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Dopamine, an exogenous quorum sensing signaling molecule or a modulating factor in *Pseudomonas aeruginosa*?

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ABSTRACT

Pseudomonas aeruginosa is recognized globally as an opportunistic pathogen of considerable concern due to its high virulence and pathogenicity, especially in immunocompromised individuals. While research has identified several endogenous quorum sensing (QS) signaling molecules that enhance the virulence and pathogenicity of *P. aeruginosa*, investigations on exogenous QS signaling molecules or modulating factors remain limited. This study found that dopamine serves as an exogenous QS signaling molecule or modulating factor of *P. aeruginosa* PAO1, enhancing the production of virulence factors and biofilms. Compared to the control group, treatment with 40 μM dopamine resulted in a 33.1 % increase in biofilm formation, 68.1 % increase in swimming mobility, 63.1 % increase in swarming mobility, 147.2 % increase in the signaling molecule 3-oxo-C12-HSL, and 50.5 %, 28.5 %, 27.0 %, and 33.2 % increases in the virulence factors alginate, rhamnolipids, protease, and pyocyanin, respectively. This study further explored the mechanism of dopamine regulating the biofilm formation and virulence of *P. aeruginosa* PAO1 through transcriptome and metabolome. Transcriptomic analysis showed that dopamine promoted the expression of virulence genes *psl, alg, lasA, rhlABC*, *rml*, and *phz* in *P. aeruginosa* PAO1. Metabolomic analysis revealed changes in the concentrations of tryptophan, pyruvate, ethanolamine, glycine, 3-hydroxybutyric acid, and alizarin. Furthermore, KEGG enrichment analysis of altered genes and metabolites indicated that dopamine enhanced phenylalanine, tyrosine, and tryptophan in *P. aeruginosa* PAO1. The results of this study will contribute to the development of novel exogenous QS signaling molecules or modulating factors and advance our understanding of the interactions between *P. aeruginosa* and the host environment.

1. Introduction

As a highly opportunistic pathogen, *Pseudomonas aeruginosa* poses a considerable threat to individuals with compromised immune systems, burn injuries, and cystic fibrosis [1]. *P. aeruginosa* produces a range of virulence factors, operating in a synchronized system to enable colonization and adaptation within the host. These virulence factors, including biofilms, pyocyanin, elastase, and rhamnolipids, are regulated through quorum sensing (QS) [2], notably acyl-homoserine lactone (AHL)-mediated LasI/R and RhlI/R signaling and 2-alkyl-4-(1H)-quinolone (AQ)-mediated signaling [3]. The LasI enzyme produces N-3-oxododecanoyl-L-homoserine lactone (3-oxo-C12-HSL), which activates the LasR regulatory protein, leading to the activation of downstream QS genes. These genes encompass the *rhl* system and those responsible for encoding virulence factors like elastase [4]. RhlI is essential for generating N-butanoyl-L-homoserine lactone (C4-HSL), with the C4-HSL and RhlR complex regulating downstream genes, such as *rhlI* and *phz* for pyocyanin synthesis [5]. These two QS pathways are interconnected with the PQS system, in which 2-heptyl-3-hydroxy-4-quinolone is used as a QS signaling molecule of the PQS system [6].

The functionality of the QS system is not only regulated by signaling molecules but also by external substances, including catecholamines, which can significantly increase under environmental stress in certain host organs, reaching concentrations of up to 0.1–1 mM [7]. And in a diseased state, dopamine levels in some organs of mice can reach concentrations of 10–100 μM and even extend to millimolar levels in individual organ diagrams [8]. When low-dose dopamine (3 μg/kg/min) is injected into patients, the plasma dopamine concentration can reach

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about 0.5 μM when reaching steady state, and the steady state concentration will also increase when using medium to high concentrations [9]. At the same time, the dopamine concentration at the injection site will also be higher. Intestinal flora can synthesize catecholamines under specific conditions [10]. Catecholamines can also be produced in wound environments, facilitating potential colonization by *P. aeruginosa* or other opportunistic bacteria, and impeding wound healing [11–13]. For example, the catecholamine epinephrine may act as a global virulence signaling molecule in enterohemorrhagic *Escherichia coli* (EHEC), playing a key role in its virulence expression [14]. Similarly, norepinephrine can promote *P. aeruginosa* proliferation and up-regulate PA-I lectin expression, crucial for its pathogenicity toward intestinal epithelial cells [15,16]. Norepinephrine can also reduce the iron-binding capacity of transferrin in the host, thus facilitating bacterial growth, and enhance virulence factor production, epithelial cell invasion, and swimming motility [17,18]. In contrast, β-adrenergic receptor antagonists have been observed to modify *P. aeruginosa* biofilm formation in vitro, thereby promoting wound healing [13].

Dopamine, a neurotransmitter, shares structural similarities with the exogenous QS signaling molecules adrenaline and norepinephrine. Despite this, no studies have explored whether dopamine acts as an exogenous QS signaling molecule or modulating factor in *P. aeruginosa*. Therefore, in the current study, we investigated the effects of dopamine on biofilm formation and virulence factors, as well as the underlying mechanisms by which dopamine regulates biofilm formation and virulence based on transcriptomic and metabolomic analyses.

2. Results

2.1. Dopamine enhances P. aeruginosa PAO1 growth

Dopamine treatment resulted in an increase in the growth rate of *P. aeruginosa* PAO1 compared to the control group after 10 h (Fig. 1). A concentration of 40 μM dopamine led to a biomass increase of approximately 12.5 % compared to the control group after 22 h.

2.2. Dopamine enhances P. aeruginosa PAO1 biofilm formation

As shown in Fig. 2, biofilm formation in *P. aeruginosa* PAO1 was significantly increased in the presence of dopamine compared to the control. While a dopamine concentration of 5 μM did not yield a notable increase in biofilm formation, the concentrations of 10, 20, and 40 μM led to significant increases in biofilm formation by 22.0 %, 28.1 %, and 33.1 % respectively.

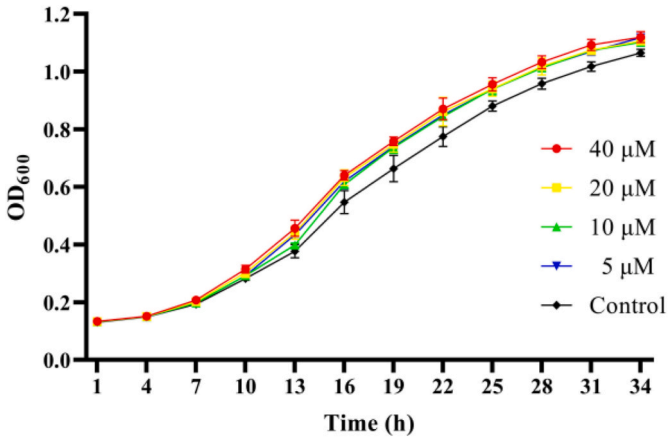

Fig. 1. Effects of dopamine on *P. aeruginosa* PAO1 growth.

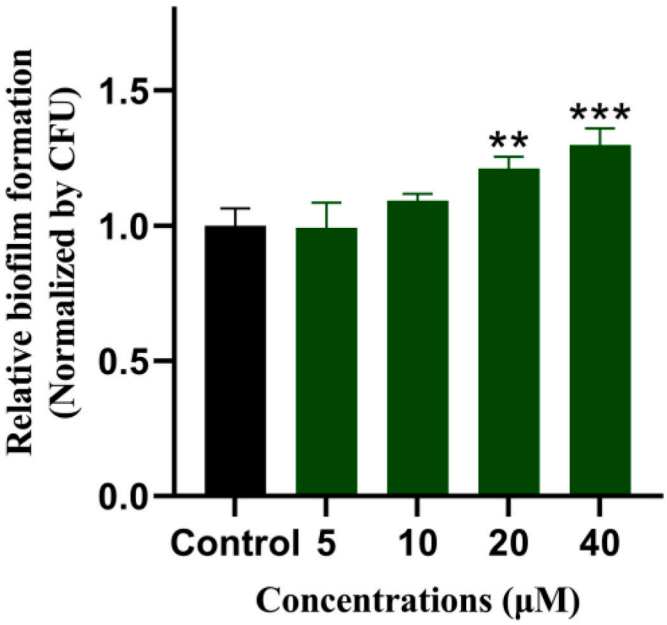

Fig. 2. Dopamine promotes *P. aeruginosa* PAO1 biofilm formation.

2.3. Dopamine enhances P. aeruginosa PAO1 motility

As shown in Fig. 3, dopamine administration markedly improved the motility of *P. aeruginosa* PAO1. Specifically, the swimming rates of *P. aeruginosa* PAO1 increased by 72.7 %, 58.3 %, and 68.1 % and the swarming rates of *P. aeruginosa* PAO1 increased by 76.6 %, 62.5 %, and 63.1 % under dopamine concentrations of 10, 20, and 40 μM dopamine, respectively.

2.4. Dopamine enhances P. aeruginosa PAO1 virulence

Dopamine treatment led to a significant rise in alginate content, with increases of approximately 40.1 % and 50.5 % observed at dopamine concentrations of 20 and 40 μM, respectively (Fig. 4A). Similarly, rhamnolipid content significantly improved following dopamine administration, with increases of 10.6 %, 18.5 %, 22.1 %, and 28.5 % at dopamine concentrations of 5, 10, 20, and 40 μM, respectively (Fig. 4B). Furthermore, protease activity significantly increased by 12.3 %, 21.0 %, and 27.0 % under dopamine concentrations of 10, 20, and 40 μM, respectively (Fig. 4C), while pyocyanin production also significantly increased by 36.0 % and 33.2 % under dopamine concentrations of 20 and 40 μM, respectively (Fig. 4D).

2.5. Dopamine promotes 3-oxo-C12-HSL production in P. aeruginosa PAO1

Dopamine significantly enhanced 3-oxo-C12-HSL synthesis in *P. aeruginosa* PAO1, with treatments at concentrations of 10, 20, and 40 μM leading to marked increases of 155.7 %, 118.5 %, and 142.7 %, respectively (Fig. 5B). Conversely, C4-HSL production in *P. aeruginosa* PAO1 was not notably influenced by dopamine exposure (Fig. 5A).

2.6. Dopamine affects expression of virulence and biofilm formation genes in P. aeruginosa PAO1

Transcriptomic analysis of groups treated with dopamine compared to the control group without dopamine revealed a total of 34 DEGs, including 11 up-regulated and 23 down-regulated DEGs (Table S1), determined based on $|\log_2\text{fold-change (FC)}| \geq 1$ and false discovery rate (FDR) ≤ 0.05 criteria.

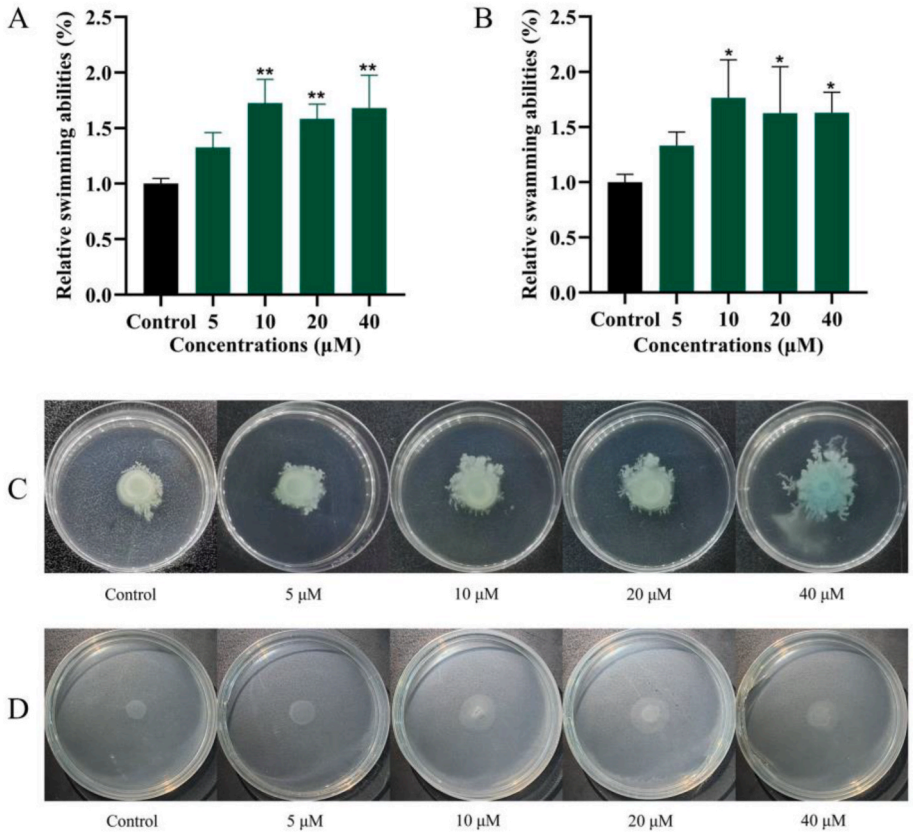

Fig. 3. Effects of dopamine on *P. aeruginosa* PAO1 motilities. A. Swimming; B. Swarming; C. Swimming; D. Swarming.

Further analysis of the genetic-level changes in QS, biofilms, and virulence factors of *P. aeruginosa* PAO1 after the addition of dopamine was conducted. All DEGs were classified into three GO categories, i.e., biological process, cellular component, and molecular function (Fig. 6A), with the distribution of DEGs across each term shown in Fig. 6B–D. Results showed that dopamine impacted the O-antigen biosynthetic process, lipopolysaccharide biosynthetic process, lipopolysaccharide metabolic process, cellular carbohydrate biosynthetic process, phenazine biosynthetic process, and cell wall of *P. aeruginosa* PAO1, thereby affecting virulence factor production, biofilm formation, and energy metabolism.

Additionally, enrichment in biological processes and functional pathways associated with the DEGs was determined based on KEGG analysis. Results revealed enrichment in pathways related to amino acid metabolism, fatty acid biosynthesis, amino sugar and nucleotide sugar metabolism, pyruvate metabolism, two-component system, and QS in *P. aeruginosa* PAO1 (Fig. 7).

Validation of virulence and biofilm formation genes through RT-qPCR showed that after dopamine treatment, the expression levels of the *fliA*, *algU*, *pslA*, *rhlA*, *phzM*, and *lasA* genes were up-regulated by 2.0-, 3.3-, 2.6-, 2.5-, 5.4-, and 9.2-fold, respectively, while *pilA* was down-regulated by 0.8-fold (Fig. 8). These findings are consistent with the transcriptome data, confirming the reliability of the transcriptomic analysis.

2.7. Metabolomic analysis

Metabolomic analysis was conducted on both the dopamine-treated groups and the control group without dopamine exposure. In total, 16 differentially expressed metabolites were identified based on a variable importance in projection (VIP) > 1, $p < 0.05$, and FC > 1.2 or FC < 0.8. Compared to the control group, six metabolites were up-regulated and 10 were down-regulated. As shown in Table 1, in the dopamine treatment group, malonamide, resveratrol, atrazine-2-hydroxy, anandamide, alizarin, and tryptophan were significantly increased, while 3-hydroxybutyric acid, glycine, aminomalonic acid, 3-hydroxyphenylacetic acid, ribose, palmitoleic acid, canavanine degr prod, di-peptide (L-Gly-L-Pro), ethanolamine, and maleimide were significantly reduced. KEGG enrichment analysis indicated that dopamine mainly affected the bacterial chemotaxis pathway, pentose phosphate pathway, and glycerophospholipid metabolism pathways in *P. aeruginosa* PAO1 (Fig. 9).

3. Discussion

P. aeruginosa represents an important pathogen, contributing to high incidence and mortality rates among immunocompromised individuals and cystic fibrosis patients. Previous studies have suggested that the presence of catecholamines, including epinephrine and norepinephrine, in the host environment can enhance the virulence and pathogenicity of *P. aeruginosa* PAO1 [19,20]. Consequently, we investigated the impact of dopamine, another known catecholamine, on the virulence of *P. aeruginosa* PAO1.

Our results indicated that dopamine promoted *P. aeruginosa* PAO1 growth, consistent with previous reports on the enhancing effects of epinephrine and norepinephrine on the proliferation of *P. aeruginosa* [19,21]. The observed elevation in metabolism of pyruvate, a critical substrate in the tricarboxylic acid (TCA) cycle, suggests enhanced energy metabolism within *P. aeruginosa* [22]. This enhanced metabolic activity may contribute to the capacity of dopamine to stimulate *P. aeruginosa* PAO1 growth. The culture medium used in this study contains ions such as Na^+, K^+, PO_4^{3-}, and Mg^{2+}, with the exogenous addition of bovine serum, simulating the human environment. In Freestone's study, the identical culture medium was employed for in vitro experiments [23]. However, the medium lacks physiological relevance to the human microenvironment, necessitating further research. In vitro experiments offer potential for subsequent in vivo studies.

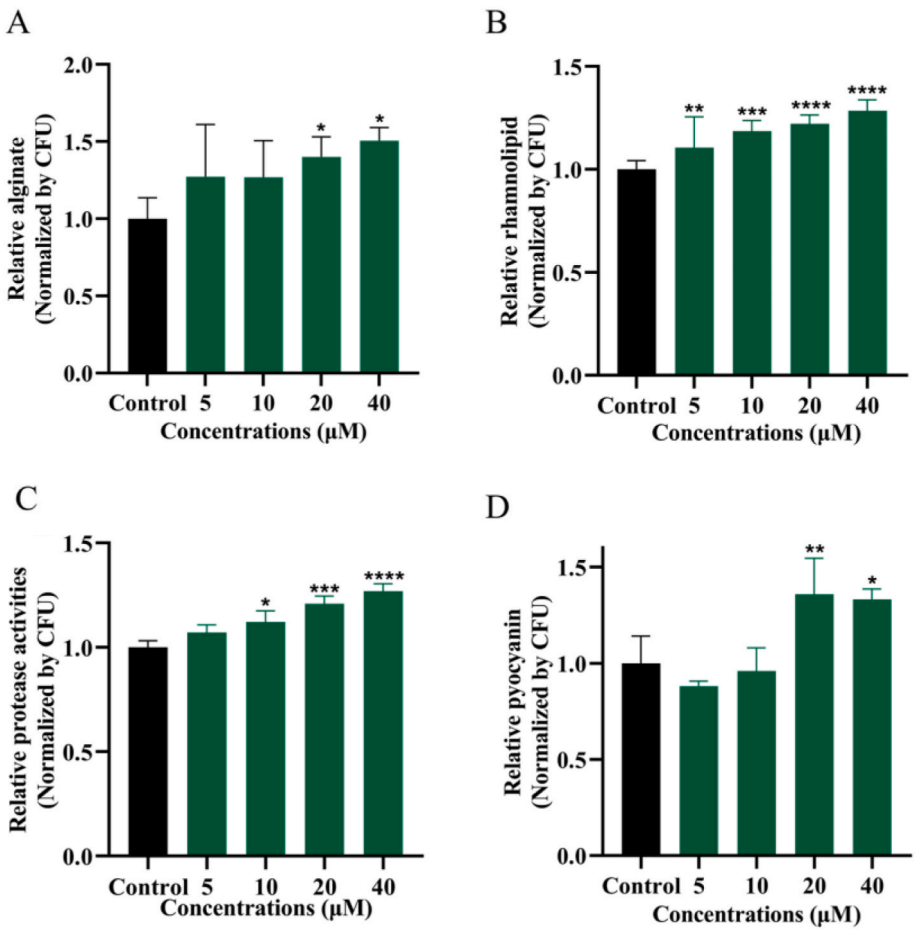

Fig. 4. Effects of dopamine on *P. aeruginosa* PAO1 virulence factors. A. Alginate; B. Rhamnolipid; C. Protease; D. Pyocyanin.

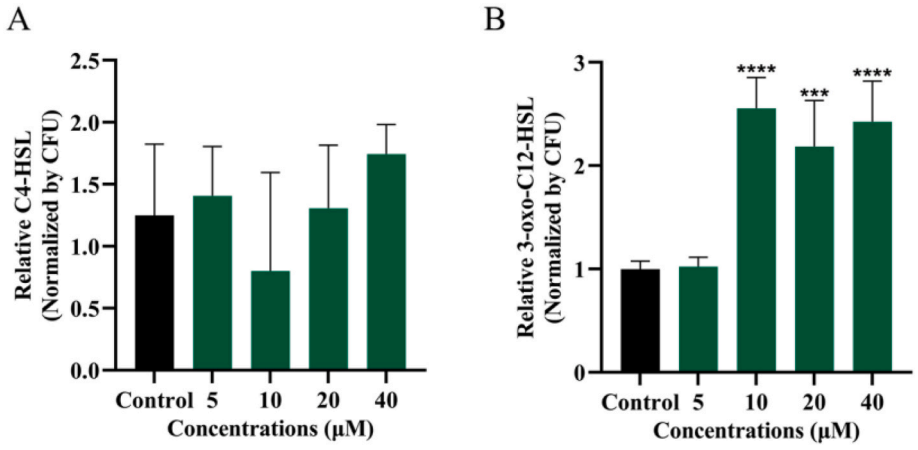

Fig. 5. Effects of dopamine on *P. aeruginosa* PAO1 signaling molecules. A. C4-HSL; B. 3-oxo-C12-HSL.

P. aeruginosa produces a variety of QS-related virulence factors, such as alginate, rhamnolipid, protease, and pyocyanin [24]. Increased levels of alginate, an extracellular polysaccharide, signal enhanced biofilm formation and virulence [25], while rhamnolipids support both biofilm formation and cell motility of *P. aeruginosa* [22]. Pyocyanin facilitates bacterial envelope formation and inhibits immune host defense, thereby enhancing the pathogenicity of *P. aeruginosa* [26]. Additionally, protease enables *P. aeruginosa* to decompose host proteins to aid in nutrient uptake [26]. In the current study, alginate, rhamnolipid, protease, and pyocyanin increased by 50.5 %, 28.5 %, 27.0 %, and 33.2 %, respectively, following dopamine (40 μM) treatment. In *P. aeruginosa*, rhamnolipid virulence factors are synthesized through the *rhlABC* regulatory system [27]. The *rmlBDAC* operon genes also participate in the rhamnolipid synthesis process [28]. Protease production is regulated by the *lasA* gene [29], while pyocyanin synthesis is regulated by the *phzABCDEFG* operon genes, with anthranilate synthase, encoded by *phnA* and *phnB*, facilitating its catalysis [30]. These operon genes are instrumental in synthesizing phenazine-1-carboxylic acid (a vital precursor of pyocyanin) from chorismic acid [31]. Our transcriptomic and RT-qPCR results showed an up-regulation in *rhlABC*, *rmlBDAC*, *lasA*, and

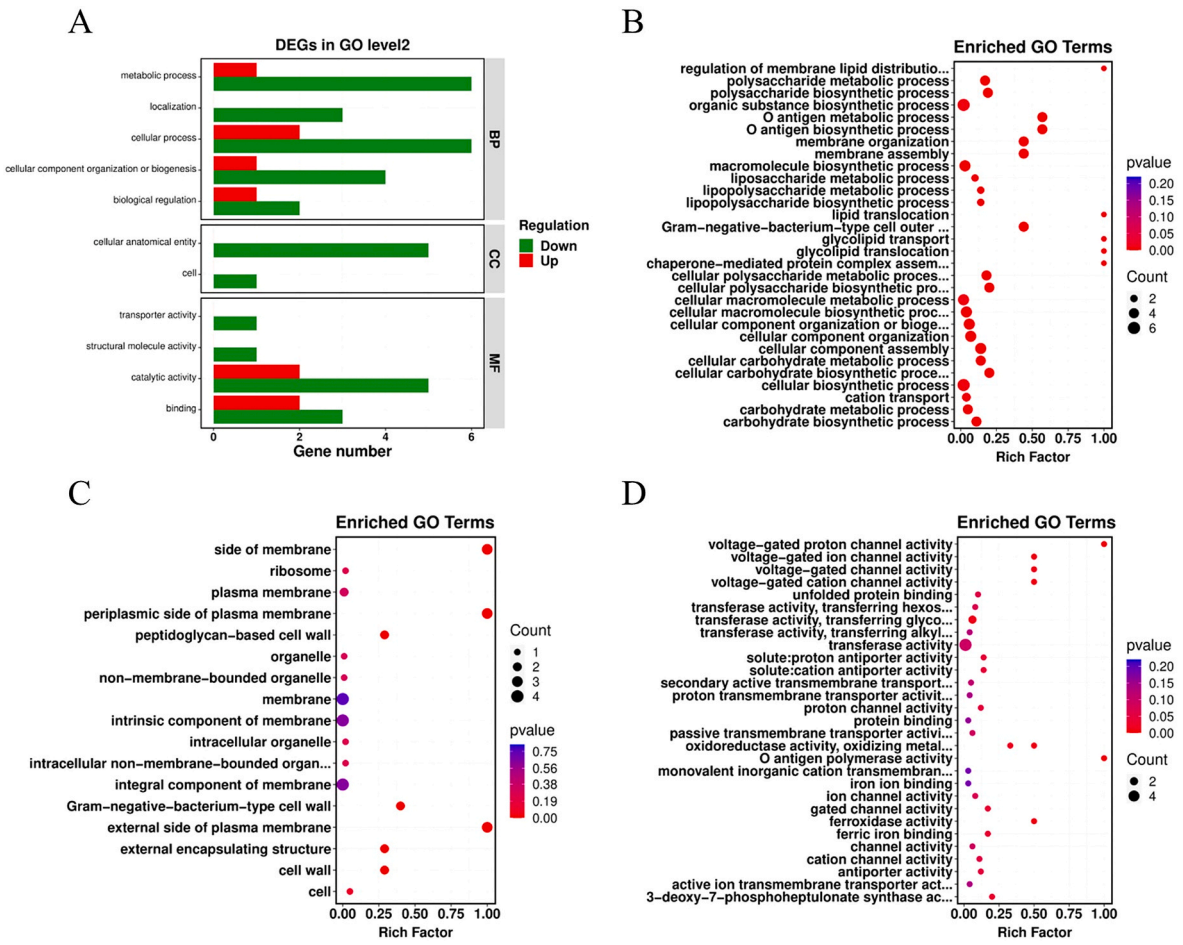

Fig. 6. GO analysis of DEGs in *P. aeruginosa* PAO1 after treatment with dopamine (40 μM). A. GO annotation statistics of DEGs; B. GO functional enrichment analysis statistics for biological processes; C. GO functional enrichment analysis statistics for cellular processes; D. GO functional enrichment analysis statistics for molecular functions.

phz-related genes (Table S3), suggesting an increase in QS-modulated virulence factors.

Biofilms formed by *P. aeruginosa* contribute to the persistence of chronic infection in hosts [24]. These biofilms are primarily composed of proteins, extracellular DNA (eDNA), and extracellular polysaccharides (EPSs) [32], which, in turn, are mainly composed of Pel, Psl, and alginate [24]. Rhamnolipids also play an important role in biofilm formation [22]. Here, dopamine exposure at 40 μM resulted in a 33.1 % increase in biofilm formation in *P. aeruginosa* PAO1, as well as a 50.5 % and 28.5 % increase in the levels of alginate and rhamnolipid, respectively. The up-regulation of *psl* and *alg*-related genes is known to enhance Psl and alginate production [33]. Notably, our RT-qPCR results confirmed the elevation of *pslA* and *algU* expression. And dopamine can not only promote the production of biofilm in *P. aeruginosa* PAO1. Simultaneously effective on both clinical strains S406 and R127 (Figs. S8 and S9).

P. aeruginosa cells utilize various motility mechanisms, including flagella and Type IV Pili (TFP) [34], enabling the bacteria to navigate toward favorable environments and target hosts [35]. The *fliA* gene is crucial for flagella assembly and motility, while *pilA*, the main subunit of TFP, plays a significant role in TFP assembly and adhesion and motility [36]. Up-regulation of *fliA* enhances flagella production and motility in *P. aeruginosa*, whereas downregulation of *pilA* may not affect, or could potentially increase, its motility under certain conditions [37]. The observed increases in *P. aeruginosa* swimming and swarming rates (68.1 % and 63.1 %, respectively) following 40 μM dopamine treatment may be attributed to the up-regulation of *fliA*. These findings suggest that *P. aeruginosa* can more effectively colonize and disseminate within the host under environmental stress, with the produced virulence factors posing a significant threat.

Studies have found that virulence factors and biofilm formation are regulated by QS systems [24], with both LasI/R and RhlI/R playing important roles in the QS system of *P. aeruginosa*. The LasI/R system uses 3-oxo-C12-HSL as its signaling molecule, while the RhlI/R system utilizes C4-HSL [4,5]. As a signaling molecule of *P. aeruginosa*, 3-oxo-C12-HSL regulates the expression of downstream genes by binding to LasR, thereby promoting the production of protease, rhamnolipids, and pyocyanin in *P. aeruginosa* [3]. This investigation revealed that while dopamine had no effect on C4-HSL levels, it promoted the production of 3-oxo-C12-HSL. The lack of effect on C4-HSL may be due to it reaching a yield threshold or undergoing metabolism and decomposition. This mechanism may explain how dopamine promotes *P. aeruginosa* PAO1 virulence. In contrast, eugenol treatment specifically reduces the expression of C4-HSL and 3-oxo-C12-HSL in *P. aeruginosa*, leading to a down-regulation in virulence and membrane functionality [38].

We further investigated the mechanism by which dopamine promotes the virulence of *P. aeruginosa* PAO1, employing transcriptomic and metabolomic analyses. Transcriptomic analysis indicated that exposure to dopamine (40 μM) resulted in a significant up-regulation in the expression of genes related to cyanoamino acid metabolism, phenylalanine, tyrosine, and tryptophan biosynthesis, carbon fixation pathways in procaryotes, nitrogen metabolism, propanoate metabolism, pyruvate metabolism, and QS in *P. aeruginosa* PAO1. Notably, in

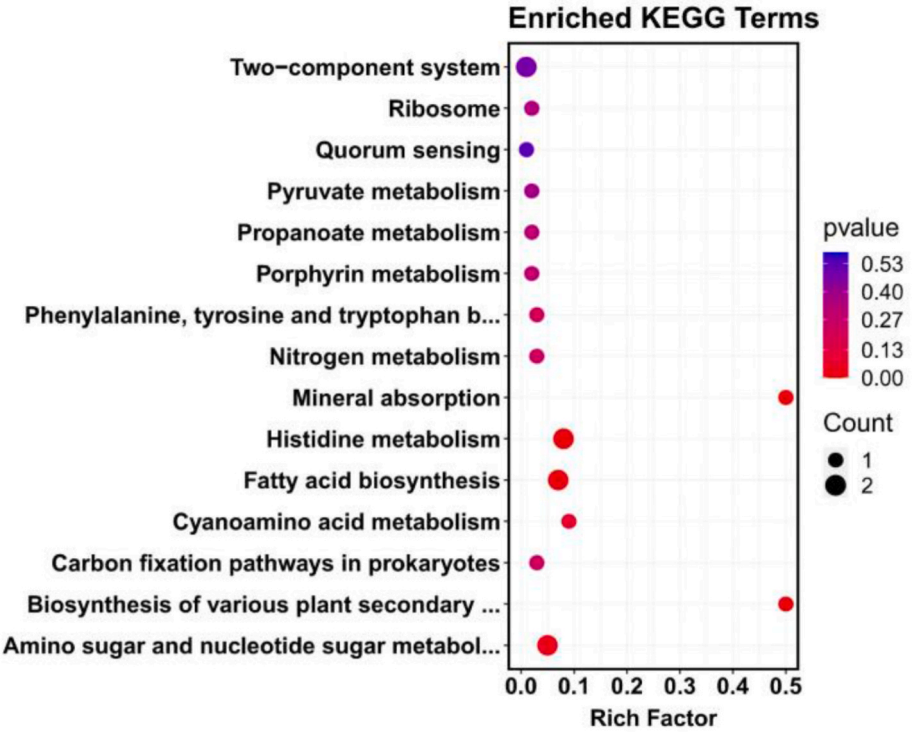

Fig. 7. KEGG enrichment analysis of DEGs in *P. aeruginosa* PAO1 after treatment with dopamine (40 μM).

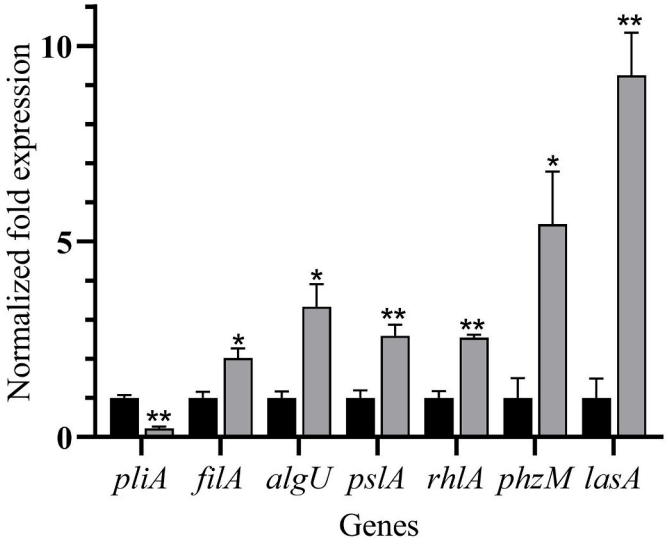

Fig. 8. Seven representative genes were evaluated for validation of transcriptomic data using RT-qPCR after treatment with dopamine (40 μM).

P. aeruginosa PAO1, pyocyanin, phenylalanine, tyrosine, and tryptophan share chorismate as a common synthetic precursor [39]. Here, the observed increase in pyocyanin and tryptophan levels based on metabolomic analysis suggests that dopamine may promote related metabolic pathways in *P. aeruginosa* PAO1, potentially linked to the structure of dopamine. The gene *phzC*, pivotal in chorismate synthesis, showed increased expression, indicating intensified metabolism and the production of related compounds [39]. This up-regulation in *phzC2* may be associated with the activation of upstream QS genes. Furthermore, ethanolamine, essential for cell membrane integrity, saw a reduction in levels, suggesting an increase in membrane permeability [40].

Table 1
Differential metabolites.

Name	VIPs	*p*-value	Fold-change
Malonamide	1.6949	0.0419	4.8746
Resveratrol	1.7282	0.0346	4.2001
Atrazine-2-hydroxy	1.7219	0.0359	4.0909
Anandamide	1.9619	0.0194	1.6510
Alizarin	1.9673	0.0132	1.5759
Tryptophan	2.0302	0.0168	1.4273
Maleimide	2.0204	0.0106	0.7559
Ethanolamine	2.1907	0.0017	0.7227
Gly-pro	2.0682	0.0201	0.6890
Canavanine degr prod	2.0056	0.0256	0.6728
Palmitoleic acid	2.0420	0.0135	0.6645
Ribose	2.1909	0.0027	0.6048
3-Hydroxyphenylacetic acid	1.9723	0.0423	0.5989
Aminomalonic acid	2.0278	0.0458	0.5413
Glycine	2.0663	0.0307	0.4870
3-Hydroxybutyric acid	1.9670	0.0480	0.3449

4. Conclusion

Dopamine, serving as an exogenous QS signaling molecule or modulating factor that significantly, enhances both biofilm development and the production of virulence factors in *P. aeruginosa* PAO1, further increasing its pathogenicity by improving energy and amino acid metabolism. Dopamine administration also induces the expression of QS-regulated genes, such as *fliA*, *algU*, *pslA*, *rhlA*, *phzM*, and *lasA*. This research contributes to a deeper understanding of the interactions between *P. aeruginosa* and dopamine, providing new evidence and explanation for the promoting effect of neurotransmitters on the virulence of *P. aeruginosa*.

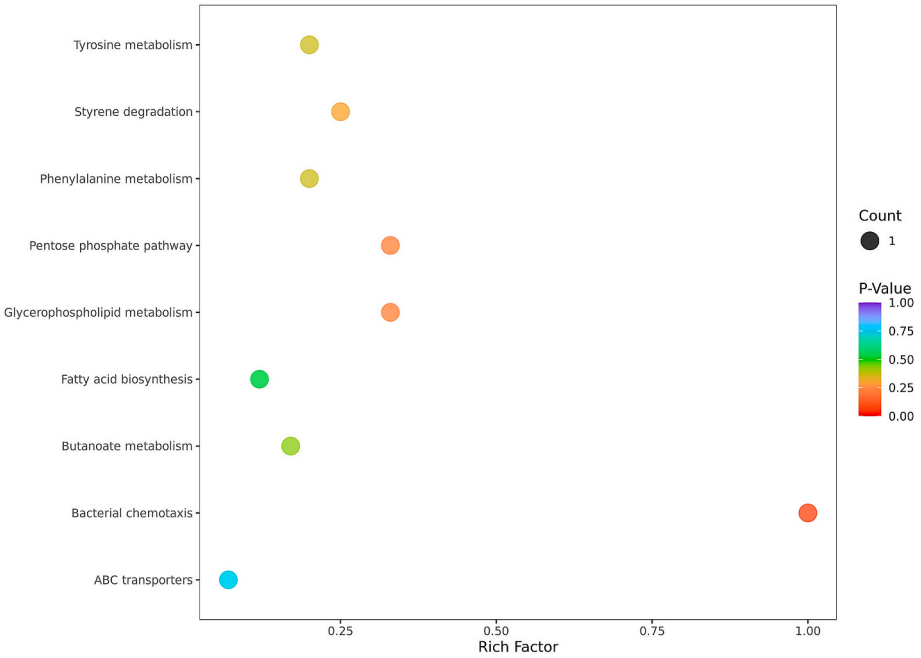

Fig. 9. KEGG enrichment analysis of differential metabolites in *P. aeruginosa* PAO1 after treatment with dopamine (40 μM).

5. Materials and methods

5.1. Chemicals and strains

Dopamine hydrochloride was purchased from Aladdin (Shanghai, China) and dissolved in ultrapure water, then filtered through a 0.22-μm sterile filter membrane. The dopamine concentration was determined following Perraud's method, with necessary adjustments [23,41]. The *P. aeruginosa* PAO1 strain (ATCC 15692) was cultured at 37 °C in Luria-Bertani broth (LB) (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) or SMI medium (85.1 mg/L $MgSO_4$, 175.3 mg/L KH_2PO_4, 174.8 mg/L KCl, 350.2 mg/L NH_4NO_3, 349.4 mg/L glucose, 30 % (v/v) bovine serum) [23].

Growth measurement of *P. aeruginosa* PAO1.

The planktonic cell growth measurement assay was performed following previous study [42], with some modifications. The *P. aeruginosa* PAO1 culture was inoculated into the SMI medium at a 1 % (v/v) ratio. Dopamine was then added to achieve final concentrations of 5, 10, 20, or 40 μM, followed by incubation at 37 °C and 180 rpm for 34 h. Growth curves were determined by measuring optical density at 600 nm (OD_{600}) (Epoch2, Biotech, USA).

5.2. Effects of biofilm formation

Biofilm formation assays were carried out according to previous research [43], with minor modifications. The overnight-grown *P. aeruginosa* PAO1 cultures were introduced into SMI medium at a 1 % (v/v) ratio [19], followed by the addition of dopamine. A 200-μL aliquot of this mixed culture was then transferred to a 96-well polystyrene microtiter plate, with final dopamine concentrations in the wells of 5, 10, 20, or 40 μM. After incubation at 37 °C for 15 h, the culture medium was carefully removed, and the wells were washed three times with sterile phosphate-buffered saline (PBS) to eliminate planktonic cells. The remaining water in the wells was evaporated under 60 °C. The biofilms adhered to the bottom of the wells were fixed with cool methanol for 15 min. The liquid was then removed, and the plate was dried at 60 °C. Each well was stained with 200 μL of a 0.05 % (w/v) crystal violet solution for 10 min, after which excess crystal violet was removed, and the wells were washed three times with PBS. After drying at 60 °C, 200 μL of 95 % ethanol was added to each well and the plate was decolorized for 15 min on a shaker at 37 °C and 180 rpm. A 150-μL sample of the decolorizing solution was taken from each well and absorbance was measured at 570 nm. The measurement results were normalized with CFU.

5.3. Motility (swimming and swarming) assays

Motility assays were performed in accordance with previous research [44]. The swimming medium consisted of 10 g/L peptone, 5 g/L NaCl, and 3 g/L agar, while the swarming medium consisted of 10 g/L peptone, 5 g/L NaCl, 5 g/L glucose, and 5 g/L agar, each supplemented with dopamine to a final concentration of 5, 10, 20, or 40 μM. The mixtures were thoroughly combined and uniformly distributed into culture dishes. Sterile water served as the negative control. After solidification, 2 μL of the *P. aeruginosa* PAO1 bacterial solution was inoculated in the center of each dish. Migration was recorded after 24 h of cultivation at 37 °C.

5.4. Measurement of alginate production

Alginate production was estimated according to established protocols [45], with minor modifications. The *P. aeruginosa* PAO1 strain was inoculated at a 0.1 % (v/v) ratio in SMI medium containing different concentrations of dopamine (5, 10, 20, 40 μM). The cultures were then incubated at 37 °C and 180 rpm for 15 h, using sterile water as the negative control. Subsequently, 1 mL of *P. aeruginosa* PAO1 in culture was mixed with 3 mL of 10 % (w/v) $CuSO_4$ and allowed to stand at room temperature for 1 h. This mixture (1 mL) was combined with 2 mL of a copper-HCl reagent (0.5 g/L $CuSO_4$, 9.6 M HCl) and 1 mL of 4 g/L 1, 3-dihydroxynaphthalene, then heated in a boiling water bath for 40 min. After cooling, 4 mL of ethyl acetate was added, and the mixture was subjected to centrifugation at 1050 × *g* for 10 min. The ethyl acetate layer was then washed twice with a 20 % (w/v) NaCl solution, with absorbance measuring at 565 nm. The measurement results were normalized with CFU.

5.5. Measurement of rhamnolipid production

Rhamnolipid content was measured based on previous study [43], with some modifications. The sample was first prepared as described earlier. After centrifugation at 4 °C and 10 000×*g* for 10 min, the supernatant was collected, boiled for 4 min, and centrifuged again at 4 °C and 10 000×*g* for 15 min. The resulting middle layer was collected and filtered through a 0.22-μm sterile filter to obtain the sample solution for testing. After centrifugation at 4 °C and 10 000×*g* for 10 min, the supernatant was boiled for 4 min and then centrifuged at 4 °C and 10 000×*g* for 15 min. The clear middle layer was filtered through a 0.22 μm sterile filter to obtain the sample solution for testing. To prepare the anthrone-sulfuric acid solution, 0.2 g of anthrone was weighed and dissolved in 100 mL of 85 % sulfuric acid. Subsequently, 200 μL of the sample solution was added to an ice bath and mixed with 800 μL of anthrone-sulfuric acid solution. The mixture was then boiled for 15 min and allowed to cool before rhamnolipid analysis, with OD measuring at 620 nm. The measurement results were normalized with CFU.

5.6. Measurement of protease production

Protease activity was quantified following methods described in previous research [46], with some modifications. In brief, skimmed milk agar was prepared by incorporating 4 % (w/v) skimmed milk powder and 1.5 % (w/v) agar into quarter-strength LB liquid medium. After creating wells, 50 μL of cultured supernatant was added, followed by incubation at 37 °C for 24 h. Protease activity was then determined by measuring the diameter of the transparent ring around the wells. The measurement results were normalized with CFU.

5.7. Measurement of pyocyanin production

Pyocyanin content was determined according to previous research [47], with some modifications. In brief, the culture supernatant was extracted using chloroform at a 5:3 (v/v) ratio. After layer separation, the lower organic phase was collected, then added to an equal volume of 0.2 M HCl and thoroughly mixed. The mixture was then centrifuged at 4 °C and 10 000×*g* for 10 min. Absorbance of the upper aqueous phase was measured at 520 nm using 200 μL of sample in a microplate reader. The measurement results were normalized with CFU.

5.8. Measurement of signaling molecule production

Overnight-cultured *P. aeruginosa* PAO1 was added to SMI medium at a 1 % (v/v) ratio and supplemented with different concentrations of dopamine (5, 10, 20, and 40 μM), with the same volume of sterile water used as the negative control. The cultures were then incubated at 37 °C and 180 rpm for 15 h, followed by centrifugation at 4 °C and 12 000 rpm for 10 min. The resulting supernatant was mixed with an equal amount of methanol and filtered using a 0.22-μm filter membrane for later use. Quantitative analysis of AHL was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS), with the detection of signaling molecules based on the peak times and ion fragments of standard reference materials. The results were quantified by integrating the peak area of the signaling molecules, normalized to the relative content of the negative control. The content of AHL was determined using the Acquity H-Class Xevo TQD LC-MS/MS system (Waters, USA) equipped with Vydac-C_{18} columns (5 μm, 4.6 × 250 mm) at an injection volume of 2 μL. Mobile phase A consisted of ddH_2O containing 0.1 % (v/v) formic acid, and mobile phase B consisted of acetonitrile, at a flow rate of 0.4 mL/min. The mobile phase gradient was set as follows: 0–5 min, 80 % (v/v) B. Analysis was conducted using positive ion mode within a scan range of 150–350 *m/z* [44]. The measurement results were normalized with CFU.

5.9. RNA sequencing (RNA-seq)

Seed solution was inoculated into SMI medium at a 1 % (w/v) ratio, supplemented with 40 μM dopamine, with sterile water used as the negative control. The mixture was incubated at 37 °C and 180 rpm for 15 h, then centrifuged at 4 °C and 10 000×*g* for 15 min. The resulting pellet was frozen in liquid nitrogen and stored at −80 °C for further analysis. Total RNA was extracted from *P. aeruginosa* PAO1 in accordance with the manufacturer's protocols (Magigene, Guangzhou, China). RNA integrity and quality were verified using a Thermo NanoDrop One and Agilent 4200 Tape Station. Upon successful quality assessment, ribosomal RNA was removed using an Epicentre Ribo-Zero rRNA Removal Kit (Illumina, USA). An RNA library was subsequently constructed using a NEBNext Ultra II Directional RNA Library Prep Kit for Illumina. Following library quality assessment, the qualified libraries were sequenced using the Illumina high-throughput sequencing platform in PE150 mode. Raw image data files were converted into raw sequencing sequences (Raw Reads) through base calling analysis. Sequencing data quality control was performed using Fastp software [48], and Bowtie2 was employed to align the quality-controlled data with ribosomal sequences from the Rfam database for ribosomal data removal [49]. RSeQC was used to determine sequencing quality [50] and RSEM was used for gene quantification [51]. Differential gene expression analysis was carried out using edgeR [52], and differentially expressed genes (DEGs) were subjected to functional analysis against the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to elucidate their roles in cellular and metabolic pathways [53].

5.10. Metabolomic experiments

Sample preparation was conducted in line with standard transcriptomic procedures. Metabolite extraction followed the guidelines provided by the manufacturer (Magigene, Guangzhou, China). Modifications were applied to certain metabolomic assay methods as indicated [54].

5.11. Quantitative real-time polymerase chain reaction (PCR)

Quantitative real-time PCR (RT-qPCR) was performed in accordance with previous study, with slight modifications [43]. Overnight-cultured *P. aeruginosa* PAO1 was introduced into SMI medium at a 1 % (v/v) ratio. The experimental group was supplemented with different concentrations of dopamine (5, 10, 20, and 40 μM), while the same volume of sterile water was used as the negative control. The culture broth was then centrifuged (8000×*g*, 4 °C, 15 min) to harvest cells, which were subsequently washed three times with diethyl pyrocarbonate (DEPC)--treated water. Total RNA from *P. aeruginosa* PAO1 was extracted using an Eastern® Super Total RNA Extraction Kit (Promega, Beijing, China) and transformed into cDNA using a Reverse Transcription Kit (Biosharp, China). Real-time PCR was performed using TB Green® Premix Ex Taq™ II FAST qPCR Mix (TaKaRa, Dalian, China) with a Bio-Rad CFX Connect System (Bio-Rad, USA). The primers used for PCR are listed in Table S2. The *rpsL* gene was used as the internal control.

5.12. Statistical analysis

Unless otherwise stated, all experiments were performed independently in triplicate. The Kolmogorov-Smirnov test was used to test the normality and homogeneity of variance for all data. The data shown in the figures are means ± standard deviation (SD) of the replicates. Statistical differences were determined using one-way analysis of variance (ANOVA) or *t*-test in GraphPad Prism v8, with $p < 0.05$ considered statistically significant.

CRediT authorship contribution statement

Shi-Liang Xiang: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Kai-Zhong Xu:** Writing – original draft, Formal analysis, Data curation. **Lu-Jun Yin:** Writing – original draft, Software, Methodology. **Yong Rao:** Methodology, Investigation. **Bo Wang:** Supervision, Funding acquisition. **Ai-Qun Jia:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare have no competing financial interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2024.100208.

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