Application Note

Microbiology

High-Throughput Determination of Bacteriophage Host Range

Using the Agilent BioTek LogPhase 600 microbiology reader

Authors

Andrew Saghir, Alisha Monsibais, Eric Adams, Sage Dunham, PhD, and Katrine Whiteson, PhD
University of California, Irvine
Department of Molecular Biology and Biochemistry

Abstract

Antimicrobial resistance (AMR) poses a significant challenge to the future of global health. Currently, infections by resistant ESKAPE pathogens are associated with greater rates of morbidity and mortality, consequently imposing great economic stress. Furthermore, as antibiotics have become increasingly ineffective in the treatment of such infections, alternative therapeutic approaches are critically needed. Bacteriophage (known as "phage") therapy represents one such alternative; however, the exquisite specificity of phages necessitates high-throughput approaches to assess host specificity. This application note demonstrates the use of the Agilent BioTek LogPhase 600 microbiology reader to ascertain lytic phage activity against a panel of potential bacterial hosts for the determination of phage host range.

Introduction

The rapidly growing burden of AMR poses a significant challenge to public health, which is only further compounded by detrimental healthcare and economic costs. Currently, it is projected that within the following three decades over two million people will succumb to AMR infections in the Western Hemisphere alone, incurring a cost of over USD$3 billion.[1] Some studies have projected that the direct healthcare cost due to AMR infections is closer to $20 billion annually in the United States.[2] Of these resistant organisms, those that pose the most severe health threats, as designated by the World Health Organization (WHO) are: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*, known as the ESKAPE pathogens.[3] As a result of overuse, the pool of viable antibiotics is shrinking, which urgently calls for alternatives to current antibiotic options.

Phages, viruses that infect bacteria with high specificity, present a promising alternative for treating antibiotic resistant bacterila infections. Although the discovery of phages predates that of antibiotics, they are experiencing a resurgence in interest, as a result of rising AMR.[4] Phages are widely abundant in the environment and highly specific in their bacterial hosts. Although the availability and specificity speak to the therapeutic potential of phages, this renders the determination of phages with therapeutic potential difficult. Thus, high-throughput methods to monitor phage–host interactions are needed for the identification of such phages.

A phage that infects a bacterial host may undergo two distinct replication pathways. Under the lysogenic cycle, a phage integrates its genetic material into the host genome, whereby it may replicate along with the host for several generations. Conversely, phages under the lytic cycle infect, replicate inside, and subsequently lyse the host cell. For this reason, lytic phages are desired for their use as bioagents against bacterial infections. When cultured with a viable host, lytic phages will hinder proliferation of the bacteria resulting in a notable decrease in optical density (OD) of the culture when compared to normal cell growth.

The Agilent BioTek LogPhase 600 microbiology reader allows for quick and confident determination of lytic phage activity. Given that the reader is able to perform OD_{600} readings on four individual microplates, the susceptibility of bacterial hosts to a particular phage can be assessed in triplicate, in conjunction with a control microplate, with all microplates experiencing the same environmental conditions.

This application note demonstrates how to use the microplate reader to assess phage host range over a broad panel of hosts using kinetic OD_{600} readings.

Experimental

Brain heart infusion (BHI) media powder was obtained from Research Products International (Mt. Prospect, IL) and used to prepare liquid media as per package instructions. SM buffer consisted of 5.8 g NaCl, 2 g $MgSO_4 6H_2O$, 50 mL of 1 M Tris-HCl pH 7.5, and 1 L ultrapurified water. Following preparation, all buffers and media were autoclaved. A 1 mL aliquot of BHI liquid media was transferred to a 2 mL round-bottom 96-well plate (Corning, NY) and subsequently inoculated with 93 unique *Enterococcus faecalis* strains, including three non-*Enterococcus* negative controls: *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. This template plate was incubated overnight at 37 ºC, stored at −80 ºC, and thawed to inoculate the 96-well plates to be used for host range determination.

Twenty-four hours prior to each assay, the template plate was allowed to thaw from −80 ºC, whereby 5 µL of each culture was transferred into 195 µL BHI liquid media in a 0.35 mL Corning flat-bottom 96-well plate, and subsequently incubated at 37 ºC overnight. Four 0.35 mL Corning flat-bottom 96-well plates were prepared with 185 µL of BHI and inoculated with 5 µL bacterial host culture. Three of the four plates had 10 µL phage suspended in SM buffer transferred to each well. One plate was prepared with
10 µL SM buffer transferred to each well in lieu of 10 µL phage stock. This allowed for a total of three biological replicates and a control plate.

All four plates were sealed with Breathe-Easy sealing membrane from Sigma-Aldrich (St. Louis, MO) and loaded into the LogPhase 600. Through the accompanying software, the reader was set to maintain a temperature of 37 ºC with continuous shaking at 600 rpm while taking OD_{600} readings every 15 minutes (Figure 1). Following each run, data was exported through the software and the average and standard deviation was plotted in R.

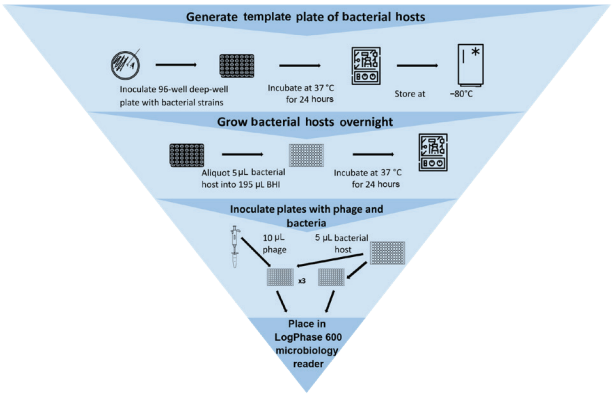

Figure 1. Overview of the host range assay pipeline. Plaques of desired host strains are picked and inoculated into wells of 96-well deep-well plates containing 1 mL BHI to generate a template plate for upcoming assays. The template plate is allowed to incubate at 37 ºC for 24 hours to allow for proliferation of host strains, and stored at −80 ºC for future runs. A volume of 5 µL liquid culture from the template plate is transferred to 195 µL BHI in a 0.35 mL 96-well round-bottom plate, which is placed at 37 ºC for 24 hours. A volume of 5 µL bacterial culture from the 0.35 mL 96-well plate is transferred to 185 µL BHI in four new 0.35 mL 96-well microplates, three of which are inoculated with phages, and one with only SM buffer. The four plates are placed in the Agilent BioTek LogPhase 600 microbiology reader and the assay is performed.

Results and discussion

Given the strain-level specificity that phages are capable of, it is often the case that only a few strains of a given bacterial taxa may be vulnerable to a particular phage. As such, we profiled the host range of five previously sequenced *Enterococcus* phages, as well as two phage cocktails against our *Enterococcus* panel. In this application note, three *Myoviridae* phages—referred to hereafter as phages Bill, Bob, and Car, one *Siphoviridae* phage referred to as CCS4, and one *Podoviridae* phage referred to as Ump—were assayed for lytic activity against various bacterial *Enterococcus faecalis* isolates. Additionally, two phage cocktails, sisters and cousins, which are mixtures of two or more phages, were similarly assayed.

To first assess the growth of the *Enterococcus* host panel and additional control taxa when uninhibited by phages, the bacteria were inoculated in BHI liquid media with SM buffer and allowed to grow in the reader while kinetic OD_{600} measurements were taken.

Here, growth of the bacterial strains within our panel presented growth curves typical to that of standard bacterial kinetic growth, with strains exhibiting generally defined lag, log, and stationary phases (Figure 2). Most strains were shown to enter the stationary phase by 10 hours, as demonstrated in Figure 2; however, a 24-hour runtime is preferred, as it allows for the observation of bacterial resistance if it were to arise.

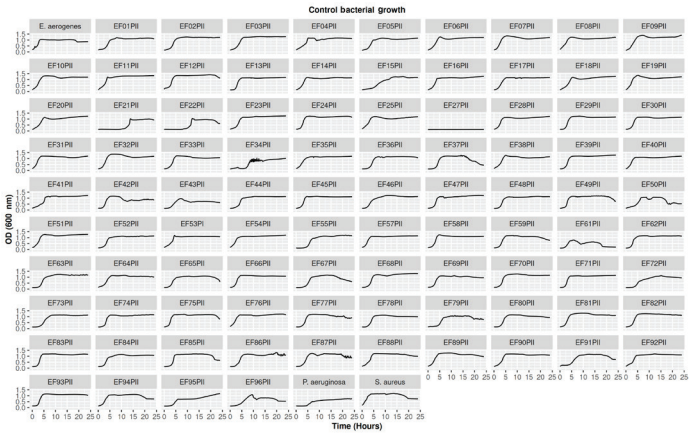

Figure 2. Bacterial growth controls in the absence of phages. Growth curves of all bacterial strains examined growing in BHI liquid media and SM buffer. The bacterial growth generally follows that of a standard bacterial growth curve.

To examine the effects of phages on bacterial growth, bacterial strains were co-inoculated with phage or a phages cocktail. Several of the tested *Enterococcus* strains within the panel grew seemingly uninhibited by the phages, however, a few of these strains exhibited stunted growth rate and reduced density (Figure 3).

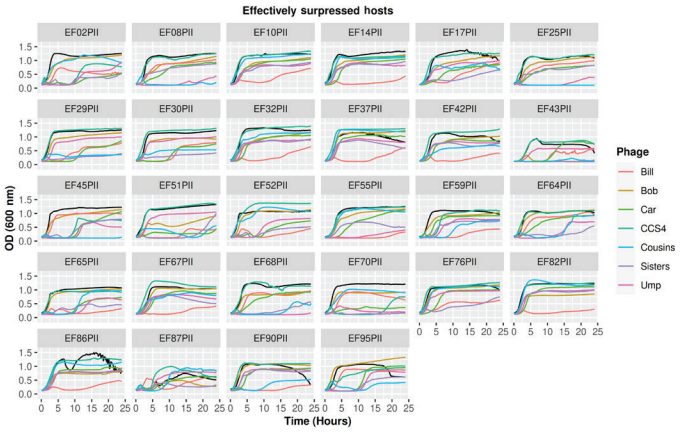

Figure 3. Growth of susceptible *Enterococcus* hosts with phages. Bacterial strains represented are those which demonstrated complete susceptibility to at least one of the phages or cocktails assayed. *Enterococcus* strains were cultured with phages in a 96-well microplate. Kinetic OD_{600} readings were taken for a period of 24 hours at 15-minute intervals. Data represent the average of three biological replicates, and a control grown in the absence of phages (black).

To assess this variation in phage effectivity on the *Enterococcus* hosts, phage host ranges were designated one of four possible categorical rankings. Rank 0 is indicative that treatment with the specific phage had no impact on bacterial growth with respect to controls. Ranks 1 and 2 are both indicative of some host suppression, whereby bacterial growth was either (1) diminished relative to controls or (2) suppressed initially only to exhibit resistance at later time points. Rank 3 indicates that phage was successful in suppressing growth of the bacterial host. Host bacterial strains and phages are differentiated according to the aforementioned rankings and, as such, each phage or cocktail combination exhibited a range of effectivity throughout the panel of hosts (Figure 4).

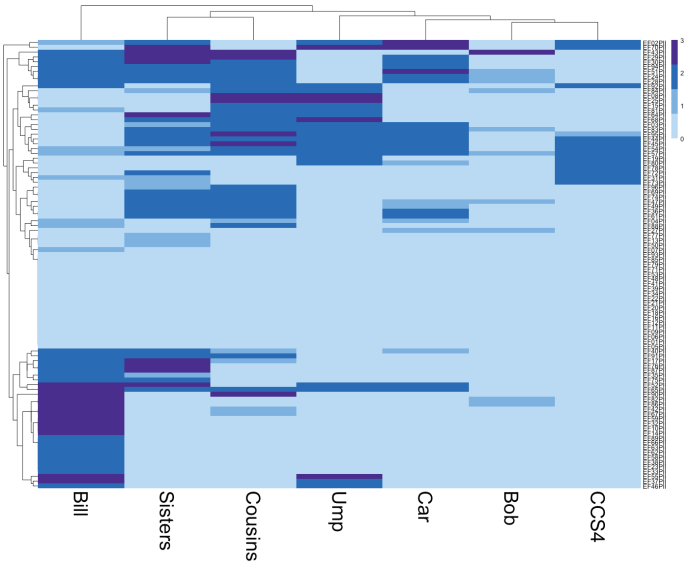

Figure 4. Suppression of *Enterococcus* growth by phage or cocktail. Effectivity of phage or cocktail against *Enterococcus* hosts. Lytic phage activity ranked from 0, whereby phage bears no impact, to 3, whereby phage is effective. *Enterococcus* strains were cultured in a 96-well microplate. Kinetic OD_{600} readings were taken at 15-minute intervals for a period of 24 hours. Data represent an average of three biological replicates.

It is again observed that the majority of bacterial hosts did not exhibit suppression of growth; however, a few strains were affected by the different phages to varying degrees. As presented by the data in Figure 4, the assayed phages maintained an effective host range that was a small fraction of the total host strains tested and, in the case of CCS4, appeared ineffective against *Enterococcus* hosts in the panel.

Throughout these experiments, it should be noted that it would be an inaccurate assessment to compare runs between phages, as phage titers were not able to be normalized to a standard PFU/mL. This may lend some explanation as to why some phages, such as Bill, demonstrated effectiveness against 12 *Enterococcus* strains, while, in contrast, phage CCS4 was unable to show effective suppression. Despite this, information regarding phage–host interaction may still be ascertained, as even at lower and albeit varied titers, all phage treatments except for one were able to successfully inhibit the growth of at least one *Enterococcus* host (Figure 4).

Conclusion

The data presented in this application note showcases the successful use of the Agilent BioTek LogPhase 600 microbiology reader to identify lytic phage activity through kinetic OD600 measurements. In a single experimental run, the host ranges of five phages and two phage cocktails were assessed against a panel of *Enterococcus* host strains in triplicate under standardized growth conditions with the LogPhase 600. Identifying lytic phages, especially those with efficacy against clinically relevant bacterial strains, is critical in the development of antibiotic alternatives. Regarding species of *Enterococcus*, there are few characterized phages.[5] Therefore, higher-throughput methods for identification of effective *Enterococcus* phages, such as the method presented in this study, may be of great benefit in bridging this gap. Furthermore, even upon determination of effective lytic phages, it may be necessary to expand host range through the development of multiphage or phage-antibiotic cocktails. In such cases, a multitude of bacterial hosts against several phages or cocktail combinations must be assayed in order to determine optimal therapeutic potential.

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RA45005.3092013889

Published in the USA, March 18, 2023
5994-5629EN

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