Virulence Factors of *Pseudomonas aeruginosa*: Investigating the Differential Expression of Pyocyanin Pigment in a Polymicrobial Setting

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1. Introduction

Bacteria almost always exist in polymicrobial communities where communication or quorum sensing between *intra*-species and *inter*-species is very important in regulating their virulence gene expression. The focus of our research is to understand the differential regulation of quorum sensing genes in the bacteria *Pseudomonas aeruginosa*. *P. aeruginosa* is an opportunistic bacterium that often results in serious infections in health-care settings especially in immunocompromised patients. One of the very important aspects of *P. aeruginosa* virulence is the production of the blue-green pigment pyocyanin and its association in the formation of biofilm. Our research is aimed to identify the role of pyocyanin on the formation of the biofilm by the bacteria. We are also interested in understanding at the genetic level how the quorum sensing circuit is modulated by *P. aeruginos*a in a polymicrobial community. We co-cultured the laboratory strain of *P. aeruginosa* with other various laboratory strains of gram positive and gram negative bacteria (*Klebsiella pneumonia, Escherichia coli, Staphylococcus aureus, Enterococcus faecalis* that are frequently associated with nosocomial infections). Our preliminary results indicate that the expression of pyocyanin by the bacteria *P. aeruginosa* is highly dependent on the specific bacterial population that *Pseudomonas* encounters. We quantified the pyocyanin expression under various polymicrobial conditions using a colorimetric assay that we optimized. The future research efforts will be focussed on generating the pyocyanin gene knockout strains with a goal to compare the relative gene expression differences between the wild type and mutant strains. Our findings would help understand how several other virulence factors of *P. aeruginosa* are co-regulated with pyocyanin. This could eventually provide valuable insight on the surveillance mechanisms of quorum sensing for *P. aeruginosa* in detection of its bacterial neighbors and response by producing antimicrobial factors. Our findings can also suggest novel therapeutic strategies for treating *P. aeruginosa* dominated polymicrobial infections.

2. Pyocyanin-A QS Phenomenon

One of the hallmark features of *P. aeruginosa* is its ability to produce the blue-green pigment pyocyanin. Pyocyanin belongs to the family of phenazines [5-methyl-1(5H)-phenazinone] which are heterocyclic compounds that are produced naturally with side chains substituted at different points around their rings by different bacterial species. At neutral pH, pyocyanin exhibits a distinctive blue color and turns red in acidic pH conditions. It is a small and highly diffusible nitrogen-containing aromatic compound with a multitude of biological activities. Pyocyanin is involved in a variety of biological activities including quorum sensing, maintaining fitness of bacterial cells and biofilm formation. It is also known to exhibit antibacterial and antifungal properties. In addition, pyocyanin plays a major role in the survival of *P. aeruginosa* under oxygen-poor conditions.

In *P. aeruginosa*, pyocyanin production involves a stepwise process, beginning with the synthesis of the primary quorum sensing (QS) molecule *N*-acyl-L-homoserine lactone (AHL) during the exponential growth phase followed by the secondary QS molecule *Pseudomonas* quinolone signaling (PQS) during the late exponential phase. PQS directly controls the expression of *phzA-G* operons resulting in the production of phenazine-1-carboxylic acid (PCA) from its precursor chorismic acid. PCA is then modified to produce three metabolites during the early stationary phase of which pyocyanin is the predominant product and is regulated by the *phzM* gene. The two other types of phenazine products are phenazine-1-carboxamide (PCN; encoded by *phzH*) and 1-hydroxyphenazine (1-OHPHZ; encoded by *phzS*).

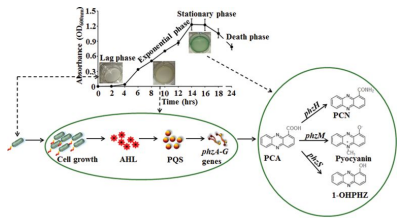


Figure 1. Stepwise Production of Pyocyanin

3. Quantification of Pyocyanin Pigment Procedure

1. Actively growing bacterial suspension was used to inoculate the tryptic soy agar plates using a sterile spreader.

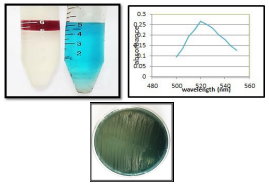
2. The plates were incubated at 37℃ for more than 60 hours to maximize the yield of pyocyanin.

3. The agar was cut aseptically into 1-cm squares and placed in a sterile bottle.

4. Pyocyanin was extracted by adding chloroform & hydrochloric acid.

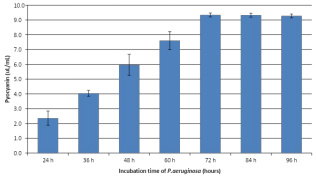
5. Absorbance at 520 nM is taken for the extracted pigment.

6. Concentration of Pyocyanin uL/mL=(OD)520 * 17.072



4. Effect of Incubation Time on the Pyocyanin Pigment Production

Using the Tryptic Soy Agar, we cultivated the bacteria at 37℃ for extended periods of time and harvested the cells every 12 hours to extract and measure the pyocyanin pigment. Approximately after 72 hours the pyocyanin production reaches maximum and stays the same after that. For all the subsequent experiments, *P. aeruginosa* was grown for 72 hours at 37℃ to maximize the expression of pyocyanin.



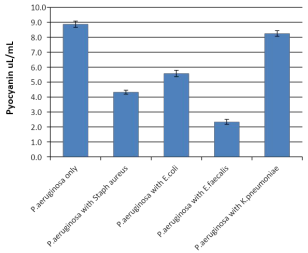
5. Differential Regulation of Pyocyanin

P. aeruginosa was co-cultured with the two commonly found nosocomial gram positive bacteria *Staphylococcus aureus* (SA), *Enterococcus faecalis* (EF) and the two commonly found nosocomial gram negative bacteria *Escherichia coli* (EC), *Klebsiella pneumonia* (KP). (All the strains used were common wild type laboratory strains).

Procedure:

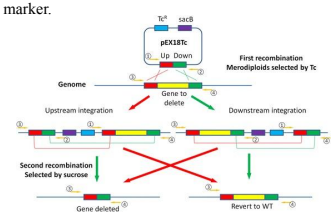
1. SA, EF, EC and KP (laboratory bacterial strains) each of the bacterial strains were grown individually on TSA plates by swabbing a freshly grown liquid culture with OD of 0.6 @ 600 nm.
2. A fresh culture of *P. aeruginosa* was suspended in a sterile Tryptic Soy broth tube to a reach a turbidity equivalent of a 0.6 OD @ 600nm.
3. 100 uL of the above *P. aeruginosa* was added at the center of each TSA plate having one of the following bacterial strains: EC, KP, EF and SA.
4. The plates were incubated at 37℃ for 72 hours and pyocyanin pigment was measured.

6. Results: *P. aeruginosa* Differentially Regulates the Production of Pyocyanin



7. Experimental Strategy for Generating *P. aeruginosa* Gene Knockouts (Work under progress):

Homologous recombination of the engineered vector: Positive selection of recombinants using antibiotic selection marker counter selection using SacB (sucrose gene) marker.



8. Conclusion

Pyocyanin is a highly versatile molecule recognized for its effect as a potent virulence factor of *P. aeruginosa* . Our results suggest that *P. aeruginosa* modulates the expression of pyocyanin in a mixed microbial population and we hypothesize that this regulation has an effect on the biofilm forming ability of *P. aeruginosa*. Biofilm formation is associated with increased resistance to antibiotic therapies and persistence of bacteria in nosocomial infections. Using versatile tools of Microbiology, Molecular Biology and Biochemistry we are working towards understanding the role of pyocyanin in the biofilm formation of *P. aeruginosa* in a polymicrobial setting. Eventually the goal is to identify novel drug targets that could serve as quorum quenchers or quorum-sensing inhibitors. This approach has a promising potential for the future in minimizing and delaying the problem of antibiotic resistance.

9. References & Acknowledgements

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