Novel Ca^{2+} Sensor Controlling Iron Uptake in *Pseudomonas aeruginosa*, a Human Pathogen

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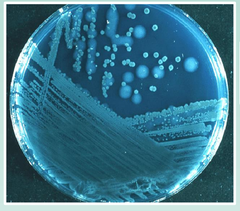
Abstract

Pseudomonas aeruginosa is a Gram negative, opportunistic pathogen which causes a wide range of severe infections in humans. These infections are difficult to treat and, especially in the lungs of cystic fibrosis (CF) patients, often become chronic. Complicating eradication of *P. aeruginosa* is pyoverdine, a virulence factor which aids bacterial iron acquisition in iron-limited environments. Previous work has shown that pyoverdine production in *P. aeruginosa* strain PAO1 is increased under elevated levels of Ca^{2+}, as are found in the pulmonary fluids of CF patients. A Ca^{2+}-binding EF-hand protein, EfhP (PA4107), was demonstrated to be critical for the Ca^{2+}-regulated virulence in PAO1. This study seeks to characterize EfhP regulation of the *P. aeruginosa* response to Ca^{2+}, particularly pertaining to critical iron uptake pathways. We have established that deletion of *efhP* significantly hinders PAO1 pyoverdine production when grown at high Ca^{2+} in BMM8 medium. Pyoverdine is a fluorescent molecule quantified by measuring fluorescence at 400 nm excitation/460 nm emission and normalized by OD600. The presence of *efhP* in several CF clinical isolates was confirmed with PCR using *efhP*-specific primers. We observed that these clinical isolates also show increased pyoverdine production at 5 mM Ca^{2+} vs. the no Ca^{2+} condition. In the clinically relevant synthetic cystic fibrosis sputum medium (SCFM), PAO1 shows increased pyoverdine production with increasing Ca^{2+} concentrations. In the future, we aim to identify specific sequences in the *efhP* gene that are most important to the regulation of pyoverdine production by testing a series of strains expressing mutated EfhP. We also plan to evaluate expression of *efhP* in CF clinical isolates and test for their pyoverdine production when cultured in SCFM. The new knowledge gained can support further studies to develop novel efficient medications to improve the quality of life of CF patients who struggle with chronic *P. aeruginosa* infections.

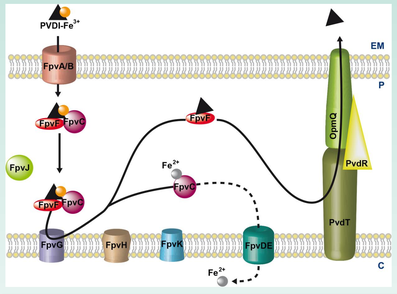
Introduction

- *Pseudomonas aeruginosa* is a deadly human pathogen, particularly known for causing chronic infections in CF patients
- Elevated calcium levels in the nasal and lung liquids is a signature feature of CF
- *P. aeruginosa* secretes many virulence factors, including iron-sequestering siderophores such as **pyoverdine**
- Dr. Patrauchan's group previously showed that calcium controls several virulence factors such as **biofilm formation**, **pyocyanin production**, and **swarming motility**
- An EF-hand protein, EfhP, was shown to **bind calcium** and play a role in *P. aeruginosa* calcium-regulated virulence
- Calcium-bound EfhP binds several protein partners to transmit calcium signals
- Preliminary data generated by my graduate mentor suggests that EfhP possibly binds PvdR, a pyoverdine pump
- The molecular details of EfhP interactions that enable calcium regulation of pyoverdine and other virulence factors are unknown



"Pseudomonas aeruginosa colonies grown from sputum of a CF patient with chronic lung infection," (Høiby, Niels, et al, 2017).

Predicted structurre of EfhP, a **Ca^{2+} sensor** that binds Ca^{2+} with its EF hands (blue)



Pyoverdine uptake and secretion pathway in *P. aeruginosa*. Iron uptake master regulator **Fur** binds Fe^{2+} in the cytoplasm and controls pyoverdine production (Bonneau et al. 2020)

Significance

The new knowledge generated in this research will help future studies aiming to develop inhibitors to silence EfhP function and reduce the ability of the pathogen to survive in a human body. New medications can then provide better quality of life for CF patients living with chronic *P. aeruginosa* infections.

Pyoverdine Production is Regulated by Calcium in PAO1

This work began with optimization of the culturing procedures and media for pyoverdine assays in order to best detect and quantify pyoverdine production in PAO1. Pyoverdine (PVD) is a fluorescent molecule which is detected at 400/460 nm excitation/emission. Additionally, both Apo-PVD and PVD-Fe have an absorbance peak at 400 nm at pH 7 (Hoegy et al. 2014). All fluorescence and absorbance reads were taken using Synergy Mx Multi Mode Microplate Reader (Biotek) and all incubations were conducted at 37 ºC and 200 rpm.

Pyoverdine production was tested in three growth media:

Biofilm Minimal Medium (BMM8)

- We tested pyoverdine production by PAO1, **Δ***efhP*, and 5 CF clinical isolates
- Pre-cultures were grown in 3 ml BMM8 0 mM Ca $^{2+}$, normalized, and used to inoculate BMM8 main cultures containing 0 or 5 mM Ca $^{2+}$
- Main cultures were grown in 96 well plates in the Biotek. Kinetic fluorescence reads (400/460 nm excitation/emission) and Abs400 reads were taken hourly and normalized by Abs600.

Synthetic Cystic Fibrosis Sputum Medium (SCFM)

- This is a clinically relevant rich medium intended to mimic the conditions present in the CF lung
- We tested pyoverdine production of mid-log PAO1 grown for 10 hours in 5 ml tubes of SCFM
- For this experiment, we used 4 different Ca^{2+} concentrations: 0 mM, 1 mM, 3 mM, and 5 mM

LB Medium

LB medium was found to not be an optimal medium for quantifying pyoverdine production in PAO1. When comparing the emission spectra at 400 nm excitation for both PAO1 in LB medium and an LB blank we observed that the growth medium itself provides a significant amount of background fluorescence.

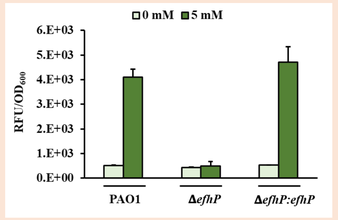
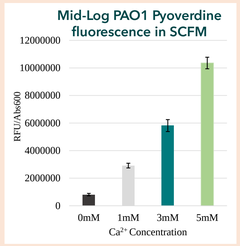


Figure 1: Pyoverdine production of wild type PAO1, Δ*efhp*, and Δ*efhP:efhP* was quantified by fluorescence (400 excitation/460 emission) and normalized by OD 600



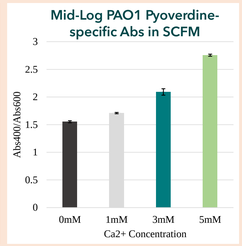


Figure 2 and 3: PAO1 unbound pyoverdine in SCFM measured by fluorescence, and PAO1 total pyoverdine in SCFM measured by Abs400

Pseudomonas aeruginosa consistently shows an increase in pyoverdine production at increasing levels of calcium (Fig. 1).

PAO1 shows increased fluorescence and Abs400 in response to elevated Ca^{2+} concentrations in SCFM, a clinically relevant medium (Fig. 2-3).

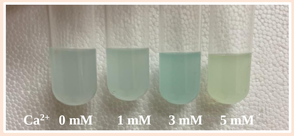


Figure 4: Visible pyoverdine pigment differences in *P. aeruginosa* grown at differing levels of calcium

CF Clinical Isolates Encode *efhP* and Respond to Calcium

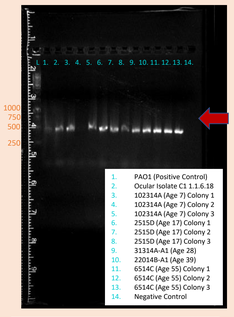


Figure 5: Amplification of the *efhp* gene in several CF sputum clinical isolates

We confirmed the presence of *efhP* in all but one clinical isolate via PCR using efhp_F and efhp_R primers and running the amplified products on ethidium bromide agarose gels (expected size 468 bp) (Fig. 5).

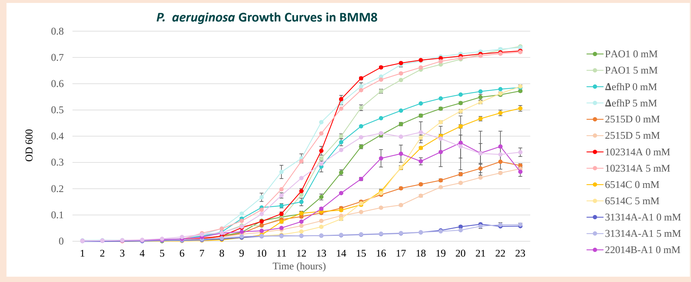
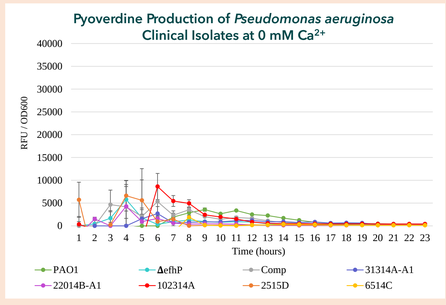


Figure 6: Growth of PAO1, Δ*efhP*, and a number of clinical isolates in BMM8 measured by Abs600



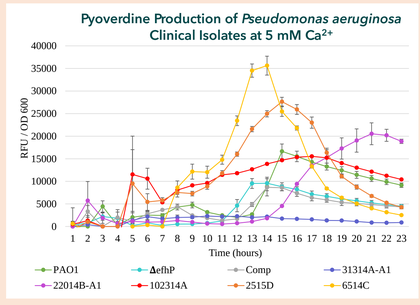


Figure 7 and 8: pyoverdine production of PAO1 and a number of clinical isolates measured by fluorescence

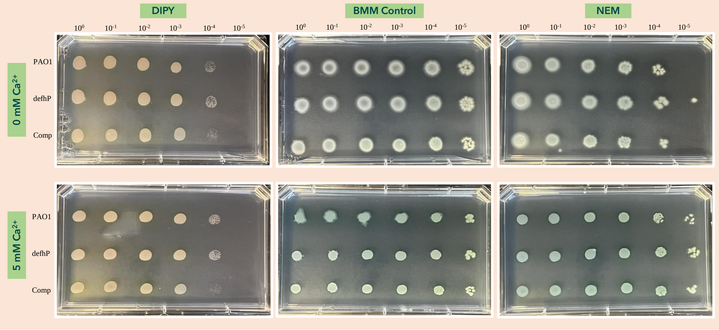
We quantified the growth and pyoverdine production of PAO1 and selected CF isolates in 0 mM Ca^{2+} and 5 mM Ca^{2+} BMM8. All selected isolates except 31314A-A1 show much higher pyoverdine production at the elevated 5 mM Ca^{2+} level as compared to isolates grown at 0 mM Ca^{2+} (Fig. 6-8).

P. aeruginosa responds to NEM and 2,2'-dipyridyl

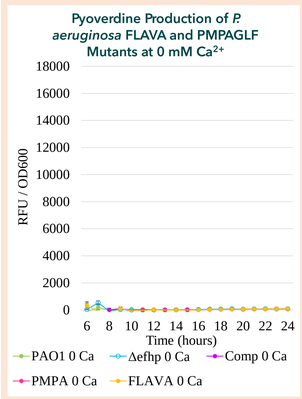
2,2'-dipyridyl is an iron-chelating molecule that removes the iron from growth media, preventing pyoverdine, and in turn, the cell, from using it. N-Ethylmaleimide (NEM) disrupts disulfide bonds, which help efhP oligomerize. This compound was relevant, since efhP is our subject of interest.

All tested strains show a decrease in growth in the presence of 2,2'-dipyridyl regardless of calcium level, whereas N-Ethylmaleimide only has a significant effect at 0 mM Ca^{2+} (Fig. 9).

Figure 9: *P. aeruginosa* sensitivity assays on BMM8 agar with 2,2'-dipyridyl (DIPY) and N-Ethylmaleimide (NEM)



Hydrophobic Patches FLAVA and PMPAGLF



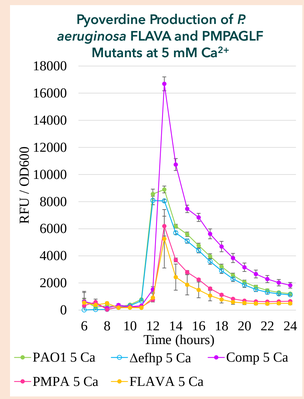


Figure 10: Comparison of PAO1 and Δ*efhP* pyoverdine production with a complement, FLAVA, and PMPAGLF in BMM8

The FLAVA and PMPAGLF sequences are highly hydrophobic and therefore predicted to bind protein partners and enable the function of EfhP, as the hydrophobicity of EfhP increases when it binds calcium. Both FLAVA and PMPGLF showed a notable decrease in pyoverdine at elevated calcium when compared to the wild type PAO1 and the deletion mutant (Fig. 10).

What's Next?

- To determine residues of EfhP which are essential for regulating pyoverdine production, we will test pyoverdine production of six mutants that were previously generated by my graduate mentor. These strains lack the native EfhP, instead expressing mutated variants of EfhP:
  - P34S
  - V41L
  - KTPA63-66QAST_G69S
  - H91R
  - D106E
  - SK148-149GR_Q154R

Each name reflects which amino acid(s) was/were replaced with which.. All other mutations occur at high frequency in clinical isolates of *P. aeruginosa*, suggesting their importance for adaptations of the pathogen in a host during infections.

Conclusions

- We have successfully confirmed the presence of *efhP* in a total of 12 clinical isolates, spanning a patient age range of 7 to 55
- Wild type PAO1 and most clinical strains show increased pyoverdine production at 5 mM Ca^{2+} BMM8 compared to the 0 mM Ca^{2+} control
- So far, it doesn't appear that age of patient has an impact of Ca^{2+}-induced pyoverdine production in isolates
- Deletion of *efhP* reduces the effect of Ca^{2+} on PAO1 pyoverdine production in BMM8
- PAO1 shows increased pyoverdine production in SCFM with increasing concentrations of Ca^{2+}
- Depletion of cellular Fe by using 2,2'-dipyridyl decreases growth of *P. aeruginosa* at both calcium conditions.
- Distortion of disulfide bonds by N-Ethylmaleimide decreases growth of *P. aeruginosa* that is recovered in the presence of calcium.
- The PMPAGLF and FLAVA hydrophobic patches play role in EfhP-dependent regulation of pyoverdine production at elevated calcium.

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