The response regulator OmpR contributes to the pathogenicity of avian pathogenic *Escherichia coli*

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ABSTRACT Avian colibacillosis is a serious systemic infectious disease in poultry and caused by avian pathogenic *Escherichia coli* (**APEC**). Previous studies have shown that 2-component systems (**TCSs**) are involved in the pathogenicity of APEC. OmpR, a response regulator of OmpR/EnvZ TCS, plays an important role in *E. coli* K-12. However, whether OmpR correlates with APEC pathogenesis has not been established. In this study, we constructed an *ompR* gene mutant and complement strains by using the CRISPR-Cas9 system and found that the inactivation of the *ompR* gene attenuated bacterial motility, biofilm formation, and the production of curli. The resistance to environmental stress, serum sensitivity, adhesion, and invasion of DF-1 cells, and pathogenicity in chicks were all significantly reduced in the mutant strain AE17Δ*ompR*. These phenotypes were restored in the complement strain AE17C-*ompR*. The qRT-PCR results showed that OmpR influences the expression of genes associated with the flagellum, biofilm formation, and virulence. These findings indicate that the regulator OmpR contributes to APEC pathogenicity by affecting the expression and function of virulence factors.

Key words: avian pathogenic *Escherichia coli*, response regulator, OmpR, pathogenicity

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INTRODUCTION

Avian pathogenic *Escherichia coli* (**APEC**) is a typical extra-intestinal pathogenic *E. coli* (**ExPEC**) that is mainly transmitted through the respiratory tract. It causes serious localized or systemic infectious diseases in poultry and significant economic losses in the poultry industry (Nakazato et al., 2009). APEC contaminates poultry meat or eggs, to cause human extraintestinal disease (Ramírez et al., 2009). Furthermore, APEC and human ExPEC share some virulence genes, suggesting that APEC is a reservoir of ExPEC virulence genes (Chanteloup et al., 2011; Griffin et al., 2012; Mellata, 2013). APEC has numerous virulence factors, such as 2-component systems (**TCSs**), quorum sensing (**QS**), and a secretion system, which are closely related to the pathogenicity and drug resistance of bacteria (Nakazato et al., 2009; Wu et al., 2017). The pathogenic mechanism of APEC is complex. Therefore, it is important to investigate the pathogenic mechanisms of APEC to increase control in poultry farming and public health safety.

Comparative genomic analysis indicates that about 62 TCSs genes are conserved in *E. coli* genomes, these include BarA/UvrY, PhoP/Q, KdpD/E, CpxR/A, BasS/R, OmpR/EnvZ, etc (Capra and Laub, 2012). In APEC, the function of some TCSs has been identified and they are involved in bacterial growth, biofilm formation, response to environmental stress, drug resistance, and pathogenicity. The BasS/R inhibits biofilm formation, colonization in chickens, and virulence of APEC (Yu et al., 2020). The CpxR/A system regulates the motility, biofilm, and production of type Ⅰ fimbriae of APEC (Matter et al., 2018). The RstA/B TCS participates in the pathogenicity of APEC and adapts to survival under stress (Gao et al., 2015). Previous studies have shown that PhoP/Q significantly reduces invasion and adhesion to primary chicken embryonic fibroblasts (**CEF**) cells and virulence (Tu et al., 2016) and KdpD/E influences flagellum formation, motility, and serum to resistance (Xue et al., 2020a). Therefore, studying the role of the TCS in APEC is conducive to elucidating the pathogenic regulatory mechanism of APEC, which is of

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great significance for the prevention and treatment of colibacillosis.

The OmpR/EnvZ TCS is a global regulation system and plays an important role in mediating signal transduction in response to environmental osmotic pressure in Gram-negative bacteria (Capra and Laub, 2012; Quinn et al., 2014). The OmpR/EnvZ consists of a histidine kinase EnvZ and a cytoplasmic response regulator OmpR (Cai and Inouye, 2002). The transcription factor OmpR directly binds to the outer membrane protein genes *ompF* and *ompC*. In response to osmotic stress, OmpC is expressed preferentially at high osmolarity and OmpF is expressed preferentially at low osmolarity (Cai and Inouye, 2002; Foo et al., 2015). In *E. coli* K-12, the transcriptional regulator OmpR also regulates the virulence-associated genes *flhD, flhC, fimB*, and *csgD* (Jubelin et al., 2005; Rentschler et al., 2013; Samanta et al., 2013). OmpR contributes to the pathogenesis of Enterohemorrhagic *Escherichia coli* (**EHEC**), and OmpR can directly bind to *ler* and *stx1* (Wang et al., 2021). In adherent-invasive *Escherichia coli* (**AIEC**), OmpR is essential for adhesion and invasion of intestinal epithelial cells and colonization of the mouse gut (Rolhion et al., 2007; Lucchini et al., 2021). OmpR regulates the expression of the SPI-2 type III secretion system and 2-component system SsrA/B in *Salmonella* (Lee et al., 2000; Feng et al., 2003; Cameron and Dorman, 2012). In *Yersinia enterocolitica*, OmpR is a regulator that controls the expression of genes involved in cellular processes and virulence (Reboul et al., 2014). OmpR also inhibits the expression of porin kdgM2 and enhances adaptability to the environment (Nieckarz et al., 2016). In *Burkholderia, ompR* leads to reduced antibiotic resistance, biofilm formation, and virulence (Cooper, 2018). However, the roles of OmpR/EnvZ in regulating the pathogenesis of APEC have not been reported.

In this study, the CRISPR-Cas9 system was used to construct an *ompR* gene mutant strain. We found that the regulator *ompR* mainly affected flagella formation, motility, biofilm formation, response to environmental stress, serum resistance, and virulence in the APEC of infected chickens, and also affected the expression of virulence genes. These results indicated that the regulator OmpR positively regulates the pathogenicity of APEC. Thus, these findings will contribute to our understanding of the function of the TCS, clarify the pathogenic mechanism in APEC, and provide potential drug targets for the treatment and prevention of APEC infection.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

APEC17 (serotype O2) was isolated from a case of avian colibacillosis in Anhui Province, China (Tu et al., 2016). Strains and plasmids used in this study are listed in Table 1. All *E. coli* strains were grown in Luria-Bertani (**LB**) medium at 37°C. When necessary, LB medium was supplemented with ampicillin (Amp, 100 μg/mL), spectinomycin (Spec, 100 μg/mL), kanamycin (Kan, 50 μg/mL), and chloramphenicol (Cm, 30 μg/mL).

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant genotype	Source
Strains		
AE17	APEC wild-type strain	Laboratory stock
AE17Δ*ompR*	AE17 *ompR* deletion mutant	This study
AE17C-*ompR*	AE17 *ompR* with the complement plasmid pCm-*ompR*, Cm^r	This study
DH5α	*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
Plasmids		
pTargetF	High copy cloning vector, pMB1 *aadA* sgRNA-cat, $Spec^r$	Addgene
pCas	Low copy cloning vector, temperature sensitive, P_{cas}-cas9 P_{araB}-Red $lacI^q$ P_{trc}-sgRNA-pMB1, Kan^r	Addgene
pSTV28	Low copy cloning vector, p15A origin, Cm^r	Takara
pCm-*ompR*	pSTV28 with *ompR* gene, Cm^r	This study

Abbreviations: Cm^r, chloramphenicol-resistant; Kan^r, kanamycin-resistant; $Spec^r$, Spectinomycin Hydrochloride-resistant.

Construction of Mutant Strain AE17ΔompR

The mutant strain AE17Δ*ompR* was constructed by using the CRISPR-Cas9 system. The nuclease Cas protein 9 (**Cas9**) and synthetic single-guide RNA (**sgRNA**) are co-transferred to target cells to induce expression of sgRNA and Cas9 protein to form crRNP complexes that recognize and direct Cas9 protein to act on genomic targets to produce DNA double-stranded breaks (**DSBs**). The target genes are edited through homologous recombination (**HR**) or non-homologous end-joining (**NHEJ**) DNA repair (Jiang et al., 2015b; Chung et al., 2017). The primers used are listed in Table 2. Briefly, the *ompR* upstream (***ompR*-L**) and downstream (***ompR*-R**) frames were amplified by PCR with primers *ompR*-L-F/R and *ompR*-R-F/R (containing *Hind* III digest site) from AE17 genomic DNA, respectively. The *ompR*-sgRNA frame was amplified with the primers *ompR*-sgRNA-F/R (containing *Spe* I digest site) from the plasmid pTargetF. The overlap method was used to combine the 3 fragments into the *ompR*-sgRNA+L+R large fragment. The large fragment and pTargetF plasmid were digested with *Hind* III and *Spe* I, respectively, and transformed into *E. coli* DH5α by chemical transformation (ice-bath for 30 min, 42°C for 90 s, and ice-bath for 2 min). The transformed *E. coli* DH5α were then spread on LB agar (with Spec) and identified with primers pTargetF-F/R to construct the recombinant plasmid pTargetF-*ompR*.

The AE17 was grown in LB medium at 37°C to an OD_{600} of 0.6, ice-bath for 30 min, centrifuged at 5,000 × *g*, and washed twice with 10% glycerol water. The plasmid pCas was extracted and transformed into AE17 competent cells by electroporation (200 Ω, 2,500

Table 2. Primers used in this study.

Primer name	Sequence (5'-3')	Product size/bp
AE17-F	GAGCGATCTTGGCTATACC	1,023
AE17-R	GTGAAGCTATCTAACAACGG	
ompR-L-F	TTTTTTTGAGGCGTTGCA CTCTTCGATATCTTT	832
ompR-L-R	AGTATAAACAGGCGATTGC GCTTCTCGC	
ompR-R-F	CGCAATCGCCTGTTTA-TACTCC CAAAGGTTCG	852
ompR-R-R	CCAAGCTTTGCGGCAGAA CGAATCAT	
ompR-sgRNA-F	AATACTAGTTGAATG-TAACGCGGATGCGCGTTTT AGAGCTAGAAATAGC	3,320
ompR-sgRNA-R	AGTGCAACGCCT-CAAAAAAAGC ACCGACTCGG	
ompR-in-F	TTCTGGTGGTCGAT GACGATATG	493
ompR-in-R	GCCTTCAGTACCGCAAA CTCAC	
ompR-out-F	CCTCAATGCTACCGGGGT	2,681
ompR-out-R	ACGCGTTTCCAGATTACGC	
*ompR-Bam*HI-F	CGCGGATCCATGCAAGA-GAACTA CAAGAT	720
*ompR-Hin*dIII-R	CCCAAGCTTTCATGCTTTA-GAACC GTCCG	
pTargetF-F	AGCGAGGAAGCGGAAGA GCG	800
pTargetF-R	CAAGATAGCCAGATCAATGT	
pCas-F	GTAACATCAGAGATTTT-GAGA CAC	4,714
pCas-R	GATGTAGCCGTCAA GTTGTCA	
pSTV28-F	TGTAAAACGACGGCCAGT	109
pSTV28-R	CAGGAAACAGCTATGACC	

Restriction sites are underlined.

V) using a Gene Pulser MX cell (Bio-Rad, Hercules, CA), spread on LB plates (with Kan), and identified with primers pCas-F/R. Then the AE17-pCas was grown in LB medium at 37°C to an OD_{600} of approximately 0.2. At this stage, 30 μg/mL of arabinose was added and cultures were continued to an OD_{600} of 0.6. The recombinant plasmid pTargetF-*ompR* was transformed into AE17-pCas by electroporation and grown in LB (with Spec and Kan). Then, the strain was cultured in LB liquid medium (with Kan and 0.5 mM IPTG) to remove the pTargetF. The primers *ompR*-out-F/R and *ompR*-in-F/R were used to identify the mutant strain AE17Δ*ompR*.

Construction of Complement Strain AE17C-ompR

We used AE17 as a template to amplify *ompR* fragment with primers *ompR-Bam*H I-F/*ompR-Hin*d III-R. *Hin*d III and *Bam*H I were used to digest the *ompR* fragment and plasmid pSTV28, respectively, and transformed into *E. coli* DH5α cells. The transformed *E. coli* DH5α cells were plated on LB plates (with Cm). The complement plasmid pSTV28-*ompR* was transformed into the AE17Δ*ompR* competent cells by electroporation. The primers *ompR*-in-F/R were used to identify the complement strain AE17C-*ompR*.

Growth Curves, Motility, and Curli Assays

To determine the effect of the *ompR* gene on bacterial growth, the AE17, AE17Δ*ompR,* and AE17C-*ompR* strains were determined on LB medium. Briefly, bacteria were incubated in LB medium at 37°C and 150 RPM, and the optical density (**OD**) of strains was monitored at 1 h intervals by spectrophotometry (Bio-Rad).

Overnight cultures of AE17, AE17Δ*ompR*, and AE17C-*ompR* strains were diluted 1:25 and statically cultured to the logarithmic phase. The bacteria were washed with phosphate buffer saline (**PBS**), plated on the LB semi-solid medium (0.25% agar), and incubated at 37°C for 8 h, then the motile cycles of the strains were observed. The LB semi-solid medium was prepared from 0.5% NaCl, 1% tryptone, 0.8% glucose, and 0.25% agar powder (Sangon, Shanghai, China).

Congo red plates were used to detect curli production. The overnight bacteria were added to Congo red plates and incubated at 37°C for 3 d. Congo red plates were LB agar plate without salt supplemented with 40 mg/L Congo red (Sangon) and 20 mg/L brilliant blue (Sangon).

Transmission Electron Microscopy

The bacterial flagella of the AE17, AE17Δ*ompR*, and AE17C-*ompR* strains were observed by transmission electron microscopy (**TEM**). The strains were cultured to a logarithmic phase and washed with PBS 3 times. The bacteria were placed on a 200 mesh Formvar-coated copper microscopy grid and stained with 2% phosphotungstic acid. After drying, the bacterial micromorphology was observed by the electron microscope (Hitachi HT-7700, Tokyo, Japan).

Biofilm Formation Assay and Scanning Electron Microscopy

The biofilm was determined using a crystal violet (**CV**) assay as described previously (Yu et al., 2020). Briefly, the AE17, AE17Δ*ompR*, and AE17C-*ompR* strains were cultured to logarithmic growth phase, the strains were diluted to an OD_{600} of approximately 0.03, and added to a sterile 96-well plate at 28°C for 48 h. Then, stained with 0.1% (w/v) crystal violet (Sangon) for 20 min, and washed three times with PBS. Finally, 33% glacial acetic acid (Sangon) was added for 10 min. The OD_{492} of strains was monitored by using a microplate reader.

Cultures were added to a sterile 12-well plate, placed in a coverslip, and incubated at 37°C for 24 h. First, the coverslip was washed with PBS. Next, 2.5% glutaraldehyde was added at 4°C for 8 h and washed three times with PBS. Then, the coverslip was serially placed in ethanol absolute at concentrations of 30, 50, 70, 80, 95, and 100%. Finally, the coverslip was transferred to acetone, vacuum freeze-dried, and observed with a scanning electron microscope (**SEM**; Hitachi S-4800).

Serum Bactericidal Assay

The determination of the survival ability of the bacteria in the serum was as described previously (Wang et al., 2015). Briefly, the AE17, AE17Δ*ompR*, and AE17C-*ompR* strains were cultured to the logarithmic growth phase, centrifuged, and washed with PBS. Specific-pathogen-free (**SPF**) chicken serum (Beyotime, Shanghai, China) was diluted with PBS to 25, 50, 75, or 100%. The heat-inactivated serum (at 56°C for 30 min) was used as a negative control. Bacteria incubated with different concentrations of serum were cultured at 37°C for 30 min. The number of surviving bacteria was counted by plating on LB agar plates.

Bacterial Resistance to Environmental Stress Assay

The survival ability of bacteria in diverse environmental stress was performed as described previously (Huang et al., 2009). Briefly, the AE17, AE17Δ*ompR*, and AE17C-*ompR* strains were grown to logarithmic phase, centrifuged at 5,000 × *g*, and washed twice with PBS. In the acid assay, bacteria were cultured in LB medium (PH = 3) at 37°C for 30 min. In the alkali assay, bacteria were incubated in 100 μL Tris-HCl (100 mmol/L, pH 10.0) at 37°C for 30 min. In the osmotic pressure assay, the bacteria were incubated in NaCl (4.8 mol/L) at 37°C for 60 min. In the oxidative stress assay, the bacteria were incubated in 10 μM hydrogen peroxide at 37°C for 60 min. All experiments were repeated 3 times. The counts of surviving bacteria were calculated after plating on LB agar plates.

Bacterial Adherence and Invasion Assays

The bacterial adherence and invasion assay were performed as described previously (Song et al., 2018). Briefly, the AE17, AE17Δ*ompR*, and AE17C-*ompR* strains were grown to the logarithmic phase and resuspended with Dulbecco's modified Eagle's medium (**DMEM**; Biological Industries, Israel). Chicken embryo fibroblast (**DF-1**) cells were incubated with bacteria at a multiplicity of infection (**MOI**) of 100 CFU per at 37°C and 5% CO_2 for 1.5 h. After washing with PBS to remove non-adherent bacteria, cells were lysed with 0.5% TritonX-100, and the counts of adherent bacteria were calculated on LB agar plates.

For the invasion assay, the bacterial infection of cells was performed according to the adhesion assay. After 1.5 h of incubation, cells were washed with PBS and resuspended with DMEM containing gentamicin (100 μg/mL) for 1 h to kill extracellular bacteria. Then, the cells were washed and lysed with 0.5% Triton X-100, and the lysate was diluted in a 10-fold gradient to count invading bacteria on LB agar plates.

Animal Infection

The Institutional Animal Care and Use Committee (**IACUC**) guidelines of Anhui Agricultural University (Number: 2020-021) were followed in the management of these chicks. One-day-old chicks were purchased from Anhui Anqin Poultry Company Ltd. (Anhui Province, Hefei, China). The chicks can freely eat food and drink water. The chicks were euthanized by injecting intravenous sodium pentobarbital (100 mg/kg) into the wing vein. An animal infection experiment was performed to determine the virulence of AE17 and AE17Δ*ompR* strains in chickens. After a week of feeding, chicks were injected intramuscularly with 10^6 CFUs of each bacterial strain (Tu et al., 2016; Song et al., 2018). Negative controls were injected with PBS. The survival and death of the chicks were observed every day for a total of 7 d, and a survival curve was drawn to compare the virulence of these strains.

RNA Isolation and Quantitative Real-Time PCR

Quantitative real-time PCR (**qRT-PCR**) experiments were performed to examine the transcript levels of genes. The primers are listed in Table 3. Briefly, bacterial total RNA was isolated using Total RNA Extractor (Trizol; Sangon). The synthesis of cDNA was performed using the PrimeScript RT reagent kit (TaKaRa, Beijing, China) according to the manufacturer's protocol. qRT-PCR was performed using SYBR

Table 3. qRT-PCR primers used in this study.

Primer name	Sequence (5′-3′)	Product size/bp
dnaE-F	GATTGAGCGTTATG TCGGAGGC	92
dnaE-R	GCCCCGCAGCCGTGAT	
cheY-F	TCCAGTCGGAGATAACA AATCCA	119
cheY-R	CATAGTGCGTAACCTGCTG AAAGA	
fimF-F	CGACGACTCCTGTTGTT CCATTT	118
fimF-R	AGTGCAAGCAGGTTG GCATTGTG	
fimG-F	ACGATCACGGTGAACGGTAAG	107
fimG-R	CCGGCAGACATAAGACT GAAAGA	
csgD-F	ATCGCCTGAGGTTATCGTTTG	126
csgD-R	AGATCGCTCGTTCGTTGTTCA	
ompA-F	CAGATCGTCAGTGATTGGGTAA	120
ompA-R	GAAATGGGTTATGACT GGTTAGG	
tolC-F	CACTTACCGACTCTGGA TTTGAC	112
tolC-R	GGCCCATATTGCTATCGTCAT	
fimH-F	CAGCGATGATTTCCAGTTTGT	136
fimH-R	AAGAGGAATCGGCACTGAACC	
mcbR-F	CTGTTGAAAACCTCACCCCG	100
mcbR-R	TTAATGATTTGTTCCA TGTCGCC	
flhD-F	AGAGTAATCGTCTGGTGG CTGTC	122
flhD-R	CGACAACATTAGCGGCACTG	
flhC-F	GGCTGGTGAGCGTGGGTAATA	119
flhC-R	AGTGCCCGCAAGCAGAAGAAG	

Premix Ex Taq (TaKaRa). Relative gene expression was normalized to the expression of the housekeeping gene *dnaE* using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

The primers were designed with the Primer 5.0 software. Statistics were analyzed using SPSS (v19.0) software. A paired *t* test was used for statistical comparisons between groups. The level of statistical significance was set at a *P*-value of ≤ 0.05.

RESULTS

OmpR Upregulates Motility by Interfering With the Expression of Flagellum Genes

The mutant strain AE17Δ*ompR* and the complement strain AE17C-*ompR* were successfully constructed by the CRISPR-Cas9 method (Figures 1A and 1B). Growth curves of the wild strain AE17, mutant strain AE17Δ*ompR*, and the complement strain AE17C-*ompR* showed no significant difference in growth, suggesting OmpR did not affect the growth of APEC (Figure 1C). The deletion of the *ompR* gene resulted in significantly inhibited motility in the mutant strain AE17Δ*ompR*, compared with AE17, and motility in the AE17C-*ompR* was not significantly different (Figure 2A-C). TEM showed that the flagella of wild strain AE17 were thin, long, and more numerous, while the flagella of the mutant strain AE17Δ*ompR* were less numerous and those of the complement strain AE17C-*ompR* were more numerous (Figure 2D-F).

OmpR Involved in Rdar Morphotype of APEC

As shown in Figure 3, the AE17 expresses a red, dry, and rough (rdar) morphotype on Congo red agar plates characterized by the extracellular matrix components cellulose and curli fimbriae. AE17Δ*ompR* display a smooth and white (saw) colony morphology on plates, indicating that deletion of the *ompR* gene resulted in a lack of curli and cellulose production in AE17. The rdar morphotype was restored in AE17C-*ompR*. The results indicate that OmpR is involved in the production of curli and cellulose in APEC.

OmpR Promotes the Biofilm Formation of APEC

In this study, the ability of AE17, AE17Δ*ompR*, and AE17C-*ompR* to form biofilm was detected by crystal violet assay. As shown in Figure 4A, the biofilms were significantly decreased in the mutant strain AE17Δ*ompR*, compared to that of the wild-type AE17, and the biofilms were restored in AE17C-*ompR*.

SEM showed that the biofilm of AE17 was thicker, with bacteria accumulating layer by layer and adhering closely to each other, while the biofilm of the AE17Δ*ompR* strain was significantly weakened, and

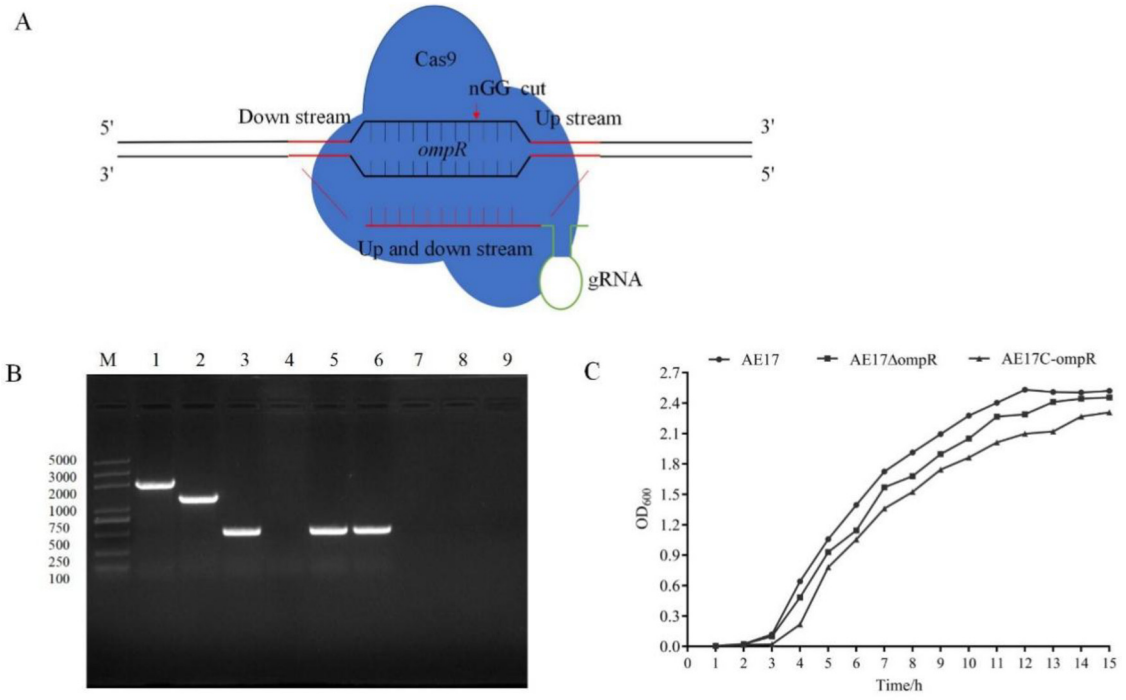

Figure 1. (A) The schematic diagram of the strategy for deleting the *ompR* gene in AE17 using CRISPR-Cas9 system. (B) Confirmation of the wild-type strain AE17, mutant strain AE17Δ*ompR*, and complement strain AE17C-*ompR*. M: 5 000 DNA marker; Lane 1: PCR product (2,617 bp) amplified from AE17 with primers *ompR*-out-F/R; Lane 2: PCR product (1,961 bp) amplified from AE17Δ*ompR* with primers *ompR*-out-F/R; Lane 3: PCR product (493 bp) amplified from AE17 with primers *ompR*-in-F/R; Lane 4: PCR product (0 bp) amplified from AE17Δ*ompR* with primers *ompR*-in-F/R; Lane 5: PCR product (493 bp) amplified from AE17 with primers *ompR*-in-F/R; Lane 6: PCR product (493 bp) amplified from AE17C-*ompR* with primers *ompR*-in-F/R; Lane 7-9: Negative control. (C) Growth curves of AE17, AE17Δ*ompR*, and AE17C-*ompR* strains in LB medium.

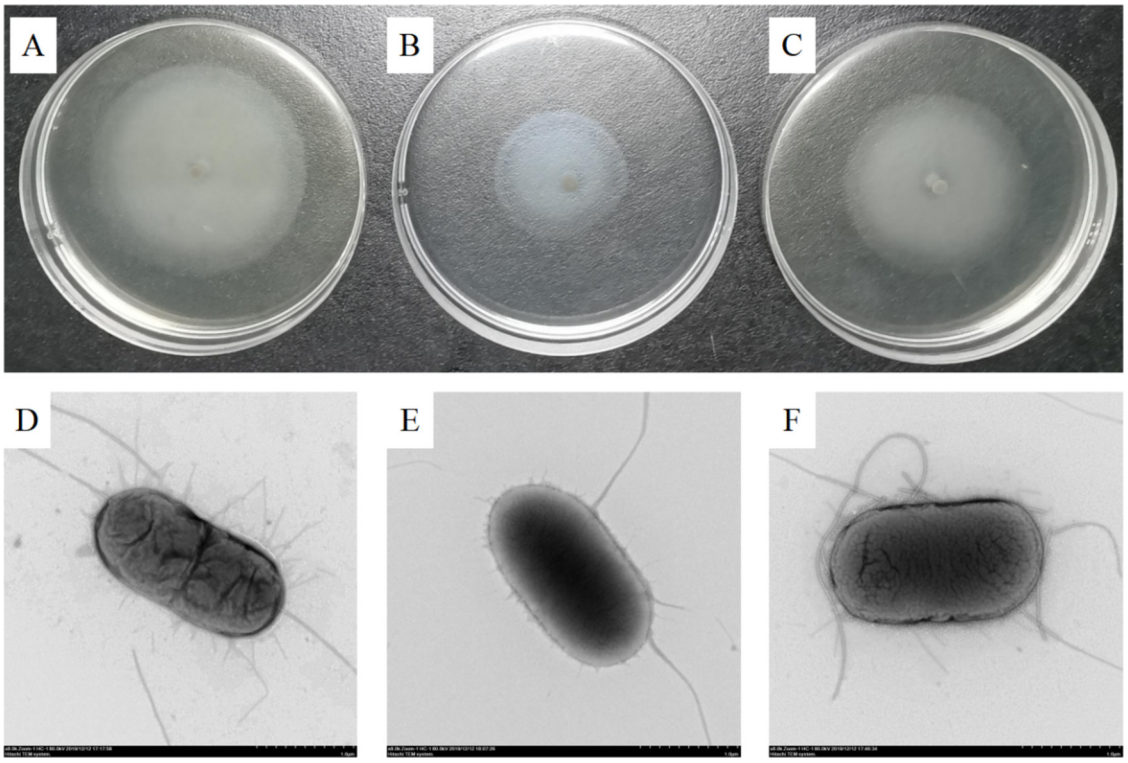

Figure 2. Bacterial motility and flagella of AE17, AE17Δ*ompR* and AE17C-*ompR* strains. (A−C) Bacterial motility was assessed by examining the migration of bacteria through the agar from the centre of the plate to the periphery. (A) the motility circle of the wild strain AE17; (B) the motility circle of the mutant strain AE17Δ*ompR*; (C) the motility circle of the complement strain AE17C-*ompR*. The diameters of the three strains of motility circles were 59.20 mm, 36.76 mm and 49.90 mm, respectively. (D, E) The flagella of AE17, AE17Δ*ompR* and AE17C-*ompR* in the transmission electron micrographs views (× 8,000). (D) The morphological observation of AE17 (× 8,000); (E) the morphological observation of AE17Δ*ompR* (× 8,000); (F) the morphological observation of AE17C-*ompR* (× 8,000).

only a partial monolayer of bacteria could be observed. The adhesion between bacteria was looser, while the biofilm of the complemented strain AE17C-*ompR* was restored to some extent, and the connection between the bacteria was tighter (Figure 4B). These results indicated that OmpR affects biofilm formation in APEC.

Deletion of the ompR Gene Was Responsible for Decreased Environmental Stresses

We compared the ability of AE17, AE17Δ*ompR*, and AE17C-*ompR* to survive in diverse environmental stress. The results showed that the ability of AE17Δ*ompR* to survive in diverse environmental stress was significantly reduced when compared to that of AE17, and the survival ability of AE17C-*ompR* was partially restored (Figure 5). These results suggested that OmpR plays an important role in bacterial adaption to different environmental stresses, including acid, alkali, osmotic, and oxidative stress.

OmpR Contributed to the Survival Ability of APEC in Serum Resistance

The AE17, AE17Δ*ompR*, and AE17C-*ompR* strains were incubated with SPF chicken serum at different

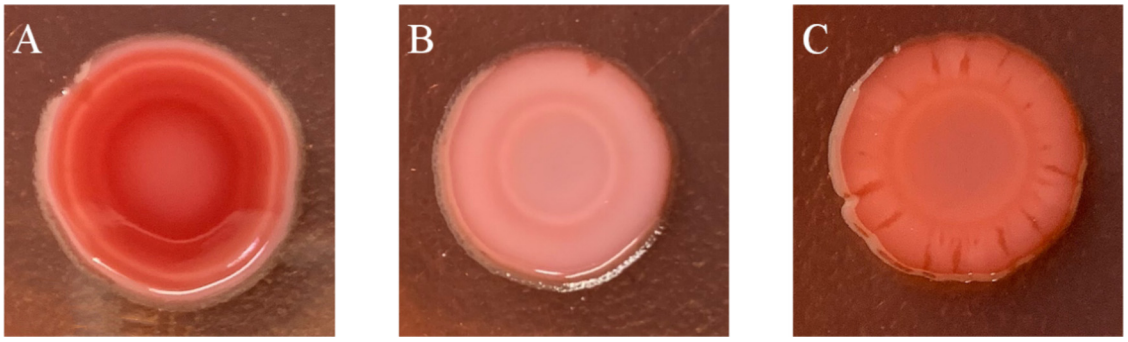

Figure 3. Biofilm formation ability by crystal violet and scanning electron microscope. (A) Analyze the biofilm formation ability of AE17, AE17Δ*ompR* and AE17C-*ompR* strains using a crystal violet assay; (B) (a) the morphological observation of AE17 (× 3,000); (b) the morphological observation of AE17Δ*ompR* (× 3,000); (c) the morphological observation of AE17C-*ompR* (× 3,000).

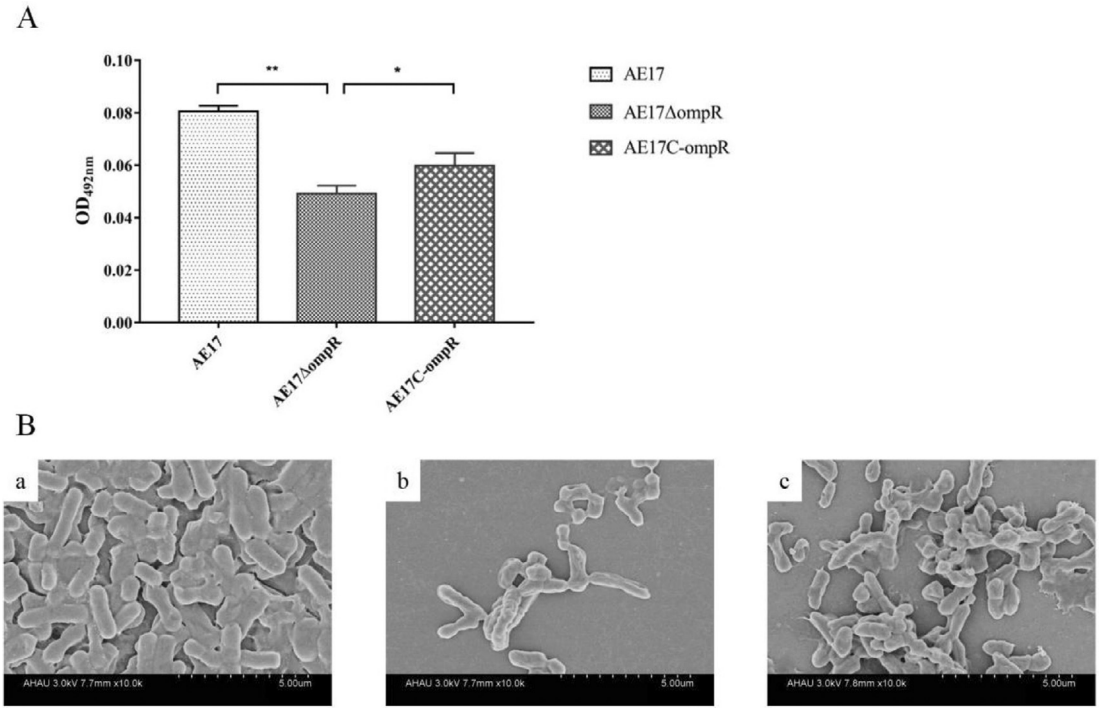

Figure 4. Morphotypes of strains was investigated at 37°C on Congo red agar plates. (A) AE17 displays the rdar morphotype indicating co-expression of curli and cellulose; (B) AE17Δ*ompR* results in the saw morphotype, indicating that neither curli nor cellulose is expressed; (C) AE17C-*ompR* also displays the rdar morphotype.

dilutions (25, 50, 75, or 100%) at 37°C for 30 min. The result showed that the mutant strain AE17Δ*ompR* was significantly susceptible to SPF chicken serum, compared with that of the wild-type strain AE17. And, the serum resistance was restored in the complement strain AE17C-*ompR* (Figure 6). The results indicated that the regulator OmpR is important for serum survival and is therefore essential for the pathogenesis of bacteria.

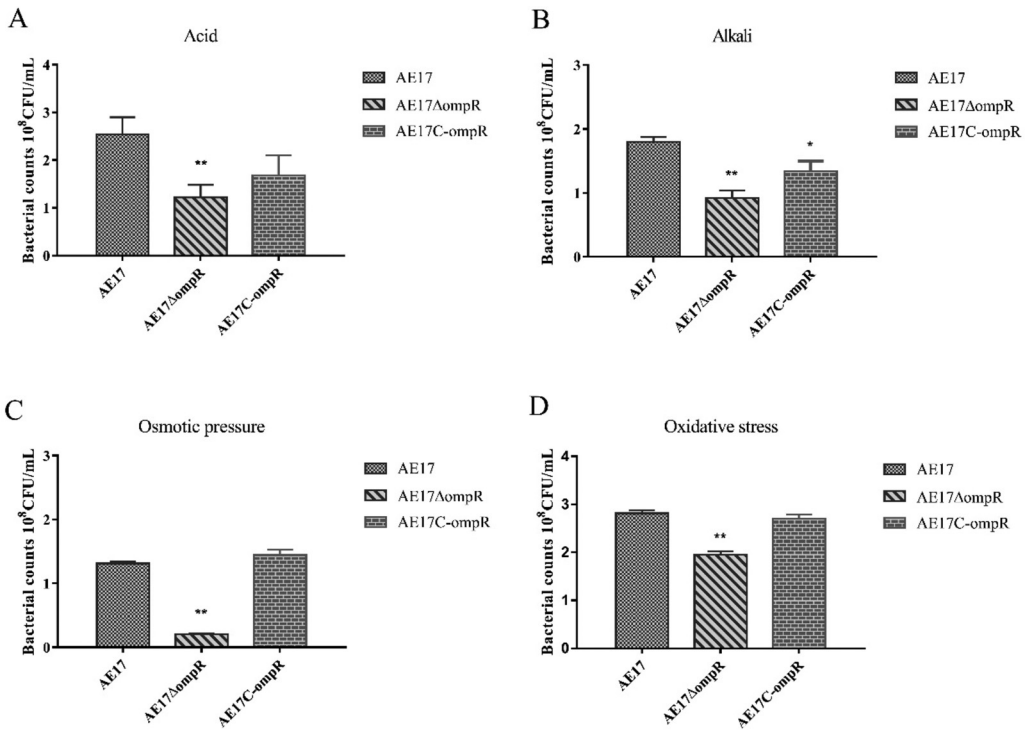

Figure 5. Determination the survival ability of AE17, AE17Δ*ompR*, and AE17C-*ompR* strains under diverse environmental stress, including acid (A), alkali (B), osmotic stress (C), and oxidative stress (D). The ability of AE17Δ*ompR* to survive in diverse environmental stress was significantly reduced when compared to that of AE17, and the survival ability of AE17C-*ompR* was partially restored($^{*}P < 0.05$, $^{**}P < 0.01$).

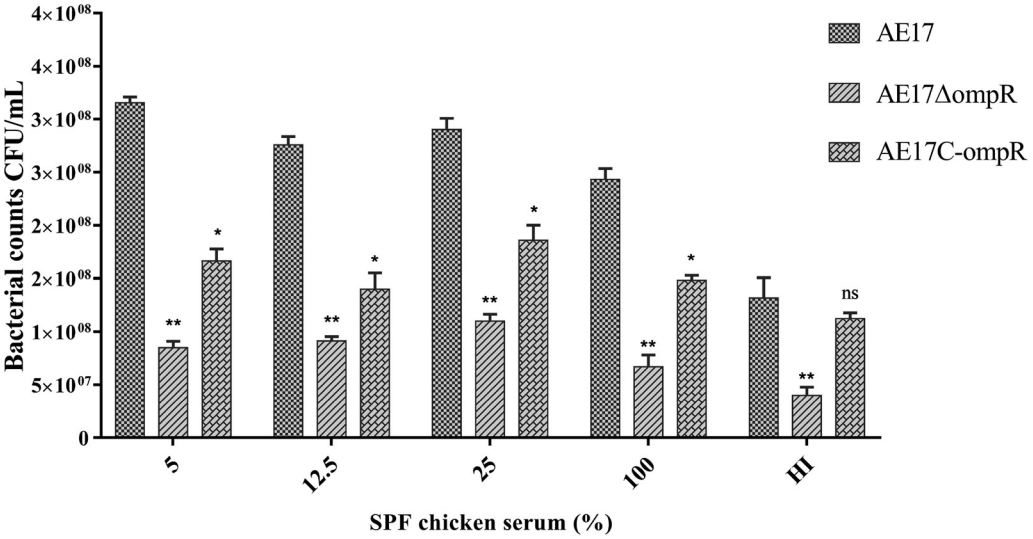

Figure 6. Bacterial resistance to SPF chicken serum. The AE17, AE17Δ*ompR*, and AE17C-*ompR* strains were incubated with different dilutions of SPF chicken serum (5, 12.5, 25, 100% and heat inactivated serum) at 37°C for 30 min. The mutant strain AE17Δ*ompR* was significantly susceptible to SPF chicken serum, compared with that of AE17. The data represent the averages of three trials ($^{*}P < 0.05$, $^{**}P < 0.01$).

OmpR Increased Adherence and Invasion to DF-1 Cells

The survival ability of AE17, AE17Δ*ompR*, and AE17C-*ompR* strains in DF-1 cells were performed. The adhesion ability of AE17Δ*ompR* to DF-1 cells was significantly decreased, compared with that of wild-type AE81 (Figure 7A). The ability of AE17Δ*ompR* to invade DF-1 cells was also significantly decreased, compared with that of wild-type AE81 (Figure 7B). These results indicate that OmpR influences the ability of APEC to adhere and invade DF-1 cells.

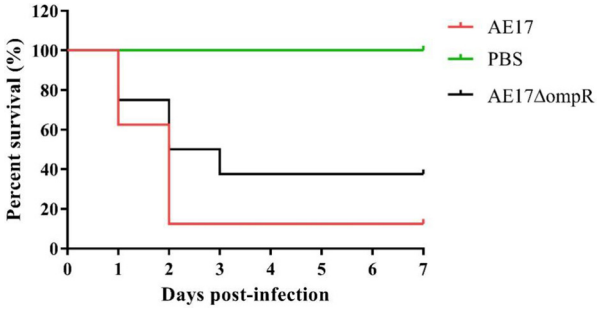

Figure 8. Determination of bacterial virulence. Seven-day-old chickens were infected intramuscularly with AE17, AE17Δ*ompR* at 10^6CFU. Negative controls were injected with PBS. Survival was monitored until 7 d postinfection.

OmpR is Necessary for APEC Virulence in Vivo

To investigate whether OmpR was involved in bacterial virulence, chicks were infected with 10^6 CFU of the wild-type strain AE17 and mutant strain AE17Δ*ompR*. Mortality was observed for 7 d post-challenge. As shown in Figure 8, the mortality of AE17 and AE17Δ*ompR* was 87.5% (7/8) and 62.5% (5/8), respectively. These results indicate that the deletion of the *ompR* gene led to the attenuation of virulence in chickens.

OmpR Upregulates the Expression of Virulence Genes

The qRT-PCR analyzed a range of virulence-related genes, including fimbriae, flagellar, and biofilm

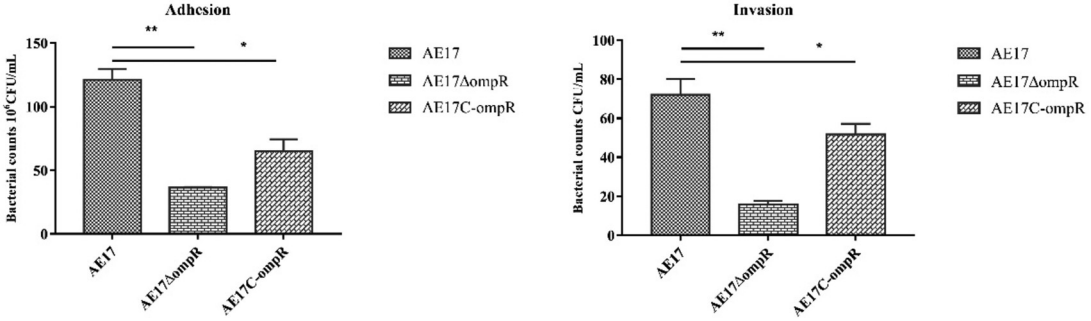

Figure 7. Adhesion and invasion to DF-1 cells. The counts of AE17, AE17Δ*ompR*, and AE17C-*ompR* to DF-1 cells were calculated. The counts of AE17Δ*ompR* to DF-1 cells were significantly lower than that of AE17 ($^{*}P < 0.05$, $^{**}P < 0.01$).

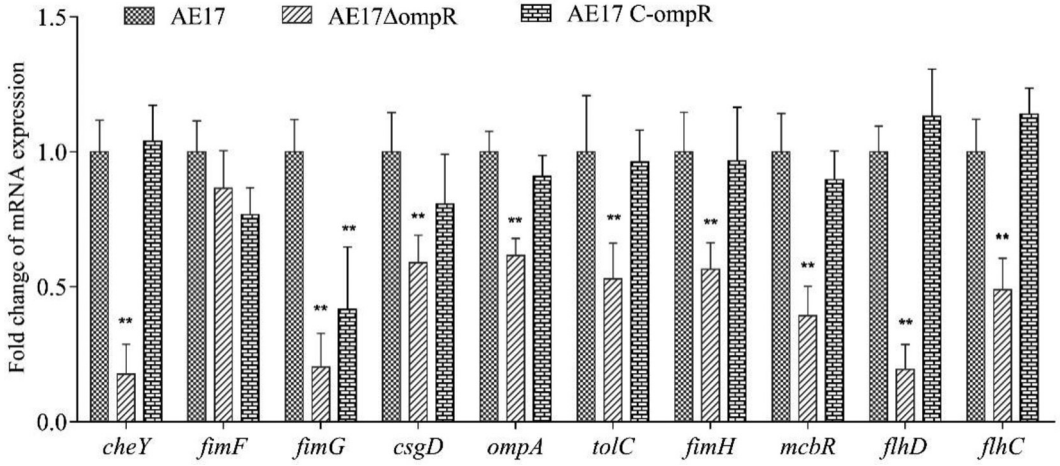

Figure 9. Relative expression of virulence genes was tested with qRT-PCR. Data were normalized to the housekeeping gene *dnaE*. X-axis represents different genes. The relative gene expression levels were calculated using the $2^{-\triangle\triangle Ct}$ method ($^{*}P < 0.05$, $^{**}P < 0.01$).

formation genes, to explore the role of *ompR* in the virulence regulation of APEC. RT-qPCR results showed that the transcription levels of the flagellar genes *flhD, flhC*, and *cheY*, the type I fimbriae gene *fimF, fimG*, and *fimH*, and the biofilm formation genes *csgD, ompA, tolC*, and *mcbR* were significantly downregulated in the mutant strain AE17Δ*ompR*, compared with those in the wild-type AE17 strain. The transcription levels of these genes were restored in the complement strain AE17C-*ompR* (Figure 9). This result suggests that OmpR plays an important regulatory role in APEC pathogenesis.

DISCUSSION

The TCS functions as a regulator control system that is involved in the response to external environment signals, bacterial drug resistance, and pathogenesis. The TCS consists of a histidine kinase that detects signals or stimuli and undergoes auto-phosphorylation, and a response regulator activated by the histidine kinase that acts as a transcriptional regulator altering gene expression (Breland et al., 2017). Different response regulators with a role in APEC pathogenesis have been reported. The regulator PhoP of PhoP/Q TCS positively regulates APEC flagella, biofilm formation, and pathogenicity, and regulates the expression of virulence genes (Yin et al., 2019). The Cpx TCS response regulator CpxR is critical to the virulence of APEC and regulates the expression of the type VI secretion system 2 (T6SS2) (Yi et al., 2019). The QseB, a response regulator of the QseBC TCS, functions as a global regulator of flagella, biofilm formation, and virulence (Ji et al., 2017). The OmpR/EnvZ system is a key TCS that plays an important role in response to environmental osmotic pressure and pathogenicity in most Gram-negative bacteria (Prigent-Combaret et al., 2001). However, the function of the response regulator OmpR is not yet understood in the APEC.

As already known, several factors have been reported to affect the flagella formation of APEC, including YqeI, ArcA, and YjjQ (Jiang et al., 2015a; Wiebe et al., 2015; Xue et al., 2020b). In this study, the *ompR* gene significantly influenced its motility in APEC O2 strain. Flagella are important motility structures and virulence factors of bacteria that participate in bacterial movement, chemotaxis, surface attachment, and invasion of host cells (Prigent-Combaret et al., 2000). The qRT-PCR results show that the OmpR regulates the flagella-associated genes *flhD, flhC, cheY, fimF*, fimG, fimH, and *csgD*. In *E. coli* K-12, the regulator OmpR regulates the flagella-associated genes *flhD, flhC, fimB*, and *csgD* (Jubelin et al., 2005; Rentschler et al., 2013; Samanta et al., 2013). OmpR directly binds to the flagellar master operon *flhDC* promoter in *Yersinia enterocolitica* (Raczkowska et al., 2011). Consistent with the results for *E. coli* K-12 and *Y. enterocolitica*, we found that OmpR also affects the expression of *flhDC* genes in APEC. The flagellar synthesis master promoter *flhDC* responds to environmental signals and transcribes under complex regulation, which in turn activates the secondary promoter and tertiary flagellar genes (Raczkowska et al., 2011).

To survive in different environments, pathogens need to adapt rapidly to different conditions (Chakraborty and Kenney, 2018). In this study, we demonstrated that OmpR is involved in responding to various external environmental changes, including acid, alkali, oxidative stress, and osmotic pressure. In non-pathogenic *E. coli* K12, the OmpR controls the expression of outer membrane porins OmpF and OmpC in response to environmental osmotic pressure. In *Y. enterocolitica*, OmpR was identified as the response regulator for osmolarity-regulated porins (Brzóstkowska et al., 2012). OmpR contributes to cytoplasmic acidification by repressing the *cadC/BA* operon during acid stress in *S. Typhimurium* and *E. coli* (Chakraborty et al., 2015, 2017). The curli fimbriae play important roles in bacterial adhesion, biofilm formation, and host pathogenesis in *E. coli* (Barnhart and Chapman, 2006). The previous study showed that EnvZ, a histidine kinase, contributes to the biofilm formation of APEC and regulates the expression of biofilm-associated genes (Feng et al., 2021). In this study, the deletion of the *ompR* gene decreased the

biofilm formation of APEC and resulted in a lack of curli and cellulose production in APEC, as well as the downregulation of transcript levels of the *csgD, ompA, tolC*, and *mcbR* genes. CsgD, a master transcriptional regulator of curli assembly, activates the production of curli fimbriae and cellulose via the positive regulation of the *csgBAC* operon and regulates the expression of biofilm formation genes (Ogasawara et al., 2011). The membrane protein TolC is involved in biofilm formation and curli production of the ExPEC strain (Hou et al., 2014). In *E. coli*, the OmpR binds the *csgD* promoter region and promotes biofilm formation and curli production (Prigent-Combaret et al., 2001).

In adherent-invasive *E. coli* (**AIEC**), OmpR is involved in vitro adhesion and invasion to human intestinal epithelial T84 cells (Lucchini et al., 2021). In this study, OmpR contributes to the adhesion and invasion of DF-1 cells and also regulates the expression of type I fimbriae genes (*fimF, fimG*, and *fimH*) and the *csgD* gene. In *E. coli*, OmpR binds the CsgD promoter and affects adhesion (Prigent-Combaret et al., 2001). Serum resistance contributes to septicemia and mortality of APEC during colonization and survival in the host (Mellata et al., 2003; Li et al., 2011). Outer membrane proteins contribute to the serum resistance of APEC (Mellata et al., 2003). In this study, the *ompR* contributed to sensitivity in SPF chicken serum, which might decrease the expression of biofilm formation. The deletion of the *ompR* gene downregulates the expression of the *ompA* gene, encoding the outer membrane proteins OmpA, associated with serum resistance (Nielsen et al., 2020). These findings suggest that OmpR plays an important role in the adhesion to host cells and serum resistance, suggesting OmpR plays an important role in the pathogenicity of APEC O2 strain.

In summary, the regulator OmpR participates in the pathogenicity of APEC O2 strain, including attenuated bacterial flagellum formation and motility, reduced biofilm formation, impaired survival in diverse environmental stress, and serum resistance, and diminished adhesion and invasion to DF-1 cells in APEC. The regulator OmpR participates in the pathogenicity of APEC and provides insight for the further research of APEC pathogenicity.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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