

## *Streptococcus suis* Serotype 2 Interactions with Human Brain Microvascular Endothelial Cells

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*Streptococcus suis* serotype 2 is a worldwide causative agent of many forms of swine infection and is also recognized as a zoonotic agent causing human disease, including meningitis. The pathogenesis of *S. suis* infections is poorly understood. Bacteria circulate in the bloodstream in the nonimmune host until they come in contact with brain microvascular endothelial cells (BMEC) forming the blood-brain barrier. The bacterial polysaccharide capsule confers antiphagocytic properties. It is known that group B streptococci (GBS) invade and damage BMEC, which may be a primary step in the pathogenesis of neonatal meningitis. Interactions between *S. suis* and human endothelial cells were studied to determine if they differ from those between GBS and endothelial cells. Invasion assays performed with BMEC and human umbilical vein endothelial cells demonstrated that unlike GBS, *S. suis* serotype 2 could not invade either type of cell. Adherence assays showed that *S. suis* adhered only to BMEC, whereas GBS adhered to both types of cell. These interactions were not affected by the presence of a capsule, since acapsular mutants from both bacterial species adhered similarly compared to the wild-type strains. Lactate dehydrogenase release measurements indicated that some *S. suis* strains were highly cytotoxic for BMEC, even more than GBS, whereas others were not toxic at all. Cell damage was related to suilysin (*S. suis* hemolysin) production, since only suilysin-producing strains were cytotoxic and cytotoxicity could be inhibited by cholesterol and antisuilysin antibodies. It is possible that hemolysin-positive *S. suis* strains use adherence and suilysin-induced BMEC injury, as opposed to direct cellular invasion, to proceed from the circulation to the central nervous system.

*Streptococcus suis* is a worldwide causative agent of many different swine diseases, such as meningitis, endocarditis, septicemia, and arthritis (21). Of the 35 official serotypes described to date for *S. suis*, serotype 2 is the most frequently isolated from diseased animals (21). This serotype is also recognized as a zoonotic agent since it has been identified as a cause of meningitis (2, 27), septicemia (15), and endocarditis (31, 49) in humans, particularly those occupationally exposed to pigs or pig products (2, 6, 31). Human survivors of *S. suis* infection often suffer sequelae such as arthritis or deafness (53).

The pathogenesis of *S. suis* infections is poorly understood. It has been recently demonstrated that the polysaccharide capsule, which is rich in sialic acid (8), confers antiphagocytic properties on *S. suis*. Acapsular mutants were readily phagocytosed by murine and porcine macrophages, unlike the wild-type strain (7, 38, 39). Furthermore, *S. suis* capsule is a virulence factor in the murine and porcine infection models since acapsular mutants were nonpathogenic and more rapidly cleared from the bloodstream than the wild-type strain (7). One hypothesis to explain *S. suis*-associated meningitis sug-

gests that virulent strains are phagocytosed, survive in macrophages, and cross the blood-brain barrier (BBB) inside the phagocytes, whereas nonvirulent strains are destroyed once phagocytosed (56). Among the proposed extracellular factors is a hemolysin (named suilysin) belonging to the family of toxins known as antigenically related cholesterol-binding cytolytic toxins (20, 23). Antibodies against this hemolysin are protective (22). However, like those of the other proposed virulence factors, its role in pathogenesis remains to be determined.

Meningitis caused by *S. suis* is often preceded by a phase of bacteremia (2). Since the presence of the capsule inhibits phagocytosis, *S. suis* circulating in the bloodstream would come in contact with brain microvascular endothelial cells (BMEC), a single layer of specialized cells forming the BBB. This barrier, responsible for maintaining biochemical homeostasis within the central nervous system, is characterized by the presence of tight junctions and regulates fluid, macromolecule, and cell trafficking on both sides of the layer (4, 50). In order to cause meningitis, bacteria would have to pass between or through these cells to circumvent the BBB. Many meningeal pathogens are known to interact with BMEC, such as *Streptococcus pneumoniae* (34), *Escherichia coli* K1 (32) and group B *Streptococcus* (GBS) (30).

We studied the interactions between *S. suis* and endothelial cells to determine if they differ from GBS-endothelial cell interactions. Using immortalized BMEC and human umbilical vein endothelial cells (HUVEC), we tested the ability of *S. suis* to invade, adhere to, and damage these cells, compared to that of GBS.

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## MATERIALS AND METHODS

**Bacterial strains and mutants.** Wild-type *S. suis* type 2 reference strain S735-SM and two isogenic derivatives, an acapsular mutant (2A [7]) and a weakly hemolytic mutant (C3P2E5 [M. Gottschalk, S. Lacouture, M. Lalonde, L. Odiermo, M. Segura, and N. Charland, Proc. Int. Pig Vet. Soc., 15:86, 1998]) (each containing a single Tn916 insertion into the chromosome), were used. In addition, selected North American (89-1591 and 89-999) and European (S735-SM and 31533) type 2 isolates (24, 33) were studied. GBS type III strain COH1 and its isogenic acapsular mutant (COH1-13 [35]), were used for comparison. *Streptococcus gordonii* strain Challis was used as a noninvasive control (30). Bacteria were grown on blood agar plates, and single colonies were used as inocula in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) for growth to mid-log phase to an optical density at 600 nm (OD<sub>600</sub>) of 0.4 (~10<sup>8</sup> CFU/ml).

**Cell cultures.** (i) **BMEC.** BMEC originated from a brain biopsy of an adult human female with epilepsy. The cells, immortalized by transfection with simian virus 40 large T antigen (41, 42), maintained their morphologic and functional characteristics (43) and were grown as previously described (30). Briefly, cells from passage 19 were grown in RPMI 1640 medium (Gibco, Burlington, Ontario, Canada) supplemented with 10% (vol/vol) fetal bovine serum (Gibco), 10% (vol/vol) Nu-Serum IV supplement (Becton Dickinson, Bedford, Mass.), L-glutamine, and penicillin-streptomycin. Falcon flasks and 24- or 96-well tissue culture plates (Becton Dickinson) were precoated with rat tail collagen to support the cell monolayers.

(ii) **HUVEC.** Immortalized HUVEC were purchased from the American Type Culture Collection (ATCC CRL-1730). Cells from passage 13 were grown in F-12K medium (Sigma, Oakville, Ontario, Canada) supplemented with 12% (vol/vol) fetal bovine serum, 50 ng of Endothelial Cell Growth Supplement (Becton Dickinson) per ml, and penicillin-streptomycin in Falcon flasks and tissue culture plates.

Both types of endothelial cells were incubated at 37°C in 5% CO<sub>2</sub> in a humid atmosphere and split twice a week with trypsin-EDTA at a ratio of 1/8 (BMEC) or 1/3 (HUVEC).

Cells were used before passage 35 for all experiments. Prior to each assay, the monolayers were washed twice with Hanks balanced saline solution (Gibco) and fresh medium without antibiotics (adherence and invasion assays) or RPMI 1640 medium alone (BMEC cytotoxicity assay) was added.

**Cell invasion assay.** The cell invasion assay was performed as previously described (30), with some modifications. Log-phase bacteria (~10<sup>8</sup> CFU/ml) were pelleted, washed once with phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3), and resuspended in fresh cell culture medium without antibiotics. Dilutions in cell culture medium were performed such that inocula between 10<sup>7</sup> and 10<sup>9</sup> CFU were added to wells of a 24-well tissue culture plate containing a monolayer (~10<sup>6</sup> cells) of endothelial cells (BMEC or HUVEC) in 0.5 ml of medium (multiplicity of infection of 10 to 0.001 bacteria/cell). The plates were centrifuged at 800 × g for 10 min to enhance the contact of bacteria with the surface of the monolayer. The plates were incubated for 2 h at 37°C in 5% CO<sub>2</sub> to allow cell invasion by the bacteria. The monolayers were then washed three times with PBS, 1 ml of cell culture medium containing 100 µg of gentamicin per ml and 5 µg of penicillin G per ml was added to each well, and the plates were incubated for 2 h at 37°C in 5% CO<sub>2</sub> to kill extracellular bacteria. The monolayers were washed three times with PBS, and cells were disrupted by the addition of 0.5 ml of sterile deionized water and repeated pipetting to liberate intracellular bacteria. One hundred microliters from each well was plated onto Todd-Hewitt agar and incubated overnight at 37°C. The percent invasion of endothelial cells was calculated as [5 × (CFU on plate count/CFU in original inoculum)] × 100%. Assays were performed in duplicate and repeated at least three times.

**Adherence assay.** Percent adherence was determined by subtracting intracellular bacteria from total cell-associated (intracellular plus surface-adherent) bacteria. Total cell-associated bacteria were quantified as for the cellular invasion assay, only without the antibiotic exposure step. Cells were washed five times with PBS, lysis was performed as described above, and 100 µl-aliquots diluted 1:10 or 1:100 in PBS were used for quantitative plating. All assays were performed in duplicate and repeated at least three times.

**Hemolysin purification.** In order to construct an affinity column to purify the hemolysin, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using the culture supernatant of strain 31533 (which produces large amounts of hemolysin) grown for 16 h in Todd-Hewitt broth produced by Oxoid (Unipath, Nepean; Ontario, Canada), a medium favoring hemolysin production. A 52-kDa band representing the hemolysin was excised, emulsified with Freund complete adjuvant, and injected into a New Zealand rabbit intramuscularly. The injection was repeated twice with Freund incomplete adjuvant. The rabbit was bled 2 weeks after the last inoculation, and the immunoglobulin G was purified with a protein A affinity column. The activity and specificity of the anti-hemolysin immunoglobulin G were confirmed by inhibition of the hemolytic activity and immunoblotting with supernatant of a hemolysin-positive strain (results not shown). An affinity column was constructed with the monospecific antibodies using the Pierce CarboLink Coupling Gel (Pierce, Rockford, Ill.), and the hemolysin was purified from culture supernatant of strain 31533 grown as described above. Hemolysin and monospecific antibody concentrations were determined by

the method of Markwell et al. (26). The purity of the hemolysin preparation was verified by silver staining.

**Cellular injury assays.** A microtiter plate assay to determine the cytolytic activity of *S. suis* was performed as previously described, with some modifications (30). Briefly, 10<sup>8</sup> CFU of log-phase bacteria were centrifuged, washed in PBS, and resuspended in 1 ml of RPMI 1640 medium without serum. A 100-µl aliquot of bacteria was added to the first well of a 96-well tissue culture plate containing a BMEC monolayer (~5 × 10<sup>4</sup> cells; multiplicity of infection of 200 bacteria/cell), and serial twofold dilutions in RPMI 1640 medium were performed across the plate. Noninfected cells and bacteria in RPMI 1640 medium without a BMEC monolayer were used as negative controls, whereas cells lysed with 100 µl of sterile deionized water were used as a positive control. The plate was incubated at 37°C in 5% CO<sub>2</sub> for 4 h in most experiments. At the end of the incubation period, a 75-µl aliquot of each supernatant was transferred to a replica plate which was centrifuged at 3,000 × g for 20 min to pellet bacteria. Lactate dehydrogenase (LDH) measurement was performed on 20-µl aliquots of each centrifuged supernatant using a miniaturized version of the Sigma colorimetric assay as previously described (29). Percent cytotoxicity was calculated as [(OD<sub>0%</sub> - OD<sub>100%</sub>) - (OD<sub>bacteria</sub> - OD<sub>100%</sub>)/(OD<sub>0%</sub> - OD<sub>100%</sub>)] × 100, where OD<sub>0%</sub> represents the OD<sub>414</sub> of noninfected cells and OD<sub>100%</sub> represents the OD<sub>414</sub> of lysed cells (adapted from reference 14). To determine if cytolytic components were produced extracellularly, culture supernatants of late-log-phase bacteria were recovered by centrifugation at 3,000 × g for 10 min and filtration through 0.22-µm-pore-size filters. Supernatants were kept at -80°C until use for LDH measurement. The assay was also performed with purified hemolysin. All of the assays were performed in duplicate and repeated at least three times.

**Inhibition of cytolytic activity.** Inhibition of cytotoxicity by heat was performed by heat killing log-phase bacteria (~10<sup>8</sup> CFU) by placing them in a 60°C water-bath for 45 min (minimal killing time and temperature as determined in our laboratory) before adding them to BMEC monolayers for LDH measurement. Inhibition of cytotoxicity by cholesterol was measured by incubating log-phase bacteria in increasing concentrations of ethanol-soluble cholesterol (Sigma) for 1 h at 37°C before adding them to BMEC monolayers. Noninfected cells with cholesterol in culture medium were used as a negative control. Inhibition of cytotoxicity by monospecific antibodies (specificity of antibodies was demonstrated by culture supernatant immunoblotting) was performed by mixing increasing concentrations of anti-hemolysin antibodies with log-phase bacteria and incubating them for 1 h at 37°C before adding them to BMEC monolayers. An anti-*Actinobacillus pleuropneumoniae* lipopolysaccharide monospecific antibody was used as a negative control.

**Electron microscopic studies.** To monolayers of BMEC in 24-well tissue culture plates, 10<sup>7</sup> CFU of log-phase *S. suis* were added in culture medium, centrifuged at 800 × g for 10 min, and then incubated at 37°C in 5% CO<sub>2</sub> for 30 min, 2 h, and 4 h. The supernatants were removed by gentle aspiration, the cells were washed once with Hanks balanced saline solution, and the monolayers were fixed for 1 h at room temperature with 3% glutaraldehyde in 0.1 M phosphate buffer (0.1 M Na<sub>2</sub>HPO<sub>4</sub>, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.3) and then postfixed for 1 h at room temperature in potassium ferrocyanide-reduced osmium tetroxide (28). Specimens were dehydrated in graded alcohols and processed for embedding in Epon. Ultrathin sections were placed on Formvar-coated nickel grids, stained with uranyl acetate and lead citrate, and then examined by transmission electron microscopy using a JEM1200 EX II operated at 60 kV.

**Statistics.** All data are expressed as means ± standard deviations (error bars). Data were analyzed by two-tailed, unpaired *t* test. A *P* value of <0.05 was considered significant.

## RESULTS

***S. suis* does not invade endothelial cells.** Before using the standard conditions for comparative assays (10<sup>5</sup> CFU, 2-h invasion time), we tested many different variables in order to find experimental conditions that promote *S. suis* invasion. Different invasion times, cell confluences, inocula, stationary and logarithmic growth phases, and serum concentrations were tested, but none induced *S. suis* invasion. *S. suis* did not invade confluent cells from the apical surface, nor did it invade subconfluent cells from the basolateral surface (results not shown). Results were comparable to those obtained with the noninvasive *S. gordonii* control regardless of the cell type (Fig. 1). The *S. suis* acapsular mutant was also noninvasive. As a comparison, GBS invaded both types of cells. The acapsular GBS mutant COH1-13 exhibited significantly greater invasiveness than wild-type strain COH1 (*P* < 0.05; Fig. 1).

***S. suis* adheres to BMEC.** Although *S. suis* does not invade endothelial cells, it appears that this bacterium has a tropism for BMEC. Figure 2A shows a significantly greater adherence

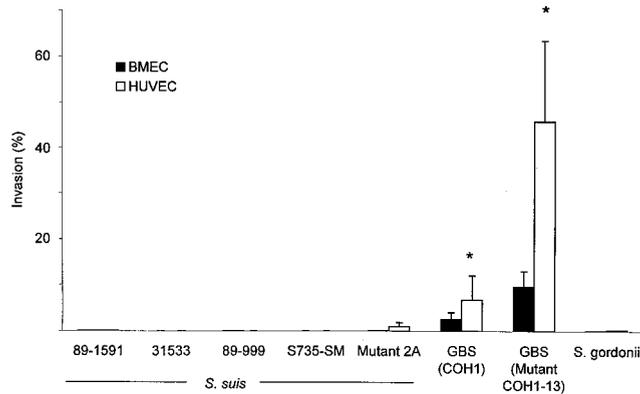


FIG. 1. Invasion of BMEC and HUVEC by *S. suis* 89-1591, 31533, 89-999, and S735-SM and acapsular mutant 2A and GBS strain COH1 and acapsular mutant COH1-13. *S. gordonii* was used as a noninvasive control. \*,  $P < 0.05$  versus wild-type strain COH1. Results were determined after a 2-h exposure with 100- $\mu$ l aliquots of  $10^6$  CFU/ml, followed by an additional 2-h incubation in the presence of penicillin-gentamicin to kill extracellular bacteria, and BMEC lysis to retrieve 100- $\mu$ l aliquots of intracellular bacteria for viable plate counts.

of all *S. suis* strains to BMEC than to HUVEC ( $P < 0.05$ ). Adherence of *S. suis* type 2 to BMEC is illustrated in Fig. 2B. GBS adherence was similar for both the encapsulated strain and the nonencapsulated mutant (Fig. 2A). Since no invasion was detected for *S. suis*, total cell-associated bacteria represent adherent organisms. Adherence levels were similar when assays were performed at 4°C, indicating that de novo protein synthesis was not required for active BMEC binding by *S. suis* (data not shown).

**Effect of *S. suis* polysaccharide capsule on adherence.** The presence of the capsule did not seem to influence *S. suis* or GBS adherence to BMEC. The adherence levels of the acapsular *S. suis* mutant 2A and GBS COH1-13 were similar to those of wild-type strains S735-SM and GBS COH1, respectively (Fig. 2;  $P > 0.05$ ).

**Some *S. suis* strains can damage BMEC.** LDH release measurements were performed to determine if *S. suis* could be cytotoxic to BMEC. Figure 3 shows that while some *S. suis* isolates did not injure BMEC at all, others were highly cytotoxic, even more than GBS ( $P < 0.05$ ). Almost 100% cell lysis was noted for strains S735-SM and 31533, whereas 55% cell lysis was obtained for GBS at the same bacterial concentration ( $10^7$  CFU per well). Cytotoxicity was directly proportional to bacterial concentration. The quantity of bacteria used for invasion and adherence assays ( $10^5$  CFU per well) was not toxic to BMEC. Indeed, little cytotoxicity was recorded for isolates after a 2-h incubation period, the incubation time used for invasion-adherence assays (Fig. 4). Cell lysis by cytotoxic *S. suis* strains increased over time and always remained higher than that due to nontoxic isolates (Fig. 4;  $P < 0.05$ ). Interestingly, all of the cytotoxic strains produce suilysin. Injury assays with a weakly hemolytic *S. suis* mutant demonstrated that the mutant was less toxic than the wild-type strain until the bacterial concentrations reached about  $10^7$  CFU per well, at which point their cytotoxicities became comparable (Fig. 3). Similar results were obtained over time, as cytotoxicity of the mutant was lower than that of the wild-type strain until the incubation time was extended to 16 h (Fig. 4).

**Suilysin-associated injury to BMEC.** It is known that suilysin is excreted in vitro in culture supernatants (20). Addition of the culture supernatant from suilysin-producing strain 31533 to BMEC monolayers induced cell injury, whereas the culture

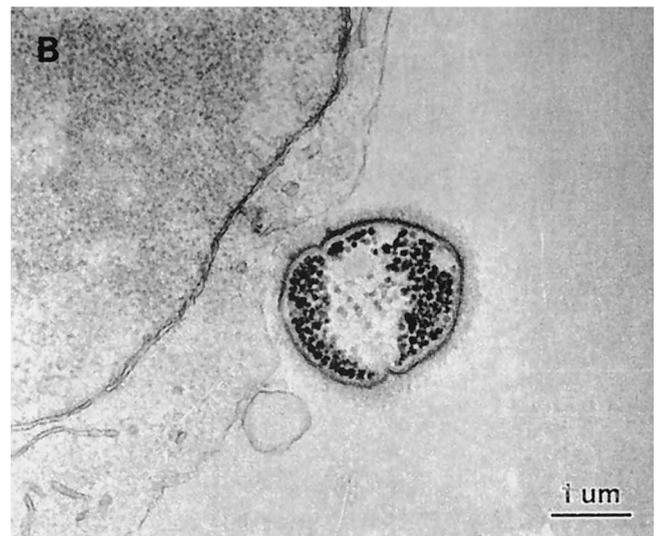
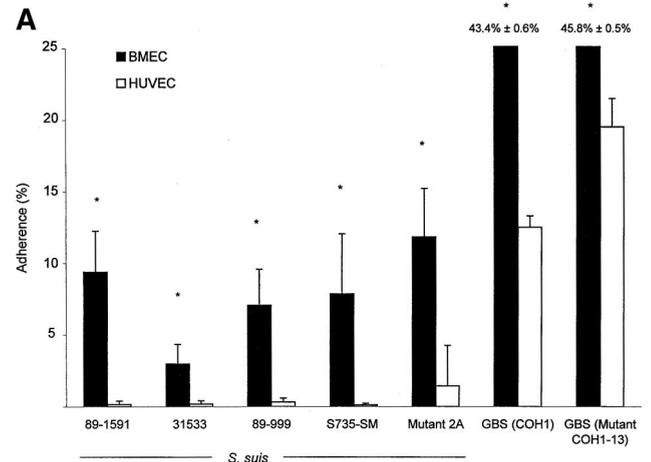


FIG. 2. (A) Adherence to BMEC and HUVEC by *S. suis* 89-1591, 31533, 89-999, and S735-SM and acapsular mutant 2A and GBS strain COH1 and acapsular mutant COH1-13. \*,  $P < 0.05$  versus adherence to HUVEC. Results were determined after a 2-h exposure with 100- $\mu$ l aliquots of  $10^6$  CFU/ml, followed by extensive washing of nonadherent bacteria and BMEC lysis to retrieve 100- $\mu$ l aliquots of total cell-associated bacteria for viable plate counts. (B) Transmission electron micrograph showing *S. suis* adherent to BMEC.

supernatant of non-suilysin-producing strain 89-1591 did not affect the cells (Table 1). Moreover, purified suilysin injured BMEC in a concentration-dependent manner, reaching a peak at  $\sim 4$   $\mu$ g of purified toxin per ml (Fig. 5). Experiments were performed to determine which treatment(s) could inhibit cytolytic activity and to confirm the involvement of suilysin in cell injury. First, to determine if cytotoxicity requires live bacteria, strain 31533 was heat killed and the suspension was added to a BMEC monolayer. Results showed that only live bacteria induced BMEC injury (Table 1). Cholesterol can inhibit suilysin activity (20). Thus, bacteria were mixed with increasing cholesterol concentrations and added to BMEC monolayers. Figure 6 shows that at 2 mg/ml, cholesterol completely inhibited the cytolytic activity of suilysin-producing strains, while 100  $\mu$ g/ml was sufficient to significantly inhibit purified suilysin toxicity ( $P < 0.05$ ). It is known that antihemolysin antibodies can inhibit suilysin hemolytic activity (20). Figure 7 shows that increasing concentrations of antisuilysin antibodies, but not a

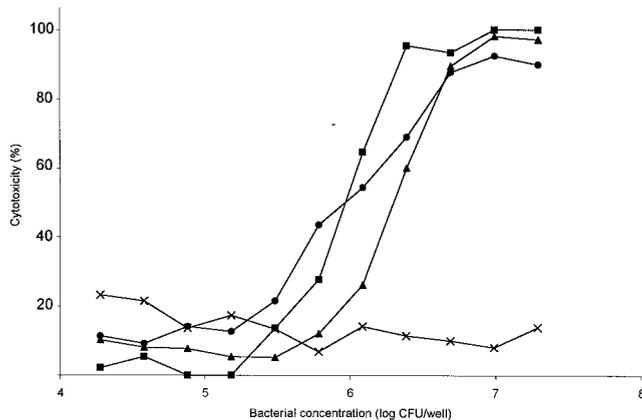


FIG. 3. Effect of *S. suis* concentration on BMCE injury. Symbols: ●, hemolytic strain 31533; ■, hemolytic strain S735-SM (wild-type strain); ▲, weakly hemolytic mutant C3P2E5; ×, nonhemolytic strain 89-1591. BMCE cytotoxicity was determined by measuring LDH release in the presence of different concentrations of *S. suis* strains after a 4-h incubation. Results of a representative experiment are shown.

nonrelated control antibody, inhibited suisysin cytotoxicity from a hemolysin-producing strain.

**Electron microscopy.** Figure 8 confirms the cytotoxicity data obtained with LDH measurements. The cytotoxicity of suisysin-producing strain 31533 increased with time, reaching a peak at 4 h (data not shown), whereas non-suisysin-producing strain 89-1591 did not affect cell integrity. After a 2-h exposure, normal cell characteristics (similar to those seen in uninfected cells; Fig. 8C), such as dense regular cytoplasmic contents, few pinocytic vesicles, and evenly distributed nuclear chromatin, were seen with strain 89-1591 (Fig. 8B). On the other hand, exposure of BMCE to strain 31533 resulted in cellular injury demonstrated by loss of cytoplasmic density, discontinuity of cytoplasmic membranes, and clumping of nuclear chromatin (Fig. 8A).

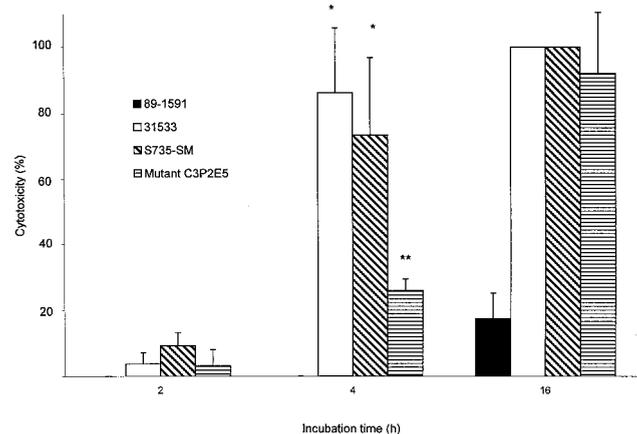


FIG. 4. Effect of time of incubation with *S. suis* hemolytic strains 31533 and S735-SM (wild type strain), weakly hemolytic mutant C3P2E5, and nonhemolytic strain 89-1591 on BMCE injury. \*,  $P < 0.05$  versus 2-h incubation; \*\*,  $P < 0.05$  versus S735-SM (4 h). BMCE cytotoxicity was determined by measuring LDH release in the presence of 100- $\mu$ l aliquots of  $10^8$  CFU/ml after different incubation times.

TABLE 1. Cytotoxicity of viable *S. suis* or of its culture supernatant to BMCE as determined by measurement of LDH release

Treatment	Hemolysin production <sup>a</sup>	% Cytotoxicity <sup>b</sup>
Strain 31533 overnight culture supernatant	Yes	73.0 $\pm$ 9.5
Strain 89-1591 overnight culture supernatant	No	0.0 $\pm$ 0.0 <sup>c</sup>
Live strain 31533	Yes	98.5 $\pm$ 2.6
Heat-killed strain 31533	No	0.0 $\pm$ 0.0 <sup>c</sup>
Live strain 89-1591	No	0.0 $\pm$ 0.0 <sup>c</sup>

<sup>a</sup> Determined by red-cell lysis assay (22).

<sup>b</sup> Values are means  $\pm$  standard deviations (at least three samples per group).

<sup>c</sup> Significantly different ( $P < 0.05$ ) from the value for nontreated suisysin-producing strain 31533 or for its culture supernatant, as calculated by Student's *t* test.

## DISCUSSION

*S. suis* is an important swine pathogen that causes significant economic losses to producers. In addition, these bacteria represent a health risk for those involved in the pig industry. Comparatively, GBS is an important human pathogen associated with meningitis. Serotype III strains are implicated in most cases of meningitis (3). GBS and *S. suis* share many characteristics, and since they produce similar diseases, their pathogeneses have often been compared. Like *S. suis*, GBS possesses a sialic acid-containing capsule and produces a hemolysin, and different virulence potentials are observed among strains (3, 25, 54). However, although the two species present similar characteristics, the pathogeneses of the infections caused by the two bacteria may be different (38). It is known that GBS can invade BMCE and that GBS hemolysin can injure lung and brain endothelial cells (18, 30). These events may be primary steps in the pathogenesis of GBS meningitis (30). GBS capsule itself attenuates endothelial cell invasion (17, 30). In contrast, the means by which *S. suis* may proceed from the circulation to the central nervous system are unknown. Understanding these mechanisms is important in unraveling *S. suis* pathogenesis in order to develop strategies for prevention or therapy.

*S. suis* did not invade endothelial cells under the conditions tested in this study, whereas GBS easily entered the cells. Many different variables were tested in order to find combina-

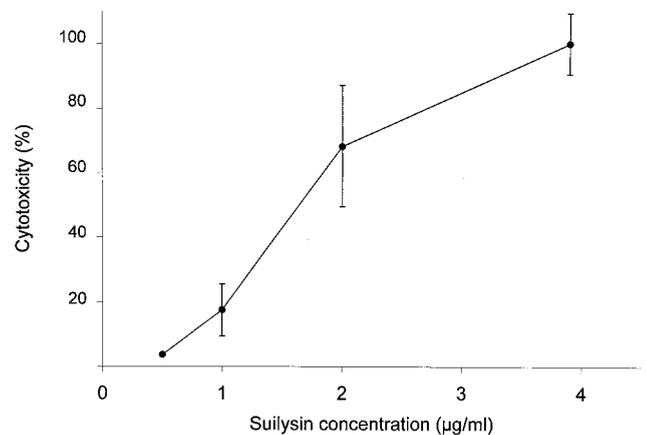


FIG. 5. Effect of increasing concentrations of purified suisysin on BMCE injury. BMCE cytotoxicity was determined by measuring LDH release in the presence of different concentrations of purified suisysin after a 4-h incubation.

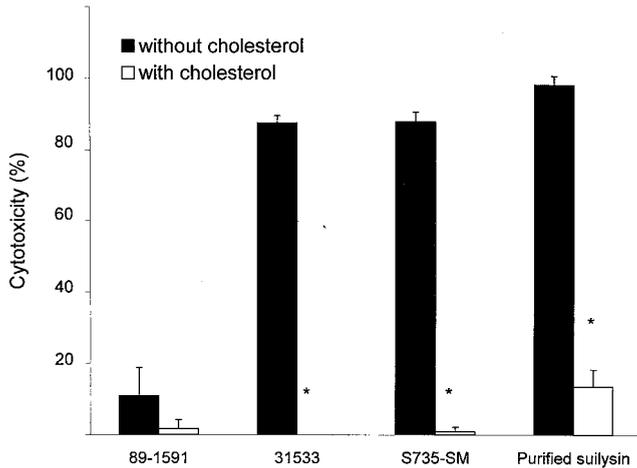


FIG. 6. Inhibition by cholesterol of *S. suis* cytotoxicity for BMEC. \*,  $P < 0.05$  versus no inhibitor. We incubated  $10^8$  CFU/ml or purified suislysin at  $4.6 \mu\text{g/ml}$  in the absence and presence of 2 mg (bacteria) or 100  $\mu\text{g}$  (suislysin) of cholesterol per ml for 1 h at  $37^\circ\text{C}$  before incubation of 100- $\mu\text{l}$  aliquots with BMEC for 4 h. BMEC cytotoxicity was determined by measuring LDH release.

tions that could favor invasion by *S. suis*; however, none induced invasion. Our results strongly suggest that entry of *S. suis* into the central nervous system follows a pattern different from pathogens such as GBS, *E. coli* K1, and *S. pneumoniae*, which invade from the apical surface (30, 32, 34), or shigellae, which invade from the basolateral surface (10). It remains possible that *S. suis* could cross the BBB by invading intercellular junctions of BMEC monolayers. This mechanism of invasion, which was described for the spirochetes *Treponema pallidum* and *Borrelia burgdorferi* (44, 47), remains to be studied for *S. suis*.

In agreement with previous reports (17, 30), the GBS capsule attenuated invasion. However, in this study, the presence of a capsule did not interfere with GBS and *S. suis* endothelial cell adherence, since both the wild-type and acapsular mutant strains bound similarly. Similar results have been obtained with pneumococci (16). St. Geme and Cutter suggested that encap-

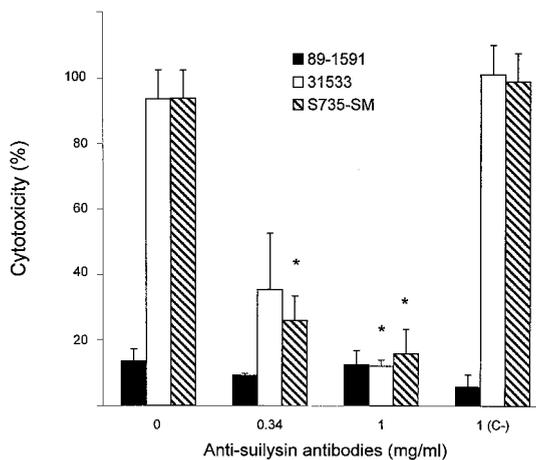


FIG. 7. Inhibition by anti-suislysin antibodies of *S. suis* cytotoxicity for BMEC. \*,  $P < 0.05$  versus the no-antibody or nonspecific antilipopolysaccharide antibody (C-) control. We incubated  $10^8$  CFU/ml in the presence of increasing antibody concentrations for 1 h at  $37^\circ\text{C}$  before incubation of 100- $\mu\text{l}$  aliquots with BMEC for 4 h. BMEC cytotoxicity was determined by measuring LDH release.

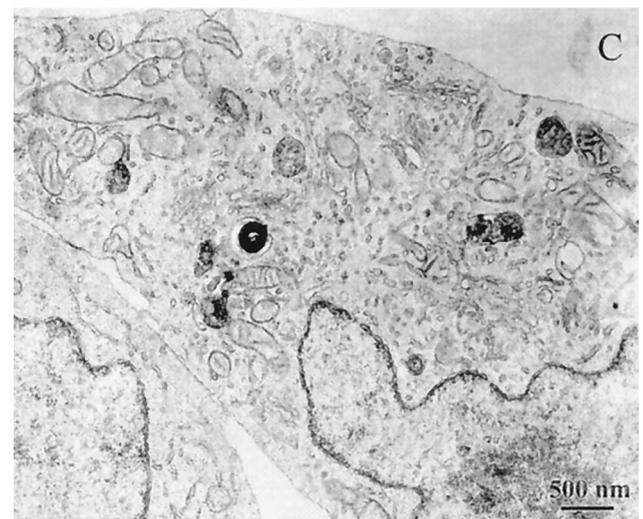
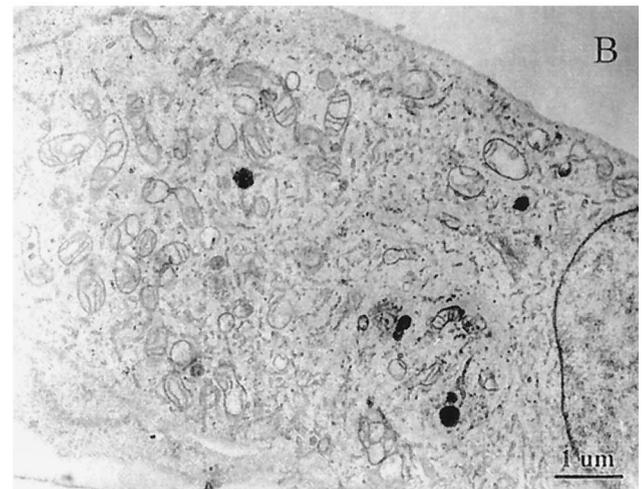
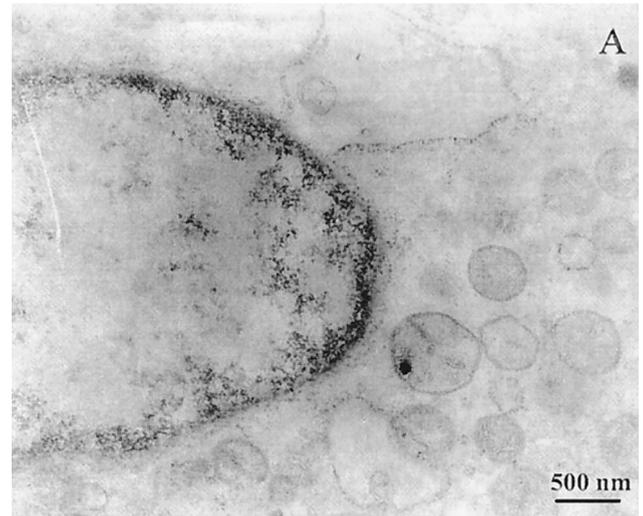


FIG. 8. Transmission electron micrographs demonstrating BMEC injury caused by *S. suis* at  $10^8$  CFU/ml. Panels: A, suislysin-producing strain 31533; B, non-suislysin-producing strain 89-1591; C, no-bacterium control. BMEC integrity after a 2-h incubation with strain 89-1591 (B) was comparable to that of the no-bacterium control (C).

sulation may be modulated depending on the infectious stage (40). Encapsulation may be down-regulated during colonization of epithelial cells (lungs, nasopharynx), and once the bacteria are in the bloodstream, up-regulation of capsule production protects them against the immune system. It was shown that *S. suis* capsule expression may vary with growth conditions (19). In addition, the presence of the capsule seems to restrict access to *S. suis* putative adhesins, limiting hemagglutination activity (48). Interestingly, capsule-attenuated adherence to lung epithelial cells has been reported for GBS (45) and the capsule also seems to significantly interfere with *S. suis* adhesion to epithelial cells (unpublished observations; 55). To date however, there is no direct evidence of such encapsulation modulation for either GBS or *S. suis*.

Despite the observation that *S. suis* does not invade endothelial cells, it does adhere to these cells, especially BMEC. As mentioned before, the presence of the capsule does not seem to interfere with adhesion. Interestingly, *E. coli* K1 also interacts with BMEC preferentially to HUVEC (32). It is possible that the preferential adherence of *S. suis* to BMEC has a role in the pathogenesis of infection. For example, it may be hypothesized that, after adherence of *S. suis* to BMEC, bacteria can secrete toxic factors which would affect the endothelial cells. Such factors could increase BBB permeability, which could lead to the development of cerebral edema, increased intracranial pressure, and cerebral blood flow blockage characteristic of bacterial meningitis (46). It was recently proposed that damage to BMEC by GBS hemolysin could contribute to increased BBB permeability (30). In the case of *S. suis*, many lines of evidence implicate suilysin as the bacterial component responsible for in vitro BMEC cytotoxicity. First, only suilysin-producing strains are toxic for BMEC and a weakly hemolytic *S. suis* mutant exhibited decreased toxicity. The observed toxicity of this mutant with longer exposures and increasing bacterial concentrations correlates with the proposed mode of action of suilysin, namely, a multihit activity (1, 20). Hence, the accumulation of suilysin molecules at the BMEC surface may have resulted in toxicity equal to that of the wild-type strain as the incubation time and bacterial concentrations increased. Second, the BMEC-cytotoxic component is present in culture supernatants. It is known that suilysin is excreted during bacterial growth (20, 23). Third, the purified suilysin is toxic to BMEC. Fourth, cholesterol inhibits BMEC cytotoxicity as it inhibits suilysin lysis of erythrocytes (20, 23). Cholesterol is required for the binding of the toxin to cell membranes, and free cholesterol acts as a competitive inhibitor (5). Finally, antisuilysin antibodies also inhibit the BMEC cytotoxicity of suilysin-producing strains, providing further evidence that suilysin was responsible for the cellular injury. Pneumolysin, a toxin with strong sequence homology to suilysin (37), is also known to damage endothelial cells (36).

While suilysin is implicated as an important virulence factor in European *S. suis* type 2 strains, the same does not seem to be the case for North American strains. In fact, unlike European strains, most virulent field strains isolated from diseased pigs or humans in the United States and Canada do not produce suilysin (9, 37); Gottschalk et al., Proc. Int. Pig Vet. Soc.; 1998). In this study, the hemolysin-negative virulent strain 89-1591 showed significant adherence to BMEC but did not exhibit any detectable toxicity to these cells. It is possible that the pathogenesis of the infection caused by suilysin-positive strains and that of the infection caused by suilysin-negative strains are different and that different virulence factors are involved in each case. It has been reported that adherence of *Neisseria meningitidis* and pneumococci to endothelial cells results in widening or disruption of intercellular junctions, effects not

related to the production of cytotoxins (16, 52). However, no BMEC morphological changes were observed after adherence of the hemolysin-negative strain of *S. suis*.

It is possible that the tropism of hemolysin-negative strains for BMEC has consequences other than direct damage to the cells. One mechanism may be the stimulation of cytokine production through bacterial adherence with resultant recruitment of inflammatory cells and/or alteration of BBB permeability. It has been shown that *E. coli* adherence is required for interleukin-6 induction from epithelial cells and that oral viridans group streptococcal adhesins and pneumococcal attachment initiate the release of various cytokines from endothelial and epithelial cells (12, 16, 51). *S. pneumoniae* invasion of unstimulated endothelial cells is low (less than 0.1%), whereas cytokine activation increased invasion levels to 2 to 3% (11). In addition, the release of cytokines from BMEC could lead to increased BBB permeability. In fact, it was recently demonstrated that tumor necrosis factor alpha opens a paracellular route for human immunodeficiency virus type 1 through the BBB (13). Preliminary studies in our laboratory indicate that BMEC stimulated with *S. suis* release more than 200 pg of interleukin-6 per ml, as measured by immunoassays (unpublished results). However, the roles of this and other cytokines in the pathogenesis of *S. suis* infection remain to be studied.

*S. suis* and GBS host cell interactions appear to differ in important ways. For example, *S. suis* and GBS interact differently with macrophages, and these interactions imply different virulence roles for capsular sialic acid and the whole capsule (8, 38). Whereas *S. suis* capsule prevents nonopsonic phagocytosis, in contrast to an acapsular mutant, the presence of capsular polysaccharides on GBS did not block phagocytosis under the same experimental conditions (38). Instead, intracellular survival of encapsulated GBS and of its isogenic acapsular mutant was higher than that of *S. suis* strains (38). The results described herein give additional evidence that pathogenesis of the infection differs between *S. suis* and GBS. In particular, it is possible that hemolysin-positive *S. suis* strains use adherence and suilysin-induced BMEC injury, as opposed to direct cellular invasion, to proceed from the circulation to the central nervous system. Since part of the toxic effect of any toxic molecule on the luminal side of the endothelial cells may be partially washed away by circulation through the cerebral vascular bed, in vivo investigation is necessary to further elucidate the pathogenesis of *S. suis* meningitis.

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