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The transcription factor YbdO attenuates the pathogenicity of avian pathogenic *Escherichia coli* by regulating oxidative stress response

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Abstract

Avian pathogenic *Escherichia coli* (APEC) is a significant pathogen infecting poultry that is responsible for high mortality, morbidity and severe economic losses to the poultry industry globally, posing a substantial risk to the health of poultry. APEC encounters reactive oxygen species (ROS) during the infection process and thus has evolved antioxidant defense mechanisms to protect against oxidative damage. The imbalance of ROS production and antioxidant defenses is known as oxidative stress, which results in oxidative damage to proteins, lipids and DNA, and even bacterial cell death. APEC uses transcription factors (TFs) to handle oxidative stress. While many TFs in *E. coli* have been well characterized, the mechanism of the YbdO TF on protecting against oxidative damage and regulating the virulence and pathogenicity of APEC has not been clarified. Here we focus on the regulatory mechanism of YbdO on the pathogenicity of APEC. The results from this study showed that YbdO attenuated the pathogenicity of APEC in chicks infection models by inhibiting the expression of virulence genes *fepG* and *ycgV* using quantitative real-time reverse transcription PCR (RT-qPCR) experiments. The electrophoretic mobility shift assays (EMSA) confirmed that YbdO specifically bound to the promoters of *fepG* and *ycgV*. Additionally, YbdO increases H_2O_2-induced oxidative damage to APEC via repressing the expression of oxidative stress response genes *sodA*, *soxR*, *ahpC*, *ahpF*, *katG*, and *oxyR* by binding to their promoter regions. The repression effect facilitates host immune response to eliminate APEC and to generate beneficial immune protection to the body, thereby indirectly attenuating the pathogenicity of APEC. These findings might provide further insights into the mechanism of oxidative damage to APEC and offer new perspectives for further studies on the prevention and control of APEC infections.

Keywords Avian pathogenic *Escherichia coli*, ROS, Antioxidant defenses, Oxidative stress, YbdO, Pathogenicity

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Introduction

Avian pathogenic *Escherichia coli* (APEC) causes diverse localized and systemic infections in different species of poultry, including chickens, ducks, turkeys, and other avian species [1–3]. Scientific evidence suggests that the outbreak of APEC in the poultry industry can lead to high levels of mortality (up to 20%) and morbidity and the considerable economic losses due to decreased meat (2% decline in live weight, and 2.7% deterioration in feed conversion ratio) and egg productions (up to 20%), declined hatch rates, and elevated condemnation of carcasses (up to 43%) of infected poultry at slaughter [1, 4, 5]. APEC generally colonizes in the avian intestinal and respiratory tracts as a commensal member of the intestinal and respiratory microbiome. However, it can result in airsacculitis in the presence of stressors, followed by a generalized infection due to inhalation or entry into bloodstream and internal organs such as heart, liver, lungs, spleen, kidneys, and reproductive organs. This can occur through blood circulation in poultry, leading to pericarditis, perihepatitis, peritonitis, cellulitis, arthritis, and salpingitis, which may progress to septicemia and even death [1, 2, 4, 6]. Although APEC infections are secondary infections, i.e., they often occur subsequent to viral or mycoplasma infections, APEC is yet considered to be a primary pathogen causing diverse infections in healthy poultry [2, 4, 6, 7].

During the course of APEC infections, neutrophils and macrophages in host produce reactive oxygen species (ROS), including superoxide anion radicals ($O_2{\cdot}^-$), hydroxyl radicals (·OH), and hydrogen peroxide (H_2O_2), which can damage to membrane lipids, proteins, and DNA, alter enzyme activity by damaging iron-sulfur (Fe-S) clusters in enzymes, and even cause bacterial cells death [8–12]. In order to survive, APEC has evolved antioxidant enzymes (superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT)) to protect bacterial cells against oxidative damage due to SOD converting $O_2{\cdot}^-$ to less toxic H_2O_2, which is further broken down into H_2O by GPx or CAT [9, 11, 13, 14]. However, the imbalance of ROS production and antioxidant defenses is called for oxidative stress, which can cause the accumulation of oxidative products, resulting in oxidative damage to proteins, lipids, and DNA, and even bacterial cells death [9, 11, 15, 16]. Therefore, oxidative stress is considered as an action mechanism of antimicrobial agents to kill bacteria.

To rapidly sense and respond to extracellular environmental fluctuations, including oxidative stress, acidic stress, temperature, and antibiotics, bacteria have developed transcription factors (TFs) and employ TFs to regulate expression of complex gene networks as well as maintain homeostasis [17]. Many studies have revealed that TFs are important components of the bacterial cellular response to ROS by regulating SOD, GPx, and CAT that facilitate bacterial cells to return to homeostasis [17–19]. YbdO is a TF belonging to the LysR-type family with an N-terminal DNA-binding helix-turn-helix (HTH) motif and a C-terminal co-factor-binding domain in *E. coli* [20–23]. Although previous studies demonstrated that YbdO contributed to *E. coli* K1 invasion of human brain microvascular endothelial cells (HBMECs) by directly activating the expression of K1 capsule encoding gene *kpsMT* and *neuDBACES* to increase K1 capsule synthesis, *ybdO* transcription was repressed by histone-like nucleoid structuring protein (H-NS) by binding to the *ybdO* promoter [20, 24–26]. Fan et al. subsequently confirmed that *ybdO* transcription repression were relieved by H-NS sensing the acidic pH within endosomes *E. coli* K1 invasion, resulting in increased YbdO-dependent capsule synthesis, thereby promoting the pathogenicity of *E. coli* K1 [20]. However, the contribution of YbdO to the virulence and pathogenicity of APEC remains largely unknown.

In this study, we investigated the contribution of *ybdO* to APEC CE1 virulence by combining oxidative stress and animal infection models. Additionally, quantitative real-time reverse transcription PCR (RT-qPCR) experiments and electrophoretic mobility shift assays (EMSA) were performed to investigate the regulatory mechanism of YbdO on the virulence and pathogenicity of APEC CE1. Hence, this study might deepen our understanding of the mechanism of oxidative damage to APEC.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains include the wild-type strain APEC CE1, the *ybdO* gene mutant strain CE1Δ*ybdO*, the *ybdO* gene complemented strain CE1Δ*ybdO*/pC*ybdO*, and the *ybdO* gene overexpressed strain CE1/pUC*ybdO* (Table 1). In order to ensure the uniformity in the cultivation conditions of these strains, the low copy plasmid pSTV28 was electroporated into CE1Δ*ybdO* and CE1 to generate CE1Δ*ybdO*/pSTV28 and CE1/pSTV28, and pUC19 was electroporated into CE1 to generate CE1/pUC19. Thus, the wild-type strain CE1/pSTV28, the mutant strain CE1Δ*ybdO*/pSTV28 and the complemented strain CE1Δ*ybdO*/pC*ybdO* were grown in Luria-Bertani (LB) medium with 16 μg/mL chloramphenicol at 37 °C, and the overexpressed strain CE1/pUC*ybdO* and its parent strain CE1/pUC19 were grown in LB medium with 100 μg/mL ampicillin at 37 °C. These strains were got from our previous research (not published).

H_2O_2 stress assays

H_2O_2 stress assays were performed to detect the effects of H_2O_2 on the survival ability of CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/pC*ybdO*, CE1/pUC19, and CE1/

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source
Strains		
E. coli		
CE1	Avian pathogenic *E. coli* (APEC) CE1, wild-type	Laboratory stock
CE1Δ*ybdO*	CE1 *ybdO*-deletion mutant	Laboratory construction
CE1/pSTV28	CE1 with the empty vector pSTV28, Cm^r	Laboratory construction
CE1Δ*ybdO*/pSTV28	CE1Δ*ybdO* with the empty vector pSTV28, Cm^r	Laboratory construction
CE1Δ*ybdO*/pC*ybdO*	CE1Δ*ybdO* with the complement plasmid pC*ybdO*, Cm^r	Laboratory construction
CE1/pUC19	CE1 with the empty vector pUC19, Amp^r	Laboratory construction
CE1/pUC*ybdO*	CE1 with the overexpression plasmid pUC*ybdO*, Amp^r	Laboratory construction
Plasmids		
pSTV28	Low copy number cloning vector, Cm^r	Takara
pC*ybdO*	pSTV28 with *ybdO* gene, Cm^r	Laboratory construction
pUC19	Cloning vector, Amp^r	Takara
pUC*ybdO*	pUC19 with *ybdO* gene, Amp^r	Laboratory construction

Cm^r, Amp^r, and Kan^r, denote chloramphenicol, ampicillin, and kanamycin resistance, respectively

pUC*ybdO*, according to described previously and modified as follows [27, 28]. Briefly, the overnight cultures of CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/pC*ybdO*, CE1/pUC19, and CE1/pUC*ybdO* were each adjusted to an OD_{600} of approximately 0.03 in 3.0 mL of fresh LB broth with the appropriate antibiotic, and then incubated at 37℃ for 4 h with shaking. After 4 h of incubation, CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/pC*ybdO*, CE1/pUC19, and CE1/pUC*ybdO* were centrifuged at 5000 g for 2 min, and washed twice with the sterile phosphate-buffered saline (PBS) buffer (pH 7.4). Subsequently, 100 μL bacterial cells were inoculated into 10 μmol/L H_2O_2 of LB broth with the appropriate antibiotic, and then these cultures were incubated at 37℃ for 1 h with shaking. After 1 h of incubation, 10-fold serial dilutions of cultures were performed by successive transfer (0.1 mL) through 8 microfuge tubes containing 0.9 mL of LB broth, and 100 μL dilutions of each microfuge tube were dropped and spread onto LB agar plates to cultivate for 18 h at 37℃. After 18 h of cultivation, the colony-forming units (CFU) of surviving bacteria were counted, and the survival rates CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/pC*ybdO*, CE1/pUC19, and CE1/pUC*ybdO* were cultivated. The survival rates of CE1/pSTV28 and CE1/pUC19 were designated as 100%, respectively. The experiments were repeated independently 3 times.

Animals

One-day-old chicks were purchased from Rizhao Langya Chicken Company Ltd. These chicks were adequately fed food and water (a complete diet without antibiotics) and a 12 h illumination period per day. Healthy 7-day-old chicks were selected for the animal infection experiment. The care and management of all chicks were in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines of Linyi University (Protocol Approval Number: LYU20240109) and the procedures adhered to the guidelines of the NIH Guide for the Care and Use of Laboratory Animals, as well as the regulations for the Administration of Affairs Concerning Experimental Animals as mandated by the State Council of the People's Republic of China regarding euthanasia. After the experiments, these chicks were euthanized by intravenous injection of pentobarbital sodium in wing vein at a dose three times higher than the anesthetic dose. Subsequently, the loss of consciousness was rapid, followed by cessation of respiration and heartbeat, and then exsanguination were performed to confirm euthanasia.

Animal infection experiments

After 7 days of feeding, a total of 48 chicks were used in the animal infection experiments to evaluate the virulence of CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/pC*ybdO*, CE1/pUC19, and CE1/pUC*ybdO*. Firstly, the above 5 bacterial strains were inoculated on fresh LB agar with the appropriate antibiotic. After overnight of cultivation, these bacterial cells were scraped down from LB agar, washed three times and resuspended using PBS, and then adjusted to 1.0×10^9 CFU/mL. Next, 48 chicks were divided randomly into 6 groups, with 8 chicks in each group, and then chicks from each group were intramuscularly injected with 1.0 mL of 1.0×10^9 CFU/mL (0.5 mL of each leg) of each strain. The negative controls was intramuscularly injected with 1.0 mL of PBS. The clinical signs of infected chicks, such as lethargy, anorexia and hypothermia, were observed, and the survival and death of chicks were recorded until 7 days post-infection. The survival curve was drawn to compare the virulence of CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/pC*ybdO*, CE1/pUC19, and CE1/pUC*ybdO*.

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

Total RNA of CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/pC*ybdO*, CE1/pUC19, and CE1/pUC*ybdO* was extracted using RNAprep Pure Cell/Bacteria Kit (Tiangen, Beijing, China) and cDNA was synthesized using HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China), according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed with RT primers following the instructions of

HiScript III All-in-one RT SuperMix Perfect for qPCR (Vazyme) on the Appliedbiosystems Quant Studio 1 plus (Thermo Fisher Scientific, Shanghai, China). Relative gene expression was normalized by subtracting the Ct value of the housekeeping gene *16 S* rRNA using the $2^{-\Delta\Delta Ct}$ method (where Ct = cycle threshold). All of reverse transcription qPCR assays were repeated at least 3 times with similar results. All primers used in this study were shown in Table 2.

Table 2 Oligonucleotide primers used in this study

Primer name	Oligonucleotide (5′-3′)[a]	Product size/bp	Tm/℃
rt-*16 S*-f	TTTGAGTTCCCGGCC	259	60
rt-*16 S*-r	CGGCCGCAAGGTTAA		
rt-*oxyR*-f	GGGAATGCTGCTGGTGGATC	210	60
rt-*oxyR*-R	GGGTCTGTGCTTCATGCAGA		
rt-*soxR*-f	GGCGACCATTGGTGAAGCGT	180	60
rt-*soxR*-r	CAATCACTGCGCGAAAGGCA		
rt-*ycgV*-f	AGCATCTTTTCCGGCGGTTC	187	60
rt-*ycgV*-r	AAATTCCCCTGGCTCCTGCC		
rt-*ahpC*-f	AGCAGCTCTGAAACCATCGC	183	60
rt-*ahpC*-r	GTCACGGCCAATGCCTTCAG		
rt-*ahpF*-f	AAACGTGCGGCAGAAGAGCT	199	60
rt-*ahpF*-r	GCCCTTCAGTCTTCGGTACA		
rt-*katG*-f	GCGCAGATGCCATTACCTCT	171	60
rt-*katG*-r	ACGGATCCGGGGATAATTTCC		
rt-*fepG*-f	TGATTTACGTCTCTCGCC	180	60
rt-*fepG*-r	GTAAACGCCATTCGGTGA		
rt-*sodA*-f	ACCACACCAAACACCATCAG	185	60
rt-*sodA*-r	ACCTTTCCAGAACAGGCTGT		
ahpC-biotin-f	TCGAGTAAAAGGCATAACCT	341	50
ahpC-r	TATACTTCCTCCGTGTTTTC		
katG-biotin-f	ATAGTGTGGCTTTTGTGAAA	328	50
katG-r	CAATGTGCTCCCCTCTACAG		
kpsM-biotin-f	CCATTTGATGATGTGATCCT	287	50
kpsM-r	TTTTCTGAGAAATTAACTCT		
oxyR-biotin-f	AACGGGCAGTGACTTCAAGG	140	50
oxyR-r	TATCCATCCTCCATCGCCAC		
sodA-biotin-f	CTTCTTATCCTCATCATTTT	284	50
sodA-r	ATTCATCTCCAGTATTGTCG		
soxR-biotin-f	ATCAATGTTAAGCGGCTGGT	160	50
soxR-r	AAATCGCTTTACCTCAAGTT		
ycgV-biotin-f	ATTCTCTGAGAAGCTCATCA	126	50
ycgV-r	ACCACTCCTATATAGTACCC		
fepG-biotin-f	CAATTGAGATGAAACGAG	201	50
fepG-r	ACGAACTTCCATGATAAT		
lacZ-biotin-f	CTGGCCGTCGTTTTACAACG	199	50
lacZ-r	AGCTTTCCGGCACCGCTTCT		

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

Purification of the YbdO protein and electrophoretic mobility shift assays

The YbdO protein was expressed, purified and preserved in 20% (v/v) sterile glycerol according to previously described methods [27]. The putative promoter regions of target genes were amplified by PCR using p-primers (Table 2) from genomic DNA of CE1 and gel-purified. Electrophoretic mobility shift assays (EMSA) were performed by incubating the biotin-labeled DNA fragments with various amounts of purified YbdO in 5×binding buffer to confirm the binding ability of YbdO to the target gene promoters according to previously described methods. The band shifts of the YbdO protein and the target gene promoters were detected and analyzed following the instructions of chemiluminescent EMSA kit (Beyotime, Shanghai, China).

Statistical analysis

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

Results

YbdO reduces the survival of APEC CE1 under H_2O_2 stress condition

To investigate the effect of YbdO on the survival of APEC CE1 under oxidative stress condition, we compared the survival ability of CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/p*CybdO*, CE1/pUC19, and CE1/pUC*ybdO* under H_2O_2 stress condition. As shown in Fig. 1A, the survival rates of CE1Δ*ybdO*/pSTV28 under H_2O_2 stress condition were increased by almost 5.06-fold when compared to that of CE1/pSTV28, and the survival rates of CE1Δ*ybdO*/p*CybdO* were restored. Likewise, the survival rates of CE1/pUC*ybdO* under H_2O_2 stress condition were reduced by almost 7.22-fold when compared to that of CE1/pUC19 (Fig. 1B). These results indicated that the deletion of *ybdO* increased the survival of APEC CE1 under oxidative stress condition, thereby implying that the deletion of *ybdO* contributes to APEC CE1 evading the invasion of host immune system.

YbdO attenuates APEC CE1 virulence in chick infection models

To investigate whether YbdO affects APEC CE1 virulence, the chick infection models were established to evaluate the virulence of CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/p*CybdO*, CE1/pUC19, and CE1/pUC*ybdO*. Chicks of the test groups were intramuscularly infected with 1.0×10^9 CFU of CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/p*CybdO*, CE1/pUC19, and CE1/pUC*ybdO*,

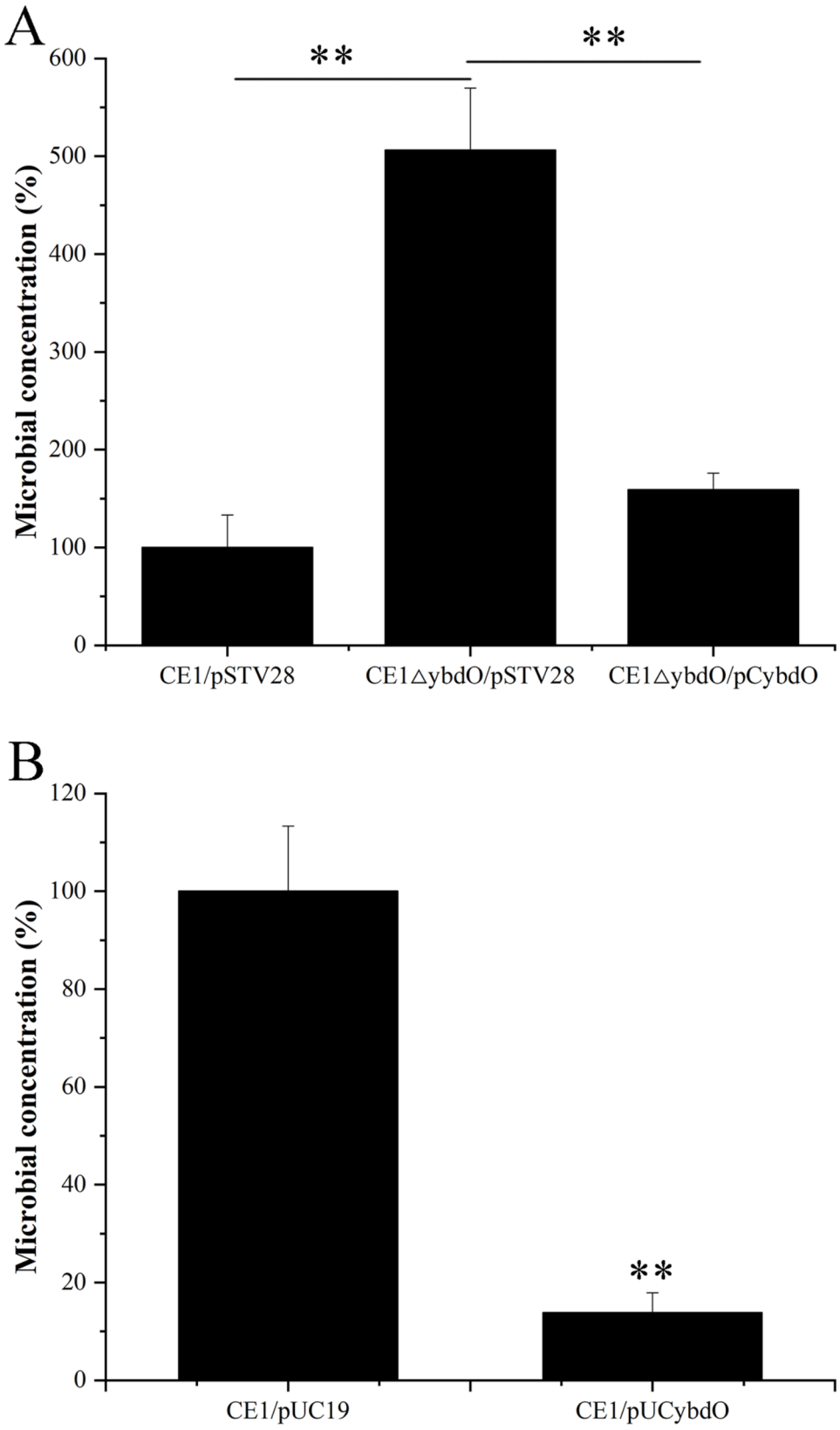

Fig. 1 The survival ability of CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/p*CybdO*, CE1/pUC19, and CE1/pUC*ybdO* under H_2O_2 stress condition. (**A**) The survival rate of CE1/pSTV28, CE1Δ*ybdO*/pSTV28, and CE1Δ*ybdO*/p*CybdO*. The survival rate of CE1/pSTV28 was designated as 100%. (**B**) The survival rate of CE1/pUC19 and CE1/pUC*ybdO*. The survival rate of CE1/pUC19 was designated as 100%. Error bars indicate standard deviations. $^{**}P < 0.01$, indicating the extremely significant difference

respectively, and the chick mortality was observed for 7 days post-infection. On the first day, they began to show lethargy, anorexia and hypothermia in the test groups with CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/p*CybdO*, CE1/pUC19, and CE1/pUC*ybdO*. Meanwhile, the mortality of chicks was recorded in CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/p*CybdO*, CE1/pUC19, and CE1/pUC*ybdO*, but not in the negative controls. As shown in Fig. 2, the mortality of CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/p*CybdO*, CE1/pUC19, and CE1/pUC*ybdO* was 75% (6/8), 100% (8/8), 87.5% (7/8), 87.5% (7/8), and 62.5% (7/8), respectively. These results demonstrated that the deletion of *ybdO* increased APEC CE1 virulence in chicks, further indicating that YbdO might be a transcription repressor of virulence genes expression in APEC CE1.

YbdO down-regulates the expression of virulence genes and oxidative stress response genes

To investigate the role of YbdO in regulation of virulence genes and oxidative stress response genes in APEC CE1, RT-qPCR experiments were conducted to detect the expression of virulence genes and oxidative stress response genes in CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/p*CybdO*, CE1/pUC19, and CE1/pUC*ybdO*, and the results were shown in Fig. 3. These genes include virulence genes *fepG* (encoding ferric enterobactin ABC transporter membrane subunit FepG) and *ycgV* (encoding autotransporter adhesin), and oxidative stress response genes, namely *sodA* (encoding superoxide dismutase (Mn)), *soxR* (encoding redox-sensitive transcriptional activator SoxR), *ahpC* (encoding alkyl hydroperoxide reductase, AhpC component), *ahpF* (encoding alkyl hydroperoxide reductase, AhpF component), *katG* (encoding catalase/hydroperoxidase KatG), and *oxyR* (encoding oxidative stress transcriptional regulator OxyR). As shown in Fig. 3A, the transcription levels of *fepG*, *ycgV*, *sodA*, *soxR*, *ahpC*, *ahpF*, *katG*, and *oxyR* were increased 3.77-fold, 4.10-fold, 2.85-fold, 1.91-fold, 2.05-fold, 1.93–fold, 4.48-fold, and 6.54-fold, respectively, in CE1Δ*ybdO*/pSTV28 when compared to that of CE1Δ*ybdO*/pSTV28, and were restored in CE1Δ*ybdO*/p*CybdO*. Likewise, the transcription levels of *fepG*, *ycgV*, *sodA*, *soxR*, *ahpC*, *ahpF*, *katG*, and *oxyR* in CE1/pUC*ybdO* were reduced 1.61-fold, 3.13-fold, 1.42-fold, 1.91-fold, 3.61-fold, 3.34–fold, 7.46-fold, and 15.63-fold, respectively, when compared to that of CE1/pUC*ybdO* (Fig. 3B). These data indicated that YbdO attenuated the pathogenicity of APEC CE1 through reducing the

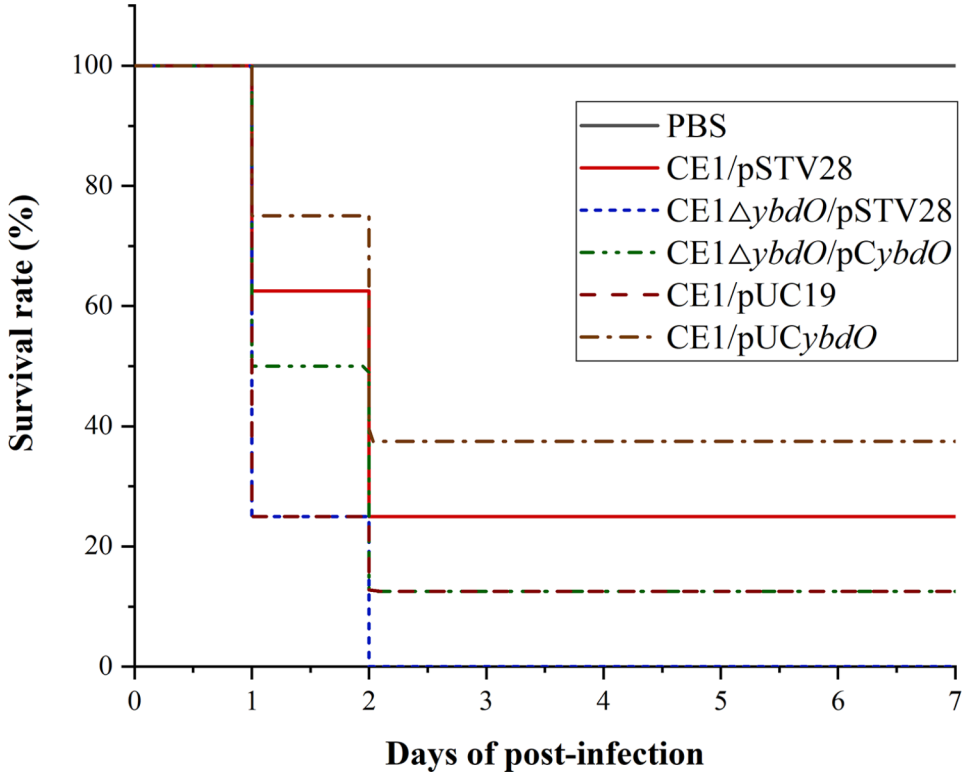

Fig. 2 The survival rates of chicks infected by of CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/p*CybdO*, CE1/pUC19, and CE1/pUC*ybdO* were detected using animal infection experiments. Seven-day-old chicks in the test groups were intramuscularly injected with 1.0×10^9 of CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/p*CybdO*, CE1/pUC19, and CE1/pUC*ybdO*, respectively. Seven-day-old chicks in the control groups were intramuscularly injected with PBS. Survival was monitored until 7 days post-infection

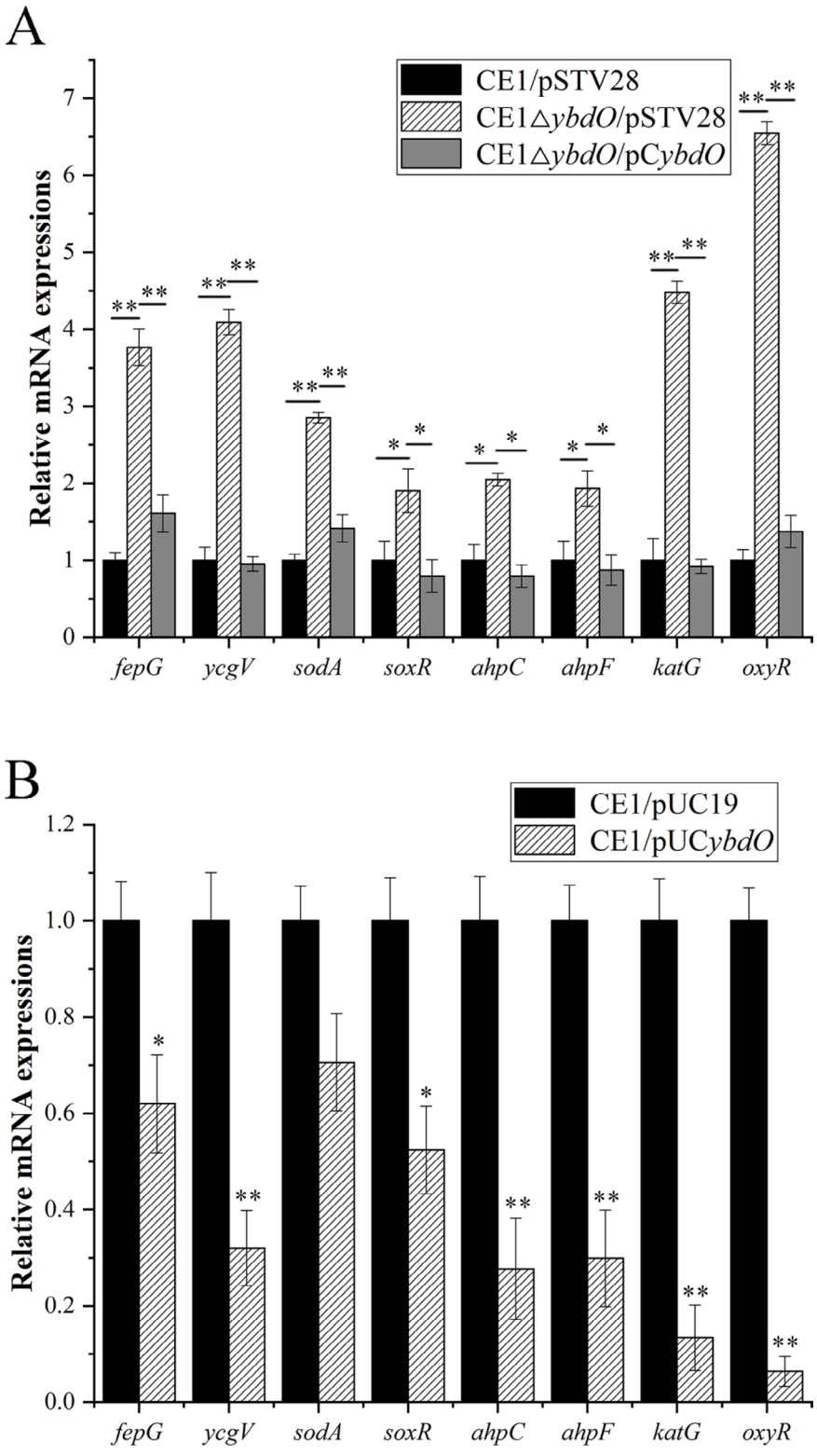

Fig. 3 Relative mRNA expressions of virulence genes and oxidative stress response genes by RT-qPCR in CE1/pSTV28, CE1Δ*ybdO*/pSTV28, CE1Δ*ybdO*/pC*ybdO*, CE1/pUC19, and CE1/pUC*ybdO*. (**A**) Relative transcription levels of *fepG*, *ycgV*, *sodA*, *soxR*, *ahpC*, *ahpF*, *katG*, and *oxyR* were determined by RT-qPCR in CE1/pSTV28, CE1Δ*ybdO*/pSTV28, and CE1Δ*ybdO*/pC*ybdO* cultured in LB broth with 16 μg/mL chloramphenicol. (**B**) Relative transcription levels of *fepG*, *ycgV*, *sodA*, *soxR*, *ahpC*, *ahpF*, *katG*, and *oxyR* were determined by RT-qPCR in CE1/pUC19 and CE1/pUC*ybdO* cultured in LB broth with 100 μg/mL ampicillin. Error bars indicate standard deviations. The relative gene expressions were calculated using the $2^{-\Delta\Delta Ct}$ method. $^{**}P < 0.01$, indicating the extremely significant difference; $^{*}P < 0.05$, indicating the significant difference

transcription levels of *fepG*, *ycgV*, *sodA*, *soxR*, *ahpC*, *ahpF*, *katG*, and *oxyR*.

YbdO directly binds to the promoters of the target genes

To determine the regulatory mechanism of YbdO on *fepG*, *ycgV*, *sodA*, *soxR*, *ahpC*, *katG*, and *oxyR*, EMSA were carried out to detect the binding ability of YbdO to the promoters *fepG*, *ycgV*, *sodA*, *soxR*, *ahpC*, *katG*, and *oxyR*. The purified His_6-tagged YbdO protein was used to bind biotin-labeled DNA fragments containing the putative promoter regions of *fepG*, *ycgV*, *sodA*, *soxR*, *ahpC*, *katG*, and *oxyR*, respectively. As shown in Fig. 4A-H (Figure S1-8), clearly shifted bands of protein-DNA complex were detected at YbdO concentrations of 2, 4, and 8 μM; the intensity of the shifted band was enhanced as the amount of YbdO increased, while the shifted band disappeared in the presence of an approximately 10-fold excess of unlabeled promoter DNA fragment as a specific competitor (Ctrl). Figure 4A (Figure S1) was the positive control in Fig. 4 (Figure S1-8). Figure 4I (Figure S9) was the negative control, the biotin-labeled encoding DNA fragment of *lacZ* was used as a probe, and the shifted band of protein-DNA complex was not detected. These results confirmed that YbdO specifically bound to the promoter regions of *fepG*, *ycgV*, *sodA*, *soxR*, *ahpC*, *katG*, and *oxyR*, indicating that YbdO attenuated the pathogenicity of

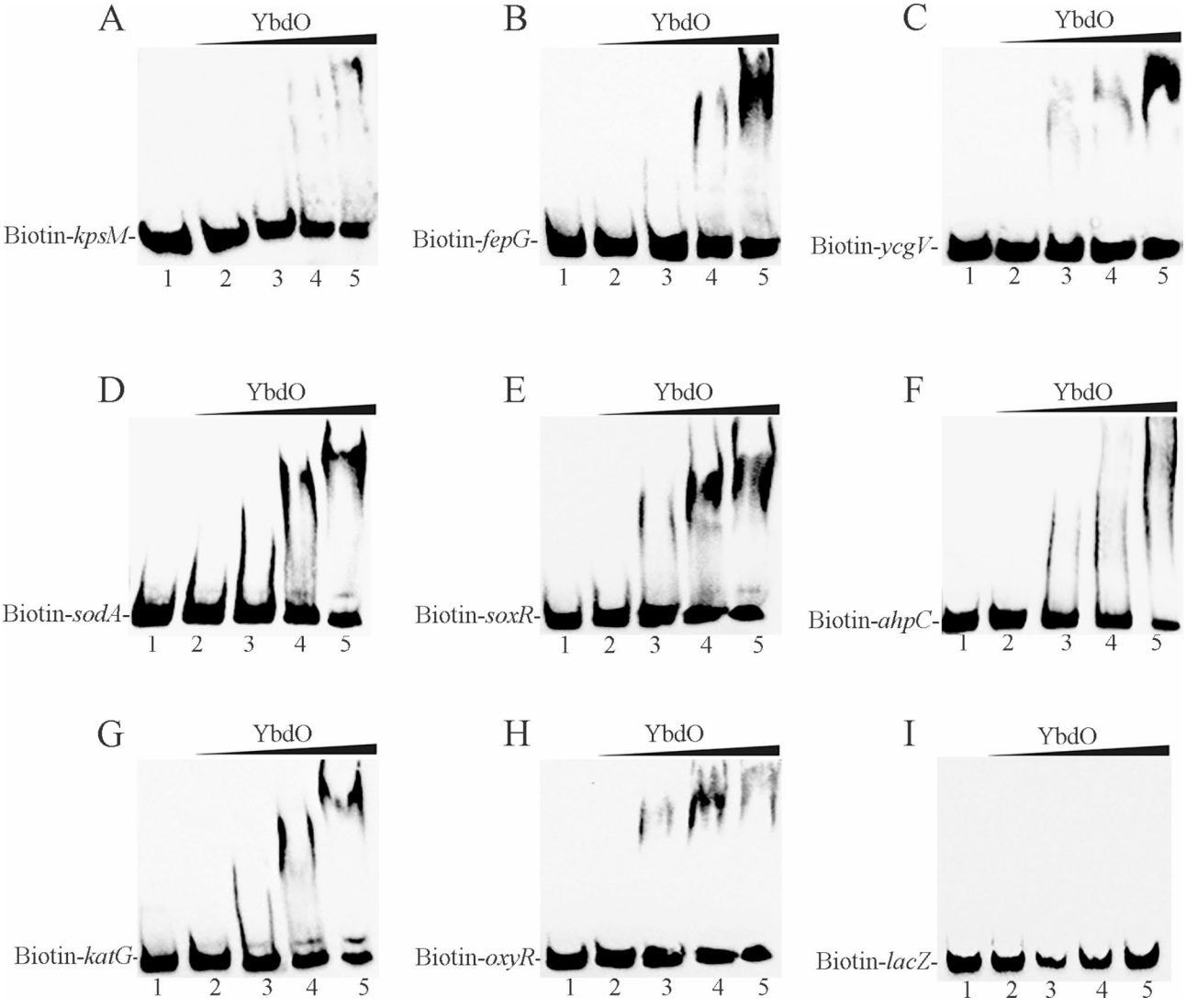

Fig. 4 The binding ability of YbdO to target gene promoters was determined by EMSA. Increasing amounts of YbdO were incubated with Biotin-labeled *kpsM*, *fepG ycgV*, *sodA*, *soxR*, *ahpC*, *katG*, *oxyR*, and *lacZ* (Biotin-*kpsM*, Biotin-*fepG* Biotin-*ycgV*, Biotin-*sodA*, Biotin-*soxR*, Biotin-*ahpC*, Biotin-*katG*, Biotin-*oxyR*, and Biotin-*lacZ*). In each panel, from lanes 1 to 5, the concentrations of YbdO were 8, 0, 2, 4 and 8 μM, respectively; the amount of Biotin-labeled probes in all lanes was 100 fmol. In lane 1, besides the labeled probes, 1 pmol of unlabeled probe was added as the competitive control (Ctrl). (**A**) The positive control, the binding ability of YbdO to the *kpsM* promoter; (**B**) the *fepG* promoter; (**C**) the *ycgV* promoter; (**D**) the *sodA* promoter; (**E**) the *soxR* promoter; (**F**) the *ahpC* promoter; (**G**) the *katG* promoter; (**H**) the *oxyR* promoter; (**I**) The negative control, the binding ability of YbdO to the *lacZ* encoding DNA fragment

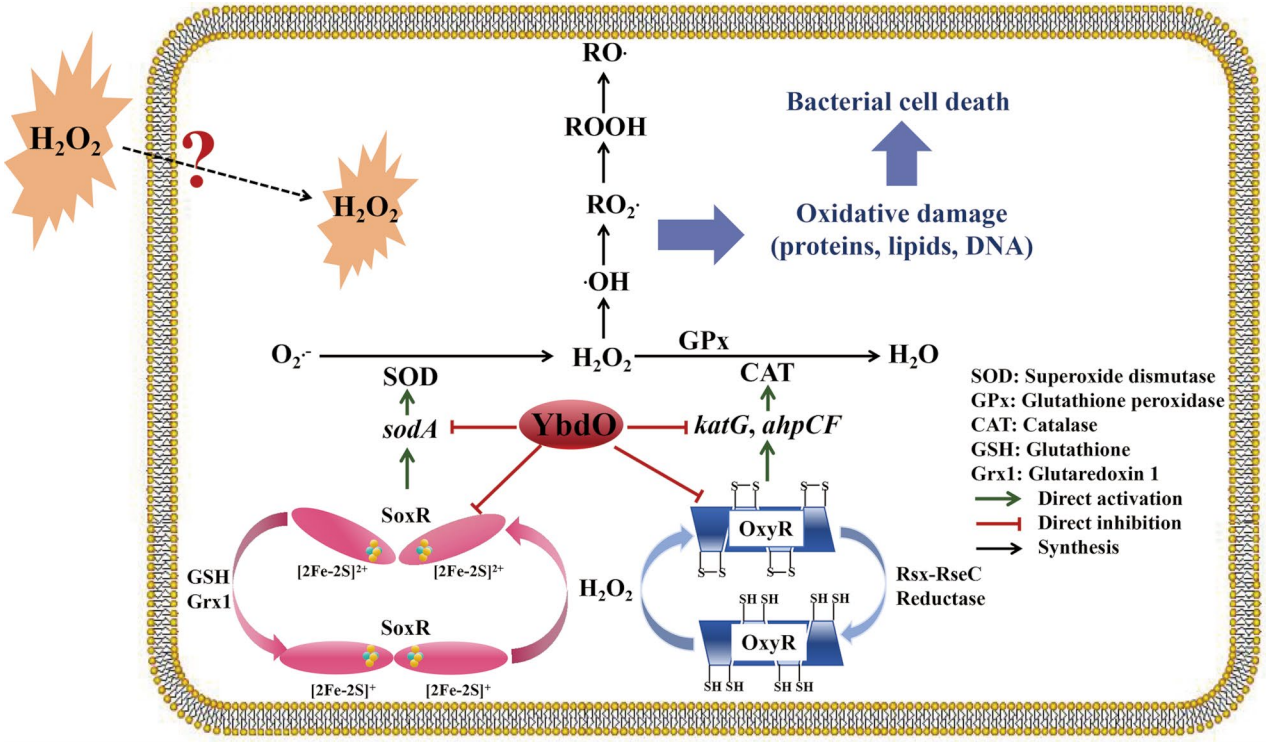

Fig. 5 Schematic diagram of the YbdO-mediated regulation on H_2O_2-induced oxidative stress in APEC [28, 33, 34]

APEC CE1 by directly inhibiting the expression of *fepG*, *ycgV*, *sodA*, *soxR*, *ahpC*, *katG*, and *oxyR*.

Discussion

YbdO is a HTH-type TF that promotes the pathogenicity of *E. coli* K1 by directly activating K1 capsule gene expression to increase K1 capsule synthesis [20]. However, whether YbdO affects the virulence and pathogenicity of APEC has not been clarified. In this study, we found that the deletion of *ybdO* contributed to APEC CE1 virulence by directly upregulating the transcription of virulence gene *fepG* and *ycgV*, thereby promoting APEC CE1 to cause infection in the chick infection models. APEC is well established as the primary cause of infecting poultry that is responsible for severe economic losses to the poultry industry globally and present significant risks to the health of poultry [2, 29, 30]. When APEC invades the host cell, the innate immune response in host is activated, i.e., the innate immune cells include neutrophils and macrophages involving the production of ROS to kill it [5, 31–33]. In order to survive, APEC has evolved defense mechanisms to evade immune response by synthesizing antioxidant enzymes to protect it against ROS damage, including three SODs, namely MnSOD (encoded by *sodA*), FeSOD (encoded by *sodB*), and CuZnSOD (encoded by *sodC*), three CATs, namely hydroperoxidase I (HPI, encoded by *katG*), hydroperoxidase II (HPII, encoded by *katE*), and alkyl hydroperoxide reductase (AhpCF, encoded by *ahpCF*), and one GPx (BtuE, encoded by *btuE*) (Fig. 5) [17, 33–35].

Oxidative stress, a consequence of the imbalance between ROS accumulation and antioxidant defenses, plays an important role in causing oxidative damage to lipids, proteins, and DNA in bacterial cells [11, 36, 37]. Indeed, many studies have investigated the role of oxidative stress in causing APEC death [38–41]. For example, OmpR and EnvZ render APEC greater tolerance to oxidative stress and facilitate the pathogenicity of APEC [40, 41]; IbeA confers increased H_2O_2 resistance to APEC strain BEN2908 [38]; and SodA protects APEC O2 strain E058 against H_2O_2-induced oxidative stress and contributes to the virulence of E058 [38]. This study focused on the effect of YbdO on the extracellular H_2O_2 stimulation under in vitro condition as a distinct ROS species. This is because of H_2O_2 being an essential substance in the ROS defense mechanism and oxidative stress-mediated bacterial cell death [37, 42]. H_2O_2, a stable molecule, diffuses across the bacterial cellular membrane with a specific carrier protein and is converted into H_2O having no damage to bacterial cells or ·OH inducing bacterial cell death [37]. Hence, this study used H_2O_2 as a extracellular stimulant for oxidative stress to investigate the effect of YbdO on H_2O_2-induced oxidative stress response using H_2O_2 stress assays. The results showed that the deletion of *ybdO* significantly increased the adaptation of APEC CE1 to H_2O_2-induced oxidative stress. Combining RT-qPCR,

EMSA and animal infection experiments, these results from this study indicated that YbdO reduced the survival ability of APEC CE1 under H_2O_2 stress condition by directly downregulating the transcription of oxidative stress response genes *sodA*, *soxR*, *ahpC*, *ahpF*, *katG*, and *oxyR*, and attenuated the pathogenicity of APEC CE1, which are not consistent with the results of Fan et al. [20]. This is probably because the strain used in this study is an APEC isolated strain, which is different from *E. coli* K1, and the hosts of the two strains and the virulence and pathogenicity of the two strains to their hosts are different.

Collectively, the results of this current study showed that YbdO attenuated the pathogenicity of APEC CE1 by reducing the expression of virulence genes *fepG* and *ycgV*, and inhibiting antioxidant defense mechanisms through downregulating the transcription of oxidative stress response genes *sodA*, *soxR*, *ahpC*, *ahpF*, *katG*, and *oxyR*. A schematic diagram was made to illustrate the regulatory mode of YbdO on H_2O_2 stress in APEC (Fig. 5). Therefore, this study has provided the evidence to show that YbdO could increase H_2O_2-induced bacterial cellular damage via repressing the expression of antioxidant enzymes. Anyhow, these findings deepen our understanding of the mechanism of oxidative damage to APEC.

The limitations of this study should be recognized. Detailed means of H_2O_2 diffusion from cellular outside to cellular inside were not captured. In other words, how does extracellular H_2O_2 diffuse from cellular outside to cellular inside? Does H_2O_2 diffusion across bacterial cellular membrane require the specific carrier proteins? How do the specific carrier proteins sense and rapidly respond to extracellular H_2O_2? And how to identify the specific carrier proteins? These above questions mentioned have been not reported. Therefore, it is necessary to investigate the mechanism of H_2O_2 diffusion across bacterial cellular membrane in future experiments.

Conclusion

The transcription factor YbdO attenuates the pathogenicity of APEC by directly inhibiting the expression of virulence genes *fepG* and *ycgV* to reduce the virulence. Moreover, YbdO increases H_2O_2-induced oxidative damage to APEC via repressing the expression of oxidative stress response genes *sodA*, *soxR*, *ahpC*, *ahpF*, *katG*, and *oxyR* by binding to their promoter regions, thereby indirectly attenuating the pathogenicity of APEC.

Abbreviations

APEC	Avian pathogenic Escherichia coli
CAT	Catalase
E. coli	Escherichia coli
EMSA	Electrophoretic mobility shift assay
GPx	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HTH	Helix-turn-helix
LB	Luria-Bertani
$O_2^{\cdot-}$	Superoxide anion radicals
·OH	Hydroxyl radicals
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
RT-qPCR	Quantitative real-time reverse transcription PCR
SOD	Superoxide dismutase
TFs	Transcription factors

Supplementary Information

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Supplementary Material 1

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Author contributions

LY: investigation, methodology, experiments, writing original draft, writing review and editing, data curation and funding acquisition. ST and YG: methodology and experiments. SZ, YZ and CX: formal analysis, methodology. XZ: conceptualization, supervision and funding acquisition. All authors read and approved the final manuscript.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

This study was carried out in compliance with the ARRIVE guidelines. Animal experiments were conducted under animal protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Linyi University (Protocol Approval Number: LYU20240109). All animal work was carried out following accordance within the guidelines of the Laboratory Animal Research Center of Linyi University.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Clinical trial number

Not applicable.

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