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Characterization of colonization-deficient mutants of *Actinobacillus suis*

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ABSTRACT

Actinobacillus suis is an important opportunistic pathogen of swine that can cause disease in pigs of all ages, especially in high-health status herds. Although *A. suis* shares many virulence factors in common with *Actinobacillus pleuropneumoniae* and can cause a haemorrhagic pleuropneumonia similar to that caused by *A. pleuropneumoniae*, *A. suis* most often causes septicaemia and diseases such as arthritis and meningitis that are sequelae to septicaemia. In a recent signature-tagged transposon mutagenesis study, 30 colonization-essential genes of *A. suis* were identified. In the current study, the attachment and invasion patterns of strains harboring Tn*10* insertions in *ompA*, *pfhaB1*, *lcbB*, and *cpxR* were evaluated using porcine palatine tonsil organ cultures, the swine kidney epithelial cell line, SK6, and a porcine brain microvascular endothelial cell line, PBMEC/C1-2. All of these mutants attached in lower numbers than wild type to the tonsillar explants and to the SK6 cells. The *ompA* mutant attached in significantly lower numbers than wild type to the porcine tonsil cells ($P = 0.02$) and to PBMEC ($P = 0.0008$) at 60 min time point. As well, the *ompA* mutant showed significantly greater sensitivity than wild type to chemical stressors and to swine serum. Using fluorescent microscopy, a GST-OmpA fusion protein could be demonstrated to interact with the crypt epithelial cells of porcine palatine tonsil.

1. Introduction

The Gram-negative bacterium *Actinobacillus suis* is a ubiquitous opportunistic pathogen of high-health status swine (Wilson and McOrist, 2000). It colonizes the upper respiratory tract early in the neonatal period and often resides asymptomatically within the tonsils of the soft palate (Sanford et al., 1990; MacInnes et al., 2008). At times of stress such as weaning, farrowing, and transportation, it may be activated and cause disease in pigs of all ages. *A. suis* can cause a haemorrhagic fibrinopleuropneumonia that is very similar to that caused by *A. pleuropneumoniae*; however, in contrast to *A. pleuropneumoniae*, *A. suis* can also cause enteritis, mastitis, metritis, abortion, and a number of invasive diseases including, arthritis, meningitis, and fatal septicaemia (MacInnes and Desrosiers, 1999).

The initial attachment and colonization of the host tissues are critical events in pathogenesis. To begin to understand the process of attachment of *A. suis* to porcine tissues, four colonization-deficient mutants identified in a prior signature tagged mutagenesis study were selected for characterization (Ojha et al., 2005). Three of the mutants (*ompA*, *pfhaB1*, and *lcbB* homologs) had Tn*10* insertions in genes known to encode, or be involved in the synthesis of cell surface structures. A mutant with a Tn*10* insertion in a *cpxR* pilin regulatory gene homolog was also selected for further study. Analysis of these mutants revealed that the *A. suis* OmpA protein might play an important role in both the initial colonization of the tonsils

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and in the invasion of the central nervous system. Further, OmpA appears to be necessary for *A. suis* to resist killing by porcine serum and other stressors.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *A. suis* strains used in this study were stored as glycerol stocks maintained at −70 °C; for routine culture, they were streaked on sheep blood agar plates (BAP) and incubated at 37 °C in an atmosphere of 5% CO_2. For adhesion assays, cells from heavily streaked BAP were harvested in phosphate buffered saline (PBS), pelleted, and resuspended in RPMI 1640 medium (Gibco, Invitrogen Corporation, Burlington, Ontario, Canada) to an optical density (OD_{600}) of ∼1.0 (∼2×10^9); the precise number of viable cells were determined by plating 10-fold serial dilutions on BAP. To enumerate the number of *A. suis* that adhered to palatine tonsil culture assays (see below), bacteria were plated on 'super-selective' (SS) brain heart infusion (BHI) agar medium (Becton Dickinson, Oakville, Canada) containing 10 μg/ml bacitracin, 1 μg/ml crystal violet, 25 μg/ml nalidixic acid and 50 μg/ml kanamycin (mutant strains) or 5 μg/ml chloramphenicol (wild type *A. suis* with pFP12). Antibiotics were obtained from Sigma–Aldrich, St. Louis, MO, USA.

2.2. Palatine tonsil organ culture

Palatine tonsils collected from weaned 3 to 4-week-old high-health status piglets (Arkell Swine Research Station, University of Guelph) were placed in warm RPMI 1640 medium. The tissues were debrided and 1.3 cm diameter pieces were incubated in 15 ml tubes in 5 ml of RPMI 1640 plus 5% heat-inactivated foetal bovine serum (FBS) (Cansera, Rexdale, Ontario, Canada) with 100 IU/ml penicillin and 100 IU/ml streptomycin (pen/strep) and 100 μg/ml gentamicin, in a roller drum at 37 °C for 2 h. Tissues were then transferred to new tubes containing RPMI 1640 plus 1% FBS without antibiotics (RPMI/WA) for 5–6 h during which time the medium was changed at least twice. The tissues were then stored overnight in RPMI/WA at 4 °C. Cell viability was measured by trypan blue exclusion.

2.3. Porcine tonsil attachment assay

For the attachment studies, wells of 24 well culture plates (1.5 cm diameter; Nunclon, Life Technologies, Burlington, Ontario, Canada) were incubated with 1% bovine serum albumin (BSA in PBS) and incubated overnight at 4 °C then washed with 1 ml sterile PBS. Pieces of tonsil were placed in nine wells, washed with warm RPMI/WA medium and the medium removed. Four pieces were infected with 1 ml of culture containing ∼2×10^9 of wild type *A. suis* and four with 2×10^9 one of the mutant strains; one piece was mock infected with 1 ml of medium (uninfected control). To limit unavoidable biological variation, the experiment was controlled within (i.e., one piece of a single tonsil was incubated with the wt a second piece with a mutant). The culture plates were then centrifuged at 1500 rpm × *g* for 10 min before incubation at 37 °C in a humidified atmosphere of 5% CO_2. To harvest attached bacteria, tissue pieces were removed to 15 ml round-bottomed tubes, washed 3 times with sterile PBS, then immersed in 1 ml of trypsin plus 1% Triton X-100 and incubated for 20 min at 37 °C. After vortexing for 30 s, the surface of the tissue was vigorously scraped with a sterile scalpel blade. The tonsil cell suspensions with their adherent bacteria were diluted 10-fold and plated on SS-BHI agar. The uninfected tonsil piece was treated similarly at the end of experiment (120 min) and serial 10-fold dilutions were plated on BAP. As the antibiotic pre-treatment of the tonsil pieces and subsequent plating on SS plates did not completely eliminate all of the normal microflora, only cells with typical *A. suis* morphology were counted. Three independent replicates ($n = 3$) of the attachment assays were done. Cell viability was measured by trypan blue exclusion.

2.4. SK6 cell attachment assay

SK6 monolayers were infected with ∼1×10^8 bacterial cells in 500 μL of antibiotic free Eagles Minimal Essential Medium (Gibco, Invitrogen Corp.) with 10% heat-inactivated FBS and 2 mM L-glutamine (Sigma–Aldrich), at a multiplicity of infection of (MOI) of 100. Adherent bacteria were harvested at 30, 60, and 120 min. Immediately before harvest, the plates were washed three times with PBS then the monolayers were incubated for 10 min with 300 μL of 0.05% trypsin/0.01% EDTA. The plates were then vortexed for 30 s and serial dilutions of the cell suspensions were plated on selective plates for enumeration of attached bacteria. Three independent assays were performed; cell viability was measured by typan blue exclusion.

2.5. PBMEC adhesion and invasion assays

PBMEC/C1-2 cells (Teifel and Friedl, 1996) were maintained as described previously (Vanier et al., 2004). For adhesion and invasion, monolayers were infected at MOI of 10.0 and assays were performed as described previously (Vanier et al., 2004). The viability of the PBMECs was monitored by measuring the release of lactate dehydrogenase (LDH) as described previously (Vanier et al., 2004).

2.6. Cloning, sequencing, and expression of GST-ompA$_{A.s.}$

The regions of *ompA* flanking the mini-Tn*10* insertion were identified by inverse PCR as described previously (Ojha et al., 2005) and by primer walking. Primers SF (5′TTTAGGAGGGGTGGGTTATTG3′) and SR (5′CGGGCGTTT-TTTTATTCTTTAG3′) were used to amplify the entire gene (95 °C for 2 min, followed by 35 cycles of 30 s at 95 °C, primer annealing at 55 °C for 30 s, and extension for 1 min at 72 °C with a final elongation step of 7 min at 72 °C). The 25 μL PCR mixture contained 2.5 μL 10× PCR buffer, 0.75 μL 50 mM $MgCl_2$, 2.0 μL 2.5 mM dNTPs, 1.0 μL 25 μM of each primer, 0.25 μL *Taq* polymerase and 1 μL of genomic DNA (100 ng). The entire *ompA* gene was cloned into pTOPO-TAr (Gibco, Invitrogen Corp.) and sequenced on both strands by primer walking. The *ompA*