

Development of a high throughput assay for indirectly measuring phage growth using the OmniLog™ system

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The conventional and most accepted method of measuring the lytic activity of a phage against its bacterial host is the plaque assay. This method is laborious, time consuming and expensive, especially in high throughput analyses where multiple phage-bacterial interactions are required to be monitored simultaneously. It can also vary considerably with the experimenter and by the growth and plating conditions. Alternatively, the lytic activity can be measured indirectly by following the decrease in optical density of the bacterial cultures owing to lysis. Here we describe an automated, high throughput, indirect liquid lysis assay to evaluate phage growth using the OmniLog™ system. The OmniLog™ system uses redox chemistry, employing cell respiration as a universal reporter. During active growth of bacteria, cellular respiration reduces a tetrazolium dye and produces a color change that is measured in an automated fashion. On the other hand, successful phage infection and subsequent growth of the phage in its host bacterium results in reduced bacterial growth and respiration and a concomitant reduction in color. Here we show that microtiter plate wells inoculated with *Bacillus anthracis* and phage show decreased or no growth, compared with the wells containing bacteria only or phage resistant bacteria plus phage. Also, we show differences in the kinetics of bacterial growth and the timing of appearance of phage resistant bacteria in the presence of individual phages or a cocktail of *B. anthracis* specific phages. The results of these experiments indicate that the OmniLog™ system could be used reliably for indirectly measuring phage growth in high throughput host range and phage and antibiotics combination studies.

Introduction

Bacteriophages are obligate intracellular parasites of bacteria, and in general they are highly host (bacteria) specific at species and often even at strain level.¹ There are four basic steps in the life cycle of a bacteriophage: (1) specific attachment of the phage to the bacterial surface, (2) injection of phage DNA into the host cytoplasm, (3) growth and multiplication of phage inside the bacterium and (4) eventual lysis of the host releasing progeny phage particles.² In nature, there is a plethora of phage-specific variations in each of these steps. Each of these steps can be monitored and measured by a variety of methods³ that can be broadly grouped into two categories: semi-solid medium and liquid assays.

An example of semi-solid medium assay is the plaque assay, which is somewhat more quantitative as compared with spot or cross-streak test.^{4,5} The plaque assay was originally described by one of the discoverers of phages, Felix d'Herelle,⁶ and later modified by a number of other phage biologists.^{7–11} The plaque assay measures the formation of a clear zone in a bacterial lawn,

resulting from amplification of a single phage particle. Although the plaque assay is simple, plaque morphologies and sizes can vary with the experimenter, media and other conditions. Plaque assay based enumeration gives the efficiency of plating (e.o.p.) of a given phage preparation in the bacterial strain in which the phage was prepared as compared with other susceptible strains. The presence of restriction modification systems in bacteria can heavily influence the efficiency of plating in a given host.¹²

Liquid lysis assays generally monitor the reduction in the optical density of a bacterial culture upon infection, growth and lysis by phage. Recently, an automated lytic assay based on Bioscreen C analyzer was reported.¹³ The drawback of this method is that bacterial cell debris resulting from lysis may contribute to the optical density values recorded and therefore might obscure or underestimate the real phage lytic activity. In contrast to methods described above, single step growth experiments offer a more precise measurement of phage growth in a given bacterial strain, giving data on each of the steps in the phage life cycle, such as adsorption efficiency, latent period, eclipse period, burst

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size, etc.^{3,8} However, single step growth experiments are not only resource and reagent intensive, for example, requiring a large number of plates, they are also labor intensive and therefore impractical in high-throughput analysis.

Automation is one potential solution for the inconsistencies and inconveniences inherent in many of the existing phage assays. Therefore, we tested the utility of the OmniLog™ instrument (Biolog, Inc.) for automated assay of phage growth. The OmniLog™ instrument is a specialized plate incubator (with a capacity of 50 microtiter plates) coupled to a camera and a computer and is marketed for use with specialized phenotypic microarray plates (PM) or specialized plates for bacterial identification assays. In the case of the PM assays, the wells of each plate contain different carbon sources, secondary metabolites and even selective or inhibitory compounds/agents like antibiotics, and redox chemistry is employed to measure respiration of input microorganisms under the various conditions present in each well.¹⁴ The PM technology has been successfully used in a variety of comparative analyses of phenotypes of organisms^{15,16} as well as to assess the accuracy of genome annotations.¹⁷

In the current study, we have utilized the OmniLog™ system to monitor lack or reduction of bacterial growth in the presence of phage as compared with untreated conditions. The OmniLog™ system is designed to rely on redox chemistry, employing cell respiration as a universal reporter. If the growth of bacteria is strongly positive, the cells respire actively, reduce a tetrazolium dye and form a strong color. In the case of bacterial lysis by phage infection, respiration is slowed or stopped, and less color or no color is formed. In the OmniLog™ system, as many as 4,800 bacterial growth assays in the presence of phage can be performed simultaneously, allowing kinetics of bacterial growth and development of resistance to be monitored continuously over the course of the experiment. Here, we demonstrate the utility of the OmniLog™ system for indirect measuring of phage lytic activity using six individual *Bacillus anthracis*-specific phages as well as a cocktail of phages in combination with different *B. anthracis* strains. The results of these experiments indicated that the OmniLog™ system could be used reliably in high-throughput host range analysis and for evaluating additive or antagonistic interactions of phages and antibiotics. As compared with more traditional phage assays, the OmniLog™ system has the distinct advantage of automatic, real-time monitoring of phage infection and bacterial growth over the entire duration of the experiment, spanning several days if desired.

Results

Morphology of *B. anthracis* phages used in this study. Six phages were used individually or as a cocktail for the in vitro indirect phage lytic assays using the OmniLog™ system. Phage Gamma is a CDC recommended diagnostic phage for *B. anthracis*. *Giraffe* was isolated from giraffe feces from a zoo on Long Island, New York and characterization of this phage will be published elsewhere (Schuch R, personal communication). The other four phages were isolated in this study from sewage collected from Great Seneca sewage treatment plant in Germantown,

Maryland. Electron micrographs of the six phages used in this study are shown in **Figure 1**. As published, Gamma (**Fig. 1B**) exhibits a typical morphology of a phage belonging to the family Siphoviridae¹⁸ with an icosahedral head and a long non-contractile tail and phage *Giraffe* exhibits a similar morphology (**Fig. 1A**). Phage BA39 (**Fig. 1E**) appears to belong to the Myoviridae family (icosahedral head and contractile tail). Phages BA21, BA28, BA51 also appear to belong to the family Myoviridae based on tail width and bacterial growth inhibition profiles (see later) but this assignment is tentative.

Monitoring the kinetics of bacterial growth using OmniLog™ system upon infection with *Giraffe* phage. In order to assess the utility of the OmniLog™ system for indirect assay of phage lysis, we tested individual phages against their host, vegetative cells and spores of *B. anthracis* Sterne 7702. The kinetics of growth in wells seeded with *B. anthracis* Sterne strain 7702 vegetative cells or spores with and without infection by *Giraffe* phage are shown in **Figure 2A and B** respectively. The growth of 7702 without phage infection followed a typical sigmoidal growth curve with a lag period of ~3 h. Addition of phage at concentrations ranging from 0.0001 to 100 MOIs completely suppressed bacterial growth for ~9 h after which growth resumed in majority of cases. There are some cases in which bacterial growth was completely suppressed for up to 24 h. However, there was no correlation with the concentration of phage and the appearance of resistance; for example, with only 0.001 or 0.01 MOI of phage, growth was completely suppressed, whereas at concentrations of 0.1 to 100 and 0.0001 MOI, growth was evident after 9 h. A similar profile was seen when spores were used as the inocula instead of vegetative cells. In this case, growth suppression was evident up to ~9 h after which growth resumed. However, complete suppression was observed up to 24 h in cases where phages were added at MOIs of 0.0001, 0.1 and 1. We hypothesize that the resurgence of growth after 9 h is the result of the presence of truly phage resistant or “persister” bacteria in the population.¹⁹ The absence of a correlation with the concentration of the phage inocula could indicate that the appearance of phage resistant/persister bacteria followed a random fluctuation model.²⁰ Experiments where the cultures were monitored for up to 48 h showed a similar trend (data not shown). In **Figure 2B**, we presume that infection is occurring post germination of the spores, as all the phages used in this study have been shown to specifically infect vegetative *B. anthracis* and not spores (unpublished data). There is rapid germination and outgrowth of spores and subsequent infection without a further lag period since this time is comparable to the lag period seen in uninfected vegetative cell controls. We also tested the kinetics of growth of *Giraffe* phage-resistant mutant upon exposure to *Giraffe* (**Fig. 2C**). This strain continued to grow even in the presence of very high concentrations of phage (100 MOI), similar to the control without any added phage. There was no evidence of “lysis from without,” even at these high MOIs.²¹

Monitoring the kinetics of bacterial growth using OmniLog™ system upon infection with Gamma phage. A slightly different profile was observed when Gamma phage was used in the kinetic experiments. Complete suppression of growth was observed in a few cases (0.01, 1 and 100 MOI) whereas in

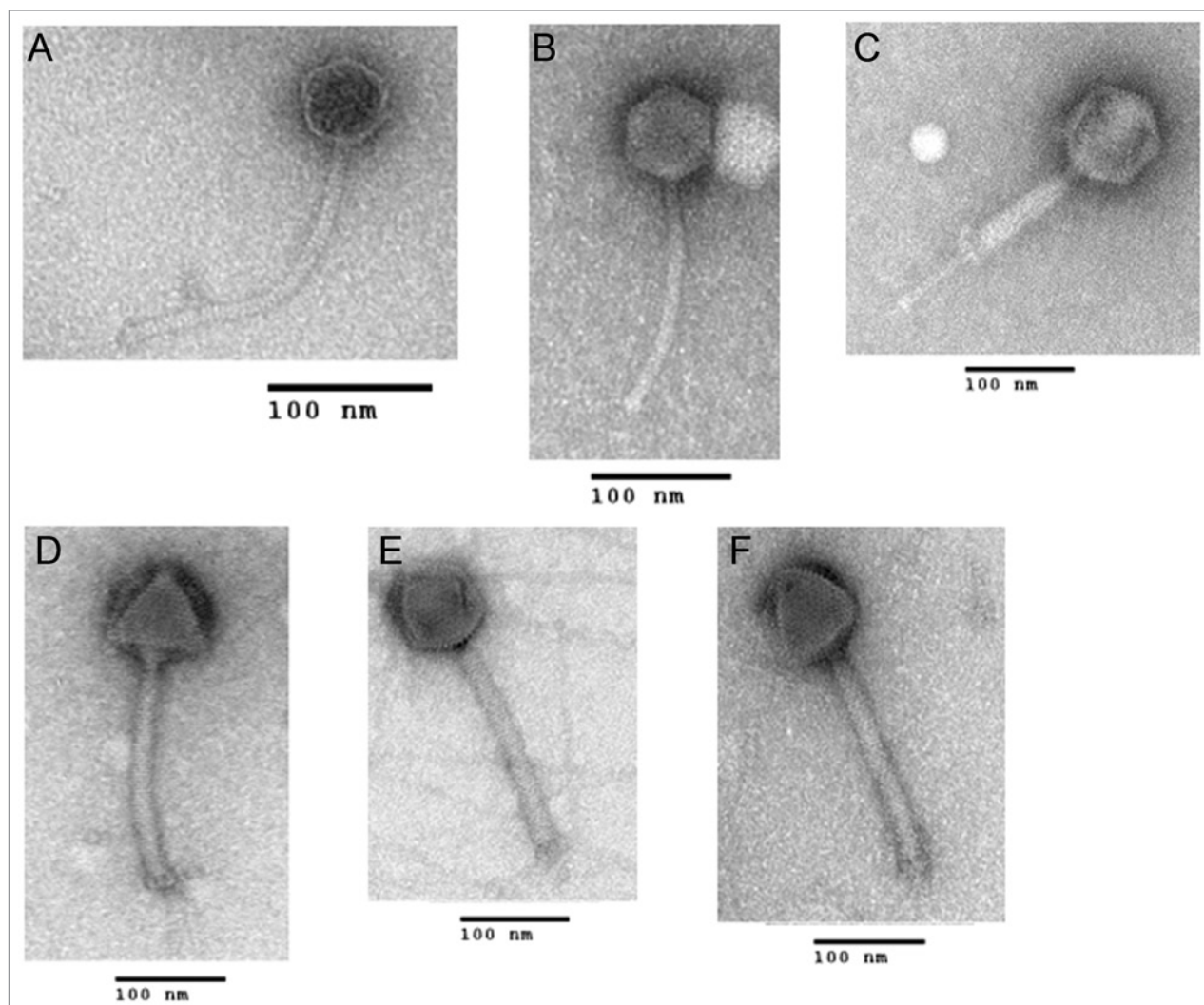


Figure 1. Electron micrographs of bacteriophages used in this study and viewed at 150,000× magnification: (A) *Giraffe*, (B) Gamma, (C) BA21, (D) BA28, (E) BA39 and (F) BA51.

other cases (0.0001, 0.001 and 0.1 MOI) bacterial growth continued for a few hours (up to 5.25 h) and then reached a plateau. In some cases (0.1 and 10 MOI) suppression was prolonged a little longer, up to ~10 h, before growth resumed again (Fig. 3A). Similar trends to those observed using vegetative cells were seen in wells seeded with spores except that the effects of various phage concentrations (i.e., the timing of resumption of growth) were more variable (Fig. 3B). Notably, a striking difference was observed between *Giraffe* and Gamma; *Giraffe* completely suppressed bacterial growth for up to 9 h in all concentrations of phage before growth resumed, whereas in the case of Gamma infection, bacterial growth continued to occur in low phage concentrations. Results from experiments with shake flask cultures suggest that *Giraffe* exhibits a rapid lysis phenotype and hence the residual resistant cells present in the population are better able to rapidly grow and populate the culture. The rapid lysis phenotype is supported by the observation in shake flask culture experiments that *Giraffe* has shorter latent and eclipse periods (~5 min each) and a burst size of ~45 (unpublished data).

We also tested the kinetics of growth of Gamma phage-resistant mutant upon exposure to Gamma phage (Fig. 3C). This strain continued to grow even in the presence of very high concentrations of phage (100 MOI), similar to the control without any added phage. There was no evidence of ‘lysis from without’ even at these high MOIs.²¹

Monitoring the kinetics of bacterial growth using OmniLog™ system upon infection with T4-like phages. As compared with *Giraffe* and Gamma phages, the T4-like phages BA21, BA28, BA39 and BA51 exhibited a very different bacterial growth profile. With these four phages, bacterial growth inhibition was observed in almost all concentrations except for BA51 at 0.0001 and 0.001 MOI (Fig. 4A–D). Also, wells that received spore inocula produced similar results as vegetative cells in that only low concentrations of phage, such as an MOI of 0.0001 to 0.001, exhibited any hint of bacterial growth (data not shown).

Monitoring the kinetics of bacterial growth using OmniLog™ system upon infection with a phage cocktail. Next we wished to test what effect a phage cocktail comprised of all

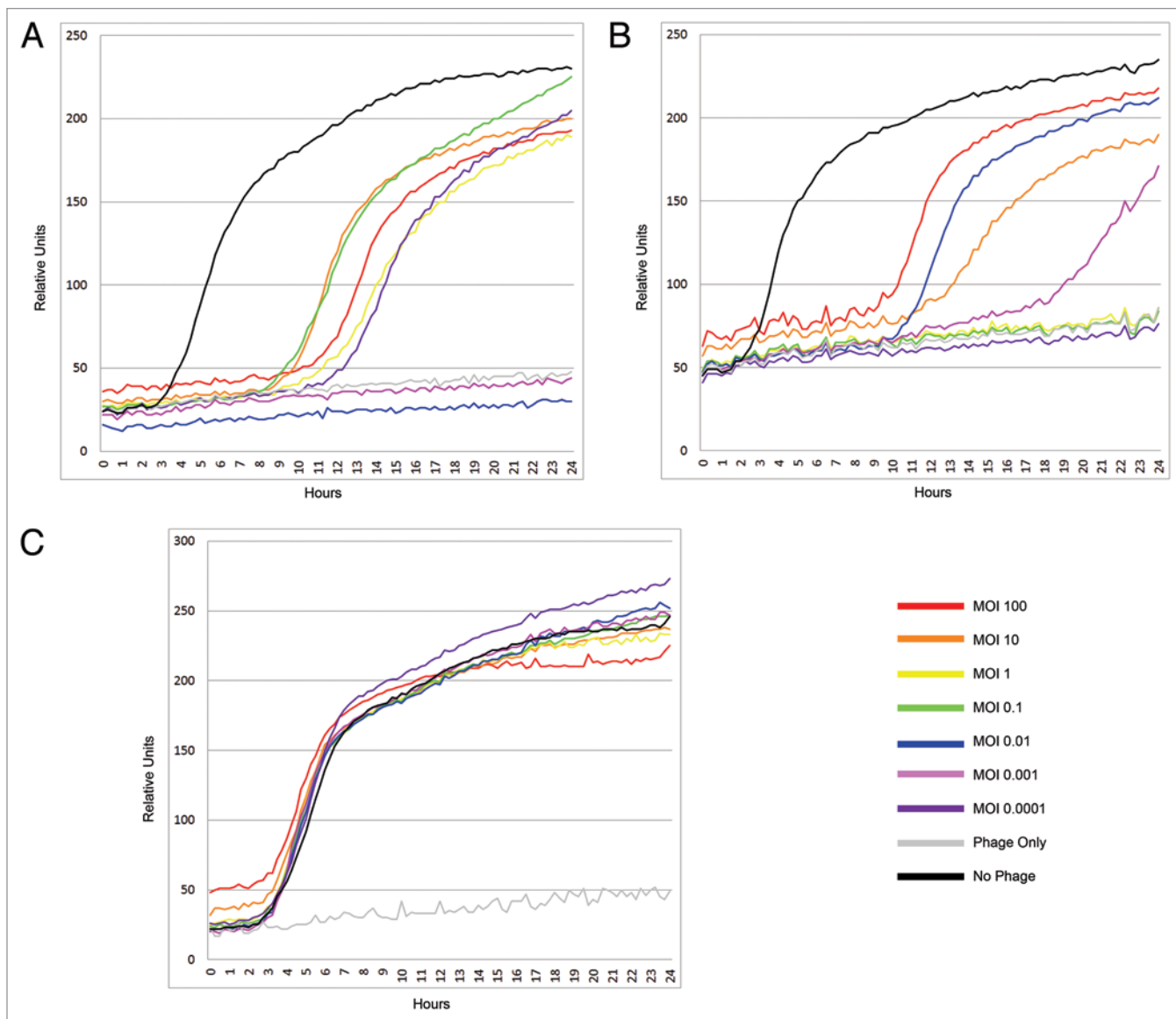


Figure 2. Kinetics of bacterial growth in the presence of phage *Giraffe*. *B. anthracis* strain 7702 was grown at 37°C in tryptic soy media and 1% (v/v) of tetrazolium dye. The wells designed to receive bacteria were seeded with 10^6 cells. Microtiter plates for the phage assay were prepared as described in materials and methods. The tests were done with (A) vegetative cells, (B) spores and (C) *Giraffe*-resistant *B. anthracis* Sterne strain 8009, a derivative of the 7702 strain.

six phages would have on growth of *B. anthracis* strain Sterne 7702. The results are shown in Figure 4E. In this experiment growth inhibition was observed at all phage concentrations for the entire duration of the experiment, indicating that the very few bacteria that are resistant to any given phage are still susceptible to infection by the other phages present in the cocktail indicating possibly different bacterial receptors are used by these phages. Experimentally, we have isolated resistant bacteria against each of the individual phages upon single phage treatment. Although these resistant mutants appear at very low frequencies (results not shown) their emergence after single phage treatment would still cause concern. Therefore, based on the apparent lack of resistant mutants post phage cocktail treatment, this experiment supports the idea of using a mixture of different phages for phage therapy

experiment. The minimum number and combination of phages needed to completely arrest the growth of the bacteria were not evaluated in this study.

Monitoring the kinetics of Ciprofloxacin resistant bacteria using OmniLog™ system upon infection with *Giraffe* phage. In order to further evaluate the utility of the OmniLog™ system for indirect phage assays, phage infection in the presence and absence of antibiotic treatment was also examined. Wild type *B. anthracis* Sterne, in the absence of phage, was exposed to varying concentrations of ciprofloxacin ranging from 0.5 ug/ml up to 32 ug/ml. As expected, bacterial growth was inhibited at all concentrations (Fig. 5A) whereas the control, without any antibiotic, exhibited a typical sigmoidal growth curve. On the other hand, a medium level ciprofloxacin resistant mutant (HS2-7),²²

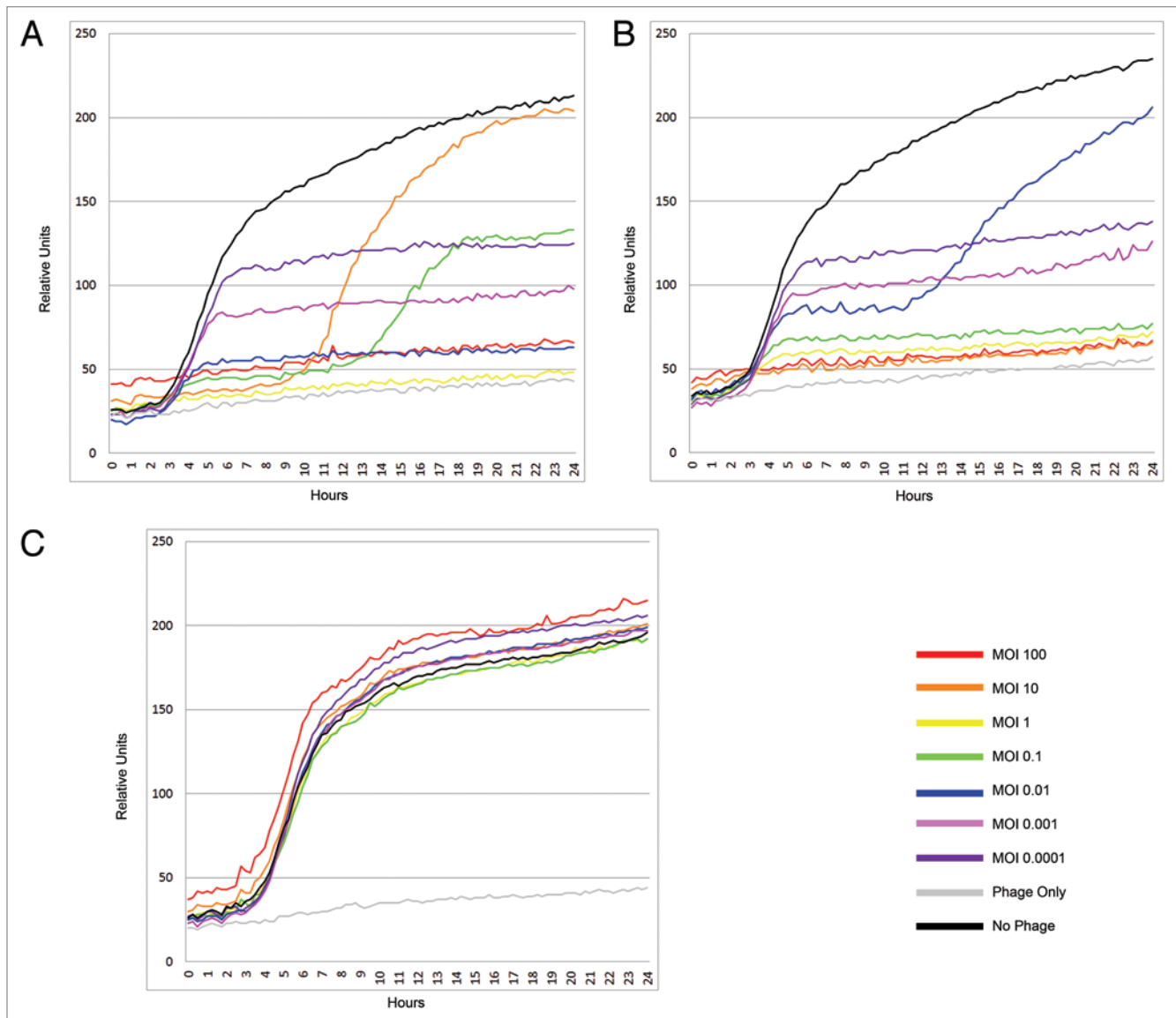


Figure 3. Kinetics of bacterial growth in the presence of phage Gamma. *B. anthracis* strain 7702 was grown at 37°C in tryptic soy media and 1% (v/v) of tetrazolium dye. The wells designed to receive bacteria were seeded with 10^6 cells. Microtiter plates for the phage assay were prepared as described in materials and methods. The tests were done with (A) vegetative cells, (B) spores and (C) gamma-resistant *B. anthracis* Sterne strain 8030, a derivative of the 7702 strain.

exhibited resistance to ciprofloxacin concentrations up to 4 ug/ml and sensitivity at concentrations above 4 ug/ml up to the highest concentration tested; i.e., 32 ug/ml (Fig. 5B). When the additive effect of phage plus antibiotic was tested, it was found that the bacterial growth was completely inhibited in all cases (Fig. 5C). Surprisingly, the *Giraffe* phage effectively suppressed the growth of HS2-7 even at very low multiplicity of infection and in the absence of ciprofloxacin treatment (Fig. 5D). In this case, appearance of resistant bacteria was not seen as was observed with the strain 7702 (Fig. 2A). We speculate that this difference is due to strain specific variations in the infectivity and growth of *Giraffe*. HS2-7 is a derivative of Δ ANR which is a *B. anthracis* strain lacking both pXO1 and pXO2 plasmids, whereas 7702 is a Sterne strain lacking only pXO2. It is not clear at this time how the

absence of the two plasmids prevents the emergence of resistance. It is possible that the absence of pXO1 and pXO2 plasmids may allow for efficient infection and killing of the host and possibly suppression of resistance.

Discussion

Although originally designed to monitor bacterial growth under a range of varying growth conditions, we have successfully adapted the OmniLog™ system for indirectly monitoring phage growth. Conventionally, one can monitor bacterial growth by following the increase in the optical density of the culture and a lack thereof; i.e., decrease in optical density due to lysis by phage. The OmniLog™ system, however, circumvents this by monitoring

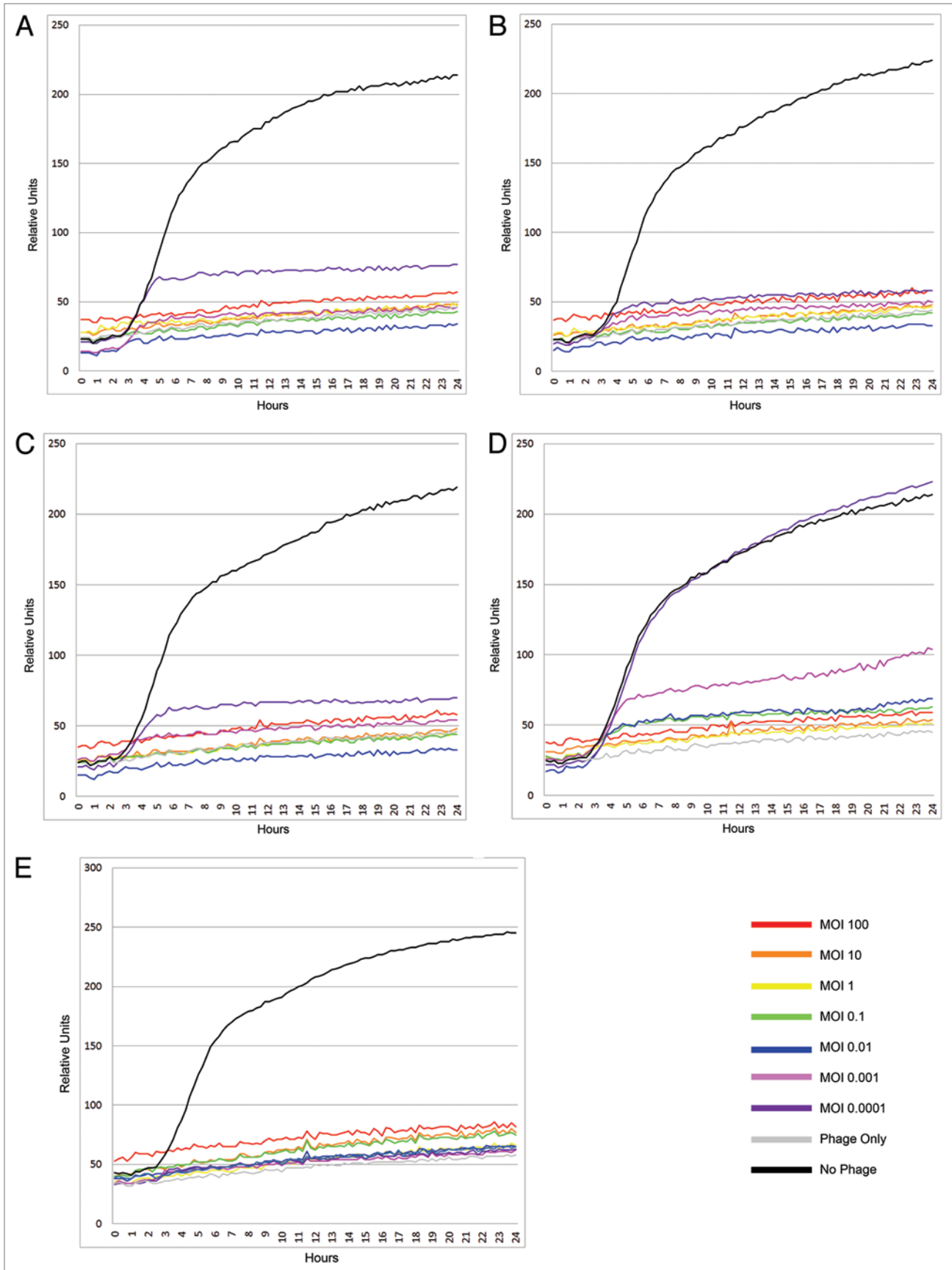


Figure 4. See figure legend on page 165.

Figure 4 (See opposite page). Kinetics of bacterial growth in the presence of T4 like phages. *B. anthracis* strain 7702 was grown at 37°C in tryptic soy media and 1% (v/v) of tetrazolium dye. The wells designed to receive bacteria were seeded with 10⁶ cells. Microtiter plates for the phage assay were prepared as described in materials and methods. These experiments were performed with vegetative cells and using the phages: (A) BA21, (B) BA28, (C) BA39, (D) BA51 and (E) a mixture of Gamma, *Giraffe*, BA21, BA28, BA39 and BA51.

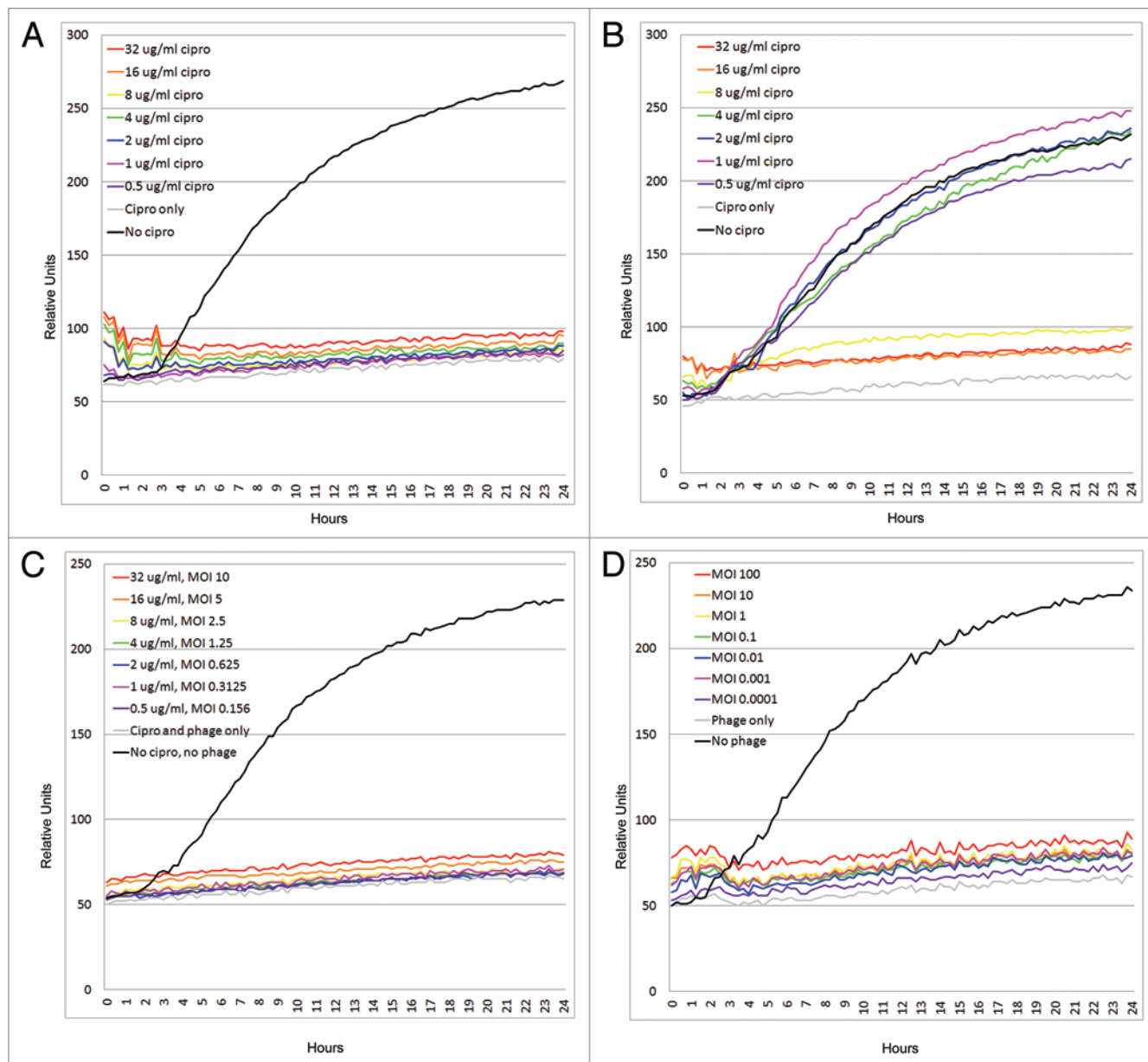


Figure 5. Kinetics of bacterial growth in the presence of phage *Giraffe* or ciprofloxacin. *B. anthracis* HS2-7, a ciprofloxacin resistant strain, was grown at 37°C in tryptic soy media and 1% (v/v) of tetrazolium dye. The wells designed to receive bacteria were seeded with 10⁶ cells. The *Giraffe* phage PFU ranged from 10⁸ PFU to 10² PFU and ciprofloxacin concentrations ranged from 32 µg/ml to 0.5 µg/ml. One well was used as a phage only control and another as a bacterial positive control. The experiments were performed with (A) *B. anthracis* Sterne 7702 plus ciprofloxacin, (B) HS2-7 plus ciprofloxacin, (C) HS2-7 plus ciprofloxacin and *Giraffe* phage and (D) HS2-7 plus *Giraffe*.

the respiration rates in the well, which is directly proportional to the number of actively growing cells. Measuring respiration, instead of optical density, allows us to measure the living cells while excluding the dead cells and other debris that may contribute to increasing the optical density in a well.¹² Also, the

OmniLog™ system provides an unparalleled high-throughput capability (4800 phage assays), with continuous and automated monitoring for the entire duration of the experiment.

The advantage of real time, continuous monitoring is evident in infections with the phage *Giraffe*. Conventional endpoint

analysis of an overnight phage growth experiment would give false impression of no lysis because of the turbidity of the culture upon overnight incubation whereas in reality *Giraffe* caused full lysis prior to the establishment of bacterial growth from founder persisters or resistant bacteria present in the population. Continuous monitoring reveals the real kinetics of the bacterial growth upon infection. Also, phage specific variations can be uncovered in long-term continuous monitoring of indirect phage assays. The ‘rapid lysis’ phenotype of *Giraffe* is distinct from that of Gamma in which there is initial growth of bacteria before phage induced killing can happen (this may be due to shorter eclipse time in *Giraffe* as compared with Gamma). Similarly, the T4-like phages that are obligatorily lytic have different profiles and are efficient bacterial killers even at low MOI.

Lastly, the additive, synergistic or antagonistic effect of a phage-antibiotic combination has been tested in this study, using the *Giraffe* phage in combination with ciprofloxacin against a ciprofloxacin-resistant strain of *B. anthracis*. The strain HS2-7 is known to exhibit medium level resistance (up to 4 µg/ml) but is completely susceptible to *Giraffe* phage.²² When combined in the same wells, the phage and antibiotic seem to work additively to produce virtually no growth in the course of 48 h. This suggests that this phage could be used therapeutically in the event of an infection from an antibiotic resistant strain of *B. anthracis*.

It is apparent that OmniLog™ cannot determine the kinetics of phage growth in each well, thereby requiring one to extract the contents of the wells and enumerate the phage present. Also, the OmniLog™ system may have to be modified for phage assays of anaerobic bacteria. Despite these caveats, we demonstrate that the OmniLog™ system can be successfully used for monitoring phage growth in high throughput experiments such as host range analysis and combination studies involving antibiotics and phages.

Materials and Methods

The bacterial strains and phages used in this study are listed in Table 1. Additionally, both vegetative cells and spores of *B. anthracis* Sterne 7702 and vegetative cells of phage resistant mutants of 7702 were used in this study. All the phage methods were followed using standard protocols.⁴ CsCl purification of phage was done according to published protocol.²³ CsCl purified phage lysate was used for electron microscopy by negative staining with 2% uranyl acetate following standard procedures.⁴ Preparation of spores was done according to published protocol with some modifications.²⁴

Phage strains: Four of the six phages used in this study were obtained from sewage water. These phages are designated as *B. anthracis* BA21, BA51, BA28 and BA39. *B. anthracis* specific Gamma phage was obtained from NMRC phage stock. *Giraffe* phage was from the collection of Dr. Raymond Schuch.

Isolation of phage from raw sewage using polyethylene glycol. Sewage samples (2 gallons total) were obtained from the Great Seneca sewage treatment plant in Germantown, MD 20874. The sewage samples were placed in six Beckman plastic bottles (380 mL volume), followed by addition of 22 g of NaCl

Table 1. List of bacterial strains and phages used in this study

Strain	Description	Source/reference
<i>B. anthracis</i>		
BA663	7702 Sterne pXO1+pXO2-	T. Koehler via Scott Stibitz
NBGD 8009	7702 Sterne Giraffe resistant	This study
NBGD 8030	7702 Sterne Gamma resistant	This study
HS2-7	ΔANR Ciprofloxacin resistant	22
Phages		
Gamma	Large plaque variant of lab isolate	B. Biswas (this study)
Giraffe	Natural isolate from giraffe feces	R. Schuch
BA21	Natural isolate from sewage	M. Henry (this study)
BA28	Natural isolate from sewage	M. Henry (this study)
BA39	Natural isolate from sewage	M. Henry (this study)
BA51	Natural isolate from sewage	B. Biswas (this study)

and then the mixture was allowed to stand on ice for 1 h. The samples were then centrifuged at 10,000 × g for 10 min. The supernatant was transferred to a separate container and mixed with polyethylene glycol (PEG, MW 8,000; Fisher Scientific) to provide a final concentration of 10% (w/v). The mixture was then allowed to sit for 1 h on ice and then centrifuged at 12,000 × g for 20 min. The supernatant was discarded and the resulting precipitate was suspended in 5ml of phage dilution buffer (10 mM NaCl, 50 mM Tris-Cl pH 8, 10 mM MgCl₂) or SM buffer (100 mM NaCl, 8 mM MgSO₄·7H₂O, 50 mM Tris-Cl, 0.002% gelatin (w/v)). This phage fraction was used as seed stock for isolation of phage against any given bacterium.

Amplification of phages in the sewage sample. In some experiments an alternative approach was used for phage isolation using the sewage directly as a source without purifying the viral fraction by ultra centrifugation. 100 ml of sewage was added to a 250 ml flask containing 3 g of Tryptic soy (TS) media and mixed thoroughly to dissolve the media with sewage water. 0.2 ml of overnight bacterial culture was added to the flask and then incubated with vigorous shaking (225 rpm) at 37°C. After 6 h incubation, a 5 ml aliquot from each flask was filtered through 0.22 µm Millex GV sterile filters (Millipore). Another 1 ml aliquot from each flask was treated with 50 µl of chloroform for 15 min at room temperature. Chloroform treated sample was centrifuged at 5,000 × g for 1 min. The aqueous phase of this processed sample was transferred to a new 1.5 ml eppendorf tube and used for further analysis. 50 µl aliquots of the filtered or chloroform treated sample were mixed with 100 µl of bacterial isolate and incubated at 37°C for 20 min prior to plating by soft agar overlay technique. Plates were incubated at 37°C overnight and the next day plates were carefully examined for appearance of phage plaques.

Preparation of microtiter plates for Biolog assay. Bacterial cultures for the microtiter plate assay were prepared from colonies grown from an overnight culture on Tryptic Soy Agar (TSA)

plates and suspended in phosphate buffered saline (pH 7.4) to an optical density of 0.1, which corresponded to roughly 10^8 colony forming units/ml. After further dilution, 10^6 CFUs were deposited in each well. Individual phages or a cocktail of *Giraffe*, BA21, BA28, BA39, BA51 and Gamma were mixed in equal proportions and used to infect the *B. anthracis* host. The phage titers used ranged from 10^8 PFUs to 10^2 PFUs per well, corresponding to a multiplicity of infection (MOI) of 100 to 0.0001.

Microtiter plates (96 well) plates were prepared as follows. In each well, 90 μ l of TS broth mixed with 1% v/v tetrazolium dye was added as growth medium followed by the addition of 10 μ l of (10^9 PFU) phage per well in one column, making a 10^8 PFU/well dilution. 10-fold dilutions were made in the subsequent columns down to 10^2 PFU per well, leaving 90 μ l left in each well. The last column received media and phage alone, with a total volume of 100 μ l. Following dilution of phage, 10 μ l of 10^8 CFU/ml of *B. anthracis* cells or spores were added to each well designed to have bacteria, increasing the volume to a total of 100 μ l and

giving a final concentration of 10^6 CFU per well. The 96 well plates were then incubated in the OmniLog™ system at 37°C for 48 or 72 h. All experiments represent biological replicates of three except the ciprofloxacin experiment which was done once.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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