



RESEARCH ARTICLE

Inner membrane protein TolA facilitates the antibiotic resistance, environment adaptability, biofilm formation and virulence of avian pathogenic *Escherichia coli*

Shuangyang Li^{1,2*}, Jiangang Hu^{1*}, Chang Liu¹, Zizhu Lin¹, Haoheng Peng¹, Beibei Zhang¹, Xinyu Wang¹, Weiqi Guo¹, Jingjing Qi¹, Mingxing Tian¹, Yanqing Bao¹, Guangdong Zhang¹, Yuxiang Shi^{2#}, Shaohui Wang^{1#}

¹ Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai 200241, China

² College of Life Science and Food Engineering, Hebei University of Engineering, Handan 056001, China

Highlights

- TolA enhances the antibiotic resistance and environment adaptability of avian pathogenic *Escherichia coli* (APEC).
- TolA promotes the biofilm formation and interbacterial competition of APEC.
- TolA facilitates the infectivity and virulence of APEC.

Abstract

Avian pathogenic *Escherichia coli* (APEC) can cause colibacillosis, which is economically devastating to poultry industries worldwide. The bacterial membrane is critical to its environment adaptability and virulence. The inner membrane protein TolA maintains membrane integrity, but its roles in the fitness and pathogenesis of APEC are not completely understood. Thus, a *tolA* gene mutant and complemented strains of APEC were constructed and characterized. Mutant strain $\Delta tolA$ showed damage in the inner and outer membranes, as well as altered morphology, impaired flagella production, reduced motility, increased outer membrane vesicle (OMV) production, and reduced resistance to antibiotics and environmental stress. Deletion of the *tolA* gene resulted in significant reductions in biofilm formation and interbacterial competition, due to the downregulated expression of biofilm-associated genes and type VI secretion system (T6SS) genes, respectively. In addition, the mutant strain exhibited reductions in serum bactericidal resistance, cell infection capacity, intracellular survival, consequently leading to attenuated bacterial survival and virulence in mice. Compared with the wild-type and complemented strains, the mutant strain induced less expression of inflammatory cytokine interleukin 1 beta (IL-1 β) in HD-11 macrophages, consistent with the pathological damage in mice. In conclusion, inner membrane protein TolA contributes to the antibiotic resistance, environmental adaptability, biofilm formation and virulence of APEC.

Keywords: avian pathogenic *Escherichia coli*, TolA, membrane integrity, biofilm, virulence

1. Introduction

Avian pathogenic *Escherichia coli* (APEC) can cause a wide range of diseases in poultry, including airsacculitis, pericarditis, perihepatitis, septicemia and other extra-intestinal diseases. APEC is seriously affecting the global poultry industry and causing huge economic losses (Hu *et al.* 2019). APEC uses a variety of mechanisms to facilitate bacterial adaptation and pathogenesis in different environmental

conditions. The bacterial cell envelope is a complex and dynamic structure that is essential for maintaining cellular integrity and defending against external threats (Sharma *et al.* 2024). Gram-negative bacteria, including APEC, possess an outer membrane that serves as a robust barrier against detrimental substances such as antibiotics (Zhou *et al.* 2023). The Tol-Pal system has been shown to regulate outer membrane integrity and enhance bacterial adaptation

Received 23 October 2024; Received in revised form 1 December 2024; Accepted 4 February 2025; Available online 11 February 2025

Shuangyang Li, E-mail: lsy12201102@126.com; Jiangang Hu, E-mail: vethjg@163.com; *Correspondence Shaohui Wang, E-mail: shwang@shvri.ac.cn; Yuxiang Shi, E-mail: hbyxshi@126.com

* These authors contributed equally to this study.

© 2026 CAAS. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>). Peer review under responsibility of Editorial Board of *Journal of Integrative Agriculture*.
doi: 10.1016/j.jia.2025.02.012

to diverse environmental conditions. It is a multi-protein complex comprised of TolQ, TolR, TolA, TolB and Pal, which traverses the inner, periplasmic, and outer membranes. The Tol-Pal system participates in many different processes within bacterial cells, including growth, morphology, antibiotic resistance, motility, and virulence (Hirakawa *et al.* 2019; Li *et al.* 2022).

The inner membrane protein TolA plays a crucial role in the Tol-Pal system of *E. coli*, influencing outer membrane integrity and vesicle formation (Kowata *et al.* 2016; Reimer *et al.* 2021). The TolQ, TolR, and TolA proteins are situated within the inner membrane, with TolA serving as the primary protein. It can form a complex with TolQ and TolR (Baccelli *et al.* 2022). TolA is comprised of three distinct domains, as observed in its structural analysis. The N-terminal domain is responsible for anchoring TolA to the inner membrane (Derouiche *et al.* 1995). The central domain facilitates interactions with outer membrane porins, while the C-terminal domain interacts with TolB (Derouiche *et al.* 1996; Walburger *et al.* 2002). TolA has been shown to interact with trimeric porins, including OmpF, OmpC, PhoE, and LamB, *via* its central domain. This interaction is indicative of the extensive contribution made by TolA to the overall functionality of the bacterial cell envelope (Derouiche *et al.* 1996). TolA plays roles in bacterial outer membrane vesicle (OMV) production, lipopolysaccharide (LPS) production and antibiotic resistance (Li *et al.* 2022).

Previous studies have underscored the pivotal role of TolA in the pathogenicity of bacteria. A *tolA* gene mutation resulted in reduced virulence of pathogenic *E. coli* (Morgan *et al.* 2014; Su *et al.* 2024), emphasizing its role in bacterial survival and pathogenesis. Nevertheless, the roles of TolA in influencing the resistance, environmental adaptation and virulence of APEC remain largely uninvestigated. In this study, we sought to elucidate the impact of TolA on the resistance, environmental adaptability and virulence of APEC. The findings demonstrated that TolA is indispensable for APEC morphology, motility, biofilm formation, survival in acidic environments and under oxidative stress, competition, serum survival, OMV production, adhesion, colonization and virulence. Elucidating the roles of TolA in APEC will help us to understand the pathogenic mechanisms and identify potential targets for controlling APEC infections.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. APEC strain AH50 was isolated and preserved in our laboratory (Yao *et al.* 2023). The bacteria were cultured in Luria-Bertani (LB) medium at a temperature of 37°C. Antibiotics ampicillin (Amp, 100 µg mL⁻¹) or chloramphenicol (Cm, 35 µg mL⁻¹) were added to the LB medium as necessary.

2.2. Construction of mutant and complemented strains

The *tolA* gene was deleted using the λ Red recombinase system as described previously (Datsenko and Wanner 2000). Briefly, a linear PCR product carrying chloramphenicol

Table 1 Bacterial strains and plasmids used in this study

Bacteria and plasmids	Characteristics ¹⁾	Reference
Bacteria		
AH50	APEC wild-type strain	(Yao <i>et al.</i> 2023)
ΔtolA	<i>tolA</i> gene deletion mutant in AH50	This study
CΔtolA	ΔtolA with plasmid pSTV28-tolA	This study
Plasmids		
pKD46	Amp, expresses λ Red recombinase	(Datsenko and Wanner 2000)
pKD3	Cm, template plasmid	(Datsenko and Wanner 2000)
pCP20	Cm, Amp, encoding the flippase recombinase	(Datsenko and Wanner 2000)
pSTV28-Pamp	Cm, Flag tag and His tag	(Zhang <i>et al.</i> 2021)
pSTV28-tolA	Cm, pSTV28-Pamp containing <i>tolA</i> gene	This study

¹⁾ APEC, avian pathogenic *Escherichia coli*; Amp, antibiotic ampicillin; Cm, chloramphenicol.

resistance was transformed into AH50. The *tolA* gene was replaced with the chloramphenicol resistance gene, which was then removed using plasmid pCP20. The mutant strain was confirmed and named ΔtolA. The *tolA* gene was amplified and then inserted into plasmid pSTV28. The resulting plasmid, pSTV28-tolA, was then transformed into the mutant strain ΔtolA for the construction of complemented strain CΔtolA.

2.3. Growth curve and motility assays

The bacterial growth and motility of the AH50, ΔtolA and CΔtolA strains were determined in LB medium and plates as described previously (Zuo *et al.* 2019). Briefly, equal amounts of each bacterial culture were transferred into 100 mL of LB at a ratio of 1:100 (v/v) and incubated at 37°C with shaking. The OD_{600 nm} was monitored at 1 h intervals using a spectrophotometer. Bacterial motility halos on LB soft agar motility plates (0.5% agar) were measured after a 12 h incubation at 37°C. These assays were performed three times.

2.4. Observation of bacterial morphology

APEC strain morphology was observed by transmission electron microscopy (TEM). Briefly, the fresh APEC culture was washed with phosphate-buffered saline (PBS). Then, the bacteria were negatively stained with 2% uranyl acetate, and observed under a transmission electron microscope (HT-7700, Hitachi, Japan).

2.5. Detection of outer membrane permeability and intracellular membrane integrity

Changes in the inner and outer membranes of APEC strains were determined using previously described methods (Liu *et al.* 2020). APEC cultures were washed and adjusted to an OD_{600 nm} of 0.5 with HEPES solution (pH 7.0, plus 5 mmol L⁻¹ glucose). Then, the bacterial solution was incubated with fluorescent dyes 1-N-phenyl-1-naphthylamine (NPN) and propidium iodide (PI) for 1 h to assess outer membrane permeability and inner membrane integrity, respectively (Liu *et al.* 2020). NPN is

a lipophilic fluorescent dye that can penetrate damaged cell membranes but not intact ones. It accumulates in the lipid bilayer of the cytoplasmic membrane and emits fluorescence when excited. Stronger fluorescence indicates a compromised membrane, suggesting cell damage. PI is a DNA-binding fluorescent dye that is unable to cross intact cell membranes but can enter cells with damaged membranes. These assays were performed three times. The fluorescence values were determined using a microplate reader (BioTek, USA).

2.6. Determination of the minimum inhibitory concentration (MIC)

The MIC determination was performed as previously described (Hu *et al.* 2022a). The fresh bacterial culture was adjusted to an OD_{600 nm} value of 0.5 using mueller hinton (MH) medium. Then, 5 µL of bacterial solution was added to each well containing various concentrations of antibiotics. After incubation, the MIC was determined as recommended by the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. This assay was performed three times.

2.7. Detection and analysis of biofilm formation

The biofilm formation of each APEC strain was measured according to a previously described method (Hu *et al.* 2022b). Briefly, fresh bacteria were cultured to an OD_{600 nm} value of 1.0, and diluted 1:100 with fresh LB plus 0.5% glucose. Then, 200 µL of the diluted culture was transferred to a 96-well plate and incubated at 37°C for 24 h. The bacterial culture was removed and rinsed three times with PBS. Then the plates were stained with 0.1% crystal violet for 30 min at 37°C and rinsed with PBS. After drying, 200 µL of 95% ethanol was added to dissolve the crystal violet. The OD_{595 nm} was measured using a microplate reader (BioTek, USA). This assay was performed three times. In addition, the biofilm was allowed to form on coverslips and stained with the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. Then, the biofilm was observed using a confocal laser scanning microscope (Zeiss, Germany).

2.8. Acid resistance test

The acid resistance levels of APEC strains were tested as previously described with minor modifications (Weber *et al.* 2010). A 50 µL aliquot of the bacterial solution was added to 2 mL of LB medium (pH 4.0), and incubated at 37°C for 1 h. Then, the cultures were serially diluted, and bacterial survival was quantified. This assay was performed three times.

2.9. Assay for bacterial resistance to H₂O₂ treatment and the measurement of reactive oxygen species (ROS)

The determination of resistance to H₂O₂ treatment in the AH50, ΔtolA and CΔtolA strains was performed as previously described with some modifications (Ju *et al.* 2021). Aliquots of 10⁸ colony-forming units (CFUs) of fresh bacteria were mixed with 0.25 mmol L⁻¹ H₂O₂. After a 30 min incubation, the surviving bacteria were enumerated by plating on LB agar plates. The intracellular ROS levels of APEC after

H₂O₂ treatment were measured using a ROS Assay Kit (Beyotime, China). The bacteria were incubated with a 2',7'-dichlorodihydrofluorescein (DCFH-DA) probe for 20 min at 37°C. Measurements were taken using a microplate reader (BioTek, USA) at wavelengths of 495 nm for excitation and 520 nm for emission. This assay was performed three times.

2.10. Serum bactericidal assay

The serum bactericidal assay was performed as previously described with some modifications (Zhang *et al.* 2021). Briefly, bacteria were incubated with different dilutions (25%, 50%, and 100%) of SPF chicken serum at 37°C for 30 min. Heat-inactivated serum was used as a negative control. Surviving bacteria were enumerated by plating on LB plates. This assay was performed three times.

2.11. Bacterial adhesion, invasion and intracellular survival assays

The influence of TolA on the adhesion to and invasion of host cells was assessed in three ways. DF-1 and HD-11 cells were used for bacterial adhesion and invasion tests as previously described (Zhang *et al.* 2021). The cells were washed with DMEM without FBS and infected with bacteria at an MOI of 100 for 2 h at 37°C in a CO₂ incubator. After washing, the cells were lysed with Triton X-100, and the bacteria were counted on LB plates. For invasion assays, APEC-infected DF-1 cells were treated with gentamicin (100 µg mL⁻¹) for 1 h to kill extracellular bacteria. The cells were then washed and lysed, and the invasive bacteria were counted. To assess intracellular survival, cells were cultured in DMEM containing gentamicin (10 µg mL⁻¹) for 0, 3, 6, 12 and 24 h. Intracellular survival was determined by comparing the change in the number of bacteria at a given time with the initial number of invading bacteria. These assays were performed three times.

2.12. Bacterial competition assays *in vitro*

Bacterial competition assays were performed as previously described with some modifications (Yi *et al.* 2019; Song *et al.* 2021). Briefly, fresh donor and recipient strains were adjusted to an OD_{600 nm} of 0.5 and mixed in a 5:1 ratio. This mixture was plated on low salt LB plates with nitrocellulose membranes for 6 h at 30°C. Bacterial spots were collected, diluted and plated on LB plates with or without antibiotics for the selection of donor or recipient strains. The competition results were then calculated as the ratio of the donor strain to the recipient strain. This assay was performed three times.

2.13. RNA extraction and quantification of gene expression

The regulatory roles of TolA on the expression of biofilm and virulence genes were investigated by qRT-PCR as previously described (Hu *et al.* 2022b). In addition, the mRNA levels of cellular inflammatory cytokines in HD-11 cells infected with the APEC strains were also measured via qRT-PCR. Briefly, total RNA was extracted from bacteria or cells with TRIzol reagent (Invitrogen, USA), and residual genomic DNA was removed with a Turbo DNase Kit (Thermo Fisher, USA). cDNA

synthesis was performed with a PrimeScript® RT Reagent Kit (TaKaRa, Japan) according to the manufacturer's protocol. qRT-PCR was conducted with SYBR®Premix Ex Taq™ (TaKaRa) and gene-specific primers (Appendix A). Relative gene transcription was normalized to the housekeeping gene *dnaE* (APEC) or β -actin (HD-11 cells) using the $2^{-\Delta\Delta Ct}$ method.

2.14. Impact of TolA on APEC virulence *in vivo*

The effect of TolA on APEC virulence was investigated in mouse models as previously described (Wei *et al.* 2023). Briefly, groups of five 6-wk-old specific pathogen-free (SPF) BALB/c mice were intraperitoneally injected with 10^8 CFUs of APEC. PBS was used as negative control. The mortality was monitored daily until 7 d after infection. The proliferation ability and virulence of bacteria were ascertained as previously described (Zhang *et al.* 2021). Specifically, groups of five 6-wk-old SPF BALB/c mice were intraperitoneally infected with 10^7 CFUs bacteria and humanely euthanized within 24 h. Their spleens were collected, and one part was weighed and homogenized for the determination of bacterial loads. The other part was subjected to histopathological analysis.

2.15. Effect of TolA on OMV production

The OMV of AH50 and Δ tolA were extracted using ultra-high-speed centrifugation as previously described (Reimer *et al.* 2021). Briefly, the bacteria were cultured at 37°C and centrifuged to separate the supernatant from the bacteria. The collected supernatant was filtered through a 0.22 μ m bacterial filter (Millipore, USA) to remove any residual bacteria. The designated supernatant portion filtered by ultracentrifugation was then centrifuged at 40,000 r min⁻¹ in polycarbonate tubes for 2 h in a Ti70 rotor using a Beckman ultracentrifuge (VWR Part of Avantor, USA). The supernatant was carefully decanted to avoid disrupting the pellet. After centrifugation of the entire filtrate, the pellets in each tube were resuspended in 50 mmol L⁻¹ HEPES buffer. The OMV were analyzed *via* SDS-PAGE and electron microscopy.

2.16. Statistical analyses

Statistical analyses were conducted using the GraphPad Software package (GraphPad Software, La Jolla, CA, USA). One-way analysis of variance (ANOVA) and two-way ANOVA were used to analyze biofilm formation, competition, oxidative stress, bacterial adherence, invasion data and bactericidal serum, and qRT-PCR results, respectively. The comparisons of bacterial loads in the animal infection study were evaluated with the nonparametric Mann-Whitney U Test. Statistical significance was considered at $P < 0.05$.

3. Results

3.1. Effects of TolA on the growth curve, motility and morphology of APEC

First, the APEC *tolA* gene mutant and complemented strains were constructed and characterized. The strains Δ tolA and C Δ tolA were confirmed by PCR (Fig. 1-A). Using the *tolA*-out-F/*tolA*-out-R primers, a 1,692 bp product was amplified from strain AH50, while a 475 bp product was amplified from Δ tolA and C Δ tolA. PCR amplification with the *tolA*-in-F/*tolA*-

in-R primers did not amplify a product in the mutant strain Δ tolA, but a 339 bp product was amplified in AH50 and C Δ tolA. Furthermore, the complemented strain was identified by Western blotting. The complemented strain was identified using an anti-Flag antibody, and the TolA protein fused to Flag was expressed in the complemented strain (Fig. 1-B). This indicated that the TolA protein was expressed in the mutant strain. The growth curve showed that the mutant strain Δ tolA grew similarly to the wild-type and complemented strains (Fig. 1-C). However, the mutant strain Δ tolA showed significantly impaired motility (Fig. 1-D). Morphologically, the wild-type and complemented strains were rod-shaped bacteria with bluntly rounded ends and flagella. In contrast, the mutant strain became rounded and no flagella were observed (Fig. 1-E). These results indicated that TolA affects the morphological characteristics and motility of APEC.

3.2. Inactivation of TolA modifies the membrane permeability and antibiotic resistance of APEC

As the morphology of APEC was altered after *tolA* deletion, we further investigated the effect of TolA on the inner and outer membranes of APEC. NPN and PI were used to determine the changes in the outer membrane and inner membrane, respectively. A positive control polymyxin treatment affected the outer and inner membranes of AH50. The results revealed that *tolA* deletion increased the outer membrane permeability and disrupted the inner membrane integrity of APEC (Fig. 2-A and B). In the complemented strains, the outer and inner membranes were not affected.

Since TolA affects the inner and outer membranes of APEC, we examined the sensitivity of the APEC strain to ampicillin and vancomycin. As shown in Fig. 2-C, the MICs of ampicillin against AH50 and C Δ tolA were 1 μ g mL⁻¹, and 0.5 μ g mL⁻¹ for Δ tolA. AH50 was resistant to vancomycin at 64 μ g mL⁻¹, while C Δ tolA was resistant at over 64 μ g mL⁻¹. Δ tolA was only resistant at 32 μ g mL⁻¹. Therefore, APEC became more sensitive to ampicillin and vancomycin after *tolA* gene deletion.

3.3. TolA contributes to biofilm formation in APEC

The biofilm assay showed that *tolA* gene deletion resulted in a significantly reduced biofilm formation capacity compared to the wild-type and complemented strain ($P < 0.01$) (Fig. 3-A). Furthermore, a confocal laser scanning microscope was used to observe the APEC biofilm. The wild-type strain could adhere to the coverslips and form a scattered distribution of biofilm (Fig. 3-B). However, only a few of the mutant strain bacteria adhered to the coverslips and barely formed a biofilm. The biofilm of the complemented strain was restored to the level of the wild-type strain.

The expression levels of biofilm-associated genes were analyzed by qRT-PCR, including the polysaccharide biosynthesis gene *wza* and biofilm poly-N-acetyl-glucosamine (PGA) biosynthesis protein gene *pgaD*. The transcript levels of biofilm-associated genes were downregulated in the mutant strain compared to the wild-type strain (Fig. 3-C), but recovered in the complemented strain. These results suggested that TolA plays a key role in biofilm formation in APEC.

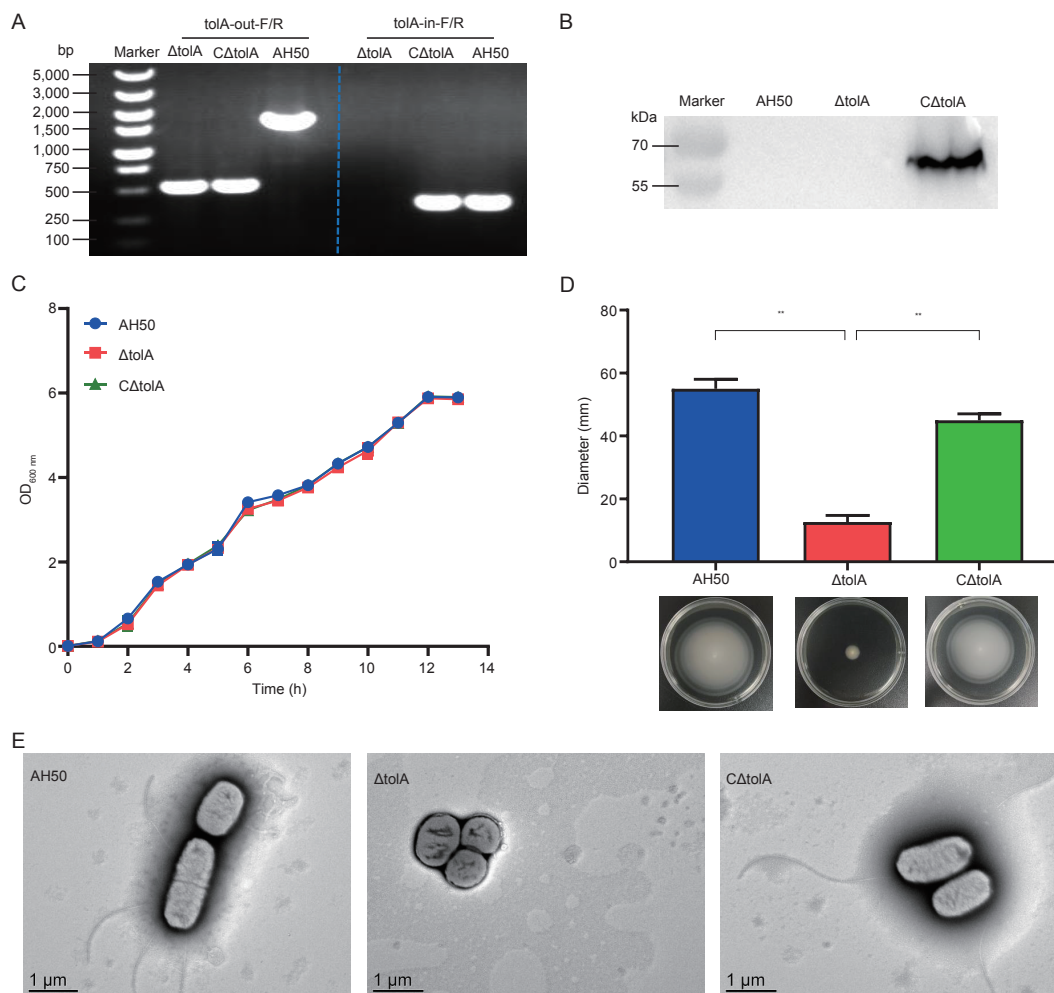


Fig. 1 Biological characterization of avian pathogenic *Escherichia coli* (APEC) strains. A, Identification of the *tolA* gene mutant and complemented strains by PCR. B, identification of the complemented strain by Western blot. C, bacterial growth curves. APEC strains were grown in Luria-Bertani (LB) medium at 37°C, and the OD_{600 nm} was measured every hour. D, bacterial motility. The motility halo of the mutant strain was significantly smaller than those of the wild-type and complemented strains. E, morphology of APEC observed by transmission electron microscopy. The morphology of the mutant was rounded and no flagella were observed, unlike to the wild-type and complemented strains. Significant differences were analyzed using one-way ANOVA (*, $P < 0.01$).

3.4. TolA is beneficial for resistance to environmental stress

APEC strains were cultured in different media to determine whether TolA affects the resistance of APEC to pH and hydrogen peroxide. The results showed that the survival of the mutant strain was significantly lower than those of the wild-type and complemented strains at pH 4.0 (Fig. 4-A). In the presence of H₂O₂, the survival of the mutant strain was also significantly lower than those of the wild-type and complemented strains (Fig. 4-A). The intracellular ROS were significantly increased in the mutant strain $\Delta tolA$ compared to the wild-type and complemented strains (Fig. 4-B). These results indicated that TolA plays an important role in APEC resistance to the external environment.

3.5. TolA promotes serum bactericidal resistance

The effect of TolA on the serum bactericidal resistance of APEC was determined. The results showed that the survival of the mutant strain $\Delta tolA$ in 100%, 50%, and 25% SPF

chicken serum was significantly reduced compared to that of the wild-type strain (Fig. 4-C). The serum bactericidal resistance was restored in the complemented strain. However, no differences were observed between the wild-type, mutant and complemented strains in heat-inactivated serum, due to the lack of a functional antibacterial factor such as complement. These observations suggested that TolA promotes the serum bactericidal resistance of APEC.

3.6. TolA facilitates interbacterial competition

To further investigate the role of TolA in antibacterial competition, the fresh APEC donor strain and recipient strain DH5 α were co-cultured, and the surviving recipient bacteria were counted. When the donor bacteria were $\Delta tolA$, the survival rate of the recipient bacteria DH5 α was significantly increased ($P < 0.01$, Fig. 5-A). The complemented strains showed stronger antibacterial competition than the mutant strain. To determine whether the reduced competition activity of $\Delta tolA$ is due to a change in T6SS expression, the

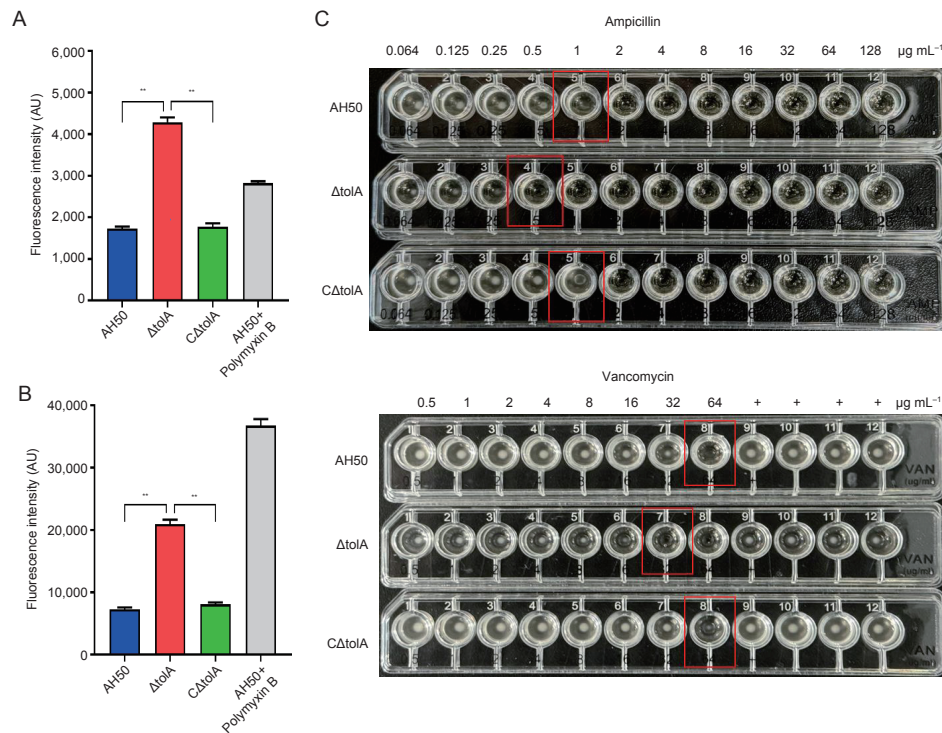


Fig. 2 Determination of outer membrane permeability, inner membrane integrity and antibiotic minimum inhibitory concentration (MIC). A, NPN probe used to determine the outer membrane permeability of avian pathogenic *Escherichia coli* (APEC). The outer membrane permeability of the mutant strain was higher than those of the wild-type and complemented strains. B, integrity of the inner membrane of APEC detected using the propidium iodide (PI) dye. The integrity of the inner membrane was disrupted in the mutant strain, unlike the wild-type and complemented strains. C, MIC assays of ampicillin and vancomycin for APEC. Significant differences were determined by one-way ANOVA (*, $P < 0.01$).

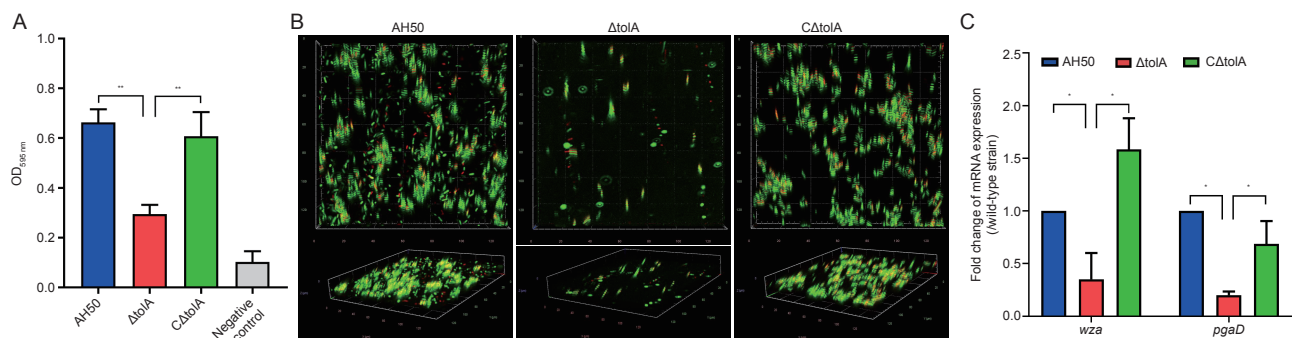


Fig. 3 Effects of TolA on avian pathogenic *Escherichia coli* (APEC) biofilm formation. A, determination of biofilm formation ability by crystal violet staining. The mutant strain showed significantly reduced biofilm formation compared to the wild-type and complemented strains. B, observation of the APEC biofilm by confocal laser scanning microscopy. The mutant strain barely formed biofilm. C, analysis of the transcript levels of biofilm-associated genes. qRT-PCR was used to measure the transcript levels of biofilm-associated genes in the wild-type, mutant and complemented strains. Transcript levels were normalized to the housekeeping gene *dnaE*. The results are presented as relative expression ratios compared to expression in the wild-type strain. Statistical significance was analyzed by one-way ANOVA or two-way ANOVA (*, $P < 0.05$; **, $P < 0.01$).

expression levels of T6SS core genes in these APEC strains were analyzed via qRT-PCR. The results showed that the deletion of *tolA* significantly downregulated the transcription of T6SS core genes *hcp1*, *vgrG1*, *clpV2*, *hcp2A* and *hcp2B* ($P < 0.05$, Fig. 5-B).

3.7. TolA is not conducive to OMV production

To investigate the effect of TolA on OMVs, electron microscopy was used to observe the OMVs extracted from the wild-type

and mutant strains. The results showed that more OMVs were extracted from the mutant strain than the wild-type strain (white arrows in Fig. 6-A). Differences in the protein contents of the OMVs from wild-type and mutant strains were further analyzed by SDS-PAGE. The OMVs extracted from the wild-type strain had almost no protein band, whereas the OMVs from the mutant strain had multiple distinct protein bands (Fig. 6-B). This result indicated a significant difference between the proteins in the OMVs of the wild-type and mutant strains.

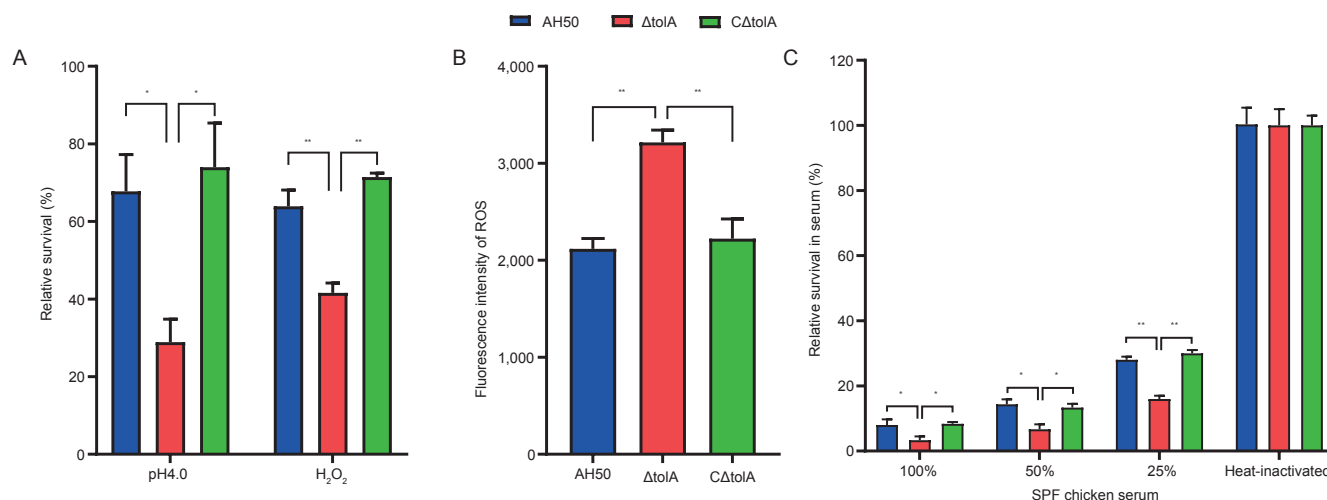


Fig. 4 Determination of the environmental adaptability of avian pathogenic *Escherichia coli* (APEC). A, APEC resistance to acidic environment and hydrogen peroxide. The survival of the mutant strain in acidic and oxidative stress conditions were significantly lower than those of the wild-type and complemented strains. B, APEC intracellular reactive oxygen species (ROS) content. Under oxidative stress conditions, the mutant strain showed significantly higher ROS production than the wild-type and complemented strains. C, bacterial resistance to serum bactericidal activity. APEC strains were incubated with different dilutions of specific pathogen-free (SPF) chicken serum at 37°C for 30 min and the surviving bacteria were counted. The mutant strain showed reduced survival compared to the wild-type and complemented strains. Two-way ANOVA was conducted to analyze statistical significance (*, $P < 0.05$; **, $P < 0.01$).

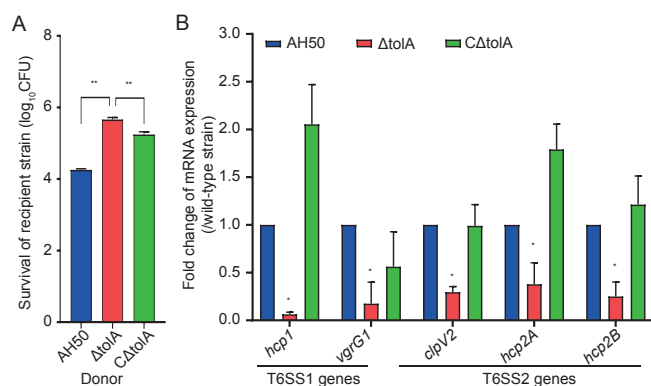


Fig. 5 The effect of TolA on interbacterial competition in avian pathogenic *Escherichia coli* (APEC). A, TolA facilitates the interbacterial competition of APEC. APEC was used as the donor bacterium and DH5 α as the recipient bacterium in a 5:1 co-incubation ratio. After incubation at 30°C for 6 h, the mutant strain showed a significantly lower competitive ability than the wild-type and complemented strains. B, TolA regulates the expression of T6SS genes in APEC. qRT-PCR was used to measure the transcript levels of T6SS-associated genes in the wild-type, mutant and complemented strains. Transcript levels were normalized to the housekeeping gene *dnaE*. The results are presented as relative expression ratios compared to expression in the wild-type strain. Statistical significance was analyzed by one-way ANOVA or two-way ANOVA (*, $P < 0.05$; **, $P < 0.01$).

3.8. TolA facilitates bacterial adhesion, invasion and intracellular survival

The adhesion, invasion and intracellular survival of APEC on avian cells were examined. The results showed that the adhesion and invasion ability of the mutant strain on DF-1 cells were significantly reduced compared to the wild-type strain (Fig. 7-A and B). The invasion ability of the mutant strain on HD-11 cells was reduced and the intracellular

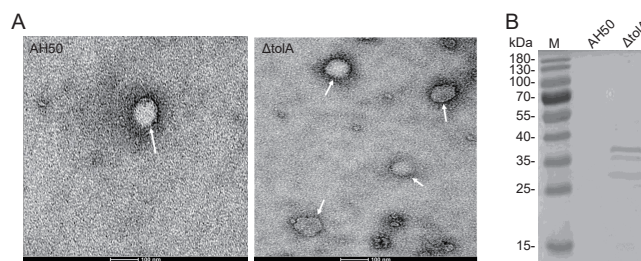


Fig. 6 Analysis of avian pathogenic *Escherichia coli* (APEC) outer membrane vesicles (OMVs). A, observation of APEC OMVs by transmission electron microscopy (TEM). The OMVs of the wild-type strain and mutant strain were extracted and then observed by transmission electron microscopy. More OMVs were extracted in the mutant strain compared to the wild-type strain, as indicated by the white arrows. B, SDS-PAGE analysis of OMVs. More protein was observed in the mutant strain OMVs.

viability in HD-11 cells was also significantly reduced at all time points (3, 6, 12 and 24 h) (Fig. 7-C and D). These capacities were restored in the complemented strain. The results revealed that TolA is required for APEC to adhere to cells, invade cells and survive intracellularly.

3.9. TolA is essential for bacterial survival and virulence in vivo

To investigate the effect of TolA on APEC pathogenicity, the virulence of APEC was evaluated. The mortalities of mice infected with AH50, Δ tolA, and C Δ tolA were 100% (5/5), 0% (0/5) and 80% (4/5), respectively. All mice died after infection with the wild-type strain, while no mice died in the mutant strain infection group (Fig. 8-A). To determine whether TolA can affect the colonization of APEC during systemic infection, the bacterial loads in the spleen were determined at 24 h after infection. The amount of recovered mutant strain Δ tolA in the

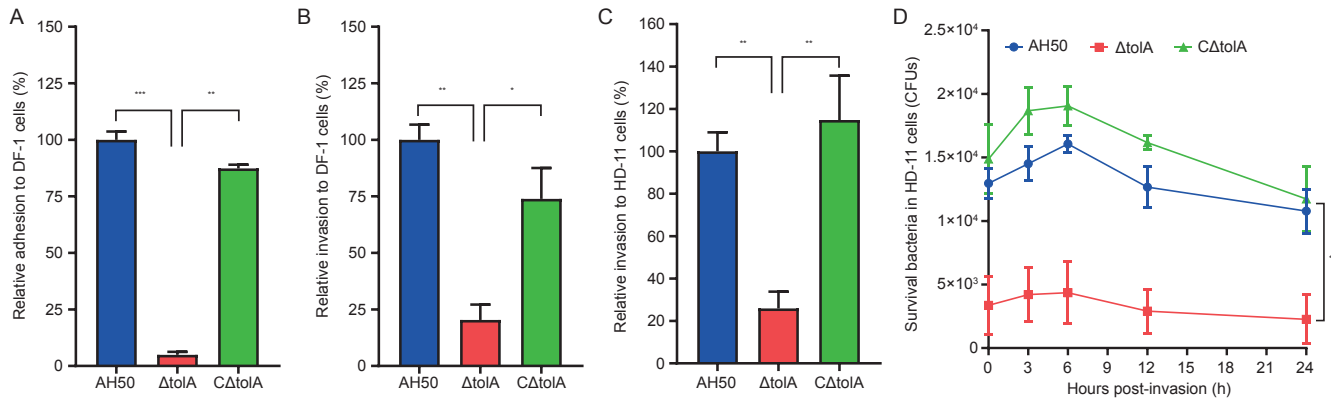


Fig. 7 Avian pathogenic *Escherichia coli* (APEC) adhesion, invasion and intracellular survival assays. A, adhesion ability of APEC to DF-1 cells. The mutant strain Δ tolA showed significantly reduced adhesion capacity compared with the wild-type and complemented strains. B, invasion capacity of APEC into DF-1 cells. The invasion capacity of the mutant strain on DF-1 cells was significantly lower than those of the wild-type and complemented strains. C, invasion capacity of APEC into HD-11 cells. The invasion capacity of the mutant strain into HD-11 cells was significantly lower than those of the wild-type and complemented strains. D, intracellular survival of APEC in HD-11 cells. The mutant strain showed reduced survival in HD-11 cells compared to the wild-type and complemented strains. CFUs, colony-forming units. Statistical significance analysis was analyzed using one-way ANOVA (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

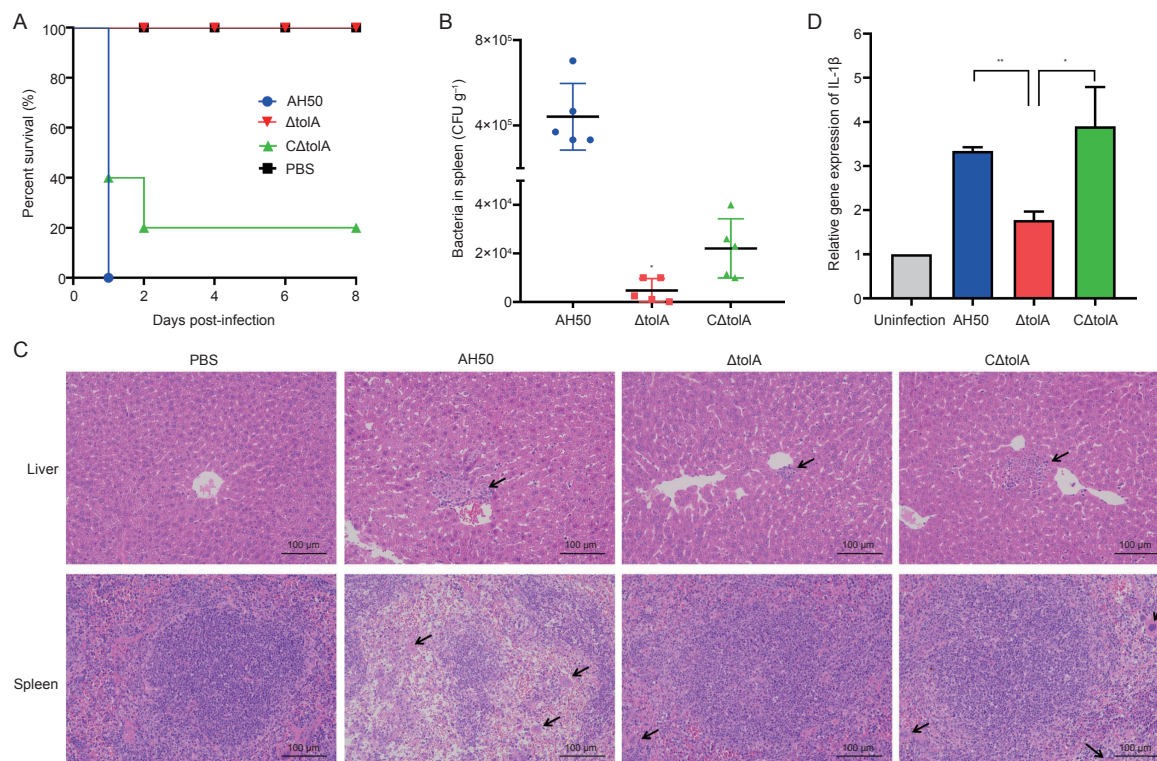


Fig. 8 TolA contributes to the colonization and virulence of avian pathogenic *Escherichia coli* (APEC). A, bacterial virulence. Mice were infected with APEC strains and mortality was monitored until 7 d after infection. Negative controls were injected with phosphate-buffered saline (PBS). B, bacterial colonization of mouse spleen. Mice were infected with APEC strains and sacrificed 24 h after infection, and bacteria were harvested from the spleens. The non-parametric Mann-Whitney U test was used to determine statistical significance (*, $P < 0.05$). C, histopathological examination. In pathological micrographs of the liver and spleen, black arrows indicate granulocyte infiltration and multinucleated giant cells, respectively. D, IL-1 β expression in avian HD-11 macrophages. HD-11 cells were infected with each APEC strain. The transcript levels of IL-1 β in bacteria-infected HD-11 cells at 3 h post-invasion were analyzed by qRT-PCR. Statistical significance was evaluated by two-way ANOVA (*, $P < 0.05$; **, $P < 0.01$).

spleen was significantly less than the wild-type strain ($P < 0.05$) (Fig. 8-B). The pathological micrographs showed increased granulocyte infiltration in the liver, reduced lymphocytes and

an increase in multinucleated giant cells in the spleens of mice infected with the wild-type and complemented strains. In contrast, the pathological lesions in mutant strain infected

mice were significantly milder (black arrows, Fig. 8-C). These results indicated that TolA is necessary for the full virulence of APEC.

3.10. TolA promotes inflammatory cytokine IL-1 β expression in HD-11 cells

The inflammatory cytokine expression in HD-11 cells infected with APEC was examined. As shown in Fig. 8-D, the expression of IL-1 β was significantly upregulated in APEC-infected HD-11 cells compared to uninfected cells. However, the expression of IL-1 β in Δ tolA-infected HD-11 macrophages was significantly downregulated compared to the wild-type at 3 h after invasion ($P < 0.01$). Furthermore, IL-1 β transcription was restored in C Δ tolA-infected macrophages.

4. Discussion

The Tol-Pal system is composed of proteins TolQ, TolR, TolA, TolB and Pal, which affect the expression of multiple genes in bacteria. Studies have demonstrated that the Tol-Pal system plays key roles in the morphology, cell membrane integrity and virulence of bacterial cells (Germon *et al.* 2001; Hirakawa *et al.* 2019). This study suggested that *tolA* gene deletion resulted in alterations in the morphology of APEC. The TolA protein is part of the Tol-Pal system, which is necessary for maintaining the outer membrane of Gram-negative bacteria. It is located on the inner membrane and works with TolQ and TolR to form the inner membrane protein complex (Baccelli *et al.* 2022). Consequently, the deletion of *tolA* not only directly impacts the integrity of the inner membrane but also affects the Tol-Pal system, resulting in alterations to both the inner and outer membranes of APEC and consequently influencing the morphology of the bacteria. The effect of TolA on the bacterial inner and outer membranes makes the Δ tolA strain more sensitive to antibiotics such as ampicillin and vancomycin.

The Tol-Pal system is also implicated in the biogenesis and release of OMVs (Li *et al.* 2022). Evidence has demonstrated that cross-linking between peptidoglycan and bacterial envelope proteins represents a pivotal mechanism for OMV biogenesis (Schwechheimer and Kuehn 2015). The Tol-Pal system encompasses 5 envelope proteins that participate in OMV biogenesis by interacting with peptidoglycan. The yield of OMVs is enhanced when the cross-linking is reduced. The observation that the *tolA* mutant strain produced more OMVs can thus be attributed to a reduction in cross-linking between peptidoglycan and bacterial envelope proteins resulting from the *tolA* deletion. OMVs play an important role in bacterial pathogenesis, as they carry adhesins, toxins and immunomodulatory compounds involved in the pathogenic process (Haurat *et al.* 2015). In this study, we found membrane damage in the mutant strains, and although the cells were able to increase their secretion of OMVs, the mutant strain Δ tolA had reduced adhesion and invasion of host cells and was more easily cleared in the host. Thus, the production of OMVs by the mutant strain is only a minor contributor to the virulence of APEC.

The mutant strain Δ tolA showed impaired flagella and reduced motility. This phenomenon may be attributed to

damage to the inner and outer membranes of APEC, which impairs the synthesis of flagella. Flagella mediate bacterial adhesion to host proteins and cells, which contributes to the virulence of pathogenic *E. coli* (Avelino-Flores *et al.* 2022). The flagella also represent a virulence factor recognized by the innate immune system that induces IL-1 β expression (Zgair 2012). This may be an explanation for the reduced expression of the inflammatory cytokine IL-1 β and the virulence of the mutant strain *in vitro* and *in vivo*.

Biofilm facilitate the persistence of APEC within the host and under harsh environmental stress (Hu *et al.* 2022b). In this study, biofilm observations revealed that TolA affects the attachment of APEC to surfaces and biofilm morphology. Furthermore, the qRT-PCR analysis results indicated that genes associated with the biofilm were downregulated. Wza is involved in the export of extracellular polysaccharide and influences biofilm structure (Li *et al.* 2007). The *pgaD* gene encodes biofilm PGA synthesis protein, and its deletion leads to a loss of the strong biofilm phenotype (Choi *et al.* 2009). The reduction in biofilm formation for the mutant strain in this study was due to the downregulation of the transcript levels of *wza* and *pgaD*. This may also explain the reduced virulence of the mutant strain.

This study further investigated the effect of TolA on the environmental adaptability of APEC. The survival of the Δ tolA mutant in bactericidal serum was reduced, maybe because of the reduced production of TraT protein (Pramoonjago *et al.* 1992). The absence of TolA compromises the structural integrity of the bacterial outer membrane, leading to reduced TraT expression. In this study, the acid and antioxidant resistance of the mutant strain Δ tolA was significantly lower than in the wild-type and complemented strains. Bacteria must regulate multiple systems to withstand unfavorable conditions in acidic and oxidative stress environments. This resistance is multifaceted, relying on various bacterial components like the lipopolysaccharide layer and specific outer membrane proteins for protection (Su *et al.* 2024). However, due to inner and outer membrane damage, the mutant strain was unable to synthesize the appropriate proteins to adapt to the extreme environmental conditions, resulting in a significant reduction in its ability to resist unfavorable environments.

The results of cellular and animal infection experiments indicated that TolA was essential for APEC to adhere to, invade, survive within the cells and produce an infection. Mutations in Tol-Pal components have been shown to lower LPS levels in the outer membrane (Lazzaroni *et al.* 1999). This reduction impairs the ability of cells to adhere to surfaces, which is a crucial step in biofilm formation. It also diminishes the capacity to adhere to cells and is likely to reduce the levels of inflammatory cytokines directed against host cells.

T6SS1 and T6SS2 play important roles in APEC pathogenicity and antibacterial competition (Wang *et al.* 2014). The T6SS core components ClpV, Hcp and VgrG promote APEC invasion of host cells and are also involved in antibacterial competition (Ma *et al.* 2014). Hcp1 and Hcp2B are required for interspecies competition (Ma *et al.* 2018). In terms of pathogenicity, the T6SS2 effector protein

Hcp1 regulates cytoskeletal rearrangement and apoptosis (Zhou *et al.* 2012). The present study observed that *tolA* deletion affected the interspecific competitive ability of APEC. However, the reduced transcript levels of the core component genes of T6SS1 and T6SS2 may be responsible for the reduced invasion capacity, survival and competitive capacity of the mutant strain $\Delta tolA$. The effector protein Hcp2A regulates the expression of IL-1 β and IL-8, thereby affecting inflammation (Song *et al.* 2020). The deletion of *tolA* resulted in lower Hcp2A transcript levels in APEC. This may also explain the reduced IL-1 β transcript levels after $\Delta tolA$ infection.

5. Conclusion

In this study, the *tolA* gene mutant and complemented strains of APEC were constructed and characterized. The deletion of the *tolA* gene resulted in altered morphology, impaired flagella production, reduced motility, and increased OMV production in APEC. Furthermore, this study demonstrated that TolA enhances antibiotic resistance and environmental adaptability, promotes biofilm formation and interbacterial competition, and facilitates the virulence of APEC. These findings contribute to our comprehensive understanding of APEC pathogenesis.

Acknowledgements

This work was supported by the National Key Research and Development Program of China (2021YFD1800402), the National Natural Science Foundation of China (32172856, 32302881), the Natural Science Foundation of Shanghai, China (22ZR1476100, 23ZR1476600), the China Postdoctoral Science Foundation (2023M743852), the Shanghai Oriental Talent Plan, China, and the Agricultural Science and Technology Innovation Program of the Chinese Academy of Agricultural Sciences (CAAS-ASTIP-2021-SHVR1-07). The funders played no roles in study design, data collection and interpretation, or submission for publication.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical approval

The animal experiments were approved by the Animal Care and Use Committee at the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences (CAAS). All animals were managed in accordance with the Guidelines of the Institutional Administrative Committee (SYXK[Hu]-2020-0027).

Appendix associated with this paper is available at <https://doi.org/10.1016/j.jia.2025.02.012>

References

- Avelino-Flores F, Soria-Bustos J, Saldaña-Ahuactzi Z, Martínez-Laguna Y, Yañez-Santos J A, Cedillo-Ramírez M L, Girón J A. 2022. The transcription of flagella of enteropathogenic *Escherichia coli* O127:H6 is activated in response to environmental and nutritional signals. *Microorganisms*, **10**, 792.
- Bacelli P, Rachedi R, Serrano B, Petiti M, Bernard C S, Houot L, Duche D. 2022. Timing of TolA and TolQ recruitment at the septum depends on the functionality of the Tol-Pal system. *Journal of Molecular Biology*, **434**, 167519.
- Choi A H K, Slamti L, Avci F Y, Pier G B, Maira-Litrán T. 2009. The pgaABCD locus of *Acinetobacter baumannii* encodes the production of poly- β -1-6-N-acetylglucosamine, which is critical for biofilm formation. *Journal of Bacteriology*, **191**, 5953–5963.
- Datsenko K A, Wanner B L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 6640–6645.
- Derouiche R, Bénédetti H, Lazzaroni J C, Lazdunski C, Llobès R. 1995. Protein complex within *Escherichia coli* inner membrane. TolA N-terminal domain interacts with TolQ and TolR proteins. *The Journal of Biological Chemistry*, **270**, 11078–11084.
- Derouiche R, Gavioli M, Bénédetti H, Prilipov A, Lazdunski C, Llobès R. 1996. TolA central domain interacts with *Escherichia coli* porins. *The EMBO Journal*, **15**, 6408–6415.
- Germon P, Ray M C, Vianney A, Lazzaroni J C. 2001. Energy-dependent conformational change in the TolA protein of *Escherichia coli* involves its N-terminal domain, TolQ, and TolR. *Journal of Bacteriology*, **183**, 4110–4114.
- Haurat M F, Elhenawy W, Feldman M F. 2015. Prokaryotic membrane vesicles: New insights on biogenesis and biological roles. *Biological Chemistry*, **396**, 95–109.
- Hirakawa H, Suzue K, Kurabayashi K, Tomita H. 2019. The Tol-Pal system of uropathogenic *Escherichia coli* is responsible for optimal internalization into and aggregation within bladder epithelial cells, colonization of the urinary tract of mice, and bacterial motility. *Frontiers in Microbiology*, **10**, 1827.
- Hu J, Che C, Jiang W, Chen Z, Tu J, Han X, Qi K. 2022a. Avian pathogenic *Escherichia coli* through Pfs affects the transcription of membrane proteins to resist β -lactam antibiotics. *Veterinary Sciences*, **9**, 98.
- Hu J, Lü X, Niu X, Yu F, Zuo J, Bao Y, Yin H, Huang C, Nawaz S, Zhou W, Jiang W, Chen Z, Tu J, Qi K, Han X. 2022b. Effect of nutritional and environmental conditions on biofilm formation of avian pathogenic *Escherichia coli*. *Journal of Applied Microbiology*, **132**, 4236–4251.
- Hu J, Zuo J, Chen Z, Fu L, Lv X, Hu S, Shi X, Jing Y, Wang Y, Wang Z, Mi R, Huang Y, Liu D, Qi K, Han X. 2019. Use of a modified bacterial ghost lysis system for the construction of an inactivated avian pathogenic *Escherichia coli* vaccine candidate. *Veterinary Microbiology*, **229**, 48–58.
- Ju X, Fang X, Xiao Y, Li B, Shi R, Wei C, You C. 2021. Small RNA GcvB regulates oxidative stress response of *Escherichia coli*. *Antioxidants* (Basel, Switzerland), **10**, 1774.
- Kowata H, Tochigi S, Kusano T, Kojima S. 2016. Quantitative measurement of the outer membrane permeability in *Escherichia coli* lpp and Tol-Pal mutants defines the significance of Tol-Pal function for maintaining drug resistance. *The Journal of Antibiotics*, **69**, 863–870.
- Lazzaroni J C, Germon P, Ray M C, Vianney A. 1999. The Tol proteins of *Escherichia coli* and their involvement in the uptake of biomolecules and outer membrane stability. *FEMS Microbiology Letters*, **177**, 191–197.
- Li J, Attila C, Wang L, Wood T K, Valdes J J, Bentley W E. 2007. Quorum sensing in *Escherichia coli* is signaled by AI-2/LsrR: Effects on small RNA and biofilm architecture. *Journal of Bacteriology*, **189**, 6011–6020.
- Li Q, Li Z, Fei X, Tian Y, Zhou G, Hu Y, Wang S, Shi H. 2022. The role of TolA, TolB, and TolR in cell morphology, OMVs production, and virulence of *Salmonella Choleraesuis*. *AMB Express*, **12**, 5.
- Liu Y, Jia Y, Yang K, Tong Z, Shi J, Li R, Xiao X, Ren W, Hardeland R, Reiter R J, Wang Z. 2020. Melatonin overcomes MCR-mediated colistin resistance in Gram-negative pathogens. *Theranostics*, **10**, 10697–10711.

- Ma J, Bao Y, Sun M, Dong W, Pan Z, Zhang W, Lu C, Yao H. 2014. Two functional type VI secretion systems in avian pathogenic *Escherichia coli* are involved in different pathogenic pathways. *Infection and Immunity*, **82**, 3867–3879.
- Ma J, Sun M, Pan Z, Song W, Lu C, Yao H. 2018. Three Hcp homologs with divergent extended loop regions exhibit different functions in avian pathogenic *Escherichia coli*. *Emerging Microbes & Infections*, **7**, 49.
- Morgan J K, Ortiz J A, Riordan J T. 2014. The role for TolA in enterohemorrhagic *Escherichia coli* pathogenesis and virulence gene transcription. *Microbial Pathogenesis*, **77**, 42–52.
- Pramoonjago P, Kaneko M, Kinoshita T, Ohtsubo E, Takeda J, Hong K S, Inagi R, Inoue K. 1992. Role of TraT protein, an anticomplementary protein produced in *Escherichia coli* by R100 factor, in serum resistance. *Journal of Immunology*, **148**, 827–836.
- Reimer S L, Beniac D R, Hiebert S L, Booth T F, Chong P M, Westmacott G R, Zhanel G G, Bay D C. 2021. Comparative analysis of outer membrane vesicle isolation methods with an *Escherichia coli* *tolA* mutant reveals a hypervesiculating phenotype with outer-inner membrane vesicle content. *Frontiers in Microbiology*, **12**, 628801.
- Schwechheimer C, Kuehn M J. 2015. Outer-membrane vesicles from Gram-negative bacteria: Biogenesis and functions. *Nature Reviews Microbiology*, **13**, 605–619.
- Sharma P, Vaiwala R, Gopinath A K, Chockalingam R, Ayappa K G. 2024. Structure of the bacterial cell envelope and interactions with antimicrobials: Insights from molecular dynamics simulations. *Langmuir*, **40**, 7791–7811.
- Song L, Pan J, Yang Y, Zhang Z, Cui R, Jia S, Wang Z, Yang C, Xu L, Dong T G, Wang Y, Shen X. 2021. Contact-independent killing mediated by a T6SS effector with intrinsic cell-entry properties. *Nature Communications*, **12**, 423.
- Song X, Hou M, Jiang H, Shen X, Xue M, Shao Y, Wang L, He Q, Zheng L, Tu J, Qi K. 2020. Hcp2a of type VI secretion system contributes to IL8 and IL1 β expression of chicken tracheal epithelium by affecting APEC colonization. *Research in Veterinary Science*, **132**, 279–284.
- Su S, Li Z, Sun Y, Gao S, Gao Q. 2024. The multifaceted role of TolA protein in promoting survival, biofilm formation and virulence of avian pathogenic *Escherichia coli*. *Poultry Science*, **103**, 104142.
- Walburger A, Lazdunski C, Corda Y. 2002. The Tol/Pal system function requires an interaction between the C-terminal domain of TolA and the N-terminal domain of TolB. *Molecular Microbiology*, **44**, 695–708.
- Wang S, Dai J, Meng Q, Han X, Han Y, Zhao Y, Yang D, Ding C, Yu S. 2014. DotU expression is highly induced during *in vivo* infection and responsible for virulence and Hcp1 secretion in avian pathogenic *Escherichia coli*. *Frontiers in Microbiology*, **5**, 588.
- Weber M M, French C L, Barnes M B, Siegle D A, Mclean R J C. 2010. A previously uncharacterized gene, *yjfO* (*bsmA*), influences *Escherichia coli* biofilm formation and stress response. *Microbiology* (Reading, England), **156**, 139–147.
- Wei J, Chen C, Feng J, Zhou S, Feng X, Yang Z, Lu H, Tao H, Li L, Xv H, Xuan J, Wang F. 2023. Muc2 mucin O-glycosylation interacts with enteropathogenic *Escherichia coli* to influence the development of ulcerative colitis based on the NF- κ B signaling pathway. *Journal of Translational Medicine*, **21**, 793.
- Yao L, Bao Y, Hu J, Zhang B, Wang Z, Wang X, Guo W, Wang D, Qi J, Tian M, Bao Y, Li H, Wang S. 2023. A lytic phage to control multidrug-resistant avian pathogenic *Escherichia coli* (APEC) infection. *Frontiers in Cellular and Infection Microbiology*, **13**, 1253815.
- Yi Z, Wang D, Xin S, Zhou D, Li T, Tian M, Qi J, Ding C, Wang S, Yu S. 2019. The CpxR regulates type VI secretion system 2 expression and facilitates the interbacterial competition activity and virulence of avian pathogenic *Escherichia coli*. *Veterinary Research*, **50**, 40.
- Zgair A K. 2012. *Escherichia coli* flagellin stimulates pro-inflammatory immune response. *World Journal of Microbiology & Biotechnology*, **28**, 2139–2146.
- Zhang Y, Wang Y, Zhu H, Yi Z, Afayibo D J A, Tao C, Li T, Tian M, Qi J, Ding C, Yu S, Wang S. 2021. DctR contributes to the virulence of avian pathogenic *Escherichia coli* through regulation of type III secretion system 2 expression. *Veterinary Research*, **52**, 101.
- Zhou G, Wang Q, Wang Y, Wen X, Peng H, Peng R, Shi Q, Xie X, Li L. 2023. Outer membrane porins contribute to antimicrobial resistance in Gram-negative bacteria. *Microorganisms*, **11**, 1690.
- Zhou Y, Tao J, Yu H, Ni J, Zeng L, Teng Q, Kim K S, Zhao G P, Guo X, Yao Y. 2012. Hcp family proteins secreted via the type VI secretion system coordinately regulate *Escherichia coli* K1 interaction with human brain microvascular endothelial cells. *Infection and Immunity*, **80**, 1243–1251.
- Zuo J, Tu C, Wang Y, Qi K, Hu J, Wang Z, Mi R, Yan H, Chen Z, Han X. 2019. The role of the *wzy* gene in lipopolysaccharide biosynthesis and pathogenesis of avian pathogenic *Escherichia coli*. *Microbial Pathogenesis*, **127**, 296–303.

Executive Editor-in-Chief Dongming Zhao
Managing Editor Yanyan Sun