Absence of TolC Impairs Biofilm Formation in Actinobacillus pleuropneumoniae by Reducing Initial Attachment

Ying Li¹, Sanjie Cao¹, Luhua Zhang¹,², Jianlin Yuan¹, Gee W. Lau², Yiping Wen¹, Rui Wu¹, Qin Zhao¹, Xiaobo Huang¹, Qigui Yan¹, Yong Huang¹, Xintian Wen¹*

1 Research Center of Swine Diseases, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, 611130, China, 2 Department of Pathobiology, University of Illinois at Urbana-Champaign, Urbana, Illinois, United States of America

☯ These authors contributed equally to this work.
* xintian3211@126.com

Abstract

Actinobacillus pleuropneumoniae is the etiologic agent of porcine contagious pleuropneumonia, a major cause of economic loss in swine industry worldwide. TolC, the key component of multidrug efflux pumps and type I secretion systems, has been well-studied as an exit duct for numerous substances in many Gram-negative bacteria. By contrast, little is known on the role of TolC in biofilm formation. In this study, a ΔtolC mutant was used to examine the importance of TolC in biofilm formation of A. pleuropneumoniae. Surface attachment assays demonstrated the essential role of TolC in initial attachment of biofilm cells. The loss of TolC function altered surface hydrophobicity, and resulted in greatly reduced autoaggregation in ΔtolC. Using both enzymatic treatments and confocal microscopy, biofilm composition and architecture were characterized. When compared against the wild-type strain, the poly-β-1, 6-N-acetyl-D-glucosamine (PGA), an important biofilm matrix component of A. pleuropneumoniae, was significantly reduced at the initial attachment stage in ΔtolC. These results were confirmed by mRNA level using quantitative RT-PCR. Additionally, defective secretion systems in ΔtolC may also contribute to the deficiency in biofilm formation. Taken together, the current study demonstrated the importance of TolC in the initial biofilm formation stage in A. pleuropneumoniae. These findings could have important clinical implications in developing new treatments against biofilm-related infections by A. pleuropneumoniae.

1. Introduction

Actinobacillus pleuropneumoniae is a Gram-negative bacterium in the family of Pasteurellaceae. It is the etiologic agent of porcine contagious pleuropneumonia, a significant respiratory disease in swine, with serious economic losses to the swine industry worldwide [1]. To date, there have been a lot of studies on virulence factors of A. pleuropneumoniae that contribute to...
colonization and lung infection. Among them, biofilm formation mediates in vivo colonization of host tissues [2]. However, not much is known about the mechanism of biofilm formation in A. pleuropneumoniae. A better understanding of the mechanisms that influence or regulate the biofilm formation would potentially improve antibiofilm strategies in this pathogen.

Biofilms are multicellular communities that cells are encased in a self-produced extracellular matrix, which could attach to biotic or abiotic surfaces. Biofilms protect pathogenic bacteria from deleterious agents and other stresses within their environment, rendering them difficult to treat. Therefore, it is not surprising that biofilms of pathogens are important for chronic infections in human and animals [3]. The biofilm matrix is known as extracellular polymeric substances (EPS), of which exopolysaccharides, extracellular DNA (eDNA) and extracellular proteins are the most important constituents. Exopolysaccharides vary greatly in their compositions, and are identified as the framework of biofilm architecture in many bacteria and play significant roles in intercellular adhesion, biofilm maturation and dispersal of biofilm cells [4]. eDNA is also a structural component of biofilm matrix in some bacterial species and often plays a crucial role in establishment and maintenance of bacterial biofilms [5]. Various kinds of proteins can be found in the biofilm matrix, including extracellular enzymes, structural proteins and proteinaceous appendages [6]. These extracellular proteins are often involved in the integrity and stabilization of the polysaccharide matrix network [7]. Collectively, the EPS provide the scaffold of the three-dimensional biofilm architecture and play crucial roles in surface adhesion and cohesion in the biofilm [7].

The development of biofilms varies between bacterial species and different living conditions. In general, biofilm formation is initiated by the attachment of cells to a surface and production of EPS, followed by recruitment of additional bacteria, and proliferation to form microcolonies which eventually develop into a mature biofilm [8]. During the early stages, a variety of extracellular adhesive substances such as eDNA, adhesins, as well as bacterial surface structures such as flagella, pili and curli fimbriae are employed to initiate the attachment. Bacterial cell surface hydrophobicity could affect the aggregation of microcolonies, and the excretion of extracellular matrix further strengthens the adherence. As the microcolony grows, bacteria switch to a distinct pattern of gene expression and are surrounded by EPS and intercellular signaling molecules, resulting in the maturation of biofilms with complex three dimensional structures [3,5].

In A. pleuropneumoniae, biofilm formation is prevalent among field isolates [9], with the extracellular polymer of PGA as a key component of the biofilm matrix [10]. Dispersin B protein, encoded by dspB, is a PGA-hydrolyzing enzyme that could dissolve the biofilm matrix and disperse cells. Multiple genes are involved in the biofilm formation or regulation, including the sigma factor σE [11], serine protease AasP [12], RNA chaperone Hfq [13], ClpP protease [14] and the recently identified O-antigen of LPS [15]. Moreover, transcriptome analysis of biofilm cells has identified more proteins as the potential regulator or cues that affect biofilm formation in A. pleuropneumoniae [16]. Despite these advancements, knowledge regarding the regulation of biofilm development processes remains limited.

TolC is an outer membrane channel component of multidrug efflux pumps and type I secretion systems in Escherichia coli. Numerous studies have revealed the importance of TolC in drug resistance and bacterial virulence [17]. Recently, the correlation between efflux pump genes, including TolC, and biofilm formation has attracted an increasing interest. Studies have showed that efflux pumps play key roles in the biofilm growth of E. coli, Klebsiella pneumoniae, Staphylococcus aureus, and Salmonella typhimurium, and efflux pump inhibitors have been identified as efficient antibiotic synergists against biofilm-related infections of these pathogens [18,19]. In our recent study, a protein TolC1 was confirmed, now named TolC, to be required for biofilm formation of A. pleuropneumoniae without a substantial growth inhibition
(Unpublished data). However, the mechanism by which TolC regulates biofilm development remained poorly understood.

The objective of this study was to determine the link between TolC and biofilm development of \textit{A. pleuropneumoniae} using a \textit{$\Delta$tolC} mutant. The inactivation of TolC was found to be deficient in initial surface attachment step during biofilm formation. Subsequent assays pinpointing the crucial role of TolC in initial attachment was carried out by analyzing the bacterial surface hydrophobicity, biofilm composition, and PGA production.

2. Results

2.1 Inactivation of TolC impairs biofilm formation in \textit{A. pleuropneumoniae}

The time course of biofilm biomass for \textit{A. pleuropneumoniae} SC1516, \textit{$\Delta$tolC} and the genetically-complemented strain \textit{$\Delta$tolC/tolC} were measured to investigate the kinetics of biofilms formation and to determine an appropriate time point to perform further studies. Biofilm biomass was quantified using a crystal violet staining at 0, 4, 6, 12, 24 and 36 h after incubation. As shown in Fig 1, the amount of biofilm in \textit{$\Delta$tolC} mutant was significantly reduced compared to that in the wild-type (WT) strain during the course of experiments. The ability of biofilm formation was restored back to the WT level in \textit{$\Delta$tolC/tolC}. The biofilm biomass peaked at 6 and 12 h, and decreased gradually over time in all groups. Based on the time course data, we tentatively designated 6 h as the time point of biofilm being fully formed and 12 h as the biofilm maturation in all subsequent studies.

2.2 Inactivation of TolC reduces initial attachment of biofilms in \textit{A. pleuropneumoniae}

To evaluate whether TolC was involved in the initial surface attachment of \textit{A. pleuropneumoniae}, the ability of adherence to the plastic substrate was determined in a 96-well microtiter

![Fig 1. Effects of \textit{tolC} deletion on biofilm formation of \textit{A. pleuropneumoniae}. Biofilm formation was determined at various time points (0, 4, 6, 12, 24 and 36h) by crystal violet staining using static broth cultures of WT SC1516, \textit{$\Delta$tolC} and the genetically-complemented strain \textit{$\Delta$tolC/tolC}. Data are means ± standard deviations (SDs) of three independent experiments with three replicates. Asterisks indicate statistical significance (P< 0.05).](doi:10.1371/journal.pone.0163364.g001)
plate by crystal violet staining. When compared against WT and complemented strains, the ΔtolC showed reduced number of attached cells in the well (Fig 2A), which was proportional to the optical density (OD) at 595 nm [10]. This result indicated that ΔtolC is defective in initial surface attachment step during biofilm formation.

2.3 Inactivation of TolC decreases bacterial cell autoaggregation of *A. pleuropneumoniae*

The aggregative ability of each strain was determined by the sedimentation rate of the bacterial cells. As shown in Fig 2B, WT and ΔtolC/tolC cells sedimented to the bottom of the culture tubes, while ΔtolC cells remained in suspension. These results showed that ΔtolC cells were less adhesive and therefore, showed a reduced autoaggregation phenotype. This finding suggested that the loss of TolC may alter cell surface hydrophobicity of *A. pleuropneumoniae*.

2.4 Inactivation of TolC decreases cell surface hydrophobicity in *A. pleuropneumoniae*

To determine whether inactivation of TolC led to alteration in the cell surface hydrophobicity, the hydrophobicity values were estimated based on the ability to bind with hexadecane. As a result, when compared against WT, a decreased hydrophobicity was observed in the tolC mutant, while the ΔtolC/tolC restored the surface hydrophobicity (Fig 2C). These results suggested that inactivation of TolC decreases cell surface hydrophobicity. The reduced cell hydrophobicity of ΔtolC may be one of the explanations for its defectiveness in initial surface attachment.

2.5 Inactivation of TolC changes the biofilm composition of *A. pleuropneumoniae*

To determine the components of biofilm matrix of *A. pleuropneumoniae* SC1516 and ΔtolC, enzymatic treatments were performed using dispersin B, DNase I and proteinase K on biofilms cultured at 4, 6 and 12 h, respectively. As shown in Fig 3, the addition of dispersin B, a glycoside hydrolase that specifically degrades PGA, resulted in significant dispersal of cells from

---

**Fig 2. Effects of TolC on initial surface adherence, autoaggregation and cell surface hydrophobicity.** (A) The surface attachment assay was determined in 96-well microtiter plates by staining with crystal violet. Attached cells in the well were quantitated by measuring the optical density at 595 nm. Data represents mean values for triplicate wells from three independent experiments. Error bars indicate SDs and the asterisks show significant differences (p<0.05). (B) The ΔtolC cells remained mostly in suspension (middle), while the WT (left) and complemented strain (right) autoaggregated and settled to the bottom of the tubes after 6 h static incubation at room temperature. (C) The surface hydrophobicity of ΔtolC cells was significantly reduced when compared against WT. Values are expressed as means ± SE, * p < 0.05.

doi:10.1371/journal.pone.0163364.g002
both the WT and the ΔtolC mutant biofilms. Digestions with proteinase K also significantly reduced the biofilm formation in all groups, and DNase I showed a significant dispersion effect on biofilms incubated for 6 h and 12 h. These results indicated that PGA was indeed a major component of *A. pleuropneumoniae* biofilm matrix, while proteins and eDNA were also involved in the formation of biofilm architecture. The data in Fig 3A showed that, at the initial attachment stage, the ΔtolC biofilms were significantly less sensitivity to dispersin B than that of WT strain. This result suggested that the loss of TolC reduced PGA production in early-stage biofilms. Similarly, at all time points analyzed, the biofilms of ΔtolC were more resistant to the digestion of proteinase K than WT, suggesting that less extracellular proteins were involved in biofilm matrix in ΔtolC.

2.6 The loss of TolC changes the biofilm morphology of *A. pleuropneumoniae*

Next, we examined if reduced PGA and extracellular proteins in ΔtolC altered its biofilm structures. The biofilms of WT and ΔtolC mutant were compared by confocal laser scanning microscopy (CLSM). Biofilms at 4 h and 6 h in microtiter plates were washed and stained with SYTO-9 (Fig 4A) and propidium iodide (Fig 4B) to label the live and dead cells, respectively. Fig 4A showed significant reduction in attached cells of ΔtolC at both of these two time points, as indicated by decreased fluorescence intensity of merge images (Fig 4C). The results were consistent with the surface attachment assays (Fig 2A). Besides, a significantly higher proportion of dead cells were observed in ΔtolC when compared against WT strain (Fig 4B). These observations suggested that TolC was required to maintain the viability of *A. pleuropneumoniae* within a biofilm. The biofilm architecture was further analyzed by using the WGA fluorescent probe that specifically labeled the PGA, the framework of the biofilm architecture. As shown in Fig 4D, ΔtolC formed thinner biofilms as seen by scattered fluorescent spots, while the biofilms produced by WT appeared exuberant that diffused across the entire field of view. These results indicated that the loss of TolC significantly changed the morphology of the biofilm when compared to WT. Overall the abundance of biofilms observed with CLSM was consistent with the quantification by crystal violet staining (Fig 2A).

2.7 The loss of TolC reduces the transcription of pgaA and cpxR genes in *A. pleuropneumoniae*

Because PGA was the major component of the biofilm matrix of *A. pleuropneumoniae*, we used quantitative reverse-transcriptase PCR (qRT-PCR) to determine whether the expression of
pgaA and cpxR genes was affected in ΔtolC. The pgaA gene is the first gene in the operon pgaABCD that encodes PGA. The cpxR gene encodes the response regulator of the CpxAR two component regulatory system, which was previously reported to regulate biofilm formation [16]. When compared against WT, the expression of both pgaA and cpxR were significantly lower in ΔtolC at both 4-h and 6-h (Fig 5A and 5B). However, no significant difference in pgaA and cpxR transcription levels was observed between the two strains at 12-h (Fig 5C). These results suggested that tolC mutation reduced the mRNA levels of pgaA and cpxR in the early, initial surface adherence stage of biofilm formation, but not at the maturation stage.

2.8 Inactivation of TolC results in the secretion deficit of certain biofilm-promoting factor in A. pleuropneumoniae

To investigate whether the deficiency of ΔtolC biofilms can be rescued by the presence of wild-type supernatant, transwell assays were performed. Three combinations of strains—WT/ΔtolC mutant, ΔtolC/ΔtolC and WT/WT (top/bottom)—were used. After 12h of incubation,
compared to the ΔtolC/ΔtolC group, biofilm formation by ΔtolC in the WT/ΔtolC was significantly rescued, though still below the wild-type level (Fig 6A). The biofilm biomass in the lower chamber was quantified by crystal violet staining. The data were consistent with the observation in the transwell plates (Fig 6B). Together, coculturing with wild-type strain appeared to have the ability to partially restore the ability of ΔtolC to form biofilms.

3. Discussion

TolC, an outer membrane channel protein, was widely studied for its crucial role in multidrug resistance and virulence in numerous Gram-negative bacteria pathogens [17,20]. TolC was reported to be involved in the biofilm formation of S. typhimurium and E. coli [21,22]. Functional TolC proteins have been found in several members of the Pasteurellaceae family whose role in antibacterial resistance are well characterized, but the function of TolC in biofilm formation of this group of important pathogens has not been examined.

In this study, we examined the importance of TolC in biofilm formation of A. pleuropneumoniae. Overall, the time course of biofilm formation in WT in this study is slower than the one previously published by others [16]. The results suggested that the kinetics of biofilm formation varies among different A. pleuropneumoniae field isolates. Interestingly, while ΔtolC showed initial attachment defects, it showed similar biofilm development schedule in which biofilms reached its maximum at 6 h after incubation and remained at 12h. This result suggested that deletion of tolC did affect the biofilm maturation but did not affect the biofilm maturation rate in A. pleuropneumoniae.

Attachment of bacterial cells to abiotic surfaces and aggregation into microcolonies are considered the first step of biofilm formation, and cell surface hydrophobicity and motility play important roles in bacterial attachment [8,23]. When compared against WT, ΔtolC had significantly reduced surface attachment by crystal violet staining (Fig 2A), which was confirmed by CLSM images that showed sparse bacteria colonies (Fig 4). We speculated that the loss of an important membrane protein such as TolC from the outer membrane of bacteria probably changed the surface hydrophobicity of the cells. Indeed, the cell hydrophobicity of ΔtolC was decreased (Fig 2C). Bacterial motility, a factor that may impact on the adherence of biofilm cells, was also determined using a soft-agar plate as published previously [24]. As a result, the ΔtolC displayed the similar motility phenotype with WT (Data not shown).

EPS are known as a conglomeration of different types of biopolymers that account for over 90% of the dry mass of biofilms. The excretion of EPS is critical for the initial adherence as well as biofilm maturation [7]. A previous study suggested that eDNA and proteins contributed little to the biofilm architecture of A. pleuropneumoniae [25]. Rather, PGA is a major surface polysaccharide that acts as the scaffold of biofilm matrix and functions in intercellular adhesion.
within the biofilm colony [10]. In this study, PGA was confirmed to be an important fraction of the biofilm matrix of WT strain SC1516. However, we found that eDNA and proteins also contributed to the biofilm architecture of SC1516 (Fig 3). This unexpected result was probably due to that the compositions and ratios of EPS might vary greatly between strains of a single species [7]. When compared against the WT, the biofilm composition of ΔtolC was always deficient in the extracellular proteins during the entire process of biofilm formation (Fig 3). One potential mechanism for this phenotype is that inactivation of TolC reduces the export of secreted proteins [26]. Importantly, we showed that ΔtolC produced lesser amount of PGA in the initial stage of biofilm formation, as demonstrated by the dispersin B treatment (Fig 3A),

Fig 6. Coculturing with WT rescued biofilm formation defects of ΔtolC. Biofilm formation was performed by coculturing ΔtolC with WT in Transwell chambers. (A) Biofilms in the lower chamber were stained with crystal violet. (B) Quantitative analysis of biofilms. Data presented here are means of triplicate experiments and error bars indicate the SDs. *, statistical significance (P< 0.05). doi:10.1371/journal.pone.0163364.g006
and also by the CLSM images (Fig 4). Considering the essential role of PGA in intercellular adhesion within the biofilm colony, we propose that the lower level of PGA production in ΔtolC at the initial stage of biofilm formation likely contributes to the reduced surface attachment. Our hypothesis is corroborated by the observation that the expression of pgaA was significantly down-regulated in ΔtolC at the initial stage of biofilm formation (Fig 5A).

Previous studies have shown that TolC of *E. coli* could directly respond to membrane stress [17]. CpxAR is a classical two-component system that is involved in envelope stress response in *E. coli* [27]. The link between TolC and CpxAR system has not been reported. Previous transcriptomic analyses of *A. pleuropneumoniae* biofilms revealed the important role of cpxAR in biofilm growth [16]. Given that regulator sigma factor σE was one of the Cpx-regulated genes and the pga operon was positively regulated by σE [28], the correlation between TolC, CpxAR system and pga operon attracted much interest. We found that cpxR was significantly down-regulated in ΔtolC. Whether the pga operon was positively regulated by the CpxAR, and whether the regulation of biofilm formation by tolC is achieved by affecting the expression of cpxAR, is currently unknown. Further experiments are needed to clarify the interplays between TolC, CpxAR and PGA biosynthesis.

TolC has been well characterized as the channel of efflux pumps and type I secretion systems, through which a wide range of substrates could be exported, such as antibiotics, toxins and signaling molecules [29,30]. In the transwell assay, TolC of the wild type likely secreted some unknown soluble biofilm-promoting factor(s) that crossfeed and partially restored biofilm formation in ΔtolC (Fig 6). This finding was unexpected, because no “rescue” effect was observed during coculturing of a ΔtolC mutant of *S. typhimurium* with its wild-type parental strain [18]. We speculate that the “biofilm-promoting factor” could be quorum sensing molecules that affect the initial adherence and maturation of biofilms.

On the other hand, we have confirmed that TolC is essential for the secretion of ApxII, an alpha-hemolysin produced by *A. pleuropneumoniae* (Data not shown). Previous studies have shown that alpha-toxins are required for biofilm formation of *S. aureus* and *Streptococcus pneumoniae* [31,32]. Therefore, it is possible that ApxII toxin is one of the factors secreted through TolC that “rescue” biofilm formation in ΔtolC. Further studies are required to clarify the mechanism.

In summary, our current study identified the importance of TolC in the formation of a competent biofilm in *A. pleuropneumoniae*. Based on our results, we propose that the absence of TolC impairs biofilm formation by reducing the initial attachment of *A. pleuropneumoniae*. Several factors contribute to the reduction of initial biofilm adherence in ΔtolC. Among them, the down regulation of pgaA expression, consequently, reduced PGA production and the changes in surface hydrophobicity of the ΔtolC cells seem to be major contributors. Besides, the reduction of extracellular proteins within biofilm matrix caused by the loss of TolC would also be an additional explanation for the deficiency of biofilm formation. Our findings demonstrate that TolC is essential for the biofilm formation of *A. pleuropneumoniae* by impacting upon the initial attachment of biofilm cells. These findings highlight the potential of targeting TolC in the antibiofilm strategies of *A. pleuropneumoniae*.

4. Materials and Methods

4.1 Bacterial strains and growth conditions

*A. pleuropneumoniae* wild-type strain SC1516 (serotype 7), an isolate from a diseased pig in Sichuan, China, was used as the parental strain. ΔtolC was derived from SC1516, in which the tolC gene was deleted. ΔtolC/tolC was the genetically complementary strain of ΔtolC. The ΔtolC and ΔtolC/tolC strains were both constructed in our recent study (Unpublished data). Briefly,
an 842-bp DNA fragment obtained from tolC and the chloramphenicol acetyl transferase gene were fused by overlap PCR and cloned into a pMD19-T vector. The resulting recombinant plasmid was electroporated into strain SC1516 to construct ΔtolC mutant. The full-length tolC with its promoter sequence was cloned into the shuttle vector pLS88, followed by electroporation into the ΔtolC to generate ΔtolC/tolC. The WT and its derivatives were cultured in Tryptic Soy Broth (TSB, Difco Laboratories, Detroit, USA) supplemented with 0.01% β-nicotinamide adenine dinucleotide (NAD). Where necessary, 50 μg/ml kanamycin or 2 μg/ml chloramphenicol were added. E. coli strains were grown in Luria–Bertani (LB, Difco) medium or on LB agar and ampicillin (100 μg/ml) was added when necessary. All strains were grown at 37°C.

4.2 Biofilm formation and surface attachment assays
To analyze the time course of biofilm formation, overnight broth cultures of WT, ΔtolC and ΔtolC/tolC were inoculated into fresh TSB medium by dilution of 1:100 and transferred to the 96-well polystyrene microtiter plates in triplicate (Costar 3599, Corning, NY, USA) for 0, 4, 6, 12, 24 and 36 h at 37°C, respectively. Biofilms were washed with water and stained with 100 μl of 0.1% crystal violet for 5 min at room temperature. After washing and drying, 100 μl of acetic acid (33%, v/v) was added to each well to release the bound dye. The optical density at 595 nm was measured using a microplate reader (Bio-Rad iMark™ microplate Reader).

For the surface attachment assays, bacteria from overnight cultures were inoculated with a dilution (1/100) into a 96-well microtiter plate in triplicate for 4h at 37°C. Attached cells in the wells were stained with crystal violet and quantified as described above. The OD 595 nm is considered to be proportional to the number of attached cells in the well [10].

4.3 Autoaggregation assay
The abilities of A. pleuropneumoniae strains to autoaggregate were evaluated as previously described with minor modifications [33]. Briefly, single colonies of the WT, ΔtolC and ΔtolC/tolC were cultured in 3 mL of TSB medium with agitation for 12h. Then, cultures were allowed to remain static at room temperature for 6h and photographed.

4.4 Cell surface hydrophobicity assay
Hydrophobicity of a bacterial surface was determined as described previously [34], with minor modifications. In brief, cells of WT, ΔtolC and ΔtolC/tolC were harvested from TSB cultures (OD550 = 0.8) by centrifugation at 5000 rpm for 3 min, respectively. Pellets were washed twice with PUM buffer and resuspended in the same buffer. 1 ml of suspensions was mixed with 200 μl of hexadecane, by vortex for 1 min. The phases were allowed to separate for 30 min at 30°C. The hydrophobicity indexes were determined by the equation: \((A_0 - A) A_0 - 1\) ×100, as described previously [23]. \(A_0\) and \(A\) were the initial and final optical densities of the aqueous phase at A550 nm, respectively. This experiment was repeated three times independently.

4.5 Expression and purification of A. pleuropneumoniae dispersin B
Genomic DNA of A. pleuropneumoniae SC1516 was amplified by PCR using the forward primer 5’- CATGCCATGGCAACTTATGCAAACGCTATGG-3’ and the reverse primer 5’-CCGCTCGAGATGCGATTTCGGATCATTAG-3’. The restriction sites were underlined in each primer sequence. The PCR product was digested with NcoI and XhoI followed by ligation into the NcoI/XhoI sites of the expression vector pET-22b (+). The resulting recombinant plasmid pEDB was transformed into E. coli BL21 (DE3). Expression of dispersin B was induced by the addition of 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and the recombinant
protein was purified from the supernatant by Ni affinity chromatography using Profinity IMAC Ni-Charged Resin (Biorad) as described previously [35]. The concentration of dispersin B was indirectly quantified using a Bradford assay as previously described [36].

4.6 Enzymatic treatments
Biofilm dispersion assays were performed on WT and ΔtolC as previously described [37]. Biofilms were cultured in a 96-well microtiter plate as described above for 4h, 6h and 12h at 37°C. Then, 100 µg/ml of dispersin B, 500 µg/ml of DNase I and 500 µg/ml of proteinase K were added to the biofilms, respectively, 50 µl per well. Meanwhile, control wells were added with 50 µl of Tris-HCl buffer without the enzyme. Wells with dispersin B were treated for 5 min, and those with DNase I and proteinase K were treated for 1 h at 37°C. After the incubation, biofilms were washed, stained with crystal violet, and quantified as described above. This experiment was repeated for three times independently with triplicate samples.

4.7 Confocal laser scanning microscopy
Static biofilms were cultured on 20-mm² coverglasses submerged in 3 ml TSB broth at the bottom of 6-well microtiter plate for 4 h or 6 h at 37°C, respectively. Then, the supernatants were removed and the biofilms were washed with water and stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit (catalog no. L13152; Molecular Probes, Invitrogen, USA) or Wheat Germ Agglutinin (WGA)–Oregon Green® 488 (Invitrogen, Molecular Probes, OR, USA) according to the manufacturer’s instructions. Stained biofilms were visualized with a Nikon A1R confocal scanning laser microscope and images were acquired using the NIS-Elements AR software (Nikon, Japan).

4.8 RNA extraction and qRT-PCR
For qRT-PCR, WT and ΔtolC were grown in TSB in 6-well plates for 4h, 6h or 12h, respectively. After the removal of supernatants, biofilms were collected with a cell scraper. Cell pellets were resuspended in PBS with 50% ice-cold methanol to prevent changes in transcript levels. RNA extraction and reverse transcription of total RNA were performed as described before [38]. 50-fold dilutions of cDNA were used as the template for qRT-PCR reactions in triplicate using the MiniOpticon™ Real-Time PCR Detection System (Bio-Rad, USA). Relative expression was normalized against the 16S rRNA gene, and the data were calculated with a threshold cycle (ΔΔCt) method as previously described [13]. Primers used were as follows: pgaA, AAGCGGTT GCCGTTTAG and ACGTTTGCTCGTATGG; cpxR, GGGCAAATTCTTTCTCGTG and AAACCAAGGGA AGTTATCGT; 16s, ACCCTTATCCTTTGTTGC and CATCTTGCT TCCCTCTGT. This experiment was repeated for three times independently with triplicate samples.

4.9 Transwell assays
A transwell assay was carried out using the 6-well polystyrene transwell plate with a 0.4-µm-pore-size polyester membrane (Corning, NY). With this permeable membrane, secretions or metabolite, but not bacterial cells in the supernatant of the upper chamber were allowed into the lower chamber where the biofilm formed. 3 ml of TSB broth was inoculated with 30 µl of an overnight culture of ΔtolC in the lower chamber, and 1 ml WT suspension (1/100 dilution) in the upper chamber. Control wells were set with WT or ΔtolC suspension in both top and bottom wells. Transwell plates were incubated at 37°C for 12h. Bacterial suspension in the upper chamber was removed and the biofilms formed in the lower chamber were stained and
quantified using crystal violet staining. This experiment was repeated independently three times with triplicate samples.

4.10 Statistical analysis
Data were analyzed with GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA). One-way analysis of variance (ANOVA) test was used for surface attachment and transwell assays. Two-way ANOVA test was used for biofilm formation, enzymatic treatments and qRT-PCR analysis. P values of < 0.05 were considered significant and indicated by an asterisk.

Author Contributions
Conceptualization: XW YW.
Data curation: YL SC LZ.
Formal analysis: YL SC LZ.
Funding acquisition: XW.
Methodology: YL SC LZ.
Project administration: XW.
Resources: XW YW.
Software: JY XH.
Supervision: XW YW.
Validation: SC.
Visualization: RW QY QZ.
Writing – original draft: YL SC LZ.
Writing – review & editing: GL YH.

References
1. Bosse JT, Janson H, Sheehan BJ, Beddek AJ, Rycroft AN, Kroll JS, et al. (2002) *Actinobacillus pleuropneumoniae*: pathobiology and pathogenesis of infection. Microbes Infect 4: 225–235. PMID: 11880056
2. Chiers K, De Waele T, Pasmans F, Ducattele R, Haesebrouck F (2010) Virulence factors of *Actinobacillus pleuropneumoniae* involved in colonization, persistence and induction of lesions in its porcine host. Vet Res 41: 65. doi: 10.1051/vetres/2010037 PMID: 20546697
3. López D, Vlamakis H, Kolter R (2010) Biofilms. Cold Spring Harbor perspectives in biology 2: a000398. doi: 10.1101/cshperspect.a000398 PMID: 20519345
4. Sutherland IW (2001) The biofilm matrix—an immobilized but dynamic microbial environment. Trends Microbiol 9: 222–227. PMID: 11336839
5. Okshevsky M, Meyer RL (2015) The role of extracellular DNA in the establishment, maintenance and perpetuation of bacterial biofilms. Crit Rev Microbiol 41: 341–352. doi: 10.3109/1040841X.2013.841639 PMID: 24303798
6. Branda SS, Vik S, Friedman L, Kolter R (2005) Biofilms: the matrix revisited. Trends Microbiol 13: 20–26. PMID: 15639528
7. Flemming HC, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8: 623–633. doi: 10.1038/nrmicro2415 PMID: 20676145
8. Stoodley P, Sauer K, Davies DG, Costerton JW (2002) Biofilms as Complex Differentiated Communities. Annu Rev Microbiol 56: 187–209. PMID: 12142477
9. Kaplan JB, Mulks MH (2005) Biofilm formation is prevalent among field isolates of *Actinobacillus pleuropneumoniae*. Vet Microbiol 108: 98–94. PMID: 15917136

10. Kaplan JB, Velliyagounder K, Ragunath C, Rohde H, Mack D, Knobloch JK, et al. (2004) Genes involved in the synthesis and degradation of matrix polysaccharide in *Actinobacillus actinomycetemcomitans* and *Actinobacillus pleuropneumoniae* biofilms. J Bacteriol 186: 8213–8220. PMID: 15576769

11. Bosse JT, Sinha S, Li MS, O'Dwyer CA, Nash JH, Rycroft AN, et al. (2010) Regulation of *pga* operon expression and biofilm formation in *Actinobacillus pleuropneumoniae* by sigmaE and H-NS. J Bacteriol 192: 2414–2423. doi: 10.1128/JB.01513-09 PMID: 20207760

12. Tegetmeyer HE, Fricke K, Baltes N (2009) An isogenic *Actinobacillus pleuropneumoniae* *AasP* mutant exhibits altered biofilm formation but retains virulence. Vet Microbiol 137: 392–396. doi: 10.1016/j.vetmic.2009.01.026 PMID: 19217220

13. Subashchandrabose S, Leveque RM, Kirkwood RN, Kiupel M, Mulks MH (2013) The RNA chaperone Hfq promotes fitness of *Multidrug resistance* during porcine pleuropneumonia. Infect Immun 81: 2952–2961. doi: 10.1128/IAI.01371-14 PMID: 23732171

14. Xie F, Zhang Y, Li G, Zhou L, Liu S, Wang C. (2013) The ClpP protease is required for the stress tolerance and biofilm formation in *Actinobacillus pleuropneumoniae*. PLoS One 8: e53600. doi: 10.1371/journal.pone.0053600 PMID: 23326465

15. Hathroubi S, Hancock MA, Bosse JT, Langford PR, Tremblay YDN, Labrie J, et al. (2016) Surface Polysaccharide Mutants Reveal that Absence of O Antigen Reduces Biofilm Formation of *Actinobacillus pleuropneumoniae*. Infect Immun 84: 127–137. doi: 10.1128/IAI.00912-15 PMID: 26483403

16. Tremblay YD, Deslandes V, Jacques M (2013) *Actinobacillus pleuropneumoniae* genes expression in biofilms cultured under static conditions and in a drip-flow apparatus. BMC Genomics 14: 364. doi: 10.1186/1471-2164-14-364 PMID: 23725589

17. Zgurskaya HI, Krishnamoorthy G, Ntreh A, Lu S (2011) Mechanism and Function of the Outer Membrane Channel ToIC in Multidrug Resistance and Physiology of Enterobacteria. Front Microbiol 2: 189.

18. Baugh S, Phillips CR, Ekanayaka AS, Piddock LJ, Webber MA (2014) Inhibition of multidrug efflux as a strategy to prevent biofilm formation. J Antimicrob Chemother 69: 673–681. doi: 10.1093/jac/dkt420 PMID: 24176982

19. Kvist M, Hancock V, Klemm P (2008) Inactivation of efflux pumps abolishes bacterial biofilm formation. Appl Environ Microbiol 74: 7376–7382. doi: 10.1128/AEM.01310-08 PMID: 18836028

20. Holland IB (2004) Translocation of bacterial proteins—an overview. Biochim Biophys Acta 1694: 5–16. PMID: 15546654

21. Baugh S, Ekanayaka AS, Piddock LJ, Webber MA (2012) Loss of or inhibition of all multidrug resistance efflux pumps of *Salmonella enterica* serovar Typhimurium results in impaired ability to form a biofilm. J Antimicrob Chemother 67: 2409–2417. doi: 10.1093/jac/dks228 PMID: 22733653

22. Hou B, Meng XR, Zhang LY, Tan C, Jin H, Zhou R, et al. (2014) ToIC promotes ExPEC biofilm formation and curli production in response to medium osmolarity. Biomed Res Int 2014: 574274. doi: 10.1155/2014/574274 PMID: 25243151

23. Di Bonaventura G, Piccolomini R, Paludi D, D’Orio V, Vergara A, Conter M, et al. (2008) Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: relationship with motility and cell surface hydrophobicity. J Appl Microbiol 104: 1552–1561. doi: 10.1111/j.1365-2672.2007.03688.x PMID: 18194252

24. Negrete-Abascal E, Reyes ME, Garcia RM, Vaca S, Giron JA, Garcia O, et al. (2003) Flagella and Motility in *Actinobacillus pleuropneumoniae*. J Bacteriol 185: 664–668. PMID: 12511514

25. Wilton M, Charron-Mazenod L, Moore R, Lewenza S (2015) Extracellular DNA Acidifies Biofilms and Induces Aminoglycoside Resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 60: 544–553. doi: 10.1128/AAC.01650-15 PMID: 26552982

26. Kanonenberg K, Schwarz CK, Schmitt L (2013) Type I secretion systems—a story of appendices. Res Microbiol 164: 596–604. doi: 10.1016/j.resmic.2013.03.011 PMID: 23541474

27. Dorel C, Lejeune P, Rodrigue A (2006) The Cpx system of *Escherichia coli*, a strategic signaling pathway for confronting adverse conditions and for settling biofilm communities? Res Microbiol 157: 306–314. PMID: 16487683

28. Price NL, Raivo TL (2009) Characterization of the Cpx regulon in *Escherichia coli* strain MC4100. J Bacteriol 191: 1798–1815. doi: 10.1128/JB.00798-08 PMID: 19103922

29. Yang S, Lopez CR, Zeichiedrich EL (2006) Quorum sensing and multidrug transporters in *Escherichia coli*. Proc Natl Acad Sci U S A 103: 2386–2391. PMID: 16467145

30. Koronakis V, Eswaran J, Hughes C (2004) Structure and function of ToIC: the bacterial exit duct for proteins and drugs. Annu Rev Biochem 73: 467–489. PMID: 15189150
31. Caiazza NC, O’Toole GA (2003) Alpha-Toxin Is Required for Biofilm Formation by *Staphylococcus aureus*. J Bacteriol 185: 3214–3217. PMID: 12730182

32. Shak JR, Ludewick HP, Howery KE, Sakai F, Yi H, Harvey RM, et al. (2013) Novel role for the *Streptococcus pneumoniae* toxin pneumolysin in the assembly of biofilms. MBio 4: e00655–00613. doi: 10.1128/mBio.00655-13 PMID: 24023386

33. Lee HS, Gu F, Ching SM, Lam Y, Chua KL (2010) CdpA is a *Burkholderia pseudomallei* cyclic di-GMP phosphodiesterase involved in autoaggregation, flagellum synthesis, motility, biofilm formation, cell invasion, and cytotoxicity. Infect Immun 78: 1832–1840. doi: 10.1128/IAI.00446-09 PMID: 20194589

34. Stipp RN, Boisvert H, Smith DJ, Hofling JF, Duncan MJ, Mattos-Graner RO. (2013) CovR and VicRK regulate cell surface biogenesis genes required for biofilm formation in *Streptococcus mutans*. PLoS One 8: e58271. doi: 10.1371/journal.pone.0058271 PMID: 23554881

35. Zhang L, Li Y, Wen Y, Lau GW, Huang X, Wu R, et al. (2016) HtrA is important for stress resistance and virulence in *Haemophilus parasuis*. Infect Immun 84: 2209–2219. doi: 10.1128/IAI.00147-16 PMID: 27217419

36. Blazquez R, Sanchez-Margallo FM, de la Rosa O, Dalemans W, Alvarez V, Tarazona R, et al. (2014) Immunomodulatory Potential of Human Adipose Mesenchymal Stem Cells Derived Exosomes on in vitro Stimulated T Cells. Front Immunol 5: 556. doi: 10.3389/fimmu.2014.00556 PMID: 25414703

37. Hathroubi S, Fontaine-Gosselin SE, Tremblay YD, Labrie J, Jacques M (2015) Sub-inhibitory concentrations of penicillin G induce biofilm formation by field isolates of *Actinobacillus pleuropneumoniae*. Vet Microbiol 179: 277–286. doi: 10.1016/j.vetmic.2015.06.011 PMID: 26130517

38. Zhang L, Wen Y, Li Y, Wei X, Yan X, Wen X, et al. (2014) Comparative proteomic analysis of the membrane proteins of two *Haemophilus parasuis* strains to identify proteins that may help in habitat adaptation and pathogenesis. Proteome Sci 12: 38. doi: 10.1186/1477-5956-12-38 PMID: 25057263