Current Research in Microbial Sciences 8 (2025) 100351

Contents lists available at ScienceDirect

Current Research in Microbial Sciences

journal homepage: www.sciencedirect.com/journal/current-research-in-microbial-sciences

Cooperative mechanisms of LexA and HtpG in the regulation of virulence gene expression in *Pseudomonas plecoglossicida*

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ARTICLE INFO

Keywords:
Adhesion
Biofilm formation
LexA
Pseudomonas plecoglossicida
Virulence regulation

ABSTRACT

LexA is a well-known transcriptional repressor of DNA repair genes induced by DNA damage in *Escherichia coli* and other bacterial species. Recently, this paradigm—that LexA solely regulates the SOS response—has been challenged as studies reveal its involvement in various biological functions linked to virulence. *Pseudomonas plecoglossicida*, a major pathogen in mariculture, causes substantial economic losses annually in China. Our previous research suggested that LexA might collaboratively regulate virulence gene expression with HtpG during infection. This study aims to elucidate the molecular mechanism by which LexA controls virulence gene expression. We employed an array of methods including molecular dynamics simulations, molecular docking, ChIP-seq, RNA-seq, mass spectrometry, gene mutagenesis, LacZ reporter assays, electrophoretic mobility shift assays, co-immunoprecipitation, and in vitro LexA degradation experiments. Our findings identified 36 downstream virulence genes regulated by LexA, define three critical LexA binding motifs, and provide an in-depth analysis of LexA's recognition and binding to promoters, thereby regulating virulence gene expression. Additionally, we confirm the cooperative regulatory roles of HtpG, RecA, and LexA in virulence gene modulation. This is the first report of an endogenous accessory factor aiding in the binding of LexA to DNA. This study enhances our understanding of LexA's role in virulence regulation and offers a valuable theoretical and practical foundation for disease prevention and control.

Introduction

The LexA protein was initially identified in *Escherichia coli* as a key regulator of the SOS response. This protein comprises 202 amino acids (Hostetler et al., 2020; Cory et al., 2024) and consists of an N-terminal DNA-binding domain (NTD) and a C-terminal catalytic core domain (CTD), which are connected by a hydrophilic linker composed of five amino acid residues (Mo, Birdwell, and Kohli, 2014). In cellular contexts, LexA predominantly exists as a dimer and can adopt both cleavable and non-cleavable conformations (Kovačič et al., 2013). Under normal physiological conditions, LexA specifically binds to promoter regions of DNA, thereby repressing the expression of genes involved in DNA repair and cell division inhibition. Upon DNA damage, activated RecA protein stabilizes the cleavable conformation of LexA, facilitating self-cleavage of the peptide bond between Ala84 and Gly85. The resulting C-terminal LexA85–202 and N-terminal LexA1–84 fragments are then rapidly degraded by proteases ClpXP and Lon. Following LexA cleavage, SOS genes are sequentially expressed to repair DNA damage (Bellio et al., 2020). Historically, SOS has been regarded as a prokaryotic checkpoint, and LexA was considered primarily responsible for maintaining genomic stability and genetic conservation.

Recent studies have, however, revealed that LexA is involved in a variety of biological processes beyond SOS repair, including bacterial pathogenicity (Fornelos, Browning, and Butala, 2016; Walter et al., 2015; Wurihan et al., 2018), antibiotic resistance (Mo et al., 2017; Bunnell et al., 2017), and environmental responses (Bellio et al., 2020; Jian et al., 2015). Notably, the regulatory role of LexA varies among different species, with some exhibiting LexA self-cleavage that is independent of RecA (Kumar, Kirti, and Rajaram, 2015). Furthermore, recent research indicates that exogenous proteins can act as accessory factors in LexA-DNA binding; however, there have been no reports of endogenous accessory factors involved in this interaction (Fornelos et al., 2015; Caveney et al., 2019). The discovery of LexA's new functions and the variability in its regulatory mechanisms across different

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https://doi.org/10.1016/j.crmicr.2025.100351

Available online 29 January 2025

bacteria have garnered increasing attention.

The large yellow croaker (*Pseudosciaena crocea*) is a major economic fish species endemic to China's coastal waters and currently holds the largest aquaculture production among single fish species in seawater (Xu et al., 2022). However, with the rapid expansion of large yellow croaker aquaculture in recent years, the industry has faced frequent outbreaks of various diseases, leading to significant economic losses (Xiu et al., 2024). Therefore, effective prevention and control of diseases in large yellow croaker are of paramount importance for the sustainable and healthy development of this industry. "visceral white spot disease" is one of the most common diseases affecting net-caged large yellow croakers in recent years, with mortality rates reaching up to 80% and causing annual direct economic losses exceeding 100 million RMB (Li et al., 2020). The disease is characterized by white nodules ranging from 0.5 to 3 mm in size found in the liver, spleen, kidneys, and other internal organs (Li et al., 2020; Zhang et al., 2014). Currently, *Pseudomonas plecoglossicida* is widely recognized as the causative agent for "visceral white spot disease" (Zhang et al., 2014; Mao, Li, and Chen, 2013; Tang et al., 2019).

Infections caused by *P. plecoglossicida* have been reported in various aquaculture species, including large yellow croaker (Sun et al., 2019; Tang et al., 2019), sweetfish (Nishimori, Kita-Tsukamoto, and Wakabayashi, 2000), and rainbow trout (Akayli, Canak, and Basaran, 2011), resulting in substantial economic damage. Additionally, preliminary research by our group has revealed that artificial infection with *P. plecoglossicida* can also induce "visceral white spot disease" in grouper (Sun et al., 2018). Given the severe threat posed by *P. plecoglossicida* to large yellow croaker aquaculture, there is considerable interest in studying its pathogenic mechanisms. Existing research indicates that *P. plecoglossicida* possesses multiple virulence factors, including type III and type VI secretion systems (Mao et al., 2024; Tao et al., 2016) and the exotoxin ExoU (Zhang et al., 2014), and can survive and proliferate within macrophages (Mao, Li, and Chen, 2013). Its pathogenicity shows notable temperature dependence (Tao et al., 2020). These findings provide valuable foundational data for further in-depth studies on the pathogenic mechanisms of *P. plecoglossicida*.

In our preliminary research utilizing dual RNA-seq, we identified that *htpG* (also known as *hsp90*) plays a crucial role in the interaction between *P. plecoglossicida* and its host (Huang et al., 2019). HtpG affects the expression of various virulence-related genes, including those involved in flagellar assembly and ribosomal assembly. Additionally, we discovered that many of these virulence genes affected by HtpG contain LexA binding sites in their promoters (Huang et al., 2019). Therefore, we hypothesize that LexA may collaborate with HtpG in the regulation of these virulence genes.

Here, we investigate the molecular mechanisms by which LexA regulates the expression of virulence genes in *P. plecoglossicida*. Utilizing a comprehensive approach that includes bioinformatic, molecular, and microbiological assays, we systematically identify downstream genes of LexA and analyze their associations with pathogenicity. This study delves into the molecular mechanisms by which LexA recognizes and binds to promoters to regulate virulence gene expression. Additionally, we explore the molecular mechanisms underlying the collaborative regulation of virulence gene expression by HtpG and LexA. Our findings identify 36 downstream virulence genes regulated by LexA, define three key LexA binding motifs, and demonstrate that LexA is not only a critical transcriptional regulator of the SOS response but also a key factor in the regulation of *P. plecoglossicida* adhesion, biofilm formation, and environmental adaptation. Importantly, we report for the first time the endogenous accessory factor HtpG associated with LexA. The results presented in this study provide valuable theoretical and practical insights into the molecular mechanisms by which LexA modulates virulence in *P. plecoglossicida*, offering a foundation for the development of strategies to combat infections caused by this bacterium.

Materials and methods

Sequence alignment and homology modeling

We performed a comparative analysis of the amino acid sequences of LexA proteins across ten bacterial species, including *P. plecoglossicida, P. aeruginosa, E. coli, S. typhimurium, K. pneumoniae, M. tuberculosis*, and *S. aureus*. The sequence alignments were generated using ClustalW and visualized with ESPript3.x and ENDscript2.x.

To identify homologous templates for the HtpG and LexA proteins, we employed the BLAST algorithm. Crystal structures with significant homology were retrieved from the RCSB Protein Data Bank (PDB IDs: 2IOP and 1JHH). Subsequent homology modeling was performed using MOE software. The protonation states and hydrogen orientations of the proteins were optimized at pH 7 and 300 K using LigX. Initially, the target sequences were aligned with the template sequences, resulting in the construction of ten distinct intermediate models. These models, differing in loop structures and side-chain orientations, were evaluated based on their scoring metrics. The model with the highest GB/VI scoring function was selected as the final model, which was then subjected to energy minimization using the AMBER10: EHT force field.

Bacterial strains and culture conditions

The pathogenic *P. plecoglossicida* strain isolated from the spleen of naturally infected large yellow croaker was designated NZBD9 and utilized in this study (Huang et al., 2021). In previous research, we constructed *P. plecoglossicida* strains *htpG*-RNAi, which exhibited 95% silencing efficiency of *htpG* (Huang et al., 2020). These strains were cultured in LB (Luria-Bertani) medium at 18 °C, with tetracycline for the RNAi strains and without tetracycline for the wild-type strain. *E. coli* DH5α, obtained from TransGen Biotech (Beijing, China), was grown in LB medium at 37 °C with shaking at 220 r.p.m.

Construction of mutant strains

Primers with homologous arms, designed based on the *P. plecoglossicida lexA* gene sequences, were synthesized using Primer Premier 5.0 (Supplemental materials 1). The 5′ ends of these primers were complementary to the 50-bp upstream and downstream flanking regions of the target genes, while the 3′ ends were homologous to the tetracycline resistance gene. After PCR amplification, targeting fragments conferring tetracycline resistance for *lexA* were generated.

The plasmid pKD46 was introduced into *P. plecoglossicida*, and the bacteria were cultured to an OD_{600} of 0.3. Addition of 30 mmol/ l-arabinose induced expression of the recombinant enzymes Exo, Bet, and Gam from pKD46. Electroporation was used to introduce the targeting fragments into *P. plecoglossicida*. Positive clones were selected on tetracycline (10 μg/mL) and further verified by PCR and gene sequencing (Huang et al., 2020).

Artificial infection

Healthy *E. coioides* weight about 145 g/tail with no scars and no parasites were used for the artificial infection. After purchasing from the farm, they were temporarily raised in a fish tank with a water temperature of 28 °C for two weeks. The strains were expanded at 28°C, 200 r.p.m. The concentration of bacterial solution was adjusted to 1.0×10^8 CFU/mL. 200 μL of bacterial solution was injected at the base of the pectoral fins of the fish (20 fish per group). One group of fish was injected with an equal amount of PBS as a negative control. The survival of the grouper was observed and recorded for about two weeks (Huang et al., 2018).

LacZ reporter gene system

To construct the LacZ reporter gene system, the promoter regions containing the LexA binding site was amplified using primers listed in Supplemental materials 1. The PCR products, digested with restriction enzymes, were ligated into the pRW50 plasmid and introduced into wild-type, *lexA* knockout, and *htpG* knockdown strains by electroporation. Positive clones were selected using colistin (12.5 μg/mL) and tetracycline (10 μg/mL). The β-galactosidase activity was measured as described previously (He et al., 2021).

Expression and purification of proteins

The mature peptide sequences of *lexA* was amplified with primers (Supplemental materials 1) and cloned into the pET-32a(+) expression vector after digestion with *EcoRI* and *XhoI* (TaKaRa). Recombinant plasmids were transformed into *E. coli* BL21 (DE3). Protein expression was induced with isopropyl-β-d-thiogalactopyranoside (IPTG), and the proteins with a TrxA-His tag were purified using a nickel-nitrilotriacetic acid (Ni-NTA) column (TaKaRa) (Huang et al., 2020).

Electrophoretic mobility shift assay (EMSA)

For EMSA, varying concentrations (0, 0.5, 1, 1.5, or 2 μM) of LexA protein was incubated with 2 μg of motif DNA fragments labeled with 6-carboxyfluorescein at the 5′ end (GenePharma Co. Ltd., Shanghai, China) in a reaction mixture (50 μl) containing 200 mM KCl, 50 mM Tris–HCl, 5% (v/v) glycerol, and 0.1 mM EDTA. The mixtures were incubated at 25 °C for 2 h and then subjected to electrophoresis on a 5% native polyacrylamide gel (Huang et al., 2020). The 5′−6-carboxyfluorescein-labeled DNA fragment used in this experiment consists of regions approximately 170 bp upstream and downstream of the LexA binding site within the promoter sequence.

DNA and RNA isolation

Genomic DNA from bacteria was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) (Wang, Li, and Huang, 2023).

Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR was performed on the QuantStudio 6 Flex real-time PCR system (Life Technologies, USA) (Wang, Li, and Huang, 2023). Gene expression levels were normalized to *gyrB*, and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Primers used are detailed in Supplemental materials 1.

Biofilm analysis

Biofilm formation by *P. plecoglossicida* was evaluated using crystal violet staining method (Wang, Li, and Huang, 2023). Overnight cultures of *P. plecoglossicida* strains in LB medium at 28 °C were adjusted to an OD_{600} of 0.2. A 150 μL aliquot of LB medium and a 50 μL aliquot of bacterial suspension were combined and incubated at 18 °C for 24 h. Following incubation, the wells were washed three times with sterile PBS, stained with 200 μL of 1% crystal violet for 15 mins, and then rinsed again with sterile PBS. The stained biofilm was allowed to air-dry. To quantify biofilm formation, the dye was solubilized using 33% acetic acid, and absorbance was measured at 570 nm ($n = 6$).

Biofilm analysis was also conducted using laser confocal fluorescence microscopy (CLSM) (Cai et al., 2023). non-adherent cells were removed by gentle washing with sterile PBS (three times). The samples were then fixed with 4% glutaraldehyde for 30 mins. Subsequently, the biofilms were washed again with sterile PBS (three times) and stained with SYBR Green I for 30 mins in the absence of light. After staining, the samples were washed three times with sterile PBS. Confocal laser scanning microscopy (Leica, Germany) was employed to capture the images.

Soft agar plate motility assay

Motility of *P. plecoglossicida* strains was assessed using the soft agar plate method (Cai et al., 2023). A 1 μL drop of cell suspension from overnight cultures ($OD_{660} = 0.03$) was placed at the center of LB agar plates (0.3% agar) and incubated at 37 °C for 20 h. Colony diameters were then measured to assess motility.

In vitro adhesion assay

Adhesion of *P. plecoglossicida* strains was determined as outlined by Liu et al. (Liu et al., 2017). Adhesion was visualized using microscopy at × 1000 magnification ($n = 6$).

Intracellular survival and immune evasion analysis

Healthy *E. coioides* were procured from Zhangzhou (Fujian, China). Head kidney macrophages were isolated using the procedure described by Zhang et al. (Zhang et al., 2018) and plated in a 6-well culture plate containing 2 mL of l-15 medium supplemented with 10% FCS and 100 IU/mL S/P per well. After a 4-hour incubation at 28 °C, the cultures were washed twice. The macrophages (10^7 cells/mL) were then exposed to wild-type and mutant *P. plecoglossicida* strains at a multiplicity of infection (MOI) of 100 (100 bacteria per macrophage) in l-15 medium with 10% FCS. After a 1-hour incubation at 18 °C, macrophages were washed twice with cold PBS, resuspended in 2 mL PBS, and treated with 250 μg/mL gentamicin for 20 mins at 18 °C to eliminate extracellular bacteria, followed by two additional washes with PBS. The supernatant was tested for sterility. Cells were then resuspended in fresh l-15 medium with 10 IU/mL heparin, 100 IU/mL S/P, and 10% FCS, with this time point designated as 0 h. Samples were further incubated for 1 hour and 3 h, denoted as 1 hour and 3 h, respectively. At each time point, cells were centrifuged for 5 mins at 100 × *g* and lysed with 1 mL of sterile distilled water for 30 mins. Colony-forming units (CFU) of the precipitate were enumerated by plating appropriate dilutions on TSA plates (Zhang et al., 2018). For the 3-hour sample, the bacterial CFU in the supernatant was also determined. Intracellular survival rate was calculated as the CFU at 1 hour divided by the CFU at 0 h. The escape rate was defined as the CFU in the supernatant at 3 h divided by the CFU at 0 h (Zhang et al., 2018).

Transcriptome sequencing analysis

Transcriptome sequencing was performed using the Illumina HiSeq4000 sequencer (Illumina, San Diego, CA, USA). Clean reads were aligned to the reference genome and gene sequences using SOAP2, allowing for up to five mismatches in the alignment. Gene expression was quantified using the RPKM (Reads Per Kilobase of transcript per Million reads) method, which normalizes for gene length and sequencing depth, facilitating comparison of expression levels across samples. Differentially expressed genes (DEGs) between mutant and wild-type strains were identified using the MA-plot-based method with unpaired class comparison, highlighting genes with significant differences ($P < 0.001$). The sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA606205.

For DEG annotation, Blast2GO was utilized to assign Gene Ontology (GO) terms to the unigenes. GO functional classification was performed with WEGO software to provide a broad overview of gene function distribution. P-values were adjusted using Bonferroni correction, with a threshold of ≤ 0.05. GO terms meeting this criterion were considered significantly enriched. COG and KEGG pathway annotations were

conducted using Blastall software against the COG (http://www.ncbi.nlm.nih.gov/COG) and KEGG (http://www.genome.jp/kegg/) databases. Pathways with a Q-value $\leq$ 0.05, where Q represents the false discovery rate (FDR), were considered significantly enriched in DEGs.

Chromatin immunoprecipitation sequencing (ChIP-seq)

The plasmids pET28a-GFP-*lexA* and pET28a-GFP, which encode LexA-GFP and GFP, respectively, were expressed in *P. plecoglossicida* for chromatin immunoprecipitation (ChIP) assays, following established protocols (Gu et al., 2016). Overnight cultures of each strain were diluted to an equivalent optical density (OD_{600}) and statically subcultured in LB medium supplemented with kanamycin at 28 °C for 24 h. Subsequently, rifampicin (Sigma) was added to achieve a final concentration of 150 μg/mL, followed by a 30-minute incubation at 28 °C. In vivo cross-linking of protein-DNA complexes was performed using formaldehyde, and the reaction was quenched with glycine solution. ChIP assays were executed as previously outlined (Gu et al., 2016). DNA was purified using phenol/chloroform extraction and precipitated with ethanol. For ChIP-sequencing (ChIP-seq), the DNA fragments were processed for library construction utilizing the VAHTS Turbo DNA Library Prep Kit (Vazyme, Nanjing, China). The sequencing methodology adhered to previously described procedures (Gu et al., 2016), and the MEME Suite (http://meme-suite.org) was employed to identify the LexA binding motif. The sequencing data have been deposited in the Genome Sequence Archive (GSA) under accession number CRA002447.

Screening out LexA interacting proteins by CoIP-MS

Approximately 10^8 *P. plecoglossicida* cells harboring either pET28a-GFP-*lexA* or pET28a-GFP were lysed overnight using 1 mL of IP Lysis/Wash Buffer (Thermo Fisher). Subsequently, 4 μg of GFP antibody (Abcam) was conjugated with Pierce Crosslink Magnetic Beads (Thermo Fisher) for each reaction and incubated with the supernatant obtained from the lysed cells to isolate the co-immunoprecipitated products. A negative control was conducted to collect co-immunoprecipitated products in the absence of GFP antibody. The enriched co-immunoprecipitation products were analyzed through mass spectrometry with support from Wuhan SeqHealth Co., Ltd. (China) using LCESI-MS/MS. Peptides with scores below 20 were excluded from analysis, with higher scores indicating a superior match to the secondary atlas. The peptides were qualitatively searched and compared using UniPro, while the UniquePep Count and Coverage Percent were assessed as supplementary metrics to support the final identification results (Chen et al., 2016).

Co-immunoprecipitation

Approximately 10^8 *P. plecoglossicida* cells expressing either pET28a-Flag-*recA* or pET28a-Flag-*htpG* were lysed using a Triton X-100 lysis buffer (40 mM Tris, 120 mM NaCl, 1% Triton X-100, 1 mM NaF, 1 mM Na3VO4), supplemented with a protease inhibitor cocktail. The total protein concentrations of the resulting lysates were determined using a BCA assay. Rabbit anti-Flag antibodies (Abcam) were incubated with protein A beads at 4 °C for 1 hour, then conjugated to the beads using a 450 μM DSS solution, following the manufacturer's protocol. Subsequently, interacting proteins were purified with the antibody-conjugated beads and analyzed by Western blotting (Chen et al., 2016).

Cleavage assays of LexA

To analysis the cleavage ability of LexA, purified protein was mixed in a 1:1 ratio with 2 × cleavage buffer (100 mM Tris-Glycine-CAPS and 300 mM NaCl) (Mo, Birdwell, and Kohli, 2014). Reaction mixtures were incubated at room temperature for 30 min. Cleavage was quenched by adding 2 × Laemmli sample buffer to the reaction mixture and by denaturation at 95 °C for 10 min. The extent of LexA cleavage was visualized by running reaction samples on 15% SDS−PAGE gels and analyzed by Western blotting.

Western blotting

Proteins were eluted from the beads by incubating them in SDS-PAGE loading buffer, followed by a 10-minute exposure in a boiling water bath. For the purpose of Western blotting, proteins resolved by SDS-PAGE were subsequently transferred to polyvinylidene fluoride (PVDF) membranes utilizing a wet electroblotting apparatus. The membranes were incubated overnight at 4 °C with primary antibodies, followed by a 1-hour incubation at room temperature with secondary antibodies. The bound antibodies were visualized using enhanced chemiluminescence (ECL) detection reagents (Chen et al., 2016).

Statistical analyses

Results are expressed as means ± standard deviation (S.D.). Statistical significance was determined using one-way ANOVA followed by Dunnett's test, with data analyzed using SPSS 13.0 software (Chicago, IL, USA). A p-value of < 0.05 was considered statistically significant.

Results

Structural and functional insights into the P. plecoglossicida LexA dimer

The self-cleavage site of the *P. plecoglossicida* LexA protein exhibits a high degree of conservation at the amino acid level (Supplemental materials 2A). We also conducted a structural analysis of the LexA dimer from *P. plecoglossicida* and identified its characteristic domains, including an alpha-helical region (NTD) and a beta-sheet region (CTD) (Supplemental materials 2B). The structural features are suggestive of *P. plecoglossicida* LexA functioning as a transcription factor, binding to DNA and undergoing self-cleavage in response to environmental stimuli to regulate gene expression, but this needs to be experimentally verified. Notably, the amino acid composition of *P. plecoglossicida* LexA differs from that of LexA proteins in other pathogens. For example, the amino acid sequence identity between *P. plecoglossicida* and *E. coli* LexA is only 60.26%. This is suggestive of potential differences in the mechanisms by which they regulate downstream gene expression. However, this still requires experimental verification.

Effects of Mitomycin C and environmental stresses on LexA expression in P. plecoglossicida

Mitomycin C, a well-known DNA damage inducing agent, was employed to investigate the level of LexA in *P. plecoglossicida*. To examine this, total RNA and protein from *P. plecoglossicida* were extracted following treatment with 1 mmol/L Mitomycin C and analyzed via qRT-PCR and Western blot. qRT-PCR analysis revealed a significant increase in *lexA* gene expression upon exposure to Mitomycin C, indicating that Mitomycin C promotes *lexA* expression (Fig. 1A). In contrast, the abundance of LexA protein decreased following Mitomycin C treatment (Fig. 1B), suggesting a response to DNA damage that affects LexA protein stability.

In addition to DNA damage, the effects of various environmental stresses on *lexA* expression were also evaluated. Hydrogen peroxide (H_2O_2) stress was investigated using three different concentrations of H_2O_2. A significant reduction in *lexA* expression was observed across all concentrations tested (Fig. 1C), which was mirrored by a decrease in LexA protein levels (Fig. 1D). Similarly, exposure to copper and lead ions led to a marked downregulation of *lexA* expression, with both metal stresses exhibiting significant effects (Fig. 1E). Correspondingly, the protein levels of LexA displayed a similar trend (Fig. 1F).

Temperature stress was examined across five different temperature

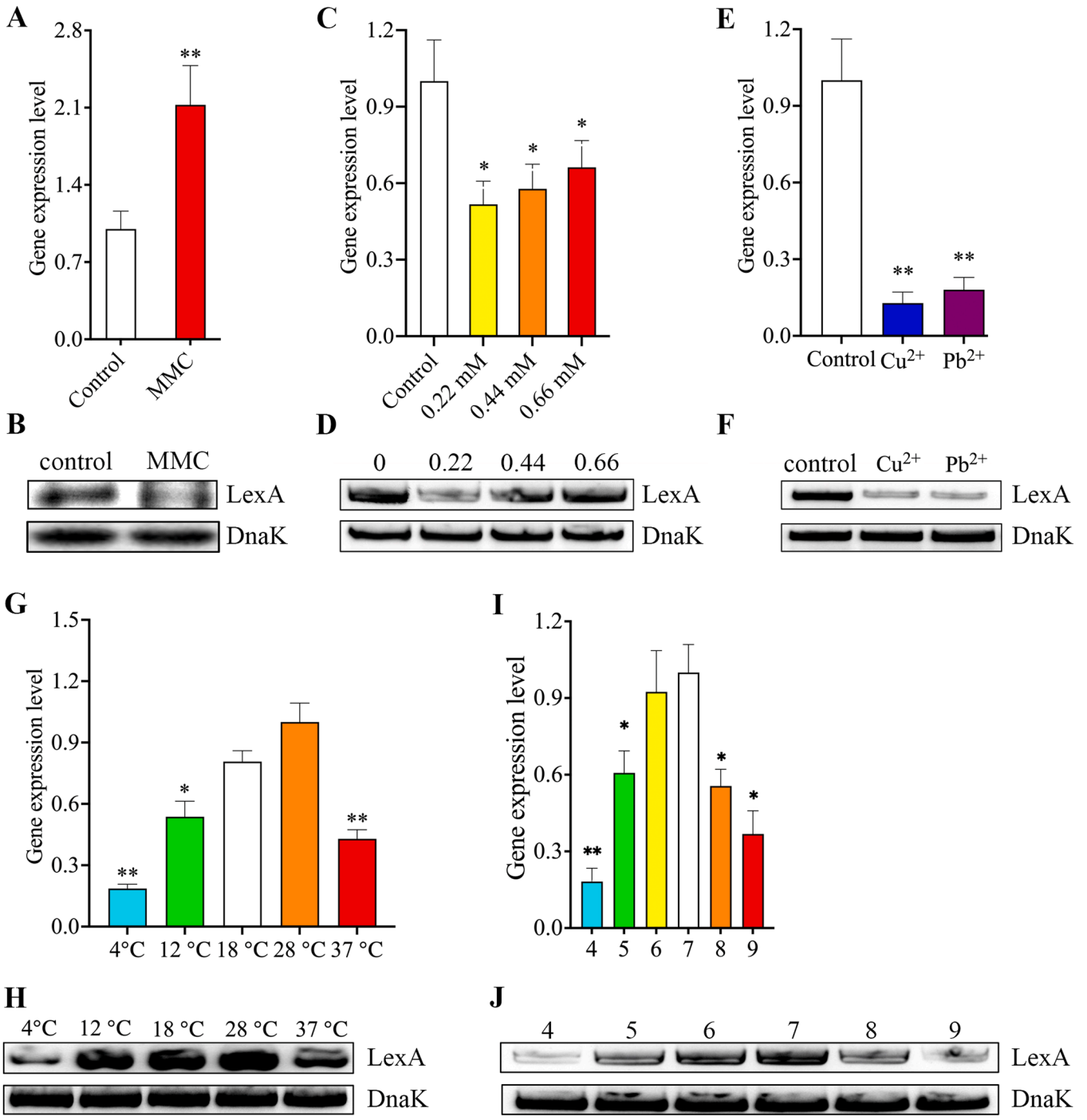

Fig. 1. Variability in the level of LexA under diverse stress conditions. **(A)** The transcription level of *lexA* was effectively increased after treated with 1 mmol/L mitomycin C, an anti-tumor antibiotic known for inducing DNA damage. **(B)** The protein expression level of LexA was significantly reduced after treated with 1 mmol/L mitomycin C. Representative graphs of western blot were displayed. **(C)** The transcription level of *lexA* was significantly affected by H_2O_2. Variability in the expression level of *lexA* under 0.22, 0.44 and 0.66 mM H_2O_2 was detected by qRT-PCR. **(D)** The protein expression level of LexA was significantly affected by H_2O_2. Representative graphs of western blot were displayed. **(E)** The transcription level of *lexA* was significantly affected by heavy metal exposure. Variability in the expression level of *lexA* after exposed to 30 mM Cu^{2+} and Pb^{2+} was detected by qRT-PCR. **(F)** The protein expression level of LexA was significantly affected by Cu^{2+} and Pb^{2+}exposure. Representative graphs of western blot were displayed. **(G)** The transcription level of *lexA* was significantly affected by temperatures. Variability in the expression level of *lexA* under 4 °C, 12 °C, 18 °C, 28 °C, and 37 °C was detected by qRT-PCR. **(H)** The protein expression level of LexA was significantly affected by temperatures. Representative graphs of western blot were displayed. **(I)** The transcription level of *lexA* was significantly affected by pH. Variability in the expression level of *lexA* at pH=6, 7, 8 and 9 was detected by qRT-PCR. **(J)** The protein expression level of LexA was significantly affected by pH. Representative graphs of western blot were displayed. For the qRT-PCR results, statistical significance is denoted by * for $P < 0.05$ and ** for $P < 0.01$, with $n = 6$ biological replicates. For the Western blot experiments, DnaK was used as an internal reference.

conditions. Compared to the control at 28 °C, *lexA* expression was significantly reduced at 4 °C, 12 °C, and 37 °C, while a slight, non-significant decrease was observed at 18 °C (Fig. 1G). LexA protein levels also exhibited changes consistent with the transcript expression patterns (Fig. 1H). Additionally, the effects of pH were evaluated using five different pH values. At pH 4, a highly acidic environment, *lexA* expression was drastically reduced. A significant reduction in *lexA* expression was noted at pH 5, while at pH 6, a minor decrease was observed, though no significant difference compared to the neutral pH control was detected. Notably, significant reductions in *lexA* expression were observed at the alkaline pH values of 8 and 9 (Fig. 1I). These results were further corroborated by changes in LexA protein levels, which aligned with the qRT-PCR findings (Fig. 1J).

Overall, these results demonstrate that various stress conditions significantly influence *lexA* gene expression at the transcriptional level, highlighting the important role of LexA in responding to diverse environmental stresses beyond DNA damage. Notably, the increased transcriptional expression of *lexA* in response to Mitomycin C suggests a potential feedback regulation mechanism where LexA may participate in DNA damage repair through self-cleavage and degradation. These findings provide new insights into how *P. plecoglossicida* adapts to environmental pressures.

Role of LexA in virulence regulation of P. plecoglossicida

Utilising λ-red recombination, we successfully engineered a *lexA* knockout mutant in *P. plecoglossicida*. Sequencing confirmation and stable inheritance through multiple passages confirmed the integrity of the mutant strain. PCR and Western blot analyses further validated the mutation. As depicted in Fig. 2A, the full-length *lexA* gene is 650 bp, which was replaced by a 1203 bp tetracycline resistance fragment through homologous recombination, indicative of a successful mutant construction. Subsequent Western blot analysis revealed the absence of LexA protein expression in the *lexA* knockout strain Fig. 2B. These findings collectively confirm the successful construction of the Δ*lexA* mutant, which was then employed for subsequent experimental investigations.

The growth curve of the Δ*lexA* mutant was comparable to that of the wild-type strain (Fig. 2C), suggesting that the mutation does not impair the growth of *P. plecoglossicida*. However, the Δ*lexA* mutant exhibited significant enhancements in key virulence attributes. Specifically, the virulence of the Δ*lexA* mutant (Fig. 2D) was markedly increased compared to the wild-type strain. In the negative control group, no mortality was observed in the fish until day 10. Fish injected with the wild-type strain first exhibited mortality on day 3, and all individuals were dead by day 5. In contrast, fish injected with the *lexA* deletion mutant strain displayed earlier onset of mortality, with the first death occurring on day 1 and all fish dying by day 3. Additionally, the Δ*lexA* mutant demonstrated enhanced biofilm formation (Fig. 2E), motility (Fig. 2G and H), intracellular survival (Fig. 2I), and immune evasion capabilities (Fig. 2J), while hemolytic activity remained unchanged (Fig. 2F). Specifically, biofilm formation was increased by approximately 1.75-fold, motility was augmented by approximately 1.46-fold, intracellular survival rate was elevated by 1.89-fold, and immune evasion capability was enhanced by roughly 3.25-fold compared to the wild-type strain. These findings suggest that the *lexA* plays a crucial role in modulating these important biological processes.

Confocal microscopy observations further revealed that the deletion of the *lexA* gene significantly accelerates the structural construction in *P. plecoglossicida* biofilm. These data underscore the pivotal role of LexA in the regulation of virulence factors in *P. plecoglossicida* (Fig. 2K).

LexA-mediated transcriptional regulation in P. plecoglossicida virulence

To delineate the molecular mechanisms by which *lexA* modulates the virulence of *P. plecoglossicida*, we conducted prokaryotic chain-specific transcriptomic sequencing on both the wild-type and *lexA* knockout strains. As depicted in Supplemental materials 3A, the *lexA* knockout strain exhibited a multitude of differentially expressed genes compared to the wild-type strain. A total of 5344 genes were identified, of which 301 genes were significantly differentially expressed in the *lexA* knockout strain, with 295 genes upregulated and only 6 genes downregulated. We randomly selected four upregulated genes (*flgN, fliE, fliO* and *fliP*) and five downregulated genes (*ttgB, DVB73_RS08030, adeC, DVB73_RS17570* and *dnaN*) from those with significant differential expression for qRT-PCR validation. The observed trends in gene expression were consistent with the results from bioinformatics analysis, thereby confirming the reliability of our transcriptomic data (Supplemental materials 3B).

The 301 differentially expressed genes were subjected to GO functional annotation, which revealed their distribution across 20 sary classifications within the three main categories of Biological Process, Cellular Component, and Molecular Function (Supplemental materials 3C). The largest groups of differentially expressed genes were associated with biofilm composition and catalytic activity, each accounting for approximately 20% of the annotated genes. This was followed by genes involved in binding, cellular processes, and metabolic processes, each constituting around 13% of the annotated genes.

KEGG pathway enrichment analysis assigned the differentially expressed genes to 30 distinct pathways (Supplemental materials 3D). Notably, these genes were significantly enriched in pathways known to be involved in the regulation of *P. plecoglossicida* virulence factors, including biofilm formation, flagellar assembly, and protein export. The biofilm formation pathway, a complex network, contained 34 *lexA*-induced differentially expressed genes, with *rmsA* and *hsiI* showing downregulated expression. In the flagellar assembly pathway, 35 *lexA*-induced genes were identified, with *fliE, fliO, fliP*, and *flgN* showing significantly upregulated expression levels in response to *lexA* knockout, indicating a pivotal role for *lexA* in the regulation of these pathways critical to *P. plecoglossicida* virulence.

The motif for LexA-DNA binding and the downstream virulence genes

To delineate the specific role of LexA, we performed ChIP-seq analysis to explore its interaction with the bacterial genome. We mapped the distribution of LexA-bound DNA library sequencing reads on the reference genome by detecting reads around transcription start sites (TSS) in both the control Input and experimental IP groups. As depicted in Supplemental materials 4A, effective binding sites for LexA were detected within a 2 kb region upstream and downstream of the TSS, with the experimental group showing significantly higher peak values compared to the control, indicating a greater number of effective binding sites in the vicinity of the TSS.

Supplemental materials 4B illustrates the GO secondary enrichment classification for 800 genes associated with LexA-binding peaks. These genes were annotated to 31 GO functions, primarily categorized into biological processes, cellular components, and molecular functions. In the biological process category, the most enriched factors were involved in cellular respiration, cell localization, and nucleoside-triphosphate metabolic processes. The cellular component category annotated only one function related to the outer membrane, while the molecular function category showed the highest enrichment in nucleotide binding, glycoside derivative binding, and nucleoside-phosphate binding. The Rich Factor indicates the degree of enrichment, with higher values signifying greater enrichment.

GO enrichment analysis of peak-associated genes (Supplemental materials 4C) revealed enrichment in 20 GO functional groups, including cellular respiration, binding, cell localization, iron chelate transport, siderophore transport, and iron ligand transport, suggesting a close relationship with iron transport functions. Iron ions are crucial regulators of bacterial virulence and pathogenicity, suggesting that the repressor protein LexA may play a role in processes such as

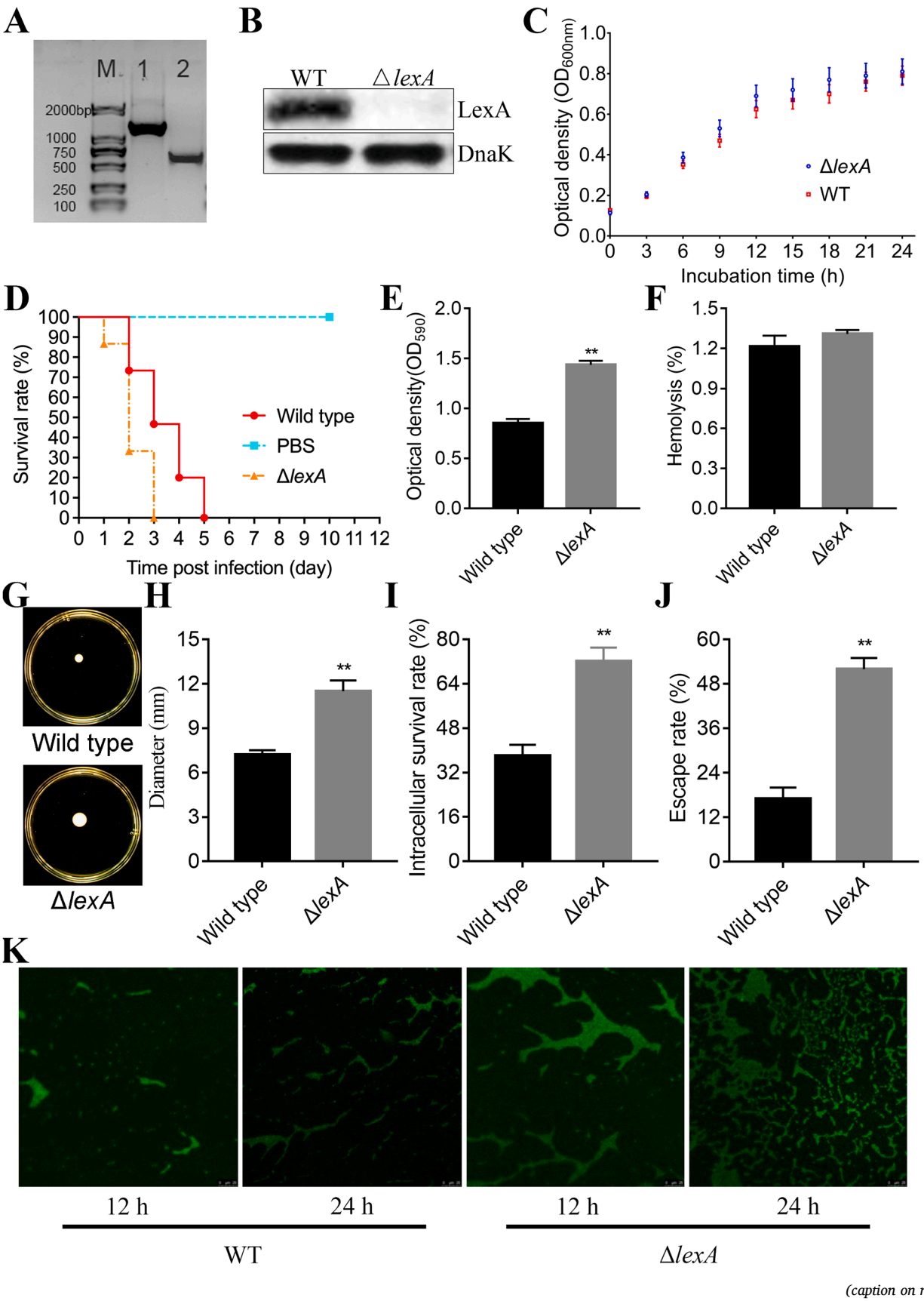

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Fig. 2. Impact of *lexA* gene knockout on the virulence of *P. plecoglossicida*. **(A)** PCR verification of *lexA* knockout strain in *P. plecoglossicida*. Lane M: DNA molecular weight marker; Lane 1: PCR product of *lexA* mutant strain; Lane 2: PCR product of wild-type strain. **(B)** Detection of LexA protein abundance in wild-type and *lexA* knockout strains. Representative graphs of western blot were displayed, while DnaK was used as an internal reference. **(C)** Measurement of growth ability between wild-type and *lexA* mutant strains. The bacterial solution was adjusted to an OD_{600} of 0.2. Then, 10 μL of the bacterial suspension was mixed with 190 μL of LB medium in a 96-well plate. The OD_{600} values were measured hourly for 24 h. Each group consisted of 6 biological replicates. Based on the OD_{600} data, a growth curve was constructed to compare the growth of the wild-type and *lexA* knockout strains. **(D)** Survival curves of *E. coioides* injected with wild type strain, PBS, and *lexA* knockout strain. The infection group was injected at the base of the pectoral fins of the fish with 200 μL 1.0×10^8 CFU/mL *P. plecoglossicida*. The negative control group was injected with an equal amount of PBS. Each group contained 20 fish. The survival of the grouper was observed and recorded for 10 days. **(E)** Biofilm formation of *P. plecoglossicida* was increased after *lexA* mutation. OD_{590} of crystal violet (1%) stained biofilm in the colony of each strain was detected. The data are presented as the means ± *S*.D., 6 independent biological replicates were performed for each data point. Statistical significance is denoted by ** for $P < 0.01$. **(F)** Mutation of *lexA* did not affect the hemolytic activity of *P. plecoglossicida*. Hemolysis assays were carried out by incubating the *P. plecoglossicida* strains with rabbit blood. After incubation, samples were centrifuged, and the released hemoglobin was measured by OD_{540} nm. The percentage of total hemolysis was calculated by comparing the OD_{540} nm of the samples with positive (100% lysis by 1% Triton X-100) and negative controls. Three independent biological replicates were performed for each data point. **(G-H)** The motility of *P. plecoglossicida* was significantly increased after *lexA* mutation. For the assay of motility, 1 μl of the *P. plecoglossicida* suspension was spotted on to the center of the LB plates (0.3% agar) at 37 °C for 20 h. Typical images of spreading of *P. plecoglossicida* strains were captured **(G)**. The diameters of the colonies were measured at 20 h **(H)**. The data are presented as the means ± S.D. (n = 3). Statistical significance is denoted by ** for $P < 0.01$. **(I-J)** *lexA* in *P. plecoglossicida* is important for intracellular survival. The intracellular viability **(I)** and immune escape **(J)** of wild type and Δ*lexA* strains were compared in *E. coioides* macrophages. Values are mean ± SD ($n = 3$, $^{**}P < 0.01$). **(K)** Influence of *lexA* knockout on the construction of a biofilm structure. The typical observation of biofilm formation of the wild type and Δ*lexA* strains were captured under CLSM at 12 and 24h.

transmembrane iron transport, potentially functioning as a regulator of virulence.

KEGG pathway enrichment analysis of peak-associated genes (Supplemental materials 4D) showed that these genes were enriched in five pathways, with the highest number of genes enriched in carbon metabolism, followed by oxidative phosphorylation. Notably, genes related to flagellar assembly, a critical factor in bacterial pathogenicity, were also enriched, suggesting that LexA may participate in this process.

To identify the DNA motifs recognized by LexA, we extracted peak interval sequences and scanned for common motifs using Homer. Fig. 3A reveals three main motifs recognized by LexA: GATGACGTCA, AGCGCGTCTT, and TTTGGCTATAAG. The sequence composition of these three motifs is significantly different from that of *E. coli* LexA box, indicating that the function and regulation of LexA in *P. plecoglossicida* may differ significantly from that in *E. coli*. At the same time, there are significant differences in sequence composition among these three motifs, indicating that LexA may regulate different genes of *P. plecoglossicida* differently.

By mapping these motifs to their corresponding peaks and associated genes, we identified multiple genes regulated by LexA. To validate the ChIP-seq results and compare them with the prokaryotic chain-specific transcriptomic sequencing data, we found seven genes that showed significant differences in both ChIP-seq and RNA-seq analyses. These genes are associated with siderophore transport, biofilm formation, and membrane transport, all of which are related to virulence. qRT-PCR validation of these seven genes' expression levels in *P. plecoglossicida* (Fig. 3B) confirmed the reliability of the ChIP-seq and RNA-seq data, with expression patterns consistent with the sequencing results, indicating LexA's regulatory role over these genes.

Using EMSA and LacZ reporter gene assays, we further verified that LexA can recognize and bind the three motifs (Fig. 3C-E) and has a regulatory effect on the promoter sequences of genes such as *ycaC, tonB, fucP, atpD*, and *duf1214* (Fig. 3F). Furthermore, confirmation of LexA binding to the promoter regions of *ycaC*-, *duf1214*-, *tonB*-, *fucP*-, and *atpD* was carried out by EMSA assays performed with LexA protein on 5′−6-carboxyfluorescein-labeled DNA fragments containing the promoter regions of *ycaC*-, *duf1214*-, *tonB*-, *fucP*-, and *atpD*, respectively. The results showed that LexA can recognize and bind these promoter regions. Therefore, we have identified, for the first time, virulence genes in *P. plecoglossicida* regulated by LexA and characterized the key motifs for LexA recognition and binding.

Identification of LexA-interacting proteins in P. plecoglossicida virulence

No studies have yet reported on the existence of an endogenous accessory factor for LexA in bacteria. To investigate whether LexA's function in *P. plecoglossicida* requires the assistance of RecA and HtpG, we conducted a co-IP-MS experiment using the Δ*lexA* mutant and wild-type strains. The precipitated proteins were subjected to in-gel digestion and analyzed using liquid chromatography-mass spectrometry (LC-MS). The analysis revealed 933 proteins in the control IgG group and 594 proteins in the experimental IP group, with 168 unique proteins identified in the IP group.

Spectrometry analysis of the co-IP samples identified interactions between LexA and both RecA (Supplemental materials 5A) and HtpG (Supplemental materials 5B). Based on the identified interacting proteins, we constructed a protein-protein interaction (PPI) network using GeneMANIA and Cytoscape visualization software (Fig. 4A). LexA was found to interact with multiple proteins, forming a complex PPI network. This analysis suggests potential interactions between RecA and HtpG with LexA.

Furthermore, we employed molecular dynamics simulations to analyze the protein structures and perform molecular docking of LexA and HtpG from *P. plecoglossicida*. The simulations revealed that amino acids Y295, Q296, E298, K382, Q418, G441, E442, T467, D468, and R469 of Hsp90 could form salt bridges or hydrogen bonds with amino acids E99, Q100, S101, C102, N103, N105, H110, S196, V199, R201, and R202 of LexA (Fig. 4B). This finding suggests that LexA may collaborate with HtpG in the regulation of virulence gene expression in this bacterium.

Role of RecA and HtpG in LexA self-cleavage and degradation in P. plecoglossicida

To validate the involvement of RecA in the self-cleavage and degradation of LexA in *P. plecoglossicida*, we performed co-IP analysis. Our results demonstrate that RecA can indeed bind to LexA (Fig. 5A), indicating its potential role in the regulatory mechanism. We further activated RecA filaments with ATP and single-stranded DNA (ssDNA) and incubated them with His 6-tagged LexA. The incubation was followed by SDS-PAGE analysis to monitor the cleavage and degradation of His 6-LexA. These results suggest that RecA can catalyze the cleavage and degradation of LexA (Fig. 5B).

Similarly, our co-IP analysis revealed that HtpG can bind to LexA (Fig. 5C). In our previous study, we constructed a *htpG*-silenced strain. Here, we measured the protein content of LexA in the *htpG*-silenced strain and observed a significant increase in the content of LexA compared to the wild-type strain (Fig. 5D). To investigate the impact of HtpG silencing on the expression of downstream virulence genes, we used LacZ reporter gene analysis in the *htpG*-silenced strain to assess the activity of the promoters of *ycaC, tonB, fucP, atpD*, and *duf1214*. The results revealed that after HtpG silencing, the activity of these promoters was inversely correlated with the trend observed in the *lexA* knockout strain (Fig. 5E).

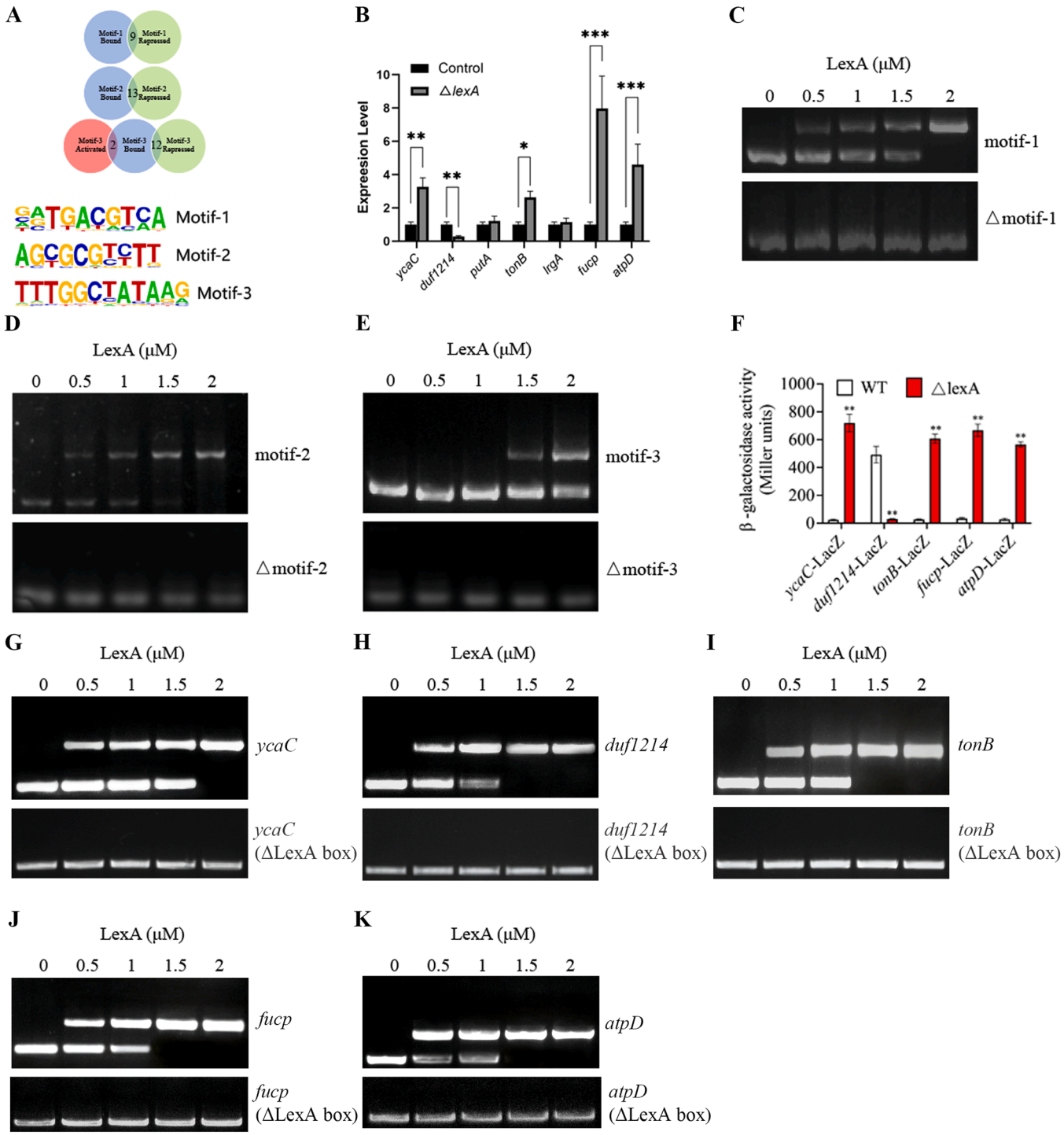

Fig. 3. LexA regulated virulence gene expression in a multi-motif interaction approach. **(A)** Identification of LexA binding motifs on virulence genes. Venn diagram illustrating genes directly bound and regulated by LexA, as identified through RNA-seq and ChIP-seq analyses. LexA binding motifs were also predicted by ChIP-seq. **(B)** qRT-PCR validation of LexA's regulatory role on downstream virulence genes. The graph compares the expression levels of selected genes in the wild-type and *lexA* knockout strains, confirming the regulatory effect of LexA on these genes. Statistical significance is denoted by * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$, with $n = 6$ biological replicates. **(C-E)** Confirmation of LexA binding to the three identified motifs. EMSA assays were performed with LexA protein on 5′−6-carboxyfluorescein-labeled DNA fragments containing motif1, motif2, and motif3, which were 353 bp, 353 bp, and 355 bp in length, respectively (upper panel). The assays were also conducted with DNA fragments of the same boundaries but with the motifs removed (lower panel). **(F)** LacZ reporter gene assay validating LexA's binding and regulatory action on the promoter sequences of downstream virulence genes. Transcription levels of the *ycaC*-, *duf1214*-, *tonB*-, *fucP*-, and *atpD*-LacZ reporter gene fusion were measured by β-galactosidase activity from bacterial cells after knockout of *lexA*. Statistical significance is denoted by * for $P < 0.05$ and ** for $P < 0.01$, with $n = 6$ biological replicates. **(G-K)** Confirmation of LexA binding to the promoter regions of *ycaC*-, *duf1214*-, *tonB*-, *fucP*-, and *atpD*. EMSA assays were performed with LexA protein on 5′−6-carboxyfluorescein-labeled DNA fragments containing the promoter regions of *ycaC*-, *duf1214*-, *tonB*-, *fucP*-, and *atpD*, which were 353 bp, 355 bp, 353 bp, 353 bp and 353 bp in length, respectively (upper panel). The assays were also conducted with DNA fragments of the same boundaries but with the motifs removed (lower panel).

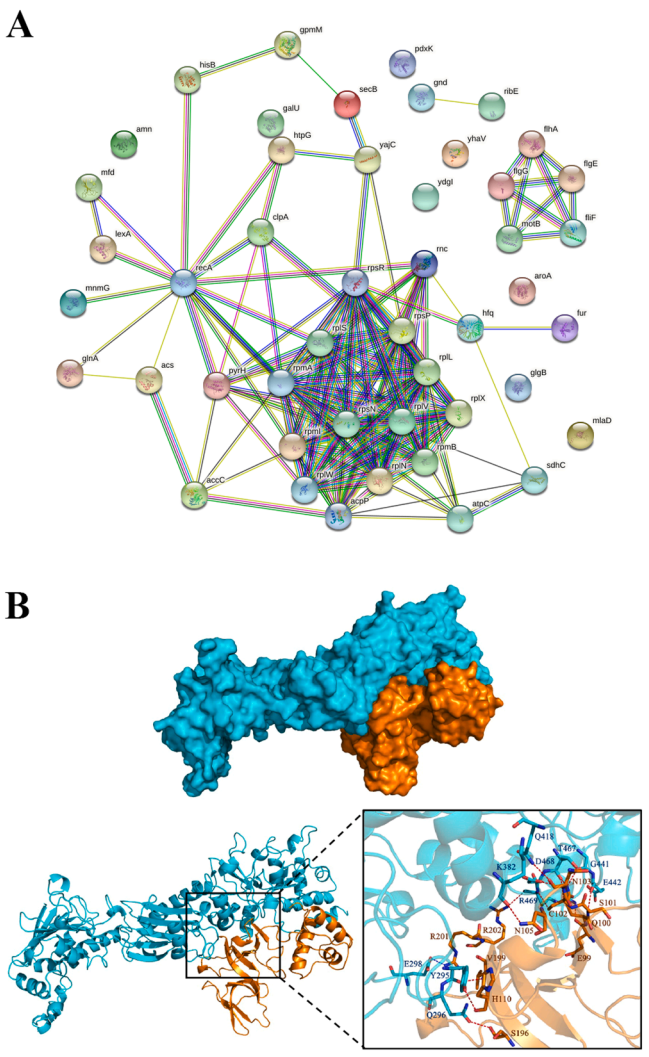

Fig. 4. LexA interaction analysis and molecular docking of HtpG in *P. plecoglossicida*. **(A)** Potential protein-protein interaction network of LexA-associated proteins. **(B)** Molecular docking model of HtpG and LexA. The backbone and residues of HtpG are shown in cyan, while those of LexA are depicted in orange. Red represents hydrogen bond interactions, and blue indicates salt bridge interactions, highlighting the molecular docking between HtpG and LexA.

These findings collectively confirm that HtpG promotes the self-cleavage and degradation of LexA, which is catalyzed by RecA, thus regulating the expression of downstream virulence genes in *P. plecoglossicida*.

Discussion

LexA can drive environmental adaptation

The SOS response in bacteria is a well-conserved DNA repair and stress response system. It is activated when the bacterial cell experiences DNA damage. Many factors can trigger the SOS response. For instance, when *E. coli* enter warm-blooded animals, they encounter various stressors, such as acidic pH, that can induce this response (Sousa et al., 2006). Another example is the production of antimicrobial agents like hydrogen peroxide by neutrophils, which causes DNA damage and aids in pathogenesis, as seen in enterohemorrhagic *E. coli* (Wagner, Acheson, and Waldor, 2001). Additionally, nitric oxide has been shown to induce the SOS response (Spek et al., 2001). Inside macrophages, *Salmonella* is exposed to DNA-damaging nitric oxide, and in the gallbladder—its site for chronic infections—bile is thought to induce the SOS response, a phenomenon also observed in *E. coli* (Prieto, Ramos-Morales, and Casadesus, 2004; Bernstein et al., 1999).

LexA, a central transcriptional regulator in the SOS response, plays a crucial role in DNA damage repair and environmental adaptation through changes in its expression levels (Hurstel, Granger-Schnarr, and Schnarr, 1988). The fact that LexA directly regulates the expression of various colicins demonstrates that LexA regulon members are involved in more than just genome maintenance (Ebina, Kishi, and Nakazawa, 1982; Jerman, Butala, and Zgur-Bertok, 2005). For example, the SOS response influences virulence factor production in *Staphylococcus aureus* (Bisognano et al., 2004) and type III secretion in enteropathogenic *E. coli* (Mellies, Haack, and Galligan, 2007). Notably, colicin production by *E. coli* in the mammalian colon (Kirkup and Riley, 2004) may help enhance microbial diversity (Walker et al., 2004). Recent investigations also revealed that in deep-sea bacteria, LexA's function is temperature-dependent, with its transcription being upregulated under cold adaptation and cold shock conditions. This suggests that high expression levels of LexA at low temperatures may underpin its temperature-dependent functionality (Jian et al., 2015).

Our study further explores the role of LexA in various stress responses. In the present study, MMC treatment led to an approximately two-fold increase in *lexA* expression, accompanied by a decrease in LexA abundance, as confirmed by qRT-PCR and Western blot analysis. This pattern is consistent with the characteristic stages of the SOS response: dissociation of LexA dimers, activation of DNA repair mechanisms, and subsequent reformation of LexA dimers post-repair. The increase in *lexA* transcription in response to DNA damage represents a feedback mechanism aimed at restoring SOS response repression after DNA repair is completed (Ben-Yehoyada, Wang, and Kozekov, 2009; Shinohara, Bando, and Sasaki, 2006; Weng, Zheng, and Jasti, 2010; Räschle, Knipscheer, and Enoiu, 2008).

Additionally, the study observed a notable decrease in LexA expression at non-optimal temperatures (4 °C, 12 °C, and 37 °C) compared to the optimal growth temperature (28 °C). These findings suggest that LexA plays a role in temperature stress response and tolerance in *P. plecoglossicida*. It is worth noting that the pathogenic temperature of *P. plecoglossicida* is 18 °C (Huang et al., 2019). Actually, *Shewanella piezotolerans* WP3 is the first bacterial species shown to have an SOS regulator significantly influenced by temperature (Jian et al., 2015). Huahua et al. (Jian et al., 2015) investigated LexA's impact on *S. piezotolerans* WP3 by constructing a *lexA* deletion strain. Comparative genome microarray analysis revealed 481 and 108 differentially expressed genes at 20 °C and 4 °C, respectively. Additionally, swarming motility and dimethylsulfoxide reduction assays indicated LexA's temperature-dependent function. LexA expression was upregulated during cold acclimatization and cold shock, suggesting its role in temperature adaptation. Our findings, along with previous studies, collectively support the hypothesis that the SOS response aids bacteria in managing temperature stresses.

Under oxidative stress induced by H_2O_2, the expression of LexA was significantly reduced, indicating its involvement in the oxidative stress response in *P. plecoglossicida*. This aligns with the known function of LexA in mitigating oxidative damage through the SOS response. High levels of LexA rendered *Anabaena* cells sensitive to oxidative stress-inducing agents such as H_2O_2, through the transcriptional down-regulation of the genes involved in alleviation of oxidative stress, such as *katA, katB, sodA*, and *petC* (Kumar, Kirti, and Rajaram, 2018). This study, along with previous research, collectively supports the hypothesis that LexA plays a critical role in the oxidative stress response. The consistent findings across studies highlight LexA's function in modulating the cellular defense mechanisms against oxidative damage, particularly through the regulation of key genes involved in oxidative stress alleviation.

The impact of pH on LexA expression was also assessed. Significant downregulation of LexA was observed under both acidic (pH=4) and

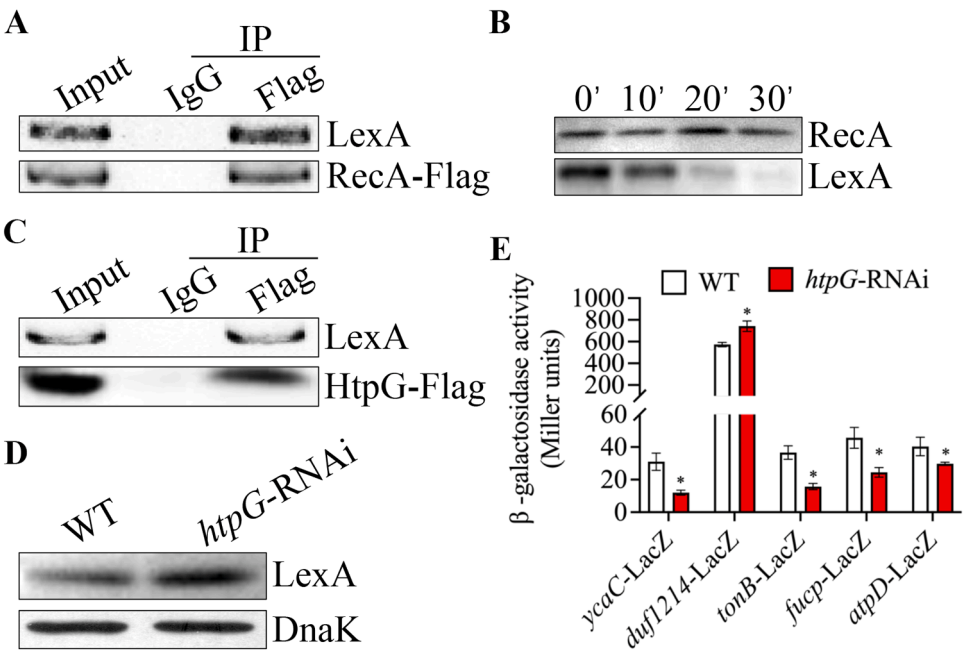

Fig. 5. Co-IP analysis and western blot confirmation of LexA-RecA and LexA-HtpG interactions in *P. plecoglossicida*. **(A)** Co-IP experiment validating the interaction between LexA and RecA. Anti-LexA and anti-Flag antibodies were used to assess the LexA-RecA interaction in *P. plecoglossicida*. IgG served as the negative control. **(B)** Time course of His 6-LexA autoproteolysis in the presence of RecA-ATP filaments. Proteins were incubated at pH 10.6, quenched at the indicated time points, and analyzed by western blotting. **(C)** Co-IP experiment confirming the interaction between LexA and HtpG. Anti-LexA and anti-Flag antibodies were used to assess the LexA-HtpG interaction in *P. plecoglossicida*. IgG served as the negative control. **(D)** Western blot confirming that HtpG can promote LexA's self-cleavage and degradation. Representative graphs of LexA expression in *htpG*-RNAi strain was displayed, while DnaK was used as an internal reference. **(E)** LacZ reporter gene analysis confirming that HtpG can regulate the activity of the promoters of *ycaC, tonB, fucP, atpD*, and *duf1214*. Transcription levels of the *ycaC*-, *duf1214*-, *tonB*-, *fucP*-, and *atpD*-LacZ reporter gene fusion were measured by β-galactosidase activity from bacterial cells after knockdown of *htpG*. Statistical significance is denoted by * for $P < 0.05$ and ** for $P < 0.01$, with $n = 6$ biological replicates.

alkaline (pH=9) conditions, with expression levels decreasing approximately fivefold in acidic conditions. This indicates that LexA is involved in the pH stress response and tolerance mechanisms in *P. plecoglossicida*. The study by Francisco et al. (Sousa et al., 2006) found that the LexA repressor undergoes structural rearrangement under acidic pH conditions, with aggregation occurring at pH 2.5 and a decrease in stability. This study suggests that the oligomerization of LexA may regulate the SOS response in *E. coli* under acidic conditions. While this study and previous research by Francisco et al. (Sousa et al., 2006) focus on different aspects of the LexA response to pH stress, both support the hypothesis that LexA plays a critical role in pH stress tolerance mechanisms. These findings contribute to the understanding that pH-induced changes in LexA expression and stability may be a common feature across different bacterial species, highlighting the broader significance of this regulator in maintaining cellular integrity under varying environmental pH conditions.

Moreover, exposure to copper and lead, two common heavy metals, resulted in a significant downregulation of LexA expression. Copper overload and lead toxicity are known to disrupt cellular functions and induce stress responses in bacteria (Li, Li, and Ding, 2019). The observed reduction in LexA expression under these conditions suggests that LexA also plays a role in heavy metal stress response and tolerance. Similar phenomenon has been observed by Kumar et al. (Kumar, Kirti, and Rajaram, 2018), who reports the global regulatory role of LexA across cyanobacteria, thereby manipulating the response to heavy metal such as Cd and As. Both this study and previous research collectively support the hypothesis that LexA plays a crucial role in the bacterial stress response to heavy metals.

LexA can regulate virulence factors

An increasing body of evidence suggests that LexA is involved in the pathogenesis of various bacteria. For example, LexA controls the production of temperate phage CTX, which encodes cholera toxin in *Vibrio cholerae* (Quinones, Kimsey, and Waldor, 2005), and colicins in *E. coli* (Žgur-Bertok, 2012; Jerman, Butala, and Žgur-Bertok, 2005). Chellappa et al. (Chellappa et al., 2013) found that the SOS response induction suppresses flagellar movement but enhances biofilm formation in *Pseudomonas aeruginosa*. Additionally, the resistance of *Bacillus subtilis* spores to DNA double-strand breaks is associated with RecA and its related proteins (Vlasic et al., 2014). Walter et al. (Walter et al., 2015) proved that LexA controls sporulation, motility and biofilm formation in *Clostridium difficile*. In this study, by constructing a *lexA* knockout strain, we found that LexA negatively regulates *P. plecoglossicida*'s virulence, biofilm formation, motility, intracellular survival, and immune evasion, particularly promoting the structural formation of biofilms. These results collectively indicate that LexA plays a critical role in *P. plecoglossicida*'s pathogenic process. To gain deeper insights into the impact and mechanisms of LexA on pathogenesis, we conducted RNA-seq and ChiP-seq analyses. Through GO and KEGG pathway enrichment analysis, we revealed that LexA may regulate virulence mechanisms by modulating the expression of virulence genes such as those in the flagellar assembly pathway and biofilm formation pathway.

This study is the first to identify three key motifs (GATGACGTCA, AGCGCGTCTT, and TTTGGCTATAAG) recognized and bound by LexA in *P. plecoglossicida* using ChIP-seq and EMSA. These motifs significantly differ from the reported LexA-box sequences in *E. coli*, suggesting that LexA's functions and regulatory mechanisms in *P. plecoglossicida* may differ from those in *E. coli*. Furthermore, the distinct sequence compositions among the motifs suggest that LexA may regulate different genes in *P. plecoglossicida* through distinct mechanisms. Unlike other widely conserved transcriptional regulators, the DNA-binding motif of LexA has experienced notable changes throughout evolution (Erill, Campoy, and Barbe, 2007). The ability of LexA to bind to distinct motifs might be

facilitated by the conformational flexibility of its dimer structure, which allows it to accommodate non-palindromic and diverse sequences (Sanchez-Alberola et al., 2015). While LexA is generally a dimer that typically binds to palindromic sequences, previous research has demonstrated that it can also recognize and bind to non-palindromic sequences under certain conditions. For instance, a similar non-palindromic motif has been observed in *B. thuringiensis*, where phage protein gp7 assists in binding to one of the two adjacent LexA binding sites: a palindromic site, dinBox1, and a neighboring non-canonical half-site, dinBox1b (Fornelos et al., 2015). Therefore, not all LexA boxes are perfectly palindromic (Candra, Cook, and Hare, 2024; Lewis, Jenkins, and Mount, 1992), and not all variations are directly related to gene regulation (Fernández De Henestrosa et al., 2000).

Correlation analysis of ChIP-seq and RNA-seq results identified seven common differentially expressed genes, namely *ycaC, duf1214, fucP, putA, atpD, lrgA*, and *tonB*. Among these, except for *duf1214*, the other six genes have been confirmed to be associated with pathogenicity in previous studies. For instance, the lysozyme hydrolase YcaC has been shown to be associated with cell membrane components in drug-resistant *E. coli* (Schmidt, Krizsan, and Volke, 2016); the regulator FucP has been reported to regulate the type III secretion system (T3SS) in *Edwardsiella tarda* and regulate virulence gene expression through intestinal fucose (Wu, Liu, and Sun, 2018); PutA, a bifunctional enzyme converting proline to glutamic acid, has been found to play a crucial role in the pathogenesis of *P. aeruginosa* (Zheng, Feng, and Wei, 2018); LrgA, whose exact function is not yet fully understood, has been associated with various virulence traits of *Streptococcus mutans*, such as biofilm formation and oxidative stress tolerance (Ishkov, Ahn, and Rice, 2020); and TonB, as a siderophore transport receptor, has been linked to virulence mechanisms in many bacteria, including *Pseudomonas fluorescens*, where its knockout led to a significant reduction in virulence (Hu, Dang, and Sun, 2012). Transcriptomic sequencing results also showed that, except for *duf1214*, the expression levels of the other genes were significantly upregulated. Subsequent qRT-PCR and LacZ reporter gene analysis results were consistent with the transcriptomic data. Therefore, we prove that LexA positively regulates the expression of the target gene *duf1214* and negatively regulates the expression of the other six virulence genes, which are likely to be key targets of LexA's regulation of *P. plecoglossicida*'s pathogenic process.

Although LexA is predominantly considered a repressive transcription factor, there are notable exceptions to this generalization. For instance, Arvind et al. (Kumar, Kirti, and Rajaram, 2018) demonstrated that in *Anabaena*, the LexA protein functions as both an activator and a repressor by binding to a palindromic AnLexA-box (AGT-N[4–11]-ACT), which is located upstream of at least 57 genes across diverse functional groups. This dual role of LexA as both an activator and a repressor is not an isolated phenomenon, but rather a common feature in bacterial systems, particularly in the regulation of virulence and environmental adaptation. A similar example is observed in *Edwardsiella piscicida*, where Yin et al. (Yin et al., 2018) identified the critical role of the promoter discriminator in bidirectional control of gene expression and virulence, mediated by RpoS. In the present study, we identified three distinct LexA recognition motifs in *P. plecoglossicida* and confirmed the direct activation or repression of several genes by LexA. However, the exact relationship between these motifs and the regulatory functions of LexA—whether they correspond to activation or repression—remains to be further investigated. This highlights the need for additional research to elucidate the mechanisms underlying LexA's complex regulatory roles in bacterial gene expression, particularly with respect to virulence factors and environmental stress responses.

LexA-Dependent transcriptional regulation is assisted by HtpG

In a previous study on *E. coli*, the combination of high-density microarray and chromatin immunoprecipitation revealed the binding of the LexA protein to atypical SOS box sites. Given that LexA binding is primarily restricted to biologically relevant sites, the authors hypothesized that LexA might cooperate with accessary factors to bind to these less conserved sites (Wade et al., 2005). Cooperative regulation of transcription is often mediated by DNA-binding proteins, which either directly interact with each other or indirectly alter DNA topology to prevent or promote the binding of RNA polymerase (RNAP) to promoters. A well-documented example of this is the cooperative interaction between the global transcriptional regulator cAMP receptor protein (CRP) and CytR in *E. coli* (Valentin-Hansen, Søgaard-Andersen, and Pedersen, 1996). Both are DNA-binding proteins; however, CytR cannot stably bind to promoter DNA independently—its efficient DNA binding relies on co-binding with CRP-cAMP. The binding of one protein can increase the affinity of another protein for adjacent binding sites through specific protein-DNA and protein-protein interactions. Similarly, CRP and MelR require cooperation to enhance MelR occupancy on DNA and effectively activate downstream gene transcription (Wade et al., 2001; Li and Biopesticides, 2024). In other systems, such as the *Bacillus subtilis* lysogenic bacteriophage phi29, the transcriptional regulator p4 interacts with the histone-like protein p6 to form a hairpin structure crucial for the switch between early and late gene transcription (Camacho and Salas, 2010). To date, there has been only one report of an exogenous accessary factor assisting LexA: Nadine et al. discovered that Bacteriophage GIL01 gp7 interacts with the host LexA repressor to enhance DNA binding and inhibit RecA-mediated auto-cleavage (Fornelos et al., 2015). No reports exist regarding endogenous accessary factors for LexA.

The study presents a comprehensive investigation into the role of LexA in *P. plecoglossicida* virulence, highlighting the identification of LexA-interacting proteins and their functional implications. co-IP-MS analysis revealed interactions between LexA and RecA, as well as HtpG, suggesting a complex protein-protein interaction network crucial for virulence regulation. Molecular dynamics simulations and molecular docking further supported the interaction between LexA and HtpG, indicating a potential collaborative role in virulence gene expression.

Moreover, the study demonstrated that RecA and HtpG are involved in the self-cleavage and degradation of LexA, with RecA catalyzing the cleavage and HtpG promoting the degradation process. This regulation is essential for controlling the expression of downstream virulence genes, including *ycaC, tonB, fucP, atpD*, and *duf1214*, as evidenced by the altered promoter activities in silenced strains. Our results, therefore, identify, for the first time, an endogenous factor that directly interacts with the transcription factor LexA to modulate virulence and environmental adaptation. Overall, the findings provide novel insights into the molecular mechanisms underlying LexA's function in *P. plecoglossicida*, paving the way for further research on bacterial virulence regulation.

Ethics statement

All animal experiments were carried out strictly under the recommendations in the 'Guide for the Care and Use of Laboratory Animals' set by the National Institutes of Health. The animal protocols were approved by the Animal Ethics Committee of Jimei University (Acceptance NO JMULAC201159).

Funding

This work was supported by the Natural Science Foundation of Fujian Province (No. 2022J02044), and the Ministry of Science and Technology of the People's Republic of China (No. 2023YFD2402600).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2025.100351.

Data availability

I have shared the link to our data at the File Attach step.

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