

Novel Insight of Transcription Factor PtrA on Pathogenicity and Carbapenems Resistance in *Pseudomonas aeruginosa*

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Introduction: Globally, *Pseudomonas aeruginosa* (PA) is emerging as a predominant nosocomial pathogen that often induces aggressive and even deadly infections. *Pseudomonas* type III repressor A (PtrA) can be activated specifically by copper ions and interacts with type-III transcriptional activator ExsA. This study aims to provide insight into the PtrA-mediated regulation of the pathogenicity and antibiotics resistance of PA.

Methods and Results: The results of transcriptome sequencing analyses and real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) showed that PtrA plays a dual regulatory role in the virulence systems of PA: negatively regulates the type-III secretion system (T3SS) and positively regulates the quorum-sensing system (QS). The *ptrA* mutant attenuated extracellular virulence related to QS like pyocyanin, elastase, rhamnolipids, proteolytic activity, and biofilm production. According to adhesion and invasion experiments, PtrA can not only contribute to the adhesiveness but also the invasive of PA. Moreover, the PtrA-mediated regulation of PA pathogenicity was determined both in vivo and in vitro through cytotoxicity and *Galleria mellonella* survival experiments. In addition, apart from virulence, PtrA was found to influence the carbapenems resistance of PA. After deleting *ptrA*, the minimum inhibitory concentration (MIC) of carbapenems antibiotics was decreased by 2-fold, while a 2–8 fold increase was noted for the complemented strain.

Conclusion: Our findings establish that PtrA exerts a regulatory role in both pathogenicity and carbapenems resistance of PA. This work may shed light on a novel target for the clinical treatment of PA.

Keywords: *Pseudomonas aeruginosa*, PtrA, T3SS, Quorum-sensing, pathogenicity, carbapenems resistance

Introduction

Pseudomonas aeruginosa (PA) is known as a common opportunistic pathogen that is widely disseminated on the skin, respiratory tract, and intestine.^{1,2} Several virulence factors,³ such as pili, flagella, lipopolysaccharides, alkaline protease, exotoxin A, and effector proteins, secreted by PA, can contribute to hospital-acquired infections in immunocompromised patients.^{4,5} Considering the ability to develop resistance against several of the presently available antibiotics, treating PA-induced infections in a clinical setting has become a challenge.⁶ PtrA is a member of the LysR-type transcription regulator (LTTR) family commonly found in prokaryotes.⁷ LTTR is involved in regulating the pathogenicity and metabolism-related functions of bacteria, including invasive virulence, QS, oxidative stress, and amino acid metabolism.^{8,9} However, how PtrA regulates the virulence of PA and is it related to host pathogenicity and antibiotics resistance remain unclear.

The secretion system is one of the important ways for bacteria to communicate and coordinate with the external environment in order to obtain the required nutrients, secrete toxic proteins, or directly attack eukaryotic cells.^{3,10} Several types of secretion systems have been discovered so far, of which T3SS is regarded as the key pathogenic factor in PA.^{11,12} Furthermore, it was demonstrated that most acute infections are related to T3SS in PA.¹³ Mainly, 4 types of toxin proteins have been studied, namely, extracellular enzyme S (ExoS), extracellular enzyme T (ExoT), extracellular enzyme U (ExoU), and extracellular enzyme Y (ExoY).^{10,13} Host tissue damage mediated by these proteins has been reported to lead to systemic infection and sepsis.¹⁴

The strong environmental adaptability and complex virulence regulation mechanism of PA are likely caused by biofilm formation and the possession of QS systems. The formation of biofilms often leads to chronic infection by PA.^{15,16} QS is a type of gene regulation mechanism that is coordinated by chemical signaling molecules to sense the communication within or between bacterial cells as well as to regulate the expression of the corresponding genes.¹⁷ At present, the QS system of PA mainly includes 4 types, Las, Rhl, Pqs, and Lqs, respectively. It has been confirmed that the phenotypic diversity of the PA strain is dependent on the intercellular signal processing regulated by the QS system.^{4,18} The QS system is closely associated with PA virulence and pathogenicity, and several extracellular virulence factors are produced by a pathogen (such as pyocyanin, elastase, rhamnolipids, proteolytic activity, biofilm production, and motility) are directly modulated by QS systems.^{17,19,20} Interestingly, QS can exert regulatory effects on the T3SS expression.^{21–24} Weina Kong demonstrated that the Rhl and Pqs system has a negative regulatory effect on T3SS-related genes, while the Las system exerts no regulatory effect on T3SS. In addition, it is proved that Pqs system and Rhl system regulate T3SS in different ways.^{22,23} The specific regulatory mechanism of T3SS is still unclear.

In addition to strong pathogenicity, PA often has a multidrug-resistant phenotype due to its strong environmental adaptability and inherent drug resistance. Carbapenems are the first choice in the treatment of multidrug-resistant PA.²⁵ With the spread of carbapenem-resistant PA, the WHO lists carbapenem-resistant PA as one of the most serious threats to humans.²⁶ It is urgent to find novel targets that can combat carbapenems resistance.

In this research, we recorded strong evidence that PtrA plays complex regulatory roles in the pathogenicity of PA via regulating T3SS and QS. Moreover, PtrA also influences the carbapenems resistance of PA. Our conclusions will supply a novel theoretical basis for preventing and controlling of PA infection in the clinic, particularly those caused by highly virulent and multidrug-resistant (MDR) PA.

Materials and Methods

Strains and Plasmids

The strains and plasmids employed in this study are summarized in [Table S1](#). The standard strain PAO1 was preserved in our laboratory, the plasmid PUCP-Red was donated by Dr. Hua Yu,¹³ and the plasmid pUCP20 was donated by Prof. Qiyu Bao.²⁷

Construction of PAO1-Derived Strain

The *ptrA* deletion mutant strain was synthesized as suggested elsewhere.²⁸ In short, the PAO1 strain genomic DNA was extracted, and the flanking primers containing the restriction sites were employed for PCR to amplify the upstream (*ptrA* Knockout Up-F and *ptrA* Knockout Up-R) and the downstream (*ptrA* Knockout Down-F and *ptrA* Knockout Down-R) homologous arms of *ptrA*. The gentamicin-resistance gene was amplified with the pJQ200SK plasmid as a template (Gmr-F and Gmr-R), and overlapping PCR was performed to construct linear target fragments (*ptrA* Knockout Up-F and *ptrA* Knockout Down-R). The PAO1/pUCP-Red strain was constructed through electrical transformation (25-μF capacitor, 600 Ω) introducing the pUCP-Red plasmid into PAO1 competent cells, and the positive transformants were determined by PCR (Red-F and Red-R). Finally, the linear target fragment was transformed into PAO1/pUCP-Red competent cells. Genomic DNA of positive clones screened on an LB agar plate containing gentamicin and carbenicillin were extracted for PCR identification (*ptrA* Knockout Up-F and *ptrA* Knockout Down-R), and the PCR products were dispatched for sequencing.

The pUCP-Red plasmid in the *ptrA* mutant strain was lost through the continuous passage in drug-free LB broth. PCR was conducted to amplify *ptrA* with upstream and downstream primers (*ptrA* Knockout Up-F and *ptrA* Knockout Down-R) designed with a restriction site (Table S2). The pUCP20 plasmid and *ptrA* PCR products were digested with *EcoRI* and *HindIII*-restriction enzyme and connected overnight. The complementation plasmid was transformed to *E. coli* DH5 α and screened on LB agar plates containing 100 μ g/mL of ampicillin at 37°C overnight. Plasmid DNA was extracted and electroporation into the competent cells of the *ptrA*-deletion mutant strain and then screened on LB plates supplemented with 300 μ g/mL of Cb. For the pUCP20 empty vector strains, the pUCP20 plasmid was electroporated into the competent cells of the PAO1 wild-type (WT) strain and PAO1 Δ *ptrA*, respectively, and then screened on LB plates supplemented with 300 μ g/mL Cb. The plasmid was extracted for PCR (*ptrA* complementary verification-F and *ptrA* complementary verification-R), and the positive-amplification products were confirmed by sequencing.

Transcriptome Sequencing Analysis

PAO1 and *ptrA* deletion mutant were cultured to the logarithmic growth stage, and total RNAs were extracted by Trizol/chloroform protocol, as described by the manufacturers (Life Technologies, CA, USA). The RNA of PAO1 and the PAO1 Δ *ptrA* strains were sent to the Beijing genomics institution (BGI) for transcriptome sequencing analysis. Raw data from sequencing was filtered to obtain a clean Read. RSEM (<http://deweylab.biostat.wisc.edu/rsem/rsem-calculate-expression.html>) was employed for the calculation of gene expression alterations in wild strains and *ptrA*-mutation strains.^{29,30} The differentially expressed genes (DEGs) were classified into different biological pathways according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation. A DEG with a false discovery rate (FDR) of ≤ 0.05 was considered to be significantly different. To acquire information on protein-protein associations, we employed DIAMOND to compare the genes to the STRING database (available online at <https://string-db.org/>),²⁰ and used a homology with known proteins to obtain the interaction relationships between the proteins. Each sample was performed using 3 biological replicates.

Quantitative Real-Time PCR (RT-qPCR)

The strain of PAO1 and PAO1 Δ *ptrA* were inoculated on a blood plate at 37°C for 16–18 h. A single colony was selected and cultured in LB medium with shaking at 180 rpm until reaching the logarithmic phase at 37°C. The bacterial suspension was then harvested for RNA extraction by using the Trizol method. Then, reverse transcription of the extracted RNA into cDNA using a Reverse Transcription kit, and diluted to the same concentration. TB Green Premix Ex Taq II (TOYOBO, Osaka, Japan) and primers (Table S2) were added, and the reaction was conducted on a fluorescence quantitative PCR instrument (7500 RT-PCR System, Marsiling, Singapore). The 16S rRNA gene was simultaneously used as a reference gene for data normalization. The $2^{-\Delta\Delta C_t}$ formula was used to calculate the relative gene levels. All experiments were performed in triplicate and repeated thrice independently.

Bacterial Motility Assay

Bacterial motility plates were prepared as described elsewhere³¹ with slight modifications. Swimming plates were prepared of LB agar containing 0.2% agar (consisted of 0.1% tryptone, 0.05% yeast extract, 0.5% NaCl, and 0.2% bacteriological agar). LB broth solidified with 1% agar was used for the twitching motility assay. The media used for the swarming motility assay consisted of 0.5% agar, 10 g/L tryptone, 5 g/L NaCl, and 5 g/L glucose. For swarming and swimming motility, 2 μ L of overnight cultured strains were inoculated in the center of the swimming plates and swarming plates. The strains were stabbed to the bottom of twitching plates and inoculated for twitching motility. Motility diameter in each plate was determined after 24h of incubation.

Pyocyanin Production

Single bacterial colonies of PAO1 and its derivative strains were dipped and overnight cultured in LB medium at 37°C. After adjusting the absorbance at OD₆₀₀ nm of the bacterial solution to 0.5 with LB medium, the culture medium was centrifuged (16,000 \times g, 10 min) and the supernatant was retrieved for analysis. In short, 1 mL chloroform was blended with 0.5mL supernatant, and pyocyanin was re-extracted with 0.5-mL HCl (0.2 M). Subsequently, a pink solution was formed, and the absorbance was measured at OD₅₂₀.

Elastolytic Activity

The elastase activity was determined using Elastin–Congo red (ECR; Sigma) as the substrate. Briefly, 10 mg of ECR was introduced to 100 μ L of the bacterial culture supernatant and 900 μ L of ECR buffer (100 mM Tris, 1 mM CaCl_2 ; pH 7.5), followed by incubation at 37°C for 18 h. Elastase, produced by bacteria, can break down elastin. After the reaction was completed, 1 mL of 0.7 M sodium phosphate buffer (pH 6.0) was added and the reaction mixture was placed in the cold water bath to stop the reaction. The undecomposed elastin was separated through centrifugation, and the supernatant absorbance value was recorded at 498nm. The experiment was repeated thrice, and the average value was calculated.

Detection of Rhamnolipid Production

Rhamnolipid production was detected by a past described method.³² Rhamnolipid test plates were prepared of M9 agar with 2 mM (wt/vol) MgSO_4 , 0.2% (wt/vol) glucose, 0.02% cetyltrimethylammonium bromide (CTAB), 0.005% (wt/vol) methylene blue, and 0.05% (wt/vol) glutamate. Then, 2 μ L of overnight bacterial culture with the OD_{600} of 0.5 was dropped on the rhamnolipid test plate, incubated at 37°C for 24h, followed by incubation at room temperature for 48h. The rhamnolipid produced by the bacteria formed a blue halo on the plate, and rhamnolipid formation was measured based on the diameter of the blue halos.

Detection of Proteolytic Activity

Proteolytic activity of each strain was detected.³³ Briefly, 2 μ L of the above-mentioned overnight culture was dropped onto the milk plate containing 1% LB agar and 2.0% skim milk (wt/vol) and inoculated at 37°C for 24 h. As the bacteria could hydrolyze the protein, they created transparent rings on the milk plate. The diameter of the clearing circles represented the proteolytic activity of the bacteria.

Growth Curve Assay

The effect of PtrA on the growth of each strain was measured. Bacteria cultured overnight at 0.5-McFarland was prepared. 200 μ L bacterial solution with 20mL fresh LB medium was incubated on a shaker at 200 rpm at 37°C. The OD_{600} value was recorded every 2h with the Microplate Reader over 24 h by Microplate Reader (Bio Tek).

Biofilm Production Assay

Crystal violet (CV) staining was performed to detect the biofilm formation ability of each strain.³⁴ Adjusted the overnight cultured bacteria to 0.5 McFarland and diluted at 1:100. Next, 100 μ L LB broth and 100 μ L bacteria solution was introduced to a 96-well plate, followed by incubation at 37°C for 20–24 h. Discard planktonic bacteria, and washed the plate with PBS. Stain the biofilm with 150 μ L of 0.1% CV at 37°C for 15 min. After washing the plate with PBS, 150 μ L of 95% alcohol containing 5% acetic acid was introduced and placed at 37°C for 15 min to solubilize the stained biofilms. Recorded the absorbance at 595nm.

Adhesion and Invasion Assays

The adhesion and invasion capacities of each strain were measured according to the previous method.^{35,36} Briefly, overnight cultures of each strain were subcultured to the OD_{600} of 1. The culture medium underwent centrifugation at 4000 \times g for 5 min, and fresh DMEM was introduced to suspend the cell pellets. Each strain was co-incubated with RAW264.7 cells (ATCC, Manassas, VA) for 3 h in a carbon dioxide incubator with an optimal multiplicity of infection (MOI) of 50:1.³⁷ The RAW264.7 cells in 24-well plates were rinsed with PBS 4 times and lysed with 1mL of 1% Triton X-100. After diluted with PBS, 100 μ L of the lysate diluents were spread on the MHA plates for CFU enumeration.

For invasion assays, polymyxin B was used to kill the extracellular bacteria of RAW264.7. After 3 h of bacterial infection, 1mL of 100 μ g/mL polymyxin B was introduced to the 24-well plate for 1h. RAW264.7 cells were washed with sterile PBS 4 times, and 1mL 1% TritonX-100 was added to lyse the RAW264.7 cells. The lysate was diluted in different multiples and smeared on the MH agar and inoculated at 37°C for 18 h. The adhesion and invasion rates were calculated according to the enumeration of the bacterial CFUs of each strain.

Cytotoxicity Assay

The cytotoxicity test was conducted on RAW264.7 cells previously cultured in DMEM medium augmented with 10% heat-inactivated fetal bovine serum (FBS) and incubated under a 5% CO₂ incubator at 37°C until reaching the desired confluence. The confluent cells were harvested with trypsin. About 100 µL of the cell suspension (1×10^4 CFU/mL) was plated into each well of a 96-well microplate, followed by incubation for 8 h. Next, 10 µL of overnight cultures of each strain were cultured with 100 µL of RAW264.7 cell suspension (1×10^4 CFU/mL) with an MOI of 10:1. Following a 5 h incubation at 37°C, 50 µL of the supernatant was collected from each well, and the activity of alkaline phosphatase (ALP) was determined by using a commercial kit for ALP assay. After cell lysis and death, the amount of ALP released reflected the toxicity of the bacteria to cells. The absorbance of OD₅₁₀ was recorded to calculate the percent of cell growth inhibition.

Galleria mellonella Infection Experiments

To test the pathogenicity of each strain in vivo, *G. mellonella* infection experiments were conducted. Briefly, insects weighing 250–350 mg were selected. PAO1 and its derivative strains were cultured overnight and the bacteria concentration was adjusted to 1×10^2 to 1×10^5 CFU/mL with PBS. Next, 10 µL bacteria solution was administered to the rear left proleg of *G. mellonella* and incubated at 37°C. Insects injected with sterile PBS served as the negative control. The survival rate of the *G. mellonella* was recorded every day until the 7th day. When the larvae did not respond to any physiological stimuli, they were considered dead. The Log rank test was conducted to assess insect mortality.

Antimicrobial Susceptibility Testing

To explore the regulatory role of PtrA on antimicrobial resistance, the MICs of antibiotics commonly used in the treatment of PA, including levofloxacin, ciprofloxacin, piperacillin, cefixime, ceftazidime, aztreonam, amikacin, tobramycin, colistin E, imipenem, meropenem, and ertapenem were measured using the microdilution broth technique. The MICs of antibiotics were defined as the lowest concentration of antibiotics that visibly inhibited bacterial growth. The interpretation of the results was made with reference to the Clinical and Laboratory Standards Institute (CLSI).

Statistical Analyses

The Graphpad 8.0.2 software was employed for all statistical analyses. Inter-group differences were assessed by the Student's *t*-test. A Log rank test was applied to evaluate the *G. mellonella* survival rate. All data represent a minimum of 3 individual experiments, and the results are presented as mean ±SD. $P < 0.05$ was set as the significance threshold.

Results

Transcriptomics Profiling of the PtrA Mutation Strain

According to RNA-Seq analysis, 493 DEGs were identified, with 114 genes up-regulated and 379 genes down-regulated in the *ptrA* deletion mutant (Figure 1A). The KEGG pathway enrichment clusters revealed that DEGs heavily contribute to the cellular community, cell motility, membrane transport, signal transduction, and the global and overview maps (Figure 1B). The protein network diagram showed that 290 downregulated DEGs and 87 upregulated DEGs revealed 1001 interactions (Figure 1C). The STRING and metabolism analyses indicated that DEGs mainly interfered with the processes of T3SS (*exoT*, *exoS*, *exoY*, *exsA*, *exsC*, *pcrV*, and *pcrH*), T6SS (*hcpA*, *hcpB*, *hcpC*, *hcpI*, *clpV1*, and *vgrG1*), QS systems (*rhlA*, *rhlB*, *lasA*, *lasB*, *pqsA*, *pqsB*, *pqsC*, *pqsD*, and *pqsE*), flagellum biosynthesis (*flgB*, *flgC*, *flgD*, *flgE*, *flgF*, *flgK*, *flgL*, *flgM*, *fliC*, and *fliD*), polysaccharide biosynthesis (*pelA*, *pelB*, *pelC*, *pelD*, *pelE*, *pelF*, and *pelG*), and chemotaxis (*cheA*, *cheD*, *cheR*, *cheV*, *cheW*, and *cheY*).

The Regulatory Role for PtrA on T3SS and QS-Related Genes of PA

To further verify the PtrA-mediated regulation of T3SS and QS, RT-qPCR was performed with 16S rRNA as an internal control. Specifically, T3SS (ie, *exsA*, *exoS*, and *exoT*) genes were found to be upregulated (>2 fold) in *ptrA* mutant strain compared with WT strain (Figure 2A). Moreover, the expression of QS genes (*rhlA*, *rhlB*, *lasA*, *lasB*, *pqsA*, and *pqsE*)

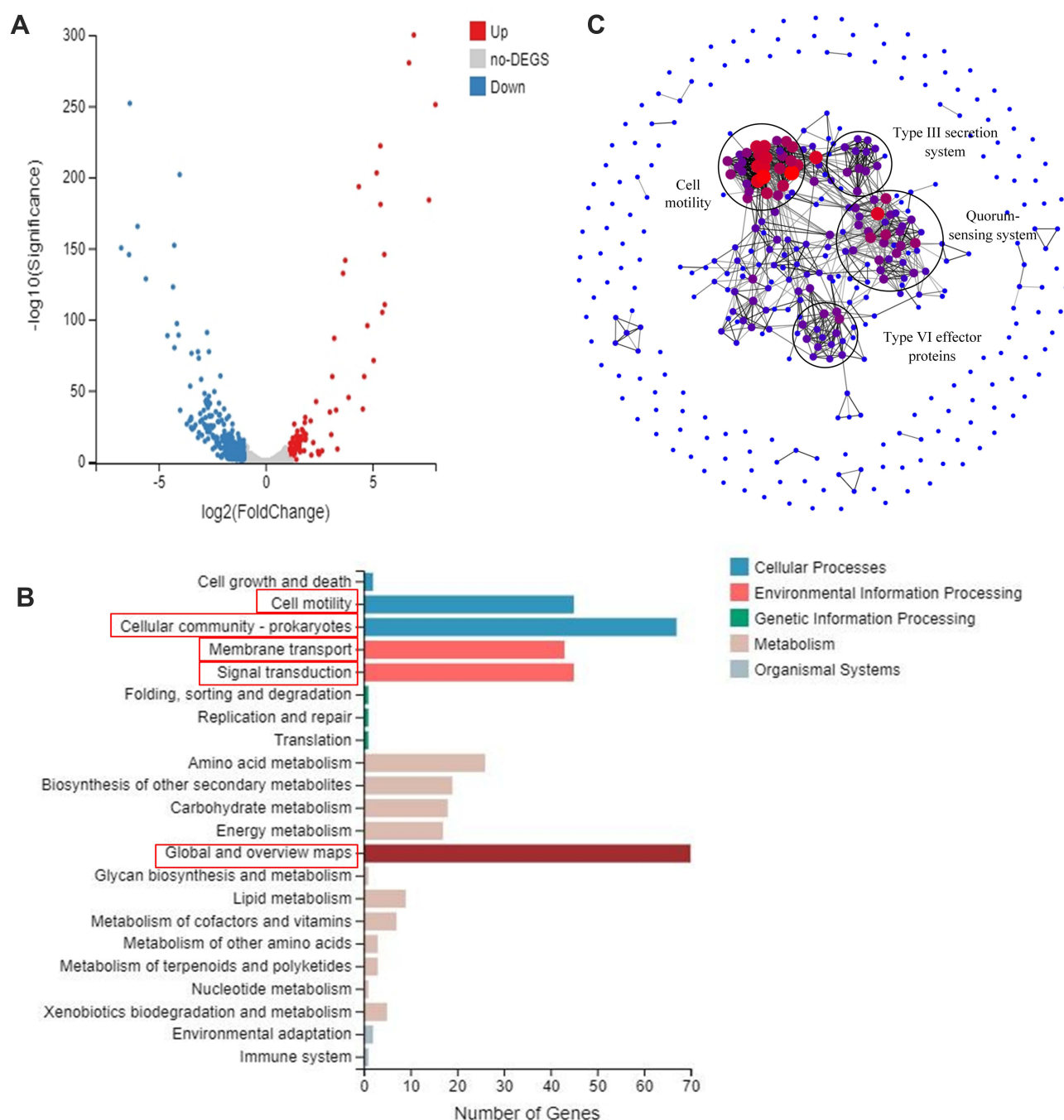


Figure 1 Transcriptome sequencing analysis of $\Delta ptrA$ and PAO1 strain. **(A)** The volcano plot of DEGs. **(B)** The KEGG pathway classification enrichment of DEGs. **(C)** STRING analysis of 290 downregulated and 87 upregulated DEGs; Lines represent reported or estimated protein-protein associations, and the nodes of genes involved in conducting similar functions are circled in black.

markedly reduced (>2 -fold) in the *ptrA* mutant strain (Figure 2B). Based on these data, PtrA serves as a negatively regulatory in T3SS while a positive regulatory in QS-related genes.

PtrA Positively Controlled the Motility of PA

The motility of PA is an important manifestation of bacterial invasiveness, which is closely related to the virulence and pathogenicity of PA. The movement of PA varies with different environmental media, such as swimming in a liquid environment, swarming on a semi-solid surface, and twitching on a solid surface. Swimming is mainly mediated by

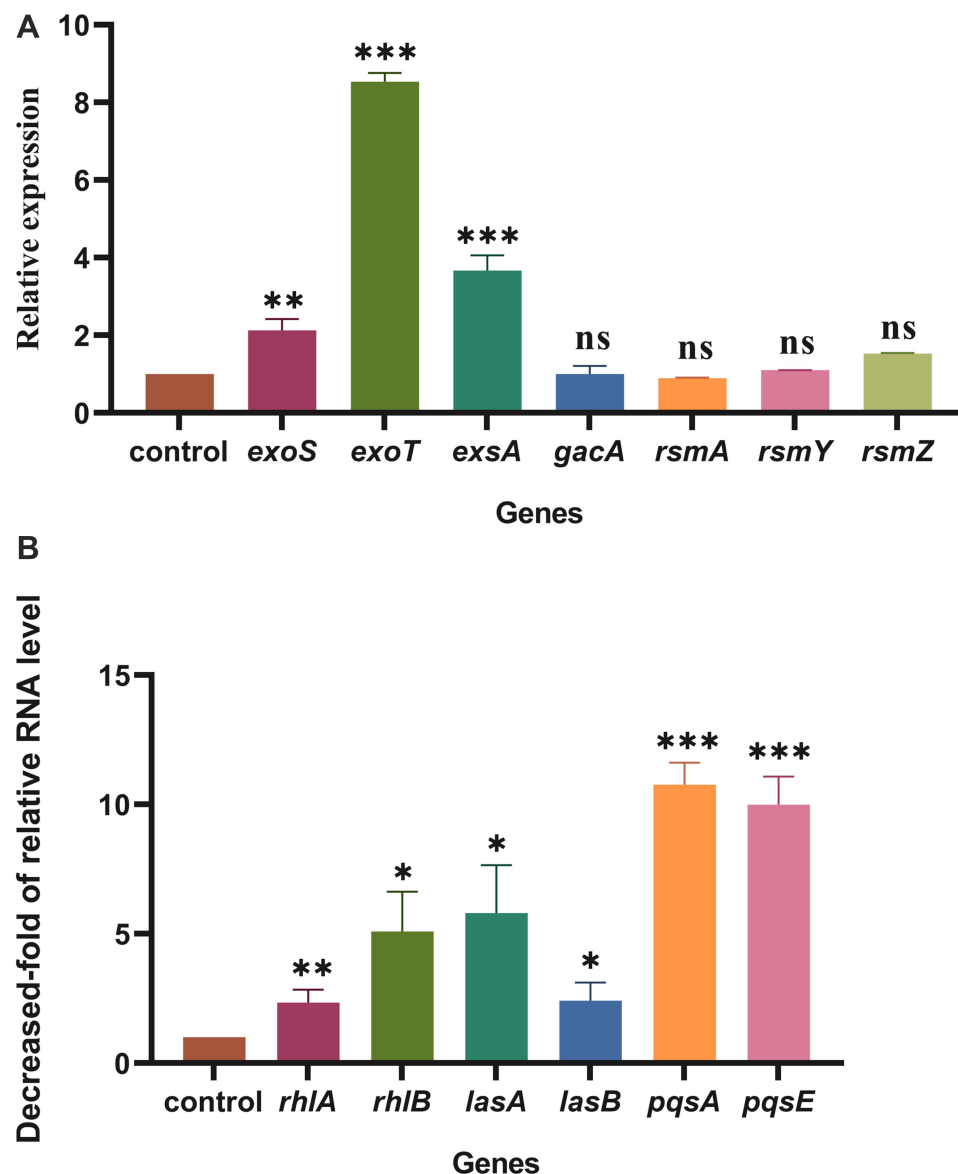


Figure 2 The relative quantitative analyses of T3SS, and QS-related genes. **(A)** The effect of PtrA on T3SS gene. **(B)** The effect of PtrA on QS gene. The gene expression level in WT strain was adjusted to 1.0 (presented as a dotted line). ns, not statistically significant, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ analyzed via Student's *t*-test. The results are expressed as mean \pm SD, and the data represent a minimum of 3 individual experiments.

flagella, whereas swarming and twitching are mediated by flagella and type IV fimbriae. To study the effect of PtrA on PA motility ability, motility experiments were carried out. As shown in Figure 3, after 24 h of incubation, the swimming, swarming, and twitching diameter of the *ptrA* mutant strains (14.7, 10.7 and 6.3 mm, respectively) was found to be significantly decreased when compared with that of the wild-type strains (54.7, 31.3 and 27.7 mm, respectively). Therefore, the mutation of *ptrA* resulted in reduced PA motility through swimming, swarming, and twitching motilities.

PtrA Positively Controlled the QS-Related Extracellular Virulence Factor of PA

A large number of extracellular virulence factors are controlled by QS, including elastase, pyocyanin, rhamnolipids, and proteolytic activity. Pyocyanin is a blue phenazine compound, that can promote infection and pathogenicity by inducing neutrophil apoptosis, inhibiting cilia movement of respiratory epithelial cells, and inhibiting the growth of other microorganisms. Both elastase and exoproteases secreted by PA can act on the host's immune regulatory system. Elastase facilitates disarming of the host immune system by degrading immunoglobulins and disrupting the complement system to reduce immune cell production. Rhamnolipid is a biosurfactant associated with PA biofilm formation, enabling bacteria to adhere to surfaces and

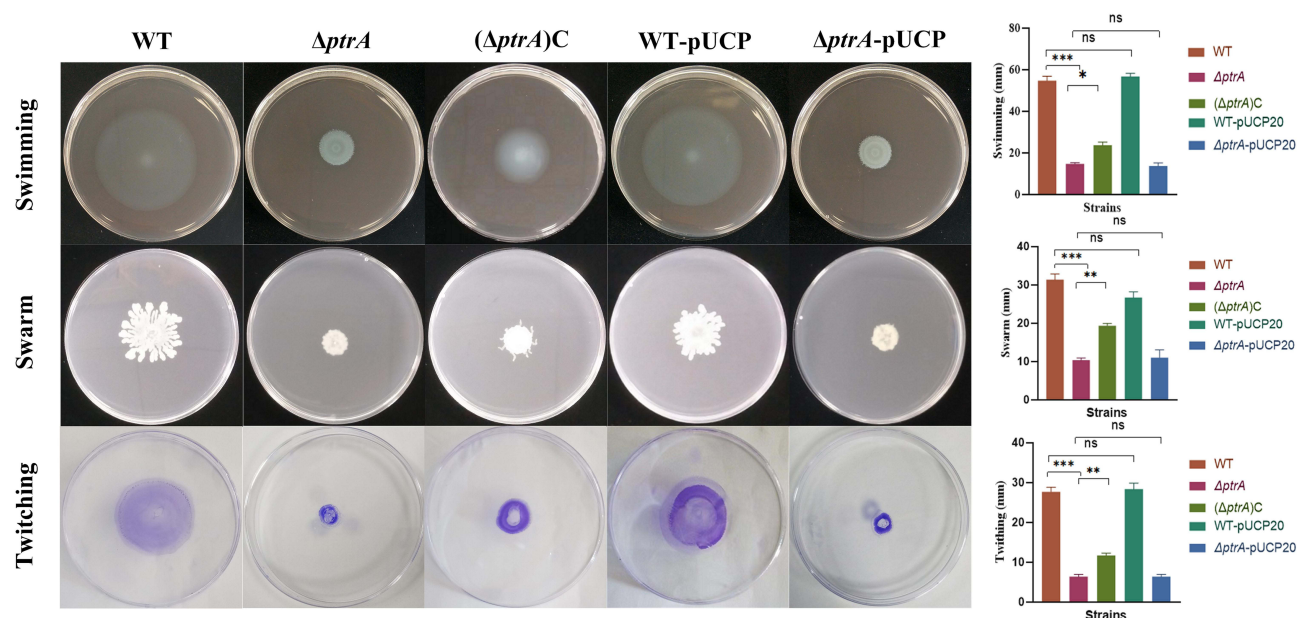


Figure 3 Effect of *PtrA* mutation on motility. ns, not statistically significant, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ analyzed via Student's *t*-test. The results are expressed as mean \pm SD, and the data represent a minimum of 3 individual experiments.

form the biofilm matrices. Proteases mediate host cell penetration and disrupt host cell immune system regulation. Figure 4A and B shows that the color of PAO1 was darker than that of the PAO1 $\Delta ptrA$ strain after overnight culturing, indicating the production of pyocyanin and elastase was significantly higher in the PAO1 strain than that in the PAO1 $\Delta ptrA$ strain, and the difference was statistically significant ($P < 0.05$) (Figure 4C and D). Figure 4E and F show that the diameter of the blue halos and the clearing

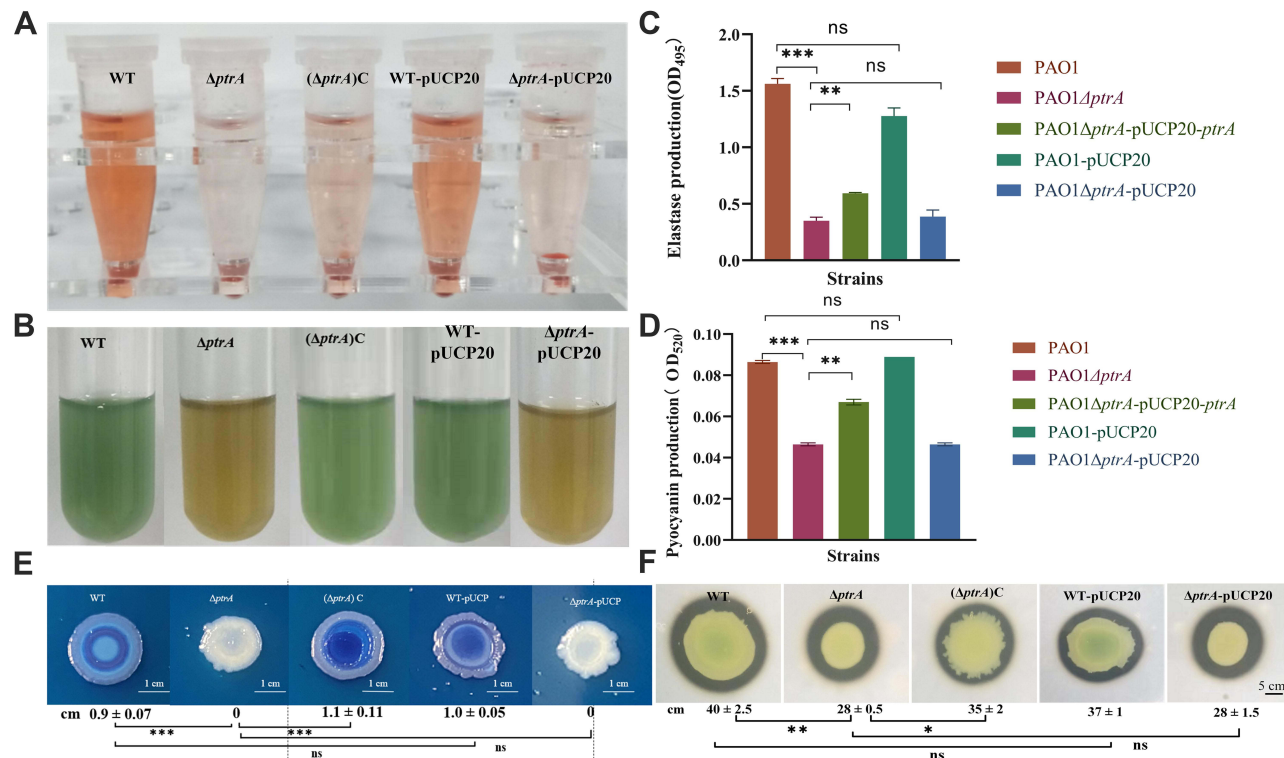


Figure 4 Effect of *PtrA* on QS-related extracellular virulence factor. (A and C) The determination of elastase production. (B and D) The determination of pyocyanin production. (E) The determination of rhamnolipid production. (F) The determination of proteolytic activity. ns, not statistically significant, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ analyzed via Student's *t*-test. The results are expressed as mean \pm SD, and the data represent a minimum of 3 individual experiments.

circles of PAO1 were bigger than that of the PAO1 $\Delta ptrA$ strain, which means that the productions of rhamnolipids and exoproteases were drastically decreased in the $\Delta ptrA$ strain. Our results signify that PtrA could positively regulate various QS-related extracellular virulence factors.

PtrA Affected Planktonic Growth and Biofilm Formation

There exists a relationship between the growth of PA planktonic cells and their spread, and emerging evidence suggests that chronic infection is correlated with biofilm formation.^{38–40} To verify whether PtrA affects the proliferation and adhesion of PA, a growth curve was drawn and a biofilm formation experiment was performed. We noted that the growth rates of each strain were comparable at 0–8 h. However, when entering the logarithmic phase, the growth rates of $\Delta ptrA$ strain were lower than the WT strain ($P < 0.05$) (Figure 5A). Concerning biofilm formation, as shown in Figure 5B–C, deletion mutation of *ptrA* caused an almost 75% reduction in biofilm-forming of the PAO1 strain. Therefore, PtrA had a positive effect on the growth of planktonic bacteria and the biofilm formation of PA.

PtrA Positively Regulated the Adherence and Invasion of PA

The adhesion and invasion abilities of bacteria are closely related to their pathogenicity. To illuminate the effect of PtrA on the adherence and invasion capacities of PA to infect the host cells, the adhesion and invasion assays were conducted. As shown in Figure 6, the capacity of the PtrA mutant to adhere and invade RAW264.7 cells was found to be weaker than that of the WT strain, and it decreased by 59.2% and 87.0%, respectively. As can be seen, PtrA is critical for adhering and invading capacities.

PtrA is Required for Bacterial Pathogenicity in PA

To explore the PtrA-mediated regulation of PA virulence in vitro and in vivo, a cytotoxicity test and the *G. mellonella*-infection experiment were performed. The result of the cytotoxicity assay indicated that the cytotoxic activity of the PAO1 WT strain against RAW264.7 cells was markedly higher than *ptrA* mutant strain ($P < 0.01$, **) (Figure 7A). In the

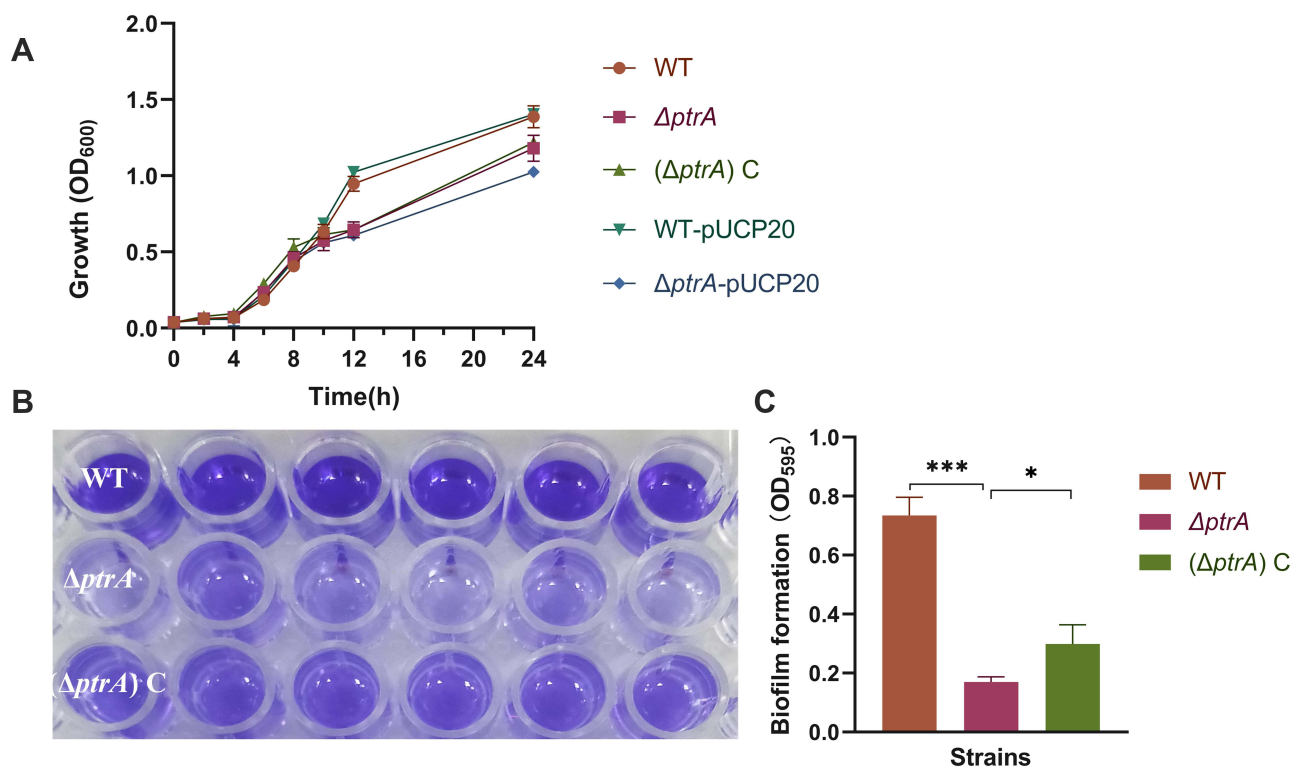


Figure 5 PtrA affected planktonic growth and biofilm formation. (A) Growth curve assay. (B and C) Biofilm formation assay. ** $P < 0.01$, and *** $P < 0.001$ analyzed via Student's *t*-test. The results are expressed as mean \pm SD, and the data represent a minimum of 3 individual experiments.

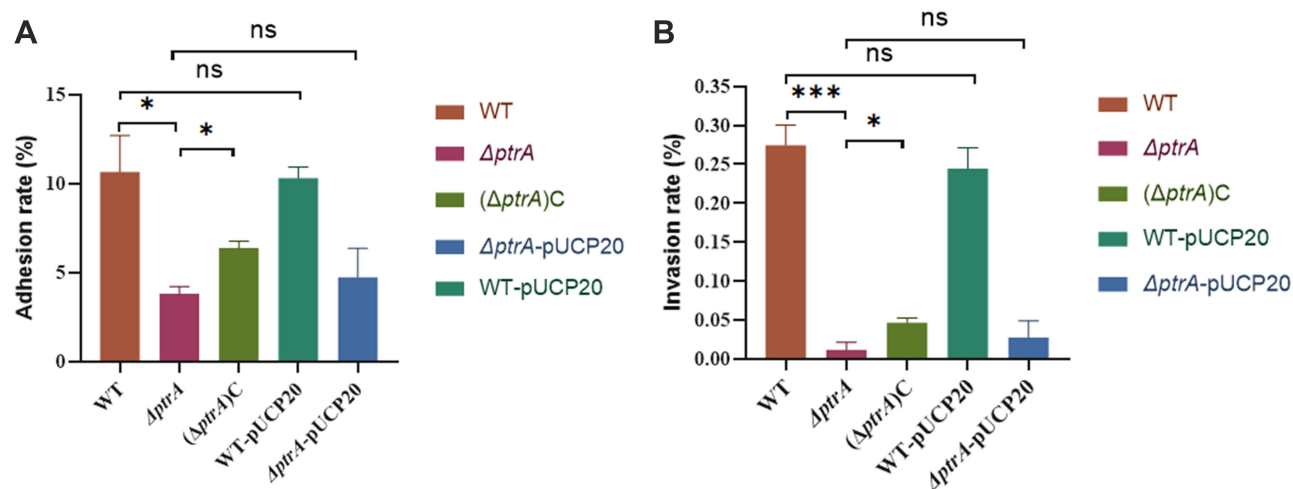


Figure 6 Analysis of the adherence and invasion ability of PA. (A) Adherence; (B) Invasion. ns, not statistically significant, * $P < 0.05$, and *** $P < 0.001$. The results are expressed as mean \pm SD, and the data represent a minimum of 3 individual experiments.

G. mellonella-infection model, the survival rates of the WT strains under the same inoculation doses were markedly reduced, compared to the *ptrA* mutation strain ($P < 0.05$). All *G. mellonella* larva administered with PBS survived after 168 h (Figure 7B). Hence, *PtrA* plays an essential regulating PA pathogenicity both in vivo and in vitro.

PtrA Mutant Was More Susceptible to Carbapenems

We noted that, other than virulence, *PtrA* had a minor influence on carbapenems resistance in PA. According to the antimicrobial susceptibility testing, the MICs of carbapenems antibiotics (ie, imipenem, meropenem, and ertapenem) decreased 2-fold in PAO1 $\Delta ptrA$, relative to the PAO1 WT strain. Interestingly, in the complementation strain, the MICs of cephalosporins and carbapenems antibiotics (ie, cefixime, ceftazidime, imipenem, meropenem, and ertapenem) increased by 2-8-fold. No changes were observed between each strain for the other tested antibiotics. The antibiotic resistance profiles are displayed in Table 1.

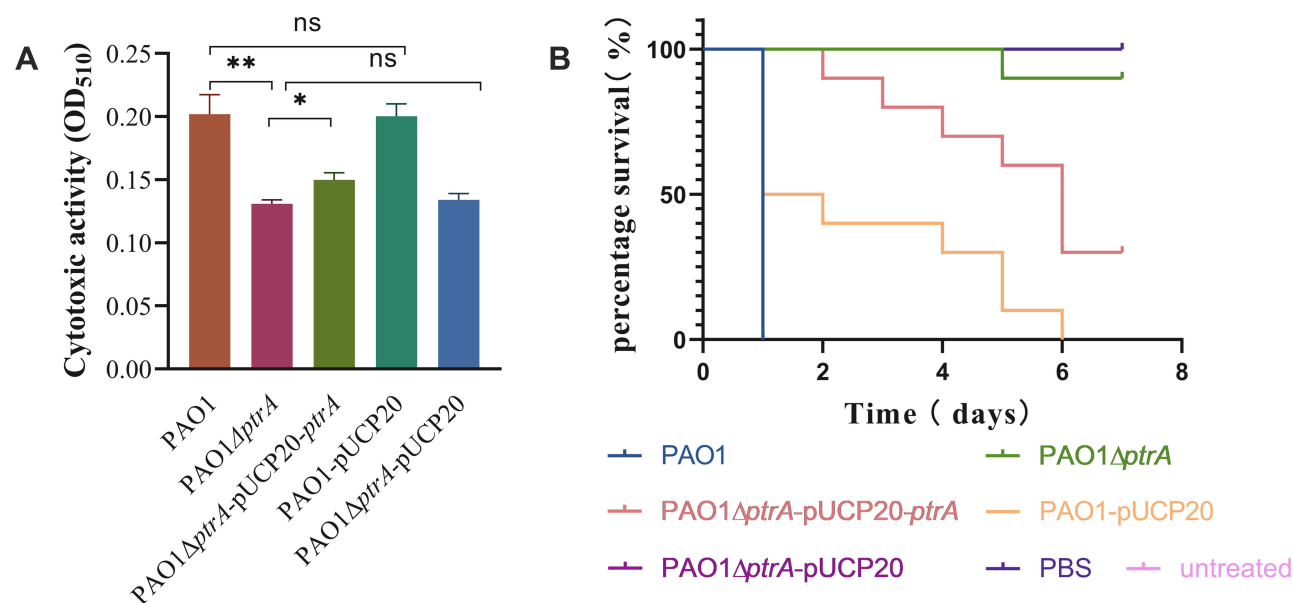


Figure 7 *PtrA* is crucial for PA virulence. (A) Cytotoxicity assay. (B) *Galleria mellonella* experiments. ns, not statistically significant, * $P < 0.05$, and ** $P < 0.01$ via Student's *t*-test. The results are expressed as mean \pm SD, and the data represent a minimum of 3 individual experiments.

Table 1 The Minimum Inhibitory Concentration (MIC) of Common Antibiotics

MICs (μg/mL)	Strains				
	PAO1	$\Delta ptrA^a$	$\Delta ptrA (C)^b$	WT-pUCP20	$\Delta ptrA$ -pUCP20
Levofloxacin (≥ 4)	0.25	0.25	0.25	0.25	0.25
Ciprofloxacin (≥ 2)	0.25	0.25	0.25	0.25	0.25
Piperacillin (≥ 128)	4	4	256	256	256
Cefixime (≥ 32)	2	2	4	2	2
Ceftazidime (≥ 32)	2	2	8	2	2
Aztreonam (≥ 32)	8	8	32	32	32
Amikacin (≥ 64)	2	2	2	2	2
Tobramycin (≥ 16)	0.25	0.25	0.25	0.25	0.25
Colistin (≥ 4)	0.125	0.125	0.125	0.125	0.125
Imipenem (≥ 8)	2	1	16	2	1
Meropenem (≥ 8)	0.5	0.25	2	0.5	0.25
Ertapenem (≥ 8)	8	4	16	8	4

Notes: ^aBold fonts represent the MIC of antibiotics decreased in the *ptrA* mutation strain. ^bBold fonts represent the MIC of antibiotics increased in the *ptrA* complementation strain.

Discussion

PA possesses strong environmental adaptability, complex virulence systems, and drug resistance mechanism, which make the infection difficult to tackle.⁴¹ *PtrA* is a newly discovered transcription-related regulator in the *Pseudomonas* genus, which belongs to the most common transcriptional regulators family in PA, the LTTR family.⁷ However, the effect of *PtrA* on T3SS expression seems to be disputed in the literature. Ha⁴² reported that *PtrA* can act as a specific inhibitor of ExsA and can hence inhibit T3SS expression in PA; Elsen opined that *PtrA* is specifically induced by copper and that it neither regulates ExsA activity nor T3SS production.^{42,43} In this study, after deleting *ptrA*, the relative transcript level of *exoS*, *exoT*, and *exsA* were significantly upregulated (Figure 2), implying that *PtrA* may be an inhibitor of T3SS.

The transcription of T3SS effector protein and structural protein-linked genes is mainly modulated by ExsA, which can recognize and bind to common sequences of upstream transcription initiation sites in the T3SS genes and regulate their expression.^{12,44} Moreover, the expression of *exsA* is mainly regulated by 3 pathways, GacS/A-RsmA, CyaB-cAMP/Vfr, and PsrA-RpoS. Among these, GacS/A-RsmA is the most common signaling pathway affecting the regulatory factors of T3SS.⁴⁵⁻⁴⁷ We, therefore, investigated whether *PtrA* regulates the ExsA expression through the GacS/A-RsmA pathway. However, according to the results of RT-qPCR, *ptrA* mutations did not affect the GacS/A-RsmA pathway (Figure 2A). Consistent with Ha,⁴² there may exist a direct association between *PtrA* and ExsA.

The QS system is associated with PA virulence and pathogenicity closely, and several virulence-related factors are strictly controlled by the QS system.¹⁷ Whether *PtrA* can regulate QS and what regulatory role it plays remains undisclosed. Based on transcriptome sequencing and qRT-qPCR, the expression levels of several crucial QS genes in the *ptrA* mutant strain were significantly decreased. Furthermore, our results show that QS-related virulence like motility, pyocyanin, elastase, rhamnolipids, proteolytic activity, and biofilm formation are both positively controlled by *PtrA*. There are four QS systems (including Las, Rhl, Pqs, and Lqs) in PA, and there is mutual regulation between them. Las can positively regulate the gene expression of RhlI, RhlR, and PqsR,^{48,49} and the Pqs system also regulates Las and Rhl through its PQS signal.⁵⁰ Previous studies have shown that the QS system of PA is often directly regulated by transcriptional regulators.⁵¹⁻⁵³ According to our result, we speculate that *PtrA* may directly act on the QS system of PA. However, which system *PtrA* mainly acts on and how it affects the interaction of each system needs to be further explored in our subsequent research.

The results of adhesion and invasion assays displayed that the adhesion and invasion ability of *ptrA* mutant strain to RAW264.7 cells was significantly decreased compared with the parental strain. Furthermore, cytotoxicity and *G. mellonella* experiment demonstrated that *PtrA* affects PA pathogenicity both in vivo and in vitro. Although both T3SS and QS are important virulence regulatory systems in PA, the *PtrA*-induced changes in PA pathogenicity should be

mainly regulated by QS. Furthermore, according to previous studies, Rhl and Pqs systems exert a negative regulatory effect on T3SS-related genes.^{21–24} Therefore, PtrA could regulate the virulence of PA in a complex manner, and the effect of PtrA on T3SS may be regulated by QS. However, several virulence phenotypes in complementation have not recovered completely compared with its parent strain strains. As previously reported,⁵⁴ we speculated that this is due to we introducing *ptrA* gene through a plasmid to *ptrA*-disrupted mutants rather than restore in their chromosome.

In addition to investigating the PtrA-mediated regulation of virulence, we also examined the function of PtrA in antimicrobial resistance. Interestingly, there was a 2-fold decrease in the MIC of carbapenems antibiotics in the *ptrA* mutation strain, and the MICs of carbapenems antibiotics increased 2–8-fold in the *ptrA* complementation strain. It is thus suggested that PtrA has a certain influence on carbapenems antibiotic resistance. There are hundreds of two-component systems (TCSs) in PA.⁵⁵ The CopS/CopR system can participate in imipenem resistance by regulating the expression of *oprD*.⁵⁶ In addition, the Mex efflux pump is an important cause of multidrug resistance in PA, and it is responsible for carbapenems resistance.^{57,58} Furthermore, evidence suggests that both PQS and the PqsE-mediated network respond to carbapenems resistance.^{51,59} However, how PtrA precisely regulates carbapenems resistance warrants further study.

Conclusion

Overall, PtrA can function as a global regulator that controls several metabolic pathways in PA. Our research revealed that, PtrA can positively regulated the growth rate of PA and a variety of QS-related extracellular virulence factors, while negatively regulating the expression of T3SS-related genes and the sensitivity of carbapenems to PA. The impact of PtrA on virulence and resistance can provide novel insights into the virulence and resistance regulatory network of PA, while PtrA is expected to become a new target for clinical treatment.

Data Sharing Statement

The transcriptomic datasets from this study are available in the NCBI Sequence Read Archive (SRA). The corresponding accession number(s) were SRR14139708, SRR14139709, SRR14139710, SRR14139711, SRR14139712, and SRR14139713.

Ethical Approval

The whole investigation protocols in this study were approved by The Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. There are no studies with humans or animals performed by any of the authors in this article. Informed consent was waived because this study with observational nature mainly focused on bacteria and did no interventions for patients.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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