RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Bacteriophages as antimicrobials for the control of *Staphylococcus aureus* – **NPB #17-108**

Investigator: Jason J. Gill

Institution: Texas A&M University

Date Submitted: March 14, 2019

INDUSTRY SUMMARY

Rationale: *Staphylococcus aureus* is an opportunistic pathogen of major concern in both human and animal health. While this bacterium can cause life-threatening illness, it more commonly asymptomatically colonizes the nasal passages and skin of healthy people. *S. aureus* can also be carried by livestock animals including swine, and there is evidence that *S. aureus* can be transmitted back and forth between humans and swine. This makes swine a potential reservoir for *S. aureus*, which may pose a risk to workers in swine production facilities, and potentially to the larger community. Bacteriophages (phages) are bacterial viruses and they are major predators of bacteria in natural environments. The continued emergence of antibiotic resistant bacteria, including resistant strains of *S. aureus*, has led to increased interest in novel antibacterial strategies, including the use of phages. **Objectives:** Our preliminary data suggested the previously isolated *S. aureus* phage may be effective in combating *S. aureus* prevalence in swine production environments. To evaluate the utility of these phage the goals of this project were to i) investigate the host range that these isolated phage have against a variety of both swine and human *S. aureus* isolates either alone or in combination and ii) to select broad host range phage and test their ability to eliminate phage-resistant *S. aureus* isolates as well as to eliminate *S. aureus* biofilms. **Methods:** 14 phage were tested at two different concentrations (10^8 and 10^6 PFU/ml) for their ability to effectively infect a panel of 17 different *S. aureus* isolates from both human and swine sources in a liquid plate reader assay. Five of these phage were previously isolated, and the other 10 were phage isolated during the first awarded NPB grant titled "Characterization of *Staphylococcus aureus* Bacteriophages in Swine Production Environments". Phage resistant *S. aureus* were isolated for four different phage and the ability of five phage to overcome these isolates were tested in a liquid plate reader assay. Five different phage cocktails were evaluated in their ability to infect six different *S. aureus* isolates in liquid culture assays and are currently being tested in their ability to control biofilm formation by the same strains. A panel of 16 *S. aureus* isolates were evaluated for their ability to form a biofilm in a 96-well tissue

culture plates, a common model system for studying biofilms. Lastly, attempts to sequence myophages that have failed sequencing on three different platforms is ongoing. **Results:** Novel isolated phage display a varied host range, with podophages displaying the most limited host range and myophages displaying the most varied host range with some infecting 80% of the tested strains. These observations are the same for the previously isolated phages K and 44AHJD. However, while 44AHJD is a podophage, it has a slightly more expanded host range than the novel podophages tested. Phage-resistant mutants, if seen, usually caused regrowth of the culture at approximately 18 hours into the 20 hour assay. Therefore, phage-resistant *S. aureus* isolates were isolated for four different phage. These phage-resistant *S. aureus* isolates were tested in the liquid culture assay against a panel of five phage to determine if other phage could still infect these phage-resistant *S. aureus* mutants. This data was then used to generate different phage cocktails to ensure that if an *S. aureus* isolate became resistant to one phage, it would be sensitive to another phage in the formulations. Biofilm 96-well plate assays for 16 *S. aureus* isolates have shown extremely varied results between strains as well as the positive control (ATCC 25923). The strain with the strongest biofilm formation ability besides the positive control is human isolate NRS70, which is an ST5, USA100 and SccMec type II strain. The strongest biofilm former of the swine isolates tested in this assay was PD18 which is a ST9 methicillin-sensitive (MSSA) nasal swine strain isolated in North Carolina. Sequencing of previously isolated podophage was finished with a total of sixteen podophage genomes sequenced. Comparison of the podophage genomes to understand the observed difference in things such as plaque morphology and host range is ongoing. Additionally, all attempts to sequence a set of previously isolated large myophages have failed, suggesting hypermodification of phage DNA. Experiments to identify which base in these phage is modified by using thin layer chromatography (TLC) is ongoing.

KEYWORDS: *Staphylococcus aureus*, MRSA, bacteriophage, phage therapy, occupational health, swine production

SCIENTIFIC ABSTRACT

Staphylococcus aureus is a pathogen of significant concern in both humans and livestock. Asymptomatic carriage of *S. aureus* by swine, particularly multidrug-resistant (MDR) strains, poses a potential risk to workers involved with livestock production and to the larger community. Bacteriophages are the most abundant form of life in the biosphere and are major predators of bacteria in natural environments. Recent interest in phages as novel antimicrobials has raised the possibility that phages infecting *S. aureus* may provide an alternative means for modulating this pathogen in the livestock environment and reducing the risk of transmission to humans.
A collection of 51 phages were isolated from swine barn environmental samples. 13 of these isolated phage were chosen as representatives and assessed for their ability to infect 17 different *S. aureus* strains from both human and swine sources at two different concentrations. These phage were tested alongside previously isolated phage as well as well-known *S. aureus* phage such as phage K. Select phage were also tested for their ability to overcome *S. aureus* phage-resistant isolates, which allowed for the formulation of phage cocktails to be assessed for the treatment of *S. aureus* isolates. Lastly, a panel of 16 different *S. aureus* isolates were tested for their ability to form a biofilm as a model to assess for phage treatment for biofilm reduction.

INTRODUCTION

There are an estimated 80,000 invasive methicillin resistant *Staphylococcus aureus* (MRSA) infections in the United States annually [1]. *S. aureus* is the leading cause of bacteremia, skin, soft tissue and device-related infections [2]. These *S. aureus* infections, particularly MRSA infections, can be extremely difficult and costly to treat. In one study, the median cost to treat a surgical site infection with MRSA was $92,363 [3]. MRSA infections are mainly divided into three main sources of infection: hospital associated (HA-MRSA), community associated (CA-MRSA) and livestock associated (LA-MRSA).

Carriage of *S. aureus* in the general public in the continental US ranges from 26% to 32% [4]. An estimated 1.3% of that *S. aureus* being MRSA [5]. However, in individuals in the US that are swine farmers, production workers or veterinarians, carriage of multi-drug resistant *S. aureus* (MDRSA) is two to six times greater than individuals in the community, or those who are not exposed to swine [6, 7]. MDRSA is defined by an *S. aureus* isolate that demonstrates resistance to three or more classes of antimicrobials [1].

While there have been few human infections from ST398 LA-MRSA recorded, there have been several cases that had severe consequences, such as a case of lethal necrotizing pneumonia and several cases of bacteremia [8, 9]. Recently, there has been growing concern for bloodstream infections caused by several ST398 isolates that have appeared in multiple hospital infections, leading the authors to investigate if ST398 has once again become a "human adapted subclone" in France [10, 11]. Therefore, LA-MRSA strains may still pose a threat to swine worker safety.

In a recent survey of *S. aureus* carriage among swine in the US, Sun et al., 2015 examined 36 different swine herds; this sample represented 11 different states and 9 different breed stock companies. Results from this study found that only two out of 1200 *S. aureus* isolates were not categorized as MLST lineages: ST9, ST398 or ST5. Additionally, for 21 out of 35 herds the prevalence of *S. aureus* exceeded 80%. However, this prevalence is *S. aureus* in general, all isolates in this study with the exception of a known MRSA positive herd were MSSA. This study indicates that MRSA prevalence in the USA appears to be currently lower than in European countries [12]. In the Netherlands, MRSA prevalence among swine herds is approximately 69% [13].

To combat the transfer of MRSA strains from swine to humans and to the community new safety measures should be employed. While elimination of *S. aureus* from swine environments as well as swine workers does not seem likely, one study found that people carrying MSSA had an 83% decreased risk of MRSA acquisition when samplings were conducted months apart [14]. Therefore, if a product could serve to decolonize MRSA carriage in swine and workers temporarily, this may allow for an MSSA strain to fill the niche. Bacteriophages, viruses that infect bacteria, could have a part in resolving this problem.

There are two known life cycles a phage can pursue: lytic or lysogenic. In the lytic life cycle a phage adsorbs to a bacteria, it then injects its DNA into the host cell and replicates, producing virions. After replicating in the cell, the phage will lyse the cell to release its progeny. In contrast, in the lysogenic life cycle a phage will adsorb to the cell, as well as inject its DNA, however, this DNA will either be integrated into the chromosome of the bacteria or exist as an episomal element. The phage DNA will replicate with the bacterial DNA; damage or stress to the bacterial cell can cause these phage to excise from the bacterial genome and begin the lytic life cycle, in a process called induction [15].

Strictly lytic phages are considered to be the best option for therapeutic applications, as lysogenic phage present safety concerns with their ability to integrate into the host genome and possibly disseminate bacterial DNA. For the *S. aureus* bacteriophages, lytic phages of serogroups D and G are of most interest for therapeutic use; other serogroups of *S. aureus* phage were commonly used to type strains o*f S. aureus* for clinical usage, however all known isolates of these phage are lysogenic [16].

However, before a phage product could be employed, it would need to show not only good results in culture, it would also need to be effective against *S. aureus* biofilms, as in nature both bacteria and bacteriophages usually exist in biofilms and it is important to investigate how these novel phage will perform in that environment [17]. A biofilm is composed of surface-associated microbial cells that are encased in an extracellular polymeric substance matrix [18]. There are four generic stages of biofilm formation: 1.) bacterial attachment, 2.) microcolony formation, 3.) biofilm maturation and 4.) detachment and dispersal [19]. Forming a biofilm provides bacteria with protection from a hostile environment, host defenses and antibiotics. How some biofilms are resistant to antibiotics is still not completely known. However, several hypotheses such as the failure of antibiotics to diffuse successfully through the polymeric substance matrix, as well as the slow-growing, starved state of the bacteria, or most likely a combination of factors have been proposed. Regardless of which reason is more correct, it is very hard to treat and eliminate biofilms successfully [20].

In *S. aureus* two different types of biofilms have been observed. The first is a polysaccharide intercellular antigen (PIA) dependent biofilm which is mediated by the *icaADBC* operon (intercellular adhesion). This type of biofilm is characterized by the production of a glycocalyx which is primarily composed of β-1,6-linked N-acetylglucosamine residues as well as a smaller proportion of non-N-acetylated D-glucosaminyl residues [21]. In contrast, PIA-independent biofilms rely on the major autolysin protein Alt, fibronectin binding protein FnBp and extracellular DNA (eDNA) to form biofilms. An important recent observation is that MSSA strains form PIA-dependent biofilms while MRSA strains form PIA-independent biofilms [22].

Phage treatment has showed great promise in reducing *S. aureus* biofilms in studies thus far [23,24,25,26,27]. A problem with phage treatment of *S. aureus* biofilms is the appearance of phage resistant *S. aureus* mutants, which usually occurs at a frequency 10^{-7}, this problem can be addressed by using a cocktail of selected phage instead of applying phage individually [28]. Therefore, these novel phage either alone or in combination (phage cocktail) could have great therapeutic potential.

OBJECTIVES

Our preliminary data suggested the previously isolated *S. aureus* phage may be effective in combating *S. aureus* prevalence in swine production environments. To evaluate the utility of these phage the goals of this project were to i) investigate the host range of these phage against a panel of both swine- and human-associated *S. aureus* isolates either alone or in combination and ii) to select broad host range phage and test their ability to eliminate phage resistant *S. aureus* isolates as well as to eliminate *S. aureus* biofilms.

MATERIALS AND METHODS

Culture and maintenance of bacteria and phages. *Staphylococcus aureus* was routinely cultured on trypticase soy broth (Bacto TSB, Difco) or trypticase soy agar (TSA, TSB + 1.5% w/v Bacto agar, Difco) aerobically at 30 ºC. Phages were cultured using the double-layer

overlay method [17] with 4 mL of top agar (10 g/L Bacto Tryptone (Difco)), 10 g/L NaCl, 0.5% w/v Bacto agar) supplemented with 5 mM each calcium chloride and magnesium sulfate over TSA bottom plates. Lawns were inoculated with 0.1 ml of a mid-log *S. aureus* bacterial culture grown to an OD_{550} of ~0.5. Bacterial strains used for phage isolation and propagation are shown in Table 1.

Creating Phage Stocks. After subculturing the phage, parent stocks of each phage were made by the confluent plate lysate method [29]. Each phage was propagated on its original isolation host and harvested from the agar overlay with 6 mL of phage buffer (100 mM NaCl, 25 mM Tris-HCl pH 7.4, 8 mM $MgSO_4$, 0.01% w/v gelatin). Phage lysates were filter-sterilized (0.2 µm) and stored in the dark at 4 ºC.

Phage Genomic DNA Extraction. High titer (>10^8 PFU/mL) stocks of each phage were made and gDNA was then extracted from 10-20 mL of phage stock. To extract gDNA, 0.5 µL of nuclease solution per ml of lysate (10 µg/mL DNase & RNase final) was added and lysates were incubated at 37 ºC for 30 minutes. Next, precipitant solution (10% PEG-8000, 1 M NaCl final) was added to each lysate at a rate of 1:2 precipitant:lysate and incubated at 4 ºC overnight. The next day the lysate was centrifuged at 10,000 x g, 4 ºC for ten minutes and the supernatant was discarded. The remaining pellet was then resuspended in 500 µL of resuspension buffer (5 mM MgSO4) and transferred to a new 1.5 ml microcentrifuge tube. To remove any insoluble particles the sample was centrifuged for 5-10 seconds and the supernatant transferred again to a new 2 mL microcentrifuge tube. To eliminate the heat stable nuclease produced by *S. aureus* to each 500 µL aliquot of resuspended phage, 10 µL of 0.5 M EDTA pH 8 and proteinase K to a final concentration of 100 µg/mL was added and incubated at 50 ºC for 30 minutes. After allowing the sample to return to room temperature 1 mL of resin from the Promega Wizard Kit ® was added and the tube was inverted approximately five times. The resin was then run through a 3 mL syringe that had a minicolumn attached to it and then rinsed twice with 2 mL of 80% isopropanol. The minicolumn was then transferred to a 1.5 mL microcentrifuge tube and centrifuged at 13,000g for 2 minutes to dry the resin. After drying the minicolumn was transferred to a new 1.5mL microcentrifuge tube and 100 µL of preheated 80 ºC water was added to the column and centrifuged at 13,000 g for 1 minute to elute the DNA. Extracted gDNA from the phage lysate was then stored at 4 ºC.

Restriction Digest of gDNA. To distinguish different phage types approximately 300 ng of extracted gDNA was digested with both DraI (5' TTTAAA 3') (New England BioLabs Inc. https://www.neb.com). 300 ng of gDNA from each phage was incubated with enzyme and CutSmart ® Buffer individually at 37 °C overnight. After overnight incubation, 4 µL of loading dye was then added and the total 24 µL for each sample was run on a 1% agarose gel at 90 V for two and half hours.

Phage genome sequencing and annotation. DNA was sequenced in an Illumina MiSeq 250-bp paired-end run with a 550-bp insert library through the generosity of Dr. H. Morgan Scott's lab at Texas A&M College of Veterinary Medicine & Biomedical Sciences (College Station, TX, USA). FastQC (bioinformatics.babraham.ac.uk), FastX Toolkit (hannonlab.cshl.edu) and SPAdes 3.5.0 [30] were then used for read quality control, read trimming, and read assembly,

respectively. Preliminary phage relationships were determined by BLASTn [31] against the nr database at NCBI. Analyses were performed via CPT Galaxy (https://cpt.tamu/edu/galaxy-pub).

Liquid host range assay.
Staphylococcus aureus was routinely cultured on trypticase soy broth (Bacto TSB, Difco) or trypticase soy agar (TSA, TSB + 1.5% w/v Bacto agar, Difco) aerobically at 30 ºC. 18 hour overnight cultures were diluted in fresh in TSB to an OD_{550} of ~1.0 and iced. This culture was then diluted 10X when added to a cell culture 96-well plate (Falcon®, Corning ®) into TSB and either 10^9 PFU/ml phage, 10^7 PFU/ml phage, or phage buffer (100 mM NaCl, 25 mM Tris-HCl pH 7.4, 8 mM $MgSO_4$, 0.01% w/v gelatin) was added to the well. 96-well plates were incubated with shaking in a plate reader (Tecan® Spark™ 10M) for 20 hours and the OD_{550} was recorded every 30 minutes.

Biofilm 96-well plate assay.
Staphylococcus aureus was routinely cultured on trypticase soy broth (Bacto TSB, Difco) or trypticase soy agar (TSA, TSB + 1.5% w/v Bacto agar, Difco) aerobically at 37 ºC. TSA plates were streaked from 24 hour TSB cultures of each *S. aureus* strain and incubated for 24 hours at 37 ºC. After 24 hours of incubation, approximately three loops of each *S. aureus* strain was scraped off a TSA plate into 500 ul of TSB, which was vortexed on high for one minute. This culture was then diluted down to an OD_{550} of 0.5 in TSB and iced. Next, cultures were vortexed for 30 seconds on high and 20 ul was used to inoculate cell culture 96-well plates (Sarstedt®) which contained trypticase soy broth with an added 1% dextrose (TSBg) (Bacto TSB, Difco) (BDH, Dextrose Anhydrous). This plate was incubated for 24 hours statically at 30 ºC. After incubation, the supernatant was removed and the formed biofilms were washed twice with PBS (Sigma-Aldrich®), heat fixed for 60 minutes at 60 ºC, stained with 1% crystal violet (VWR™) for 15 minutes, washed with PBS five times, air dried for 30 minutes and then the dye was resolubilized with 95% EtOH which was allowed to sit for 25 minutes, before transferring the dye to a new 96-well plate (Falcon®, Corning ®) and finally the OD_{570} was measured in a plate reader (Tecan® Spark™ 10M).

RESULTS

14 phage total were tested at two different concentrations (10^8 and 10^6 PFU/ml) for their ability to effectively infect a panel of 17 different *S. aureus* isolates from both human and swine sources in a liquid plate reader assay. Five of these phage were previously isolated by our group or obtained from collections, and the other 10 were all phage isolated during the first awarded NPB grant titled "Characterization of Staphylococcus aureus Bacteriophages in Swine Production Environments".

Phage resistant *S. aureus* were isolated for four different phage and the ability of five phage to overcome these isolates were tested in a liquid plate reader assay. Five different phage cocktails were evaluated in their ability to infect six different *S. aureus* isolates in the liquid plate reader assay and are currently being tested in their ability to control biofilm formation by the same strains. A panel of 16 *S. aureus* isolates were evaluated for their ability to form a biofilm in a 96-well tissue culture plate.

Sequencing of previously isolated podophage was finished with a total of sixteen podophage genomes sequenced. Comparison of the podophage genomes to understand the

observed difference in things such as plaque morphology and host range is ongoing. Additionally, all attempts to sequence previously isolated large myophages have failed.

DISCUSSION

Novel isolated phage display a varied host range, with podophages displaying the most limited host range and myophages displaying the most varied host range with some infecting 80% of the tested strains. These observations are the same for the canonical phage assessed, with phage K displaying a much broader host range than phage 44AHJD. However, while 44AHJD is a podophage, it has a bit more of an expanded host range than the novel podophages tested (Table 1).

Phage resistant mutants if seen, usually caused regrowth of the culture at approximately 18 hours into the 20 hour assay (Figure 1A). However, some phage were able to completely suppress the growth of a strain for 20 hours (Figure 1B). Due to the appearance of phage resistant *S. aureus* mutants, various combinations of phage were tested against different *S. aureus* strains to prevent the regrowth of the culture at 18 hours. Figure 1C displays one of these combinations, showing that if phage RP1 is combined with phage P4 we do not see regrowth of culture at 18 hours, as RP1 is able to infect the P4-resistant NRS653 *S. aureus* strain.

Biofilm 96-well plate assays for 16 *S. aureus* isolates have shown extremely varied results between strains as well as the positive control (ATCC 25923). The strain with the strongest biofilm formation ability besides the positive control is human isolate NRS70, which is an ST5, USA100 and SccMec type II strain. The strongest biofilm former of the swine isolates tested in this assay was PD18 which is a ST9 methicillin-sensitive (MSSA) nasal swine strain isolated in North Carolina (Figure 2). Phage treatment of these biofilms is currently ongoing, with the hypothesis that various combinations of phage will be able to decrease the amount of bioflm biomass after 8, 12 and 24 hour treatments.

Prior to this study, three major classes of *S. aureus* phages had been described: large (~140 kb) virulent K-like myophages, small (~18 kb) virulent phi29-like podophages, and a slightly more diverse group of temperate siphophages with genomes of ~45 kb [16]. Currently, a total of 16 podophages have been sequenced and are being annotated and analyzed for small changes that could possibly account for plaque morphology and host range differences.

While the DNA of other K-like myophages infecting *S. aureus* have been sequenced by conventional methods, there are known to be more distantly-related myophages that infect other members of the Firmicutes that are refractory to DNA sequencing due to the presence of highly modified bases in the phage DNA. Perhaps the most striking recent example of this is *Bacillus subtilis* phage CP51, which contains hydroxymethyluracil (hmU) in place of thymidine (T) in its DNA; this substitution rendered the phage DNA resistant to most restriction enzymes and Illumina and Sanger sequencing approaches [32]. This phage was eventually sequenced by using the PacBio platform, which was able to process this modified DNA, however, attempts at this preparation have failed for the large myophages in our collection. Additionally, we attempted to use the MinION platform from Oxford Nanopore Technologies (ONT), and while the run was informative that the DNA does appear to be modified, these modifications appear to have made the obtained data uninterpretable by the ONT basecalling algorithm. Currently, a representative of these large myophages is being prepared by a new version of the Nextera™ Flex chemistry to attempt to sequence through Illumina, as this new library preparation is most similar to the ONT MinION preparation and could provide some resolution of the sequence if successful.

We hypothesize that these myophages in our collection represent a novel subtype of K-like myophage infecting *S. aureus.* To our knowledge this would be the first incidence of *S. aureus* phage containing such hypermodified DNA. Other phage such as *Bacillus* phage SPO1 have been documented to modify their DNA as well but this has not yet been recorded in *S. aureus* phages [16].

REFERENCES

1. Magiorakos, A.P., et al., *Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance.* Clinical Microbiology and Infection, 2012. **18**(3): p. 268-281.
2. Tong, S.Y., et al., *Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management.* Clin Microbiol Rev, 2015. **28**(3): p. 603-61.
3. Engemann, J.J., et al., *Adverse clinical and economic outcomes attributable to methicillin resistance among patients with Staphylococcus aureus surgical site infection.* Clin Infect Dis, 2003. **36**(5): p. 592-8.
4. Sivaraman, K., N. Venkataraman, and A.M. Cole, *Staphylococcus aureus Nasal Carriage and its Contributing Factors.* Future microbiology, 2009. **4**: p. 999-1008.
5. Salgado, C.D., B.M. Farr, and D.P. Calfee, *Community-acquired methicillin-resistant Staphylococcus aureus: a meta-analysis of prevalence and risk factors.* Clin Infect Dis, 2003. **36**(2): p. 131-9.
6. Neyra, R.C., et al., *Multidrug-resistant and methicillin-resistant Staphylococcus aureus (MRSA) in hog slaughter and processing plant workers and their community in North Carolina (USA).* Environ Health Perspect, 2014. **122**(5): p. 471-7.
7. Wardyn, S.E., et al., *Swine Farming Is a Risk Factor for Infection With and High Prevalence of Carriage of Multidrug-Resistant Staphylococcus aureus.* Clin Infect Dis, 2015. **61**(1): p. 59-66.
8. Rasigade, J.P., et al., *Lethal necrotizing pneumonia caused by an ST398 Staphylococcus aureus strain.* Emerg Infect Dis, 2010. **16**(8): p. 1330.
9. van Belkum, A., et al., *Methicillin-resistant and -susceptible Staphylococcus aureus sequence type 398 in pigs and humans.* Emerging Infectious Diseases, 2008. **14**(3): p. 479-483.
10. Valentin-Domelier, A.S., et al., *Methicillin-susceptible ST398 Staphylococcus aureus responsible for bloodstream infections: an emerging human-adapted subclone?* PLoS One, 2011. **6**(12): p. e28369.
11. van der Mee-Marquet, N., et al., *Emergence of Unusual Bloodstream Infections Associated with Pig-Borne–Like Staphylococcus aureus ST398 in France.* Clinical Infectious Diseases, 2011. **52**(1): p. 152-153.
12. Sun, J., et al., *Prevalence and Characterization of Staphylococcus aureus in Growing Pigs in the USA.* PLoS One, 2015. **10**(11): p. e0143670.

13. Broens, E.M., et al., *MRSA CC398 in the pig production chain.* Prev Vet Med, 2011. **98**(2-3): p. 182-9.
14. van Cleef, B.A., et al., *Livestock-associated MRSA in household members of pig farmers: transmission and dynamics of carriage, a prospective cohort study.* PLoS One, 2015. **10**(5): p. e0127190.
15. Campoy, S., et al., *Induction of the SOS response by bacteriophage lytic development in Salmonella enterica.* Virology, 2006. **351**(2): p. 360-367.
16. Lobocka, M., et al., *Genomics of Staphylococcal Twort-like Phages - Potential Therapeutics of the Post-Antibiotic Era.* Adv Virus Res, 2012. **83**: p. 143-216.
17. Abedon, S. T. *Ecology of Anti-Biofilm Agents II: Bacteriophage Exploitation and Biocontrol of Biofilm Bacteria*. Pharmaceuticals, 2015. **8**(3): 559-589.
18. Donlan, R. M. *Biofilms: Microbial Life on Surfaces.* Emerg Infect Dis, 2002 **8**(9): 881-890.
19. Crouzet, M., C. Le Senechal, V. S. Brözel, P. Costaglioli, C. Barthe, M. Bonneu, B. Garbay and S. Vilain. *Exploring early steps in biofilm formation: set-up of an experimental system for molecular studies.* BMC Microbiology, 2014. **14**(1): 253.
20. Costerton, J. W., P. S. Stewart and E. P. Greenberg. *Bacterial biofilms: a common cause of persistent infections.* Science, 1999 **284**.
21. Archer, N. K., M. J. Mazaitis, J. W. Costerton, J. G. Leid, M. E. Powers and M. E. Shirtliff. *Staphylococcus aureus biofilms.* Virulence, 2011. **2**(5): 445-459.
22. McCarthy, H., J. K. Rudkin, N. S. Black, L. Gallagher, E. O'Neill and J. P. O'Gara. *Methicillin resistance and the biofilm phenotype in Staphylococcus aureus*. Front Cell Infect Microbiol, 2015. **5**.
23. Del Pozo, J. L., M. Alonso, C. R. Arciola, R. Gonzalez, J. Leiva, I. Lasa and J. Penades. *Biotechnological war against biofilms. Could phages mean the end of device-related infections?* Int J Artif Organs, 2007 **30**(9): 805-812.
24. Kelly, D., O. McAuliffe, R. P. Ross and A. Coffey. *Prevention of Staphylococcus aureus biofilm formation and reduction in established biofilm density using a combination of phage K and modified derivatives.* Lett Appl Microbiol, 2012. **54**(4): 286-291.
25. Lungren, M. P., R. M. Donlan, R. Kankotia, B. E. Paxton, I. Falk, D. Christensen and C. Y. Kim. *Bacteriophage K antimicrobial-lock technique for treatment of Staphylococcus aureus central venous catheter-related infection: a leporine model efficacy analysis*. J Vasc Interv Radiol, 2014. **25**(10): 1627-1632.
26. Son, J.-S., S.-J. Lee, S. Y. Jun, S. J. Yoon, S. H. Kang, H. R. Paik, J. O. Kang and Y.-J. Choi. *Antibacterial and biofilm removal activity of a podoviridae Staphylococcus aureus bacteriophage SAP-2 and a derived recombinant cell-wall-degrading enzyme.* Applied Microbiology and Biotechnology, 2010. **86**(5): 1439-1449.
27. Gutiérrez, D., B. Martínez, A. Rodríguez and P. García. *Genomic characterization of two Staphylococcus epidermidis bacteriophages with anti-biofilm potential.* Bmc Genomics, 2012. **13**(1): 228.

28. Drilling, A., S. Morales, C. Jardeleza, S. Vreugde, P. Speck and P. J. Wormald. *Bacteriophage reduces biofilm of Staphylococcus aureus ex vivo isolates from chronic rhinosinusitis patients.* Am J Rhinol Allergy, 2014. **28**(1): 3-11.
29. Kropinski, A.M., et al., *Enumeration of bacteriophages by double agar overlay plaque assay.* Methods Mol Biol, 2009. **501**: p. 69-76.
30. Bankevich, A., et al., *SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing.* J Comput Biol, 2012. **19**(5): p. 455-77.
31. Camacho, C., et al., *BLAST+: architecture and applications.* BMC Bioinformatics, 2009. **10**: p. 421
32. Klumpp, J., et al., *The odd one out: Bacillus ACT bacteriophage CP-51 exhibits unusual properties compared to related Spounavirinae W.Ph. and Bastille.* Virology, 2014. **462-463**(Supplement C): p. 299-308.

FIGURES AND TABLES

Table 1. Host ranges and virulence of 14 *S. aureus* phages against a panel of 17 *S. aureus* strains of various origins. Sequence type (ST) is denoted in parentheses below each bacterial strain name if available. Phages were assayed against strains at two initial phage concentrations, 10^6 and 10^8 PFU/ml. Higher values in each cell indicate stronger ability of the phage to control the growth of the bacterial strain; a value of 100 indicates complete control (i.e., no bacterial growth), and a value of 0 indicates the strain is completely resistant to the phage. Cell shading reflects value, with higher values shown with more intense color.

		Human *S. aureus* isolates											Swine *S. aureus* isolates					
Phage	Phage conc. (PFU/ml)	USA300-0114 (ST8)	NRS103 (ST 508 SLV)	NRS255 (ST80)	NRS253 (ST398)	NRS70 (ST5)	HFH-30364 (NA)	HFH-30522 (NA)	NRS648 (ST8)	NRS653 (ST5)	NRS689 (ST72)	NRS232 (ST22)	PD6 (ST9)	PD10 (ST398)	PD17 (ST398)	PD18 (ST9)	PD19 (ST5)	PD32 (ST9)
TP1	10^8	81	90	84	8	28	89	68	90	6	92	91	82	5	92	5	8	87
TP1	10^6	60	11	34	0	4	1	0	0	1	52	0	0	0	0	0	N/A	N/A
K	10^8	98	4	1	5	98	98	1	98	2	22	1	3	0	60	98	10	97
K	10^6	93	0	0	0	88	92	0	0	0	2	0	0	0	0	0	0	50
P4	10^8	0	1	0	80	0	0	0	0	93	19	0	0	0	78	84	0	86
P4	10^6	0	1	0	84	0	0	0	0	73	0	0	1	1	81	78	0	92
PD32-F19-S-2	10^8	0	0	9	19	0	1	0	0	83	21	1	1	0	75	32	0	89
PD32-F19-S-2	10^6	0	0	6	1	1	1	0	0	94	3	0	2	1	8	1	0	93
PD18-F16-S	10^8	0	2	1	4	1	2	1	0	1	0	0	1	1	0	1	0	0
PD18-F16-S	10^6	0	2	0	0	9	0	0	0	0	0	0	0	0	0	0	1	0
PD17-F11-S	10^8	0	6	1	2	0	0	1	98	1	0	0	1	1	1	98	0	0
PD17-F11-S	10^6	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PD17-F7-S-2	10^8	0	3	0	84	0	0	0	0	40	4	0	1	0	88	83	0	92
PD17-F7-S-2	10^6	0	4	0	80	0	0	0	0	0	0	0	1	1	91	78	0	85
PD17-F4-S-2	10^8	0	5	6	82	0	1	4	0	84	7	0	2	0	85	84	0	87
PD17-F4-S-2	10^6	0	4	2	86	2	0	1	0	34	2	0	3	1	90	89	2	94
44AHJD	10^8	10	83	44	61	0	61	0	1	67	68	95	2	1	80	52	0	93
44AHJD	10^6	2	6	15	6	6	0	0	0	0	10	84	0	0	10	1	4	69
RP1	10^8	98	62	1	2	0	2	2	99	97	42	2	11	0	87	99	1	97
RP1	10^6	83	2	0	0	0	0	0	83	65	5	0	0	0	24	0	0	54
PD32-F19-S-1	10^8	0	1	12	48	0	1	0	0	73	10	0	0	0	56	39	0	81
PD32-F19-S-1	10^6	0	2	4	14	0	0	1	0	91	0	0	0	0	6	0	0	92
PD32-F19-S-2	10^8	0	4	34	86	0	0	2	0	76	35	0	1	1	96	84	1	91
PD32-F19-S-2	10^6	0	4	9	81	1	0	0	0	92	5	0	2	1	85	81	2	93
PD32-F14-S	10^8	0	0	3	83	0	0	0	0	79	21	0	0	0	98	91	0	96
PD32-F14-S	10^6	0	0	1	26	0	0	0	0	88	0	0	0	0	74	57	0	88
4018-F19-H	10^8	0	0	1	65	0	0	0	0	86	0	0	0	0	81	76	0	81
4018-F19-H	10^6	0	0	0	78	0	0	0	0	0	0	0	0	0	80	73	0	93
4023-F15-H 107	10^7	0	0	0	53	0	0	0	0	90	9	0	0	1	80	70	0	93
PD17-F16-S-2-S13	10^8	33	95	49	7	4	89	14	87	7	91	99	77	84	98	92	92	98
PD17-F16-S-2-S13	10^6	18	57	33	0	0	6	1	2	0	77	88	23	0	65	53	11	73

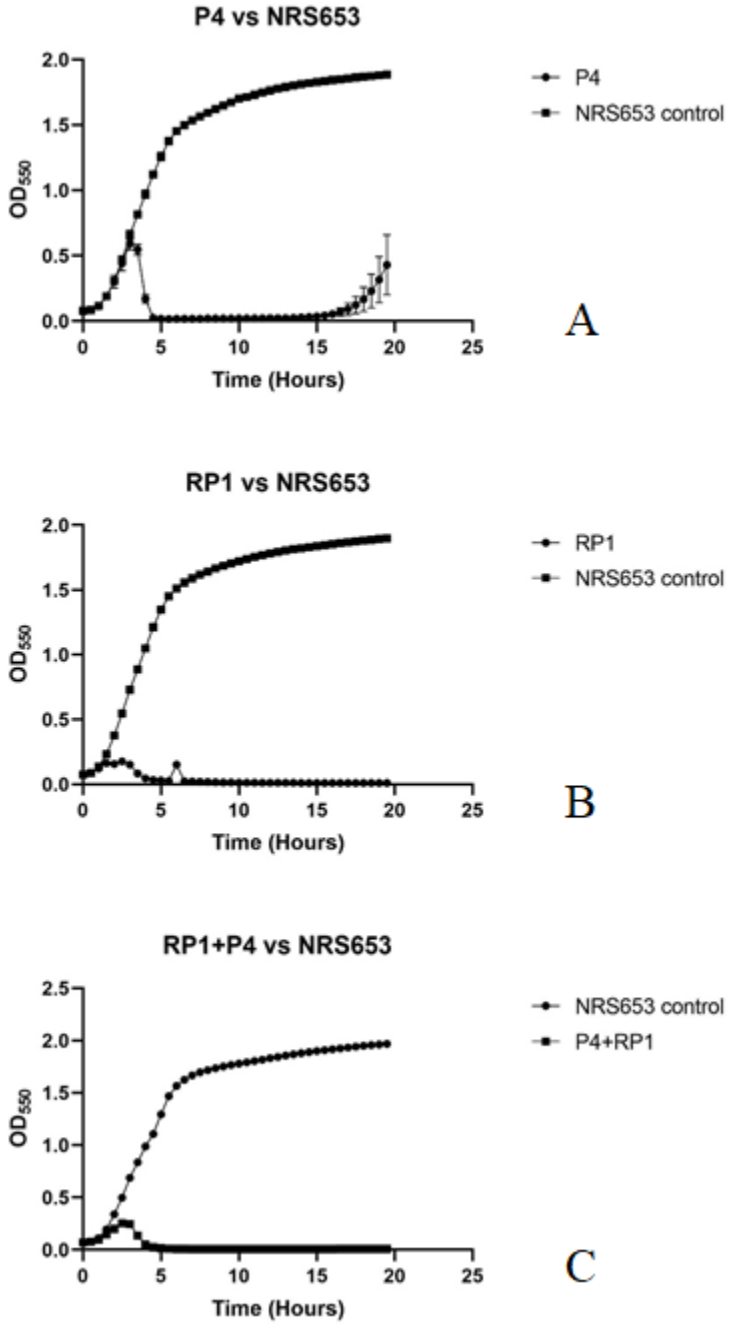

Figure 1. Sample results from liquid culture host range assays using 10^8 PFU/ml of phage. A) Phage P4 vs *S. aureus* isolate NRS653; a resistant mutant of NRS653 starts to regrow at approximately 18 h. B) Phage RP1 vs *S. aureus* isolate NRS653, no regrowth of culture is noted, this phage was found to infect the P4 resistant *S. aureus* mutant. C) P4 and RP1 phage are combined and RP1 is able to overcome the P4 resistant NRS653 mutant, so no regrowth of the culture is observed.

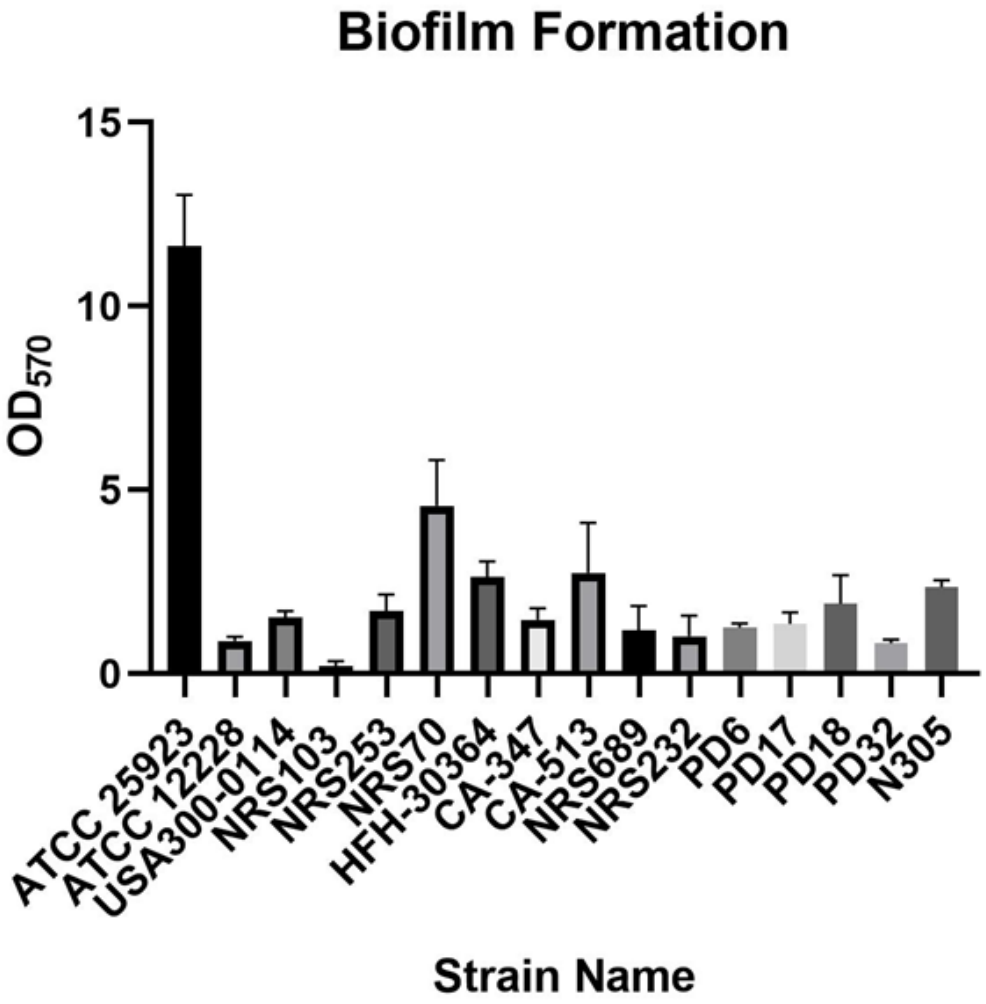

Figure 2. Assays of 24 hour static biofilm formation ability by 16 different *S. aureus* isolates using 1% crystal violet staining. ATCC 25923 is a known *S. aureus* biofilm-positive control, while ATCC 12228 is a known *Staphylococcus epidermidis* biofilm negative strain. Of the panel tested, strain NRS70 shows the highest biofilm forming ability, this strain is a human clinical *S. aureus* isolate.