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Short communication

An isogenic *Actinobacillus pleuropneumoniae* AasP mutant exhibits altered biofilm formation but retains virulence

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ABSTRACT

AasP, an autotransporter serine protease of *Actinobacillus pleuropneumoniae*, has been shown to be expressed in necrotic porcine lung tissue. Based on the hypothesis that AasP might play an important role in *A. pleuropneumoniae* adhesion and virulence by processing other surface-associated proteins, the predicted catalytic site of AasP was deleted and the isogenic mutant, AP76Δ*aasP*, was compared to the wild-type strain in a biofilm assay as well as an aerosol infection model. AP76Δ*aasP* showed increased adherence compared to the wild-type strain under standard culturing conditions as well as under NAD restriction. No significant differences between AP76 wild-type and AP76Δ*aasP* were observed upon experimental infection of pigs, indicating that AasP does not play a crucial role in *A. pleuropneumoniae* virulence.

1. Introduction

Actinobacillus (*A.*) *pleuropneumoniae* is the causative agent of porcine pleuropneumonia, an economically important disease of fattening pigs. The ability to persistently colonize pigs after infection and even after vaccination with bacterins is a major obstacle for eradication of the organism (Fenwick and Henry, 1994; Haesebrouck et al., 1997; Chiers et al., 1998). We recently identified an autotransporter serine protease, AasP (GenBank accession no. DQ490067), which appears to remain associated with the membrane and is present in membrane fractions in large amounts (Baltes et al., 2007). Its function is largely unknown to date, but one possible role might be the processing of other autotransporter proteins, namely adhesins, similar to SphB1 in *Bordetella pertussis* (Coutte et al., 2001). AasP has recently been found to be involved in the processing of OmlA (Ali et al., 2008). While autotransporter adhesins have not been characterized in *A. pleuropneumoniae* to date, two have been identified, one of which is interrupted by the insertion of an IS element (Baltes and Gerlach, 2004; Tegetmeyer et al., 2008). *A. pleuropneumoniae* has been shown to form biofilms, and genes responsible for matrix polysaccharide production have been identified (Kaplan et al., 2004; Kaplan and Mulks, 2005). However, the role of biofilms in *A. pleuropneumoniae* infection has not been extensively studied in the pig. A regulatory mutant lacking regulator protein *arcA* was shown to be impaired in biofilm formation and virulence (Buettner et al., 2008). In the present study, we investigate the influence of AasP on *A. pleuropneumoniae* adherence in vitro, and on virulence.

2. Materials and methods

2.1. Bacterial strains, plasmids, and primers

The strains, plasmids, and primers used in this work are listed in Table 1.

2.2. Media and growth conditions

Escherichia coli strains were cultured in LB medium supplemented with ampicillin (100 μg/ml). *A. pleuropneumoniae* strains were routinely cultured in PPLO medium

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Table 1
Strains, plasmids and primers used in this study.

	Characteristics	Source
Strains		
AP76	*A. pleuropneumoniae* serotype 7 strain kindly provided by the Western College of Veterinary Medicine, Saskatoon, Canada	Anderson et al. (1991)
AP76Δ*aasP*	Unmarked *aasP* deletion mutant of *A. pleuropneumoniae* AP76	This work
Plasmid		
pSER750	Transconjugation vector pEMOC2 (Baltes et al., 2003) containing truncated *aasP* fragment, lacking the putative catalytic site	This work
Primers		
oSER101-Psp	5′ GACGACGGGCCCAAAACCCGTCGAACAGAATG 3′	This work
oSER102-Not	5′ GTAAGTGCGGCCGCGCTCAAGCCAAACTGACGTT 3′ primer pair containing PspOMI and NotI restriction sites, respectively, surrounding the putative catalytic site of *aasP*, resulting in a 1750 bp amplification product in the wild-type, and a 1627 bp product in the *aasP* mutant	
oSER105-Bsm	5′ ACAAGTGGGCCCAAAACCTGTTGAACAGAATG 3′ downstream primer containing BsmBI restriction site, binds upstream of putative catalytic site	This work
oSER106-Bsm	5′ TGGACCCGTCTCAGTTGCATTAAGTATTGCTAGATTGC 3′ upstream primer containing BsmBI restriction site, binds downstream of putative catalytic site	This work

(Difco GmbH, Augsburg, Germany) supplemented with nicotinamide dinucleotide (NAD; 10 μg/ml; Merck, Darmstadt, Germany), L-glutamine (100 μg/ml; Serva, Heidelberg, Germany), L-cysteine-hydrochloride (260 μg/ml; Sigma–Aldrich), L-cystine-dihydrochloride (10 μg/ml; Sigma–Aldrich), dextrose (1 mg/ml), and Tween® 80 (0.1%).

2.3. Manipulation of DNA

DNA-modifying enzymes were purchased from New England Biolabs (Bad Schwalbach, Germany) and used according to the manufacturer's instructions. *Taq* polymerase was purchased from Gibco-BR Life Technologies (Karlsruhe, Germany). Chromosomal DNA for PCR and Southern blotting as well as plasmid DNA was prepared by standard protocols (Sambrook et al., 1989). PCR, Southern blotting, transformation and gel electrophoresis were done by standard procedures (Sambrook et al., 1989).

2.4. Sequence analyses

Nucleotide sequencing was performed by SeqLab GmbH, Göttingen, Germany. Promoter prediction was performed using the Virtual Footprint promoter analysis tool (Munch et al., 2005) available at http://prodoric.tu-bs.de/vfp/.

2.5. Construction of transconjugation plasmid pSER705

Primer pairs oSER101-Psp/oSER106-Bsm and oSER102-Not/oSER105-Bsm were used to generate PCR products which were ligated together after digestion with BsmBI. The ligation product was amplified using primer pair oSER101-Psp/oSER102-Not, which was then digested with PspOMI and NotI and ligated into pEMOC2 to generate transconjugation plasmid pSER705.

2.6. Adherence assay

Adhesion of *A. pleuropneumoniae* to abiotic surfaces under different conditions was tested in an adherence assay that has been described for *Listeria monocytogenes* (Djordjevic et al., 2002). Overnight cultures of AP76 and AP76Δ*aasP* were diluted 1:100 with fresh PPLO media containing additives for the respective conditions (standard: 10 μg/ml NAD; reduced NAD: 0.1 μg/ml; iron restriction: 0.1 mM dipyridyl and 10 μg/ml NAD). Sterile 96 well flat bottom tissue culture plates (Sarstedt, Germany) were seeded with 4× 200 μl of bacterial suspension or the respective supplemented media per growth condition, and incubated at 37 °C, 5% CO_2 for a total of 6 or 24 h. Optical density (OD) was measured in a microtiter plate reader (GENios Pro, Tecan GmbH, Crailsheim, Germany) at 630 nm. After incubation, the medium was removed and non-adherent bacteria were removed by rinsing the wells with distilled water three times. After drying, biofilms were stained with 80 μl of 1% crystal violet solution per well for 30 min, followed by four washing steps with distilled water. After at least 30 min of air drying, crystal violet was dissolved by adding 150 μl destaining solution (50% ethanol, 30% glacial acetic acid and 20% acetone) and OD was measured at 550 nm after 15 min of incubation. Average OD values from control wells were subtracted from average OD values from respective inoculated wells both for bacterial culture (630 nm) and crystal violet (550 nm) ODs. For quantitative comparison of adherence, OD values of crystal violet readings were divided by culture OD values to correct for different growth OD values between the two *A. pleuropneumoniae* strains. Four wells were set up for each strain and each growth condition, and the experiment was repeated three times.

2.7. Animal experiments

Animal experiments were carried out using an aerosol infection model previously described (Baltes et al., 2001). 16 *A. pleuropneumoniae* seronegative male landrace pigs of 7–8 weeks of age were randomly divided into two groups. The challenge dose was prepared from cultures at an OD_{600} of 0.49 (AP76*wt*) and 0.48 (AP76Δ*aasP*). 13 ml of a 1:30,000 dilution of these cultures were aerosolized per four pigs. Body temperatures were monitored daily. Pigs