The RhlR mutant of the bacterium *Pseudomonas aeruginosa* shows attenuated virulence and increased antibiotic susceptibility compared to the wild type strain

Kayla Tram, Anish Gayam and Dr. Kokila Kota
Ramapo College of New Jersey, Mahwah, NJ, 07430

Introduction

Pseudomonas aeruginosa is an opportunistic bacterium that is frequently found in patients with cystic fibrosis, burn wounds, and various immunocompromised conditions. The bacterium is known to produce various virulence factors through group communication behaviors known as quorum sensing. Quorum sensing and the related bacterial communication is highly regulated at the genetic level and our research focus is to understand the mechanisms involved in the process. Quorum sensing behaviors of the bacterial strains contribute to the global problem of antibiotic resistance and the study will contribute to our understanding of some of the fundamental concepts of the field.

RhlR is a quorum sensing receptor and transcriptional regulator in *Pseudomonas aeruginosa t*hat activates the transcription of various virulence factors. RhlR promotes the expression of genes coding for pyocyanin, biofilm formation, elastases, along with genes for many other virulence factors. The goal of our research is to use biofilm formation and pyocyanin production as biomarkers to compare the pathogenicity of wild type versus the RhlR mutant strains. While comparing the two strains for their virulence, we observed that the bacterium exhibits a novel mechanism known as "autolysis or sacrificial killing" which is bacterial apoptosis. Both the virulence phenotypes-pyocyanin and biofilm contributed to the autolysis of the bacteria but what was very interesting was that the wild-type strain showed much higher autolysis than the RhlR mutant strain. These results were consistent with our experimental observations that the mutant formed weaker biofilms and expressed lesser pyocyanin than the wild type strain. We also tested various gram-negative bacterial antibiotics on the wild-type and the mutant strains for their susceptibility/resistance. These results provide an important insight into the transcriptional activator RhlR of the *Pseudomonas aeruginosa* and in the future studies, might provide some novel gene targets for designing quorum quenchers or quorum sensing inhibitors as drug molecules.

RhlR Mutant

RhlR is a quorum sensing receptor and transcriptional regulator in *Pseudomonas 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 quorum-sensing genes naturally occur in patients. This implies 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 the RhlR mutant strains. 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.

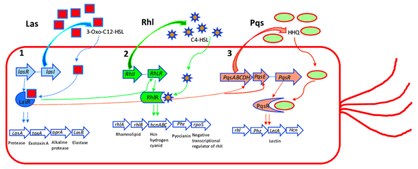


Figure 1. Major quorum sensing circuits in *P. aeruginosa*

Guzzo, Francesca et al. "Plant Derived Natural Products against *Pseudomonas aeruginosa* and *Staphylococcus aureus*: Antibiofilm Activity and Molecular Mechanisms." *Molecules (Basel, Switzerland)* vol. 25,21 5024. 29 Oct. 2020, doi:10.3390/molecules25215024

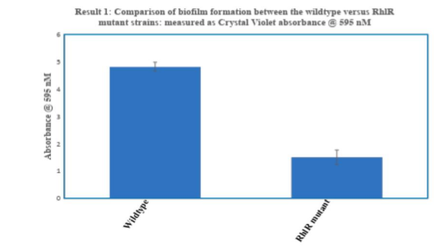
Methods

Biofilm formation in wild type versus mutant:
Overnight bacterial cultures of wild type and RhlR mutant were added to the sterile Tryptic Soy Broth (TSB) media in 12-well plates and were incubated at 37 ºC for 24-48 hours. The supernatant was then discarded and the adhered cells were with distilled water before adding 0.1% Crystal Violet solution to each well. The dye was incubated for some time and organic solvent was added to release the dye bound to the biofilm. The amount of biofilm was quantified using a spectrophotometer by measuring the absorbance at 595 nM. Experiments were performed in triplicates to ensure reproducibility.

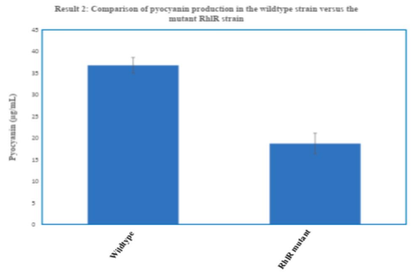
Pyocyanin quantitation in wild type versus mutant:
Wildtype and mutant strains of *Pseudomonas* were grown at 37ºC for 24 hours. Supernatants were collected after centrifugation at 10,000 rpm for 10 minutes and they were filter-sterilized using a 0.45uM syringe filter. The filtered samples were again centrifuged for 10 minutes at 10,000 rpm. 3 mL of the blue layer was transferred to a new tube and 1.5 mL of 0.2 M Hcl was added. The blue layer turned to pink and the samples were centrifuged to separate the pink layer that extracted the pyocyanin pigment. Absorbance was measured at 520 nM and the concentration of pyocyanin(µg/mL) was calculated using the extinction coefficient.

Effect of antibiotic treatment on the *Pseudomonas* wild type versus RhlR mutant biofilms:
Pseudomonas biofilms were established by adding actively growing overnight culture of the bacteria into the 12 well plates. After 24 hours, planktonic cells were removed and fresh Tryptic Soy Broth media was added to each well. The wells were then supplemented with various antibiotics at their MIC values for planktonic *Pseudomonas aeruginosa.* The 12-well plates were incubated at 37 ºC for 24 hours. Biofilms were quantified using the Crystal Violet Dye absorbance method as mentioned previously

Results



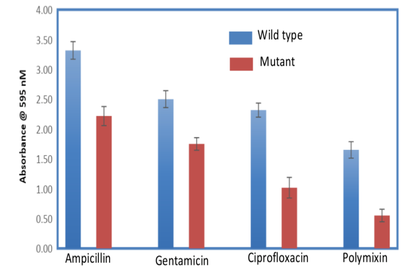
Result 1: As seen from the above graph, the biofilm formation was drastically reduced in the RhlR mutant strain compared to the wildtype strain. As RhlR is a QS transcription factor that controls the virulence of the bacteria and as biofilm formation is a critical step in the process, the mutant clearly fails to form stronger biofilm.



Result 2: As seen from the above graph, the pyocyanin production is attenuated in the RhlR mutant. As the pyocyanin operons *phz*A-Z are under the direct regulation of the quorum-sensing 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.

Results

Result 3: Effect of antibiotic treatment on the *Pseudomonas* wild type versus RhlR mutant biofilms:



As seen from the above graph, the biofilm formation was inhibited significantly in the RhlR mutant compared to the wild type. This could indicate that the bacteria could be more susceptible to the already available antibiotic treatments provided that they are supplemented with drugs that can target the RhlR QS circuit

Conclusions / Future Directions

- RhlR mutants show significantly attenuated biofilm formation and the pyocyanin production. These are some of the virulence factors that contribute to the pathogenicity of the *P.aeruginosa* and that is the reason we propose that RhlR transcription factor controlled genes might be valuable drug targets
- Antibiotics tested at their MICs effectively inhibited the biofilm formation in the mutant strain compared to the wild type strain
- Future experiments will include a dose dependence testing of the antibiotics and gene expression analysis of wild type versus mutant to identify valid gene targets

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