Efficacy and Durability of *Bacillus anthracis* Bacteriophages Used Against Spores

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Abstract

Antibiotics and vaccines help fight anthrax disease, but there are no anthrax spore control methods suitable for use in environments where humans are present. The work reported in this article indicates that bacteriophages may help reduce risk from anthrax spores. Dose-response studies demonstrated that higher concentrations of mixed *Bacillus anthracis* bacteriophages (3.5×10^8 plaque-forming units per milliliter) inhibited subsequent growth of bacteria when sprayed on *B. anthracis* spores. Phages also were tested for durability under conditions designed to simulate environments possibly encountered during mass phage production, storage, and use against anthrax spores. They remained infectious at temperatures from –20°C to 37°C, under filtration, aerosolization, and treatments with perspiration and blood. Phages were sensitive to temperatures over 55°C and to desiccation. Ultraviolet light reduced spore viability more than phage infectivity under similar conditions. The potential for personal or environmental decontamination of anthrax spores with phages is discussed.

Introduction

Current medical strategies against anthrax are limited and may fail if they encounter *Bacillus anthracis* strains that are antibiotic resistant or not targeted by vaccines. Naturally occurring viruses of bacteria (called bacteriophages, or phages) may help augment anti-anthrax strategies and have recently been investigated as sources for bacteriolytic agents useful against anthrax (Schuch, Nelson, & Fischetti, 2002).

Anthrax is caused by *B. anthracis,* a Gram-positive*,* rod-shaped, spore-forming bacterium closely related to the soil bacterium *B. cereus* (Harrell, Andersen, & Wilson, 1995; Helgason et al., 2000). Long-lasting anthrax spores can be killed only by relatively harsh methods, because the spores are not metabolically active and must be physically disabled rather than poisoned. Gamma irradiation, ultraviolet (UV) light, high-pressure steam, nanoscale powder biocides (Koper et al., 2002), and various gases kill spores, but none of these approaches is safe for humans.

The next best method is to ensure that effective antibacterials are present when spores germinate. Current medical strategies include vaccinations and antibiotics that attack metabolically active bacteria (those emerging from spores) or their products. More recent research indicates that a phage bacterial lysin (PlyG, from phage-γ) can lyse vegetative *B. anthracis* cells (not spores) and may help control anthrax disease (Schuch et al., 2002). Antibiotics and vaccinations are more effective against lower initial concentrations of bacteria (inoculum dose), so anthrax is best treated promptly. During the 2001 anthrax attacks, vaccinations and antibiotics against anthrax were given to many exposed or symptomatic persons well after the initial exposure to spores (Jernigan et al., 2001) and helped limit mortality and morbidity (Brookmeyer & Blades, 2002). Unfortunately, anthrax vaccination is relatively rare in civilian populations, so future anthrax releases will again affect mostly unvaccinated individuals.

To best augment current anti-anthrax strategies, new treatments must lower the initial amount of bacteria (the inoculum dose) by immediately attacking emerging bacteria as spores germinate and by targeting a potentially wider variety of anthrax strains. Like many viruses, some phages naturally attack and burst (lyse) a wider array of bacterial species and strains than

others. In addition, combinations of different phages (addressing a broader host range) could be applied to skin surfaces or clothing or introduced into the respiratory tract. Phages proximal to spores during subsequent spore germination would infect and lyse the target bacteria, lowering the inoculum dose targeted by antibiotics and vaccines. Because phages infect only certain types of bacteria, their use can reduce destruction of needed intestinal flora by antibiotics.

To be used against anthrax spores, phages must be durable and effective under a variety of conditions, including dryness, ultraviolet and solar radiation, and extremes of temperature. Phages also must maintain bacteria-killing ability in the presence of various body fluids and cells internal and external to the human body.

Potentially useful phages are abundant wherever bacteria are naturally found, as in soil. Phage-based antibacterial therapies have recently been reviewed (Duckworth & Gulig, 2002; Summers, 2001), and phages have been evaluated for a variety of antibacterial applications (Payne & Jansen, 2001; Weber-Dabrowska, Zimecki, & Mulczyk, 2000). Phages have been developed for therapeutic and prophylactic uses against *Salmonella* (Akimkin, Bondarenko, Voroshilova, Darbeeva, & Baiguzina, 1998), *Escherichia coli* O157:H7 (Kudva, Jelacic, Tarr, Youderian, & Hovde, 1999), *E. coli* in chickens (Barrow, Lovell, & Berchieri, 1998), and vancomycin-resistant *Enterococcus faecium* (Biswas et al., 2002). Phage lytic enzyme has been used to control *Streptococcus* in mice (Nelson, Loomis, & Fischetti, 2001) and as a treatment for burn infections by *Pseudomonas spp.* (Ahmad, 2002). Early phage therapy studies showed somewhat decreased anthrax in mice, depending on treatment of bacteria and phage before injection (Cowles & Hale, 1931). More recently, PlyG lysin, purified from phage-γ (specific to *B. anthracis*) has demonstrated efficacy against anthrax in mice (Schuch et al., 2002).

Bacillus anthracis and its nonlethal close relatives are widespread in soils. There are many different soil phages that infect and lyse *B. anthracis* (Ackermann & Dubow, 1989; Ackermann et al., 1994; Brown & Cherry, 1955). Soil probably contains a variety of naturally occurring phages active against both avirulent *B. anthracis* Sterne and virulent strains. Because of safety and legal considerations, the avirulent *B. anthracis* Sterne strain served as a surrogate for virulent *B. anthracis* in this study. This substitution does not seriously limit the value of the study, because the members of the *B. cereus* bacterial group (including *B. anthracis*) are very closely related. *Bacillus cereus* and *B. anthracis* share many phage parasites. Only phages W and γ (Brown & Cherry, 1955; McCloy, 1951) are known to distinguish *B. cereus* from *B. anthracis*. Therefore most naturally occurring phages capable of infecting *B. anthracis* Sterne will also infect pathogenic *B. anthracis* strains, although some variation in host susceptibility may occur between encapsulated and non-encapsulated strains. The wide variety of phages in soil can be used to thwart bacterial resistance and also permits selections of phages based on physical durability and rapid growth in culture, which are important to pharmaceutical production.

The purpose of this study was to use standard techniques to select and amplify populations of naturally occurring *B. anthracis* phages. A dose-response experiment was employed to test the effectiveness of the phage assemblage against *B. anthracis* Sterne, and the extent of phage attack was evaluated by monitoring of bacterial growth. Finally, phages were subjected to several conditions similar to those they would probably encounter during manufacture or application to human tissues. These approaches demonstrated the potential of easily obtainable, high concentrations of phage to kill bacteria emerging from anthrax spores. The presence of multiple phage strains indicated the potential for selecting effective phages for different bacterial strains in diverse environments.

Because of the natural capacity of many lytic phages, phages of *B. anthracis* were expected to tolerate artificial growth and maintain their bacteria-killing capacity when proximal

to anthrax spores. This study demonstrated that a mixed-phage assemblage amplified from soil contained phages that can kill bacteria originating from *B. anthracis* spores. The survival of phages was also determined under several conditions that phages could encounter during manufacture, purification, storage, or use.

Methods

Selecting and Amplifying Soil Phages

To obtain sufficient quantities of phages for dose-response and durability tests, it was first necessary to increase concentrations of phages occurring in soil bacteria related to *B. anthracis*. Naturally occurring mixtures of soil phages were initially grown as follows: 5 grams (5 g) of finely ground topsoil (from Blackhawk County, Iowa) was combined with 30 milliliters (30 mL) of NBY broth (DIFCO Nutrient Broth, 8 g/L, with DIFCO Yeast Extract: 3 g/L, pH 6.8, [DIFCO Laboratories, Detroit, Michigan]) and 3 mL log-phase *B. anthracis* Sterne, an avirulent vaccine strain. This slurry was shaken at 150 revolutions per minute for 12 hours at 37°C. *Bacillus* bacteria (and spores) that contain phages (Moreno, 1979) germinate and grow during this incubation and release phages into the media. Some of these phages infect *B. anthracis* and grow through many cycles of reproduction along with host bacteria. After 12 hours, the phages were separated as follows: The soil-bacteria mixture was stirred for five minutes with chloroform (5 mL), and this step was followed by centrifugation (10,000*g* for 10 minutes at 4°C). Supernatants were stored at 4°C with 1/20th volume of chloroform. Then *B. anthracis*–specific soil phages were further amplified by standard agar plate lysis (Adams, 1959; Thorne, 1968). *Bacillus anthracis* Sterne was used as host. Growth on plates, for eight to 12 hours, was followed by harvest and centrifugation/chloroform clarification as described above. Mixed soil phage assemblages were typically grown to approximately 10^6 plaque-forming units (PFU)/mL by soil slurry culture, then to 10^{10}–10^{12} PFU/mL by agar plate lysis. Plate lysates were used in all of the experiments described below.

Spore Decontamination by Phages

Spore decontamination trials were designed as simple dose-response experiments to determine the efficacy and limits of using phages to kill anthrax bacteria. Bacterial spores (measured as colony-forming units [CFU]) were combined with phages (measured in PFU) by spraying of phages onto dried spores. Spraying is a method very likely to be used for skin applications of phage-based decontamination techniques. Closely comparable procedures were not found in previous literature, so methods were improvised with commonly available materials. Phage-to-spore ratios of approximately10^4–10^5 (PFU to CFU) were used because similar ratios had been used in early animal experiments (Cowles & Hale, 1931) and because ratios below 10^3 had no visible effect on spores in preliminary experiments (data not shown). Bacterial growth from spores sprayed with higher numbers of phages was compared with growth from spores treated with lower numbers of phages. Spores of *B. anthracis* Sterne (in sterile, distilled water) were applied in 10-microliter (10-μL) aliquots into depressions of glass microscope slides (Fisher Scientific), dried, and treated under a Biosafety Level 2 laminar flow hood. Phages were diluted in NBY broth and sprayed on the spore-containing glass slides (lying flat) from a distance of 15 centimeters (15 cm) and at an angle of approximately 45 degrees from the horizontal. Phage spraying consisted of pumping 0.5 mL of phage dilution through a metered nasal aerosol spray bottle (Apothecary Products, Inc., Burnsville, Minnesota). The phage was

pumped through an opening 0.2 millimeters (2 mm) in diameter at 0.23 mL per second, covering the slide with fine droplets (without causing running). After five minutes, spores and phages were recovered from the slide depression in 100 μL of NBY broth, stirred briefly, and assayed immediately for production of vegetative bacteria. Negative controls were sprayed with broth only.

It was also important to know how many spores a given quantity of phage could kill. The limits of a fixed concentration of phages (1.4×10^8 PFU/mL) were tested by spraying on different quantities of spores (4.4×10^1 to 4.4×10^5 CFU) as described above.

Assessments of bacterial growth were made visually and by standard colony counts. Visual evaluations of bacterial growth from spores consisted of spotting 20-μL aliquots of recovered spore-phage mixture as replicates on NBY agar plates, followed by incubation at 37°C for eight to 18 hours. Relative growth of bacteria was monitored visually and recorded electronically by scanning of agar plates on an HP Scanjet 5100C (Hewlett-Packard, Palo Alto, California).

Tests of Phage Durability

Physical tests of individual phage durability are somewhat standardized (Ackermann & Dubow, 1989), but phages are rarely tested in mixed assemblages. In this study, conditions were established that simulated conditions expected during manufacturing, storage, or use of phages against anthrax spores on humans.

The UV-light resistance experiment was conducted to evaluate phage durability with respect to the UV component of sunlight. Higher UV exposure than standard (Ackermann & Dubow, 1989) was used in order to kill the majority of spores.

Phage durability experiments were carried out in various volumes depending on the need for simple temperature treatments or for treatments combining fluids, drying, filtering, or spraying. All treatments were replicated three times. Immediately after each treatment, the phages were tested for infectivity by standard plaque assay (Adams, 1959; Thorne, 1968) on *B. anthracis* Sterne, with least three subsamples from each replicate. Treatment volume was included in calculations of final phage infectivity (PFU/mL).

Resistance to deactivation by filtration was tested as follows: 2–3 mL of phage suspension (in NBY broth) was passed through 0.45-micrometer nylon filters (Fisher Scientific), which allow passage of most phages but block bacteria. Resistance to aerosolization was tested as follows: phage suspension was pumped three times through a nasal aerosol sprayer as described above for decontamination treatments. Temperature resistance experiments involved incubating 100 μL of phage suspension at various temperatures for 12 hours in sealed glass tubes. Desiccation resistance was tested as follows: 100 μL of phage suspension was spotted onto a sterile glass slide, then dried at 37°C for 12 hours, recovered in 100 μL NBY, and assayed. Resistance to some body fluids was determined as follows: 100 μL of phage suspension was incubated with 100 μL of body fluid at 37°C for eight hours. These fluids consisted of calf serum or erythrocytes (Colorado Serum, Denver, Colorado) or human perspiration. Appropriate procedures for testing the effect of human perspiration on phages were unavailable, so novel methods were employed. It was recognized that skin surface application of phages would probably bring phages into contact with a mixture of perspiration, dust, and dead skin cells naturally found on skin surfaces. Therefore, the author collected a mixture of these

components (approximately 1 mL) by scraping his forearm and facial skin surfaces following one hour of rigorous outdoor exercise. The perspiration mixture was stored in sterile, 1-mL microcentrifuge tubes at 4°C for one hour, then used in phage tests. The effect of UV light on phage suspensions was tested by direct exposure of 100 µL of phages (in depressions of glass slides) to UV light at a distance of 10 cm from a standard Shortwave UV Sterilization Lamp (Model C81, 0.70 amps, Ultra-Violet Products, Inc., San Gabriel, California) over a 30-minute period. The effect of UV light on dried *B. anthracis* Sterne spores was addressed in order to give a relative measure of the viability of spores under UV exposure. The spores also were dried as 10-µL spots on glass slides as described for decontamination trials and treated with UV light simultaneously with phage. Then the spores were recovered in 200 µL of sterile, distilled water and assayed with standard plate counts (Adams, 1959).

Results

Spore Decontamination by Phages

Phages could be considered effective if spraying with phages decreased bacterial growth from spores. Figure 1A depicts bacterial growth typical of spores treated with phages. Bacterial growth was decreased when the spores were sprayed with higher levels of phages, and only a perimeter ring of growth was present after approximately eight hours. Colony counts (not shown) from the same experiments indicated that bacteria dropped from approximately 4×10^4 to 0 in response to treatments with phages in the range of 0 to 3.5×10^8 PFU/mL. Spore treatments with phage in amounts of 1.4×10^8 PFU/mL or fewer allowed bacterial growth at the centers of assay spots. A variation was included to test the effect of postspray drying (simulating drying of spore-contaminated clothing or other surfaces), in which slides were allowed to dry for four hours following spraying with phages. Bacterial growth was not inhibited following this treatment (Figure 1B).

Sprays with 1.4×10^8 PFU/mL of phages did not decrease growth in spore amounts of 4.4×10^4 CFU or more. Growth was eliminated from spore levels at or below 4.4×10^3 CFU (Figure 1C). In this system, phages decreased bacterial growth at initial phage-spore ratios of approximately 3×10^4 or greater. This ratio is similar to the 1.6×10^4 ratio used in early animal experiments (Cowles & Hale, 1931).

Phage Durability

Phages were most sensitive to high temperatures, desiccation, and UV light. Incubation of phages at 55°C or higher (not shown) reduced or eliminated phage infectivity. Desiccation also eliminated phage infectivity. Phages survived filtration, aerosolization, and treatments with blood and perspiration (Figure 2). UV light reduced phage infectivity by about four orders of magnitude over 30 minutes. Under similar conditions, however, target spores were nearly completely inactivated within two minutes (Figure 3). Thus, phage susceptibility to limited UV light may not compromise bacteria-killing ability.

Discussion

Spore treatments with phages were designed to simulate surface spray application of phages against *B. anthracis* spores and to demonstrate the efficacy and limits of known phage-

spore ratios in killing bacteria originating from treated spores. Spray treatments are the method most likely to be used in application of phages to skin surfaces or lungs invaded (or threatened) by spores. The reductions in bacterial growth following phage spray treatments indicate that if the spores are treated with enough phages, growth of vegetative bacteria will be reduced. This concept is not new, but it has not previously been applied to reduction of risk from environmental sources of anthrax spores.

The spore decontamination experiments were designed with the assumption that many naturally occurring phages have adapted to infect *B. anthracis* under conditions favoring the germination and growth of vegetative host bacteria and not under the more harsh and varied conditions experienced by spores between hosts. Therefore the decontamination experiments conducted for this study demonstrate phage efficacy only if spore germination is induced shortly after treatment with phage. In fact, the ability of phages to reduce the risk from anthrax spores may be limited by the conditions experienced by the spores after they have been treated with phages and before they have entered a potential host cell, conditions of dryness and heat being the most deleterious to phages. Should phage-treated spores be inhaled or enter a wound relatively soon after treatment, phages may face other deactivating conditions also not modeled by spray decontamination experiments in this study. Blood, for example, might contain antibodies (depending on previous exposure to specific phages) that could bind phages and decrease their efficacy against bacteria. Factors that reduce phage infectivity may be encountered during macrophage uptake of spores. Such factors were not tested in the work reported here. It is assumed that *B. anthracis* phages occur in diverse abundance because they have overcome many such barriers.

In spore decontamination experiments, peripheral rings of bacterial growth surrounding assay spots indicated that bacterial "escapes" had occurred in treatments with higher phage concentrations (Figure 1). Infection and burst cycles probably produce higher concentration of phages in the center of assay spots. Bacteria at the perimeter may then encounter fewer phages and could escape infection. Even with escapes, spraying of phages on spores substantially reduced bacterial growth. This effect has not previously been demonstrated for *B. anthracis*.

Experimental simulations of various conditions included in phage durability tests were, at best, artificial and were limited by lack of similar, previously published studies. Conditions in the phage durability experiments were assumed to be somewhat mild (compared with real situations), because phages deployed against spores on skin surfaces or wounds would probably face combinations of various factors, such as perspiration, blood, and sunlight, and results might be more deleterious to phages. Conversely, UV-light intensities and exposures in these experiments were much higher than those likely to be found under natural conditions. High UV-light levels were used to emphasize that phages remained viable even after bacterial spores had been inactivated under experimental conditions, as discussed below. Temperature, filtration, and aerosolization resistance experiments were included to address possible production, storage, or deployment conditions. Filtration and aerosolization treatments made use of standard bacterial filters and pharmaceutical spray applicators probably similar to devices likely to be used in production and deployment of phages. Temperature regimes encountered in storage or transport might be less predictable, but heat treatments to remove bacteria and other contaminants from phage preparations during manufacture might be of shorter duration, similar to flash pasteurization (approximately 72°C, 15 seconds), as opposed to the 12-hour test times to which phages were subjected in this study. Finally, conditions experienced simultaneously or in series might more seriously decrease the viability of phages. Although combinations of factors may deactivate phages, it is still important to determine the effects of single factors so that the results can be used to design future screening and selection procedures for phages.

Phage infectivity could be decreased by sunlight, UV light, or other conditions encountered during use. Early work indicated that UV light completely inactivated some single species of phages within minutes (Adams, 1959; Baker & Nanvutty, 1929; Luria & Delbrück, 1942). The present work focused on a mixed phage assemblage that appeared more resistant to UV inactivation under test conditions. The apparent UV resistance (relative to spores) was, however, probably due to phage suspension in UV-absorbing broth (100 μL) during exposure. These phages received a somewhat lower UV dose than the dried bacterial spores. Drying inactivated phages much more rapidly than UV radiation (Figure 1B and durability data, not shown). It was difficult, therefore, to subject dried phages to precisely the same conditions to which spores were exposed.

Soil contains phages that can reduce bacterial production from spores. In preliminary studies of mixed *B. cereus* phage assemblages, 14 of 24 virulent *B. anthracis* strains were killed by phages (M. Longnecker, Johns Hopkins University Applied Physics Laboratory, personal communication, and Walter, unpublished data). This result opens possibilities for treating skin surfaces and the respiratory tract against anthrax with defined phage preparations. Mass preparation of pure, toxin free, concentrated suspensions of phage will require purification, filtration, and other treatments designed to eliminate impurities. Such treatments could potentially lower the infectivity of some phages, but the phage assemblage in this study was generally durable under similar treatments.

Recent research indicates that phage-γ bacterial lysin (PlyG) can lyse vegetative *B. anthracis* cells (independent of whole phage) and may control anthrax disease in mice (Schuch et al., 2002). In addition, PlyG appears to bind tenaciously to vegetative cells, opening possibilities for affinity reagent applications for the enzyme. A therapeutic administration of PlyG would be subject to dilution on surfaces, in the bloodstream, and in lymph because, unlike whole phages, PlyG has no means to replicate and replenish itself in the presence of high levels of host bacteria. Still, this timely development in phage-based approaches to anthrax further underscores the importance of research on phages as antibacterials.

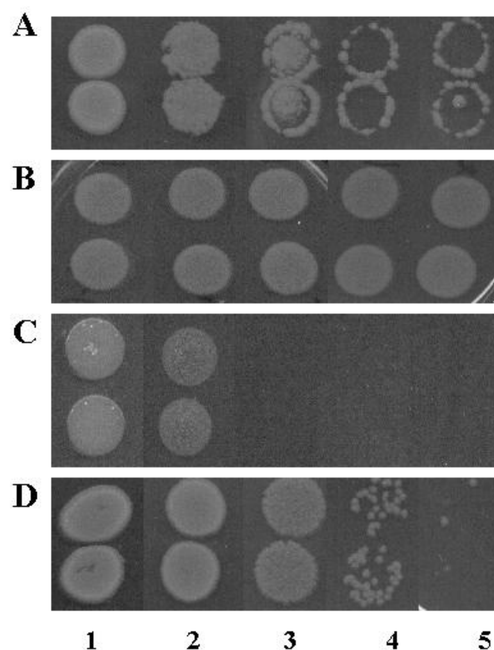
Phages selected for applications in or on humans must remain infectious despite contact with bodily fluids and cells. In addition, internal phage therapeutics must be minimally antigenic in order to circulate for longer periods without being removed by the immune system. Long-circulating enteric phage isolates have been selected through animal systems (Merril et al., 1996). This process requires expensive animal handling that may produce a very narrow selection of phages, possibly unsuited for the final application. Initial screening of a broader array of phages (if available) for durability and effectiveness—before animal testing of single species or combinations of phages—would minimize economic risk.

The 2001 anthrax attacks on the Senate Hart Building and U. S. Postal Service facilities exemplified a situation in which local, contained environmental contamination with anthrax spores posed a direct threat to public health. Similar situations will no doubt recur. The decontamination, prophylaxis, and therapy approaches suggested in this study might best be applied at the very earliest stages of such attacks, before and during evacuation. Future phage-based applications may range from personal-inhalation or topical-spray devices to larger, area spore treatment systems and may be deployed in combination with other technologies before and during evacuation. After evacuation, a broader choice of decontamination methods is available. Phage-based techniques applicable to detection and location of sources of environmental contaminants (anthrax spores) are the subjects of ongoing research. In addition, the author continues to investigate the "species richness" of *B. anthracis* phages in topsoil.

Conclusions

Some topsoil contains assemblages of *B. anthracis* phages that reduce bacterial growth when sprayed on spores at high phage-to-spore ratios. Since phage production and actual deployment against true pathogenic spores are still undocumented, the experiments described herein can only approximate relevant conditions. Under model conditions (and the absence of desiccation), however, inhibition of bacterial growth followed a basic dose-response pattern. Limited, single tests of phage durability suggested that some phages can maintain infectivity under conditions assumed to be similar to those that phages might experience if manufactured in quantity and deployed against anthrax spores on or around humans. Phages therefore may be useful in anthrax therapy or in reducing spore inoculum in combination with vaccines and antibiotics. Phage-based technologies also may be appropriate methods of reducing environmental threats from anthrax spore attacks. The results of this study suggest that some naturally occurring phages may be suitable for such applications.

Figure 1
Bacterial Growth from Spores Sprayed with Phages



The images show representative duplicate subsamples (oriented vertically) typical of bacterial growth from spores sprayed with phages. *Bacillus anthracis* Sterne spores (4.4×10^4 CFU in 10 μL aliquots) were applied on glass slides, sprayed with different concentrations of phages, recovered in NBY. Also shown are 20-μL duplicate subsamples of spores grown on NBY agar plates at 37°C.

A Spores treated with 1) no phage, 2) 3.5×10^7 PFU/mL, 3) 1.4×10^8 PFU/mL, 4) 2.8×10^8 PFU/mL, and 5) 3.5×10^8 PFU/mL of mixed phage lysate.

B Spores treated as in A, but allowed to dry following spraying with phage.

C Spores in various amounts (CFU) were dried on glass slides and sprayed with 1.4×10^8 PFU/mL phages, recovered in NBY, and assayed as in A:

1) 4.4×10^5 CFU of spores,
2) 4.4×10^4 CFU,
3) 4.4×10^3 CFU,
4) 4.4×10^2 CFU, and
5) 4.4×10^1 CFU

D As in C, but without phage treatment.

Figure 2.
Infectivity (Average PFU/mL) of *Bacillus anthracis* Sterne Phages Following Various Treatments.

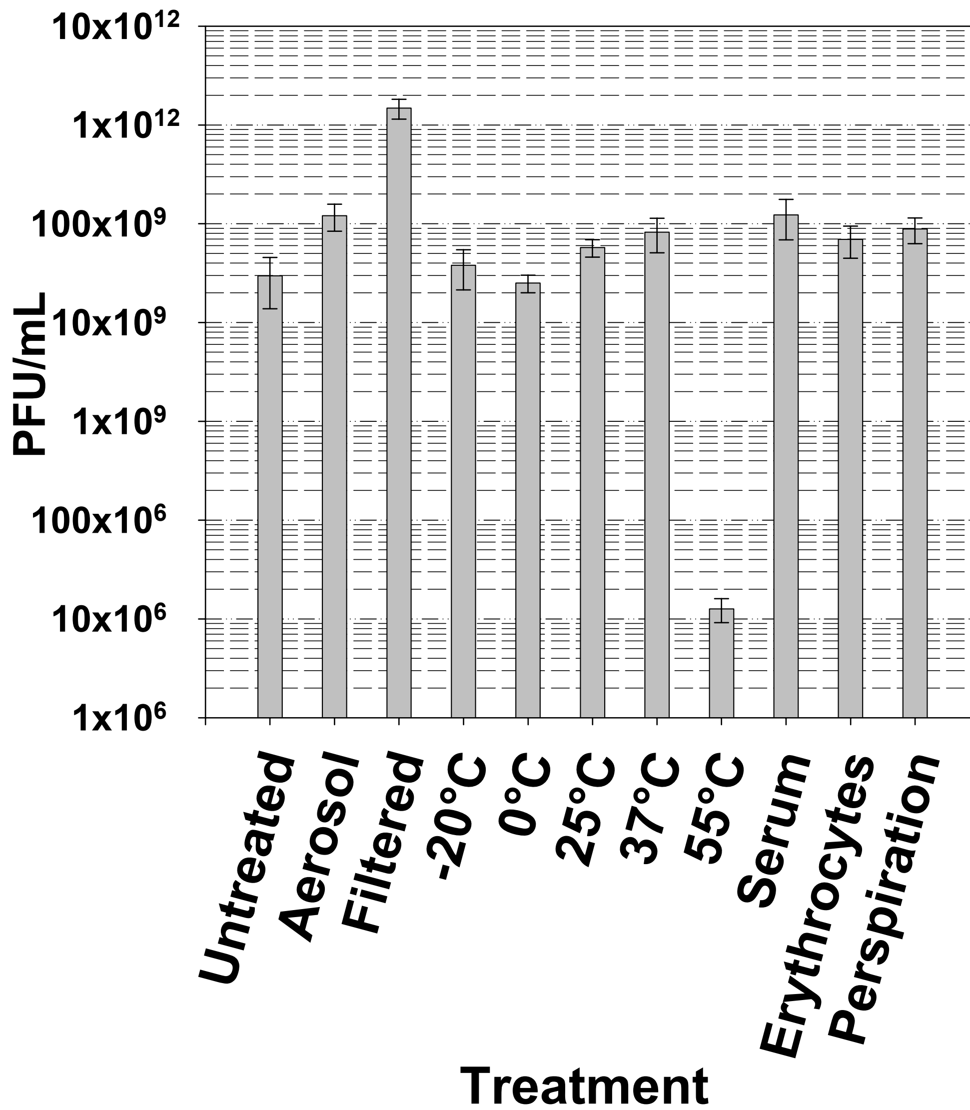
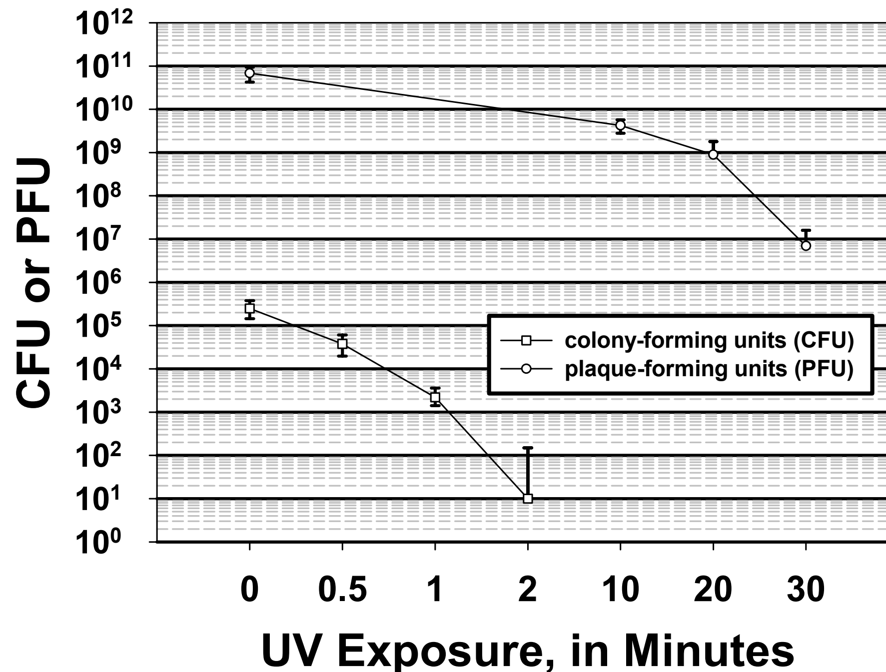


Figure 3. Effect of Ultraviolet Light on Viability of Spores and Phages.



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