Role of LsrR in the regulation of antibiotic sensitivity in avian pathogenic Escherichia coli

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ABSTRACT Avian pathogenic Escherichia coli (APEC) is a specific group of extraintestinal pathogenic E. coli that causes a variety of extraintestinal diseases in chickens, ducks, pigeons, turkeys, and other avian species. These diseases lead to significant economic losses in the poultry industry worldwide. However, owing to excessive use of antibiotics in the treatment of infectious diseases, bacteria have developed antibiotic resistance. The development of multidrug efflux pumps is one important bacterial antibiotic resistance mechanism. A multidrug efflux pump, MdtH, which belongs to the major facilitator superfamily of transporters, confers resistance to quinolone antibiotics such as norfloxacin and enoxacin. LsrR regulates hundreds of genes that participate in myriad biological processes, including mobility, biofilm formation, and antibiotic susceptibility. However, whether LsrR regulates mdtH transcription and then affects bacterial resistance to various antibiotics in APEC has not been reported. In the present study, the lsrR mutant was constructed from its parent strain APECX40 (WT), and high-throughput sequencing was performed to analyze the transcriptional profile of the WT and mutant XY10 strains. The results showed that lsrR gene deletion upregulated the mdtH transcript level. Furthermore, we also constructed the lsrR- and mdtH-overexpressing strains and performed antimicrobial susceptibility testing, antibacterial activity assays, real-time reverse transcription PCR, and electrophoretic mobility shift assays to investigate the molecular regulatory mechanism of LsrR on the MdtH multidrug efflux pump. The lsrR mutation and the mdtH-overexpressing strain decreased cell susceptibility to norfloxacin, ofloxacin, ciprofloxacin, and tetracycline by upregulating mdtH transcript levels. In addition, the lsrR-overexpressing strain increased cell susceptibility to norfloxacin, ofloxacin, ciprofloxacin, and tetracycline by downregulating mdtH transcript levels. Electrophoretic mobility shift assays indicated that LsrR directly binds to the mdtH promoter. Therefore, this study is the first to demonstrate that LsrR inhibits mdtH transcription by directly binding to its promoter region. This action subsequently increases susceptibility to the aforementioned four antibiotics in APECX40.

Key words: avian pathogenic Escherichia coli, multidrug efflux pump, MdtH, LsrR, the antibiotic susceptibility

INTRODUCTION

Avian colibacillosis is considered one of the leading causes of economic loss in the poultry industry worldwide because of high morbidity and mortality (Dho-Moulin and Fairbrother, 1999; Holden et al., 2012; Davide et al., 2013; Li et al., 2016). It is caused by avian pathogenic Escherichia coli (APEC), a specific group of extraintestinal pathogenic E. coli (Holden et al., 2012; Li et al., 2016). Avian pathogenic E. coli strains cause airsacculitis, pericarditis, perihepatitis, polyserositis, septicemia, and other mainly extraintestinal diseases in chickens, ducks, pigeons, turkeys, and other avian species (Ewers et al., 2004; Davide et al., 2013; Yu et al., 2018a). The most common form of colibacillosis is characterized as an initial respiratory tract infection (airsacculitis), which is usually followed by a generalized infection with characteristic fibrinous lesions (perihepatitis and pericarditis) and fatal septicemia (Melha et al., 2003; Herren et al., 2006; Davide et al., 2013). It has necessitated the use of antimicrobial therapy to prevent and control APEC infection outbreaks (Watts et al., 1993; Aggad et al., 2010; Yu et al., 2018a). However, owing to inappropriate use and misuse of antimicrobial agents in APEC infections, the emergence and dissemination of antibiotic-resistant strains
resistance bacteria has become a critical issue (Davide et al., 2013; Niesch-Inderbinen and Stephan, 2016; Yu et al., 2018c). One important mechanism that generates drug or multidrug resistance in bacteria is the expression of multidrug efflux pumps that expel antimicrobial agents from the cytoplasm into the periplasmic space (Nikaido 1996; Su et al., 2007; Hong-Suk et al., 2010).

Bacterial multidrug efflux pumps are currently classified into 5 families on the basis of AA sequence similarities, predicted secondary protein structures, and phylogenetic relationships. The families include ATP-binding cassette (ABC), major facilitator superfamily (MFS), multidrug and toxic compound extrusion, small multidrug resistance, and resistance nodulation cell division (Nikaido, 1996; Putman et al., 2000; Nishino and Yamaguchi, 2001; Kumar et al., 2013; Zhang et al., 2018). The E. coli K-12 genome encodes at least 20 drug efflux pump genes (11 MFS, 2 small multidrug resistance, 6 resistance nodulation cell division, and 1 ABC) that confer drug resistance when they are overexpressed (Nishino and Yamaguchi, 2001; Hirakawa et al., 2005; Kobayashi et al., 2006; Nishino & Zhang et al., 2011). These drug efflux pumps genes are slr, mdfA (formerly cmr), mdtG (formerly yceE), mdtH (formerly yceL), bcr, emrKY, emrAB, emrD, mdtL (formerly yidY), mdtM (formerly yijO), mdtK (formerly ydbE), acrAB, cusA (formerly ybdE), mdtABC (formerly yegMNO), acrD (an acrB homolog), acrEF (formerly envCD), mdtEF (formerly yhiUV), emrE, mdtJI (formerly ydgFE), and macAB (formerly ybjYZ) (Nishino and Yamaguchi, 2001; Hirakawa et al., 2005). Among these drug efflux pumps, MdtH, which belongs to the MFS family, increases E. coli resistance to quinolone antibiotics, including norfloxacin (2-fold) and enoxacin (2-fold) (Nishino and Yamaguchi, 2001). Moreover, MdtH overexpression increases the E. coli tolerance to short- and medium-carbon-chain alcohols (an advanced biofuel) (Zhang et al., 2018). However, whether the MdtH multidrug efflux pump affects APEC tolerance to various antibiotics has not been reported.

Quorum sensing (QS) is a process in which bacteria use chemical molecules as a signaling language to change their behaviors to adapt to specific environments (Bassler 1999, 2002; Miller and Bassler, 2001). Many studies have reported that QS is a regulator of cellular processes, including bioluminescence production, cell division, carbohydrate metabolism, virulence gene expression, and antibiotic responsiveness (Bassler et al., 1997; Lyon et al., 2001; Roderick et al., 2003; Ahmed et al., 2007). Autoinducer 2 (AI-2), a signal molecule, is produced by both gram-negative and gram-positive bacteria to mediate both intraspecies and interspecies communication; it is synthesized by the highly conserved S-ribosylhomocysteine lyase (LuxS) (Schauder et al., 2001; Xue et al., 2009). S-ribosylhomocysteine lyase converts S-ribosylhomocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione, which cyclizes spontaneously and then undergoes further rearrangements to form AI-2 signals (Esteban et al., 2006; Xue et al., 2009). In E. coli, extracellular AI-2 accumulates in the midexponential phase but decreases drastically on entry into the stationary phase (Li et al., 2007; Xue et al., 2009; Yu et al., 2018b). The rapid disappearance of extracellular AI-2 is because of its import by an ABC transporter, the luxS-regulated (lsr) transporter (Wang et al., 2005a; Xavier and Bassler, 2005; Xavier et al., 2007). The transporter proteins are part of the lsr operon, which is composed of 6 genes (lsrACDBFG).

The first 4 genes, lsrACDB, encode the ABC transporter for AI-2 internalization, and the remaining 2 genes, lsrFG, are required for AI-2 modification after uptake into cells (Xavier and Bassler, 2005; Xavier et al., 2007; Xue et al., 2009). The regulatory network for AI-2 uptake comprises 2 genes, lsrR and lsrK, located immediately upstream of the lsr operon and divergently transcribed in their own lsrRK operon (Wang et al., 2005b; Li et al., 2007; Xue et al., 2009). LsrR represses the expression of the lsr operon and its own lsrRK operon by binding to the promoters; the DNA sequences recognized by LsrR are known (Xue et al., 2009). LsrK is a cytoplasmic kinase responsible for phosphorylating AI-2 into an activated molecule (phospho-AI-2), which is required for releasing LsrR repression and then activating lsr operon expression (Li et al., 2007; Xue et al., 2009). In addition to regulating lsr operon expression, LsrR regulates hundreds of other genes that participate in myriad biological processes, including mobility, biofilm formation, and antibiotic susceptibility (Parra-Lopez et al., 1993; Li et al., 2007). Our previous study confirmed that exogenous AI-2 increases the β-lactam antibiotic resistance of a clinical E. coli strain isolated from a dairy cow with mastitis. This mechanism involves upregulating the expression of a TEM-type enzyme in an LsrR-dependent manner (Xue et al., 2016). However, whether LsrR regulates the expression of the MdtH multidrug efflux pump and then affects bacterial resistance to various antibiotics in APEC has not been reported.

In this study, we constructed an isogenic lsrR-deficient mutant using the λ Red recombinase system, as described previously (Datsenko and Wanner, 2000) and lsrR- and mdtH-overexpressing strain using the pUC19 vector. The antibiotic susceptibility of the mutant and overexpressing strains to quinolone antibiotics and tetracycline was tested using antibiotic susceptibility testing and CFU assays. Besides, real-time reverse transcription PCR (RT-qPCR) and electrophoretic mobility shift assays (EMSA) were performed to further investigate the regulatory mechanism of LsrR on the MdtH multidrug efflux pump in APECX40. While LsrR is a crucial global regulator of the AI-2 QS system, this study might provide a potential drug target for the prevention and treatment of APEC infections by inhibiting cell–cell communication.
MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* cultures were routinely grown at 37°C in Luria–Bertani (LB) broth (Oxoid, Basingstoke, UK) or on LB solid medium with 2.0% agar (Oxoid, Basingstoke, UK) 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 monitored by measuring the turbidity at 600 nm by using a UV/Vis spectrophotometer (DU730; Beckman Coulter, Miami, FL). The appropriate antibiotics for plasmid selection and maintenance were used at the following final concentrations: 16 μg/mL chloramphenicol (Cm), 50 μg/mL kanamycin, and 100 μg/mL ampicillin (Amp).

General DNA Manipulation

Genomic DNA from *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), as per the manufacturer’s instructions. PCR amplification was performed using Taq or Pfu DNA polymerases (Transgen, Beijing, China). PCR product and DNA fragment purification was performed using a gel purification kit (Promega, Madison, WI), as per the manufacturer’s instructions. DNA restriction enzyme (TaKaRa, Dalian, Liaoning, China) digestion, and T4 DNA ligase (TaKaRa, Dalian, Liaoning, China) ligation were performed using standard methods. Sequence analyses were performed using Vector NTI Advance 11 software to predict conserved *lsrR* domains and to design the primers. Primer nucleotide sequences are listed in Table 2.

### 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 ΔlacU169Δ(lacZΔM15) bsdR17 recA1 endA1 gprA96 thi-1 relA1 | Invitrogen |
| BL21              | Expression strain, F’ ompT hsdS(rB2 mB2) gal dcm(DE3) | Invitrogen |
| WT                | Avian pathogenic *E. coli* (APEC) 40, wild-type |
| XY10              | APECX40 *lsrR*-deletion mutant |
| WT/pSTV28         | WT with the empty vector pSTV28, Cm’ |
| XY10/pClsR        | XY10 with the empty vector pSTV28, Cm’ |
| WT/pUC19          | WT with the empty vector pUC19, Amp’ |
| WT/pUCmdtH        | WT with the overexpression plasmid pUCmdtH, Amp’ |
| WT/pUClsR         | WT with the overexpression plasmid pUClsR, Amp’ |
| **Plasmids** |
| pKD46             | Expresses λ Red recombinase Exo, Bet and Gam, temperature sensitive, Amp’ |
| pKD3              | cat gene, template plasmid, Amp’ Cm’ |
| pCP20             | FLP’ λcl857’, λpRep(Ts), temperature sensitive, Amp’ Cm’ |
| pSTV28            | Low copy number cloning vector, Cm’ |
| pClsR             | pSTV28 with *lsrR* gene, Cm’ |
| pUC19             | Cloning vector, Amp’ |
| pUClsR            | pUC19 with *lsrR* gene, Amp’ |
| pUCmdtH           | pUC19 with *mdtH* gene, Amp’ |
| pET28a(+)         | Expression vector, Kan’ |
| pET-IsrR          | pET28a(+) with *lsrR* gene, Kan’ |

1Abbreviations: Amp’, ampicillin-resistant; Cm’, chloramphenicol-resistant; Kan’, kanamycin-resistant.

Construction of the *lsrR* Gene Mutant

The isogenic *lsrR*-deficient mutant was constructed using homologous recombination methods based on the λ Red recombinase system (Datsenko and Wanner, 2000). The Cm-resistance cassette flanked by 40 bp homology arms located upstream and downstream of the *lsrR* gene was PCR amplified from pKD3 using primers APECO2-*lsrR*-f and APECO2-*lsrR*-r. Subsequently, PCR products were gel purified and suspended in sterilized distilled deionized water. The purified PCR products were transformed into the competent cells of WT strain that carried plasmid pKD46. The mutant was screened and confirmed by PCR amplification and DNA sequencing using primers check-*lsrR*-f and check-*lsrR*-r. The *cat* was cured by transforming plasmid pCP20 and selecting the sensitive strain, which was designated as XY10.
Complementation of the lsrR Mutant

For functional complementation of the lsrR mutant strain, the lsrR open reading frame (ORF) was amplified from WT strain genomic DNA using primers lsrR-EcoRI-f and lsrR-KpnI-r. The fragment was gel purified and cloned into the EcoRI and KpnI sites of low-copy-number plasmid pSTV28 (TaKaRa, Dalian, Liaoning, China) to use the lacZ promoter in the plasmid to drive lsrR expression, and then transformed into E. coli DH5α chemically competent cells. The transformed cells were spread on LB agar with 16 mg/mL Cm. Positive colonies were selected and confirmed by PCR using primers M13-f and M13-r, and the recombinant plasmid pSTV28-lsrR was extracted and further confirmed by DNA sequencing (data not shown). The purified recombinant plasmid pSTV28-lsrR and control vector pSTV28 were transformed into mutant strain XY10 and its parent WT strain to generate strains XY10/pClsrR, XY10/pSTV28, and WT/pSTV28, respectively. The growth rates of each strain were determined by detecting the cell density (600 nm) at each time point.

Construction of the lsrR- and mdtH-Overexpressing Strains

The lsrR- and mdtH-overexpressing strains were constructed in accordance with the followed methods. Briefly, the lsrR and mdtH ORF were amplified by PCR using primers lsrR-KpnI-f and lsrR-EcoRI-r and mdtH-KpnI-f and mdtH-EcoRI-r, respectively, from WT chromosomal DNA. The fragment was gel purified and cloned into the KpnI and EcoRI sites of pUC19 (TaKaRa, Dalian, Liaoning, China) to use the lacZ promoter in the plasmid to drive lsrR and mdtH expression, and then transformed into E. coli DH5α chemically competent cells. The transformed cells were then spread on LB agar with 100 μg/mL Amp. Positive colonies were selected and confirmed

| Oligonucleotide primers used in this study. |
|---------------------------------------------|
| Primer name | Oligonucleotide (5'-3') |
|------------|------------------------|
| lsrR-f     | ATGACAATCAACGATTCG     |
| lsrR-r     | TTAACTACGTAACATGCC     |
| APECO2-1srR-f | ATCGTCTCGGCCTGACCCGTTTGAGTTCTGGAGCTGCTT |
| APECO2-1srR-r | GTAAATTCACGCTGCACTCCGCGTTAAGCTGCCCGATACCTGAATATCCTCCTTAGTTC |
| CHECK-lsrR-f | GCGGAAAGACTGAAACATTTGG |
| CHECK-lsrR-r | CTTATTTGATTGCCTTCCAGG |
| CM-f | TGTAGAGCTGGACGCTGTT |
| CM-r | CATGAAATATGCTCTGTTAGT |
| lsrR-KpnI-f | CCGGAACTCATTGAAACTCCGCAAGTTGGGTACCTAGTCGTGCGCGATTACTTGTAGGCTGGAGCTGCTT |
| lsrR-KpnI-r | GGGGTACCCTAACTAGTAAATACTGCGG |
| lsrR-EcoRI-f | CCGGAACTCATTGAAACTCCGCAAGTTGGGTACCTAGTCGTGCGCGATTACTTGTAGGCTGGAGCTGCTT |
| lsrR-EcoRI-r | GGGGTACCCTAACTAGTAAATACTGCGG |
| mdtH-KpnI-f | CCGGAACTCATTGAAACTCCGCAAGTTGGGTACCTAGTCGTGCGCGATTACTTGTAGGCTGGAGCTGCTT |
| mdtH-KpnI-r | GGGGTACCCTAACTAGTAAATACTGCGG |
| mdtH-EcoRI-f | CCGGAACTCATTGAAACTCCGCAAGTTGGGTACCTAGTCGTGCGCGATTACTTGTAGGCTGGAGCTGCTT |
| mdtH-EcoRI-r | GGGGTACCCTAACTAGTAAATACTGCGG |
| lsrR-NcoI-f | CATGCCATGGACAATCAACGATTCGGT |
| lsrR-XhoI-r | CCGCTGAGACTCGTAAATACTGCGG |
| M13-f | TGTAAACCGACGCGCAGT |
| M13-r | CAGGAAACACCTATAGCC |
| T7-f | TAATACGACTCATATAGGG |
| T7-r | TGCTAGTTATGCTACGCGG |
| rt-16s-f | TTTGAGTTCCCGGCCCAAG |
| rt-16s-r | CGCCGCCAAGAGTAA |
| rt-mdtH-f | GCGGGAACCTGGGTAAAG |
| rt-mdtH-r | CGGGCCAAAATACCCAGA |
| p-yfgF-biotin-f | CATGACTACGCGAGATCC |
| p-yfgF-r | TATGAGCTGGAGCTGCTT |
| p-mdtH-biotin-f | TCCGCTCCTCCGGGAAATAAA |
| p-mdtH-r | TCTATACCTACCTGCTCGG |

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

Table 3. Differentially expressed genes whose mRNA levels (log2FC) displayed >0.6-fold decreases in the lsrR-deficient mutant XY10 compared with the WT strain.

| Gene            | Product                                       | log2FC |
|-----------------|-----------------------------------------------|-------|
| APECO2_RS03445  | prpB                                          | -1.30 |
| APECO2_RS16285  | ygoA                                          | -1.25 |
| APECO2_RS03310  | -                                              | -1.57 |
| APECO2_RS03450  | prpCz                                         | -1.03 |
| APECO2_RS17500  | xanQ                                          | -1.60 |
| APECO2_RS07530  | flagH                                         | -2.25 |
| APECO2_RS07010  | highC                                         | -0.99 |
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Table 4. Differentially expressed genes whose mRNA levels (log2FC) displayed >0.6-fold increases in the lsrR-deficient mutant XY10 compared with the WT strain.

| Gene     | Product                                                                 | log2FC |
|----------|--------------------------------------------------------------------------|--------|
| APECO2_RS10070 | lsrA  Autoinducer 2 ABC transporter ATP-binding protein LsrA | 5.36   |
| APECO2_RS10075 | lsrC  Autoinducer 2 ABC transporter permease LsrC | 5.74   |
| APECO2_RS10090 | lsrF  3-hydroxy-5-phosphonooxypentane-2,4-dione thiolase LsrF | 4.61   |
| APECO2_RS10085 | lsrR  MULTISPECIES: autoinducer 2 ABC transporter substrate-binding protein | 4.30   |
| APECO2_RS10095 | lsrG  MULTISPECIES: autoinducer 2-degrading protein LsrG | 4.50   |
| APECO2_RS10080 | lsrD  MULTISPECIES: autoinducer 2 import system permease LsrD | 4.97   |
| APECO2_RS10060 | lsrK  Autoinducer-2 kinase | 3.09   |
| APECO2_RS10065 | lsrR  Transcriptional regulator | 2.07   |
| APECO2_RS09645 | ydcH  MULTISPECIES: hypothetical protein | 1.10   |
| APECO2_RS10525 | yofA  MULTISPECIES: hypothetical protein | 3.10   |
| APECO2_RS17345 | ygdR  MULTISPECIES: YgdI/YgdR family lipoprotein | 1.03   |
| APECO2_RS088740 | ykiA  MULTISPECIES: hypothetical protein | 1.68   |
| APECO2_RS25335 | famB  Fumarate hydratase | 2.81   |
| APECO2_RS26595 | -  Hypothetical protein | 1.65   |
| APECO2_RS17745 | cmtA  PTS mannitol transporter subunit IIIBC | 3.76   |
| APECO2_RS07460 | mdtH  MFS transporter, response to antibiotic | 1.14   |
| APECO2_RS19525 | yghI  MULTISPECIES: tRNA-binding protein | 2.00   |
| APECO2_RS06250 | ybiW  MULTISPECIES: formate C-acetyltransferase/glycerol dehydratase family glycol radical enzyme | 1.06   |
| APECO2_RS15950 | yflL  MULTISPECIES: ferredoxin | 1.07   |
| APECO2_RS25660 | ybiA  MULTISPECIES: hypothetical protein | 1.01   |
| APECO2_RS19370 | -  MULTISPECIES: hypothetical protein | 1.13   |
| APECO2_RS11595 | yeaQ  MULTISPECIES: GlsB/YeaQ/YmgE family stress response | 0.95   |
| APECO2_RS03705 | gaiA  MULTISPECIES: hypothetical protein | 0.93   |
| APECO2_RS03835 | yqiE  MULTISPECIES: GlsB/YeaQ/YmgE family stress response | 0.95   |
| APECO2_RS19155 | ybdS  MULTISPECIES: glycogen synthase | 0.85   |
| APECO2_RS16460 | cvrA  MULTISPECIES: carbon storage regulator | 0.81   |
| APECO2_RS20295 | yheO  MULTISPECIES: barnase inhibitor | 0.89   |
| APECO2_RS04755 | yceI  MULTISPECIES: hypothetical protein | 0.72   |
| APECO2_RS091755 | expE  MULTISPECIES: cold-shock protein CspE | 0.72   |
| APECO2_RS11830 | yebV  MULTISPECIES: hypothetical protein | 0.70   |
| APECO2_RS12035 | cutC  MULTISPECIES: copper homeostasis protein CutC | 0.78   |
| APECO2_RS09925 | rpsV  MULTISPECIES: stationary-phase-induced ribosome-associated protein | 0.71   |
| APECO2_RS08655 | yeiG  MULTISPECIES: hypothetical protein | 0.70   |
| APECO2_RS15075 | yidY  MULTISPECIES: hypothetical protein | 0.68   |
| APECO2_RS15160 | yecJ  MULTISPECIES: hypothetical protein | 0.70   |
| APECO2_RS08735 | yciN  MULTISPECIES: hypothetical protein | 0.70   |
| APECO2_RS11755 | yofF  MULTISPECIES: DUF5217 domain-containing protein | 0.69   |
| APECO2_RS24175 | tusB  Sulfurtransferase TusB | 0.78   |
| APECO2_RS08985 | yccX  MULTISPECIES: acylphosphatase | 0.75   |
| APECO2_RS24945 | ygbJ  MULTISPECIES: CsbD family protein | 0.64   |
| APECO2_RS24590 | ygbE  Hypothetical protein | 0.78   |
| APECO2_RS03940 | bolA  MULTISPECIES: protein BolA | 0.62   |
| APECO2_RS11270 | ydiZ  MULTISPECIES: hypothetical protein | 0.61   |
| APECO2_RS11775 | yobH  MULTISPECIES: hypothetical protein | 0.98   |
| APECO2_RS02455 | yaeP  MULTISPECIES: hypothetical protein | 0.75   |
| APECO2_RS04770 | tatA  MULTISPECIES: twin-arginine translocase subunit TatA | 0.61   |
| APECO2_RS08085 | ydhH  MULTISPECIES: HxIR family transcriptional regulator | 0.87   |
| APECO2_RS10875 | ygbL  MULTISPECIES: DUF1289 domain-containing protein | 0.73   |
| APECO2_RS06140 | ybiL  MULTISPECIES: DksA/TraR family C4-type zinc finger protein | 0.61   |
| APECO2_RS17580 | cptB  MULTISPECIES: antitoxin CptB | 0.64   |
| APECO2_RS11915 | edd  MULTISPECIES: phosphogluconate dehydratase | 0.67   |

by PCR using primers M13-f and M13-r, and the recombinant plasmid pUC19-lsrR and pUC19-mdtH were extracted and further confirmed by DNA sequencing (data not shown). The purified recombinant plasmid pUC19-lsrR and pUC19-mdtH and the control vector pUC19 were transformed into the WT parent strain to generate strains WT/pUClsrR, WT/pUCmdtH, and WT/pUC19, respectively. The growth rates of each strain were determined by detecting the cell density (600 nm) at each time point.

RNA Sequencing, Library Generation, and Bioinformatics Analysis

RNA sequencing and library construction were performed at the Shanghai Biotechnology Corporation, Shanghai, China. High-quality reads for sequence analysis and bioinformatic data analysis were also supplied by the Shanghai Biotechnology Corporation. The detailed methods and processes were delineated in the Supplementary Materials section.
Table 5. Susceptibility of Escherichia coli strains to various antibiotics.

| Strains            | CIP    | Nor    | Of     | TET    |
|--------------------|--------|--------|--------|--------|
| WT/pSTV28          | 2      | 2      | 2      | 128    |
| XY10/pSTV28        | 2      | 2      | 2      | 256    |
| XY10/pClsrR        | 2      | 2      | 2      | 128    |
| WT/pUC19           | 4      | 4      | 4      | 256    |
| WT/pUClsrR         | 4      | 4      | 4      | 256    |
| WT/pUCmdtH         | 4      | 4      | 4      | 256    |

Abbreviations: CIP, ciprofloxacin; MIC, minimal inhibitory concentration; NOR, norfloxacin; OF, ofloxacin; TET, tetracycline.

Antibiotic Susceptibility Testing

Broth-dilution antibiotic susceptibility tests were performed to examine the changes in antibiotic susceptibility of the lsrR mutant XY10/pSTV28, the complementation strain XY10/pClsrR, and their parent strain WT/pSTV28, or the overexpression strains WT/pUClsrR and WT/pUCmdtH and their parent strain WT/pUC19. The assays followed Clinical and Laboratory Standards Institute standards and used Mueller–Hinton broth with modifications in the concentrations of the toxic compounds (shown in the following) from those recommended. Experiments were repeated 3 times. The overnight cultures of WT/pSTV28, XY10/pSTV28, and XY10/pClsrR or WT/pUC19, WT/pUClsrR, and WT/pUCmdtH were diluted to an OD600 of approximately 0.03 in fresh LB broth with 16 μg/mL Cm or 100 μg/mL Amp, respectively (Wang et al., 2014). 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. Subsequently, total RNA was extracted from the cells using TRIzol reagent (Transgen). Reverse transcription was performed using the EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (Transgen), in accordance with the manufacturer’s instructions. Reverse transcription 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). Differences in gene expression were calculated using the ∆∆Ct (where Ct = cycle threshold) method, 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-qPCR assays were repeated at least 3 times with similar results, and the PCR amplification efficiency was between 1.93 and 2.09.

Purification of the LsrR Protein

The His6-tagged LsrR was cloned and purified using standard procedures (Wang et al., 2015). Briefly, the lsrR ORF was amplified by PCR with primers lsrR-NcoI-f and lsrR-XhoI-r from WT genomic DNA. It was cloned into expression vector pET28a (+) (Novagen) and then transformed into E. coli DH5α chemically competent cells, which were then spread on LB agar with 50 μg/mL kanamycin. Positive colonies were selected and confirmed by PCR using primers T7-f and T7-r, and the recombinant plasmid pET-lsrR was extracted and further confirmed by DNA sequencing (data not shown). Subsequently, E. coli BL21 (DE3) was transformed with the purified complement plasmid...
pET-lsrR. The transformant was grown in 200 mL LB medium at 37°C to an OD_{600} of approximately 0.3, transferred to 16°C, and induced overnight with a final concentration of 0.6 μg/mL isopropyl-β-D-1-thiogalactopyranoside. Cells were collected by centrifugation and washed 2 times with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_{2}HPO_{4}, 2 mM KH_{2}PO_{4}, pH 7.4). The cells were resuspended in 35 mL lysis buffer (20 mmol Tris, 1 M NaCl, pH 8.0), disrupted for 50 min by sonication, and centrifuged at 5,000 rpm for 30 min at 4°C. The supernatant was loaded onto a column with 2 mL nickel-nitrilotriacetic acid agarose solution (Transgen) to bind for 1 h at 4°C. Subsequently, the column was washed with 250 mL washing buffer I (5 mmol imidazole, 20 mmol Tris, 1 mol NaCl, pH 8.0), 100 mL washing buffer II (20 mmol imidazole, 20 mmol Tris, 1 mol NaCl, pH 8.0), and 50 mL washing buffer III (100 mmol imidazole, 20 mmol Tris, 1 mol NaCl, pH 8.0). Bound protein was eluted with 3 mL elution buffer (250 mmol imidazole, 20 mmol Tris, 1 mol NaCl, pH 8.0) and dialyzed with 300 mL dialysis buffer (20 mmol Tris, 1 mol NaCl, pH 8.0). The LsrR 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 the standard.

**Electrophoretic Mobility Shift Assays**

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

**Statistical Analysis**

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

**RESULTS**

**lsrR Deletion Did Not Affect XY10 Growth**

The lsrR mutant strain XY10 was generated by λ Red-mediated recombination. Complementation of the lsrR mutant was accomplished by expressing the lsrR ORF in the pSTV28 vector. To ensure that the growth conditions for the mutant XY10 strain and its parent WT strain were consistent with the complementation strain, WT and XY10 were transformed with the empty vector pSTV28. The colony morphology of XY10/pSTV28 and XY10/pClsrR on the LB agar plates with 16 μg/mL Cm were similar to those of WT/pSTV28: circular, convex, moist, smooth, and 1 to 2 mm in diameter (data not shown). Furthermore, the XY10/pSTV28 and XY10/pClsrR growth curves in LB broth with 16 μg/mL Cm were similar to that of WT/pSTV28 (Supplementary Figure 1).

**lsrR and mdtH Overexpression Did Not Affect Strain Growth**

The lsrR- and mdtH-overexpressing strains were generated by expressing the lsrR or mdtH ORF in pUC19. To ensure that the growth conditions of the parent WT strain was consistent with the overexpression of WT/pUClsrR and WT/pUCmdtH strains, the WT strain was transformed with the empty vector pUC19. The colony morphology of WT/pUClsrR and WT/pUCmdtH on the LB agar plates with 100 μg/mL Amp were similar to those of WT/pUC19: circular, convex, moist, smooth, and 1–2 mm in diameter (data not shown). Furthermore, the WT/pUClsrR and WT/pUCmdtH growth curves in LB broth with 100 μg/mL Amp were similar to that of WT/pUC19 (Supplementary Figure 2).

**Transcriptional Profile of the lsrR-Deficient Mutant Strain**

To investigate the molecular regulatory mechanism of lsrR, high-throughput sequencing was performed to analyze the transcriptional profile (RNA-seq) of WT and XY10 using total RNA from the exponential growth stage. The transcript levels of 3,872 genes were quantified for WT and the lsrR-deficient mutant strain XY10. Differentially expressed genes (DEGs) were identified to search for functional genetic differences between WT and XY10. A total of 59 gene transcripts were identified as DEGs between WT and XY10; of which, 52 genes were upregulated and 7 genes were downregulated ($\log_{2}$ [fold-change] $> 0.6, P < 0.05$) in XY10, compared with WT (Table 3 and 4). In addition, to describe gene functions and products relevant to DEGs between WT and XY10, all transcripts were functionally characterized into Gene Ontology (GO) categories, namely biological processes, cellular components, and molecular functions. As shown in Supplementary Figure 3A, GO enrichment analysis identified a total of 87 terms, including 30 related to biological processes, 42 for cellular components, and 15 for molecular functions (XY10 vs. WT). In the XY10 strain, the top 30 genes were highly enriched in classifications related to plasma membrane, membrane, integral component of
membrane, cytoplasm, transport, and metabolic process when compared with WT (Supplementary Figure 3B). The GO enrichment classification may provide new clues for investigating the regulatory mechanism of LsrR in the bacterial physiological process.

To further explore the DEG pathway enrichments, a KEGG analysis was performed to examine the biological systems and their relationships at the molecular, cellular, and organismal levels. Comparison of XY10 and WT strains revealed that the genes were associated with cellular processes, environmental information processing, and metabolism (Supplementary Figure 4A). The top 30 enriched genes were involved in 16 KEGG pathways, including phosphotransferase system, microbial metabolism in diverse environments, flagellar assembly, biosynthesis of secondary metabolism, biofilm formation, and ABC transports, among others (Supplementary Figure 4B). In general, the KEGG pathways were similar to the GO terms for XY10 and WT and provided further information for selecting candidates that may be regulators of bacterial adaptability to diverse environment.

**lsrR Gene Deletion Decreased Antibiotic Susceptibility**

The MIC of 4 antibiotics were determined and interpreted in accordance with Clinical and Laboratory Standards Institute standards. The broth-dilution MIC results for the *lsrR* mutant XY10/pSTV28, the complementation strain XY10/pClsrR, and their parent strain WT/pSTV28 are shown in Table 5. The tetracycline MIC increased 2 times in the mutant XY10/pSTV28 strain, but the MICs of other 3 antibiotics were unchanged, when compared with that of WT/pSTV28. Furthermore, the tetracycline MIC of the complement strain was restored. To further determine whether *lsrR* affects antibiotic susceptibility in APECX40, the survival rates of the *lsrR*-deficient mutant strain XY10/pSTV28 with that of the WT strain WT/pSTV28 and the complement strain XY10/pClsrR were confirmed using CFU assays. Specifically, cultures were grown in LB broth with 16 μg/mL Cm and then challenged with a final concentration of 2 μg/mL norfloxacin, 2 μg/mL ofloxacin, 2 μg/mL ciprofloxacin, or 64 μg/mL tetracycline. As shown in Figure 1,
in the presence of norfloxacin, ofloxacin, ciprofloxacin, or tetracycline, the survival rates of XY10/pSTV28 were increased approximately 2.36-fold, 2.12-fold, 2.07-fold and 2.59-fold ($P < 0.01$), respectively, when compared with WT/pSTV28. Furthermore, the survival rates were restored in XY10/pClsrR ($P < 0.01$). These data indicated that $lsrR$ gene deletion significantly decreases susceptibility to tetracycline and quinolones in APECX40.

### LsrR Overexpression Increased Antibiotic Susceptibility

The broth-dilution MIC results for the $lsrR$-overexpressing strain WT/pUClsrR and its parent strain WT/pUC19 are shown in Table 5. $lsrR$ gene overexpression did not alter the MIC of the 4 antibiotics compared with the parent strain WT/pUC19. To further determine the effect of $lsrR$ on antibiotic susceptibility in APECX40, the survival rates of the overexpression strain WT/pUClsrR and that of the WT strain WT/pUC19 were confirmed in CFU assays, when cultures grown in LB broth with 100 μg/mL Amp were challenged with a final concentration of 2 μg/mL norfloxacin, 2 μg/mL ofloxacin, 2 μg/mL ciprofloxacin, or 64 μg/mL tetracycline. As shown in Figure 2, in the presence of norfloxacin, ofloxacin, ciprofloxacin, or tetracycline, the WT/pUClsrR survival rates were decreased 6.26-fold, 7.56-fold, 6.45-fold and 5.07-fold ($P < 0.01$), respectively, when compared with those of WT/pUC19. These data indicated that $lsrR$ overexpression significantly increases susceptibility to tetracycline and quinolones in APECX40 and further suggest that LsrR plays an important role in antibiotic susceptibility.

### mdtH Gene Overexpression Decreased Antibiotic Susceptibility

The broth-dilution MIC results for the $mdtH$-overexpressing strain WT/pUCmdtH and its parent strain WT/pUC19 are shown in Table 5. $mdtH$ overexpression did not change the MIC of the 4 antibiotics compared with the parent strain WT/pUC19. To further determine the effect of $mdtH$ on antibiotic susceptibility in APECX40, the survival rates of the overexpression

**Figure 2.** CFU assays of the $lsrR$-overexpressed strain WT/pUClsrR, and its parent strain WT/pUC19 in the presence of 4 antibiotics. Data are shown for (A) ciprofloxacin, (B) norfloxacin, (C) ofloxacin, and (D) tetracycline. The survival rate of WT/pUC19 was designated as 100%. The WT/pUClsrR colony counts were compared with those of WT/pUC19. Error bars indicate SD; **$P < 0.01$ and indicates a significant difference between WT/pUC19 and WT/pUClsrR.**
strain WT/pUCmdtH and that of the wild-type strain WT/pUC19 were confirmed in CFU assays, when cultures grown in LB broth with 100 μg/mL Amp were challenged with a final concentration of 2 μg/mL norfl oxacin, 2 μg/mL ofloxacin, 2 μg/mL ciprofloxacin, or 64 μg/mL tetracycline. As shown in Figure 3, in the presence of norfl oxacin, ofloxacin, ciprofloxacin, or tetracycline, the WT/pUCmdtH survival rates were increased 2.23-fold, 2.02-fold, 2.38-fold and 3.53-fold (P, 0.01), respectively, when compared with those of WT/pUC19. These data indicated that overexpression of the mdtH gene significantly decreases susceptibility to tetracycline and quinolones in APECX40.

The Regulatory Effect of lsrR on the Multidrug Efflux Pump–Encoding Gene mdtH

To investigate how LsrR affects the susceptibility of norfl oxacin, ofloxacin, ciprofloxacin, and tetracycline in APECX40, the transcript levels of the multidrug efflux pump–encoding gene mdtH were examined. As shown in Figure 4A, the mdtH transcript level was increased 7.62 times (P < 0.01) in the lsrR-deficient strain XY10/pSTV28 when compared with that of its parent strain WT/pSTV28. The mdtH transcript level was restored in the complement strain XY10/pClsrR (P < 0.01). However, the mdtH transcript level in the lsrR-overexpressing strain WT/pUClsrR were decreased 10.73 times (P < 0.01), and the mdtH transcript level in the mdtH-overexpressing strain WT/pUCmdtH were increased 9.96 times (P < 0.01), when compared with that of their parent strain WT/pUC19 (Figures 4B, 4C). These results indicated that lsrR affects the susceptibility of APECX40 to norfl oxacin, ofloxacin, ciprofloxacin, and tetracycline by regulating mdtH.

Figure 3. CFU assays of the mdtH-overexpressing strain WT/pUCmdtH, and its parent strain WT/pUC19 in the presence of 4 antibiotics. Data are shown for (A) ciprofloxacin, (B) norfl oxacin, (C) ofloxacin, and (D) tetracycline. The survival rate of WT/pUC19 was designated as 100%. The WT/pUCmdtH colony counts were compared with those of WT/pUC19. Error bars indicate SD; **P < 0.01 and indicates a significant difference between WT/pUC19 and WT/pUCmdtH.

Binding of LsrR to the mdtH Promoter

To determine whether LsrR regulates mdtH transcription by directly binding to its promoter regions, we performed EMSA. The purified His6-tagged LsrR protein was used to bind biotin-labeled DNA amplification fragment with the putative mdtH promoter. As shown in Figure 5B, a clearly shifted band indicative of a protein–DNA complex was detected at 1, 2, 4, and 8 μmol LsrR concentrations. Furthermore, the shifted
band intensity was enhanced as the amount of LsrR increased; the shifted band disappeared in the presence of an approximately 10-fold excess unlabeled promoter DNA fragment as a specific competitor. These results confirmed that LsrR directly regulates mdtH transcription by binding to the mdtH promoter region.

**DISCUSSION**

The AI-2 QS system reportedly increases susceptibility to erythromycin and Amp, 2 groups of antibiotics with different antibacterial mechanisms, in *Streptococcus anginosus* by comparing the susceptible changes of wild type strain and its isogenic luxS mutant (deficient in AI-2 production) (Ahmed et al., 2007). A subsequent study indicated that the luxS mutant in *Staphylococcus aureus* results in decreased susceptibility to cell wall synthesis inhibitor antibiotics, including penicillin, oxacillin, vancomycin, and teicoplanin. The susceptibility was restored by genetic complementation or addition of AI-2 (Xue et al., 2013). Moreover, our previous study demonstrated that the AI-2 QS system increases resistance to β-lactam antibiotics, including Amp, oxacillin, and penicillin-G, in an extended-spectrum β-lactamase-positive E. coli that was isolated from a dairy cow with mastitis, by addition exogenous AI-2. This regulation relies on the function of the intracellular AI-2 receptor (LsrR) (Xue et al., 2016). Another study revealed that the addition of exogenous AI-2 increases trimethoprim-sulfamethoxazole susceptibility in APEC through downregulating the transcription of folate synthesis–associated genes *folA, folC, luxS, metE,* and *metH* by a feedback inhibition effect but not in the LsrR-dependent pathway (Yu et al., 2018a). These aforementioned studies support a role of AI-2 in adaptation to environmental challenges. However, whether the transcriptional regulator LsrR affects antibiotic susceptibility by regulating the expression of multidrug efflux pump systems in APEC has not been reported.

To further investigate the molecular regulatory mechanism of LsrR on antibiotic susceptibility, we constructed an isogenic *lsrR*-deficient mutant using the *λ* Red recombinase system in APECX40, which is a clinical *E. coli* strain isolated from a pigeon with airsacculitis. We performed RNA-seq and RT-qPCR experiments. These results showed that *lsrR* deletion significantly increased the transcript levels of *mdtH*, a gene that encodes the MdtH multidrug efflux pump. These data suggested that the transcriptional regulator LsrR affects antibiotic susceptibility by regulating the expression of multidrug efflux pump systems in APEC has not been reported.

Figure 4. Comparative measurement of transcription (complementary DNA [cDNA] abundance) of the multidrug efflux pump–encoding gene *mdtH* in the *lsrR*-deficient strain, the *lsrR*- and *mdtH*-overexpressing strains, and their parent strain. (A) The relative *mdtH* transcript level was determined by RT-qPCR in WT/pSTV28, XY10/pSTV28, and XY10/pClsrR bacteria cultured in LB broth with 16 μg/mL chloramphenicol. (B) The relative *mdtH* transcript level was determined by RT-qPCR in WT/pUC19 and WT/pUClsrR bacteria cultured in LB broth with 100 μg/mL ampicillin (Amp). (C) The relative *mdtH* transcript level was determined by RT-qPCR in WT/pUC19 and WT/pUCmdtH bacteria cultured in LB broth with 100 μg/mL Amp. Error bars indicate SD; **P < 0.01 and indicates a significant difference between WT/pSTV28, XY10/pSTV28, and XY10/pClsrR or WT/pUC19, WT/pUClsrR, and WT/pUCmdtH. Abbreviations: LB, Luria–Bertani; RT-qPCR, reverse transcriptase PCR.
and tetracycline, and deletion of the lsrR gene also decreased the susceptibility of APECX40 to norfloxacin, ofloxacin, ciprofloxacin, and tetracycline. Moreover, our results demonstrated that deletion of lsrR upregulates mdtH transcription. LsrR binds to the mdtH promoter, a finding that indicates LsrR inhibits the expression of the MdtH multidrug efflux pump by binding to the promoter region of mdtH. Therefore, this study is the first to demonstrate that the transcriptional regulator LsrR decreases the E. coli strain APECX40 susceptibility to norfloxacin, ofloxacin, ciprofloxacin, and tetracycline by inhibiting transcription of mdtH, which encodes the MdtH multidrug efflux pump.

**CONCLUSIONS**

This study is the first to demonstrate that LsrR inhibits transcription of the MdtH multidrug efflux pump by binding directly to its promoter region, and then decreases susceptibility to norfloxacin, ofloxacin, ciprofloxacin, and tetracycline in E. coli strain APECX40. Hence, this study provides a molecular basis for investigation on the effect of LsrR on bacterial resistance, and this study might provide a potential drug target for the prevention and treatment of APEC infections by inhibiting cell–cell communication.

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**SUPPLEMENTARY DATA**

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2020.03.064.

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