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*Original article*

# Modulation of virulence genes by the two-component system PhoP-PhoQ in avian pathogenic *Escherichia coli*

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## Abstract

Avian pathogenic *Escherichia coli* (APEC) infections are a very important problem in the poultry industry. PhoP-PhoQ is a two-component system that regulates virulence genes in APEC. In this study, we constructed strains that lacked the *PhoP* or *PhoQ* genes to assess regulation of APEC pathogenicity by the PhoP-PhoQ two-component system. The *PhoP* mutant strain AE18, *PhoQ* mutant strain AE19, and *PhoP*/*PhoQ* mutant strain AE20 were constructed by the Red homologous recombination method. Swim plates were used to evaluate the motility of the APEC strains, viable bacteria counting was used to assess adhesion and invasion of chick embryo fibroblasts, and Real-Time PCR was used to measure mRNA expression of virulence genes. We first confirmed that AE18, AE19, and AE20 were successfully constructed from the wild-type AE17 strain. AE18, AE19, and AE20 showed significant decreases in motility of 70.97%, 83.87%, and 37.1%, respectively, in comparison with AE17. Moreover, in comparison with AE17, AE18, AE19, and AE20 showed significant decreases of 63.11%, 65.42%, and 30.26%, respectively, in CEF cell adhesion, and significant decreases of 59.83%, 57.82%, and 37.90%, respectively, in CEF cell invasion. In comparison with AE17, transcript levels of *sodA*, *polA*, and *iss* were significantly decreased in AE18, while transcript levels of *fimC* and *iss* were significantly decreased in AE19. Our results demonstrate that deletion of *PhoP* or *PhoQ* inhibits invasion and adhesion of APEC to CEF cells and significantly reduces APEC virulence by regulating transcription of virulence genes.

**Key words**: APEC, PhoP-PhoQ, pathogenicity, mutant

## Introduction

Avian pathogenic *Escherichia coli* (APEC) is a common pathogen in poultry, and its main clinical manifestations are airsacculitis, perihepatitis, pericarditis, and encephalitis, as well as other symptoms. APEC can eventually lead to death by a range of diseases, leading to serious economic losses. APEC infection is currently the most economically important bacterial disease in the poultry industry (Kaper et al. 2004).

Two-component regulatory system occur widely in bacteria, where they control responses to environ-

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mental conditions, including virulence and growth, and these systems are essential for bacterial survival. PhoP-PhoQ is a ubiquitous two-component regulatory system in Gram-negative bacteria that is composed of the histidine protein kinase PhoQ (the sensor kinase) and the response regulator protein PhoP (a transcription factor). Upon sensing an external stimulus, PhoQ autophosphorylates and subsequently phosphorylates Asp51 in the regulatory domain of PhoP, which then regulates transcription of target genes by binding to PhoP boxes ((T/G)GTTTA) in the promoter regions of bacterial DNA. The kinase activity of PhoQ can be activated by $Mg^{2+}$, $Ca^{2+}$, and $H^{+}$, but is more likely to be activated by defensins (Shprung et al. 2012). Activation of PhoP-PhoQ improves the acid resistance of bacteria, increasing their ability to survive within macrophages and resistance to mammalian antimicrobial peptides (Bader et al. 2003). The PhoP-PhoQ two-component system has been studied extensively in the context of *Yersinia pestis* and *Salmonella* virulence. However, few studies exist on PhoP-PhoQ as a regulator of APEC virulence.

In this study, we successfully constructed single-gene knockout APEC strains for PhoP-PhoQ, as well as a double-gene knockout APEC strain. To gain a better understanding of the APEC PhoP-PhoQ two-component system and provide a theoretical basis for research into APEC virulence, the wild-type APEC strain AE17 and the 3 mutant strains were subjected to assessments of motility, adhesion and invasion of chicken embryo fibroblasts (CEFs), and mRNA transcription levels of virulence genes.

## Materials and Methods

### Bacterial strains, plasmids, and culture conditions

The wild-type strain was a clinical isolate of APEC strain AE17 (serotype O2) that was collected from a chicken in Anhui Province, China in 2008. The bacteria were cultured in Lennox broth (LB) at 37°C throughout the experiments. Homologous recombinant plasmid pKD46 (containing a temperature-sensitive replicon), plasmid pKD3 (providing a chloramphenicol resistance gene), and plasmid pCP20 (containing FRT sites to eliminate chloramphenicol resistance) were provided by the Shanghai Veterinary Research Institute of the Chinese Academy of Agricultural Sciences. The low-copy expression plasmid PSTV-28 was obtained from Takara Bio Inc. (Otsu, Shiga, Japan). Chicken embryo fibroblasts were provided by the China Animal Husbandry and Veterinary Research Institute of the Anhui Academy of Agricultural Sciences. All chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Animals

In the experiments, we used the one-day-old Roman hens which we purchased from Anhui Anqin Poultry Company Ltd. (Anhui Province, China) as the experimental animals. We are very sorry to do the experiments at the cost of their lives. We tried our best to provide a comfortable and clean environment for them. They were housed in cages with enough water and feed, and the indoor temperature was kept at 20-28°C. These hens made the biggest contribution to scientific experiments, we must make full use of our experimental results to save more poultry in future. Animals were euthanized using intravenous sodium pentobarbital (100 mg/kg), Dolethal, Vetoquinol, Cedex, France) in the wing vein.

### Ethics statement

One-day-old Roman hens were obtained from Anhui Anqin Poultry Company Ltd (Anhui Province, China) and kept under controlled temperature conditions (28-30°C). Care and maintenance of all animals were in accordance with the Institutional Animal Care and Use Committee (IACUS) guidelines by Shanghai Veterinary Research Institute, the Chinese Academy of Agricultural Sciences (CAAS).

### Construction and identification of mutant strains

The AE18, AE19, and AE20 mutant strains were generated using the lambda Red recombinase method as described previously (Datsenko and Wanner 2000) with some modifications. Briefly, upstream and downstream homologous recombination fragments (566 bp and 661 bp, respectively) of the PhoP gene were amplified by PCR using primers Phop-UP-F/Phop-lap--cm-UP-R and PhoP-lap-cm-Down-F/PhoP-Down-R (Table 1). The chloramphenicol resistant cassette (CAT) was amplified from plasmid pKD3 by PCR with primers pKD3-cm-PhoP-R/pKD3-cm-PhoP-F with a short complementary sequence (Table 1). Targeting fragment PhoP-UP-cat-Down (2258 bp) was amplified by PCR using primers PhoP-UP-F/PhoP-Down-R (Table 1). The homologous recombination fragment PhoQ-Up-cat-Down (1232 bp) of PhoQ was amplified by PCR using primers

Table 1. Primers used in the gene mutagenesis.

| Primers | Oligonucleotide sequence (5' to 3') | Product size/bp |
|---|---|---|
| PhoQ-UP | ATGAAAAAA TTACTGCGTC TTTTTTTCCC GCTCTCGCTG CGGGTACGTT TTCTGTTGGC AACGGCAGCG GTAGTACTGG TGCTTTCGCT TGCCTACGGA TGTAGGCTGGAGCTGCTTCGA | 1232 |
| PhoQ-Down | TTATTCATCTTTCGGCGCAGAATGCTGGCGACCAAAAATCACCTCCAT CCGCGCACCGCCCAGCATGCTCTCTCCGGCGACGATTTTACCCTCATA TTGCTC CATATGAATATCCTCCTTAG | |
| PhoP-UP-F | GTGACCTGACCGACTCCAC | 566 |
| PhoP-lap-cm-UP-R | CCAGCCTACACGCATTGTCTTCAACAACC | |
| PhoP-lap-cm-Down-F | TATTCATATGAGTGATTACCACCGTTCGC | 661 |
| PhoP-Down-R | ACAGCAGATTGGCTGAGAG | |
| pKD3-cm-PhoP-F | AGACAATGCGTGTAGGCTGGAGCTGCTT | 1051 |
| pKD3-cm-PhoP-R | GGTAATCACTCATATGAATATCCTCCTTAGTTC | |
| PhoPQ-UP-F | ATGGCTAATGTCGCCTACC | 1068 |
| PhoPQ-lap-cm-UP-R | CCAGCCTACACTGATGACCAGCATCCTGT | |
| PhoPQ-lap-cm-Down-F | TATTCATATGGGATGGAGGTGATTTTTGGTC | 992 |
| PhoPQ-Down-R | CTGATAAGGCGGCTCTGGC | |
| pKD3-cm-PhoPQ-F | TGGTCATCAGTGTAGGCTGGAGCTGCTT | 1051 |
| pKD3-cm-PhoPQ-R | ACCTCCATCCCATATGAATATCCTCCTTAGTTC | |
| PhoP-in- F | CAGTAAAGTTCAGATTCAGGATGCT | 383 |
| PhoP-in- R | TAGAAAATACACGGCGAGAGAGAT | |
| PhoQ-in- F | CTAACTTATGATGAGAACGGGC | 931 |
| PhoQ-in- R | ACACGAAGCAGTATTTACAGGC | |
| PhoQ-out-F | GGCAAAGTGGTGAGCAAAG | 711 |
| PhoQ-out-R | AAAATCGGGCCAGTTAAGAG | |
| PhoP-out-F | GTGAGCAAACTGGAAGTGAAC | |
| PhoP-out-R | GAATTCGCGGTTATGTTCTT | |
| PhoPQ-in- F | AGTAGTATTGGTGCTTTCGCT | |
| PhoPQ-in- R | CTTTTTAACAGTCGGTTCAGG | |
| PhoPQ-out-F | GCACGCATCAAAACCATCG | |
| PhoPQ-out-R | CGCACCAGCACATCACCTT | |
| PhoP- EcorI | ccggaattcATCGCCCTTAACCACTTCA | 1677 |
| PhoP-HindIII | ccgaagcttGCTGCCCGTTCTCATCATA | |
| PhoPQ- EcorI | CCGGAATTCGACTCCACCGTGCTGCGT | 2776 |
| PhoPQ-SalI | CGCGTCGACCGTTCAAGAAAATCGGGC | |
| M13-R | TTGTAAAACGACGGCCAG | |
| M13-F | CAGGAAACAGCTATGACC | |

a EcorI restriction sites are underlined respectively.
b HindIII restriction sites are underlined respectively.
c SalI restriction sites are underlined respectively.
d A 1835 bp PCR product was amplified from wild strain AE17, and 1341bp product was amplified from mutant strain AE18, using primers PhoP-out-F/PhoP-out-F.
e A 1748 bp PCR product was amplified from wild strain AE17, and 488bp product wasamplified from mutant strain AE19, using primers PhoQ-out-F/PhoQ-out-F.
f A 4432bp PCR product was amplified from wild strain AE17, and 2377bp product was amplified from mutant strain AE20, using primers PhoPQ-out-F/PhoPQ-out-F.
g A 1719bp,2776bp or 2776bp PCR product was amplified from wild strains complementation strains CAE18, CADE19, CADE20, respectively, using primers M13-R/M13-F.

PhoQ-UP/PhoQ-Down (Table 1) and plasmid pKD3 as a template. Upstream and downstream fragments (1068 bp and 992 bp, respectively) of the PhoP and PhoQ genes were amplified by PCR using primers PhoPQ-UP-F/PhoPQ-lap-cm-UP-R and PhoPQ-lap-cm-Down-F/PhoPQ-Down-R, respectively. The PhoPQ-UP-cat-Down fragment (2258 bp) was amplified using the same method as that used for the Phop homologous recombination. The homologous recombination fragments were purified using a Gel Extraction Kit (Sangon Biotech, Shanghai, China).

Next, 10 μL of each PCR products was added to 100 μL of AE17 competent cells containing the lambda Red recombinase expression plasmid pKD46, and electroporation was performed with a Gene Pulser II transfection apparatus (Bio-Rad, Hercules, CA, USA) at 25 μF, 2.5 kV, and 200 Ω. After electroporation, the cells were immediately added to 1 mL of SOC medium and centrifuged at 200 rpm for 2 h at 37°C. Next, the cells were plated on LB plates containing 10 μg/mL chloramphenicol. After incubation for 24 h, chloramphenicol-positive recombinants were identified and subjected to PCR amplification with primers PhoP-out-F/PhoP-out-R, PhoQ-out-F/PhoQ--out-R, and PhoPQ-out-F/PhoPQ-out-R (Table 1), in order to determine whether homologous recombination occurred in the right arm position. The mutant strain was transformed further with the pCP20 plasmid (Datsenko et al., 2000) and chloramphenicol-sensitive mutant strains were selected, identified by PCR with primers pKD3-F/pKD3-R (Table 1), and designated AE18, AE19, and AE20.

### Complementation of the gene mutant strains

PhoP and PhoQ share a promoter (Kasahara et al. 1992). An expression plasmid was constructed containing the PhoP and PhoP/Q gene fragments. The target fragments of PhoP (1677 bp) and PhoP/Q (4432 bp) were amplified by PCR using primers PhoP-EcorI/PhoP-HindIII and PhoPQ-EcorI/PhoPQ--SalI (Table 1). The target fragments were ligated to the PSTV-28 vector using a restriction enzyme. The constructed vector was introduced into mutant strain competent cells, which were identified by PCR using primers 13-R/M13-F (Table 1) and designated CAE18, CAE19, and CAE20.

### Motility assay

The motility assay was performed in swim plates (1% tryptone, 0.5% NaCl, 0.8% glucose, and 0.3% agar) as described previously (Ling et al. 2010), with some modifications. AE17 and the mutant strains were grown in LB medium. The culture supernatants were collected by centrifugation, washed 3 times with sterile PBS, and adjusted to an $OD_{600}$ value of 1.0. A total of 10 μL of the bacterial suspension was loaded onto each swim plates and incubated at 37°C overnight. Motility was assessed by examining the migration of bacteria through the agar from the center of the plate toward the periphery.

### Bacterial adherence and invasion assay

To identify whether deletion of PhoP and PhoQ influenced bacterial adhesion and invasion, assays were performed based on previously described method (Wang et al. 2011). CEF cells were grown in 24-well plates to 90% confluence in 5% $CO_2$ at 37°C. The AE17 or mutant strains were added to each well at 200 MOI (multiplicity of infection), followed by low speed centrifugation for 5 min at 400 *g*. Next, 1 mL of cell culture medium was added and the cells were incubated in 5% $CO_2$ at 37°C for 1.5 h, after which the cells were washed thoroughly with sterile PBS 3 times to remove nonadherent bacteria. The adhesive cells were dissolved by adding 200 μL of 0.5% Triton 0X-100 to each well for 10 min, after which bacteria were counted to determine the adhesive frequency (Yoshida et al. 2005). Another group of cells received 1 mL DMEM complete medium containing gentamicin for 1 h at 37°C to kill extracellular bacteria. The cells were dissolved by adding 200 μL of 0.5% Triton X-100, and bacteria were counted (Yoshida et al. 2005) to determine the invasion frequency.

### RNA isolation, reverse transcription, and real-time PCR

Total RNA was extracted from the AE17 and mutant strains using the Trizol method according to the manufacturer's protocol. RNA was reverse transcribed into cDNA as described previously (Han and Lu 2009). Briefly, random primers (100 pmol) were added to 8 μL of total RNA and incubated at 70°C for 5 min. The solution was cooled on ice, and 5 μL of 5x M-MLV reaction buffer, 4 μL of dNTPs (10 mM), 1 μL of RNase inhibitor (Promega, USA), 1 μL of M-MLV reverse transcriptase (Promega, USA), and 5 μL of RNase-free ddH2O were added, and the mixture was maintained at 42°C for 1 h. The RNA integrity was assessed using 1% agarose gel electrophoresis.

The 13 virulence genes of APEC (see Table 2 for primers) were assessed by RT-PCR with the *dnaE* gene as a reference. SYBR Green I-based two-step

Table 2. Primers used in Real-Time PCR.

| Primers | Oligonucleotide sequence (5' to 3') | Product size/bp |
|---|---|---|
| dnaE-R<br>dnaE-F | GATTGAGCGTTATGTCGGAGGC<br>GCCCCGCAGCCGTGAT | 80 |
| MarA-R<br>MarA-F | CGCAAAAGCTGAAGGAAAGT<br>CGGCGGAACATCAAAGTAAT | 113 |
| YhiD-R<br>YhiD-F | GGTTATTGGCAGCGGTATGT<br>GTTCATCAGGCGGAAGGTTA | 100 |
| SodA-R<br>SodA-F | TCGGCTCCGTTGATAACTTC<br>CAGCGGAGAGTCCTGGTTAG | 134 |
| metJ-R<br>metJ-F | AAAATCCTCACCGATGAACG<br>TCACGCATGATCTCTTTGC | 182 |
| polA-R<br>polA-F | AGTTCAAACGCTGGACTGCT<br>CTTTTTCCAGCTTCGCAATC | 198 |
| tsh-R<br>tsh-F | GCACGAACTGGGAAGTATGGA<br>GGCATAGAAACCACCACCCC | 118 |
| ibeA-R<br>ibeA-F | TTGTTTTGGCGGAATGATG<br>CATTGATTTTGCCGTTTCTTCT | 118 |
| Stx2F-R<br>Stx2F-F | GTGTAAAACTACGCCATCCG<br>AAGCCCAGAACCAGACTCCC | 186 |
| fimC-R<br>fimC-F | GCCGATGGTGTAAAGGATGG<br>AACTTTCCCGATCCTGTGGC | 127 |
| OmpA-R<br>OmpA-F | TCCAGAGCAGCCTGACCTTC<br>GCTGAGCCTGGGTGTTTCCT | 152 |
| iss-R<br>iss-F | CCGACAGCAGTAACACCAAAGG<br>TTCTGCACCGCCAACAAATT | 105 |
| LuxS-R<br>LuxS-F | ACGCCATTACCGTTAAGATG<br>AGTGATGCCAGAAAGAGGGA | 81 |
| pfs-R<br>pfs-F | CGGCAACAGCCAGGAACTCA<br>GCGAAAATCCGCCACAACTT | 169 |

Table 3. Determination of the median lethal dose of the bacteria.

| Groups | Dilution degree (CFU/mL) | | | | | | | $LD_{50}$ (CFU/mL) |
|---|---|---|---|---|---|---|---|---|
| | $1 \times 10^9$ | $1 \times 10^8$ | $1 \times 10^7$ | $1 \times 10^6$ | $1 \times 10^5$ | $1 \times 10^4$ | Normal saline | |
| AE17 | 8/8 | 8/8 | 8/8 | 8/8 | 2/8 | 0/8 | 0/8 | $1.78 \times 10^5$ |
| AE18 | 8/8 | 8/8 | 5/8 | 1/8 | 0/8 | 0/8 | 0/8 | $5.62 \times 10^6$ |
| AE19 | 8/8 | 8/8 | 7/8 | 3/8 | 0/8 | 0/8 | 0/8 | $1.78 \times 10^6$ |
| AE20 | 8/8 | 8/8 | 6/8 | 3/8 | 0/8 | 0/8 | 0/8 | $2.37 \times 10^6$ |

RT-PCR was performed using the Step One Plus™ Real-Time PCR System (Applied Biosystems®, Thermo Fisher Scientific, Waltham, MA, USA). Real-Time PCR was performed in a total volume of 25 μL, containing 12.5 μL of PCR Master Mix (SYBR Green PCR Master Mix, Applied Biosystems®), 1 μL of cDNA (or water as a negative control), 0.5 μL each of 10 mM upstream and downstream primers (Table 2) and 10.5 μL of water. Real Time PCR was performed with an initial denaturation step of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min annealing/extension at 65°C. Data were analyzed using the 2-ΔΔCT method to calculate relative mRNA transcript levels of virulence genes.

### Determination of the median lethal dose of the bacteria

AE17 and the mutant strains were grown in LB medium to the logarithmic phase. The bacterial concentration was adjusted to $1 \times 10^9$ CFU/mL by adjusting the $OD_{600}$, and a 10-fold serial dilution was per-

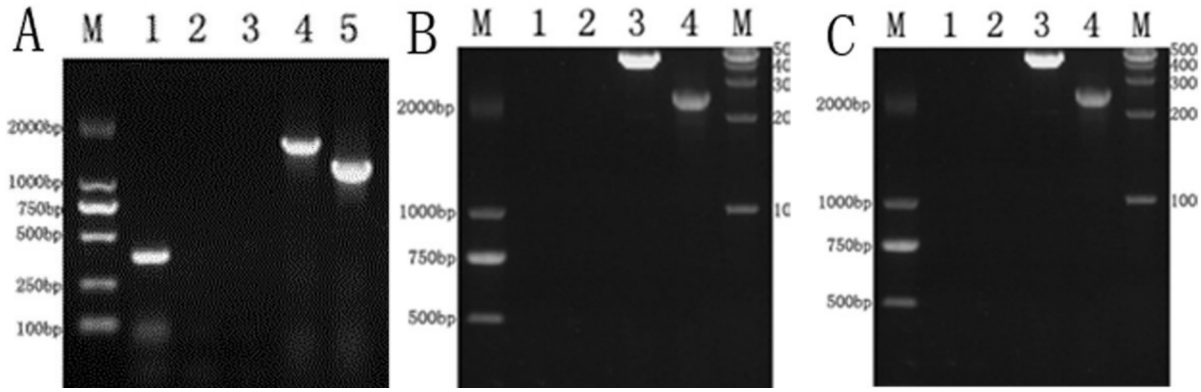


Fig. 1. PCR analysis of the mutant strain AE18, AE19 and AE20. **A)** PCR analysis of the mutant strain AE18. Lane 1: The wild strain AE17 showed a 383bp PCR product using primers PhoP-in-F/PhoP-in-R. Lane 2: The mutant strain AE18 showed no PCR product using primers PhoP-in-F/PhoP-in-R.Lane 3: Negative control. Lane 4: The wild strain AE17 showed a 1835bp PCR product using primers PhoP-out-F/PhoP-out-R. Lane 5: The mutant strain AE18 showed a 1341bp PCR product using primers PhoP-out-F/PhoP-out-R. **B)** PCR analysis of the mutant strain AE19. Lane 1: The wild strain AE17 showed a 931bp PCR product using primers PhoQ-in-F/PhoQ-in-R. Lane 2: The mutant strain AE19 showed no PCR product using primers PhoQ-in-F/PhoQ-in-R.Lane 3: Negative control. Lane 4: The wild strain AE17 showed a 1748bp PCR product using primers PhoQ-out-F/PhoQ-out-R. Lane 5: The mutant strain AE19 showed a 488bp PCR product using primers PhoQ-out-F/PhoQ-out-R. **C)** PCR analysis of the mutant strain AE20. Lane 1: The mutant strain AE20 showed no PCR product using primers PhoP-in-F/PhoP-in-R. Lane 2: The mutant strain AE20 showed no PCR product using primers PhoQ-in-F/PhoQ-in-R.Lane 3: Negative control. Lane 4: The wild strain AE17 showed a 4432bp PCR product using primers PhoPQ-out-F/PhoPQ-out-R. Lane 5: The mutant strain AE20 showed a 2377bp PCR product using primers PhoPQ-out-F/PhoPQ-out-R.

formed to obtain 6 different concentrations. Seven-day-old Roman hens were randomly divided into groups (n=8 each). Each group received an intramuscular injection of 1 mL of the appropriate concentration of bacteria. The control group received an intramuscular injection of 1 mL saline. The number of deaths in each group was counted and compared.

## Statistical analysis

Data were expressed as mean±SEM of three independent assays (n=3). Statistical assays were analyzed using one-way or two-way analysis of variance (ANOVA) by SAS software version 9.2. For one-way ANOVA analysis, a Tukey varianciple comparison test was used for post-test analysis when significant differences existed. Differences with P-values less than 0.05 were considered statistically significant. The direction and degree of each correlation were studied by Pearson's coefficient (±r). At the same time, the significance of r was tested at the 5% significance level for the appropriate degrees of freedom using a two-tailed test.

## Results

### Generation and identification of the mutant strains

The mutant PhoP gene in mutant strain AE18 was identified by PCR (Fig. 1A). As shown in Fig. 1A, a 383 bp PCR product was amplified from wild-type strain AE17 using the PhoP-in-F/PhoP-in-R primers (lane 1), while no product was amplified from mutant strain AE18 using these primers (lane 2). When the PhoP-out-F/PhoP-out-R primers were used, a 1835 bp PCR product was amplified from wild-type strain AE17 (lane 4) and a 1341 bp PCR product was amplified from mutant strain AE18 (lane 5). The mutant PhoQ gene in mutant strain AE19 was identified by PCR (Fig. 1B). As shown in Fig. 1B, a 931 bp PCR product was amplified from wild-type strain AE17 using the PhoQ-in-F/PhoQ-in-R primers (lane 1), while no product was amplified from mutant strain AE19 using these primers (lane 2). When the PhoQ-out-F/PhoQ-out-R primers were used, a 1748 bp PCR product was amplified from wild-type strain AE17 (lane 4) and a 488 bp PCR product was amplified from mutant strain AE19 (lane 5). The mutant

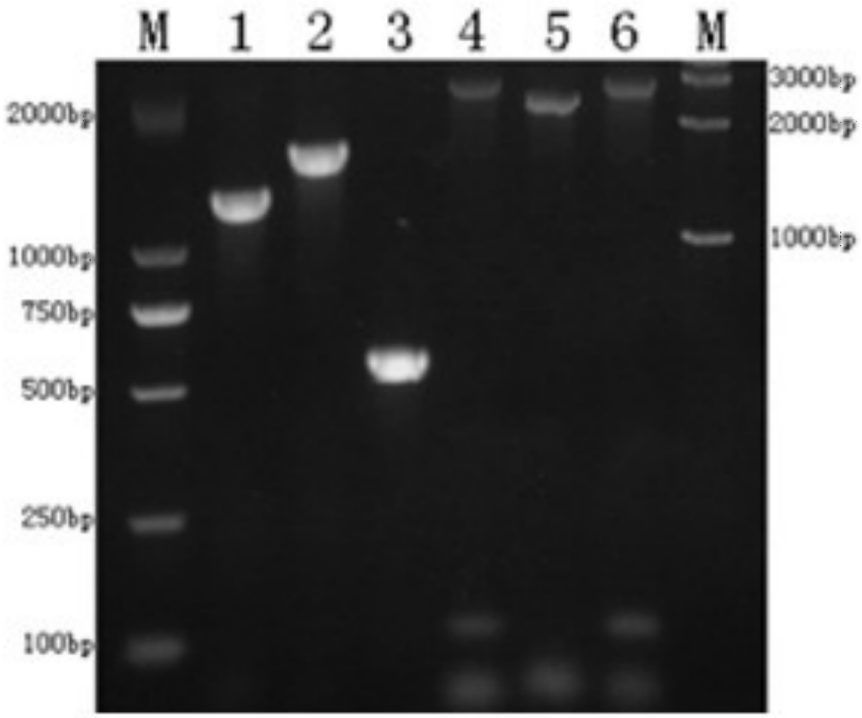


Fig. 2. PCR analysis of the complementation strains CAE18,CAE19 and CAE20. Lane 1: The complementation strain CAE18 showed 1341bp PCR product using primers PhoP-out-F/PhoP-out-R. Lane 2: The strain CAE18 showed a 1719bp PCR product using primers M13-F/M13-R. Lane 3: The strain CAE19 showed a 488bp PCR product using primers PhoQ-out-F/PhoQ-out-R. Lane 4:The strain CAE19 showed a 2776bp PCR product using primers M13-F/M13-R . Lane 5: The strain CAE20 showed a 2377bp PCR product using primers PhoPQ-out-F/PhoPQ-out-R. Lane 6: The strain CAE20 showed a 2776bp PCR product using primers M13-F/M13-R

PhoP and PhoQ genes in mutant strain AE20 were identified by PCR (Fig. 1C). As shown in Fig. 1C, no product was amplified from mutant strain AE20 using the PhoP-in-F/PhoP-in-R primers (lane 1) or the PhoQ-in-F/PhoQ-in-R primers (lane 2). When the PhoPQ-out-F/PhoPQ-out-R primers were used, a 4432 bp PCR product was amplified from wild-type strain AE17 (lane 4) and a 2377 bp PCR product was amplified from mutant strain AE20 (lane 5).

### Generation and identification of complementation strains

Complementation strains were constructed using plasmid PSTV-28 and confirmed using PCR (Fig. 2). As shown in Fig. 2, a 1341 bp PCR product was amplified from strain CAE18 using the PhoP-out-F/PhoP-out-R primers (lane 1), while a 488 bp PCR product was amplified from strain CAE19 using the PhoQ-out-F/PhoQ-out-R primers (lane 2). When the PhoPQ-out-F/PhoPQ-out-R primers were used, a 2377 bp PCR product was amplified from the wild-type complementation strain CAE20 (lane 3). When the M13-R/M13-F primers were used, PCR products of 1719 bp (lane 4), 2776 bp (lane 5), and 2776 bp (lane 6) were amplified from the CAE18, CAE19, and CAE20 strains, respectively.

### Assessment of mutant strain motility

As shown in Fig. 3, the motility of the AE18, AE19, and AE20 mutant strains was significantly reduced by 70.97%, 83.87%, and 37.1%, respectively, in comparison with that of wild-type strain AE17, while the complementation strains showed partially restored motility by 109.56%, 101.61%, and 78.06%, respectively.

### Comparison of CEF adhesion and invasion ability

The adhesive cell counts for the AE17, AE18, AE19, and AE20 strains were $3.47 \times 10^4$ CFU/well, $2.19 \times 10^4$ CFU/well, $2.27 \times 10^4$ CFU/well, and $1.05 \times 10^4$ CFU/well, respectively. The AE18, AE19, and AE20 mutant strains showed significantly decreased adhesion ($p<0.01$) in comparison with wild-type strain

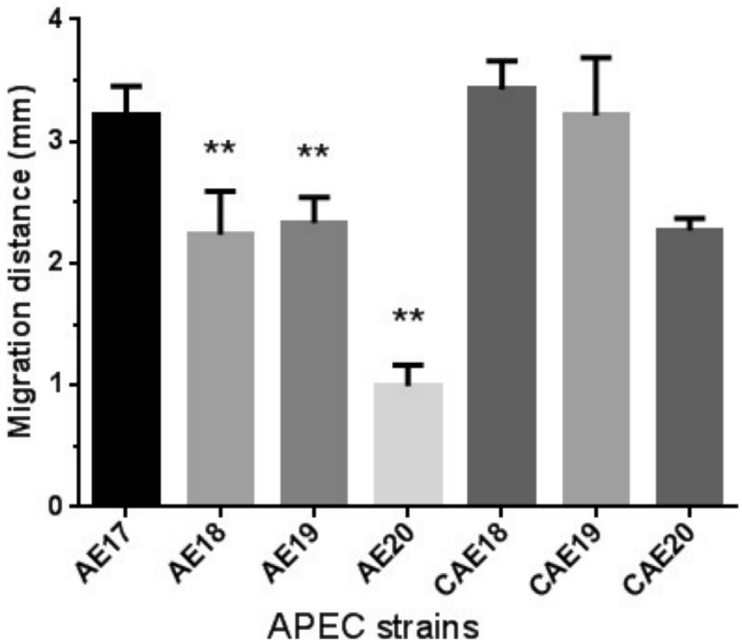


Fig. 3. Bacterial motility assay. The motility was assessed by examining migration of bacteria through the agar from the center toward the periphery of the plate. The swimming motility of mutant strains was significantly decreased compared with that of the wild strain AE17 (p<0.05), while the swimming motility of the complementation strains was restored.

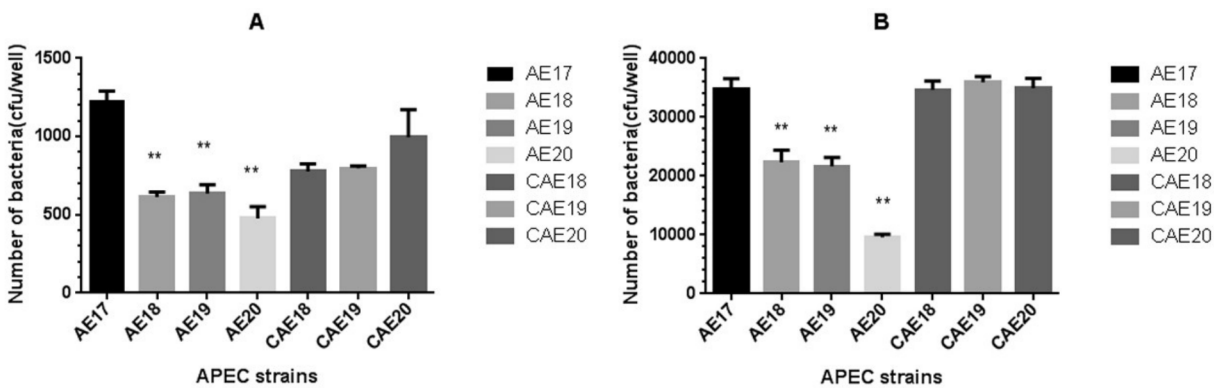


Fig. 4. Adherence assay. **A.** Adherence to CEF cells by mutant strains AE18, AE19 and AE20 were significantly reduced, compared with AE17. **B.**The ability of mutant strains adhesion to CEF cells was significantly decreased, compared with AE17. while the adhesion and invasion abilities of the complementation strains was restored.

AE17 (decreased to 63.11%, 65.42%, and 30.26%, respectively, the number of adherent AE17 cells) (Fig. 4A). The invasive cell counts for the AE17, AE18, AE19, and AE20 strains were $1.19 \times 10^3$ CFU/well, $7.12 \times 10^2$ CFU/well, $6.88 \times 10^2$ CFU/well, and $4.51 \times 10^2$ CFU/well, respectively. The AE18, AE19, and AE20 mutant strains showed significantly decreased invasion (p<0.01) in comparison with wild-type strain AE17 (decreased to 59.83%, 57.82%, and 37.90%, respectively, of the number of invasive AE17 cells) (Fig. 4B). The adhesion and invasion capacities of double-mutant strain AE20 were significantly decreased in comparison with those of single gene mutant strains AE18 and AE19.

## mRNA transcript levels of virulence-related genes

The mRNA transcript levels of 13 virulence genes were detected in the wild-type and mutant strains using Real-Time PCR. In comparison with strain AE17, strain AE18 showed significantly increased expression of *MarA*, *metJ*, *ibeA*, and *stx2F* (p<0.01), and *YhiD* (p<0.05), and significantly decreased expression of *sodA*, *polA* (p<0.01), *tsh*, *fimC*, and *pfs* (p<0.05), while *OmpA* expression was not changed (Fig. 5). In comparison with strain AE17, strain AE19 showed significantly increased expression of *metJ*, *polA*, *ibeA*, *stx2F*, *OmpA*, *luxS* (p<0.01), *MarA*, *sodA*, and *tsh* (p<0.05),

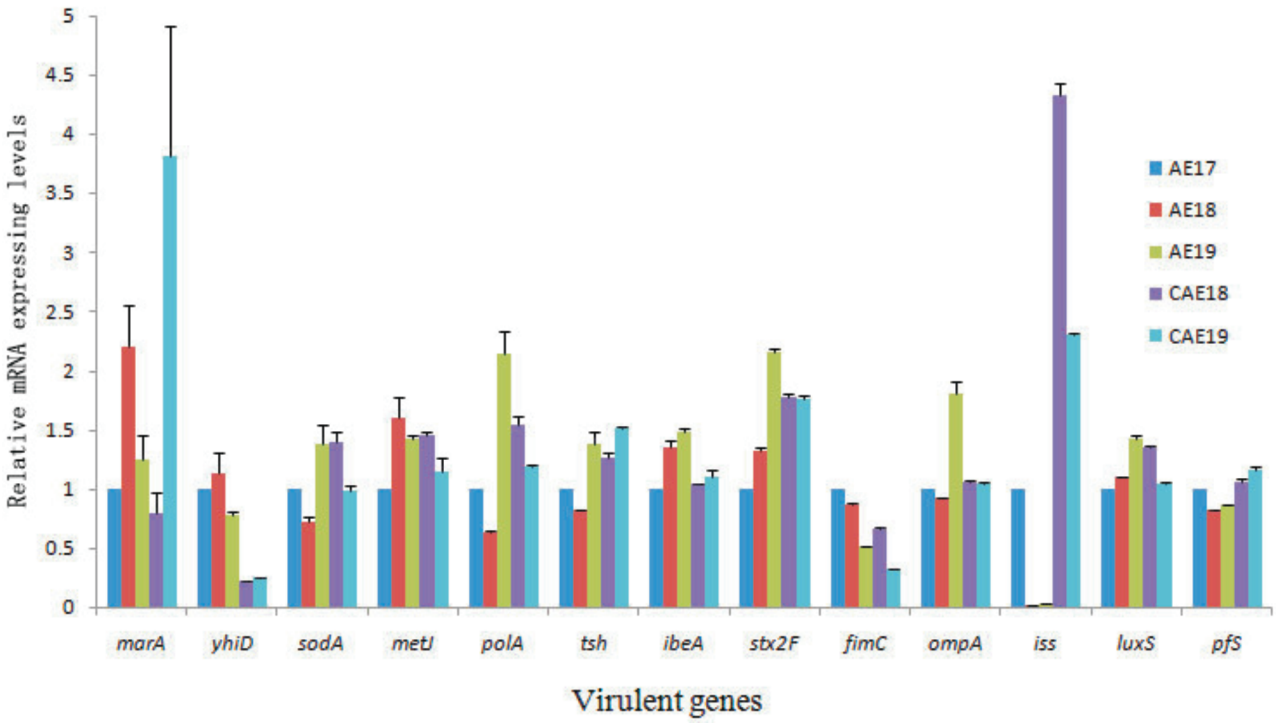


Fig. 5. The virulence genes expression levels of AE17 and mutant strains are differences. The five columns represent AE17, AE18, AE19, CAE18 and CAE19 virulence genes mRNA transcription level. The mRNA transcription level of each gene was normalized to that of dnaE. Results are shown as relative expression ratios compared to expression in the wild strain AE17. Each value shown represents the means and standard deviations. The mRNA transcript levels of 13 virulence genes were detected in the wild-type, mutant and reverant strains using Real-Time PCR.

and significantly decreased expression of *fimC*, *iss*, ($p<0.01$), *YhiD*, and *pfs* ($p<0.05$). In comparison with strain AE17, strain AE20 showed significantly increased expression of *MarA*, *polA*, *tsh*, *OmpA* ($p<0.01$), and *stx2F* ($p<0.05$), and significantly decreased expression of *sodA*, *iss* ($p<0.01$), *YhiD*, and *fimC* ($p<0.05$). However, there were no significant differences between the mRNA levels of the other measured genes in the AE17 and AE20 strains.

### Determination of the median lethal dose of the bacteria

The $LD_{50}$ values of the AE17, AE18, AE19, and AE20 strains were $1.78 \times 10^5$ CFU/mL, $5.62 \times 10^6$ CFU/mL, $1.78 \times 10^6$ CFU/mL, and $2.37 \times 10^6$ CFU/mL. There were no deaths in the control group. The virulence of the mutant strains in chickens was significantly reduced in comparison with that of wild-type strain AE17.

## Discussion

Virulence genes in bacteria are often regulated by two-component systems, including PhoP-PhoQ, the QS system, and the Fur system. Recent studies have shown that PhoP-PhoQ two-component system is an important regulator of pathogenic gene expression and is involved in the regulation of bacterial adaptation to the external environment (Nam et al. 2010, Ogasawara et al. 2012).

In this study, the Red homologous recombination system was used to construct the PhoP single gene mutant APEC strain AE18, the PhoQ single gene mutant APEC strain AE19, and the PhoP/PhoQ double-gene mutant APEC strain AE20, providing an experimental basis for further study of the APEC PhoP-PhoQ two-component system. The Red recombination system has many advantages, including rapid and simple use and high efficiency, and it has been widely used in published studies (Muyrers et al. 2001). Bacterial adhesion and invasion are the first steps in the process of colonization, while bacterial motility has a major impact on pathogenicity (Shen et al. 2010, Lertsethtakarn et al. 2011). By comparing motility, adhesion, and invasion capacity of the AE17 and mutant strains for CEF cells, we showed that the mutant

strains had significantly reduced motility, adhesion capacity, and invasion capacity in comparison with wild-type strain AE17. Real-Time PCR indicated that the mutant strains showed different degrees of up- and down-regulation of the *OmpA*, *fimC*, and *tsh* transcripts, which encode adhesion molecules, *ibeA*, which encodes an invasion molecule, and *luxS*, which affects bacterial motility, demonstrating potential mechanisms underlying reduced adhesion, invasion, and motility.

Real-Time PCR was conducted to measured mRNA transcript levels of 13 virulence genes. We found that the 13 measured virulence genes showed varying degrees of up- and down-regulation in the mutant strains, and the manner of regulation was not consistent among the single gene and double-gene mutant strains. These results suggest that other mechanisms in addition to the PhoP-PhoQ two-component system participate in the regulation of transcription of virulence genes (Bijlsma and Groisman 2005, Garcia et al. 1996).

In this study, we successfully constructed single gene mutant strains AE18 and AE19 and double-gene mutant strain AE20. We showed that mutations in PhoP and PhoQ regulate transcription of virulence genes and significantly reduce APEC motility and CEF cell invasion. Our *in vitro* experiments showed that mutations in PhoP and PhoQ significantly reduced APEC virulence, and our *in vivo* experiments indicated that these mutations reduced the ability of the bacteria to invade the body. Our results provide theoretical basis for further study of the function of the PhoP-PhoQ two-component system as a regulator of APEC pathogenicity.

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