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Original Article

THE EFFECT OF KNOCKING OUT OF PVDP GENE IN THE VIRULENCE OF *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Objective: The aim of the study is to investigate the role of PvdP enzyme in the virulence of *Pseudomonas aeruginosa* both *in vitro* and *in vivo*.

Methods: In this study, we investigate the effect of deletion of pvdP gene on *P. aeruginosa* by observing its phenotypes. The observed phenotypes are the growth, pyoverdine production, motilities, 3-oxo-C12-Homo Serine Lactone (3-oxo-C12-HSL) accumulation and biofilm formation. The growth and pyoverdine production were investigated under a low iron condition, while the motilities of the mutant were investigated in a semi-solid media. The accumulation 3-oxo-C12-HSL was facilitated by a biosensor strain and biofilm formation was investigated using a spectrophotometer through a crystal violet staining method. The *in vivo* study was performed to *Galleria mellonella* larvae as an infection model.

Results: The deletion of the pvdP gene does not affect the growth of the *P. aeruginosa* but significantly reduces the production of pyoverdine. The motility properties of the bacteria were not affected by the deletion of the pvdP gene. The *P. aeruginosa* PvdP knock-out mutant also showed a reduction in the biofilm formation and the accumulation of 3-oxo-C12-HSL at low iron concentrations. In an *in vivo* experiment, the PvdP knock-out mutant caused a significantly reduced death rate of *G. mellonella* larvae infection model compared to the control group.

Conclusion: The findings underscore the major role of PvdP in pyoverdine production, its contribution to biofilm formation, and the motility of *P. aeruginosa.* Those results confirm the important role of PvdP in the virulence of *P. aeruginosa in vitro* and *in vivo*.

Keywords: Iron, *P. aeruginosa*, Pyoverdine, PvdP, Virulence factor, Biofilm, *G. mellonella*

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INTRODUCTION

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium. It can infect almost every organ of immunocompromised patients suffering from diseases such as cystic fibrosis, burn wounds, and Acquired Immunodeficiency Syndrome (AIDS) [1]. In addition, it is known as one of the multi-drug resistant (MDR) pathogens due to its resistance to many antibiotics [2].

Like any other organism, bacteria need iron for survival and growth. This requirement conflicts with the conditions that bacteria must cope with in the process of infecting their hosts, which are characterized by low iron concentrations. To resolve the lack of iron, bacteria secrete iron chelators named siderophore to capture and secure sufficient iron supply from the environment. The major siderophore of *P. aeruginosa* is known as pyoverdine [3], which causes the green fluorescence phenotype upon its secretion having a high affinity for iron [4], whereas the second siderophore is pyochelin, having a low affinity for iron [5].

The biosynthesis of pyoverdine is a complex process involving several enzymes that are contained partly in the cytoplasm and the periplasm. At least 14 enzymes have been identified to be involved in the biosynthesis of pyoverdine. In the cytoplasm, there are 4 Non-Ribosomal Peptide Synthetases (NRPs): PvdL, PvdI, PvdJ, and PvdD catalyze the assembly of the backbone of pyoverdine. In addition, the involvement of PvdA, PvdF, and PvdH catalyzes the subsequent formation of acylated ferribactin as the precursor of pyoverdine [6]. Next, the acylated ferribactin is transported into the periplasm, and PvdQ deacetylates it [3]. Subsequently, PvdP catalyzes the oxidative cyclization [7] and PvdO facilitates the final oxidation [8]. Finally, the side-chain modification is catalyzed by PvdN [9] or PtaA [10], resulting in mature pyoverdine that is secreted via the transport system PvdRT-OmpQ [11, 12].

Recently, PvdP has been investigated as a target of interest to develop a novel treatment against *P. aeruginosa* infections. Even though the role of this enzyme in pyoverdine biosynthesis has been elucidated [7] and the crystal structure has been solved [13]. However, the correlation between the existence of the PvdP enzyme and the virulence factor related to the pyoverdine production of *P. aeruginosa* remains uninvestigated.

Therefore, in this study, the phenotypes of the PvdP knock-out mutant were analyzed for pyoverdine production, biofilm formation, and motility. In addition, the effect of the PvdP knock-out mutant on production of 3-oxo-C12-HSL as bacterial cell-to-cell communication medium was investigated under low iron conditions. Furthermore, to know the effect of the absence of PvdP in the infection setting, the mutant was injected in a *Galleria mellonella* infection model.

MATERIALS AND METHODS

Bacterial strains and media

The bacterial strains used in this study were *P. aeruginosa* PAO1, *P. aeruginosa* PAO1_PvdP knockout [14], and *Escherichia coli* (pSB1075) [15]. The bacterial cells were inoculated in Luria-Bertani (LB) media and LB agar. The low iron media is Casamino Acids (CAA) media containing 5 g casamino acids, 1.54 g $K_2HPO_4.3H_2O$, and 0.25 g $MgSO_4.7H_2O$ [15].

Bacterial growth and pyoverdine production

To investigate the effect of knocking out of PvdP on growth and pyoverdine production, we inoculated the strain (*P. aeruginosa* PAO1_PvdP knock out) in CAA media. The overnight culture of the strain in CAA media was diluted at 1:50 by adding fresh CAA media. White clear round-bottom 96-well plates (VWR®, The US) were used to measure the growth and production of pyoverdine. The growth (OD_{600}) and the pyoverdine production (A_{405}) were measured every hour for 20 h at 37 °C. The experiments were done in triplicate.

Swimming assay

Swimming assays were performed in LB media containing 0.3% agar (w/v). Two µl** of an overnight culture of the bacteria strains (*P. aeruginosa* PAO1 and *P. aeruginosa* PAO1_PvdP knock out) were

spot inoculated in separate 6-well flat-bottom plates (VWR®, The US) and the swimming zone was observed after 24 h incubation at 37 °C. The experiments were done in triplicate.

Swarming assay

Swarming assays were performed in LB media containing 0.5% agar (w/v). To the solid media on the plates, 2 µl** of each overnight bacterial culture was inoculated in separate 6-well flat-bottom plates (VWR®, The US). After 24 h incubation at 37 °C, the swarming area was measured, and the experiments were done in triplicate.

Biofilm formation

The *P. aeruginosa* PAO1 and *P. aeruginosa* PAO1_PvdP knock-out strains were grown overnight in LB liquid medium at 37 °C. The optical density (OD_{600}) of each strain was measured with a cell density meter (Biochrom Ultrospec 10, UK). Bacterial cells were harvested from the overnight cultures and resuspended to OD_{600} = 1.6 in 2-times concentrated LB medium. Clear round-bottom polystyrene 96-well plates (VWR®, The US) were used to test biofilm formation. The experiments were started by adding 50 µl** of the bacterial solution to the designated wells and 50 µl** of Phosphate-Buffered Saline (PBS) buffer was used as the control. After incubation for 18 h at 37 °C, the planktonic cells were removed from the wells. The wells were washed 3 times with 110 µl** of PBS and heat fixated at 60 °C for 1 h. Subsequently, the wells were stained with 110 µl** of 0.1% crystal violet for 15 min. The excess crystal violet was removed under tap water. Next, the plates were air-dried, and the wells were eluted with 110 µl** of 30% acetic acid to release the crystal violet. New plates were prepared to dilute (1:10) of the biofilm solution. The absorbance of the biofilm solution was measured at 585 nm using a microplate reader (SPECTROstar® Omega, BMG Labtech, Germany). Afterward, the results were evaluated.

Determination of N-acyl homoserine lactones (AHLs)

To investigate the effect of knocking out of PvdP on the production of signaling molecule (3-oxo-C12-HSL), we inoculated the strains (*P. aeruginosa* PAO1 and *P. aeruginosa* PAO1_PvdP knock out) in CAA media. The production of 3-oxo-C12-HSL was measured through a bioassay facilitated by a biosensor strain *E. coli* (pSB1075) where the amount of 3-oxo-C12-HSL is correlated to the amount of light produced by the strains. After 24 h incubation at 37 °C in CAA media, biofilm supernatants of the *P. aeruginosa* PAO1 and *P. aeruginosa* PAO1_PvdP knockout were collected. Following centrifugation for 10 min, the supernatants were filtered using a 0.2 µm pore filter and stored at-20 °C. The biosensor assay was started by adding 20 µl** of each supernatant to 180 µl** of overnight 1:100 dilution *E. coli* (pSB1075) culture in a round-bottom 96-well plate (VWR®, The US). The amount of light produced by the strain was recorded every 60 min for 20 h using a microplate reader (FLUOstar® Omega, BMG Labtech, Germany) at 37 °C.

Preparation of bacterial inoculum for testing in an animal model

The procedure followed the previously published protocol by Koch *et al.* [16]. The LB media of overnight *P. aeruginosa* PAO1 culture was spined down (5,000 rpm, 10 min, 4 °C) and replaced with fresh LB media then reinoculated at 37 °C in a 200-rpm shaking incubator to reach OD_{600} = 0.1 (~10^8 cfu/ml). Subsequently, the inoculum was centrifuged again, the media was replaced with PBS, and the concentration of the cell was adjusted to ~10^3 cfu/ml. The suspension was stored on ice until further use. The same procedure was conducted to prepare the inoculum of the PvdP knockout mutant.

***Galleria mellonella* larvae injection**

Twelve larvae per group were selected randomly (weight 250-300 mg, purchased in Fritz Kuiper Fishing Shop, Groningen). Each bacterial suspension (*P. aeruginosa* PAO1 and *P. aeruginosa* PAO1_PvdP knock out) was injected (1 ml insulin syringe, needle 25G, volume 20 µl**) into every larva of the groups via the last left proleg with the addition of two groups (PBS and untreated). Subsequently, the larvae were stored in separate petri dishes according to their treatments and incubated at 37 °C for four days, and the death rate was recorded daily.

RESULTS AND DISCUSSION

The antibiotic resistance of is still a serious health problem nowadays. In addition, the trend between the finding of new antibiotics does not able to catch up the development of bacterial resistance to antibiotics [17]. Even though some studies have been performed to find new antibiotics, including candidates from natural source [18] or repurposing the existing Food Drug Administration (FDA-approved) drugs [19] but this approach is still not enough to solve the problem. Therefore, an alternative treatment is urgently needed. To find an alternative treatment for infection especially caused by *P. aeruginosa,* we need a novel drug target. PvdP, a tyrosinase that is known for its role for the maturation of pyoverdine [7], serves as a promising chance to be targeted. To further confirm the effect of PvdP enzyme to the virulence of *P. aeruginosa* both *in vitro* and *in vivo*, we investigated the role of PvdP enzyme through a *P. aeruginosa*_PvdP knockout mutant.

Influence of PvdP on bacterial growth and pyoverdine production in low iron condition

The effect of PvdP on the growth and pyoverdine production was investigated in CAA media. It is clearly shown in the absence of PvdP, after an overnight incubation, the strain grew normally at the same level as the wildtype (fig. 1A). Instead, the pyoverdine production of the knockout mutant was significantly impaired compared to the wild type (fig. 1B). The mutant demonstrates that the deletion of the pvdP gene resulted in the reduction of pyoverdine. Regardless of the absence of pyoverdine, the mutant is still able to grow normally. A similar result was also reported for the PvdQ knock-out mutant [15].

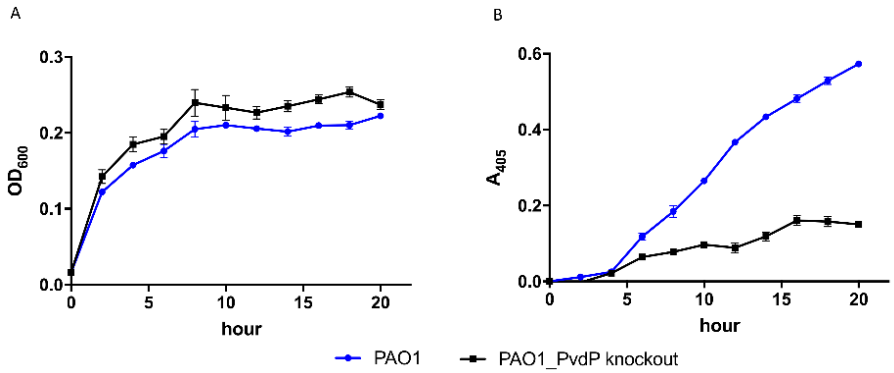

Fig. 1: Comparison between the PvdP knockout mutant and the wild type on the growth and the pyoverdine production in low iron conditions. The knocking out of the pvdP gene does not influence the growth of the *P. aeruginosa* mutant (A), while its pyoverdine production is significantly reduced (B). Error bars stand for SD

Effect of PvdP on swimming and swarming motility

It is known that *P. aeruginosa* has three different types of motilities. In this report, we investigated the effect of PvdP on swimming and swarming motility. On the swimming assay, we did not see any change between the mutant strain and the wild type (fig. 2A). This suggests that there is no direct correlation between pyoverdine production and the swimming motility of the bacteria. On the other hand, the swarming motility enhanced slightly in the mutant compared to the wild type (fig. 2B). This is in good correlation with a previous report where the biofilm-deficient mutant showed enhanced swarming motility [20].

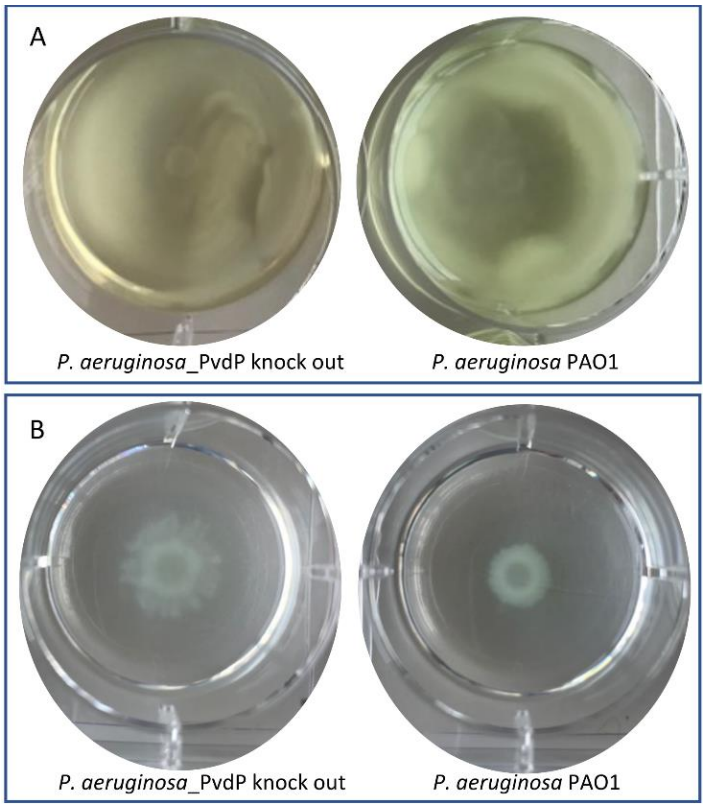

Fig. 2: The effect of PvdP on the motilities of *P. aeruginosa*. The swimming motility of *P. aeruginosa* PAO1 wild type and *P. aeruginosa* PAO1_PvdP knockout mutant was studied in the LB media added with 0.3% agar with no obvious difference between the 2 strains (A), the swarming motility was performed in the media supplemented with 0.5% agar and the *P. aeruginosa* PvdP knockout shows enhanced swarming motility (B). Error bars stand for SD

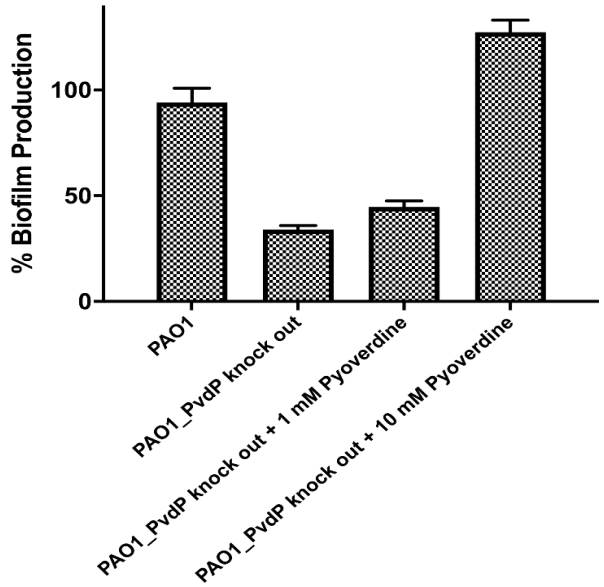

Fig. 3: The effect of PvdP on biofilm formation. The biofilm formation in the mutant is significantly reduced compared to the wild type. The supplementation of purified pyoverdine could restore the biofilm formation on the mutant strain. The strains were grown in CAA media at 37 °C. Error bars stand for SD

Further, the mutation does not influence the motility of the mutant. The same phenomenon was also reported previously on the PvdQ deletion mutant [15]. It could be expected since PvdP and PvdQ are working on the same pathway for the production of pyoverdine. Therefore, the knockout of both enzymes independently gives the same effect on the swimming and swarming motility.

Influence of PvdP on biofilm formation

Previous studies have demonstrated the reduction of biofilm formation in a pyoverdine-deficient mutant and the biofilm could be restored by the addition of an iron source [21]. In another study, Jimenez *et al.* reported their PvdQ knock-out mutant was also unable to form biofilm and the addition of an iron source was unable to restore the ability of the strain to form biofilm [15]. In our study and similar to previous studies mentioned above, the biofilm formation of the PvdP-knock-out mutant in CAA media is significantly reduced in comparison to the wild type based on the crystal violet staining. To gain a better understanding of the correlation between the iron acquisition facilitated by pyoverdine and biofilm formation, we did a follow-up experiment. In this experiment, we supplemented purified pyoverdine to the PvdP knock-out mutant culture. The supplementation of pyoverdine could fully restore the biofilm formation (fig. 3).

The absence of biofilm formation in the mutant confirms the regulation of biofilm formation is facilitated by the iron acquisition system. In addition, the supplementation of purified pyoverdine in the media enables the mutant to restore biofilm formation. Meaning the mutant can capture the iron from the environment regardless of its ability to produce pyoverdine on its own. Consequently, the mutant can obtain a sufficient level of intracellular iron. When a sufficient level of intracellular iron is reached, it serves as the signal for biofilm formation in *P. aeruginosa* [22]. Therefore, the biofilm formation is restored.

Detection of 3-oxo-C12-HSL

Quorum-sensing communication of *P. aeruginosa* involves a signaling molecule. The 3-oxo-C12-HSL is known as the quorum-sensing communication molecule of *P. aeruginosa* [23]. It was reported that PvdQ is an Ntn-hydrolase having a role to deacetylate the 3-oxo-C12-HSL as confirmed by two other studies [24, 25]. The deacetylation of 3-oxo-C12-HSL causes the bacteria losing the ability to "communicate". A study reported an increase of 3-oxo-C12-HSL levels in the ΔPvdQ mutant [15].

To know the effect of PvdP to the production of 3-oxo-C12-HSL as well, we performed a bioassay facilitated by a biosensor strain *E. coli* (pSB1075). Our bioassay results also showed a significant increase of 3-oxo-C12-HSL levels in the PvdP knock-out mutant (fig. 4). As shown, in the absence of PvdP, the level of 3-oxo-C12-HSL increases significantly.

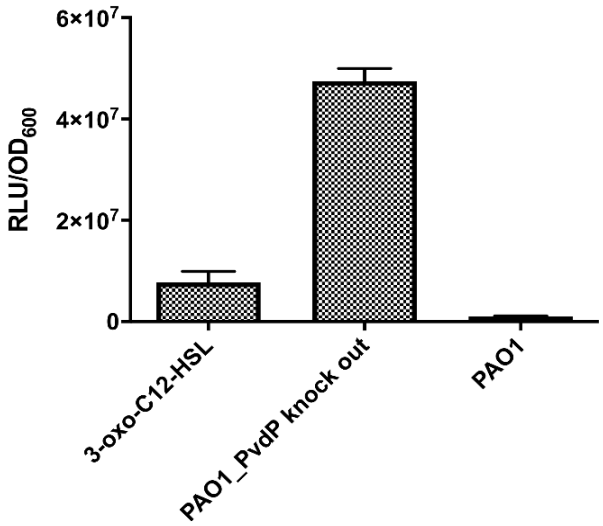

Fig. 4: The 3-oxo-C12-HSL levels of PAO1 and PAO1_PvdP knock-out mutant. The light produced by biosensor strain (*E. coli* pSB1075) in response to the amount of 3-oxo-C12-HSL produced by the strains. The assays were performed overnight at 37 °C. Error bars stand for SD

The finding of this study, which is the deletion of the pvdP gene influences the production of 3-oxo-C12-HSL is unpredictable. One might speculate that the increase of 3-oxo-C12-HSL levels is caused by the deletion of the pvdP gene, which would also affect the expression of the pvdQ gene, resulting in less degradation of 3-oxo-C12-HSL level. As both genes, however, are not located next to each other on the genome, this explanation is less likely. Possibly the accumulation of non-cyclized ferribactin has an inhibiting effect on the PvdQ enzyme.

Effect of PvdP on the virulence of P. aeruginosa in *G. mellonella*

To know the effect of PvdP on an infection model, we did a follow-up investigation. We studied the effect of knocking out PvdP on the virulence of *P. aeruginosa* in the *G. mellonella* larvae infection model. The same amount (~10^3 cfu/ml) of both strains (PAO1 and PvdP knockout mutant) was injected into two different groups in addition to two other groups (untreated, PBS) as control groups of *G. mellonella* larvae. The larvae were incubated for 4 days at 37 °C and the death rate was recorded daily.

Our results show the PvdP-knock-out mutant is not virulent to the Galleria larvae. When applying the wild type to the larvae, the death rate reaches more than 50%; in contrast, the death rate decreases significantly in the mutant (fig. 5). The mutant strain is avirulent to the infection model regardless of the enhancement of 3-oxo-C12-HSL levels.

At last, the deletion of PvdP, which is responsible for the biosynthesis of pyoverdine has been proven to reduce the virulence of *P. aeruginosa in vivo*. Apparently, the PvdP-related virulence factors (pyoverdine, biofilm, and motility) have a more dominant effect on the virulence of *P. aeruginosa* than the existence of 3-oxo-C12-HSL. This result is in line with the result of the previous report where the reduction of pyoverdine production, disruption of biofilm formation, and the increased level of 3-oxo-C12-HSL leads to the avirulent mutant in the *Caenorhabditis elegans* infection model [15].

Knowing the result where the mutant is avirulent in the Galleria larvae and the pharmacokinetic data of Galleria is directly correlated to the human data [26], this implies that PvdP is an important enzyme to regulate the virulence of *P. aeruginosa in vivo*. Our finding is a starting point to implement the concept of inhibition of PvdP in humans. The synthesis and development of a compound that specifically binds to PvdP should disrupt the production of pyoverdine and biofilm formation, resulting in the reduction of the virulence of *P. aeruginosa.*

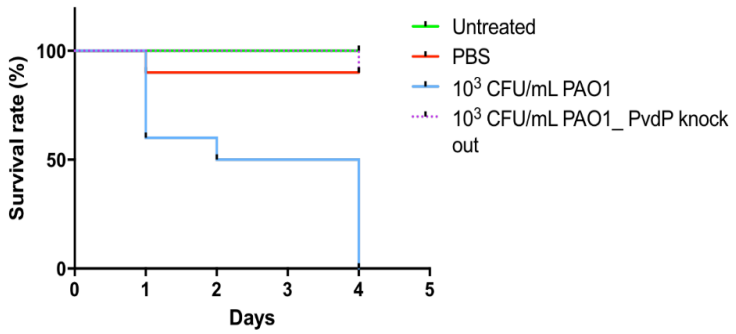

Fig. 5: The survival rate of *G. mellonella* larvae. The larvae were divided into 4 groups based on the treatment (green line= untreated; red line= PBS; sky blue line= 10^3 cfu/ml *P. aeruginosa* PAO1, purple dotted line= 10^3 cfu/ml *P. aeruginosa* PAO1_PvdP knock out)

CONCLUSION

Pyoverdine is a major siderophore in *P. aeruginosa.* The biosynthesis of pyoverdine involves the catalytic activity of tyrosinase PvdP. The knockout of pvdP gene resulted in the reduction of pyoverdine production and biofilm formation, changes in motilities, and the increase of 3-oxo-C12-HSL. However, bacterial growth is unaffected, probably due to the ability of another iron uptake system to provide a sufficient amount of iron for the growth. PvdP knock-out mutant caused a significantly reduced death rate in the *G. mellonella* larvae infection model. Those results confirm the role of PvdP in the virulence of *P. aeruginosa in vitro* and *in vivo*.

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AUTHORS CONTRIBUTIONS

JPW executed the work, analysed the results and wrote the manuscript; FJD planned the experiments and supervise the execution; WJQ reviewed and approved the final manuscript.

CONFLICTS OF INTERESTS

The authors declare no conflict of interest.

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