Comparison of the anti-microbial and anti-biofilm synergistic activities of the bacterial pigment *Prodigiosin* against *Pseudomonas aeruginosa*

Dr. Kokila Kota, Mariam Tinawi
Ramapo College of New Jersey, Mahwah, NJ, 07430

Introduction

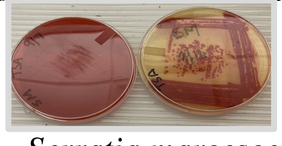
Biofilms are a hallmark feature of various pathogenic and opportunistic pathogenic bacteria that allow them to communicate with each other. *Pseudomonas aeruginosa* is an opportunistic bacterium that often results in serious infections in health-care settings especially in immunocompromised patients. Our previous experimental results indicated that the polymicrobial biofilms formed by *P. aeruginosa* are not only more complex and stronger than the single species biofilms but also show much more resistance to the antibiotic treatment. There is an urgent need to work on new methods and ways to supplement our current antibiotics. Novel antimicrobial peptides and pigments isolated from various bacterial strains are gaining immense focus keeping in view of their relative ease of isolation and selective toxicity. Two such pigments that we have been studying in the lab are Prodigiosin - a red colored pigment from the laboratory bacterial strain *Serratia marcescens* and carotenoid - a yellow colored pigment from the laboratory bacterial strain *Micrococcus luteus.*

In the current study we optimized the production of the red pigment Prodigiosin from the bacteria *S. marcescens a*nd partially purified it using organic solvents. Maximum production of the pigment was observed after 48 hours and at 25ºC. Prodigiosin showed significant antimicrobial activity against *P. aeruginosa*, *E. coli, and Klebsiella pneumoniae*. Prodigiosin is known to inhibit the enzymes involved in DNA replication, such as topoisomerase IV and DNA gyrase, which inhibit cell growth (Berlanga et al. 2000). Pre-treatment with the crude extract of Prodigiosin also resulted in an increased susceptibility of the *Pseudomonas aeruginosa* biofilms to the antibiotic gentamicin. Together, our results indicate that the bacterial pigments like Prodigiosin might provide a promising avenue for new anti-biofilm agents.

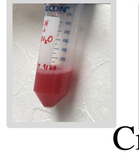
Experimental Methods

Extraction & Quantification of the Prodigiosin Pigment

Overnight actively growing *Serratia marcescens* was heavily inoculated onto a TSA plate and incubated at 25°C for 48 hours. The culture was then scraped and suspended in 20 mL distilled water. For the extraction process, 5 mL from above broth was taken in a test tube, and 4 mL of methanol was added. The mixture was vigorously vortexed for 2 min. The solution was then centrifuged for 10 min at 6000 rpm. To quantitate the amount of Prodigiosin, 0.8 ml supernatant was further mixed with 0.2 mL of 0.05 N HCl: methanol mixture (4:1 v/v). Prodigiosin displays a characteristic absorption spectrum in acidified methanol, with a strong maximum at 534 nm. The absorbance of the resulting solution was then measured at 534 nm. The remaining supernatant was dried by evaporation at 60°C and the dry mass of prodigiosin was determined before it was dissolved in 95% methanol.



Serratia marcescens

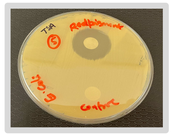


Crude Extract *Serratia marcescens*

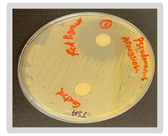
Biofilm Assay

Overnight bacterial cultures were added to the sterile Tryptic Soy Broth (TSB) media in 12-well plates and were incubated at 37 °C for 48-72 hours. The supernatant was discarded, and the adhered cells were rinsed with distilled water. 0.1% Crystal Violet (CV) solution was added to each well to stain the adhered biomass and organic solvent was used to release the bound CV dye from the biofilm, and the biofilm was quantified by reading the absorbance at 595 nm. Experiments were performed in triplicates to ensure reproducibility.

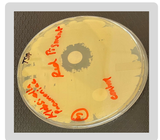
Agar Disk Diffusion Method



Escherichia coli



Pseudomonas aeruginosa



Klebsiella pneumoniae

Antimicrobial Assay of Prodigiosin

The antimicrobial property of Prodigiosin was evaluated using the Kirby-Bauer standardized disc-diffusion assay. 500 µg/µL of Prodigiosin was used in this assay. Sterile Whatman filter paper was used as discs (6 mm) and impregnated with the red pigment and 99% methanol was used as a control (as this was the solubilizing solvent for the pigment). Each bacterial species (*E. coli, K.pneumoniae, P.aeruginosa*) was inoculated heavily on a sterile TSA plate and the sterile impregnated filter discs were aseptically placed onto the bacterial lawn using sterile forceps. The plates were then incubated at 37˚C for 24 hours. Any zones of inhibition formed around the discs were then measured and recorded. For each bacterial sample, three biological replicates were performed.

Results-1

Figure 1: Effect of temperature on Prodigiosin production

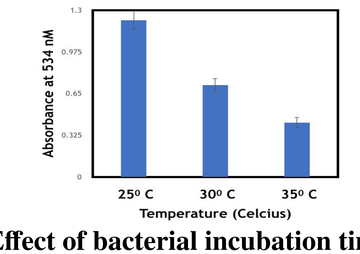
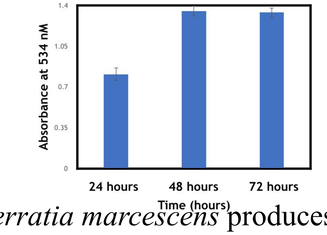


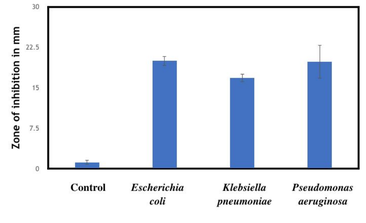
Figure 2: Effect of bacterial incubation time on *Prodigiosin production*



Results-1 *Serratia marcescens* produces maximum pigment at 25 C and the incubation time needed is 48 hours to reach this optimal production phase

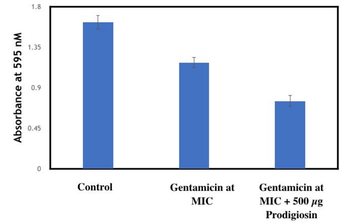
Results-2

Figure 3: Anti-microbial activity of Prodigiosin



Results-2 Prodigiosin exhibits anti-bacterial activity on the gram negative bacteria *E.coli, K. Pneumoniae and P. aeruginosa*

Figure-4: Anti-biofilm effects of Prodigiosin on *Pseudomonas aeruginosa*



Result-3: Pre-treatment of the *Pseudomonas aeruginos*a biofilms resulted in an increased antibiotic susceptibility

Conclusion

Prodigiosin pigment isolated from the wild-type *Serratia marcescens* strain shows a broad antimicrobial activity against the gram-negative bacterial strains and also against *P. aeruginosa*. It is especially promising to note that the *P, aeruginosa* biofilm sensitivity to the antibiotic gentamicin has increased upon the pre-treatment of the bacterial cells with Prodigiosin. Most antibiotics are tested on the planktonic bacteria to calculate their MICs but in the complex microbial communities, biofilms and the group behaviors of the bacteria are very common. To combat the increasing problem of antibiotic resistance, an approach to identify anti-biofilm agents is very much needed and our work will be a contribution to those efforts. Prodigiosin pigment will need to be further purified using specialized chromatography techniques and dose-response experiments will be performed to further confirm the anti-biofilm activity against *Pseudomonas aeruginosa*.

Select References

Berlanga, Mercedes & Ruiz, Neus & Borrell, Jordi & Montero, Teresa & Vinas, Miguel. (2000). Role of the outer membrane in the accumulation of quinolones by Serratia marcescens. Canadian journal of microbiology. 46. 716-22. 10.1139/w00-052.

Bhagwat A, Padalia U. Optimization of prodigiosin biosynthesis by Serratia marcescens using unconventional bioresources. J Genet Eng Biotechnol. 2020 Jul 9;18(1):26. doi: 10.1186/s43141-020-00045-7. PMID: 32648013; PMCID: PMC7347734.

Darshan, N., Manonmani, H.K. Prodigiosin inhibits motility and activates bacterial cell death revealing molecular biomarkers of programmed cell death. *AMB Expr* 6, 50 (2016)

Yip CH, Mahalingam S, Wan KL, Nathan S. Prodigiosin inhibits bacterial growth and virulence factors as a potential physiological response to interspecies competition. PLoS One. 2021 Jun 23;16(6):e0253445.

Acknowledgements

Our sincere thanks to the Foundations Research Grant from Ramapo College of New Jersey, TAS Research Honors Program, Biology Department Staff and Dean Saiff for the support of our work.