in 24-well tissue culture plates. Monolayers were used after 24 to 48 h of incubation.

Bacteria were grown to mid-log phase in TH broth to an optical density at 600 nm of 0.4 (approximately 10^8 CFU/ml), washed once with PBS, and resuspended in Dulbecco's modified Eagle medium. Cells were infected at a multiplicity of infection of 10 bacteria per cell for 2 h at 37°C in 10% CO2. The monolayers were then washed four to five times with PBS, and the cells were disrupted by the addition of 1 ml of sterile deionized ice-cold water and repeated pipetting. Serial dilutions of the lysate were plated onto TH agar for counts of viable bacteria. The percent adherence was calculated as follows: (CFU on plate/CFU in original inoculum) \times 100. Assays were performed in duplicate and were repeated at least three times.

Binding of \textit{S. agalactiae} to human fibronectin. Bacteria were grown to stationary phase in TH broth (approximately 3 \times 10^8 CFU/ml), washed once with PBS, and resuspended in PBS containing 1% bovine serum albumin (BSA; fraction V; Sigma) and 0.75% Tween 20 (Sigma) to give 0.5 \times 10^9 to 1.0 \times 10^9 CFU/ml. A standard enzyme-linked immunosorbent assay (ELISA) was performed by using microtiter plates (Maxisorb; Nunc) that had been coated overnight at 4°C with 100 \mu l of fibronectin (Sigma). The plates were washed in 1 ml of 0.01 M carbonate buffer, pH 9.6, in the range of 0.5 to 8 mg/ml. The wells were rinsed four times with 0.75% Tween 20 in PBS, blocked with 100 \mu l of 3% BSA in PBS for 1 h at 37°C, and rinsed once with 0.75% Tween 20 in PBS. One hundred microliters of the bacterial suspension was then added to each well, and the plates were incubated for 2 h at 37°C. After four washes with 100 \mu l of 0.75% Tween 20 in PBS to remove the unbound bacteria, 100 \mu l of a rabbit polyclonal anti-GBS antibody in PBS containing 1% BSA and 0.75% Tween 20 was added to each well. The plates were then incubated for 30 min at room temperature and washed four times with 0.75% Tween 20 in PBS, and 100 \mu l of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (TEBU, Le Perray-en-Yvelines, France) at a dilution of 1:3,000 was added to each well. After 30 min of incubation at room temperature, the plates were washed four times with 0.75% Tween 20 in PBS and one time with 0.05 M citrate buffer, pH 5.0. One hundred microliters of O-phenylenediamine dihydrochloride (Sigma) (0.4 mg/ml in citrate buffer containing 0.015% H2O2) was added to each well, and after 20 min at room temperature, the yellow color that developed was read as the \textit{A}490 nm of a ELISA plate reader (Multiskan RC, ThermoLife Sciences, Cergy-Pontoise, France). Assays were performed in triplicate and were repeated three times.

In vivo virulence studies. Neonatal Sprague-Dawley rat pups (48 h old) were used for 50% lethal dose (LD50) studies. Randomized groups of five rat pups were inoculated intraperitoneally (i.p.) with serial log dilutions of mid-log-phase bacteria (0.1 ml of each strain in 0.9% NaCl). The LD50 was calculated after 72 h for survival or 96 h for mortality. Statistical analysis of the mortality data was performed with the Mann-Whitney test as calculated with GraphPad Prism (San Diego, Calif.).

RESULTS

The \textit{srtA} gene of \textit{S. agalactiae} NEM316. The \textit{srtA} protein (GBS 0929) of NEM316 was previously identified by its similarity with other sortase proteins (25). This 248-amino-acid (aa) protein possesses a molecular mass of 27.5 kDa and displays significant sequence identity with the \textit{srtA} proteins of \textit{S. pyogenes} (60%), \textit{S. gordonii} (54.4%), \textit{S. suis} (54%), \textit{S. pneumoniae} (53%), \textit{L. monocytogenes} (35.5%), and \textit{S. aureus} (27.3%) (Fig. 1). This protein contains an NH2-terminal hydrophobic signal peptide that could also serve for membrane anchoring (37) and, at the COOH terminus, the critical cysteyl residue within the catalytic TLXTC signature sequence (Fig. 1). As is the case for other streptococcal species (37), the \textit{srtA} start site of the \textit{srtA} gene of \textit{S. agalactiae} NEM316 is located downstream of the housekeeping gene \textit{gyrA} encoding the DNA gyrase subunit A (http://genolist.pasteur.fr/SagaList/). The ATG translational start site of \textit{srtA} is preceded by a poorly conserved ribosome binding site (ATTAGGaagTATG) which includes the TAG stop codon of \textit{gyrA} (the RBS sequence and ATG start site are in uppercase letters). This genetic organization may result in translational coupling of both genes to provide coordinated synthesis of the corresponding proteins. A 411-bp long open reading frame, \textit{orfX}, was located 15 bp downstream from \textit{srtA}, but the function of the corresponding putative cytoplasmic protein is not known. An ortholog of \textit{orfX} is present down-
stream from \textit{srtA} in \textit{S. pyogenes}, \textit{Streptococcus equi}, and \textit{S. mutans} but not in \textit{S. gordonii}, \textit{Streptococcus mitis}, \textit{S. pneumoniae}, and \textit{S. suis} (data not shown). A palindromic sequence forming a possible stem-loop transcriptional terminator (\( \Delta G = -16.9 \) kcal/mol) was detected immediately downstream of \textit{orfX} (http://genolist.pasteur.fr/SagaList/). It is therefore likely that \textit{gyrA}, \textit{srtA}, and \textit{orfX} are transcribed from the \textit{gyrA} promoter and that the corresponding transcript ends at this palindromic.

**Inactivation of \textit{S. agalactiae srtA}.** To characterize the function of \textit{SrtA}, we constructed the \textit{S. agalactiae} mutant NEM2135 by replacing the internal fragment of \textit{srtA}, encoding amino acid residues 121 to 208 of \textit{S. agalactiae} with a 841-bp DNA fragment containing the promoterless kanamycin resistance gene \textit{aphA-3} (data not shown). In NEM2135 (NEM316\textsuperscript{aphA-3}), the \textit{aphA-3} cassette is thought to be transcribed from the promoter directing \textit{srtA} transcription in NEM316. To exclude the possibility that the phenotypes associated with \textit{srtA} inactivation were due to a polar effect on the expression of the downstream gene \textit{orfX}, we constructed the complemented strain NEM2136 by replacing the internal fragment of \textit{srtA} in NEM2135 with a functional copy of \textit{srtA} from the chromosome of NEM2135 (see Materials and Methods). A phenotypic comparison (morphology, growth rate, hemolysis, and antibiotic resistance) of NEM2135 (\textit{srtA} mutant) and NEM2136 (\textit{srtA} complemented) was performed in TH broth or RPMI 1640 at 37°C. The critical cysteyl residue within the catalytic TLXTC motif is indicated with a black arrow. The single letter code is that recommended by IUPAC/IUB. Dots represent gaps introduced into the sequences to ensure optimal homology.

**Role of \textit{S. agalactiae SrtA} in sorting of cell wall proteins.** To test if the \textit{SrtA} mutant was defective in cell wall anchoring of LPXTG-containing proteins, we focused our studies on two unrelated surface proteins that were previously extensively characterized, namely, Alp2 (GBS 0470) and ScpB (GBS1308). Alp2 (1,126 aa) is an \( \alpha\)-C-like protein which contains an internal series of tandem repeats and a carboxylic sorting signal including a canonical LPXTG motif (32). This protein, which likely contributes to the genesis of antigenic diversity, is highly related (99.3% identity between the first 400 aa constituting their NH\textsubscript{2}-terminal extremities) to the R28 protein originally described for \textit{S. pyogenes} (53). Therefore, antibodies directed against R28 cross-react with Alp2. The cell wall-anchored C5a peptidase ScpB (1,150 aa) cleaves the complement factor C5a, the major neutrophil chemoattractant produced by activation of the complement cascade (12, 55). More recent studies have shown that ScpB is also a fibronectin binding protein (4) which contributes to epithelial cell invasion by GBS, although it is surprisingly not involved in bacterial adhesion (11). ScpB and its homolog ScpA in \textit{S. pyogenes} both contain a carboxylic sorting signal with an LPXTN motif, and the cell wall anchor- ing of ScpA was shown to be \textit{SrtA} dependent (3). The presence of Alp2 and ScpB at the bacterial surface or in the culture supernatant was studied by immunoblotting using specific anti-Alp2 and anti-ScpB polyclonal antibodies. As shown in Fig. 2A (panel 1), a band of approximately 124 kDa corresponding to Alp2 and ScpB was detected in the cell wall extracts from the wild-type and complemented strains but not in the cell wall extract of the \textit{SrtA}\textsuperscript{mutant}. In contrast, Alp2 was not detected in culture supernatants of the wild-type and complemented strains but was present in a large amount in the cell wall anchoring of ScpA as shown in Fig. 2A (panel 3). We also observed that this protein was only present in the culture supernatant of the mutant strain (Fig. 2A, panel 4) and not in the wild-type and complemented strains. In a \textit{SrtA}\textsuperscript{mutant}, the LPXTG-containing proteins conserve their carboxylic extremities containing the hydrophobic segment and might be transiently retained at the bacterial surface through hydrophobic interactions with the membrane. Consistent with this hypothesis, solubilization of Alp2 and ScpB was observed with the \textit{SrtA}\textsuperscript{mutant} but not with the...
wild-type and complemented strains following incubation with hot SDS (Fig. 2B). The multiple band pattern detected with the R28/Alp2 antibodies was likely due to partial protein degradation (Fig. 2B, panel 1). The presence of Alp2 on the surfaces of bacteria incubated with SDS at room temperature was further analyzed by immunofluorescence using specific anti-R28/Alp2 polyclonal antibodies. As shown in Fig. 3, Alp2 was not detected on the surfaces of SrtA mutant cells, whereas it was clearly detected on the surfaces of cells of the wild-type and complemented strains. Taken together, these results suggest that S. agalactiae SrtA is required for cell wall anchoring of proteins bearing an LPXTG signature sequence.

**SrtA contributes to adherence of S. agalactiae to cultured epithelial cells and to fibronectin.** We investigated whether the SrtA mutant displayed a defect in the ability to adhere to various tissue-cultured epithelial cell lines of human (A549, Caco-2, and HeLa) and murine (L2) origins. As shown in Table 1, the adherence of the SrtA mutant was reduced approximately 10-fold compared to that of the parental strain in the four cell lines tested. Complementation of the SrtA mutant fully restored the defect in adherence in human epithelial A549, Caco-2, and HeLa cells but only partially restored the defect in the rat cell line L2 (Table 1). Many bacteria bind to host tissues by adhering to extracellular matrix proteins, and Tamura and Rubens have clearly established that GBS binds to immobilized human fibronectin (56). Up to now, ScpB was the only fibronectin binding protein identified for this bacterial species (4). To determine if fibronectin binding was affected in the SrtA mutant strain, we used a simple binding assay (ELISA) to compare its binding properties to those of the wild-type and complemented strains. As shown in Fig. 4, the S. agalactiae SrtA-expressing strains (NEM316 and NEM2136) bound in significantly larger numbers to fibronectin than did the isogenic SrtA mutant strain (NEM2135). In a similar assay, we observed that the SrtA mutant displayed reduced binding to fibrinogen compared to the wild-type and complemented strains (data not shown). It is thus conceivable that the major GBS fibrinogen-binding protein FbsA (GBS1087) is a SrtA-dependent LPXTG-containing protein (49, 50).

Taken together, these results indicate that SrtA activity in S. agalactiae is essential for bacterial adherence to eucaryotic cells and for binding to fibronectin and fibrinogen.

**Virulence of the S. agalactiae SrtA mutant in neonatal rats.** The role of SrtA in the virulence of S. agalactiae was first studied by determining the LD<sub>50</sub> of the wild-type strain NEM316 and the SrtA mutant NEM2135 in an intraperitoneal injection model using neonatal rats. In two separate experiments, the LD<sub>50</sub> of NEM316 (wild type) (6.8 × 10<sup>5</sup> and 5.8 × 10<sup>5</sup> CFU/animal; mean = 6.3 × 10<sup>5</sup> CFU/animal) was found to be slightly inferior (fourfold increase) to that of NEM2135 (SrtA<sup>−</sup>) (2.9 × 10<sup>6</sup> and 2.1 × 10<sup>6</sup> CFU/animal; mean = 2.5 × 10<sup>6</sup> CFU/animal). The virulence of these strains was more accurately compared by following, over a period of 7 days, the mortality of rat pups infected i.p. with 5 × 10<sup>5</sup> bacteria (Fig. 5). On day 3 postinfection, 80% and 60% of the rats infected with NEM316 and NEM2135, respectively, were dead (Fig. 5). No additional death was subsequently recorded for the SrtA mutant, whereas all pups infected with NEM316 died by day 5. However, this slight difference in virulence was not considered statistically significant by the Mann-Whitney test (P = 0.0849).

We hypothesized that SrtA might be required during early stages of infection such as during the colonization of the lung. To test this possibility, we inoculated rat pups intranasally with approximately 10<sup>5</sup> CFU of NEM316 and NEM2135 and monitored bacterial clearance in the lung over a 72-h period. The initial colonization levels, determined 6 h after inoculation, were identical with both strains and their clearance from the lungs occurred at similar rates (data not shown). We therefore concluded that S. agalactiae SrtA does not play a major role in virulence in neonatal rats.

**Role of SrtA in colonization of the mouse gut.** Colonization of the intestine and vagina of the mother by GBS constitutes an essential step for the development of disease in neonates. To address whether SrtA-dependent cell surface adhesins might be required for colonization of the intestines, we monitored the implantation of NEM2093 (a SrtA<sup>−</sup> spectinomycin resistant derivative of NEM316) and of the SrtA<sup>−</sup> kanamycin-resistant mutant NEM2135 in the intestinal tracts of axenic mice. Preliminary experiments revealed that the oral inoculation of 10<sup>8</sup> CFU of either strain singly enabled their stable establishment at an average level of 9.0 log<sub>10</sub> CFU/g of feces throughout a 10-day experiment (data not shown). A competitive colonization assay in which 10<sup>5</sup> CFU of each strain were simultaneously inoculated into axenic mice was then carried out. On day 1 of the experiment, the SrtA<sup>−</sup> and SrtA<sup>−</sup> strains were established at similar levels, of 9.2 and 9.0 log<sub>10</sub> CFU/g of feces, respectively, to give a competitive index (CI) of 0.98.
For 23 days, the SrtA\(^{+}\) population was maintained at the same level, whereas from day 2 of the experiment, the SrtA\(^{-}\) population regularly declined to reach 3.1 log\(_{10}\) CFU/g of feces at the end of the experiment to give a CI of 3.9\(\times\)10\(^{-7}\) (Fig. 6). Mice were sacrificed, and their intestines were collected for bacterial counts 3 weeks after inoculation. The mean log counts of NEM2093 (SrtA\(^{+}\)) and of NEM2135 (SrtA\(^{-}\)) were 9.1\(\pm\)0.2 and 1.9\(\pm\)0.3 log\(_{10}\) CFU/g of intestine, respectively, confirming that the number of bacteria in mouse feces reflects the number of bacteria in the cecum. In contrast, when NEM2093 (SrtA\(^{+}\)) and NEM2135 (SrtA\(^{-}\)) were serially cocultivated over a 23-day period in TH broth, the CI remained approximately constant throughout the experiment, varying from 0.92 (day 1) to 0.4 (day 23) (Fig. 6). We therefore concluded that in a competitive situation the SrtA\(^{-}\) mutant is unable to stably colonize the intestines of mice.

**DISCUSSION**

The availability of the whole genome sequence of the human pathogen *S. agalactiae* has provided insights into the repertoire of the cell surface proteins present in this bacterial species (25, 57). Thirty-five cell wall-associated proteins that may play a role in adhesion, invasion, the inhibition of phagocytosis, and the evasion of the host immune defense mechanisms have been annotated. Since most of these proteins are thought to be substrates of the universal sortase SrtA, evaluations of the role of their cell wall anchoring in virulence can be tested globally by inactivating the *srtA* gene. Previous studies have shown that
SrtA is required for the virulence of the intracellular pathogen *L. monocytogenes* (5, 22) and the extracellular pathogen *S. aureus* (30). Among the streptococci, a SrtA-deficient mutant of *S. gordonii* was shown to exhibit reduced adhesive properties in vitro and a decreased ability to colonize the oral mucosa of mice (9). Similarly, a SrtA/H11002 mutant of *S. mutans* was also found to display a decrease in colonization of the oral mucosae and teeth of rats (36). The inactivation of *srtA* in *S. pneumoniae* decreased its adherence to human pharyngeal cells but had no effect on the virulence of a capsular type III strain in an intraperitoneally inoculated mouse model (31).

**TABLE 1.** Comparison of capacities of *S. agalactiae* NEM316 (wild-type strain), NEM2135 (SrtA– mutant), and NEM2136 (SrtA–/SrtA+ complemented strain) to adhere to various epithelial cell lines

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative % adherence to cell linea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A549</td>
</tr>
<tr>
<td>NEM316</td>
<td>100</td>
</tr>
<tr>
<td>NEM2135</td>
<td>13.4 ± 4.2</td>
</tr>
<tr>
<td>NEM2136</td>
<td>91 ± 10</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> MG1363</td>
<td>3.1 ± 0.8</td>
</tr>
</tbody>
</table>

a Cells were infected at a multiplicity of infection of 10 bacteria per cell for 2 h at 37°C, and adherence frequencies were calculated from the numbers of bacteria remaining attached to cells after the incubation period with respect to the total number of inoculated bacteria. The level of adherence of the wild-type strain is arbitrarily reported as 100, and the levels of adherence of the derivative strains are relative values. The results are presented as mean values (± SD) from at least three experiments performed in duplicate. For these experiments, *L. lactis* MG1363 was used as a negative control for bacterial adherence. NT, not tested.

**FIG. 4.** Adherence of *S. agalactiae* strains to immobilized fibronectin. Microtiter wells were coated with various concentrations of fibronectin, and 10⁷ CFU of the wild-type strain NEM316 (○), the SrtA– mutant NEM2135 (●), or the complemented strain NEM2136 (■) was added. The wells were washed and bound bacteria were assayed as described in Materials and Methods. The results are presented as mean values (± standard deviations [SD]) of one experiment performed in triplicate. The curves are representative of three independent experiments.

**FIG. 5.** Mortality curves for rat pups infected with the wild-type strain NEM316 (○) and the SrtA– mutant NEM2135 (●). Groups of 20 neonatal Sprague-Dawley rat pups (48 h old) were inoculated i.p. with 5 × 10⁶ bacteria, and mortality was observed over a 7-day period. The difference in virulence of the two strains was considered not quite significant, with a two-tailed *P* value of 0.0849 by the Mann-Whitney test, as calculated with GraphPad Instat (version 3.0).

**FIG. 6.** Competitive index analysis of mixed cultures and colonization with the SrtA– strain NEM2093 (Sp' SrtA–) and the SrtA–/SrtA+ mutant NEM2135 (Kmr SrtA–/SrtA+). For in vitro experiments (●), 20 ml of TH broth was inoculated with 10⁶ CFU each of NEM2093 and NEM2135, and the bacterial mixture was subcultivated over a 23-day period. The two bacterial populations were enumerated daily on agar plates containing spectinomycin (NEM2093) or kanamycin (NEM2135). For in vivo experiments (○), germfree mice were inoculated on day zero with 10⁸ CFU each of the SrtA–/SrtA+ strain NEM2093 and the SrtA– mutant NEM2135. The bacterial numbers in homogenates were determined at various intervals by plating on TH agar plates containing the appropriate antibiotic. The competitive indices were calculated by dividing the CFU of NEM2093 by the CFU of NEM2135. The in vitro competitive index was the average of values from three independent experiments, whereas the in vivo competitive index was the average of values from five individual mouse experiments. The vertical bars represent one SD.
In this study, we showed that an SrtA− mutant of *S. agalactiae* is unable to anchor two unrelated LPXTG-containing proteins, Alp2 and ScpB, possessing different sorting signals (LPXTG and LPXTN, respectively). We also showed that this mutant displays a defect in adherence to human fibronectin, fibrinogen, and various epithelial cells. These observations are consistent with the finding that the SrtA-dependent LPXTG-containing protein ScpB mediates fibronectin binding (4). Our results also suggest that the major GBS fibrinogen binding protein, FbsA, which promotes adherence to human epithelial cells (27, 49), is also anchored to the cell wall by SrtA. Restoration of the wild-type phenotype (cell wall anchoring of Alp2 and ScpB and adherence) was observed upon complementation of the mutant strain, excluding the possibility that the mutant phenotype was due to a polar effect on the downstream gene of X. However, the inactivation of *srtA* did not dramatically alter the virulence of NEM316 in a neonatal rat sepsis model, as we only observed a fourfold increase in the LD₅₀ of the mutant strain. Until now, only two LPXTG-containing proteins, ScpB and CspA, have been associated with GBS virulence by promoting resistance to phagocytosis (8, 29). These two enzymes, which are likely substrates for SrtA, are members of the cell envelope-associated proteases and belong to the subtilisin-like serine protease subfamily (29). The role of ScpB in virulence has been demonstrated in a model of reconstitution of C5-deficient mice with human C5a (8) since this enzyme does not cleave murine C5a (7). A recent report indicated that a CspA− mutant of the GBS strain COH1 displayed a 10-fold increase in its LD₅₀ in a neonatal rat model (29). However, it is not known if these enzymes need to be anchored to the bacterial cell wall to fulfill their biological roles during the infectious process. We hypothesize that these enzymes remain enzymatically active and are either retained in the membrane of the SrtA− mutant or released into the culture medium.

We recently reported that the virulence of a D-alanylatedpoteichoic acid mutant of NEM316 was severely impaired in the same animal model (with a 2-log increase in the LD₅₀) (45). The fact that the virulence of the SrtA− mutant was not so dramatically reduced suggests that the anchoring of SrtA-dependent LPXTG-containing proteins does not constitute a key event for GBS virulence, at least in the neonatal rat intraperitoneal model. The adherence of GBS to epithelial cells is required for colonization of the rectal and vaginal tracts of mice. *S. agalactiae* is part of the normal digestive flora of humans and animals, and both SrtA− and SrtA+ GBS strains can singularly colonize the intestines of axenic mice at similarly high levels. However, in a competitive assay with equal numbers of both strains, the SrtA-expressing strain was stably established at a high level throughout a 23-day experiment, whereas the SrtA−deficient strain was cleared rapidly and was almost entirely eliminated by the end of the experiment. These results strongly suggest that the cell wall anchoring of SrtA-dependent LPXTG-containing proteins is required for colonization and persistence in the digestive tracts of mice. These results correlate with our in vitro data showing that the SrtA− mutant was about 10-fold less adherent to the human epithelial cell lines Caco-2, from a colorectal adenocarcinoma, and HeLa, from a cervical carcinoma. It is therefore tempting to speculate that SrtA is required for rectal and vaginal GBS carriage in humans.

Surprisingly, after intranasal inoculation the wild-type and SrtA− GBS strains were similarly cleared from the lungs of neonatal rat pups, although in vitro the mutant strain was significantly less adherent to rat and human pulmonary epithelial cell lines. Lung homeostasis is achieved via the phagocytosis of inhaled foreign material by alveolar macrophages, and neonates manifest an increased susceptibility to lung infections which may possibly be due to a deficiency in the function of alveolar macrophages (35). Several studies have demonstrated a deficient phagocytic capacity of alveolar macrophages in neonates compared to that in adults (2, 28), including humans (26), but these results are controversial (14, 35, 52). Our results indicate that the phagocytic capacity of alveolar macrophages from newborn rats enables the rapid clearance of a massive lung infection with GBS. This finding is consistent with the observation that the CR3 receptor, which is known to mediate opsonin-independent GBS phagocytosis (1), is highly expressed by neonatal alveolar macrophages (35). Thus, it is likely that in our model of lung infection the phagocytic killing of bacteria masked the SrtA-dependent colonization of the pulmonary epithelial surfaces by GBS. Whether these results can be extrapolated to human pathogenesis remains to be demonstrated.

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