



Association of furanone C-30 with biofilm formation & antibiotic resistance in *Pseudomonas aeruginosa*

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Received December 20, 2016

Background & objectives: *Pseudomonas aeruginosa* is an opportunistic pathogen that can cause nosocomial bloodstream infections in humans. This study was aimed to explore the association of furanone C-30 with biofilm formation, quorum sensing (QS) system and antibiotic resistance in *P. aeruginosa*.

Methods: An *in vitro* model of *P. aeruginosa* bacterial biofilm was established using the standard *P. aeruginosa* strain (PAO-1). After treatment with 2.5 and 5 µg/ml of furanone C-30, the change of biofilm morphology of PAO-1 was observed, and the expression levels of QS-regulated virulence genes (*lasB*, *rhlA* and *phzA2*), QS receptor genes (*lasR*, *rhlR* and *pqsR*) as well as QS signal molecule synthase genes (*lasI*, *rhlI*, *pqsE* and *pqsH*) were determined. Besides, the *AmpC* expression was quantified in planktonic and mature biofilm induced by antibiotics.

Results: Furanone C-30 treatment significantly inhibited biofilm formation in a dose-dependent manner. With the increase of furanone C-30 concentration, the expression levels of *lasB*, *rhlA*, *phzA2*, *pqsR*, *lasI*, *rhlI*, *pqsE* and *pqsH* significantly decreased in mature biofilm bacteria while the expression levels of *lasR* and *rhlR* markedly increased. The *AmpC* expression was significantly decreased in both planktonic and biofilm bacteria induced by imipenem and ceftazidime.

Interpretation & conclusions: Furanone C-30 may inhibit biofilm formation and antibiotic resistance in *P. aeruginosa* through regulating QS genes. The inhibitory effect of furanone C-30 on *las* system appeared to be stronger than that on *rhl* system. Further studies need to be done with different strains of *P. aeruginosa* to confirm our findings.

Key words *AmpC* - antibiotic resistance - biofilm formation - furanone C-30 - *Pseudomonas aeruginosa* - quorum sensing

Pseudomonas aeruginosa is an opportunistic pathogen that can cause nosocomial bloodstream infections in humans, and its infection is also reported to be associated with deterioration of lung function and

reduced life expectancy^{1,2}. Multiple drugs such as beta (symbolic)-lactam antibiotics and aminoglycosides can control the *P. aeruginosa* infections³. However, the *P. aeruginosa* infections are difficult to be cured by

current antibacterial drugs, and the drug resistance is increasingly becoming a common problem^{4,5}.

Recently, the key role of the quorum sensing (QS) system in preventing the *P. aeruginosa* infection has gained more attention. *P. aeruginosa* is found to use three known QS systems, namely las, rhl and pqs, to regulate a large number of genes, including many virulence factors⁶. Moreover, it is reported that QS system may contribute to the antimicrobial resistance of *P. aeruginosa*⁷. QS system inhibitors exhibit broad prospects in the treatment of *P. aeruginosa* infection⁸. To inhibit many virulence factors induced by QS system, some researchers have focused on the halogenated furanone compounds, which have strong inhibitory effect on the QS system⁹. Bukholm *et al*¹⁰ have also confirmed that use of furanone C-30, instead of large concentrations of antibiotics, can suppress the QS system of planktonic and biofilm bacteria. However, the association of furanone C-30 with the antibiotic resistance of *P. aeruginosa* is largely unknown.

In the present study, we established a dynamic model of *P. aeruginosa* using the standard *P. aeruginosa* strain PAO-1 *in vitro*. Biofilm generation and the expression of QS genes and antibiotic-induced *AmpC* gene in PAO-1 were investigated after treatment with different concentrations of furanone C-30. The present study aimed to explore the association of furanone C-30 with biofilm formation and antibiotic resistance in *P. aeruginosa*.

Material & Methods

Bacterial strains and culture conditions: The study was performed between October 2014 and December 2015 in the Central Laboratory of The Affiliated Hospital of Quindao University. The standard *P. aeruginosa* strain (PAO-1) was provided by the National Engineering Research Center for Marine Drugs, Ocean University of China (Qingdao, PR China). PAO-1 monoclonal colonies were grown in Luria-Bertani (LB) medium (NaCl 5 g/l, tryptone 10 g/l, yeast extract 5 g/l and agar 15 g/l) at 37°C in a thermostated bacteriological incubator for 8 h up to logarithmic phase. The bacterial concentration was adjusted to 1.5×10^8 cfu/ml (equivalent to 0.5 McFarland tube turbidity) by nephelometry.

Establishment of a biofilm model: The biofilm model was established as previously described with some modifications¹¹. Briefly, the bacteria were seeded into the tube used for nutrient solution infusion, and the biofilms were grown in the nutrient solution infusion tube by a once-flow through the system. With a peristaltic

pump (Beijing LingZe Pharmaceutical Technology Development Company, PR China), the flow velocity was controlled at 45 ml/h, and the temperature of LB medium inside the tube was maintained at 37°C. Then 2.5 and 5 µg/ml of furanone C-30¹² was added into the LB medium in infusion bag. Biofilm generation was visualized using argention method as described previously¹³. The morphology of three-day biofilm (maturation-1 stage) and six-day biofilm (maturation-2 stage) was observed by optical microscopy.

Determination of minimum inhibitory concentration (MIC): Planktonic bacteria were cultivated in LB medium in a 37°C thermostat bacterial incubator for 8 h to logarithmic phase. The MIC of planktonic bacteria and biofilm bacteria to imipenem and ceftazidime was measured using a semi-automatic susceptibility analyzer (API ATB plus system, BioMerieux, France) according to the protocols described previously¹⁴.

Induction of *AmpC* expression: The medium for incubating planktonic and biofilm bacteria (three and six days) was supplemented with imipenem and ceftazidime to induce the expression of *AmpC* gene. Imipenem concentrations used for incubating the planktonic bacteria, three and six days biofilm bacteria were set as 2 (1/2 MIC), 16 (4 MIC) and 16 mg/l (4 MIC), respectively, and ceftazidime concentrations were 2 (1/2 MIC), 40 (10 MIC) and 48 mg/l (12 MIC), respectively¹⁵. To further evaluate the effect of furanone C-30 in *P. aeruginosa*, furanone C-30 (2.5 and 5 µg/ml) was added to the medium containing different concentrations of imipenem and ceftazidime. The induced biofilm bacteria were removed using ultrasonic oscillation (100 W×15 min).

Quantitative real-time polymerase chain reaction (q-PCR) analysis: Bacterial total RNA was extracted using RNA isolation kit (Qiagen, USA). To confirm RNA integrity, agarose gel (1.0%) electrophoresis was performed. Moreover, the concentration and purity (Absorbance at 260 nm/Absorbance at 280 nm; A₂₆₀/A₂₈₀) were detected by ultraviolet-visible spectroscopy (SMA 400 UV-VIS, Merinton, Shanghai, PR China). The purified RNA was reverse transcribed into cDNA to be used as a template for PCR amplification. The q-PCR reaction was performed as follows: 95°C for 10 min followed by 40 cycles of 95°C for 5 sec and 60°C for 60 sec, which was carried out by the SuperScript III first-strand synthesis system (Invitrogen, USA). At the end of each PCR reaction, the melting curve was

Table. Primer sequences for polymerase chain reaction amplification

Gene	Forward (5'-3')	Reverse (5'-3')
16s RNA	GCGCAACCCTTGTCTTAGTT	TGTCACCGGCAGTCTCCTTAG
<i>AmpC</i>	GGTGATGAAGGCCAATGACAT	CGGCCGTCCTCTTTTCGA
<i>lasB</i>	CAGGTAGAACGCACGGTTGT	GATCGGCTACGACATCAAGAA
<i>phzA2</i>	CCGACAACCTGGAATTGCGT	CCGCTTTCCGTGGTCCAGTT
<i>rhIA</i>	TGGTGTATTTCGCCCAGCTG	TGAAAGCCAGCAACCATCAG
<i>lasR</i>	TCTTGAGCTGGAACGCTCAAG	GATGAAGGCGTTCTCGTAGT
<i>lasI</i>	AATTGGTCGGCGGAAGAGTTC	AGGTGTTCTTCAGCATGTAGG
<i>rhIR</i>	TTGAGGATCGCCGGATCCAC	TGCAGCCGATCCACGACAGC
<i>rhII</i>	TGCAGCCATTCCGGGTGGTA	TTGCAACGGCGTGATCCTC
<i>pqsE</i>	GATGATGACCTGTGCCTGTT	CGCCCAAACCAATTCCGGCAT
<i>pqsR</i>	CACGCACAGGTTCGATTTCCA	CGTGAACATGTTCTCCAGGT
<i>pqsH</i>	GCGAACGAGGGTATTCCTCA	GATCCTGTTCCAGCAGCA

AmpC, AmpC β -lactamase gene (cephalosporinase gene); *lasB*, lasB elastase precursor gene; *phzA2*, virulence factor gene phzA2; *rhIA*, virulence factor gene rhIA; *lasR*, lasR receptor gene; *lasI*, lasI gene; *rhIR*, rhIR receptor gene; *rhII*, rhII gene; *pqsE*, PqsE gene; *pqsR*, pseudomonas quinolone signal transcriptional regulator gene; *pqsH*, PqsH gene

analyzed to confirm the specificity of PCR product. The primers for PCR amplification (Table) were designed by Premier Express 3.0 software (Applied Biosystems, Thermo Fisher, USA) and synthesized by Shanghai Sangon Biotechnology Co. (Shanghai, PR China). Each experiment was carried out in triplicate, and 16S rRNA of PAO-1 was used as internal control. The relative gene expression levels were calculated with the comparative threshold (Ct) cycle ($2^{-\Delta\Delta C_t}$) method¹⁶.

Statistical analysis: The data were expressed as mean \pm standard deviation and tested for the normal distribution using one-sample Kolmogorov-Smirnov test. Statistical analysis was tested with SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). In each PCR reaction, each sample was tested for three parallels, and each strain was repeated 10 times using 10 different monoclonal colonies within it. The difference in gene expression levels across the different furanone C-30 treatments, including 2.5 versus 0, 5 versus 2.5 and 5 versus 2.5 $\mu\text{g/ml}$ of furanone C-30, was tested by two-sided *t* test. Statistical power calculations for testing the sample size were conducted with two independent samples *t* test using SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA, USA) and $P > 0.80$ indicated a good sample size.

Results

Identification of biofilm model: As shown in Fig. 1, biofilm generation of PAO-1 was identified by

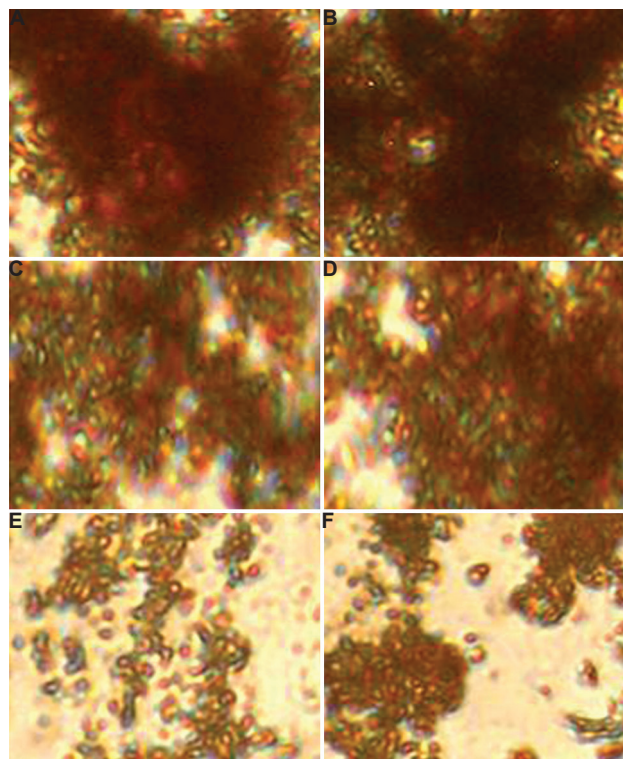


Fig. 1. Silver staining results of PAO-1 biofilm at different stages. (A) PAO-1 three-day biofilm (maturation-1 stage); (B) PAO-1 six-day biofilm (maturation-2 stage); (C) PAO-1 maturation-1 stage after treatment with 2.5 $\mu\text{g/ml}$ of furanone C-30; (D) PAO-1 maturation-2 stage after treatment with 2.5 $\mu\text{g/ml}$ of furanone C-30; (E) PAO-1 maturation-1 stage after treatment with 5 $\mu\text{g/ml}$ of furanone C-30; (F) PAO-1 maturation-2 stage after treatment with 5 $\mu\text{g/ml}$ of furanone C-30 ($\times 400$).

argentation method. Black-stained cottony patches were identified as positive results indicating relatively thick biofilm; scattered black spots or dark spots were considered as negative results, suggesting relatively thin or immature biofilm. The results showed that the cottony and unevenly distributed biofilm were dyed dark brown, and mature biofilms could be seen at three day (maturation-1 stage, Fig. 1A) and six day (maturation-2 stage, Fig. 1B) after inoculation. The PAO-1 biofilm at maturation-2 stage was more dense and mature than at maturation-1 stage. After treatment with 2.5 $\mu\text{g/ml}$ of furanone C-30, the dark dyed biofilm in both maturation-1 and -2 stages was less dense and unevenly distributed (Fig. 1C and D). After treatment with 5 $\mu\text{g/ml}$ furanone C-30, short rod-shaped bacteria gathered in piles and little of the black dyed cottony wool biofilm was observed in the two maturation stages (Fig. 1E and F).

MIC analysis: The MIC of imipenem and ceftazidime to both planktonic bacteria and various stages of biofilm bacteria was 4 mg/l. There was no change in the MIC of these two drugs to biofilm bacteria.

Analysis of the association between furanone C-30 and quorum sensing (QS) gene expression: As shown in Fig. 2A, the association of furanone C-30 with the expression of virulence genes was investigated by q-PCR assays. The expression levels of virulence genes *lasB*, *rhlA* and *phzA2* were all significantly decreased in mature biofilm bacteria after treatment with 2.5 $\mu\text{g/ml}$ of furanone C-30 ($P < 0.05$). The expression levels of *lasB*, *rhlA* and *phzA2* were further decreased after the treatment with 5 $\mu\text{g/ml}$ of furanone C-30, and the differences were significant ($P < 0.05$). The expression levels of *lasR* and *rhlR* in mature biofilm bacteria were significantly increased with the increase of furanone C-30 concentration ($P < 0.05$), while that of *pqsR* was markedly decreased. The increased level of *lasR* expression was significantly higher than that of *rhlR* ($P < 0.05$) (Fig. 2B). The expression levels of *lasI*, *rhlI*, *pqsE* and *pqsH* significantly decreased in mature biofilm bacteria with the increase of furanone C-30 concentrations ($P < 0.05$) (Fig. 2C).

Analysis of the association of furanone C-30 with *AmpC* expression after induction by antibiotics: As shown in

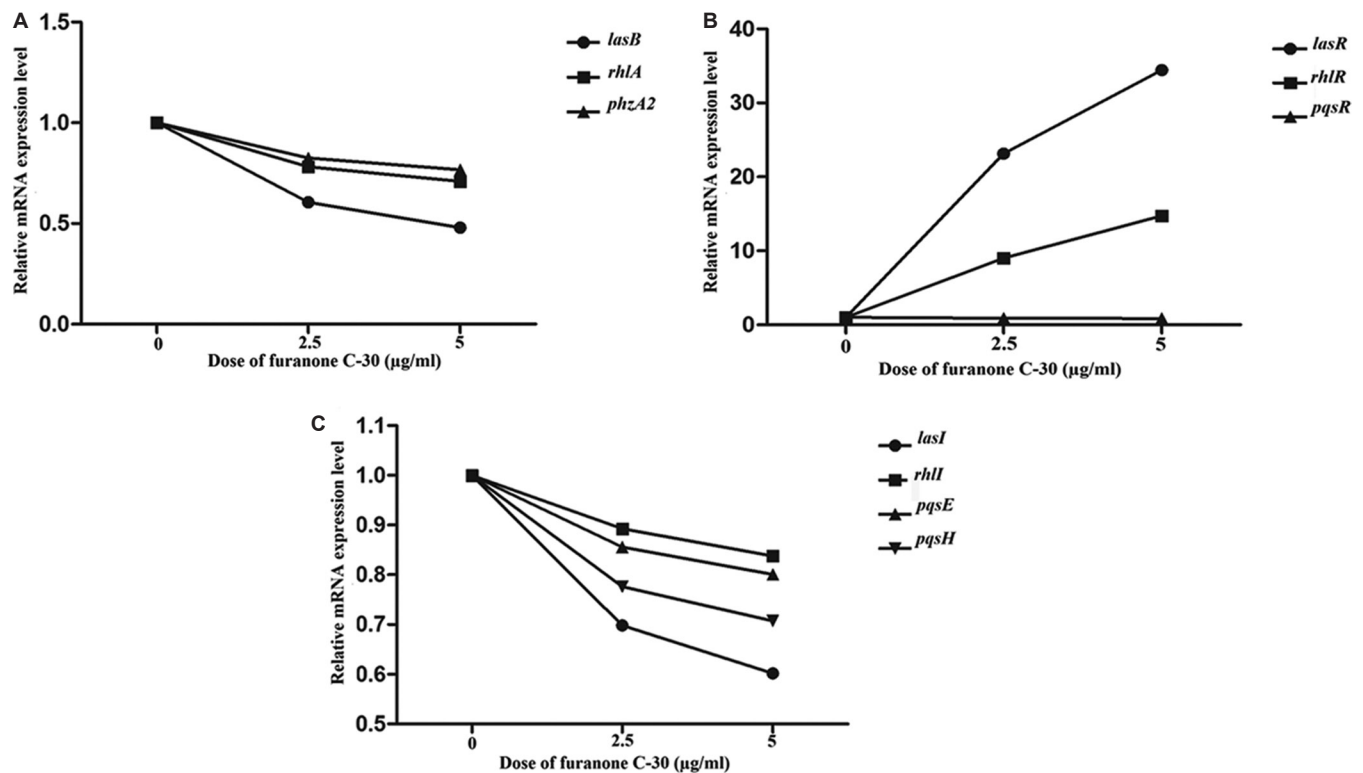


Fig. 2. Effect of furanone C-30 on the expression of quorum sensing genes. (A) expression levels of *lasB*, *rhlA* and *phzA2* in mature biofilm after treatment with 2.5 and 5 $\mu\text{g/ml}$ of furanone C-30; (B) expression levels of *lasR*, *rhlR* and *pqsR* in mature biofilm after treatment with 2.5 and 5 $\mu\text{g/ml}$ of furanone C-30; (C) expression levels of *lasI*, *rhlI*, *pqsE* and *pqsH* in mature biofilm after treatment with 2.5 and 5 $\mu\text{g/ml}$ of furanone C-30.

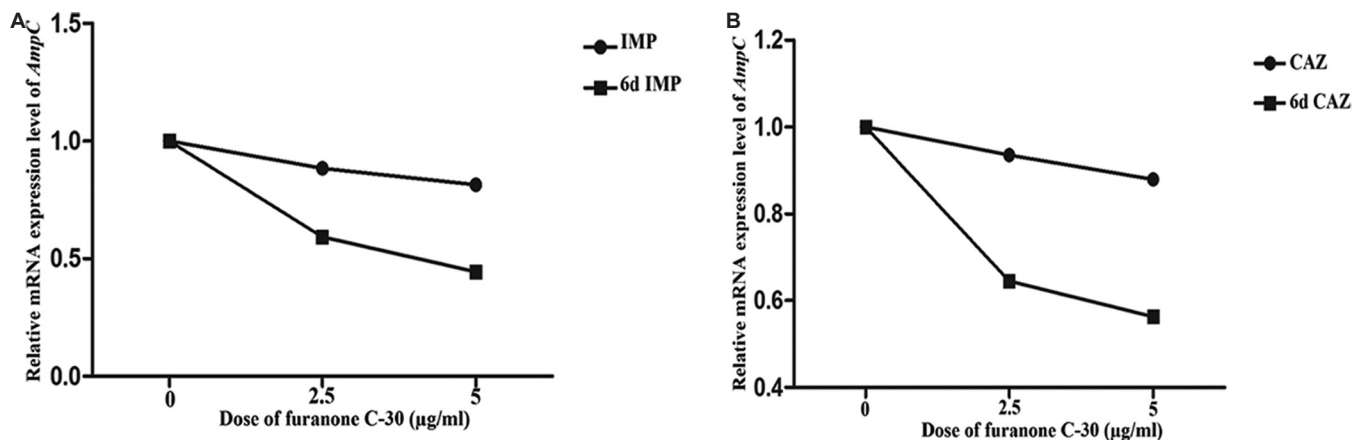


Fig. 3. Effect of furanone C-30 on PAO-1 *AmpC* gene expression levels in planktonic and biofilm bacteria induced by antibiotics. (A) expression levels of *AmpC* gene in PAO-1 planktonic (IMP) and biofilm bacteria (6 day IMP) induced by imipenem; (B) expression levels of *AmpC* gene in PAO-1 planktonic and biofilm bacteria induced by ceftazidime (CAZ) after 6 day (6d CAZ), maturation-2 stage of biofilm bacteria induced by antibiotics.

Fig. 3A and B, *AmpC* expression induced by imipenem and ceftazidime was significantly decreased in PAO-1 planktonic and biofilm bacteria with the increase of furanone C-30 concentrations ($P < 0.05$). With the treatment of the same concentration of the furanone C-30, the down regulation of *AmpC* expression in PAO-1 mature biofilm was significantly larger than that in planktonic stage ($P < 0.05$).

Discussion

In the current study, we investigated the association of furanone C-30 with biofilm formation, QS-controlled gene expression and/or antibiotic resistance in PAO-1. The results showed that biofilm formation was obvious at both maturation-1 and -2 stages, and the biofilm thickness at maturation-2 stage was more than that at maturation-1 stage. Furanone C-30 significantly inhibited the formation of PAO-1 biofilms in a dose-dependent manner. The expression levels of *lasB*, *rhIA*, *phzA2*, *pqsR*, *lasI*, *rhlI*, *pqsE* and *pqsH* were significantly decreased in mature biofilm bacteria with the increase of furanone C-30 concentrations, while the expression levels of *lasR* and *rhlR* markedly increased. Besides, the antibiotic-induced *AmpC* expression in PAO-1 planktonic and biofilm bacteria also significantly decreased with the increase of furanone C-30 concentrations. These findings imply the important potential of furanone C-30 in preventing *P. aeruginosa* infections.

In a previous study, Gambello and Iglewski¹⁷ found that multilayer clustered samples of bacterial biofilm structure were observed at maturation-1 stage (cluster

thickness $> 10 \mu\text{m}$), and the maximum cell cluster occurred at maturation-2 stage (cluster thickness up to $100 \mu\text{m}$). The change of biofilm morphology, seen in our study, was in line with previous findings of biofilm formation at maturation-1 and -2 stages and the thickness of biofilm at maturation-2 stage being more than that at maturation-1 stage. The formation of PAO-1 biofilms was significantly inhibited at higher concentrations of furanone C-30.

The formation of *P. aeruginosa* biofilm is shown to be closely related to the key function of the las QS system¹⁸. *LasI* expression is observed to progressively decrease during the course of eight-day biofilm development¹⁹. In addition, the expression of *P. aeruginosa* virulence factor can be regulated by the lasR-lasI system²⁰. *P. aeruginosa* virulence is shown to be markedly reduced by interfering with the QS system, without affecting its growth^{12,21}. In this study, the increase in the levels of *lasR* expression was higher than that of *rhlR*, suggesting that negative regulation on *lasR* expression may be higher than on *rhlR*, due to the decrease of virulence factor generation. Similarly, the lower *pqsR* gene expression in this study was considered to be related to the weakened regulation by las system. Furanone C-30 as a signal molecule analogue can inhibit lasR and rhlR activation, thus regulating the expression of *lasB*, *rhIA* and *pqsR*²². It is reported that halogenated furanones (particularly C-30 & C-56) exhibit biofilm reduction and abilities of targeting las and rhl systems in *P. aeruginosa*²³. Hentzer *et al*¹² found that the majority of QS-induced genes, including major virulence factors such as *lasA*, *lasB*, *hcnAB*, *rhlAB* and *phzABCDEFG*, were repressed by furanone C-30, which might control

the infectious bacteria through acting on these QS regulators at the post-transcriptional level. Paterson²⁴ has also reported that furanone C-30 can inhibit LasR protein activation and elastase production through competing with N-(3-oxododecanoyl)-L-homoserine lactone to combine with LasR protein. In our study, the expression levels of *lasB*, *rhlA*, *phzA2*, *lasI*, *rhlI*, *pqsE* and *pqsH* were significantly decreased in mature biofilm bacteria with the increase of furanone C-30 concentrations. It can be speculated that furanone C-30 may inhibit the formation of *P. aeruginosa* biofilms through interfering with QS systems.

AmpC lactamases are known to be the key players that resist β -lactam antibiotics in *P. aeruginosa*²⁵. The expression of *AmpC* can be induced by antibiotics and its overproduction may result in cephalosporin resistance in a variety of bacteria²⁶. Since AmpC enzyme is inducible, antibiotics and furanone C-30 were simultaneously added to the bacterial culture medium in this experiment. Our results showed that *AmpC* expression, induced by imipenem and ceftazidime significantly decreased in both planktonic and mature biofilm phases with increase of furanone C-30 concentration. With the same concentration of furanone C-30, downregulation of *AmpC* expression in *P. aeruginosa* mature biofilm stage occurred to a greater extent than that in planktonic stage, which was consistent with the previous findings that QS system of *P. aeruginosa* always expressed in the late logarithmic growth stage or plateau stage of planktonic bacteria, and could regulate other genes at mature biofilm period^{27,28}. Our findings suggest that the higher expression level of *AmpC* in mature biofilm bacteria, relative to that in planktonic bacteria, may be involved in different regulatory role of QS system in planktonic and biofilm stages. However, we did not detect *AmpC* expression before testing inducibility with the drugs, which could, limit the reliability of our study.

The strength of our study was the elucidation of the association between furanone C-30 and decreased antibiotic resistance in *P. aeruginosa*. The inhibitory effect of furanone C-30 on las system was stronger than rhl system. There were some limitations in our study. First, only one strain, PAO-1, was used in this study. Second, we did not design many analytical approaches or experiments, such as multiple linear regression analysis, to further explore the effect of furanone C-30 on biofilm formation, QS system and antibiotic resistance in *P. aeruginosa*. Therefore, more

experiments and data analyses would be needed to confirm our observations.

In conclusion, our study showed that furanone C-30 inhibited biofilm formation, QS and antibiotic resistance in *P. aeruginosa* in a dose dependent manner. The inhibitory effect of furanone C-30 on las system was stronger than on rhl system. Considering the prominent role of furanone C-30 in the growth inhibition of *P. aeruginosa* clinical isolates from patients with cystic fibrosis²⁹, it may be considered as a potential drug for the treatment of *P. aeruginosa* infection. Further studies would be required to validate the clinical application of furanone C-30.

Financial support & sponsorship: This work was financially supported by the Chinese Medicine Science and Technology Development Project Fund of Shandong Province (grant No. 2017-200), Postdoctoral Applications Research Project Fund of Qingdao (grant No. 2016055), and The Affiliated Hospital of Qingdao University Youth Research Fund (2016), PR China.

Conflicts of Interest: None.

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