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Role of ArcA in the regulation of antibiotic sensitivity in avian pathogenic *Escherichia coli*

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ABSTRACT Avian pathogenic *Escherichia coli* (**APEC**) is one of the common extraintestinal infectious disease pathogens in chickens, geese, and other birds, inducing serious impediments to the development of the poultry industry. Hence, investigating how bacteria regulate themselves amidst different challenging conditions is immense essential in prevention and treatment for bacterial pathogen infections. The ArcA regulatory factor has been reported to regulate oxygen availability in strains, but its role in regulation of antibiotics resistance in APEC is unclear. This study delved into understanding how ArcA regulates antibiotic resistance in APEC. An *E. coli* APEC40 *arcA* knockout strain was constructed, and the regulatory mechanism of *arcA* on APEC antibiotic susceptibility was identified by drug sensitivity test, colony counting assay, real-time quantitative PCR, β-galactosidase assays and electrophoretic mobility shift assay (**EMSA**). The results showed that *ArcA* directly binds to the promoter region of the outer membrane protein OmpC/OmpW and regulates bacterial susceptibility to kanamycin and penicillin G. At the same time, the double knockout of *ompW and ompW/arcA* resulted in an increase in resistance to kanamycin compared to the deletion of the *arcA* gene. This outcome provided experimental proof suggesting that the outer membrane protein OmpW could serve as a crucial pathway for the ingress of kanamycin into cells. These results confirmed the important regulatory role of ArcA transcription factors under APEC antibiotic stress.

Key words: avian pathogenic *Escherichia coli*, ArcA, the antibiotic susceptibility, outer membrane protein

2024 Poultry Science 103:103686
https://doi.org/10.1016/j.psj.2024.103686

INTRODUCTION

One of the major causal agents of extraintestinal infectious illnesses in birds is avian pathogenic *Escherichia coli* (**APEC**), which is a form of extraintestinal pathogenic *Escherichia coli* (**ExPEC**) (Dho-Moulin and Fairbrother, 1999; Giovanardi, et al., 2013). The high incidence rate and mortality rate of colibacillosis bring heavy economic losses to the poultry industry every year. Therefore, worldwide, colibacillosis is a serious issue in domestic poultry industry, which hinders the development of poultry breeding industry (Guabiraba and Schouler, 2015).

Due to overprescription and overuse of antibiotics, more and more antimicrobial resistance (**AMR**) pathogens have emerged (Andersson and Hughes, 2010). Antimicrobial resistance is a bacterium's ability to resist antibiotic growth inhibition and killing (Brauner, et al., 2016). The clinical burden of infections caused by AMR pathogens is a major risk to public health (Nolan, et al., 2023). To find new potential antibiotics is effective but also expensive and being outpaced by the rate at which new resistant strains are emerging (Fernandes and Martens, 2017). Therefore, to figure out the mechanism of antibiotics-resistant in bacteria is a replace way to treatment APEC infection.

Previous studies have shown that bacteria synthesize stress proteins to resist external environmental pressures, including antibiotics, acid-base, and osmotic pressure, in order to survive (Fernández and Hancock, 2012; Bremer and Krämer, 2019; Xu, et al., 2020). Gram-negative bacteria's outer membrane (**OM**) primarily functions as a permeability barrier, blocking the entry of harmful substances while simultaneously allowing the inflow of nutritional molecules (Nikaido, 2003). The outer membrane contains various protein channels, called porins, which participate in the influx of various compounds, including several antibiotics and the

Received February 6, 2024.
Accepted March 18, 2024.

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transmembrane pore proteins of barrels are the most common OMPs in Gram-negative bacteria (Pagès, et al., 2008). There is research to have proved that ompW is involved in the efflux of multidrug-resistant protein EmrE towards specific substrates to increase environmental tolerance (Beketskaia, et al., 2014a). Therefore, outer membrane proteins are one of the important factors contributing to bacterial resistance.

The two-component signal transduction system (**TCS**) is a key mechanism for bacteria to perceive external signals and respond corresponding changes to adapt to environmental stress (Capra and Laub, 2012). The ArcAB system, consisting of the kinase sensor ArcB and response regulator ArcA, is one of the mechanisms by which *E. coli* to adapts to changes in oxygen availability (Gunsalus and Park, 1994; Lynch and Lin, 1996). A previous study has reported that under anaerobic or aerobic conditions, the arcA gene can directly or indirectly control the expression of 1,139 genes in the *E. coli* K-12 genome with a 99% confidence level (Salmon et al., 2005). More recently, however, the research showed that arcAB can affect the cellular metabolism, biosynthesis, and motility of Salmonella typhimurium, while regulating the chemotaxis of APEC (Morales et al., 2013; Jiang et al., 2015). In Hemophilus influenzae, *arcA* is an important regulatory factor for its virulence and serum drug resistance (De Souza-Hart et al., 2003).

At present, there remains a significant knowledge gap regarding the influence of *arcA* on antibiotic susceptibility. In this study, we investigated the changes in antibiotic susceptibility of APEC40 strain through the knockout and complementation of *arcA* gene. Our investigation extended to finding its regulatory pathways. By antibiotic sensitivity experiments, rt-qPCR, and EMSA experiments, it was found that ArcA selectively regulates genes for the outer membrane protein contributing to APEC antibiotic susceptibility.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

Table 1 lists the plasmids and bacterial strains that were used in this investigation. *E. coli* strains were routinely grown aerobically in Luria-Broth (**LB**) medium at 37°C or maintained on LB agar plates, except when indicated. All cultures for temperature-sensitive plasmid were incubated at 30°C. Plasmid selection and maintenance necessitated the use of ampicillin (**Amp**, 100 μg/mL), kanamycin (**Kan**, 50 μg/mL), and chloramphenicol (**Cm**, 30 μg/mL).

Genetic and Molecular Biology Techniques

According to the standard protocol for molecular manipulation of Gram-negative bacteria. The genome and plasmid extraction were performed by the extraction kit (Sangon Biotech, Shanghai, China), respectively. The PCR product and DNA restriction fragment were purified using the gel purification kit (Transgen, Beijing, China). Use DNA restriction endonucleases (Thermo Fisher Scientific, Waltham, MA) and DNA ligases (Takara Bio Inc., Dalian, China) according to standard methods for enzyme digestion connections. Sequence analysis and primer design were using Primer premier 5.0 software. The primer sequences used are shown in Table 2.

Construction of the arcA-Deficient Mutant and Complemented Strains

The construction of *arcA* deletion mutant of APEC40 based on the λ Red recombinase system (Datsenko and Wanner, 2000). In brief, a fragment containing chloramphenicol-resistance cassette (**cat**) was amplified using pKD3 as a template and 40-arcA-f/40-arcA-r as a primer, and then electroporated into APEC40 competent cells containing pKD46 plasmid. Subsequently, 900 μL LB was immediately added, and then incubated at 37°C for 1 h. For screening, the cultures were plated on LB plates containing 30 μg/mL chloramphenicol. The arcA mutant was selected with PCR amplification by primers Check-*arcA*-out/in. Plasmid pCP20 was then electroporated into mutant strain for deleting the Cm cassette. The PCR amplification was performed using Check-*arcA*-out-f and Check-*arcA*-out-r primers to confirm cm deletion, and were further confirmed by DNA sequencing, and the mutant strains were named APEC40/Δ*arcA* (XM2). Strains APEC40/Δ*ompW* (XM3), APEC40/Δ*arcA*/Δ*ompW* (XM4) were obtained by using the same method.

For complementary strain construction, the *arcA* gene was amplified from the APEC40 strain using primers pC*arcA* − *EcoR* I-f /pC*arcA*- *Hind* III-r, and cloned into the low-copy plasmid pSTV28 (Takara, Dalian, China), named plasmid pCarcA. It was then electroporated into the mutant strain XM2 to achieve functional complementation of the *arcA* gene. As a control, the empty pSTV28 plasmid was electroporated into WT and XM2 strains to obtain WT/pSTV28 and XM2/pSTV28.

Bacterial Growth Curves

The overnight cultured strains were diluted to 100 mL fresh LB broth containing 30 μg/mL chloramphenicol (OD_{600} of approximately 0.03), and then were grown at 37°C for 14 h with shaking. Detect the cell density at 600 nm of ultraviolet light every 2 h using the plate reader (TECAN, Infinite 200 PRO). The experiment was repeated 3 times.

Antibiotic Susceptibility Tests and assays

According to the requirements of the Clinical and Laboratory Standard Institute (**CLSI**), the antimicrobial susceptibility changes of the strains were detected

Table 1. Strains and plasmids were used in this study.

Strains or plasmids	Description	Reference or source
Strains		
E. coli		
DH5α	Clone host strain, supE44 ΔlacU169(φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1relA1	Invitrogen
BL21(DE3)	Expression strain, F-ompT hsdS(rB- mB-) gal dcm (DE3)	Invitrogen
WT	Avian pathogenic *E. coli* (APEC) 40, wild-type	Laboratory stock
XM2	APEC40 *arcA*-deletion mutant	This study
WT/pSTV28	WT with the empty vector pSTV28, Cm^r[1]	This study
XM2/pSTV28	XM2 with the empty vector pSTV28, Cm^r	This study
XM2/p*CarcA*	XM2 with the complement plasmid p*CarcA*, Cm^r	This study
XM3	APEC40 *ompW*-deletion mutant	This study
XM4	APEC40 *arcA*-deletion and *ompW*-deletion mutant	This study
WTΔlacZ	APEC40 *lacZ*-deletion mutant	This study
XM2/ΔlacZ	APEC40 *lacZ* and *arcA* deletion mutant	This study
WTΔlacZ/pRCL-p_{ompC}	WTΔlacZ with plasmid pRCL-p_{ompC}, Cm^r	This study
XM2/ΔlacZ/pRCL-p_{ompC}	XM2/ΔlacZ with plasmid pRCL-p_{ompC}, Cm^r	This study
WTΔlacZ/pRCL-p_{ompF}	WTΔlacZ with plasmid pRCL-p_{ompF}, Cm^r	This study
XM2/ΔlacZ/pRCL-p_{ompF}	XM2/ΔlacZ with plasmid pRCL-p_{ompF}, Cm^r	This study
WTΔlacZ/pRCL-p_{ompW}	WTΔlacZ with plasmid pRCL-p_{ompW}, Cm^r	This study
XM2/ΔlacZ/pRCL-p_{ompW}	XM2/ΔlacZ with plasmid pRCL-p_{ompW}, Cm^r	This study
Plasmids		
pKD3	*cat* gene, template plasmid, Cm^r Amp^r[1]	(Datsenko and Wanner, 2000)
pKD46	Expresses λ Red recombinase Exo, Bet and Gam, temperature sensitive, Amp^r	(Datsenko and Wanner, 2000)
pCP20	FLP^+ $\lambda cI857^+$ λ_{pR}Rep(Ts), temperature sensitive, Cm^r Amp^r	(Datsenko and Wanner, 2000)
pSTV28	Low copy number cloning vector, Cm^r	Takara
p*CarcA*	pSTV28 with *arcA* gene, Cm^r	This study
pET28a(+)	Expression vector,Kan^r	Novagen
pET-*arcA*	pET28a(+) with *arcA* gene, Kan^r[1]	This study
pRCL	Cm^r, promoterless *lacZ*	(Wang et al., 2019a)
pRCL-P_{ompC}	pRCL harboring *ompC* promoter	This study
pRCL-P_{ompF}	pRCL harboring *ompF* promoter	This study
pRCL-P_{ompW}	pRCL harboring *ompW* promoter	This study

[1]Cm^r: chloramphenicol-resistant; Amp^r: ampicillin-resistant; Kan^r: kanamycin-resistant.

by using Mueller−Hinton (**MH**) (hopebiol, Qingdao, China) medium dilution antimicrobial susceptibility tests. The concentration that completely inhibits the growth of the strain is defined as the minimum inhibitory concentration (**MIC**). The experiment was repeated 3 times.The stock solutions of the following antibiotics were prepared, and their classes are listed in parentheses: ofloxacin (fluoroquinolone, Sangon) at 20 mg/mL; erythromycin (macrolide, Sangon) at 30 mg/mL; Kanamycin (aminoglycoside, Sangon) at 50mg/mL; penicillin G (β-lactams, Sangon) at 100mg/mL.

Based on previous methods (Yu et al., 2020), the antibiotic susceptibility of the strains was determined by antibacterial activity assays. The overnight cultured strains were diluted into fresh MH containing 30 μg/mL chloramphenicol and incubate in 37°C for 2 h with shaking. After incubation, different antibiotics (final concentration 1/2MIC) were added separately and incubate for another 2 h. Then, they were diluted continuously by 10-fold and three appropriate dilutions (0.1 mL) were dropped onto LB agar plates and colony counts (CFU/mL) were counted after incubation at 37°C. The experiments were repeated 3 times.

Total RNA Isolation, cDNA Generation, and Real-Time PCR Processing

The overnight cultured strains were diluted 100 times using fresh LB broth containing specific antibiotics and then incubated at 37°C to logarithmic growth phase. And the cells were centrifuged and collected for total RNA extraction. The total RNA was extracted from the cells using Trizol reagent (Transgen, Beijing, China). Reverse transcription was performed using the EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (Transgen, Beijing, China) and follow the manufacturer's instructions. RT-qPCR was performed with RT primers on the CFX96 Real-Time System (Bio-Rad, California) according to the instructions of the TransStart Tip Green qPCR SuperMix kit (Transgen, Beijing, China). Normalize the quantity of the target genes with the 16S cDNA gene as a housekeeping gene. All of the RT-qPCR assays were repeated 3 times.

β-Galactosidase Assays

The enzyme activity assay was experimented with reference to the reported methods (Wang, et al., 2021). Briefly, the promoters of ompC, ompF, and ompW were amplified and cloned into the promoterless lacZ plasmid pRCL separately to Construct the lacZ fusion transcription report plasmid, and then the constructed lacZ fusion reporter plasmids were introduced into the strain WT/ΔlacZ and XM2/ΔlacZ respectively. The strains containing the lacZ fusion reporter plasmid were incubated in 100mL of LB broth containing 30 μg/mL chloramphenicol and the samples were collected every 2 h. Collected cells were diluted to 1 mL Z-buffer containing

Table 2. Oligonucleotide primers were used in this study.

Primer name	Oligonucleotide (5′-3′)
40-*arcA*-F	TCGATTTAGTTGGCAATTTAGGTAGCAAACATGCAGACCCTGTAGGCTGGAGCTGCTT
40-*arcA*-R	CGCCGTTTTTTTTGACGGTGGTAAAGCCGATTAATCTTCCTGAATATCCTCCTTAGTTC
40-ompW-F	TATAACCATAACGACGGAGCGGATATGAAAAAGTTAACAGTGTAGGCTGGAGCTGCTT
40-ompW-R	TTACGGGGTCGTTTTTGTGCGGAATTAAAAACGATATCCTGTGAATATCCTCCTTAGTTC
lac-F	ATGGTAAGCCGCTGGCAAGCGGTGAAGTGCCTCTGGATGTTGTAGGCTGGAGCTGCTT
lac-R	GACAATGGTTAAATTGAAATTTGCATAAAAATTCCTCATATGAATATCCTCCTTAGCGG
CM-f	TGTAGGCTGGAGCTGCTT
CM-r	CATATGAATATCCTCCTTAGTTC
Check- arcA -in-f	AAGAACGGTCTTCTGTTAGCG
Check- arcA -in-r	AGTAGGTTGCGTGCACGA
Check-arcA-out-f	CCCACGACCAAGCTAATG
Check- arcA -out-r	GAAGTTACAACGGACGATGAG
Check-ompW-in-f	CGTTAGGAAGTCTGGGTGG
Check-ompW-in-r	CATTATCAAAGAAGGTGGTGTAGTT
Check-ompW-out-f	CCGTGATAGACATGCACTCT
Check-ompW-out-r	GGCGTAGAAGCGAAGAAA
Check-pRCL-F	AGAAAGTTAAAATGCCGCGC
Check-pRCL-R	GCCAGGCATCAAATTAAGCAG
pET-*arcA* -NcoI-f	CATGCCATGGAGACCCCGCACATTCTTATC[1]
pET-*arcA*-XhoI-r	CCGCTCGAGATCTTCCAGATCACCGCAG[1]
pC*arcA* - EcoRI-f	CCGGAATTCATGCAGACCCCGCACATTCT[1]
pC*arcA*- HindⅢ-r	CCCAAGCTTAATCTTCCAGATCACCGCAG[1]
pompC-HindIII-F	CCAAGCTTGGGAGTTATTCTAGTTGCGAG[1]
pompC-BamHI-R	CGGGATCCGTTATTAACCCTCTGTTATATGC[1]
pompF-HindIII-F	CCAAGCTTATTTAGCGTCTTCAAGAGC[1]
pompF-BamHI-R	CGGGATCCTATTTATTACCCTCATGGTT[1]
pompW-HindIII-F	CCAAGCTTTGCACTCTCCTTGAGTTT[1]
pompW-BamHI-R	CGGGATCCATCCGCTCCGTCGTTATG[1]
M13-f	TGTAAAACGACGGCCAGT
M13-r	CAGGAAACAGCTATGACC
T7-f	TAATACGACTCACTATAGGG
T7-r	TGCTAGTTATTGCTCAGCGG
rt-16s-f	TTTGAGTTCCCGGCC
rt-16s-r	CGGCCGCAAGGTTAA
rt-ompC-f	AGTAGTAGGTAGCACCAACATCA
rt-ompC-r	TGGGCGAACAAAGCACAG
rt-ompF-f	TGGTGTAAGCGATGGACG
rt-ompF-r	TCTACCTGGCAGCGAACTA
rt-ompW-f	ATTATCTGATTAACCGTGACTGG
rt-ompW-r	TACGCTATCGTGTTGCTGTG
p-ompC-bio-F	GTTATTAACCCTCTGTTATATGC
p-ompC-R	GGGAGTTATTCTAGTTGCGAG
p-ompW-bio-F	TGCACTCTCCTTGAGTTT
p-ompW-R	ATCCGCTCCGTCGTTATG
p-motA-bio-F	AAACTGTACCGAGAACAACCAG
p-motA-R	AGACGTAAACTTTCCCAGAATC

[1]The sequences with the underline refer to the restriction endonuclease recognition sites.

16.1 g/L Na_2HPO_4, 5.50 g/L NaH_2PO_4, 0.75 g/L KCl, and 0.246 g/L $MgSO_4$ and then assayed for β-galactosidase activity using ortho-Nitrophenyl-β-galactosidase (**ONPG**) as a substrate. Calculate units of enzyme activity: Miller Units: $[OD_{420} \times 1{,}000)/(OD_{600} \times \text{Volume (mL)} \times \text{Time (min)}]$. The experiments were repeated 3 times.

His6-ArcA Protein Purification

The arcA gene was amplified from the genome of WT strain using primers pET-arcA-*Nco* I-f / pET-*arcA*-*Xho* I-r, and then the arcA gene was cloned into the pET-28a plasmid, renamed pET-arcA. pET-arcA plasmid was transferred into BL21(DE3) strain and cultured in LB broth containing 50 μg/mL kanamycin to OD_{600} is 0.5 to 0.8. Sequently, isopropyl β-D-thiogalactopyranoside (**IPTG**) with a final concentration of 0.5 mM was added to induce protein expression for 14 h at 16°C. Ni-NTA resin affinity chromatography used to purify His6-ArcA. The concentration of purified protein was measured using the BCA Protein Assay Kit (Beyotime, Shanghai, China).

Electrophoretic Mobility Shift Assays

The DNA fragments containing the promoters were amplified by p-*motA*-biotin-f/p-*motA*-r, p-*ompC*-biotin-f/p-*opmC*-r, and p-*ompW*-biotin-f/p-*ompW*-r using the chromosomal DNA of APEC40 as a template. Incubate biotin-labeled promoter DNA with different concentrations of His6-ArcA protein in 4 μL of 5 × binding buffer (50 mM Tris−HCl (pH 7.5), 100 mM NaCl, 3 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM dithiothreitol) for 30 min at 25°C. After incubation, bromophenol blue loading buffer was added to the mixture, followed by electrophoresis in a 4% native polyacrylamide gel. In accordance with the manufacturer's chemiluminescent EMSA kit instructions, DNA bands were detected and

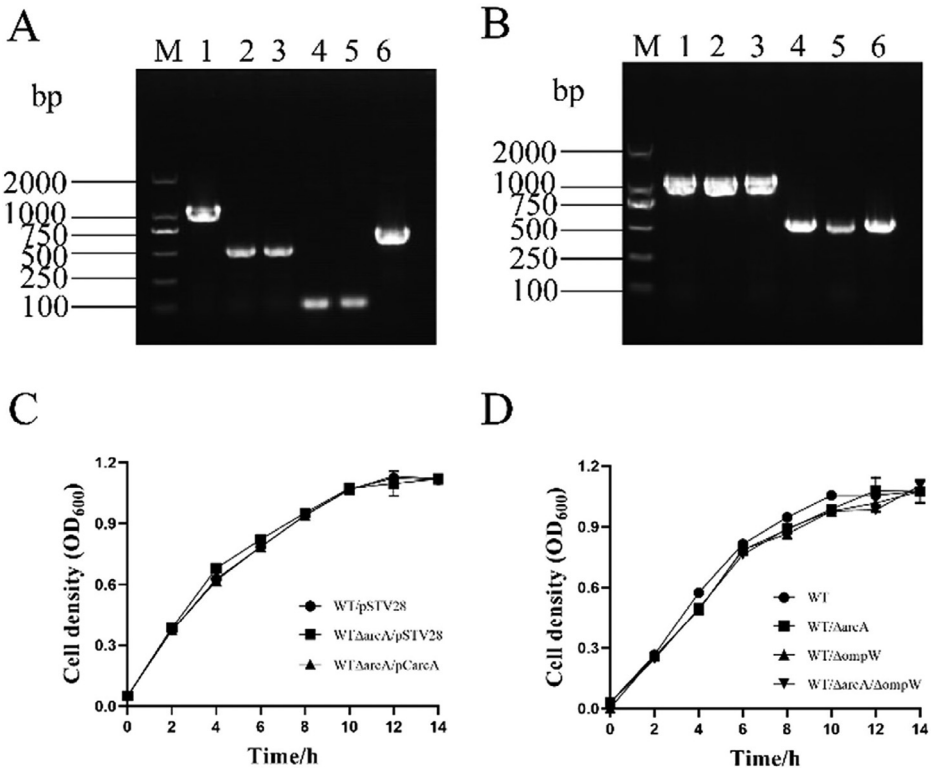

Figure 1. WT, arcA mutant, ompW mutant, arcA and ompW mutant, and arcA complementary strains PCR identification and growth curve assays. (A) Molecular identification of WT/pSTV28, XM2/pSTV28, XM2/p*C*arcA strains. M: 2000 bp marker, lanes 1−3 for the identification of *arcA* gene, lanes 4−6 for the identification of pSTV28 plasmid and complement plasmid p*C*arcA. (B) Molecular identification of WT, XM3, XM4 strains. M: 2000 bp marker, lanes 1,3,5 for the identification of arcA gene, lanes 2,4,6 for the identification of ompW gene. (C) Growth curves of WT/pSTV28, XM2/pSTV28, XM2/p*C*arcA strains. (D) Growth curves of WT, XM2, XM3, XM4 strains.

analyzed according to the manufacturer's instructions for the chemiluminescent EMSA kit (Beyotime, Shanghai, China).

Statistical Analyses

For all studies, statistically analyzed using GraphPad Prism 8.0 (GraphPad Software Inc., GraphPad Prism 8.0.2, San Diego, CA). P-values ≤ 0.05 were considered significant difference. The test results were shown as the mean ± SD. Comparison between two groups was conducted by using a t-test.

RESULTS

Identification of arcA, ompW Mutations and arcA Complementary Strains of APEC40

All mutant strains were achieved through homologous recombination technology. The *arcA* gene knockout and complementary were identified by PCR (Figure 1A). Lanes 1 to 3 represent identification of the *arcA* gene. Lane 1 shows a 1,145 bp product amplified from WT/pSTV28, while lanes 2 and 3 show 570 bp products amplified from XM2/pSTV28 and XM2/pCarcA strains, respectively. The plasmid pSTV28 and the complementary plasmid pCarcA were confirmed by PCR. Lanes 4 to 5 were 150 bp products amplified from WT/pSTV28 and XM2/pSTV28 strains, respectively, and lane 6 was 860 bp product amplified from XM2/pCarcA.

The molecular identification of ompW knockout and *ompW*/*arcA* double knockout is shown in Figure 1B. Lanes 1, 3, 5 were identification of the *arcA* gene, the products of lanes 1,3,5 were 1,145 bp amplified from strain WT, 1,145 bp amplified from strain XM3, 570 bp amplified from strain XM4. Lanes 2,4,6 are identification of the *ompW* gene, the products of lanes 2,4,6 were 1,031 bp amplified from strain WT, 1,031 bp amplified from strain XM3, 540 bp amplified from strain XM4.

Therefore, we successfully constructed the required knockout strains and *arcA* gene complementary strains.

arcA, ompW Deletion Did Not Affect Strains Growth

To verify whether arcA, ompW gene mutation influences strain growth, the growth curves of WT strains, knockout strains, and measure complementary strains were measured under the same conditions. The results showed that the growth trends of WT/pSTV28, XM2/pSTV28, and XM2/p*C*arcA strains in LB broth containing 30 μg/mL Cm were similar (Figure 1C), and there are no growth differences between WT, XM2, XM3, XM4 (Figure 1D), indicating that the arcA, ompW gene knockout did not affect the growth of the strains.

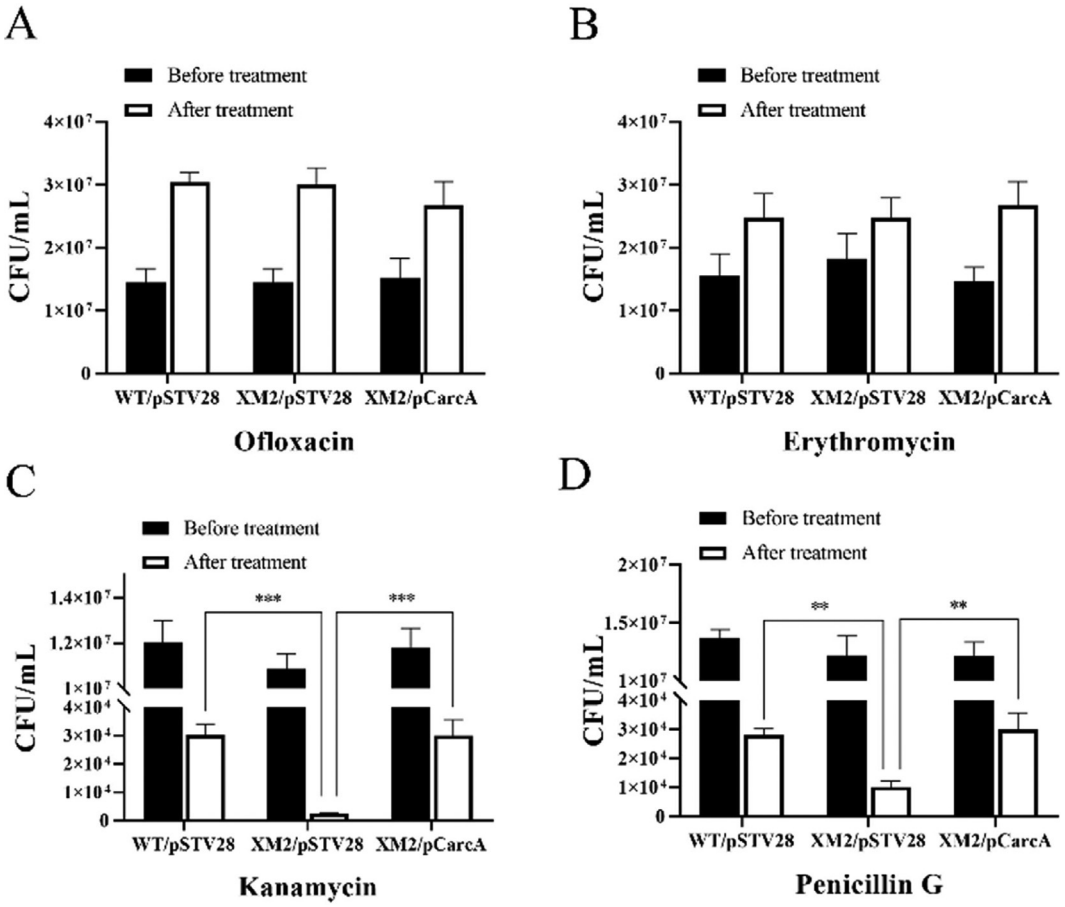

Figure 2. Colony counts assays of WT/pSTV28, XM2/pSTV28, and XM2/pC*arcA* under different antibiotic pressures (A) ofloxacin, (B) erythromycin, (C) kanamycin, (D) penicillin G; Error bars indicate SD; ** represents $P < 0.01$, *** represents $P < 0.001$.

Deletion of the arcA Gene Changed Bacteria Antibiotic Susceptibility

The MIC values of experimental bacteria to 4 antibiotics were measured according to the CLSI standards. The MICs were shown in Table 3. The results showed that strain XM2/pSTV28 was more sensitive to kanamycin compared to WT/pSTV28 strain. Moreover, the sensitivity of complementary strain XM2/pCarcA was reinstated.

To further determine the effect of *arcA* on antibiotic sensitivity, colony counts experiment was conducted. As shown in Figures 2A and B, the deletion of *arcA* gene did not significantly affect the sensitivity of APEC40 to erythromycin and ofloxacin. In the presence of kanamycin and penicillin G, the viable counts of XM2/pSTV28 were significantly lower than that of WT/pSTV28, while the viable bacterial counts of complementary strain XM2/pCarcA returned to the similar level as that of WT/pSTV28 (Figures 2C and D), indicating that deletion of the *arcA* gene can affect the susceptibility of the APEC40 to kanamycin as well as penicillin G.

Table 3. Susceptibility of *Escherichia coli* strains to various antibiotics.

	MIC (μg/mL) of Three *E.coli* strains		
Antibiotics	WT/pSTV28	XM2/pSTV28	XM2/pCarcA
Erythromycin	15	15	15
Kanamycin	25	12.5	25
Penicillin G	50	50	50
Ofloxacin	0.25	0.25	0.25

Abbreviation: MIC, minimal inhibitory concentration.

ArcA Down-Regulated the Transcription Level of ompC and Up-Regulated the Transcription Level of ompW

To investigate the effect of ArcA at the RNA level, we performed RT-qPCR experiments to detect the transcript levels of genes encoding outer membrane proteins, including *ompC, ompF ompW*. The results showed that the transcript level of *ompC* was reduced by 5.8-fold and *ompW* was elevated by 2.2-fold in XM2/pSTV28, compared to WT/pSTV28 (Figure 3). The transcript levels of these 2 genes were restored in XM2/pCarcA. However, there was no significant difference in transcription levels of *ompF*. These data suggested that the *arcA* gene affects the antibiotic sensitivity of APEC40 by regulating outer membrane protein-related genes.

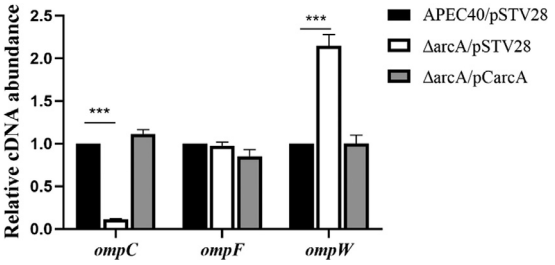

Figure 3. Transcript levels (cDNA abundance) of genes encoding outer membrane proteins were determined. The relative transcript levels of *ompC, ompF* and *ompW* in WT/pSTV28, XM2/pSTV28 and XM2/pCarcA were determined using RT-qPCR. Error bars indicate SD; *** represents $P < 0.001$.

Deletion of arcA Increased the Expression of ompW, and Decreased the Expression of ompC

To verify the transcriptional regulation of *arcA* on *ompC ompF* and *ompW*, a β-galactosidase report plasmid system was constructed for detecting the expression levels of the target genes promoters. The data showed that the deletion of arcA had no effect on the transcriptional activity of the *ompF* promoter (Figure 4B), reducing the transcriptional activity of the *ompC* promoter (Figure 4A) and enhancing the transcriptional activity of the *ompW* promoter (Figure 4C). Collectively, these results consolidate our understanding that *arcA* effects outer membrane proteins expression and decreases the bacterial ability to respond to antibiotic.

Deletion of ompW Decreased Bacteria Kanamycin Susceptibility

There were research reports that the outer membrane protein OmpW is involved in the efflux of specific substrates (Park et al., 2021) and affects kanamycin resistance (Zhang et al., 2015). Therefore, we constructed *ompW*-mutant strain and double knockout strains of *ompW* and *arcA* to verify their effect on kanamycin sensitivity. As shown in the Figure 5, *ompW*-knockout

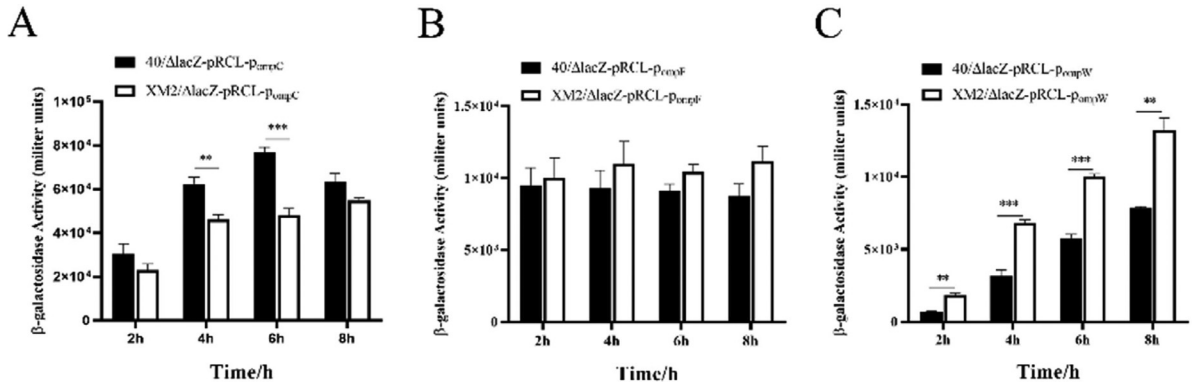

Figure 4. Measurement of expression activities of the ompC, ompF, ompW promoters. The β-galactosidase activities expressed in strains WT/ΔlacZ and XM2/ΔlacZ containing plasmid (A) pRCL-p_{ompC} (B) pRCL-p_{ompF} (C) pRCL-p_{ompW}. ** $P < 0.01$, *** $P < 0.001$.

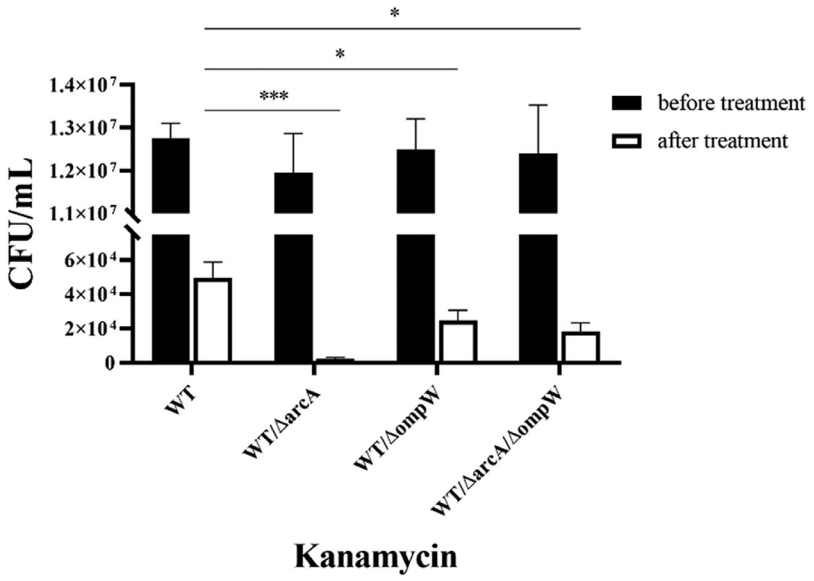

Figure 5. Colony counts assays of WT, XM2, XM3, and XM4 under kanamycin antibiotic pressures; Error bars indicate SD; * represents $P < 0.05$, *** represents $P < 0.001$.

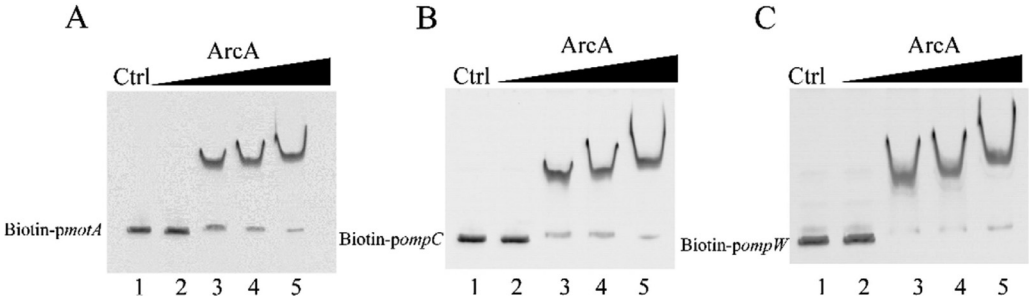

Figure 6. The binding ability of ArcA to the *ompC* and *ompW* promoter was determined by EMSA. In each panel, Lanes 1−5: the ArcA concentrations in order of 8, 0, 2, 4, and 8 μM, the amounts of biotin-labeled probes in all lanes were 200 fM, with an additional 2 pM of unlabelled probe added to lane 1 as a competition control (Ctrl). (A) The positive control. (B) The binding ability of ArcA to the *ompC* promoter. (C) The binding ability of ArcA to the *ompW* promoter.

strain and *arcA/ompW* double knockout strain have reduced the susceptibility abilities to kanamycin compared to that of arcA-knockout strain, indicating that *ompW* is an important gene for susceptibility to kanamycin in APEC40 strain. Our results provided some experimental evidence that the outer membrane pore protein OmpW may be the main channel for kanamycin to enter cells.

ArcA Binding to ompC and ompW Promoters

Based on the above results, we predicted that ArcA regulates the transcriptional levels of *ompC* and *ompW* by directly binding to their promoter regions, and we performed EMSA experiments to verify the speculation. The positive control showed that ArcA was able to bind to *motA* promoter (Jiang et al., 2015). It is evident from the depicted Figure 6 that there are significant band shifts of protein-DNA complexes. Furthermore, with the progressive augmentation of ArcA protein concentration, there was a corresponding increase in the intensity of the shifted bands. The results confirmed that ArcA can specifically bind the promoter of *ompC* and *ompW*, indicating that ArcA can directly regulate the transcription of the outer membrane protein OmpC and OmpW.

DISCUSSION

APEC is a major pathogen in poultry infections, causing millions of dollars in losses to the poultry industry each year (Guabiraba and Schouler, 2015), which needs to be addressed urgently. At present, antibiotics are still the main major of treating bacterial infections, but the non-standard use of antibiotics leaded to the emergence of drug-resistant strains (Agunos et al., 2012). It has been demonstrated that the three major classes antibiotics stimulated the production of highly harmful hydroxyl radicals by Gram-negative and Gram-positive bacteria through the involvement of the tricarboxylic acid cycle, a transient depletion of NADH, and the destabilization of iron-sulfur clusters, which ultimately leads to cell death (Kohanski et al., 2007). The ability of APEC resistance to almost all classes of antibiotics, including carbapenems, has already been reported (Kathayat et al., 2021). Therefore, it is necessary to find an effective method to treat APEC infection.

Exploring the regulatory mechanisms of bacteria under various unfavorable conditions is important for the prevention and treatment of bacterial pathogen infections. The two-component system is one of the important mechanisms by which bacteria resist environmental stress, and many two-component systems have been proven to regulate biological processes, including pathogenesis, stress response (Groisman, 2016). ArcAB two-component system was initially discovered to be related to oxygen perception and regulation of the transformation in strains towards fermentation (Malpica et al., 2006; Brown et al., 2022). In the past few decades, the structure and function of ArcAB two-component systems have been extensively studied. ArcA regulates cell metabolism, flagella biosynthesis, and motility of *Salmonella* under many in vitro conditions, including the presence of anaerobic and H_2O_2 (Evans, et al., 2011; Morales et al., 2013). As one of the main transcription regulators, ArcA can promote the metabolic transition of bacteria from anaerobic to aerobic, and activate their defense against reactive oxygen species (**ROS**) (Nyström, et al., 1996). Although the extensive research has been conducted regarding the regulation of ArcA in *E. coli* K-12 strain, there is still limited research of the involvement of ArcA antibiotic sensitivity in APEC (Oshima, et al., 2002).

In this study, we explored the effect of deletion of *arcA* gene in APEC40 strain on antibiotic sensitivity and the molecular mechanisms underlying its regulation. Our results showed that the *arcA* gene plays a role in the resistance of the APEC40 strain to antibiotic pressure. The deletion of the *arcA* gene leads to an increased sensitivity of APEC40 strains to kanamycin and penicillin G. This finding may help to discover the antibiotic sensitivity patterns of APEC infection and further provide some new insights for the treatment and prevention of APEC infection.

Outer membrane proteins (**OMP**) exist in many bacteria, affecting their virulence and material transport (Ojima et al., 2020; Wang et al., 2019b). We found that in APEC ArcA can directly bind to the promoter region to inhibit the transcription level of the outer membrane protein OmpW and enhance the transcription level of

the outer membrane protein OmpC. The outer membrane protein OmpW functions as a multidrug resistance transporter, contributing to the expulsion of substrates alongside the ethidium multidrug resistance protein E (**EmrE**) (Beketskaia, et al., 2014b). It serves as a cation channel in strain Caulobacter crescentus (Benz et al., 2015). Therefore, we investigated the role of OmpW in kanamycin resistance. The results showed that in the APEC40 strain, both *ompW* gene deletion and *ompW/arcA* gene double deletion decreased sensitivity to kanamycin compared to *arcA* gene deletion, and this is similar to previous research results (Zhang et al., 2015). In general, multiple mechanisms coordinate and work together to lead to bacterial resistance, such as modifying the targets of antibacterial drugs in microorganisms to reduce drug affinity; alteration of membrane permeability to lessen drug uptake; pump drugs from the cell through the activation of efflux pump genes; and the cellular adaptation process controlled by global transcription regulatory factors (Munita and Arias, 2016; Camp et al., 2021). One typical method of bacterial multidrug resistance is efflux pump gene expression (Kim et al., 2010). However, our current study has not demonstrated whether or not the ArcA regulatory factors regulate the exocytosis pump genes, or elucidated the specific mechanisms involved in such regulation. Hence, our future research objective is to determine whether OmpW functions as an independent channel facilitating the entry of kanamycin in cells or if it plays a role within the efflux pathway associated with efflux genes.

In summary, our study suggests that ArcA affects APEC sensitivity to kanamycin and penicillin G by directly binding to the promoter region of outer membrane pore protein OmpC, OmpW and regulates their transcription level. Among them, outer membrane pore protein OmpW may be the main channel for kanamycin to enter cells. Our results provide some experimental data for improving the regulatory network of ArcA. However, direct evidence as to whether OmpW is a major channel for kanamycin entry from cells in APEC40, as well as how kanamycin enters cells via OmpW, require further explored. Furthermore, the impact of ArcA on the susceptibility to other antibiotics and its potential influence on the expression levels of efflux pump genes remains ambiguous. Hence, there is a need for further exploration to elucidate the intricate molecular mechanisms by which ArcA governs antibiotic sensitivity.

CONCLUSIONS

This study showed that ArcA can directly bind to the promoter region of outer membrane pore protein OmpC and, OmpW, regulating their transcription level, thereby affecting the sensitivity of APEC to kanamycin and penicillin G. Therefore, this study provides a molecular basis for studying the effect of ArcA on bacterial resistance and may provide potential drug targets for the prevention and treatment of APEC infection.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (grant number 32270194).

DISCLOSURES

The authors declare no conflicts of interest.

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