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D-Alanylation of Lipoteichoic Acid Contributes to the Virulence of *Streptococcus suis*▽

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We generated by allelic replacement a Δ*dltA* mutant of a virulent *Streptococcus suis* serotype 2 field strain and evaluated the contribution of lipoteichoic acid (LTA) D-alanylation to the virulence traits of this swine pathogen and zoonotic agent. The absence of LTA D-alanylation resulted in increased susceptibility to the action of cationic antimicrobial peptides. In addition, and in contrast to the wild-type strain, the Δ*dltA* mutant was efficiently killed by porcine neutrophils and showed diminished adherence to and invasion of porcine brain microvascular endothelial cells. Finally, the Δ*dltA* mutant was attenuated in both the CD1 mouse and porcine models of infection, probably reflecting a decreased ability to escape immune clearance mechanisms and an impaired capacity to move across host barriers. The results of this study suggest that LTA D-alanylation is an important factor in *S. suis* virulence.

Streptococcus suis is a major swine pathogen and a zoonotic agent that is responsible for, among other diseases, meningitis and septicemia (15). In swine, *S. suis* causes severe losses to the industry (15), while human *S. suis* infection is emerging as an important public health issue (13). Very recently, more than 200 cases of human *S. suis* infection were reported during an outbreak in China, and 38 of these cases resulted in death (39). *S. suis* is considered the primary cause of adult meningitis in Vietnam (20), and human *S. suis* infection resulting in death or in severe postinfection sequelae has been reported in different Asian and European countries, as well as in New Zealand, Australia, Argentina, Canada, and the United States (13). Of the *S. suis* serotypes, serotype 2 is responsible for most cases of disease in both swine and humans, and almost all studies on virulence factors and pathogenesis of the infection have been carried out with this serotype (13, 15). Despite the increasing number of studies, our understanding of the pathogenesis of *S. suis* infection remains limited. The polysaccharide capsule is known to play a critical role in the pathogenesis of *S. suis* infection (15). It has been shown that unencapsulation of *S. suis* correlates with increased phagocytosis by porcine macrophages and killing by porcine neutrophils (4, 6, 29) and that it severely impairs virulence in a porcine model of infection (29). Recently, an isogenic serum opacity-like factor mutant was found to be highly attenuated in pigs (2). Other proposed putative virulence factors, such as the suilysin, the extracellular protein factor, the muramidase-released protein, and a fibronectin/fibrinogen-binding protein, were found to be associated with and/or partially involved in, but not essential for, virulence (7, 15).

S. suis can affect the viability of porcine blood brain barrier (BBB)-forming cells, such as porcine choroid plexus epithelial cells, through necrotic and apoptotic mechanisms (34). It also can adhere to and invade in vitro-cultured porcine brain microvascular endothelial cells (BMEC), another type of BBB-forming cells (35). The ability of *S. suis* to interact with these cells is thought to be important for gaining access to the central nervous system (CNS) and causing meningitis in swine (13). In a recent study (11), selective capture of transcribed sequences was used to elucidate genes that this pathogen preferentially upregulates during its interactions with porcine BMEC. Among other genes, the study identified a member of a putative *S. suis dlt* operon (11). In all bacteria in which this operon has been studied, it has been found to be responsible for the incorporation of D-alanine residues into lipoteichoic acids (LTA), which are surface-associated amphiphilic molecules found in most gram-positive bacteria (23).

The cell wall of *S. suis* has been proposed to be an important virulence factor. Several studies have shown that the cell wall or purified components of the cell wall, such as the LTA, contribute to exacerbation of the host inflammatory response to infection (13, 15). However, the structure and composition of *S. suis* LTA are poorly known. It has been proposed that *S. suis* LTA may have a backbone structure similar to that of group A streptococcal teichoic acid, but with differences in the attachment of glucosyl substituents (9). Besides its involvement in inflammation, LTA may also play a direct role in *S. suis* virulence. Indeed, a recent study showed that the adherence of *S. suis* to porcine BMEC can be inhibited by preincubation of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Bacterial strains		
E. coli Top 10	General strain for cloning, F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL*(Str^r) *endA1 nupG*	Invitrogen
S. suis 31533	Serotype 2 field strain, highly virulent	35
S. suis Δ*dltA*	Δ*dltA* mutant strain derived from strain 31533	This study
S. suis BD102	Unencapsulated mutant strain derived from strain 31533	12
Plasmids		
pCR4	*E. coli* vector for cloning PCR fragments	Invitrogen
pSmall	*E. coli-S. suis* shuttle vector, Sp^r, source of *aad9*	P. Willson, unpublished results
pSET5s	Temperature-sensitive suicide vector for *S. suis* mutagenesis, Cm^r (*cat*)	32
p5ΔdltA	pSET5s carrying the construct for allelic exchange	This study

the BMEC with purified LTA (36). In addition, it has been proposed that *S. suis* may D-alanylate its LTA and that a high ratio of D-alanine to glycerol phosphate in this molecule may be important for the interaction of this pathogen with host cells (11). It is known from previous reports that D-alanylation of the LTA is important for the virulence of gram-positive pathogens based on findings indicating that it enables these organisms to modulate their surface charge, to regulate ligand binding, and to control the electromechanical properties of the cell wall (23). In addition, formation of D-alanyl-LTA is required to resist the action of cationic antimicrobial peptides (CAMPs) (1, 17, 18, 26). The D-alanylation of *S. suis* LTA and its contribution to the pathogenesis of infection have not been documented previously. In this study, we demonstrated that *S. suis* D-alanylates its LTA and that this modification is important for the virulence traits of this pathogen.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, culture conditions, and chemicals. Bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, *S. suis* strains were grown in Todd-Hewitt broth (THB) (Becton Dickinson, Sparks, MD) or on Todd-Hewitt agar (THA) at 37°C under 5% CO_2. *Escherichia coli* strains were cultured in Luria-Bertani broth or on Luria-Bertani agar (Becton Dickinson) at 37°C. When needed, antibiotics (Sigma, Oakville, Ontario, Canada) were added to the culture media at the following concentrations: for *S. suis*, 5 μg/ml chloramphenicol (Cm) and 100 μg/ml spectinomycin (Sp); and for *E. coli*, 50 μg/ml kanamycin, 50 μg/ml Sp, and 10 μg/ml Cm. Unless otherwise indicated, all chemicals were purchased from Sigma.

DNA manipulations. Restriction enzymes, DNA-modifying enzymes, and the *Taq* and *Pwo* DNA polymerases were purchased from GE Healthcare (Piscataway, NJ) or Takara Bio (Otsu, Shiga, Japan) and used according to the manufacturers' recommendations. *S. suis* genomic DNA was prepared by the guanidium thiocyanate method (24). Mini-preparation of recombinant plasmids from *E. coli* and transformation of *E. coli* were performed by using standard procedures (27). Southern hybridizations were performed by the procedures described previously (28), except that hybridizations were carried out at 68°C. For preparation of probes, DNA fragments were labeled with digoxigenin using a digoxigenin-PCR labeling mixture (Roche Diagnostics, Laval, QC, Canada) according to the manufacturer's instructions. Oligonucleotide primers were obtained from Invitrogen (Burlington, Ontario, Canada).

Allelic replacement. (i) Construction of the knockout vector. DNA fragments corresponding to regions upstream and downstream of the *dltA* gene (Fig. 1A) were amplified from genomic DNA of *S. suis* strain 31533 by PCR using primers 2872F and 3765R (left arm) and primers 5250F and 5809R (right arm). An Sp resistance cassette (*aad9* gene) was amplified from plasmid pSmall with primers specF3 and specR. All three primer sets introduced unique restriction sites (Table 2). PCR amplicons were digested using the appropriate restriction enzymes and sequentially ligated in the order left arm-Sp cassette-right arm using T4 DNA ligase. The resulting fragment was amplified by PCR using primers 2872F and 5809R, cloned into vector pCR4 (TOPO TA PCR cloning kit; Invitrogen), excised with HindIII and BamHI, and recloned into the HindIII and BamHI sites of the temperature-sensitive *S. suis-E. coli* shuttle vector pSET5s, which carries the gene *cat* conferring Cm resistance (32), giving rise to knockout vector p5ΔdltA (Fig. 1B).

(ii) Generation of *S. suis* Δ*dltA* mutant. Procedures for selection of mutants by allelic exchange via double crossover have been described previously (32). Briefly, *S. suis* strain 31533 was transformed with p5ΔdltA by electroporation as previously described (31). The cells were grown at 28°C in the presence of Cm and Sp for selection. Bacteria at mid-logarithmic growth phase were diluted with THB containing Sp and grown at 28°C to early logarithmic phase. The cultures were then shifted to 37°C and incubated for 4 h. Subsequently, the cells were spread onto THA containing Sp and incubated at 28°C. Temperature-resistant, Sp-resistant colonies were screened for the loss of vector-mediated Cm resistance to detect putative mutants in which the wild-type (WT) allele had been exchanged for a genetic segment containing the *aad9* gene as a consequence of homologous recombination via a double crossover. Allelic replacement in candidate clones was verified by PCR and Southern hybridization, which confirmed the expected genotype (data not shown).

Transmission electron microscopy. Transmission electron microscopy was performed as previously described (12). Briefly, overnight (ON) cultures of the *S. suis* WT or mutant Δ*dltA* strains were mixed with rabbit anti-*S. suis* serotype 2 polyclonal serum and incubated at room temperature for 1 h. Cells were then fixed in cacodylate buffer (0.1 M cacodylate, 5% glutaraldehyde, 0.15% ruthenium red; pH 7.2) for 2 h. After fixation, cells were immobilized in 4% agar, washed in cacodylate buffer, and postfixed ON at 4°C in 2% osmium tetroxide. Samples were dehydrated using a graded ethanol series and embedded in Spurr low-viscosity resin. Thin sections were poststained with uranyl acetate and lead citrate and examined with a transmission electron microscope (model 420; Philips Electronics, The Netherlands).

Determination of LTA D-alanine content. *S. suis* WT and Δ*dltA* mutant strains were cultured in tryptic soy broth (Becton Dickinson) containing beef extract (5 g/liter) and glucose (8 g/liter) at 37°C for 18 h with shaking. After incubation, bacteria were harvested by centrifugation at 4,225 × *g* for 20 min. The integrity of bacteria and potential contamination by gram-negative species were checked by Gram staining and microscopy. LTA were prepared by butanol extraction (which preserved the integrity of the D-alanine substitutions) and hydrophobic interaction chromatography as previously described (22). Nuclear magnetic resonance (NMR) spectra for LTA were recorded with a Bruker Avance DRX 600 spectrometer (Bruker BioSpin, Ettlingen, Germany) equipped with an inverse TXI-H/C/N triple-resonance probe at 300 K using 3-mm Bruker Match sample tubes. Measurements were carried out in D_2O using sodium 3-trimethylsilyl-3,3,2,2-tetradeutero-propanoate as an internal standard for ^1H NMR (δ_H 0.00 ppm).

Antimicrobial peptide sensitivity. Assays were carried out in sterile 96-well microtiter plates. The concentrations of logarithmic-phase *S. suis* cells were adjusted to approximately 10^4 CFU/ml in 100 μl THB containing serial dilutions of one of the following antimicrobial compounds: colistin (0 to 200 μg/ml), polymyxin B (0 to 300 μg/ml), or magainin II (0 to 45 μg/ml). Plates were incubated for 24 h at 37°C. The MIC was defined as the lowest antimicrobial concentration yielding no detectable bacterial growth as determined by measurement of the optical density at 600 nm.

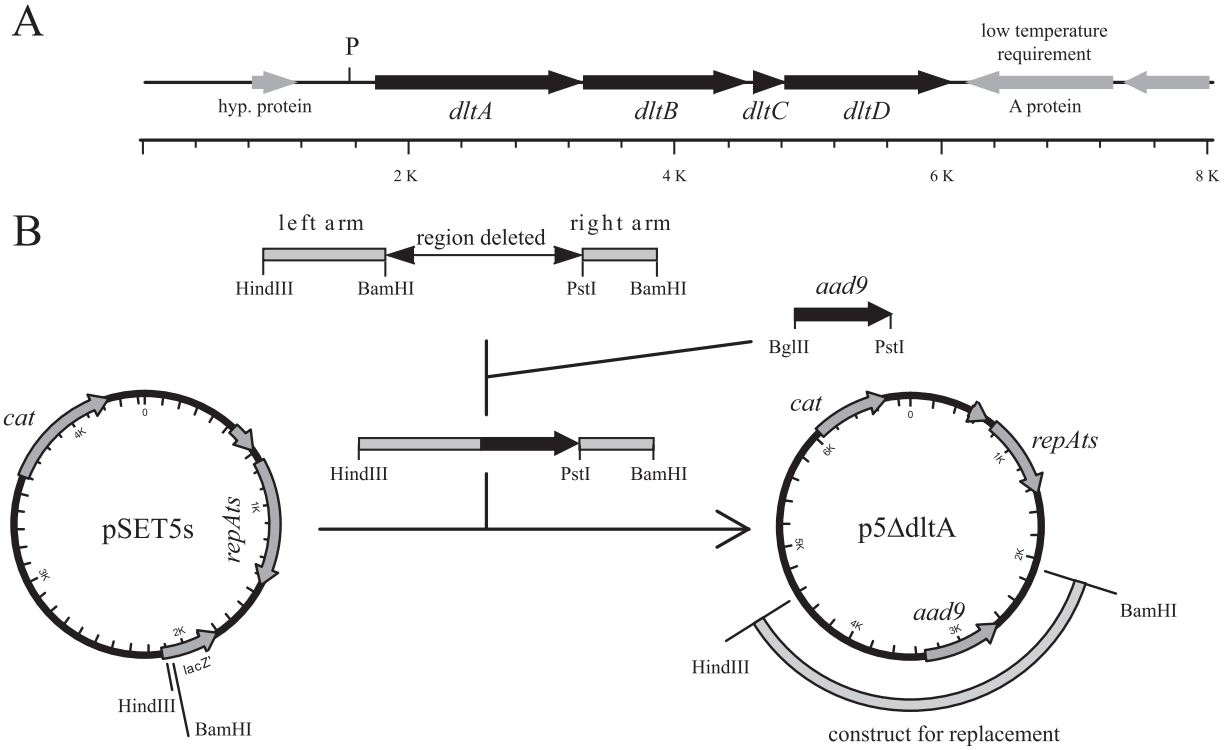

FIG. 1. (A) Genetic organization of the *S. suis dlt* operon as determined by sequencing of the region in strain 31533 and comparison with data from sequenced strain P1/7 available at the Sanger Institute (http://www.sanger.ac.uk/Projects/S_suis/). The *S. suis dlt* operon is 4,340 bp long and comprises four genes, *dltA* (1,563 bp), *dltB* (1,242 bp), *dltC* (240 bp), and *dltD* (1,266 bp). A putative strong promoter (indicated by P) was predicted 228 bp upstream of the start codon for *dltA* using the software package Softberry BProm (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). hyp. protein, hypothetical protein. (B) Strategy used in this study to construct the knockout vector used to generate the Δ*dltA* mutant. See Materials and Methods for details.

Killing by porcine neutrophils. Killing experiments were carried out as described previously (4). Briefly, blood samples were collected by venous puncture from high-health-status pigs considered to be free of *S. suis* serotype 2 as determined by an enzyme-linked immunosorbent assay (19). Cell populations were separated by Ficoll-Hypaque (GE Healthcare) density gradient centrifugation, and neutrophils were isolated by sedimentation in 6% dextran. Contaminating erythrocytes were removed by lysis with 0.83% ammonium chloride. Neutrophils were resuspended in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated porcine serum at a final concentration of 5×10^6 cells/ml. Bacteria (WT or Δ*dltA* mutant strain at a concentration of approximately 1×10^4 CFU/ml) were opsonized with complete normal porcine serum for 30 min at 37°C and then mixed in microtubes with neutrophils at a concentration of 5×10^6 cells/ml. The mixture was incubated for 90 min at 37°C under 5% CO_2. Under these conditions bacteria are not toxic to neutrophils (4). After incubation cells were lysed with sterile water, and viable bacterial counts were determined by plating onto THA.

Experimental infections. All experiments involving animals were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care.

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence (5′–3′)[a]	Restriction site
2872F	GCAGTTACCTCT**AAGCTT**GCGACAACGG	HindIII
3765R	CTGCTAATCATTT**GGATCC**TCTCCTC	BamHI
5250F	CTTCCTTTGA**CTGCAG**ATGGGAAGATT	PstI
5809R	CGTCTATAA**GGATCC**ATAGGG	BamHI
specF3	GCCAATG**AGATCT**ATAAATAAAC	BglII
specR	AAAGTGTTTC**CTGCAG**TTTTTCAA	PstI

[a] Restriction sites are indicated by bold type.

(i) Pigs. A total of 20 4-week-old, second-generation caesarean-derived pigs were used in this study. Strict biosecurity measures were used to avoid undesirable contamination of the pigs; these measures included an air filtration system and airlocks for each unit. The pigs were divided into three groups. Six of seven animals in groups 1 and 2 were inoculated by intravenous injection of 10^8 CFU of the *S. suis* WT 31533 and mutant Δ*dltA* strains, respectively. The remaining animal in both the WT and Δ*dltA* mutant groups was not inoculated, although it was housed with inoculated animals and served as a sentinel. The group 3 animals ($n = 6$) were sham inoculated. Clinical signs and the presence of *S. suis* in blood were monitored during the trial. Surviving animals in all three groups were sacrificed 7 days postinfection (p.i.) and examined for pathological lesions. Isolation of bacteria from different organs (liver, spleen, lungs, heart, and articulations) was performed as described below for mice.

(ii) CD1 mice. A recently described murine model of *S. suis* infection was used (8). A total of 60 female CD1 mice that were 6 weeks old (Charles River Laboratories, Wilmington, MA) were used to assess virulence. At zero time, animals were divided into four groups of 15 mice. Group 1 was inoculated by intraperitoneal injection of 1 ml of an *S. suis* strain 31533 suspension (5×10^7 CFU/ml), while group 2 received the same dose of mutant strain Δ*dltA*. Groups 3 and 4 received 1 ml of a 5×10^6-CFU/ml suspension of the WT and mutant strains, respectively, using the same route of inoculation. Mice were monitored three times per day for 10 days for clinical signs, and clinical scores were assigned as previously described (8). Blood samples (5 μl) were collected daily (from the tail vein) and at the time of euthanasia (by cardiac puncture) and used to evaluate the bacterial load by plating onto sheep blood agar plates. Isolated tiny alpha-hemolytic colonies were counted and assigned to *S. suis* by serotyping as previously described (16). Surviving animals in both groups were sacrificed at day 10, and macroscopic examination was performed. Bacterial colonization of the liver, spleen, and brain of infected animals was also evaluated. Briefly, small pieces of these organs weighing 0.5 g were trimmed, placed in 500 μl of phosphate-buffered saline (PBS), pH 7.3, and homogenized. After this, 50 μl of each suspension was plated as described above. In addition, enrichment of the samples

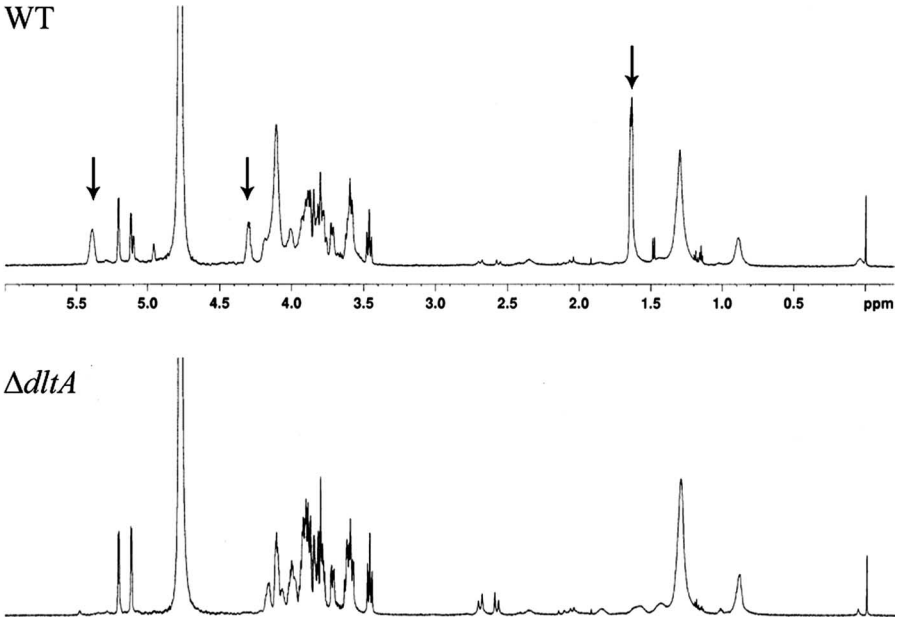

FIG. 2. NMR spectra of WT (upper panel) and Δ*dltA* mutant (bottom panel) LTA. The arrows indicate the peaks for D-alanine residues in the WT strain spectrum. These peaks are not present in the Δ*dltA* mutant spectrum. No other differences between the LTA of the two strains were found.

was carried out by inoculation of 300 μl of homogenized organ sample or 100 μl of blood into THB, followed by ON incubation at 37°C and subsequent dilution and plating onto sheep blood agar plates as described above.

Adherence to and invasion of porcine BMEC. The porcine BMEC line PBMEC/C1-2 (33) was grown in Primaria 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) using IF culture medium (a 1:1 mixture of Iscove's modified Dulbecco's and Ham's F-12 media; Invitrogen) supplemented as previously described (35). *S. suis* was grown in THB for 16 h at 37°C, harvested by centrifugation, washed twice in PBS (pH 7.3), and resuspended in fresh IF culture medium. The invasion assays were performed as described previously (35). Briefly, confluent monolayers of porcine BMEC (10^5 cells/well) were infected with 1-ml aliquots of bacterial suspensions (10^5 CFU/ml; multiplicity of infection, 1). The plates were centrifuged at 800 × *g* for 10 min and incubated for 2 h at 37°C with 5% CO_2. The monolayers were then washed twice with PBS, 1 ml of cell culture medium containing 100 μg/ml of gentamicin and 5 μg/ml of penicillin G was added to each well, and the preparations were incubated for 1 h. After incubation, the monolayers were washed three times with PBS, trypsinized, and disrupted by repeated pipetting. Serial dilutions of the cell lysates were plated onto THA and incubated ON at 37°C. To confirm that 100% of the extracellular bacteria were killed after the antibiotic treatment, a 100-μl sample of the last PBS wash was plated onto THA (results not shown). Adherence assays were performed essentially as described above for invasion, but neither antibiotic treatment nor extended incubation was performed. After 2 h of incubation, cells were vigorously washed five times with PBS, trypsinized, and disrupted, and serial dilutions of the cell lysates were plated as described above.

RESULTS AND DISCUSSION

The *dlt* operon is responsible for LTA D-alanylation in *S. suis*. The genetic organization of the *S. suis dlt* operon is shown in Fig. 1A. Sequence comparison at The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/), as well as previous reports (17, 18, 25), showed that the *S. suis dlt* operon is organized in a fashion similar to that of all *dlt* operons reported for pathogenic streptococci so far, with the exception of the *dlt* operon of *Streptococcus agalactiae*, which also includes two regulatory genes upstream of the *dltA* gene (25). Accordingly, the deduced proteins showed a high degree of similarity to streptococcal Dlt proteins (data not shown). To assess the contribution of the *dlt* operon to LTA D-alanylation, we constructed by allelic replacement a Δ*dltA* mutant strain and analyzed the content of D-alanine in purified LTA of the WT and Δ*dltA* mutant strains by NMR. Figure 2 shows the NMR spectra for LTA of the two strains. Both LTA showed the expected peaks for fatty acids (0.85 and 1.3 ppm) and sugars (3.5 to 4.5 ppm). However, peaks for D-alanine (1.65, 4.3, and 5.4 ppm) were absent in the Δ*dltA* mutant spectrum, suggesting that the LTA of the mutant lacks this amino acid. The in vitro growth of the Δ*dltA* mutant was comparable to that of the WT strain (Fig. 3A), and no other major phenotypic changes were observed. In contrast to previous reports on cells of *S. agalactiae* and *Streptococcus pyogenes* Δ*dltA* mutants, which were either poorly separated or multiseptate in the stationary phase of growth (18, 26), the *S. suis* Δ*dltA* mutant cells were encapsulated and well separated and exhibited normal septation (Fig. 3B).

***S. suis* LTA D-alanylation contributes to antimicrobial peptide resistance and decreases susceptibility to neutrophil killing.** CAMPs kill bacteria by forming pores in the cytoplasmic membrane (30). Introduction of positively charged D-alanine residues into the LTA would reduce the global negative charge of the *S. suis* envelope, thus providing the bacterium with a physical mechanism for resistance to the action of CAMPs (23). To assess this hypothesis, we evaluated the sensitivities of the WT and Δ*dltA* mutant strains to selected CAMPs. The *S. suis* Δ*dltA* mutant was more sensitive than the WT strain to the bacterium-derived cationic peptide polymyxin B and colistin and the frog-derived peptide magainin II (Table 3). These results were in agreement with previous reports of inactivation of the *dltA* gene in streptococcal species (17, 18, 26) and indicate that D-alanylation of LTA is an important component of

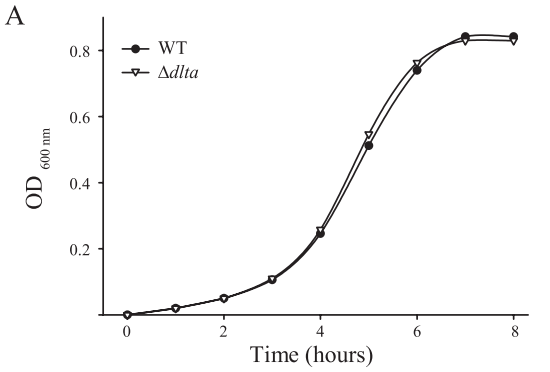

FIG. 3. (A) Growth curves for the *S. suis* WT and Δ*dltA* mutant strains. The growth of the Δ*dltA* mutant was similar to the growth of the WT parent strain under normal laboratory conditions. $OD_{600\ nm}$, optical density at 600 nm. (B) Morphology of the Δ*dltA* mutant (right panel) and WT (left panel) strains. Transmission electron microscopy showed that cells of both strains were well separated, had normal septation, and were surrounded by a thick polysaccharide capsule. Bars = 0.5 μm.

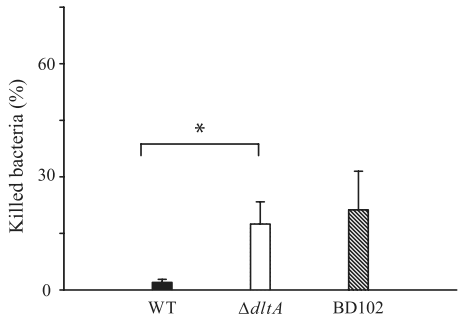

FIG. 4. Percentages of bacteria killed after 90 min of incubation with porcine neutrophils. The different strains were opsonized with complete porcine sera before incubation. The level of killing of the Δ*dltA* mutant was similar to that of the unencapsulated mutant BD102 and significantly higher than that of the WT strain. The data are data from at least three independent experiments. The error bars indicate standard deviations. The asterisk indicates significant differences ($P < 0.05$, *t* test).

the intrinsic resistance of *S. suis* to CAMP killing. On the other hand, the WT and *dltA* mutant strains had equivalent susceptibilities to the antibiotics gentamicin and penicillin G and to lysozyme (data not shown). Functional homologues of the CAMPs tested in this study are secreted by neutrophils both into the phagosome and extracellularly (21). When we compared killing of the WT and Δ*dltA* mutant strains by purified porcine neutrophils, in agreement with a previous study (4), the WT strain avoided killing by neutrophils when it was opsonized with normal complete porcine sera. On the other hand, 20% of the Δ*dltA* mutant bacteria were killed by neutrophils (Fig. 4). This level of killing was similar to that of the unencapsulated mutant strain BD102, despite the fact that the Δ*dltA* mutant does not have altered capsule expression (Fig. 4). This was surprising, since encapsulated WT *S. suis* has been shown to resist phagocytosis by porcine neutrophils (unless it is opsonized by specific antibodies) (4). However, it is known that neutrophils are also able to destroy infecting microorganisms in the absence of phagocytosis in the so-called neutrophil extracellular traps (NETs) (3). Interestingly, it has recently been shown that in *Streptococcus pneumoniae* the absence of LTA D-alanylation results in enhanced extracellular killing in NETs by neutrophils but not in increased phagocytosis of this organism by these polymorphonuclear cells (37). Although our killing assay is not able to discriminate between intra- and extracellular killing, taking all these findings together, it might be proposed that the encapsulated *S. suis* Δ*dltA* mutant is killed by porcine neutrophils extracellularly, perhaps after being trapped in NETs. In addition, we speculate that the enhanced killing of the *S. suis* Δ*dltA* mutant might be a consequence of the absence of LTA D-alanylation, which results in increased susceptibility to CAMPs released by neutrophils. Further experiments are needed to evaluate this hypothesis.

TABLE 3. Sensitivity of the *S. suis* WT and Δ*dltA* mutant strains to the action of selected antimicrobial peptides

Peptide (origin)	Net charge	MICs (μg/ml)	
		31533 (WT)	Δ*dltA* mutant
Colistin (*Bacillus colistinus*)	+5	50	25
Polymyxin B (*Bacillus polymyxa*)	+5	75	18.75
Magainin II (claw frog skin)	+4	45	5.6

The virulence of the Δ*dltA* mutant is attenuated in pigs. Several Δ*dltA* mutants of different gram-positive pathogens have been described, and almost all of these mutants were highly susceptible to CAMPs and killing by neutrophils and/or macrophages (1, 5, 10, 14, 17, 18, 26, 37, 38). However, only a limited number of studies have analyzed in vivo the contribution of LTA D-alanylation to the virulence of these pathogens. In these cases, the virulence of the Δ*dltA* mutants tested varied greatly between bacterial species, preventing conclusions regarding the contribution of LTA D-alanylation to the virulence traits of pathogens to be drawn from previous studies (1, 26, 37, 38). Finally, for various valid reasons, previous studies on the virulence of Δ*dltA* mutants in gram-positive species used surrogate models of infection instead of the natural hosts (1, 26, 37, 38). *S. suis* shares certain characteristics with pathogens for which Δ*dltA* mutants have been described. However, its pathogenesis of infection is essentially different (15). In this study, we evaluated for the first time the virulence of a gram-positive Δ*dltA* mutant in the context of its natural host by using intravenous inoculation of pigs. Animals in the sham-inoculated group did not present any clinical signs during the trial. In contrast, severe clinical signs were recorded for five of the six animals inoculated with the WT strain during the first 4 days of

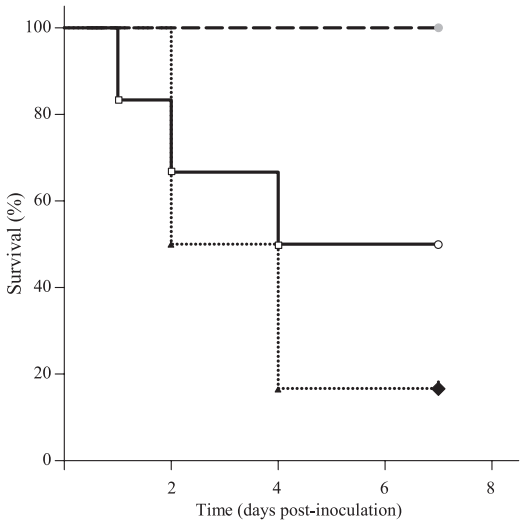

FIG. 5. Survival of pigs inoculated with the WT (dotted line) or Δ*dltA* mutant (solid line) strain and survival of pigs that were sham inoculated (dashed line). All the sham-inoculated animals survived the trial. The survival rate of the pigs in the Δ*dltA* mutant group was 50%, while most animals in the WT group died from septicemia during the first days of the trial (survival rate, 17%). The sentinel animals were not considered in this analysis. See the text for details.

the trial. These five pigs died or were sacrificed for ethical reasons at day 2 p.i. (three animals) and at day 4 p.i. (two pigs). The remaining inoculated animal and the sentinel pig in this group survived until the end of the trial (Fig. 5). Animals infected with the Δ*dltA* mutant presented, on average, less severe clinical signs during the first 4 days p.i. However, two animals died, and an additional animal in this group was euthanized for ethical reasons (Fig. 5). Nevertheless, the remaining inoculated animals recovered noticeably starting at day 4 p.i. and, along with the sentinel pig in the Δ*dltA* group, survived until the end of the trial. Hyperthermia (>40.5°C) was observed in all pigs infected with either the WT or Δ*dltA* strain at 24 h p.i. The temperatures returned to normal values after day 4 in both groups. However, in the WT group, the sentinel pig developed hyperthermia starting on day 6 p.i. *S. suis* serotype 2 could be isolated from the blood of all inoculated pigs in both groups and the sentinel animal in the WT group. The pigs in the latter group had higher bacterial counts (as high as 1×10^{10} CFU/ml in some cases) than the pigs infected with the Δ*dltA* mutant (average, 1×10^8 CFU/ml) during the first 4 days p.i. Similar to the results for the blood, the bacterial titers in organs were slightly lower in pigs inoculated with the Δ*dltA* mutant than in animals inoculated with the WT strain. However, examination at necropsy did not reveal major differences between the WT and Δ*dltA* mutant groups regarding damage to tissues or organs. After euthanasia, macroscopic lesions typical of *S. suis* infection were found in most animals infected with the WT strain or the Δ*dltA* strain, especially at the pleura, pericardium, and peritoneum. Fibrin deposits were observed in the liver and spleen of most animals in both groups. Pneumonia and fibrinal pleurisy were also observed in some animals. Additionally, the meninges showed inflammation consistent with meningitis. Lameness was observed in all pigs infected with the WT or Δ*dltA* strain. At necropsy, articulations showed inflammation, along with fibrin deposits and excess synovial liquid. The results of this experimental infection showed that the Δ*dltA* mutant is attenuated in the pig and suggest that LTA D-alanylation provides an advantage to the WT strain. However, this conclusion is mitigated by the fact that dissemination of the bacterium was not prevented and by the fact that mortality was observed among animals inoculated with the Δ*dltA* mutant. Since clearance of the mutant from the circulation might rely primarily on neutrophil activity, the high dose used to inoculate the animals may explain, at least in part, the mortality observed. Indeed, it has been proposed that suilysin may affect complement activity, and suilysin-producing *S. suis* strains, such as the WT and mutant strains used in this study, have been shown to be toxic to neutrophils at high titers (4). In addition, since CAMP activity occurs primarily at mucosal surfaces, the extremely aggressive intravenous route of administration may also have influenced the clinical onset of disease observed in pigs.

The absence of LTA D-alanylation impairs *S. suis* virulence in mice. To better evaluate the attenuation of the *S. suis* Δ*dltA* mutant observed in pigs, we performed additional in vivo trials using the CD1 mouse model of infection in which the intraperitoneal route of inoculation is used (8). We performed two different trials using high and intermediate doses. At the high dose (5×10^7 CFU per animal) most mice in both the WT and Δ*dltA* mutant groups presented severe clinical signs associated with septicemia, such as depression, swollen eyes, weakness, and prostration during the first 72 h p.i. At this dose we did not observe a clear reduction in the ability of the Δ*dltA* mutant to successfully initiate infection and induce septicemia in mice. In fact, several mice in both groups died from septicemia during the first 3 days of the trial (Fig. 6A). High titers of *S. suis* were obtained for blood samples ($>1 \times 10^7$ CFU/ml) and for organs, such as the liver and spleen, of septicemic animals ($>1 \times 10^7$ CFU/0.5 g of tissue in some animals). Starting on day 5 p.i., some mice in both the WT and Δ*dltA* groups developed clinical signs associated with *S. suis* meningitis in the mouse (8), such as hyperexcitation, episthotonus, opisthotonus, bending of the head, and walking in circles. It has been proposed that maintaining a high level of bacteremia is essential for CNS disease to appear at later stages of the infection (13). Interestingly, the number of meningitis-presenting mice was lower for the Δ*dltA* group ($n = 1$) than for the WT group ($n = 6$), and this observation was consistent with the reduction in the bacterial load in the blood of animals inoculated with the Δ*dltA* mutant compared to the animals that received the WT strain (data not shown). Therefore, we performed a second trial with mice using an intermediate dose (5×10^6 CFU per animal) in order to avoid development of septicemia. Mice in both groups presented moderate clinical signs during the first 72 h p.i., but no animal in either group died from septicemia. However, starting at day 7 p.i., several mice in the WT group developed clinical signs associated with meningitis. High titers of *S. suis* were isolated from the brains of these animals at ($>1 \times 10^6$ CFU/0.5 g of tissue). In strong contrast, no clinical signs of meningitis were observed in the Δ*dltA* group, nor was *S. suis* isolated from the brain of any animal infected with the Δ*dltA* mutant. There were significant differences in the mortality rate between the mice inoculated with the WT and the mice inoculated with the

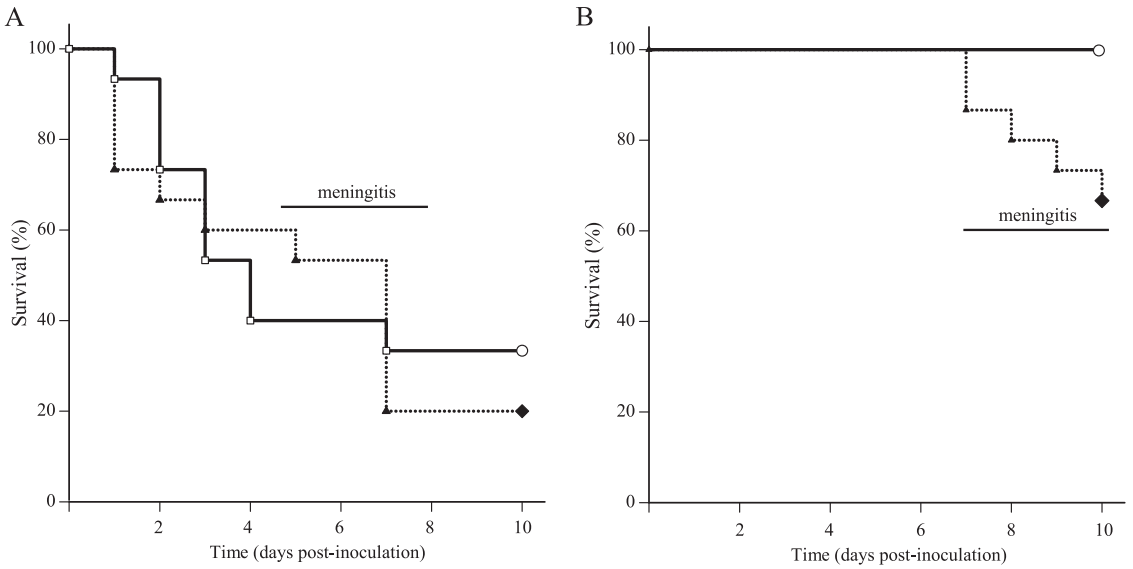

FIG. 6. Survival of mice inoculated with the WT (dotted line) or Δ*dltA* mutant (solid line) strain. (A) No significant differences in survival between groups were observed when the high dose was used for inoculation. However, fewer animals in the mutant group died from meningitis. (B) When the intermediate dose was used for inoculation, all mice in the Δ*dltA* mutant group survived, while 35% of the mice in the WT group died from meningitis. There were significant differences in survival ($P < 0.05$, Kaplan-Meier test).

Δ*dltA* mutant strain ($P < 0.05$, Kaplan-Meier test) at the intermediate infection dose (Fig. 6B).

***S. suis* LTA D-alanylation promotes adherence to and invasion of porcine BMEC.** Experimental infection of mice using the intermediate dose clearly demonstrated that the Δ*dltA* mutant is less able to induce CNS disease. A recent study of *S. suis* meningitis in the mouse showed that cells lining the choroid plexus and the brain endothelium are potential CNS entry sites for this pathogen (8). In addition, previous studies demonstrated the ability of *S. suis* to adhere to and invade immortalized porcine BMEC (35, 36). Recently, it has been shown that expression of the *dlt* operon is upregulated upon interaction of *S. suis* with porcine BMEC (11). Therefore, to assess the contribution of the LTA D-alanyl modification to adherence to and invasion of porcine BMEC, we compared the interactions of WT and Δ*dltA* mutant strains with cultured monolayers of these cells. After 2 h of incubation of *S. suis* with porcine BMEC at a multiplicity of infection of 1, followed by vigorous washing, we observed a marked decrease in the total number of cell-associated Δ*dltA* mutant bacteria compared with the number of cell-associated WT parent strain bacteria (Fig. 7). Using antibiotic protection to quantify bacteria which had invaded the intracellular compartment, a similar reduction in internalization of the Δ*dltA* mutant was observed (Fig. 7). Therefore, LTA D-alanylation itself plays a role in facilitating *S. suis* adherence to and invasion of porcine BMEC, and we speculate that this occurs mainly through cell envelope charge stabilization that allows efficient display of proteinaceous adhesins and/or invasins (23). Porcine BMEC are one of the main cellular types forming the porcine BBB, a structure that successful pathogens must cross in order to cause meningitis. Interestingly, a previous report proposed that the diminished resistance to killing by leukocytes was responsible for impairment of the ability of an *S. agalactiae* Δ*dltA* mutant to induce meningitis in the mouse (26). Based on our results for porcine BMEC and the observed outcome of the experimental infections in both the murine and porcine models of infection, we speculate that in addition to the failure of the Δ*dltA* mutant to maintain a high level of bacteremia, the impaired interactions with BMEC are also responsible for the reduced ability of the Δ*dltA* mutant to induce meningitis.

In summary, *S. suis* LTA D-alanylation mediated by the *dlt* operon contributes phenotypically to resistance to CAMPs, likely through an increased net positive surface charge. It also enhances the resistance of *S. suis* to neutrophil killing, as well as the capacity of this organism to adhere to and invade porcine BMEC. In addition, LTA D-alanylation contributes to *S. suis* virulence in both the murine and porcine models of infection, probably through interference with innate immune clearance mechanisms and by facilitating penetration of host barri-

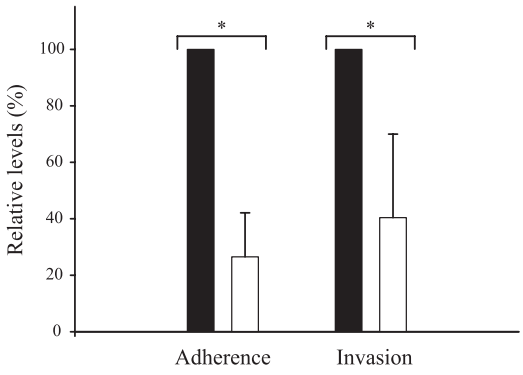

FIG. 7. Interactions of the Δ*dltA* mutant and WT strains with porcine BMEC. The Δ*dltA* mutant showed reduced levels of adherence to and invasion of porcine BMEC. The data for the WT strain were normalized to 100%. The data are data from at least four independent experiments. The error bars indicate standard deviations. The asterisks indicate significant differences ($P < 0.05$, *t* test).

ers. The results of this study strongly suggest that LTA D-alanylation is an important virulence factor of this swine pathogen and zoonotic agent.

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