# Role of McbR in the regulation of antibiotic susceptibility in avian pathogenic *Escherichia coli*

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**ABSTRACT** Avian pathogenic *Escherichia coli* (**APEC**) causes a variety of bacterial infectious diseases known as avian colibacillosis leading to significant economic losses in the poultry industry worldwide and restricting the development of the poultry industry. The development of efflux pumps is one important bacterial antibiotic resistance mechanism. Efflux pumps are capable of extruding a wide range of antibiotics out of the cytoplasm of some bacterial species, including β-lactams, polymyxins, tetracyclines, fluoroquinolones, aminoglycosides, novobiocin, nalidixic acid, and fosfomycin. In the present study, we constructed the *mcbR* mutant and the *mcbR*-overexpressing strain of *E. coli* strain APECX40 and performed antimicrobial susceptibility testing, antibacterial activity assays, real-time reverse transcription PCR, and electrophoretic mobility shift assays (**EMSA**) to investigate the molecular regulatory mechanism of McbR on the genes encoding efflux pumps. Our results showed that McbR positively regulates cell susceptibility to 12 antibiotics, including clindamycin, lincomycin, cefotaxime, cefalexin, doxycycline, tetracycline, gentamicin, kanamycin, norfloxacin, ofloxacin, erythromycin, and rifampicin by activating the transcription of *acrAB*, *acrD*, *emrD*, and *mdtD* ($P < 0.01$). Additionally, EMSA indicated that McbR specifically binds to the promoter regions of *acrAB*, *acrD*, *acrR*, *emrD*, and *mdtD*. This study suggests that, in APECX40, McbR plays an important role in the regulation of bacterial susceptibility by directly activating the transcription of efflux pumps genes.

**Key words:** avian pathogenic *Escherichia coli*, McbR, efflux pump, antibiotic susceptibility

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## INTRODUCTION

Avian pathogenic *Escherichia coli* (**APEC**) ranks among the frequent causative agents of extraintestinal infectious bacterial disease, collectively called avain colibacillosis, in chickens, ducks, geese, pigeons, and other avian species (Lamarche et al., 2005; Han et al., 2015). Avian colibacillosis usually causes a variety of severe systemic and localized extraintestinal infections, with a complex syndrome characterized by multiple organ lesions like airsacculitis, pericarditis, perihepatitis, peritonitis, salpingitis, osteomyelitis, polyserositis, and septicemia in poultry (Germon et al., 2005; Schouler et al., 2012; Yu et al., 2018). It is not only responsible for significant economic losses in the poultry industry due to high morbidity and mortality rates caused by APEC, but also restricts the development of the poultry industry (Altekruse et al., 2002; Giovanardi et al., 2013; Saidi et al., 2013). Although the use of antibiotics as feed additives in animal production has changed in recent years, antibiotics are commonly used in poultry farms as disease treatment measures to prevent and control APEC infections outbreaks (Saidi et al., 2013; Subedi et al., 2018; Yu et al., 2018). However, due to the excessive and inappropriate use of antibiotics in the poultry industry, several adverse effects have occurred, such as changes in intestinal microflora, impact on public environment, and emergence of antimicrobial resistance in microorganisms (Miles et al., 2006; Subedi et al., 2018). The emergence of antibiotic-resistant microbes has challenged the treatment of APEC infections, and the dissemination of antibiotic-resistant microbes from animals to humans could lead to alarming consequences in the treatment of potential zoonotic diseases (Miles et al., 2006; Subedi et al., 2018; Yu et al., 2018).



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Several important mechanisms of antimicrobial resistance in bacteria have been elucidated, such as generation of inactivating enzymes, alteration of drug targets, decrease of bacterial outer membrane permeability, and overexpression of efflux pumps (Putman et al., 2000; Munita and Arias, 2016). Efflux pumps are capable of extruding a wide range of antimicrobial agents out of the cytoplasm of some bacterial species, including β-lactams, polymyxins, tetracyclines, fluoroquinolones, and protein synthesis inhibitors (Kobayashi et al., 2006; Blair et al., 2014; Munita and Arias, 2016). There are 5 major families of efflux pumps that have been currently identified based on amino acid sequence similarity, predicted secondary protein structures, and phylogenetic relationships, including the small multidrug resistance family, the multidrug and toxic compound extrusion family, the major facilitator superfamily (**MFS**), the resistance-nodulation-cell-division family (**RND**), and the ATP-binding cassette family (Sulavik et al., 2001; Kobayashi et al., 2006; Munita and Arias, 2016). In *E. coli* K-12 chromosome, at least 20 efflux pumps encoding genes (11 MFS, 2 small multidrug resistance family, 6 RND, and 1 ATP-binding cassette family) such as *acrAB*, *acrD*, *emrD*, and *mdtD* could confer antibiotic resistance when they were overexpressed (Nishino and Yamaguchi, 2001; Hirakawa et al., 2005; Kobayashi et al., 2006; Kumar et al., 2013). Among these efflux pumps, AcrAB, functions as a proton antiporter, is composed of a transporter protein located in the inner membrane (AcrB) and a linker protein located in the periplasmic space (AcrA). AcrAB could transport a wide array of substrates, conferring resistance to some β-lactams, fluoroquinolones, tetracyclines, chloramphenicol, rifampicin, and novobiocin (Nishino and Yamaguchi, 2001; Perez et al., 2012; Munita and Arias, 2016). AcrD, which is paralogous to AcrB belonging to transporters of the RND family, confers resistance to tetracycline, novobiocin, nalidixic acid, norfloxacin, and SDS in addition to aminoglycosides (Nishino and Yamaguchi, 2001; Aires and Nikaido, 2005). EmrD, a multidrug efflux pump from the MFS family, confers resistance to uncouplers of oxidative phosphorylation such as meta-chlorocarbonylcyanide phenylhydrazone and tetrachlorosalicylanilide, and antimicrobial agents such as erythromycin, chloramphenicol, oxytetracycline, rifampicin, tetracycline, nalidixic acid, and SDS (Naroditskaya et al., 1993; Nishino and Yamaguchi, 2001; Smith et al., 2009). Besides, MdtD, an MFS family efflux pump, is involved in the zinc stress response in *E. coli*, and expression of MdtD can result in citrate efflux, reduced intracellular iron content, and reduced susceptibility to oxidative stress, nitrosative stress, and antimicrobial agents of diverse classes in *Salmonella typhimurium* (Frawley et al., 2013; Wang and Fierke, 2013). However, whether these efflux pumps mentioned above affect APEC tolerance to a variety of antibiotics has been rarely reported (Li et al., 2020; Yu et al., 2020).

A helix-turn-helix-type transcriptional regulator, McbR, is a DNA binding protein of the GntR/FadR superfamily and represses the expression of periplasmic protein YbiM by binding the promoter region of *ybiM*, which prevents overproduction of colanic acid (excess colanic acid causes mucoidy) and inhibits biofilm formation in *E. coli* K-12 (Zhang et al., 2008; Lord et al., 2014). Subsequently, some studies indicated that the *yciGFE* operon plays a critical role in the adaption of *E. coli* to adverse environments, and McbR activates the transcription of the *yciGFE* operon by specifically binding the *yciG* promoter in *E. coli* K-12 (Hindupur et al., 2006; Beraud et al., 2010; Yu et al., 2019). In the *yciGFE* operon, *yciG* encodes an uncharacterized protein YciG; *yciF* and *yciE* are paralogues and encode the stress proteins YciF and YciE, respectively (Beraud et al., 2010; Yu et al., 2019). Moreover, YciF has been identified as being produced by bacteria in response to stress conditions such as osmotic stress and acid stress, and YciF plays a functional role in protecting cells against oxidative damage (Hindupur et al., 2006; Beraud et al., 2010). Our previous study demonstrated that deletion of *mcbR* increases biofilm formation by upregulating the transcription of *bcsA*, *fliC*, *wcaF*, and *fimA*, and decreases $H_2O_2$ stress response by downregulating the transcription of *yciF* and *yciE* by specifically binding to the *yciF* promoter in APECX40 (Yu et al., 2019). However, whether McbR affects antibiotic resistance or regulates the expression of antibiotic resistance genes has not been reported in *E. coli*.

In this study, we constructed an isogenic *mcbR*-deficient strain using the λ red homologous recombination methods and *mcbR*-overexpressing strain using the pUC19 vector as previously described (Datsenko and Wanner, 2000; Yu et al., 2019). High-throughput sequencing (RNA-seq) was performed to analyze the transcriptional profile of the *mcbR* mutant and its parent strain. Besides, the antibiotic susceptibility of the mutant to various groups of antibiotics was tested using antibiotic susceptibility testing and antibacterial activity assays. Real-time reverse transcription-PCR (**RT-qPCR**) experiments and electrophoretic mobility shift assays (**EMSA**) were performed to investigate the regulatory mechanism of McbR on efflux pumps such as AcrAB, AcrD, EmrD, and MdtD in APECX40. Therefore, this study was conducted to find the pattern of antibiotic susceptibility in APEC, which in turn would be helpful to prevent the development of antibiotic resistance and ensure safe treatment.

## MATERIALS AND METHODS

### Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are described in Table 1. Cultures of *E. coli* were routinely grown at 37°C in Luria-Bertani (**LB**) broth or on LB agar containing 2.0% agar under aeration with shaking at 150 rpm or without shaking. All cultures for pKD46 or pCP20 temperature-sensitive plasmid maintenance were incubated at 30°C. Cell growth was

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

| Strain or plasmid | Relevant genotype | Reference or source |
|---|---|---|
| Strains | | |
| *Escherichia coli* | | |
| DH5α | Clone host strain, *supE44 ΔlacU169*(ϕ80 *lacZΔ*M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* | Invitrogen |
| BL21 | Expression strain, $F^-$ *ompT hsdS*($r_B^-$ $m_B^-$) *gal dcm* (DE3) | Invitrogen |
| WT | APECX40, wild-type | Laboratory stock |
| XY7 | APECX40 *mcbR*-deletion mutant | This study |
| WT/pSTV28 | WT with the empty vector pSTV28, $Cm^r$ | This study |
| XY7/pSTV28 | XY7 with the empty vector pSTV28, $Cm^r$ | This study |
| XY7/pCmcbR | XY7 with the complement plasmid pCmcbR, $Cm^r$ | This study |
| WT/pUC19 | WT with the empty vector pUC19, $Amp^r$ | This study |
| WT/pUCmcbR | WT with the overexpression plasmid pUCmcbR, $Amp^r$ | This study |
| Plasmids | | |
| pKD46 | Expresses λ red recombinase Exo, Bet, and Gam, temperature sensitive, $Amp^r$ | Datsenko and Wanner, 2000 |
| pKD3 | *cat* gene, template plasmid, $Amp^r$ $Cm^r$ | Datsenko and Wanner, 2000 |
| pCP20 | $FLP^+$ λ*c*I857$^+$ λ$p_R$Rep(Ts), temperature sensitive, $Amp^r$ $Cm^r$ | Datsenko and Wanner, 2000 |
| pSTV28 | Low copy number cloning vector, $Cm^r$ | Takara |
| pCmcbR | pSTV28 with *mcbR* gene, $Cm^r$ | This study |
| pUC19 | Cloning vector, $Amp^r$ | Takara |
| pUCmcbR | pUC19 with *mcbR* gene, $Amp^r$ | This study |
| pET28a(+) | Expression vector, $Kan^r$ | Novagen |
| pET-mcbR | pET28a(+) with *mcbR* gene, $Kan^r$ | This study |

$Cm^r$, $Amp^r$, and $Kan^r$ denote chloramphenicol, ampicillin, and kanamycin resistance, respectively.
Abbreviation: APEC, avian pathogenic *E. coli*.

monitored by measuring the turbidity at 600 nm using a UV/Vis spectrophotometer (DU730, Beckman Coulter, Miami, FL). Appropriate antibiotics for plasmid selection and maintenance were used at the following final concentrations: chloramphenicol at 16 μg/mL, kanamycin at 50 μg/mL, and ampicillin at 100 μg/mL.

## General DNA Manipulation

Genomic DNA of *E. coli* APECX40 (WT) was prepared by a standard protocol for Gram-negative bacteria. Plasmid DNA was extracted using a plasmid extraction kit (Promega, Madison, WI), according to the manufacturer's instructions. PCR amplification was carried out using *Taq* or *Pfu* DNA polymerases (Transgen, Beijing, China). Purification of PCR products and DNA fragments was performed using a gel purification kit (Promega), according to the manufacturer's instructions. DNA restriction enzyme (Takara, Dalian, Liaoning, China) digestion and T4 DNA ligase (Takara) ligation were carried out by standard methods. Sequence analyses were performed using Vector NTI Advance 11 software (InforMax, MA) to predict conserved domains of *mcbR* and to design the primers. Nucleotide sequences of primers are listed in Table 2.

## Construction of the mcbR Mutant

The isogenic *mcbR*-deficient mutant was constructed using homologous recombination methods based on the λ red recombinase system (Datsenko and Wanner, 2000; Yu et al., 2019). The chloramphenicol-resistance cassette gene (*cat*) flanked by 40 base pairs homology arms located upstream and downstream of the *mcbR* gene was PCR amplified from pKD3 using primers APECO2-mcbR-f and APECO2-mcbR-r and then PCR products were gel purified and suspended in sterilized distilled deionized water. The purified PCR products were transformed into competent cells of strain WT containing plasmid pKD46. The mutant was screened and confirmed by PCR amplification and DNA sequencing using primers check-mcbR-f and check-mcbR-r. The *cat* was cured by transforming plasmid pCP20 and selecting the chloramphenicol- and ampicillin-susceptibility strain, which was designated as XY7.

## Complementation of the mcbR Mutant

For functional complementation of the *mcbR* mutant strain, the *mcbR* open reading frame (**ORF**) and its putative promoter region were amplified from chromosomal DNA of wild-type strain WT using primers mcbR-EcoRI-f and mcbR-KpnI-r, and the fragment was gel purified and cloned into the *Eco*RI and *Kpn*I sites of the low copy number plasmid pSTV28 (TaKaRa), and then transformed into *E. coli* DH5α chemically competent cells, which were then spread on LB agar with 16 μg/mL chloramphenicol. Positive colonies were selected and confirmed by PCR using primers M13-f and M13-r and the recombinant plasmid pCmcbR was extracted and further confirmed by DNA sequencing (data not shown). Then the purified recombinant plasmid pCmcbR and control vector pSTV28 were

transformed into mutant strain XY7 and its parent strain WT to generate strains XY7/pCmcbR, XY7/pSTV28, and WT/pSTV28, respectively.

## Construction of the mcbR-Overexpressing Strain

The *mcbR*-overexpressing strain was constructed according to the method described earlier. Briefly, the *mcbR* ORF and its putative promoter region were amplified by PCR using primers mcbR-KpnI-f and mcbR-EcoRI-r from chromosomal DNA of wild-type strain WT, and the fragment was gel purified and cloned into the *Kpn*I and *Eco*RI sites of pUC19 (TaKaRa), and then transformed into *E. coli* DH5α chemically competent cells, which were then spread on LB agar with 100 μg/mL ampicillin. Positive colonies were selected and confirmed by PCR using primers M13-f and M13-r and the recombinant plasmid pUCmcbR was extracted and further confirmed by DNA sequencing (data not shown). Then the purified recombinant plasmid pUCmcbR and the control vector pUC19 were transformed into the parent strain WT to generate strains WT/pUCmcbR and WT/pUC19, respectively.

## Bacterial Growth Curves

Growth curves of WT/pSTV28, XY7/pSTV28, and XY7/pCmcbR or WT/pUCmcbR and WT/pUC19 were monitored, as described previously, with some modifications (Yu et al., 2019). Briefly, the overnight cultures of WT/pSTV28, XY7/pSTV28, and XY7/pCmcbR or WT/pUCmcbR and WT/pUC19 were each diluted to an $OD_{600}$ of approximately 0.03 in 50 mL of fresh LB broth with 16 μg/mL chloramphenicol or 100 μg/mL ampicillin, and grown at 37°C for 26 h with shaking. The cell density was detected 3 times every 2 h using a UV/Vis spectrophotometer. The growth curves of each strain were determined by calculating the mean of the cell density (600 nm) at each time point.

## Antibiotic Susceptibility Testing

Broth dilution antibiotic susceptibility tests were performed according to Clinical and Laboratory Standards

**Table 2.** Oligonucleotide primers used in this study.

| Primer name | Oligonucleotide (5′–3′)[1] |
|---|---|
| mcbR-f | ATGCCTGGAACGGAAAAAAT |
| mcbR-r | TTAACGATTGTATTGCTGGT |
| APECO2-mcbR-f | TGAACTCCTTCTGCCATGCAGGCAGGGTTGGACAGAAAACTGTAGGCTGGAGCTGCTT |
| APECO2-mcbR-r | ATATTGCGTGGCGATTTGAGTAATTACCTTGATGCCCGGTTGAATATCCTCCTTAGTTC |
| check-mcbR-f | ACACCAGGTGAACTCCTTCT |
| check-mcbR-r | GCGTGGCGATTTGAGTAATT |
| CM-f | TGTAGGCTGGAGCTGCTT |
| CM-r | CATATGAATATCCTCCTTAGTTC |
| mcbR-EcoRI-f | CGGAATTCAAAGTTTCAACGGCGGCGTA |
| mcbR-KpnI-r | GGGGTACCTTAACGATTGTATTGCTGGT |
| mcbR-EcoRI-f | CGGAATTCATGCCTGGAACGGAAAAAAT |
| mcbR-HindIII-r | CCAAGCTTTTAACGATTGTATTGCTGGT |
| mcbR-KpnI-f | CGGGGTACCAAAGTTTCAACGGCGGCGTA |
| mcbR-EcoRI-r | CCGGAATTCTTAACGATTGTATTGCTGGT |
| M13-f | TGTAAAACGACGGCCAGT |
| M13-r | CAGGAAACAGCTATGACC |
| T7-f | TAATACGACTCACTATAGGG |
| T7-r | TGCTAGTTATTGCTCAGCGG |
| rt-16s-f | TTTGAGTTCCCGGCC |
| rt-16s-r | CGGCCGCAAGGTTAA |
| rt-acrA-f | GCAGCCAATATCGCGCAA |
| rt-acrA-r | ATGCGACCGCTAATCGGA |
| rt-acrB-f | TTGCCAAAGGCGATCACG |
| rt-acrB-r | TTGGCAGACGCACGAACA |
| rt-acrD-f | TGTTCCTGCGTTTGCCGA |
| rt-acrD-r | CATTCGCGCCACGTTTTG |
| rt-emrD-f | GTATTACTCGTGGCCGTC |
| rt-emrD-r | ATTCCGACGAGGATCACC |
| rt-acrR-f | GCGAGATTGCAAAAGCAG |
| rt-acrR-r | CACCGTGGATTCAAGAAC |
| rt-mdtD-f | CGCAAAGCCTCGGGGAAAGT |
| rt-mdtD-r | TCGTTCAGCGTGCCGGAAAG |
| p-yciF-biotin-f | CAGGAAAATCCTGATTCA |
| p-yciF-r | AGGTATCTGAAAGCAGGT |
| p-acrAB-biotin-f | ATGTTCGTGAATTTACAGG |
| p-acrAB-r | ATGTAAACCTCGAGTGTC |
| p-emrD-biotin-f | CCGCTTTTGTTTACATAT |
| p-emrD-r | TATCACGGATGCTTTTAT |
| p-acrD-biotin-f | TGCCTCCTACTGACCAAAGAA |
| p-acrD-r | TAAAAGAGGACCTCGTGTTTC |
| p-mdtD-biotin-f | GACCCTTTCCTTATTTAAGG |
| p-mdtD-r | CGTTAAGAGTTTCTCTTCCT |

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

Institute standards. The changes in antibiotic susceptibility of WT/pSTV28, XY7/pSTV28, and XY7/pCmcbR, or WT/pUC19 and WT/pUCmcbR were examined using Mueller-Hinton broth with the following modification: the overnight cultures of WT/pSTV28, XY7/pSTV28, and XY7/pCmcbR, or WT/pUC19, WT/pUCmcbR were diluted to an $OD_{600}$ of approximately 0.03 in fresh Mueller-Hinton broth with 16 μg/mL chloramphenicol or 100 μg/mL ampicillin, respectively, contained in 96-well plates (Costar, Corning, Steuben, NY) with 2-fold serial dilutions of the antibiotics listed in Table 3. The 96-well plates were incubated for 24 h at 37°C. The lowest concentration of antibiotics that completely inhibited growth was identified as the minimal inhibitory concentration (**MIC**). Experiments were repeated 3 times.

## Antibacterial Activity Assays

Antibacterial activity assays were performed to examine the changes in antibiotic susceptibility of WT/pSTV28, XY7/pSTV28, and XY7/pCmcbR or WT/pUC19, WT/pUCmcbR according to previously described methods and some modifications (Yu et al., 2018, 2019). The overnight cultures of WT/pSTV28, XY7/pSTV28, and XY7/pCmcbR or WT/pUC19, WT/pUCmcbR were diluted to an $OD_{600}$ of approximately 0.03 in 3 mL of fresh LB broth with 16 μg/mL chloramphenicol or 100 μg/mL ampicillin, respectively, contained in polystyrene tubes at 37°C for 2 h with shaking. After incubation, 12 antibiotics used were added to polystyrene tubes containing the bacterial cultures of WT/pSTV28, XY7/pSTV28, and XY7/pCmcbR or WT/pUC19, WT/pUCmcbR, respectively. The antibiotic test concentration used is derived from the MIC results. Subsequently, the cultures continued to incubate at 37°C for 3 h with shaking. After incubation, 10-fold serial dilutions of cultures were obtained by successive transfer (0.1 mL) through 4 Eppendorf tubes containing 0.9 mL of LB broth. Next, 100 μL dilutions were dropped on LB agar plates with appropriate antibiotic. After cultivating for 18 h at 37°C, the viable colonies were counted via CFU on LB agar plates with appropriate antibiotic. The survival rates of WT/pSTV28 or WT/pUC19 were designated as 100%, and the experiments were repeated 3 times with similar results.

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

For total RNA isolation, the overnight cultures of WT/pSTV28, XY7/pSTV28, and XY7/pCmcbR or WT/pUC19, WT/pUCmcbR were diluted to an $OD_{600}$ of approximately 0.03 in fresh LB broth with 16 μg/mL chloramphenicol or 100 μg/mL ampicillin, respectively. The cultures were grown to the exponential phase at 37°C with shaking. The cells were collected by centrifugation and resuspended in RNase-free water, and subsequently, total RNA was extracted from the cells using Trizol reagent (Transgen). Reverse transcription was carried out using the EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (Transgen), according to the manufacturer's instructions. Real-time PCR was performed with RT primers following the instructions of the TransStart Tip Green qPCR SuperMix kit (Transgen) on the CFX96 Real-Time System (Bio-Rad, Hercules, CA). Then differences in gene expression were calculated by the **ΔΔ**Ct method (where Ct = cycle threshold), using the *16S* rDNA gene as a housekeeping gene, normalized by subtracting the Ct value of *16S* cDNA from that of the target cDNA. All of the real-time RT-PCR assays were repeated at least 3 times with similar results, and the PCR amplification efficiency was between 1.93 and 2.09 (Xue et al., 2016).

## Purification of the McbR Protein

The $His_6$-tagged McbR was cloned according to previously described methods, with some modifications (Yu et al., 2019). Briefly, the *mcbR* ORF was amplified by PCR with primers mcbR-EcoRI-f and mcbR-HindIII-r from WT genomic DNA and cloned into expression vector pET28a(+) (Novagen, Darmstadt, GER) and then generated a recombinant plasmid pET-mcbR. pET-mcbR was transformed into *E. coli* DH5α chemically competent cells, which were incubated for 1 h at 37°C with shaking and then spread on LB agar with 50 μg/mL kanamycin. pET-mcbR was extracted from positive colonies and confirmed by PCR amplification and DNA

**Table 3.** Antibiotics used in this study.

| Antibiotics | Classes | Dilutions | Store concentrations (mg/mL) | Test concentrations (μg/mL) |
|---|---|---|---|---|
| Clindamycin | Lincosamides | Distilled water | 32 | 156 |
| Lincomycin | Lincosamides | Distilled water | 32 | 750 |
| Cefalexin | β-Lactams | Distilled water | 100 | 500 |
| Cefotaxime | β-Lactams | Distilled water | 10 | 1.5 |
| Doxycycline | Tetracyclines | Distilled water | 10 | 30 |
| Tetracycline | Tetracyclines | Distilled water | 32 | 320 |
| Gentamicin | Aminoglycosides | Distilled water | 10 | 2.5 |
| Kanamycin | Aminoglycosides | Distilled water | 50 | 5 |
| Norfloxacin | Fluoroquinolone | Acetic acid | 20 | 3 |
| Ofloxacin | Fluoroquinolone | Acetic acid | 20 | 3 |
| Erythromycin | Macrolide | Dehydrated ethanol | 32 | 25 |
| Rifampicin | Rifamycins | Methanol | 25 | 50 |

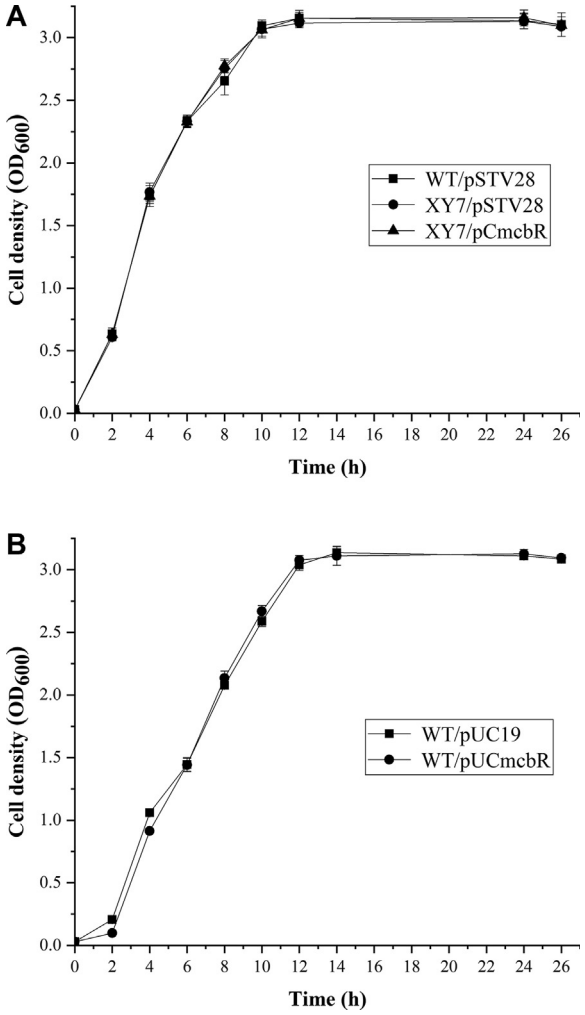


**Figure 1.** Growth curves of 5 strains. (A) Growth curves of the *mcbR*-deficient strain XY7/pSTV28, the complement strain XY7/pCmcbR, and their parent strain WT/pSTV28 grown in LB broth with 16 μg/mL chloramphenicol at 37°C for 26 h with shaking. (B) Growth curves of the *mcbR*-overexpressing strain WT/pUCmcbR and its parent strain WT/pUC19 grown in LB broth with 100 μg/mL ampicillin at 37°C for 26 h with shaking. These growth curves were determined by measuring the cell density (OD) at 600 nm, and the data represent the means of 3 independent assays. Abbreviation: LB, Luria-Bertani.

sequencing using primers T7-f and T7-r (data not shown). pET-mcbR was transformed into expression strain *E. coli* BL21 (DE3), and then McbR was purified using standard procedures (Yu et al., 2019). The McbR protein solution was preserved in 10% glycerol and stored at −80°C until use. The purity of the protein was analyzed by SDS-PAGE, and the protein concentration was measured using the Bradford assay with BSA as a standard.

### EMSA

The DNA fragments containing the promoters were amplified by PCR using p-primers from WT genomic DNA. The biotin-labeled DNA fragments were incubated with various amounts of purified McbR protein in 4 μL of 5×binding buffer (100 mM Tris, 5 M NaCl, pH 8.0) at 25°C for 30 min, and when required, the unlabeled DNA fragments were added as competitive probes. After incubation, 5 μL of 5×loading buffer with bromophenol blue was added to the mixtures, which were then electrophoresed in a 4% native polyacrylamide gel in 0.5 × Tris-borate EDTA buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.3). The band shifts were detected and analyzed according to the manufacturer's instructions of the chemiluminescent EMSA kit (Beyotime, Shanghai, China).

### Statistical Analysis

All data were analyzed using the SPSS statistical software (version 19.0, IBM Corp., Armonk, NY) by a one-way ANOVA method; the test results are shown as mean ± SD. The paired $t$ test was used for statistical comparisons between groups. The level of statistical significance was set at a $P$-value of $\leq 0.05$.

## RESULTS

### Deletion of mcbR Did Not Affect Growth of XY7

The isogenic *mcbR*-deficient mutant XY7 was generated by λ red-mediated recombination. Complementation of the *mcbR* mutant was accomplished by expressing the ORF of *mcbR* gene and its putative promoter in pSTV28 vector. To assure that the growth

**Table 4.** Susceptibility of *Escherichia coli* strains to various antibiotics.

| | MIC (μg/mL) of 5 *E. coli* strains | | | | |
|---|---|---|---|---|---|
| Antibiotics | WT/pSTV28 | XY7/pSTV28 | XY7/pCmcbR | WT/pUC19 | WT/pUCmcbR |
| Clindamycin | 156 | 156 | 156 | 39 | 39 |
| Lincomycin | 750 | 750 | 750 | 375 | 375 |
| Cefalexin | 250 | 250 | 250 | 1,000 | 2,000 |
| Cefotaxime | 0.78 | 0.39 | 0.78 | 0.78 | 3.12 |
| Doxycycline | 12 | 12 | 12 | 24 | 24 |
| Tetracycline | 125 | 125 | 125 | 125 | 125 |
| Gentamicin | 0.78 | 0.39 | 0.78 | 0.78 | 1.56 |
| Kanamycin | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 |
| Norfloxacin | 0.039 | 0.039 | 0.039 | 0.039 | 0.039 |
| Ofloxacin | 0.039 | 0.039 | 0.039 | 0.039 | 0.039 |
| Erythromycin | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 |
| Rifampicin | 9.75 | 9.75 | 9.75 | 9.75 | 9.75 |

Abbreviation: MIC, minimal inhibitory concentration.

conditions of the mutant strain XY7 and its parent strain WT were consistent with the complementation strain, WT and XY7 were transformed with the empty vector pSTV28. The colony morphology of XY7/pSTV28 and XY7/pCmcbR was similar to that of WT/pSTV28 on the LB agar plates with 16 μg/mL chloramphenicol. They were circular, convex, moist, smooth, translucent, and 1 to 2 mm in diameter (data not shown). The growth curves of XY7/pSTV28 and XY7/pCmcbR were similar to that of WT/pSTV28 in LB broth with 16 μg/mL chloramphenicol (Figure 1A).

## Overexpression of mcbR Did Not Affect Bacterial Growth

The *mcbR*-overexpressing strain was obtained by expressing the ORF of *mcbR* gene and its putative promoter in pUC19 vector. To assure that the growth conditions of the parent strain WT were consistent with the overexpression strain WT/pUCmcbR, WT was transformed with the empty vector pUC19. The colony morphology of WT/pUCmcbR was similar to that of WT/pUC19 on the LB agar plates with 100 μg/mL ampicillin. They were circular, convex, moist, smooth, translucent, and 1 to 2 mm in diameter (data not shown). The growth curves of WT/pUCmcbR were similar to that of WT/pUC19 in LB broth with 100 μg/mL ampicillin (Figure 1B).

## Deletion of the mcbR Gene Increased Antibiotic Susceptibility

The MIC of 12 antibiotics was determined and interpreted according to the Clinical and Laboratory Standards Institute standards. The broth dilution MIC results of WT/pSTV28, XY7/pSTV28, and XY7/pCmcbR are shown in Table 4. These results indicated that the MIC of XY7/pSTV28 to cefotaxime and gentamicin were decreased 2-fold, the MIC of XY7/pSTV28 to the other 10 antibiotics were unaltered, when compared to that of WT/pSTV28, and the MIC of XY7/pCmcbR was restored. To further determine

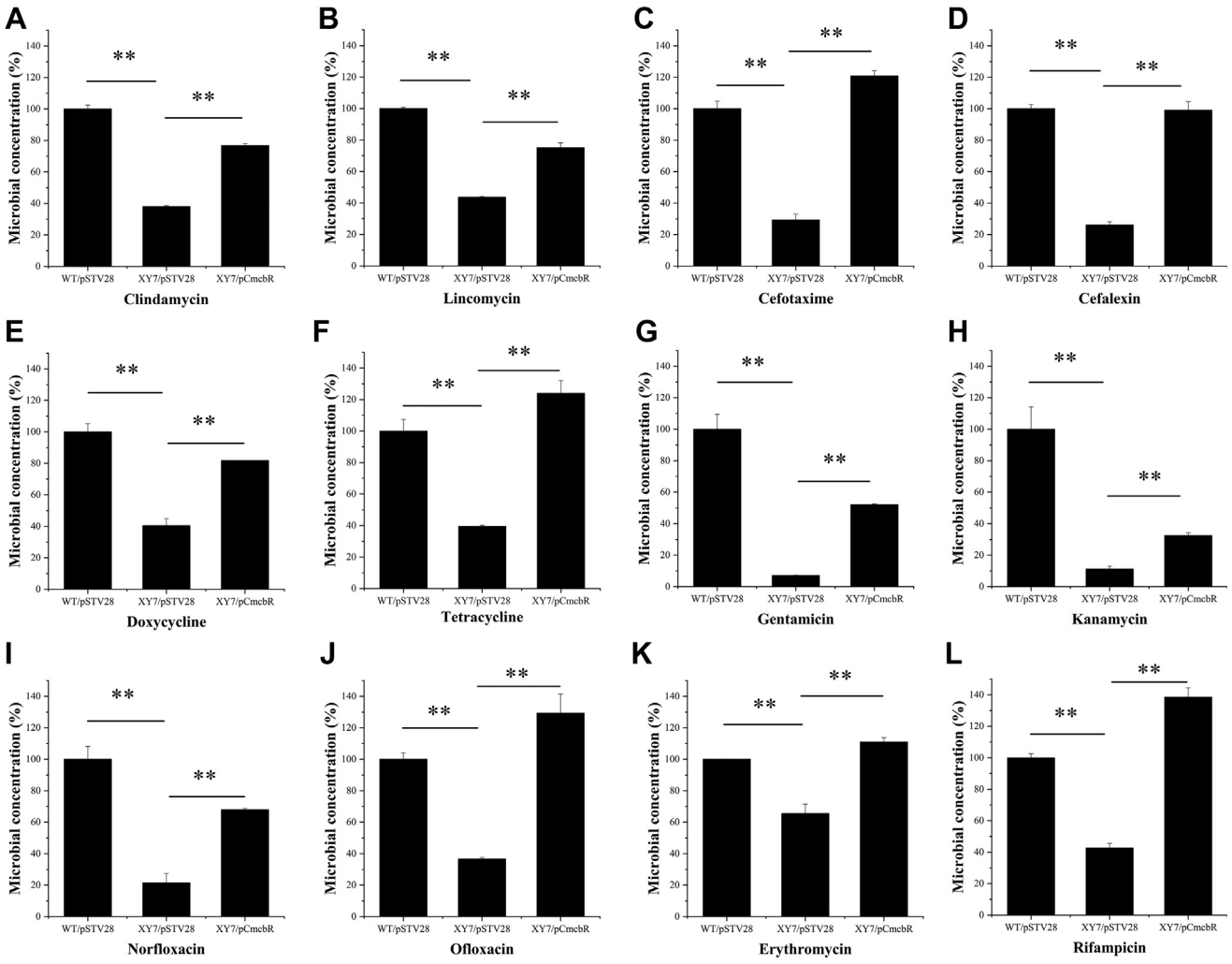


**Figure 2.** CFU assays of the *mcbR*-deficient strain XY7/pSTV28, the complement strain XY7/pCmcbR, and their parent strain WT/pSTV28 in the presence of the 12 antibiotics tested: (A) clindamycin, (B) lincomycin, (C) cefotaxime, (D) cefalexin, (E) doxycycline, (F) tetracycline, (G) gentamicin, (H) kanamycin, (I) norfloxacin, (J) ofloxacin, (K) erythromycin, and (L) rifampicin. The survival rate of WT/pSTV28 was assigned as 100%. The colony counts of XY7/pSTV28 and XY7/pCmcbR were all compared with that of WT/pSTV28. Error bars indicate SD; $^{**}P < 0.01$, demonstrate significant differences in WT/pSTV28, XY7/pSTV28, and XY7/pCmcbR.

whether or not *mcbR* affects antibiotic susceptibility in APECX40, the survival rates of WT/pSTV28, XY7/pSTV28, and XY7/pCmcbR were confirmed using CFU assays when cultures grown in LB broth with 16 μg/mL chloramphenicol were challenged with the test concentration of each antibiotic of the 12 antibiotics used. As shown in Figure 2, in the presence of 12 antibiotics, the survival rates of XY7/pSTV28 were decreased almost 2.6-fold (clindamycin), 2.3-fold (lincomycin), 3.4-fold (cefotaxime), 3.8-fold (cefalexin), 2.5-fold (doxycycline), 2.5-fold (tetracycline), 14.1-fold (gentamicin), 8.9-fold (kanamycin), 4.7-fold (norfloxacin), 2.7-fold (ofloxacin), 1.5-fold (erythromycin), and 2.3-fold (rifampicin) ($P < 0.01$), respectively, when compared to that of WT/pSTV28, and the survival rates of XY7/pCmcbR were restored. These data indicated that deletion of the *mcbR* gene significantly increases susceptibility to the above 12 antibiotics in APECX40.

## Overexpression of the mcbR Gene Decreased Antibiotic Susceptibility

The broth dilution MIC results of WT/pUCmcbR and WT/pUC19 are shown in Table 4. The results indicated that the MIC of WT/pUCmcbR to cefalexin, cefotaxime, and gentamicin increased 2-fold, 4-fold, and 2-fold, respectively; the MIC of WT/pUCmcbR to the other 9 antibiotics were unaltered, when compared to that of WT/

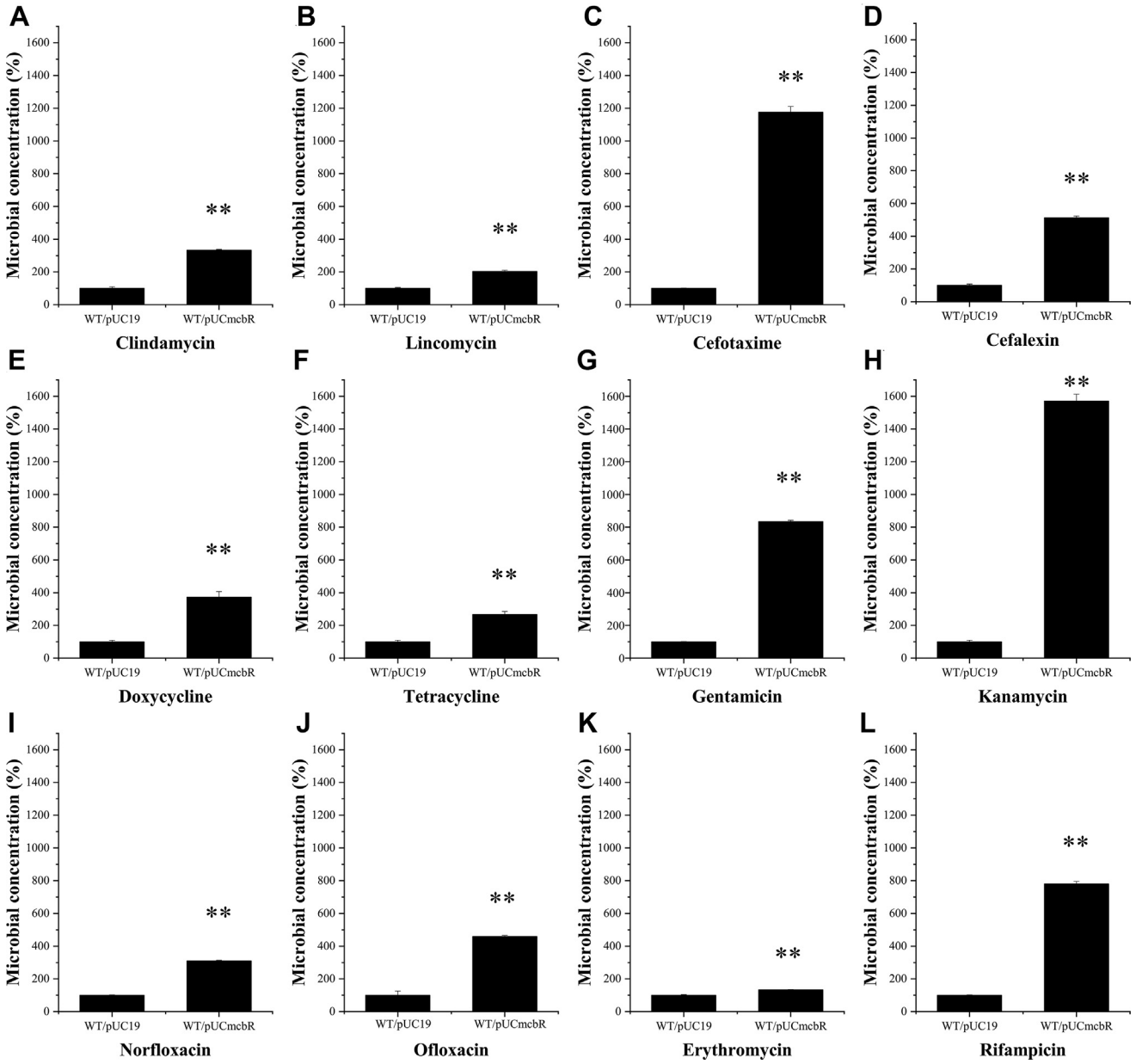


**Figure 3.** CFU assays of the *mcbR*-overexpressing strain WT/pUCmcbR and its parent strain WT/pUC19 in the presence of the 12 antibiotics tested: (A) clindamycin, (B) lincomycin, (C) cefotaxime, (D) cefalexin, (E) doxycycline, (F) tetracycline, (G) gentamicin, (H) kanamycin, (I) norfloxacin, (J) ofloxacin, (K) erythromycin, and (L) rifampicin. The survival rate of WT/pUC19 was assigned as 100%. The colony counts of WT/pUCmcbR were compared with that of WT/pUC19. Error bars indicate SD; $^{**}P < 0.01$, demonstrate significant differences between WT/pUC19 and WT/pUCmcbR.

pUC19. To further determine the effect of *mcbR* on antibiotic susceptibility in APECX40, the survival rates of WT/pUCmcbR and WT/pUC19 were confirmed in CFU assays when cultures grown in LB broth with 100 μg/mL ampicillin were challenged with the test concentration of each antibiotic of the 12 antibiotics used. As shown in Figure 3, in the presence of 12 antibiotics, the survival rates of WT/pUCmcbR were increased almost 3.33-fold (clindamycin), 2.03-fold (lincomycin), 11.75-fold (cefotaxime), 5.12-fold (cefalexin), 3.73-fold (doxycycline), 2.66-fold (tetracycline), 8.34-fold (gentamicin), 15.71-fold (kanamycin), 3.10-fold (norfloxacin), 4.59-fold (ofloxacin), 1.33-fold (erythromycin), and 7.80-fold (rifampicin) ($P < 0.01$), respectively, when compared to that of WT/pUC19. These data indicated that overexpression of the *mcbR* gene significantly decreases susceptibility to the above 12 antibiotics in APECX40, and further suggested that McbR plays an important role in the regulatory process of antibiotic susceptibility.

## Regulatory Effect of McbR on the Multidrug Efflux Pump

To investigate how McbR affects the susceptibility of APECX40 to the 12 antibiotics used, real-time RT-PCR experiments were performed to examine the transcript levels of a range of multidrug efflux pumps encoding genes, including *acrA* (encoding multidrug efflux pump membrane fusion lipoprotein AcrA), *acrB* (encoding multidrug efflux pump RND permease AcrB), *acrD* (encoding aminoglycoside/multidrug efflux pump RND permease AcrD), *acrR* (encoding multidrug efflux transporter transcriptional repressor AcrR), *emrD* (encoding multidrug efflux pump EmrD involved in adaptation to low-energy shock), and *mdtD* (encoding multidrug efflux pump MdtD). As shown in Figure 4A, the transcript levels of *acrR* were increased 2.26-fold, and the transcript levels of *acrA*, *acrB*, *acrD*, *emrD*, and *mdtD* were decreased 2.22-fold, 2.0-fold, 2.5-fold, 2.32-fold, and 2.71-fold ($P < 0.01$), respectively, in XY7/pSTV28 when compared to that of WT/pSTV28. In XY7/pCmcbR, the transcript levels of *acrA*, *acrB*, and *acrR* were restored, and the transcript levels of *acrD*, *emrD*, and *mdtD* exceeded the levels from WT/pSTV28. However, the transcript levels of *acrR* in WT/pUCmcbR were decreased 6.41-fold, and the transcript levels of *acrA*, *acrB*, *acrD*, *emrD*, and *mdtD* in WT/pUCmcbR were increased 54.68-fold, 25.42-fold, 40.91-fold, 19.45-fold, and 38.38-fold ($P < 0.01$), respectively, when compared to that of WT/pUC19 (Figure 4B). These results indicated that among others *mcbR* affects the susceptibility of APECX40 to the antibiotics tested by regulating *acrA*, *acrB*, *acrD*, *acrR*, *emrD*, and *mdtD*.

## Binding Ability of McbR to Target Genes Promoters

To determine whether or not McbR regulates the transcription of *acrAB* (*acrA* and *acrB* are co-transcript in AcrAB efflux pump), *acrD*, *acrR*, *emrD*, and *mdtD* by directly binding to their promoter regions, we performed EMSA. The purified $His_6$-tagged McbR protein was used to bind biotin-labeled DNA amplification fragments containing the putative promoters of these target genes. As shown is Figure 5, clearly shifted bands of protein-DNA complex were detected at McbR concentrations of 3, 6, and 12 μM, and the intensity of the shifted band increased as the amount of McbR increased. However, the shifted band disappeared in the presence of an approximately 10-fold excess of unlabeled promoter DNA fragment as a specific competitor. The results showed that McbR can specifically bind to the promoter regions of *acrAB*, *acrD*, *acrR*, *emrD*, and *mdtD*, indicating that McbR directly regulates the transcription of *acrAB*, *acrD*, *acrR*, *emrD*, and *mdtD*. Therefore, these results indicated that McbR regulates bacterial susceptibility to clindamycin, lincomycin, cefotaxime, cefalexin, doxycycline, tetracycline, gentamicin, kanamycin, norfloxacin, ofloxacin, erythromycin, and

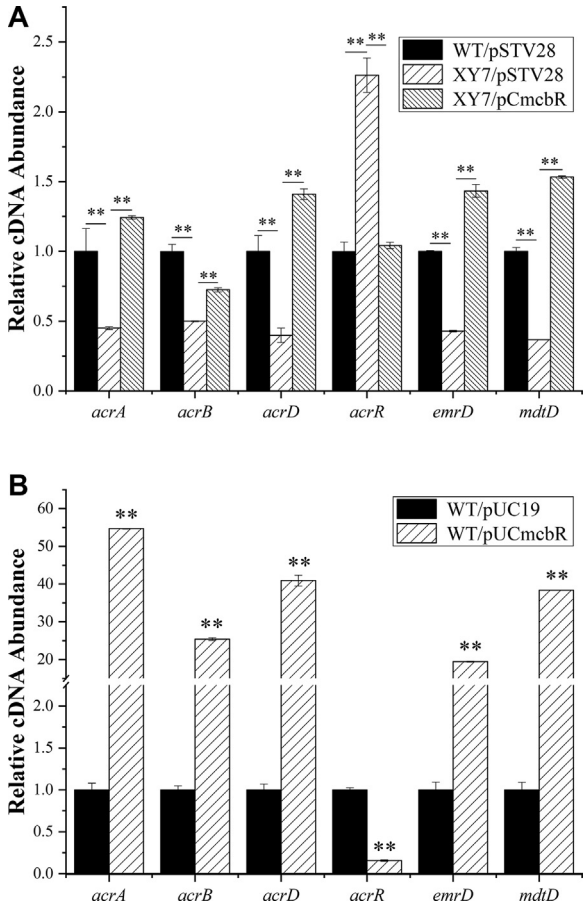


**Figure 4.** Comparative measurement of transcription (cDNA abundance) of efflux pumps encoding genes in 5 strains. (A) Relative transcript level of efflux pumps encoding genes was determined by RT-qPCR in WT/pSTV28, XY7/pSTV28, and XY7/pCmcbR incubated in LB medium with 16 μg/mL chloramphenicol. (B) Relative transcript level of efflux pumps encoding genes was determined by RT-qPCR in WT/pUC19 and WT/pUCmcbR incubated in LB medium with 100 μg/mL ampicillin. Error bars indicate SD; $^{**}P < 0.01$, demonstrate significant differences in WT/pSTV28, XY7/pSTV28, and XY7/pCmcbR, or WT/pUC19 and WT/pUCmcbR. Abbreviations: LB, Luria-Bertani; RT-qPCR, real-time reverse transcription-PCR.

rifampicin in APECX40 by directly binding to the promoter regions of *acrAB*, *acrD*, *acrR*, *emrD*, and *mdtD*.

## DISCUSSION

Previous studies indicated that the transcriptional factor McbR affects biofilm formation and a mucoidy phenotype and protects the cell from stress damage (Hindupur et al., 2006; Zhang et al., 2008; Beraud et al., 2010; Lord et al., 2014). Additionally, our previous study also verified that McbR affects biofilm formation and $H_2O_2$ stress response in APECX40 (Yu et al., 2019). However, whether or not McbR affects susceptibility or resistance to various groups of antibiotics in *E. coli* had not been reported. In the present study, we constructed the *mcbR* mutant and the *mcbR*-overexpressing strain in APECX40, which is a clinical *E. coli* isolated from a pigeon with airsacculitis, and performed RNA-seq to analyze the transcriptional profile of the *mcbR* mutant and its parent strain as in our previous study (Yu et al., 2019). RNA-seq results showed that deletion of *mcbR* affects the transcription of multidrug efflux pump MdtD and multidrug efflux pump transcriptional repressor AcrR. We found that the MIC values of XY7/pSTV28 to cefotaxime and gentamicin were decreased 2-fold using antibiotic susceptibility testing when compared to that of WT/pSTV28, and the MIC values were restored in XY7/pCmcbR (Table 4). Moreover, the MIC of WT/pUCmcbR to cefalexin, cefotaxime, and gentamicin was increased 2-fold, 4-fold, and 2-fold, respectively, when compared to that of WT/pUC19 (Table 4). However, in the presence of the above antibiotics, the survival rates of XY7/pSTV28 were significantly decreased by antibacterial activity assays, the survival rates of WT/pUCmcbR were significantly increased when compared with that of their parent strains WT/pSTV28 and WT/pUC19, respectively, and the survival rates of XY7/pCmcbR were restored. Overall, this work is the first to report that the transcriptional regulator McbR increases the susceptibility of APECX40 to the 12 antibiotics tested. However, the molecular mechanism of McbR affecting the susceptibility to various groups of antibiotics in *E. coli* has not been reported.

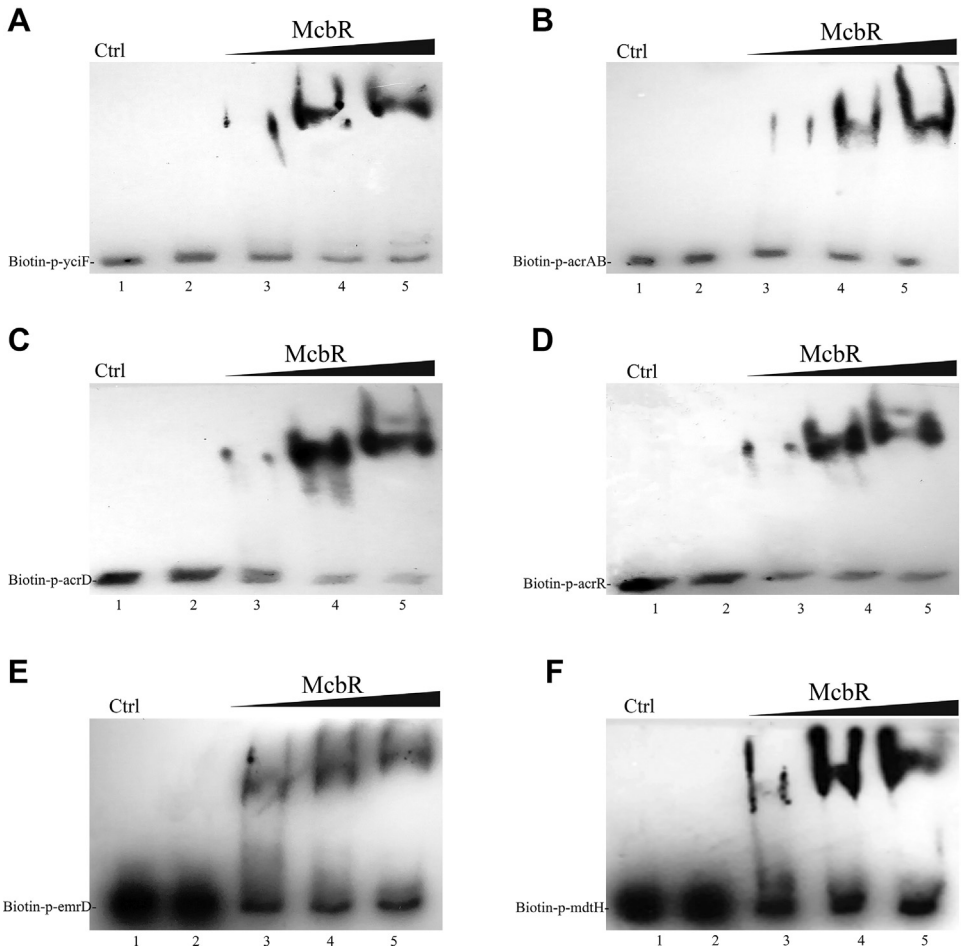


**Figure 5.** The binding ability of McbR to the target promoters was determined by electrophoretic mobility shift assay. Increasing concentrations of McbR were incubated with biotin-labeled *yciF*, *acrAB*, *acrD*, *acrR*, *emrD*, and *mdtD* promoters (biotin-p-yciF, biotin-p-acrAB, biotin-p-acrD, biotin-p-acrR, biotin-p-emrD, and biotin-p-mdtD). In panels A to F, the concentrations of McbR were 12, 0, 3, 6, and 12 μM, respectively, from lanes 1 to 5; the amounts of biotin-labeled promoter fragments in all samples were 200 fmol. In lane 1, besides the labeled probes, 2 pmol of unlabeled promoter fragment was added as the specific competitor (Ctrl). (A) The positive control, indicating the binding ability of McbR to the *yciF* promoter, (B) the *acrAB* promoter, (C) the *acrD* promoter, (D) the *acrR* promoter, (E) the *emrD* promoter, and (F) the *mdtD* promoter.

Previous studies revealed that the AcrAB efflux pump actively expels different classes of antimicrobial agents such as chloramphenicol, tetracyclines, fluoroquinolones, rifampin, β-lactams, and nalidixic acid (Piddock, 2006; Subhadra et al., 2018). On the AcrAB efflux pump, *acrA* and *acrB* form a polycistronic operon which is under the control of the *acrR* gene upstream of *acrA*, and AcrR functions as an important repressor of the AcrAB efflux pump. The AcrD efflux pump not only has distinctive substrate profiles with respect to aminoglycoside antibiotics, but also captures aminoglycosides from the periplasm to extrude them into the medium in intact cells, acting as a "periplasmic vacuum cleaner" (Aires and Nikaido, 2005; Buckner et al., 2016). The EmrD efflux pump can alter the cell susceptibility to 8 antimicrobial agents, such as ciprofloxacin, norfloxacin, doxycycline, tetracycline, clindamycin, lincomycin, erythromycin, and SDS, in *E. coli* strains APECX40 and MG1655 (Yu et al., 2020). Expression of the MdtD efflux pump can protect *S. typhimurium* against the antibiotics ampicillin and ciprofloxacin (Frawley et al., 2013). Basing on these findings, we hypothesized that McbR could cause changes in resistance to a broad range of antibiotics by regulating the expression of efflux pumps, including AcrAB, AcrD, AcrR, EmrD, and MdtD. To confirm the hypothesis mentioned above, we performed RT-qPCR experiments and EMSA assays. Our results showed that McbR regulates the expression of efflux pumps AcrAB, AcrD, EmrD, and MdtD, and AcrAB efflux pump repressor AcrR, and then alters the susceptibility to various groups of antibiotics such as lincosamides, tetracyclines, quinolones, rifampin, β-lactams, and aminoglycosides. Therefore, this study first demonstrates that McbR regulates the transcription of *acrAB*, *acrD*, *acrR*, *emrD*, and *mdtD* by directly binding to their promoter regions, and then increases the susceptibility to various groups of antibiotics in APECX40. However, whether or not deletion of *mcbR* and overexpression of *mcbR* change bacterial susceptibility to various groups of antibiotics in the host has not been detected in animal models; this is one aspect of this study that needs further research. In future work, we will examine the effect of McbR on antibiotic susceptibility of bacteria *in vivo*, and further investigate the detailed molecular mechanism of how McbR regulates efflux pumps.

## CONCLUSIONS

This study is the first to report that McbR could increase susceptibility to various groups of antibiotics by regulating the transcription of *acrAB*, *acrD*, *acrR*, *emrD*, and *mdtD* by specifically binding to their promoter regions in APECX40. Overall, this study may help find the pattern of antibiotic susceptibility in APEC and further provide some new insights for the treatment and prevention of APEC infection.

## ACKNOWLEDGMENTS

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## DISCLOSURES

The authors report no conflicts of interest in this work.

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