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Original article

Association of *OprF* mutant and disturbance of biofilm and pyocyanin virulence in *pseudomonas aeruginosa*

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ABSTRACT

Outer membrane porin F (OprF) is a major structural membrane protein of *Pseudomonas aeruginosa*, a recognised human opportunistic pathogen which is correlated with severe hospital-acquired infections. This study investigating a multiphenotypic approach, based on the comparative study of a wild type strain of *P. aeruginosa*, its isogenic OprF mutant. Both *P. aeruginosa* PAO1 and OprF mutant strains were grown in same condition and cultures were subjected to further analysis by SDS PAGE, pyocyanin production and biofilm formation that was analysed using scanning electron microscopy. Based on biofilm formation assay and pyocyanin production, the study showed that OprF plays a dynamic role in *P. aeruginosa* virulence. The absence of OprF results in slow growth rate corresponded to elongated lag phase and reduced biofilm production also a significance reduction in the production of the quorum-sensing-dependent virulence factors pyocyanin. Accordingly, in the OprF mutant scanning electron microscope “SEM” images showed impaired cellular niche and detached cells when compared to regular attached *P. aeruginosa* wild type cells in the niche. Taken together, this study shows the contribution of OprF in *P. aeruginosa* virulence, at least partly through impairment of biofilm, cell to cell attachment in niche and pyocyanin production. This study show a vital link between OprF and virulence factor production, providing novel insights for its role in pathogenicity and future could provide the basis for the development of novel drug targets for antibiotics and vaccines.

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1. Introduction

Pseudomonas aeruginosa is an ubiquitous opportunistic Gram-negative pathogenic bacteria that has a large genome (6.3 megabytes, Mb), which confers environmental versatility (Stover et al., 2000). *P. aeruginosa* is adapted to cause diseases in different hosts and additionally shows antibiotic resistance by variety of virulence factors including motility, biofilm formation and production of pyocyanin and exo-proteins such as protease, phospholipases and elastase that allowed its tissue invasion and systemic dissemination (Gómez and Prince, 2007). *P. aeruginosa* is able to grow under a wide range of oxygen tensions. A well-known example of anaerobic

conditions developing under infection occurs when the bacterium colonizes the lungs of cystic fibrosis (CF) patients (Worlitzsch et al., 2002; Govan and Deretic, 1996; Hassett et al., 2002). In addition to infecting the CF lung, anaerobic or microaerobic conditions have been found in wound injuries, otitis, and in infections within the upper respiratory tract (Portier et al., 1999; Rumbaugh et al., 1999; Brook, 2002; Carenfelt and Lundberg, 1977; Lyczak et al., 2000). In addition to naturally anaerobic environments, the bacterium often changes its environment directly to bring about anaerobic conditions. One example of this occurring is the generation of biofilm structures. Biofilms are complex bacterial communities formed on attachment to surfaces to allow increased resistance to various environmental insults (Costerton et al., 1999; O'Toole and Kolter, 1998; Xu et al., 1998). It appears that anaerobiosis is the preferred environment for biofilm bound *P. aeruginosa* as well as having other regulatory mechanisms (Yoon et al., 2002; Filiatrault et al., 2006; Eschbach et al., 2004; Sawers, 1991; Kuchma et al., 2005). Bacteria living in biofilms can be up to 1000 times more resistant to antibacterial compounds than planktonic bacteria because of the biofilm nature and structural composition (Nickel et al., 1985). Biofilm production is a major virulence that has been linked to quorum sensing (QS) system (De Kievit,

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2009) were other studies relates *P. aeruginosa* virulence and QS and to some structural component as Outer membrane proteins (OM) proteins (Fito-Boncompte et al., 2011). OprF is among the very few general porins of *P. aeruginosa* exposed on the external surface of the bacteria, functioning as a host-pathogen interactions facilitator and involved in various virulence abilities (Fito-Boncompte et al., 2011). The aim of this study is to understand the association of OprF membrane protein in biofilm formation and other virulence factors. However, this could provide the basis for development of novel drug targets for antibiotics and vaccines.

2. Methods

2.1. Bacterial strains, plasmids and media conditions

The strains and plasmids used in this project are *P. aeruginosa* PAO1 wild type, and *P. aeruginosa* OprF mutant were obtained from the *Pseudomonas aeruginosa* PAO1 Transposon Mutant Library (Manoil Lab, University of Washington). Strains were cultured routinely at 37 °C for 24 h in Luria Bertani (LB) broth or on LB agar plates or cetrimide agar, (Thermo Scientific Oxoid, UK). LB with tetracycline 50 µg/ml was used to selectively culture the mutant strain.

2.2. Growth curve analysis

From an overnight culture of PAO1 and OprF mutant strains, were inoculated to an OD₆₀₀, using spectrophotometer (biochrom libra s22), of 0.01 in 250 ml conical flask containing 25 ml of LB broth. OD₆₀₀ was measured each hour for 24 h.

2.3. SDS PAGE

Total protein was extracted from *P. aeruginosa* PAO1 and OprF mutant strains, this was achieved by growing the strains in LB broth and the pellet was centrifuged then resuspended in 20 µL of 2 × SDS loading buffer, it was incubated at 50 °C for 5 min before being sonicated for a few seconds, 10 µL of each sample and protein standards (Invitrogen, USA), were electrophoresed on 3% polyacrylamide SDS stacking gel and 12% polyacrylamide SDS resolving gel at 40 mA per gel (Bio-Rad, USA) in SDS running buffer. Proteins were stained with coomassie brilliant blue. The size of the protein bands was determined by comparison with protein standard marker (Invitrogen, USA).

2.4. Biofilm formation assays

Biofilm formation assay were performed in 96-wells polystyrene microtiter plates as previously described with some modifications (Fletcher, 1977). Strains were grown overnight in LB broth at 37 °C. Next day the cultures were diluted in LB to 10⁷ cfu/ml and dispensed in 96-well microtiter plate (Thomas science). The microtiter plates were incubated at 37 °C for 24 h. After that the cell suspension was removed and the plates were washed twice with 0.9% NaCl and inverted to dry at room temperature for 1 h. Following this 150 µL of crystal violet solution (CV; Prolab Diagnostics) was added to the wells and was allowed to stain for 15 min. After staining, CV was removed and the wells were washed 3 times with 0.9% NaCl. The bound CV was then solubilized by adding 200 µL of ethanol-acetone (80:20 v/v). The absorbance of CV was read at 595 nm on the microplate reader (BioTek Instruments, Winooski, VT).

2.5. Scanning electron microscopy (SEM)

To visualize pseudomonas strains biofilm formation, bacteria were grown overnight in LB at 37 °C. Next day the cultures were diluted in LB to 10⁷ cfu/ml. Sterile coverslips (polyvinyl; Fisher Scientific) were placed in each well of a 6-well plate (Thomas science) and then 2 ml of diluted culture plus 2 ml LB medium was added. Biofilms were then grown on the coverslips at 37 °C for 24 h. biofilm samples were fixed with using 3% glutaraldehyde in phosphate buffer pH 7.2 for 24 h. after three washes with phosphate buffer, samples were postfix with 1% osmium tetroxide (in H₂O) for 1 hr then the samples were applied in to an ethanol dehydration series of 50, 60, 70, 80, 90, and 2 × 100% (v/v) ethanol, for 5 min at each concentration (Marrie and Costerton, 1984). All samples were then dried for 1 day and sputter-coated with a palladium-gold thin film. The produced biofilm were viewed with a SEM/EDS system (FEI Quanta 400FEG ESEM/EDAX Genesis X4M, FEI Company, USA) in high-vacuum mode at 20 kV.

2.6. Pyocyanin production

Pyocyanin produced by *P. aeruginosa* was determined as previously described. Briefly, cultures were inoculated with shaking, 200 rpm using (Cheimika, Italy). Starting with OD₆₀₀ of 0.02 were grown in LB medium at 37 °C, and a humidity of 75% for 16 h. For pyocyanin determination, cultures were extracted with chloroform and re-extracted with 0.2 M HCl. The OD₅₂₀ was determined using microplate reader and normalized to cell growth measured as OD₆₀₀. For each sample, cultivation and extraction were performed at least in triplicate and 0.2 M HCl used as a control.

3. Results and discussion

3.1. Growth rate of *P. aeruginosa* PAO1 wild type, and *P. aeruginosa* PAO1 OprF mutant

To recognize the effect of mutating OprF gene on the growth rate of *P. aeruginosa*, the growth of the wild type and OprF deficient strain were compared. Observation of colonies that grown for the same period of time in LB broth, both strains had the same viability by the end of 24 h; time of colonization, but OprF mutant strain was significantly slower in growth rate of colonizing demonstrated by elongated lag phase and slower generation time compared to the wild type *P. aeruginosa* as shown in Fig. 1. The protein content of both strains were analyzed using SDS page in which the wild type *P. aeruginosa* showed the presence of OprF protein size around 37 kDa which are missing in the OprF mutant strains as shown in Fig. 2.

3.2. Biofilm formation assay

In this study we investigate the association between OprF protein and the biofilm production comparing *P. aeruginosa* PAO1 wild type, and *P. aeruginosa* PAO1 mutant, deficient in OprF gene. Fig. 3 showed the significant decline in biofilm production (with $p \leq 0.01$) between *P. aeruginosa* PAO1 and OprF mutant indicating the important role of OprF in the biofilm virulence.

3.3. SEM of *P. aeruginosa* biofilm

A scanning electron microscope used to visualize both strains biofilm and cells morphology in the niche. With both strains as shown in Fig. 4, biofilm was recognized at 24 h of incubation on glass coverslips as mentioned previously (Marrie and Costerton, 1984). SEM images displayed regular morphology and intense

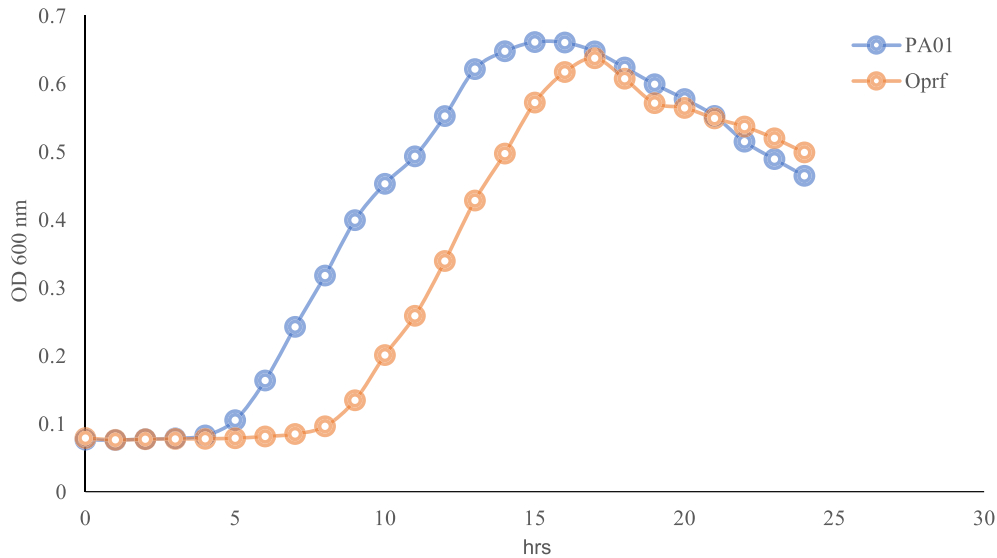


Fig. 1. Comparing bacterial growth of *P. aeruginosa* PAO1 and OprF mutant strain on LB Broth. It is demonstrated that generation time of the OprF deficient strain was slower and the elongated lag time of inoculation, than the *P. aeruginosa* wild strain, however, decline phase showed similar pattern for both strains.

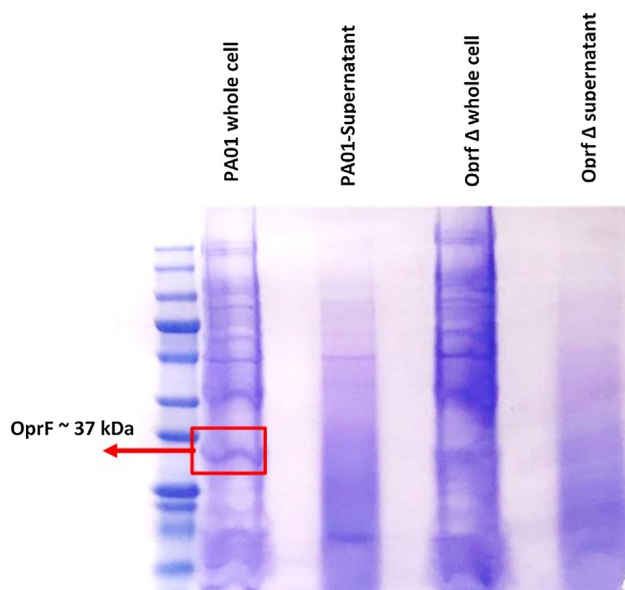


Fig. 2. SDS PAGE Coomassie blue stained comparing *P. aeruginosa* PAO1 and OprF mutant strain for OprF detection. OprF porin protein size 37 kDa shown in *P. aeruginosa* wild strain whole cell content and missing in the OprF mutant strain.

distribution of *P. aeruginosa* wild type cells in the niche and attached to biofilm, whereas, in OprF mutant the cells were detached in an irregular niche. These images supported the results of biofilm impairment by the absence of OprF.

3.4. Pyocyanin production

Major virulence factor of *P. aeruginosa* is the quorum-sensing-dependent virulence factors pyocyanin production. As it was noticed the effect of OprF on culture growth rate and biofilm formation, it was still not yet established if inactivation of the OprF have a direct effect on other virulence factors production. Bacterial culture strains were also checked for the effect of OprF on major virulence factors; exclusively studied the pyocyanin production as a marker of virulence. Measurement of pyocyanin production

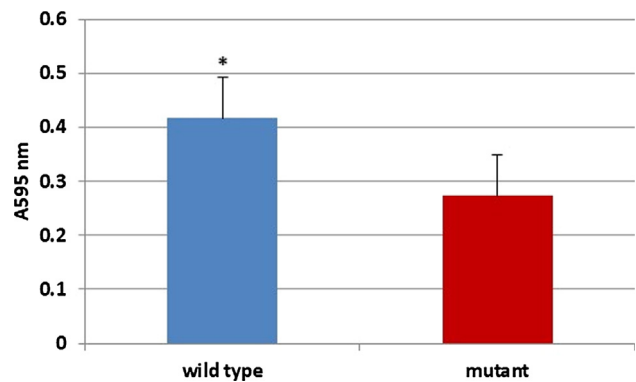


Fig. 3. Biofilm detection assay of *P. aeruginosa* shows significant decline in biofilm formation from *P. aeruginosa* PAO1 wild type strain and the OprF mutant strain. The error bars represent the mean \pm standard error. (n = 6, *P < 0.01).

by *P. aeruginosa* wild type strain and OprF mutant strain were gathered and compared showing a significant decline (with $p = 0.00069$) in pyocyanin production among the mutated strains in comparison to wild strain. This clearly magnifies the direct role of OprF gene on the production of pyocyanin as virulence factor.

4. Discussion

OprF is a cornerstone gene in the expression of biological functions of *Pseudomonas aeruginosa* (Stover et al., 2000; Fito-Boncompte et al., 2011). In this study we demonstrate the association of OprF and its role in obtaining full virulence in *P. aeruginosa* using a multiphenotypic approach, based on the comparative study of a wild-type strain of *P. aeruginosa*, its isogenic oprF mutant. Growth rates, biofilm formation and pyocyanin production of different *P. aeruginosa* strains are followed up and obtained, their affection by presence or absence of OprF was investigated.

The growth of *P. aeruginosa* PAO1 wild strain and *P. aeruginosa* PAO1 mutant strain with deficient OprF monitored in a culture for 24 h were both strains verified similar viability for the culturing condition but demonstrated slower growth rate in OprF mutant strain (Fig. 1). This derives in agreement with previous study studying the link of OM proteins in virulence of *P. aeruginosa*, they

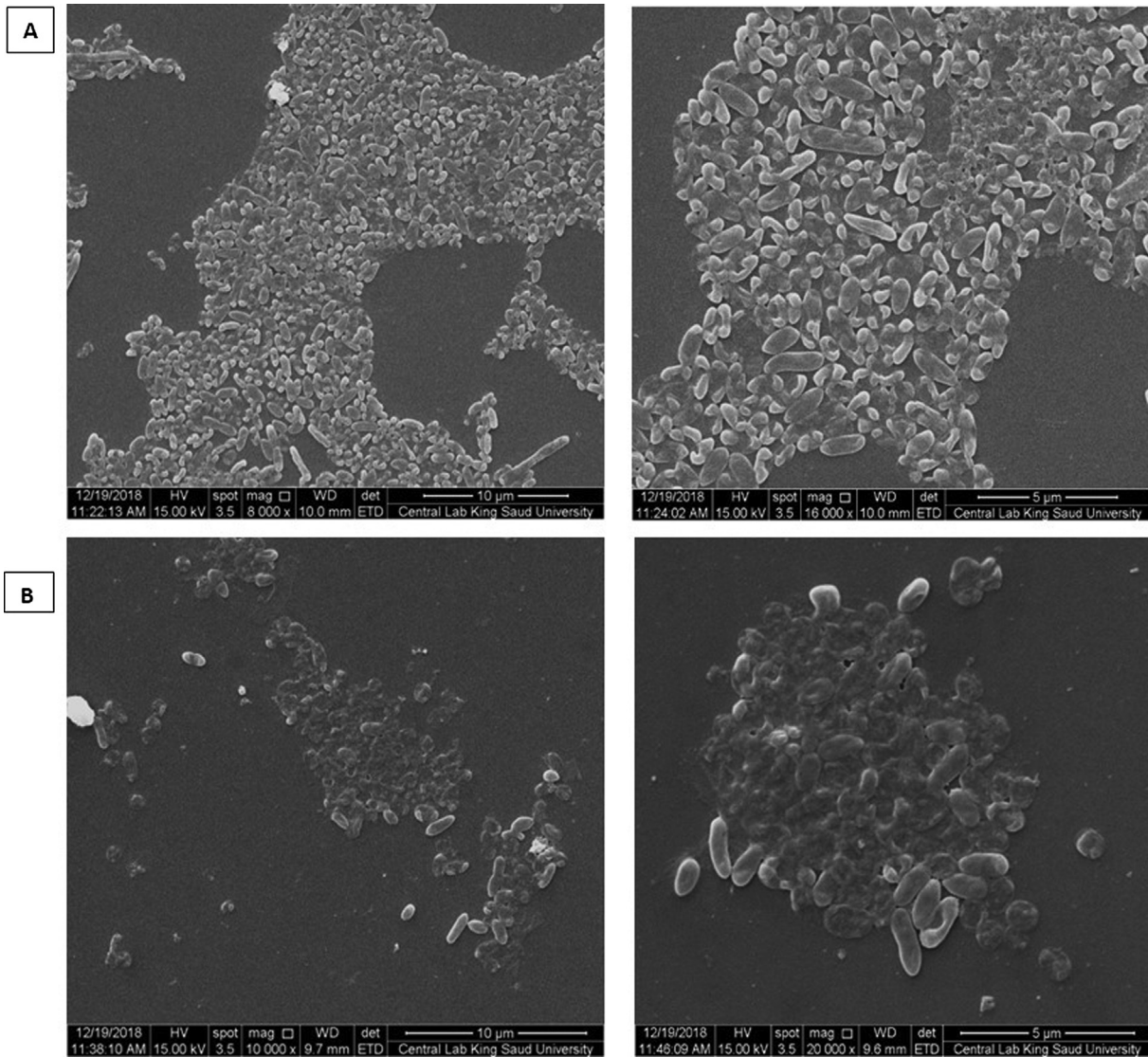


Fig. 4. SEM images at different magnification for (A) *P. aeruginosa* PAO1 wild type, and (B) *P. aeruginosa* OprF mutant detecting biofilm and cells attachment.

hypothesized that OprF is involved in cellular adhesion and/or production of virulence-related factors and the study results showed that the decreased toxicity of the *oprF* mutant is at least partially due to its lowered ability to adhere to cells. This confirms the role of OprF as a cellular adhesin (Fito-Boncomppte et al., 2011). On the other hand biofilm production was not studied or associated to the OprF porin which in this study was directly investigated and our results showed significant reduction in the biofilm formation and these results were supported by SEM images that showed detached in colonizing niche in absence of OprF. SEM images showed the morphology of *P. aeruginosa* PAO1 wild bacterial cells and their adhesion to each other in a steady niche and intense biofilm in contrast to the OprF mutant were irregular niche biofilm and detached cells were noticed. This may attributed not only to reduced biofilm production in OprF mutants but also to the altered biofilm component which is a future item to be investigated.

The bacterial culture supernatants were also investigated for the presence of major virulence factors, namely, pyocyanin. Altered pyocyanin production by OprF mutant (Fig. 5). Our result comes in agreement with the reduced virulence phenotype of the *oprF* mutant from previous study (Fito-Boncomppte et al., 2011). Inactivation of OprF strongly affected all these virulence factors and

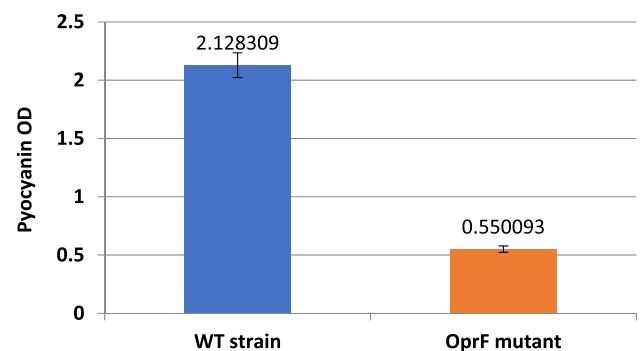


Fig. 5. OprF mutant strains showing altered pyocyanin production. Compared to the relative amounts of pyocyanin extracted from strain *P. aeruginosa* PAO1 after the bacteria were grown in LB broth. Experiments were repeated three times and pyocyanin measurements at OD520 was obtained using microplate reader and normalized to cell growth measured as OD600.

revealed vital role in the observed phenotypes. This could be due to the lack of OprF can change the OM composition on the cell surface, transporting a stress signal, which sequentially could be

responsible for the observed phenotypes, all leading to less pathogenic strain. Targeting the OprF porin or gene in *P. aeruginosa* pathogenic strains would render them less pathogenic and more susceptible strains so this could be a novel drug targets for antibiotics and vaccines.

Declaration of Competing Interest

We declare that we have no conflict of interest.

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