Histone-like protein H-NS regulates biofilm formation and virulence of *Actinobacillus pleuropneumoniae*

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**Abstract**

*Actinobacillus pleuropneumoniae* is the causative agent of porcine contagious pleuropneumonia, a very important swine respiratory infectious disease causing great economic losses worldwide. The pathogenesis of this disease is still not completely understood. Biofilm formation contributes to full virulence in many Gram-negative bacterial pathogens. In the present study, two biofilm-producing mutants were identified from the transposon mutagenesis mutant pools of a pleuropneumonia strain of serovar 1 (a non-biofilm forming strain). Inverse PCR and sequencing analysis revealed that the hns gene encoding the histone-like nucleoid structuring protein (H-NS) was inactivated by the mini-Tn10 transposon in both mutant strains. Further analysis revealed that the virulence was attenuated in the mutant strains when their haemolytic activity and 50% lethal doses in mice were compared with the parental strain. Real-time RT-PCR analysis suggested that the down-regulation of the exotoxin genes in the hns mutants may partly contribute to the virulence attenuation. Our data indicate that H-NS plays important roles in regulating biofilm formation and virulence in *A. pleuropneumoniae*.

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1. Introduction

*Actinobacillus pleuropneumoniae*, a member of the family of *Pasteurellaceae*, is the etiologic agent of porcine contagious pleuropneumonia that causes important economic losses in the pig industry worldwide. *A. pleuropneumoniae* invades the porcine tonsil and upper respiratory tract, and can be isolated from nasal cavities, tonsils, the middle ear cavity and the lungs of infected pigs [1,2]. Depending on the number of bacteria reaching the lung, the particular serovar of the infection and the immunological status of the host, the course of the disease can be divided into peracute, acute and chronic forms [2]. Peracute and acute cases usually show high mortality with pulmonary lesions characterized by severe oedema, inflammation, haemorrhage and necrosis, whereas the chronic form of disease is characterized by haemorrhagic, fibrous and necrotic pleuritis, pericarditis and pneumonia [2]. The pathogenesis mechanism of this disease is still not completely understood, although exotoxins (Apx toxins), capsular polysaccharide (CPS), lipopolysaccharide (LPS), urease and iron uptake proteins have been implicated as putative virulence factors of this bacterium [2].

It has been demonstrated that many species of bacteria can live as a free-living unicellular organism or in a form of sessile multicellular communities known as biofilms, which are defined as a bipolymer matrix-enclosed bacterial population adherent to each other and/or surfaces or interfaces [3]. Biofilms play a very important role in the pathogenesis of many bacterial infections [4]. Bacteria within biofilms are notorious for their resistance towards the host immune response and antibacterial agents compared to their free-living planktonic counterparts, making most biofilm infections difficult or impossible to eradicate from the host, and consequently leads to persistent/chronic infections [5].

Biofilm formation was reported to be a prevalent phenotype among field isolates of *A. pleuropneumoniae* with only two reference strains (serovars 5b and 11) exhibiting this phenotype *in vitro* [6]. Like *Escherichia coli* a major component of the *A. pleuropneumoniae* biofilm matrix was reported to be poly-N-acetylglcosamine (PGA), a linear polymer of N-acetyl-D-glucosamine residues in (1,6) linkage, and the PGA functions as a major biofilm adhesin in *A. pleuropneumoniae* [7,8]. Biofilm formation is a highly complex and dynamic process involving the switch from a planktonic existence to a sessile mode of growth, and this process may regulated by a number of genes. However, little is known about the genes involved in the biofilm formation of most veterinary bacteria including *A. pleuropneumoniae*. Recently, Buettner et al. reported that arcA gene is essential for biofilm formation of...
A. pleuropneumoniae, and the arcA deletion mutant shows decreased virulence [9]. Our previous report demonstrated that LuxS also regulates biofilm formation and virulence in A. pleuropneumoniae [10]. In the present study, we report another gene namely hns encoding the histone-like nucleoid structuring protein (H-NS) regulates both biofilm formation and virulence in A. pleuropneumoniae as well. This finding may highlight our understanding of the relationship between biofilm formation and pathogenesis of A. pleuropneumoniae.

2. Results

2.1. Identification of biofilm-forming mutants

From the mini-Tn10 transposon mutant pools of A. pleuropneumoniae strain 4074 (parental strain) generated in our laboratory, two biofilm-forming mutant strains (1-21 and 6-42) were identified using the glass tube biofilm assay (Fig. 1A). Strains 1-21 and 6-42 were from the mutant pools generated by the transposomal plasmid pLOF/TAG-1, and pLOF/TAG-6 respectively. Biofilm formation was quantitatively analyzed using microtiter plate assay (Fig. 1B) and observed under the confocal scanning laser microscopy (CSLM) (Fig. 1C). Both mutant strains formed obvious biofilms at air-liquid interface in glass tubes and on the polystyrene/glass surfaces, while the parental strain 4074 generated no visible biofilm. The biomass of the biofilms formed by the mutant strains was significantly increased ($P < 0.01$) during middle to late exponential phases (24–42 h) in both mutant strains compared with the parental strain (Fig. 1B). In the CSLM assay, an analysis of the X–Z plane revealed that not only the surface of the structures was stained, but also there was staining throughout the depth of the structure. The average thickness through the X–Z plane of the mutant strains 1-21 ($10 \pm 0.3 \mu m$) and 6-42 ($8 \pm 0.3 \mu m$) was increased up to 10- and 8-fold respectively (Fig. 1C).

2.2. Cloning and expression of the mutant gene in E. coli

The DNA sequences flanking the mini-Tn10 transposon in the both biofilm-forming mutants were amplified by inverse PCRs. Two flanking fragments of approximately 150 bp and 350 bp were obtained (Fig. 2A). DNA sequencing and BLAST analysis revealed that the transposons carrying the TAG-1 and TAG-6 were inserted...
in the same locus of the \textit{A. pleuropneumoniae} genome, between position 392 and 393 nucleic acid of the coding sequence of the \textit{hns} gene encoding a histone-like nucleoid structuring protein (H-NS) (Fig. 2C). To further verify the transposon insertion, the genomic DNA was digested with \textit{EcoRV} and transferred onto nylon membrane. Southern blot analysis was performed using a DIG-labeled 385 bp fragment from the coding sequence of \textit{hns} gene (Fig. 2B). The results indicated that both mutant strains 1-21 and 6-42 are identical, differing only by carrying different tags.

2.3. Enhanced biofilm formation in the \textit{hns} mutants

To complement the \textit{hns} deletion in the mutants, a recombinant plasmid carrying an \textit{hns}-expressing cassette (pJN-hns) was constructed and transformed into the mutant strain 1-21, obtaining a complementary strain C-3 by antibiotics selection and PCR confirmation. At the same time, pJN-hns was also transformed into the wild-type strain 4074 to obtain an \textit{hns} over-expressing strain O-2 as a control.

The ability of biofilm formation in the \textit{hns} mutant and complementary strains was tested. Unlike the mutant strains 1-21 and 6-42, strains C-3 and O-2 could not form visible biofilm at the air–liquid interfaces in the glass tube assay (Fig. 1). Quantitative microtiter plate assay also demonstrated that there was no obvious difference in biofilm formation among the wild-type (parental) strains (1-21 and 6-42), while both mutant strains formed high biomass of biofilms on the polystyrene surfaces (Fig. 1B). Under the CLSM, both mutant strains grew on the glass surface in a multiple-layer of structure, whereas strains 4074, C-3 and O-2 grew in a monolayer (Fig. 1C).

2.4. Attenuated virulence in the \textit{hns} mutant and down-regulated strains

To characterize the potential role of \textit{hns} gene in the pathogenesis of \textit{A. pleuropneumoniae}, the virulence was compared among the parental, mutant, complementary strains. Compared with the parental strain 4074, the haemolytic activity was slightly increased in both mutant strains 1-21 and 6-42, and significantly reduced in the complementary strains C-3 and O-2 (Fig. 3A). The 50\% lethal doses (LD\textsubscript{50}) were determined in a mouse infection model. The LD\textsubscript{50} of the parental strain 4074 is about 2.8 \texttimes 10\textsuperscript{6} CFU, and that of strains 1-21, 6-42, C-3 and O-2 was increased about 19.1-, 16.5-, 5.4- and 14.3-fold respectively (Fig. 3B). This indicated that the virulence was decreased in the mutant and complementary strains.

To explain the virulence attenuation in the mutant and complementary strains, the relative transcription levels of \textit{hns} gene and two major exotoxin genes (\textit{apxIA} and \textit{apxIIA}) in the mutant and complementary strains were compared with those of parental strain at both exponential and stationary phases by quantitative real-time RT-PCR (qRT-PCR). The transcription of \textit{hns} gene was confirmed in the strains 4074, C-3 and O-2, but not in the strain 1-21. However, the expression levels in the strains C-3 and O-2 were unexpectedly lower (\textit{P} < 0.01) than that in the wild-type strain 4074 (Fig. 4A). This indicated that the \textit{hns} transcription was down-regulated in strains C-3 and O-2.

Compared with the wild-type strain 4074, the transcription level of \textit{apxIA} gene was slightly but not significantly increased (\textit{P} > 0.05) in the \textit{hns} mutant strain 1-21, but significantly reduced (\textit{P} < 0.01) in the \textit{hns} down-regulated strains C-3 and O-2 at both exponential and stationary phases. The transcription of \textit{apxIIA} gene was significantly repressed in strains 1-21, C-3 and O-2 (Fig. 4B). The relative expression levels of the exotoxin genes were in agreement with the reduced virulence in strains 1-21, C-3 and O-2.

3. Discussion and conclusion

Biofilm formation is a common feature of \textit{A. pleuropneumoniae} and may be involved in the pathogenesis of this important pathogen [6,10]. In the present study, two \textit{A. pleuropneumoniae} mutant strains (1-21 and 6-42) were identified from the mini-Tn10 transposon mutant pools, and exhibited an increased ability to form biofilms (Fig. 1). Further analysis revealed that the \textit{hns} gene encoding the histone-like nucleoid structuring protein (H-NS) was inactivated by the transposition in both of the biofilm-forming mutant strains (Fig. 2), and that the virulence was decreased.
slightly in the \textit{hns} mutant strains, but significantly in both of the wild-type (parental) and mutant strains carrying \textit{hns}-expressing plasmids (Fig. 3).

H-NS belongs to the family of histone-like proteins widespread in Gram-negative bacteria and has been well characterized in \textit{E. coli} and other enterobacteria [11]. In \textit{E. coli}, H-NS is a small protein consisting of 134 amino-acid residues (approximately 15.4 kDa). It consists of an N-terminal dimerization domain and a C-terminal nucleic acid-binding domain that are separated by a linker [12]. The \textit{hns} gene in \textit{A. pleuropneumoniae} encodes a 135 amino-acid protein with an additional proline residue in its linker region compared with many other species such as \textit{E. coli}, \textit{Haemophilus influenzae} and \textit{Haemophilus parasuis} (data not shown). Although the H-NS protein of \textit{A. pleuropneumoniae} shares only 48\% sequence identity to the homologue of \textit{E. coli}, the predicted secondary structures of its N- and C-terminal domains show a very high similarity [13,14].

H-NS protein plays a dual role, both as an architectural protein contributing to the chromatin/nucleoid structure and as a global regulator of gene expression [12,15]. H-NS can bind all types of nucleic acids in a relatively sequence-independent manner but

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig3.png}
\caption{Virulence comparison among the wild-type (P), \textit{hns} deletion (1-21 and 6-42) and complementary (C-3 and O-2) strains of \textit{A. pleuropneumoniae}. (A) Haemolytic activity. (B) The 50\% lethal doses (LD50) of the strains in BALB/c mice. Data are shown as means ± SEM of three independent tests.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Transcription levels of \textit{hns} (A) and \textit{apx} toxin genes (B) in the wild-type (P), \textit{hns} deletion (1-21) and complementary (C-3 and O-2) strains of \textit{A. pleuropneumoniae} in different growth stages. Samples were collected from both exponential phase and early stationary phases. The relative quantification of the transcripts of each sample was obtained from six repetitions using its own 16S rRNA as internal control. The transcripts of each gene in the wild-type strain grown in the exponential phase were adjusted to 1. Data are means ± SEM of three independent assays. Significant difference (\(P < 0.05\)) was indicated with different letters (a, b, c, d) or numbers (1, 2, 3, 4) on the top of each column in the graph. No significant difference (\(P > 0.05\)) was indicated with same letters or numbers.}
\end{figure}
with a preference for intrinsically curved AT-rich DNA, which is commonly found in promoters. It also bends DNA and affects its topology upon binding, properties apparently involved in DNA packaging. In addition, H-NS influences recombination and genome stability [16]. The H-NS protein is not only capable of interacting with DNA but also with itself and other proteins. The generation of homodimers, tetramers, and oligomers appears to be a key process in allowing H-NS to modulate gene expression [12].

As a global regulator, H-NS has been shown to modulate the expression of a large number of genes involved in diverse biological processes, and hns mutants exhibit pleiotropic phenotypes, such as growth lag, loss of motility, increased resistance to acid stress and osmolarity, and reduced virulence [13,17]. In the present study, the hns mutants of *A. pleuropneumoniae* gained the ability to form biofilms and displayed attenuated virulence. To complement the hns disruption, the recombinant plasmid pJN-hns carrying an hns-expression cassette under the control of its native promoter was transformed into the mutant strain 1-21 (resulting a complementary strain C-3), and also the parental strain 4074 (resulting an over-expressing strain O-2) as a control. The hns transcript was detected in the strains 4074, C-3 and O-2 but not in 1-21, and the transcription levels were increased 2-fold as the cells entered stationary phase compared with the exponential phase (Fig. 4A). This growth-phase regulation was also observed previously in *E. coli* [18–20]. Unexpectedly, the hns transcript in the strains C-3 and O-2 never reached the level in the wild-type strain 4074, even much lower than that. This indicated that the hns deletion was not completely complemented in the strain C-3, and hns was not over-expressed but down-regulated in the strain O-2 (Fig. 4A). The results indicated that both strain C-3 and O-2 are hns down-regulated strains. This may be due to the auto-regulation mechanism of hns expression. This negative auto-regulation has been demonstrated at the transcriptional level in *E. coli* [18,19,21]. H-NS is a very abundant protein (>20,000 copies per cell), the auto-regulation may contribute to maintenance of a constant H-NS to DNA ratio under normal growth condition [13]. Thus, it is difficult to over-produce H-NS in bacterial cells under normal growth conditions.

In contrast to the hns mutant strains, both wild-type strain 4074 and hns down-regulated strains C-3 and O-2 could not form visible biofilm (Fig. 1). The virulence of the hns down-regulated strains C-3 and O-2 reduced more extensively than the mutant strains 1-21 and 6-42 (Fig. 3). In order to explain this phenomenon, the relative transcription levels of two major virulence genes apxA and apxIIA were compared among the strains 4074, 1-21, C-3 and O-2 at the exponential and stationary growth phases. The in vitro haemolytic activity was slightly increased in the hns mutant strains, and significantly decreased in the down-regulated strains C-3 and O-2 (Fig. 3A). This was in agreement with their transcriptional levels of apxA gene (Fig. 4B) that encodes the major haemolytic RTX toxin (ApXLX) in *A. pleuropneumoniae* [21]. In contrast, the virulence of either the mutant strains or the down-regulated strains C-3 and O-2 was attenuated in mice (Fig. 3B), which may be partly due to the down-regulation of apxIIA gene in strains C-3 and O-2, and of apxA gene – encoding the ApxI with the strongest cytotoxic activity and moderate haemolytic activity [21] – in all these strains (Fig. 4B). The up-regulations of hemolysins (ClyA, SheA, HlyE and HlyA) by hns mutation have also reported in *E. coli* [22–26], but its effect on virulence is ambiguous [27].

As a global regulator, H-NS plays its role through affecting the synthesis of a large number of gene products that are involved in different biological processes [28]. In most cases, H-NS negatively regulates target gene expression at transcription level [13,29]. However, positive regulation of target genes by H-NS was also observed [21,30], but most of these genes have not been identified, and the regulation mechanism is largely unknown, probably through binding with other transcription factors [15]. In the present study, up-regulation of apxA genes was observed in the hns deletion mutant 1-21, while the transcription of apxIIA gene was repressed in both hns deletion and down-regulated strains. This indicated that the H-NS protein could either repress or stimulate the expression of different target genes in *A. pleuropneumoniae*, and the regulation seems to be in a dose-dependent manner. These genes warrant further identification.

In conclusion, mutation disrupting the hns gene in *A. pleuropneumoniae* resulted in increased biofilm formation and attenuated virulence, while the hns down-regulated strains displayed even more attenuated virulence but could not form biofilms. Several virulence associated genes have been up- or down-regulated by H-NS protein in a dose dependent manner. Further identification of the regulated genes may contribute to our understanding of the roles of H-NS in regulating metabolism and pathogenesis of this important pathogen.

4. Materials and methods

4.1. Bacteria, plasmids and primers

The bacterial strains, plasmids and primers used in this work are listed in Table 1. *A. pleuropneumoniae* strains were grown on Tryptic Soy Agar (TSA) or in Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, MI, USA) supplemented with 10 μg/ml of nicotinamide adenine dinucleotide (NAD) and 10% (v/v) filtered bovine serum. Luria-Bertani (LB) medium was routinely used to culture *E. coli* strains. Antibiotics were used at the following concentrations as appropriate: 20 μg/ml for nalidixic acid (Nal), 25 μg/ml for kanamycin (Km) or gentamicin (Gm).

4.2. Transposon mutagenesis and screen for biofilm-forming mutants

Mini-Tn10 transposon mutant pools of *A. pleuropneumoniae* strain 4074 (a non-biofilm forming and nalidixic acid resistant strain of serovar 1) was generated by mating with *E. coli* S17, pir harboring the tag plasmids pLOF/TAG1-48 as described previously [31]. Biofilm-forming mutants were screened from the mutant pool using the glass tube biofilm assay as described previously [32]. Microtitre plate biofilm assay and CSLM observation of bacterial biofilms were carried out according to our previous report [10].

4.3. Inverse PCR and Southern blot

To identify the genes in which the mini-Tn10 transposition was inserted in the biofilm-forming mutants, the DNA fragments flanking the site of transposon insertion were amplified by inverse PCR as described previously [33] with a minor modification. Briefly, genomic DNA from the mutants was digested with SspI that cuts once in the transposon, and purified using QIAGEN Nucleotide Removal Kit (QIAGEN GmbH, Hilden, Germany). Approximately 150 ng of the purified DNA was self-ligated overnight at 16 °C in a volume of 20 μl using 2 units of Taq DNA ligase (TaKaRa, Dalian, China). A 5 μl aliquot of the self-ligation mixture was directly used as template in a PCR reaction containing 200 μM each dNTP, 0.2 μM each primer STM10 and STM11 or STM10 and STM12, 1 × PCR buffer and 2.5 units of Taq DNA Polymerase (TaKaRa). PCR was carried out as follows: 94 °C for 1.5 min; 35 cycles of 94 °C for 30 s, 60 °C for 45 s and 72 °C for 4 min; 72 °C for 7 min. Primer STM10 was used to sequence the inverse PCR products using standard Sanger sequencing method. The online BLSAT programs were used to search for homologous sequences.
To further confirm the transposon insertion in the targeting genes identified above, Southern blot was carried out. Chromosomal DNA was digested with an appropriate restriction endonuclease (EcoRI), separated by agarose gel electrophoresis and transferred to a nylon membrane. A DNA fragment from the identified gene was labeled using the PCR DIG Probe Synthesis Kit (Roche) according to the manufacturer's instructions. These transformants were compared with the parental and hns mutant strains. The signals were developed using the DIG Nucleic Acid Detection Kit (Roche) according to the manufacturer’s instructions. A broad-host-range expression vector, gentamicin resistant (Gm’), was used to integrate the hns-expression cassette under the control of its native promoter between the Sal and EcoRI cloning sites, Gm’.

4.5. Expression of hns gene in A. pleuropneumoniae

A 788 bp hns-expression cassette containing its coding sequence (408 bp) and its native promoter and terminator sequences was amplified by PCR from A. pleuropneumoniae 4074 genomic DNA using primers HNS03 and HNS04 (Table 1), and then cloned into the shuttle vector pJN105 carrying an hns-expression cassette under the control of its native promoter between the Sal and EcoRI cloning sites, Gm’. 4074 genomic DNA was separately transformed into A. pleuropneumoniae mutant strains by electroporation as described previously [34].

4.6. Virulence determinants in a mouse model

Animal experiments were carried out according to the International Guiding Principles for Biomedical Research Involving Animals – 1985. A. pleuropneumoniae strains were grown in TSB supplemented with 10 μg/ml NAD, 10% bovine serum and 20 μg/ml nalidixic acid overnight, and re-inoculated to fresh medium to culture for about 6 h. Based on viable count, different groups of eight-week-old female BALB/c mice were intraperitoneally injected with 0.3 ml of a series of different dose of bacteria respectively. The control mice were injected with the equal volume of medium. Immediately after the inoculation in mice, the vital cells of inoculum were counted again. Mice were observed in the following 3 days at 8 h intervals and the survival numbers of each group were calculated according to Karber's method.

4.7. Haemolytic assay

Haemolytic activity of A. pleuropneumoniae strains was tested on the TSA plates containing 10 μg/ml NAD, 5 μg/ml nalidixic acid, and recorded. The 50% lethal doses (LD50) of different strains were compared with that of the strains tested.

Table 1

<table>
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<th>Characteristics of the bacterial strains, plasmids and primers used in this study.</th>
<th>Source/reference</th>
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<tr>
<td>1-21</td>
<td>4074 NaF Δhns: Tn10(tag 1): Km</td>
</tr>
<tr>
<td>6-42</td>
<td>4074 NaF Δhns: Tn10(tag 6): Km</td>
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<tr>
<td>C-3</td>
<td>Strain 1-21 carrying pJN-hns</td>
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<tr>
<td>O-2</td>
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4.4. RNA isolation and quantitative real-time RT-PCR (qRT-PCR)

Total RNA of A. pleuropneumoniae strains was isolated from cells obtained from different exponential and stationary phase cultures using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The contaminated DNA in the RNA samples was removed by treatment with RNase-free DNase I (Fermentas, Burlington, Canada). Reverse transcription was performed using the RevertAid™ First Strand cDNA Synthesis Kits (Fermentas) according to the manufacturer’s instructions. Quantitative real-time PCR was performed using the SYBR Green II® (Amersham Biosciences, Piscataway, NJ, USA) on the ABI PRISM 7500 system (Amersham Biosciences). The primers used are listed in Table 1. The thermal cycling conditions were 10 min at 95 °C, 40 cycles of 95 °C 30 s, 60 °C 30 s and 72 °C 45 s. Data collection was performed during each extension phase. 16S rRNA was used as an internal control [10].
acid and 5% fresh defibrinated sheep erythrocytes as described previously [35].

4.8. Statistic method

Two-tailed paired Student’s t test was used in qRT-PCR.

Acknowledgements

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