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4-Imidazolecarboxaldehyde attenuates virulence and pathogenicity of Pseudomonas aeruginosa by targeting las and rhl systems

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Abstract

Pseudomonas aeruginosa is an important pathogen which often causes serious and difficult-to-treat infections in humans due to its high intrinsic and acquired antibiotic resistance, highlighting an urgent need for novel therapeutic strategies or agents. QS system serves as the core regulatory network governing virulence and antibiotic resistance of PA, has been recognized as important targets for anti-virulence therapy. In this study, 4-Imidazolecarboxaldehyde, a 4-formyl imidazole derivative, was identified as a promising QS inhibitor that could significantly suppress the expression of the QS-associated genes of *lasI/R*, *rhlI/R*, *pqsA/R*, *lasA*, *rhlA* and *phzA1*, and inhibit the corresponding virulence factor production of *lasA* encoded protease, *rhlA* related rhamnolipid and *phzA1* associated pyocyanin, as well as the motility and biofilm formation in *Pseudomonas aeruginosa* PAO1 at sub-MICs of 80 and 160 μg/mL. The pathogenicity of PAO1 could also be attenuated by 4-imidazolecarboxaldehyde at 80 and 160 μg/mL, verified by *Chinese cabbage*, *Drosophila melanogaster* and *Caenorhabditis elegans* infection analysis. 4-Imidazolecarboxaldehyde might target *las* and *rhl* systems to interfere with QS system based on the molecular docking analysis and the infection analysis in mutants. Less drug resistance tendency to PAO1 was observed, at least 20 generations tested. Furthermore, 4-Imidazolecarboxaldehyde could enhance the sensitivity of tetracycline and azithromycin to PAO1 obviously. Therefore 4-imidazolecarbaldehyde was identified as a promising QSI agent of PA targeting *las* and *rhl* systems, used independently or synergistically with tetracycline and azithromycin to attenuate PA infections without obviously detectable resistance.

Introduction

World Health Organization (WHO) has classified antimicrobial resistance (AMR) of bacteria, viruses, fungus and parasites as one of the top ten global public health threats[1]. Infections caused by antibiotic-resistant bacteria currently result in approximately 700,000 deaths worldwide annually and will exceed 10 million by 2050[2]. The novel drug development strategies or targets are urgently needed worldwide, including developing antibiotic adjuvants, utilizing generative artificial intelligence to design novel antibiotics, and repurposing existing drugs[3]. Quorum Sensing Inhibition (QSI) offers a promising strategy by targeting bacterial communication rather than cell viability to attenuating pathogenicity of pathogens. QSI impose a less selective pressure on bacteria compared to traditional antibiotics, reducing the possibility of drug resistance development efficiently.

Quorum sensing (QS) system is a cell density dependent intercellular communication system to regulate the gene expression and coordinate the bacteria behavior finally. QS system consists of signaling molecule and their cognate receptor. The signaling molecules are released into the extracellular environment[4] and bind to the cognate receptors as reaching the responding threshold to regulate gene expression and physiological activities through a series of cascade reactions[5], like antibiotic resistance and pathogenesis[6].

Pseudomonas aeruginosa (PA) is a multi-drug resistant (MDR) opportunistic pathogen that has been recognized by WHO as a critical threat to global public health[7]. As a leading cause of nosocomial infections, PA contributes substantially to morbidity and mortality, particularly in immunocompromised patients[8]. Treatment of PA infections is severely challenged by both the intrinsic and acquired resistance to most antibiotics, including carbapenems[9].

Three quorum sensing systems are well identified in PA, *lasI*/*lasR*, *rhlI*/*rhlR*, and *pqsA/pqsR* system[10]. *LasI*/*lasR* and *rhlI*/*rhlR* systems are two typical Acyl-homoserine lactone (AHL)-based QS systems in PA. LasI /RhlI synthesize the signal molecules of N-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL) respectively to bind to their cognate cytoplasmic receptors of LasR and RhlR, regulating their downstream gene expression, like protease coding gene *lasA*, elastase coding gene *lasB*, and *rhlAB* encoding virulence factor rhamnolipids accordingly[11]. In *pqs* system, signaling molecule 2-heptyl-3-hydroxy-4-quinolone (PQS) derived from *pqsABCDE-phnAB* binds to the corresponding receptor PqsR (MvfR)[12] to regulate the downstream gene expression, like *phzA1* and *phzA2*[13] which are responsible for pyocyanin biosynthesis to promote oxidative stress and host tissue damage[14]. The three systems coordinate hierarchically to regulate acute and chronic infections induced by kinds of virulence factors, such as acute infection associated motility, chronic infection and drug resistance associated biofilm formation in PA[15].

Compounds which could inhibit QS system are termed as QS inhibitors (QSIs). Numerous QSIs have been identified from both natural and synthetic sources[16], like flavonoids derived from various plants[17] and synthetically designed analogues of N-acyl homoserine lactones (AHLs)[18]. However, considering synthetic feasibility and structural diversity[19], identifying smaller, synthetically accessible scaffolds as alternative QSI chemotypes has become a parallel support strategy[20].

4-Imidazolecarboxaldehyde (4-IC) is a very important pharmaceutical precursor, due to its unique dual-functional structure comprising a reactive aldehyde group and a bioactive imidazole group. The imidazole group serves as a pharmacophore fundamental to azole compounds, while the aldehyde group can participate in various electrophilic reactions, such as condensation, cyclization, and reductive amination, offering access to diverse molecular architectures to synthesize a wide range of imidazole-based bioactive molecules[21]. For example, C17,20-lyase inhibitors derived from 4-imidazolecarboxaldehyde has been utilized in prostate cancer therapy[22]. More recently, Davood et al. reported that 1-(4-chlorophenyl)-N-[(1H-imidazol-4-yl)methyl] methanamine, a derivative synthesized from 4-imidazolecarbaldehyde, exhibits both antifungal activity against *Candida albicans* and significant antibacterial effects against *Staphylococcus aureus* and *Escherichia coli*[23].

In this study, 4-imidazolecarbaldehyde was identified as an efficient QSI to downregulate the expression of QS-associated genes, suppress the correspond QS-regulated virulence factors, biofilm formation, swarming motility, and the pathogenicity of PA at sub-MICs of 80 and 160 μg/mL with undetectable resistance to PA over at least 20 generations. 4-imidazolecarboxaldehyde may interfere with QS system

of PA, mainly by *las* and *rhl* systems. Moreover, 4-imidazolecarbaldehyde could enhance the sensitivity of tetracycline and azithromycin against PA, to be used individually or synergistically with any of the two antibiotics to tackle the PA infection.

Materials and methods

Bacterial strains and culture conditions

All bacterial strains and plasmids utilized in this study are listed in Supplementary Table 1. PAO1 and its derivatives were cultured in Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) at 37°C with shaking at 200 rpm. The antibiotics, like trimethoprim (Tmp, 300 μg/mL) or kanamycin (Kan, 50 μg/mL), was supplemented when necessary.

4-imidazolecarboxaldehyde (≥ 98% purity; Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China) was dissolved in dimethyl sulfoxide (DMSO) to prepare a 20 mg/mL stock solution. The stock solution was diluted to the final concentration of 80 and 160 μg/mL via DMSO for the further test by using DMSO of 0.1% (v/v) as a control.

Minimum inhibitory concentration (MIC) measurement

The MIC of 4-imidazolecarbaldehyde or antibiotics against PAO1 were determined by the growth monitoring in a transparent 96-well plate (Costar, New York, NY, USA) via microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines[14] The growth was monitored by measuring OD_{600} every 30 min for 24 h at 37°C using a Synergy H1 microplate reader (Bio Teck, Winooski, VT, USA). DMSO was used as the negative control. MIC was determined as the lowest concentration that prevented the visible bacterial growth over the 24 h incubation.

Bacterial growth and the QS-associated gene expression determination

The expression of the QS-associated genes, like *lasR*, *lasI*, *rhlI*, *rhlR*, *pqsA*, *pqsR*, *lasA*, *rhlA* and *phzA1*, was determined by using the *luxCDABE* based promoter-reporter fusion system as previously reported[24]. The promoter region of the QS-related genes was cloned into the upstream of the promoterless *luxCDABE* operon in plasmid pMS-402 and then electroporated into PA or the derivatives to obtain promoter-reporter strains, like PAO1(pKD-*lasI*). The transcriptional expression of the genes was measured as the chemiluminescence derived from the promoter in a Synergy H1 microplate reader. Briefly, overnight cultures of the reporter strains were diluted to OD_{600} of 0.5 and cultivated for an additional 3 h. 5 μL of the culture was inoculated into the parallel wells containing 95 μL LB broth with 4-imidazolecarbaldehyde at different concentrations (0, 80 and 160 μg/mL) in the black clear-bottom 96-well plate. 50 μL of sterilized liquid paraffin was added to each well to prevent evaporation. Luminescence (counts per

second, CPS) as well as OD_{600} were measured every 30 min for 24 h by a Synergy H1 microplate reader (BioTeck, Winooski, VT, USA).

Production of the QS-associated virulence factor

Protease activity

Protease activity was assessed using a 2% skim milk powder according to a previously described method[25]. Briefly, PAO1 were grown in 5mL LB broths containing 4-imidazolecarboxaldehyde at different concentrations of 0, 80 and 160 μg/mL at 37°C for 12 h. The harvested supernatants were sterilized via 0.22-μm membrane filter. An aliquot of 200 μL supernatant was mixed with 800 μL of phosphate buffer (pH 7.8) containing 2% skim milk powder and then incubated at 37°C for 30 min. The protease activity was quantified by measuring the absorbance at 440 nm.

Elastase activity

The elastase activity of PA was evaluated using the elastin-Congo red method as previously described[24]. 200 μL of the above filtered supernatant was added into 800 μL of Elastin-Congo red (ERC) buffer containing 5 mg/mL elastin Congo red, 100 mM Tris-HCl, 1 mM of $CaCl_2$ with pH 7.5 and then incubated at 37°C for 6 h with shaking. After eliminating the undigested elastin-Congo red, supernatant was used for Elastase activity determination by measuring the absorbance at 495 nm.

Pyocyanin

The quantification of pyocyanin followed the methodology established by Jayaseelan and Ramaswamy[26]. PAO1 was cultured in LB broth supplemented with 4-imidazolecarboxaldehyde at concentrations of 0, 80, and 160 μg/mL at 37°C for 16 h to obtain cell-free supernatant via centrifugation at 8,000 rpm. A 5 mL portion of the supernatant was mixed with 3 mL of chloroform thoroughly. The organic phase was collected by centrifugation and acidified with 1 mL of 0.2 N HCl. After removing the HCl layer, the pink phase solution was used to determine OD_{520} for pyocyanin quantification.

Rhamnolipid

The production of rhamnolipids was evaluated by using the mineral salt cetyl trimethyl ammonium bromide (CTAB)-methylene blue (MB) agar method[27]. Overnight culture of PAO1 was adjusted to an OD_{600} of 0.2. 2 μL of the culture was spot inoculated at the center of CTAB-MB agar plates (0.2 mg/mL CTAB, 5 μg/mL methylene blue) supplemented with 4-imidazolecarboxaldehyde at various concentrations (0, 80 and 160 μg/mL). After 72 h incubation at 37°C, the transparent zone around the colony was determined for rhamnolipid qualification.

Motility determination

Motility of PAO1, including swimming, swarming and twitching, were determined as the previous report[28]. Overnight cultures of PAO1 were adjusted to an OD_{600} of 0.5 by LB broth. For swimming and swarming evaluations, 2 μL of the aliquot was spotted onto the center of the swimming (1% nutrient broth, 0.5% glucose, 0.3% agar) or swarming (0.3% agar, 1% tryptone, 0.5% yeast extract, 0.5% NaCl) agar plates supplemented with 4-imidazolecarboxaldehyde at various concentrations. The twitching capacity was evaluated in the LB agar plates containing the various concentration of 4-imidazolecarboxaldehyde. 2 μL of the inoculum was stabbed into the bottom of the twitching agar plates. All the plates were incubated at 37°C for 24 h and the diameter of the bacterial motility was measured.

Biofilm formation

Biofilm formation was assessed as the previous report[29]. The aliquots of 20 μL overnight culture for PAO1 was transferred into the wells of the 12-well plate (Corning/Costar, Corning, NY, USA) containing 2 mL of LB broth with 4-imidazoleformaldehyde at various concentrations. After a 24 h incubation at 37°C and elimination of the supernatant, the remaining cells in the wells were gently rinsed with PBS for three times to eliminate the planktonic cells. The remaining adherent biofilm was fixed and stained via 1% crystal violet for 20 min and then washed by PBS to remove the excess crystal violet. The stained biofilms were washed by 1 mL of 95% ethanol and the biomass was determined at OD_{595}.

Pathogenicity assay in vivo

Chinese cabbage (*B. pekinensis*), fruit fly (*D. melanogaster Canton S*), and nematode (*C. elegans* N2) infection models were used for in vivo pathogenicity assay according to the previous report[30–32].

For the pathogenicity assay on Chinese cabbage infection model, the cell pellets from the overnight cultures of PAO1 were washed and resuspended in 10 mM $MgSO_4$ to OD_{600} of 2.0. The stems of Chinese cabbage were sterilized with 0.1% H_2O_2 and 10 μL of the suspension containing 4-imidazolecarboxaldehyde at different concentrations of 0, 80 and 160 μg/mL was inoculated into the stem using DMSO of 0.1% (v/v) as control. The cabbage was transferred into the petri dishes containing a Whatman filter soaked with 10 mM $MgSO_4$. The plates were kept at 30°C for 5 d and the rotten area of cabbage was monitored for the triple replication.

For the performance on *Drosophila melanogaster* infection model, the cell pellets of PAO1 were resuspended in 5% sucrose solution to an OD_{600} of 2.0. 100 μL of the suspension with or without 4-imidazolecarboxaldehyde was spotted onto the sterilized filter which fully covered the surface of the solidified LB agar containing 5% sucrose on a 50 mL Erlenmeyer flask. 25 male fruit flies (3–5 d old) which have been starved for 3 h were transferred into the flask and then kept at 25°C for 10 d. The survived rates were determined for the triple replication.

For the nematode infection examination, NGM plates (0.3% NaCl, 0.25% tryptone, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 μg/mL cholesterol, 100 μg/mL FUDR, and 2% agar) with or without 4-imidazolecarboxaldehyde was utilized. The synchronized nematodes in L4 phase were transferred to the NGM plates, and the survival rate of nematodes was monitored every 12 h for 5 d.

The synergy effect of 4-imidazolecarboxaldehyde combined with the antibiotics was investigated similarly.

Molecular docking analysis

Molecular docking analysis was performed according to the previous report[33]. The structures of 4-imidazolecarboxaldehyde and the QS signal molecules were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and the three-dimensional models of the receptors LasR (PDB ID: 6D6A) together with PqsR(PDB ID: 4JVI), and RhlR (Uniprot: AlphaFill) were obtained from the online protein database (https://www.rcsb.org/), and predicted using AlphaFold3 respectively. AutoDock Vina was employed to assess the binding interactions between 4-imidazolecarboxaldehyde or the signal molecules and the receptor proteins. The optimal conformation was confirmed by the number of hydrogen bonds, hydrophobic interaction force as well as binding energy. Docking results were visualized using the PyMOL molecular graphics system (v. 2.3.4).

Target detection of 4-imidazolecarboxaldehyde on QS system

The three quintuple mutants of PA, PAO1 (Δ*lasI*Δ*rhlI*Δ*pqsA*Δ*rhlR*Δ*pqsR*), PAO1 (Δ*lasI*Δ*rhlI*Δ*pqsA*Δ*lasR*Δ*pqsR*) and PAO1 (Δ*lasI*Δ*rhl*IΔ*pqsA*Δ*lasR*Δ*rhlR*) which contained only one receptor coding gene of *lasR*, *rhlR* or *pqsR* was constructed and named as QM-1, QM-2 and QM-3 respectively. The reporter strains of QM-1 (pKD-*lasB*), QM-2 (pKD-*lasB*) and QM-3 (pKD-*pqsA*) were constructed as described above. The expression of *las* and *rhl*-regulated gene *lasB*, and *pqs*-regulated gene *pqsA* was determined in LB broth with different concentrations of 4-imidazolecarboxaldehyde and 10 mM of the corresponding exogenous signaling molecules (odDHL, BHL, PQS) as described above.

The three QS receptor deletion mutants, PAO1 (Δ*lasR*), PAO1 (Δ*rhlR*), and PAO1 (Δ*pqsR*) were further used to validate the effect of 4-imidazolecarboxaldehyde on the pathogenicity of PA via Chinese cabbage infection analysis.

Synergy test of 4-imidazolecarboxaldehyde with antibiotics

According to the previous report[24], the MICs of antibiotics were determined. Synergistic effects were assessed by analyzing bacterial growth curves treated with antibiotics at 1/2 MIC combined with 4-imidazolecarboxaldehyde at 80 or 160 μg/mL according to the growth detection method.

Resistance induction assay

Drug resistance induction assay was performed as previously described[20]. PAO1 was cultured in LB broth supplemented with 1/2 MIC (640 μg/mL) of 4-imidazolecarboxaldehyde or polymyxin B (poly B) at 1/2 MIC (2 μg/mL) at 37°C for 24 h. The bacterial culture was standardized to 5×10^5 CFU/mL, and 50 μL of the standardized culture was transferred to 5 mL of fresh LB broth supplemented with 4-imidazolecarboxaldehyde at 640 μg/mL or polymyxin B at 2 μg/mL to assess the MIC in the subsequent round. The serial passage was conducted for 20 passages with triplicate biological replicates.

Statistics analysis

All experiments were carried out in triplicate, and the results were presented as mean ± standard deviation (SD). Statistical analyses were conducted by using Graph Pad Prism version 5.0.1 to determine significant differences (ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$). Statistical differences in the survival rate were analyzed using log rank and others by t-test.

Results

4-imidazolecarboxaldehyde reduced the expression of QS-associated genes in PA

To assess the effect of 4-imidazolecarbaldehyde on QS-associated gene expression independent of antibacterial activity, the MIC of 4-imidazolecarbaldehyde against PA was determined as 1,280 μg/mL by using the broth microdilution method. 4-imidazolecarbaldehyde at concentrations of 80 or 160 μg/mL exerted no significant bacteriostatic effects as shown in Fig. 1A and used for the further tests.

Then the effect of 4-imidazolecarbaldehyde on the QS associated genes, the autoinducer synthase and the cognate receptor encoding genes of *lasI/ lasR*, *rhlI/ rhlR* and *pqsA/ pqsR* as well as the QS system regulated genes of *lasA*, *rhlA* and *phzA1*, were evaluated. As shown in Fig. 1H-J, treatment with 4-imidazolecarbaldehyde at 80 and 160 μg/mL resulted in a robust downregulation for all the tested genes compared with the group without 4-imidazolecarbaldehyde. The results suggested that 4-imidazolecarbaldehyde might negatively interfere the QS circuitry at the transcriptional level without imposing selective pressure on bacterial survival at tested concentrations (Fig. 1A).

(A) Growth of PA treated with 4-imidazolecarboxaldehyde at sub-MIC concentrations. The transcriptional expression of the QS-associated genes treated with 4-imidazolecarboxaldehyde at sub-MIC concentrations: **(B)** *lasR*, **(C)** *rhlR*, **(D)** *pqsR*, **(E)** *lasI*, **(F)** *rhlI*, **(G)** *pqsA*, **(H)** *lasA*, **(I)** *rhlA*, **(J)** *phzA1*. ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

4-imidazolecarboxaldehyde inhibits the production of the virulence factors

To determine the impact of 4-imidazolecarbaldehyde on the functional output of the QS pathway, the production of the several hallmark virulence factors in PA, like protease responsible for breaking down various plasma proteins, pyocyanin associated with oxidative stress and inflammatory responses, and rhamnolipid corresponding to disrupting epithelial polarity and facilitating biofilm maturation, was determined. As shown in Fig. 2, compared with the untreated control, treatment with 4-

imidazolecarboxaldehyde at sub-MICs of 80 and 160 μg/mL obviously inhibit the production of the *phzA1* operon encoded pyocyanin by 21.3% and 42.9%, the *rhlA* encoded rhamnolipid by 28.8% and 43.0%, and *lasA* encoded protease by 53.7% and 63.7% respectively. The results indicated that 4-imidazolecarboxaldehyde could suppress the virulence factor production in PA in a dose-dependent pattern.

(A) pyocyanin; **(B)** protease; **(C)** rhamnolipid. All data were expressed as mean ± SD values with three independent experiments performed in triplicate. *: $p < 0.05$, **: $p < 0.01$, ***$p < 0.001$.

4-imidazolecarboxaldehyde decreases the motility and biofilm formation in PA

Motility and biofilm formation represent other key virulence traits regulated by QS. Motility and biofilm are associated with the acute and chronic as well as antibiotic resistance in PA. The motility and biofilm formation of PAO1 were assessed in absence and presence of 4-imidazolecarboxaldehyde at 80 and 160 μg/mL. As shown in Fig. 3, compared with the control group, treatment of 4-imidazole formaldehyde at 80 and 160 μg/mL reduced the swimming by 28.45% and 36.33%, swarming by 57.04% and 63.14%, and twitching by 16.08% and 44.72% respectively, as well as the biofilm formation by 45.67% and 55.44% respectively. The results indicated that 4-imidazolecarboxaldehyde at the tested concentrations could significantly reduce the biofilm formation and the motility without impacting the growth of PAO1.

(A) swimming; **(B)** swarming; **(C)** twitching; **(D)** biofilm formation. All data were expressed as mean ± SD values with three independent experiments performed in triplicate. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

4-imidazolecarboxaldehyde attenuates the pathogenicity of PA.

Three infection models, *Chinese cabbage*, *Caenorhabditis elegans* and *Drosophila melanogaster*, were utilized to investigate the effect of 4-imidazolecarboxaldehyde at the sub-MICs on the pathogenicity of PAO1. As shown in Fig. 4, treatment with 4-imidazolecarboxaldehyde at 80 and 160 μg/mL decreased the rotten area in the stem of the *Chinese cabbage* derived from PAO1 by 32.0% and 52.8% respectively, suggesting that 4-imidazolecarboxaldehyde at sub-MICs could decrease the virulence of PAO1 in a dose dependent patter. Also, the survival rates of fruit flies infected by PAO1 were increased by 29% and 39% respectively in presence of 4-imidazolecarboxaldehyde at 80 and 160 μg/mL compared with the untreated group in *Drosophila melanogaster* infection model. Similarly, 4-imidazolecarboxaldehyde at 160 μg/mL could enhance the survive of the nematodes in *Caenorhabditis elegans* infection model by 20% compared with the control group. All the results indicated that 4-imidazolecarboxaldehyde at sub-MICs could attenuate the pathogenicity of PAO1.

(A) rotten area of Chinese cabbage; **(B)** survival rate of fruit flies; **(C)** survival rate of nematodes.

(+): with PAO1; (−): without PAO1. All data were expressed as mean ± SD values with three independent experiments performed in triplicate. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

4-Imidazolecarboxaldehyde interfere with the QS system by targeting las and rhl systems

Molecular docking analyses in silico was performed to detect the interaction of 4-imidazolecarboxaldehyde with the receptor proteins of LasR, RhlR and PqsR of the QS system by using response regulator CarR, a non-QS-associated regulator in PA as a control. As shown in Table 2 and Fig. 5, 4-imidazolecarboxaldehyde form hydrogen bonds with several residues involved in ligand recognition, like Tyr56, Thr75, Tyr93, Thr115 and Ser129 in LasR, Trp68 and Ser135 in RhlR, Gln194, Ser196 and Leu197 in PqsR, and Leu7 and Ala5 in PqsA. The binding energies of 4-imidazolecarboxaldehyde toward LasR, RhlR and PqsR were − 3.68, − 2.99, − 3.83 kcal/mol respectively. However, the binding affinity for 4-imidazolecarboxaldehyde with CarR was − 2.92 kcal/mol, lower than that of 4-imidazolecarboxaldehyde with other protein receptors, indicating the poor interaction of 4-imidazolecarboxaldehyde with CarR. All RMSD values were below 2.0 Å (Table 2), indicating reliable docking parameters. The results suggested that 4-imidazolecarboxaldehyde might bind to LasR, RhlR and PqsR to negatively interfere with the QS-associated circuit.

To further validate the main targets, three quintuple mutants of PAO1 (*ΔlasIΔrhlIΔpqsAΔrhlRΔpqsR*), PAO1 (*ΔlasIΔrhlIΔpqsAΔlasRΔpqsR*) and PAO1 (*ΔlasIΔrhlIΔpqsAΔlasRΔrhlR*) which contained only one receptor LasR, RhlR or PqsR was constructed and named as QM-1, QM-2 and QM-3 respectively. The reporter strains of QM-1 (pKD-*lasB*), QM-2 (pKD-*lasB*) and QM-3 (pKD-*pqsA*) were constructed to determine the expression of *las* and *rhl*-regulated gene *lasB* and *pqs*-regulated gene *pqsA* in LB broth with different concentrations of 4-imidazolecarboxaldehyde supplemented with 10 mM of the corresponding exogenous signaling molecules (odDHL, BHL, PQS). As shown in Fig. 6A-F, treatment with 4-imidazolecarboxaldehyde significantly decreased the expression of *lasB* in both QM-1(pKD-*lasB*) and QM-2(pKD-*lasB*), especially in QM-2(pKD-*lasB*). However, 4-imidazolecarboxaldehyde could not inhibit the expression of *pqsA* in QM-3(pKD-*pqsA*) at the tested concentrations compared with the control group.

Then the mutants of PAO1 (Δ*lasR*), PAO1 (Δ*rhlR*) and PAO1 (Δ*pqsR*) were used to further determine the virulence by cabbage infection analysis treated with or without 4-imidazolecarboxaldehyde. As shown in Fig. 6D, 4-imidazolecarboxaldehyde at 80 and 160 μg/mL could decrease the rotten area in all the three single mutants, 42.1% and 64.6% for PAO1(Δ*lasR*), 44.3% and 52.9% for PAO1(Δ*rhlR*), and 44.5% and 72.1% for PAO1(Δ*pqsR*). The most vigorously attenuated virulence, observed in PAO1(Δ*pqsR*) which contain both *las* and *rhl* systems compared with those in PAO1(Δ*lasR*) and PAO1(Δ*rhlR*) containing only *las* or *rhl* system, suggested the involvement of both *las* or *rhl* in QS inhibition of 4-imidazolecarboxaldehy.

Table 2
The docking energy and binding sites between the QS receptors and ligands

Receptors	Ligands	Docking energy (kcal/mol)	RMSD(Å)	H-bonds
LasR	4-IC	-3.68	0.777	Tyr56, Thr75, Tyr93, Thr115, Ser129
	3-oxo-C12-HCL	-5.37	0.558	Trp60, **Thr115**, **Ser129**
LasI	4-IC	-3.62	0.797	Asp73
RhlR	4-IC	-2.99	0.808	Trp68, Ser135
	3-oxo-C4-HSL	-3.43	0.419	Asn76
PqsR	4-IC	-3.83	0.792	Gln194, Ser196, **Leu197**, Ile236
	PQS	-5.48	1.002	**Leu197**
PqsA	4-IC	-3.77	0.756	Leu7, Ala5
CarR	4-IC	-2.92	0.816	Thr100, Arg111

Three-dimensional (left) and 3D partial enlarged graphics (right) of molecular docking analysis. **(A)** LasR, PDBID: 6D6A; **(B)** RhlR (Uniprot: AlphaFill); **(C)** PqsR, PDBID: 4JVD; **(D)** CarR, PDBID: 8GC6

(A-C) Expression of *lasB* in QM-1(pKD-*lasB*) or QM-2(pKD-*lasB*), *pqsA* in QM-3(pKD-*pqsA*); **(D)** Rotten area of Chinese cabbage infected by PAO1 (Δ*lasR*), PAO1 (Δ*rhlR*) or PAO1 (Δ*pqsR*) with treatment of 4-imidazolecarboxaldehyde. All data were expressed as mean ± SD values with three independent experiments performed in triplicate. ns: $p > 0.05$, *: $p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ***$p < 0.0001$.

4-imidazolecarboxaldehyde could increase the sensitivity of tetracycline and azithromycin to PA

Seven antibiotics belonging to different structural classes, including tetracyclines, macrolides, β-lactams, fluoroquinolones, aminoglycosides, sulfonamides, and glycopeptides, were selected to determine the sensitivity of them against PAO1. As shown in Fig. 7A-B, 4-imidazolecarboxaldehyde at sub-MICs together with tetracycline or azithromycin at sub-MIC could significantly inhibit the growth of PAO1 while the separated 4-imidazolecarboxaldehyde at sub-MICs or tetracycline or azithromycin at sub-MIC showed no obvious inhibition on the growth of PAO1. However, no such synergistic effect was observed with levofloxacin (Fig. 7C). The results demonstrated that 4-imidazolecarboxaldehyde could be an antibiotic adjuvant to enhance the sensitivity of tetracycline and azithromycin against PA to synergistically challenge PA infection by combining usage at both sub-MIC.

Resistance induction assay was performed to evaluate the drug resistance development of 4-imidazolecarboxaldehyde to PA, using polymyxin B as control. As shown in Fig. 7D, a continuous

culturing of PA in the presence of 4-imidazolecarboxaldehyde for 20 passages did not result in detectable alteration in MIC values. In contrast, the MIC of polymyxin B increased progressively after the eighth passage and eventually reached to approximately threefold higher than the initial value. The results suggested that 4-imidazolecarboxaldehyde exhibits a relatively low propensity to induce the resistance against PA at least in the 20 passages.

(**A**): Tetracycline (32 μM); (**B**): Azithromycin (32 μM); (**C**): Amikacin (8 μM); (D): Bacterial resistance induction test of 4-imidazolecarboxaldehyde and polymyxin B against PA. ns: $p > 0.05$, **** $p < 0.0001$

Discussion

The emergence of bacterial resistance has led to great difficulty in infection treatment and increased mortality, causing a global public health crisis. QS is an intercellular communication system that is widely present in bacteria controlling various physiological activities, like antibiotic resistance, virulence and pathogenicity. Inhibition of the QS system has been reported as a promising strategy to tackle infections caused by multidrug resistant bacteria including PA.

This study found that 4-imidazolecarboxaldehyde could be a promising QSI targeting *las* and *rhl* systems to decrease the expression of the QS related genes, production of corresponding virulence factor and the acute and chronic infection associated motility and biofilm formation, and finally attenuate the pathogenicity of PA significantly without inducing obvious resistance to PA at least in 20 passages.

Numerous QSIs have been reported, including natural and synthetic compounds. Natural QSIs are derived from plants, animals, and micro-organisms[34], like essential oils, flavonoids and alkaloids, marine algae extract and QS quenching enzymes (QQEs)[35].They are widely available from natural sources and possess pharmacological properties, yet difficult to extract and poor pharmacokinetics somehow[36, 37].On the other hand, synthetic QSIs are artificially designed compounds that interfere with the specific QS pathways or bacterial species[38]. The current repertoire of synthesized QSIs primarily includes analogues of autoinducers and nanomaterials, like meta-bromo-thiolactone, the AHL analogue of PA[39] and gold nanoparticles exhibiting anti-QS against drug-resistant PA[40]. Synthetic QSIs circumvent the extraction complexities, but limited by potential cytotoxicity and low bioavailability[16]. 4-Imidazolecarboxaldehyde is a small, hydrophilic molecule (MW: 96.09 g/mol, XLogP3-AA = -0.1), which may facilitate its delivery in aqueous environments. The imidazole ring and aldehyde group provide potential sites for hydrogen bonding and other non-covalent interactions, facilitating its interaction with quorum sensing-related targets. Moreover, 4-Imidazolecarboxaldehyde could be synthesized through simple and cost-effective chemical routes, facilitates its potential application as QSI and development of its derivatives. 17-(4'-imidazolyl)androsta-5,16-dien-3β-ol (2e), a derivative of 4-imidazolecarboxaldehyde, exhibited potent CYP17A1 inhibitory activity with an IC_{50} value as low as 24 nM[22]. Although no direct cytotoxicity data are currently available for this compound, 4-imidazolecarboxaldehyde at used the concentrations (80 and 160 μg/mL) showed no observable growth inhibition on PA (Fig. 1A), nor did it

compromise the viability of *Caenorhabditis elegans* or *Drosophila melanogaster* (Fig. 4B-C), suggesting its less cytotoxicity but needing further investigation.

The QSIs function by disrupting the bacterial QS system through three main mechanisms: (i) inhibiting the synthesis of signal molecules; (ii) degrading signal molecules; and (iii) utilizing signal molecule analogues to competitively bind with natural signal molecules for receptor binding. QSIs could target multiple targets, like isoliquiritigenin acting on three QS receptor proteins, Linalool affecting two systems of *las* and *rhl*, and *ortho*-vanillin only targeting one QS receptor protein RhlR[41–43]. 4-Imidazolecarboxaldehyde targets *las* and *rhl* systems based on the expression analysis by QS quintuple mutants and virulence evaluation by Chinese cabbage infection models infected by single QS receptor mutant (Fig. 6). 4-Imidazolecarboxaldehyde targets LasR by sharing two identical interacting residues with the LasR corresponding signal molecule, suggesting the probably competitive binding between them, while the lack between 4-imidazolecarboxaldehyde–RhlR and RhlR–autoinducer suggesting a probably allosteric function for RhlR inhibition (Table 2), need further studying. It has been reported that simultaneous inhibition of both *las* and *rhl* systems may be a more promising therapeutic strategy[35].

Many but not all QSIs could impact antibiotics sensitivity due to its target(s) toward QS system which regulate antibiotics resistance as well, serving as antibiotic adjuvants sometimes[44]. For instance, allicin could act as adjuvant of cefoperazone, tobramycin, and ciprofloxacin, and isoliquiritigenin could act as adjuvant of aminoglycoside antibiotics to synergistically tackle the bacterial infection[41, 45]. 4-Imidazolecarbaldehyde, as a promising QSI, could be used independently or synergistically with antibiotics of tetracycline and azithromycin to attenuate infections caused by PA (Fig. 7A-B).

Collectively, 4-imidazolecarboxaldehyde in this study has been identified as an QSI, attenuated QS-regulated genes expression and virulence factor production, biofilm formation, motility, and pathogenicity of PA at sub-MICs 80 and 160 μg/mL without inducing resistance. It may interfere with QS signaling mainly by targeting *las* and *rhl* systems. It could also enhance the sensitivity of tetracycline and azithromycin against PA. 4-Imidazolecarboxaldehyde could be used individually or synergistically with any of the two antibiotics to tackle the PA infection. The QSI activity of 4-imidazolecarboxaldehyde against other Gram-negative and Gram-positive bacteria warrants further research.

Declarations

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Data availability: All data generated or analyzed during this study are included in this article.

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Figures

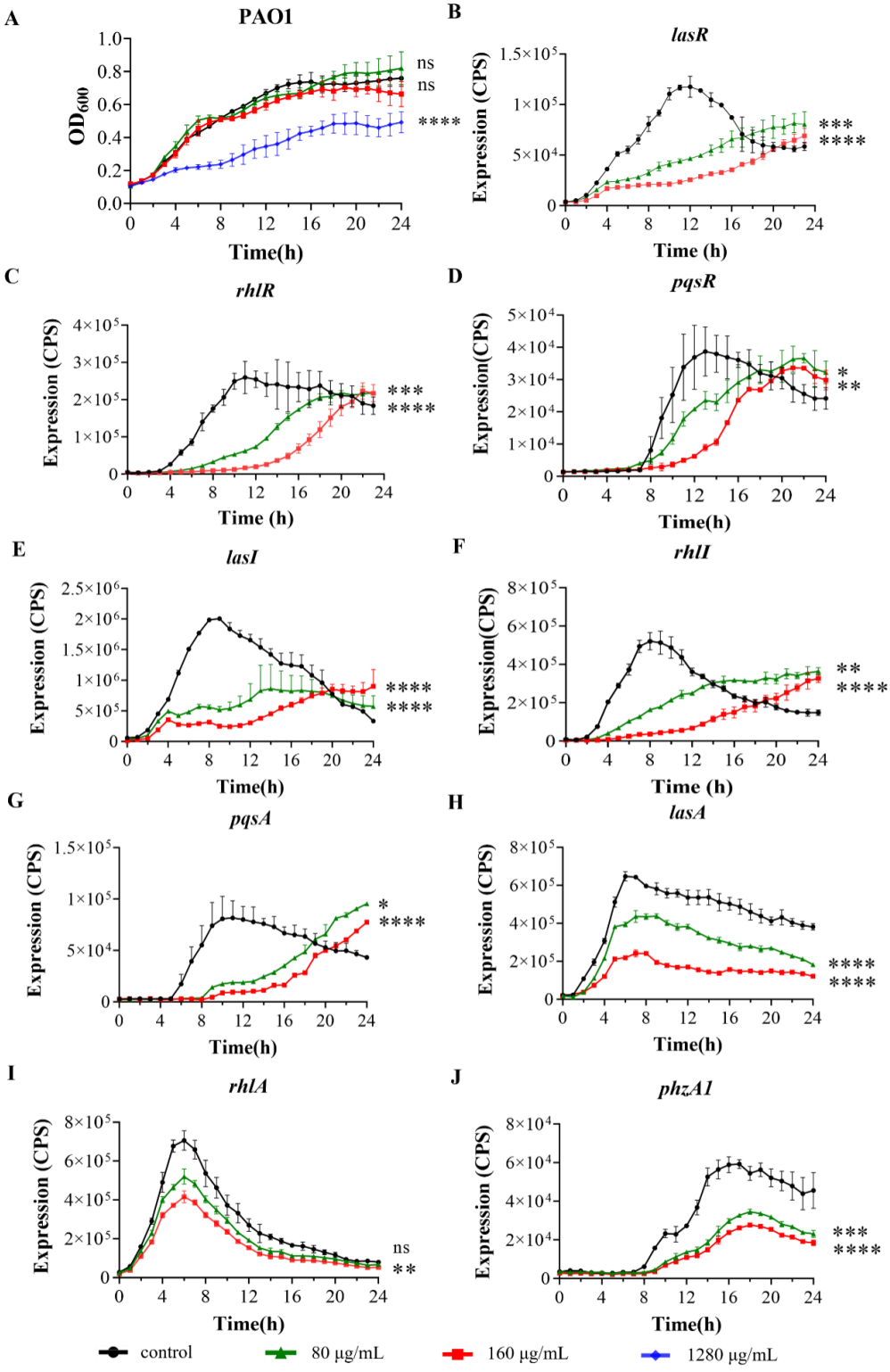


Figure 1

Effect of 4-imidazolecarboxaldehyde on the transcriptional expression of QS-related genes in PA

(A) Growth of PAtreated with 4-imidazolecarboxaldehyde at sub-MIC concentrations. The transcriptional expression of the QS-associated genes treated with 4-imidazolecarboxaldehyde at sub-MIC

concentrations **(B)** *lasR*, **(C)** *rhlR*, **(D)** *pqsR*, **(E)** *lasI*, **(F)** *rhlI*, **(G)** *pqsA*, **(H)** *lasA*, **(I)** *rhlA*, **(J)** *phzA1*. ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

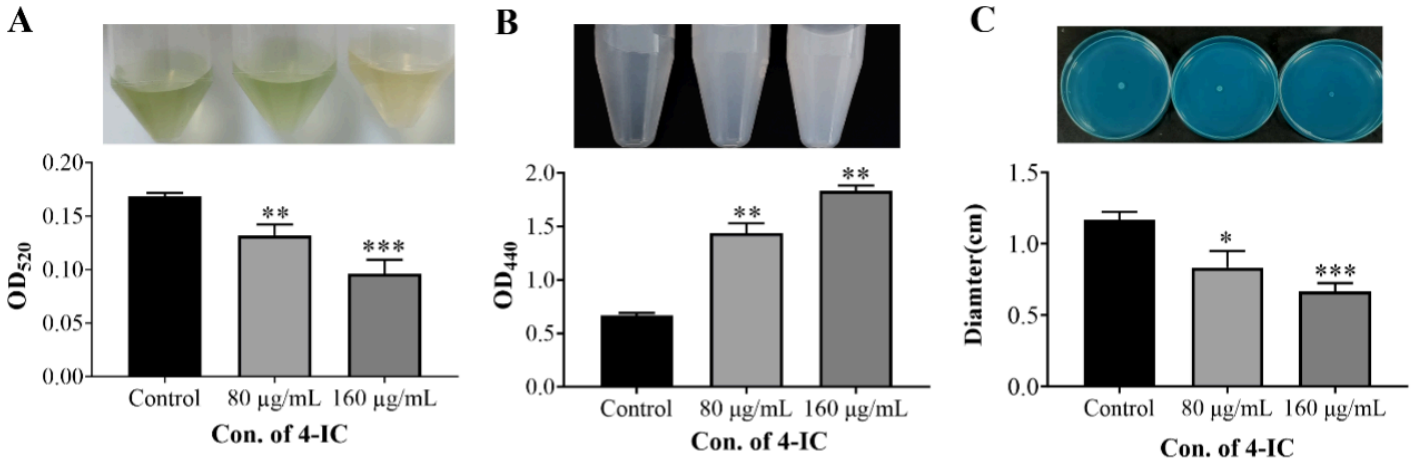


Figure 2

Inhibition of 4-imidazolecarboxaldehyde at sub-MICs on the production of virulence factors in PAO1

(A) pyocyanin; **(B)** protease; **(C)** rhamnolipid. All data were expressed as mean ± SD values with three independent experiments performed in triplicate. *: $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

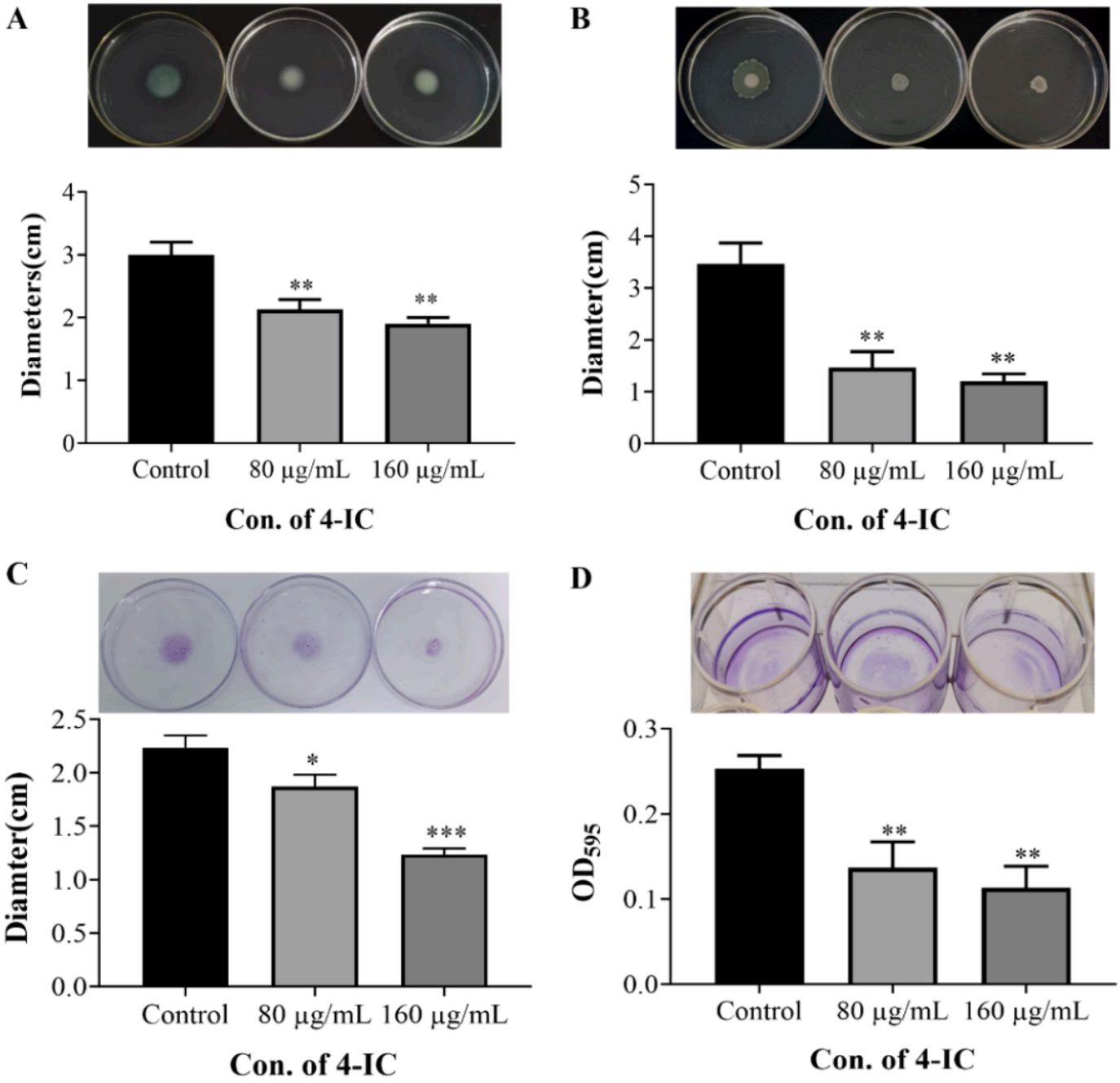


Figure 3

Inhibition of 4-imidazolecarboxaldehyde on the motility and biofilm formation in PAO1

(A) swimming; **(B)** swarming; **(C)** twitching; **(D)**biofilm formation. All data were expressed as mean ± SD values with three independent experiments performed in triplicate. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

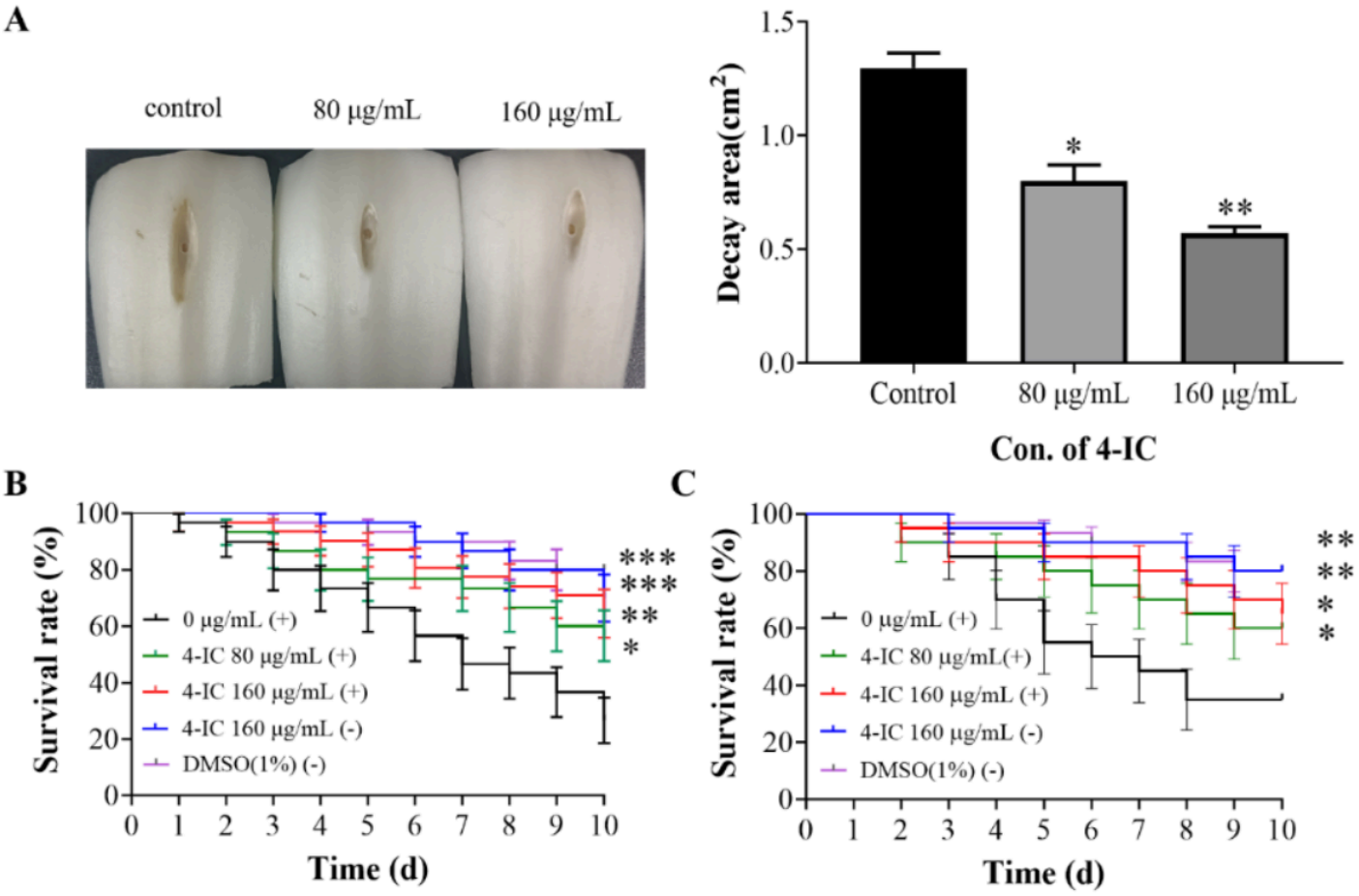


Figure 4

4-imidazolecarboxaldehyde attenuates the pathogenicity of PAO1

(A) rotten area of Chinese cabbage; **(B)** survival rate of fruit flies; **(C)** survival rate of nematodes.

(+): with PAO1; (–): without PAO1. All data were expressed as mean ± SD values with three independent experiments performed in triplicate. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

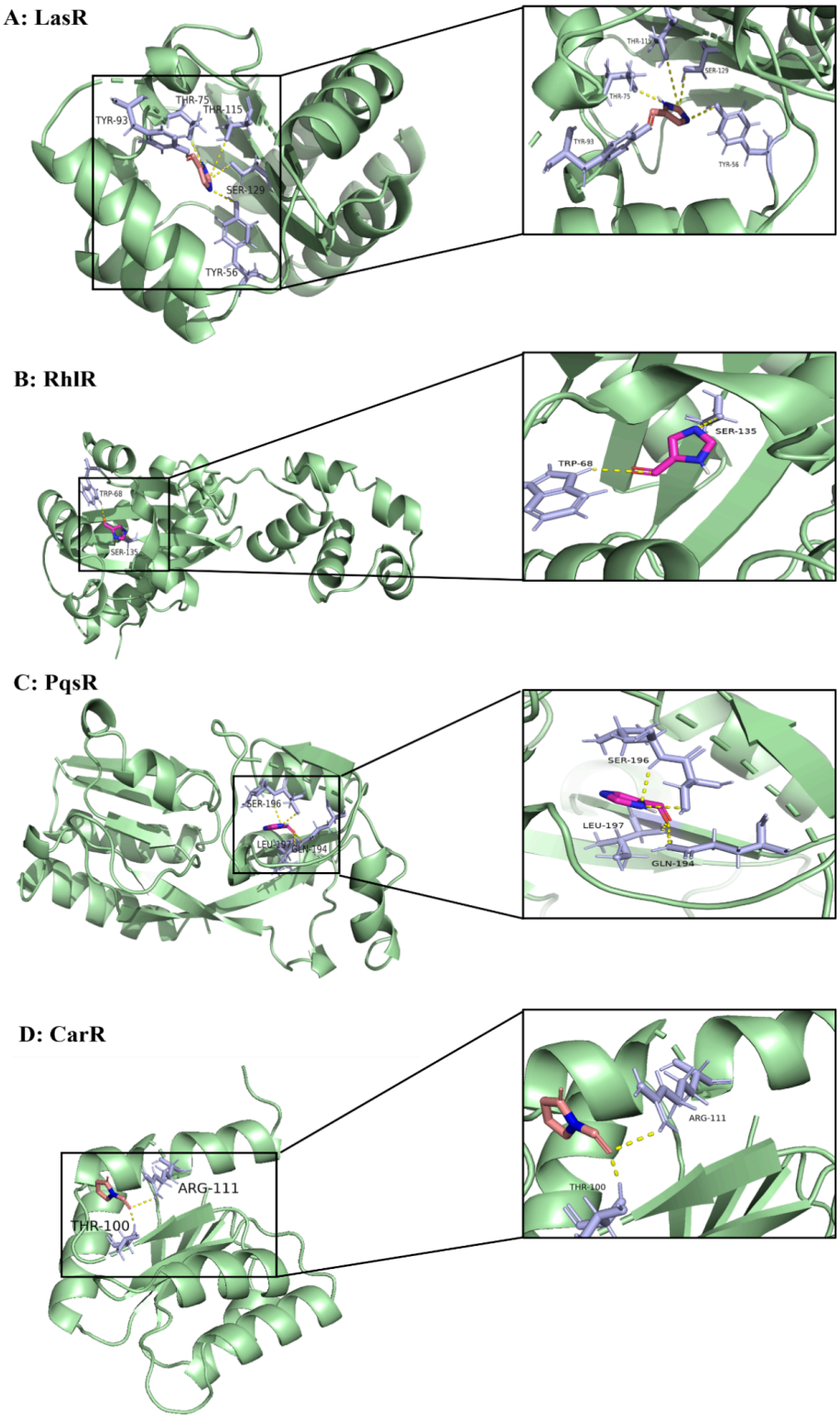


Figure 5

Molecular docking analysis of 4-imidazolecarboxaldehyde with the QS receptor proteins of PA

Three-dimensional (left) and 3D partial enlarged graphics (right) of molecular docking analysis. **(A)**LasR, PDBID: 6D6A; **(B)** RhlR (Uniprot: AlphaFill); **(C)** PqsR, PDBID: 4JVD; **(D)** CarR, PDBID: 8GC6

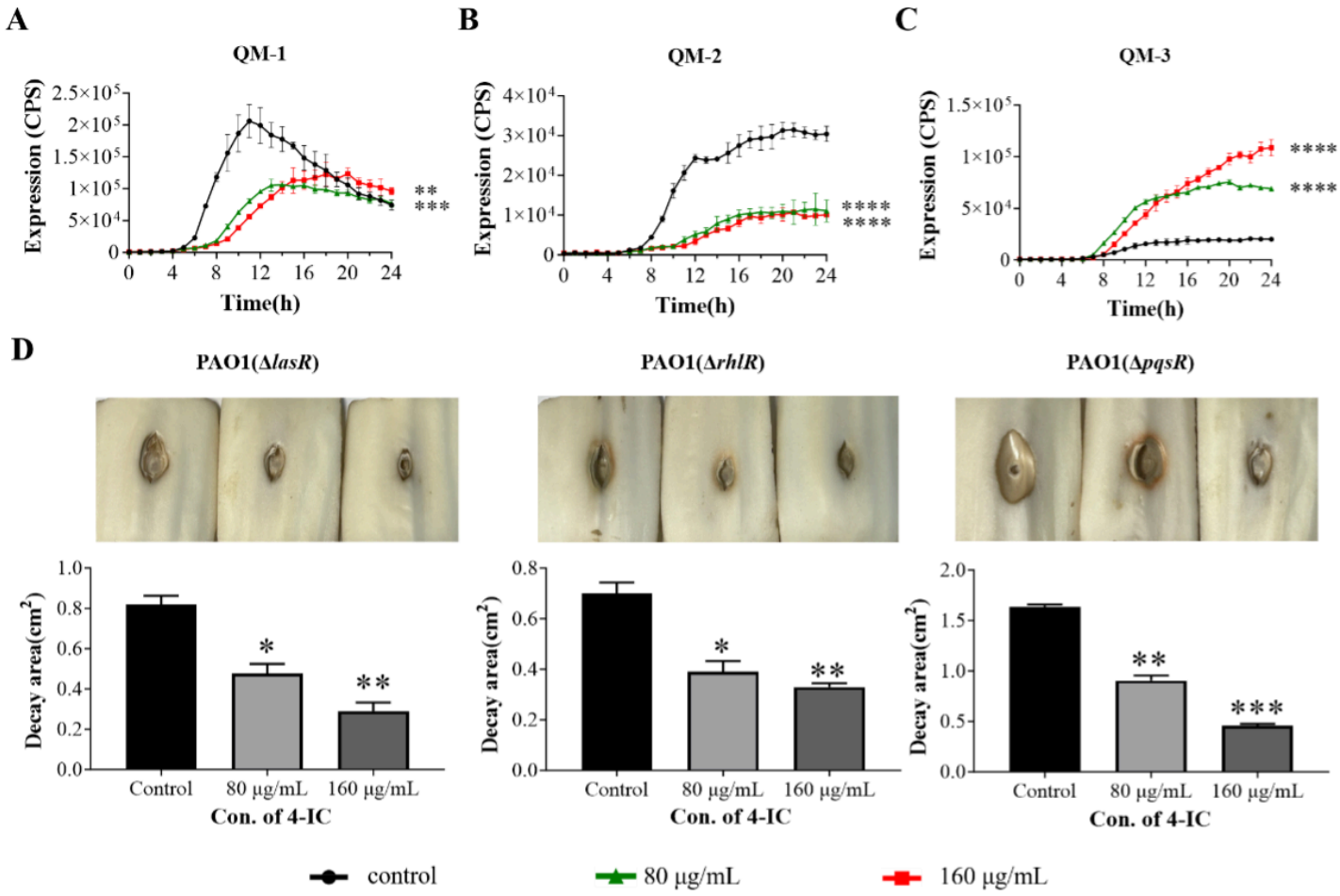


Figure 6

Effect of 4-imidazolecarboxaldehyde on the expression of *lasB* and *pqsA*in the QS quintuple mutants and the virulence in the single mutants

(A-C) Expression of *lasB* in QM-1(pKD-*lasB*) or QM-2(pKD-*lasB*), *pqsA* in QM-3(pKD-*pqsA*); **(D)** Rotten area of Chinese cabbage infected by PAO1 (Δ*lasR*), PAO1 (Δ*rhlR*) or PAO1 (Δ*pqsR*) with treatment of 4-imidazolecarboxaldehyde. All data were expressed as mean ± SD values with three independent experiments performed in triplicate. ns: $p > 0.05$, *: $p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ***$p < 0.0001$.

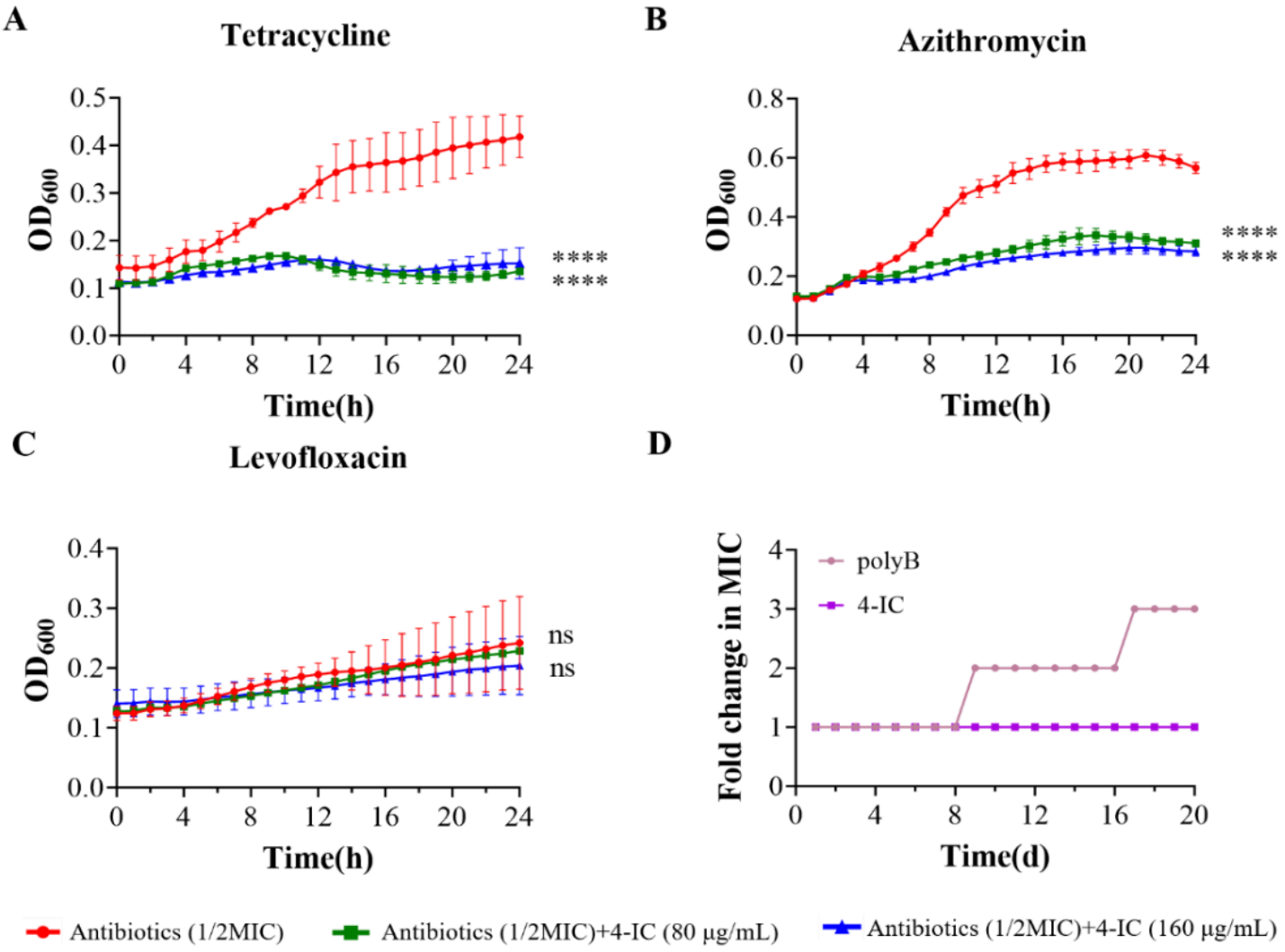


Figure 7

Growth of PAO1 treated by 4-imidazolecarboxaldehydecombined with antibiotics at sub-MICs and the resistance analysis

(**A**): Tetracycline(32 μM); (**B**): Azithromycin (32 μM); (**C**): Amikacin (8 μM); (D): Bacterial resistance induction test of 4-imidazolecarboxaldehyde and polymyxin B against PA. ns: $p > 0.05$, **** $p < 0.0001$

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