University of Groningen

# Controlling the virulence of Pseudomonas aeruginosa through inhibiting the synthesis of pyoverdine

Wibowo, Joko

*DOI:*
10.33612/diss.218605187

**IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.**

*Document Version*
Publisher's PDF, also known as Version of record

*Publication date:*
2022

Link to publication in University of Groningen/UMCG research database

*Citation for published version (APA):*
Wibowo, J. (2022). *Controlling the virulence of Pseudomonas aeruginosa through inhibiting the synthesis of pyoverdine*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen. https://doi.org/10.33612/diss.218605187

**Copyright**
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

**Take-down policy**
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

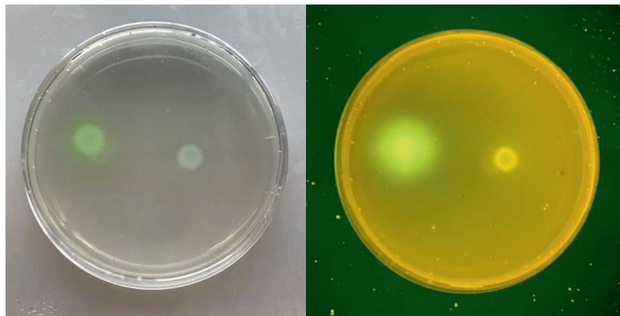
*Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.*

Download date: 25-04-2025

Chapter 2

# The role of PvdP in the virulence of *Pseudomonas aeruginosa*

Joko P. Wibowo, Frank J. Dekker, Wim J. Quax



*Manuscript in preparation.*

**Abstract**

Iron is an important substance for every living organism, including the pathogen *Pseudomonas aeruginosa*. However, the bioavailability of iron is very low in its host. To gain access to enough iron, *P. aeruginosa* developed an iron uptake mechanism by secreting a major siderophore named pyoverdine. The production of pyoverdine proceeds via a biosynthetic pathway involving several enzymatically catalyzed steps. One of those enzymes is PvdP, which is known for its role in maturating pyoverdine in the final step of pyoverdine biosynthesis. Nevertheless, the correlation between PvdP, pyoverdine production, and other virulence factors of *P. aeruginosa* remains uninvestigated. In this study, we report the role of PvdP in pyoverdine production and its influence on other virulence factors. The deletion of the *pvdP* gene does not affect the growth of the bacteria but significantly reduces the production of pyoverdine. The PvdP knockout mutant also showed a reduction in biofilm formation and the accumulation of 3-oxo-C12-HSL at low iron concentrations. In addition, a *Galleria mellonella* infection model showed the deletion of the *pvdP* gene results in the reduction of virulence. Taken together the data underscore the major role of PvdP in pyoverdine production, its contribution to biofilm formation, and the motility of *P. aeruginosa*.

Keywords: *P. aeruginosa*, iron, pyoverdine, PvdP

## 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 HIV. In addition, it is known as one of the multi-drug resistant (MDR) pathogens due to its resistance to many antibiotics.

Like any other organism, bacteria need iron for survival and growth. This requirement conflicts with the conditions that bacteria have to 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[1], which causes the green fluorescence phenotype upon its secretion having a high affinity for iron[2], whereas the second siderophore is pyochelin has a low affinity for iron[3].

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[4]. Next, the acylated ferribactin is transported into the periplasm then PvdQ deacetylates it[1]. Subsequently, PvdP catalyzes the oxidative cyclization[5] and PvdO facilitates the final oxidation[6]. Finally, the side-chain modification is catalyzed by PvdN[7] or PtaA[8] resulting in mature pyoverdine that is secreted out of the cell via the transport system PvdRT-OmpQ[9], [10].

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 the pyoverdine biosynthesis has been elucidated[5] and the crystal structure has been solved[11], the role of this enzyme in the virulence-related to pyoverdine production of *P. aeruginosa* remains uninvestigated. Therefore, knowing the role of PvdP in the virulence of *P. aeruginosa* could be a proof of concept in the inhibition of PvdP as a novel strategy against the infections.

In this study, the phenotypes of a PvdP knockout mutant were analyzed for pyoverdine production, biofilm formation, and motilities. In addition, the effect of the PvdP knockout mutant on homoserine-mediated bacterial cell-to-cell communication was investigated under low iron conditions. Furthermore, the effect of PvdP knockout in infection was investigated in a *Galleria mellonella* infection model. This study demonstrates the importance of PvdP as an essential enzyme to regulate the virulence of *P. aeruginosa* by controlling pyoverdine production, biofilm formation, and cell-to-cell communication; the absence of this enzyme showed the reduced virulence in the *G. mellonella* infection model.

## Materials and Methods

### Bacterial strains and media

The bacterial strains used in this study were *P. aeruginosa* PAO1, PAO1_PvdP knockout[12], and *Escherichia coli* (pSB1075)[13]. The bacterial cells were inoculated in LB (Luria-Bertani) media and LB agar. The low iron media is CAA media containing 5 g casamino acids, 1.54 g $K_2HPO_4.3H_2O$, 0.25 g $MgSO_4.7H_2O$[13].

### Growth and pyoverdine production

To investigate the effect of knocking out PvdP on the growth and the pyoverdine production, we inoculated the strain (PAO1_PvdP knockout) 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 the production of pyoverdine. The growth ($OD_{600}$) and the pyoverdine production ($A_{405}$) was measured every hour for 20 h at 37 $^oC$. 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 PAO1_PvdP knockout) 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 $^oC$. 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, two µL of each overnight bacterial culture was inoculated in separate 6-well flat-bottom plates (VWR, The US). After 24 h incubation at 37 $^oC$, the swarming area was measured, and the experiments were done in triplicate.

### Biofilm formation

The P. *aeruginosa* PAO1 and PAO1_PvdP knockout 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). 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-wells 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 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 three 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 minutes. 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) 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)**

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 strain. After 24 h incubation at 37 $^{o}$C in LB media, biofilm supernatants of the PAO1 and 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 $^{o}$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 $^{o}$C.



**Preparation of inoculum for testing in an animal model**

The procedure was following the previously published protocol by Koch *et al.*[14]. The LB media of overnight *P. aeruginosa* PAO1 culture was spined down (5000 rpm, 10 minutes, 4 $^{o}$C) and replaced with fresh LB media then reinoculated at 37 $^{o}$C in a 200-rpm shaking incubator to reach $OD6_{00}$ = 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 knockout) 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 $^{o}$C for four days and the death rate was recorded daily.

**Results and Discussions**

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

The effect of PvdP on growth and pyoverdine production was investigated in CAA media. It is clearly shown in the absence of PvdP, the strain grew normally at the same level as the wildtype (**Figure 1A**). Instead, the pyoverdine production was significantly impaired in the knockout mutant compared to the wildtype (**Figure 1B**). The results confirmed the deletion of the *pvdP* gene resulted in the reduction of pyoverdine in the mutant strain but in the absence of pyoverdine, the strain is still able to grow normally. A similar result was also reported for the PvdQ knockout mutant[13].

It means another iron chelator, possibly pyochelin is still sufficient to provide iron for the growth of the bacteria.

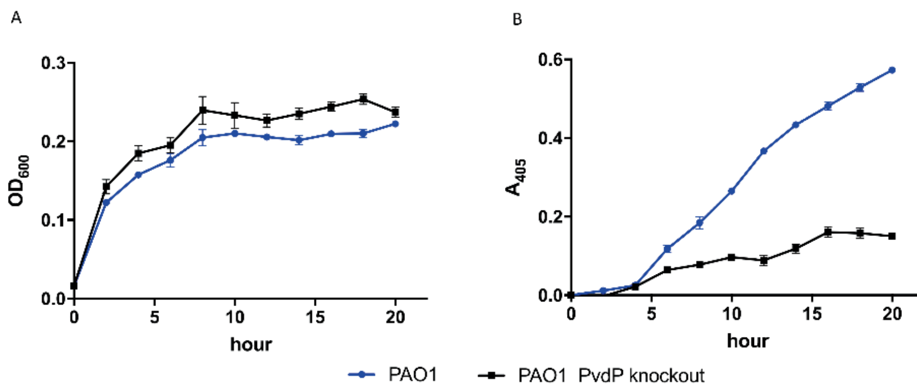



**Figure 1.** Comparison between the PvdP knockout mutant and the wildtype of *P. aeruginosa* PAO1 on the growth (A) and the pyoverdine production (B) in low iron condition.

**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 wildtype (**Figure 2A**). This is suggesting 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 wildtype (**Figure 2B**). This is in good correlation with a previous report where the biofilm-deficient mutant showed enhanced swarming motility[15]. The same phenomenon was also previously reported on the PvdQ deletion mutant[13]. It could be expected since PvdP and PvdQ are working on the same pathway for the production of pyoverdine. Therefore, the knockout of each enzyme independently gave the same effect on the 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[16]. In another study, Jimenez et al. reported their PvdQ knockout 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[13]. In our study and similar to previous studies mentioned above, the biofilm formation of the PvdP-knockout mutant in CAA media is significantly reduced in comparison to the wildtype based on the crystal violet staining.

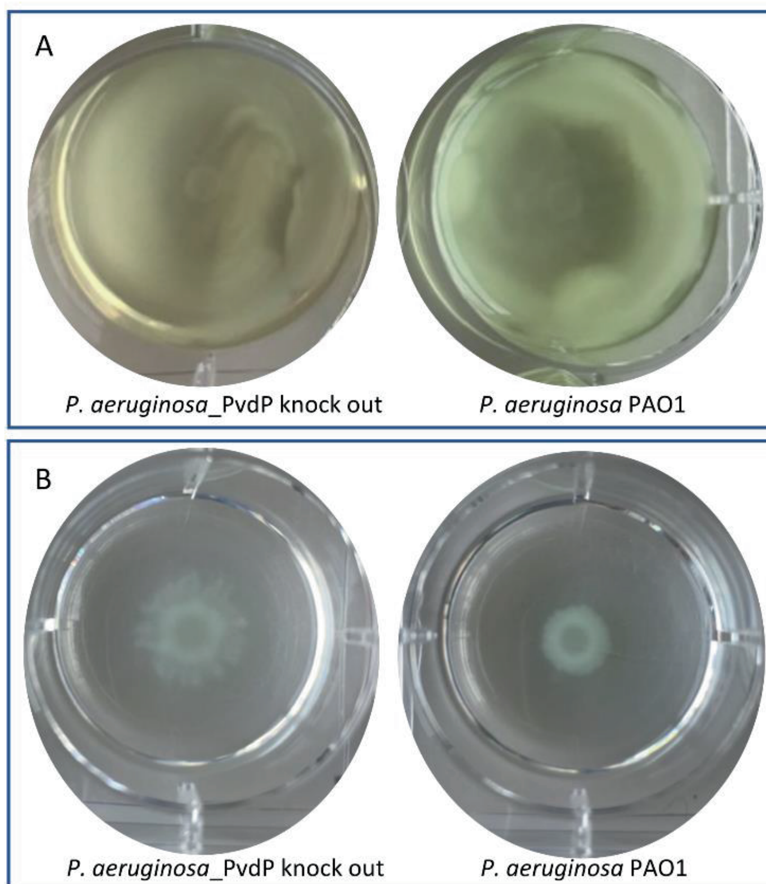



**Figure 2.** The effect of knocking out of PvdP on the motility of *P. aeruginosa.* The swimming motility of PAO1 wild type and PAO1_PvdP knockout mutant was studied in the LB media added with 0.3 % agar with no obvious difference between the 2 strains (A), the swarming motility was performed in the media supplemented with 0.5 % agar and it can be seen that the PvdP knockout shows enhanced swarming (B).

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 PAO1_PvdP knockout mutant culture. The supplementation of pyoverdine could fully restore the biofilm formation (**Figure 3**). It confirms the regulation of biofilm formation facilitated by the iron acquisition system. The supplementation of purified pyoverdine enables the mutant to capture the iron from the environment regardless of its ability to produce pyoverdine. In consequence, the mutant can obtain a sufficient level of intracellular iron. Where a sufficient level of intracellular iron serves as the signal for biofilm formation[17].

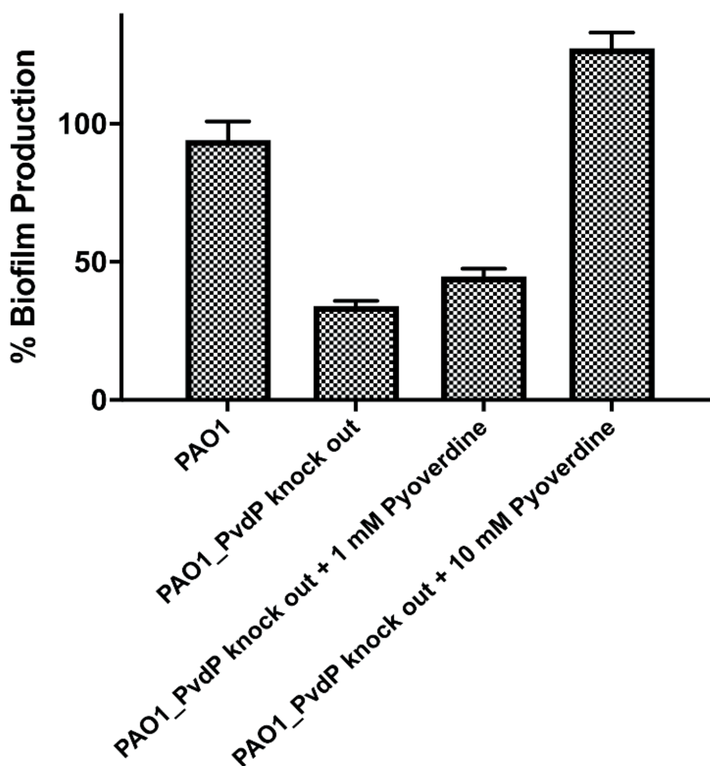



**Figure 3**. The effect of knocking out of PvdP on biofilm formation, the strains were grown in CAA media at 37 °C. The biofilm formation on the PAO1_PvdP knockout mutant is significantly reduced. The supplementation of purified pyoverdine could restore the biofilm formation on the mutant strain.

**Detection of 3-oxo-C12-HSL**

The 3-oxo-C12-HSL has been characterized as the quorum sensing communication molecule of *P. aeruginosa*. A study reported the increase of 3-oxo-C12-HSL levels in the ΔPvdQ mutant[13]. In that study, it was clearly demonstrated that PvdQ is an Ntn-hydrolase that deacetylates the long-chain AHLs as confirmed by two other studies[18], [19]. Therefore, in the absence of PvdQ the level of 3-oxo-C12- HSL increases significantly.

Our bioassay results also showed a significant increase of 3-oxo-C12-HSL levels in the PvdP knockout mutant (**Figure 4**). 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 as a result the inhibition of PvdQ caused the significant increasing concentration of 3-oxo-C12-HSL.

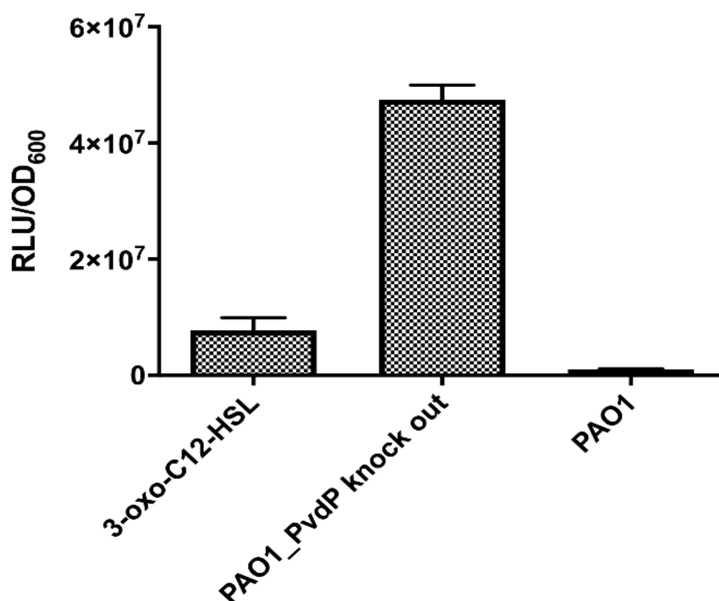



**Figure 4**. The 3-oxo-C12-HSL levels of PAO1 and PAO1_PvdP knockout mutant in comparison to the pure 3-oxo-C12-HSL. The light produced by the biosensor strain (*E. coli* pSB1075) in response to the amount of 3-oxo-C12-HSL produced by the strains. The assays were performed overnight at 37 °C.

**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) were 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 d at 37 °C and the death rate was recorded daily.

Our results show the PvdP-knockout 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 (**Figure 5**). The mutant strain is obviously avirulent to the infection model regardless of the enhancement of 3-oxo-C12-HSL levels. Apparently, the PvdP-related virulence factors (pyoverdine, biofilm, and motility) have a more dominant effect on the virulence of *P. aeruginosa* than 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[13].

Finally, the deletion of an enzyme that is responsible for the biosynthesis of pyoverdine has been proven to reduce the virulence of *P. aeruginosa in vitro* and *in vivo*. 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[22], this implies that PvdP is an important enzyme to regulate the virulence of *P. aeruginosa in vivo*. The application of this concept (the inhibition of PvdP) in humans by developing 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*.

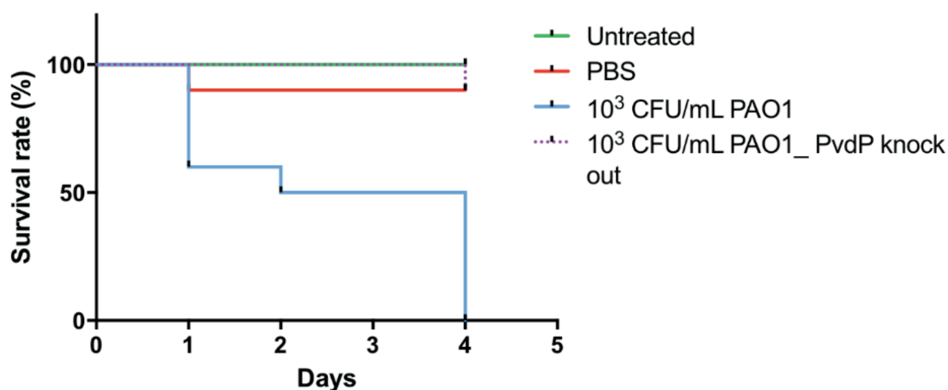



**Figure 5.** The survival rate of *G. mellonella* larvae. The larvae were divided into 4 groups (green line: untreated; red line: PBS; sky blue line: $10^3$ CFU/mL PAO1, purple dotted line: $10^3$ CFU/mL PAO1_PvdP knockout).

## Conclusions

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

## Acknowledgments

The authors thank Pol Nadal Jimenez for providing the P. *aeruginosa* PAO1_PvdP knockout and Jan G.T. Vogel for providing biosensor strain (E. *coli* pSB1075) and

sharing the skill to perform the biofilm and biosensor assay. JPW thanks to Indonesia Endowment Fund for Education (LPDP) Republic of Indonesia for the financial support of the project.

**References**

[1] P. Visca, F. Imperi, and I. L. Lamont, “Pyoverdine siderophores: from biogenesis to biosignificance,” *Trends Microbiol.*, vol. 15, no. 1, pp. 22–30, 2007, doi: 10.1016/j.tim.2006.11.004.

[2] C. Gessard, “On the Blue and Green Coloration that Appears on Bandages,” *Rev. Infect. Dis.*, vol. 6, no. Supplement_3, pp. S775–S776, Sep. 1984, doi: 10.1093/CLINIDS/6.SUPPLEMENT_3.S775.

[3] C. D. Cox, “Effect of pyochelin on the virulence of Pseudomonas aeruginosa,” *Infect. Immun.*, vol. 36, no. 1, pp. 17–23, 1982.

[4] I. J. Schalk and L. Guillon, “Pyoverdine biosynthesis and secretion in Pseudomonas aeruginosa: implications for metal homeostasis,” *Environ. Microbiol.*, vol. 15, no. 6, pp. 1661–1673, 2013, doi: 10.1111/1462-2920.12013.

[5] P. Nadal-Jimenez *et al.*, “PvdP is a tyrosinase that drives maturation of the pyoverdine chromophore in Pseudomonas aeruginosa,” *J. Bacteriol.*, vol. 196, no. 14, pp. 2681–2690, 2014, doi: 10.1128/JB.01376-13.

[6] M. T. Ringel, G. Dräger, and T. Brüser, “PvdO is required for the oxidation of dihydropyoverdine as the last step of fluorophore formation in Pseudomonas fluorescens,” *J. Biol. Chem.*, vol. 293, no. 7, pp. 2330–2341, 2018, doi: 10.1074/jbc.RA117.000121.

[7] M. T. Ringel, G. Dräger, and T. Brüser, “PvdN enzyme catalyzes a periplasmic pyoverdine modification,” *J. Biol. Chem.*, vol. 291, no. 46, pp. 23929–23938, 2016, doi: 10.1074/jbc.M116.755611.

[8] M. T. Ringel, G. Dräger, and T. Brüser, “The periplasmic transaminase PtaA of Pseudomonas fluorescens converts the glutamic acid residue at the pyoverdine fluorophore to -ketoglutaric acid,” *J. Biol. Chem.*, vol. 292, no. 45, pp. 18660–18671, 2017, doi: 10.1074/jbc.M117.812545.

[9] E. Yeterian, L. W. Martin, I. L. Lamont, and I. J. Schalk, “An efflux pump is required for siderophore recycling by Pseudomonas aeruginosa,” *Environ. Microbiol. Rep.*, vol. 2, no. 3, pp. 412–418, Jun. 2010, doi: 10.1111/j.1758-2229.2009.00115.x.

[10] M. Hannauer, E. Yeterian, L. W. Martin, I. L. Lamont, and I. J. Schalk, “An efflux pump is involved in secretion of newly synthesized siderophore by Pseudomonas aeruginosa,” *FEBS Lett.*, vol. 584, pp. 4751–4755, 2010, doi: 10.1016/j.febslet.2010.10.051.

[11] J. P. Wibowo, F. A. Batista, N. van Oosterwijk, M. R. Groves, F. J. Dekker, and W. J. Quax, “A novel mechanism of inhibition by phenylthiourea on PvdP, a tyrosinase synthesizing pyoverdine of Pseudomonas aeruginosa,” *Int. J. Biol. Macromol.*, vol. 146, pp. 212–221, Mar. 2020, doi: 10.1016/j.ijbiomac.2019.12.252.

[12] I. L. Lamont and L. W. Martin, “Identification and characterization of novel pyoverdine synthesis genes in Pseudomonas aeruginosa,” *Microbiology*, vol. 149, pp. 833–842, 2003, doi: 10.1099/mic.0.26085-0.

[13] P. Nadal-Jimenez *et al.*, “Role of PvdQ in Pseudomonas aeruginosa virulence under iron-limiting conditions,” *Microbiology*, vol. 156, no. 1, pp. 49–59, 2010, doi: 10.1099/mic.0.030973-0.

[14] G. Koch, P. N. Jimenez, R. H. Cool, and W. J. Quax, “Assessing Pseudomonas Virulence with Nonmammalian Host: Galleria mellonella,” in *Pseudomonas Methods and Protocols, Methods in Molecular Biology*, vol. 1149, A. Folloux and J.-L. Ramos, Eds. New York: Humana Press, 2014, pp. 681–688.

[15] N. C. Caiazza, R. M. Q. Shanks, and G. A. O'Toole, “Rhamnolipids Modulate Swarming Motility Patterns of Pseudomonas aeruginosa,” *J. Bacteriol.*, vol. 187, no. 21, pp. 7351–7361, Nov. 2005, doi: 10.1128/JB.187.21.7351-7361.2005.

[16] G. M. Patriquin, E. Banin, C. Gilmour, R. Tuchman, E. P. Greenberg, and K. Poole, “Influence of Quorum Sensing and Iron on Twitching Motility and Biofilm Formation in Pseudomonas aeruginosa,” *J. Bacteriol.*, vol. 190, no. 2, pp. 662–671, Jan. 2008, doi: 10.1128/JB.01473-07.

[17] E. Banin, M. L. Vasil, and E. P. Greenberg, “Iron and Pseudomonas aeruginosa biofilm formation,” *PNAS*, vol. 102, no. 31, pp. 11076–11081, 2005.

[18] P. K. Singh, A. L. Schaefer, M. R. Parsek, T. O. Moninger, M. J. Welsh, and E. P. Greenberg, “Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms,” *Nature*, vol. 407, no. 6805, pp. 762–764, Oct. 2000, doi: 10.1038/35037627.

[19] M. Hentzer, L. Eberl, and M. Givskov, “Transcriptome analysis of Pseudomonas aeruginosa biofilm development: anaerobic respiration and iron limitation,” *Biofilms*, vol. 2, no. 1, pp. 37–61, Jan. 2005, doi: 10.1017/S1479050505001699.

[20] A. Latifi, M. Foglino, K. Tanaka, P. Williams3, and A. Lazdunski, “A hierarchical quorum-sensing cascade in Pseudomonas aeruginosa links the transcriptional activators LasR and RhlR (VsmR) to expression of the stationary-phase sigma factor RpoS,” *Mol. Microbiol.*, vol. 21, no. 6, pp. 1137–1146, 1996.

[21] J.-M. Meyer, A. Neely, A. Stintzi, C. Georges, and I. A. Holder, “Pyoverdin Is Essential for Virulence of Pseudomonas aeruginosa,” *Infect. Immun.*, vol. 64, no. 2, pp. 518–523, 1996.

[22] R. J. Thomas *et al.*, “Galleria mellonella as a model system to test the pharmacokinetics and efficacy of antibiotics against Burkholderia pseudomallei,” *Int. J. Antimicrob. Agents*, vol. 41, no. 4, pp. 330–336, Apr. 2013, doi: 10.1016/j.ijantimicag.2012.12.009.