



The effects of LL-37 on virulence factors related to the quorum sensing system of *Pseudomonas aeruginosa*

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Background: Antimicrobial peptides (AMPs) have shown promise in the treatment of multi-resistant pathogens. It was therefore of interest to analyze the effects of the AMP LL-37 on the regulation of several virulence factors related to the quorum sensing (QS) system of *Pseudomonas aeruginosa* (*P. aeruginosa*) *in vitro*.

Methods: The minimum inhibitory concentration (MIC) was evaluated by the micro broth dilution method. The expression of QS-related and QS-regulated virulence factor genes was also evaluated. Exotoxin A activity was measured with the nicotinamide adenine dinucleotide (NAD) (Coenzyme I) method; Elastase activity was detected with the elastin-Congo red (ECR) method; Pyocyanin detection was performed using the chloroform extraction method. The effects of LL-37 were assessed by measuring the expression changes of the virulence protein-encoding genes of the strains with quantitative polymerase chain reaction (PCR).

Results: The MIC of LL-37 against both *P. aeruginosa* reference strain (ATCC 15692 PAO1) and *PA-ΔlasI/rbII* was therefore determined to be 256 µg/mL. LL-37 at sub-minimum inhibitory concentrations (sub-MICs) had no significant effects on *P. aeruginosa* bacterial growth ($P > 0.05$), but significantly downregulated the expression of all 3 virulence factors.

Conclusions: Interestingly, this effect appeared to be dose-related. These findings suggest that LL-37 could be a potential candidate for QS inhibition against bacterial infection and may have significant clinical potential in the treatment of *P. aeruginosa* biofilms.

Keywords: Antimicrobial peptide LL-37; virulence factor; *Pseudomonas aeruginosa* (*P. aeruginosa*); quorum sensing system

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Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a complex Gram-negative conditional pathogen that is responsible for both hospital-acquired and community-acquired infections (1-4). Quorum sensing (QS) is a cell density-based intercellular communication system. *Pseudomonas* has a QS system (5-7) and exhibits significant resistance to antibiotics and the innate immune system. The QS system of *Pseudomonas* is one of its most important signal transduction systems and is the key to its pathogenicity (8,9). QS has a cascade regulation mechanism. The Las system is located at the top of the regulatory network. It is initially activated after receiving a stimulus signal and can positively regulate the Rhl system (10,11). The synthesis of the *Pseudomonas* quinolone signal (PQS) (12) is positively regulated by the Las system. PQS regulates the expression of the *rhlI*-associated genes that depend on the Rhl system, thus linking the Las system and the Rhl system. In this way, the QS system can regulate the expression of virulence factors and trigger biofilm formation (13,14).

The virulence of *P. aeruginosa* is closely related to its cellular structure (pili, lipopolysaccharides, and flagella) and secreted factors (exotoxins, elastases, pyocyanin, and extracellular polysaccharides). The QS system involves the cell-density dependent accumulation of signal molecules that enable bacteria to modulate the expression of virulence-associated genes (15,16) including elastase, alkaline protease, exotoxin A, rhamnolipid, pyocyanin, and exogenous lectins. A host of virulence factors are regulated by 2 acyl-homoserine lactone (AHL) QS systems: the LasI-LasR (*las* system) and the RhlI-RhlR (*rhl* system) (17,18). Virulence factors can alter the balance between host defenses and bacterial virulence, leading to damaged blood vessels, exotoxin spread, systemic inflammatory response syndrome, and ultimately host injury or death (19). This means that the virulence factors in the QS system are key to the pathogenicity of *P. aeruginosa*.

Previous study found that exotoxin A was a type II secreted extracellular enzyme encoded by the *toxA* gene. This enzyme alone or synergistically with other hydrolases causes cell death, severe tissue damage, and necrosis in humans (20). Elastase, which is encoded by the *lasA* gene (staphylolysin), *lasB* gene (pseudolysin), and secreted extracellularly (21,22), plays an important role in the infection process of *P. aeruginosa*. Pyocyanin is a redox-active virulence factor that is produced by *P. aeruginosa* and can easily penetrate biological membranes. Pyocyanin has

been shown to play a major role in animal models of acute and chronic infection caused by *P. aeruginosa* (23,24).

The unreasonable use of antibiotics makes bacteria resistant to these treatments, which can lead to repeated or persistent infections. *P. aeruginosa* resistance is an issue of public concern. As the prevalence of *Pseudomonas* bacterial resistance increases, it is critical to find new methods for treating infections. Interfering with the expression of virulence genes regulated by the QS system can effectively regulate the pathogenicity and drug resistance of *P. aeruginosa*, and may represent a new and promising direction for potential drug therapies.

Since antimicrobial peptides (AMPs), also known as host defense peptides, were discovered in the 1980s, they have been viewed as a promising alternative to conventional antibiotics. AMPs show promise against multi-resistant pathogens due to their combined antibacterial, immunomodulatory, and anti-biofilm effects (25,26). The AMP LL-37 is the only member of the Cathelicidin family that is produced by epithelial cells, macrophages, neutrophils, and lymphocytes in the human body (27,28). LL-37 has been shown to exhibit a broad-spectrum of antimicrobial and immunomodulatory activities, and promotes damage repair and other restorative functions. It is also involved in important physiological functions including cell proliferation, invasion, cell cycle regulation, and apoptosis. LL-37 is highly expressed at barrier sites, serving as an important first-line defense against pathogenic microorganisms (29). Like other AMPs, the primary mechanism of action is membrane disruption. Dorschner *et al.* (30) reported that LL-37 had an inhibitory effect on *Escherichia coli*, *P. aeruginosa*, *Enterococcus*, and *Staphylococcus aureus* (*S. aureus*) *in vitro*. LL-37 has also been found to inhibit the activity of *Staphylococcus epidermidis* (31), *Pseudomonas* (32), and *Acinetobacter baumannii* (33). The ability to form biofilms is a critical component of chronic *P. aeruginosa* infection (34,35). LL-37 can inhibit the formation of *P. aeruginosa* biofilms and destroy already formed biofilms, suggesting that it may be a potential therapy against chronic *P. aeruginosa* infection (36). Nagant *et al.* showed that not only LL-37 but also some of its fragments blocked the formation of a biofilm. LL-37 also affected *PAO1* within an established biofilm (37). Overhage *et al.* (38) demonstrated using microarrays that LL-37 can inhibit biofilm formation by reducing cell adhesion, stimulating the twitching motility of bacteria, and influencing the QS system to downregulate genes essential to biofilm development. However, how LL-37 affects the

QS system of *P. aeruginosa* to exert its antibacterial effects has not been well reported. Although several studies have provided promising evidence of the effectiveness of LL-37 in the treatment of infections, further studies are still needed. The goal of this work is to understand the influence of LL-37 on the virulence factors related to the QS of *P. aeruginosa*. We present the following article in accordance with the MDAR reporting checklist (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-617/rc>).

Methods

Bacterial strains, media, reagents, and instruments

The wild-type *P. aeruginosa* strain PAO1 was donated by Zou Lin from Children's Hospital of Chongqing Medical University. The double mutant *PA-ΔlasI/rbII* (39) strain was constructed via gene homologous recombination and verified with polymerase chain reaction (PCR) sequencing, southern blot, and high performance liquid chromatography mass spectrometry (HPLC MS). PAO1 and *PA-ΔlasI/rbII* were grown at 37 °C in Luria-Bertani broth (LB). Total RNA was extracted using RNAiso Plus reagent (Takara, Dalian, Liaoning, China). Reverse transcription was performed with the PrimeScript RT Reagent Kit (Takara, Dalian, Liaoning, China; code No. RR047A). Azithromycin (AZM) (Guangdong Drug Inspection Institute, Guangzhou, China), and real-time PCR kit (Thermo Fisher Scientific, Waltham, MA, USA) were used. LL-37 (LLGDFFRKSK EKIGKEFKRIVQRIKDFLRNLVPRTEs; lot: JT69395) was purchased from Nanjing Jptide Biotechnology Co. A Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) and Rotor-Gene Q real-time quantitative PCR (Thermo Fisher Scientific, Waltham, MA, USA) were used.

Determination of MIC

The MICs of LL-37 for the PAO1 and *PA-ΔlasI/rbII* strains were measured using the micro broth dilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) M07-A9 guidelines: (I) MIC was measured during the logarithmic growth phase; (II) 100 μL of bacterial solution at a concentration of 1×10^5 CFU/mL was added to 96-well microtiter plates; (III) two-fold serial dilutions of LL-37 ranging from 1,024 to 0.25 μg/mL were tested; (IV) the final culture concentrations of LL-37 were 1,024, 512, 256, 128, 64, 32, and 16 μg/mL; (V) sterile physiological

saline was used as a blank control and a bacterial solution well with no AMP was used as a negative control; (VI) the plates were incubated in a 35 °C carbon dioxide incubator for 18–20 h. Following the incubation period, the plates were visually evaluated for the presence of bacterial growth. The lowest concentration of LL-37 that inhibited bacterial growth was defined as the MIC value. Measurement of the MIC values of the QS inhibitor AZM was then performed in the same manner so that AZM could be used as a positive control.

P. aeruginosa growth curve

Single colonies of PAO1 and *PA-ΔlasI/rbII* were inoculated in 3 mL of LB medium and cultured overnight at 37 °C with continuous shaking (200 r/min). Various concentrations of LL-37 were combined with a range of *P. aeruginosa* cultures: (I) 20 μL of bacterial culture was added into 3 mL of LB medium and cultured with continuous shaking at 37 °C; (II) the culture was then treated with LL-37 at concentrations of 250, 125, 62.5, and 31.25 μg/mL. OD_{600nm} values (performed in triplicate) were measured 2, 4, 6, 8, 10, 12, 14, and 16 h after treatment to draw growth curves.

The detection of exotoxin A

Exotoxin A activity was measured with the NAD (Coenzyme I) method: (I) 1 mL of the bacterial solution was centrifuged at 10,000 r/min at 4 °C for 1 h; (II) 10 μL of the supernatant and 2 μL of NAD solution was added to each Eppendorf tube and mixed via pipetting; (III) the mixture was incubated at 25 °C for 30 min; (IV) in order to prepare for the corresponding solution in each measuring tube of the color development system, 0.2 mL of nitroretazolium blue (NTB), 0.8 mL of phenazine dimethyl ester (PMS), 0.1 mL of lactate dehydrogenase (LDH), and 1.8 mL of sodium lactate were added in advance; (V) the mixture was then placed in a water bath at 37 °C for 10 min; (VI) supernatant-treated NAD was then added to the corresponding test tubes of the color development system in addition to 0.2 mL of 10 mmol/L phosphate buffered saline; (VII) after mixing each of the above measuring tube solutions with the previously prepared solution, the samples were placed in a water bath at 37 °C for 5 min; (VIII) finally, 0.1 mL of hydrochloric acid (2 mol/L) was added to each tube to stop the reaction. The experiment was set with negative and positive controls. The negative control tube did not contain NAD, while the positive control tube contained NAD but

Table 1 Primer sequences

Gene	Forward (5'-3')	Reverse (5'-3')
<i>lasA</i>	CTGCTGGCTTTCAAGGTTTC	CCAGCAAGACGAAGAGGAAC
<i>lasI</i>	CGTGCTCAAGTGTTC AAGGA	AAAACCTGGGCTTCAGGAGT
<i>rhIA</i>	AGCTGGGACGAATACACCAC	GA CTCCAGGTCGAGGAAATG
<i>rhIB</i>	GAGCGACGAACTGACCTACC	CGTACTTCTCGTGAGCGATG
<i>toxA</i>	TGCTGCACTACTCCATGGTC	ACACCTTGATGTTCGAAGGC
<i>rpoD</i>	CTGATCCAGGAAGGCAACAT	TGAGCTTGTGATCGTCTCG

did not include a toxin. When the OD_{490nm} colorimetric measurement was performed, the negative control tube was used as the zero, and the color inhibition rate was calculated according to the formula RI = 100 - (OD_{490nm} value of toxin-treated tube/OD_{490nm} value of positive reference tube) × 100%. Three parallel experiments were performed for statistical analysis.

Elastase detection

Elastase activity was detected with the elastin-Congo red (ECR) method (40,41): (I) 1 mL of bacterial solution was centrifuged at 12,000 r/min at 4 °C for 1 h; (II) 100 µL of the supernatant was added into a new Eppendorf tube; (III) 900 µL of ECR and ECR buffer was added and mixed by pipetting to achieve an ECR concentration of 20 g/L; (IV) the reaction was placed in a 37 °C incubator for 16 h; (V) the reaction was stopped by adding 100 µL of ethylenediaminetetraacetic acid (EDTA), then centrifuging at 10,000 r/min at 4 °C for 20 min. OD_{495nm} absorbance was measured with a microplate reader. LB medium was used as a negative control. All experiments were performed in triplicate.

Pyocyanin detection

Pyocyanin detection was performed using the chloroform extraction method (42): (I) 5 mL of bacterial solution was centrifuged at 5,000 r/min at 4 °C for 1 h; (II) 3 mL of chloroform was added to 3 mL of the supernatant for extraction; (III) the blue layer was re-extracted into 1 mL of 0.2 mol/L hydrochloric acid for the extract, yielding a red layer on the top. Absorbance was measured at 520 nm with a microplate reader, and the concentration of pyocyanin in the absence and presence of LL-37 was recorded.

Quantitative PCR (qPCR)

The RNA of *P. aeruginosa* strains was extracted for qPCR analysis according to the RNA reagent instructions (Promega, Madison, WI, USA): (I) 1 µg of the extracted RNA was reverse transcribed into cDNA with a reverse transcription reagent kit (TaKaRa, Dalian, Liaoning, China); (II) following the instructions of the SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), relative real-time PCR was performed with the ABI ViiA™ 7Dx system (Applied Biosystems, Foster, CA, USA) to detect cDNA; (III) the housekeeping gene ribosomal protein *rpoD* was used as an internal control for normalization. Relative changes in gene expression levels were calculated with the 2^{-ΔΔCt} method. Primer sequences are shown in *Table 1*.

Statistical analysis

All treatments were performed in triplicate to confirm reproducibility. SPSS 16.0 (IBM, Armonk, NY, USA) statistical software was used for analysis. Data are expressed as mean ± standard deviation. Comparisons between two groups were performed using grouped *t*-tests. Comparisons between three groups were performed using one-way ANOVA. Differences were considered statistically significant at P < 0.05.

Results

Minimum inhibitory concentration (MIC) of LL-37 against *P. aeruginosa*

A sub-minimum inhibitory concentration (sub-MIC) was used to test the effect of LL-37 on the virulence factors regulated by the QS system. The wells exposed to LL-37

Table 2 Identifying the minimum inhibitory concentration of LL-37

Group	1	2	3	4	5	6	7	Negative control	Blank
LL-37 concentration ($\mu\text{g/mL}$)	1,024	512	256	128	64	32	16	0	0
PAO1	-	-	-	+	+	+	+	+	-
PA- <i>AlasI/rhII</i>	-	-	-	+	+	+	+	+	-

"+": turbidity and bacterial growth; "-": clear fluid and bacterial inhibition. PAO1, wild-type *P. aeruginosa* strain; PA-*AlasI/rhII*, double mutant *P. aeruginosa* strain; *P. aeruginosa*, *Pseudomonas aeruginosa*; Negative control, untreated group; Blank, sterile saline.

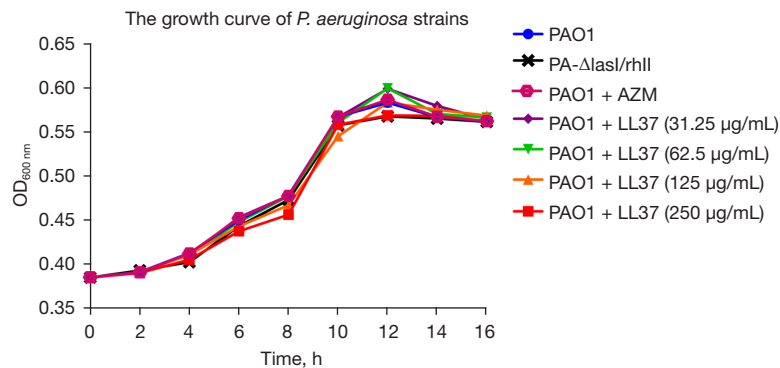


Figure 1 Growth curves of PA-*AlasI/rbII* and their parent strain PAO1. The relationship between OD_{600nm} to viable cell count was equivalent for all strains examined. Each point represents the mean OD_{600nm} value. PAO1, wild-type *P. aeruginosa* strain; PA-*AlasI/rbII*, double mutant *P. aeruginosa* strain; *P. aeruginosa*, *Pseudomonas aeruginosa*.

concentrations of 1,024, 512, and 256 $\mu\text{g/mL}$ were transparent, while turbidity appeared in wells treated with a concentration of 128 $\mu\text{g/mL}$, as shown in Table 2. The MIC of LL-37 against both PAO1 and PA-*AlasI/rbII* was therefore determined to be 256 $\mu\text{g/mL}$. As a positive control, the MIC of azithromycin (AZM) was 128 $\mu\text{g/mL}$, and the concentration of AZM used against all of the strains in this study was 2 $\mu\text{g/mL}$.

The growth curves of *P. aeruginosa* strains in the setting of sub-MIC LL-37

Four different concentration levels of LL-37 sub-MIC were selected as the treatment group, and PAO1, PA-*AlasI/rbII*, and PAO1 + AZM without LL-37 were used as controls. Since AZM is a QS inhibitor, it was used as a positive control. A concentration of 2 $\mu\text{g/mL}$ of AZM did not affect the growth of the strains. PA-*AlasI/rbII* had a complete deletion of the coding domain. As shown in Figure 1, there were no significant differences in proliferation between the groups at any timepoint ($P > 0.05$).

Influence of LL-37 on the expression of *P. aeruginosa* virulence factors

To confirm the expression of *P. aeruginosa* virulence factors, we examined exotoxin A, elastase, and pyocyanin expression induced by LL-37. As shown in Figure 2, the expression of exotoxin A, elastase, and pyocyanin was significantly reduced in the PA-*AlasI/rbII* strain compared with the wild-type PAO1 strain ($P < 0.05$). There were no significant differences between the PA-*AlasI/rbII* strain and the negative control group. Notably, all of the different concentrations of LL-37 influenced the expression of exotoxin A, elastase, and pyocyanin, as shown in Figure 2A-2C. After interference with LL-37, the PAO1 strain exposed to a different sub-MIC concentration of LL-37 showed a significant decrease in exotoxin A, elastase, and pyocyanin expression compared with the PAO1 control group ($P < 0.05$). With increasing concentrations of LL-37, reduced activity of exotoxin A, elastase, and pyocyanin were again demonstrated, but differences between the groups were not significant ($P > 0.05$).

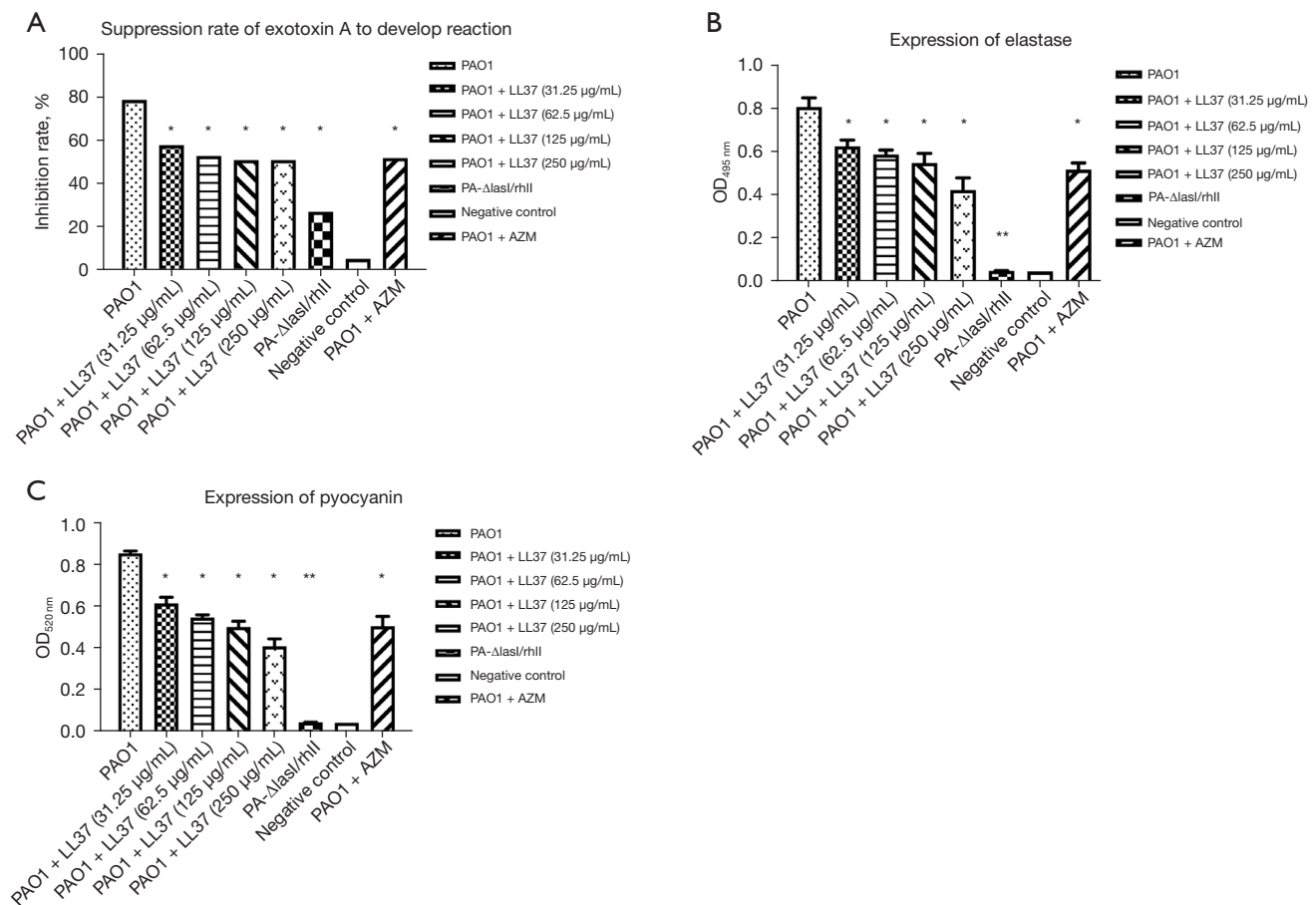


Figure 2 The interference of different concentrations of LL-37 on exotoxin A (A), elastase (B), and pyocyanin (C) in *P. aeruginosa* when compared with *PAO1* alone. LL-37 at doses of 250, 125, 62.5, and 31.25 µg/mL were used. *, statistical significance ($P < 0.05$) compared with *PAO1*; **, statistical significance ($P < 0.01$) compared with *PAO1*. *PAO1*, wild-type *P. aeruginosa* strain; *PA-ΔlasI/rhII*, double mutant *P. aeruginosa* strain; *P. aeruginosa*, *Pseudomonas aeruginosa*.

Influence of LL-37 on the expression of *PAO1* virulence genes

The expression of the QS-related virulence genes of *P. aeruginosa* were significantly decreased compared with the *PAO1* strain at a sub-MIC concentration of LL-37 (Figure 3A,3B). With increased LL-37 concentrations, the expression levels of *lasA*, *lasI*, *toxA*, *rbIA*, and *rbIB* genes significantly decreased ($P < 0.05$) to a degree that was consistent with the expression of virulence factors at sub-MIC concentrations of LL-37. Low QS-related gene expression of the *PA-ΔlasI/rhII* group further suggested that a double gene deletion strain model was successfully constructed ($P < 0.01$).

Discussion

This study sought to understand whether the antibacterial effects of LL-37 are through the regulation of virulence factors associated with the QS system. Human cathelicidin was found to be more potent than conventional antibiotics at killing extra- and intracellular *S. aureus* (43). LL-37 was previously found to inhibit the inflammatory pathway by reducing the release of IL-6 and TNF- α and inhibiting the phosphorylation of Akt and JNK (44). Furthermore, Uhlmann *et al.* showed that LL-37 affected the virulence factors of *Streptococcus pyogenes* (45). It was therefore of interest to determine whether the potency of LL-37 translated to the treatment of *P. aeruginosa*, which commonly

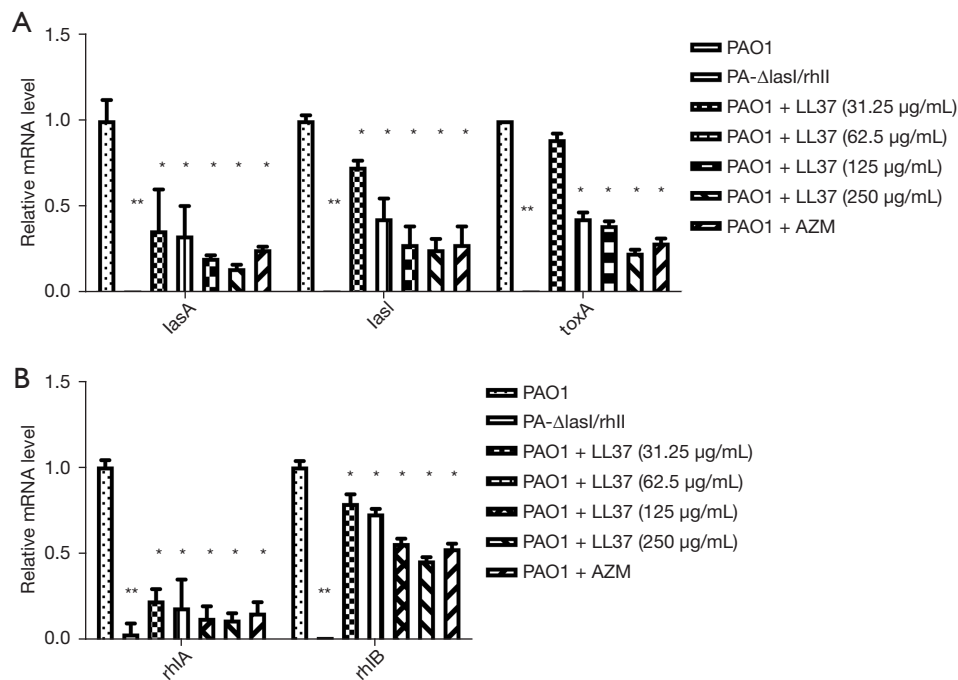


Figure 3 The effect of LL-37 on quorum sensing system of *P. aeruginosa*. (A) The effect of LL-37 on Las system (*lasA*, *lasI*, *toxA*)-related gene expression. *P<0.05, **P<0.01, compared with the *PAO1* group. (B) The effect of LL-37 on Rhl system (*rhlA*, *rhlB*)-related gene expression. *P<0.05, **P<0.01, compared with the *PAO1* group. *PAO1*, wild-type *P. aeruginosa* strain; *PA-Δ*lasI*/*rhlI**, double mutant *P. aeruginosa* strain; *P. aeruginosa*, *Pseudomonas aeruginosa*.

develops multidrug resistant strains and would therefore benefit from novel adjunct treatments.

To develop an accurate model to evaluate the impact of LL-37 on *Pseudomonas* function, it was necessary to confirm that sub-MIC concentrations of this vector did not affect the growth characteristics of the *PA-Δ*lasI*/*rhlI** and wild-type *PAO1* strains. The present work showed that LL-37 at concentrations below 256 μg/mL did not affect the growth curve of *P. aeruginosa* in its early stages (0–16 h in culture) of proliferation. It is therefore possible to attribute changes in virulence factor expression to the effects of LL-37 alone, not altered proliferation.

Patients with *P. aeruginosa* have increased expression of virulence factors. Inhibiting their activity is one of the main targets for *P. aeruginosa* treatment. The present work suggests that LL-37 has an inhibitory effect on *P. aeruginosa* exotoxin A, elastase, and pyocyanin. *P. aeruginosa* possesses the ability to form biofilms to facilitate the colonization of medical devices, thereby contributing to the spread of drug-resistant strains in the hospital environment (46). Pyocyanin is one of the primary virulence factors responsible for biofilm formation (47). In addition to biofilm development,

QS has been linked to the regulation of other physiological processes, including virulence factor production, metabolic adjustment, and host-microbe interactions (48). The expression of pyocyanin was significantly inhibited by LL-37. Mishra and Wang (49) in their study of *P. aeruginosa* emphasized the importance of antibiofilm peptides for early infections.

The results of the present work suggest that LL-37 plays a vital role in the antibiofilm process. It is also possible that the antibiofilm effects of LL-37 shown against *P. aeruginosa* may be similarly effective against other bacteria that produce biofilms (50). LL-37 significantly reduced *Pseudomonas*-associated QS virulence factors, and as the concentration of LL-37 increased, its absorbance gradually decreased and its toxicity was reduced. This may suggest that the anti-infective effect of LL-37 can only be achieved by inhibiting the production of *P. aeruginosa* virulence factors. The effects of LL-37 on other virulence factors may be of importance as well, and will be evaluated in future work.

Using *PAO1* as a control group, the mRNA transcription levels of the virulence factor genes *lasA*, *lasI*, *toxA*, *rhlA*, and *rhlB*, which are involved in QS systems, were significantly

reduced by LL-37. The transcription level of these factors also decreased with increased concentrations of LL-37, suggesting that there is a dose-dependent antibacterial effect. This change is consistent with the altered phenotype of virulence factors caused by LL-37. As these virulence factors are Las (*lasA*, *lasI*, *toxA*) and Rhl (*rhlA*, *rhlB*) system-related genes (51), it is possible that if both the Las and rhl cell-to-cell signaling systems are congested, *P. aeruginosa* may not be able to re-establish the production of cell-to-cell signaling-dependent virulence factors. This approach may efficiently reduce virulence factor production and the high death rates associated with *P. aeruginosa*.

In agreement with published data (52), the present work demonstrates that LL-37 can downregulate the expression of *P. aeruginosa* QS system virulence factors *in vitro*, and that these inhibitory effects are dose-dependent. Our study emphasizes the importance of LL-37 in the pathogenesis of *P. aeruginosa*. LL-37 may therefore be of clinical potential in the future.

There are several limitations to this study. For example, it only focused on a single bacterial species and tested a relatively small number of virulence factors. Future work should include *S. aureus*, *Klebsiella pneumoniae*, and other biofilm-producing bacteria. However, the experimental data presented in this work provides valuable insights into the function of LL-37, especially on *P. aeruginosa*.

Conclusions

In conclusion, the AMP LL-37 is a potential inhibitor of the QS system and the pathogenicity of *P. aeruginosa*. The newly uncovered therapeutic potential of LL-37 against this challenging pathogen merits further investigation (53,54). In-depth study of the QS system will not only help to enrich our understanding of the mechanism of *P. aeruginosa* resistance, but also how to optimize the antimicrobial properties of LL-37, which may improve our ability to treat bacterial resistance. Future work will seek to further evaluate the effects of sub-antimicrobial concentrations of LL-37 on other regulatory pathways under the control of the *lasRI/rhlRI* system, clarify the regulatory pathway that contributes to this drug's efficacy, and identify additional new therapeutic targets for treating clinical bacterial infections.

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Footnote

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Data Sharing Statement: Available at <https://atm.amegroups.com/article/view/10.21037/atm-22-617/dss>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-617/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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