Role of McbR in the regulation of antibiotic susceptibility in avian pathogenic \textit{Escherichia coli}

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ABSTRACT Avian pathogenic \textit{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 \(\beta\)-lactams, polymyxins, tetracyclines, fluoroquinolones, aminoglycosides, novobiocin, nalidixic acid, and fosfomycin. In the present study, we constructed the \textit{mcbR} mutant and the \textit{mcbR}-overexpressing strain of \textit{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 \textit{acrAB}, \textit{acrD}, \textit{emrD}, and \textit{mdtD} \((P < 0.01)\). Additionally, EMSA indicated that McbR specifically binds to the promoter regions of \textit{acrAB}, \textit{acrD}, \textit{acrR}, \textit{emrD}, and \textit{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 \textit{Escherichia coli}, McbR, efflux pump, antibiotic susceptibility

INTRODUCTION

Avian pathogenic \textit{Escherichia coli} (APEC) ranks among the frequent causative agents of extraintestinal infectious bacterial disease, collectively called avian 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).
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, AcrB, 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; Hirakawa 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-chloroarboxylcyanide 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 H2O2 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
The chloramphenicol-resistance red recombinase system (Datsenko and Wanner, 2000) using homologous recombination methods based on the λ red recombinase system. Construction of the mcbR mutant

**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-susceptible 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 EcoRI and KpnI 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 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.

Abbreviation: APEC, avian pathogenic *E. coli.*
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 KpnI and EcoRI 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<sub>600</sub> 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

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

| Primer name | Oligonucleotide (5’–3’) |
|-------------|------------------------|
| mcbR-f      | ATGCCTGGAACGGAAAAAAT   |
| mcbR-r      | TTAACGATTGTATTTGCTGTT |
| APECO2-mcbR-f | TGAACTCCTTCTGCCATGCAGGGTTGGACAGAAAACTGTAGGCTGGAGCTGCTT |
| APECO2-mcbR-r | ATATTGCGTGGCGATTTGAGTAATTACCTTGATGCCCGGTTGAATATCCTCCTCTTAGTTC |
| check-mcbR-f | ACACCAGTTGAACCTCCTCTTCTT |
| check-mcbR-r | GCGTGGCCGATTGTAGTAAAT |
| CM-f        | TGATGCGCTAGCTTGCTT |
| CM-r        | CATATGAAATATCTCCCTAGTTC |
| mcbR-EcoRI-f | CGAATTTCAAGTTTCAACGCGCCGCTGA |
| mcbR-KpnI-r | GGGGTACCCTTTAACGATTGTATTGCTGTT |
| mcbR-EcoRI-f | GGGGTCACCTTTAACGATTGTATTGCTGTT |
| mcbR-HindIII-r | CGAATTTCAAGTTTCAACGCGCCGCTGA |
| mcbR-KpnI-f | ATGCCTGGAACGGAAAAAAT |
| mcbR-EcoRI-r | TTAACGATTGTATTTGCTGTT |
| T7-f        | TTAACGATTGTATTTGCTGTT |
| T7-r        | TTAACGATTGTATTTGCTGTT |
| M13-f       | TGTAACGATGCTACGTCGT |
| M13-r       | CGAAGTGGGTAGCAGGAGG |
| rt-16s-f    | CAGGAAAATCTGATTCAAGGT |
| rt-16s-r    | CTGGCGCGAACGTTTA |
| rt-acrA-f   | GAACACATATCCGAGGAC |
| rt-acrA-r   | CTGGCGCGAACGTTTA |
| rt-acrB-f   | TTCTGGAGTGCTAAGAC |
| rt-acrB-r   | CGGAGAGGTCGACAGGACG |
| rt-acrD-f   | TCAGATGCTCCGAGTGA |
| rt-acrD-r   | GCTTCGGCTAGCTGCT |
| rt-emrD-f   | GAAGATGCTCTGCTG |
| rt-emrD-r   | ACGGCGCCGCAAGT |
| rt-acrB-f   | TTAACGATTGTATTTGCTGTT |
| rt-acrB-r   | GGCGCGCCGCAAGT |
| rt-mdtD-f   | TTAACGATTGTATTTGCTGTT |
| rt-mdtD-r   | CGAAGTGGGTAGCAGGAGG |
| p-yciF-biotin-f | TTAGTTACGCGTACGTC |
| p-yciF-r    | AGGTATCTGAAAGCAGGT |
| p-acrAB-biotin-f | TTAGGTATCTGAAAGCAGGT |
| p-acrAB-r   | TTAGGTATCTGAAAGCAGGT |
| p-emrD-biotin-f | CAGGAAAATCTGATTCAAGG |
| p-emrD-r    | TTAACGATTGTATTTGCTGTT |
| p-acrD-biotin-f | TTAGGTATCTGAAAGCAGGT |
| p-acrD-r    | TTAGGTATCTGAAAGCAGGT |
| p-mdtD-biotin-f | TTAGGTATCTGAAAGCAGGT |
| p-mdtD-r    | TTAGGTATCTGAAAGCAGGT |

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 OD600 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 OD600 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 were used to add 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 OD600 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 ΔΔCt 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 His6-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

| 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| 30                          | 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                          |

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

| Antibiotics      | MIC (μg/mL) of 5 *E. coli* strains |
|------------------|-----------------------------------|
|                  | 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 WT/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...
whether or not \textit{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 \( \mu \)g/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 \textit{mcbR} gene significantly increases susceptibility to the above 12 antibiotics in APECX40.

**Overexpression of the \textit{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/pUC19.

![Figure 2](image_url)

Figure 2. CFU assays of the \textit{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) norflaxacin, (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 acrA, acrB, and 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<sub>6</sub>-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 unlabelled 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
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 H2O2 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](image-url)
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 Escherichia 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 (Germon, P., Y. H. Chen, L. He, J. E. Blanco, A. Bree, C. Schouler, S. H. Huang, and M. Moulin-Schouleur. 2005. “AcrD pump can alter the susceptibility to 8 antimicrobial agents.”). Basing on these observations, we hypothesized that McbR could cause changes in resistance against the antibiotics ampicillin and ciprofloxacin (Frawley et al., 2013). Basing on these observations, we hypothesized that McbR could cause changes in resistance against the antibiotics ampicillin and ciprofloxacin (Frawley et al., 2013). Basing on these observations, we hypothesized that McbR could cause changes in resistance against the antibiotics ampicillin and ciprofloxacin (Frawley et al., 2013).

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 directly 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

This work was supported by the National Natural Science Foundation of China (grant number 31672571).

DISCLOSURES

The authors report no conflicts of interest in this work.

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