Veterinary Microbiology 228 (2019) 61–68

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Unexpected transcriptome p*ompT′* contributes to the increased pathogenicity of a p*ompT* mutant of avian pathogenic *Escherichia coli*

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ARTICLE INFO

Keywords:
Lambda red recombination system
Avian pathogenic *Escherichia coli*
Outer membrane protease T located on plasmid (pOmpT)
Chloramphenicol (*cat*) resistance gene
Pathogenicity

ABSTRACT

The lambda red recombination system makes it suitable for screening virulence gene utility in avian pathogenic *Escherichia coli* (APEC) on account of its wide applicability, simplicity and high efficiency. In APEC E058 (O2 serogroup), there are two copies of the outer membrane protease (*ompT*) gene, c*ompT* encoding cOmpT that is located on the chromosome and p*ompT* encoding pOmpT that is located on a ColV plasmid. However, the relationship between pathogenesis and p*ompT* expression in APEC E058 has yet to be elucidated. Here, we successfully constructed two p*ompT* gene mutants: E058Δp*ompT* containing a chloramphenicol (*cat*) resistance gene and E058Δp*ompT′* without the *cat* gene. By RT-PCR and sequencing analysis, an unexpected transcriptome p*ompT′* was detected in mutant strain E058Δp*ompT′* after deletion of the *cat* gene induced by the lambda red recombination system. Surprisingly, the pathogenicity of mutant E058Δp*ompT* was significantly attenuated compared to its parental strain in the chicken infection model and HD11 cell model then the p*ompT* gene was knocked out, while the pathogenicity of the other mutant strain E058Δp*ompT′* had no difference. Furthermore, the presence of unexpected transcriptome p*ompT′* influenced the bactericidal activity of SPF chicken serum and decreased the transcription level of TLR2 in the heart tissue of chickens. Our study identifies the p*ompT* gene plays an important role in the virulence of APEC E058, and the unexpected transcriptome p*ompT′* contributes to the increased pathogenicity of APEC E058 mutants following deletion of the *cat* gene induced by the lambda red recombination system, which suggests that this system still has some limitations for construction of mutant strains particularly where these are used in development of live vaccine.

1. Introduction

In recent years, the λ red homologous recombination system has been widely applied to study gene function and has played a significant role in the gene modification of a series of pathogenic bacteria.

In 1998, Murphy (1998) first reported a method of gene replacement in *Escherichia coli* that used the λ bacteriophage red recombination system. The recombinant λ phage genes *exo*, *beta* and *gam,* harbored on a multi-copy plasmid, were expressed for gene replacement in wild-type *E. coli* host bacteria. The λ red homologous recombination system is an effective technique for *E. coli* targeted gene replacement and provides a simple and effective method for chromosomal DNA, plasmid or bacterial artificial chromosome targeted gene integration, deletion or point mutation (Mosberg et al., 2010). At present, the two-step homologous recombination method reported by Datsenko and Wanner is the most widely reported and employed (Datsenko and Wanner, 2000). The advantages of this method are that it is simple, quick and accurate (Shen-Cong et al., 2013). However, one defect of the red recombination system is that a FLP recognition target (FRT) site scar is left in the genome of the target strain after gene knockout (Doublet et al., 2008), affecting subsequent operations. To date, red homologous recombination technology has been widely applied in *E.*

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https://doi.org/10.1016/j.vetmic.2018.11.011
Received 29 June 2018; Received in revised form 10 November 2018; Accepted 15 November 2018

coli. For example, using this knockout system, Broach and Hicks (Broach and Hicks, 1980) successfully knocked out more than 40 genes in *E. coli*. In poultry, APEC acting either as a primary or secondary agent can infect all types of birds at all ages, causing localized and systemic infections, often referred to as avian colibacillosis{Dozois and Curtiss, 1998 #3027; Kaper, 2004 #3059} (Dozois and Curtiss, 1998). At present, avian colibacillosis is one of the leading causes of mortality and morbidity associated with economic losses in the poultry industry throughout the world (Kabir, 2011). The virulence factors in APEC are numerous and complex (Guabiraba and Schouler, 2015). OmpT is located in the outer membrane of *Escherichia coli* and is a member of the typical outer membrane protease family. In APEC, there are two copies of the *ompT* gene, c*ompT* encoding cOmpT that is located on the chromosome and p*ompT* encoding pOmpT that is located on a ColV plasmid. The nucleic acid homogeneity between c*ompT* and p*ompT* is 72.2% in APEC E058 (O2 serogroup). Previous studies have reported that cOmpT can degrade the antimicrobial peptides secreted by host cells (Thomassin et al., 2012), and promote *E. coli* to colonize the urinary tract epithelium (Hritonenko and Stathopoulos, 2007; Hui et al., 2010), and that this may be related to the secretion of some virulence factors (Hanke et al., 1992). Previous studies in our laboratory found that the transcription level of *ompT* in APEC strain E058 was significantly up-regulated in a chicken infection model (Zhao et al., 2009). This finding suggested that the *ompT* gene contributes to the pathogenicity of APEC E058.

We found that the pathogenicity of APEC E058 *neuC* (encoding a capsular protein) mutant strain with a deletion of the chloramphenicol resistance (*cat*) gene which resulted in an unexpected transcriptome *neuC'* was increased compared with that of the mutant with the *cat* gene; both of the mutants were developed using λ red homologous recombination technology (Unpublished). There were no previous reports that the pathogenicity of the mutant with an unexpected transcriptome is significantly increased after the removal of the *cat* resistance gene using the λ red recombination system. Certainly, this effect could potentially due to the transcription of the up- and/or downarm of *neuC* gene residuals, other than the removal of *cat* gene per se. Therefore, to investigate the universality of this phenomenon, and confirm the pathogenesis of pOmpT in APEC, p*ompT* mutants of APEC strain E058 with or without the *cat* gene were developed using the λ red recombination system, and their pathogenicity was evaluated both *in vitro* and *in vivo*. Importantly, the contribution of the unexpected transcriptome p*ompT′* to the increased pathogenicity of the APEC E058Δp*ompT* mutant was elucidated.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Supplement 1 (S1). Oligonucleotide primers and probes are listed in S2. All primers and probes used for the amplification of genes were obtained from Sangon (Shanghai, China). APEC strain E058 was isolated from a chicken with the typical clinical symptoms of colibacillosis in China (Gao et al., 1999). All *E. coli* strains were grown in Luria–Bertani (LB) broth or plates at 37°C with aeration. When necessary, antibiotics were added at the following concentrations: 60 μg/mL ampicillin, 30 μg/mL chloramphenicol and 50 μg/mL tetracycline.

2.2. Construction of mutant strains

Isogenic p*ompT* mutant strains of APEC E058 were constructed based on the λ red recombinase system (Datsenko and Wanner, 2000). Briefly, the p*ompT* gene was replaced with a chloramphenicol resistance cassette (*cat* gene) which was amplified from plasmid pKD3 using polymerase chain reaction (PCR). The primers for PCR amplification are listed in **S2**. The p*ompT* gene of the mutant strain was replaced with the *cat* gene, and the mutant was designated E058Δp*ompT*. Then, the *cat* cassette was cured by plasmid pCP20 and the mutant strain was designated E058Δp*ompT′*. The modified p*ompT* gene in these mutants was confirmed by PCR and sequencing.

2.3. Complementation of the E058ΔpompT mutant with an unexpected transcriptome of pompT′ or a native pompT

For the complementation study, both the native p*ompT* gene and the unexpected transcriptome-encoding p*ompT′*, along with their native putative promoters, were amplified and cloned into plasmid pACYC184, using the primers Rep*ompT*-F/R and Rep*ompT′*-F/R, respectively (S2). Promoter prediction analyses were conducted using the prediction program tools available at http://www.fruitfly.org/seq_tools/promoter. The resulting plasmids, p184-p*ompT′* and p184-p*ompT*, were transformed into the mutant strain E058Δp*ompT* to generate the complementation strains by electroporation.

2.4. RNA extraction and reverse transcription (RT)-PCR analysis

Expression of the *ompT* genes in APEC strain E058 and its mutant derivatives was investigated by RT-PCR as described previously (Mu et al., 2013). In brief, wild-type strain E058, the mutant derivatives and their complementation strains were grown to log phase in LB medium at 37°C. Cells were then harvested, washed twice and suspended in phosphate-buffered saline (PBS). Total RNA was isolated using an RNAiso Plus kit (TaKaRa, Dalian, China) according to the manufacturer's recommendations. Contaminating DNA was removed from the samples with gDNA Eraser (TaKaRa, Dalian, China). Then, cDNA synthesis was performed using the PrimeScript RT reagent kit (TaKaRa) according to the manufacturer's protocol. Primer sets for PCR amplification of the target gene p*ompT* and the unexpected transcriptome encoding gene p*ompT′* in cDNA samples are shown in S2. In parallel, PCRs were performed with pAPEC-O2-ColV-like plasmid DNA as the positive controls and cDNA samples without activation of the reverse transcription (RT) as negative controls. The PCR products were resolved on 0.8% agarose gels and visualized by GoodView™ nucleic acid stain (SBS Genetech, Beijing, China). Next, fragments corresponding to the PCR-amplified unexpected transcriptome encoding gene p*ompT′* were excised from the agarose gels using an Axygen DNA gel extraction kit (Corning, Suzhou, China) and were then sent for sequencing verification.

2.5. Determination of LD_{50}

The 50% lethal dose (LD_{50}) was determined in 1-day-old specific-pathogen-free (SPF) chickens (White Leghorn; Jinan SPAFAS Poultry Co., Ltd., Jinan, China) in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (approved by the State Council on 31 October 1988). Briefly, cultures of the wild-type strain and its mutant derivatives were grown to logarithmic phase at 37°C. The bacteria of each strain were then harvested, washed twice, and suspended in sterile PBS prior to dilution to an appropriate concentration (10^8 colony forming units [CFU]/ml), then further diluted to 10^7, 10^6, 10^5 , and 10^4 CFU/ml. Six birds in each group were challenged *via* the air sac with 0.1 mL of each culture suspension containing 10^7, 10^6, 10^5, 10^4 or 10^3 CFU of bacteria. The mock group was injected with sterile PBS. The chickens were observed for 7 days until survival rates were steady. The LD_{50} results were analyzed using the Reed–Muench method and the SPSS statistics software (IBM SPSS statistics version 22, United States).

2.6. Bacterial colonization and persistence assay

Birds were infected with the wild-type, mutant or complementation strains in accordance with the Regulations for the Administration of

Affairs Concerning Experimental Animals (Approved by the State Council on October 31, 1988). Briefly, 15 5-week-old SPF chickens in each group were inoculated with a bacterial suspension containing 10^8 CFU *via* the left thoracic air sac. Birds were euthanized and necropsied at 24 h post-infection. To determine the number of bacteria colonizing the internal organs, the cardiac blood, liver, spleen, lungs and kidneys of every bird were individually and aseptically collected, weighed and homogenized by plating 10-fold serial dilutions on LB agar plates with or without chloramphenicol or tetracycline. The number of bacteria colonizing the tested organs of birds during systemic infection was enumerated.

2.7. In vivo competition assay

Briefly, 5-week-old white leghorn SPF chickens were inoculated with cultures of the mutant strains E058Δp*ompT* and E058Δp*ompT′* mixed in a 1:1 ratio (1×10^8 CFU for each strain) *via* the left air sac. Birds housed in an animal facility were provided with sterile food and water *ad libitum*. At 24 h post-challenge, the cardiac blood, liver, spleen, lungs and kidneys of inoculated birds were collected, weighed and homogenized, and serial dilutions were plated on LB medium with or without antibiotics to differentiate the mutant E058Δp*ompT* from the mutant E058Δp*ompT′*. Serial dilutions of homogenates were spread onto LB plates with or without chloramphenicol and were incubated at 37 °C for 18 h before enumerating the CFUs.

2.8. Bactericidal activity of SPF chicken serum

Complement-sufficient SPF chicken serum was prepared and pooled from 10 SPF chickens (White Leghorn, Jinan SPAFAS Poultry Co., Ltd.). A bactericidal assay was performed in a 96-well plate. SPF chicken serum was diluted to 0.5%, 2.5%, 5.0%, 12.5% and 25.0% in PBS, pH 7.2. Bacteria (10 μL containing 10^6 CFUs) were inoculated into 190 μL reaction wells containing the diluted SPF chicken serum, 25% heat-inactivated SPF chicken serum or PBS alone, and were incubated at 37°C for 30 min. Serial dilutions (1:10) of each well were plated onto LB agar plates. The resulting colonies were counted after 24 h of incubation. This assay was repeated at least twice on at least two separate occasions (Mu et al., 2013).

2.9. Invasion assay in the HD11 cell line

For the invasion assay, avian macrophage cell line HD11 was grown in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Los Angeles, CA, USA) with 10% fetal bovine serum (FBS, PAA, Pasching, Australia) at 37°C in a 5% CO_2 atmosphere at 2×10^5 cells per well in a 24-well cell culture plate. Cells were incubated for 24 h prior to the invasion assays. Bacteria were inoculated into cells with a multiplicity of infection (M.O.I.) of 100. Inoculated cells were incubated at 37°C for 1 h under 5% CO_2 to allow the bacteria to invade the cells. The cells were washed with PBS and then extracellular bacteria were eliminated by incubating cells with DMEM medium containing gentamicin (100 μg/mL) at 37°C for 1.5 h prior to washing the cells with PBS. Intracellular bacteria were treated with 1 mL of 0.1% Triton X-100. Next, 100 μL of the suspension was inoculated into 900 μL of PBS. Serial dilutions (1:10) of each well were plated onto LB agar plates. The resulting colonies were counted after 24 h of incubation. Wells containing only HD11 cells were used as negative controls. The invasion ratio was determined by dividing the number of invading bacteria by the initial inoculum. This assay was repeated three times on at least two separate occasions (Mu et al., 2013).

2.10. Quantification of TLR-2, TLR-21 and NF-κB1 in the organs of challenged birds using real-time PCR

Approximately 15 mg of the heart, liver, spleen and lung tissue of each bird (used in the bacterial colonization and persistence assay, Section 2.6) was collected and separately homogenized in 1 mL of Trizol regent (Invitrogen, Carlsbad, CA, USA). Total RNA extraction and cDNA synthesis was performed as described in Section 2.4. Real-time quantitative RT-PCR (qRT-PCR) was used to quantify mRNA levels of the three Toll-like receptor (TLR)- nuclear factor kappa B (NF-κB) signaling pathway genes: TLR-2, TLR-21 and NF-κB subunit 1 (NF-κB1). Primers and probes specific for quantifying chicken mRNAs are shown in S2. qRT-PCR was performed using SYBR® Premix Ex Taq™ (TaKaRa) and relative gene expression was normalized to the expression of the housekeeping gene β-actin *via* the ΔΔCT method. The assay was performed in duplicate and repeated three times (Mu et al., 2013).

2.11. Statistical analysis

The significance of differences between groups was analyzed using the GraphPad Prism® software program (GraphPad Prism version 5.01, USA). All data were analyzed by the Mann–Whitney test.

3. Results

3.1. The pompT and pompT′ mutants and their complementation derivatives were constructed correctly in APEC E058

The p*ompT* gene situated on the pAPEC-O2-ColV-like plasmid from APEC E058 was sequenced and confirmed to be 954 bp in length. The APEC E058 mutants were created by the two-step method described by Datsenko and Wanner (Datsenko and Wanner, 2000), as shown in S3.

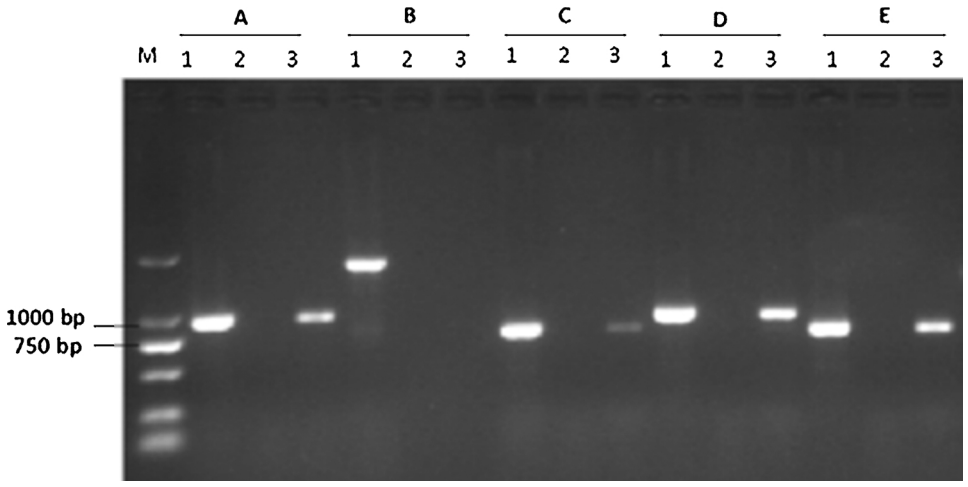

Fig. 1. RT-PCR analysis of the transcription of the p*ompT* or p*ompT′* genes. Lanes: A, E058; B, E058Δp*ompT*; C, E058Δp*ompT′*; D, ReE058Δp*ompT*-p*ompT*; E, ReE058Δp*ompT*-p*ompT′*. Sample identities: lanes 1, genomic DNA from E058, E058Δp*ompT*, E058Δp*ompT′*, ReE058Δp*ompT*-p*ompT* or ReE058Δp*ompT*-p*ompT′*; lanes 2, total RNA from E058, E058Δp*ompT*, E058Δp*ompT′*, ReE058Δp*ompT*-p*ompT* or ReE058Δp*ompT*-p*ompT′* without RT after genomic DNA was removed; lanes 3, cDNA derived from the total RNA of E058, E058Δp*ompT*, E058Δp*ompT′*, ReE058Δp*ompT*-p*ompT* or ReE058Δp*ompT*-p*ompT′*. DL2000 marker (TaKaRa) was used as the molecular size standard (lane M).

RT-PCR analysis showed that the p*ompT* gene has been disrupted by the chloramphenicol resistance cassette, and was not normally transcribed in the mutant strain E058Δp*ompT* (Fig. 1B). Meanwhile, in the mutant E058Δp*ompT′* which p*ompT* gene was disrupted other than replaced by the chloramphenicol resistance cassette, an unexpected transcriptome (p*ompT′*) consisted of up- and down-arm of p*ompT* gene residuals was transcribed together with the FRT site scar after the removal of *cat* gene (Fig. 1C). Furthermore, the unexpected transcriptome (p*ompT′*) was found to be normally transcribed in the complementation strain ReE058Δp*ompT*-p*ompT′* (Fig. 1E). Sequence alignment of the p*ompT* gene and the unexpected transcriptome of p*ompT′* is shown in S4, which further confirmed that an unexpected chimeric p*ompT′* consisted of up- and down-arms of p*ompT*, primers of *cat* gene and FLP recognition target sites (FRT) residual during deleting the chloramphenicol resistance gene (*cat*) following the lambda red recombination system. In the mutant E058Δp*ompT′*, 258 bp fragment in the p*ompT* open reading frame was deleted, and 117 bp of FRT residual remained, so the size of unexpected transcriptome of p*ompT′* was 813 bp which was smaller than that of p*ompT* gene (954 bp) (Figs. 1C, 1E; S4).

3.2. E058ΔpompT is significantly attenuated compared with its wild-type parental strain, but the pathogenicity of mutant E058ΔpompT′ is increased compared with mutant E058ΔpompT

The results of the LD_{50} assay are shown in Table 1. At 7 days post-infection, the LD_{50} values of E058, E058Δp*ompT,* E058Δp*ompT′,* ReE058Δp*ompT*-p*ompT* and ReE058Δp*ompT*-p*ompT′* were $10^{3.005}$, $10^{4.897}$, $10^{3.298}$, $10^{4.484}$ and $10^{3.926}$, respectively. Compared with the wild-type strain E058, the pathogenicity of mutant strain E058Δp*ompT* was almost 78 times lower ($p < 0.001$), but there was no obvious difference in virulence between the mutant strain E058Δp*ompT′* and wild-type strain E058 ($p > 0.05$). The LD_{50} of birds infected with the complementation strain ReE058Δp*ompT*-p*ompT′* was nearly ten times greater than that of the mutant strain E058Δp*ompT* ($p < 0.01$), which suggested that the unexpected transcriptome p*ompT′* potentially affects the virulence of APEC mutants. Unfortunately, the LD_{50} value of complementation p*ompT* gene was not restored to that of wildtype strain E058 ($p < 0.01$) (Table 1).

3.3. The colonization and persistence of mutant strain E058ΔpompT decreased in vivo, whereas its derivative E058ΔpompT′ promoted colonization and persistence

Compared with the wild-type strain, the bacterial burden in the cardiac blood, liver, spleen, lungs and kidneys of birds infected with mutant strain E058Δp*ompT* was ~10^3-fold lower than that of the parental E058 strain (Fig. 2A–E) (all $p < 0.001$), whereas loads in the liver, spleen, lungs and kidneys of birds infected with the mutant strain E058Δp*ompT′* have no difference with that of wildtype strain E058 (Fig. 2B–E) ($p > 0.05$), except the cardiac blood (Fig. 2A) ($p < 0.05$). In contrast, the bacterial load in these tissues of birds infected with mutant strain E058Δp*ompT′* was 10^2-fold higher than that of the mutant strain E058Δp*ompT* (Fig. 2A–E) (all $p < 0.01$). The increased capacity for colonization and persistence of the complementation strain with the unexpected transcriptome p*ompT′* was indicated by the number of bacteria recovered from the liver (Fig. 2B) ($p < 0.01$), spleen (Fig. 2C) ($p < 0.05$), lungs (Fig. 2D) ($p < 0.05$) and kidneys (Fig. 2E) ($p < 0.05$) compared with the mutant strain E058Δp*ompT*, whereas loads from the complementation strain with *ompT* could not be recovered to the same level as the wild-type strain E058 in all of the organs tested and exhibited no difference with those in the mutant strain E058Δp*ompT* (Fig. 2A–E) ($p > 0.05$).

Table 1
LD_{50} of the wild-type and mutant/complementation strains.

Strains	Deaths/birds Inoculated					LD50
	Inoculated Doses(CFU /bird)					
	10^7	10^6	10^5	10^4	10^3	
E058	6/6	6/6	5/6	5/6	4/6	$10^{3.005}$
E058Δp*ompT*	6/6	4/6	2/6	1/6	1/6	$10^{4.897}$
E058Δp*ompT′*	6/6	6/6	6/6	4/6	3/6	$10^{3.298}$
ReE058Δp*ompT*-p*ompT*	6/6	5/6	4/6	2/6	1/6	$10^{4.484}$
ReE058Δp*ompT*-p*ompT′*	6/6	6/6	5/6	2/6	2/6	$10^{3.926}$

3.4. Mutant strain E058ΔpompT′ showed more competitive growth in chicken tissues than E058ΔpompT

At 24 h post-challenge, the mutant E058Δp*ompT′* showed significantly improved growth in the blood, liver, spleen and lungs ($p < 0.01$) (Fig. 2F). In the kidneys, the growth of mutant strain E058Δp*ompT′* was also enhanced compared with mutant strain E058Δp*ompT* ($p < 0.05$) (Fig. 2F).

3.5. The anti-phagocytic ability of the mutant E058ΔpompT strain on HD11 macrophages was slightly attenuated compared with the wild-type and E058ΔpompT′ mutant strain

At 1 h post-infection, the percent of intracellular bacteria (in HD11 macrophages) observed with strains E058, E058Δp*ompT,* E058Δp*ompT′* and ReE058Δp*ompT*-p*ompT′* were 0.00147%, 0.00896%, 0.00314% and 0.00422%, respectively. The anti-phagocytic ability of the E058Δp*ompT* mutant on HD11 macrophages was significantly reduced compared with the wild-type strain (Fig. 3A) ($p < 0.05$), and compared with the E058Δp*ompT* mutant, the anti-phagocytic ability of the E058Δp*ompT′* mutant and the complementation strain ReE058Δp*ompT*-p*ompT′* was also significantly weakened (Fig. 3A) ($p < 0.05$).

3.6. The unexpected transcriptome pompT′ had a slight effect on the bactericidal activity of SPF chicken serum in the E058 mutants

In a bactericidal assay, strains E058, E058Δp*ompT*, E058Δp*ompT′* and ReE058Δp*ompT*-p*ompT′* all rapidly adapted to growth in the presence of chicken serum (Fig. 3B), indicating that pOmpT might be unrelated to serum complement resistance. However, compared with mutant strain E058Δp*ompT*, the growth of strain E058Δp*ompT′* was slightly elevated under serum concentrations of 2.50% and 5.00%, respectively (Fig. 3B).

3.7. pompT and the unexpected transcriptome pompT′ can decrease the transcription level of TLR2 in the heart tissue of chickens

The relative transcription levels of the *tlr-2, tlr-4* and *nf-kb1* genes in the tested organs were up-regulated to some extent, but there were no significant differences between them (Fig. 4A–D) ($p > 0.05$), except for *tlr-2* in the heart tissue of challenged chickens. Compared with mutant E058Δp*ompT*, the relative transcription level of the *tlr-2* gene in the heart of birds infected with the wild-type strain E058 and mutant strain E058Δp*ompT′* was significantly lower than that in birds infected with the E058Δp*ompT* mutant (Fig. 4A) ($p < 0.05$).

4. Discussion

The lambda red recombination system makes it suitable for screening virulence gene utility in avian pathogenic *Escherichia coli* (APEC) on account of its wide applicability, simplicity and high efficiency. Furthermore, this system allows mutants with no antibiotic resistance genes to be generated, which is advantageous for vaccine development. However, the system has potential limitations that remain relatively unexplored, such as whether the antimicrobial

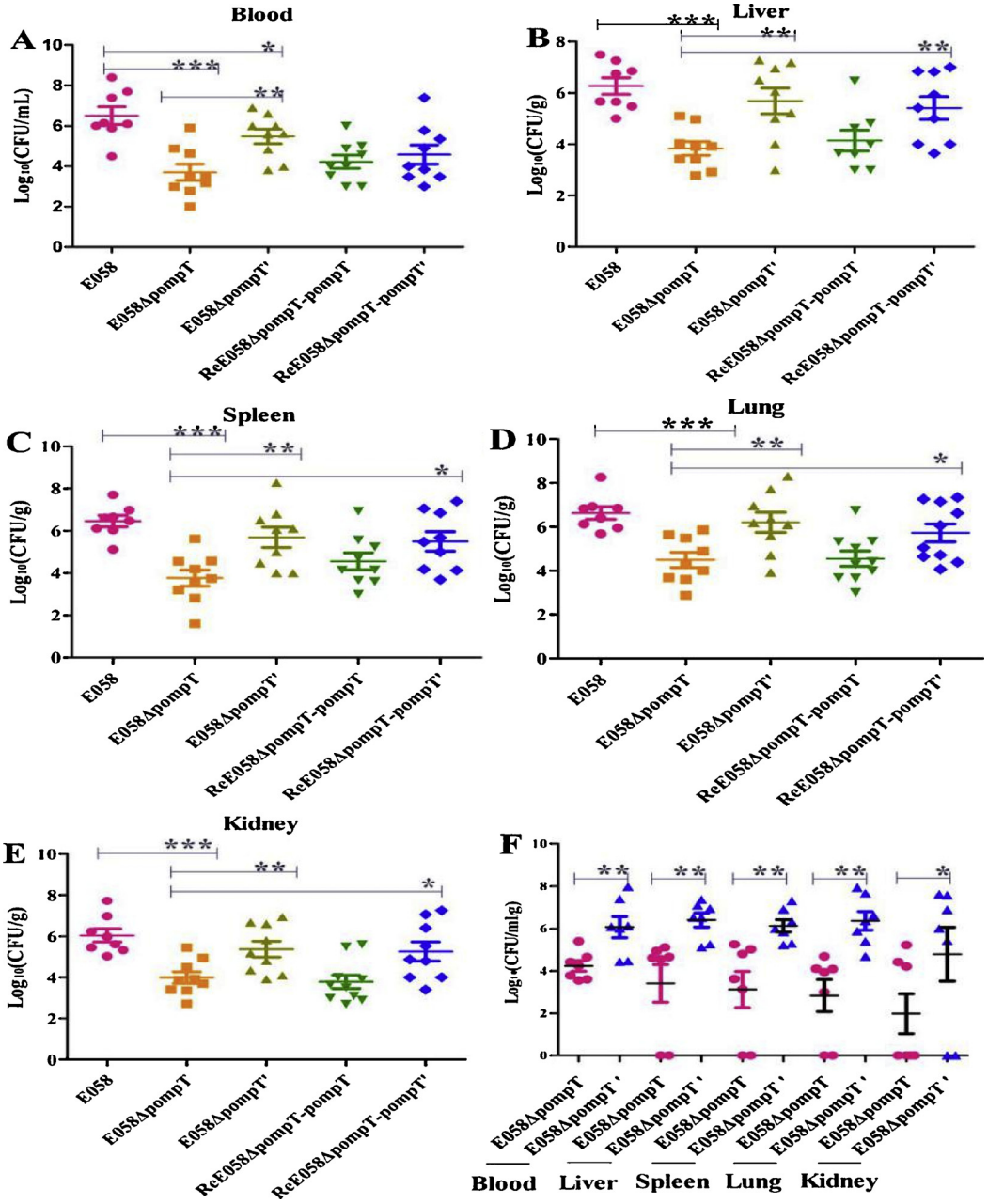

Fig. 2. *In vivo* colonization and persistence assay and competition assay. A–E: Colonization and persistence of the wild-type strain E058 (Pink), mutant strain E058Δp*ompT* (Yellow), mutant strain E058Δp*ompT′* (Reseda), complementation strain ReE058Δp*ompT*-p*ompT* (Green) and complementation strain ReE058Δp*ompT*-p*ompT′* (Blue) during systemic infection. F: Mutant strains E058Δp*ompT* (pink) and E058Δp*ompT′* (blue) were inoculated simultaneously. Each data point represents a single sample from an individual bird, and data are presented as the $\log_{10}$ CFU/g/mL of tissue. Horizontal bars indicate the mean $\log_{10}$ CFU/g/mL values. The data were analyzed using a Mann–Whitney test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

resistance gene, when left in or removed from the target gene, will interrupt normal transcription and expression of this gene, and conversely, what happens when the antimicrobial resistance gene is removed from the mutant. These questions formed the basis of the current study.

In a recent study, the role of cOmpT in pathogenicity of APEC strain TW-XM has been explored (Hejair et al., 2017). In this study, pOmpT and its pathogenesis in APEC strain E058 was focused. The protein pOmpT encoded on the *ColV* plasmid of the O2 serotype strain APEC E058 belongs to the omptin protein family, as does the cOmpT protein, which is an outer membrane defense protein expressed by Gram-negative bacteria. We found that pOmpT was unable to inactivate the protamine, which is the main substrate of the outer membrane protease encoded by the c*ompT* gene of *E. coli*. However, the role of pOmpT in the pathogenesis of APEC remains unclear. Therefore, the plasmid harboring the *ompT* gene was selected for mutation, and the pathogenicity of mutants before and after removal of the resistance gene was studied.

In this study, we successfully constructed two mutant strains of the p*ompT* gene of APEC E058, with and without antibiotic resistance genes, using the λ red recombination system, namely E058Δp*ompT* and E058Δp*ompT′*. An unexpected transcriptome p*ompT′* could be identified after deletion of the cat^r gene from mutant E058Δp*ompT*. The assumed promoter of the p*ompT* gene and the unexpected transcriptome p*ompT′* encoding fragment were cloned together into a low copy plasmid pACYC184, and p*ompT′* could also be transcribed normally under the

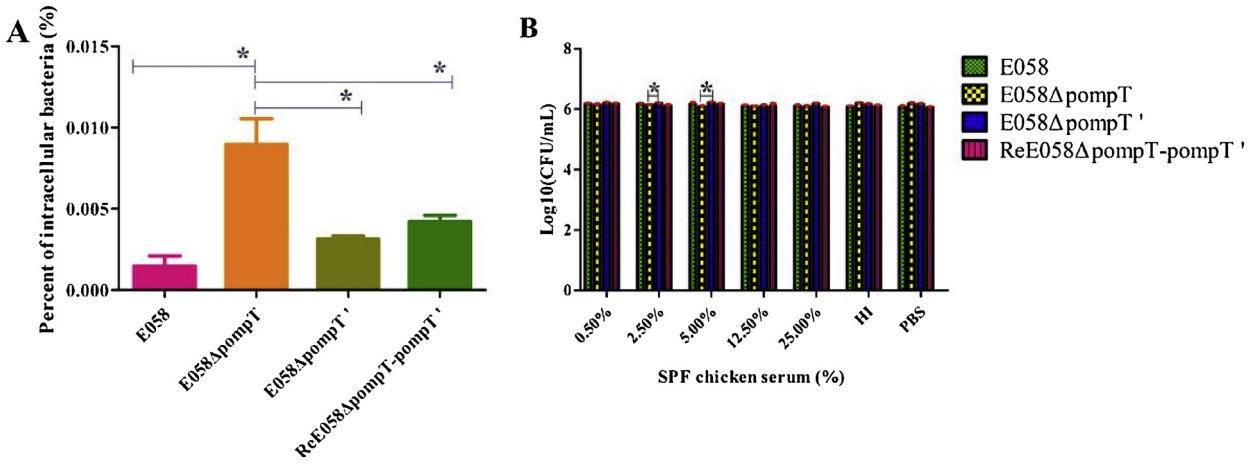

Fig. 3. *In vitro* invasion assay and bacteriacial assay. A. Ability of bacterial strains to invade the chicken macrophage HD11 cell line. Bacterial strains: E058 (pink bar), E058Δp*ompT* (orange bar), E058Δp*ompT′* (Reseda bar) and ReE058Δp*ompT*-p*ompT′* (green bar). B. Bactericidal activity of SPF chicken serum against the wild-type, mutant and complementation strains. Strains: E058 (green bar), E058Δp*ompT* (yellow bar), E058Δp*ompT′* (blue bar), ReE058Δp*ompT*-p*ompT′* (red bar). HI represents the group of heat-inactivated 25% SPF chicken serum used as a control for each strain. PBS was used as a negative control. The data represent the averages of three independent assays. Statistically significant differences in values between E058Δp*ompT* and its derivative are indicated with asterisks (*, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

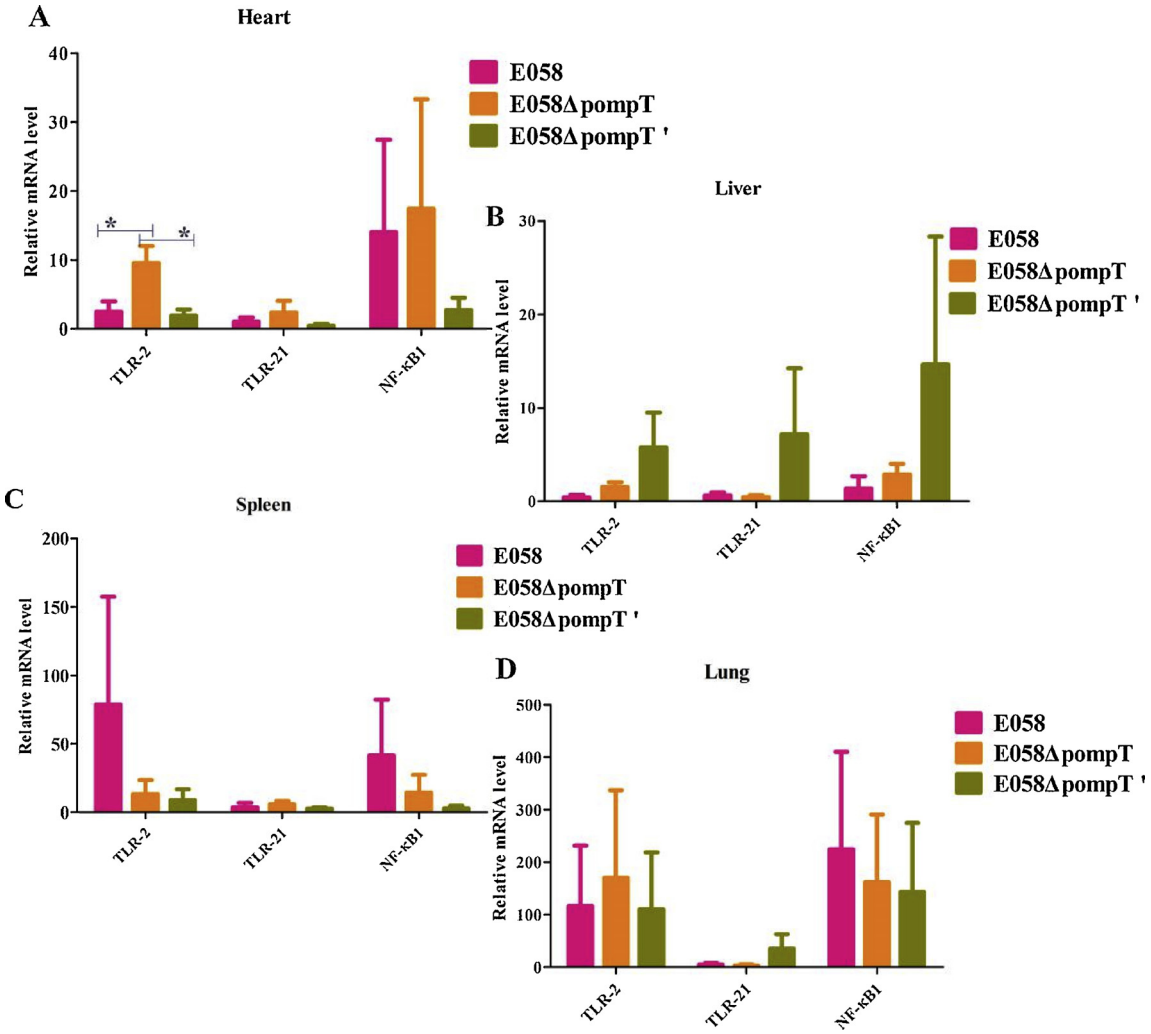

Fig. 4. Quantitative RT-PCR analysis of the relative transcription levels of the *tlr2*, *tlr21* and *nf-κb1* genes in the tested organs of birds infected with wild-type E058, or mutant strains E058Δp*ompT* or E058Δp*ompT′*. Error bars indicate the standard deviations of triplicate measurements. Asterisks indicate statistically significant differences (*, $p < 0.05$).

control of the native p*ompT* putative promoter (Fig. 1E). This result suggested that the up- and down-arms of the original p*ompT* gene together with the introduced foreign fragment (FRT sequence) in the unexpected transcriptome p*ompT′* may be re-encoded and transcribed normally. We therefore speculated that the unexpected transcriptome p*ompT′* may play an important but undefined role.

In one-day-old SPF chickens, the LD_{50} of the E058Δp*ompT* mutant was 78 times higher than that of the wild-type strain ($p < 0.001$). Unexpectedly, we found that the LD_{50} of the E058Δp*ompT′* mutant was lower than that of the E058Δp*ompT* mutant, with the LD_{50} of E058Δp*ompT′* being reduced 40 times after removal of the antibiotic resistance gene ($p < 0.01$). Compared with that of the E058Δp*ompT* mutant, the LD_{50} of ReE058Δp*ompT*-p*ompT′*, which was complemented with the unexpected transcriptome p*ompT′*, recovered by nearly 10 times ($p < 0.05$)(Table 1). These findings were confirmed by a colonization and persistence test in 35-day-old chickens. Compared with the parental strain, the ability of the mutant E058Δp*ompT* to colonize and persist in the target organs of chickens, including the cardiac blood, liver, spleen, lungs and kidneys, decreased dramatically (Fig. 2A–E) ($p < 0.001$), which indicated that p*ompT* greatly contributes to the pathogenicity of APEC. However, when mutants E058Δp*ompT* and E058Δp*ompT′* and the complementation strain ReE058Δp*ompT*-p*ompT′* were introduced into the same animal model, unexpectedly the bacterial burden in the cardiac blood, liver, spleen, lungs and kidneys of birds infected with the E058Δp*ompT′* mutant was higher than that observed with the E058Δp*ompT* mutant (Fig. 2A–E) ($p < 0.01$). Similar CFUs were recovered from the cardiac blood, liver, spleen, lungs and kidneys of birds inoculated with the complementation strain ReE058Δp*ompT*-p*ompT′* and the mutant strain E058Δp*ompT′*, suggesting that enhancement of the pathogenicity of a mutant with the antibiotic resistant gene deletion may indeed be due to the unexpected transcriptome. To further verify this result, the capacity of the E058Δp*ompT* mutant to compete for growth with the mutant strain E058Δp*ompT′* in the tissues of 35-day-old SPF chickens was also evaluated in a co-infection chicken model. The results showed that the mutant E058Δp*ompT* was significantly attenuated compared with the mutant strain E058Δp*ompT′* in the co-infection model, with bacterial loads significantly reduced compared with E058Δp*ompT′* in the cardiac blood ($p < 0.01$), liver ($p < 0.01$), spleen ($p < 0.01$), lungs ($p < 0.01$) and kidneys ($p < 0.05$) (Fig. 2F).

For *E. coli* to survive and proliferate in the host, it must first evade the complex defense mechanisms of the host. Previous studies have shown that *E. coli* has evolved some complex escape defense mechanisms (Fetherston et al., 2010; Kwon et al., 2013), (Caza et al., 2011; Gao et al., 2012), one of which is phagocytosis{Brubaker, 2003 #3109; Brubaker, 2003 #3109; Heesemann et al., 2006 #3115}(Brubaker, 2003; Heesemann et al., 2006). The results of this study showed that the anti-phagocytic ability of the E058Δ*ompT* mutant on HD11 macrophages was significantly reduced compared with the wild-type strain (Fig. 3A) ($p < 0.05$). This may suggest that pOmpT can affect the anti-phagocytic ability of APEC E058 on macrophages. Compared with the E058Δp*ompT* mutant, the anti-phagocytic ability of the E058Δp*ompT′* mutant and the complementation strain ReE058Δp*ompT*-p*ompT′* was also significantly elevated (Fig. 3A) ($p < 0.05$), which was consistent with the results of the LD_{50} test, the *in vivo* colonization and persistence test and the *in vivo* competition test.

The bactericidal effect of complement in serum represents another defense mechanism. In fact, serum resistance has been proven to be an important virulence factor (Mellata et al., 2003). Our results showed that compared with the E058Δp*ompT* mutant, E058Δp*ompT′* showed significantly strengthened antiserum bactericidal ability under serum concentrations of 2.50% and 5.00% (Fig. 3B) ($p < 0.05$). This result may suggest that the presence of the unexpected transcriptome p*ompT′* affects the serum resistance of APEC in some tissues.

The innate immune system is the first line of defense against invading pathogens and activation of the TLR/NF-κB signaling pathway plays an important role in this defense system. Two major types of TLRs are involved in recognition of the conserved region of bacteria in chickens: TLR-2, which recognizes peptidoglycan, and TLR-21, which recognizes unmethylated CpG DNA usually found in bacteria (Keestra et al., 2010). Then TLRs initiate a series of signal transduction intermediators and activate NF-κB to induce the production of pro-inflammatory cytokines (Luo et al., 2012). In this study, the results showed that compared with mutant strain E058Δp*ompT*, the expression of the *tlr-2* gene in birds infected with wild-type strain E058 and mutant strain E058Δp*ompT′* was significantly lower in the heart (Fig. 4A) ($p < 0.05$), which indicated that p*ompT* and the unexpected transcriptome p*ompT′* can decrease the transcription level of TLR-2 in the heart. A previous study showed that inactive *E. coli* can also induce TLR-2 (Zhong et al., 2014), which was in accordance with our results.

In summary, these results suggest that the single p*ompT* gene mediating outer membrane protein T biosynthesis is likely to play an important role in the virulence of APEC E058, and deletion of the *cat* gene in mutant strain E058Δ*ompT* induced by the λ red recombination system contributes to the increased pathogenicity of the APEC mutant strain E058Δp*ompT* mutant. This effect might due to the transcription of the up- and/or down-arm of p*ompT* gene residual consisted in the unexpected transcriptome *pompT'*, not due to the removal of *cat* gene per se. However, the actual mechanism of this effect needs to be elucidated in the future. Meanwhile, the λ red recombination system should be noted that deletion of the cat^r gene in the system can enhance the pathogenicity of the derived mutant strains, which has particularly important implications for the development of attenuated vaccines using this system.

Acknowledgements

We are grateful to Prof. Xin'an Jiao of the Jiangsu Key Laboratory of Zoonosis at Yangzhou University for kindly providing the HD11 cell line, and Prof. Guoqiang Zhu at Yangzhou University for kindly providing the plasmid pACYC184. This study was supported by grants from the National Natural Science Foundation of China (31672553, 31272559), the National Key R&D Program of China (2017YFD0500203-2, 2017YFD0500705), the National Program for High Technology Research and Development in China (2003AA 222141), the Special Fund for Agroscientific Research in the Public Interest (201303044 to S.G.), the Natural Science Foundation of Jiangsu Province (BK20151308), and the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). We thank Kate Fox, DPhil, from Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2018.11.011.

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