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Novel genes associated with biofilm formation of *Actinobacillus pleuropneumoniae*

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ABSTRACT

Actinobacillus pleuropneumoniae is a Gram-negative bacterium and is the causative agent of swine pleuropneumonia, a highly contagious respiratory disease. Biofilm formation is an important ability possessed by numerous bacterial pathogens. The purpose of this study was to identify and characterize biofilm mutants of *A. pleuropneumoniae* serotype 1 strain S4074 created using a mini *Tn-10* transposon. The transposon library was screened to identify mutants with a modified ability to form biofilms in polystyrene microtiter plates. A total of 1200 mutants were screened and the analysis identified 24 mutants that exhibited abnormal biofilm formation, at least 16 unique genes were identified. Most genes identified in the enhanced-biofilm mutants encoded proteins with unknown functions, whereas most genes identified in the biofilm-reduced mutants encoded proteins related to transport, protein synthesis and nucleic acid synthesis. Approximately 50% of genes, including *hns*, *potD2*, *ptsI, tig* and *rpmF*, identified in our screen have been previously associated with biofilm formation in *A. pleuropneumoniae* and other bacterial species, and thus validated the screening method. The rest of genes identified, such as APL_0049, APL_0637 and APL_1572, have not been previously associated with biofilm formation. Interestingly, gene APL_0049 was previously seen among the genes differentially expressed during a natural infection of pig lungs. Preliminary characterization of the mutants was also initiated by assessing their hydrophobicity, their biofilm matrix composition and their ability to adhere to a polystyrene surface or NPTr cells. Based on the preliminary characterization, some of the mutants identified appear to have deficiencies during the initial attachment or growth of the biofilm. In conclusion, transposon mutagenesis analysis allowed the identification of new genes associated with biofilm formation in *A. pleuropneumoniae*.

1. Introduction

Actinobacillus pleuropneumoniae is a Gram-negative bacterium from the *Pasteurellaceae* family and is the causative agent of swine pleuropneumonia, a highly contagious respiratory disease. The virulence factors of this microorganism involved in colonization and induction of lung lesions have been thoroughly studied and some have been well characterized (for a recent review see Chiers et al., 2010). Biofilm formation is involved in the virulence of numerous bacterial pathogens of human (Donlan and Costerton, 2002) and veterinary (Jacques et al., 2010) importance. It has been reported that *A. pleuropneumoniae* has the ability to form biofilms under certain static growth conditions (Kaplan and Mulks, 2005; Labrie et al., 2010). Additionally, many field isolates of *A. pleuroneumoniae* have the ability to form biofilms.

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In *A. pleuropneumoniae*, biofilm formation on polystyrene microtiter plates depends on the production of a polymer of β-1,6-N-acetyl-D-glucosamine (PGA) (Izano et al., 2007; Kaplan et al., 2004). Biosynthesis of PGA is dependent of the proteins encoded by the *pgaABCD* operon (Kaplan et al., 2004). H-NS regulates biofilm synthesis by repressing the *pga* operon (Bossé et al., 2010) and *hns* mutants exhibit enhanced biofilm formation (Dalai et al., 2008). The expression of the *pga* operon can also be positively regulated by the alternative sigma factor σ^E, indicating that biofilm formation is part of the extracytoplasmic stress response (Bossé et al., 2010). Other genes are associated with biofilm formation in *A. pleuropneumoniae*. For example, enhanced biofilm formation was observed in a quorum sensing (*luxS*) mutant (Li et al., 2008). On the other hand, an ArcAB two-component regulatory system mutant (Δ*arcA*) (Buettner et al., 2008) and an autotransporter serine protease mutant (Δ*aasP*) (Tegetmeyer et al., 2009) were unable to form biofilms.

The objective of this study was to identify additional genes associated with biofilm formation in *A. pleuropneumoniae* using transposon mutagenesis and a microtiter plate screening assay.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A nalidixic acid-resistant mutant derived from the *A. pleuropneumoniae* reference strain serotype 1 (4074NalR) was used as the recipient strain for transposon mutagenesis (Rioux et al., 1999). *A. pleuropneumoniae* strains were routinely cultured on Brain Heart Infusion agar (BHI; Oxoid Ltd, Basingstoke, Hampshire, England) supplemented with 15 μg/mL nicotinamide adenine dinucleotide (NAD); this medium is referred to as BHI-NAD. When necessary, 30 μg/mL of nalidixic acid (Nal), and 75 μg/mL of kanamycin (Km) were added to BHI-NAD. Plates were incubated at 37 °C in a 5% CO_2 atmosphere for 18–24 h. *Escherichia coli* S17.1 λpir (pLOF/Km) was used as donor strain (Tascon et al., 1993) and was routinely cultured on LB agar supplemented with 50 μg/mL of Km.

2.2. Transposon mutagenesis

Mutagenesis of *A. pleuropneumoniae* with a *Tn10* derivative was performed as described by Tascon et al. (1993). Briefly, overnight cultures of the donor and recipient strains were diluted to an OD_{650nm} of 0.8 in 0.85% (w/v) NaCl. A volume (100 μL) of each suspension was removed and added to 5 mL of sterile 10 mM $MgSO_4$ solution. The resulting mixtures were then filtered through a 0.45 μm membrane (Millipore type HA, Millipore; Boston, MA, USA). Mating filters containing donor and recipient bacteria were placed onto BHI-NAD agar, bacteria side up, and incubated at 37 °C for 4 h. Membranes were transferred onto BHI-NAD agar containing 100 μM IPTG to induce expression of the transposase. After an overnight incubation at 37 °C, the bacterial cells were resuspended in sterile NaCl (0.85% [w/v]), diluted and spread on solid counter-selection medium (BHI-NAD-Nal-Km).

2.3. Biofilm assays

2.3.1. Crystal violet staining

The assay was performed as described by Labrie et al. (2010). Briefly, the wells of a sterile 96-well microtiter plate (Costar® 3599, Corning, NY, USA) were filled in triplicate with a dilution (1/100 in BHI-NAD) of an overnight culture of *A. pleuropneumoniae*. Following a 6 h incubation at 37 °C, the wells were washed by immersion in water. Excess water was removed by inverting plates onto a paper towel. The wells were then filled with 100 μL of crystal violet (0.1% [w/v]) and the plate was incubated for 2 min at room temperature. After removal of the crystal violet solution, the plate was washed and dried at 37 °C for 30 min and 100 μL of ethanol (70% [v/v]) were added to the wells to release the stain. The amount of stain released was quantified by measuring the absorbance at 590 nm using a microplate reader (Powerwave, BioTek Instruments, Winooski, VT, USA). To measure the optical density (OD) of the culture, bacteria in the wells were thoroughly mixed and the absorbance at 600 nm was measured using a microplate reader (Powerwave, BioTek Instruments).

In addition to the microtiter plate technique, biofilm formation was also assessed in glass tubes. Briefly, 2 mL of a dilution (1/100 in BHI-NAD) of an overnight culture were transferred to a glass tube and incubated for 6 h at 37 °C with shaking (160 rpm). After the incubation, the tubes were washed gently with water and were then filled with 3 mL of crystal violet (0.1% [w/v]). After 2 min of incubation at room temperature, the crystal violet solution was removed and the tubes were washed with water and dried at 37 °C for 30 min. The amount of biofilm present was evaluated by comparing the thickness of ring stained with crystal violet.

2.3.2. Safranin staining

The biofilm assay was carried as described in Section 2.3.1 with some modifications. After the first wash, 96-well plates were air dried and stained with 0.1% (w/v) safranin for 10 min. After staining, the plates were rinsed with distilled water to remove excess dye and air dried for 1 h and 100 μL of ethanol (70%) was added to the wells. Biofilms were quantified by measuring the absorbance of stained biofilms at 490 nm using a microplate reader (Powerwave, BioTek Instruments).

2.3.3. Reduction of resazurin

The ability of different mutants to reduce resazurin to resorufin was measured using the CellTiter-Blue® Reagent (Promega Corporation, Madison, MI, USA). The biofilm was prepared as described in Section 2.3.1. Once the 100-μL aliquots were added to wells, the recommended volume of CellTiter-Blue® Reagent (20 μL) was added to each well. After thorough mixing, the microplates were incubated at 37 °C, and the absorbance (λ_{ex} 570 nm λ_{em} 600 nm) was measured at 1-h intervals for 6 h using a microplate reader Synergy HT (BioTek, Instruments).

2.4. Cell surface hydrophobicity

Bacterial cell surface hydrophobicity was measured as described by Labrie et al. (2002). Cell surface hydropho-