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Mr. Kuhn

Antibiotic Resistance Loss In Co-evolving Bacteria With Bacteriophages

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Research Question

To what extent does co-evolution between carbenicillin-resistant *Mycobacterium smegmatis* and bacteriophages restore carbenicillin sensitivity in *M. smegmatis*?

Introduction/Background

- *Mycobacterium smegmatis* (abbreviated as *M. smegmatis* or *M. smeg*) is a species of bacteria that belongs to the Mycobacterium genus, a group that includes well-known pathogens like Mycobacterium tuberculosis. However, *M. smeg* is generally considered non-harmful.
- Because of its structural similarities to pathogenic mycobacteria but its non-pathogenic nature, much faster growth rate (1), and environmental resilience, *M. smegmatis* is widely used as a model organism in microbiological research.
- Bacteriophages are viruses that specifically infect bacteria. They are the most common biological entities on Earth.
- Scientists have been exploring how phages can be used to fight antibiotic-resistant bacteria. This approaches known as phage therapy, and it involves using phages to target and kill bacteria that no longer respond to antibiotics.
- Phage therapy has already been used to successfully treat dangerous infections caused by antibiotic-resistant bacteria like *Pseudomonas aeruginosa* and *Staphylococcus aureus* (2).
- However, another way to fight antibiotic-resistant infections is by using bacteriophages to restore bacterial antibiotic sensitivity.
- When the bacteria attempt to protect themselves from phages, they end up damaging their own antibiotic resistance system.
- As a result, they can no longer pump out antibiotics effectively, making them susceptible to antibiotics.

Hypothesis:

It will take at least 50 generations (6-7 passages) of *M. smegmatis* evolving against the bacteriophages for it to lose any amount of resistance to carbenicillin.

Methodology

1. **LB Agar Synthesis** – The LB (lysogeny broth) agar contains the nutrients to supplement bacterial growth.
2. **LB++ Agar Plate Pouring** – The LB Agar must be poured onto petri dishes and mixed with antibiotics.
3. **M. Smegmatis Trial Growth on Agar Plates** – This is to ensure the current m. smegmatis stock cultures are viable. If there are colonies present on the agar plates after 2-3 days of incubation, they are viable,
4. **7H9 Preparation** – This is a liquid growth medium specifically for *M. smegmatis*.
5. **ADC Preparation** – This must be mixed with 7H9 in liquid culture to provide nutrition for *M. smegmatis*.
6. **M. Smegmatis 1st and 2nd Generation Liquid Cultures** – After the first *M. smeg* liquid culture is grown, a second liquid culture must be grown from this for better plaque formation on plates.
7. **MBTA (Middlebrook Top Agar) Preparation** – When finished, this is a type of agar that will be mixed with liquid culture + bacteriophages and poured on top of the existing agar layer on an LB++ plate.
8. **Phage Titering Using MBTA Agar** – This step is used to quantify the density of plaque-forming units (PFUs) in a phage lysate, which indicates the number of active phage particles in that lysate. This is important to adjust the phage concentration for the co-evolution passages.
9. **Streaking Colonies to Ensure Viability** – After seeing colony growth on the plates that were used for phage titering, individual colonies near the PFUs were streaked onto separate LB++ plates to ensure they were viable for further co-evolution passages.
10. **Co-Evolution Passages –** Colonies from the previous streak plates were suspended in PBS buffer and mixed with MBTA like before. This step was only repeated for 3 passages due to time constraints.
11. **Liquid Culture Growth for MIC Assay –** Individual colonies were taken from one plate with good bacterial growth, but there were no PFUs on the plate. A control liquid culture was grown from the 2nd generation liquid culture prepared in step 6.
12. **MIC Assay –** This is used to quantify antibiotic sensitivity. Since the bacteria grew on plates treated with carbenicillin, kanamycin was used for this assay instead.

Visual Results

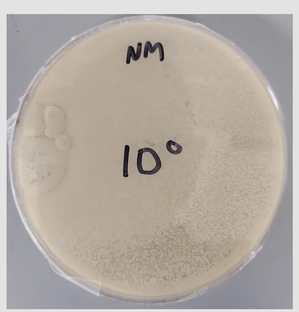

Figure 1: First attempt at phage titering. All plates yielded lawns of bacteria and no plaques like shown above.

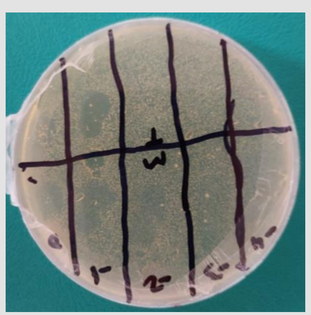

Figure 2: The 2nd attempt at phage titering yielded some plaques at the 10^-1 dilution for both phages, but the area was too small for PFU count.

Figure 3: 4th attempt at phage titering. 6 PFUs were spotted on the 10^-2 dilution using the Twigg phage.

Results

- The phage titer of the Twigg lysate was found to be 6.0*10^4 PFU/mL. This was calculated by finding 6 PFUs in 10 uL of bacteriophage lysate in a 10^-2 dilution. Also change uL to mL.
- So, 6/10*10^-2*10^3 = 6.0*10^4 PFU/mL.
- The streaking of the colonies near PFUs on the 10^-2 plate yielded individual colonies for all the colonies streaked.
- After 3 passages, *M. smegmatis* was still growing on plates treated with carbenicillin.
- OD600 values from the MIC assay were higher in the *M. smeg* culture that was evolving against bacteriophages, compared to the control culture.

Discussion/Setbacks

- It took 4 attempts to properly calculate phage titer. The 1st attempt indicated the phages were not selecting for the bacteria, so a new *M. smegmatis* culture needed to be grown.
- The 2nd attempt for phage titer calculation yielded 1 large plaque for the 10^-1 twigg dilution and 1 smaller plaque for the 10^-1 millipede dilution.
- The 3rd attempt for phage titer calculation yielded no bacterial growth at all.
- After the 3rd passaging of *M. smegmatis* colonies, the bacteria was still growing on the plates treated with carbenicillin. This indicates it did not lose any resistance.
- Time spent on attempting to calculate the phage titer prevented enough time to do enough passages to assess if antibiotic resistance would be lost.
- However, there was one useful result: OD600 values may indicate that bacteria evolving stronger resistance to bacteriophages may also increase its resistance to some antibiotics, such as kanamycin.

References

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2 - Wiśniewska, K., Szewczyk, A., Piechowicz, L., Bronk, M., Samet, A., & Swieć,K. (2012). The use of spa and phage typing for characterization of clinical isolates of methicillin-resistant Staphylococcus aureus in the University Clinical Center in Gdańsk, Poland. Folia microbiologica, 57(3), 243–249. https://doi.org/10.1007/s12223-012-0148-z