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RESEARCH ARTICLE

A novel antibiotic 3-isopropylhexahydro-4*H*-pyrido[1,2-α]pyrazine-1,4(6*H*)-dione isolated from the thermophilic bacterium *Bacillus licheniformis* QX928

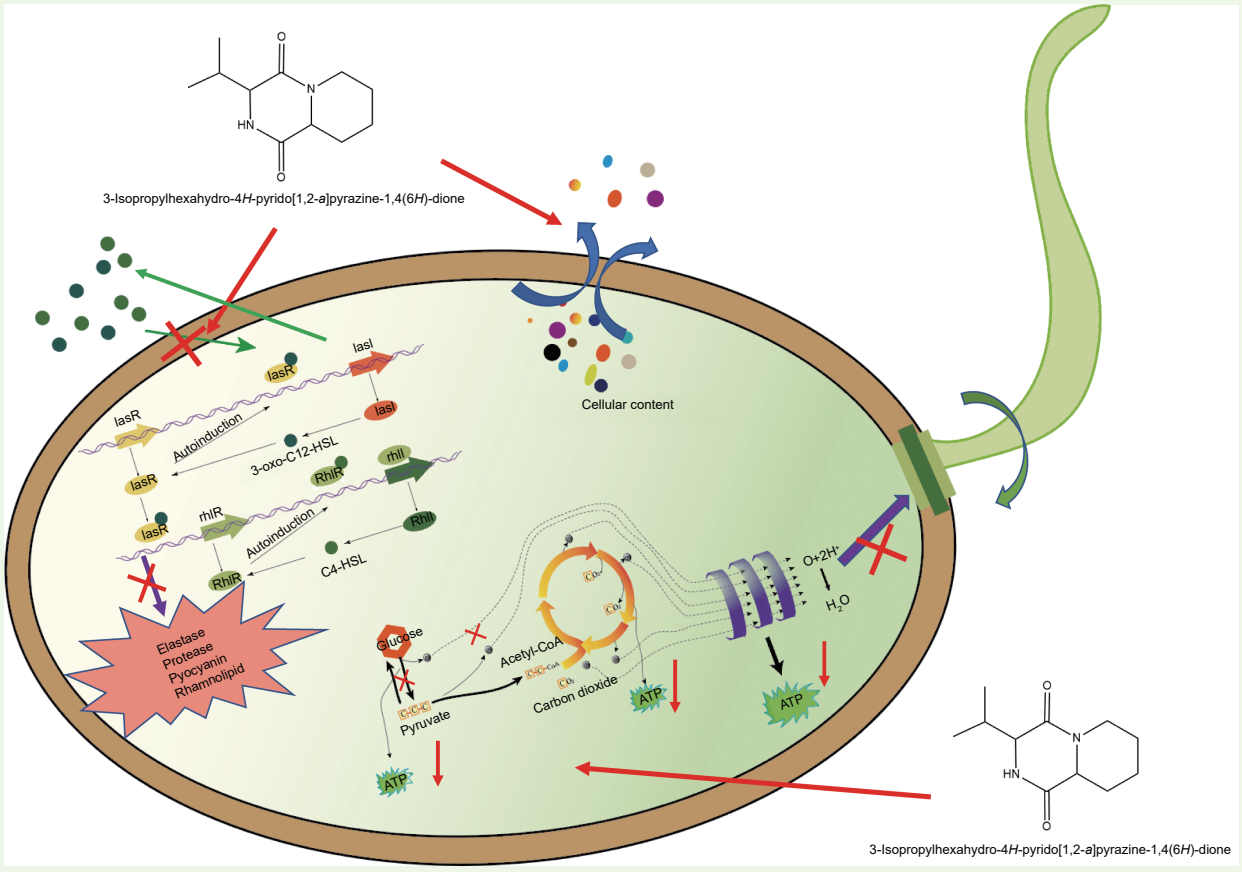
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Highlights

- A new microbial source compound with a “dual resistance” mechanism has been discovered.
- It reveals the concentration-dependent multi-target synergy mechanism of IPHPPD.

Graphical abstract



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Abstract

A microbial strain designated *Bacillus licheniformis* QX928 was screened from hot springs in Sichuan Province, China, and a compound generated in the culture of this strain clearly inhibited *Pseudomonas aeruginosa* ATCC27853. The measured minimum inhibitory and lowest bactericidal concentrations were (13±0.17) and (22±0.72) mg L^{-1}, respectively. The compound was identified as 3-isopropylhexahydro-4*H*-pyrido[1,2-α]pyrazine-1,4(6*H*)-dione (IPHPPD). A SciFinder search revealed that IPHPPD could be the first compound synthesized by microorganisms that had both antibacterial and anti-quorum sensing properties. At low concentrations, IPHPPD interfered with the signaling factors and population effects of *P. aeruginosa*, thereby altering the biofilm morphology and structure. IPHPPD more strongly inhibited *P. aeruginosa* at high concentrations, primarily by reducing its virulence factors, cell membrane permeability and energy metabolism. A transcriptome analysis highlighted the role of IPHPPD in the transcriptional regulation of cellular metabolism and quorum sensing. Thus, the results of this study provide critical evidence that IPHPPD is a potential target for drug development to prevent and treat diseases in animals.

Keywords: thermophilic bacteria, antibacterial mechanism, quorum sensing, *Pseudomonas aeruginosa*

1. Introduction

Pseudomonas aeruginosa is one of the six "ESKAPE" pathogens on the antibiotic resistant "key pathogens" list of the World Health Organization (Holbrook and Garneau-Tsodikova 2018). *Pseudomonas aeruginosa* can not only infect humans but also animals and plants. As an important pathogen, *P. aeruginosa* possesses multiple virulence factors that enable it to damage host cells and regulate their adaptation to new immune mechanisms, which leads to the emergence of new infections (Gellatiy *et al.* 2013). The resistance of pathogens to two or more types of antibiotics is known as multidrug resistance (MDR) (Magiorakos *et al.* 2012). An overwhelming number of MDR strains of *P. aeruginosa* have developed. The rate of incidence and mortality of these strains are very high and account for 10% of hospital infections (Aloush *et al.* 2006). For example, *P. aeruginosa* is the primary cause of infections, such as ventilator-associated pneumonia and urinary tract, blood flow, and chronic infections (Ferrara *et al.* 2006; Mesaros *et al.* 2007; Parkins *et al.* 2018). In immunocompromised hosts, such as AIDS or diabetes patients, the bacteremia caused by *P. aeruginosa* is a common complication (Mesaros *et al.* 2007). Diseases in livestock and pets can also be caused by *P. aeruginosa*. Simultaneously, *P. aeruginosa* is naturally resistant to many types of antibiotics.

Faced with the global health threat of increasing resistance to antibiotics, researchers are exploring interventions that target bacterial virulence factors, with quorum sensing (QS) is a particularly attractive target (Fleitas Martínez *et al.* 2019). QS is the ability of bacteria to directly enhance their pathogenesis and endow them with drug resistance by regulating the formation of biofilm, the level of expression of a variety of virulence factors, and their movement. Furthermore, attacking the QS signaling network is less likely to select for resistant strains than using conventional antibiotics. In the QS system, Gram-negative bacteria communicate using the homoserine lactone, while Gram-positive bacteria communicate by induced peptides. Biofilms are usually composed of single or multiple microorganisms and develop on biotic or abiotic surfaces. Their infections are usually related to medical devices, particularly medical implants (O'Toole *et al.* 2000). The existence of biofilms can cause resistance to a variety of conventional antibiotics that are used to treat *P. aeruginosa*. Thus, eradicating or inhibiting the formation of biofilms could substantially improve the ability to treat this type of infection (Lee and Zhang 2015). The use of a strategy to prevent QS could be an effective way to treat *P. aeruginosa* infections.

With the development and utilization of antibiotics during the past few decades, it has become increasingly difficult to isolate new ones from the environment (Trischman *et al.* 1994). Increasing amounts of attention have focused on screening microorganisms from special habitats to obtain new biological drugs that are antibacterial (Wilson and Brimble 2009) because the ecological competitors of microorganisms that live in highly competitive or severe environmental conditions are usually limited or killed by the secondary metabolites (Mearns-Spragg *et al.* 1998). In recent years, with the continuous improvement of an understanding of the origin, function and structural diversity of antibiotics, the research on antibiotics has become increasingly prominent. However, bacteria are still the most promising source of antibiotics in the future. Hot springs contain a complete ecosystem and stable biochemical components, so they have higher temperatures and greater biodiversity (Adigüzel *et al.* 2011; Genc *et al.* 2015). Currently, thermophilic microorganisms have been considered as the source of secondary metabolites with a substantial potential for applications in biotechnology and functional diversity (Selvarajan *et al.* 2017). Many *Bacillus* strains have been isolated from thermophilic environments of 45°C–70°C (Noorollahi *et al.* 2009) and can produce biologically active fatty acids, alkaloids, sterols, cyclic peptides, peroxides and amino acids (Selvin *et al.* 2012). *Bacillus* is well-known for its high probiotic potential, and members of this genus can synthesize a variety of antibacterial compounds (Matsunaga *et al.* 1985). More than 200 antibiotics have been isolated from *Bacillus*, and they often differ in structure and function. They contain compounds, such as bacteriocin inhibitors, surfactants and bacteriocins. Compared with antibiotics whose mode of action is to damage the cell wall, the secretion of virulence factors of pathogenic bacteria can be reduced by antibiotics that inhibit the activity of ribosomes

(Sansinenea and Ortiz 2011). Multidrug resistance is primarily related to the drug resistance of *Staphylococcus aureus*, *Pneumococcus* and *P. aeruginosa*. In particular, serious infection and death can be caused by *P. aeruginosa* because of its virulence, pathogenicity, and adaptability to different environments (Hodille *et al.* 2017).

Therefore, this study aimed to screen the previously unidentified compounds with antibacterial activity from the microorganisms in hot springs. A strain designated *Bacillus licheniformis* QX928 was isolated from hot springs and produces a compound with antibacterial activities that inhibits the formation of biofilm in *P. aeruginosa* ATCC27853. The structure of this compound was characterized using high-pressure liquid chromatography (HPLC), high resolution mass spectrometry (HRMS), Fourier transform infrared spectroscopy (FTIR), gas chromatography-mass spectrometry (GC-MS), ^{1}H NMR, and ^{13}C NMR techniques. A search of SciFinder was also used to determine whether this compound might be the first biosynthetic compound with both antibacterial and anti-QS activities synthesized by microbial metabolism.

2. Materials and methods

2.1. Isolation and identification of the bacteria

The bacteria were isolated from hot springs in the National Geopark of Reefs in Anxian County, Mianyang City, southwestern China (latitude 104°35′09.9′′, longitude 31°58′83.6′′). *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella enteritidis*, *Clostridium* and *E. coli* were used as test bacteria to screen the antibacterial activity using an agar well diffusion assay on brain heart infusion (BHI) solid medium. The bacterial strains with spectral antibacterial activity were selected for subsequent experiments. The strain with the most effective antibacterial activity was selected for further identification. The bacterial genomic DNA was isolated as previously described (Logan and Berkeley 1984), and the universal primers (fD1-5′-CAGAGTTTGATCCTGGC TAGGAGGTGATCCAGCCGCA -3′ and rP2-5′-AGTTTGATC MTGGCTCAGGGTTACCTTGTTACGACTT-3′) were used to amplify the 16S rRNA using optimized PCR conditions. The amplified PCR products were sequenced, analyzed and then submitted to the NCBI GenBank. MEGA6.0 was used for the systematic analyses, while the phylogenetic tree was derived by the evolutionary distance. A bootstrap analysis was performed, and the maximum likelihood algorithm was used to determine the evolutionary distance.

Pseudomonas aeruginosa ATCC27853 was obtained from the Shanghai Microbial Technology Preservation Center (Shanghai, China) and used throughout this study. The strain was cultured in BHI agar to confirm the cell viability and propagation under standard growth conditions in LB broth at 37°C. The same amount of inoculum (OD_{600} of 2) was used in all the experiments.

2.2. Preparation, fractionation, and identification of the active compound

The bacterial culture was cultured in 300 mL of BHI broth at 50°C for 12 h and inoculated into the fermentation medium after activation. The medium was incubated at 50°C for 72 h and then centrifuged at 100,000×g for 15 min to obtain a cell-free supernatant, which was filtered with a 0.22 μm filter to remove residual cells. The supernatant was extracted with an equal volume of ethyl acetate, and the extract was evaporated to dryness at 50°C on a rotary evaporator. The dried residue was then dissolved in absolute ethanol to remove the insoluble glycoproteins. The crude extract of *B. licheniformis* QX928 was dissolved in water, adsorbed on a macroporous adsorption resin (Model D101; Beijing Ruida Henghui Technology Development Co., Ltd., Beijing, China), and eluted with ethanol. The eluent of each component was collected using 40% ethanol. The eluent had bacteriostatic activity, and the fraction was lyophilized to obtain a solid. The solid was dissolved in methanol and subjected to silica gel column chromatography (silica gel 200–300 mesh; Qingdao Ocean Chemical Co., Ltd., Shandong, China) with chloroform: methanol (v/v) as the solvent. Fractions were collected and evaporated on a rotary evaporator at 50°C to produce a solid. The solid phase was dissolved in methanol, and the fractions that contained 40% methanol were collected on a C18 preparative column (column height 180 mm; column diameter 25 mm). These fractions showed maximal antibacterial activity. After lyophilization, the components were separated using HPLC (Agilent 5 HC-C18 250×4.6 mm; Agilent Technologies, Santa Clara, CA, USA). After the antibacterial activity of each part had been determined (Singh *et al.* 2017), the purified active compounds were analyzed using HPLC, liquid chromatography (LC-MS), NMR, HRMS, and FTIR (Kiran *et al.* 2017). The data was processed with GC-MS, and the ChemStation (Agilent Technologies) column conditions were set as follows: The oven temperature was 80°C (4 min), and the temperature increased by 4°C min^{-1}. The temperatures of the injection port and detector port were 250°C and 280°C, respectively. The GC peaks were analyzed by mass spectrometry, while the spectra were analyzed by an NIST MS search (version 2.0). The purified compound was dissolved in deuterated chloroform at a concentration of 20 mg mL^{-1} to obtain the NMR spectra. Both ^{1}H and ^{13}C were obtained using a Bruker Advance II 500 (Bruker, Billerica, MA, USA) at 22°C.

2.3. Assays of the minimum inhibitory/bactericidal concentrations (MIC/MBC) and lethality over time

The MIC was determined by a gradient dilution method on 96-well microtiter plates based on the European Committee for Antimicrobial Susceptibility Testing (EUCAST) standard (Leclercq *et al.* 2013) to test the susceptibility to antimicrobial compounds. *Pseudomonas aeruginosa* that had grown overnight was diluted 1:300 in LB broth. The IPHPPD was dissolved in water at different concentrations, which were dissolved in culture to assess the MIC (Leclercq *et al.* 2013). A volume of 200 μL diluted bacterial suspension and IPHPPD at various concentrations were added to sterile 96-well flat bottom microtiter plates, and the cultures were incubated at 37°C for 24 h with an equal volume of methanol as the negative control. The MBC was determined by examining the lowest concentration of IPHPPD. When there was no bacterial growth at 24 h, it was considered to be the lowest

inhibitory concentration. The concentration of IPHPPD with no obvious bacterial growth was spread on the plate, and the number of colonies was evaluated as the minimum sterilization amount. All the experiments were conducted in triplicate. Based on the recommendations of EUCAST of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), the minimum extract concentration that showed a reduction in the number of viable cells by at least three orders of magnitude was regarded as the MBC (EUCAST of ESCMID 2000).

The time for lethality was determined as previously described (Sharafutdinov *et al.* 2017). An overnight culture of *P. aeruginosa* was diluted 1:300 with LB broth and inoculated into six test tubes. The bacteria were treated with different concentrations of IPHPPD and incubated at 37°C for 24 h. The bacteria were then serially diluted with phosphate-buffered saline (PBS), and the number of colonies was counted on a trypticase soy agar (TSA) plate. The experiment was conducted in triplicate.

2.4. Determination of thermal stability

IPHPPD was treated at 55°C–80°C for 21 h, and *Pseudomonas aeruginosa* was used as the test bacteria. The thermal stability of the compound was evaluated by the diameter of inhibition zone.

2.5. Influence of IPHPPD on the signal factors in quorum sensing

Chromobacillus violaceum CV026 was used as the reporting strain to assay the anti-QS activity. The anti-biofilm activity was evaluated by quantifying the production of violet and the diameter of bacteriostatic circle. The different concentrations of IPHPPD (0.1×MIC to 1.0×MIC, MIC=(13±0.17) mg L^{-1}) were used to test the ability of extract to quench the population. *Chromobacillus violaceum* CV026 was diluted to an OD_{600} of 0.1 in nutrient broth (NB) supplemented with hexyl homoserine lactone (0.0625 μg mL^{-1}). The mixture was incubated at 30°C and shaken at 180 r min^{-1} for 24 h. A volume of 10 mL of culture in each flask was centrifuged for 10 min at 16,000×g to precipitate the insoluble violetin. After the culture supernatant had been discarded, 10 mL of DMSO was added to the precipitate, thoroughly vortexed for 30 s to completely dissolve the violetin, and then centrifuged at 16,000×g for 10 min to remove the cells. Finally, the absorbance at 585 nm was measured.

The conductivity method was used to evaluate the influence of *B. licheniformis* QX928 on cell permeability. The bacteria were collected, washed three times with PBS, and diluted to an OD_{600} of 2 (Kim *et al.* 2013). An equal volume of PBS that contained 1×MIC, 2×MIC and 3×MIC concentrations of IPHPPD was added to the culture wells using an equal volume of PBS as the control. The conductivity was measured after the cells had been cultured for 0, 15, 30, 60, and 120 min at 20°C. All the experiments were conducted in triplicate and repeated several times.

Conductivity increase =(Conductivity increase at different moments-Conductivity value at 0 min/Conductivity value at 0 min)×100%

2.6. Effect of IPHPPD on the structure of *P. aeruginosa* biofilm

Fluorescence microscopy *Pseudomonas aeruginosa* was cultured in 24-well polystyrene plates with different concentrations of IPHPPD to evaluate the development of biofilms on 11 mm sterile glass coverslips. The sensitivity of *P. aeruginosa* to IPHPPD was assayed in a 24-well polystyrene plate in which the bacteria in 1 mL LB had reached an OD_{600} of 0.1. The cultures were then added to each well that contained different concentrations of IPHPPD. One glass coverslip was submerged into the 24-well polystyrene plate, which was statically incubated at 37°C. Simultaneously, when *P. aeruginosa* was cultured for 24 h to form a normal film, certain amounts of 16 mg L^{-1} of IPHPPD were added to the culture and incubated for 1 h in the other group, while the biofilm was stained with the fluorescent dyes propidium iodide and fluorescein diacetate. Both living and dead cells were observed under a drop fluorescence microscope. The effect of IPHPPD on the formation of biofilm was evaluated with an equal volume of methanol as the negative control.

Scanning electron microscopy and atomic force microscopy Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to observe the effect of IPHPPD on the topology of biofilm formed by *P. aeruginosa*. For SEM, after the biofilm of *P. aeruginosa* had grown on sterile coverslips, they were soaked in BHI and then immersed in broth on a 24-well polystyrene plate. The culture plate and cover glass were placed at 37°C for 24 h. Different concentrations of IPHPPD (12 and 22 mg L^{-1}) were added, while the plate was incubated at 37°C for 6 h to remove the plankton. Moreover, the cover glass was gently rinsed with 0.9% PBS. The samples were treated with 2.5% glutaraldehyde for 20 min, dehydrated with an ethanol gradient (10 to 95%) for 10 min, and the dehydrated and dried biofilm were covered with gold and observed under SEM.

Pseudomonas aeruginosa that had been cultured overnight in BHI broth was diluted to an OD_{600} of 0.1. The sterile coverslips were immersed in 24-well polystyrene plates, and the plates were incubated at 37°C for 24 h. Different concentrations of IPHPPD were added to the media and incubated for 6 h. After the incubation period, the biofilm formed on the coverslips was gently rinsed with phosphate buffer (pH=7.4) and then placed under a dryer until it had completely dried. Finally, the biofilm was scanned in half contact mode under AFM with the same volume of methanol as the negative control in this study.

2.7. Assays of swarming and swimming motility

The swarming movement was tested in the presence of 0.5×MIC IPHPPD (Overhage *et al.* 2007). An overnight culture of *P. aeruginosa* was diluted to an OD_{600} of 1.0 and inoculated on a plate that contained BM2 colony medium (62 mmol L^{-1} PBS at pH=7, 2 mmol L^{-1} $MgSO_4$, 10 μmol L^{-1} $FeSO_4$, 0.4% glucose, 0.1% casein amino acids and 0.5% agar). The plates were incubated at 37°C for 24 h, and the colony area was observed with an equal volume of methanol

as the negative control.

An overnight culture of *P. aeruginosa* was diluted to an OD_{600} of 1.0, inoculated in trypsin broth (10 g L^{-1} trypsin, 5 g L^{-1} NaCl and 0.3% agar), which was supplemented with 12.0 mg L^{-1} IPHPPD. The culture was analyzed after incubation at 37°C for 24 h with an equal volume of methanol as the negative control. The effect of IPHPPD on the movement of *P. aeruginosa* was then observed.

2.8. Effect of IPHPPD on the energy metabolism and expression of *P. aeruginosa* virulence factors

Determination of soluble protein Activated *P. aeruginosa* was diluted 1:300 with TSA and cultured at 37°C for 12 h. IPHPPD was added to the culture medium at the 1×MIC concentration and incubated at 37°C for 3 h. A total of 0.6 g bacterial cells were ground with 1 g of sterilized quartz sand and 2 mL of PBS in an ice bath. The mixture was then centrifuged in a pre-cooled centrifuge at 12,000 r min^{-1} for 10 min, while the supernatant was collected. A volume of 5 mL Coomassie brilliant blue was added to 0.1 mL of the collected supernatant and diluted to 6 mL with distilled water. The absorbance of the supernatant at 595 nm was measured with 1 mL of distilled water and 5 mL of Coomassie brilliant blue as controls to calculate the content of soluble protein.

Determination of reducing sugars A volume of 2 mL of the supernatant and the same amount of 3,5-dinitrosulfuric acid (DNS) reagent were mixed. The absorbance of the mixture was measured, and the contents of reducing sugars in the bacteria were calculated using a standard curve.

Determination of pyruvate A volume of 2 mL of trichloroacetic acid (8%) was added to 1 mL of the collected supernatant and 1 mL of 0.1% 2,4-dinitrophenylhydrazine. A volume of 5 mL of NaOH (1.5 mol L^{-1}) was added, and the A_{540} value of the solution was measured. The content of pyruvate per unit mass of bacteria could be calculated based on a standard curve.

Determination of the virulence factors The effects of 12.0 mg L^{-1} IPHPPD on the production of virulence factors by *P. aeruginosa* were assayed by measuring the levels of pyocyanin (McClure and Schiller 1992) and rhamnolipid (McClure and Schiller 1992) and analyzing the activities of elastase (Zhu *et al.* 2002) and protease (Bjorn *et al.* 1979). The amount of pyocyanin was measured by treating an overnight culture of *P. aeruginosa* that was diluted to an OD_{600} of 0.1 with 5 mL of PB medium (20 g L^{-1} peptone, 1.4 g L^{-1} $MgCl_2$ and 10 g L^{-1} K_2SO_4). A volume of 10 ml of PB was added to the culture supplemented with 0.5×MIC of IPHPPD and incubated at 37°C for 24 h. An equal volume of methanol was used as the control group. After centrifugation for 10 min at 10,000×g to collect the supernatant of 5 mL of the PB media (OD_{600} of 0.1), the pyocyanin was extracted with 3 mL of chloroform. Next, 1 mL of 0.2 mol L^{-1} HCl was added to the pyocyanin that was quantified by spectroscopy at 520 nm. The rhamnolipid was determined by culturing *P. aeruginosa* overnight at 37°C in NB, and 10 mg L^{-1} of IPHPPD (0.5×MIC) was added. An equal volume of methanol was used for the negative control. The culture supernatant was collected by centrifugation for 10 min at 10,000×g and acidified to pH=2 with HCl. The absorbance was then measured at 570 nm.

Pseudomonas aeruginosa was cultured overnight in the media that contained IPHPPD (0.5×MIC) to assay the activity of elastase. The supernatant of the culture (750 μL) was incubated with 250 μL of elastin Congo (5 mg mL^{-1}) (0.1 mol L^{-1} Tris-HCl, pH=8, and 1 mmol L^{-1} $CaCl_2$) at 37°C for 16 h with an equal volume of methanol as the control. The supernatant of the reaction mixture was collected by centrifugation at 3,000×g for 10 min, and the absorbance was measured at 495 nm with an equal volume of methanol as the negative control. The experiments were conducted three times in parallel.

The protease activity was assayed using 2% of azocasein prepared in 50 mmol L^{-1} PBS (pH=7). *Pseudomonas aeruginosa* was cultured overnight in media that contained IPHPPD (0.5×MIC). A volume of 400 μL of culture supernatant was mixed with an equal volume of 2% azocasein and incubated at 37°C for 1 h with an equal volume of methanol as the negative control. Finally, 500 μL of 10% trichloroacetic acid was added to the mixture to stop the reaction. The reaction mixture was centrifuged at 8,000×g for 5 min to remove the residual azocasein, and the absorbance of the supernatant was measured at 400 nm.

2.9. Analysis of the gene expression of *P. aeruginosa* that had been treated with IPHPPD

A concentration of 1×MBC of IPHPPD was added to the *P. aeruginosa* cultured overnight, while an equal volume of methanol was added to the culture of the control group. After 3 h of incubation, the cells were collected by centrifugation at 12,000×g for 5 min. TRIzol® was used to extract the total RNA from the cells, but DNaseI (TaKaRa, Dalian, China) was used to remove the genomic DNA. The experimental samples and controls were then sent to Shanghai Majorbio BioPharma Technology Co., Ltd., (Shanghai, China) for transcriptome gene testing and data analysis. Statistical analyses were performed using GraphPad Prism (San Diego, CA, USA). A one-way analysis of variance (ANOVA) followed by a Tukey post hoc test was utilized to compare the differences in data between the experimental and control samples.

3. Results

3.1. Microbial diversity and identification of the thermophilic bacteria with anti-bacterial potential

Overall, 136 bacterial strains were isolated from the hot spring and identified as thermophilic bacterial strains. In a pH range of 7.2–8.1, the number of these strains decreased with the increase in temperature, and more importantly, no strain could be isolated when the temperature was increased to 80°C. Among the strains isolated, the strain designated QX928 produced an obvious antibacterial circle and an antibacterial effect on *P. aeruginosa* (Fig. 1-A). The QX928 strain was identified as *B. licheniformis* after 16S RNA sequencing and an alignment of sequence alignment in concert with the analysis of biochemical characteristics. *Bacillus licheniformis* QX928 was designated with the accession number MT994992 of 16S rRNA in GenBank

(Fig. 1-B) and then submitted to the China General Microbial Culture Preservation Management Center (CGMCC) for preservation under the number 62062. After the culture medium of *B. licheniformis* QX928 had been extracted with ethyl acetate, the extract that had been washed with water was found to have broad-spectrum antibacterial activity as shown by significant inhibition of the growth of *P. aeruginosa*, *E. coli*, *Salmonella enteritis* and *Staphylococcus aureus* following treatment with this extract (Fig. 1-C).

3.2. Purification, separation and identification of the active compound

The antibacterial activity was determined, and a 6:4 ratio of chloroform: methanol (v/v) had the greatest amount of antibacterial activity (35 mm) (Fig. 2-A). The largest elution peak had a retention time of 10.52 min (Fig. 2-B; Appendix A). The molecular structure of the bioactive compound was identified by GC-MS. Compared with the NIST library, there was a primary compound in the sample on the GC-MS spectrum based on its molecular weight, retention time and molecular formula. A main peak that appeared at 24.37 min was visible in the GC-MS spectra, and the content of the compound in the sample corresponded to the peak area. Mass spectrometry showed that the mass was 210 Da, and the molecular formula was $C_{11}H_{18}N_2O_2$ (Fig. 2-D). Simultaneously, HRMS was utilized to analyze the active section, and the results showed that there was a main spectral peak at M/Z 211.14338 (Fig. 2-C). Thus, the molecular weight of the active compound was considered to be near 210.1. In this study, the absorption peaks of the compounds were studied in more detail using FTIR (Fig. 2-E). The FTIR spectrum indicated that the amide N-H group corresponded to the characteristic peak at 3,400–3,140 cm^{-1}; the alkyl C-H group corresponded to 2,940 cm^{-1}; C–O corresponded to 1,638 cm^{-1}, and the stretching of amide and C=O corresponded to 1,450–1,244 cm^{-1}. The display signal of the ^1H NMR (400 MHz, $CDCl_3$) (Fig. 2-F) spectrum indicated that the secondary amide protons corresponded to ^1H NMR (400 MHz, $CDCl_3$) δ 6.28 (s, 1H); the methylene protons corresponded to 2.42–2.30 (m, 1H) and 0.96 (D, j=6.5 Hz, 3H), and the piperidine protons corresponded to 3.63–3.51 (m, 2h), 2.13–1.99 (m, 3H), 1.98–1.85 (m, 1H) and 1.86–1.72 (m, 2h). The display signal of the ^{13}C NMR (101 MHz, $CDCl_3$) (Fig. 2-G) spectrum also showed that the amide carbon atoms corresponded to δ170.33 and δ166.24; the cyclohexane aliphatic carbon atoms corresponded to δ 58.99 and δ 53.40; the piperidine carbon atom corresponded to δ 45.51, δ 23.30 δ 24.66, δ22.75 and δ 28.10, and the aliphatic carbon atoms corresponded to δ 38.57 and δ 21.22. Based on the MS data, the molecular ion peak was 211.14338, which was consistent with the theoretical value of $[M+H^+]$=211.14410, and the chemical formula was $C_{11}H_{18}N_2O_2$. All of these analyses identified this compound as 3-isopropylhexahydro-4*H*-pyrido[1,2-α] pyrazine-1,4(6*H*)-dione (IPHPPD). Two chiral centers (asterisks) are present in the molecule, so there may be a

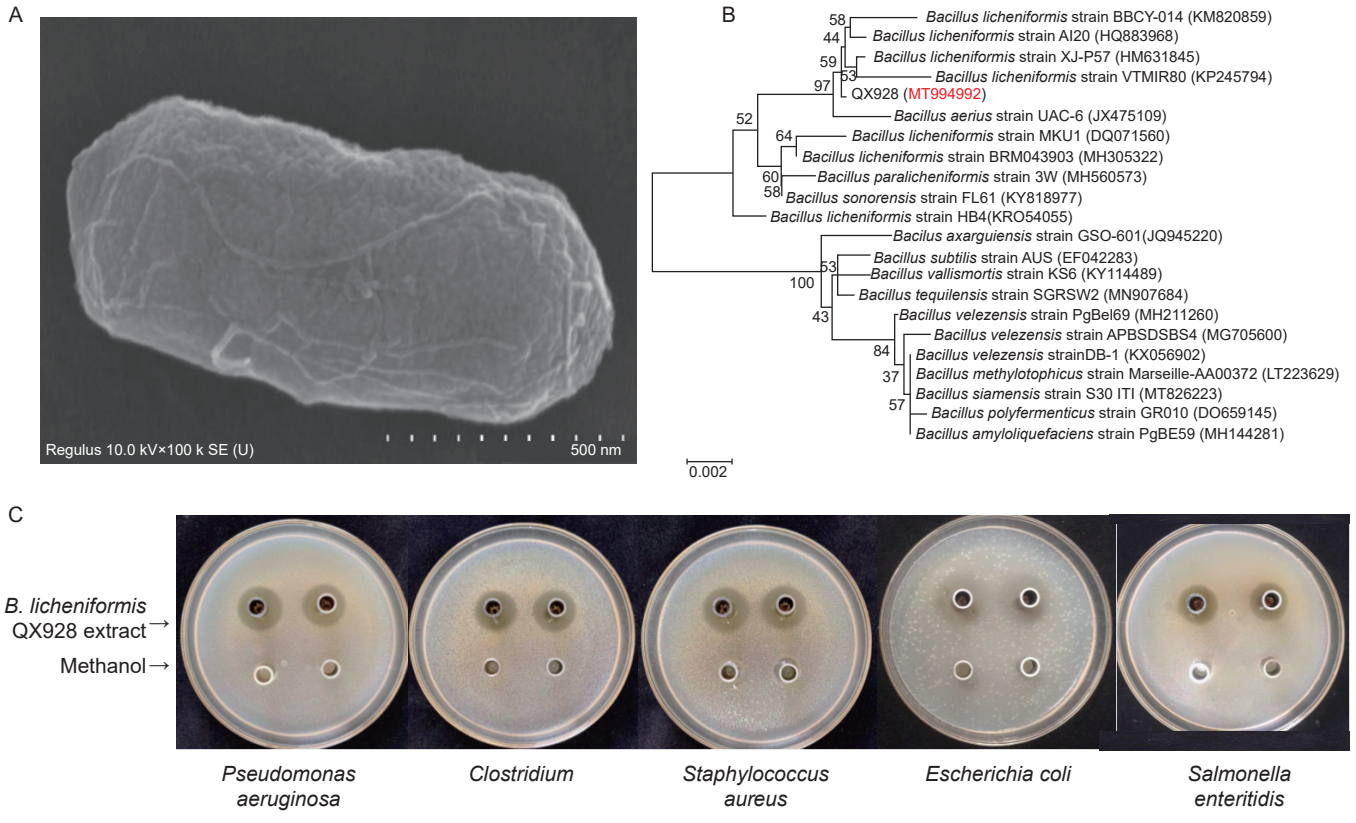


Fig. 1 Identification and antibacterial characteristic analysis of *Bacillus licheniformis* QX928. A, electron microscopy of *B. licheniformis* QX928. B, phylogenetic tree of *B. licheniformis* QX928. C, determination of the broad-spectrum antibacterial activity of the *B. licheniformis* QX928 extract.

small number of corresponding isomers. Moreover, there were some peaks of impurities in the NMR spectrum, and there were many accompanying small peaks in the carbon spectrum. Because the substance was extracted from the fermentation products of a microorganism, it was effectively non-corresponding selective. A query using SciFinder indicated that this compound is the first biosynthetic compound with antibacterial activity and anti-QS activity generated by microbial fermentation and metabolism.

3.3. MIC, MBC and the time of lethality of IPHPPD

It was determined that IPHPPD could inhibit the growth of *P. aeruginosa* with measured MIC and MBC values of (13±0.17) and (22±0.72) mg L^{-1}, respectively. The calculated ratio of MIC to MBC was 0.59, which suggests that IPHPPD is highly bactericidal.

The time at which IPHPPD was lethal to *P. aeruginosa* was prolonged with a reduced concentration of MBC. In detail, 1×MBC of IPHPPD was completely lethal to *P. aeruginosa* within 14 h. When 4×MBC of IPHPPD was used, the solution was completely lethal to *P. aeruginosa* within 1 h, while the number of colony forming units (CFU) was reduced by 7 log units (Fig. 3-A). This fact indicates that IPHPPD rapidly produces a bactericidal effect.

3.4. Thermal stability of IPHPPD

IPHPPD was also treated at different temperatures with *P. aeruginosa* as the test bacterium to verify its thermal stability (Fig. 3-B). IPHPPD was observed to highly stable thermally because the treatment of IPHPPD at 60°C for 24 h or 80°C for 21 h still exhibited strong antibacterial activity to cause an antibacterial circle of 8 mm. Thus, IPHPPD had

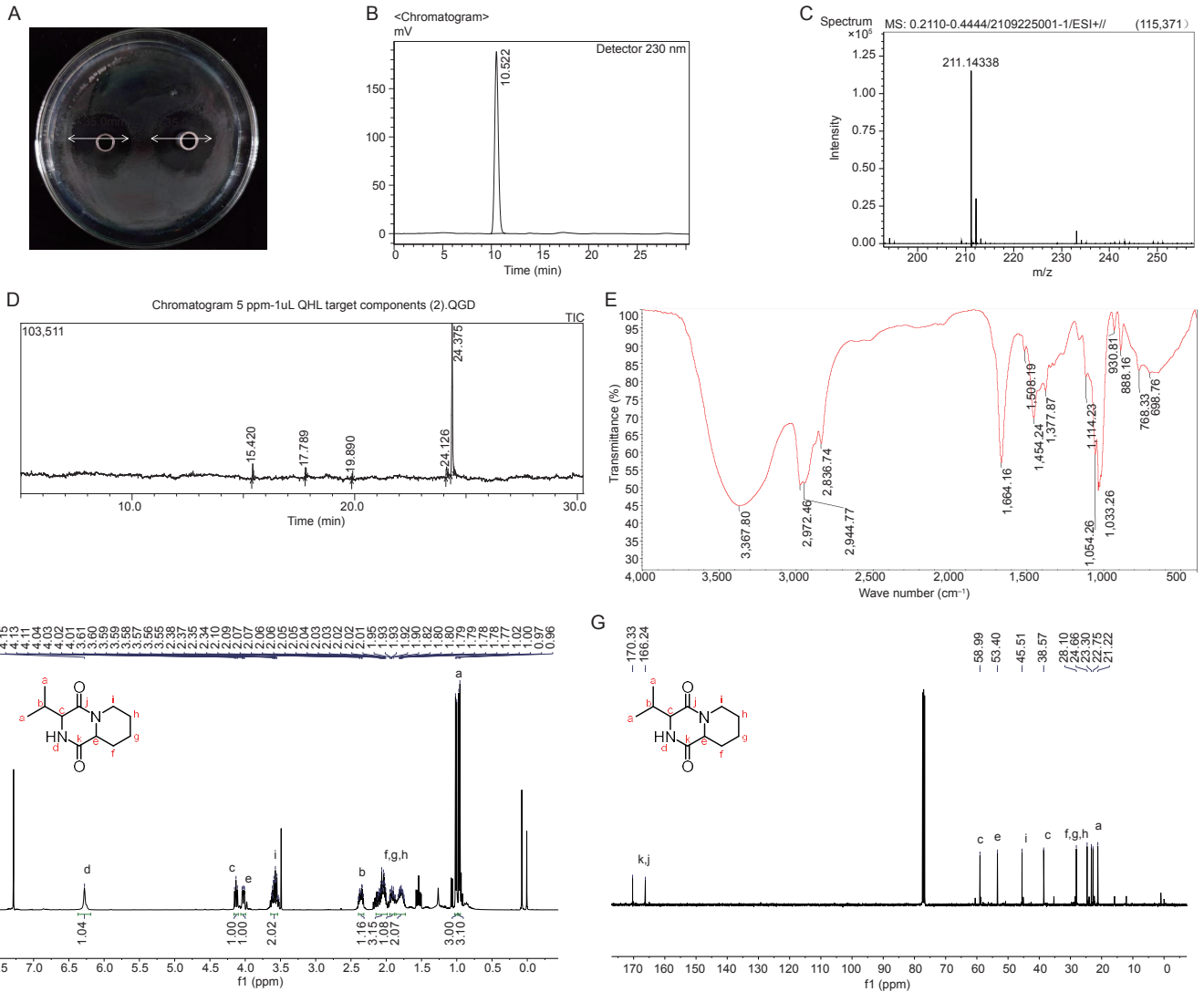


Fig. 2 Characterization and determination of the purified product of *Bacillus licheniformis* QX928. A, antibacterial activity of the purified compounds against *Pseudomonas aeruginosa*. B, HPLC was used to detect the purified compounds. C, HRMS shows the molecular weight of the compound. D, GC-MS was used to detect the purified compounds. E, an FTIR analysis shows the functional groups of the compound. F, ^{13}C NMR shows the carbon spectra of the compound. G, ^{1}H NMR shows the hydrogen spectra of the compound. FTIR, Fourier transform infrared spectroscopy; GC-MS, gas chromatography-mass spectrometry; HPLC, high-pressure liquid chromatography; HRMS, high resolution mass spectrometry; NMR, nuclear magnetic resonance.

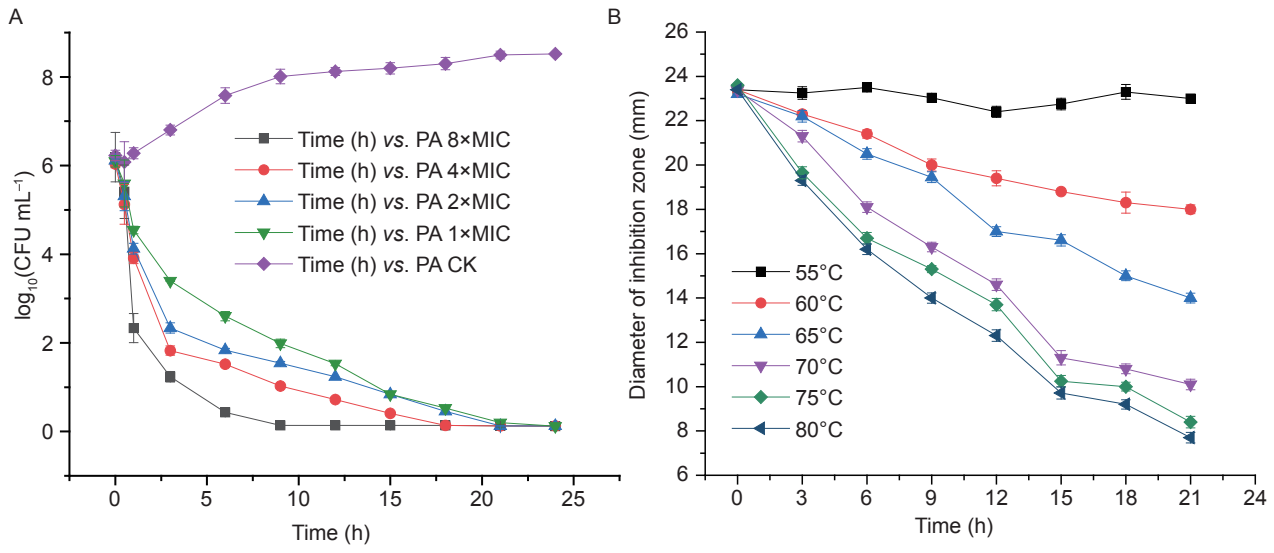


Fig. 3 Determination of lethal time and thermal stability of IPHPPD. A, determination of sterilization time of IPHPPD at different concentrations. B, thermal stability of IPHPPD. IPHPPD, 3-isopropylhexahydro-4*H*-pyrido[1,2-α]pyrazine-1,4(6*H*)-dione. Data are mean±SD.

strong thermal stability with potential application.

3.5. Influence of IPHPPD on signal factors in quorum sensing

Chromobacillus violaceum CV026 is a mutant strain that lacks a signal factor. The strain turned purple in the medium supplemented with homoserine, while it remained colorless in the medium without homoserine. When the signal factor was inhibited, the strain would not turn purple even if supplemented with homoserine endogenous lipids. Cinnamaldehyde was used as the positive control and methanol as the negative control. There were white and purple regions of the plate after 0.2×MIC of IPHPPD was added to the *C. violaceum* CV026 plate that contained acyl homoserine lactone. On the white area of the plate, *C. violaceum* CV026 grew normally without any color change, indicating that IPHPPD had interrupted the response of *C. violaceum* CV026 to the acyl homoserine lactones (Fig. 4-A). Compared with the previous QS inhibitor cinnamaldehyde, IPHPPD had a significant rate of antibacterial activity as shown by the 1×MBC, and no antibacterial circle was detected when methanol was used as a negative control (Fig. 4-B). This fact indicated that the yield of violacein positively correlated with the activity of QS. Thus, different concentrations of IPHPPD could be used to evaluate the level of QSI. The inhibitory rate of IPHPPD on violacein was found to be dose-dependent. A higher concentration of IPHPPD caused a decrease in the content of violacein by 94% when a treatment of 0.7×MIC IPHPPD was utilized (Fig. 4-C), i.e., the induction of population between cells could be interrupted by IPHPPD by inhibiting the bacterial response to the signal factor acyl homoserine lactone.

Owing to the damage to the cell membranes, the cell permeability changed, while the cytoplasm leaked from the cells and penetrated into the liquid, thus, causing increased conductivity of the solution. Compared with the control group (Fig. 4-D), the conductivity of PBS in the experimental group increased with time after IPHPPD was added, and simultaneously, the conductivity of the solution was directly proportional to the dose of IPHPPD. In contrast, the conductivity of control group basically remained unchanged. Thus, treatment with IPHPPD destroyed the cell membrane of *P. aeruginosa* and enhanced the permeability of its cells.

3.6. Effect of IPHPPD on the biofilm structure of *P. aeruginosa*

Pseudomonas aeruginosa was labeled with propidium iodide and fluorescein oxalate and then treated with IPHPPD. The cells were evaluated for the inhibitory and lethal activity of IPHPPD to biofilms by observing the dead and living cells with red and green fluorescence, respectively. The cells in the experimental group were treated with 0.5×MIC IPHPPD for 24 h (Fig. 5-A). The biofilm structure of the experimental group was looser than that of the control group. There were developmental defects, and the cell connections were not tight. In contrast, the complete membrane structure was present in the normally developed control group. Therefore, there was a significant difference between the control and experimental groups, which was that the number of microbial deaths in the experimental group was much higher than that of the control group. Compared with the control group, the normally developed cell membrane structure of *P. aeruginosa* in the experimental group was damaged by 1×MIC of IPHPPD and perforated and torn. This resulted in a large number of cell deaths. However, the cell membrane structure of *P. aeruginosa* in the control group remained normal, and the intercellular connections were tight. The results showed that a lower concentration of IPHPPD inhibited the growth of *P. aeruginosa*, while higher ones were lethal and able to destroy the biofilms.

The effects of IPHPPD on the biofilm topology of *P. aeruginosa* were also observed using both scanning electron microscopy (SEM) and atomic force microscopy (AFM). Compared with the control group, scattered cells

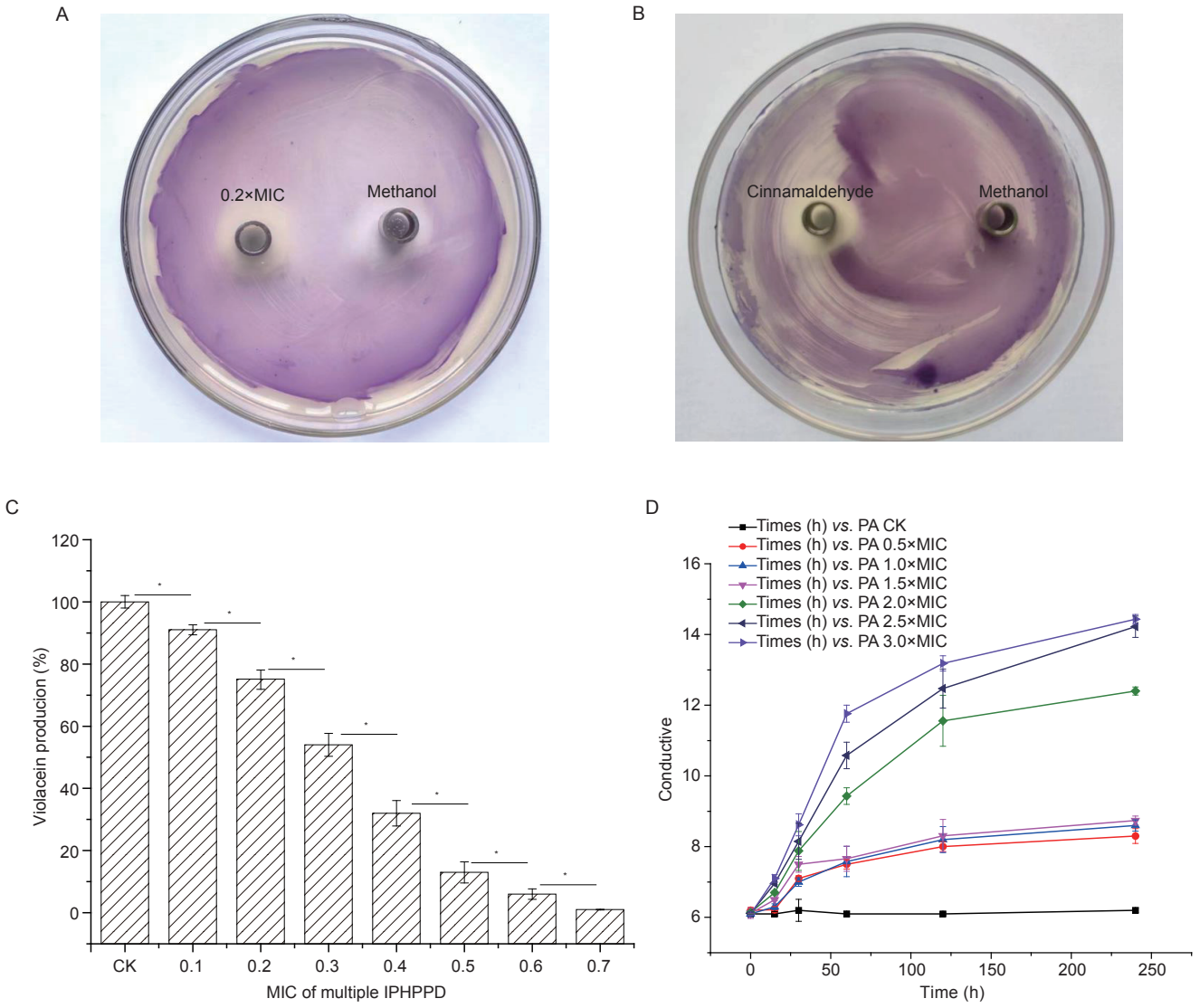


Fig. 4 Anti-quorum sensing activity of IPHPPD. A, biosensor plates that contained the reference strain *Chromobacterium violaceum* CV026. A total of 0.2×MIC produced a bright white opaque zone of inhibition that showed anti-QS activity. B, a positive control of cinnamaldehyde and a negative control of methanol. C, influence of different concentrations of IPHPPD for violacein producion. D, the effect of IPHPPD on cell membrane permeability. IPHPPD, 3-isopropylhexahydro-4*H*-pyrido[1,2-α]pyrazine-1,4(6*H*)-dione. MIC, minimum inhibitory concentration. Data are mean±SD. *, $P<0.05$.

and immature cell membranes could be observed under SEM in the presence of IPHPPD (Fig. 5-C). The scanning diagram of AFM showed that the cell membrane structure of *P. aeruginosa* had changed significantly under the action of IPHPPD (Fig. 5-B). The biofilm surface was uneven; the adhesion on the cover glass surface was poor, and the biofilm had shrunk. This appearance was in clear contrast with the normal biofilm structure with a flat distribution and a compact and uniform structure. The structure of *P. aeruginosa* biofilm was atrophic, and the thickness was significantly reduced when the scanning pattern of AFM was observed following treatment with 0.5×MIC of IPHPPD compared with the control group. This study proved that the biofilm formed could be destroyed, and the formation of biofilm could be prevented using IPHPPD to interfere with the *P. aeruginosa* signal factors.

3.7. Effect of IPHPPD on motility, energy metabolism and expression of the virulence factors of *P. aeruginosa*

The motility of the bacteria and their initial adhesion to the surface are critical factors for the formation of biofilm. Thus, the ability of IPHPPD to inhibit these two aspects of *P. aeruginosa* was investigated. The results showed that the movement and swimming of *P. aeruginosa* were significantly inhibited by IPHPPD (Fig. 6-A).

Analytical results also indicated that the virulence factors of *P. aeruginosa* decreased significantly after the bacteria were treated with IPHPPD (Fig. 6-B). IPHPPD caused a decrease in the contents of pyocyanin and rhamnolipid by 85.3 and 41.8%, respectively. Moreover, IPHPPD reduced the activities of elastase and protease by 65.7 and 34.3%, respectively. All the results showed that IPHPPD could efficiently inhibit the

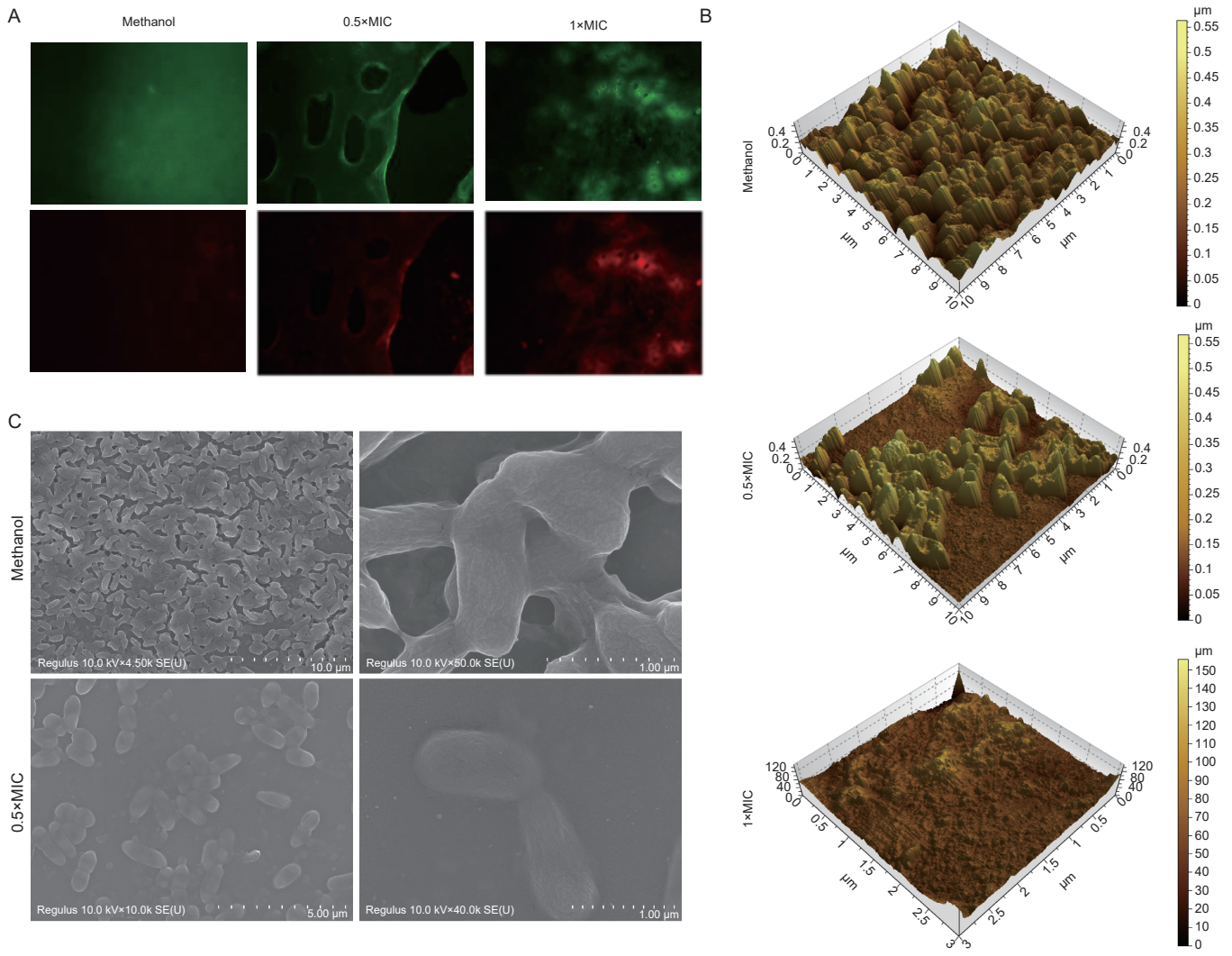


Fig. 5 Effect of IPHPPD on cell viability and the topology of biofilms developed by *Pseudomonas aeruginosa* ATCC27853. A, biofilm structure under fluorescence microscope. B, biofilm structure under atomic force microscopy. C, biofilm structure under electron microscopy. IPHPPD, 3-isopropylhexahydro-4*H*-pyrido[1,2-α]pyrazine-1,4(6*H*)-dione.

intercellular signal channels and formation of biofilm.

Pyruvate is one of the basic products involved in biology, and changes in its contents can be used to reflect the effect of targeted compounds on bacterial metabolism. The effect of IPHPPD on *P. aeruginosa* was measured by assaying the contents of pyruvate, soluble proteins and reducing sugars (Fig. 6-C). All the analyses indicated that IPHPPD decreased the contents of pyruvate, reducing sugars and soluble protein by 16.9, 31.0 and 41.5%, respectively. Thus, it was considered that the biosynthesis of soluble proteins was affected, or the cell membrane was also destroyed by IPHPPD. Therefore, IPHPPD could inhibit the cell metabolism of *P. aeruginosa* in addition to its inhibition of cell growth and biofilm formation.

3.8. Differential gene expression in response to exposure to IPHPPD

The 6,465 gene probe sets contained in the *P. aeruginosa* ATCC27853 genome array chip were used to study the changes in gene expression in response to treatment with IPHPPD. Overall, 402 out of the 6,465 genes (NCBI accession number PRJNA781762) had changed in expression by more than 2-fold (Fig. 7-A; Appendix B). In particular, 156 and 211 genes were downregulated and upregulated by IPHPPD, respectively (Fig. 7-B). Thus, several genes were identified that are the key participants in the QS network of *P. aeruginosa* ATCC27853 and play a key role in the general metabolic pathway of bacteria.

Moreover, 20 key genes of *P. aeruginosa* were selected to verify the effect of IPHPPD on gene expression (Table 1). Ten of them were upregulated or downregulated (Fig. 7-C and D). The differential expression of genes was examined with real-time quantitative reverse transcription PCR (qRT-PCR) after IPHPPD treatment for 3 h. The results showed that the levels of the genes related to the transporters decreased significantly among the downregulated genes affected by IPHPPD, including PA2932, PA2933, PA2934 and PA4356, and the levels of expression of the genes related to nucleic acid metabolism, such as PA4338 and PA0506, decreased significantly in metabolic biosynthesis. These findings indicate that IPHPPD

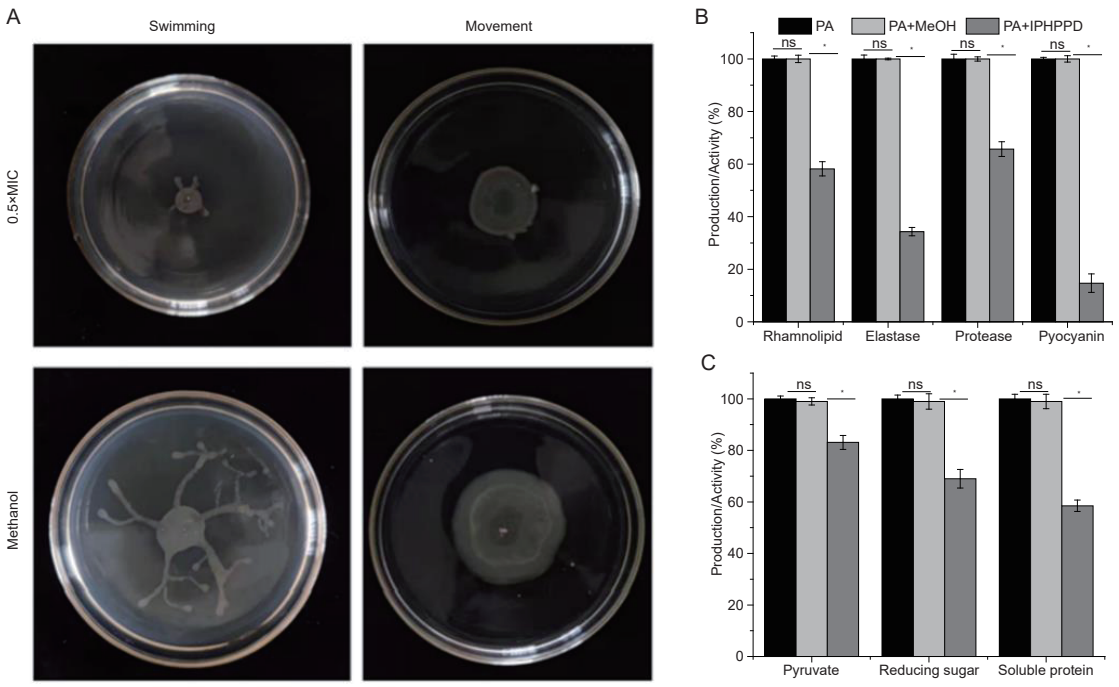


Fig. 6 IPHPPD interferes with the movement virulence factors, and energy metabolism of *Pseudomonas aeruginosa*. A, interference of IPHPPD on the movement of *P. aeruginosa* (MIC=(13±0.17) mg L^{-1}). B, interference of IPHPPD (MIC=(13±0.17) mg L^{-1}) on the virulence factors of *P. aeruginosa*. C, interference of IPHPPD (MIC=(13±0.17) mg L^{-1}) on the energy metabolism of *P. aeruginosa*. IPHPPD, 3-isopropylhexahydro-4*H*-pyrido[1,2-α]pyrazine-1,4(6*H*)-dione; MIC, minimum inhibitory concentration. Data are mean±SD. ns, no significant difference. *, $P<0.05$.

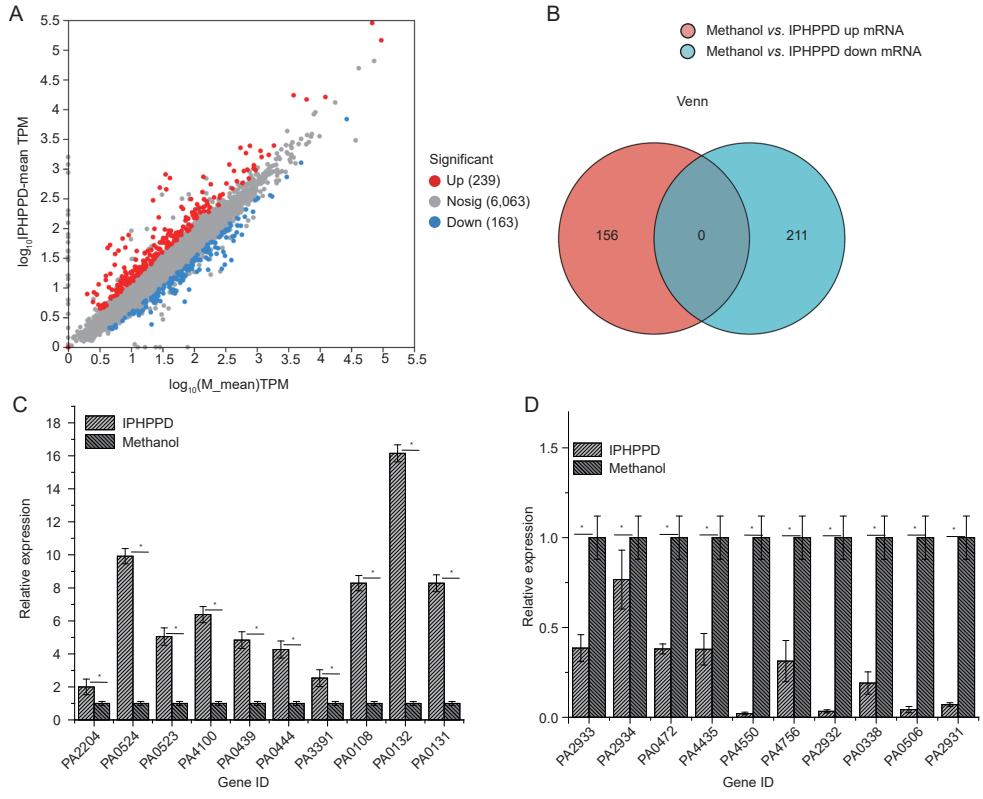


Fig. 7 Analysis of the differential expression of the *Pseudomonas aeruginosa* ATCCC27853 genes after treatment with IPHPPD. A, scattered plot showing the genes that were differentially expressed in *P. aeruginosa* as determined by a microarray. B, Venn diagram of differentially expressed genes. C and D, relative gene expression of *P. aeruginosa*. *Pseudomonas aeruginosa* grown in the presence and absence of IPHPPD. IPHPPD, 3-isopropylhexahydro-4*H*-pyrido[1,2-α]pyrazine-1,4(6*H*)-dione. Data are mean±SD. *, $P<0.05$.

can affect the metabolism of nucleic acids in the cells. There was a decrease in the levels of expression of PA0500 and PA455 related to virulence factors, which was consistent with the previous results. This study verified that the biosynthesis of pili protein and biotin of *P. aeruginosa* could be affected by IPHPPD at the gene level, which then affected the movement and expression of virulence factors. The upregulated genes were primarily concentrated in the expression of the proteins involved in the degradation of amino acids, such as PA0440, PA0180 and PA0523, which indicated that the decomposition of intracellular proteins and material consumption would be accelerated in the presence of IPHPPD. Cellular respiration would be accelerated by the overexpression of PA0524 and PA4100, which resulted in the production of a large amount of reactive oxygen species (ROS) in the cells. All these would accelerate the damage to cell membrane and the consumption of intracellular substances. Under the combined action of these processes, the production of QS system and signal factors were reduced by IPHPPD owing to its inhibition of the activity of transporters, intracellular nucleic acid metabolism and the expression of virulence factors. The failure of the las/rhl cascade reaction of *P. aeruginosa* was affected by the lack of signal factors. This led to a reduction in the number of virulence factors, such as pyocyanin, elastin and rhamnolipid, and the decreased bacterial activity was caused by the inhibition of flagellin. Furthermore, the consumption of compounds and accumulation of ROS were accelerated with the overexpression of amino acid degradation and enzymes related to respiration in *P. aeruginosa* cells. Consequently, the decrease in transportation capacity and the acceleration of consumption led to the rapid death of *P. aeruginosa* (Graphical abstract).

Table 1 Genes related to upregulated and downregulated metabolism of *Pseudomonas aeruginosa* under IPHPPD[1)]

Gene ID	Annotation	Up and down	$\log_2$ratio
PA2204	ABC transfer truck	Up	2
PA0524	Nitric oxide reductase subunit B	Up	9.917432
PA0523	Nitric oxide reductase subunit C	Up	5.051225
PA4100	Dehydrogenase	Up	6.381667
PA0439	Dihydropyrimidine dehydrogenase	Up	4.836399
PA0444	Allantoic acid amide hydrolase	Up	4.269104
PA3391	Regulatory protein nosr	Up	2.531513
PA0108	Cytochrome c oxidase subunit	Up	8.284033
PA0132	β-Alanine pyruvate aminotransferase	Up	16.15229
PA0131	β-Alanine degrading protein baub	Up	8.284033
PA2933	Major facilitator super family transporter	Down	0.385197
PA2932	Transcriptional regulator	Down	0.033423
PA0472	RNA polymerase σ factor	Down	0.380684
PA4435	Acyl CoA dehydrogenase	Down	0.378929
PA4550	Fimbriae type 4 protein	Down	0.021295
PA4756	Aminomethyl phosphate synthase large subunit	Down	0.312805
PA2934	CFTR inhibitor	Down	0.766646
PA4338	Acyltransferase	Down	0.190782
PA2931	CIRF protein	Down	0.042707
PA0506	Acyl CoA dehydrogenase	Down	0.070835

[1)] IPHPPD, 3-isopropylhexahydro-4*H*-pyrido[1,2-α]pyrazine-1,4(6*H*)-dione.

4. Discussion

4.1. Utilization of extreme microorganisms and novel drug development

Owing to their ability to live in highly harsh environments, extreme microorganisms possess special adaptation mechanisms, which could help them adapt to diverse and extreme environments, and obtain the characteristic changes in cell structure and function. To date, although terrestrial and aquatic organisms have provided a variety of secondary metabolites with antibacterial activity for humans, there has been relatively little research on hot spring microorganisms compared with those in marine and soil environments. Therefore, it should be promising to study the bioactive compounds of the microorganisms in hot springs. As the primary niche on earth, a variety of heat-resistant microorganisms in hot springs and geysers have been found by researchers. Thus, this should increase the possibility of obtaining new bioactive compounds from microorganisms in hot springs that can tolerate extreme environmental conditions. Organisms in extreme environments could have a unique ability to over-accumulate some metabolites or secrete particular compounds. Heat-induced microbial compounds have a variety of biological activities and properties, and some compounds can help to alleviate the pain that afflicts humans. Natural products are often more novel in chemical structure than synthetic drugs, and combining them with molecular informatics and the emerging artificial intelligence technology in recent years would be a boon to the pharmaceutical industries (Bender and Glen 2004). In the field of drug development, including animal husbandry and veterinary medicine, extracts from microorganisms have always been an enormous source of new and effective therapies (Gillespie *et al.* 2002; Courtois *et al.* 2003). New structural compounds with unique bioactivity could be used not only as antibacterial agents but also as veterinary medicine to treat various bacterial and viral diseases and parasitic diseases.

4.2. Biological characteristics of IPHPPD from *B. licheniformis* QX928

In this study, the antibacterial component of *B. licheniformis* QX928 was extracted, purified, analyzed with HRMS, NMR, GC-MS, FT-IR and HPLC, and identified as IPHPPD with a molecular weight of 211.14410. A query of SciFinder determined that this compound is the latest biosynthetic compound with antibacterial activity and anti-QS activity synthesized by microbial metabolism. The active molecule in IPHPPD is pyrrole, and its substitutes are pyrrolo[1,2-α] pyrazine-1,4-dione and hexahydrogen, and its peaks and functional groups in FTIR are similar to those that have been reported (Madigan and Marrs 1997).

Pyrrole is well known for its various therapeutic uses and has been used as an antibiotic and as antifungal, antitumor, anti-inflammatory and cholesterol-lowering drugs. Pyrrole can also inhibit the activities of HIV-1 virus, DNA polymerase and protein kinase (Sen *et al.* 2010; Demirjian *et al.* 2011; Mohammad *et al.* 2017). Pyrrole can prevent epileptic seizures, and its derivative pyrazine can be used as an effective anticonvulsant (Piliego *et al.* 2010). Pyrrolo[1,2-α]

pyrazine-1,4-dionehexahydro, which is similar to the molecular structure of IPHPPD, was extracted from the newly discovered strain *Streptomyces mangrovisoli* MUSC 149T. The compound has significant antioxidant and free radical scavenging activities. Antioxidants may play an important role in the prevention and treatment of chronic diseases in humans and animals, which is very important to promote the sustainable and healthy development of livestock and the aquatic animals farmed in aquaculture (Ser *et al.* 2015). *Exiguobacterium indicum* Sj16 that was isolated from the roots of aconite (*Aconitum* spp.) can produce the compound 3-benzyl-hexahydro-pyrrolo[1,2-α]pyrazine-1,4-dione, which could inhibit QS but had no bactericidal activity (Singh *et al.* 2019). The compound has a molecular formula that is similar to that of IPHPPD. However, its chemical structure is different from that of IPHPPD, which suggests that the 6-membered ring in IPHPPD plays an important role in its bactericidal activity. The metabolite pyrrolo[1,2-α] pyrazine-1,4-dione, hexahydro of *B. tequilensis* MSI45 is similar in structure to IPHPPD and has a bactericidal effect on *Staphylococcus aureus* with a minimum inhibitory concentration of (15±0.172) mg L^{-1}. In the timed lethal assay, *S. aureus* was completely inhibited after 24 h of incubation with the 1×MIC compound, and the bacteria were reduced by seven orders of magnitude within 2 h under the action of the 4× and 8×MIC compounds. Thus, these compounds are highly bactericidal. However, this compound did not display any anti-QS activity at either low or high concentrations (Kiran *et al.* 2018). The pyrazine-1,4-dione extracted from *B. seminalis* JRBHU6, which was isolated from a drought-stricken field, exhibited strong antimicrobial activities (Gohar *et al.* 2010; Geraldine *et al.* 2013). *Bacillus seminalis* JRBHU6 was also highly effective at producing chitinase and cellulase, which helped its ability to control pathogenic organisms. An analysis of the binding energy indicated that these compounds extracted from *B. seminalis* JRBHU6 were highly adaptable when interacting with many microbial and human proteins and enzymes and might play a key role in the inhibition of microbial growth (Prasad *et al.* 2021). In this study, the results indicated that the MIC and MBC of IPHPPD on *P. aeruginosa* were (13±0.17) and (22±0.72) mg L^{-1}, respectively, which were lower than those described above. Simultaneously, IPHPPD possessed stronger anti-QS activity.

4.3. The inhibition and destructive mechanism of IPHPPD on *P. aeruginosa* and its prospects for application

The lethal effects on *P. aeruginosa* were shown by a high concentration of the IPHPPD from *B. licheniformis* QX928 in a dose-dependent manner. The response of *C. violaceum* CV026 to the signal factor acetyl homoserine lactone could be inhibited by the extract of *B. licheniformis* QX928. Thus, it was proven that IPHPPD can interfere with the signal factor recognition between bacteria, and a high concentration of IPHPPD has a strong antibacterial effect on *P. aeruginosa*. Further antibacterial tests on *P. aeruginosa* ATCC27853 with 0.5×MIC IPHPPD indicated that this novel compound interferes with the signal transduction and release of virulence factors, thus, hindering the QS regulatory cascade and inhibiting the formation of biofilm. As described above, the SEM and AFM image analyses showed that 0.5×MIC IPHPPD could inhibit the formation of biofilm by *P. aeruginosa*.

Challenges encountered in relapse of illness caused by resistance of microorganisms to antimicrobial agents (drugs) are due to factors of severe stress initiated by random use of antibiotics and insufficient beneficial approaches (Odularu *et al.* 2022). These challenges have resulted to MDR and, subsequently, biofilm formation. Since the QS can regulate the activity of virulence factors and the formation of biofilm, destroying the biofilm structure would be a promising strategy to inhibit the formation of drug-resistant biofilms of *P. aeruginosa*. To form biofilm, the bacteria need to attach to a surface; thus, swarming motility, swimming motility and surge movement play an important role in the formation of biofilm by *P. aeruginosa* (O'May and Tufenkji 2011). Once attached to a surface, the bacteria would spread through swarms and swimming activities, which would eventually lead them to form the biofilms on biological or abiotic surfaces and induce persistent infection. In the presence of IPHPPD, the colony formation and swimming activity of *P. aeruginosa* were inhibited. The ability of IPHPPD to inhibit the initial adsorption of *P. aeruginosa* may be by preventing the bacteria from moving to the surface. This process may develop a new way to prevent bacterial transmission to minimize the ability of microorganisms to form biofilms (Singh *et al.* 2017).

In this study, the results showed that IPHPPD extracted from *B. licheniformis* QX928 reduced the virulence of *P. aeruginosa* by regulating the transcriptional regulatory ion system at low concentrations and exhibited bactericidal effects by interfering with energy and material metabolism at high concentrations. In conclusion, the IPHPPD described in this study is a bioactive molecule with a variety of antibacterial and bactericidal functions.

5. Conclusion

QS regulates the formation of virulence factors, and biofilms are important mechanisms that bacteria can use to develop drug resistance. Anti-QS compounds provide an effective strategy to solve the critical issue of pathogen infection. *Bacillus licheniformis* QX928 is a thermophilic microorganism isolated from hot springs. It is active against QS and bacteria. A low concentration of the active IPHPPD extracted from *B. licheniformis* QX928 can inhibit the formation of biofilms by *P. aeruginosa* ATCC27853. It manifests these effects by reducing their swimming and group movement and regulating the production of virulence factors, such as anthocyanins, rhamnolipids, elastase and protease, to inhibit the growth of strains. Moreover, high concentrations of IPHPPD can interfere with the ability of bacteria to produce energy and affect their material metabolism. This study showed that the movement of microorganisms, production of virulence factors and formation of biofilms could be strongly inhibited by low concentrations of IPHPPD. A key factor is that this data showed that low concentrations of IPHPPD could not only interfere and destroy the QS system of *P. aeruginosa* but also interfere with the response of homoserine lactone between cells and thus, limit

their intercellular communication (QSI). Therefore, in our efforts to enrich our antimicrobial weapons and equipment, the compounds identified would have substantial potential for drug development in the prevention and treatment of animal diseases, such as the development of specific inhibitors that target specific bacteria or fungi or the development of next-generation antibiotics with lower toxicity and better pharmacokinetics. Further research on this topic is merited.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical statements

This article does not contain any studies with human participants or animals performed by any of the authors.

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