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Isolation and Host Range of *Staphylococcus aureus* Bacteriophages
and Use for Decontamination
of Fomites

Kyle C. Jensen

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Master of Science

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ABSTRACT

Isolation and Host Range of *Staphylococcus aureus* Bacteriophages and Use for Decontamination of Fomites

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Master of Science

Staphylococcus aureus is a common bacterium found on the skin and mucosal membranes of about 20% of the population. *S. aureus* growth on the skin is harmless, but if it bypasses the skin it can cause life-threatening diseases such as pneumonia, meningitis, bacteremia, and sepsis. Antibiotic-resistant strains of *S. aureus*, called Methicillin Resistant *S. aureus* (MRSA), are resistant to most antibiotics except vancomycin. However, vancomycin resistant strains of MRSA are becoming more common. In this study, 12 phages were isolated capable of infecting human *S. aureus* and/or MRSA strains. Five phages were discovered through mitomycin C induction of prophages and seven phages were found through enrichment of environmental samples. Primary *S. aureus* strains were also isolated from environmental sources to be used as tools for phage discovery and isolation as well as to examine the target cell host range of the phage isolates. *S. aureus* isolates were tested for susceptibility to oxacillin in order to determine methicillin-resistance. Experiments were performed to assess the host range and killing potential of newly discovered phage. The M1M4 phage had the broadest host range and lysed 12% of the *S. aureus* strains that were tested. The host ranges were reinforced by spectrophotometric assay data which showed a reduction in bacterial optical density of 1.3 OD₆₀₀. The phages were used to decontaminate MRSA from fomites (glass and cloth) and successfully reduced colony forming units by 1-2 logs, including tests of a phage cocktail against a cocktail of MRSA isolates. Our findings suggest that phage treatment can be used as an effective tool to decontaminate human MRSA from both hard surfaces and fabrics.

Key words: *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, bacteriophage, phage therapy

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Introduction

Staphylococcus aureus infections are the most frequent hospital-acquired infections reported in developed countries [2]. *S. aureus* is a gram positive coccus commonly found colonizing the skin and mucosal membranes of humans and many livestock species [3, 4]. *S. aureus* is very specialized in its ability to colonize and infect humans with an average of 38 different proteins specifically for host innate immune evasion [5]. These immune evasion mechanisms allow *S. aureus* to be more opportunistically effective as a pathogen. Methicillin-Resistant *S. aureus* (MRSA) is a variant of *S. aureus* resistant to methicillin and is responsible for more than 50% of *S. aureus* infections in European intensive care units [6]. Recently MRSA strains have become more common in community settings and more difficult to treat due to increasing levels of multiple-antibiotic resistance [6-8].

Bacteriophages (phages) are viruses capable of infection and replication in bacterial cells. Phages are the most abundant biological entity found on the planet and as such represent a huge portion of the world's genetic diversity [9, 10]. They also represent a huge—mostly untapped—source of new genes and protein products with potential use as medical treatments [11]. Phages discovered in 1915 were quickly commercialized and used for medical phage therapy. Early phage therapy was fraught with many problems: purifying phage treatments, exaggerated healing claims, and a failure to scientifically prove efficacy [12]. These problems, plus a lack of funding during World War II and the invention of the easily usable antibiotic penicillin, caused most scientists to abandon phage research in favor of pursuing new antibiotics. Recent increases in multi-antibiotic-resistant bacterial infections have begun to renew interest in phage therapy [13].

The purpose of this introduction is to provide a foundation for the significance *S. aureus* plays in nosocomial infections, community-associated colonizations, and the difficulties in treating MRSA infections. It will also introduce phages as a potential source of bactericidal treatments for MRSA-contaminated fomites.

Staphylococcus aureus

S. aureus colonizes 20% of the human population, and can persist anywhere between 70 days to more than 8 years [14, 15]. *S. aureus* growth on the skin and mucous membranes is harmless, but if it bypasses the skin it can cause life-threatening diseases such as skin and soft tissue infections, bacteremia, sepsis and endocarditis [16]. Immune evasion mechanisms such as protein A inactivate immunoglobulin by binding to the Fc γ domain and thus disable complement fixation, and enable *S. aureus* to become more virulent and fit [17]. *S. aureus* bacteremia mortality rate is 43% [16, 18]. *S. aureus* infections are the most commonly acquired nosocomial infections [19]. These infections can occur in many different tissue types including bones, joints, blood, lungs, heart, and brain. Those who are immunocompromised are more susceptible to these dangerous infections [20]. In 2005, approximately 11,000 deaths were attributed to *S. aureus* in the United States [21].

Livestock are also commonly colonized and infected with *S. aureus* [7, 22-24]. Bovine mastitis in cattle is the most well-known disease caused by *S. aureus*, infecting the udder and inducing inflammation [23]. The indirect cost of bovine mastitis is difficult to calculate, but direct costs in the U.S. are estimated at \$1.7-2 billion [25, 26]. *S. aureus* also causes disease in poultry such as bacterial chondronecrosis, which causes lameness in chickens [27]. Studies have shown that *S. aureus* jumped from humans to poultry approximately 38 years ago and has since become a near ubiquitous in poultry flora [27]. During the butchery process *S. aureus* flora are

released contaminating the raw poultry. For instance, 77% of raw turkey and 41% of raw chicken is contaminated with these bacteria [28]. Handling contaminated raw meat is another route of transmission for *S. aureus* to enter the community [29]. Livestock-associated *S. aureus* strains have been shown to move from animals to humans. This is exemplified by MRSA sequence type 398 which moved from pigs to humans in the early 2000s and now colonizes many humans worldwide [23, 24].

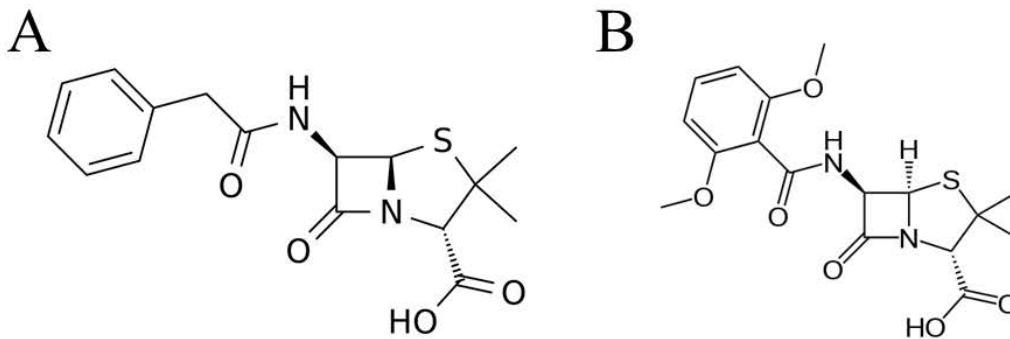


Figure 1: Structure of penicillin and methicillin

Penicillin (A) and methicillin (B) are both β -lactam antibiotics. Here the structure of both is shown. The active region on both molecules is the β -lactam ring which competitively binds to DD-transpeptidase and is the site where penicillinase cleaves penicillin.

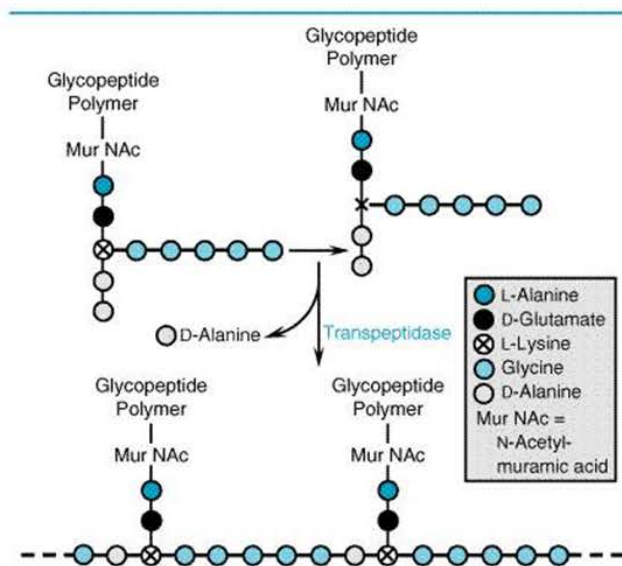


Figure 2: Transpeptidase catalysis of peptidoglycan

DD-transpeptidase assists in synthesizing new peptidoglycan by crosslinking the enzyme-OH to the D-Ala-D-Ala end of the peptidoglycan chain releasing one of the D-Ala residues. The enzyme then covalently binds the peptidoglycan chain with an adjacent chain at the Gly residue and releases the enzyme. β -lactam antibiotics mimic the D-Ala-D-Ala region of peptidoglycan and competitively bind DD-transpeptidase.

Methicillin-Resistant Staphylococcus aureus

β -lactam antibiotics function through the binding of the four-membered β -lactam ring to the bacterial enzyme DD-transpeptidase [30]. DD-transpeptidase assists in synthesizing new peptidoglycan by crosslinking the enzyme-OH to the D-Ala-D-Ala end of the peptidoglycan chain releasing one of the D-Ala residues [31]. The enzyme then covalently binds the peptidoglycan chain with an adjacent chain at the Gly residue and releases the enzyme (Fig. 2). β -lactam antibiotics mimic the D-Ala-D-Ala region of peptidoglycan, and competitively bind DD-transpeptidase (Fig. 1A) [30, 31]. Cell death occurs because of an imbalance between cell wall production and natural degradation. Penicillinases are enzymes which confer penicillin-resistance. The enzyme cleaves the β -lactam ring of β -lactam antibiotics thus preventing cell death [32]. Methicillin is a penicillinase-resistant β -lactam antibiotic created in response to penicillin-resistance [32]. Methicillin has a similar mode of action as other β -lactams, but is unaffected by penicillinases because of stoichiometric interference (Fig 1B) [33].

Methicillin-resistant isolates of *S. aureus* were isolated in British hospitals in 1959, only two years following the introduction of methicillin [34]. Methicillin-resistant *S. aureus* (MRSA) resists methicillin by employing a new transpeptidase with a low affinity for penicillinase-resistant β -lactams [35]. The *mecA* gene encodes for the new transpeptidase, commonly known as Penicillin-Binding Protein 2A (PBP2A). PBP2A replaces the function of the original transpeptidase and, because PBP2A has low affinity for β -lactams, it is unaffected by methicillin [36]. The original copy of the *mecA* gene is hypothesized to have come from *Staphylococcus fleurettii*, which contains a chromosomal copy of *mecA*, but has none of the other genes usually associated with *S. aureus* *mecA* [37].

In addition to methicillin-resistance, many MRSA strains are resistant to nafcillin, tobramycin, gentamicin, amikacin, ceftiofur, clindamycin, erythromycin, and chloramphenicol [38-40]. In the past antibiotic resistance was not considered a significant problem because new antibiotics were introduced to counter resistant bacterial strains. After the initial flurry of antibiotic discovery in the 1960s, new antibiotic derivatives have been slow in their creation, and almost no new antibiotics with novel mechanism have been discovered [41]. The lack of novel antibiotics has increased the difficulty in treating multi-antibiotic bacterial strains [42].

MRSA easily moves between different reservoirs causing community-associated MRSA (CA-MRSA) and hospital-associated-MRSA (HA-MRSA) interchange to occur often (Fig. 3) [24]. MRSA isolates becoming resistant to antibiotics have been shown to occur in both the hospital and on livestock farms [43-45]. In both settings, MRSA antibiotic resistances can quickly spread. MRSA colonizing livestock can transfer to the community through farm workers working closely with the animals, or through butchers working with raw meat contaminated with MRSA [46, 47]. Once in the community, MRSA can cause infections which often leading to hospitalization and potentially introducing the new strain to other patients as a nosocomial infection [48]. CA-MRSA infections are quickly increasing and changing the dynamics of MRSA infections [48]. This changing dynamic is alarming because of the few new antibiotics created to stop these infections. As an alternative to discovering new antibiotics, many scientists have begun investigating phages as a medical treatment for MRSA [49].

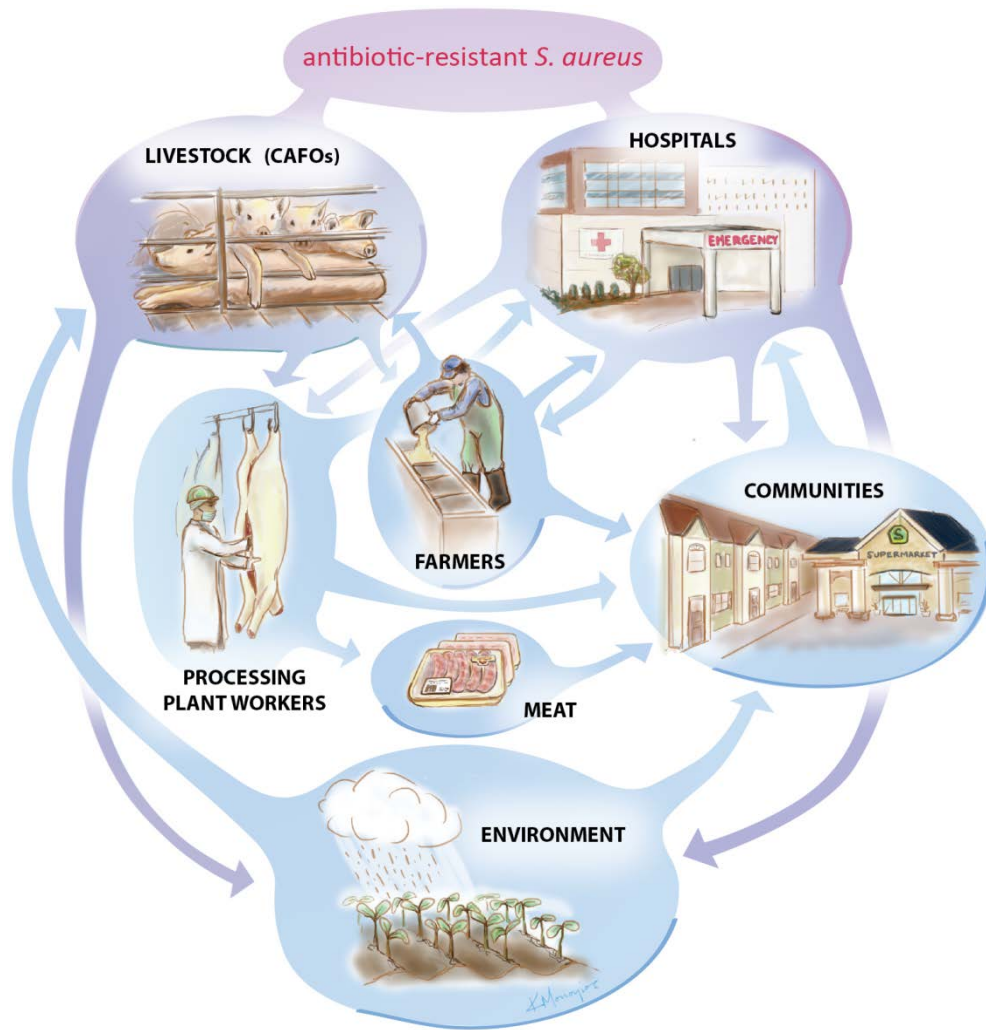


Figure 3 *MRSA Routes of Transmission*

MRSA is uniquely able to colonize many different host organisms. MRSA strains often transfer from one organism to another. CA-MRSA can easily move from farms to the community via raw meat and farm workers. From the community MRSA is then easily introduced into the hospitals. The movement of MRSA is dynamic and can flow in nearly every direction [18].

Between the years of 2003 to 2008, MRSA deaths in the U.S. doubled, outnumbering the combined mortality rate from HIV-positive and influenza hospital patients [50]. In 2011, 80,461 people in the United States were diagnosed with a MRSA infection and about 19,000 died due to the infection [51]. Despite the increasing difficulty in treating MRSA infections, hospitals have increased their efforts at controlling MRSA, decreasing the overall rates of MRSA in the United States and in Europe decreasing [6, 52]. Despite nosocomial infection decreases CA-MRSA infections are becoming increasingly more common outside the hospital [48]. CA-MRSA have distinct lineages from HA-MRSA and can be differentiated from nosocomial infections [48]. USA300 and USA400 are the most common CA-MRSA strains in the U.S. [53]. HA-MRSA and CA-MRSA infections are increasingly being treated with vancomycin. The increased use of vancomycin raises concerns that vancomycin-resistance will be increasingly selected for creating vancomycin-resistant *S. aureus* (VRSA) more often [54]. Vancomycin is one of a few antibiotics available to treat MRSA. Despite worldwide distribution of VRSA, isolates are still rare [55, 56].

Fomite contamination in hospitals is a large source of nosocomial infections [57]. MRSA from serum contaminating surfaces is detectable for 41 days on glass, 45 days on tile, and greater than 60 days on countertops [58]. *S. aureus* remained viable on cleaning cloths for 24 hours and were transferred to objects it touched [59]. In the community MRSA regularly moves from person to person, or fomite to person. Everyday items, such as computers and basketballs, act as a fomite reservoir for MRSA with 2 of 24 public computer keyboards being contaminated with MRSA [60]. The use of a cleansing wipes to decontaminate a fomite does not guarantee sterility. Fomites inoculated with 10^9 CFUs *S. aureus* and cleaned with disinfecting wipes only reduced bacterial CFUs by 4.5 logs leaving 10^5 viable *S. aureus* cells, enough to cause infection [61].

Bacteriophages

Estimates place the population of phage worldwide at about 10^{31} particles [10]. Phages are viruses capable of infecting bacteria, and as such are obligate parasites using the cellular machinery to create new phage particles [12, 62]. Phages use the host machinery to either produce a lytic or a lysogenic infection. Lytic infections immediately move to phage replication and lysis of the bacterial cell. Phage induced cell lysis represent a significant force on bacterial populations being implicated in approximately 20-90% of bacterial deaths [63]. Phage infections can follow one of two paths. Virulent infections immediately move to phage participle replication and lysis of the host cell. Temperate phages integrate the phage genome into the host chromosome creating a prophage. It then waits until conditions are right to resume a lytic infection and escape from the host. Of the two types of phage infection lytic phage are more efficient for medical treatments due to their immediate lysing of bacterial cells [64].

Phages were first discovered around the turn of the 20th century and initially studied for their anti-bacterial capabilities. D'Herelle was one of the first scientists to use phages as an antimicrobial. He administered phages to a boy with dysentery and within 24 hours the boy began to recover [65]. In addition, d'Herelle administered phage to three more patients with dysentery, all who subsequently began to recover. However, before d'Herelle published his experiments regarding phage treatments, Richard Bruynoghe and Joseph Maisin used phage to successfully treat a patient with a staphylococcal skin infection, somewhat obscuring d'Herelle's work at the time [12]. With the discovery of penicillin, and subsequent antibiotics, Western science turned away from phage therapy largely in favor of the more convenient antibiotics [12]. Despite Western science's abandonment of phage therapies, Eastern European scientists

continued using medical phage therapy [66, 67]. With the emergence of multi-antibiotic-resistant bacteria Western scientists have finally begun to renew their interest in phage [13, 68, 69].

One of the greatest advantages offered by phages is the coevolution between phages and host bacteria, which allows phages to infect resistant host strains [70, 71]. Werts et al. used phage lambda to show phage/*E. coli* coevolution. Phage lambda's protein J uses the *E. coli* cell receptor protein LamB to attach. Treating *E. coli* with phage lambda increased selective pressure for *lamB* mutations in order for *E. coli* to escape phage attachment. In turn this introduces selective pressure for lambda protein J mutants to coevolve and bind to the new LamB receptor [72]. Additional studies have shown that bacteria can quickly become resistant to current populations of phages. Phages then face increased selection for phage mutants with infectivity for the new strain of bacteria, and have been shown to increase infectivity to all past strains of the bacterium [73].

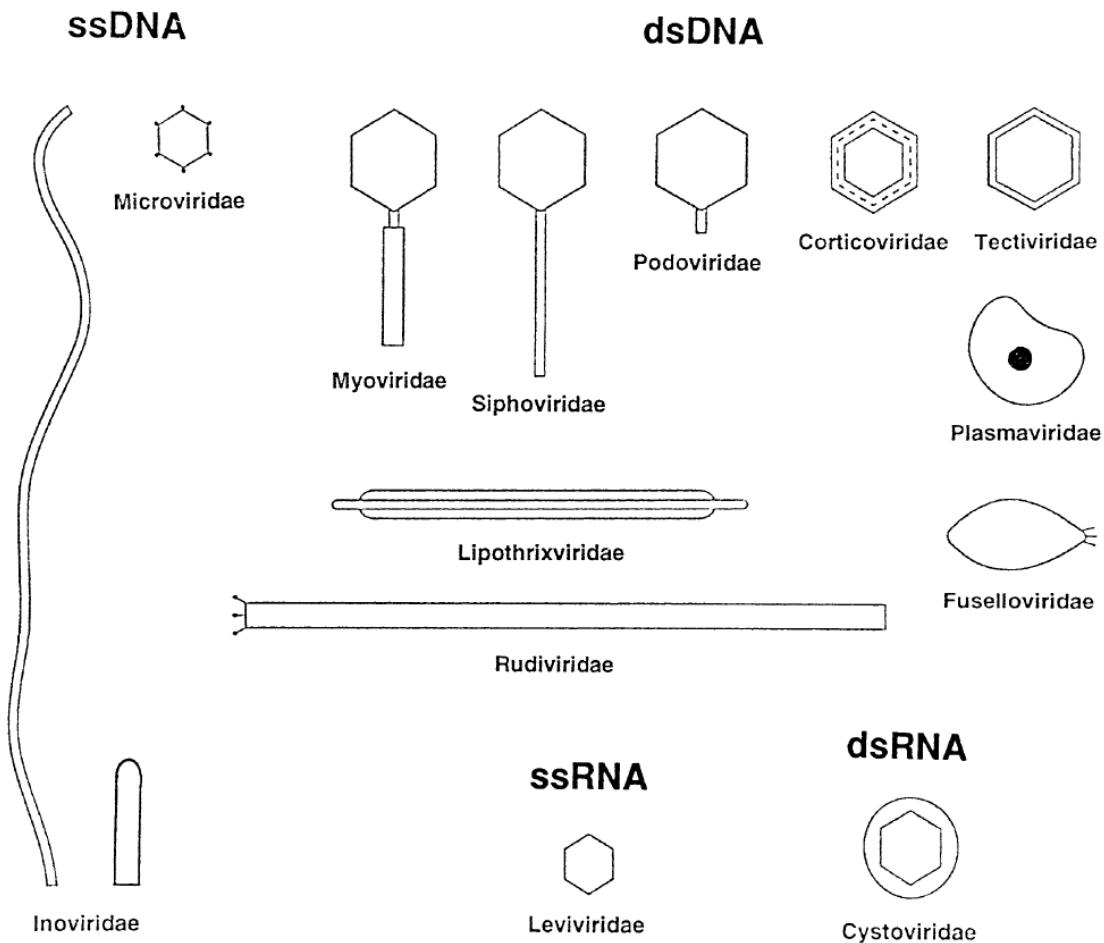


Figure 4 Phage Morphology

Phage morphology comes in many unique varieties. *S. aureus* phages are usually one of three classes: Myoviridae, Siphoviridae, and Podoviridae. As such most are also double stranded DNA phage. It is thought the most clinically relevant strains of phage are the Myoviridae class because of their inability to enter the lysogenic cycle. Image from H.-W. Ackermann's review [1]

MRSA Bacteriophage

Phages have many different morphological types. Their genomes are usually composed of dsDNA but occasionally are ssDNA, ssRNA, or dsRNA [1]. Though there are more phage categories, *S. aureus* phages are generally separated into one of three different dsDNA classes: Podoviridae, Siphoviridae, and Myoviridae (Fig. 4). Podoviridae have short tails and genomes of approximately 20kb. Siphoviridae are non-contractile tailed phage and have genomes of approximately 50kb. Myoviridae class phages have contractile tails consisting of a sheath and a central tube and genomes of greater than 125 kb. Myoviridae *S. aureus* phages are also obligate virulent and are hypothesized to be more useful for medical phage therapy [1, 74].

There are several obstacles to overcome before medical phage therapy can become a viable treatment. Those obstacles include limited phage host range, possible carriage of bacterial virulence genes, temperate phage induced protection of the bacterial cell from other phage lysing the cell and immunogenicity. Phage host range is defined as the number of bacterial host strains the phage infects. Phage host ranges vary dramatically between phage strains. Medical phage therapy can adapt to limited host range by using a cocktail of phage with a combined larger host range. A phage's host range can be increased through passaging in the presence of resistant bacterial strains [75, 76]. After successive rounds of replication, mutant phages with infectivity towards the resistant bacterial strain are selected. Temperate phages present multiple problems due to their ability to enter the lysogenic cycle. During this cycle the phage integrates its genome into the host DNA, often introducing virulence factors and phage resistance mechanisms into the host bacterium [77, 78]. Many virulence factors attributed to *S. aureus* are actually phage encoded, such as Panton-Valentine leukocidin, staphylokinase, enterotoxin A and Toxic Shock Syndrome Toxin 1 [79]. Virulence factors benefit both the *S. aureus* host and phage by

increasing the fitness of *S. aureus* inside a human host. In return the prophage multiplies along with its bacterial host. Lysogens also increase host fitness by introducing phage-resistance to the prophage. These mechanisms are classified as abortive infection, adsorption inhibition, injection blocking, or restriction/modification systems [78]. Phage antibacterial effects are also dramatically affected by antibodies, which cause a loss of antibacterial effect [80]. Phage immunogenicity is an important issue which dictates the success or failure of phage therapy [81]. In addition, natural bacterial flora contributes to phage immunogenicity because antibodies are created as a result of contact with natural phages which infect the natural flora. This natural vaccine creates antibodies against phages which may be used in phage therapy, limiting the therapy's efficacy [82]. In fact, phage immunogenicity is being taken advantage of in current research using phage as a vehicle for HIV proteins in an attempt to create a HIV vaccine [83]. Despite the enormous obstacles to overcome, phage therapy has been successfully used by Eastern European countries and is still used with great success today [84].

S. aureus can remain on a hard surface for three months and on for fabric three weeks [85]. Hard surfaces can easily transmit *S. aureus* to skin for at least 70 days post inoculation [86]. Effective reductions in *S. aureus* nosocomial infections require hospitals to carefully manage fomite decontamination. Phages are a natural decontaminator; therefore several studies have used phages to quantify phage-mediated fomite decontamination. Decontamination using phage was shown to be viable in two studies attempting to remove *Yersinia pestis* or *Listeria monocytogenes* from hard surface fomites. Each study showed bacterial load reductions of between 3.5-5 logs [87, 88].

The purpose of this research was to isolate phages which can be used to reduce MRSA loads on fomites. With this in mind, a three aim plan was created to accomplish this goal: 1) to

isolate phages and purify them into single strains; 2) Identify the host range and lytic efficiency of each of the phages; and 3) to perform experiments testing decontamination efficacy of the phages. Herein is described the isolation of 12 phages with lytic activity against MSSA and MRSA isolates. The M1M4 phage had the broadest host range and lysed 12% of the *S. aureus* strains it was tested against. Spectrophotometry assay results reinforced the host range results and showed reductions of cellular densities of about 1.3 OD₆₀₀ for most phage tested. Decontamination of fomites (glass and cloth) yielded between one and two log reductions in MRSA colony forming units.

Materials and Methods

Media

Bacterial cultures were grown in LB, LB broth supplemented with magnesium and calcium (LB-MC), LB-MC top agar, or Mannitol Salt Agar. LB broth contained the following: 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% 2 N NaOH and was sterilized by autoclaving for one hour. LB agar was prepared as LB broth above with the addition of 1.2% agar. LB-MC was prepared similar to the LB broth above. However, after autoclaving sterile 4mM MgCl₂ and 4mM CaCl₂ was added. LB-MC top agar was prepared as LB agar with a reduction of agar to 0.4% and supplemented with sterile 4mM MgCl₂ and 4mM CaCl₂.

Mannitol Salt Agar was purchased from Fluka Analytical and contained 1.0% D-mannitol, 0.1% meat extract, 1.0% peptone, 0.0025% phenol red, 7.5% NaCl, and 1.5% agar and was sterilized by autoclaving for one hour.

Phage Buffer was used to for storage of phage particles. It contained 500ml ddH₂O, (100mM) 2.92 g NaCl, (10mM) 1.016g MgCl₂·6H₂O, (50mM) 3.94 g Tris-HCl, (0.01%) 50 mg gelatin. pH was adjusted to 7.5 and the phage buffer was autoclaved for one hour.

S. aureus and Methicillin-Resistant S. aureus Isolation

Samples were taken from locations commonly associated with *S. aureus*: athletic facilities, hospitals, nasal swabs, chicken coups, raw meat, and from collaborators. *S. aureus* was selected for by adding equal an amount 2X LB broth to wet samples and suspending dry samples in LB broth and thoroughly vortexing to remove *S. aureus* cells. A MSA plate was spotted with 10μL of a sample, streaked to isolation and incubated at 37°C for 48h. Fermentation positive colonies

were further tested by Gram stain, catalase testing, and coagulase tube tests to confirm isolation of *S. aureus* (Gram positive cocci which is positive for catalase, coagulase and mannitol fermentation). Methicillin-resistance was determined by plating on MSA plates with 2µg/mL oxacillin, followed by incubation at 37°C for 48h (note that oxacillin-resistance is considered to be equivalent to methicillin-resistance in many studies; [89, 90]). Additional strains were acquired from the American Type Culture Collection (Manassas, VA, USA) and from BEI Resources (Manassas, VA) (table 1).

Phage Isolation

Temperate Phage Induction

S. aureus was inoculated into LB broth and grown overnight at 37°C at 200rpm. The overnight culture was used to inoculate four hour cultures *S. aureus* sub-cultures grown for 30 min at 37°C at 200rpm (insuring log phase growth), followed by exposure to mitomycin C (Sigma-Aldrich) at 0.5µg/mL for 8h at 37°C. Cells were pelleted by centrifugation at 3000xg for 10 min and supernatants passed through a 0.45µm filter and stored at 4°C until plaque assays were performed. 100µL of phage sample was added to 100µL of bacteria and incubated overnight at 37°C before adding to molten LB top agar for plaque production. To purify the phage into single strains isolated plaques were picked and crushed to release phage particles and re-inoculated onto *S. aureus*. Positive plaque forming samples were repicked and overlaid three successive rounds to purify phage into single strain isolates. The bacterial strain used to isolate each phage is indicated in Table 2.

Virulent Phage Isolation

Environmental samples were obtained and stored at 4°C until phage enrichment could be performed. LB broth was added to dry samples and thoroughly vortexed to dislodge phage particles. An equal volume of 2x LB broth was added to liquid samples. All samples were then filtered using 0.45µm filters to remove bacteria. Five different strains of *S. aureus* four hour cultures were combined: M1, M5, *S. aureus* 29213, DH1 and HA1 (see Table 1), to which the filtrate was added. The enrichment samples were incubated overnight at 37°C shaking at 60rpm. The overnight enrichment was centrifuged at 5,000xg for 12 min to pellet bacteria, and then filtered using a 0.45µm filter. A bacterial overlay was created by mixing 100µl four hour *S. aureus* culture into 3ml molten LB-MC top agar and poured over a LB agar plate. Once solidified 10µl of enrichment was spotted on the overlay and dried. The overlay was incubated overnight at 37°C. Each overlay was inspected for plaque formation in the area where samples were spotted. All plaques were picked and mixed in 3ml molten LB-MC top agar with 100µl *S. aureus* and overlaid on LB agar. Positive plaque forming samples were repicked and overlaid three successive rounds to purify phage into single strain isolates. The bacterial strain used to isolate each phage is indicated in Table 2.

High Titer Phage Lysates

High titer phage lysates were prepared by adding 100µl host bacteria and varying amounts of phage lysate into 3ml molten LB top agar and overlaid onto LB agar plates. Overlays with near complete lysis, or a webbed plaque distribution, were treated with 4ml of phage buffer and the top agar overlay was crushed, followed by 90 min incubation at room temperature. Phage buffer was removed and centrifuged at 5,000rpm for 10 min and filtered at 0.45µm to remove bacterial

cells, then stored at 4°C with chloroform. Phage stocks were tittered by serial dilution and plating similarly described above.

Host range

Spot Test

Overnight cultures were prepared in LB broth and then sub-cultured by addition of 100µL to 3ml LB broth and grown at 37°C for 90 min. 100µL of sub-culture was inoculated into 3mL of molten LB top agar and overlaid onto LB agar plates. Each overlay was allowed to solidify for 15min. All phage lysates (original titers approximately 10⁸pfu/mL) were diluted in 10 fold increments and 10µL of each dilution was spotted onto the bacterial overlay [91], dried, then incubated at 37°C overnight. As a control, each bacterial strain was also mock infected with sterile phage buffer. Results were analyzed based on detection of any lysis and further dilutions were checked for single plaques to ensure phage lysis rather than bacteriocin induced lysis. All spot tests were repeated in triplicate to confirm results.

Spectrophotometric Assays

500µl of *S. aureus* overnight culture was used to inoculate 3ml LB-MC broth and incubated at 37°C with 200rpm shaking, until the culture reached an optical density (O.D.) of 1.40, between 2.5-3h post inoculation. A high titer phage lysate was diluted with phage buffer to a concentration of 10⁸ pfu/ml. 100µl phage and 20µl bacteria were inoculated into 3.5ml LB broth. For use as a control a parallel mock treated run was also performed using sterile phage buffer. Samples were removed at 2, 3, and 4 hours post infection and an OD₆₀₀ was measured (Ultraspec 10 spectrophotometer, Amersham Biosciences, Piscataway, NJ, USA) using sterile LB broth as a blank. All experiments and mock treatments were run in triplicate.

Decontamination Assays

Cloth decontamination

To mimic the condition where nosocomial infections may occur, we used lab coat material composed of 35% cotton and 65% polyester as a fomite. 1.5 x 1.5cm pieces of lab coat material were cut and autoclaved to achieve sterility. Four hour sub-cultured MRSA samples were diluted 1:10⁴ and 100μL was inoculated onto the lab coat and allowed to remain for 30 min at 37°C. The initial bacterial load inoculated and subsequently recovered from untreated cloth was ~1-5x10⁶ Colony Forming Units (CFU). 100μL of phage lysate was then added and incubated at 37°C for 30min. Phage titers added to the cloth ranged from 1x10⁷ to 1x10⁸ PFU for a range of multiplicity of infection of 200 to 50,000. As a control, sterile phage buffer instead of phage lysate was added as a mock treatment. Viable bacteria were removed by placing the cloth into 500μL LB broth followed by vortexing at high speed for 10s. 10μl of the broth was serially diluted, then another 10μl was spotted onto an LB agar plate and incubated at 37°C overnight; colonies were counted the next morning. Colony-forming units were calculated for both mock-treated and phage treated. Mock-treated bacterial loads were then divided by phage-treated in order to determine the decontamination capability of the phage. These assays were performed in triplicate.

Glass coverslip decontamination

To mimic conditions possibly more suitable to phage decontamination we performed decontamination assays using glass coverslips. 22 x 22 mm glass coverslips were used to test decontamination on hard surfaces. 10μL of sterile milk was spread onto the sterile coverslip surface and dried, giving the coverslips a better surface for bacterial adherence [88]. The test MRSA strain was sub-cultured for 4h at 37°C, with 200rpm shaking, to achieve logarithmic

growth and then diluted 1:10³, giving a final concentration of approximately 10⁶ cfu/mL. 10µL of the cell culture was spread onto the coverslips and incubate at room temperature for 30 min. Coverslips were then treated with 100µL of phage lysate at a multiplicity of infection of 200 to 50,000 and incubated at room temperature for 30min. As a control, sterile phage buffer instead of phage lysate was added as a mock treatment. Viable bacteria were removed by placing the coverslip into 500µL LB broth followed by vortexing at high speed for 10 seconds. 10µl of the broth was serially diluted, then another 10µl was spotted onto an LB agar plate and incubated at 37°C overnight; colonies were counted the next morning. Colony-forming units were calculated for both mock-treated and phage treated. Mock-treated bacterial loads were then divided by phage-treated in order to determine the decontamination capability of the phage. These assays were performed in triplicate.

Results

Isolation and Host Range of Bacteriophage with Lytic Activity against Methicillin-Resistant *Staphylococcus aureus* and Potential use as a Fomite Decontaminant

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In lieu of the results section I have included a first author publication containing the results of the previously described experiments. The publication was accepted to PLoS One on June 9, 2015.

Abstract

Staphylococcus aureus is a commensal bacterium and opportunistic pathogen commonly associated with humans and is capable of causing serious disease and death including sepsis, pneumonia, and meningitis. Methicillin-resistant *S. aureus* (MRSA) isolates are typically resistant to many available antibiotics with the common exception of vancomycin. The presence of vancomycin resistance in some *S. aureus* isolates combined with the current heavy use of vancomycin to treat MRSA infections indicates that MRSA may achieve broad resistance to vancomycin in the near future. New MRSA treatments are clearly needed. Bacteriophages (phages) are viruses that infect bacteria, commonly resulting in death of the host bacterial cell. Phage therapy entails the use of phage to treat or prevent bacterial infections. In this study, 12 phages were isolated that can replicate in human *S. aureus* and/or MRSA isolates as a potential way to control these infections. 5 phages were discovered through mitomycin C induction of prophage and 7 others as extracellular viruses. Primary *S. aureus* strains were also isolated from environmental sources to be used as tools for phage discovery and isolation as well as to examine the target cell host range of the phage isolates by spot testing. Primary isolates

were tested for susceptibility to oxacillin in order to determine which were MRSA. Experiments were performed to assess the host range and killing potential of newly discovered phage, and significant reductions in bacterial load were detected. We explored the utility of some phage to decontaminate fomites (glass and cloth) and found a significant reduction in CFUs of MRSA following phage treatment, including tests of a phage cocktail against a cocktail of MRSA isolates. Our findings suggest that phage treatment can be used as an effective tool to decontaminate human MRSA from both hard surfaces and fabrics.

Introduction

Staphylococcus aureus infections are the most frequent type of hospital-acquired infections reported in developed countries [2]. *S. aureus* is a common commensal bacterium capable of colonizing the nose and skin and is found transiently in ~50% of the human population and ~20% permanently [3, 92]. Nasal colonization has been linked to surgical site infections [93] and *S. aureus* can cause life-threatening diseases in many different tissue types including bones, joints, blood, lungs, heart, and brain [16]. *S. aureus* is the bacterium most commonly associated with bloodstream, soft tissue, lung and skin infections [94]. Many of these infections are treated using antibiotics; however, bacterial evolution has resulted in strains of *S. aureus* resistant to multiple antibiotics.

Methicillin Resistant *Staphylococcus aureus* (MRSA) represents a group of *S. aureus* isolates commonly resistant to methicillin as well as erythromycin, levofloxacin, tetracycline, clindamycin, mupirocin, gentamicin, trimethoprim, and/or doxycycline but is typically susceptible to vancomycin [90]. Serious MRSA infections are increasingly difficult to treat using current antibiotics [95]. While MRSA infections rates have recently trended downwards,

community acquired MRSA infections are now more common requiring treatment using antibiotics such as vancomycin [51, 96]. The concern is that vancomycin resistance seen in other bacteria (including some *S. aureus* isolates) may be acquired by MRSA, thus leaving clinicians without any viable treatment options [97]. In April 2014 Rossi et al. highlighted this problem when they reported a case of MRSA resistant to vancomycin in Brazil [98]. There is a valid concern that vancomycin-resistant MRSA could become predominant in the near future and such infections may be untreatable. In 2012, there were an estimated 75,309 cases of invasive MRSA with 9,670 resulting in deaths within the United States [99]. A 2011 study estimated that the case fatality rate of invasive MRSA in the United States was about 25% [51] and another showed that *S. aureus* bacteremia in New York City had a 30% mortality rate from 2002-2007 [18]. Researchers seeking new treatments for antibiotic-resistant bacteria have increasingly begun to look towards bacteriophage as a viable option in treating these infections, either in tandem with or as a replacement for antibiotics [64, 100].

Bacteriophages (phages) are viruses capable of infection and replication in bacterial cells. Phages are the most common organism found on the planet and as such represent great diversity in their overall host range [9, 10]. Since virus infectivity requires binding to a specific receptor, phage are specific for a small host range and are thus unable to infect human cells. Thus, the side effects associated with phage therapy of eukaryotic hosts are thought to be minimal [12]. The idea of using phage as a potential therapeutic tool has been around for as long as phage have been known to exist [12, 101] though some eastern European countries continued using phage as medical treatments and in some countries physicians still regularly practice phage therapy [66, 67]. Phage were used in the early 1900s to treat bacterial infections, but phage treatment was largely abandoned in favor of antibiotics in the 1940s [102]. Although bacteria

can evolve to escape from phage-mediated killing, the use of a biological agent such as phage allows for evolution to also work in favor of phage re-acquiring the ability to lyse target cells [72, 100, 103]. Thus, it is thought that phage therapy could be superior to antibiotic therapy in terms of the ability of the treatment to evolve in response to the development of resistance by the target bacterium. Off-target effects of antibiotic therapy can have detrimental effects on non-pathogenic normal flora, but such effects are expected to be minimal with phage therapy [12].

In this report, we describe the isolation of 12 phages with lytic activity towards human MSSA/MRSA isolates. Virulent and temperate phages were found, isolated and purified using MRSA strains as hosts. We analyzed the lytic host range and lytic ability of each phage using spot tests and lytic culture assays of a panel of *S. aureus* and MRSA cultures isolated from various human, livestock and environmental locations. In order to demonstrate the efficacy of our phage for clearing MRSA, we used our new phage to decontaminate MRSA from fomites and found a significant reduction in MRSA load from both a glass surface as well as fabric, which could be associated with nosocomial transmission. Further, we found significant reduction of MRSA loads when mixtures of MRSA isolates were treated with either single phage or with phage cocktails. Our results suggest that phage can be used as an effective way to decontaminate materials contaminated with MRSA.

Materials and Methods

Isolation of Staphylococcus aureus strains

Bacterial strains were isolated on Mannitol Salt agar (MSA) plates (Fluka Analytical). Plaque assays and spot tests for host range were performed on Luria-Bertani (LB)

agar (10g tryptone, 5g yeast extract, 5g NaCl, 1mL 2N NaOH, 12g/L agar). LB broth contained 10g tryptone, 5g yeast extract, 5g NaCl, and 1mL 2N NaOH per liter. Top LB agar contained 4g/L agar, supplemented with 4mM MgCl₂ and 4mM CaCl₂. Phage buffer was made with 100mM NaCl, 10mM MgCl₂, 50mM Tris-HCl, and 0.01% gelatin (pH 7.5).

Some strains were acquired from the American Type Culture Collection (Manassas, VA, USA) and others from BEI Resources (Manassas, VA). Additional strains of *S. aureus* were obtained from athletic facilities, hospitals, nasal swabs, environmental sampling, and from collaborators. To select for *S. aureus*, each sample was suspended in LB broth and thoroughly vortexed. 10µL of each sample was spotted onto a MSA plate, streaked to isolation and incubated at 37°C for 48h. Gram-staining, catalase and coagulase tube tests were performed on single colonies isolated on MSA plates to confirm *S. aureus* (gram positive cocci positive for catalase, coagulase and mannitol fermentation). Methicillin resistance was determined by plating on MSA plates with 2 µg/mL oxacillin, followed by incubation at 37°C for 48h (note that oxacillin resistance is considered to be equivalent to methicillin resistance in many studies; [89, 90]).

Isolation of bacteriophage

Virulent phage isolation

Samples were obtained from the environment and stored at 4°C until phage enrichment. LB broth was added to dry samples and thoroughly vortexed to dislodge phage particles. An equal volume of 2x LB broth was added to liquid samples. All samples were then filtered using 0.45µm filters to remove bacteria. The filtrate was then added to a 4h culture of five different strains of *S. aureus*: M1, M5, *S. aureus* 29213, DH1 and HA1 (see Table 1). Samples were

incubated overnight at 37°C shaking at 60rpm for phage enrichment. The overnight culture was centrifuged for 12 min at 5,000xg to pellet bacteria, and then filtered using a 0.45µm filter.

100µL of phage sample was added to 100µL of bacteria and incubated overnight at 37°C before adding to LB top agar for plaque production. Three rounds of successive plaque purifications were performed to isolate each phage. The bacterial strain used to isolate each phage is indicated in Table 2.

Temperate phage isolation

Log-phase *S. aureus* or MRSA sub-cultures were grown for 30 min at 37°C at 200rpm, followed by exposure to mitomycin C (Sigma-Aldrich) at 0.5µg/mL for 8h at 37°C. Cells were pelleted by centrifugation and supernatants passed through a 0.45µm filter and stored at 4°C until plaque assays were performed, as above.

High titer phage lysates were prepared by adding host bacteria and phage into LB top agar and overlaying onto LB agar plates. Overlays with near complete lysis, or a webbed plaque distribution, were treated with 4ml of phage buffer and the top agar overlay was crushed, followed by 90 min incubation at room temperature. Phage buffer was removed and centrifuged at 5,000rpm for 10 min and filtered at 0.45µm to remove bacterial cells, then stored at 4°C with chloroform. Phage stocks were tittered by limiting dilution, using a similar protocol as used for plaque purification above.

Host range analysis

Spot testing

Overnight cultures were prepared in LB medium and then sub-cultured by addition of 100 μ L to 3ml LB broth and grown at 37°C for 90min. 100 μ L of sub-culture was inoculated into 3mL of molten LB top agar and overlaid onto LB agar plates. Each overlay was allowed to solidify for 15min. All phage lysates (original titers approximately 10⁸pfu/mL) were diluted in 10 fold increments and 10 μ L of each dilution was spotted onto the bacterial overlay [91], dried, then incubated at 37°C overnight. As a control, each bacterial strain was also mock infected with sterile phage buffer. Results were analyzed based on detection of any lysis and further dilutions were checked for single plaques to ensure phage lysis rather than bacteriocin induced lysis. All spot tests were repeated in triplicate to confirm results.

Spectrophotometric assay of phage-treated liquid cultures

Overnight bacterial samples were sub-cultured in LB medium supplemented with 5mM CaCl₂ and MgCl₂ until reaching an OD₆₀₀ of 1.4 by shaking at 200rpm at 37°C. At that point, 20 μ L of bacteria was inoculated into 3.5mL LB medium. Phage samples were diluted to 10⁸ pfu/mL in phage buffer. Bacterial samples were treated with 100 μ L of phage (or mock-treated with sterile phage buffer alone) into 3.5mL of culture. Samples were removed at 2, 3, and 4h after infection and the OD₆₀₀ was measured on an Ultraspec 10 spectrophotometer (Amersham Biosciences, Piscataway, NJ, USA) using growth medium as a blank. Experiments were run in triplicate.

Decontamination assays

Glass coverslip decontamination

Decontamination assays were performed to assess the ability of phage to clear MRSA from surfaces. 22 x 22 mm glass coverslips were used to test decontamination on hard surfaces. Coverslips were autoclaved and allowed to cool. 10 μ L of sterile milk was spread onto the surface and allowed dry, giving the coverslips a better surface for bacterial adherence [88]. The test MRSA strain was sub-cultured for 4h at 37°C to achieve logarithmic growth and then diluted 1:10³, giving a final concentration of approximately 10⁶ CFU/mL. 10 μ L of the culture was spread onto the coverslips and allowed to dry at room temperature (approx. 30min). 100 μ L of phage lysate was then added to the surface at a multiplicity of infection of 200-50,000 and incubated at room temperature for 30min. A mock treatment was performed using sterile phage buffer in place of phage lysate. To remove remaining bacteria from the surface, each coverslip was placed in a sterile 50mL conical tube containing 500 μ L of LB medium and vortexed at high-speed for 10s. Serial dilutions of each test were then plated on LB agar and incubated overnight at 37°C. Colonies were counted to determine bacterial load, and the ability of each phage to decontaminate the surface was calculated by dividing the mock-treated bacterial loads by the phage-treated bacterial loads.

Cloth decontamination

To test the utility of our phage for clearing MSSA/MRSA from items associated with nosocomial transmission, we tested lab coat material which was composed of 35% cotton and 65% polyester. 1.5 x 1.5cm pieces were prepared and autoclaved to achieve sterility. MRSA samples were sub-cultured for 4h to achieve logarithmic growth, then diluted 1:10⁴ and 100 μ L was added to the lab coat sample and allowed to remain for 30 min at 37°C. Our baseline

bacterial load recovered from untreated cloth was $\sim 1\text{-}5 \times 10^4$ CFU. 100 μ L of phage lysate was then added and incubated at 37°C for 30min. Phage titers added to the cloth ranged from 1×10^7 to 1×10^8 PFU for a range of multiplicity of infection of 200 to 50,000. As a control, sterile phage buffer alone was added as a mock treatment. Bacteria were removed by placing the cloth into 500 μ L LB medium followed by vortexing at high speed for 10s. The resulting medium was serially diluted, then plated onto an LB agar plate and incubated at 37°C overnight; colonies were counted the next morning. To determine the decontamination capability of the phage, colony-forming units were calculated and mock-treated bacterial loads were divided by phage-treated bacterial loads. These assays were performed in triplicate.

Statistical analysis

Unpaired, one-tailed student's t tests were performed in experiments from Figures 5, 6, and 7 in order to determine if significant differences existed between phage-treated and mock-treated samples. A value of $p \leq 0.05$ was considered to be statistically significant.

Ethics Statement

Brigham Young University Institutional Review Board (IRB) approval was granted for this study (protocol X14403). Informed written consent was obtained for each participant, and minors were not included in this study. Other human samples were provided by collaborators without any patient identifiers and were classified as exempt by the IRB.

Results

Isolation of S. aureus and methicillin-resistant S. aureus (MRSA) strains

A diverse group of *S. aureus* and MRSA isolates were acquired during the course of these experiments in order to be used as tools for phage discovery and characterization. Some isolates were purchased from ATCC, some were acquired from collaborators, and others were isolated from the environment including from human, animal, and environmental sources. The following criteria were used to confirm isolation of *S. aureus* or MRSA: growth on mannitol salt agar plates, ability to ferment mannitol, positive coagulase and catalase tests, and gram stains to confirm gram-positive cocci. Growth in the presence of 2 μ g/mL oxacillin confirmed that some isolates were MRSA. A summary of the *S. aureus* and MRSA isolates used in these studies is found in Table 1. In total, 42 *S. aureus* strains were used including 30 MRSA and 12 methicillin-susceptible strains. 34 isolates were from human sources and 8 were from animal sources.

Table 1 *S. aureus* isolates

MSSA/MRSA isolates used in these studies. Isolates were confirmed as *S. aureus* or MRSA as detailed in Methods. Methicillin resistance was tested by growth on MSA plates in the presence of 2µg/mL oxacillin. ATCC = American Type Culture Collection, NS = human nasal swab, HA = hospital acquired, DH = dog hair, CJ = raw chicken, RB = raw beef, TK = raw turkey.

Strain ID	Source	Oxacillin
M1-9	Sports Training Center	Resistant
SA 29213, 6538, 4651	Purchased from ATCC	Susceptible
MRSA 43300	Purchased from ATCC	Resistant
USA300 LAC	Collaborator	Resistant
USA300 strains 0114, CA-127, CO-34, GA-92, NY-336, JE2	Acquired from BEI	Resistant
USA400 MW2	Collaborator	Resistant
USA400 HFH-30364	Acquired from BEI	Resistant
NS 6, 15	Nasal Swabs	Resistant
NS 13-14, 16, 22-23	Nasal Swabs	Susceptible
HA 1-5	Hospital	Resistant
DH 1-2	Dog Hair	Resistant
DH 3	Dog Hair	Susceptible
CJ 11	Raw Chicken	Susceptible
CJ 9	Raw Chicken	Resistant
RB 1	Raw Beef	Susceptible
TK 11	Raw Turkey	Resistant
TK 9	Raw Turkey	Susceptible

Isolation of S. aureus and/or MRSA-specific bacteriophage

Phages were isolated as described in methods, either from environmental samples or via induction of prophages. In total we isolated 12 *S. aureus*-specific phages, all of which were isolated on and have lytic activity against MRSA strains (see Table 2). 5 of these were temperate phages induced from human *S. aureus* isolates by mitomycin C treatment. The remaining 7 were virulent phages isolated from environmental samples. These samples were first suspended in LB broth and then filtered to remove bacteria. This filtrate was then incubated with a mixture of 5 MSSA/MRSA strains to amplify any phage present. 6 of the 7 virulent phage originated from poultry samples with the 7th originating from sewage influent. All phages were purified through three rounds of plaque purification, with the *S. aureus* strain used listed in Table 2. Following plaque purification, high titer phage stocks were produced and tittered by limiting dilution.

Table 2 *Phage Isolates*

Phage isolates discovered in these studies. Temperate phages have a bipartite name where the first part reveals the bacterial strain used for isolation and the second part indicates the host strain. NS = human nasal swab, SEW = sewage influent, DH = dog hair, CJ = raw chicken, CF = chicken feces.

Strain ID	Source	Isolating Strain
M1M5	Temperate/MRSA 5	M1
M1M4	Temperate/MRSA 4	M1
M1NS15	Temperate/NS 15	M1
M5NS22	Temperate/NS 22	M5
M5NS6	Temperate/NS 6	M5
SEW	Virulent/Sewage Influent	M1
CJ11	Virulent/Raw Chicken	M1
CJ12	Virulent/Raw Chicken	M1
CJ16	Virulent/Raw Chicken	DH1
CJ17	Virulent/Raw Chicken	DH1
CJ18	Virulent/Raw Chicken	DH1
CF6	Virulent/Chicken Feces	DH1

Assessment of host range of phage isolates

One goal of this study was to determine the specificity of phage for particular MSSA/MRSA isolates, with the expectation that some phage would have a broader tropism than others due to presence/absence of phage receptor molecules or intracellular restriction mechanisms. To assess the tropism of the phage described in Table 2, spot tests were performed on plates containing lawns of various MSSA/MRSA isolates as described in Methods. Plates were assessed for the lytic ability of each phage isolate by monitoring for clearing of bacterial lawns (Table 3) and all spot tests were repeated to confirm the validity of the results. All bacterial isolates shown in Table 1 were tested for host range, although many isolates were not lysed by any of the phages and so those results are not shown in Table 3. Based upon spot testing results, at least 6 unique phages were isolated: CJ12, SEW, M1M4, CJ17, a cluster including M5NS6 and M5NS22, and a cluster including M1M5, CJ18, CJ11, CF6, M1NS15, and CJ16. Interestingly, some members of the latter cluster (M1M5 and M1NS15) were found via mitomycin C induction of prophage while the others were found as virulent phages (CJ18, CJ11, CF6, CJ16).

Table 3 Phage Host Range

Phages were assessed for host range by spot testing and plaque formation. Following plaque purification, phages were tested for host range by spot testing on lawns of *S. aureus* and MRSA isolates. Detection of any lawn clearing is indicated by an X. All testing was performed in triplicate, and virus stocks were serially diluted to confirm presence of phage lysis (discreet plaque formation) rather than bacteriocin activity.

Strain ID	Virulent phages							Temperate phages				
	SEW	CJ12	CJ16	CJ11	CJ18	CF6	CJ17	M1M4	M1M5	M1NS15	M5NS6	M5NS22
M1	X	X	X	X	X	X		X	X	X		
M5								X			X	X
M7	X	X	X	X	X	X	X	X	X	X		
DH1	X	X	X	X	X	X	X	X	X	X		
NS13	X							X				
HA1	X	X						X				
HA3											X	X

Phage M1M4 had the broadest host range, with lytic ability against 6 different bacterial strains. CJ17 and the M5NS6 cluster had the narrowest host range, with only 2 different bacterial strains lysed. Of the isolated phages, all had activity against human MRSA isolates, possibly because a cocktail of human MRSA isolates were used to enrich phage in the early steps of this study. We also noted that 3 bacterial strains (M1, M7, and DH1) were lysed by most of the new isolated phages, possibly due to the lack of prophage in these isolates. The majority of the bacterial isolates examined were not lysed by any of the phages. One possible explanation for this finding is that half of the phages (6 of 12) were isolated from an animal source while only 8 of the 42 *S. aureus* strains tested came from animal sources.

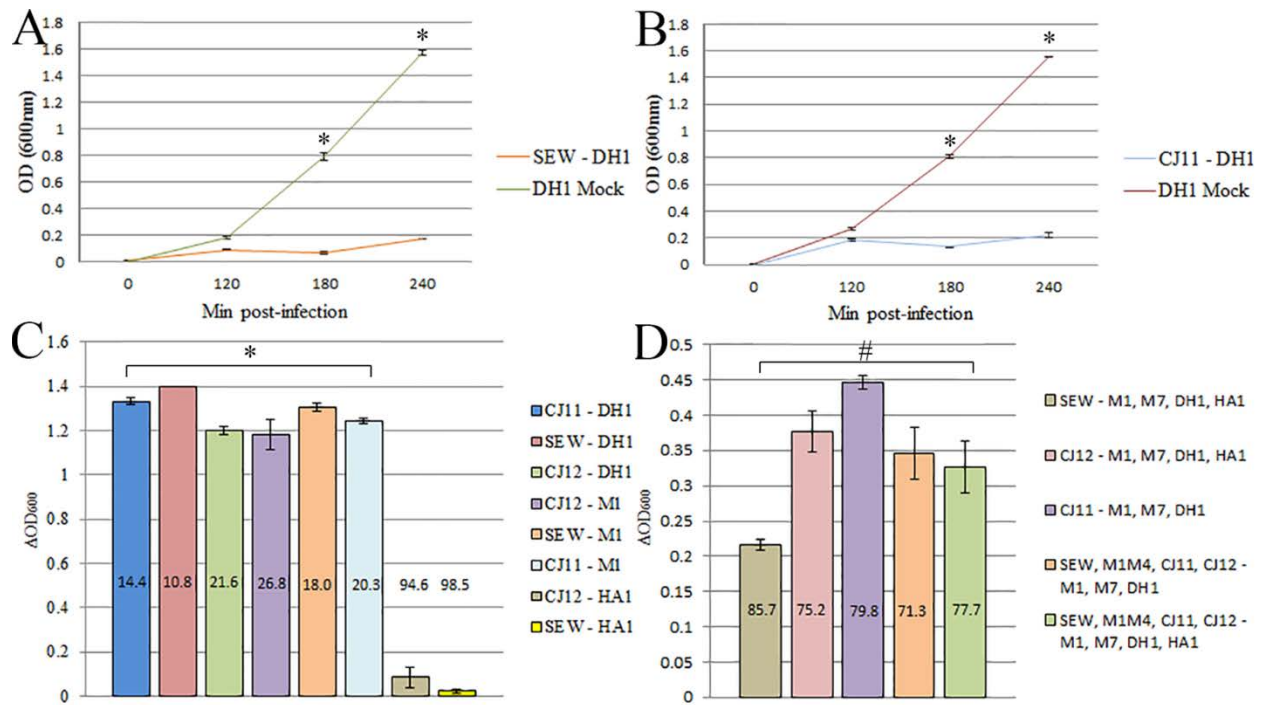


Figure 5 Assessment of host range by lysis of bacterial cultures

Phages were added to log phase bacterial cultures to assess host range and to determine the killing efficiency of the phages. Optical density was measured at 600nm to quantify cell density of the culture. A) MRSA strain DH1 infected with phage strain SEW across a time course. B) MRSA strain DH1 infected with phage strain CJ11 across a time course. C) A variety of bacterial strains were challenged with different phage and OD₆₀₀ readings were taken at 4h. Results are reported in ΔOD₆₀₀ units which were calculated as the difference between the OD₆₀₀ of mock-treated cultures (sterile phage buffer only) and phage-treated cultures. D) Various combinations of bacterial strains were treated with either single phage strains or combinations phage. In panels C and D, the percent difference in OD₆₀₀ readings (phage-treated divided by mock-treated) is also shown. All experiments were performed in triplicate; standard error is indicated. * $p \leq 0.002$ by student's t test. # $p \leq 0.01$ by student's t test when evaluating phage treated vs mock treated samples.

In order to confirm the host range results, and to determine the relative lytic ability of the various phages, liquid bacterial cultures were exposed to phage and spec. assays were performed in log phase cultures (Fig. 5). Since some bacterial isolates produce toxins, it is possible that phage stocks contained such toxins which could lead to false positive results on a spot test. Such toxins are expected to be too dilute to be effective in the large volumes used for a spec. assay but could be active in the spot test procedure, thus the spot tests were diluted until single plaques were visible. We found that changes in the optical density of bacterial cultures were routinely noted at 180 and 240 min post-infection (Figs. 5A, B). In subsequent experiments, readings were only taken at the 240 min time point. We calculated the difference in OD₆₀₀ readings between mock-treated and phage-treated cultures for a variety of phage and bacterial strain combinations and results are reported in Δ OD₆₀₀ units in Fig. 5C, D. We found significant reductions in OD₆₀₀ readings for many different combinations of phage and bacterial targets, including significant differences when one phage was targeted to multiple bacterial isolates or if multiple phages were used to target multiple bacterial isolates (Fig 5D).

Assessment of phage ability to decontaminate fomites

Nosocomial transmission of MSSA/MRSA is a major problem, especially when the immunocompromised and those with underlying health issues become infected. We sought to determine if our phages could effectively decontaminate fomites associated with nosocomial transmission. We used the results of the spot tests to design combinations where the phages were predicted to have a host range that would include the bacterial targets. Decontamination of fabric was also analyzed as a more likely source of nosocomial transmission. We used glass coverslips to represent decontamination of solid surfaces.

To imitate a contaminated fomite we inoculated a single MRSA strain onto sterile cloth (from a lab coat similar to one worn by clinicians) and then added a single phage and determined the bacterial load following either phage treatment or mock treatment. Multiple phage attachment incubation time points were tested to find the ideal time point for target cell lysis (5min, 10 min, 15 min, and 30 min) and results are presented in Appendix Figure 1. The only significant results detected were that a 30 min. phage cocktail treatment showed a significant reduction in colony forming units (CFUs) in comparison to the 10 min. phage treatment on lab coat fabric, and the 10 min. phage treatment showed a significant reduction in CFU in comparison to the 30 min. phage treatment on glass coverslips. Subsequent experiments were performed at 30 min. Mock treatments were performed using sterile phage buffer alone.

We found significant reductions in the numbers of MRSA CFUs in tests where one phage/one MRSA was used to decontaminate lab coat fabric (Fig. 6). Decontamination of MRSA strain M1 inoculated fabric with one of four phages yielded a 1-1.5 log reduction of CFUs ml⁻¹ compared to mock treated samples (Fig. 6A). Each different phage treatment had a p-value ≤ 0.01 . MRSA DH1 was similarly inoculated and treated with phages (Fig. 6C) and resulted in 0.5-1 log reductions in CFUs in each test with a p-value ≤ 0.05 .

We inoculated sterile glass coverslips with MRSA (see Methods) and then treated with singular phages or mock treatments and measured the ability to decontaminate bacteria (Fig. 6B, D). Glass slides inoculated with MRSA strain M1 and treated with singular phage showed highly significant reductions in CFUs ($p \leq 0.005$) compared to mock treatments (Fig. 6B). Each phage treatment yielded at least a 1.5 log reduction in CFUs with two phages nearly achieving a 2 log reduction. Coverslips inoculated with MRSA strain DH1 also showed significant decreases in CFUs as compared to the mock treatment (Fig. 6D), with the exception of M1M4 treatment.

CJ11 treated coverslips achieved a 0.5 log reduction ($p \leq 0.02$), while SEW and CJ12 achieved a 1 log ($p \leq 0.01$) and 1.5 log reduction ($p \leq 0.007$), respectively.

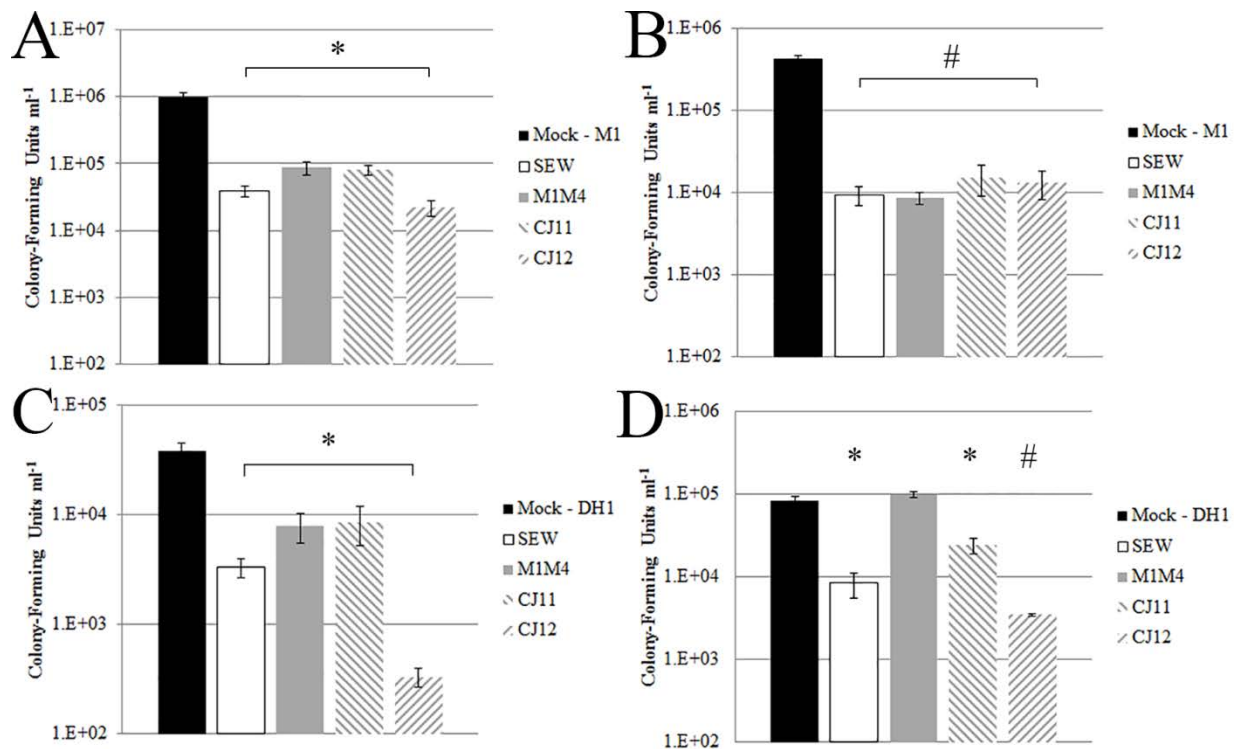


Figure 6 Decontamination of lab coats and glass coverslips

MRSA samples were inoculated onto either lab coat fabric or glass coverslips and then exposed to either a single phage or a mock phage treatment (sterile phage buffer only). The multiplicity of infection ranged from 200 to 50,000. Bacteria were recovered and viable bacterial counts were determined by serial dilution and growth on LB agar plates. Results are reported as colony-forming units ml⁻¹. A) MRSA strain M1 treated with either SEW, M1M4, CJ11 or CJ12 recovered from lab coat fabric. B) MRSA strain M1 treated with either SEW, M1M4, CJ11 or CJ12 recovered from glass coverslips. C) MRSA strain DH1 treated with either SEW, M1M4, CJ11 or CJ12 recovered from lab coat fabric. D) MRSA strain DH1 treated with either SEW, M1M4, CJ11, or CJ12 recovered from glass coverslips. Assays were performed in triplicate; standard error is indicated. * $p \leq 0.05$ by unpaired, one-tailed student's t test when evaluating phage-treated vs mock-treated samples. # $p \leq 0.005$ by same student's t test.

We combined phages M1, M1M4, CJ11, and CJ12 to create a phage cocktail to measure potential synergistic ability to decontaminate MRSA from lab coat material or glass coverslips (Fig. 7). The phage cocktail was designed to include the most efficiently lytic phages as well as those with the broadest tropism. Using the phage cocktail we found significant reductions in CFUs when decontaminating either lab coat fabric (Fig. 7A) or glass coverslips (Fig. 7B). In treating the lab coat fabric we observed a nearly 2 log reduction in CFUs for both M1 and DH1 strains. In studies using MRSA strain DH1, we found a highly significant decrease in CFUs on fabric with a p value ≤ 0.008 (Fig. 7A), similar to the phage cocktail treatment of MRSA strain M1 on a glass coverslip with a p value ≤ 0.003 (Fig 7B).

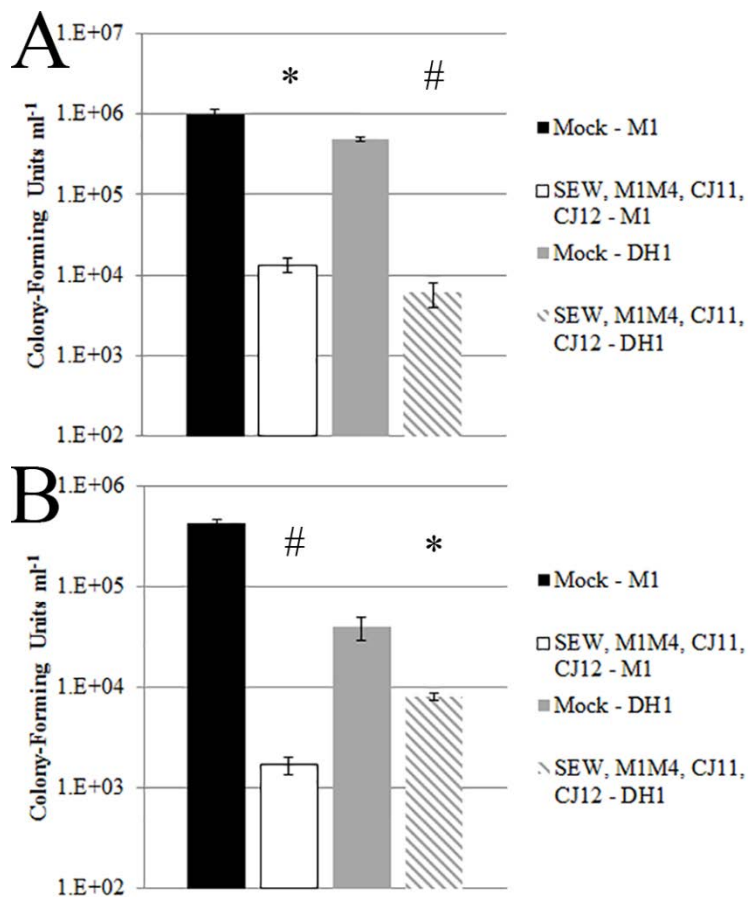


Figure 7 Decontamination of lab coats and glass coverslips with a phage cocktail

MRSA samples were inoculated onto either lab coat fabric or glass coverslips and then exposed to a phage cocktail consisting of SEW, M1M4, CJ11, CJ12, or mock phage treatment (sterile phage buffer only). The combined multiplicity of infection ranged from 300 to 1,300. Bacteria were recovered from treated materials and viable bacterial counts were determined by serial dilution and growth on LB agar plates. A) MRSA strain M1 and DH1 treated with phage cocktail or mock treatment recovered from lab coat fabric. B) MRSA strain M1 and DH1 treated with phage cocktail or mock treatment recovered from glass coverslips. Assays were performed in triplicate; standard error is indicated. * $p \leq 0.05$ by unpaired, one-tailed student's t test when evaluating phage-treated vs mock-treated samples. # $p \leq 0.005$ by same student's t test.

Discussion

In this study, we isolated 12 new phages with lytic activity against *S. aureus* and MRSA. We determined the host range of these phages by both spot testing and spec. assays of phage-treated bacterial cultures. We then examined the ability of single phages or phage cocktails to decontaminate MRSA from a glass surface as well as from fabric. We found that our phages were able to significantly reduce MRSA growth in culture, and that they were able to significantly reduce colony-forming units of human MRSA from both glass and fabric.

The host range tests carried out in these studies showed that our newly isolated phage tended to have greater lytic activity against human *S. aureus* strains as compared to non-human isolates, and to also lyse MRSA more commonly than methicillin-susceptible *S. aureus*. Most of our virulent phage isolates were found in sources related to chickens. Phage isolated from chicken sources would not necessarily be predicted to have activity against MRSA or human *S. aureus* isolates. However, these findings may be related to the protocol used to initially enrich phage, wherein 5 *S. aureus* strains were used: 4/5 was MRSA and 4/5 was human *S. aureus* isolates. Other phage may have been present during enrichment, but we selected for those with lytic activity against human MRSA in our enrichment protocol.

Measurement of the optical densities of phage-treated MRSA cultures revealed that very efficient lysis occurred. The density of phage-treated cultures after 4h was essentially the same as 2h. After comparing the results of the spot tests and spec. assays, we found that the results were mostly in agreement with one another. Some anomalies were seen in this comparison, such as when spot tests indicated lytic ability for a given phage/bacterial strain but culture assays failed to show significant lysis. Two examples are shown in Fig 5C (e.g., SEW and CJ12 treatment of isolate HA1). When we tested more challenging scenarios for phage reduction of

bacterial samples (e.g., one phage targeted to 3-4 bacterial strains in Fig. 5D) we still detected significant reductions in bacterial cell density. The cell density was higher in such experiments, despite the fact that spot testing predicted that all bacterial strains could be lysed. We partially attributed these findings to MRSA strain HA1, which was not lysed efficiently in culture assays. When strain HA1 was removed from one such experiment, the cell density dropped (Fig. 5D and data not shown) indicating that HA1 was less susceptible to clearing in a culture assay.

We opted to use glass as a model test surface for MRSA decontamination, as has been tested previously by others [88]. Significant reductions in bacterial CFUs were detected in nearly all experiments when using either glass or fabric. One experiment failed to show a significant reduction for phage strain M1M4 (see Fig. 6D), but this strain was discovered as a temperate phage and so the lytic potential is expected to be less than that seen for virulent phages. Since M1M4 had the broadest host range of our phages, we still included this phage in subsequent testing. Many decontamination experiments showed a reduction in CFUs greater than 1 log, and occasionally 2 log reductions were seen. Cocktails of phage were typically more effective at decontaminating MRSA than single phage.

We conclude that we have isolated at least 6 unique phages with lytic activity against human MRSA isolates. These phages have robust activity in both liquid culture and in decontaminating hard surfaces and fabrics associated with nosocomial transmission. Our future plans include further characterization of these phages by genome sequencing and transmission electron microscopy, and further testing for decontamination potential under varying circumstances.

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Discussion

Phages have some very attractive attributes for medical phage therapy and especially for fomite decontamination. The results of the preceding research show that fomite decontamination using phages is a viable option. However, increased phage host range and lytic ability need to be achieved before widespread disinfectant use can occur. The three-fold hypothesis stated that phages could be found and isolated into pure strains, the host range and lytic efficiency for each of the phage could be identified, and using phages to decontaminate fomites is possible and reduced bacterial loads from common fomites harboring MSSA or MRSA was achieved. The phage cocktail treatments achieved 2 log reductions in bacterial load and show promise for future use in cleaning fomites.

Using induction and enrichment techniques, 15 phage isolates were initially found. Three of the phages lacked the ability to produce a strong lytic infection. They could not be maintained in high enough titers to perform all three steps of plaque purification and were subsequently abandoned. In addition, 42 *S. aureus* isolates were found and all were used in determining the phage host ranges.

Through host range tests and lytic ability the phages were assed for future use in decontamination assays. The spot test was designed to analyze each phage's ability to lyse each of the 42 *S. aureus* isolates. Each phage isolate was able to lyse at least 2 primary *S. aureus* isolates. Unfortunately, none of the phages were able to lyse any USA300 and USA400 strains—much lower than initial expectations. This lack of lytic ability towards the most common MRSA isolates in the United States limits the efficacy of the phages use outside of the lab. However, in the future, consistent passaging of the phages in the presence of the USA300 and USA400 strains can increase the host range to eventually lyse these strains [70, 73]. Using

this method, future research should be able to create a phage cocktail with a much larger host range than the current host range of the cocktail presented in this document.

Based on host range results the phages were categorized into groups with similar patterns indicating that there were at least six different phage strains isolated. CJ12, SEW, and M1M4 all had unique host ranges, while M1M5 and CJ18; CF6, M1NS15, CJ16, CJ17, and CJ11; M5NS6 and M5NS22 were grouped together respectively. The differences in host range did allow us to combine the phage in a cocktail to create a phage lysate with a host range larger than any single phage. The phage host ranges only included human *S. aureus* strains rather than the non-human isolates. This oddity was not predicted based on the locations where most of the virulent phages were isolated; the raw meat sources would not necessarily indicate a preference towards lysis of human strains of *S. aureus* but rather the raw meat strains. The limited host range may be related to the protocol used to enrich the phages [104]; wherein five different *S. aureus* strains were used. The enriching hosts were 4/5 MRSA and 4/5 human *S. aureus* isolates. Other phages may have been present during enrichment, but by using mostly human MRSA strains the enrichment may have inadvertently enriched only the phages with lytic activity against human MRSA and without any lytic activity against the raw meat *S. aureus*. Secondly, the enrichment process did not use any USA300 or USA400 strains potentially leading to enrichments without phages ability to lyse the most common MRSA strains.

Optical density (OD) differences in bacterial growth were observed between phage treated and mock treated liquid MRSA cultures. In most cases the spec. assay results reinforced the host range results. HA1 lysis proved to be the exception with phages being unable to significantly reduce bacterial growth (Fig. 5C). CJ12 and SEW treatment reduced MRSA cell density by less than 1 OD₆₀₀. Other MRSA strains were significantly reduced by phage

treatment. HA1 may have stronger defense mechanisms for protection of phage resulting in decreased phage lysis and thus positive results during spot testing, but little other lysis in liquid cultures [11].

When tested with more challenging scenarios for phage reduction of bacterial samples (e.g., one phage targeted to 3-4 bacterial strains in Fig. 5D), we still detected significant reductions in bacterial cell density. The cell density was higher in such experiments compared to single phage-single host pairings. All host strains in the *S. aureus* cocktail were within each phage's host range, but increased cell density may be partially attributed to HA1's low lysis rates in liquid cultures. When strain HA1 was removed from one such experiment, the cell density dropped (Fig. 5D and data not shown) indicating that HA1 was less susceptible to clearing in a culture assay. Importantly, single phage strain treatments were able to show significant reductions in cell density of at least 0.2 OD₆₀₀, indicating that phage treatment against contamination with multiple MRSA strains can still be reduced using phage treatment.

The final aim was to realistically simulate fomites in hospital settings and use the phages to decrease MRSA load. Lab coat material mimicked the passage of *S. aureus* from patient to doctor to other patients. Using small strips of lab coat material we showed approximately two log reductions in bacterial CFUs with all phages used. CJ12 was the most efficient single phage in reducing bacterial load on lab coat material with reductions of about 2 logs against both DH1 and M1 (Fig 6). When phages SEW, M1M4, CJ11, and CJ12 were combined into a cocktail, synergistic reductions in CFUs were observed. Approximately two log CFU reductions were observed in every instance when using the phage cocktail to decontaminate lab coat fabric (Fig 7A).

Glass coverslips provided a model system for simulating hard surface contamination in hospital settings and has previously been used for bacterial decontamination using phage [88]. The glass surface was thought to be a more hospitable environment for the phage than fabric, and when compared to lab coat material decontamination we expected to see higher levels of bacterial load reduction. Unexpectedly, we didn't see much difference between the two decontamination surfaces indicating that neither surface was better or worse for phage use. Using the glass slides we did detect significant reductions in bacterial CFUs in nearly all experiments (Fig. 6 B & D). M1M4 had the broadest host range; however, its lytic ability was not as robust as some of the other phages (Fig 6 B & D). Lack of robust lysis from M1M4 is not unusual because M1M4 is a known temperate phage and likely entered the lysogenic cycle, halting cell lysis. Despite being a known temperate phage it still significantly reduced M1 bacterial load on the glass slides. SEW and CJ12 phages were able to show significant reductions in all the experiments on glass slides. Similar to lab coat fabric decontamination we did see synergistic reduction in MRSA CFUs (Fig. 7B).

The phage cocktail showed a significant 2 log decrease in bacterial loads on fomites. Despite the significant reduction, using the phage cocktail as a decontaminate for MRSA is likely still insufficient reason to warrant switching from cleansing wipes able to reduce bacterial loads 5 logs [61]. An important factor which may be playing a role in preventing greater log reductions in MRSA CFU may be the temperate natures of the phages [101]. Based on morphological data not shown in this thesis I suspect that most of the phage may belong to the Siphoviridae class of phages. Siphoviridae are typically temperate phage and as such may be entering the lysogenic cycle during the decontamination test, reducing the ability of the phage to decontaminate a surface. Lysogen formation should be tested in the future by treating surviving

MRSA colonies again with the same phage. Phages with higher lytic ability and less preponderance to enter the lysogenic cycle can also be selected for by continuous passaging. The phages within the cocktail could be selected for increased host ranges using previously discussed methods and increased lysis efficiency [76]. If the host range and lytic efficiency is increased to 5-6 log reductions of MRSA load, the phage cocktail would become a viable cleanser for hospital surfaces.

Future experiments are needed to more fully characterize the phages through gene sequencing. Phage DNA will be isolated and used for Sanger sequencing to identify novel strains of phage. Once novelty has been assured more thorough sequencing using next generation sequencing can take place. The resulting genetic data will be annotated and analyzed for interesting phage genes. Genes of special interest are lysogenic genes, *S. aureus* virulence genes, and antibiotic resistance genes. Ideal phages will not harbor any of these genes.

S. aureus biofilms also present a significant problem because of their formation on fomites inserted into the body. This introduces *S. aureus* into the body and acts as a reservoir for infections. A follow-up project we are working on uses bacteriophage and silver nanoparticles to eliminate *S. aureus* biofilms. Silver nanoparticles have previously been shown to have a bactericidal effect and can reduce *S. aureus* biofilms [44]. Biofilm forming *S. aureus* will be identified using three different techniques: congo red-agar biofilm detection, PCR of biofilm-associated genes, and crystal violet staining of biofilms. *S. aureus* colonies on congo red-agar turn black when producing a biofilm. Initial testing shows that 98% of the 42 *S. aureus* strains form at least a weak biofilm. Initial testing has also shown that many of the *S. aureus* strains harbor known biofilm forming genes, for example *icaD*. We will also use crystal violet staining to measure the efficacy of biofilm removal using phage therapy, silver nanoparticle treatment, or

a combined approach, but as of yet have not yet begun working on this aspect of the project. The end goal of the project is a decrease in biofilm formation when both phages and silver nanoparticles are present.

An additional follow-up research project will look at *S. aureus* contamination in raw meat. Many *S. aureus* strains found in meat are multi-antibiotic resistant and represent further danger to the community. The follow-up project will look at the prevalence of MRSA contaminating raw meats with the endpoint of identifying contamination rates in raw beef, poultry, and pork. Minimum inhibitory concentrations for eight common antibiotics will also be performed to identify multi-antibiotic resistant strains and their prevalence. Each *S. aureus* isolate will also be genotyped for specific virulence factors, biofilm production, and immune evasion genes. As we move forward with research into MSSA, MRSA and *S. aureus* phages we hope to find new methods for treating MRSA infections and controlling its transmission through fomite decontamination.

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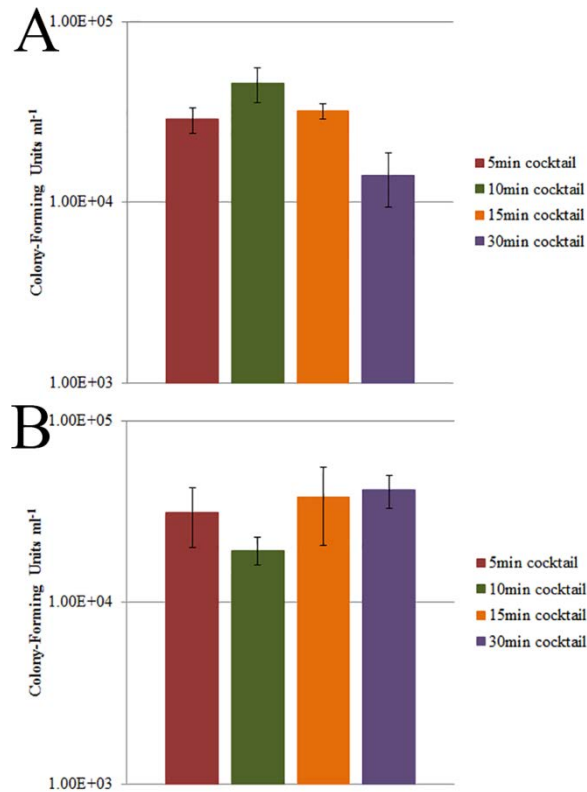
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Appendix



Supplementary Figure 1 Time course decontamination of lab coats and glass coverslips

A decontamination time course assay was done to identify the optimal time to expose the bacterial to phage before recovering cells. MRSA samples were inoculated onto either lab coat fabric or glass coverslips and then exposed for 5 min., 10 min., 15 min. or 30 min. to either a phage cocktail or a mock phage treatment (sterile phage buffer only; results not shown). The multiplicity of infection ranged from 200 to 50,000. Bacteria were recovered and viable bacterial counts were determined by serial dilution and growth on LB agar plates. Results are reported as colony-forming units ml⁻¹ A) MRSA strain DH1 inoculated onto lab coat fabric and then treated with a phage cocktail of SEW, M1M4, CJ11 and CJ12. The 30 min. phage treatment showed a significant reduction in CFUs in comparison to the 10 min. phage treatment. B) MRSA strain DH1 inoculated onto glass coverslips and then treated with a phage cocktail of SEW, M1M4, CJ11 and CJ12. The 10 min. phage treatment showed a significant reduction in CFU in comparison to the 30 min. phage treatment. Assays were performed in triplicate; standard error is indicated. * $p < 0.05$ by unpaired, one-tailed student's t test when evaluating the various time points relative to each other.