Phages, on the Rocks: Development of a Bacteriophage Cocktail Infecting *Pseudomonas chlororaphis 14B11*

THE COLLEGE OF WOOSTER

Clara Becker ’22 • Advisor: Dr. Stephanie Strand

Department of Biology, The College of Wooster, Wooster OH

Abstract

In an era of increasing multidrug resistance, phage therapy is a promising alternative to conventional antibiotics. However, when phages are administered, there is a high likelihood that a subset of phage-resistant organisms will emerge. To prevent phage resistance from emerging, phages may be combined in a cocktail of several phages. The aim of this thesis was to evaluate two previously described phage cocktail development methods to constrain the growth of *Pseudomonas chlororaphis* 14B11, a nonpathogenic environmental bacterial isolate. (i) The “Step-by-step” approach incorporated phages that infect a wildtype organism and its probable phage-resistant mutants, and (ii) the “host range” approach incorporated random combinations of phage isolates with unique host-range specificity. Subsequently, each cocktail was assayed for its ability to constrain the growth of wildtype *Pseudomonas chlororaphis* 14B11, and the proportion of phage-resistant isolates was assessed after 10 hours of co-culturing bacteria and phage. Phage-resistant mutants were also assessed for their phenotypic and genotypic characteristics. The “Step-by-step” approach was significantly more effective at constraining the growth of wildtype 14B11 compared to the host-range approach. Sequencing efforts and analysis of mutations in putative phage resistance genes are ongoing.

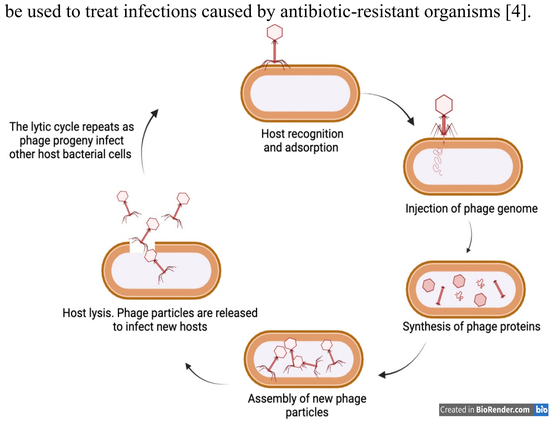
Phages and Phage Therapy

The Rise of Antibiotic Resistance

- The World Heath Organization predicts that antibiotic resistant will be one of the biggest public health threats in the twenty-first century [1], and antibiotic resistance is predicted to cause up to 300 million premature deaths by 2050 [2].
- Despite the recent rise of antibiotic resistance, the newest class of antibiotics was discovered in the 1980’s. The following decades have since been dubbed the “discovery void” [3]. Because the development of new antibiotic compounds is unlikely to match the rise of antibiotic resistance, alternative therapies are of increasing interest.

Bacteriophages and Phage Therapy

- Phage therapy uses bacteriophages (“phages”), the viruses that infect bacteria, to eradicate infections caused by bacterial organisms. During phage therapy, phages kill bacterial organisms but do not harm human cells. Phage therapy is a potential alternative to conventional antibiotics.
- Because phages use different mechanisms than antibiotics to infect their hosts, phages can be used to treat infections caused by antibiotic-resistant organisms [4].



The replication cycle of a lytic bacteriophage. Adapted from [5].

Phage Cocktails

- Resistance to phage can emerge over the course of phage therapy, rendering phage ineffective as a “living” medicine [6]. To prevent phage resistance from arising, phage cocktails can be administered, which are formulations that consist of several different phages that infect an organism of intertest.
- The most effective phage cocktails will include phages that target different cell-surface receptors on their hosts. This way, if a bacterial organism evolves resistance to one phage such that the phage is unable to bind, the bacterial organism may remain susceptible to other phages that target different receptors.

Research Aims

1. Compare two methods of phage cocktail development and assess their ability to constrain the growth of *Pseudomonas chlororaphis 14B11,* a nonpathogenic bacterial soil isolate.
2. Characterize phenotypic characteristics of bacterial organisms that have evolved to resist infection by the phages included in a phage cocktail.

Selecting Phages for Phage Cocktails: Two Approaches

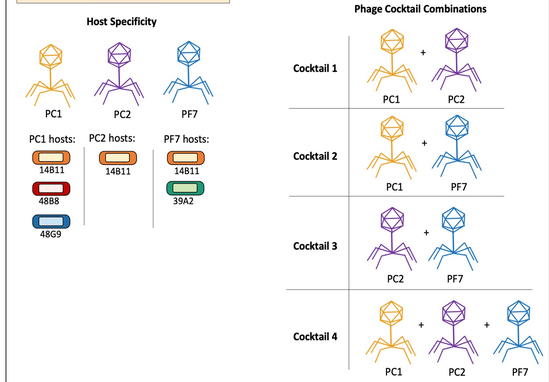
Two contrasting phage cocktail development methods (the “Host Range” approach and the “Step-by-Step” approach) were used to formulate a phage cocktail infecting *P. chlororaphis* 14B11.

Method 1: The “Host Range” Approach

The “Host Range” approach relies on the assumption that phages that infect a different range of hosts are likely to be genetically different from each other and to bind to different host-cell receptors.

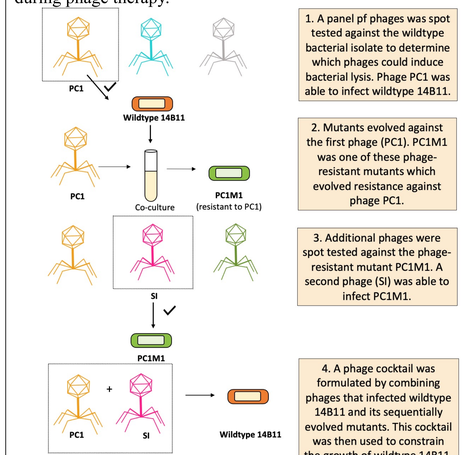
1. The host range-specificity of phages PC1, PC2, and PF7 was determined using spot tests. Each phage infected a different range of hosts; however, all 3 phages infected wildtype 14B11.

2. Phages PC1, PC2, and PF7 were combined in 2- to 3- phage combinations. Each combination was tested for its ability to inhibit the growth of wildtype 14B11



Method 2: The “Step-by-Step” Approach

The “Step-by-Step” approach selects phages for a cocktail that infect a wildtype organism and its phage-resistant mutants. This method preempts the emergence of phage-resistant organisms during phage therapy.



1. A panel pf phages was spot tested against the wildtype bacterial isolate to determine which phages could induce bacterial lysis. Phage PC1 was able to infect wildtype 14B11.

2. Mutants evolved against the first phage (PC1). PC1M1 was one of these phage-resistant mutants which evolved resistance against phage PC1.

3. Additional phages were spot tested against the phage-resistant mutant PC1M1. A second phage (SI) was able to infect PC1M1.

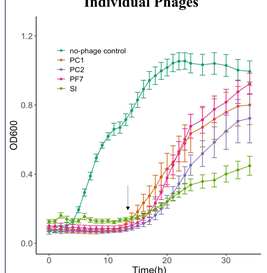
4. A phage cocktail was formulated by combining phages that infected wildtype 14B11 and its sequentially evolved mutants. This cocktail was then used to constrain the growth of wildtype 14B11.

Subsequently, the efficacy of each formulation was assessed by measuring how well each cocktail type constrained bacterial growth over 34 hours.

“Step-by-Step” Phage Cocktails Constrain 14B11 Growth More Effectively than “Host Range” Cocktails

Phages PC1, PC2, PF7, and SI were selected for inclusion in phage cocktails. Each phage was assessed individually for its ability to constrain growth of wildtype 14B11. Subsequently the four phages were combined in “Host Range” or “Step-by-Step” phage cocktails, which were assessed for their ability to constrain 14B11 growth. “Step-by-Step” formulations were most effective at constraining bacterial growth over time.

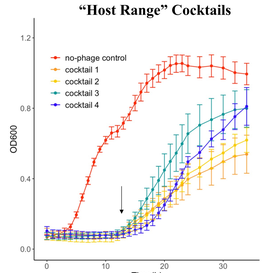
Individual Phages



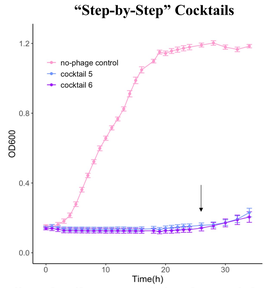
Phage Cocktail Formulations

	Phage			
Cocktail ID	PC1	PC2	PF7	SI
Cocktail 1	◦	◦		
Cocktail 2	◦		◦	
Cocktail 3		◦	◦	
Cocktail 4	◦	◦	◦	
Cocktail 5	◦			◦
Cocktail 6	◦		◦	◦

“Host Range” Cocktails



“Step-by-Step” Cocktails

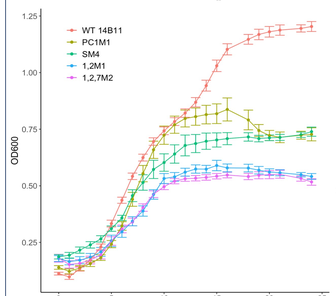


Growth of 14B11 is inhibited in the presence of individual phages and phage cocktails, with “Step-by-Step” cocktails constraining bacterial density for longest. Bacterial growth was initially assessed in the presence of four individual phages in separate cultures. Subsequently, six phage cocktails were assayed for their ability to constrain growth of 14B11. Arrows indicate the timepoint at which bacterial optical density began to increase from baseline levels.

Phenotypic and Genotypic Characteristics of Phage-Resistant Mutants

Growth of Phage-Resistant Mutants is Reduced in the Absence of Phage

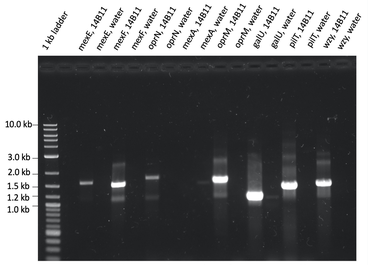
Several phage-resistant mutants (PC1M1, SM4, 1,2M1, and 1,2,7M2) evolved resistance against different combinations of phage. Compared to wildtype 14B11, phage-resistant mutants exhibited growth deficiencies in liquid media, even when no phage was present. This trend indicates that phage resistance may be accompanied by fitness costs in *P. chlororaphis.*



Growth kinetics of wildtype 14B11 and phage-resistant mutants in liquid media

Sequencing Efforts of Putative Phage-Resistance Genes are Ongoing

Previous work has shown that mutations in genes responsible for efflux mechanisms, LPS synthesis, and type IV pili may confer phage resistance. PCR primers were designed to amplify putative phage-resistance genes in wildtype 14B11 and phage-resistant mutants. Seven genes associated with phage-resistance were PCR amplified and sequenced using capillary sequencing. Computational analysis of amplicons for mutations is ongoing.



Agarose gel electrophoresis of PCR amplicons in wildtype 14B11

Conclusions and Future Directions

Conclusions

- “Step-by-Step” phage cocktails were more effective at constraining bacterial growth than “Host Range” cocktails or individual phages. However, the mechanism promoting the efficacy of “Step-by-Step” cocktails was not confirmed in this work.
- Putative phage-resistance genes have been PCR amplified in wildtype 14B11 and four phage-resistant mutants. Computational analysis of mutations is ongoing.

Future Directions

- Receptor-binding assays may reveal whether the phages included in each cocktail bind to different receptors.
- Whole-genome sequencing of phage-resistant mutants may provide a more holistic picture of phage-resistance mutations.

References

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Acknowledgements

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