Mutation of \textit{pfm} affects the adherence of \textit{Pseudomonas aeruginosa} to host cells and the quorum sensing system

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Received 22 May 2011; revised 17 August 2011; accepted 26 August 2011. Final version published online 21 September 2011.

DOI: 10.1111/j.1574-6968.2011.02401.x

Abstract

The \textit{Pseudomonas aeruginosa} quorum sensing (QS) system is controlled by the signal molecules acyl homoserine lactones (AHLs) that are synthesized from acyl enoyl-acyl carrier proteins (acyl-ACPs) provided by the fatty acid biosynthesis cycle. \textit{Pfm} (PA2950), an enoyl-CoA reductase, has previously been shown to affect swimming mobility and fatty acid biosynthesis. In this report, we further show that \textit{pfm} influences bacterial adherence to human cells. Microarray assay results suggest that \textit{pfm} affects bacterial adherence through its influence on the QS system. Further experiments confirmed that the \textit{pfm} mutant strain produces significantly less QS signal molecules than the corresponding wild-type strain. Using strains \textit{Escherichia coli} DH5\textalpha\(\textsuperscript{a}(pECP64, lasB'	extsuperscript{-}lacZ)\) and \textit{E. coli} DH5\textalpha\(\textsuperscript{a}(pECP61.5, rhlA'	extsuperscript{-}lacZ)\), biosensors for \textit{N}-(3-oxododecanoyl) homoserine lactone (3O-C12-HSL) and \textit{N}-butyryl homoserine lactone (C4-HSL), respectively, we found that \textit{pfm} mutant strain produces decreased amounts of both signal molecules. Elastase activity and pyocyanin measurements further confirmed the reduced levels of 3O-C12-HSL and C4-HSL in the \textit{pfm} mutant. Finally, bacterial virulence, as assessed by the \textit{Caenorhabditis elegans} worm killing assay, is decreased in the \textit{pfm} mutant. Taken together, these data indicate that \textit{pfm} can be an important target for the control of \textit{P. aeruginosa} infectivity.

Introduction

\textit{Pseudomonas aeruginosa}, a versatile Gram-negative bacterium, is a major opportunistic human pathogen. It is present in almost all ecological niches, including soil, marshes, and coastal marine habitats, as well as on plants and animal tissues (Hardalo & Edberg, 1997). The genome of \textit{P. aeruginosa} strain PAO1 contains 6.3 million base pairs, with 5572 predicted open reading frames (ORFs) (Stover et al., 2000). The genome complexity of this organism reflects its evolutionary adaptation to various hosts and environmental conditions (Dobrindt & Hacker, 2001). As an opportunistic human pathogen, \textit{P. aeruginosa} is commonly found in hospitals and often causes chronic infections. Many factors contribute to its infectivity and pathogenicity. It encodes a series of virulent effectors, including flagella, pilus, exotoxin A, endotoxin, pigments, protease, etc. (Bell & Robinson, 2007; Harrison, 2007; Vanegas \textit{et al}., 2009). It also takes advantages of many antibiotic resistance pathways that are readily activated during host infection (Hancock, 1998). These characteristics make it difficult to completely cure patients infected by \textit{P. aeruginosa}.

In \textit{P. aeruginosa}, there are two separate quorum sensing (QS) systems, \textit{lasR-lasI} and \textit{rhlR-rhlI} (Parsek & Greenberg, 2000). Both systems are controlled by autoinducer signal molecules, \textit{N}-(3-oxododecanoyl) homoserine lactone (3O-C12-HSL) and \textit{N}-butyryl homoserine lactone (C4-HSL), respectively (Parsek & Greenberg, 2000). In the \textit{lasR-lasI} QS system, the signal molecule 3O-C12-HSL is synthesized by LasI. In turn, the accumulated 3O-C12-HSL acts as the ligand for its receptor LasR, leading to the activation of LasR. Activated LasR functions as a transcriptional activator to upregulate downstream target genes, most of which are associated with the virulence of \textit{P. aeruginosa}, such as exoproteases, siderophores, exotoxins, and lipases (Fuqua
& Greenberg, 1998; Van Delden & Iglewski, 1998; Kiratisin et al., 2002). In a similar manner, RhlII is responsible for the generation of the signal molecule C4-HSL, which binds to its receptor RhlR and activates the transcription of its target genes, many of which are involved in the production of secondary metabolites, such as pyocyanin (Pesci & Iglewski, 1997).

According to the current knowledge, acyl homoserine lactone (AHL) signals, like 3O-C12-HSL and C4-HSL mentioned in the QS systems, are produced from acyl enoyl-acyl carrier proteins (acyl-ACPs) and S-adenosylmethionine (More et al., 1996), where the acyl-ACPs are intermediates in the fatty acid biosynthesis pathway (Schaefer et al., 2000). One well-characterized enoyl-CoA reductase, FabI, plays an important role in the fatty acid biosynthesis, like regulating the proportion of the saturated to unsaturated fatty acids, coordinating fatty acid and phospholipid syntheses, and controlling the elongation of fatty acid (Heath & Rock, 1995). Furthermore, FabI has been implicated in providing an acyl group in the synthesis of AHLs (Hoang & Schweizer, 1999).

In our previous work, the product of the PA2950 gene (we named it pfm) was shown to affect the swimming motility of P. aeruginosa. This was not attributable to the lack of intact flagellum production but to the loss of its ability to rotate (Bai et al., 2007). Recently, pfm was shown to encode an enoyl-CoA reductase, which also participates in the fatty acid biosynthesis (Zhu et al., 2010), similar to that of FabI. In this report, we show that pfm influences the QS system through the production of AHL molecules, and disruption of the pfm gene results in decreased bacterial ability to adhere to host cells and a delayed killing of Caenorhabditis elegans.

Materials and methods

Bacterial strains, plasmids, and growth media

Escherichia coli and P. aeruginosa were cultured in Luria–Bertani medium at 37 °C. Antibiotics were used as follows: for E. coli, 50 μg mL⁻¹ ampicillin, 25 μg mL⁻¹ kanamycin, and 25 μg mL⁻¹ tetracycline; for P. aeruginosa, 50 μg mL⁻¹ kanamycin, 100 μg mL⁻¹ tetracycline, and 300 μg mL⁻¹ carbenicillin. P. aeruginosa strains PA68 (a clinical isolate) and PA68 pfm::Mu (Kb²) (named I69) have been described previously (Bai et al., 2007). The biosensor strain JB525 was provided by Dr Liang Yang, Technical University of Denmark (Wu et al., 2000).

Bacterial adherence assay

The assay for bacterial adherence to a human cell line was performed as described previously (de Bentzmann et al., 2006). Human lung cell line A549 was cultured in DMEM medium with 10% FBS. Before infection, the medium was replaced with serum and antibiotic-free medium. Then, cells were incubated at 37 °C for 4 h. Bacteria were then added into the cultured cells at a multiplicity of infection (MOI) of 30 for 1 h. Cells were washed twice with PBS, fixed with 4% formaldehyde, and then washed twice with PBS again. The fixed cells were stained with 0.1% crystal violet for 5 min, washed with PBS, and observed with Axioskop 40 microscope. For counting the number of bacterial cells adhering onto the lung cells, 15 lung cells per assay were randomly chosen in microscopic fields from three independent experiments.

Microarray and semiquantitative RT-PCR

Wild-type PA68 and pfm mutant strain (I69) were cultured at 37 °C in a rotating shaker at 200 rpm overnight. The culture was diluted to OD₆₀₀ nm = 0.05 with fresh LB medium and grew at 37 °C, 200 rpm for 6 h. RNA samples were prepared at OD₆₀₀ nm = 1.5 by Tianjin Biochip Corporation (China) who also provided both technical and bioinformatic analyses. The transcriptional profiles of the clinical strain PA68 and I69 were analyzed using Affymetrix P. aeruginosa DNA chip, and microarray data were analyzed following the manufacturer’s recommendation (www.affymetrix.com). Target signals of probes used to test the transcription level were set to 500. Two independent experiments were performed. Student’s t-test was applied to analyze the significance of individual transcripts (The microarray data shown in this study corresponded to P value <0.05). Semiquantitative RT-PCR was used to confirm the results. Primer pairs: lasR-s, CAGAAGATGGCGAGCACC and lasR-anti, ATGGAC GTTCCCCAGA AAATC; lasI-s, CAAGTTGCGTGCTGTCG ATGTTT and lasI-anti, AGTTCCCAGAAATC; lasI'-lacZ reporter gene fusions to lacZ reporter

To construct the lasI-lacZ operon fusion, 487-bp fragment, upstream of lasI coding sequence, including the potential lasI promoter, was ligated into of pDN19lacΦ between EcoRI and BamHI restriction sites (the plasmid harboring promoterless lacZ). Similarly, rhlI-lacZ reporter

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that harbored 559-bp DNA fragment including the potential rhlI promoter, lasR'-lacZ reporter that harbored 660-bp DNA fragment including the potential lasR promoter, and rhlR'-lacZ reporter that harbored 742-bp DNA fragment including the potential rhlR promoter were constructed.

Detection of AHLs with the biosensor strain

Acyl homoserine lactones were detected using a method modified from a previous report (Teasdale et al., 2009). The P. aeruginosa cultures were grown overnight and pelleted by centrifugation at 10 000 g for 10 min. One mL of the cell-free supernatant was collected for further experiments. Meanwhile, 1 mL culture of indicator strain JB525-gfp (ASV; E. coli MT102 harboring recombinant plasmid pBA132) (Wu et al., 2000) was centrifuged at 10 000 g for 10 min. The JB525-gfp (ASV) cell pellet was resuspended with the supernatant of P. aeruginosa culture. The suspension was then incubated at 30 °C for 90 min with shaking. Fluorescence intensity of the suspension was measured by fluorescence spectrophotometer (λ = 480 nm excitation, λ = 515 nm emission) to indicate the relative amount of AHLs in the supernatant of P. aeruginosa culture.

The biosensor strains E. coli DH5α(pECP61.5, rhlA'-lacZ) and E. coli DH5α(pECP64, lasB'-lacZ) were used to detect the levels of C4-HSL and 3O-C12-HSL, respectively (Pearson et al., 1997). The supernatant of P. aeruginosa overnight cultures was collected as the AHL source, and the AHLs were extracted as previously described (Pearson et al., 1995). Biosensor strains were cultured overnight and then diluted to OD_{600 nm} of 0.1. The supernatant of P. aeruginosa was mixed with biosensor strains. To monitor C4-HSL, the mixture of E. coli DH5α(pECP61.5) and the P. aeruginosa AHLs extraction was incubated at 37 °C to OD_{600 nm} = 0.3, then 1 mM IPTG was added, and the mixture was cultured for another 5 h. To monitor 3O-C12-HSL, E. coli DH5α(pECP64) was used, and IPTG was also added when OD_{600 nm} reached 0.3; the mixture was incubated at 37 °C for 90 min. After incubation, β-galactosidase activity of biosensor strains was measured as described by Miller (1972).

Quantitation of pyocyanin and elastase activities

Pyocyanin was determined according to the method described previously (Essar et al., 1990). Pseudomonas aeruginosa strains were grown in LB at 37 °C for 16 h with shaking at 200 rpm. The P. aeruginosa culture was pelleted at 10 000 g for 10 min. Three mL of chloroform was added to 5 mL of the supernatant to extract pyocyanin. The chloroform phase was collected and mixed with 1.5 mL of 0.2 M HCl. Absorption of the aqueous phase at 520 nm was measured.

The elastase activity was measured as described previously (Ohman et al., 1980). Bacterial strains were inoculated on LB plates that were spread with 0.4% elastin (Sigma). Following incubation at 37 °C for 24–48 h, the size of the hydrolysis ring was measured to evaluate the capacity of Type II secretion system.

Worm fast killing assay

Bacteria were cultured overnight in LB broth at 37 °C, and 20 μL cultures were seeded onto PGS plate (1% peptone, 1% NaCl, 1% glucose, 0.15 M sorbitol, 1.7% agar, 1 mM MgCl2, 1 mM CaCl2, 25 mM KPO4, pH 6.0) and incubated at 37 °C for 24 h. After additional incubation for 8–24 h at room temperature (25 °C), 60 of L4 stage N2 worms were placed on 4 PGS plates seeded with each bacterium and then grown at 25 °C again. Surviving worms were scored at the indicated time points and transferred to a fresh plate every day. The worm was considered as dead when it gave no response to touch, and worms that died of accidental events were eliminated.

pfm knockout in PAO1 background

Upstream primer pair: Full2950k1-s, TATCCTGTTATC GGTAGGACAAAC and Full2950k1-anti, GTCCGCTT GTAATGGGGTC and downstream primer pair: Full2950k2-s, GCTCCGCTCCTCCCCGAAAC and Full2950k2-anti, GGCGTCCTACTTCCGTCCCG were used to generate pfm knockout construct. Two 943-bp fragments, upstream and downstream of the pfm, were amplified by PCR and ligated into EcoRI and HindIII restriction sites of pEX18Tc plasmid, resulting in a construct that is deleted of the pfm. This construct was introduced into PAO1, and a pfm deletion mutant was selected using the method described previously (Schweizer, 1992).

Results and discussion

pfm influences bacterial adherence

Strain I69 is a mutant derivative of the clinical strain PA68 with a Mu transposon inserted into pfm and was found to be defective in swimming mobility (Bai et al., 2007). We found that pfm also influences bacterial adherence. As shown in Fig. 1, the number of wild-type PA68 bacteria adhering to the surface of human lung cell line A549 was significantly (P < 0.001) higher than that of mutant strain I69. The I69 complemented with a plasmid pDN18 encoding pfm (strain I69C) recovered much of
the lost adherence ($P < 0.001$). These results indicated that *pfm* affects bacterial adherence to the host cells.

**pfm affects the bacterial adherence through QS system**

To further test the role of *pfm* on the bacterial adherence, we performed a microarray assay to obtain transcriptional profiles of wild-type PA68 and the isogenic *pfm* mutant strain I69. Most strikingly, all the genes of the *flp-tad-rcp* gene cluster were severely downregulated in the I69 (Table 1). The *flp-tad-rcp* gene cluster is well known to be required for the assembly of type IVb pili that are responsible for the bacterial adherence (de Bentzmann et al., 2006). Therefore, the dramatic impact of *pfm* on the *flp-tad-rcp* gene cluster is the most likely reason for the decreased bacterial adherence of the *pfm* mutant strain I69. Interestingly, most of genes in the *flp-tad-rcp* gene cluster were reported to be quorum-activated genes, including *PA4296, PA4297, PA4298, PA4300, PA4302, PA4304, PA4305,* and *PA4306* (Schuster et al., 2003). Furthermore, focusing on the genes whose transcriptional level had been changed more than twofold with confidence level higher than 99.5%, we found that the majority of those genes had previously been reported as the quorum-controlled genes, including those upregulated genes as well as downregulated genes as shown in Table S1 and Table S2. The results showed that with the exception of those genes whose confidence degree was < 99.5%, almost all quorum-activated genes reported in the previous report were downregulated in the *pfm* mutant (Table S1; Schuster et al., 2003). Conversely, all quorum-repressed genes were upregulated (Table S2). These results suggested that the product of the *pfm* gene might affect bacterial adherence through the QS system.

**pfm regulates the production of AHLs**

To further explore whether *pfm* affects the QS system of *P. aeruginosa*, we determined the production of AHLs that contain both the signaling molecules 3O-C_{12}-HSL and C_{12}-HSL. The amount of AHLs can be reflected with the biosensor strain JB525, which harbors a plasmid encoding GFP under the control of the AHLs responsive promoter (Wu et al., 2000). *Pseudomonas aeruginosa* cultures were pelleted, and the supernatants were used as the AHL sources to incubate with the indicator strain JB525. The GFP fluorescence intensity was then determined (Materials and methods). As shown in Fig. 2, the fluorescence intensity of the *pfm* mutant strain I69 was about twofold lower compared to that of the wild-type strain PA68. The I69C strain, a complemented strain, partially recovered the decreased fluorescence of I69. The residual fluorescence of I69 is likely due to the AHLs

![Fig. 1. Bacterial adherence assay. After human lung cells were replaced with the serum and antibiotic-free medium, bacteria were added into the cell culture at a MOI of 30 at 37 °C for 1 h. (a) Views of bacteria (including the wild strain PA68, *pfm* mutant strain I69, and the complemented strain I69C) adhering on human lung cells. (b) The number of bacteria adhering on human lung cells. Fifteen lung cells/assay were randomly chosen in microscopic fields from three independent assays. The data of each bacterial strain were compared by t-test. **$P < 0.001$. Error bars indicated standard deviations of triplicate assays.](image-url)
generated via FabI which is another enoyl-CoA reductase of *P. aeruginosa*.

To further determine which of the two AHLs (3O-C12-HSL and C4-HSL) have been disturbed, we tested their activities separately. *E. coli* strain DH5α(pECP64) and *E. coli* strain DH5α(pECP61.5) were used to detect 3O-C12-HSL and C4-HSL, respectively, in PA68, I69, and I69C. Both 3O-C12-HSL and C4-HSL were significantly decreased in I69 (Fig. 3), indicating that *pfm* affects the production of both signaling molecules (3O-C12-HSL and C4-HSL) of the QS system.

Furthermore, we detected the elastase (LasB) activity that was used to indicate the content of 3O-C12-HSL (Seed et al., 1995) and monitored the content of phenazine pyocyanin, the terminal signal factor in the QS network of *P. aeruginosa* and the important sign of C4-HSL content (Dietrich et al., 2006). We found that both the elastase activity and the phenazine pyocyanin of I69 were significantly lower than those in PA68 (data not shown).

In addition, the microarray results showed that AHL synthetic genes lasI and rhlI were expressed at the similar level in both PA68 and I69, while the expression levels of the QS system signal receptors lasR and rhlR were about 3.5 times and 4 times lower, respectively, in I69 compared to those in PA68. These results suggested that a feedback regulation might exist between AHLs and the signal receptors LasR and RhlR. The exact mechanism needs further investigation. To confirm these results, we performed semiquantitative RT-PCR and constructed lasI'-lacZ, rhlI'-lacZ, lasR'-lacZ, and rhlR'-lacZ operon fusions. Semiquantitative RT-PCR showed that mRNA levels of lasI and rhlI were similar and lasR and rhlR were decreased in I69 (Fig. 4a). Furthermore, lasI'-lacZ and rhlI'-lacZ reporters showed similar β-galactosidase activity, while lasR'-lacZ and rhlR'-lacZ reporters showed decreased β-galactosidase activity in I69 (Fig. 4b), which was consistent with our microarray results.

**pfm is essential for the virulence of *P. aeruginosa***

The worm model has been successfully applied to test the virulence of mammalian bacterial pathogens (Tan et al., 1999). We performed worm fast killing assays to assess the influence of the *pfm* mutation on the bacterial virulence. When one or more QS genes were deleted in *P. aeruginosa*, the resulting mutants showed decreased virulence compared to wild type (Rumbaugh et al., 1999;
Pearson et al., 2000; Smith et al., 2002; Smith & Iglewski, 2003; Mittal et al., 2006). As shown in Fig. 5, a significantly lower worm death rate was observed following infection with I69 compared to that with PA68, suggesting that the pfm is required for the full virulence of P. aeruginosa. A defect in the QS system is the most likely cause of the reduced virulence, although whether the pfm mutation also caused other defects that influence the virulence awaits further study.

Characterization of pfm mutant in strain PAO1

To confirm that pfm is an essential gene of bacterial adherence, we also knocked out pfm in the background of PAO1, resulting in mutant strain PAO1Δ2950. As shown in Fig. S1, significantly more PAO1 cells adhered to lung cells compared to the PAO1Δ2950. Strain PAO1Δ2950 complemented with a plasmid pDN18 encoding pfm (strain Δ2950C) recovered much of the lost adherence. Furthermore, we also detected C4-HSL and 3O-C12-HSL of the PAO1 and the PAO1Δ2950 by E. coli DH5α (pECP61.5, rhlA’-lacZ) and E. coli DH5α (pECP64, lasB’-lacZ), respectively, and the PAO1Δ2950 displayed similar defect as I69 in the QS system (data not shown), demonstrating that the influence of pfm on the bacterial adherence and QS is not a strain-specific phenomenon.

In conclusion, pfm affected the adherence of P. aeruginosa and the synthesis of QS signals C4-HSL and 3O-C12-HSL which had no effect on the swimming mobility of P. aeruginosa (Reimmann et al., 2002). As the QS system was shown to influence the adherence of P. aeruginosa, our results suggested that PFM might regulate the adherence of P. aeruginosa via controlling the QS system. Considering that PFM and FabI have been reported to be involved in the biosynthesis of fatty acids (Zhu et al., 2010), we believed that pfm might be involved in energy metabolism which supplies energy for bacterial swimming. On the other hand, pfm affected the production of acyl groups which provided acyl groups supporting the synthesis of AHLs. However, knockout of pfm did not eliminate the generation of AHLs, possibly because the fabI gene product also supports the synthesis of AHLs. Unfortunately, deletion of both fabI and pfm seems to be lethal as we tried multiple times to obtain the double mutant without success. Thus, it should be plausible to obtain a conditional double knockout mutant to uncover their roles in the pathology of P. aeruginosa in the future.

Acknowledgements

This project was supported in part by National Basic Research Program of China (973 Program, 2012CB518700). We thank Yuehe Ding (National Institute of Biological Sciences, Beijing, China) and Zhihong Wang...
(Nankai University, Tianjin, China) for their assistants in carrying out experiments and Dr Barbara H. Iglewski (University of Rochester, USA) for providing biosensors pECP64 and pECP61.5.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The influence of pfm on the bacterial adherence in strain PAO1. (a) Views of bacteria adhering onto A549 human lung cells (including wild type PAO1, pfm knockout strain PAO1Δ2950 and the complement strain Δ2950C). (b) The number of bacteria adhering to the A549 human lung cells. The data from each bacterial strain were compared through t test. **P < 0.001. Error bars indicated standard deviations of triplicate assays.

Table S1. Changes of quorum-activated genes in I69.

Table S2. Changes of quorum-repressed genes in I69.

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