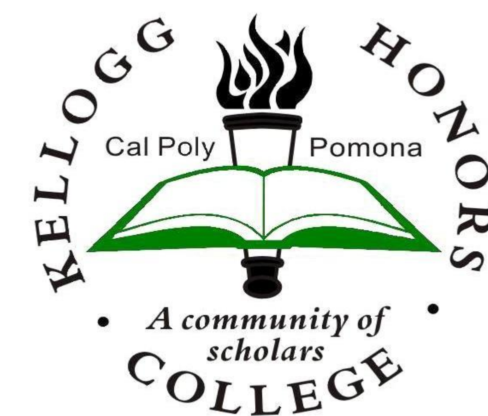
Antibiotic Resistance of Amphibian Skin-Associated Bacteria

Collin Knight, Biotechnology

Mentor: Dr. Wei-Jen Lin

Kellogg Honors College Capstone Project



Abstract

Batrachochytrium dendrobatidis (Bd) is a fungal pathogen that has been devastating global frog populations for decades. Bd causes the disease chytridiomycosis, and is transmitted amongst amphibians worldwide. One possible tool to halt the decline of these populations lies in the naturally secreted bacterial pigment violacein. Violacein is produced by *Janithinobacterium lividum*, a native skin-associated bacterium of several amphibian species and appears to confer a natural resistance to Bd. If the genes for this compound can be successfully transformed into the native bacteria of vulnerable amphibian species, it may be possible to induce resistance to Bd in species of amphibians that do not naturally carry antifungal bacteria. In order to observe successful transformations, the bacteria must initially be susceptible to at least one of two antibiotics, ampicillin and trimethoprim. We tested the antibiotic resistance of bacteria cultured from *Anaxyrus boreas halophilus* toads using Minimum Inhibitory Concentration assays (MICs). Three plasmids are available for use, containing violacein genes and one of two antibiotic resistance genes. Through testing, we found four bacteria that were vulnerable to one or both of the antibiotics. These susceptible bacterial candidates can now be used for violacein plasmid transformation experiments.

Introduction

Minimum Inhibitory Concentration assays are used to determine how effective a compound is at inhibiting the growth of a microorganism. They start with a base amount of compound in the first well, which undergoes a dilution into each of the following wells, creating a wide spectrum of concentrations. The minimum inhibitory concentration is then determined based on the concentration of the last well to completely eliminate microorganism growth(1).

In order to visualize the growth or death of the microorganism, another compound is placed within each well that will react to the presence of the living microorganism in some way. For our experiment we used resazurin. resazurin is a blue compound that is irreversibly reduced by bacteria into a pink compound called resorufin. This color change to pink indicates that the bacteria in the well is alive and able to reduce the resazurin, while wells that remain blue indicate that the bacteria is dead. During the 24 hour incubation, partial amounts of the resazurin may be reduced, giving a purple color and indicating partial inhibition of the bacteria. Full inhibition can only be concluded from a well that has no pink color change. (2)

This assay is an important step in determining potential candidates for transformation of the violacin gene. The process used most often to determine transformation success is to include within the transformed plasmid a gene that provides resistance to an antibiotic. The cells are grown on a plate containing the antibiotic and bacteria that survive on the plate are determined to carry the transformed plasmid. In order for this method to give reliable data, the bacteria must be susceptible to antibiotic being used. If it is resistant to the antibiotic, either the bacteria or the antibiotic must be changed, as there will be no way to determine transformation success(3). For this transformation, we have a choice of trimethoprim or ampicillin. Assays for each antibiotic were performed on each of the bacteria.

Materials and Methods

Bacteria were grown in Mueller Hinton (Becton, Dickinson and Company. ©2012 BD) broth at 27 C overnight, shaking at 220 rpm. On the day of the assay, bacteria were then diluted to an Optical Density (OD) of 0.05 nm., or a general concentration of 5x10^5 CFU/mL. Diluted bacteria samples were stored on ice while the MIC was prepared.

MIC assays were performed in 96 well plates, in triplicate. The top well for each assay was filled with 100 ul of 1024 ug/ml of antibiotic. 12 two-fold dilutions in MH broth were performed in the wells for final concentrations of 512 ug/mL to 0.25 ul/ml.

After dilution, a master mix was created for the tested bacteria. Each master mix contained 60% MH broth, 20% of the diluted bacteria culture, and 20% sterile resazurin (0.01%). The MH broth provides nutrients for bacteria, while the resazurin acts as a marker for bacteria death. Living bacteria metabolize the resazurin, leaving behind a compound that colors the well pink, while dead bacteria leave the well as the blue color of the resazurin.

50 ul of the master mix is then added to each of the test wells via multi channel pipette for a final volume of 100 uL. Positive and negative control wells were made in triplicate, one set for each bacteria and each antibiotic wells used. MH control wells were also made in triplicate, containing 80 ul of MH and 10 ul of resazurin, to ensure that the MH was not contaminated.

MIC plates were incubated at 27 C overnight and growth observations were recorded after 20 – 24 hours.

Antibiotic MIC Results

Bacterial Strain	Gram Stain	Ampicillin	Trimethoprim
E. coli NEB5-alpha	-	16	16
E. coli vio+	-	600	320
E. coli vio++	-	2	600
Klebsiella oxytoca	-	600	87.3
Chryseobacterium indologenes	-	600	600
Ralstonia	-	16	256
Comamonas	-	2	600
Brevundimonas	-	600	600
Acidovorax ebreus	-	512	600
Lysinibacillus	+	0.25	600
Micrococcaceae	+	2	600
Microbacterium	+	256	192
Bacillus cereus	+	600	600

Table 1: Combined results of all MIC assays. E. Coli NEB5-alpha used as baseline for resistance tolerances. Viable candidates for transformation outlined in black. Concentrations in ug/ml. .

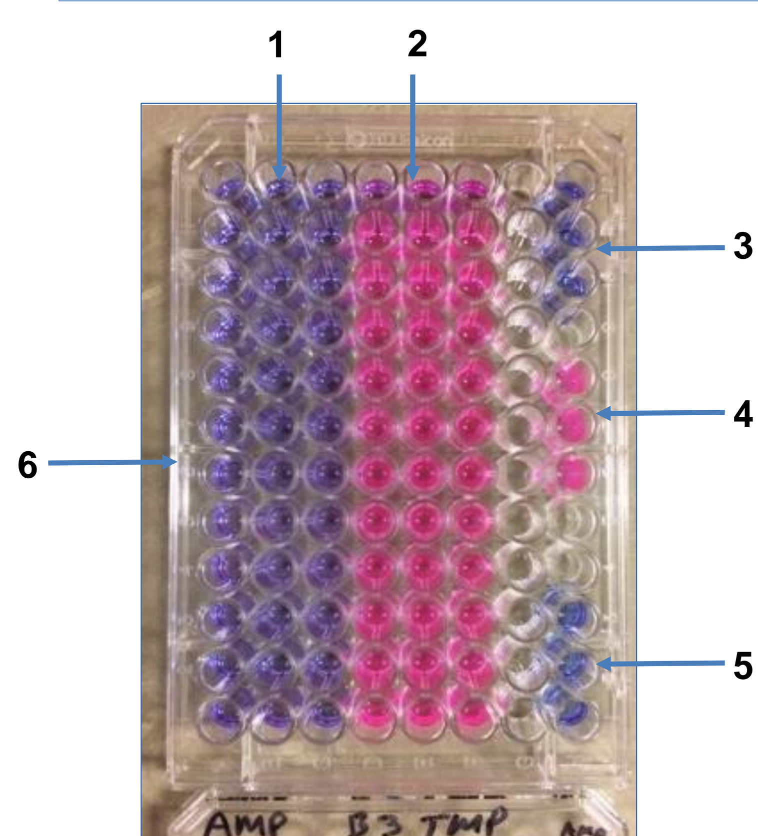


Figure 1: MIC of ampicillin (Left, inhibition at 0.25 ug/ml) and trimethoprim (Right, no inhibition) on Lysinibacillus.

Anatomy of a Minimum Inhibitory Concentration Assay

1. Dead Bacteria: resazurin remains unprocessed, leaving a blue color

2. Living Bacteria: resazurin is reduced to resorufin, creating a pink color

3. Ampicillin Control: Corresponds to left experimental wells, contains only ampicillin, broth, and resazurin. Should always be blue.

4. Bacterial Control: Contains only bacteria, broth, and resazurin. Should always be pink.

5. Trimethoprim Control: Corresponds to right experimental wells, contains only trimethoprim, broth, and resazurin. Should always be blue.

6. Dilution scale: Top well concentration is 512 ug/ml, two fold dilution of each successive well, concentration of final well is 0.25 ug/ml.

Conclusion

E.coli NEB5-alpha was used as a control bacteria to determine the upper acceptable limit of the MIC for transformation viability. This set the limit of 16ug/ml for both ampicillin and trimethoprim. By these parameters, four viable transformation candidates were identified: *Ralstonia, Comamonas, Lysinibacillus* and *Micrococcaceae.* All four were selected based on their susceptibility to ampicillin. *Ralstonia* and *Comamonas* are gram negative, while *Lysinibacillus* and *Micrococcaceae* are gram positive. Gram negative bacteria are better suited for transformation, as the electroporation technique we are planning to use is designed for the cell structure of gram negative bacteria. Gram positive bacteria would require a different transformation method.

Discussion

This experiment has produced four possible transformation targets, with two of these being compatible with our current transformation strategy. A future experiment will use *Ralstonia* and *Comamonas* in an electroporation procedure in an attempt to introduce a violacein plasmid. The process of electroporation uses an electric field to increase the permeability of the bacterial cell, allowing plasmids to pass through the membrane. These bacteria will then be grown on ampicillin plates, where they will be monitored for successful growth and for the characteristic purple color of violacein.

If neither transformation is successful, another transformation procedure my be used for the other bacteria. Alternatively, a new set of bacteria could be collected from a test frog and analyzed for antibiotic resistance. If the transformation is successful, it will provide an important proof of concept for the viability of a violacein plasmid and for the transformation capability of native frog bacteria, bringing the possibility of a treatment for chytridiomycosis closer to realization.

References

1. Andrews, Jennifer M. "Determination of minimum inhibitory concentrations." Journal of antimicrobial Chemotherapy 48, no. suppl 1 (2001): 5-16.

2. Bueno, C., M. L. Villegas, S. G. Bertolotti, C. M. Previtali, M. G. Neumann, and andM V. Encinas. "The Excited-State Interaction of Resazurin and Resorufin with Aminesin Aqueous Solutions. Photophysics and Photochemical Reaction." Photochemistry and photobiology 76, no. 4 (2002): 385-390.

3. Calvin, N. M., and P. C. Hanawalt. "High-efficiency transformation of bacterial cells by electroporation." Journal of bacteriology 170, no. 6 (1988): 2796-2801.

Acknowledgments

Marina DeLeón for providing me with this project opportunity

Dr. Wei-Jen Lin for use of her lab and equipment