D-Alanlyation of Lipoteichoic Acid Contributes to the Virulence of Streptococcus suis

Nahuel Fittipaldi, Tsutomu Sekizaki, Daisuke Takamatsu, Josée Harel, María de la Cruz Domínguez-Punaro, Sonja Von Aulock, Christian Draing, Corinne Marois, Marylène Kobisch, and Marcelo Gottschalk

Groupe de Recherche sur les Maladies Infectieuses du Porc and Centre de Recherche en Infectiologie Porcine, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Quebec J2S 7C6, Canada; Research Team for Bacterial/Parasitic Diseases, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-0856, Japan; United Graduate School of Veterinary Sciences, Gifu University, 501-1193 Gifu, Japan; Department of Biochemical Pharmacology, University of Konstanz, D-78457 Konstanz, Germany; and Agence Française de Sécurité Sanitaire des Aliments, Laboratoire d’Études et de Recherches Avicoles et Porcines, Unité de Mycoplasmologie-Bactériologie, 22440 Ploufragan, France

Received 27 November 2007/Returned for modification 10 April 2008/Accepted 6 May 2008

We generated by allelic replacement a ΔdltA mutant of a virulent Streptococcus suis serotype 2 field strain and evaluated the contribution of lipoteichoic acid (LTA) D-alanlyation to the virulence traits of this swine pathogen and zoonotic agent. The absence of LTA D-alanlyation resulted in increased susceptibility to the action of cationic antimicrobial peptides. In addition, and in contrast to the wild-type strain, the ΔdltA mutant was efficiently killed by porcine neutrophils and showed diminished adherence to and invasion of porcine brain microvascular endothelial cells. Finally, the ΔdltA 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-alanlyation 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 encapsulation 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 fibrinectin/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 transcripted 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 dtl 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...
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 electrophysiological 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% CO₂. *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. Southern hybridizations were performed by using standard procedures (27). DNA manipulations were performed by double crossover recombination in *E. coli* and the temperature-sensitive shuttle vector pSET5s, which carries the gene conferring Cm resistance (32), giving rise to knock-out vector pSADltA (Fig. 1B).

**i) Generation of S. suis ΔdltA 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 pSADltA 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 ΔdltA 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).

**Antimicrobial peptide sensitivity.** Assays were carried out in sterile 96-well microtiter plates. The concentrations of log-phase bacteria were adjusted to approximately 10⁶ 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 irradiated 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.

### TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli Top 10</td>
<td>General strain for cloning, F' <em>mcrA</em> Δ(<em>mrr-hsdRMS-mcrBC</em>) Δ880lacZAM15 Invitrogen</td>
<td></td>
</tr>
<tr>
<td>S. suis 31533</td>
<td>Serotype 2 field strain, highly virulent</td>
<td>35</td>
</tr>
<tr>
<td>S. suis ΔdltA</td>
<td>Δ<em>dltA</em> mutant strain derived from strain 31533</td>
<td>This study</td>
</tr>
<tr>
<td>S. suis BD102</td>
<td>Unencapsulated mutant strain derived from strain 31533</td>
<td>12</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCR4</td>
<td><em>E. coli</em> vector for cloning PCR fragments</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pSmall</td>
<td><em>E. coli</em>-S. suis shuttle vector, Sp', source of <em>aad9</em></td>
<td>P. Willson, unpublished results</td>
</tr>
<tr>
<td>pSET5s</td>
<td>Temperature-sensitive suicide vector for <em>S. suis</em> mutagenesis, Cm² (car)</td>
<td>32</td>
</tr>
<tr>
<td>pSADltA</td>
<td>pSET5s carrying the construct for allelic exchange</td>
<td>This study</td>
</tr>
</tbody>
</table>
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=glmdb). hyp. protein, hypothetical protein. (B) Strategy used in this study to construct the knockout vector used to generate the ΔdltA mutant. See Materials and Methods for details.

**TABLE 2. Oligonucleotide primers used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)*</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>2872F</td>
<td>GCAGTTACCTTAAGGCTCGAGAAAACGG</td>
<td>HindIII</td>
</tr>
<tr>
<td>3765R</td>
<td>CTGCTAATCTTGGATCTTCTCCTT</td>
<td>BamHI</td>
</tr>
<tr>
<td>5250F</td>
<td>CGTCCTTATGGATCGAGATGGAGATT</td>
<td>PstI</td>
</tr>
<tr>
<td>5809R</td>
<td>CGTCCTAATCGGATCCATAGG</td>
<td>BamHI</td>
</tr>
<tr>
<td>specF3</td>
<td>GCCAATGAGATCTTACATAAAC</td>
<td>BglII</td>
</tr>
<tr>
<td>specR</td>
<td>AAAGTTGTTCCTGAGTTTCTCAA</td>
<td>PstI</td>
</tr>
</tbody>
</table>

* Restriction sites are indicated by bold type.
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% CO2. 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 dltA operon to LTA d-alanylation, we constructed by allelic replacement a ΔdltA mutant strain and analyzed the content of d-alanine in purified LTA of the WT and ΔdltA 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 ΔdltA mutant spectrum, suggesting that the LTA of the mutant lacks this amino acid.

The in vitro growth of the ΔdltA 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* ΔdltA mutants, which were either poorly separated or multiseptate in the stationary phase of growth (18, 26), the *S. suis* ΔdltA 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 ΔdltA mutant strains to selected CAMPs. The *S. suis* ΔdltA 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...
VOL. 76, 2008 LTA D-Alanylation and S. suis Virulence 3591

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 ΔdltA 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 ΔdltA mutant bacteria were killed by neutrophils (Fig. 4). This level of killing was similar to that of the unencapsulated mutant BD102, despite the fact that the ΔdltA 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 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 ΔdltA 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 ΔdltA 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.

The virulence of the ΔdltA mutant is attenuated in pigs. Several ΔdltA 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 ΔdltA 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 ΔdltA 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 ΔdltA 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 ΔdltA 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 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. 3. (A) Growth curves for the S. suis WT and ΔdltA mutant strains. The growth of the ΔdltA mutant was similar to the growth of the WT parent strain under normal laboratory conditions. OD600, optical density at 600 nm. (B) Morphology of the ΔdltA 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 ΔdltA 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).

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

<table>
<thead>
<tr>
<th>Peptide (origin)</th>
<th>Net charge</th>
<th>MICs (μg/ml)</th>
<th>WT</th>
<th>ΔdltA mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colistin (Bacillus colistinus)</td>
<td>+5</td>
<td>50</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Polymyxin B (Bacillus polymyxa)</td>
<td>+5</td>
<td>75</td>
<td>18.75</td>
<td></td>
</tr>
<tr>
<td>Magainin II (claw frog skin)</td>
<td>+4</td>
<td>45</td>
<td>5.6</td>
<td></td>
</tr>
</tbody>
</table>
The remaining inoculated animal and the sentinel pig in this group survived until the end of 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).

FIG. 5. Survival of pigs inoculated with the WT (dotted line) or ΔdltA 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 ΔdltA 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 absence of LTA β-alanylation impairs S. suis virulence in mice. To better evaluate the attenuation of the S. suis ΔdltA 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 × 10⁷ CFU per animal) most mice in both the WT and ΔdltA 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 ΔdltA 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 × 10⁷ CFU/ml) and for organs, such as the liver and spleen, of septicemic animals (>1 × 10⁷ CFU/0.5 g of tissue in some animals). Starting on day 5 p.i., some mice in both the WT and ΔdltA 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 ΔdltA 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 ΔdltA 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 × 10⁶ 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 × 10⁶ CFU/0.5 g of tissue). In strong contrast, no clinical signs of meningitis were observed in the ΔdltA group, nor was S. suis isolated from the brain of any animal infected with the ΔdltA mutant. There were significant differences in the mortality rate between the mice inoculated with the WT and the mice inoculated with the
ΔdltA 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 ΔdltA 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 ΔdltA 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 ΔdltA 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 ΔdltA 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* ΔdltA mutant to induce meningitis in the mouse (26). Based on our results for porcine

**FIG. 6.** Survival of mice inoculated with the WT (dotted line) or ΔdltA 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 ΔdltA 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).

**FIG. 7.** Interactions of the ΔdltA mutant and WT strains with porcine BMEC. The ΔdltA 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.

ACKNOWLEDGMENTS

We are indebted to P. Willson for the generous gift of plasmid pSmall and to P. Friedl for kindly providing the porcine PBMEC/C1-2 cell line. We thank D. Montpetit for performing the electron microscopy and G. Vanier for help with the porcine BMEC tests. We also thank S. Lacourte for useful suggestions and M. Takahashi and M. P. Lecours for assistance.

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and by the Centre de Recherche en Immunologie Porcine (CRIP-FQRNT). N.P. and M.C.D-P. are recipients of NSERC postgraduate scholarships.

REFERENCES


Editor: V. J. DiRita

Infect. Immun.
ERRATUM

D-Alanylation of Lipoteichoic Acid Contributes to the Virulence of \textit{Streptococcus suis}

Nahuel Fittipaldi, Tsutomu Sekizaki, Daisuke Takamatsu, Josée Harel, María de la Cruz Domínguez-Punaro, Sonja Von Aulock, Christian Draing, Corinne Marois, Marylène Kobisch, and Marcelo Gottschalk

Groupe de Recherche sur les Maladies Infectieuses du Porc and Centre de Recherche en Infectiologie Porcine, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Quebec J2S 7C6, Canada; Research Team for Bacterial/Parasitic Diseases, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-0856, Japan; United Graduate School of Veterinary Sciences, Gifu University, 501-1193 Gifu, Japan; Department of Biochemical Pharmacology, University of Konstanz, D-78457 Konstanz, Germany; and Agence Française de Sécurité Sanitaire des Aliments, Laboratoire d’Études et de Recherches Avicoles et Porcines, Unité de Mycoplasmologie-Bactériologie, 22440 Ploufragan, France

Volume 76, no. 8, pages 3587–3594, 2008. Page 3587: Because of a printing error, the bottom third of this page did not print in the print version of the journal. The online version was not affected. Page 3587 should appear as shown on the following page.
d-Alanylation of Lipoteichoic Acid Contributes to the Virulence of *Streptococcus suis*

Nahuel Fittipaldi, 1 Tsutomu Sekizaki, 2, 3 Daisuke Takamatsu, 2 Josée Harel, 1 María de la Cruz Domínguez-Punaro, 4 Sonja Von Aulock, 4 Christian Draing, 4 Corinne Marois, 5 Marylène Kobisch, 6 and Marcelo Gottschalk 1, 6

Groupe de Recherche sur les Maladies Infectieuses du Porc and Centre de Recherche en Infectiologie Porcine, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, QC J2S 7C6, Canada; 2 Research Team for Bacterial/Parasitic Diseases, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-0856, Japan; 3 United Graduate School of Veterinary Sciences, Gifu University, 501-1193 Gifu, Japan; 4 Department of Biochemical Pharmacology, University of Konstanz, D-78457 Konstanz, Germany; 5 and Agence Française de Sécurité Sanitaire des Aliments, Laboratoire d’Études et de Recherches Avicoles et Porcines, Unité de Mycopathologie-Bactériologie, 22440 Ploufragan, France

Received 27 November 2007/Returned for modification 10 April 2008/Accepted 6 May 2008

We generated by allelic replacement a ΔdltΔ 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 ΔdltΔ mutant was efficiently killed by porcine neutrophils and showed diminished adherence to and invasion of porcine brain microvascular endothelial cells. Finally, the ΔdltΔ 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 septicaemia (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 sllysin, 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
AUTHOR’S CORRECTION

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

Nahuel Fittipaldi, Tsutomu Sekizaki, Daisuke Takamatsu, Josée Harel, María de la Cruz Domínguez-Punaro, Sonja Von Aulock, Christian Draing, Corinne Marois, Marylène Kobisch, and Marcelo Gottschalk

Groupe de Recherche sur les Maladies Infectieuses du Porc and Centre de Recherche en Infectiologie Porcine, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Qc, J2S 7C6, Canada; Research Team for Bacterial/Parasitic Diseases, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-0856, Japan; United Graduate School of Veterinary Sciences, Gifu University, 501-1193 Gifu, Japan; Department of Biochemical Pharmacology, University of Konstanz, D-78457 Konstanz, Germany; and Agence Française de Sécurité Sanitaire des Aliments, Laboratoire d’Études et de Recherches Avicoles et Porcines, Unité de Mycoplasmologie-Bactériologie, 22440 Ploufragan, France

Volume 76, no. 8, p. 3587–3594, 2008. Page 3591: Figure 3B should appear as shown below.

Page 3591, legend for Fig. 3: Lines 4 and 5 should read “(B) Morphology of the ΔdltA mutant (left panel) and WT (right panel) strains.”