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Functional role of *ompF* and *ompC* porins in pathogenesis of avian pathogenic *Escherichia coli*

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ABSTRACT

Avian pathogenic *Escherichia coli* is an important pathogen causes systemic infections in avian species and large economic losses in poultry industry worldwide. The functional role of porins during the infection and their mechanisms of interaction with host tissues for adhesion to and invasion are poorly understood. However, whether porins play a role in infection remains unclear. In this study we evaluated the potential of *ompF* and *ompC* outer membrane porins in the pathogenesis of avian pathogenic *E. coli* (APEC) strain TW-XM. The *ompF* and *ompC* were deleted to generate a series of mutants. We found that, Δ*ompF* and Δ*ompC* reduced significantly the adherence by 41.3% and 46.1% and invasion capabilities of APEC to mouse brain microvascular endothelial cell (BMEC) bEnd.3 cells *in vitro* by 51.9% and 49.7% respectively, compared with the wild strain TW-XM. I*n vivo* experiment based on the measurement of the LD_{50} have also shown that, Δ*ompF* and Δ*ompC* reduced the bacterial virulence by 9.8-fold, 12.3-fold in ducklings and 9-fold, 10.2-fold in mouse models. Animal infection experiments further revealed that, loss of *ompF* and *ompC* reduced TW-XM colonization and invasion capacity in brains, lungs and blood compared to wild-type strain TW-XM ($P > 0.01$). These virulence-related phenotypes were partially recoverable by genetic complementation. The results of the quantitative real-time reverse transcription-PCR (qRT-PCR) indicated that, the loss of *ompF* and *ompC* significantly decreased the expression levels of *ompA*, *fimC* and *iBeA* genes in the mutant strains, compared to wild-type strainTW-XM ($P < 0.01$). Collectively, our data demonstrate that inactivation of these two porins decreased adhesion, invasion, colonization, proliferation capacities, possibly by reduced expression levels of *ompA*, *fimC* and *iBeA*, which may indicate the involvement of *ompF* and *ompC* in APEC pathogenesis.

1. Introduction

Extra-intestinal pathogenic *Escherichia coli* (ExPEC) is a group of strains that cause a large range of infections in humans and animals, including neonatal meningitis, urinary tract infections (UTIs), pneumonia, and sepsis [1]. Avian pathogenic *Escherichia coli* (APEC), is most important ExPEC subgroup, which causes systemic infections that affects the poultry industry worldwide [2]. Virulence factors associated with APEC, include iron uptake systems [3], autotransporter genes [4,5], lipopolysaccharide (LPS) O antigens [3], K1 capsule, secretion systems [6] and certain fimbrial adhesins [3]. However, outer membrane proteins were also involved in these processes [3].

The bacterial outer membrane presents in cytoplasmic membrane and peptidoglycan layer which forms the interface between the host cell and its external environment. It's permeable to small, polar molecules but not the large or charged molecules. Pathogenicity is largely dependent on its surface structures. Among the components of the bacterial outer membrane proteins, porins play a crucial role in bacterial pathogenesis [7]. Porins can be divided into monomeric and trimeric [8]. The monomeric includes *ompA* of *E. coli* and *oprF* of *Pseudomonas aeruginosa* while the trimeric includes *ompF* of *E. coli* and porin of *Rhodobacter capsulatus* [9,10].

OmpC and OmpF are two major porin proteins of *E. coli*, which function as passive diffusion channels for small molecules, nutrients, antibiotics and toxic salts. Expression of *ompC* and *ompF* is regulated by osmolarity [11]. The loss of *ompC* in *E. coli* reduce adherence and the ability to invade intestinal cells [12,13]. The

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ompF porin is an integral membrane protein located in the outer membrane of the bacteria *E coli*. *It* has putative antigenic epitopes located on various loops [14,15], indicating that it may have some immuno-properties and may play a role in bacterial pathogenesis, such as adherence, invasion, and serum resistance [16,17]. Studies have demonstrated that loss of *ompC* and *ompF* of *Salmonell typhimurium* attenuated its virulence [18]. However, whether the *ompF and ompC* contribute to APEC pathogenesis remains unclear. To investigate potential of the APEC *ompF* and *ompC* in the pathogenicity, *ompF* and *ompC* deleted APEC mutant strains TW-XM and complementary strains were constructed and the invasion capacity, virulence and expression of invasion and adhesion associated virulence factors were estimated to describe the contributing of *ompF* and *ompC* in APEC pathogenesis.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

A total of 144 APEC strains were isolated from affected brains of diseased ducks with septicaemia and neurological symptoms as previously described [19] (Table S1). The plasmids used in this study are listed in Table 1. *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 °C. Kanamycin, Ampicillin and Chloramphenicol were used at 50 μg/ml, 100 μg/ml and 30 μg/ml respectively.

2.2. DNA manipulations

Pure Plasmid Mini Kit (OMGA.bio-tek, Shanghai, China) were used to isolate and purify plasmids. Primers were synthesized by (Sunny Biotech, Shanghai, China) while restriction enzymes were purchased from TaKaRa (Dalian, China). The DNA and amino acid sequence analysis was performed using online BLAST program of the National Center for Biotechnology Information (NCBI) and DNASTAR Lasergene 7 software.

2.3. Expression and purification of recombinant protein OmpF and OmpC

The ompF and ompC open reading frames (ORF) were amplified with PCR using primers *ompF*-F, *ompF*-R and *ompC*-F, *ompC*-R that contains *EcoR* I and *BamH* I restriction sites and their sequences were shown in Table 2. The PCR product were digested with *EcoR* I and *BamH* I and cloned into pET28a (+) vector (Novagen, Madison, WI, USA) previously digested with the same enzymes. The cloning was verified by *EcoR* I and *BamH* I digestion and analysis of DNA sequencing. The resulting plasmids were designated pET28a-OmpF and pET28a-OmpC. The plasmids were transformed into competent *E. coli* BL21 (DE3) cells, and the OmpF, OmpC proteins were expressed by adding 1 mM IPTG (isopropyl-ß-D-thiogalactopyranoside). Proteins purification was prepared using a HisTrap HP column which was purchased from GE Healthcare, Shanghai, China. The purified OmpF and OmpC proteins were dialyzed overnight at 4 °C against 500 ml of dialysis buffer (50 mM sodium phosphate, pH 7.4–7.6) followed by a concentration step using an Amicon Ultra-4 centrifugal filter (10,000-Da cutoff; Millipore, Billerica, MA, USA). Proteins concentration were measured using SmartSpec3000 (Bio-Rad, Shanghai, China) [20]. Initial expression and purity of the fusion proteins were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Polyclonal antibodies production

Polyclonal antibodies against OmpF and OmpC were generated in New Zealand White rabbits by inoculating subcutaneously with purified protein (300 μg) emulsified with an equal amount of adjuvant ISA 206 (SEPPIC, Lyon, France) [21]. Immunizations were done three times at 2-week intervals. Finally, the rabbits were sacrificed after ten days from last immunization and serums were collected.

2.5. Western blot analysis

Western blotting was performed as previous described [22]. Protein samples were separated by 10% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane using a semi-dry blotting apparatus (TE77; Amersham Pharmacia Biotech) and a buffer containing 0.037% SDS, 48 mM Tris base, 39 mM glycine and 20% methanol. Immune serum (anti-OmpF and OmpC) were used as the primary antibodies and horseradish peroxidase-conjugated anti-rabbit immunoglobulin as the secondary antibody. Diaminobenzidine chromogenic was used as the substrate.

Table 1
Bacterial strains and plasmids used in this study.[a]

Strain, plasmid	Description or sequence	Reference
***E. coli* strains**		
TW-XM	O2:K1	Lab Collection
ΔOmpF	Deletion mutant of OmpF with TW-XM background	This study
CΔOmpF	APEC TW-XM ΔOmpF with the vector pUC19-*OmpF*, Ampr	This study
ΔOmpC	Deletion mutant of OmpCwith TW-XM background	This study
CΔOmpC	APEC TW-XM ΔOmpC with the vector pUC19-*OmpC*, Ampr	This study
DH5α	Cloning host for maintaining the recombinant plasmids	Invitrogen
BL21	Host for expressing the recombinant proteins	Invitrogen
Plasmids		
pET-28a (+)	Expression vector, Kanr	Invitrogen
pET28a-OmpF	pET28a inserted in frame with the OmpF gene for expressing OmpF	This study
pET28a-OmpC	pET28a inserted in frame with the OmpC gene for expressing OmpC	This study
pUC19	*E. coli* shuttle vector; Ampr	TaKaRa
pUC19-OmpF	pUC19 containing the promoter followed by the full-length *OmpF*ORF	This study
pUC19-OmpC	pUC19 containing the promoter followed by the full-length OmpCORF	This study
pKD46	Δred recombinase expression plasmid	Datsenko and Wanner (2000)
pKD4	pANTSΔ derivative containing FRT-flanked kanamycin resistance	Datsenko and Wanner (2000)
pCP20	TS replication and thermal induction of FLP synthesis	Haldimann and Wanner

[a] ORF, open reading frame; FRT, FLP recombination target; TS, temperature sensitive.

Table 2
Primers and their sequences used in this study.

Primer	Sequence (5′–3′)[a]	Target gene
OmpF-F	GGCAATGGTGACATGACCTAT	OmpF
OmpF-R	ACTCTTCCTGCAGGTTGGTAC	OmpF
OmpF-F	**CGCGGATCC**GATCTGTACGGTAAAGCTGTCG	OmpF
OmpF-R	**CCGCTCGAG**CTGGTAAACGATACCCACAGCA	OmpF
OmpF-F	TGACAGAACTTATTGACGGCAGTGGCAGGTGTCATAAAAAAA CCATGAGGGTAATAAATAGTGTAGGCTGGAGCTGCTTC	pKD46
OmpF-R	GGCATAAAAAAAACAGGACCAAAGTCCTGTTTTTTCG GCATTT AACAAAGAGGTGTGCTACATATGAATATCCTCCTTAG	pKD46
OmpF-F	AAGATGCCTGCAGACACATAA	Upstream region of OmpF
OmpF-R	TCAGGGTAACGGGAGATTTAC	Downstream region of OmpF
OmpC-F	GGCGTACAATGAAAAAATTAA	OmpC
OmpC-R	R GAGCAGCAAAGTTCAGGCCAT	OmpC
OmpC-F	**CGCGGATCC**AAGCTGGATCTATACGGGAAAG	OmpC
OmpC-R	**CCGCTCGAG**AACCAGACCTACAGCGACGATG	OmpC
OmpC-F	CTACTTCACAAATTAAATGAGAAACTAAAACTTACATCTT GAA ATAATCACATTGATTAGGTGTAGGCTGGAGCTGCTTC	pKD46
OmpC-R	GCTCCACTTATATGTTGCGGAGGCAAAGCCTCCCGCAACATAT CTTTTTCGTAAGTCAGACATATGAATATCCTCCTTAG	pKD46
OmpC-F	GACAAGAGCCATGAATAGGAT	Upstream region of OmpC
OmpC-R	TTCTAAAGGAAGTGGCTTGAG	Downstream region of OmpC
k1	CAGTCATAGCCGAATAGCCT	pKD46
k2	CGGTGCCCTGAATGAACTGC	pKD46
dnaE RT-F	ATGTCGGAGGCGTAAGGCT	DnaE
dnaE RT-R	TCCAGGGCGTCAGTAAACAA	DnaE
OmpA RT-F	TGGGTGTTTCCTACCGTTTC	OmpA
OmpA RT-R	GAGTGAAGTGCTTGGTCTGT	OmpA
fimC RT-F	CCACAGGATCGGGAAAGTTTAT	FimC
fimC RT-R	TGCGAGCTGTAGCGTATTC	FimC
tsh RT-F	CCACAGGATCGGGAAAGTTTAT	Tsh
tsh RT-R	TTGCGAGCTGTAGCGTATTC	Tsh
ibeA RT-F	CGCGATATGTTTAGCCCTTATCT	IbeA
ibeA RT-R	CCGCCTAACGTCACATCTTT	IbeA
LusX RT-F	CGCGATATGTTTAGCCCTTATCT	LusX
LusX RT-R	CCGCCTAACGTCACATCTTT	LusX

[a] Restriction sites are underlined.

2.6. Construction of ompF and ompC isogenic mutants

The *ompF* and *ompC* in-frames deletion mutants were constructed using the lambda Red recombinase as previously described [23,24] The *ompF* and *ompC* were replaced with a kanamycin resistance gene, which was amplified from plasmid pKD4 using PCR with primers o*mpF*-F, o*mpF*-R and *ompC-F, ompC-R* (Table 2). The 5′ regions of the primers were homologous to the corresponding flanking region of *ompF* and *ompC*. The PCR products were transformed by electroporation into TW-XM strain including pKD46 as the lambda Red recombinase expression plasmid. After electroporation, samples were incubated for 1 h at 37 °C in SOC broth and plated on LB agar containing kanamycin to pick *ompF* and *ompC* mutants. Mutants were verified by PCR and sequencing using primers K1 and K2 in mixture with primers o*mpF*-F, o*mpF*-R and *ompC-F, ompC-R* which flanked *ompF* and *ompC*. The Kanamycin resistance cassette was recovered by transforming the pCP_{20} plasmid into the mutants and selecting for a Kanamycin sensitive mutant strains, which were finally constructed mutants TW XM.

2.7. Complementation of ompF and ompC mutants

For complementation studies, the coding sequences of *ompF, ompC* and their putative promoters region, were amplified from the TW-XM genome independently and cloned into the *pMD19*-T vector using primer pairs o*mpF*-F, o*mpF*-R and *ompC-F, ompC-R* with restriction enzyme recognition sites (*Hind* III and *BamH* I), and then transformed into *E. coli* DH5α cells. Positive colonies were chosen and determined by PCR and sequencing. The resulting plasmid *pMD19*-T-o*mpF, pMD19*-T-*ompC* and *pUC19* were then digested with restriction enzymes *Hind* III and *BamH* I, and ligated using T4 DNA Ligase for 2 h at 16 °C. Five microliter aliquots of ligation mixture were then transformed into *E. coli* DH5a cells and plated on LB agar containing Ampicillin. Colonies were assayed for the presence of o*mpF* and *ompC*. The mutant strain ΔOmpF and ΔOmpC were transformed with control vector *pUC19* and the recombinant plasmid *pUC19-ompF* and *pUC19-ompC* were designated as strain CΔOmpF and CΔOmpC.

2.8. Adhesion and invasion assays

For adherence assay briefly, bEnd.3 cells were cultured at about 1×10^5 cells per well in 24-well tissue culture trays (Shanghai Fuxiang Biotechnology Co., Ltd). The cells were grown in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 and incubated for 36 h before the invasion assays. Monolayers were washed and incubated with DMEM without FBS containing bacteria with a multiplicity of infection (MOI) of 100. Infected monolayers were incubated in 5% CO_2 incubator for 2 h at 37 °C to allow invasion into the cells. The extracellular bacteria were excluded by incubation of the monolayers with experimental medium containing Gentamycin (100 mg/ml) for 1 h. The monolayers were washed again with PBS and lysed with Trypsin. The released intracellular bacteria were enumerated by plating on MacConky agar plates. Negative control wells containing only bEnd.3 cells were used in all experiments. Results were expressed as percentage of adhesion and invasion which were calculated by dividing the number of invaded bacteria with initial inoculation bacterial numbers. The assay was done three times in triplicate. Adherence assays were performed similarly to the

invasion assay, except that the gentamicin treatment step was omitted. To analyze the effects of anti-OmpF and OmpC in strain TW-XM invasion, an invasion inhibition experiments were performed as described previously with minor modifications [21]. Briefly, bacteria were pre-treated with specific anti-OmpF, OmpC antibodies and pre-immune serum for 1 h at 37 °C. Pre-treated bacteria were used to infect bEnd.3 monolayers as described above.

2.9. Virulence testing

The 50% lethal dose (LD_{50}) was performed as described previously [25] to assess virulence of each strain in ducks and mouse models. Groups of ten 7-day-old ducks were injected intramuscularly with bacterial suspensions containing 10^3–10^7 CFU of bacteria in sterile PBS. The negative control group was injected with PBS. The ducks were observed for 7 days until survival rates were steady. The LD_{50} of each strain was also tested with a mouse model. The LD_{50} results were estimated by Reed-Muench method. Furthermore, 10^7 CFU/ml of each strain was injected into the leg muscles of ducks (15 ducks per strain) to obtain the survival curve. The groups were monitored for a 10-day post-infection, and survival condition was recorded with 12 h interval.

2.10. Animal infection studies

To determine the colonization and invasion capacities during systemic infection, animal infection studies were carried out as described previously [25]. Briefly, groups of 8-day-old ducks were intramuscularly injected with a dose containing 10^7 CFU. Ducks were euthanized and anatomized after 24 h post-infection. To determine the number of bacteria colonizing the internal organs, bacteria were re-isolated from the brains, lungs, spleens and blood, homogenized and plated on MacConkey agar using10-fold serial dilutions.

2.11. RNA extraction and qRT-PCR analysis

Bacterial cultures were grown to the logarithmic phase and total RNAs were extracted using TRIzol reagent (Invitrogen), and treated with RNase-free DNase I to exclude DNA contamination. The cDNA synthesis was performed using the PrimeScript1 RT reagent kit (Takara, Dalian, China). The qRT-PCR was carried out to quantify the levels of virulence genes using SYBR1 Premix Ex TaqTM (Takara, Dalian, China) and gene specific primers (Table 2) with 0.5 mg of total RNA. The data was normalized to the housekeeping gene dnaE transcript and relative fold change was calculated by using the threshold cycle $2^{-\mathbf{\Delta\Delta CT}}$ method [26].

2.12. Statistical analysis

Statistical Package for the Social Science (SPSS) version 18.0 (SPSS Inc., Chicago, IL) was used to analyze *in vitro* and *in vivo* experiments. The one-way ANOVA was used in analysis of the adhesion results *in vitro* and two-way ANOVA was performed on the qRT-PCR data. Non-parametric Mann-Whitney *U* Test was used for analysis of animal infection study and the duck survival data were analyzed by the Kaplan-Meier estimator method [27]. A *P*-value less than or equal to 0.05 was considered as statistical significance.

3. Results

3.1. Prevalence of the ompF and ompC in APEC O1 strain TW-XM

The prevalence of o*mpF* and *ompC* were screened by PCR using APEC strains available in our laboratory. 64 strains were positive for o*mpF* accounting for 44.4% out of the144 APEC strains. For all strains examined in this study, our results were compared with the phylogenetic ECOR group data of the isolates positive for o*mpF*, 2 isolates belonged to ECOR group A (3.1%), 4 to B1 (6.2%), and 45 to B2 (70.3%) and 13 to D (20.3%). 75 strains were positive for *ompC* accounting for 52% out of the 144 APEC strains, representing one isolate belonged to group A (1.3%), 7 to B1 (5.8%), and 20 to B2 (26.7%) and 47 to D (62.7%) [28]. Thus, group B2 and D seems to be the predominant groups for isolates harboring *ompF and ompC* (Fig. S1).

3.2. Expression and purification OmpF and OmpC porins

For expression of OmpF and OmpC, the pET28a-o*mpF* and pET28a-*ompC* were constructed and used to express the proteins. The proteins were induced by IPTG and purified from *E. coli* system (+) using HisTrap HP column. The proteins were characterized with 10% SDS-PAGE and Western blotting which showed that both the OmpF and OmpC proteins could solubley be purified at a band of approximately 40 KDa (Fig. 1A), which were consistent with their theoretical molecular weight. The purified OmpF and OmpC proteins were dialyzed at 4 °C against dialysis buffer containing diminishing concentrations of urea, where the final concentrations were 0.5 mg/ml.

3.3. Analysis of OmpF and OmpC expression

The expressions of *ompF* and *ompC* in the wild-type, mutant and complementation strains were compared with 10% SDS-PAGE. No difference between the wild-type and mutant strain were detected in protein patterns (data not shown), which may indicates the low *ompF* and *ompC* expression. To determine whether *ompF* and *ompC* were transcribed in wild-type strain TW-XM under laboratory conditions, qRT-PCR was performed. An expression of *ompF* and *ompC* were detected in *ompF* and *ompC*-positive strains when grown in LB medium. Our analysis revealed considerably lower transcription levels of *ompF* and *ompC* in strain ΔOmpF and ΔOmpC compared to the constitutively expressed housekeeping gene dnaE. An increased *ompF* and *ompC* transcription in complementation strains were observed, while as expected, no specific transcriptions were detected for *ompF* and *ompC* in mutant strains (Fig. 2). Immunoblotting was performed using our anti-OmpF and OmpC serum. As shown in (Fig. 1B), incubation with anti-OmpF and OmpC led to the detection of protein bands of the expected size for OmpF and OmpC in the total lysate extract of the wild-type and complementation strain, but not in mutant type, indicating that *ompF* and *ompC* were expressed under laboratory conditions.

3.4. Expression profiling of virulence genes

To determine the influence of *ompF* and *ompC* on adhesion and invasion, expression levels of virulence genes associated with adhesion and invasion were quantified by qRT-PCR. As shown in (Fig. 3), the expression levels of virulence factors *ompA* and *fimC* were significantly decreased in the *ompF* associated mutants ($P > 0.01$; $P > 0.05$). The expression levels of the virulence genes were restored in the complementation strains CΔOmpF. On the other hand, *iBeA* and *fimC* of OmpC associated mutants showed a significant decrease in gene expression levels compared with those in TW-XM ($P > 0.01$; 0.05), and its level was restored in the complementation strain CΔOmpC.

3.5. Deletion of ompF and ompC did not affect growth kinetics

A clean *ompF* and *ompC* deletion mutants of TW-XM were

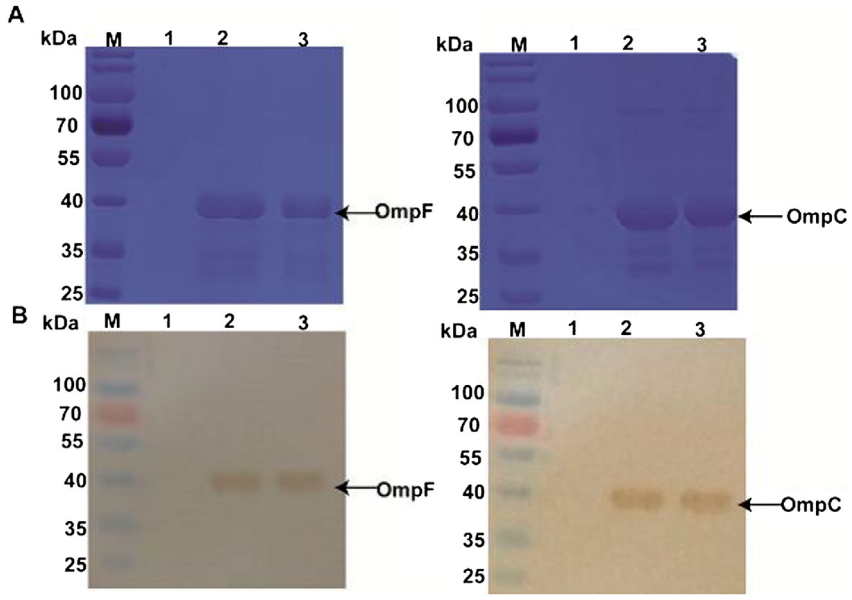

Fig. 1. (**A**) Purification of *ompF* and *ompC* expressed in *E. coli* BL21 (DE3). The whole open reading frames (ORF) were cloned into pET28a (+) leading to the expression of a 40 KDa fusion proteins. BL21 (DE3) cells were incubated in LB at 37 °C without (Lane 1) or with (Lane 2) addition of IPTG, which induces the expression of OmpF and OmpC. Proteins of the total bacteria extracts (Lanes 1 and 2) and the elution of the purified OmpF and OmpC (Lane 3) were separated on an 10% SDS-PAGE and stained with coomassie stain. Lane M: Protein marker. (**B**) Immunoblotting analysis of total cells lysate prepared from different *E. coli* strains using antibodies anti-OmpF and OmpC. Expression of OmpF and OmpC were detected in wild-type strains TW XM and complementation strains TW XM, Lane M, Prestained Protein marker; Lane 1, TW XM (wild-type); Lane 2, TW XM (complementation strains).

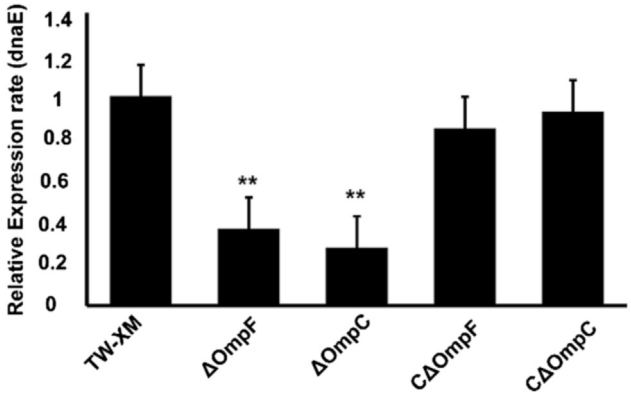

Fig. 2. Quantification of *ompF* and *ompC* expression. Expression levels of *ompF* and *ompC* in different strains were measured by qRT-PCR and differences between wild type and mutants were statistically significant at a *P* value of 0.05. Data were normalized to the housekeeping gene *dnaE*. Results are shown as relative expression ratios compared with expression in the wild type strain (TW-XM). Data from three independent assays are presented as the means standard deviations.

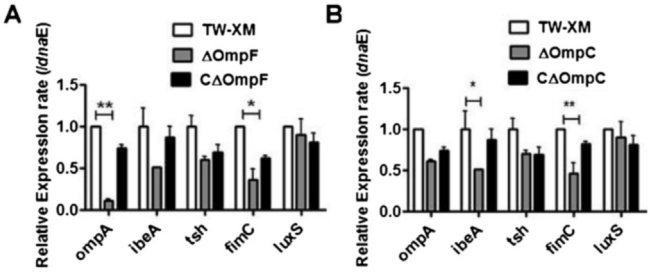

Fig. 3. Quantification of virulence gene expression. Expression levels of *ompA*, *ibeA*, *tsh*, *fimC* and *luxs* in strains wild-type (TW-XM), OmpF associated mutant and OmpC associated mutants were measured by qRT-PCR, data were normalized to the housekeeping gene dnaE. Results are shown as relative expression ratios compared to expression in the wild-type strain. The expression levels of genes *ompA*, *fimC* and *ibeA* were significantly decreased in the mutant strains, respectively (*, $P < 0.05$; **, $P < 0.01$).

created using previously described method [29] The deletion encompasses the entire *ompF* and *ompC*. For genetic complementation, the ORF and putative promoter of *ompF* and *ompC* were amplified from TW-XM chromosomal DNA and cloned into plasmid pUC19. The resulting vector pUC19-*ompF*C and pUC19-*ompC*C were transformed into the mutant strain TW-XM, yielding the complementation strains. No significant differences in generation times and final optical densities were observed for all strains during growth in LB medium (Fig. S2).

3.6. *ompF and ompC deletion attenuate virulence in vivo*

Duck and mouse models were used to assess the virulence of wild-type, mutant and complementation strains. Animals were infected with different strains, and the LD_{50} values of the mutant strains ΔOmpF and ΔOmpC, wild-type and complementation strain were, 2.04×10^6, 1.62×10^6, $2 \times 10^5$and 3.19×10^5 CFU/duck, respectively, representing a 9.8-fold and 12.3-fold decrease in

Table 3
Calculations of LD_{50} in duck model for different strains.

Dose (/duck)	No. of dead ducks/total no. tested ducks				
	TW-XM	ΔOmpC	ΔOmpF	CΔOmpF	CΔOmpC
2×10^7 CFU	10/10	9/10	7/10	10/10	10/10
2×10^6 CFU	8/10	4/10	4/10	7/10	6/10
2×10^5 CFU	5/10	1/10	1/10	5/10	4/10
2×10^4 CFU	2/10	1/10	0/10	1/10	2/10
2×10^3 CFU	0/10	0/10	0/10	0/10	0/10
LD_{50} value CFU	2×10^5	$2.04 \times 10^{6*}$	$1.62 \times 10^{6*}$	3.19×10^5	3.91×10^5

According to the ducks survival data, the differences of LD_{50} were analyzed by the Kaplan–Meier Estimator method. $^*p < 0.05$.

virulence for mutant strains compared with the wild strain TW-XM (Table 3). The virulence of the o*mpF and ompC* mutants was lower than that of the wild-type strain and the complementation strains have recovered the virulence to the level of wild strain. Similar results were also observed in the mouse model (Table 4). To determine the effect of o*mpF and ompC*-associated mutants on the overall bacterial virulence, ducks infected with TW-XM, ΔOmpF, ΔOmpC, CΔOmpF, CΔOmpC and PBS as negative control. The results showed that the survival rates were significantly longer. Within 60 h, only 6.7% of ducks infected with the wild-type strain survived, 12.5% of ducks remained alive in the CΔOmpF, whereas 80% of ducks infected with ΔOmpF survived. A similar patern was observed for OmpC-associated mutants, Within 48 h, only 7.1% of ducks infected with the wild-type strain survived, 12.5% of ducks remained alive in the CΔOmpC, whereas 80.3% of ducks infected with ΔOmpC survived (Fig. 4A). The mouse model displayed a similar result (Fig. 4B).

3.7. Effect of ompF and ompC during systemic infection in vivo

To determine the effect of o*mpF* and *ompC in vivo*, systemic infection experiments were conducted according to the method previously described [25]Bacteria were re-isolated from the brains, lungs, blood and spleens of infected ducks at 24 h post-infection. As shown in Fig. 5, when ducks were infected with OmpC-associated mutant, the recovered bacteria in the four studied organs were significantly reduced compared with the wild-type strain TW-XM ($P > 0.01$). In addition, the recovered complementation strains in the organs were restored so that the differences between strain TW-XM and complementation were not statistically significant, and the numbers of strains complementation were significantly greater than that of strain mutant ($P < 0.05$).

The strain OmpF-associated mutants showed that, the recovered bacteria in the brain, blood and lung were significantly reduced compared with the wild-type strain TW-XM (Fig. 5). These results suggested the involvement of the *ompF and ompC* in the approach of systemic infection of APEC strain TW-XM.

3.8. Adhesion and invasion were reduced in the isogenic mutant of ompF and ompC

To determine the role of *ompF* and *ompC* in adhesion, the capacities for adherence to and invasion of host cells were compared between the wild type, mutants, and complementation strains under the same conditions. The Δ*ompF* and Δ*ompC* significantly reduced the abilities of adherence to by 41.3% and 46.1% and invasion capabilities of APEC to mouse brain microvascular endothelial cell (BMEC) bEnd.3 cells *in vitro* by 51.9% and 49.7% respectively, compared to wild strain TW-XM (Fig. 6). These results indicated that *ompF* and *ompC* may be involved in APEC adherence to and invasion of bEnd.3 cells.

Table 4
Calculations of LD_{50} in mouse model for different strains.

Dose (/mice)	No. of dead mice/total no. tested mice				
	TW-XM	ΔOmpF	ΔOmpC	CΔOmpF	CΔOmpC
2×10^7 CFU	10/10	7/10	8/10	9/10	10/10
2×10^6 CFU	8/10	5/10	4/10	7/10	7/10
2×10^5 CFU	4/10	2/10	2/10	3/10	3/10
2×10^4 CFU	2/10	1/10	1/10	1/10	2/10
2×10^3 CFU	0/10	0/10	0/10	0/10	1/10
LD_{50} value CFU	2.45×10^5	$2.7 \times 10^{6}{}_{*}$	$2.4 \times 10^{6}{}_{*}$	6.5×10^5	2.9×10^5

According to the mouse survival data, the differences of LD_{50} were analyzed by the Kaplan–Meier Estimator method. $^{*}p < 0.05$.

4. Discussion

The *ompF* and *ompC* are two major porin proteins of *E. coli*, which function as passive diffusion channels for small molecules, nutrients, antibiotics and toxic salts. The *ompF* has a wide pore and is preferentially expressed under lower osmolar pressure compared with *ompC* [30]. Both *ompF* and *ompC* porin genes of *E. coli* have putative antigenic epitopes located on various loops [14,15], indicating that it may have some immune properties and play a role in bacterial pathogenesis, such as adherence, invasion, and serum resistance [16,17]. It has been shown that the loss of *ompC* and *ompF* of *Salmonella Typhimurium* attenuated virulence [18]. However, whether o*mpF* and *ompC* contributes to APEC pathogenicity remains un-clear.

The distribution of *ompF and ompC* in the APEC collection was examined, and the distribution rate were significantly higher in group B2 (70.3%) and D (62.7%), suggesting that OmpF and OmpC were mainly encoded in virulent isolates of groups B2 and D [28,31] and thus made contribution to APEC pathogenicity (Fig. S1).

The APEC adherence to and invasion of mouse brain microvascular endothelial cells (bEnd.3) was previously observed [19], which demonstrated the usefulness of these cells as a model to study the APEC adhesion and invasion mechanism. Here, we evaluated the role of o*mpF* and *ompC* on APEC adhesion and invasion using bEnd.3 cells model. The results showed a significant reduction in the adhesion and invasion capacity of mutant strains compared to wild-type strain. Moreover, the adhesion and invasion capability were partially restored by genetic complementation, which indicated that o*mpF* and *ompC* were involved in APEC adhesion and invasion of bEnd.3 cells. A parallel finding was reported for the loss of *ompC* in *E. coli* reduce adherence and the ability to invade intestinal cells [12,13] which might imply that *ompC* mutant strain TW-XM also maintained its capability to adhere and invade bEnd.3 cells.

To determine the influences of *ompF* and *ompC* on APEC adherence to and invasion of bEnd.3 cells, expression profiles of virulence genes related to adhesion, invasion and other known virulence genes were analyzed by qRT-PCR. The results indicated that, the adherence and invasion abilities of the mutant strains were significantly reduced compared with the wild strain. This is maybe caused by down-regulation of the genes (*fimC* and *ompA*), that are involved in adhesion to and invasion. The *fimC* gene is adhesion factors, which facilitate the adherence of *E. coli* to host respiratory tract epithelial cells to allow colonization [32]. Outer membrane A (OmpA) is essential for the invasion of meningitic strains of *E. coli* into human brain micro-vascular endothelial cells (HBMEC) [33]. Recent studies have demonstrated that OmpA contribute to binding and invasion of *E. coli* K1 strain RS218 into HBMEC [34]. Furthermore, *ompA*, *fimC* and *ibeA* expression levels were restored in the complementation strains TW-XM which might result in an increase of invasion capacity towards bEnd.3 cells and animal models. Reduced expression of adhesion associated genes *ompA*, *fimC* and *ibeA* in *ompF* and *ompC* mutants may be a major reason for the decreased colonization capacity in the brains, blood and lungs.

The influence of o*mpF* and *ompC* on APEC pathogenicity was also examined in this study. There were differences in mortality for the wild-type and the mutant strains. The LD_{50} of both *ompF* and *ompC*-associated mutants were increased when compared with the parent strain in the duck and mouse models. Furthermore, virulence was restored for the complementation strains. The survival time of the animal models infected with *ompF* and *ompC*-associated mutants were significantly longer than those for the wild-type or complementation strains, which indicated that *ompF* and *ompC* were necessary for the virulence of APEC strain TW-XM.

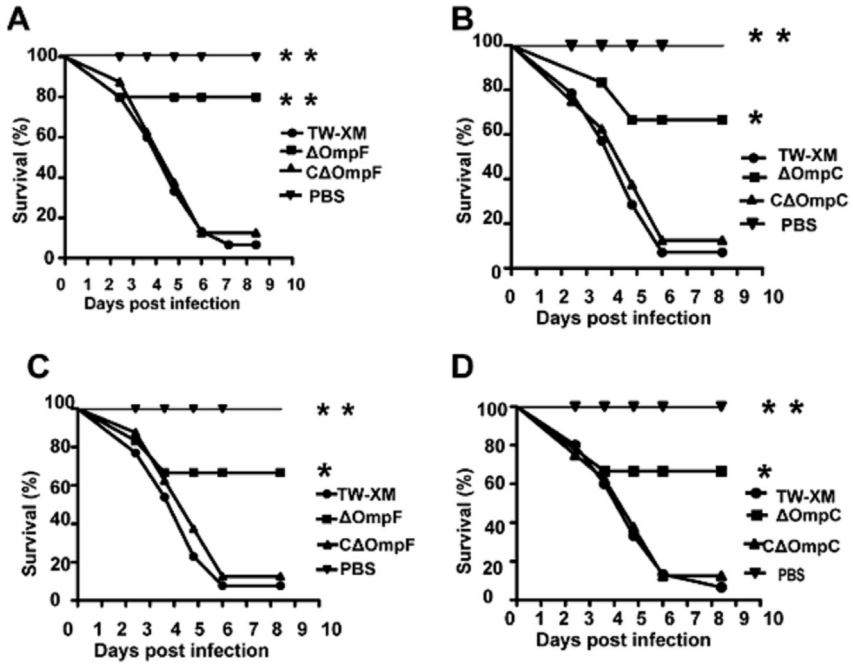

Fig. 4. Survival curves of ducks (**A**) and mouse (**B**) infected with 10^7 CFU/ml (10 LD_{50}) bacteria. Survival data were analyzed by using the Kaplan-Meier estimator method. *, $P < 0.05$; **, $P < 0.01$ (based on comparisons with strain TW-XM [wild type]).

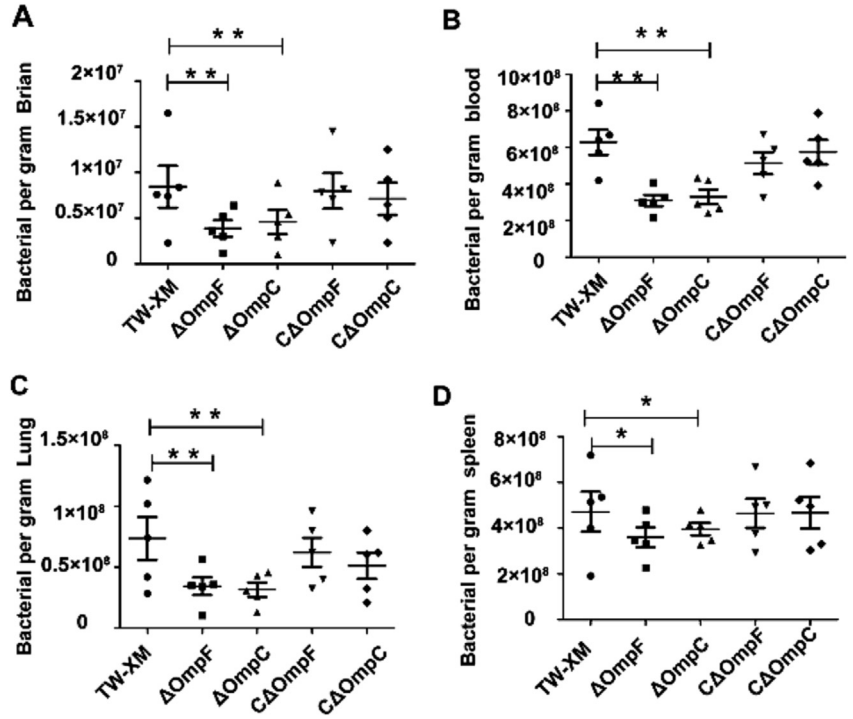

Fig. 5. Animal systemic infection experiments to determine the effect of OmpF and OmpC *in vivo*. Bacterial reisolations of TW-XM, OmpF-assosiated mutants, OmpC-assosiated mutants, and complemented strains from at 24 h postinfection were calculated by plate counting. The bars in the middle of columns indicate the average number of bacteria recovered from the organ for each group of animals. Statistical significance was determined by comparisons with strain wild type (*, $P > 0.05$; **, $P > 0.01$).

The role of *ompF* and *ompC* in the pathogenicity of APEC strain TW-XM were investigated *in vivo* by comparing its effects with those of the mutant strains. The ducks infected with different strains and the colonization and proliferation capacities of bacteria in the brains, lungs and blood were compared. The results indicated that loss of *ompF* and *ompC* significantly reduced numbers of recovered bacteria in the tissues compared to those of the wild-type strain. The number of recovered bacteria in the brains, lungs and blood were restored with the complementation strains *in vivo* and were significantly different than that for the mutant strains (Δ*ompF* and Δ*ompC*), which indicated that *ompF* and *ompC* are involved in colonization and proliferation during systemic infection.

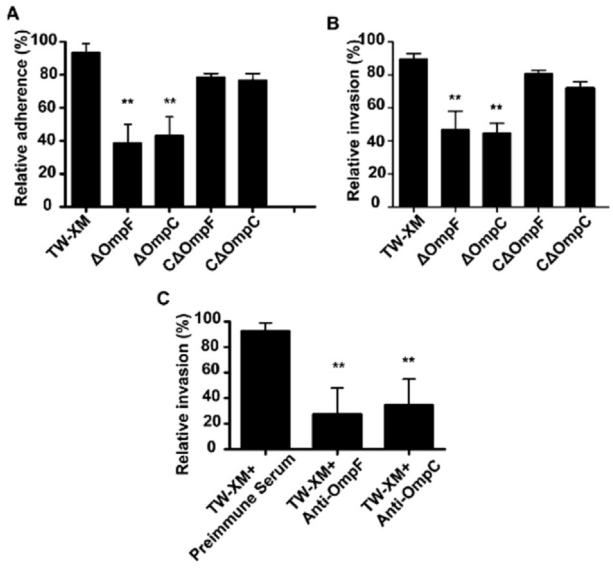

Fig. 6. Effects of *ompF and ompC* on APEC adherence to **(A)** and invasion (**B**) of bEnd.3 cells (MOI, 100). All assays were done in triplicate. Statistical significance was determined by one-way analysis of variance (ANOVA) based on comparisons with strain TW-XM (wild type) (**, $P > 0.01$). **(C)** The anti-OmpF and OmpC antibodies inhibit invasion capacity of TW-XM to bEnd.3 cells. TW-XM was incubated with pre-immune serum (control) and anti-antibodies, respectively. Then bacteria of each experiment were used to infect bEnd.3 cells. The anti antibodies significantly reduced APEC TW-XM invaded into bEnd.3 cells compared to the negative control (**, $P > 0.01$).

5. Conclusions

This study has demonstrated that *ompF* and *ompC* genes are conserved among *E. coli* isolates irrespective of pathotypes. The *ompF* and *ompC* are involved in APEC invasion and pathogenicity in cultured bEnd.3 cells and in duck and mouse models, probably by influencing adhesion, invasion, colonization and proliferation capacities, and maybe related with the expression of the *ompA* and *fimC*.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Hassan M. A., and Huochun Yao: conceived and designed the experiments. Hassan M. A., Zhang Yue., and Yingchu Zhu.,: performed the experiment. Hassan M. A., Jiale Ma., and Zihao Pan.,: analyzed the data. Hassan M. A., Hassan. Z. A., Wei Zhang., and Huochun Yao: wrote and edited the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2017.02.033.

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