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Research Article

Comparison of Group Behaviors in the Wild Type Versus Mutant Strain of the Bacteria *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is an opportunistic bacterium that is frequently found in patients with cystic fibrosis, burn wounds and various immuno-compromised conditions. The bacterium is known to produce various virulence factors through group communication behaviors known as quorum sensing. Here we report that autolysis or self-killing is a socially beneficial behavior of the bacteria and compare the wild-type and a mutant strain (deficient in one of the transcriptional activators of quorum sensing). Some of the group behaviors that are compared in the study are the ability of the bacteria to undergo programmed cell death or autolysis, the ability of the bacteria to produce the blue-green pigment pyocyanin which is toxic to many Gram-positive bacteria and animal cells, and the ability of the bacteria to form biofilms.

Keywords: *Pseudomonas aeruginosa;* Biofilms; *RhlR*

Introduction

Pseudomonas aeruginosa is an aerobic, Gram-negative, non-spore-forming rod that can cause multiple infections in both immunocompromised and immunocompetent hosts. These pathogenic bacteria are often difficult to treat in hospital settings due to their ability to develop resistance to drugs and flourish in ambient hospital temperatures without requiring any nutritional sources for up to 16 months. It is most commonly seen as an opportunistic pathogen and is an important cause of nosocomial infections like catheter-associated urinary tract infections, and ventilator-associated pneumonia, amongst others. Two of the mechanisms which allow it to become pathogenic are 1) its production of virulence factors and 2) its ability to form biofilms. *P. aeruginosa* virulence factors counteract host defenses, increase the bacterium's competitiveness, and directly damage host tissues. The most notable virulence factor produced by *P. aeruginosa* is the toxic blue-green pigment, pyocyanin, which plays a role in biofilm production. The ability of *P. aeruginosa* to form a biofilm is a critical mechanism in increasing antibiotic resistance and resisting host defenses. This is specifically important in cystic fibrosis patients, as most patients acquire infections in the first year from healthcare facilities or the environment. *P. aeruginosa* uses quorum sensing (QS), a form of cell-to-cell chemical signaling, to induce biofilm secretion and formation. QS monitors bacterial cell densities to promote communication between bacteria to regulate the expression of genes involved in competition, pathogenicity, resistance, and virulence. All the virulence factors including biofilm formation and the production of pyocyanin pigment are under the regulation of the QS gene circuit in the bacteria. A very important question is if the production of pyocyanin and the formation of biofilms in the bacteria are dependent on each other or not. In addition to this, *P. aeruginosa* also undergoes a sacrificial autolysis

known as bacterial cell death and the current study is an attempt to understand the phenomenon and its role in the bacteria's virulence properties.

Bacterial cell death is an interesting phenomenon because it plays a crucial role in many important processes. Bacterial cell death does not just occur in the late stationary phase when nutrient limitation can lead to starvation. But instead begins in the early stationary phase for some bacteria because of the phenomenon of autolysis. Our hypothesis is that there is a direct relationship between QS and programmed cell death of the bacteria and there are community benefits of the sacrificial suicide by autolysis of the bacteria *P. aeruginosa*. To address this, we are comparing the wild type and a mutant strain (*rhlR* mutant) of the bacteria in their abilities to undergo autolysis and biofilm formation (along with pyocyanin production). This study will provide a basic understanding of some of the fundamentally misunderstood concepts of bacterial behaviors. Group behaviors and community benefits are underestimated in bacteria and to this end; these studies provide an important insight into the global problem of antibiotic resistance.

RhlR is a QS receptor and transcriptional regulator in *P. aeruginosa* that activates the transcription of various virulence factors. Specifically, *rhlR* promotes the expression of genes coding for pyocyanin, biofilm formation, elastases, along with genes for many other virulence factors. Clinically, *rhlR* mutants almost never exist while bacteria containing mutations in other QS genes like *lasR* naturally occur in patients. This suggests that *rhlR* mutants are not as virulent and that this gene is essential to the pathogenicity of *P. aeruginosa*. The goal of our research is to use biofilm formation and pyocyanin production as biomarkers to compare the pathogenicity of wild type versus a *rhlR* mutant. Also, there is very limited information on the role of pyocyanin production on biofilm formation and we hypothesize that *P. aeruginosa* uses a novel mechanism known as "autolysis or sacrificial killing" which is bacterial apoptosis. Pyocyanin contributes to autolysis to form stronger biofilms. To test this hypothesis, we looked at the autolysis ability of the wild type versus mutant strains along with the other biomarkers: biofilm formation and pyocyanin production.

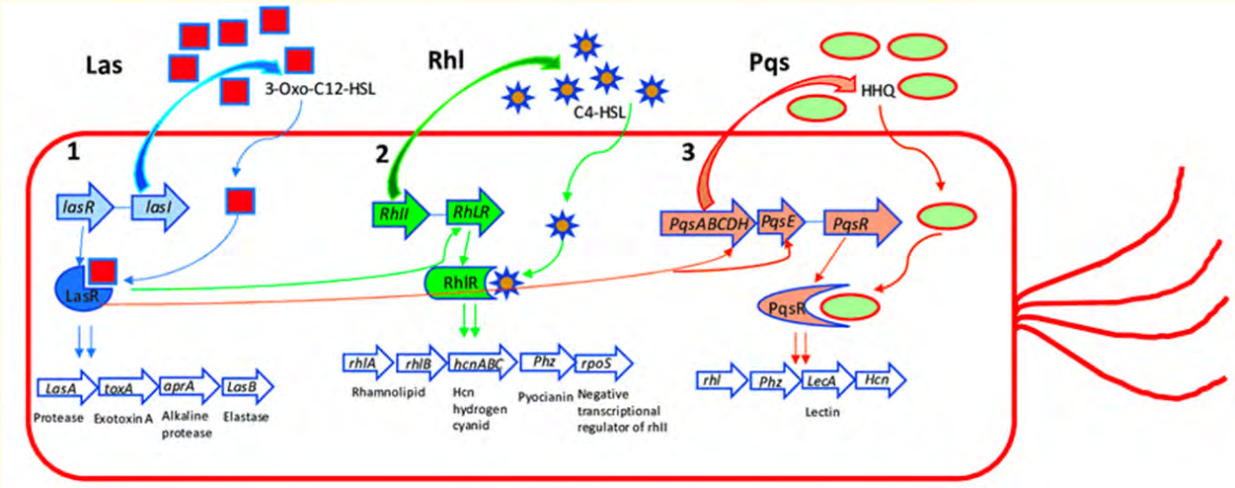

Figure 1: Major quorum sensing circuits in *P. aeruginosa* (Guzzo, Francesca., *et al.* 2020).

Experimental Materials and Methods

Bacterial culture and media: The bacteria utilized for this study was a laboratory strain of *P. aeruginosa* (PA-01) (wild type and RhlR mutant); TSA (Tryptic Soy Agar) media: To make one liter of TSA, 15 g of pancreatic digest, 5 g of enzymatic digest of soybean, 5 g sodium chloride, and 15 g agar were added. These were dissolved in a liter of water via boiling briefly. Once dissolved and mixed, this mixture was autoclaved for about 15-20 minutes

at 121°C, 15 psi; King's Agar A media: To make one liter of the King Agar A media, 20g of gelatine peptone, 1.4 g magnesium chloride, 10g of potassium sulfate and 15.0 g of agar were added to 990 mL of distilled water. After the ingredients were dissolved, 10 mL of glycerol was added and the mixture was autoclaved for about 15-20 minutes at 121°C, 15 psi.

Pyocyanin Assay- Overnight cultures were standardized to OD 600 1.0 before inoculating in 25 mL of the liquid media with 1:100 dilution. Cultures were grown at 37°C for 72 hours for maximal pyocyanin production. Supernatants were collected after centrifugation and filter sterilized. 4.5 mL of chloroform was added to 7.5 mL of supernatant and the mixture was briefly vortexed. Samples were again centrifuged and the resulting blue layer at the bottom (chloroform + pyocyanin) was transferred to a new tube. 0.2 M HCl was added to each tube and briefly vortexed. Spectrophotometric measurements were done at 520 nm to quantitate the amount of pyocyanin produced. Pyocyanin concentration (ug/mL) was calculated by multiplying the value you get at 520 nm with 17.072, which is the extinction coefficient of pyocyanin.

Biofilm Assay - Cultures were inoculated by adding an overnight culture of *P. aeruginosa* into 1 mL of sterile media, and the cultures were incubated in 12-well plates at 37 °C for 48-72 hours. The supernatant was then discarded and the adhered biofilm was quantitated using 0.1% crystal violet dye.

Autolysis Assay - Wild type and mutant strain were grown at 37°C for 24 hours. Samples were collected every 6 hours to monitor growth using a spectrophotometer. Maximal absorbance indicating 100% growth was around 24 hours. Strains continued to be incubated at 37°C over a six day period. Samples were collected every 12 hours and absorbances were checked at 600 nm. Autolysis was measured as a decrease in the percentage of maximal absorbance at 600 nm.

Results and Discussion

Biofilms are structured, surface-anchored microbial communities that contain sessile cells (fungi and/or bacteria) that are embedded in a self-produced extracellular matrix made up of DNA, polysaccharides, and other elements. Biofilms provide a perfect environment for the exchange of extrachromosomal DNA due to their high cell density, mobile genetic components, and genetic competence. *P. aeruginosa* uses QS, a form of cell-to-cell chemical signaling, to induce biofilm secretion and formation. QS monitors bacterial cell densities to promote communication between bacteria to regulate the expression of genes involved in competition, pathogenicity, resistance, and virulence. As seen in Figure 2, the biofilm formation was drastically reduced in the *rhlR* mutant strain compared to the wild-type strain. As *rhlR* is a QS transcription factor that controls the virulence of the bacteria and as biofilm formation is a critical step in the process, the mutant clearly fails to form a stronger biofilm.

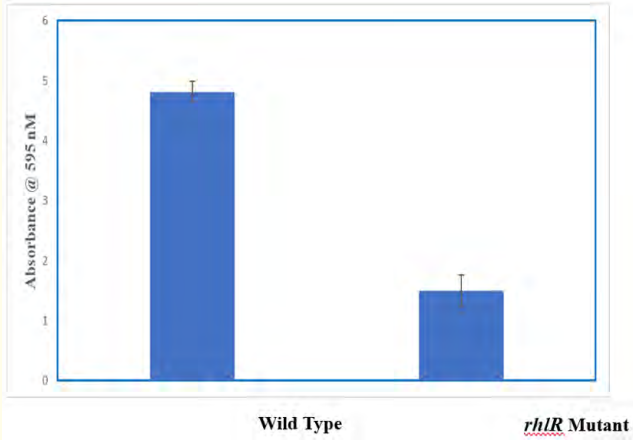

Figure 2: Comparison of biofilm formation between the wildtype versus *rhlR* mutant strains: measured as Crystal Violet absorbance @ 595 nM.

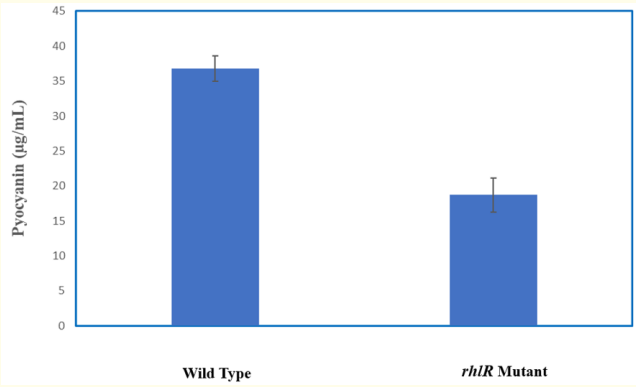

Figure 3: Comparison of Pyocyanin production in the wildtype strain versus the mutant *rhlR* strain.

Many virulence factors are responsible for the pathogenicity of *P. aeruginosa*. Virulence factors may include toxins, enzymes, and pigments. 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 naturally-produced heterocyclic compounds with side chains substituted at different points around their rings by different bacterial species. It is a small and highly diffusible nitrogen-containing aromatic compound with a multitude of biological activities. Pyocyanin pigment is produced by about 95% of *P. aeruginosa* strains and therefore is considered a biological marker for identifying this bacterium. In *P. aeruginosa*, pyocyanin production involves a stepwise process, beginning with the synthesis of the primary 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. As seen from the Figure 3, the pyocyanin production is attenuated in the *rhlR* mutant. As the pyocyanin operons *phz*A-Z are under the direct regulation of the QS gene circuit of *rhlR* transcription factor, it can be deduced that *rhlR* mutant has a reduced virulence and thus becomes a very important drug target to combat the antibiotic resistance issue.

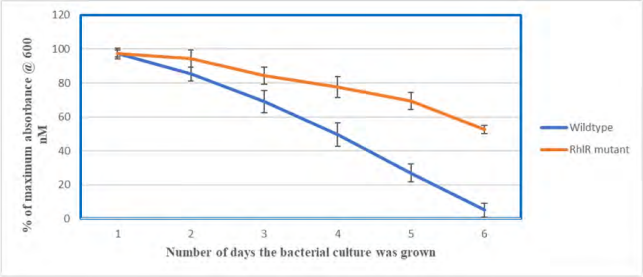

Figure 4: Autolysis of the wildtype bacteria versus the *rhlR* mutant: maximum absorbance @ 600 nM for the bacterial cells is considered as 100%. As seen from the figure, the cells start autolysis after the initial 24 hours which is plotted as the percentage of maximum absorbance.

As seen from Figure 4, the *rhlR* mutant strain shows a much-reduced autolysis compared to the wild-type strain. This is a very interesting result as it supports our hypothesis that the production of pyocyanin formation triggers the autolysis of some of the bacteria's own cells releasing various molecules like eDNA and eventually contributing to the formation of stronger biofilms that we see in the wildtype strain. The fact that the pyocyanin production is reduced in the mutant strain along with the autolysis and biofilm formation serves as preliminary evidence of the relationship between pyocyanin production and biofilm formation [1-20].

Conclusion

Pyocyanin is involved in a variety of biological activities including QS, maintaining the fitness of bacterial cells, and biofilm formation. Phenazines such as pyocyanin induce H_2O_2 production and subsequently trigger cell death in mammalian hosts and competing fungal and bacterial cells by releasing extracellular DNA (eDNA) which is a very important component of bacterial biofilms. Pyocyanin-mediated eDNA production, which likely occurs because of cell lysis via H_2O_2 generation, could possibly assist biofilm formation. *rhlR* mutants have much attenuated virulence compared to the wild-type strain and various genes that are under the control of *rhlR* can be evaluated as potential drug targets. Future experiments are required to test some antibiotics on the wild type and the mutant strains. Also it is very important to carry out the gene expression analysis of the wild type and the mutant strains to identify the differentially expressed genes in the QS circuit of the *rhlR* transcription factor.

Acknowledgement

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