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Inhibition of the *pqsABCDE* and *pqsH* in the *pqs* quorum sensing system and related virulence factors of the *Pseudomonas aeruginosa* PAO1 strain by farnesol

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ABSTRACT

Farnesol, a 15-carbon, naturally occurring sesquiterpene alcohol, exists widely in plants and *Candida albicans*. It inhibits the production of the Pseudomonas Quinolone Signal (PQS) and pyocyanin of the opportunistic pathogen *Pseudomonas aeruginosa*, but the underlying mechanisms remained unknown. In this study, the actions of farnesol on the growth, production of virulence factors (including PQS signals, elastase, pyocyanin, biofilm, and swarming motility), and the transcription of some essential key genes of three Quorum Sensing (QS) systems and other related virulence genes in the *P. aeruginosa* PAO1 strain were investigated. Moreover, the qRT-PCR and high throughput transcriptome/proteome sequencing techniques were used to differentiate the transcriptomic/proteomic expressions of the PAO1 strain following farnesol exposure. The experimental results showed that farnesol did not inhibit PAO1 growth or affect its elastase production. However, farnesol did suppress the transcription and protein expression of the *pqsABCDE* and *pqsH* genes in the strain's *pqs* QS system and then inhibit the PQS production, while leaving the transcription and protein expression of *pqsR* in the *pqs* system, of *lasI* and *lasR* in the *las* QS system, and of *rhlI* and *rhlR* in the *rhl* QS system unaffected. Farnesol also inhibited the expression of some related virulence genes and the production of the related virulence factors. For example, it inhibited the protein expression of key genes in the phenazine biosynthesis pathway, including key genes related to pyocyanin synthesis (*phzM*, phzS), inhibiting the strain's capacity for biofilm formation. In addition, farnesol enhanced the transcription and protein expression of key genes in the geraniol degradation pathway. Farnesol also stimulated the PAO1 strain with regard to swarming motility. As a result, we concluded that farnesol can suppress the virulence by inactivating the *pqs* QS system in pathogen *P. aeruginosa*, which would be significant to develop a kind of effective drug control the virulence and pathogenesis of this bacteria.

1. Introduction

Antimicrobial resistance is a steadily increasing global problem, with drug-resistant pathogens already killing at least 25,000 people annually in the European Union alone (Belkum et al., 2019). The issue of antimicrobial resistance has attracted the attention of the United Nations General Assembly and World Health Organization (WHO), and many countries have already recognized that tackling antimicrobial resistance to prevent and control infection in humans and animals is a major global challenge (Hengel and Marin, 2019; Roope et al., 2019). The abuse of antibiotics promotes the spread of super resistant and multi resistant pathogen strains. Regular use of existing antibiotics will help avoid disasters, but the root cause of the problem is still the historical neglect of new drug development (Gilbert, 2018; Chen and Huang, 2020). Therefore, human beings need to speed up the research and development of new drugs, find new drug targets, and discover new drug action mechanisms different from those of the past.

Recently, the bacterial Quorum Sensing (QS) system has caused widespread attention, and has become a promising new target for human control of pathogenic bacteria (Defoirdt, 2018). QS is a form of cellular communication between bacteria, which regulates bacterial pathogenicity and some virulence factors such as LasB elastase, LasA protease, exotoxin A, pyocyanin, hydrogen cyanide, rhamolipids, cytotoxic lectins, and over bacterial motility in *Pseudomonas aeruginosa* (Winson et al., 1995; Latifi et al., 1996; Stintzi et al., 1998; Glessner et al., 1999; Winzer and Williams, 2001; Diggle et al., 2002; Kiymaci

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et al., 2018; Pattnaik et al., 2018; Ahator and Zhang, 2019). *P. aeruginosa* is a bacterium that uses QS to regulate its pathogenic processes (Whiteley et al., 1999; Galloway et al., 2012; Asif et al., 2019). *P. aeruginosa* is a ubiquitous environmental opportunistic human pathogen that can cause intrinsic resistance to antibiotics and disinfectants (Stover et al., 2000; Lekbach et al., 2018). As is the case with many other pathogenic bacteria, the virulence and pathogenicity of *P. aeruginosa* is closely related to its QS systems, including its biofilm formation, bacterial resistance, and other virulence factors (Pustelny et al., 2009; Lee et al., 2018). These factors are very important for the formation of infection and drug resistance of *P. aeruginosa in vivo*. There are three recognized QS systems in *P. aeruginosa*: *las*, *rhl*, and *pqs*, which are hierarchically arranged (Lee et al., 2013; Lee and Zhang, 2015; Defoirdt, 2018; Cornelis, 2019). The composition of the *las* system is similar to that of the *rhl* system. The *las* system consists of genes *lasR* and *lasI*, while the *rhl* system consists of genes *rhlR* and *rhlI* (Passador et al., 1993; Pearson et al., 1995; Schuster and Greenberg, 2007). LasI and RhlI are synthetases that synthesize the Homoserine Lactone (HSL) signal molecule, synthesizing 3-Oxododecanoyl Homoserine Lactone ($3OC_{12}$-HSL) and butyl-Homoserine Lactone (C_4-HSL), respectively, while LasR and RhlR are corresponding signal molecule receptors (Latifi et al., 1996; Finch et al., 1998; Rampioni et al., 2007). The *pqs* system uses 2-heptyl-3-hydroxy-4-Quinolone (PQS) as a signaling molecule, in which the *pqs* operon *pqsABCDE*, together with *pqsH*, codes for PQS production. PQS then binds to the transcriptional regulator PqsR (also called MvfR) to activate the expression of the PQS synthesis genes (Kostylev et al., 2019).

Farnesol, a 15-carbon, naturally occurring sesquiterpene alcohol, exists widely in essential oils such as ambrette seeds and citronella (Khan and Sultana, 2011; Ku and Lin, 2016), herbs such as lemon grass and chamomile (Horn et al., 2005; Khan and Sultana, 2011; Ku and Lin, 2016), fruits such as peaches and strawberries, and vegetables such as tomatoes and corn (Tatman and Mo, 2002; Duncan and Archer, 2008). Farnesol also exists in the fungus *Candida albicans*, which produces several signaling molecules such as farnesol, tyrosol, tryptophol and phenylethyl alcohol (Han et al., 2011; Sharma and Jangid, 2016). Farnesol is the best characterized of these molecules, and has been found to suppress filamentous formation in *C. albicans* (Langford et al., 2010; Han et al., 2012; Gupta et al., 2018). Farnesol has also been reported to inhibit the production of PQS signaling molecules and pyocyanin in *P. aeruginosa* (Cugini et al., 2007). However, it is not known whether farnesol can inhibit all the three hierarchal QS systems or inhibit only one of them. Moreover, the underlying mechanism of farnesol's inhibition of the three hierarchal QS systems in the PAO1 strain of *P. aeruginosa* is still unknown.

In order to investigate the inhibitory efficacy and mechanisms of farnesol on *P. aeruginosa* QS systems, and reveal a potential mechanism, our research examined the effects of farnesol on growth, virulence factor formation, and the transcription level of key genes in the QS systems and related virulence genes of *P. aeruginosa* PAO1, and analyzed gene expression differences between farnesol-treated and untreated populations, based on their transcriptomes and proteomes. We hope that this study will serve the important role of deepening our understanding of the bacterial QS regulation process.

2. Materials and methods

2.1. Reagents, bacterial strain, media, and cultivation

Farnesol was bought from Adamas Reagent Co., Ltd. (Shanghai, China), a mixture of isomers with a purity of 95% and a relative density of 0.889. *P. aeruginosa* PAO1, Luria-Bertani (LB) medium, experimental techniques and methods were the same or with slight modifications as was used in the previous report (Li et al., 2018, 2019).

2.2. Real-time q-PCR on the transcription of key QS and virulence genes of the PAO1 strain

LB media were inoculated with *P. aeruginosa* PAO1 cells during the logarithmic phase. The total volume of cultures was 50 mL, with the cell density of 1–2 × 10^8 Colony Forming Unit (CFU)/mL, the concentration of farnesol treatment group of 0 (control) and 0.56 mg/mL (2500 μmol/L). The experiments were performed in triplicate. The control and farnesol treatment groups were both incubated for 5 h at 37 °C and 180 rpm. Bacteria were then collected from each of the six groups and were snap-frozen at −80 °C.

Total RNA was extracted from bacteria using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA). Primers of 15 genes were designed using Primer Premier 5.0 software (Premier Biosoft Int.; USA) according to the GenBank sequences. Primer sequences were synthesized at BGI Genomics (Shenzhen, China) as shown in Table S1 of Supplement 1, and all sample and primer combinations were assessed in triplicate.

2.3. Farnesol on transcriptome of the PAO1 strain, based on high-throughput RNA sequencing

The bacteria culture and farnesol treatment experiments are the same as described above in 2.2.

2.4. RNA sample preparation and detection

RNA sample preparation was performed as above. Agilent 2100 Bioanalyzer (Agilent Technologies, United States) allows to evaluate the quality and integrity of RNAs. Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) allows to quantify the RNAs concentration.

2.3.2. Library construction and sequencing

RNA sequencing was performed at BGI Genomics (Shenzhen, China). The detailed experimental procedures were described as a previous work (Li et al., 2019).

2.4. Proteome of the PAO1 strain by iTRAQ high-throughput sequencing analysis

The bacteria culture and farnesol treatment experiments are the same as described above in 2.2.

Sample preparation and Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) labeling, high-pH reversed-phase chromatography, RPLC-MS analysis, and data analysis were all performed as previous (Li et al., 2019). Proteomic analysis was performed at Guangzhou Fitgene Biotechnology Co., Ltd. (Guangzhou, China).

2.5. Farnesol on the growth curves of the PAO1 strain

The bacteria culture and farnesol treatment experiments are the same as described above in 2.2 with slight modification. The concentration gradient of farnesol were 0 (for the control), 0.14, 0.28, 0.56, or 1.11 mg/mL (0, 625, 1250, 2500, or 5000 μmol/L). The cell inoculation concentration of the PAO1 strain was 1–2 × 10^6 CFU/mL.

2.6. Farnesol on the virulence factors of the PAO1 strain

2.6.1. Farnesol on the PQS production of the PAO1 strain

The bacteria culture and farnesol treatment experiments are the same as above in 2.2. The PQS production was determined as follow according to a previous report (Gallagher et al., 2002; Cugini et al., 2007; Xu et al., 2016). The cultures both in control and farnesol group were incubated for 72 h. The PQS yields were determined at 24, 48, and 72 h 10 mL of cultures were sampled and 30 mL of acidified ethyl

acetate was added. The samples were then vortexed for 2 min and centrifuged for 3 min at 12,000 rpm 25 mL of ethyl acetate layer was transferred to a flask for rotary evaporation with a 45 °C of water bath temperature and a −10 °C of condensation temperature. Extracts were resuspended in 1 mL of 1:1 acidified ethyl acetate: acetonitrile by vortexing for 10 min. The solutions were then transferred to a chromatographic flask after filtration. The concentration of PQS molecules were determined using a Shimadzu HPLC-20 A system, with a C18 column (InertSustain 5 μm, 4.6 × 250 mm). PQS was eluted at a flow rate of 1 mL/min using a mobile phase consisting of 90% methanol and 10% water. The absorption wavelength was detected at 343 nm. The PQS standard sample (2-heptyl-3-hydroxy-4-quinolone, purity ≥ 96%, Sigma-Aldrich, USA) was diluted to 10, 20, 50, 100 μg/mL with 1:1 acidified ethyl acetate: acetonitrile. The regression curves were then drawn based on the data. The PQS concentrations of the samples were calculated by comparing with the peak area of the standard sample.

2.6.2. Farnesol on elastase activity of the PAO1 strain

An elastase activity assay was performed as above. The test concentrations of farnesol were 0, 0.14, 0.56, or 1.11 mg/mL (0, 625, 2500, or 5000 μmol/L). The cell inoculation concentration of the PAO1 strain was 1–2 × 10^6 CFU/mL. Sterilized deionized water was used in negative control. The elastolytic activity of LasB elastase was determined according to previously described methods (Karatuna and Yagci, 2010).

2.6.3. Farnesol on the pyocyanin yield of the PAO1 strain

This experiment was performed as above. The test concentrations of farnesol and the cell inoculation quantity were the same as above in 2.6.2. The cultures were incubated for 7 days. The cultures were then sampled, and their pyocyanin production determined, daily. Sterilized deionized water was used in negative control. The relative production of pyocyanin was based on absorbance at OD_{520}.

2.6.4. Farnesol on biofilm yield of the PAO1 strain

This experiment was carried out as above. The test concentrations of farnesol and the cell inoculation quantity were the same as above in 2.6.2. The relative biofilm productions of the groups were determined based on the OD_{590} of the ethanol extracts, and the cell growths were determined based on the OD_{600} of the cultures, using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, USA).

2.6.5. Farnesol on swarming motility of the PAO1 strain

The swarming motility of the PAO1 strain following farnesol exposure was accessed according a method previously described (Rashid and Kornberg, 2000). The concentrations of farnesol used were the same as above in 2.6.2. The plates were incubated at 30 °C for 1 day.

2.7. Accession number

The transcriptomic datasets are available at the NCBI Gene Expression Omnibus (GEO) database under accession number GSE138731. The proteomic datasets are available at the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD015803.

3. Results

3.1. Transcriptional level of key QS and related virulence genes of the PAO1 strain

Differences in the relative expressions of key QS genes and their regulated virulence genes in the PAO1 strain populations exposed to various farnesol concentrations are shown in Fig. 1. The results showed that the six key genes *pqsABCDE*, and *pqsH* of the *pqs* QS system, were down-regulated (the gene expression differences were more than two fold) upon farnesol exposure, while other key genes of QS systems

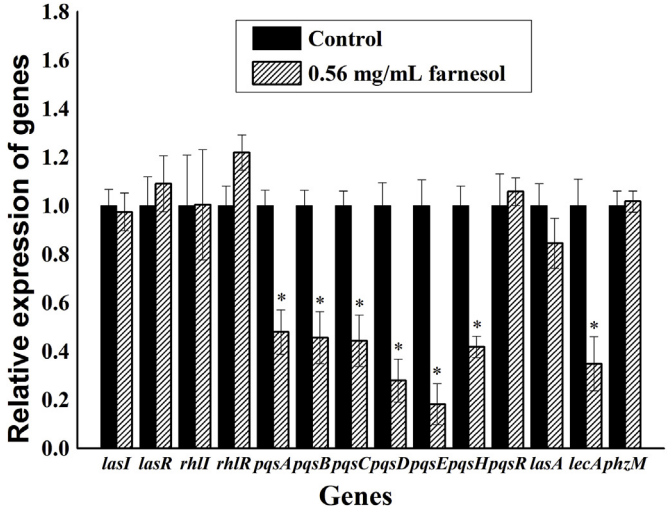

Fig. 1. Effects of farnesol on transcritption levels of key Quorum Sensing (QS) genes and virulence genes regulated by the QS systems. An asterisk denotes gene expression differences that were found to be more than two-fold and less than 10-fold.

remained unchanged (the gene expression differences were less than two fold), including *pqsR* of the *pqs* system; *lasI/lasR* of the *las* system; *rhlI/rhlR* of the *rhl* system. In addition, the virulence genes *lasA*, *phzM*, and *pslA* all remained unchanged. Therefore, we concluded that farnesol can inhibit the transcription of *pqsABCDE* and *pqsH* of the *pqs* QS system of the PAO1 strain. These experimental results are consistent with a previous report that farnesol inhibited the transcription of *pqsA* but not the transcription of *pqsR* of the *P. aeruginosa* PA14 strain (Cugini et al., 2007).

3.2. Transcriptome of the PAO1 strain

Our RNA-seq results showed that less than 100 genes of the PAO1 strain were different in transcriptional level after farnesol exposure. About 1.7% of all detected genes (96 of 5554 genes) were detected more than two-fold differentially expressed after farnesol exposure. Our volcano plots (Fig. 2A) showed that 45 genes were downregulated and 51 genes were upregulated more than two-fold in the differentially expressed genes.

The Gene Ontology (GO) categories that were significantly enriched ($q < 0.05$) are shown in Fig. S1A of Supplement 2. The GO category mainly consists of three parts: namely, biological category, cellular component, and molecular function. Eleven terms, six terms and seven terms were enriched in the biological process category, the cellular component category, and the molecular function category, respectively (Fig. S1A in Supplement 2).

The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis result are shown in Fig. 2B, which shows that the differentially expressed genes with fold changes of > 2 were enriched in 17 KEGG pathways. These pathways rank from high to low according to the significance of their enriched differentially expressed genes: Geraniol degradation, Quorum sensing, Valine, leucine and isoleucine degradation, Fatty acid degradation, Metabolic pathways, Fatty acid metabolism, Butanoate metabolism, Lysine degradation, Arginine and proline metabolism, beta-Alanine metabolism, Propanoate metabolism, Carbon metabolism, Pentose phosphate pathway, Biofilm formation – *Pseudomonas aeruginosa*, Synthesis and degradation of ketone bodies, Alanine, aspartate and glutamate metabolism, and Biosynthesis of antibiotics.

3.3. Proteome of the PAO1 strain

Our proteome sequencing results showed that about 1.7% of all

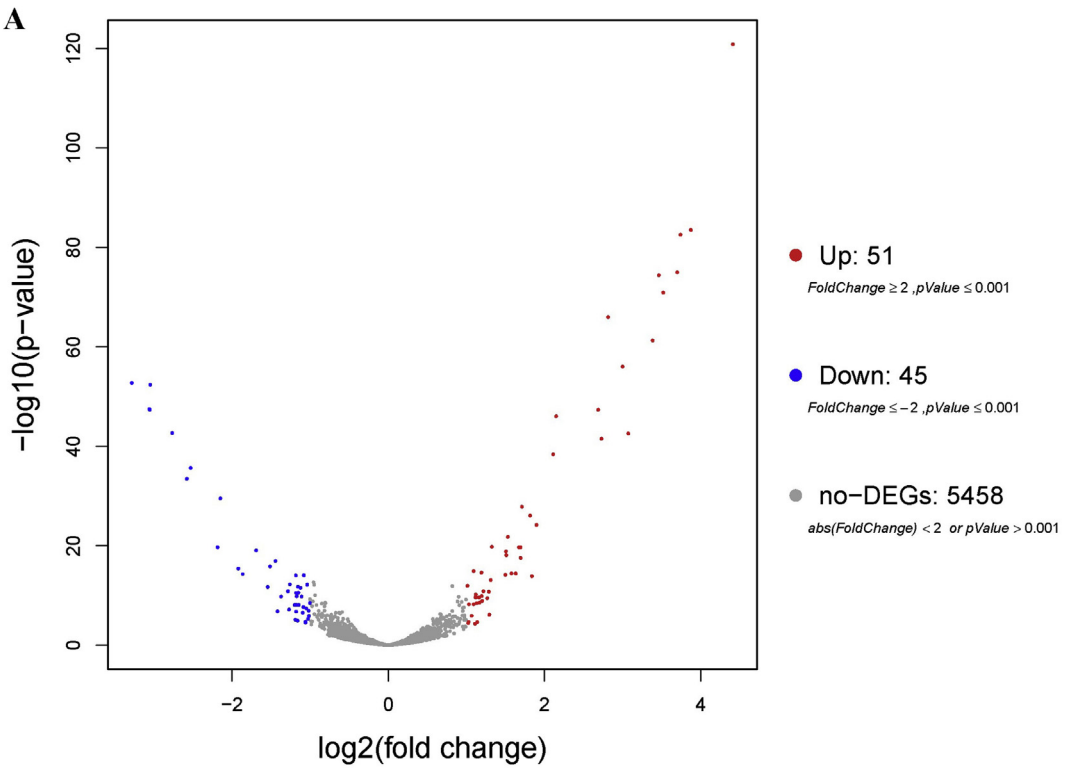

Fig. 2. Diagrams of transcriptomic analysis of untreated and farnesol-treated *P. aeruginosa* PAO1 populations. (A) Volcano plots of the differentially expressed genes based on RNA-seq analysis. Each gene is represented by a dot in the graph; The x-axis and y-axis represent the $\log_2$ value of the fold change and the t-statistic as $-\log_{10}$ of the p-value, respectively; The genes represented in red (upregulated) and blue (downregulated) are differentially expressed genes with > 2-fold change and a p-value of < 0.05. (B) Significantly enriched KEGG pathway (0.00 < q value < 1.00) of differentially expressed genes based on RNA sequencing of the farnesol-treated group compared to the control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

detected proteins (47 of 2846 proteins) were differentially abundance > 2-fold after farnesol exposure. The volcano plots (Fig. 3A) show that 26 proteins were downregulated (red dots) and 21 proteins were upregulated (blue dots) > 2-fold after farnesol exposure.

The GO categories significantly enriched (q < 0.05) are shown in Fig. S1B of Supplement 2. Nineteen terms were enriched in the biological process category, six terms were enriched in the cellular component category, and twelve terms were enriched in the molecular function category, respectively.

The KEGG pathways are shown in Fig. 3B. The differentially abundant proteins with fold change > 2 were enriched in 27 KEGG pathway terms (ranked from high to low according to the significance of the enriched differentially abundant proteins): Phenazine biosynthesis, Geraniol degradation, Quorum sensing, Synthesis and degradation of ketone bodies, Terpenoid backbone biosynthesis, Fatty acid degradation, Biosynthesis of antibiotics, Lysine degradation, Biofilm formation, Valine, leucine and isoleucine degradation, Propanoate metabolism, Fatty acid metabolism, beta-Alanine metabolism, Tryptophan metabolism, beta-Lactam resistance, Cationic Antimicrobial Peptide (CAMP) resistance, Pyruvate metabolism, Benzoate degradation, Glycolysis/Gluconeogenesis, Butanoate metabolism, Bacterial chemotaxis, Glyoxylate and dicarboxylate metabolism, Carbon metabolism, Two-component system, Biosynthesis of secondary metabolites, Microbial metabolism in diverse environments, and Metabolic pathways.

3.4. The conjoint analysis of transcriptome and proteome of the PAO1 strain

Both transcriptome and proteome analyses results showed that about 1.7% of all the detected genes/proteins were differentially expressed > 2-fold after farnesol exposure, with 96 of 5554 genes (45 downregulated and 51 upregulated genes) detected in RNA-seq and 47 of 2846 proteins (26 downregulated and 21 upregulated proteins) detected in iTRAQ seq. Sixteen genes/proteins with significant differences after farnesol exposure were identified at both the transcriptomic and proteomic levels, as shown in our Venn diagram (Fig. 4). The detailed information of the shared 16 differentially expressed genes/proteins is shown in Table 1. These results indicated that the genes/proteins expression of *P. aeruginosa* PAO1 was affected by farnesol exposure.

3.5. KEGG pathways in transcriptomic and proteomic analysis

The KEGG and GO enriched analyses of the differentially expressed genes/proteins of *P. aeruginosa* PAO1 following farnesol exposure based on transcriptome and proteome dataset were consistent. The pathways of quorum sensing and geraniol degradation were the most significantly enriched in both the iTRAQ proteomic analysis and the RNA-seq analysis. The KEGG quorum sensing pathways (including *las*, *rhl*, and the *pqs* system) in RNA-seq and proteome analysis are shown in Fig. 3A (transcriptome) and Fig. S2 (proteome) of Supplement 2. Fig. 5A and Fig. S2 indicated that the key genes in the *pqs* system were downregulated in PAO1 following farnesol exposure, including *pqsABCDE*, and *pqsH* - but that *pqsR* was not downregulated. The shared downregulated genes/proteins are also shown in Table 1. These results were consistent with the real time RT-qPCR results in Fig. 1. These results revealed that farnesol inhibited the expression of PQS signal synthase coding genes (*pqsABCDE* and *pqsH*) in the *pqs* system, but did not inhibit the expression of the PQS signal receptor coding gene (*pqsR*).

The KEGG pathway of geraniol degradation was also significantly enriched in both transcriptome and proteome analyses. The differentially expressed genes/proteins in this pathway enriched in the transcriptome and proteome are consistent, with eight key genes

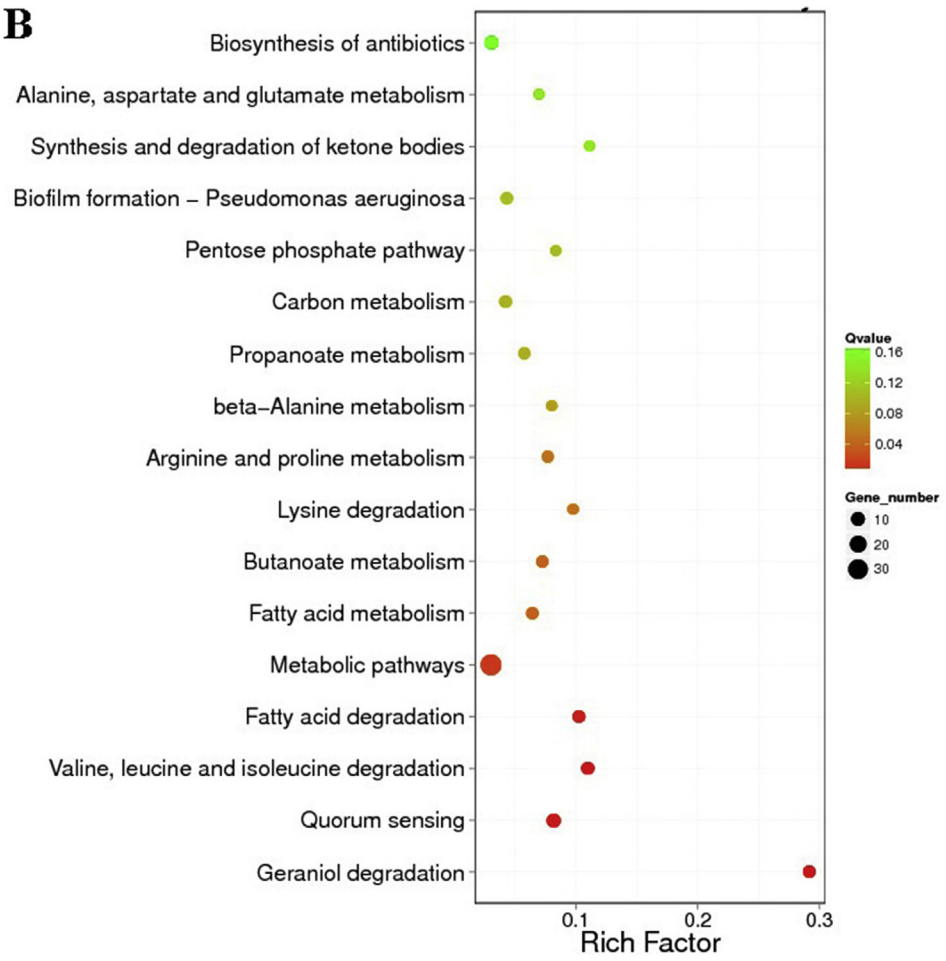

Fig. 2. (*continued*)

upregulated enriched in the transcriptome analysis (Fig. 5B), and seven of these key genes upregulated enriched in the proteome analysis (Fig. S3 in Supplement 2). The shared seven upregulated genes/proteins in this pathway are also shown in Table 1, which are *atuA*, *atuB*, *atuC*, *atuD*, *atuE*, *atuF*, and *atuG*. Fig. 5B and Fig. S3 showed that in the strengthened geraniol degradation pathway, nerol, geraniol, and citronellol undergo a series of enzymatic steps, finally entering the valine, leucine and isoleucine degradation pathway, in which upregulated genes were also enriched in both RNA-seq and Proteome-seq results. Several key genes of PAO1 were upregulated in the geraniol degradation pathway after farnesol exposure. This result suggests that farnesol or its intermediate metabolites may enter the geraniol degradation pathway.

The most significantly enriched KEGG pathway in the proteomic analysis was the phenazine biosynthesis, but this pathway was not found to be enriched in our RNA-seq analysis. In this pathway, 14 of the 21 key proteins detected were significantly downregulated (Fig. S4 in Supplement 2). Among these downregulated genes, *phzM* and *phzS* were key genes of pyocyanin biosynthesis, and some other downregulated key genes were related to PQS and phenazine synthesis.

The KEGG pathway of the biofilm formation was enriched both in transcriptome (Fig. S5 in Supplement 2) and in proteome (Fig. S6 in Supplement 2) analyses. In this pathway, 7 genes were differentially expressed based on RNA-seq, including *phnA*, *phnB*, *pqsA*, *pqsB*, *pqsC*, *pqsD*, and *lecA*, and 5 proteins were differentially abundant based on proteomic analysis, including PqsA, PqsB, PqsC, PqsD, and PqsH. The differentially expressed genes and differentially abundant proteins were basically consistent, which are some genes/proteins related to quorum sensing.

3.6. On the growth dynamics of PAO1

The growth curves of the PAO1 strain following different concentrations of farnesol treatment are shown in Fig. 6A. All the PAO1 populations showed characteristic growth curves, with an about 4 h lag period, followed by a logarithm period, a stability period, and a decay period in both the control group and the farnesol treatment groups. There was almost no difference in the growth curves of PAO1 between the control group and all the farnesol treated groups (at the concentration range from 0.14 mg/mL to 1.11 mg/mL). Therefore, we concluded that farnesol does not inhibit the growth of the PAO1 strain. This experimental result is consistent with a previous report that farnesol did not affect the growth kinetic of the *P. aeruginosa* PA14 strain (Cugini et al., 2007).

3.7. On PQS production of the PAO1 strain

The graph for PQS production of the PAO1 strain treated and untreated with farnesol is shown in Fig. 6B. The graph showed that the PQS production in control group increased with the incubation time, from tens of to hundreds of μg/mL. However, the PQS productions in the farnesol-treated group were all much lower than control in the three sampling time points, which remained close to zero. Therefore, farnesol can inhibit the PQS production of the PAO1 strain.

3.8. On elastase activity of the PAO1 strain

The LasB elastase activity of the PAO1 strain following farnesol treatment is shown in Fig. 6C. The elastase activities of the PAO1 strain

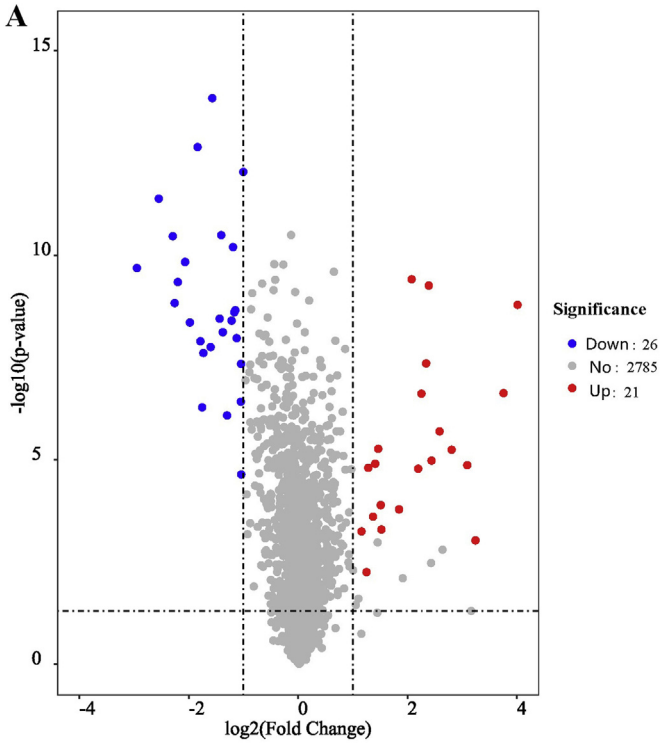

Fig. 3. Diagrams of proteomic analysis of untreated and farnesol-treated *P. aeruginosa* PAO1 populations. (A) Volcano plots of the differentially expressed genes based on proteomic analysis. Each protein is represented by a dot in the graph; The x-axis and y-axis represent the $\log_2$ value of the fold change and the t-statistic as $-\log_{10}$ of the p-value, respectively; The proteins represented in red (upregulated) and blue (downregulated) are differentially expressed proteins with > 2-fold change and a p-value of < 0.05. (B) Significantly enriched KEGG pathway (0.00 < q value < 1.00) of differentially expressed proteins based on proteomic analysis of the farnesol-treated group compared to the control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

populations incubated for one day in 0.14, 0.56, and 1.11 mg/mL farnesol-treated groups were basically identical, and was basically identical to that of the control (+) group, while no elastase activity was observed in the control (−) group. Therefore, we concluded that farnesol has no effect on the elastase activity of the PAO1 strain.

3.9. On pyocyanin yield of the PAO1 strain

The kinetic curves of the pyocyanin production of the PAO1 strain exposed to various farnesol concentrations are shown in Fig. 6D. The pyocyanin productions in the control (+) were more than that in the farnesol treatment groups at all time-point. The pyocyanin kinetics in the control (+) and control (−) groups were consistent with a previous work (Li et al., 2018). The dynamic curves of *P. aeruginosa* PAO1 populations in the 0.14 and 0.56 mg/mL farnesol-treated groups showed a similar trend to that of the control (+) group, with maximum yield also being observed on the third incubation day. However, pyocyanin yields in the two farnesol-treated groups were both much less than in the control (+) group. Moreover, the pyocyanin yields in the 1.11 mg/mL farnesol-treated group were negligible at all incubation day, similar to those of the control (−) group. In addition, the pyocyanin yield was lower and lower with increasing farnesol concentration. Therefore, we concluded that farnesol can inhibit the pyocyanin yield of the PAO1 strain. This experimental result is consistent with a previous report that farnesol decreased the pyocyanin production of PA14 strain (Cugini et al., 2007).

3.10. On biofilm volume of the PAO1

The biofilm and planktonic cell yields of the PAO1 strain populations following farnesol treatment are shown in Fig. 6E. Results showed that there was no obvious difference in the concentrations of planktonic cell incubated for one day between the control and farnesol-treated groups. In contrast, the biofilm yield of *P. aeruginosa* PAO1 in the control group was more than the three farnesol-treated groups. The biofilm yield of PAO1 in the control group was nearly double that of the 0.14, 0.56, and 1.11 mg/mL farnesol-treated groups. However, there was little difference in the biofilm yield between the 0.14, 0.56, and 1.11 mg/mL farnesol-treated groups. Therefore, we concluded that farnesol can inhibit the biofilm yield of the PAO1 strain, but the effect was small and there was no significant difference between different farnesol concentrations treated groups.

3.11. On swarming motility of the PAO1 strain

Fig. 6F is the photographs demonstrating the swarming motility of the PAO1 strain following farnesol treatment. The single bacterial colony in the control group displayed an irregular dendritic morphology, with several extensions branching from the center of the colony on the inoculum. Surprisingly, the swarming motility of PAO1 populations in the 0.14, 0.56, and 1.11 mg/mL farnesol-treated groups were enhanced to varying degrees compared to the control group. The numbers of extensions that emerged from the center of the colony on the inoculum were more with increasing farnesol concentrations. The tendrils became more and more developed and covered larger and larger areas. Therefore, we concluded that farnesol enhanced the swarming motility of the PAO1 strain.

4. Discussion

Farnesol is slightly toxic to the PAO1 strain. The 1.11 mg/mL farnesol concentration did not inhibit the growth of PAO1 strain, therefore, or affect its elastase production. However, farnesol did inhibit the PAO1 strain in terms of pyocyanin production and biofilm formation. These results are consistent with a previous reports that farnesol did not affect the growth kinetics of PA14 strain but lead to decreased production of pyocyanin of the PA14 strain (Cugini et al., 2007). Moreover, compared to the garlic diallyl disulfide study published in a previous paper (Li et al., 2019), the effect of farnesol on *P. aeruginosa* PAO1 is much milder. Not only the iTRAQ and RNA-seq data in farnesol experiments in this paper, but also the iTRAQ and RNA-seq data in the diallyl disulfide (DADS) experiments of our previous work (Li et al., 2019) suggest that transcriptomic analysis is more sensitive at detecting gene expression than proteome, with about two (farnesol experiments) or three (diallyl disulfide experiments) times higher levels of detected genes in RNA-seq compared to iTRAQ.

The quorum sensing pathway that showed some downregulated genes/proteins was the most significant enriched in both the RNA-seq analysis and the proteomic analysis. The omics results showed that farnesol inhibit the transcription and protein expression of the key genes in the *pqs* QS system. Farnesol inhibited the transcription of the PQS biosynthetic operon, including *pqsABCDE*, and *pqsH*. However, the transcription of *pqsR* which is the coding gene of PQS receptor was not affected by farnesol. These omics data were confirmed by the experimental results of RT-qPCR. Moreover, these experimental results were consistent with a previous research that farnesol inhibited the transcription of *pqsA* and the production of PQS signal molecules, but not the transcription of *pqsR* (Cugini et al., 2007). However, they are different from another previous report that farnesol produced from *Candida albicans* stimulated PQS production in LasR-defective *P. aeruginosa* strains (Cugini et al., 2010). The difference between these two experiments is that the experimental strain studied in the previous report is a LasR-defective *P. aeruginosa* strain, while our experimental strain in this

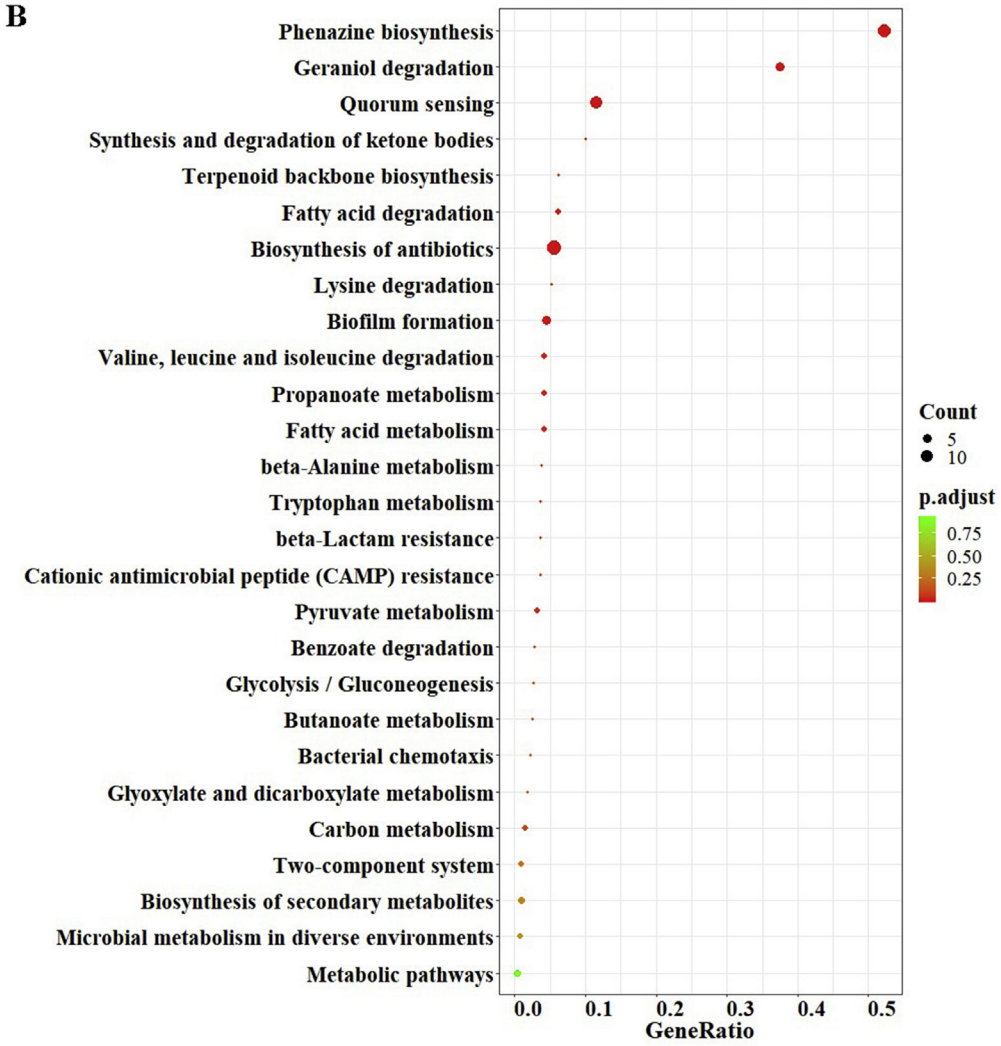

Fig. 3. (*continued*)

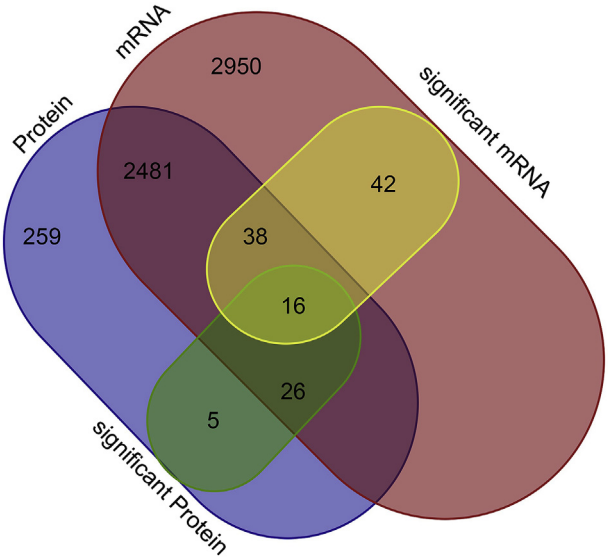

Fig. 4. The Venn diagram shows all identified, as well as significantly enriched, mRNAs, proteins, and their overlaps.

Table 1
The shared sixteen genes/proteins differentially expressed and two unshared genes/proteins based on transcriptome and proteome.

Gene ID	Gene name	Log2FoldChange (transcriptome)	Log2FoldChange (proteome)	Up/Down regulation
PA0996	*pqsA*	−3.05	−3.07	Down
PA0997	*pqsB*	−3.05	−4.64	Down
PA0998	*pqsC*	−2.18	−7.80	Down
PA0999	*pqsD*	−2.76	−5.91	Down
PA1000	*pqsE*	−3.28	Fold change < 2	Down
PA2587	*pqsH*	Fold change < 2	−2.24	Down
PA2886	*atuA*	4.41	5.93	Up
PA2887	*atuB*	3.88	4.71	Up
PA2888	*atuC*	3.70	5.00	Up
PA2889	*atuD*	3.74	13.33	Up
PA2890	*atuE*	3.52	9.35	Up
PA2891	*atuF*	3.47	5.36	Up
PA2892	*atuG*	3.39	6.91	Up
PA0364	PA0364	3.00	8.42	Up
PA1617	PA1617	1.70	4.52	Up
PA3038	PA3038	1.17	3.55	Up
PA3925	PA3925	1.12	2.21	Up
PA4435	PA4435	1.82	5.17	Up

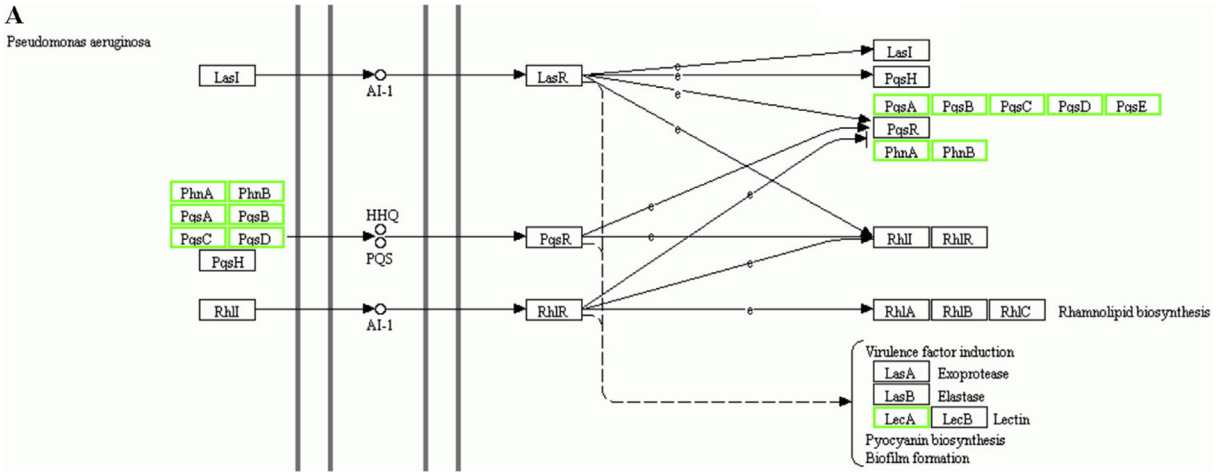

Fig. 5. The KEGG pathways based on transcriptomic or proteomic analysis. (A) The quorum sensing pathway based on RNA-seq analysis. The green box RNA indicate that the RNA expression of the gene is down regulated, while the black box RNA show no changes in RNA expression. (B) The geraniol degradation pathway based on RNA-seq analysis. The red box RNA indicate that the RNA expression of the gene is upregulated, while the black box RNA show no changes in RNA expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

experiment was the PAO1 wild-type strain. Cugini and coworkers proposed that farnesol promoted the production of reactive oxygen species (ROS), and ROS then strengthened PQS production by activated the RhlR in LasR mutants of PA (Cugini et al., 2010). Therefore, we concluded that farnesol can inhibit the production of PQS signal molecules in PAO1 by inhibiting the transcription of *pqsABCDE* and *pqsH*, but did not inhibit the recognition and transmission of PQS signal molecules in PAO1. This conclusion was also confirmed by the experimental results of the PQS determination which showed that farnesol inhibits the PQS production of the PAO1 strain. Cugini et al. (2007) also demonstrated that farnesol abolishes the PQS production of the PA14 strain chiefly due to the transcription inhibition of the *pqsA*.

The toxicity of farnesol to PAO1 was found to be lower than that of DADS which showed more differentially expressed genes (> 3000), more differentially abundant proteins (1133), and more inhibited virulence factors (Li et al., 2018, 2019), probably because the bacteria already had degradation and detoxification channels in place for farnesol. The geraniol degradation pathway that enriched in most upregulated genes/proteins based on both transcriptomic and proteomic analyses indicated that farnesol can induce this pathway. This result suggests farnesol may be degraded through this pathway. As early as 1960, a bacterium named *P. citronellolis* was reported to be able to utilize acyclic terpenes (citronellol) (Tozoni et al., 2010). Subsequently, some *Pseudomonas* species were found to be able to utilize citronellol, geraniol, and other acyclic monoterpenes as the sole carbon source, including *P. aeruginosa*, *P. menodcina*, *P. delhiensis*, and some strains of *P. fluorescens* (Cantwell et al., 1978; Fall et al., 1979; Förster-Fromme et al., 2006; Tozoni et al., 2010). After forty years, the molecular biological techniques were available to investigate and reveal the acyclic terpenes catabolism on the gene level most contributed by J. Campos-Garcia's group and D. Jendrossek's group (Förster-Fromme and Jendrossek, 2010). Consistent with the above research report, our experimental results suggested that PAO1 strain have the ability to degrade and utilize farnesol. It also showed that farnesol or its intermediate metabolites is catabolized through the KEGG pathway of geraniol degradation.

The phenazine biosynthesis pathway was significantly enriched in the proteomic analysis, but it was not enriched in the transcriptomic analysis. Among these downregulated proteins, PhzM and PhzS were key proteins of pyocyanin biosynthesis. Pyocyanin, a blue-green phenazine pigment, is an important pathogenic virulence factor of *P. aeruginosa*, which inhibits the oxidative burst of phagocytic cells by triggering apoptosis in host cells (Heidari et al., 2017; Guan et al., 2019). The downregulation of *phzM* and *phzS* were consistent with the experimental results of pyocyanin production determination, which showed that pyocyanin production was inhibited after farnesol exposure. However, the differentially expressed genes were not enriched in the phenazine biosynthesis pathway, according to RNA-seq analysis. The *phzM* gene was also not downregulated in our real time q-PCR results. The pyocyanin synthesis genes are coactivated by *las*, *rhl*, and the *pqs* system (Fig. 5A). Farnesol inhibited the transcription of *pqsABCDE* and *pqsH*, but failed to inhibit the transcription of pyocyanin synthesis genes. This may be due to the compensatory effect of *rhl* system regulation. These results suggest that the downregulation of key genes expression in pyocyanin biosynthesis and the phenazine biosynthesis pathway of PAO1 after farnesol exposure were post translational regulation.

The KEGG pathway of biofilm formation was enriched both in our RNA-seq and proteome analyses, which showed that some key genes in this pathway were downregulated. These results were consistent with the experimental result of biofilm yield determination. *P. aerugninosa* biofilm is much more resistant to antibiotics than the planktonic cells (Liu et al., 2018; Gökalsın et al., 2019). *P. aeruginosa* biofilm is also a virulence factor regulated by QS (Wang et al., 2019). Our experimental results suggested that farnesol inhibits the transcription and protein expression of key genes in the *pqs* QS system of the PAO1 strain, and therefore inhibits its biofilm production. However, the effect of farnesol on biofilm production was relatively small and there was no dose dependent effect. A previous work reported that farnesol increased the efficiency of ciprofloxacin against *P. aeruginosa* biofilms (Bandara et al., 2016). Another previous work also reported the inhibitory potential of farnesol against *Burkholderia pserdomallei* biofilms (Castelo-Branco et al., 2015). However, the RNA-seq, proteome analysis, real time q-PCR result, and the elastase activity determination result all showed that elastase (*lasB* coding gene) in PAO1 was not affected after farnesol exposure. Thus, farnesol inhibited the expression of key genes of the *pqs* system, but could not inhibit the production of elastase, which was co-regulated by the three QS systems (Fig. 5A).

Interestingly, the swarming motility experimental result showed that the swarming motility of PAO1 was enhanced after farnesol exposure, and that this was dose-dependent. This was different compared to the case in DADS exposure, wherein swarming motility was inhibited (Li et al., 2018). Swarming motility is the movement of a group of bacteria, which is dependent on flagella (Harshey, 2003). Rhamnolipid also plays an important role in the swarming motility of the PAO1 strain (Daniels et al., 2004). However, the KEGG pathway of flagella assembly

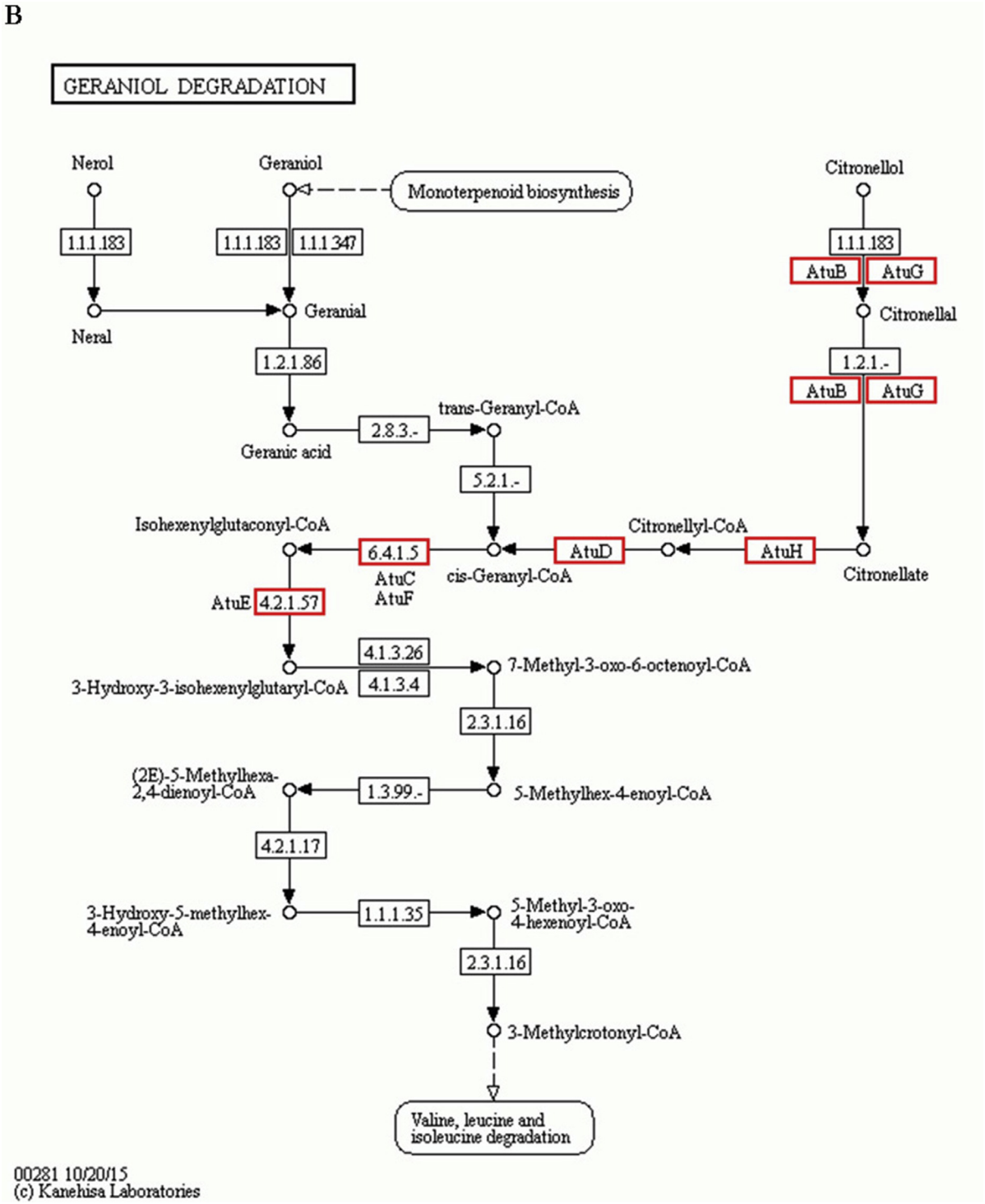

Fig. 5. (*continued*)

was not found to be enriched in either our RNA-seq or proteome analyses. The rhamnolipid synthesis genes (*rhlA*, *rhlB*, *rhlC*), which are regulated by the *rhl* system (Fig. 5A, Fig. S2), were not shown to exhibit any differential expression. These results suggest that the enhanced swarming motility may be due to the farnesol hormesis. Hormesis is a term used in toxicology to describe the biphasic dose effect of toxic factors or stimuli, which characterized by stimulatory or beneficial response to low doses of toxic factors (Calabrese and Baldwin, 1999; Saitanis and Agathokleous, 2019). Farnesol has a low toxicity to PAO1, which may stimulate some of its related metabolic activities such as geraniol degradation, and swarming motility. This bacterial motility plays a key role in its bacterial pathogenicity and biofilm formation (Pattnaik et al., 2018). This result was consistent with the experimental results concerning biofilm formation. Bacterial swarming motility was enhanced, while biofilm formation was weakened.

5. Conclusions

Farnesol showed low toxicity to the *P. aeruginosa* PAO1 strain and it did not inhibit the growth of the PAO1 strain or affect its elastase production. However, farnesol did significantly inhibit the transcription and protein expression of the *pqsABCDE* and *pqsH* genes in the strain's

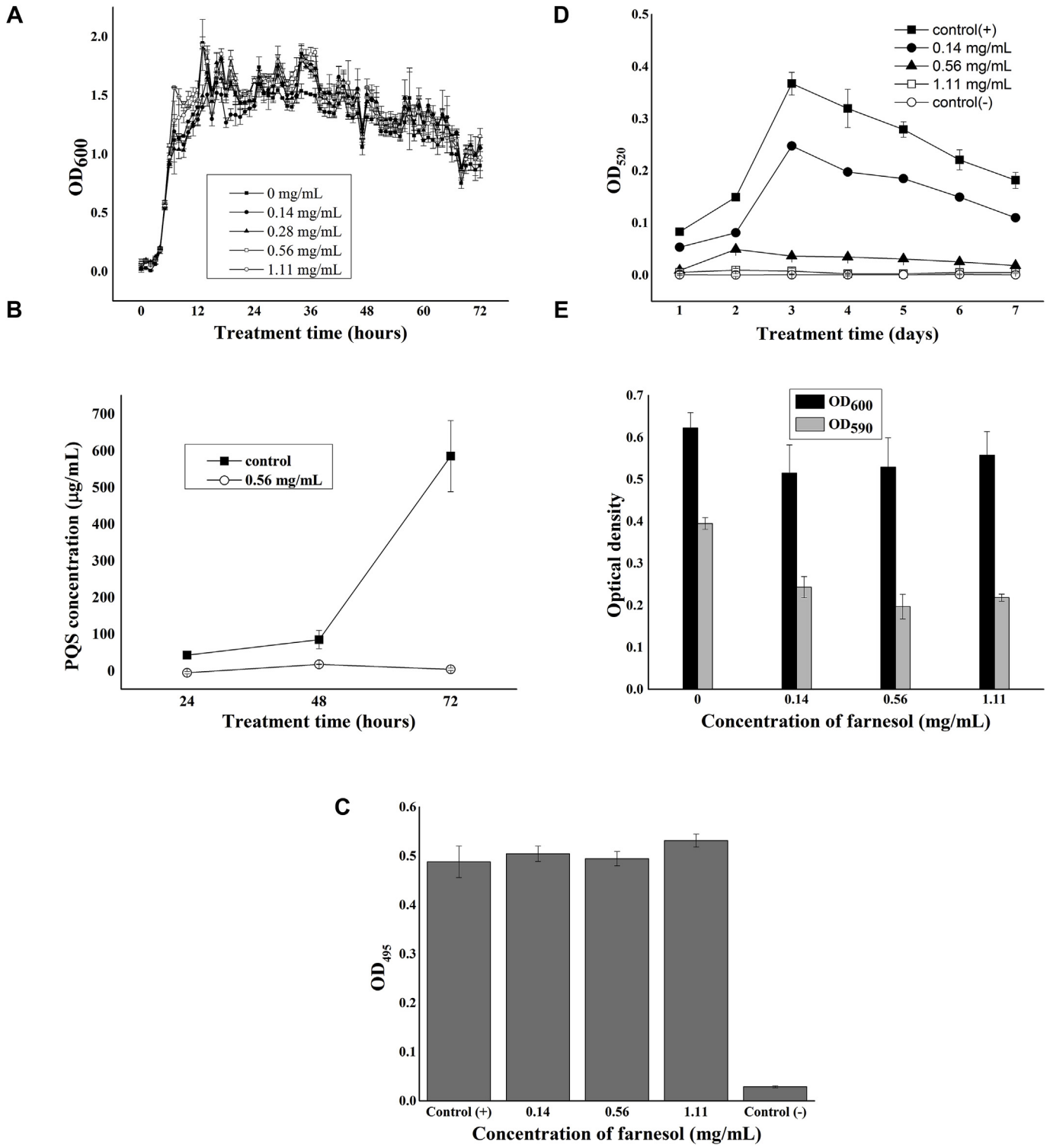

Fig. 6. The phenotype analysis charts of untreated and farnesol-treated *P. aeruginosa* PAO1 populations. (A) Growth curves of *P. aeruginosa* PAO1 cells exposed to farnesol at different concentrations. (B) Graph of relative production of PQS molecules in *P. aeruginosa* PAO1 cultures exposed to farnesol and an unexposed positive control. (C) Relative activity of LasB elastase of *P. aeruginosa* PAO1 exposed to farnesol for one day and an unexposed positive control. Sterile deionized water was used to replace the bacterial supernatant in the control (−). (D) Relative production of pyocyanin by *P. aeruginosa* PAO1 cells exposed to farnesol at different concentrations. Sterilized deionized water was used to replace the culture in control (−). (E) Relative yields of cells and biofilms of *P. aeruginosa* PAO1 exposed to farnesol at different concentrations for one day. OD_{600} values represent the cell yields, and OD_{590} values represent the biofilm yields. (F) Images displaying the swarming motilities of *P. aeruginosa* PAO1 cells exposed to farnesol at different concentrations. a, control; b, 0.14 mg/mL; c, 0.56 mg/mL; d, 1.11 mg/mL farnesol group.

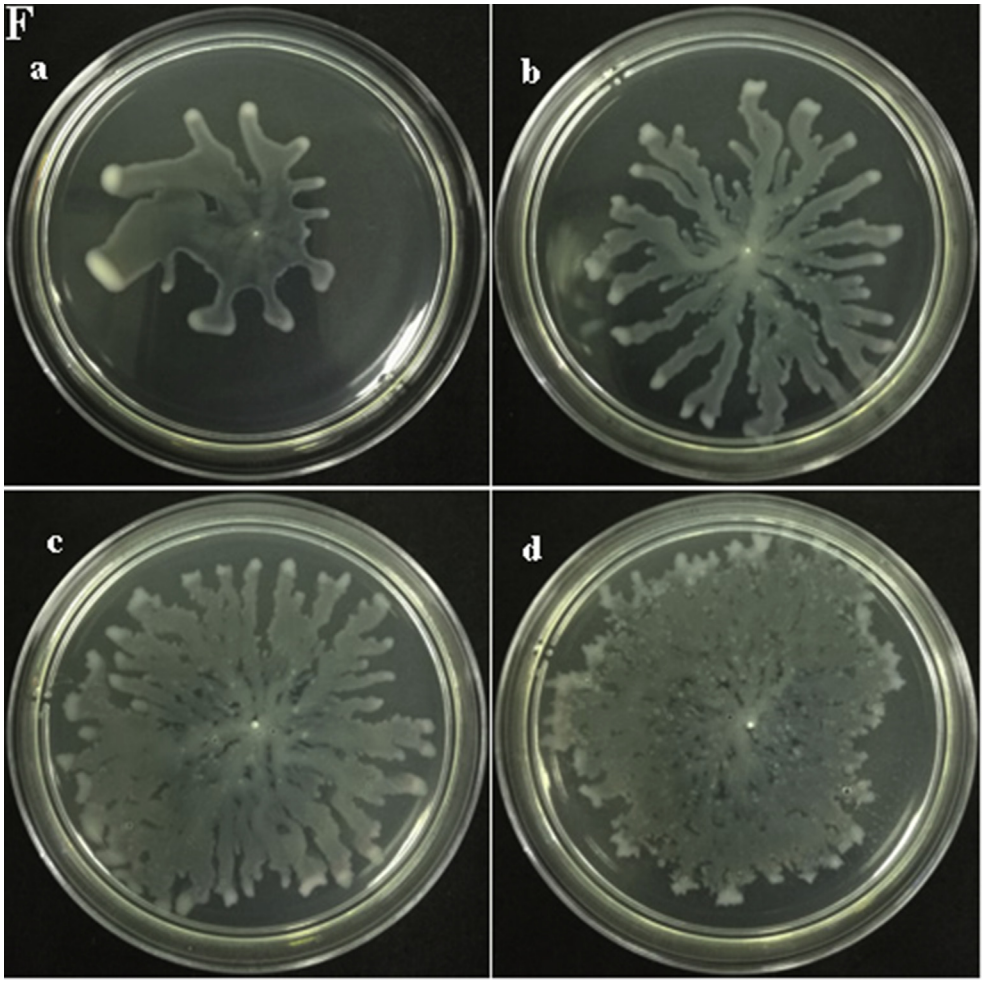

Fig. 6. (*continued*)

pqs QS system, although it did not inhibit the *pqsR* gene in the *pqs* QS system, nor the *lasI/lasR* genes in the *las* QS system, or the *rhlI/rhlR* genes in the *rhl* QS system. Farnesol also inhibited some virulence genes regulated by the *pqs* QS system, as well as the production of some related virulence factors, such as inhibiting the protein expression of key genes in the phenazine biosynthesis pathway, including the key genes related to phenazine, PQS, and pyocyanin synthesis (*phzM*, *phzS*), inhibiting the strain's biofilm formation. In addition, farnesol enhanced the transcription and protein expression of key genes in the geraniol degradation pathway. The low toxicity of farnesol also stimulated the PAO1 strain with regard to swarming motility. Therefore, we concluded that farnesol suppress the virulence by inactivating the *pqs* QS system in pathogen *P. aeguginosa*.

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2020.104956.

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