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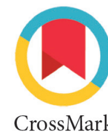

The character of PA3235 virulence factors of *Pseudomonas aeruginosa* PAO1 – a preliminary study

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ABSTRACT

Introduction: Many virulence factors of *Pseudomonas aeruginosa* PAO1 are regulated by temperature and host conditions upon infection. Based on microarray data, the PA3235 gene is one of the upregulated genes during cell growth at 37 °C. Until now, no information about its role in PAO1 pathogenicity.

Methods: The PAO1ΔPA3235 strain was constructed by overlapping polymerase chain reaction (PCR) method and through biparental mating. The deletion was confirmed by PCR and restriction analyses. The virulence factors of both *P. aeruginosa* PAO1 wild type and ΔPA3235 mutant strains were examined, which consisted of the amount of pyocyanin and pyoverdine, swarming, swimming, and twitching motility, biofilm formation, 3-oxo-dodecanoyl-homoserine lactone concentration, and growth curve profile. Data were analyzed using Student's t-tests to determine differences between treatments. P-value < 0.05 were considered significant.

Results: The ΔPA3235 strain was successfully constructed. At 37 ºC, the mutant produced less pyocyanin (p-value 0.0004), pyoverdine (p-value 0.0009), and swarm area than the wild-type. The dendrites pattern of both strains was similar. The mutant and parental strains showed no differences in swimming and twitching motility when incubated at 22 ºC and 37 ºC. The mutant produced more biofilm compared to the wild-type strain (p-value 0.0013). The AHL was higher in the mutant than in wild type strain (p-value 0.0095) after 24 h incubation. Both the wild type and mutant strains exhibited similar growth patterns in LB broth. The mutant colonies also showed the same morphology as the wild type on the LB plate (not shown here).

Conclusion: The deletion of the PA3235 gene from the *Pseudomonas aeruginosa* genome caused some changes in virulence factors production, as the bacterium grew at body temperature 37°C. We predicted that the PA3235 gene might function to transport molecules involved in the early infection of this bacterium to humans.

Keywords: pathogenicity, pyocyanin, pyoverdine, swarming, swimming, twitching.
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INTRODUCTION

Pseudomonas aeruginosa PAO1 is a Gram-negative bacterium that is commonly used as a model microorganism for studying biofilm, quorum sensing and pathogenesis. Although commonly found in soils and aquatic habitats, it can occupy a wide range of hosts, including humans, plants, and animals.[1-3] *P. aeruginosa* is a major human nosocomial pathogen, responsible for acute or chronic infections in immune-compromised patients, especially in cystic fibrosis patients.

The ability of *P. aeruginosa* to colonize and grow under a wide range of habitats relies on a few factors, such as its large genome size which provides complexity and adaptability properties, as well as a broad metabolic capacity.[4,5] *P. aeruginosa* that colonizes patients persistently usually has unique regulatory gene features by producing manifold receptors for adherence to the target cells or by expression of adaptable genes which escort the cell growth in diverse habitats, including in blood.[1,6-9]

P. aeruginosa PAO1 and *P. aeruginosa* isolated from patients express the virulence genes at 37 ºC.[10] Some virulence factors, including those involved in quorum sensing, are temperature dependent.[6,10,11] One of the genes regulated by temperature is the PA3235 gene. PA3235 is a locus tag on *P. aeruginosa* PAO1 genome. The PA3235 genes are predicted to encode a hypothetical membrane protein, *yjcH*.[12,13]

Information about the roles of the PA3235 gene in nature or the pathogenicity of *P. aeruginosa* cells is scarce. Currently, information about this gene is based on the similarities of the nucleotide and protein sequences to other known sequences, and from transcriptomic studies. Microarray data from Barbier et al. exhibited that this gene belongs to temperature and quorum-sensing-dependent genes.[10] When the cells were cultured in the whole blood of human volunteers, the gene was expressed 16 times higher compared to cells grown in the common medium Luria Bertani Broth.[8] All those studies indicate that the PA3235 gene might be involved in *P. aeruginosa's* pathogenicity against

mammals or when the bacterium infects the human body.

To investigate the involvement of the gene in the pathogenicity of *P. aeruginosa* at 37 °C, we constructed a deleted PA3235 (ΔPA3235) mutant from the parental strain. The ΔPA3235 mutant was subjected to some assays to understand the roles of the gene. In this study, we proved that the PA3235 gene might be involved in some virulence factors level of *P. aeruginosa* during cell growth at 37 °C, the human body temperature.

MATERIALS AND METHODS

Bacterial strains and growth

Bacterial strains and plasmids used in this study are listed in Table 1. Otherwise mention else, cells were grown in Luria Bertani (LB) broth or LB agar (Merck). Gentamicin (Sigma) was added to the medium at a final concentration of 10 mg.mL^{-1} for Escherichia coli or 30 mg.mL^{-1} for *P. aeruginosa* cultures.

Construction of PAO1ΔPA3235 strain

The upstream region of PA3235 was first amplified using ForA and RevA primers, whereas the downstream region was amplified using ForB and RevB primers pair (Table 2). Both amplicons were purified and ligated using T4 DNA Ligase (Promega), forming a flanking fusion region without the PA3235 gene. The fusion fragment was further amplified using RevA and ForB primers pair and digested using *Sac*I and *Eco*RI (Thermo Scientific). The purified fragment was then cloned into pEX18Gm plasmid and transformed into E. coli S17-1λpir cell.[14,15] The pEX-ΔPA3235 recombinant plasmid was transferred into *P. aeruginosa* through biparental mating. Overnight culture of *P. aeruginosa* PAO1 (diluted 10-fold) was incubated at 42 °C prior to mating. The mixture of donor and recipient cells (3:1) was incubated on an LB agar plate for 16 h at 30 °C. The selection of transconjugants was done on an LB plate containing 60 mg of L^{-1} gentamicin. After the allelic exchange mechanism, transconjugants were counter-selected in an LB agar plate supplemented with 10 % sucrose. The deletion was confirmed by PCR using RevA and ForB primers pair and by restriction analyses.

Pyocyanin production

Pyocyanin was extracted using the method described by Essar et al.[16] Overnight culture was first adjusted to OD_{600} value of 1.0 in LB broth and diluted 100-fold in 25 mL LB. The culture was then incubated at 37 °C or 22 °C for 24 h with 200 rpm shaking. The pyocyanin was extracted from 5 mL supernatant of the culture using 3 mL chloroform. Two milliliters of the organic layer were transferred to a new tube and extracted again using 1 mL 0.2 M HCl. The re-extracted pyocyanin was measured at 520 nm. The concentration of pyocyanin (μg.mL^{-1}) was determined as (OD_{520} x 17.072/OD_{600}). The assay was done in triplicates.

Pyoverdine concentration measurement

Overnight culture of wild-type and ΔPA3235 mutant strain was adjusted to OD_{600} of 1.0 and diluted 100-fold in 25 mL LB. After 24 h incubation at 37 °C or 22 °C, cell-free supernatant containing pyoverdine was measured at 405 nm.

Motility Assay

Swarming, swimming, and twitching motility assays were evaluated for both wild-type and ΔPA3235 mutant strains to see whether the deletion affects bacterial motility. A swarming medium was made by mixing 8 g.L^{-1} Nutrient Broth (Merck) and 0.5 % agar. The pH of the medium was adjusted to 7.5. The medium was supplemented with 5 g.L^{-1} filter-sterilized glucose solution added separately after autoclaving. As much as 2 μL culture (OD_{600} of 0.5) was inoculated on the surface of the medium and let dry for several minutes. The plates were incubated upside-down for 24 h.[17] Swimming medium was made of 10 g.L-1 tryptone and 5 g.L-1 NaCl, mixed with 0.3 % agar. Both strains were inoculated as 1 μL culture (OD_{600} of 0.5) in LB broth on the surface of the medium. The halo diameter was measured after 24 h incubation at 22 °C and 37 °C.[18] Twitching motility was done using LB medium mixed with 1 % agar. The overnight culture of both strains in LB broth was adjusted to OD_{600} 0.5 and inoculated by stabbing into the LB agar. After 24 h incubation at 22 °C and 37 °C, the medium was stained using Coomassie Brilliant Blue (CBB) R-250 and the diameter of the stained area was recorded.

Table 1. Bacterial strains and plasmids used in the study.

Strains	Relevant characteristic	Source or reference
P. aeruginosa PAO1	Wild-type	Holloway collection
P. aeruginosa PAO1ΔPA3235	Derivative of PAO1, PA3235 deleted	This study
E. coli S17-1 λ*pir*	Δ*ara-leu7697* Δ*lacX74* Δ*phoAPvuII phoR araD139 ahpC galE galK rpsL* F′[lac^{+} (lacI^{q}) pro] gor522::Tn10 (Tc^{R}) *trxB*::kan	[15]
E. coli JM109 pSB1075	Biosensor for sensing 3O-C12-HSL	[20]
Plasmid		
pEX18Gm	Gm^{R}, *oriT*$^{+}$ *sacB*$^{+}$, vector for gene replacement	[14]
pEX-Δ*pa3235*	pEX18Gm holding flanking region of PA3235 gene	This study

Table 2. Primers used in this study.

Primers Name	Sequence (5'→3')	Position (NC_002516)	Target
ForA	AATACTGCAGCCTCAAGGAGGCGCAGCAATGAT	3623178-3623156	Upstream of PA3235 region
RevA EcoRI	CCTAGAATTCGACATGGTAGTTGAGGCCGAACAG	3622608-3622632	
ForB SacI	TGCCGAGCTCCTGGATTCCGCACTGGATGTTC	3623962-3623941	Downstream of PA3235 region
RevB	CGGCAAGCTTCGCTGGTAGATGCTGTCGTTCA	3623446-3623467	

Crystal violet biofilm assay

Biofilm formation was measured using crystal violet staining as described by Merritt et al. (2005) with modifications.[19] Overnight culture of both wild-type and ΔPA3235 mutant was adjusted to OD_{600} of 0.001 in LB broth. The culture was then inoculated in 96-well polystyrene microtiter as much as 100 µL per well and incubated at 22 °C and 37 °C for 24 h. Biofilm was washed using NaCl 0.9 % three times and fixated with methanol. Staining was done using 1 % crystal violet solution for 30 minutes. The rest of the crystal violet was washed using 0.9 % NaCl solution. Stained biofilm was diluted in 100 µL of 33 % acetic acid and the absorbance was measured at 595 nm.

Growth curve profiling

Cells were refreshed overnight on LB broth at 37 °C. The overnight culture was diluted and used as inoculum to a final OD_{600} of 0.01 in LB broth. The cell suspensions were taken every hour and the cell density was measured at OD_{600}.

Acyl-homoserine lacton (AHL) measurement

The production of 3-oxo-dodecanoyl-homoserine lactone (3O-C12-HSL), one of the AHLs, was evaluated using *E. coli* harbouring pSB1075 plasmid as a biosensor.[20] Overnight culture of wild-type and ΔPA3235 mutant strain was adjusted to OD600 of 1.0 and diluted 100-fold in 10 mL LB. After 5 h and 24 h incubation at 37 °C, cell-free supernatant was acidified using 1 M HCl (9:1). AHL was extracted from 1 mL acidified supernatant using two times 250 µL of chloroform. The organic phase was moved to a fresh tube and allowed to evaporate. The AHL was then diluted in 100 µL methanol. As much as 2 µL AHL solution was added to a 96-well microtiter plate. Then, two hundred microliters of the 10-fold diluted overnight biosensor were then added. Bioluminescence was measured using a microplate reader (Omega Star).[21]

Data analysis

Data were analyzed using Student's t-tests to determine differences between treatments. P-value < 0.05 were considered significant.

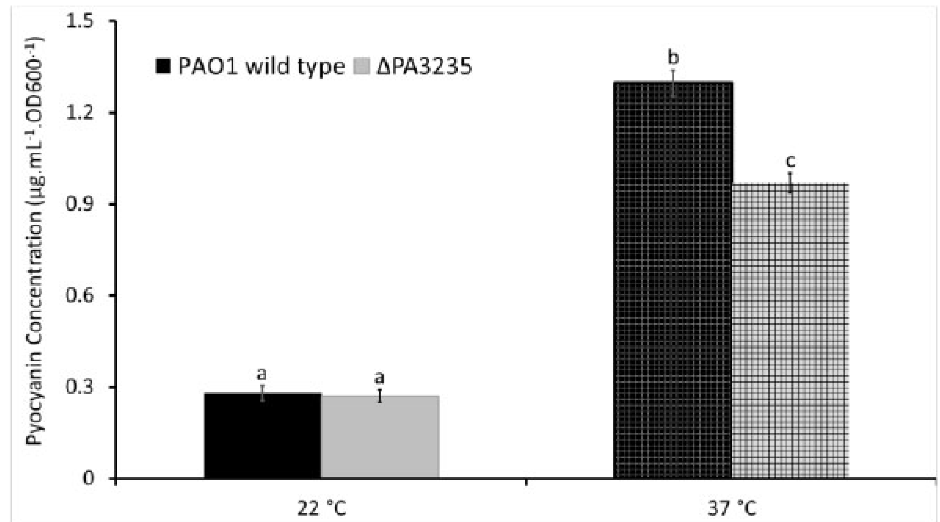

Figure 1. Production of pyocyanin by ΔPA3235 mutant (grey bar) and parental strain (black bar).

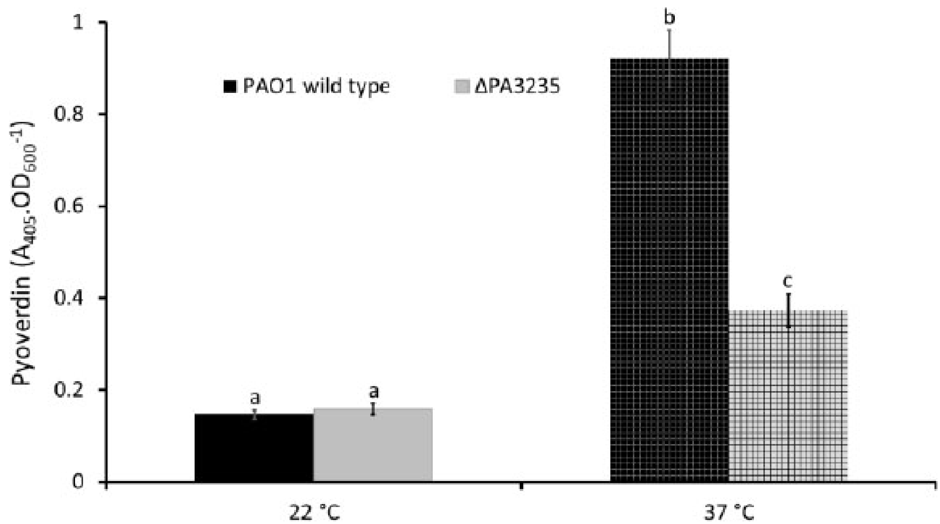

Figure 2. Pyoverdine production by ΔPA3235 mutant (grey bar) and parental strain (black bar).

RESULTS

To understand the gene's involvement in the virulence factor production, several assays such as pyocyanin and pyoverdine production, swarming, swimming, twitching motilities, and biofilm formation were performed. Deletion of the PA3235 gene from the PAO1 genome is a good way to study the role of PA3235 in the pathogenicity of this bacterium. In this study, an incubation temperature of 22 °C was used to observe the phenotypes of *P. aeruginosa* PAO1 at room temperature, while 37 °C represents the human body temperature, as stated by Barbier.[10]

Pyocyanin production

The result showed that both wild-type and mutant strains demonstrate no statistical differences in pyocyanin production at 22 °C. A significant difference was observed in 37 °C cultures, where the mutant produced less pyocyanin (p-value 0.0004) (Figure 1).

Pyoverdine concentration measurement

Pyoverdine is an important protein for the iron acquisition of *P. aeruginosa* during infection in the hosts, such as the human body. In comparison to the wild-type strain, the pyoverdine production was

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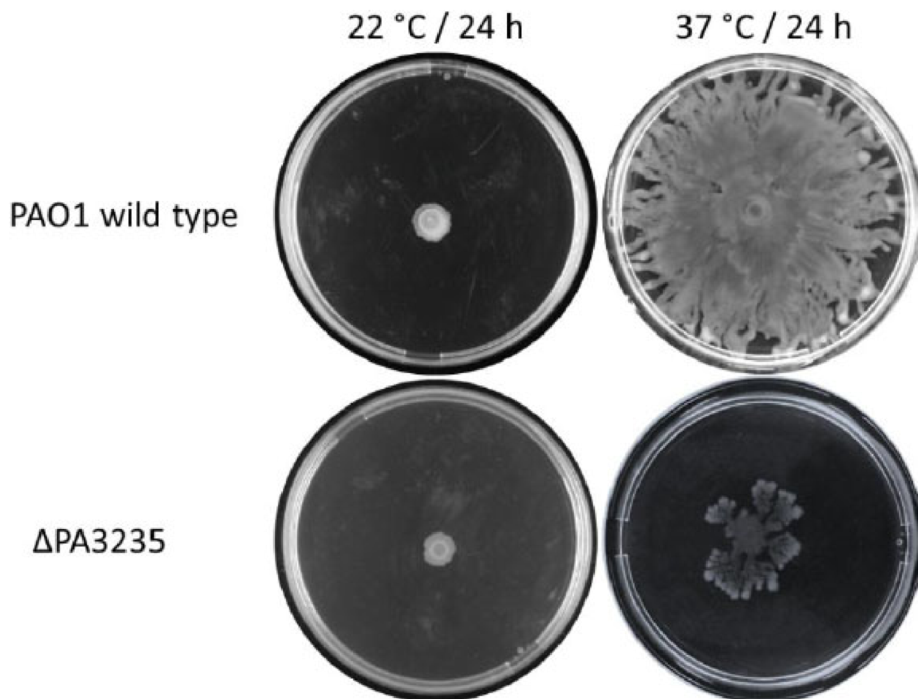


Figure 3. Swarming motility of ΔPA3235 mutant and parental strain.

lower in the ΔPA3235 mutant when it was grown at 37 °C (p-value 0.0009). No significant difference was observed in the 22 °C cultures (Figure 2).

Effects of PA3235 deletion on *P. aeruginosa* PAO1 motility

Three types of motilities were observed in this study: swarming, swimming, and twitching motilities. Incubation at 22 °C did not promote any swarming motility for both strains. The swarm area of ΔPA3235 mutant was narrower compared to PAO1 when incubated at 37 °C for 24 h (Figure 3). The dendrites pattern of both strains was similar. Both the mutant and parental strains showed no differences in swimming and twitching motility when incubated at 22 °C and 37 °C (Figure 4).

Deletion of PA3235 increased biofilm formation

Biofilm formation of *P. aeruginosa* was determined as the A_{590} of de-stained crystal violet. Interestingly, ΔPA3235 mutant produced more biofilm on the microtiter wall, compared to the wild-type strain (p-value 0.0013). A lower quantity of biofilm was observed after 24 h incubation at 22 °C in both strains (Figure 5).

AHL level is altered at 37 °C in the absence of PA3235

Virulence determinants are known to be regulated by signal molecules. To confirm whether the differences in virulence factor production were influenced by AHL production from the strains at 37 °C, 3-Oxo-C12-HSL was quantified using a biosensor system. The result demonstrated no differences in 3-O-C12-HSL quantity between mutant and wild-type strains after 5 h incubation. However, after 24 h incubation, the AHL was higher in the mutant than in wild type strain (p-value 0.0095) (Figure 6). This may indicate that the reduced or increased virulence factors were not due to the increased level of AHL in ΔPA3235 strain.

The mutation did not change the growth profile of *P. aeruginosa*

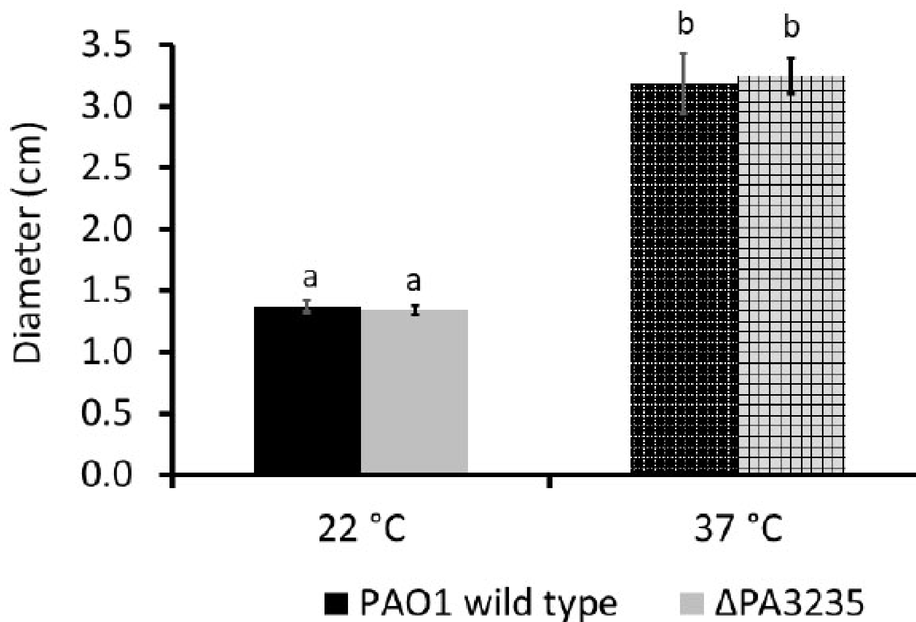
To investigate whether the mutation on PA3235 gene affects cell growth, the cells were grown in LB broth at 37 °C with 250 rpm shaking. Both the parental and mutant strains exhibited similar growth patterns in LB broth (Figure 7). The mutant colonies also showed the same morphology as the wild type on the LB plate (not shown here).

DISCUSSIONS

Many genes of *P. aeruginosa* cells are upregulated or downregulated when the cell is grown at the human body temperature, 37 °C.[6] Since all these factors are modulated by the quorum sensing system, then signal molecule expression is also important for the pathogenicity of this bacterium. Many of the quorum sensing and virulence determinants genes are modulated by the *lasI-rhlR* quorum sending system and other regulons.[22,23] Based on microarray data, the *lasI* gene, one of the signal-molecules synthesizing genes, was upregulated in PAO1 strain during cell growth at human body temperature.[10] The *lasI* gene from rhizospheres isolates also increases at the temperature.[24] The PA3235 transcript was increased during cell growth at human body temperature.[6,10,11]

Although many genes are influenced by temperature, however the result showed that there were differences in phenotypes between wild type and the mutant. The ΔPA3235 mutant showed an increase in biofilm (Figure 5) and the level of 3O-C12-HSL (Figure 6) compared to the PAO1 wild type. The twitching and swimming motility was unchanged (Figure 4), whilst pyocyanin, pyoverdine, and swarming motility were less in this mutant than in the wild type (Figures 1, 2, and 3). These differences were not caused by differences in the growth phases since the wild type and mutant exhibited the same growth patterns in LB broth at 37 °C (Figure 7).

Pyocyanin is partly modulated by the *lasI-rhlR* quorum sensing system,[25] and mostly by pseudomonas quinolone signal (pqs) signal and a complex circuit.[26] The presence of iron also influences pyocyanin production levels.[6] Several enzymes are involved in the biosynthesis and secretion of this phenazine, such as those encoded by *phzA-G*, *phzM* and *phzS* genes.[27] Pyocyanin is produced at a higher level at 37 °C by PAO1 and Pseudomonas sp, isolated from the rhizosphere.[28] As the PAO1 grows at 37 °C, the *phzA-G*, *phzM* and *phzS* biosynthesis genes and type 3 secretion systems are upregulated.[6] Conversely, Barbier's microarray data revealed that not all of those pyocyanin transcripts were upregulated, only *phzS*



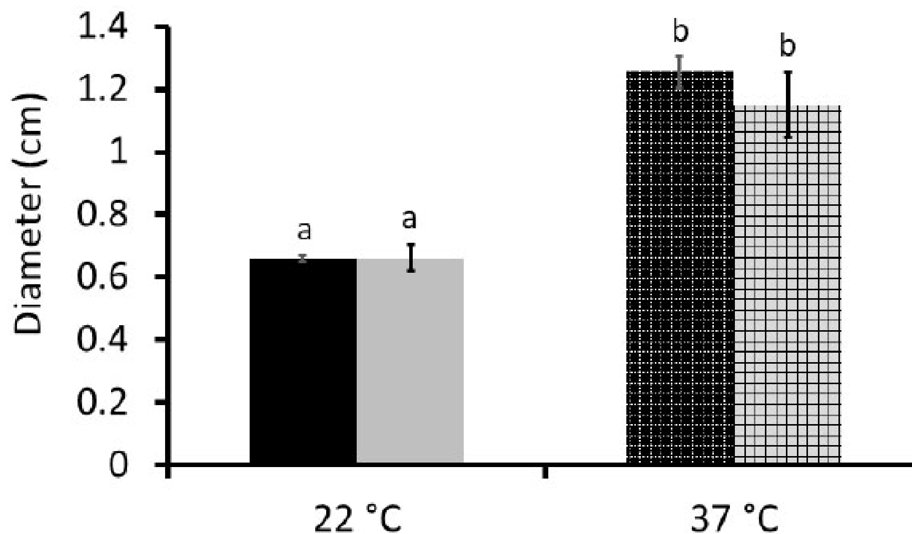


Figure 4. Swimming (A) and twitching motility (B) of ΔPA3235 mutant and PAO1.

was increased and *phzH* was decreased.[10] It is likely that PA3235 is important for pyocyanin production. Although the 3-OC12-HSL level of the mutant was higher than the wild type, however pyocyanin concentration was lower in ΔPA3235 mutant than in wild-type strains at 37 °C (Figure 1). By looking at the prediction that PA3235 protein is a membrane protein, it may be true that this protein is involved in the exports of molecules crucial for phenazine metabolism.

Like pyocyanin, pyoverdine was also produced in a lower quantity in ΔPA3235 than in the wild-type strains (Figure 2). Pyoverdine is expressed under iron-limiting conditions,[29] not by signal molecules,[25] nor body temperature.[10] It is interesting to note that the deletion of PA3235 caused reducing of pyoverdine in the mutant, although it grew in rich, non-iron limited, medium, and under higher 3-O-C12-HSL concentration (Figure 6).

Motility is important for virulence, and biofilm attachment, development, maturation, and dispersion.[30] Swimming and swarming motility rely on flagella whilst twitching on motility type IV pili. In this research, swimming and twitching motility are unaffected by the mutation. The swarming motility of *P. aeruginosa* PAO1 is modulated by quorum sensing.[31] Interestingly, in this study, we found that the 3O-C_{12}-HSL level was significantly increased in ΔPA3235 strain although the swarming motility was reduced (Figure 3 and Figure 6). Based on microarray data, the PA3235 does not belong to genes involved in swarming motility,[32] and the transcript was downregulated in tendrils compared to non-swarming cells.[33] The swarming motility is also regulated by multifactorial determinants, for instance rhamnolipid, nitrogen starvation as well as particular amino acids,[31-34] and nutrition availability in the environment, especially iron.[29,31] The decreased swarming area exhibited by the mutant might be attributed to the lessen of pyoverdine production which in turn causes less iron uptake necessary for PAO1 cells to swarm.

Although the PA3235 gene was known to be expressed less in biofilm compared to planktonic cells (E-GEOD-10030)[35], however the result of this study revealed that deletion of this gene caused increasing biofilm quantity. Based on microarray data, the PA3235 belongs to the genes that are downregulated in biofilm,[25] and expressed less in biofilm compared to the planktonic cells (E-GEOD-10030).[35] During the growth of *P. aeruginosa* in LB Broth, the PA3235 gene was upregulated at the early stationary phase and then decreased at the stationary phase on static incubation (E-GEOD-4026).[26] The result of this study revealed that deletion of this gene caused increasing in biofilm quantity. It seems that the PA3235 gene might involve in the early stage of biofilm formation. Initiations of biofilm formation are driven by complex factors, such as antimicrobials, motility, and iron.[36-39] Other studies have proven that swarm behaviour and biofilm formation are inversely regulated in *P. aeruginosa* cells.[40] Swarming motility is important for early biofilm development, defect in swarming motility impairs biofilm formation.[36,41] This was confirmed in this study that the biofilm of ΔPA3235 was increased at 37 °C, whilst swarming was decreased (Figure 5 and Figure 3).

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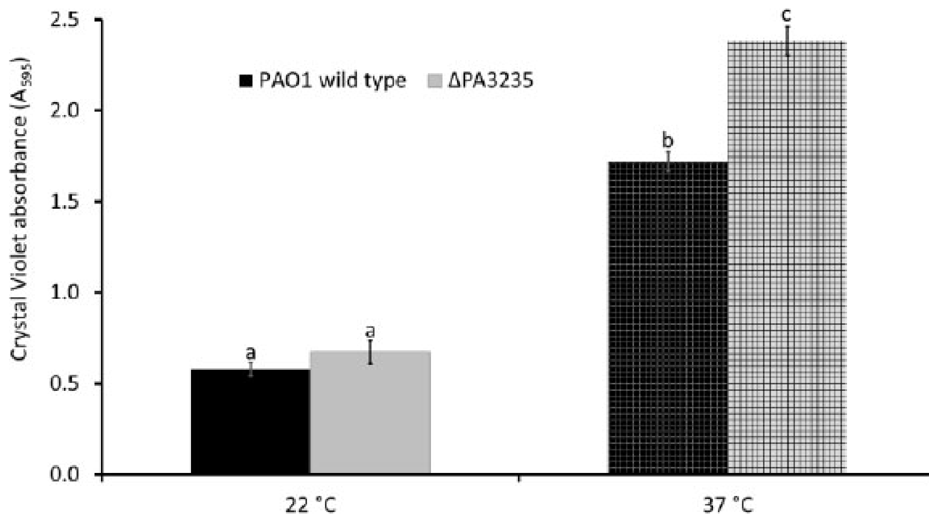

Figure 5. Biofilm of ΔPA3235 mutant and PAO1.

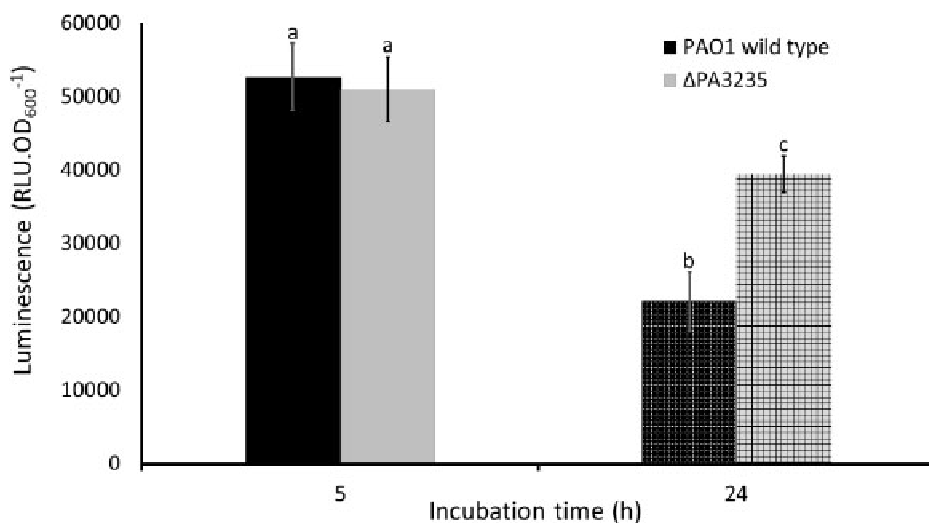

Figure 6. The production of signal molecule by ΔPA3235 mutant and PAO1.

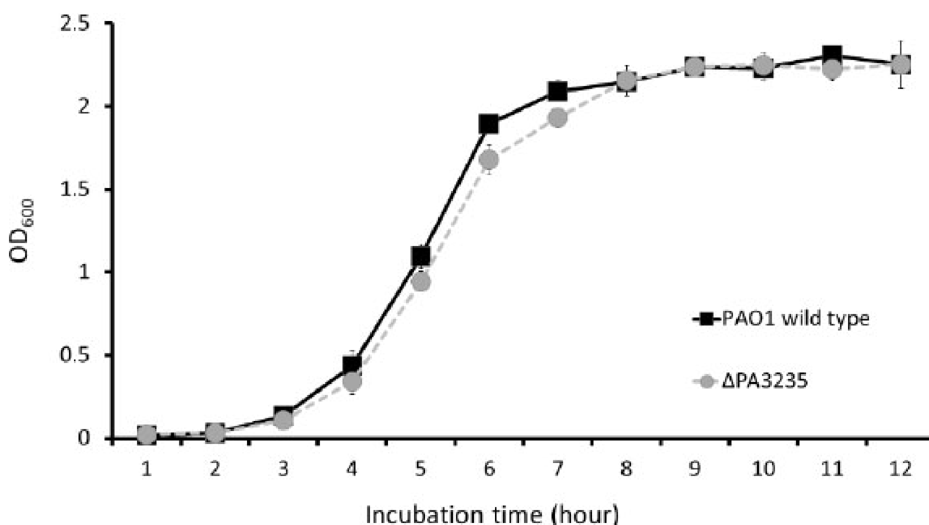

Figure 7. The growth pattern of PAO1 and ΔPA3235 mutant.

The increased level of AHL in ΔPA3235 might be responsible for the modulation of many virulence factors which are quorum-sensing dependence genes. The mutant showed the same amount of 3-O-C12-HSL as the PAO1, until 5 h incubation and then the signal molecule was less in the extracellular environment after the culture reached the stationary stage. The 3-O-C12-HSL was produced constitutively in small amounts, and deliberately from the cell. When this molecule reaches a certain threshold amount in the environment, it is taken back to the cell by facilitated diffusion. We predicted that PA3235 is important for transporting this AHL and certain nutrients into the cell. There are four main reasons. First, the PA3235 protein is predicted as a transmembrane protein according to TMHMM-2.0 analyses.[42] Until now, no information about the transport protein of the AHL, nor the real role of the PA3235 protein. Second, no AHL synthesis genes were detected in the stationary phase.[25] The PA3235 gene was categorized as a quorum sensing modulated gene;[25,43] which expression was upregulated at the early stationary phase and decreased at the stationary phase (E-GEOD-4026).[26] This means that this protein might be involved in transporting the AHL into the cells. So that, the absence of PA3235 protein might cause problems in the uptake of 3-O-C12-HSL into the cell and the molecule getting abundant in the extracellular medium. Third, the lower amount of 3-O-C12-HSL inside the cell might cause changes in a few phenotypes. Fourth, the availability of certain nutrients is important for the phenotypic traits of the colony. For example, iron impacted the pyoverdine level of cells, pyocyanin, swarming and biofilm. The presence of amino acid and other nutrients are also important for swarming motility. This means that the protein might involve in the metabolism or transportation of certain molecules.

PA3235 is predicted to encode a hypothetical membrane protein with an unknown function. It is comprised of 312 nucleotides, and encodes a protein with a domain similar to *E. coli* K12 YjcH, an inner membrane protein.[13] Analysis of the genome structures showed that this protein is highly conserved amongst other *P. aeruginosa* strains.[44] This gene is not known as a protein related to efflux pump, siderophore or pyochelin. However, during the growth of *P. aeruginosa* in iron limiting medium, the PA3235

gene was expressed at the logarithmic phase (E-GEOD-2885).[45] Furthermore, microarray data revealed that the expression was downregulated in response to siderophore/β-lactam conjugate addition (E-MTAB-1381,[46] and the PA3235 expression is not regulated by Fe in iron depletion condition.[45] This hypothesis was supported by the evidence that pyoverdine and swarming motility were reduced in ΔPA3235 strain, whereas the biofilm was increased compared to the wild type. Low iron concentration in the environment impedes biofilm formation and lowers the swarming motility.[47,39] However, high iron concentration can disrupt the biofilm.[48] The abundant quantity of ferric ions reduces rhamnolipid production which in turn represses swarming motility. A study by Schmidberger et al. (2014) has proven that excess ferric ion decreases the expression of the genes involved in rhamnolipid production, such as *rhlA*, and *rhlC*.[49] Rhamnolipid was known to be crucial in surface motility, and biofilm formation.[50] PvdQ, that is important for pyoverdine synthesis, is also expressed in iron-limiting condition, which then related to swarming motility and biofilm development. Looking at the previous evidence, we believe that the deletion of PA3235 enables cells to uptake more Fe3+ ions from the medium than the wild type, resulting in less pyoverdine production and swarming motility as the formation of thicker biofilm. The association between this gene and iron uptake still needs to be confirmed. We predicted that PA3235 might contribute to the uptake and release of molecules.

Furthermore, the PA3235 might be involved in the early pathogenesis of this bacterium in host cells. When the cells were harvested at the early stationary phase, the gene was upregulated at 37 °C compared to 22 °C,[10] and then downregulated at the stationary growth phase.[6] The results of microarray data indicate that this gene might be involved in the early infection stage of *P. aeruginosa* in mammalian host cells. The PA3235 was expressed in response to epithelial cells (GDS2502)[51] and in human whole blood cells,[8] mimicking the condition when the bacterium infects humans, such as cystic fibrosis patients. However, the expression of this gene was downregulated when this bacterium was grown on mouse tumors, cystic fibrosis lung and burned wound mouse,[11] or from infected human, such as sputum and burn wound.[52] Until now, no information about factors which may inhibit its expression in these cells.

Based on this evidence, we proposed that the PA3235 might involve in the pathogenesis of *P. aeruginosa* during infecting the human body. It is highly likely that the PA3235 is modulated by quorum sensing and involved in the transport of molecules out or into the *P. aeruginosa* cells, which are important for early pathogenesis. Future works are needed to find the precise molecules transported by the PA3235 protein and to identify the exact roles of this gene in the pathogenicity of the bacterium during infection in mammalian and human cells. Given that the gene has not been previously characterized and the influence of the gene on the increase of biofilm and 3O-C12-HSL, we hypothesize that the modulation of this gene might be used to control P. aeruginosa pathogenicity when this bacterium infects the warm-blood hosts.[53] Biofilm is the persistent form of PAO1 against antibiotics and immune cells.[22,54-56]

Infections by *P. aeruginosa* are still an issue until now. However, the bacterium has been known as an opportunistic pathogen however infected patients are rarely survived. The severe effects are caused by these factors: virulence factors, resistance towards antibiotics, emerging of multidrug resistance antibiotics. The finding of anti-infectives against this bacterium is important. In this study, we prove that the PA3235 gene influences increasing biofilm and 3O-C12-HSL, lowering pyocyanin, pyoverdine, and swarming motility. Biofilm is the persistent form of PAO1 against antibiotics and immune cells. Several reported microarray data gave the impression that this gene might be involved in the early pathogenesis of *P. aeruginosa*, given the facts that PA3235 gene has not been previously characterized and its influence on several virulence factors, we hypothesize that the modulation of this gene might be used to control *P. aeruginosa* pathogenicity in human.

CONCLUSIONS

The ΔPA3235 mutant showed some differences when grown at 37 °C compared to the wild type. Both strains showed no differences in growth patterns, twitching or swimming motilities. We predicted that the PA3235 gene might play a role in the pathogenesis of PAO1 to the human body by facilitating the transport of molecules out or into PAO1 cells.

ETHICAL CLEARANCE

This research received ethical approval from Universitas Surabaya, with certificate number 33A/KE/VIII/2018.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR'S CONTRIBUTION

Mariana Wahjudi is involved in conceptualization, funding acquisition, analysis and interpretation of results, and manuscript preparation and finalization. Samuel Stefanus Widodo: study conception, design, and data collection. Ida Bagus Made Artadana: data analysis and interpretation of results. Yulanda Antonius: manuscript finalization and review

REFERENCES

1. Paulsson M, Su YC, Ringwood T, Uddén F, Riesbeck K. Pseudomonas aeruginosa uses multiple receptors for adherence to laminin during infection of the respiratory tract and skin wounds. Sci Rep. 2019;9(1):18168. Published 2019 Dec 3. doi:10.1038/s41598-019-54622-z.
2. Chahtane H, Nogueira Füller T, Allard PM, et al. The plant pathogen Pseudomonas aeruginosa triggers a DELLA-dependent seed germination arrest in Arabidopsis. Elife. 2018;7:e37082. Published 2018 Aug 28. doi:10.7554/eLife.37082.
3. Bachta KER, Allen JP, Cheung BH, Chiu CH & Hauser AR. Systemic infection facilitates transmission of Pseudomonas aeruginosa

in mice. Nat Commun. 2020;11(543). doi: 10.1038/s41467-020-14363-4.
4. Stover CK, Pham XQ, Erwin AL, et al. Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. Nature. 2000;406(6799):959-964. doi:10.1038/35023079.
5. Klockgether J, Cramer N, Wiehlmann L, Davenport CF, Tümmler B. Pseudomonas aeruginosa Genomic Structure and Diversity. Front Microbiol. 2011;2:150. Published 2011 Jul 13. doi:10.3389/fmicb.2011.00150.
6. Wurtzel O, Yoder-Himes DR, Han K, et al. The single-nucleotide resolution transcriptome of Pseudomonas aeruginosa grown in body temperature. PLoS Pathog. 2012;8(9):e1002945. doi:10.1371/journal.ppat.1002945.
7. Kruczek C, Kottapalli KR, Dissanaike S, et al. Major Transcriptome Changes Accompany the Growth of Pseudomonas aeruginosa in Blood from Patients with Severe Thermal Injuries. PLoS One. 2016;11(3):e0149229. Published 2016 Mar 2. doi:10.1371/journal.pone.0149229.
8. Beasley KL, Cristy SA, Elmassry MM, Dzvova N, Colmer-Hamood JA, Hamood AN. During bacteremia, Pseudomonas aeruginosa PAO1 adapts by altering the expression of numerous virulence genes including those involved in quorum sensing. PLoS One. 2020;15(10):e0240351. Published 2020 Oct 15. doi:10.1371/journal.pone.0240351.
9. Moradali MF, Ghods S, Rehm BH. Pseudomonas aeruginosa Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. Front Cell Infect Microbiol. 2017;7:39. Published 2017 Feb 15. doi:10.3389/fcimb.2017.00039.
10. Barbier M, Damron FH, Bielecki P, et al. From the environment to the host: re-wiring of the transcriptome of Pseudomonas aeruginosa from 22°C to 37°C. PLoS One. 2014;9(2):e89941. Published 2014 Feb 24. doi:10.1371/journal.pone.0089941.
11. Bielecki P, Komor U, Bielecka A, et al. Ex vivo transcriptional profiling reveals a common set of genes important for the adaptation of Pseudomonas aeruginosa to chronically infected host sites. Environ Microbiol. 2013;15(2):570-587. doi:10.1111/1462-2920.12024.
12. Lewenza S, Gardy JL, Brinkman FS, Hancock RE. Genome-wide identification of Pseudomonas aeruginosa exported proteins using a consensus computational strategy combined with a laboratory-based PhoA fusion screen. Genome Res. 2005;15(2):321-329. doi:10.1101/gr.3257305.
13. Marchler-Bauer A, Bo Y, Han L, et al. CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. Nucleic Acids Res. 2017;45(D1):D200-D203. doi:10.1093/nar/gkw1129.
14. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked Pseudomonas aeruginosa mutants. Gene. 1998;212(1):77-86. doi:10.1016/s0378-1119(98)00130-9.
15. Simon R, Quandt J, Klipp W. New derivatives of transposon Tn5 suitable for mobilization of replicons, generation of operon fusions and induction of genes in gram-negative bacteria. Gene. 1989;80(1):161-169. doi:10.1016/0378-1119(89)90262-x.
16. Essar DW, Eberly L, Hadero A, Crawford IP. Identification and characterization of genes for a second anthranilate synthase in Pseudomonas aeruginosa: interchangeability of the two anthranilate synthases and evolutionary implications. J Bacteriol. 1990;172(2):884-900. doi:10.1128/jb.172.2.884-900.1990.
17. Diggle SP, Winzer K, Lazdunski A, Williams P, Cámara M. Advancing the quorum in Pseudomonas aeruginosa: MvaT and the regulation of N-acylhomoserine lactone production and virulence gene expression. J Bacteriol. 2002;184(10):2576-2586. doi:10.1128/JB.184.10.2576-2586.2002.
18. Atkinson S, Chang CY, Sockett RE, Cámara M, Williams P. Quorum sensing in Yersinia enterocolitica controls swimming and swarming motility. J Bacteriol. 2006;188(4):1451-1461. doi:10.1128/JB.188.4.1451-1461.2006.
19. Merritt JH, Kadouri DE, O'Toole GA. Growing and analyzing static biofilms. Curr Protoc Microbiol. 2005;Chapter 1:Unit-1B.1. doi:10.1002/9780471729259.mc01b01s00.
20. Winson MK, Swift S, Fish L, et al. Construction and analysis of luxCDABE-based plasmid sensors for investigating N-acyl homoserine lactone-mediated quorum sensing. FEMS Microbiol Lett. 1998;163(2):185-192. doi:10.1111/j.1574-6968.1998.tb13044.x.
21. Wahjudi M, Papaioannou E, Hendrawati O, et al. PA0305 of Pseudomonas aeruginosa is a quorum quenching acylhomoserine lactone acylase belonging to the Ntn hydrolase superfamily. Microbiology (Reading). 2011;157(7):2042-2055. doi:10.1099/mic.0.043935-0.
22. Girard G, Bloemberg GV. Central role of quorum sensing in regulating the production of pathogenicity factors in Pseudomonas aeruginosa. Future Microbiol. 2008;3(1):97-106. doi:10.2217/17460913.3.1.97.
23. Veetilvalappil VV, Manuel A, Aranjani JM, Tawale R, Koteshwara A. Pathogenic arsenal of Pseudomonas aeruginosa: an update on virulence factors. Future Microbiol. 2022;17:465-481. doi:10.2217/fmb-2021-0158.
24. Wu D. Genomic analysis and temperature-dependent transcriptome profiles of the rhizosphere originating strain Pseudomonas aeruginosa M18. BMC Genomics. 2011;12:1–17.
25. Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH. Microarray analysis of Pseudomonas aeruginosa quorum-sensing regulons: effects of growth phase and environment. J Bacteriol. 2003;185(7):2080-2095. doi:10.1128/JB.185.7.2080-2095.2003.
26. Lequette Y, Lee JH, Ledgham F, Lazdunski A, Greenberg EP. A distinct QscR regulon in the Pseudomonas aeruginosa quorum-sensing circuit. J Bacteriol. 2006;188(9):3365-3370. doi:10.1128/JB.188.9.3365-3370.2006.
27. Mentel M, Ahuja EG, Mavrodi DV, Breinbauer R, Thomashow LS, Blankenfeldt W. Of two make one: the biosynthesis of phenazines. Chembiochem. 2009;10(14):2295-2304. doi:10.1002/cbic.200900323.
28. Huang J, Xu Y, Zhang H, et al. Temperature-dependent expression of phzM and its regulatory genes lasI and ptsP in rhizosphere isolate Pseudomonas sp. strain M18. Appl Environ Microbiol. 2009;75(20):6568-6580. doi:10.1128/AEM.01148-09.
29. Jimenez PN, Koch G, Papaioannou E, et al. Role of PvdQ in Pseudomonas aeruginosa virulence under iron-limiting conditions. Microbiology (Reading). 2010;156(1):49-59. doi:10.1099/mic.0.030973-0.
30. Burrows LL. Pseudomonas aeruginosa twitching motility: type IV pili in action. Annu Rev Microbiol. 2012;66:493-520. doi:10.1146/annurev-micro-092611-150055.
31. Köhler T, Curty LK, Barja F, van Delden C, Pechère JC. Swarming of Pseudomonas aeruginosa is dependent on cell-to-cell signaling and requires flagella and pili. J Bacteriol. 2000;182(21):5990-5996. doi:10.1128/JB.182.21.5990-5996.2000.
32. Overhage J, Bains M, Brazas MD, Hancock RE. Swarming of Pseudomonas aeruginosa is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. J Bacteriol. 2008;190(8):2671-2679. doi:10.1128/JB.01659-07.
33. Tremblay J, Déziel E. Gene expression in Pseudomonas aeruginosa swarming motility. BMC Genomics. 2010;11:587. Published 2010 Oct 20. doi:10.1186/1471-2164-11-587.
34. Cai Y. Differential impact on motility and biofilm dispersal of closely related phosphodiesterases in Pseudomonas aeruginosa. Sci. Rep. 2020;10:6232.
35. Anderson GG, Moreau-Marquis S, Stanton BA, O'Toole GA. In vitro analysis of tobramycin-treated Pseudomonas aeruginosa biofilms on cystic fibrosis-derived airway epithelial cells. Infect Immun. 2008;76(4):1423-1433. doi:10.1128/IAI.01373-07.
36. Thi MTT, Wibowo D, Rehm BHA. Pseudomonas aeruginosa Biofilms. Int J Mol Sci. 2020;21(22):8671. Published 2020 Nov 17. doi:10.3390/ijms21228671.
37. Chellappa ST, Maredia R, Phipps K, Haskins WE, Weitao T. Motility of Pseudomonas aeruginosa contributes to SOS-inducible biofilm formation. Res Microbiol. 2013;164(10):1019-1027. doi:10.1016/j.resmic.2013.10.001.
38. Zaitseva J, Granik V, Belik A, Koksharova O, Khmel I. Effect of nitrofurans and NO generators on biofilm formation by Pseudomonas aeruginosa PAO1 and Burkholderia cenocepacia 370. Res Microbiol. 2009;160(5):353-357. doi:10.1016/j.resmic.2009.04.007.
39. Banin E, Vasil ML, Greenberg EP. Iron and Pseudomonas aeruginosa biofilm formation. Proc Natl Acad Sci U S A. 2005;102(31):11076-11081. doi:10.1073/pnas.0504266102.
40. Caiazza NC, Merritt JH, Brothers KM, O'Toole GA. Inverse regulation of biofilm formation and swarming motility by Pseudomonas aeruginosa PA14. J Bacteriol. 2007;189(9):3603-3612. doi:10.1128/JB.01685-06.

41. Shrout JD, Chopp DL, Just CL, Hentzer M, Givskov M, Parsek MR. The impact of quorum sensing and swarming motility on Pseudomonas aeruginosa biofilm formation is nutritionally conditional. Mol Microbiol. 2006;62(5):1264-1277. doi:10.1111/j.1365-2958.2006.05421.x.
42. Krogh A, Larsson B, von Heijne G, Sonnhammer EL. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol. 2001;305(3):567-580. doi:10.1006/jmbi.2000.4315.
43. Schuster M, Lostroh CP, Ogi T, Greenberg EP. Identification, timing, and signal specificity of Pseudomonas aeruginosa quorum-controlled genes: a transcriptome analysis. J Bacteriol. 2003;185(7):2066-2079. doi:10.1128/JB.185.7.2066-2079.2003.
44. Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FS. Enhanced annotations and features for comparing thousands of Pseudomonas genomes in the Pseudomonas genome database. Nucleic Acids Res. 2016;44(D1):D646-D653. doi:10.1093/nar/gkv1227.
45. Zheng P, Sun J, Geffers R, Zeng AP. Functional characterization of the gene PA2384 in large-scale gene regulation in response to iron starvation in Pseudomonas aeruginosa. J Biotechnol. 2007;132(4):342-352. doi:10.1016/j.jbiotec.2007.08.013.
46. van Delden C, Page MG, Köhler T. Involvement of Fe uptake systems and AmpC β-lactamase in susceptibility to the siderophore monosulfactam BAL30072 in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2013;57(5):2095-2102. doi:10.1128/AAC.02474-12.
47. Kang D, Kirienko NV. Interdependence between iron acquisition and biofilm formation in Pseudomonas aeruginosa. J Microbiol. 2018;56(7):449-457. doi:10.1007/s12275-018-8114-3.
48. Musk DJ, Banko DA, Hergenrother PJ. Iron salts perturb biofilm formation and disrupt existing biofilms of Pseudomonas aeruginosa. Chem Biol. 2005;12(7):789-796. doi:10.1016/j.chembiol.2005.05.007.
49. Schmidberger A, Henkel M, Hausmann R, Schwartz T. Influence of ferric iron on gene expression and rhamnolipid synthesis during batch cultivation of Pseudomonas aeruginosa PAO1. Appl Microbiol Biotechnol. 2014;98(15):6725-6737. doi:10.1007/s00253-014-5747-y.
50. Glick R, Gilmour C, Tremblay J, et al. Increase in rhamnolipid synthesis under iron-limiting conditions influences surface motility and biofilm formation in Pseudomonas aeruginosa. J Bacteriol. 2010;192(12):2973-2980. doi:10.1128/JB.01601-09.
51. Chugani S, Greenberg EP. The influence of human respiratory epithelia on Pseudomonas aeruginosa gene expression. Microb Pathog. 2007;42(1):29-35. doi:10.1016/j.micpath.2006.10.004.
52. Cornforth DM, Dees JL, Ibberson CB, et al. Pseudomonas aeruginosa transcriptome during human infection. Proc Natl Acad Sci U S A. 2018;115(22):E5125-E5134. doi:10.1073/pnas.1717525115.
53. Brindhadevi K. Biofilm and Quorum sensing mediated pathogenicity in Pseudomonas aeruginosa. Process Biochem. 2020; 96:49–57.
54. Dewi Yana, Widodo, A. D. W., & Pepy Dwi Endraswari. (2023). Comparison between the time to detection of pathogenic and nonpathogenic microorganisms from blood culture specimens using the BACTECTM FX machine to determine the optimal incubation time. Bali Medical Journal, 12(1), 909–915. https://doi.org/10.15562/bmj.v12i1.4199.
55. Syaiful, I., Widodo, A. D. W., Endraswari, P. D., Alimsardjono, L., Utomo, B., & Arfijanto, M. V. (2023). The association between biofilm formation ability and antibiotic resistance phenotype in clinical isolates of gram-negative bacteria: a cross-sectional study. Bali Medical Journal, 12(1), 1014–1020. https://doi.org/10.15562/bmj.v12i1.4101.
56. Bramardipa, A. A. B., Sukrama, I. D. M., & Budayanti, N. N. S. (2019). Bacterial pattern and its susceptibility toward antibiotic on burn infection in Burn Unit Sanglah General Hospital. Bali Medical Journal, 8(1), 328–333. https://doi.org/10.15562/bmj.v8i1.1456

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