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

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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-

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resistance bacteria has become a critical issue (Davide et al., 2013; Nüesch-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 *fsr*, *mdfA* (formerly *cmr*), *mdtG* (formerly *yceE*), *mdtH* (formerly *yceL*), *bcr*, *emrKY*, *emrAB*, *emrD*, *mdtL* (formerly *yidY*), *mdtM* (formerly *yjiO*), *mdtK* (formerly *ydhE*), *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.

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.

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*(*ϕ*80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
BL21	Expression strain, F⁻ *ompT hsdS*(r_B^- m_B^-) *gal dcm* (DE3)	Invitrogen
WT	Avian pathogenic *E. coli* (APEC) 40, wild-type	Laboratory stock
XY10	APECX40 *lsrR*-deletion mutant	This study
WT/pSTV28	WT with the empty vector pSTV28, Cm^r[1]	This study
XY10/pSTV28	XY10 with the empty vector pSTV28, Cm^r	This study
XY10/pClsrR	XY10 with the complement plasmid pClsrR,Cm^r	This study
WT/pUC19	WT with the empty vector pUC19, Amp^r	This study
WT/pUCmdtH	WT with the overexpression plasmid pUCmdtH, Amp^r	This study
WT/pUClsrR	WT with the overexpression plasmid pUClsrR, Amp^r	This study
Plasmids		
pKD46	Expresses λ Red recombinase Exo, Bet and Gam, temperature sensitive, Amp^r	Reference 41
pKD3	*cat* gene, template plasmid, Amp^r Cm^r	Reference 41
pCP20	FLP^+ λ*c*I857$^+$ λp_RRep(Ts), temperature sensitive, Amp^r Cm^r	Reference 41
pSTV28	Low copy number cloning vector, Cm^r	TaKaRa
pClsrR	pSTV28 with *lsrR* gene, Cm^r	This study
pUC19	Cloning vector, Amp^r	Takara
pUClsrR	pUC19 with *lsrR* gene, Amp^r	This study
pUCmdtH	pUC19 with *mdtH* gene, Amp^r	This study
pET28a(+)	Expression vector, Kan^r	Novagen
pET-lsrR	pET28a(+) with *lsrR* gene, Kan^r	This study

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

Table 2. Oligonucleotide primers used in this study.

Primer name	Oligonucleotide (5′-3′)[1]
lsrR-f	ATGACAATCAACGATTCG
lsrR-r	TTAACTACGTAAAATCGC
APECO2-lsrR-f	ATCGTCTCGGCCTGACCCGTTTGAAAGTGTCGCGATTACTTGTAGGCTGGAGCTGCTT
APECO2-lsrR-r	TAATATTCACGCTGCACTCCGCGTTAAGCTGCCCGATACCTGAATATCCTCCTTAGTTC
CHECK-lsrR-f	GCGCAAGAACTGAACAATTG
CHECK-lsrR-r	CTATTTGATTGCCTTCCAGG
CM-f	TGTAGGCTGGAGCTGCTT
CM-r	CATATGAATATCCTCCTTAGTTC
lsrR-EcoRI-f	CGGAATTCATGACAATCAACGATTCGGT
lsrR-KpnI-r	GGGGTACCTTAACTACGTAAAATCGCCG
lsrR-KpnI-f	GGGGTACCATGACAATCAACGATTCGGT
lsrR-EcoRI-r	CGGAATTCTTAACTACGTAAAATCGCCG
mdtH-KpnI-f	GGGGTACCATGTCCCGCGTGTCGCAG
mdtH-EcoRI-r	CGGAATTCTCAGGCGTCGCGTTCAAG
lsrR-NcoI-f	CATGCCATGGACAATCAACGATTCGGT
lsrR-XhoI-r	CCGCTCGAGACTACGTAAAATCGCCG
M13-f	TGTAAAACGACGGCCAGT
M13-r	CAGGAAACAGCTATGACC
T7-f	TAATACGACTCACTATAGGG
T7-r	TGCTAGTTATTGCTCAGCGG
rt-16s-f	TTTGAGTTCCCGGCC
rt-16s-r	CGGCCGCAAGGTTAA
rt-mdtH-f	GCGAGGAACCTGGGTAAA
rt-mdtH-r	CCGCCGAAAATACCCAGA
p-yfgF-biotin-f	AGATGCTCAGCAGAATCC
p-yfgF-r	CATGATAAACGTAATAAT
p-mdtH-biotin-f	TTCCCCTCCCGGGAAATAAA
p-mdtH-r	TCTATACCTACTCCTTCCCG

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

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 *Eco*RI and *Kpn*I sites of the 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 μg/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 primers mdtH-KpnI-f and mdtH-EcoRI-r, respectively, from WT chromosomal DNA. The fragment was gel purified and cloned into the *Kpn*I and *Eco*RI 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

Table 3. Differentially expressed genes whose mRNA levels (log_2FC) displayed >0.6-fold decreases in the *lsrR*-deficient mutant XY10 compared with the WT strain.

Gene		Product	log_2FC
APECO2_RS03445	*prpB*	Methylisocitrate lyase	−1.30
APECO2_RS16285	*ygaF*	Hydroxyglutarate oxidase	−1.25
APECO2_RS03310	-	LuxR family transcriptional regulator	−1.57
APECO2_RS03450	*prpCz*	2-methylcitrate synthase	−1.03
APECO2_RS17500	*xanQ*	MULTISPECIES: xanthine permease XanQ	−1.60
APECO2_RS07530	*flgH*	MULTISPECIES: flagellar L-ring protein	−2.25
APECO2_RS07010	*hybC*	Hydrogenase 2 large subunit	−0.99

Table 4. Differentially expressed genes whose mRNA levels (log_2FC) displayed >0.6-fold increases in the *lsrR*-deficient mutant XY10 compared with the WT strain.

Gene		Product	log_2FC
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	*lsrB*	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	*ynfA*	MULTISPECIES: hypothetical protein	3.10
APECO2_RS17345	*ygdR*	MULTISPECIES: YgdI/YgdR family lipoprotein	1.03
APECO2_RS08740	-	hypothetical protein	1.40
APECO2_RS25980	*ykiA*	MULTISPECIES: hypothetical protein	1.68
APECO2_RS25335	*fumB*	Fumarate hydratase	2.81
APECO2_RS26595	-	Hypothetical protein	1.65
APECO2_RS17745	*cmtA*	PTS mannitol transporter subunit IIBC	3.76
APECO2_RS07460	*mdtH*	MFS transporter , response to antibiotic	1.14
APECO2_RS19525	*ygjH*	MULTISPECIES: tRNA-binding protein	2.00
APECO2_RS06250	*ybiW*	MULTISPECIES: formate C-acetyltransferase/glycerol dehydratase family glycyl radical enzyme	1.06
APECO2_RS15950	*yfhL*	MULTISPECIES: ferredoxin	1.07
APECO2_RS25665	-	MULTISPECIES: hypothetical protein	1.01
APECO2_RS19370	-	DNA-binding protein	1.13
APECO2_RS11595	*yeaQ*	MULTISPECIES: GlsB/YeaQ/YmgE family stress response membrane protein	0.95
APECO2_RS03705	*yaiA*	MULTISPECIES: hypothetical protein	0.93
APECO2_RS08305	*ymgE*	MULTISPECIES: GlsB/YeaQ/YmgE family stress response membrane protein	0.95
APECO2_RS19155	*glgS*	MULTISPECIES: glycogen synthase	0.85
APECO2_RS16460	*csrA*	MULTISPECIES: carbon storage regulator	0.81
APECO2_RS20295	*yhcO*	MULTISPECIES: barnase inhibitor	0.89
APECO2_RS04795	*ybeD*	MULTISPECIES: hypothetical protein	0.75
APECO2_RS04755	*cspE*	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	*yciG*	MULTISPECIES: hypothetical protein	0.70
APECO2_RS15075	*yfdY*	MULTISPECIES: hypothetical protein	0.68
APECO2_RS07160	*yccJ*	MULTISPECIES: hypothetical protein	0.70
APECO2_RS08735	*yciN*	MULTISPECIES: hypothetical protein	0.70
APECO2_RS11755	*yobF*	MULTISPECIES: DUF2527 domain-containing protein	0.69
APECO2_RS24175	*tusB*	Sulfurtransferase TusB	0.78
APECO2_RS06985	*yccX*	MULTISPECIES: acylphosphatase	0.75
APECO2_RS24945	*yjbJ*	MULTISPECIES: CsbD family protein	0.64
APECO2_RS24850	*yjbE*	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_RS00685	*ytfH*	MULTISPECIES: HxlR family transcriptional regulator	0.87
APECO2_RS10875	*ydhL*	MULTISPECIES: DUF1289 domain-containing protein	0.73
APECO2_RS06140	*ybiI*	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	MIC (μg/mL)			
	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.

The RNA-seq data were deposited in the NCBI gene expression database (https://www.ncbi.nlm.nih.gov) with the SRA accession number: SRR10145196.

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 OD_{600} of approximately 0.03 in fresh Mueller–Hinton broth with 16 μg/mL Cm or 100 μg/mL Amp, respectively, in Eppendorf (**EP**) tubes with 2-fold serial dilutions of the toxic compounds listed as follows. These EP tubes were incubated for 24 h at 37°C. The lowest antibiotic concentrations that completely inhibited growth were identified as the minimal inhibitory concentrations (**MIC**). The stock solutions of the following antibiotics were prepared, and their classes are listed in parentheses: tetracycline (Sangon) at 32 mg/mL in water; ciprofloxacin (fluoroquinolone, Sangon) at 20 mg/mL in water; norfloxacin (fluoroquinolone, Sangon) at 20 mg/mL in acetic acid; ofloxacin (fluoroquinolone, Sangon) at 20 mg/mL in acetic acid.

Antibacterial Activity Assays

Antibacterial activity assays were performed to examine the changes in antibiotic susceptibility of WT/pSTV28, XY10/pSTV28, and XY10/pClsrR or WT/pUC19, WT/pUClsrR, and WT/pUCmdtH, in accordance with previously described methods and modified as follows (Yu et al., 2018a). The overnight cultures of WT/pSTV28, XY10/pSTV28, and XY10/pClsrR or WT/pUC19, WT/pUClsrR, and WT/pUCmdtH were diluted to an OD_{600} of approximately 0.03 in 3 mL fresh LB broth with 16 μg/mL Cm or 100 μg/mL Amp, respectively, in polystyrene tubes at 37°C for 2 h with shaking. After incubation, different norfloxacin, ofloxacin, ciprofloxacin, and tetracycline concentrations were added to polystyrene tubes that contained the bacterial cultures. The cultures were incubated at 37°C for 2 h with shaking, after which 10-fold serial dilutions of cultures were performed by successive transfer (0.1 mL) through four EP tubes that contained 0.9 mL LB broth. Next, 100 μL dilutions were dropped on LB agar plates. After cultivating for 18 h at 37°C, viable colonies were counted via CFUs on LB agar plates with the appropriate antibiotic. The survival rates of WT/pSTV28 or WT/pUC19 were designated as 100%; the experiments were repeated 3 times with similar results.

Total RNA Isolation, Complementary DNA Generation, and RT-qPCR Processing

For total RNA isolation, the overnight cultures of WT/pSTV28, XY10/pSTV28, and XY10/pClsrR or WT/pUC19, WT/pUClsrR, and WT/pUCmdtH were diluted to an OD_{600} 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 ΔΔCt (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 His_6-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_2HPO_4, 2 mM KH_2PO_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 × Tris-borate EDTA buffer (45 mmol Tris-borate, 1 mmol 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

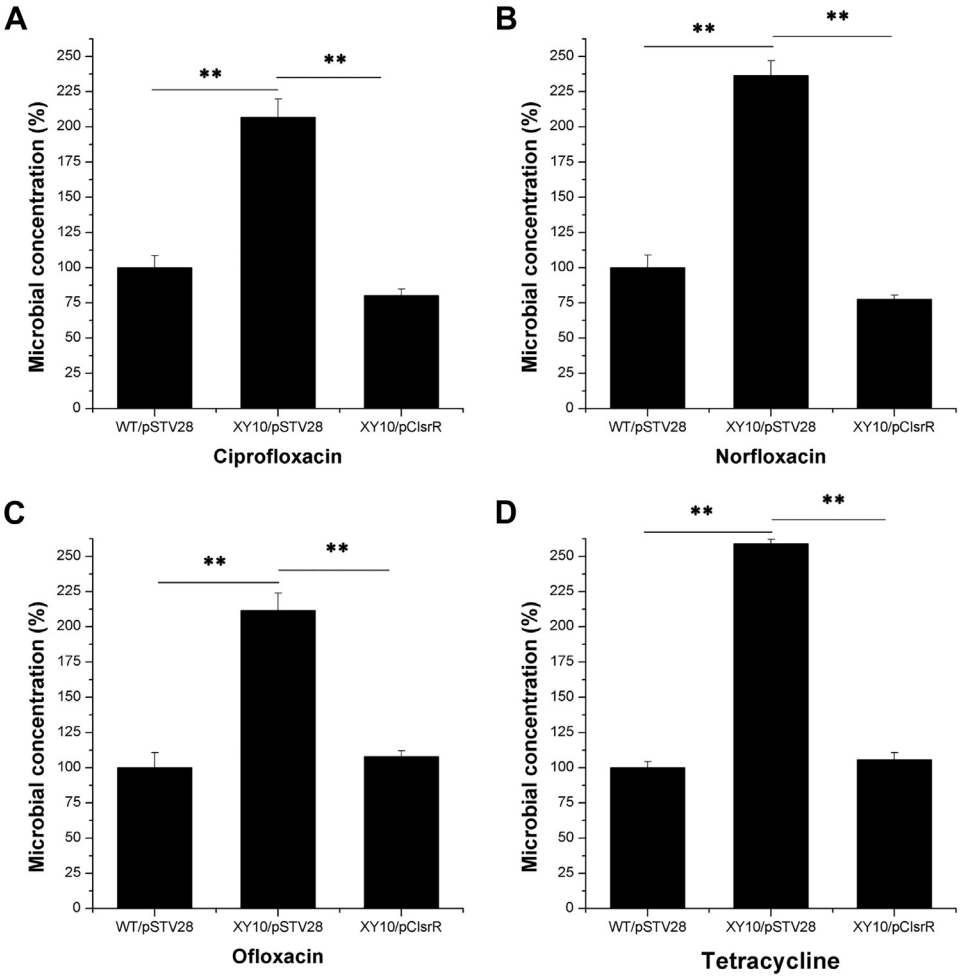

Figure 1. CFU assays of the *lsrR*-deficient strain XY10/pSTV28, the complement strain XY10/pClsrR, and their parent strain WT/pSTV28 in the presence of 4 antibiotics. Data are shown for (A) ciprofloxacin, (B) norfloxacin, (C) ofloxacin, and (D) tetracycline. The survival rate of WT/pSTV28 was designated as 100%. The XY10/pSTV28 and XY10/pClsrR colony counts were compared with those of WT/pSTV28. Error bars indicate SD; $^{**}P < 0.01$ and indicates a significant difference between WT/pSTV28, XY10/pSTV28, and/or XY10/pClsrR.

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,

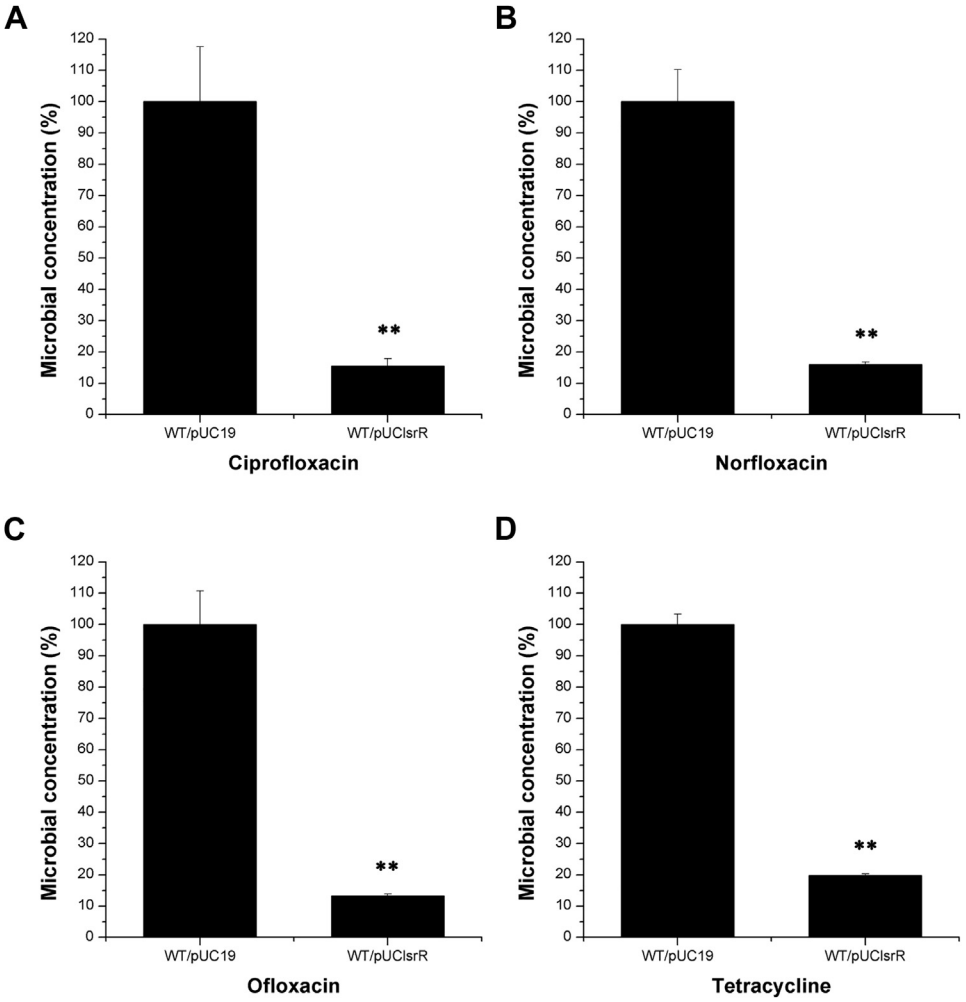

Figure 2. CFU assays of the *lsrR*-overexpressed strain WT/pUClsrR, and its parent strain WT/pUC19 in the presence of 4 antibiotics. Data are shown for (A) ciprofloxacin, (B) norfloxacin, (C) ofloxacin, and (D) tetracycline. The survival rate of WT/pUC19 was designated as 100%. The WT/pUClsrR colony 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.

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

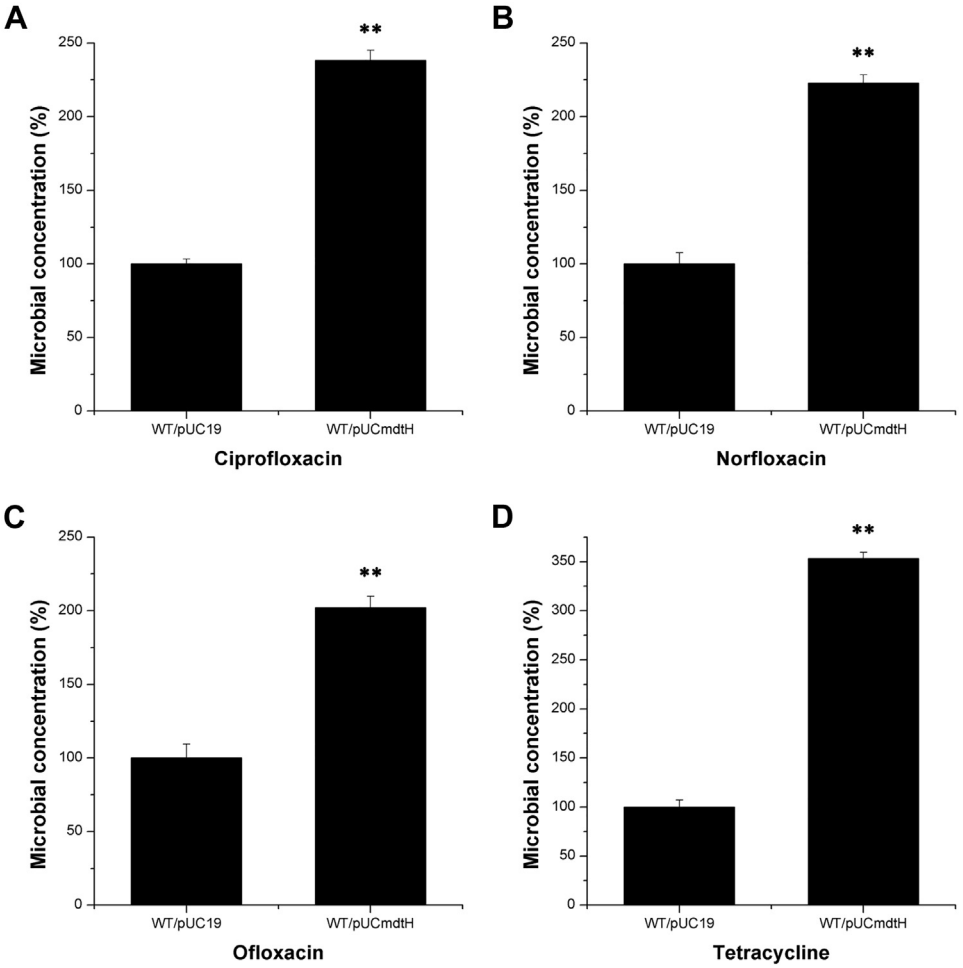

Figure 3. CFU assays of the *mdtH*-overexpressing strain WT/pUCmdtH, and its parent strain WT/pUC19 in the presence of 4 antibiotics. Data are shown for (A) ciprofloxacin, (B) norfloxacin, (C) ofloxacin, and (D) tetracycline. The survival rate of WT/pUC19 was designated as 100%. The WT/pUCmdtH colony 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.

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 an final concentration of 2 μg/mL norfloxacin, 2 μg/mL ofloxacin, 2 μg/mL ciprofloxacin, or 64 μg/mL tetracycline. As shown in Figure 3, in the presence of norfloxacin, 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 norfloxacin, 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 norfloxacin, ofloxacin, ciprofloxacin, and tetracycline by regulating *mdtH*.

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 His_6-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

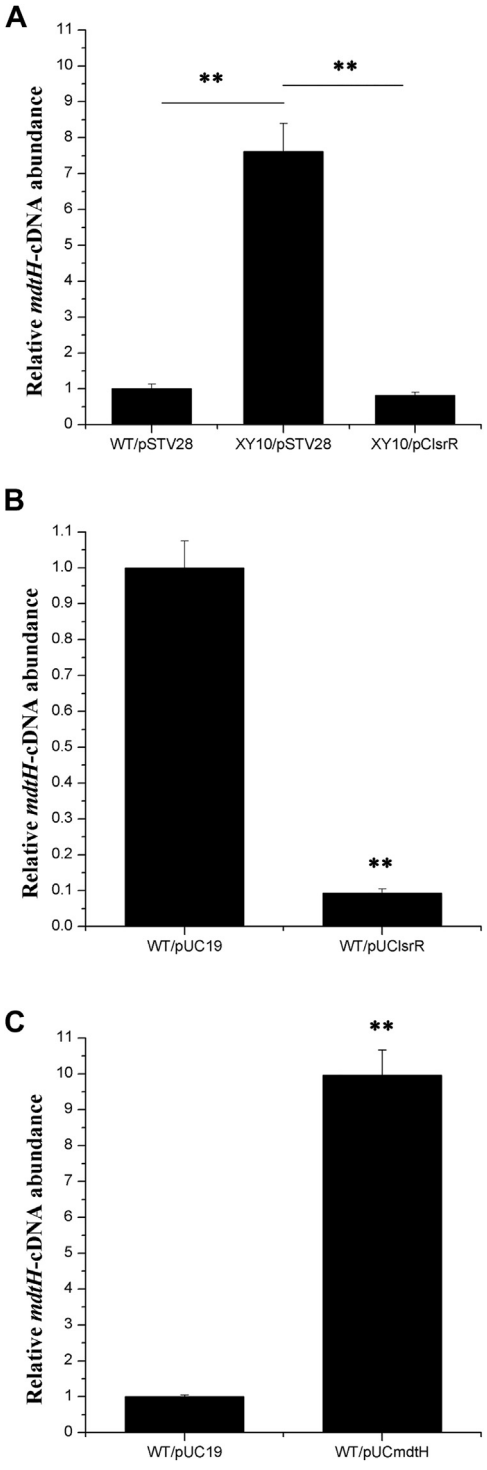

Figure 4. Comparative measurement of transcription (complementary DNA [cDNA] abundance) of the multidrug efflux pump–encoding gene *mdtH* in the *lsrR*-deficient strain, the *lsrR*- and mdtH-overexpressing strains, and their parent strain. (A) The relative *mdtH* transcript level was determined by RT-qPCR 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.

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 system. These data suggested that the transcriptional regulator LsrR negatively regulates expression of the MdtH multidrug efflux pump. To further confirm the regulatory mechanism of LsrR on the MdtH multidrug efflux pump system, we also constructed the *lsrR*- and *mdtH*-overexpressing strains using pUC19 and detected the susceptibility of these strain to norfloxacin, ofloxacin, ciprofloxacin, and tetracycline. Our results revealed that only the tetracycline MIC was altered (increased 2 times) in the mutant strain XY10/pSTV28 compared with WT strain WT/pSTV28; the MIC value of the

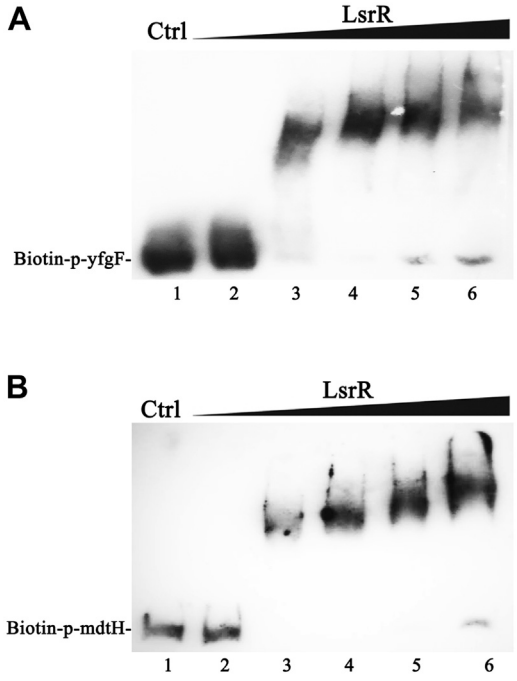

Figure 5. The binding ability of LsrR to the *mdtH* promoter was determined by gel shift assays. Increasing LsrR amounts were incubated with biotin-labeled *yfgF* and *mdtH* promoters (biotin-p-yfgF and biotin-p-mdtH). In each panel, from lanes (1) to (6), the LsrR concentrations were 8, 0, 1, 2, 4, and 8 μmol, respectively; the amount of biotin-labeled probes in all lanes was 200 fmol. In lane 1, besides the labeled probes, 2 pmol unlabeled probe was added as the competitive control (Ctrl). (A) The positive control, which demonstrates the binding ability of LsrR to the *yfgF* promoter and (B) the *mdtH* promoter.

complement strain was restored. Furthermore, the overexpression of *lsrR* or *mdtH* did not alter the MIC of the overexpressing strains XY10/pUClsrR and XY10/pUCmdtH to norfloxacin, ofloxacin, ciprofloxacin, and tetracycline when compared with the parent strain WT/pUC19 (Table 5). However, in the presence of antibiotics, the survival rates of the *lsrR* mutant and the *mdtH*-overexpressing strains significantly increased, and the survival rate of the *lsrR*-overexpressing strain was significantly decreased, when compared with that of their parent strains. It is reasonable that the discrepancy in results can be attributed to the use of a less sensitive broth dilution MIC method compared with the more sensitive efficiency of plating method (Sulavik et al., 2001).

Nishino and Yamaguchi cloned the *mdtH* ORF into the pTrc6His expression vector and found that *mdtH* increases 2 times the norfoxacin and enoxacin MIC of *E. coli* KAM3, a derivative of K-12 that lacks a restriction system and *acrB* (Nishino and Yamaguchi, 2001). A subsequent study reported that MdtH overexpression increases cell tolerance and production of 1,2,4-butanetriol, an important precursor in the synthesis of various drugs and chemicals such as 1,2,4-butanetriol trinitrate, in *E. coli* (Zhang et al., 2018). Until now, whether *mdtH* affects the antibiotic susceptibility in APEC has not been reported. In this study, we found that MdtH overexpression decreased the susceptibility of APECX40 to norfloxacin, ofloxacin, ciprofloxacin, 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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Conflict of Interest Statement: The authors did not provide a conflict of interest statement.

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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