

RESEARCH ARTICLE

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LsrR, the effector of AI-2 quorum sensing, is vital for the H₂O₂ stress response in mammary pathogenic *Escherichia coli*

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Abstract

Mammary pathogenic *Escherichia coli* (MPEC) is an important causative agent of mastitis in dairy cows that results in reduced milk quality and production, and is responsible for severe economic losses in the dairy industry worldwide. Oxidative stress, as an imbalance between reactive oxygen species (ROS) and antioxidants, is a stress factor that is common in most bacterial habitats. The presence of ROS can damage cellular sites, including iron-sulfur clusters, cysteine and methionine protein residues, and DNA, and may cause bacterial cell death. Previous studies have reported that Autoinducer 2 (AI-2) can regulate *E. coli* antibiotic resistance and pathogenicity by mediating the intracellular receptor protein LsrR. This study explored the regulatory mechanism of LsrR on the H₂O₂ stress response in MPEC, showing that the transcript levels of *IsrR* significantly decreased under H₂O₂ stress conditions. The survival cell count of *IsrR* mutant XW10/pSTV28 was increased about 3080-fold when compared with that of the wild-type WT/pSTV28 in the presence of H₂O₂ and overexpression of *IsrR* (XW10/pUCIsrR) resulted in a decrease in bacterial survival rates under these conditions. The β-galactosidase reporter assays showed that mutation of *IsrR* led to a remarkable increase in expression of the promoters of *ahpCF*, *katG* and *oxyR*, while *IsrR*-overexpressing significantly reduced the expression of *ahpCF* and *katG*. The electrophoretic mobility shift assays confirmed that LsrR could directly bind to the promoter regions of *ahpCF* and *katG*. These results revealed the important role played by LsrR in the oxidative stress response of MPEC.

Keywords: cow mastitis, mammary pathogenic *Escherichia coli*, H₂O₂, AI-2 quorum sensing, LsrR

Introduction

Escherichia coli is one of the main pathogenic bacteria causing clinical mastitis [1, 2]. Mammary pathogenic *E. coli* (MPEC) is a class of extraintestinal pathogenic *E. coli* that usually adheres to and infects dairy cow mammary epithelial cells together with other pathogens, forming biofilms. The biofilm can help pathogens to evade the host immune system and continue to multiply in the mammary gland, leading to persistent intramammary

infections [3–5]. As such, research on the role of biofilms in the pathogenesis of mastitis has become important.

Previous studies showed that bacteria express a series of stress proteins to protect themselves from environmental stimuli and to resist adverse environmental pressures such as antibiotics, oxidative stress, acid–base, and osmotic pressure [6–10]. It is generally understood that bacterial infection can trigger innate immune response [11, 12]. Once the pathogenic bacteria infect the host, they are phagocytosed by the host immune cells and macrophages and heterophile cells may produce oxidative bursts in response to pathogens [13, 14]. The rapid production of reactive oxygen species (ROS) including superoxide anions (O₂⁻), hydrogen peroxide (H₂O₂)

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and the highly reactive hydroxyl radical ($\cdot\text{OH}$) can damage many cellular sites, including iron-sulfur clusters, cysteine and methionine protein residues and DNA, and may eventually cause cell senescence and death [15–17]. H_2O_2 is a widely used bactericide for inactivating food-borne pathogens. Over time, a variety of *E. coli* regulatory pathways has evolved to adapt to H_2O_2 stress including synthesizing catalases (encoded by *katG* and *katE*), alkyl hydroperoxide reductase (Ahp, encoded by *ahpCF*), and stress protein YciF (encoded by *yciF*). A range of transcriptional regulators including the transcriptional regulator OxyR and the sigma factor RpoS could regulate the synthesis of these enzymes [6, 8, 18–20]. Previous studies showed that *E. coli* resistance to oxidative stress affects both survival and pathogenic potential in the host [21, 22]. However, the detailed molecular mechanism of bacterial adapting to H_2O_2 stress needs to be further explored.

Autoinducer 2 (AI-2) is produced by many Gram-negative and Gram-positive bacteria and is considered to be a quorum sensing (QS) signaling molecule involved in interspecies communication [23]. Previous studies have reported that the AI-2 QS system is involved in the bacterial response to oxidative stress [24–26]. The absence of *luxS* (involved in the synthesis of AI-2) in *Streptococcus suis* significantly enhanced its tolerance to H_2O_2 , and the mutation of *luxS* in *Deinococcus radiodurans* resulted in a significantly higher susceptibility to H_2O_2 than wild-type strains [25, 26]. In *E. coli*, the process of AI-2 uptake involves an ATP-binding transporter complex encoding by the *lsrACDB* operon. The expression of the *lsr* operon was regulated by LsrR, a DNA-binding repressor and LsrK, a cognate signal kinase, whose genes (*LsrRK*) are located immediately upstream of the *lsr* operon and divergently transcribed. The LsrR represses the expression of the *lsr* operon and its own *lsrRK* operon by binding to the promoters, while phospho-AI-2 can release LsrR repression and then activating *lsr* operon expression [27–29]. The role of LsrR as a transcriptional regulator mediating QS signal AI-2-related cellular functions has been identified in previous studies and its significant influence on gene expression control has been revealed. According to a previous study, 146 genes were significantly affected by LsrR deletion [30], but whether there is a relationship between LsrR and the regulation of bacterial oxidative stress response has not yet been reported.

In the present study, we demonstrated that the survival ability of *lsrR* mutant under H_2O_2 stress was significantly increased compared to that of the wild type. The β -galactosidase reporter assays indicated that LsrR had an obvious inhibitory effect on the expression of *ahpCF*, *katG*, and *oxyR*. Electrophoretic mobility shift assays (EMSA) confirmed that LsrR inhibited the expression

of *ahpCF* and *katG* by direct binding to their promoter regions. This study is therefore helpful to understand the bacterial response to host-derived ROS in MPEC, and might provide potential drug targets for the treatment and prevention of *E. coli* infection.

Materials and methods

Bacterial strains, plasmids, and culture conditions

All the strains and plasmids used in this study are listed in Table 1. The *E. coli* strain DCM5 was isolated from milk samples in dairy cows with mastitis and was identified by 16S rDNA sequencing. The *E. coli* cultures were routinely grown at 37 °C in Luria–Bertani (LB) broth (Oxoid, Basingstoke, UK) or on LB agar plates containing 1.5% agar (Oxoid). When needed, antibiotics (Sangon Biotech, Shanghai, China) were used at the following final concentrations: ampicillin (100 $\mu\text{g}/\text{mL}$), chloramphenicol (15 $\mu\text{g}/\text{mL}$), and kanamycin (50 $\mu\text{g}/\text{mL}$).

General DNA manipulation

Genomic DNA from *E. coli* DCM5 was prepared according to the instructions of TIANamp Bacteria DNA Kit (TianGen Biotech, Bei Jing, China). Plasmid DNA was extracted using a plasmid extraction kit (Sangon Biotech, Shanghai, China). PCR testing was carried out using Taq or PrimeSTAR[®] Max DNA Polymerase (Takara Bio Inc., Dalian, China). The PCR products and DNA restriction fragments were purified using a gel purification kit (Sangon Biotech, Shanghai, China). DNA restriction endonuclease (Thermo Fisher Scientific, Waltham, MA, USA) digestion and DNA ligation (Thermo Fisher Scientific) were performed by standard methods. Primer premier 5.0 software was used for sequence analysis and primer design. The primer sequences used are listed in Table 2.

Construction of the *lsrR*-deficient mutant

Homologous recombination was used for deletion of *lsrR* from the chromosome of DCM5 based on λ red recombinase system [31]. Briefly, using pKD3 plasmid as a template, the fragment containing the chloramphenicol-resistance cassette (*cat*) inserted within the *lsrR* gene was amplified with primers DCM5-*lsrR*-f and DCM5-*lsrR*-r and then transformed into DCM5 competent cells containing lambda Red recombinase expressed by pKD46 plasmid using electroporation. After electroporation, the cells were added immediately to 900 μL super optimal broth with catabolite repression broth (SOC) without antibiotics, incubated at 37 °C for 60 min, and then mutants were selected by plating cultures on LB plates with 15 $\mu\text{g}/\text{mL}$ chloramphenicol. Following incubation for 24 h, the *lsrR* mutant was selected by PCR amplification using the primers check-*lsrR*-f and check-*lsrR*-r. Plasmid pCP20 was subsequently transformed into the

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Reference or source
Strains		
<i>E. coli</i>		
DH5α	Clone host strain, supE44 ΔlacU169(φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Invitrogen
BL21	Expression strain, F-ompT hsdS(rB- mB-) gal dcm (DE3)	Invitrogen
WT	<i>MPEC</i> (DCM5), wild-type	Laboratory stock
XW10	DCM5 <i>lsrR</i> -deletion mutant	This study
WT/pSTV28	WT with the empty vector pSTV28, Cm ^r	This study
XW10/pSTV28	XW10 with the empty vector pSTV28, Cm ^r	This study
XW10/pClsrR	XW10 with the complement plasmid pClsrR, Cm ^r	This study
WT/pUC19	WT with the empty vector pUC19, Amp ^r	This study
WT/pUClsrR	WT with the overexpression plasmid pUClsrR, Amp ^r	This study
WTΔlacZ	DCM5 lacZ-deletion mutant	This study
XW10ΔlacZ	DCM5 lacZ and <i>lsrR</i> mutant strain	This study
WTΔlacZ/pRCL-p _{ahpCF}	WTΔlacZ with plasmid pRCL-p _{ahpCF} , Cm ^r	This study
XW10ΔlacZ/pRCL-p _{ahpCF}	XW10ΔlacZ with plasmid pRCL-p _{ahpCF} , Cm ^r	This study
WTΔlacZ/pRCL-p _{oxyR}	WTΔlacZ with plasmid pRCL-p _{oxyR} , Cm ^r	This study
XW10ΔlacZ/pRCL-p _{oxyR}	XW10ΔlacZ with plasmid pRCL-p _{oxyR} , Cm ^r	This study
WTΔlacZ/pRCL-p _{katG}	WTΔlacZ with plasmid pRCL-p _{katG} , Cm ^r	This study
XW10ΔlacZ/pRCL-p _{katG}	XW10ΔlacZ with plasmid pRCL-p _{katG} , Cm ^r	This study
WTΔlacZ/pRCL-p _{katE}	WTΔlacZ with plasmid pRCL-p _{katE} , Cm ^r	This study
XW10ΔlacZ/pRCL-p _{katE}	XW10ΔlacZ with plasmid pRCL-p _{katE} , Cm ^r	This study
WTΔlacZ/pRCL-p _{yciF}	WTΔlacZ with plasmid pRCL-p _{yciF} , Cm ^r	This study
XW10ΔlacZ/pRCL-p _{yciF}	XW10ΔlacZ with plasmid pRCL-p _{yciF} , Cm ^r	This study
WTΔlacZ/pRCL-p _{rpoS}	WTΔlacZ with plasmid pRCL-p _{rpoS} , Cm ^r	This study
XW10ΔlacZ/pRCL-p _{rpoS}	XW10ΔlacZ with plasmid pRCL-p _{rpoS} , Cm ^r	This study
WTΔlacZ/pRCL-p _{katGM6}	WTΔlacZ with plasmid pRCL-p _{katGM6} , Cm ^r	This study
WTΔlacZ/pRCL-p _{ahpCFM6}	WTΔlacZ with plasmid pRCL-p _{ahpCFM6} , Cm ^r	This study
Plasmids		
pKD46	Expresses λ red recombinase Exo, Bet and Gam, temperature sensitive, Amp ^r	[31]
pKD3	cat gene, template plasmid, Amp ^r Cm ^r	[31]
pCP20	FLP + λci857 + λpRRep(Ts), temperature sensitive, Amp ^r Cm ^r	[31]
pSTV28	Low copy number cloning vector, Cm ^r	Takara
pUC19	Cloning vector, Amp ^r	Takara
pUClsrR	pUC19 with <i>lsrR</i> gene, Amp ^r	This study
pClsrR	pSTV28 with <i>lsrR</i> gene, Cmr	This study
pRCL	Cm ^r , promoterless <i>lacZ</i>	[46]
pRCL-p _{ahpCF}	pRCL harboring <i>ahpCF</i> promoter	This study
pRCL-p _{oxyR}	pRCL harboring <i>oxyR</i> promoter	This study
pRCL-p _{katG}	pRCL harboring <i>katG</i> promoter	This study
pRCL-p _{katE}	pRCL harboring <i>katE</i> promoter	This study
pRCL-p _{yciF}	pRCL harboring <i>yciF</i> promoter	This study
pRCL-p _{rpoS}	pRCL harboring <i>rpoS</i> promoter	This study
pRCL-p _{katGM6}	Mutational pRCL-p _{katG} with 6-bp mutation ('AACAAAT' to 'GCGCGC')	This study
pRCL-p _{ahpCFM6}	Mutational pRCL-p _{ahpCF} with 6-bp mutation ('AAAACCT' to 'GCGCGC')	This study
pET28a (+)	Expression vector, Kan ^r	Novagen
pET-lsrR	pET28a (+) with <i>lsrR</i> gene, Kan ^r	This study

^a Cm^r: chloramphenicol-resistant; Amp^r: ampicillin-resistant; Kan^r: kanamycin-resistant.

Table 2 Oligonucleotide primers used in this study

Primer name	Oligonucleotide (5'-3')
DCM5-lsrR-f	ATCGTCTCGGCCTGACACGTTTGAAAGTGTCCGATTGCTTGTAGGCTGGAGCTGCTT
DCM5-lsrR-r	TAATATTACACTGCACGCCGCTTAAGCTGCCGATTCCTGAATATCCTCTTAGTTC
DCM5-lacZ-f	ATGGTAAGCCGCTGGCAAGCGGTGAAGTGCCCTCTGGATGTTGTAGGCTGGAGCTGCTT
DCM5-lacZ-r	GACAATGGTTAAATGAAATTTGCATAAAAATTGCGGCCTCATATGAATATCCTCCTTA
Check-lsrR-f	GTTGTTGCCCTCAATCTCC
Check-lsrR-r	ACTCCGCCGTCCCACT
check-lacZ-f	GGCGGTGATTTTGGCGATAC
check-lacZ-r	GTAACGTTGGGTGCAAT
CM-f	TGTAGGCTGGAGCTGCTT
CM-r	CATATGAATATCCTCCTTAGTTC
p _{katG} -KpnI-f	GGGGTACC GTGAAAATCACACAGTGATC
p _{katG} -BamHI-r	CGGGATCCCAATGTGCTCCCCTCTACAG
p _{ahpCF} -HindIII-f	CCAAGCTTTAGATCAGGTGATTGCCCTT
p _{ahpCF} -BamHI-r	CGGGATCCCTATACTCCTCCGTGT
p _{oxyR} -HindIII-f	CCAAGCTTTCCGCAAAAGTTCACGTTGG
p _{oxyR} -BamHI-r	CGGGATCCATCCATCCTCCATCGCCAC
p _{katE} -HindIII-f	CCAAGCTTTACTGGCTTCACTAAACGCA
p _{katE} -BamHI-r	CGGGATCC TGAACCTCGTCTCCTAATTT
p _{yciF} -HindIII-f	CCAAGCTTACCGGAACAGTTCAACAG
p _{yciF} -BamHI-r	CGGGATCCAAGGTGGCTCCTACCCGTGA
p _{rpoS} -HindIII-f	CCAAGCTTCTGATTACCCGTTAATAT
p _{rpoS} -BamHI-r	CGGGATCC TTTTGCCAGTGCCCGGTT
M6-p _{katG} -f	TTAACCGCGCGCATGTAAGATCTCAACTATCGCATCCG
M6-p _{katG} -r	TTACATGCGCGCGTTAAAGAGATGTAGATCAAATTGATCT
M6-p _{ahpCF} -f	TTGTAAGGTGCGCGCTATCGATTGATAATGGAAACGCA
M6-p _{ahpCF} -r	ATAGCGCGCACCTTACAACCTTCGTAAGACAACCTT
rt-16 s-f	TTTGAGTTCCCGGCC
rt-16 s-r	CGGCCGCAAGGTTAA
rt-lsrR-f	CGGATCGCGTGTTTT
rt-lsrR-r	TCAACATATGCGCCGC
rt-ahpCF-f	AGGCATCAAAAACGCGCGAA
rt-ahpCF-r	CGTAGTGGTCAGCAACGTCA
rt-katG-f	GGTGTGAGAAAGCCGCAAG
rt-katG-r	AGACGAGCGCGATAGTTACG
M13-f	TGTAAAACGACGGCCAGT
M13-r	CAGGAAACAGCTATGACC
T7-f	TAATACGACTCACTATAGGG
T7-r	TGCTAGTTATTGCTCAGCGG
lsrR-EcoRI-f	CCGGAAATTCGTTTCAGTTTGCAGGTGAG
lsrR-KpnI-r	CGGGGTACC TTAAC TACGTAAAATCGCCG
lsrR-KpnI-f	GGGGTACC ATGACAATCAACGATTCGGT
lsrR-EcoRI-r	CGGAATTC TTAAC TACGTAAAATCGCCG
lsrR-NcoI-f	CATGCCATGGACAATCAACGATTCGGT
lsrR-XhoI-r	CCGCCTCGAGACTACGTAAAATCGCCG
p-lsrR-biotin-f	ATTTCCCCGTTTCAGTTTTG
p-lsrR-r	AATTCATTCTCACTTTGAA
p-oxyR-biotin-f	TCCGCAAAAGTTCACGTTGG
p-oxyR-r	TATCCATCCTCCATCGCCAC
p-ahpCF-biotin-f	TAGATCAGGTGATTGCCCTT
p-ahpCF-r	CTATACTTCTCCGTGTT
p-katG-biotin-f	GTGAAAATCACACAGTGATC
p-katG-r	CAATGTGCTCCCCTACAG

^a The underlined sequences refer to the restriction endonuclease recognition sites.

mutant strain to delete the Cm cassette. The Cm-sensitive mutant strain was confirmed by PCR amplification using the primers check-*lsrR*-f and check-*lsrR*-r, and the PCR products were further confirmed by DNA sequencing, with the mutant strain was designated as DCM5Δ*lsrR* (XW10).

Complementation of the *lsrR* mutant

To construct the complemented plasmid pSTV28-*lsrR*, the *lsrR* gene and its promoter region were amplified from the DCM5 strain using the primers *lsrR*-KpnI-f and *lsrR*-BamHI-r, and then the PCR products were gel-purified and ligated into the BamHI and KpnI restriction sites of the plasmid pSTV28. The recombinant plasmid pSTV28-*lsrR* was extracted and further confirmed by PCR using primers M13-f and M13-r, and DNA sequencing. To obtain the complemented strain XW10/pCl*lsrR*, the plasmid pSTV28-*lsrR* was electroporated into the mutant strain XW10. The wild-type strain (WT) and the mutant strain XW10 were transformed with the empty vector pSTV28 to obtain WT/pSTV28 and XW10/pSTV28, respectively.

Construction of the *lsrR* overexpressing strain

The strain overexpressing *lsrR* was constructed as previously described [32]. The *lsrR* gene was amplified from the DCM5 strain using the primers *lsrR*-KpnI-f and *lsrR*-EcoRI-r, and then the PCR products were gel-purified and ligated into the KpnI and EcoRI restriction sites of the plasmid pUC19 to construct the overexpressing plasmid pUC19-*lsrR*. Subsequently, the recombinant plasmid pUC19-*lsrR* was extracted and further confirmed by PCR using primers M13-f and M13-r, and DNA sequencing. The purified recombinant plasmid pUC19-*lsrR* and the control empty vector pUC19 were electroporated into the wild-type strain WT to obtain strains WT/pUC*lsrR* and WT/pUC19, respectively.

Bacterial growth curves

All strains were cultured overnight in fresh LB broth. Then each sample was diluted to an OD₆₀₀ of approximately 0.03 in 100 mL of fresh LB broth with 15 μg/mL chloramphenicol or 100 μg/mL ampicillin and grown at 37 °C for 28 h with shaking. The OD₆₀₀ was detected at 2 h intervals using a UV/Vis spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA), and the growth curves of these strains were established. The experiment was repeated three times and all samples were measured in triplicate.

H₂O₂ stress assays

The overnight cultures were diluted to an OD₆₀₀ of approximately 0.03 in 3 mL of fresh LB broth with

15 μg/mL chloramphenicol or 100 μg/mL ampicillin, and incubated in culture tubes at 37 °C for 2 h with shaking. Then, 6 μL of 30% H₂O₂ was added in each experimental group cultures, and the cells continued to be incubated at 37 °C for 1 h with shaking. All cultures from the experimental group and control group (without addition of H₂O₂) were then diluted by serial ten-fold dilution with LB medium, and 3 μL of each dilution was dropped onto the LB agar plates and cultured at 37 °C overnight. At the same time, to perform the viable colony-forming units (CFUs) assays, 100 μL of each dilution was spread onto the LB agar plates and cultured at 37 °C overnight. The colonies on each plate were counted. The results were compared between the test groups and the control groups and all experiments were repeated three times.

β-galactosidase assays

The promoters of *oxyR*, *katG*, *katE*, *rpoS*, *yciF*, and *ahpCF* were amplified and cloned into the promoterless *lacZ* plasmid pRCL by restriction enzyme digestion and DNA ligation to construct the transcriptional *lacZ* fusion reporter plasmid. To avoid the *lacZ* gene on the chromosome of the strain from affecting the experimental results, the *lacZ* genes from the WT strain and the *lsrR*-deficient strain were mutated using homologous recombination methods based on λ red recombinase system, and then the constructed *lacZ* fusion reporter plasmids were introduced into strain WT/Δ*lacZ* and XW10/Δ*lacZ*, respectively. The *E. coli* cells containing the *lacZ* fusion reporter plasmid were cultured in 100 mL LB broth containing a final concentration of 15 μg/mL chloramphenicol for the specified time. After cultivation, cells were collected and diluted in Z-buffer containing 16.1 g/L Na₂HPO₄, 5.50 g/L NaH₂PO₄, 0.75 g/L KCl, and 0.246 g/L MgSO₄ to 1 mL and assayed for β-galactosidase activity using ortho-Nitrophenyl-β-galactoside (ONPG) as a substrate. The following equation was used to calculate units of enzyme activity: Miller Units: [OD₄₂₀ × 1000]/(OD₆₀₀ × Volume (mL) × Time (min)]. The experiment was repeated three times independently.

Total RNA isolation, cDNA generation, and real-time reverse transcription-quantitative PCR processing

Real-time reverse transcription-quantitative PCR (RT-qPCR) assays were performed according to a previous study [32]. To detect the transcript levels of *lsrR* in the presence or absence of H₂O₂, the overnight cultures of DCM5(WT) cells were diluted at 1:100 and transferred into two parallel fresh 100 mL LB broth. When the cell density reached OD₆₀₀ of 1, the cultures were cultured for another 40 min with or without 50 μL of 30%

H₂O₂, and then collected for total RNA extraction. To verify the effect of *lsrR* overexpression on the expression of *katG* and *ahpCF*, the overnight cultures of WT/pUC19, and WT/pUCLsrR were diluted to an OD₆₀₀ of approximately 0.03 in fresh LB broth with 100 µg/mL ampicillin. 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 Biotech (Beijing) Co. Ltd., Beijing, China]. Reverse transcription was carried out using the EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen) according to the manufacturer's instructions. RT-qPCR was performed using the TransStart Tip Green qPCR SuperMix kit (TransGen) with the CFX96™ Real-Time System (Bio-Rad, USA). The 16S cDNA abundance was used to normalize the quantity of the target genes. All the real-time RT-qPCR assays were repeated at least three times.

Expression and purification of the LsrR protein

The C-terminal His6-tag LsrR protein was cloned and purified according to a previous study [27]. The open reading frame of the *lsrR* gene of *E. coli* DCM5 was amplified with primers *lsrR*-NcoI-f and *lsrR*-XhoI-r from DCM5 genomic DNA, and then cloned into the pET28a (+) vector. The recombinant plasmid pET-*lsrR* was confirmed by PCR using primers T7-f and T7-r, and further confirmed by DNA sequencing. The pET-*lsrR* vector was then transferred into *E. coli* BL21(DE3) by a chemical transformation method. The LsrR protein was induced to express in *E. coli* BL21(DE3) cells by adding isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM. The purification of His6-tagged LsrR fusion protein was performed with a HisTrap high-performance column as previously described [32]. The purified LsrR protein was stored in 10% glycerol at -80 °C until use. The LsrR protein purity was determined by SDS-PAGE, and the protein concentration was measured by an enhanced BCA protein assay kit (Beyotime, Shanghai, China).

Electrophoretic mobility shift assay (EMSA)

Four promoter DNA fragments, p-*lsrR*, p-*oxyR*, p-*ahpCF* and p-*katG*, were amplified from the WT genome by biotin-labeled probe primers (p-*lsrR*-biotin-f/p-*lsrR*-r, p-*ahpCF*-biotin-f/p-*ahpCF*-r, p-*katG*-biotin-f/p-*katG*-r, and p-*oxyR*-biotin-f/p-*oxyR*-r) respectively. The biotin-labeled promoter fragments were incubated at 25 °C for 30 min with various amounts of LsrR proteins in 4 µL 5 × binding buffer

containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 3 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM dithiothreitol. After incubation, 2 µL 10 × loading buffer with bromophenol blue was added to the mixture and then electrophoresed in a 4% native polyacrylamide gel in a 0.5 × Tris-borate EDTA buffer. The band shifts were detected and analyzed according to the manufacturer's EMSA kit instructions (Beyotime, Shanghai, China).

Mutation of *ahpCF* and *katG* promoter sites and enzyme activity detection

Mutation of binding sequences of the *ahpCF* and *katG* promoter was performed according to the previous method [27]. There were 6-bp nucleotide bases (AACAAT) in the *katG* promoter region, and 6-bp nucleotide bases (AAAAC) in the *ahpCF* promoter region, which may be necessary for LsrR binding. High ratios of A and T within these motifs also suggest that LsrR might preferentially bind to AT-rich sequences [27] so these 6-bp nucleotide bases in plasmids pRCL-p_{ahpCF} and pRCL-p_{katG} were replaced by the GCGCGC sequence to obtain plasmids pRCL-p_{ahpCFM6} and pRCL-p_{katGM6} (Table 1). The mutations were performed by using Mut Express II Fast Mutagenesis Kit V2 (Vazyme Biotech, Nanjing, China). A DNA fragment about 6.0 kb, containing the full length of the plasmid except the 6-bp nucleotide bases, was amplified by PCR using the plasmid pRCL-p_{ahpCF} or pRCL-p_{katG} as the template and M6-p-*ahpCF*-f/M6-p-*ahpCF*-r or M6-p-*katG*-f/M6-p-*katG*-r as the primers. The remaining steps were performed according to the Mut Express II Fast Mutagenesis Kit V2 instructions. The constructed mutant plasmid was identified by DNA sequencing then the mutant reporter plasmids were introduced into strain WT/Δ*lacZ*. The strains WTΔ*lacZ*/pRCL-p_{ahpCFM6} and WTΔ*lacZ*/pRCL-p_{katGM6} were obtained by screening and the expression activity of the mutant promoter was detected by β-galactosidase assays.

Statistical analyses

Statistical analyses were conducted using the GraphPad Primer 8.0 (GraphPad Software Inc., GraphPad Prism 8.0.1.244, San Diego, CA, USA, 2018) using a one-way ANOVA method. The test results are shown as the mean ± SD. A paired t test was used for statistical comparisons between groups. The level of statistical significance was set at a *P* value of ≤ 0.05.

Results

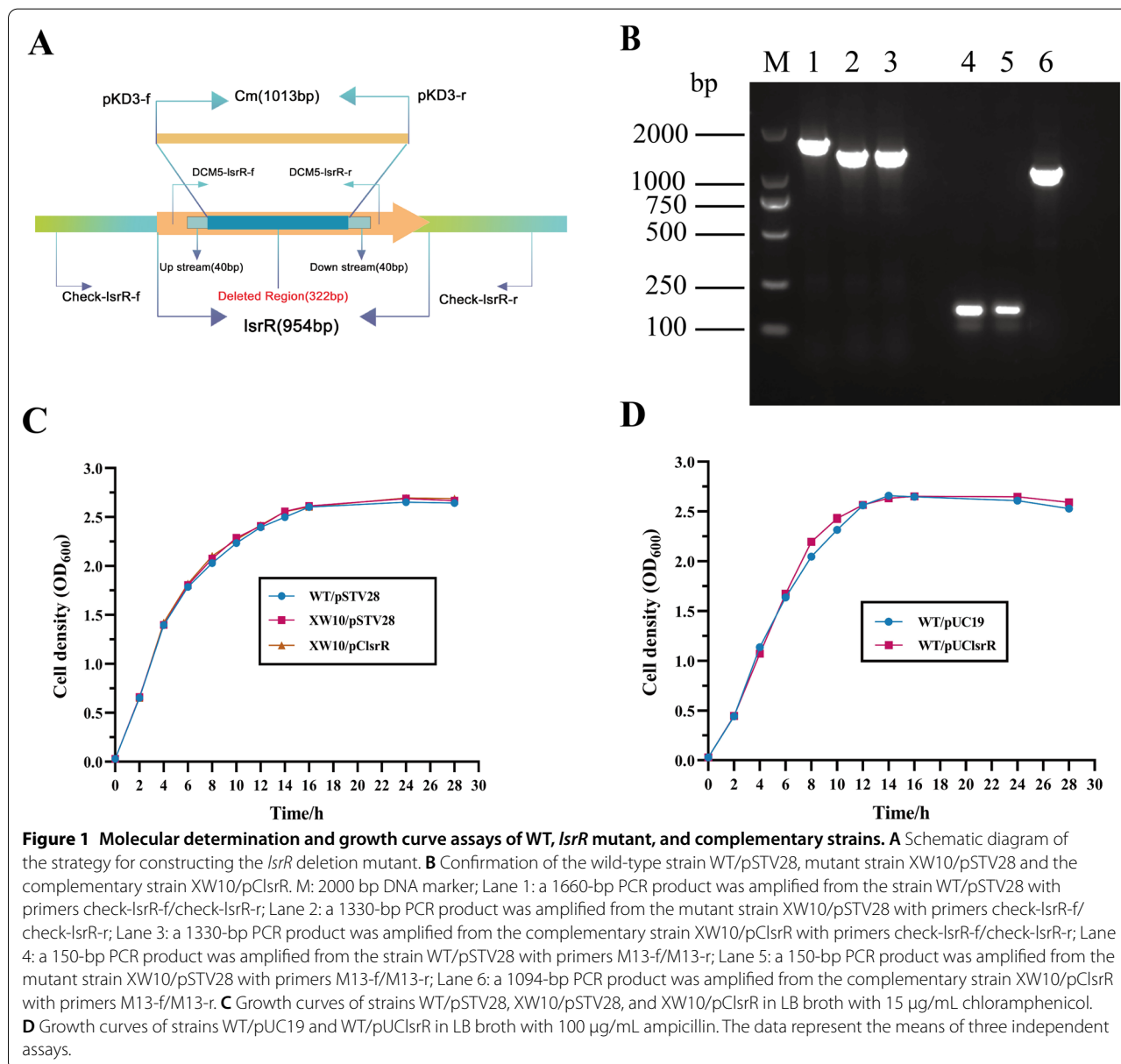
Identification of *lsrR* mutant and complementary strains of DCM5

The *lsrR* gene was knocked out using homologous recombination to obtain the *lsrR* deletion mutant. The schematic diagram of the strategy for deleting the *lsrR* gene in DCM5 and the primers used for confirmation of the *lsrR* deletion are shown in Figure 1A. The mutant and complementary strains of *lsrR* were confirmed by PCR (Figure 1B). A 1660 bp product was amplified from the wild type strain WT/pSTV28, while 1330 bp products were respectively amplified from the mutant strain XW10/pSTV28 and the complementary strain XW10/pClSrR by

using primers check-*lsrR*-f and check-*lsrR*-r (Lane 1–3). The plasmid pSTV28 and the complementary plasmid pClSrR were also confirmed by PCR (Figure 1B, Lane 4–6). The PCR products of 100 bp were amplified from strains WT/pSTV28 and XW10/pSTV28 using primers M13-f/M13-r, and a 1094 bp product was amplified from strain XW10/pClSrR.

lsrR deletion and overexpression did not affect strains growth

To ensure that the growth conditions of the mutant XW10 strain and its parent strain were consistent with that of the complementary strain, the empty



vector pSTV28 was transformed into the WT and XW10 strains. Growth curve assays were performed and results showed that the growth curves of XW10/pSTV28 and XW10/pClSrR were like that of WT/pSTV28 when cells were cultured in LB broth with 15 $\mu\text{g}/\text{mL}$ chloramphenicol (Figure 1C). Likewise, to ensure that the growth condition of the WT strain was consistent with that of the *lsrR* overexpression strain, the empty vector pUC19 was transformed into the WT strain. The growth curve of strain WT/pUC19 in LB broth with 100 $\mu\text{g}/\text{mL}$ ampicillin was like that of strain WT/pUC19 as shown in Figure 1D.

Deletion of *lsrR* increased bacterial survival ability under the H_2O_2 stress

With the tolerance of pathogenic bacteria to H_2O_2 stress being increased, the infecting ability of pathogenic bacteria to the host is also increased. To evaluate the effect of *lsrR* on the survival of strain DCM5 in the presence of H_2O_2 , the survival ability of the *lsrR*-deficient mutant strain XW10/pSTV28 was compared with that of the wild-type strain WT/pSTV28 and

the complementary strain XW10/pClSrR. The results showed that there was no difference in the number of colonies grown on the plates of each group in absence of H_2O_2 (Figure 2A). However, the tolerance of strain XW10/pSTV28 to H_2O_2 stress was increased when compared with that of strain WT/pSTV28 after treating with H_2O_2 for 1 h, and the tolerance was partially restored in strain XW10/pClSrR (Figure 2B). The CFU assays were performed to further analyze the survival rates of strains WT/pSTV28, XW10/pSTV28, and XW10/pClSrR, and the colonies of hydrogen peroxide treatment group and control group on the plates were counted and compared after cultivating in LB broth with 15 $\mu\text{g}/\text{mL}$ chloramphenicol overnight. In the absence of H_2O_2 , there was no difference in the survival rate between the groups as shown in Figure 2C, D. However, with the addition of H_2O_2 , the survival rate of strain XW10/pSTV28 was increased 3080-fold when compared with that of strain WT/pSTV28, and the survival rate was partially recovered in strain XW10/pClSrR. These results suggested that *LsrR* played a vital role in the bacterial oxidative stress response.

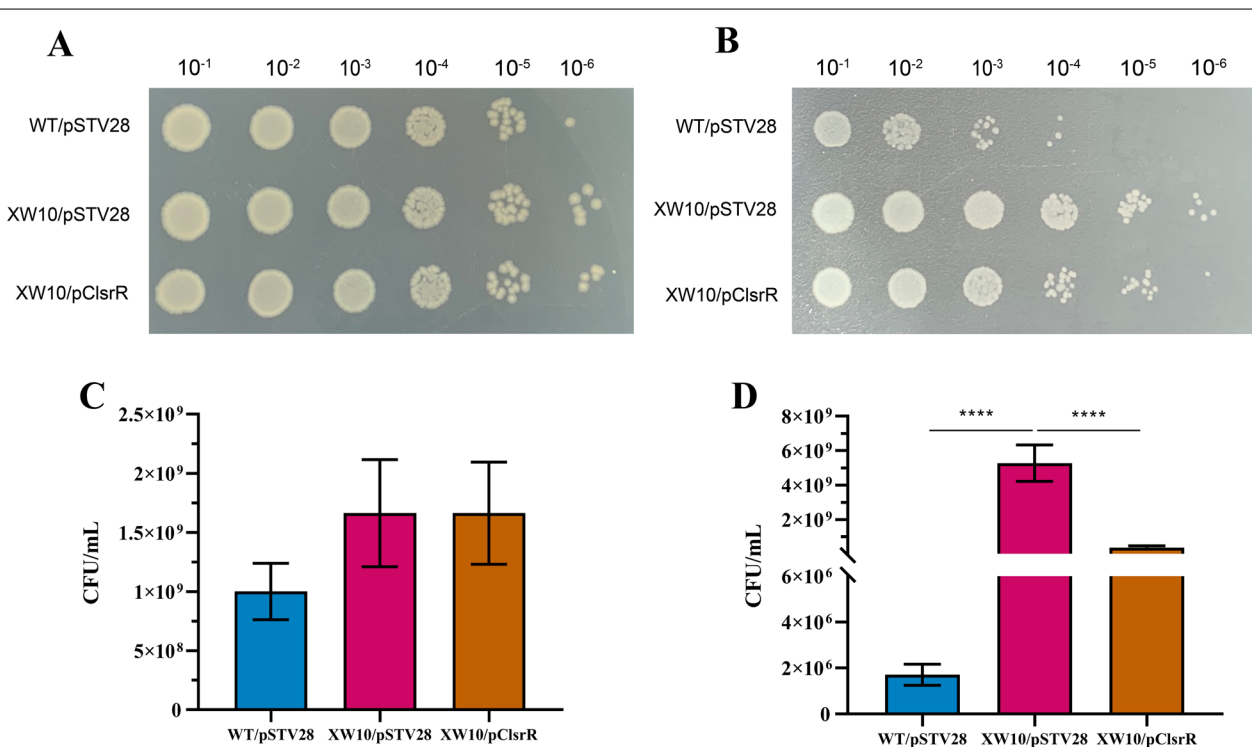


Figure 2 Analysis of the H_2O_2 stress response of strains WT/pSTV28, XW10/pSTV28, and XW10/pClSrR. The strains WT/pSTV28, XW10/pSTV28 and XW10/pClSrR with an initial OD_{600} of 0.03 were inoculated into LB broth containing 15 $\mu\text{g}/\text{mL}$ chloramphenicol and incubated for 2 h. Then the cultures of the control group (A) and the experimental group (treated with 6 μL 30% hydrogen peroxide for 1 h) (B) were ten-fold serially diluted, and 3 μL of each dilution was dropped onto LB agar plates and cultured overnight. The above dilutions of the control group (C) and the experimental group (D) were spread onto the LB agar plates. After incubation overnight, the colony forming units were analyzed. (**** $P < 0.0001$).

Overexpression of *lsrR* decreased bacterial survival ability under the H₂O₂ stress

To further evaluate the effect of *lsrR* on the survival of strain DCM5 in the presence of H₂O₂, the survival ability of the *lsrR* overexpression strain WT/pUC*lsrR* and its parental strain WT/pUC19 were compared under the H₂O₂ stress condition. The results showed that there was no difference in the number of colonies grown on the plates of each group in absence of H₂O₂ (Figure 3A), but strain WT/pUC*lsrR* showed reduced tolerance to H₂O₂ stress compared with WT/pUC19 after being treated with H₂O₂ for 1 h (Figure 3B). To further analyze the survival rates of strains WT/pUC19 and WT/pUC*lsrR*, CFU assays were performed. There was no difference in the survival rate between the groups in the absence of H₂O₂ as shown in Figures 3C, D. In the presence of H₂O₂, the survival rate of WT/pUC*lsrR* was decreased approximately 21-fold when compared with that of WT/pUC19. This data further supported the important role of LsrR on the oxidative stress response of MPEC.

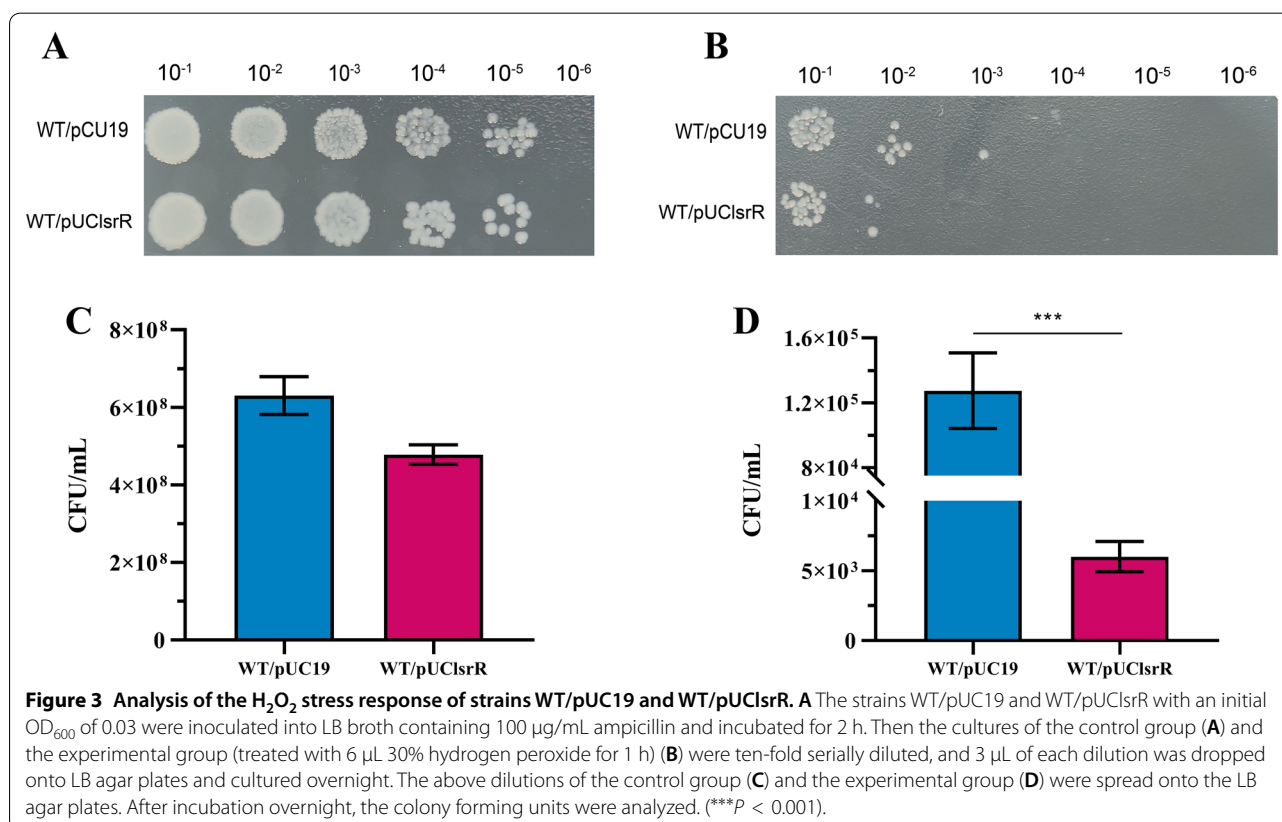
Deletion of *lsrR* increased the expression of *oxyR*, *ahpCF*, and *katG*

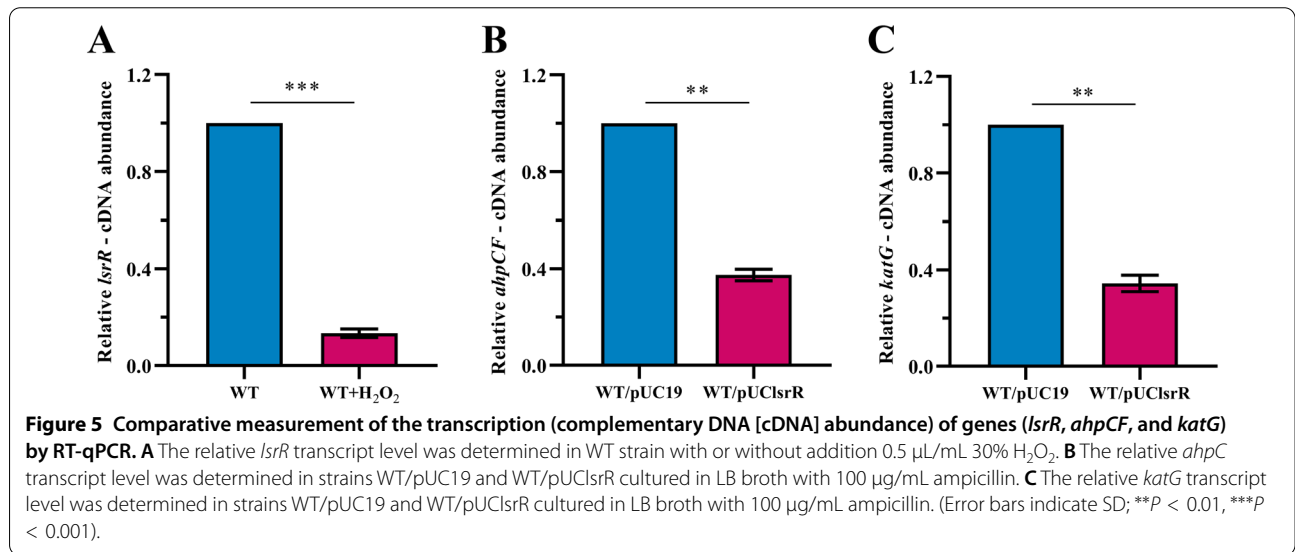
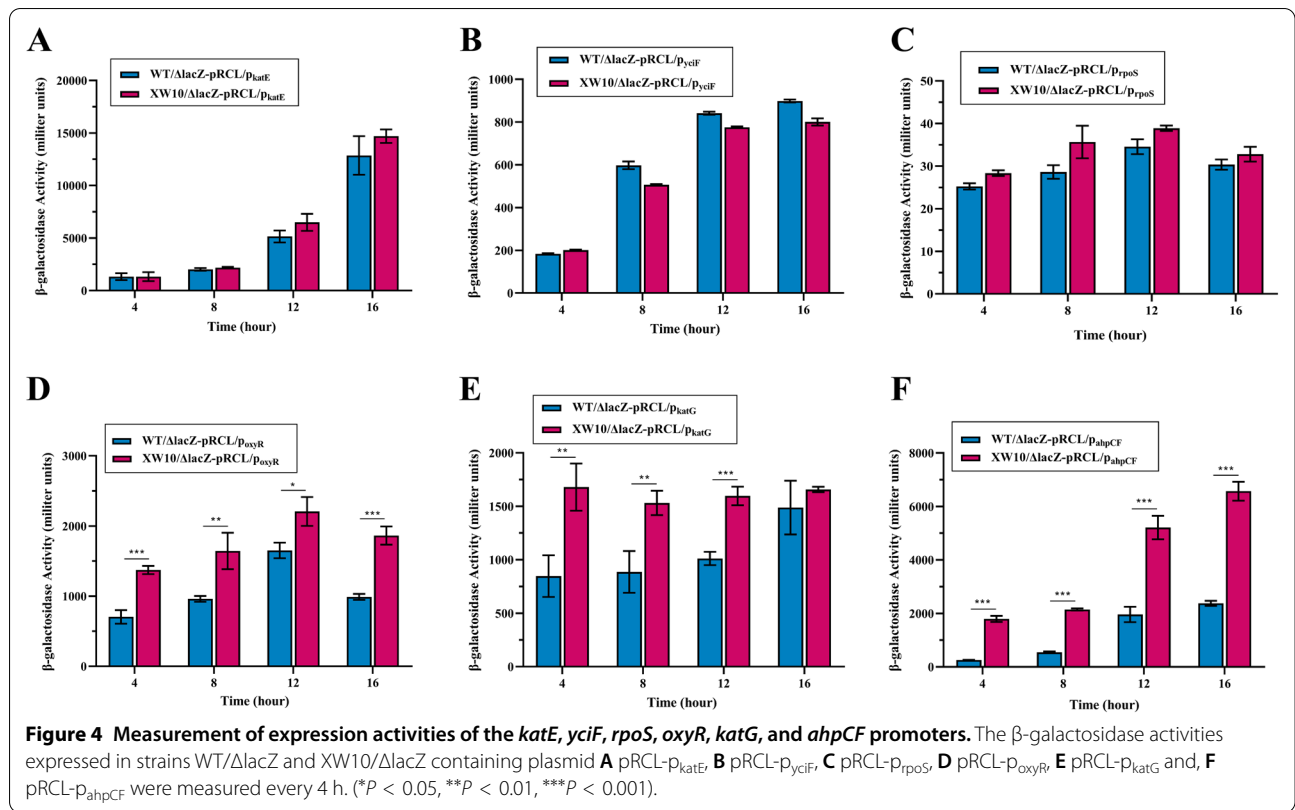
To assess whether the regulatory effects of LsrR on *oxyR*, *rpoS*, *ahpCF*, *katE*, *yciF*, and *katG* were transcriptionally

mediated, a system of β -galactosidase report plasmids was constructed to measure expression levels of the promoters of target genes. The data showed that the deletion of *lsrR* had no effect on the transcriptional activities of the *katE*, *yciF*, and *rpoS* promoters (Figures 4A–C). Compared with the WT strain, the deletion of *lsrR* significantly increased the transcription activity of the promoters of *oxyR* (1.95-, 1.71-, and 1.88-fold at 4, 8 and 16 h, respectively), *katG* (1.98-, 1.73-, and 1.58-fold at 4, 8, and 12 h, respectively), and *ahpCF* (7.0-, 3.94-, 2.66- and 2.76-fold at 4, 8, 12 and 16 h, respectively) seen in Figures 4D–F. Taken together, these results suggested that LsrR decreases the bacterial ability to respond to hydrogen peroxide stress by downregulating the expression of hydrogen peroxide stress-associated genes *oxyR*, *katG* and *ahpCF*.

H₂O₂ stress decreased the expression of *lsrR*, and overexpression of *lsrR* decreased the expression of *ahpCF*, and *katG*

To investigate whether the expression level of *lsrR* in ECM5 was affected by H₂O₂ stress, 0.5 μ L 30% H₂O₂ was added and the transcript level of *lsrR* decreased by 7.5-fold as shown in Figure 5A. The RT-qPCR experiments were performed to determine whether





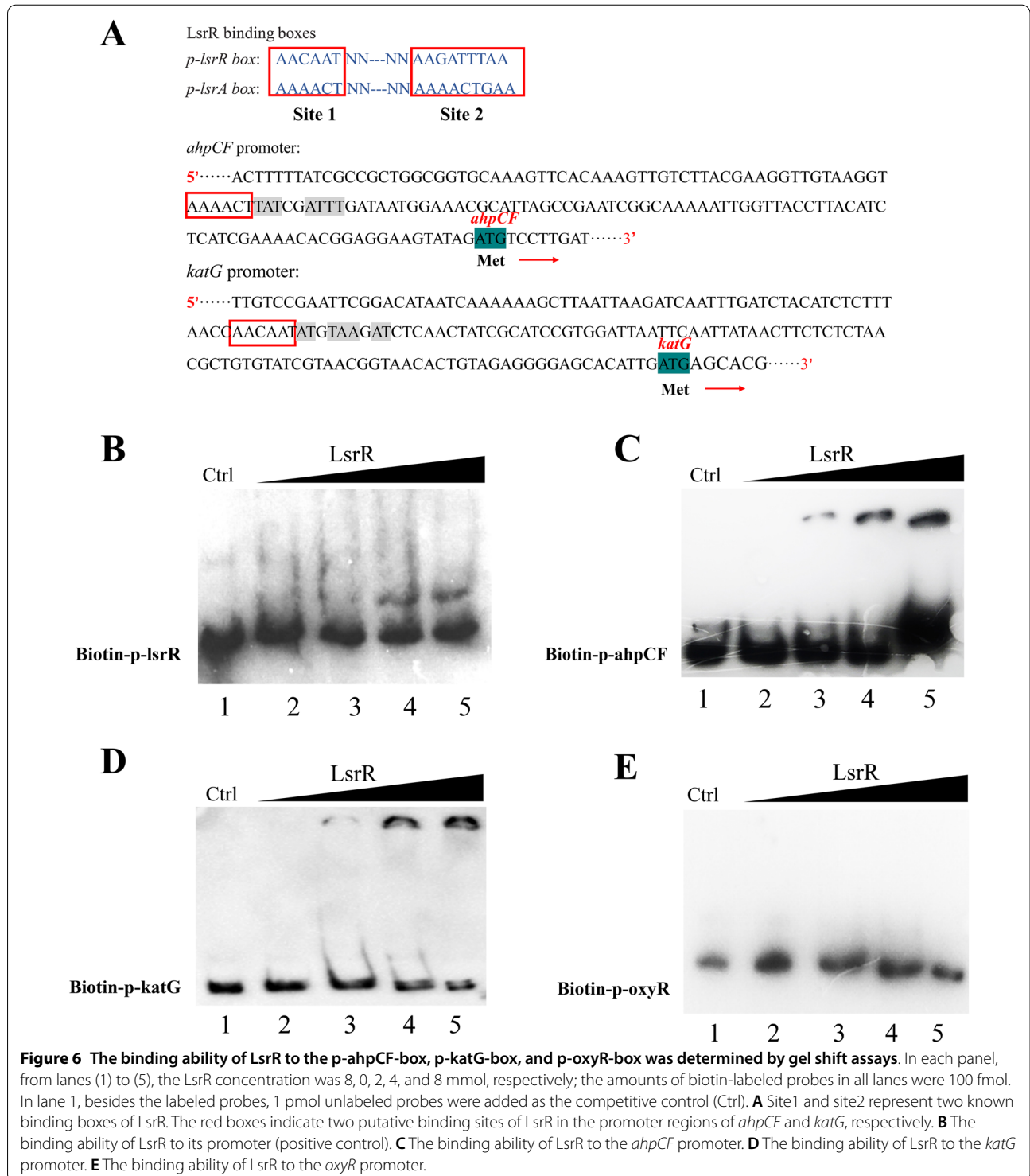
overexpression of *lsrR* affected the transcript levels of the catalase encoding gene *katG* and alkyl hydroperoxide reductase encoding gene *ahpCF*. The data showed that the transcript level of *ahpCF* and *katG* of the *lsrR*-overexpressing strain WT/pUC1sR was decreased by 2.7- and 2.9-fold when compared with its parental

strain WT/pUC19 as seen in Figures 5B, C. The expression level of *lsrR* was inhibited by H_2O_2 stress and *lsrR* reduced the expression of *ahpCF* and *katG*, so it can be inferred that MPEC can adapt to the H_2O_2 stress conditions by mediating *LsrR* expression.

LsrR binding to *ahpCF* and *katG* promoters

Our previous study showed that *lsrR* and *lsrA* promoter regions have two LsrR binding boxes, AAAACT and AAAACTGAA in the p-*lsrA*-box and AACAAT and

AAGATTTAA in the p-*lsrR*-box, and the motifs contain high ratios of A and T [27]. On this basis, it was speculated that a putative binding site of LsrR existed in the promoter regions of *ahpCF* and *katG* genes (Figure 6A)



and the results suggested that LsrR might repress the two genes by direct binding to their promoter regions. According to sequence alignment, the putative binding site of LsrR in the *oxyR* promoter region was not found.

To verify the above hypothesis, electrophoretic mobility shift assays were performed to confirm whether LsrR binds to the *oxyR*, *ahpCF* and *katG* promoter regions. A positive control assay confirmed that LsrR could bind to its own promoter region (Figure 6B). As shown in Figure 6C, D, clearly shifted bands of protein-DNA complex were detected at LsrR concentrations of 4, 8 and 12 mM, and the intensity of the shifted band was enhanced as the amount of LsrR was increased. The results confirmed that LsrR could specifically bind to the promoter regions of *ahpCF* and *katG*, indicating that LsrR could directly regulate the transcription of *ahpCF* and *katG*. However, the shifted band of the protein-DNA complex was not detected when the promoter region of *oxyR* was used as a probe (Figure 6E), suggesting that LsrR cannot bind to the *oxyR* promoter regions. This suggested that LsrR might regulate the expression of *oxyR* through several indirect pathways and indicated that it had a complicated regulatory pattern in the regulation of bacterial oxidative stress response.

The β -galactosidase assays of the mutated promoters of *ahpCF* and *katG*

Based on sequence alignment, it was speculated that a putative binding site of LsrR existed in both promoter regions of *ahpCF* and *katG* genes. The EMSA results showed that LsrR could directly bind to the promoter regions of *ahpCF* and *katG*, which led to the hypothesis that these two putative sequences may be crucial for LsrR binding. To further verify this, mutant β -galactosidase

reporter plasmids pRCL-P_{ahpCFM6} and pRCL-P_{katGM6} were constructed and the data in Figure 7A showed that compared with pRCL-P_{ahpCF}, pRCL-P_{ahpCFM6} was associated with an apparent increase in the expression of *lacZ* throughout the growth cycle. The expression of *lacZ* in pRCL-P_{katGM6} increased only before 4 h compared with pRCL-P_{katG} as shown in Figure 7B, so these results suggested that the sequence AAAACT in the promoter region of *ahpCF* was essential for the binding of LsrR in DCM5 strain. However, mutation of AACAAAT in the promoter region of *katG* did not reduce the binding ability of LsrR throughout the growth cycle.

Discussion

Bacteria can precisely regulate gene expression to promote their survival in various environments or hosts. Bacterial AI-2 quorum sensing is common and is involved in a variety of biological processes, including signal transduction, drug resistance, pathogenicity, motility, and interbacterial competition [23, 33–36]. In *E. coli*, AI-2 can regulate some physiological processes through the AI-2 receptor protein LsrR and the AI-2 metabolic pathway. The previous study showed that AI-2 relies on the intracellular receptor LsrR to increase the β -lactam antibiotic resistance of a broad-spectrum β -lactamase-positive *E. coli* isolated from cows with mastitis [37]. Another subsequent study indicated that exogenous AI-2 can down-regulate the transcription of folate biosynthesis-related genes *folA*, *folC*, *luxS*, *metE*, and *metH* through non-LsrR-dependent pathways, increasing avian pathogenic *Escherichia coli* (APEC) sensitivity to trimethoprim-sulfamethazole [38]. These studies supported the regulatory role of AI-2 or LsrR in bacterial adaption to environmental stimulus. However, whether

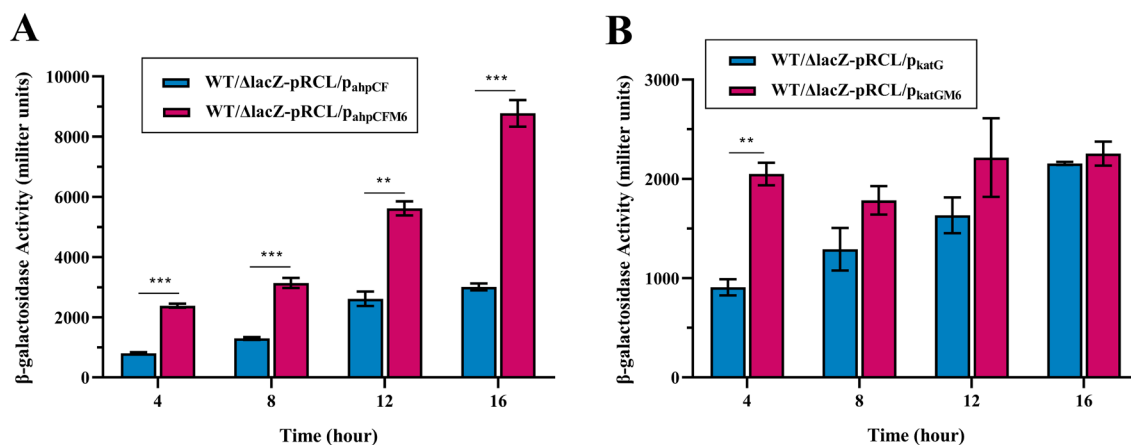


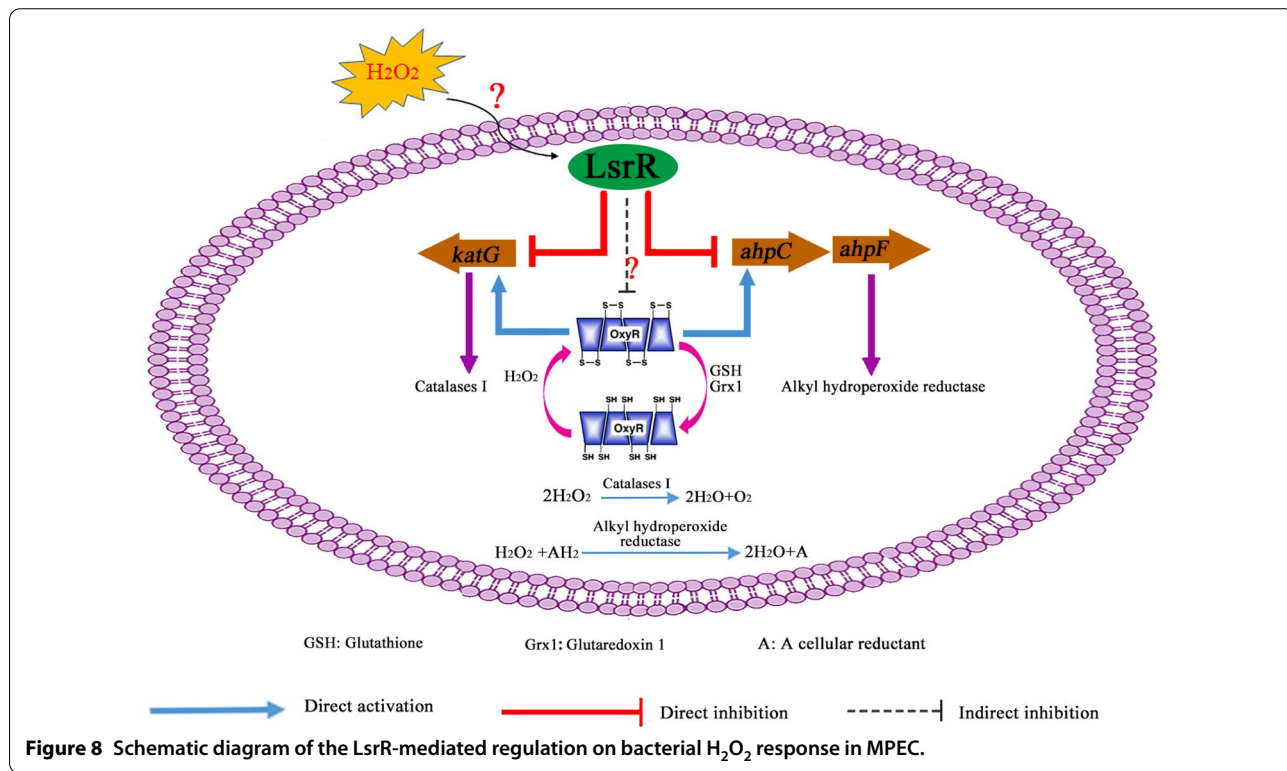
Figure 7 Expression activities of the *ahpCF* and *katG* promoters and their mutant promoters. β -galactosidase activities of the strains with plasmids **A** pRCL-P_{ahpCF} or pRCL-P_{ahpCFM6}, **B** pRCL-P_{katG} or pRCL-P_{katGM6} were measured every 4 h. (** $P < 0.01$, *** $P < 0.001$).

there is a relationship between LsrR and the regulation of bacterial oxidative stress response has not been reported yet. This study explored the relationship between LsrR and bacterial oxidative stress regulation in MPEC and clarified the regulatory details of LsrR on the oxidative stress-related genes.

Oxidative stress can trigger multiple responses that have evolved as complex regulatory systems in bacterial pathogens, aiding bacteria to cause persistent infections [39–41]. A previous study indicated that the LsrR controls invasiveness of *Salmonella* Typhimurium by regulating *Salmonella* pathogenicity island-1 (*SPI-1*) and flagella genes [42]. A subsequent study demonstrated that the *lsr* operon is involved in mediating the virulence of APEC but the regulatory mechanism remains unclear [43]. In *Xanthomonas oryzae* pv. *oryzae*, OxyR degrades hydrogen peroxide in rice by regulating the expression of catalase CatB, and then promotes bacterial infectivity [44]. The data from this study showed that the expression of LsrR was affected by H₂O₂ stress in the environment and that LsrR can directly regulate the expression of oxidative stress response associated genes *ahpCF* and *katG* suggesting that MPEC might respond to the H₂O₂ stress in an LsrR-dependent pathway. Because the ability to respond to oxidative stress of bacteria is very important for its infection process in the host, it was speculated

that LsrR may also be associated with the regulation of bacterial pathogenicity in MPEC, so it was important to explore the regulatory role of LsrR on bacterial pathogenicity or other important physiological processes in future work.

The EMSA results showed that LsrR directly binds to the promoter regions of *ahpCF* and *katG*, which led to the hypothesis that p_{ahpCF}: AAAACT and p_{katG}: AACAAT may be crucial for LsrR binding. To verify this, mutant β-galactosidase enzyme activity reporter plasmids pRCL-P_{ahpCF}M6 and pRCL-P_{katG}M6 were constructed and their β-galactosidase activities were measured. The results suggested that the AAAACT sequence in the promoter region of *ahpCF* was essential for the binding of LsrR in DCM5 strain, which was consistent with our previous results [27], but the mutation of the AACAAT sequence in the promoter region of *katG* did not promote the transcriptional activity of *katG* promoter throughout the growth cycle. The previous study also has shown that LsrR binding sequences contained high ratios of A and T, but do not need to share a high level of sequence homology [27]. A subsequent study indicated that mutation of nucleotides in the vicinity of p-lsrR-box sequence AAC AAT–AAGATTTAA also affected the transcriptional activity of the *lsrR* promoter, which might be associated with binding of CytR repressor [45], therefore it was



speculated that the mutation of the binding site in the *katG* promoter region may cause the recognition and binding by other transcriptional repressors.

According to present and previous studies, a schematic diagram was made to illustrate the regulatory mode of LsrR on the H₂O₂ stress response of MPEC (Figure 8). These data showed that LsrR decreased H₂O₂ stress tolerance ability of MPEC by directly or indirectly inhibiting the transcriptions of H₂O₂ stress response associated genes including *ahpCF*, *katG*, and *oxyR*. The transcription of *lsrR* exhibited a significant decrease in response to the H₂O₂ stress. These results led to the conclusion that MPEC can adapt to H₂O₂ stress conditions by reducing the transcription of *lsrR*, which contributed to increased expression of H₂O₂ stress response associated genes. But how this bacterium regulates the *lsrR* expression under the H₂O₂ stress conditions remains obscure and needs to be further explored.

Abbreviations

E. coli: *Escherichia coli*; MPEC: mammary pathogenic *E. coli*; APEC: avian pathogenic *Escherichia coli*; ROS: reactive oxygen species; H₂O₂: hydrogen peroxide; QS: quorum sensing; IPTG: isopropyl β-D-thiogalactopyranoside; LB: Luria–Bertani; AI-2: autoinducer 2; ONPG: ortho-Nitrophenyl-β-galactoside; CFU: colony-forming units; PCR: polymerase chain reaction; RT-qPCR: real-time reverse transcription-quantitative PCR.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant Number 31672571) and the Doctoral Start-Up Foundation of Linyi University (Grant Number LYDX2020BS031). We thank International Science Editing for editing this manuscript.

Authors' contributions

HW: investigation, methodology, experiments, writing—original draft, writing—review and editing, data curation. FS: methodology, data curation, writing—review and editing. JS and JX: methodology and experiments. XC: investigation, data curation. JN: formal analysis, writing—review and editing. LY: methodology, writing—review and editing. TX: conceptualization, writing—review and editing, supervision and funding acquisition. All authors read and approved the final manuscript.

Declarations

Competing interests

The authors declare that they have no competing interests.

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Received: 16 May 2021 Accepted: 11 September 2021

Published online: 02 October 2021

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