PREVALENCE AND CHARACTERIZATION OF STAPHYLOCOCCUS AUREUS BACTERIOPHAGES ISOLATED FROM SWINE FARMS ACROSS THE UNITED STATES

A Dissertation

by

ABBY MARIE KORN

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Jason J. Gill
Committee Members,	Sara D. Lawhon
	H. Morgan Scott
	Chad B. Paulk
Head of Department,	Cliff G. Lamb

December 2020

Major Subject: Animal Science

ABSTRACT

Swine farm environments are a reservoir for *Staphylococcus aureus* and therefore may also be a reservoir for *S. aureus* phages. The diversity and ecology of these phages has not been investigated previously. Phage isolated from swine environments could potentially be used to control *S. aureus* in swine environments or used for phage therapy for human *S. aureus* infections. The objective of this work was to 1) determine the prevalence of *S. aureus* phages in swine environments across the United States; 2) isolate and characterize phages by restriction digestion of gDNA, morphological classification and host range; 3) examine the genomic diversity of isolated phages in comparison to each other and well-known *S. aureus* phages.

20 swine farms from across the US were sampled for the prevalence of *S. aureus* phages. One location was sampled in-depth at two different time points, six months apart. Of the 19 farms sampled only once, 12 farms (63%) were positive for phage capable of infecting at least one of the ten *S. aureus* host strains used during enrichment. These enrichment panels included *S. aureus* strains relevant to human health and recently acquired swine nasal isolates from across the US.

Of the virulent phages tested in this study, *Podoviridae* displayed the narrowest host ranges and *Myoviridae*, both K-like and Mars Hill-like, displayed the widest range. Both types of *Myoviridae* were also able to infect strains of *S. pseudintermedius* and *S. epidermidis* although this ability was very strain specific. Overall, twenty-six isolated

phages were sequenced, with four of them having genomes of ~44 kb, seventeen with ~18 kb, one with ~141 kb and four with ~ 270 kb.

The genomic diversity among isolated *Podoviridae* and *Siphoviridae* was low. However, one type of *Myoviridae* with ~ 270 kb genomes (Mars Hill-like) represents a new type of *S. aureus* phage. These results show that *S. aureus* phages are prevalent within swine environments in the US. Additionally, the characterization of these phages provides new insights into the diversity of *S. aureus* phages and the possible application they may have for controlling *S. aureus* in swine environments or for phage therapy in human medicine.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Jason Gill, for taking a chance on me and providing support throughout my graduate career. I would like to thank my committee members, Dr. Lawhon, Dr. Scott and Dr. Paulk for their guidance, support and sense of humor throughout the course of this research. I would like to thank Dr. Hillhouse for being an awesome scientist who was willing to help me finally obtain a genomic sequence for a particularly difficult phage to sequence.

Thanks also go to my friends and colleagues for their support. I would like to thank Denish Piya, Yicheng Xie and Tram Le, specifically, for their friendship and support. My experience in the Gill Lab would not have been the same without them. Also, Chelsea Corson and Amanda Moreland for their constant friendship and support. These two women I know will always answer my phone calls, no matter the time and I can't put into words what their friendship means to me.

I would like to thank my fiancé, Jeremy, for always being able to make me laugh and loving me unconditionally. I would like to thank the Brumley family for adopting me as one of their own and supporting me in so many ways. Finally, I would like to thank my family for always loving and supporting me, even though my life choices led me far away from them. I would not be the person I am today without their love and sacrifices.

CONTRIBUTORS AND FUNDING SOURCES

This work was supervised by a dissertation committee consisting of Drs. Jason Gill (chair) Department of Animal Science, Sara Lawhon, H. Morgan Scott Department of Veterinary Pathobiology and Chad Paulk (Kansas State University) Department of Grain Science and Industry.

Genomic sequencing of bacteriophages was supported by Dr. H. Morgan Scott Department of Veterinary Pathobiology and Dr. Andrew Hillhouse Department of Veterinary Pathobiology.

Bioinformatic analysis and support was provided by the Center for Phage Technology at Texas A&M University. Genomic annotation of phage Portland was completed by Rachele M. Bonasera. Genomic annotation of phage Maine was completed by Russell Moreland. Genomic annotation of phage Sebago was completed by Kathryn Klotz.

Funding Sources

Graduate study was supported by a fellowship from Texas A&M AgriLife Research, an excellence fellowship and Ron Richter scholarship from the Department of Animal Science. This work was also made possible in part by the National Pork Board under grant numbers 16-143, 17-108 and 19-051 as well as the National Science Foundation Advances in Biological Informatics grant number 1565146.

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CHAPTER I

INTRODUCTION

Staphylococcus aureus

Staphylococcus aureus belongs to the genus *Staphylococcus,* which was first described by Scottish surgeon Sir Alexander Ogston in 1880. Ogston observed staphylococci in pus from an abscess and noted that: “the masses looked like bunches of grapes.” Later, in 1884 a physician named Friedrich Julius Rosenbach differentiated species of *Staphylococcus* by the color of the colonies produced by each; *S. aureus* coming from the Latin “*aurum*” meaning gold and *Staphylococcus albus,* meaning white (Licitra, 2013).

S. aureus is a common colonizer of the skin and mucous membranes of humans and many different animal species. While *S. aureus* is considered to be a commensal, it is also an opportunistic pathogen in both humans and animals (Stewart et al., 2017, Tong et al., 2015). *S. aureus* is a gram-positive, non-motile, facultative anaerobe that grows using aerobic respiration or fermentation. Morphologically, *S. aureus* is shaped like cocci that are approximately 0.5 um- 1.0 µm in diameter. These cocci tend to group together to form pairs, tetrads or small clusters.

Diagnostically, *S. aureus* is a coagulase and catalase positive bacteria, however some *S. aureus* may be coagulase negative and these are harder to characterize (Foster 1996). Additionally, *S. aureus* is able to survive in high salt environments and therefore Mannitol Salt Agar can be used to isolate *S. aureus,* as the high salt selects for *S. aureus* and the fermentation of mannitol produces acid, producing a color change in the

medium. The ability to ferment mannitol will distinguish *S. aureus* from *Staphylococcus epidermidis* (Harris and Foster et al., 2002). Another media commonly used to isolate *S. aureus* is Baird-Parker agar which selects for coagulase positive *Staphylococcus* species (Baird-Parker 1962). For everyday culturing of *S. aureus,* blood agar, tryptic soy broth and agar and brain heart infusion media are frequently used.

Methicillin-resistant *S. aureus* (MRSA) and Impact of MRSA

S. aureus is an opportunistic pathogen of both humans and animals. Like many bacteria, *S. aureus* strains associated with human infections were quick to adapt to antibiotic treatment and the prevalence of MRSA increased dramatically as penicillin became the main treatment for *S. aureus* infections. For example, the first isolates of methicillin-resistant *S. aureus* (MRSA) were isolated only two years after the initial clinical use of methicillin in 1960 (Moellering et al., 2012). The spread and expansion of MRSA strains has proved to be a great burden to the health care system (Valiquette et al., 2014).

Even today, β-lactam antibiotics are still used for the treatment of Gram-positive infections, including *S. aureus*. β-lactam antibiotics work by inhibiting the functions of the so-called penicillin binding proteins, which are responsible for construction and maintenance of the peptidoglycan cell wall. The synthesis and linkage of peptidoglycan chains is vital for cell wall integrity, and cells without this function will have a compromised cell wall which renders them susceptible to death by plasmolysis. *S. aureus* employs two main strategies for β-lactam resistance. One is the production of β-lactamases, which are secreted from the cell and degrade β-lactam antibiotics. The other

strategy is to express an allele of the β-lactam target that has less affinity for these drugs (Fuda et al., 2005). In MRSA strains the *mecA* gene encodes an alternate penicillin binding protein termed PBP2a. PBP2a has a broadly reduced affinity for β-lactam antibiotics, therefore MRSA strains are still able to synthesize peptidoglycan normally in the presence of these drugs and persist through antibiotic treatment (Pantosti et al., 2007).

MRSA currently poses a serious risk to human health, with 323,700 cases of MRSA estimated in hospitalized patients annually in the United States (Center for Disease Control, 2019) *S. aureus* is the leading cause of bacteremia, skin, soft tissue and device-related infections (Tong et al., 2015). 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 (Engemann et al., 2003). MRSA infections are mainly divided into three main roots of infection: hospital associated (HA-MRSA), community associated (CA-MRSA) and livestock associated (LA-MRSA).

A study examining the national burden of invasive MRSA infections in the United States in 2011 concluded that the next step in MRSA prevention is to prevent infections from occurring in people who have not had any recent health care exposures (CA-MRSA infections) (Dantes et al., 2013). While MRSA infections in the past were typically associated with hospital infections, community acquired infections are becoming a new concern, especially since CA-MRSA infections are associated with fatalities in healthy individuals (Dukic et al., 2013). Additionally, the need for new

strategies to combat *S. aureus* infections is becoming even more urgent with the development of *S. aureus* that are intermediately or completely resistant to vancomycin (VISA and VRSA). Vancomycin is reserved as one of the only treatments for MRSA infections, so *S. aureus* that are resistant to vancomycin present a huge problem in human healthcare (Loomba et al., 2010).

Classification of *S. aureus*

There are multiple molecular methods used to type *S. aureus* isolates. One method focuses on the *spa* gene which encodes protein A. The repeat regions in the *spa* gene are used to assign *S. aureus* isolates a *spa* type as this region is prone to mutation as well as loss and gain of repeats (Harmsen et al., 2003). Multilocus sequence typing (MLST) is based on the comparison of ~450 bp internal sequences from seven housekeeping genes. The most commonly used genes for *S. aureus* include: carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*), acetyl coenzyme A acetyltransferase (*yqiLI)* (Enright et al., 2000). A study by O'Hara et al., in 2016 found that *spa* types and MLST types had similar discriminatory power, with spa typing being slightly higher. Another method used for *S. aureus* typing is pulsed-field gel electrophoresis (PFGE). This method uses restriction enzyme *SmaI* to digest *S. aureus* genomic DNA and a very specific type of agarose gel which is run on a contour-clamped homogenous electric field (CHEF) capable device. The results from this gel are photographed and compared to reference *S. aureus* pulse-field types (PFT). The naming system for isolates typed this way will be displayed as "USAX000" and example of this

is the USA300 *S. aureus* PFT (McDougal et al., 2003). While PFGE is used for *S. aureus* typing, many of the other methods are used instead, as this method is both time consuming to run as well as interpret (Tenover et al., 2009). It is important to note that members of *S. aureus* ST398 are not typeable using this method (Monaco et al., 2017). Another method used for typing MRSA isolates is by sequencing the staphylococcal cassette chromosome *mec* element (SCC*mec*). This element carries two important gene complexes: the *mec* gene complex (includes *mecA*, responsible for methicillin resistance) and the *ccr* gene complex. SCC*mec* types are assigned by the type of *mec* and *ccr* gene complexes (Chongtrakool et al., 2006).

The most common HA-MRSA lineages in North America are USA100, USA500 and USA200. For CA-MRSA, USA400 was the most prevalent clone in the US until about 2001, when USA300 became the most prevalent clone, this clone is responsible for a large portion of skin and soft tissue infections (SSTI) as it carries genes for the Panton-Valentine leukocidin (PVL) and the arginine catabolic mobile element (ACME) (Monaco et al., 2017). While ST398 has been the dominant LA-MRSA clone in European countries, recent surveys in the US showed that while ST398 is present, ST5 and ST9 are also prevalent MLST types found but most of these are MSSA not MRSA (Smith et al., 2018). Both MSSA and MRSA ST398 is a common colonizer of pigs and infections in pigs from this sequence type are rare (Aires-de-Sousa 2017). However, for dairy cattle, MSSA and MRSA often cause mastitis with MLST type CC97 being the most common type isolated from dairy cattle worldwide (Spoor et al., 2013).

S. aureus and MRSA in the Swine Industry

Over approximately the last fifteen years researchers have begun to quantify *S. aureus* carriage in swine production workers and farmers. Carriage of *S. aureus* in the general public in the continental US ranges from 26% to 32% (Sivaraman et al., 2009). An estimated 1.3% of that *S. aureus* being MRSA (Salgado et al., 2003). 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 (Neyra et al,.2014;Wardyn et al., 2015). MDRSA is defined by an *S. aureus* isolate that demonstrates resistance to three or more classes of antimicrobials (Magiorakos et al., 2012). The presence of MRSA in livestock is not a new phenomenon with one of the earliest reports being from dairy cow milk in 1972 (Devriese et al., 1972). However, swine farming environments in particular seem to provide a unique reservoir for *S. aureus*.

A study by You et al. in 2018 of 76 typable *S. aureus* isolates in North Carolina found that *S. aureus* isolates from hog workers were more likely to have more than two antibiotic resistance genes when compared to *S. aureus* isolates from community members. Specifically, the prevalence of the penicillin resistance gene *blaZ* was higher with 84.2% of worker's *S. aureus* isolates vs 54.2% of community members. Additionally, a study conducted by Parisi et al., (2019) sampling both swine and farmers at 85 swine farms and 85 abattoirs in Italy via nasal swab found that 59.1% of the pigs were positive for MRSA while 17.3% of the farmers were positive for MRSA.

The epidemiology surrounding the transfer of *S. aureus* from swine to humans and vice versa is not well understood. However, one study conducted by Price et al., in 2012 comparing 89 isolates of *S. aureus* CC398 from livestock and human origin strongly suggests that LA-MRSA CC398 was originally MSSA in humans and then later acquired methicillin resistance in food animal production environments. The authors note the livestock CC398 isolates appear to have lost phage-carried human virulence genes but acquired tetracycline and methicillin resistance. The incidence of LA-MRSA ST398 in humans can be seen as a reestablishment, as humans were the original host (Price et al., 2012).

While there have been few recorded human infections from ST398 LA-MRSA, there have been several cases that had severe consequences, such as a case of lethal necrotizing pneumonia and several cases of bacteremia (Rasigade et al., 2010; van Belkum et al., 2008). 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 (Valentin-Domelier et al., 2011;van der Mee-Marquet et al., 2011).

As with humans, *S. aureus* is considered to be part of the normal bacterial flora of swine and normal carriage is asymptomatic. While nasal swabs have been the most common anatomical site sampled to assess *S. aureus* prevalence in a swine herd, tonsils and skin samples are also likely to have similar prevalence of *S. aureus.* Swine can be

colonized with multiple *spa*-types in these different anatomical sites (Linhares et al., 2015).

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 belonging to MLST lineages ST9, ST398 or ST5. Additionally, for 21 out of 35 herds the prevalence of *S. aureus* exceeded 80%. However, despite this general prevalence of *S. aureus,* 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 and confined to particular regions or operations (Sun et al. 2015).

In the Netherlands, MRSA prevalence among swine herds is estimated to be much higher than the US at approximately 69% (Broens et al., 2011a). An interesting difference between North American and European *S. aureus* carriage in swine is the most commonly seen sequence types among herds. European studies, particularly the Netherlands, mainly report *S. aureus* isolates of sequence type ST398 in swine herds and this is considered to be the main LA-MRSA that is a risk to the public in those systems (Broens et al., 2011b, Denis et al., 2009). However, in the United States the five most frequent sequence types of *S. aureus* isolated from swine environments are ST398, ST9, ST5, ST8 and ST30 (Smith et al., 2018). The ST9 lineage is seen in swine herds in Asia (Smith 2015) and is a common sequence type seen in Chinese swine herds. *S. aureus* ST5 lineage is considered to be a hospital-associated lineage that is seen in hospitals

around the world (Takano et al., 2013). Therefore, it is concerning to see that ST5 is present among US swine herds. A SNP analysis of 82 LA-MRSA swine associated isolates vs 72 human clinical ST5 MRSA isolates from the US found that all clinical isolates with no recorded swine contact with the exception of one did not cluster with LA-MRSA isolates. Suggesting that LA-MRSA swine isolates are phenotypically unique. However, the authors noted the next step is to examine hospital ST5 isolates where swine production is prevalent to determine if swine environment isolates are phenotypically unique in that setting as well (Hau et al., 2018).

Certain activities on swine farms such as pressure washing and tail docking generate particle sizes capable of depositing primarily in human upper airways but also the primary and secondary bronchi as well as terminal bronchi and alveoli (Madsen et al., 2018). LA-MRSA isolates have been found to have a half-life of five days in settled barn dust with an approximate die-off of 99.9% after 66-72 days (Feld et al., 2017). Therefore, mitigation of MRSA in swine production facilities would be of benefit to farmers and workers for safety reasons, as MRSA isolates are able to persist and possibly spread throughout the environment and workers. While elimination of *S. aureus* colonization 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 (Cleef et al., 2015). 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.

Additionally, another application that a product to combat MRSA might have is to decontaminate swine housing before a tested MRSA negative herd is moved into the housing. To maintain MRSA-free swine herds vertical integration has been suggested, as well as making sure that when bringing new animals onto a farm everything down to the transport vehicles are MRSA free (Grøntvedt et al., 2016). Therefore, a MRSA targeting product could be useful in decontaminating buildings and other production equipment.

Biofilm Formation of *S. aureus*

Currently, *S. aureus* and *S. epidermidis* are opportunistic pathogens that are frequently associated with biofilms on medical implants such as catheters, orthopedic or other implants such as breast implants (Chessa et al., 2016). The ability of *S. aureus* to attach to prosthetic devices is mediated by the MSCRAMM family of bacterial proteins such as fibronectin-binding protein FnBPA. This protein allows *S. aureus* to bind to fibronectin which coats newly implanted medical devices (Tong et al., 2015). Approximately four out five cases of implant infections are caused by staphylococci, with two-thirds of those infections being caused by *S. aureus* or *S. epidermidis* (Oliveira et al., 2018). From the infected patient's perspective, this could add many monetary costs, as well as the possible removal of the device, or amputation.

A biofilm is composed of surface-associated microbial cells that are encased in an extracellular polymeric matrix (Donlan 2002). There are four generic stages of biofilm formation: 1.) bacterial attachment, 2.) microcolony formation, 3.) biofilm maturation and 4.) detachment and dispersal (Figure 1) (Crouzet et al., 2014). Biofilm formation provides bacteria with protection from a hostile environment, host defenses

and antibiotics. In particular, persister cells are a small subpopulation of the bacterial cells in a biofilm that are characterized by their tolerance to high antibiotic concentrations and a non-growing state (Bigger 1944). While these cells are nondividing cells, transcriptome data indicates that stress responses such as the SOS response are triggered. Persister cells are able to return to a normal, growing, antibiotic susceptible phenotype once the antibiotic pressure has been removed (Peyrusson et al., 2020).

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 (*ica* for 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 (Archer et al., 2011). In contrast, PIA-independent biofilms rely on the major autolysin protein Alt, fibronectin binding protein FnbpA and extracellular DNA (eDNA) to form biofilms. An important recent observation is that MSSA strains are more likely to form PIA-dependent biofilms while MRSA strains are more likely form PIA-independent biofilms (McCarthy et al., 2015). Overall, the structure of the biofilm and protection it provides from antibiotics these biofilms provide can make it is very hard to treat and eliminate biofilms successfully (Costerton et al., 1999).

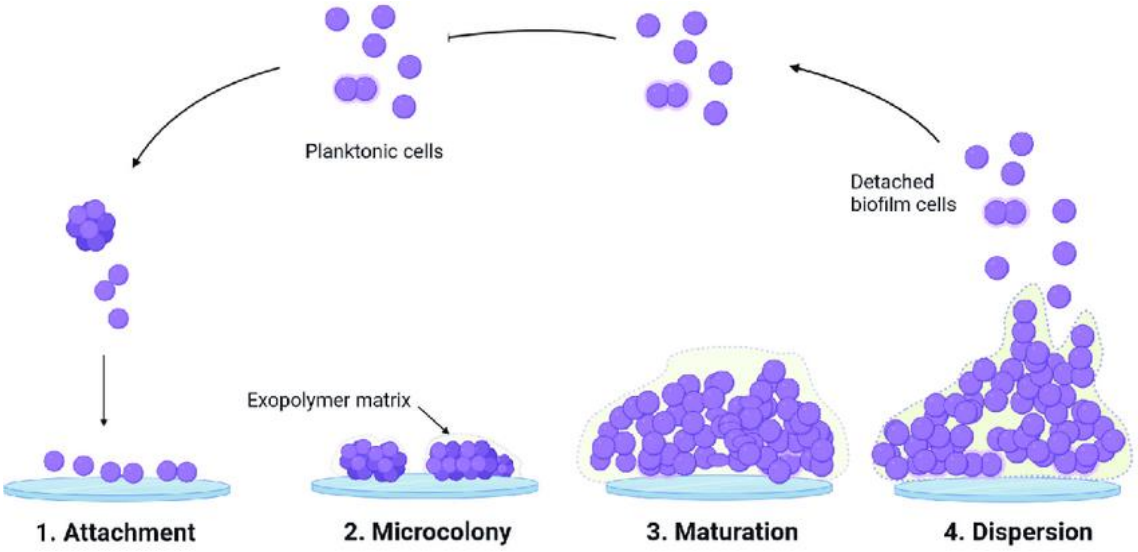

Figure 1. Bacterial biofilm formation steps. Reprinted from Carneiro et al., 2020.

Bacteriophages and *S. aureus* Phages

Bacteriophages (phages) are viruses that infect bacteria and are the most abundant organisms on Earth with an estimated 10^{31} in the biosphere (Comeau et al., 2008). Phages play an important role in bacterial biology as well as microbial diversity and evolution in the biosphere (Clokie et al., 2011). Bacteriophages were discovered independently by Frederick Twort in 1915 and by Felix d'Herelle a few years later in 1917. d'Herelle would be the one to coin the term "bacteriophage" which literally means "bacteria-eater" (Twort 1915;D'Herelle 1917;Salmond and Fineran 2015).

There are two known life cycles a phage can pursue: lytic or lysogenic (Figure 2). In the lytic life cycle a phage adsorbs to a bacteria, it then ejects 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 and eject its DNA but the expression of the phage lytic cycle will be repressed and the phage chromosome will exist as a prophage, integrated into the bacterial chromosome or as its own entity such as a plasmid. Either way, the phage DNA will replicate each time the bacterial DNA is replicated and may excise themselves and re-enter the lytic cycle in response to stress signals from the host (Campoy et al., 2006). There are many different types of phage, from double stranded DNA to single stranded RNA phage. The size of a phage genome can be very variable, some can be small, such as ssRNA phage MS2 which is only 3.2kb, while others can be extremely large, like dsDNA *Bacillus* phage G1 which has a 500kb genome (Salmond and Fineran 2015).

Tailed phages are members the order *Caudovirales* which comprise 96% of all described phages. *Caudovirales* is divided into three different morphologies: *Podoviridae* which have short noncontractile tails, *Siphoviridae* which have long noncontractile tails and *Myoviridae* which have long contractile tails (Ackermann 1998).

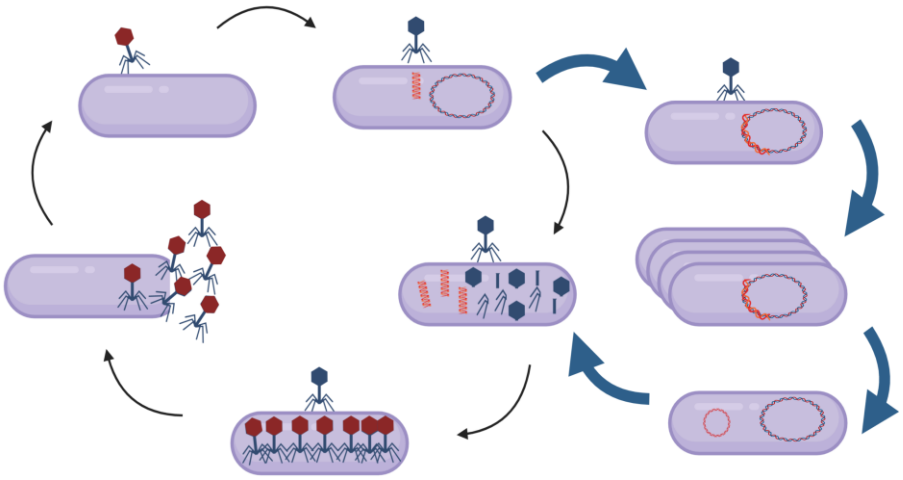

Figure 2. The two life cycles a phage can pursue.
A strictly lytic life cycle is indicated by the black arrows and a lysogenic life cycle is shown by the thick blue arrows, these phages can also pursue the lytic cycle.

All known staphylococcal phages belong to *Caudovirales*. Historically, *S. aureus* phages were characterized into serogroups, which were based on the results of phage neutralization by antisera from rabbits which had been injected with specific phage filtrates prior (Rountree 1949, Rippon 1952, Rippon 1956). Currently, in *S. aureus* phages, there are three different genome size classes seen and each corresponds to a certain morphological group. The three classes of size with their corresponding morphology are: class I: < 20 kb *Podoviridae*, class II: ≈ 40 kb *Siphoviridae* and class

III: >125 kb *Myoviridae* (Kwan et al., 2005). The genomes of *S. aureus* phages are arranged into different modules for each morphology type (Figure 3) (Deghorain et al., 2012).

In *S. aureus*, lytic phages belonging to the morphogenic classification of *Myoviridae* have an icosahedral head with a long contractile tail and possess the largest genomes known in *S. aureus* phage. Twort-like phages belong to the *Spounavirinae* subfamily of myoviruses and have all of characteristics listed above as well as a baseplate at the end of their tail. The canonical member of Twort-like phages for *S. aureus* is phage K, a large, polyvalent myophage. (Figure 4).

In contrast, *S. aureus* phages belonging to the *Podoviridae* group have an icosahedral head with a short non-contractile tail and have the smallest genomes. Most *S. aureus Podoviridae* phages show homology to 44AJHD phage and *Bacillus* phage phi29 (Lobocka et al., 2012) Lastly, *S. aureus Siphoviridae* have an isocahedral head but have

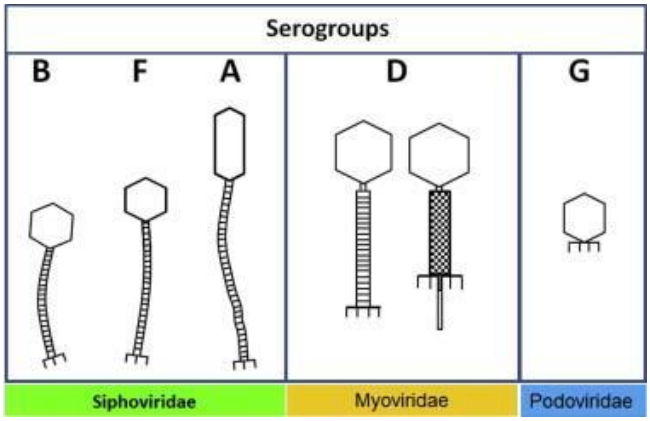

Figure 3. Common serotypes and morphology of *S. aureus* phage. Reprinted from Xia and Wolz 2014.

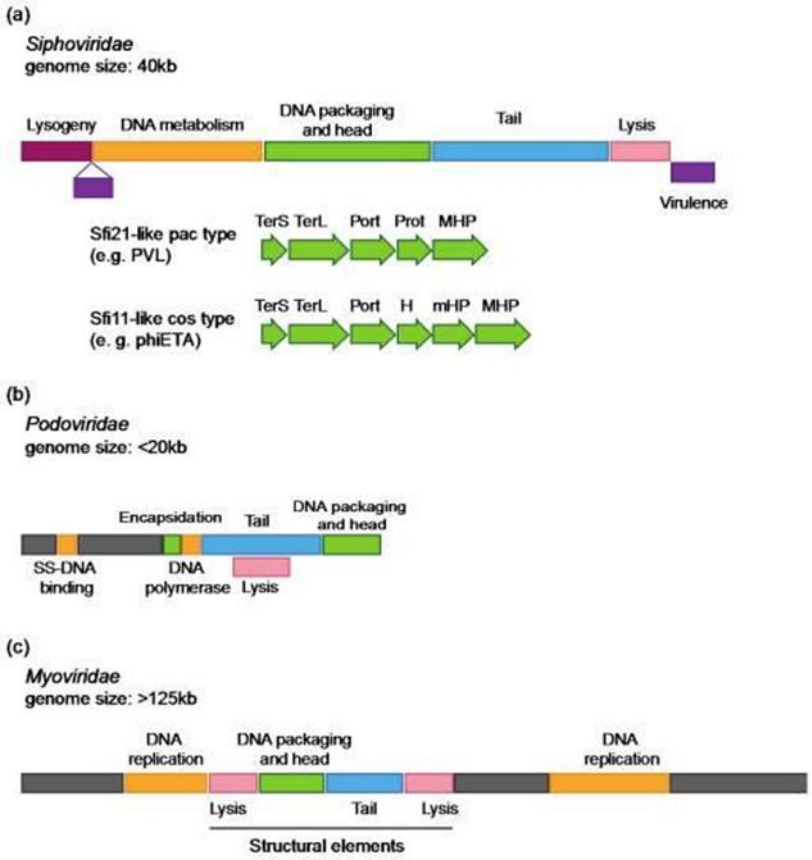

Figure 4. Size of genome and organization of modules for each morphological *S. aureus* phage type. Reprinted from Deghorain and Melderen 2012.

a non-contractile long tail with a baseplate at the end. These phages have and are in some cases still used to type *S. aureus* strains (Barber and Whitehead 1949, Paul-Satyaseela et al., 2011). For the typing of a strain, a collection of phages, sometimes in

excess of 24 *Siphoviridae* lysates would be spotted onto the lawn of *S. aureus* that needed to be typed (LOCATCHER and GUTIERREZ 1960). All *S. aureus Siphoviridae* characterized to date are temperate, so while useful for the typing of *S. aureus* strains and other applications, they are considered unsuitable for phage therapy (Deghorain et al., 2012).

All three types of *S. aureus* phages use different parts of the *S. aureus* wall teichoic acid (WTA) as a receptor. In general, Twort-like phages adsorb to the backbone of the WTA with a possible unknown secondary receptor, *Siphoviridae* use the α-glyceryl-GlcNAc modifications on WTA and 44AHJD-like *Podoviridae* use β-glyceryl-GlcNAc modifications on WTA. *S. aureus* strains lacking WTA are insensitive to phage infection by all *S. aureus* phage types. (Lobocka et al., 2012, Xia et al., 2011, Moller et al., 2019).

Strictly lytic phages are considered to be the best option for therapeutic applications, as lysogenic phages present safety concerns with their ability to integrate into the host genome and possibly disseminate bacterial DNA (Gill and Hyman 2010). For the *S. aureus* phages, lytic phage of serogroups D and G are of most interest for therapeutic use (Figure 4); other serogroups of *S. aureus* phages were commonly used to type strains o*f S. aureus* for clinical usage, however these phages are lysogenic (Lobocka et al., 2012).

Phage Therapy

Phage therapy — or the treatment of bacterial infections with phage — is not a new concept. Since their initial therapeutic application by D'Herelle in 1917 to treat

dysentery in soldiers there has been interest in using phage to treat common human and animal bacterial infections (Chan et al., 2013). However, with the mass production of antibiotics in the 1940's, the interest in phage therapy declined. Phage therapy research persisted in what was then the Soviet Union, but the volume of phage therapy research declined in many other areas of the world. Currently, with the rise of many multi-drug resistant bacteria and the lack of new antibiotics being produced there has been a renewed interest in phage therapy and the place it could have in Western medicine (Nobrega et al., 2015).

Phage as Agents Against *S. aureus*

In theory, the best suited type of *S. aureus* phage for combating *S. aureus* in an applied manner is the Twort-like group of phages, as they are strictly lytic and typically have a wide host range. Additionally, their wide host range is very appealing for therapy usage as one phage could potentially infect and be used on many different *S. aureus* strains (Lobocka et al., 2008). Also of interest, are small virulent 44AJHD-like *Podoviridae* as these phages are also strictly lytic, but generally have a more limited host range than the Twort-like phages.

Many different studies have recently been conducted using *S. aureus* lytic phages for phage therapy. Studies using *S. aureus* phages showed that certain phage were able to prevent abscesses in mice and rabbits (Wills et al., 2005;Capparelli et al., 2007) and treat diabetic foot ulcers in mice and humans, which was equilibrated to treatment with linezolid in one study (Chhibber et al., 2013; Fish et al., 2016). Also, to

rescue mice after a lethal dosage of *S. aureus* was delivered IP to mice (Matsuzaki et al., 2003).

Another area of interest is treating *S. aureus* biofilms. Because *S. aureus* biofilms are able to persist through antibiotic treatment it is important to come up with new strategies to combat these biofilms. The largest issue brought up with phage treatment of any bacterial biofilm is that it is thought that most phage do not replicate well in stationary phase bacteria (Kadavy et al., 2000). However, this does not mean that phage cannot have an impact on a newly formed biofilm or be used to prevent the bacterial microcolonies from forming a more robust biofilm on newly implanted devices or on exposed bone structures. Additionally, phage progeny could be disseminated throughout the body to find bacteria that have been dispersed from the biofilm (Abedon 2016).

While phage treatment of a biofilm is certainly challenging, there are several studies that have reported that phage treatment has resulted in significant reduction of biofilms. Phage treated biofilm studies have been conducted in *Pseudomonas fluorescens* (Sillankorva et al., 2008), *S. epidermidis* (Cerca et al., 2007) and *Escherichia coli* (Lu et al., 2007) among others. In *S. aureus* specifically, there has been great promise shown in reducing biofilms via phage treatment (Kelly et al., 2012;Lungren et al., 2014; Son et al., 2010;Gutiérrez et al., 2012) with all studies results showing that *S. aureus* phage treatment can reduce *S. aureus* biofilm mass both in vitro and in vivo models. A problem with phage treatment of *S. aureus* biofilms addressed by Drilling et al., 2014 was the appearance of phage resistant *S. aureus* mutants at a frequency 10^{-7}, this problem was overcome by using a cocktail of selected phages instead of applying phage individually.

Overcoming Phage Resistant Bacteria

The constant arms race between bacteria and phages means that there are many different defense strategies that have evolved in both bacteria and phages to defend one from the other. To defend themselves against phage infection bacteria can prevent phage attachment, block DNA entry, employ restriction-modification systems as well as abortive infection, interfere with phage assembly and most likely many more strategies that have yet to be discovered (Seed, 2015). To avoid these bacterial defense strategies phages have adopted several strategies, such as changing the surface receptor they use, having no restriction sites in their DNA, using enzymes to degrade EPS and using toxin and antitoxin systems (Hazan et al., 2001). As stated above, it is common to see phage-resistant mutants appear during application and this problem can be solved by using a phage cocktail in which you know that the resistant mutant is sensitive to another phage. Secondly, if no other phage is available, the original phage can be plated onto the phage-resistant strain to possibly isolate new mutant phage that can infect the resistant strain.

CHAPTER II

ISOLATION AND CHARACTERIZATION OF *S. AUREUS* BACTERIOPHAGES FROM SWINE PRODUCTION FACILITIES ACROSS THE UNITED STATES

Introduction

Bacteriophages (phages) are the most abundant organism on Earth (Comeau et al., 2008). Phages occupy many different niches on Earth from extreme environments, such as deep-sea hydrothermal vents, to the human gastrointestinal tract (Castelán-Sánchez et al., 2019; Sutton and Hill 2019). Phages can be isolated from both solid and liquid samples and these samples are commonly enriched to further amplify phage of interest from the sample (Van Twest and Kropinski 2009). To isolate phage that infect a particular bacterial species, or host, it is advised to collect samples in which the bacterial host is usually present in abundance (Hyman 2019). Historically, in isolating *S. aureus* phages, untreated sewage is one of the most common sample types that scientists attempt to isolate phages from (Synnott et. al., 2009). One study conducted by Plano et al. in 2010 found that participants bathing for 15 minutes in marine water shed an average of 10^5 to 10^6 CFU of *S. aureus* per person. Therefore, it is easy to imagine how *S. aureus* would be abundant in sewage waters and why sewage is commonly used when sampling to isolate *S. aureus* phages. Another commonly used method to isolate *S. aureus* phages is through inducing resident prophages in an *S. aureus* strain. These phages are lysogenic in nature as they have the ability to integrate and persist in the host genome or pursue the lytic cycle should the bacterial cell be stressed. Each *S. aureus* strain is estimated to have between one and four resident prophages in its genome (Moller et al., 2019). These

phages are most commonly isolated from *S. aureus* cells by induction with UV light, antibiotics or mitomycin C to stress the cells and induce the phage (Wagner and Waldor 2002; Goerke et al., 2009).

All previously described *S. aureus* phage can be separated into three morphological groups: *Podoviridae*, *Siphoviridae* and *Myoviridae* (Xia and Wolz 2014). Thus far, in *S. aureus* phages, all reported *Podoviridae* and *Myoviridae* are lytic phages and *Siphoviridae* are lysogenic. The *Podoviridae* have the smallest genomes at approximately 18kb, *Siphoviridae* have genomes of ~40kb and *Myoviridae* have the largest genomes at an approximate 130kb in length. (Lobocka et al., 2012). In general, *S. aureus Podoviridae* share protein homology with *Bacillus* phage ϕ29 and two well characterized *S. aureus Podoviridae*, 44AHJD and P68 (Lobocka et al., 2012, Vybiral et al., 2003). *Siphoviridae* are categorized into multiple groups and are more diverse than the reported *Podoviridae* and *Myoviridae*. This diversity can be attributed to several factors, as *S. aureus Siphoviridae* are lysogenic and have the opportunity to integrate into the host chromosome which enables them to potentially engage in horizontal gene transfer (HGT). Additionally, as these phages are able to excise from the host chromosome and then pursue the lytic cycle to then infect a new strain of *S. aureus* this makes them a major vector for HGT (Dion et al., 2020). Lastly, the described *S. aureus Myoviridae* are members of the Twort-like phages. The best characterized of this group is phage K, which is a lytic phage with a genome of ~140kb (Gill, 2014). While these phages do share some similarity with the original phage Twort, phages belonging to this

group are more homologous to phage K and therefore the subgroup Kayvrius was created.

In the current study, *S. aureus* phage prevalence was surveyed from swine farm environmental swabs collected from farms across the US. The intent of this study was to assess not only the prevalence of these phages, but also the diversity of *S. aureus* phages isolated in comparison to known *S. aureus* phages. Additionally, the potential these phages could have as an antimicrobial was assessed using a microtiter 96 well plate method developed by Xie et al., 2018 to measure the host range and virulence of select phage. The knowledge gained from this study adds greatly to what is known about the diversity of *S. aureus* phages and also suggests several different phages which could be used in the future to help decontaminate swine farms of *S. aureus*.

Methods and Materials

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 (Kropinski et al., 2009) 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. After subculturing the phage, parent stocks of each phage were made by the confluent plate lysate method (Adams, 1959). Each phage was propagated on its original isolation host and harvested

from the agar overlay with 4-5 mL of lambda diluent (100 mM NaCl, 25 mM Tris-HCl pH 7.4, 8 mM $MgSO_4$, 0.01% w/v gelatin). Phage lysates were vortexed, sterilized by passage through a 0.2 µm syringe filter (Millipore) and stored in the dark at 4 ºC.

Formation of mixed S. aureus *panels for phage enrichment*

To test for active prophages in each *S. aureus* strain a single colony from each strain was inoculated into 2 ml of TSB which was incubated aerobically overnight at 30 ºC. After 18-24 hours the cultures were centrifuged at 13,000 RPM for two minutes and 750 µl of the supernatant was removed. Chloroform was added to the supernatant and samples were briefly vortexed and allowed to sit for five minutes. The samples were centrifuged again at 13,000 RPM and 500 µl of the supernatant was removed, avoiding the chloroform pellet. This supernatant was spot titered onto lawns of *S. aureus*. A single plaque formed on the test lawn would be interpreted as the sampled strain having an active prophage which could infect the other *S. aureus* strain. Only strains in which the test results were negative in triplicate would be combined for mixed enrichment.

Phage Portland

Thirty-two environmental swabs were collected from a swine farm located in Kansas, USA. Swabs were sourced from drainage, lagoon, barn and gutter areas of the farm with particular interest paid to areas where biofilms and or sediment had collected. These swabs were transported to Texas A&M University and upon arrival each swab was aseptically clipped into a 50 ml conical tube (BD Falcon). Swabs were shaken with 5 ml TSB for 1.5 hours at room temperature. All samples were then filter sterilized (0.2 µm, Millipore) into a 15 ml conical tube (BD Falcon) and stored at 4 ºC. Swab eluate (4

ml) was inoculated with 20 µl of a prepared inoculum containing seven *S. aureus* strains: USA300-0114, HT20020371, HT20020354, CA-513, 22S, 2N and 5T (Table 1). Enrichment inocula were prepared by mixing equal volumes of seven overnight *S. aureus* cultures. These test tubes were incubated at 30 ºC aerobically overnight. An aliquot if enrichment culture was sterilized with 2% (v/v) chloroform and centrifuged at 13,000 x g. After centrifugation 500 µl of the supernatant was removed and 10 µl of each sample was spotted onto the appropriate *S. aureus* host bacterial lawn. These TSA plates were incubated at 30 ºC overnight. A sample was considered "positive" if one phage plaque or more was observed on the bacterial lawn.

Phage Maine

At a Texas abattoir, post-mortem skin and nasal swabs were taken from five different hogs. The samples were eluted in 30 ml TSB with shaking at room temperature for two hours. The samples were centrifuged at 8,000 x g at 4 °C for 10 minutes and the supernatant was sterilized by passage through a 0.2 µm syringe filter (Millipore) and stored at 4 ºC. Enrichment inocula were prepared by mixing equal volumes of four overnight *S. aureus* cultures: USA300-0114, HT20020371, HT20020354 and CA-513 (Table 1). Enrichment cultures were composed of either 20 or 25 ml of sample supernatant in a 250 mL flask and inoculated 1:100 with the appropriate *S. aureus* inoculum. Enrichments were incubated overnight in a table top shaker at 30 °C 160 RPM. The next day, 10 ml of the enrichment culture was centrifuged at 8,000 x g at 4 °C, for 10 minutes and the supernatant was sterilized by passage through a 0.2 µm

Table 1. *S. aureus* strains used in this study.
For nationwide swine farm survey, four human clinical isolates representing the first enrichment panel and swine nasal isolates indicated by the black border included for the second enrichment panel used for isolating *S. aureus* phages.

Strain No.	Strain Name	ST	CC or PFGE	SCCmec	Geographic Region	Isolation source
NRS384	USA300-0114	8	USA300	IV	United States, Mississippi	Human Clinical
NRS255	HT20020371	80	80	IV	France	Human Clinical
NRS253	HT20020354	398	398	NONE	France	Human Clinical
NRS653	CA-513	5	USA800	IV	United States, California	Human Clinical
N/A	PD6	9	N/A	NONE	United States, Illinois	Swine Nasal
N/A	PD10	398	N/A	YES	United States, Iowa	Swine Nasal
N/A	PD17	398	N/A	NONE	United States, Texas	Swine Nasal
N/A	PD18	9	N/A	NONE	United States, North Carolina	Swine Nasal
N/A	PD19	5	N/A	NONE	United States, North Carolina	Swine Nasal
N/A	PD32	9	N/A	NONE	United States, Alabama	Swine Nasal
N/A	22S	N/A	N/A	YES	United States, Kansas	Swine Skin
N/A	2N	N/A	N/A	YES	United States, Kansas	Swine Nasal
N/A	5T	N/A	N/A	YES	United States, Kansas	Swine Tonsil

syringe filter (Millipore) and stored at 4 °C. 10 µl of each enrichment was then spotted onto lawns of the four *S. aureus* host strains. These TSA plates were incubated at 30 °C overnight. A sample was considered positive if one phage plaque or more was observed on a *S. aureus* lawn after incubation.

Phage Mars Hill

Five barn floor and pen environmental swabs were collected from a Texas swine farm. These individual swabs were aseptically clipped into a 50ml Falcon ® tube to and eluted in TSB with shaking for 2 hours at room temperature. Afterwards, the sample was centrifuged at 8,000 x g at 4 °C, for 10 minutes and the supernatant was sterilized by passage through a 0.2 µm syringe filter (Millipore). Enrichment inocula was prepared by mixing equal volumes of four overnight *S. aureus* cultures: USA300-0114,

HT20020371, HT20020354 and CA-513 (Table 1). Enrichment cultures were composed of a pooled 12 ml of supernatant from all swabs, 8 ml of TSB in a 250 mL flask, inoculated 1:100 with the appropriate *S. aureus* inoculum. Enrichments were incubated overnight in a table top shaker at 30 °C 210 RPM. The next day, 8 ml of the enrichment culture was centrifuged at 8,000 x g at 4 °C, for 10 minutes and the supernatant was sterilized by passage through a 0.2 µm syringe filter (Millipore) and stored at 4 ºC. 10 µl of each enrichment was then spotted onto lawns of the four *S. aureus* host strains. These TSA plates were incubated at 30 °C overnight. A sample was considered "positive" if one phage plaque or more was observed on the bacterial lawn.

Collection and transportation of survey samples

Samples for this study were collected from volunteer participant sites distributed across the continental United States from July 2016 through May 2017. Participants received a Styrofoam cooler, freezer packs, gloves, swabs and an instructional packet on how to collect samples. The instructions detailed that participants should identify areas with visible residue within the barn they were sampling, such as in between slatted floors and on top of water lines. Once a site was identified the participant broke the seal on a sterile BD BBL™ swab containing liquid Stuart's media (BD BBL™, VWR Scientific), wetted the swab with the sponge in the tube and then collected the sample by swabbing approximately a 2x2 inch area. Each swab collected was labeled with the type of site the participant collected it from. These swabs were then shipped overnight in the same provided cooler on ice to Texas A&M University. The participants were also asked to

answer five questions regarding the number of animals and type of operation being sampled.

Processing of survey environmental samples

The five environmental swabs from each site were pooled by clipping the swab heads into a 50 ml Falcon ® tube containing 30 ml sterile TSB. The sample was eluted by shaking at room temperature for 2 h, followed by centrifugation at 8,000 x g at 4 °C for 10 minutes followed by sterilization of the supernatant by passage through a 0.2 µm syringe filter (Millipore). This sterilized sample was then divided and enriched with two different panels of *S. aureus* strains in a mixed-strain enrichment approach {Gill, 2003 #1304}. One enrichment panel consisted of a mixture of four human-associated *S. aureus* isolates and the other contained six swine-associated isolates as shown in Table1. Enrichment inocula were prepared by mixing equal volumes of *S. aureus* cultures that had been adjusted to OD_{550} of ~0.5 as described above. Enrichment cultures were composed of 10 ml of swab eluate and 10 ml of TSB in a 250 ml flask, inoculated 1:100 with the appropriate *S. aureus* inoculum. Enrichments were incubated overnight at 30 °C with aeration. After incubation, a 10 ml aliquot of each enrichment was centrifuged at 8,000 x g at 4 °C for 10 minutes after which the sample was sterilized by passage through a 0.2 µm syringe filter (Millipore) and stored at 4 ºC. Enriched samples were screened for the presence of phage by spotting 10 µl aliquots onto soft agar lawns inoculated with each individual *S. aureus* strain used for enrichment; samples were scored as positive for phage if they produced clearing zones or visible plaques on a lawn of any *S. aureus* host. Phages were isolated from phage-positive samples by dilution and

plating to lawns of *S. aureus* followed by picking of well-isolated plaques. Each phage was subcultured three times to ensure clonality.

In-depth survey sampling

30 swabs were collected from a single site (Farm 16) at recorded sites within a barn using the same methodology as described above, except that swab samples were not pooled. Swabs were individually clipped into 15 ml Falcon ® tubes and eluted in 5 ml TSB, filter-sterilized and enriched against the swine-derived *S. aureus* host panel in a total volume of 4 ml, and then screened for the presence of phage as described above. This sampling was repeated six months later at the same recorded sample sites as the first.

Phage genomic DNA extraction

High titer (>10^8 PFU/mL) stocks of each phage were produced as described above and gDNA was then extracted from 10-20 mL of phage stock. To extract gDNA, a nuclease solution (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 5 mM $MgSO_4$ 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'), EcoRI-HF (5' GAATTC 3') (New England BioLabs). gDNA samples that showed indistinct banding patterns or could not be digested by the enzymes listed above were then digested with TaqαI (5' TCGA 3'). For DraI and EcoRI-HF 300 ng of gDNA from each phage was incubated with each enzyme and CutSmart ® Buffer individually at 37 °C overnight. For gDNA treated with TaqαI, 300 ng of gDNA was incubated with TaqαI and CutSmart ® Buffer at 65 °C for two hours. 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 2.5 h.

Microtiter plate host range assay

Phage stocks were adjusted using lambda diluent to 10^9 and 10^7 PFU/ml. *S. aureus* overnight cultures were diluted to an OD_{550} of approximately 1 using TSB and placed on ice. Both phage and bacterial cultures were diluted 10-fold when combined in 96-well plates (Sarstedt). Bacterial controls were inoculated with lambda diluent and several wells were inoculated with lambda diluent and TSB to allow for adjustment of the background caused by media absorbance. Each 96-well plate was incubated at 30 °C with double orbital shaking in a Tecan Spark 10 M plate reader (Tecan Group Ltd., Mannedorf, Switzerland) and growth was monitored by measuring OD_{550} at 30-min intervals for 18 h. Analysis and calculations of plate reader data were conducted as described in Xie et al., 2018.

Results and Discussion

Kansas research farm sampling

Out of the 32 different environmental swabs taken from a Kansas swine farm, 20 were positive for phage against at least one of the seven *S. aureus* enrichment strains used (Table 2). Swabs collected from drainage areas of the farm were least likely to be positive for phage with five out of the 11 swabs containing phage that infected at least one of the enrichment hosts. Swabs taken from both the barn and gutter areas had more phage positive swabs, with eight out of 11 and seven out of ten swabs positive respectively (Table 2). From these positive swabs, 20 phage isolates were collected, subcultured three times, and propagated for gDNA extraction. Restriction digests from these 20 phages revealed that there was only one restriction digest pattern observed when using DraI or EcoRI-HF (Figure 5). Due to the similarity of all phage isolates restriction patterns, one phage isolate, initially labeled as "P4" was chosen to be characterized. It was determined via TEM imaging that this phage was of *Podoviridae* morphology and therefore the phage was renamed as phage Portland (Figure 6).

Table 2. Kansas swine farm swab locations and phage prevalence results.
A "+" sign indicates at least one phage plaque was formed on the denoted *S. aureus* strain after the swab enrichment process.

		***S. aureus* enrichment strains**						
	Location Sampled	USA300-0014	NRS255	NRS253	CA-513	5T	2N	22S
Swab								
1	Drainage	-	-	-	-	-	-	-
2	Drainage	-	-	-	-	-	-	-
3	Drainage	-	-	-	-	-	-	-
4	Drainage	-	-	+	+	-	-	-
5	Drainage	-	-	+	+	-	-	-
6	Drainage	-	-	-	-	-	-	-
7	Drainage	-	-	-	-	-	-	-
8	Drainage	+	+	+	-	+	+	-
9	Drainage	-	-	+	+	-	-	-
10	Drainage	-	-	-	+	-	-	-
11	Drainage lagoon	-	-	-	-	-	-	-
12	Barn	+	+	+	+	+	+	-
13	Barn	-	-	-	-	-	-	-
14	Barn	-	-	+	+	-		-
15	Barn	-	-	-	-	-	-	-
16	Barn	-	-	+	+	-	-	-
17	Barn	-	+	+	+	-	-	-
18	Barn	-		+	+	-	-	-
19	Barn	-	-	-	-	+	+	-
20	Barn	+	+	+	+	+	+	-
21	Barn	-	-	-	-	-	-	-
22	Barn lagoon pit	+	+	+	+	+	+	-
23	Gutter	+	+	+	+	+	+	-
24	Gutter	+	+	+	+	-	-	-
25	Gutter	-	-	+	+			-
26	Gutter	-	-	-	-	-	-	-
27	Gutter	+	+	+	+	+	+	-
28	Gutter	-	-	-	-	-	-	-
29	Gutter	-	-	-	-	-	-	-
30	Gutter	+	+	+	+	+	+	-
31	Gutter	-	+	+	+	-	-	-
32	Gutter	-	-	+	+	-	-	-

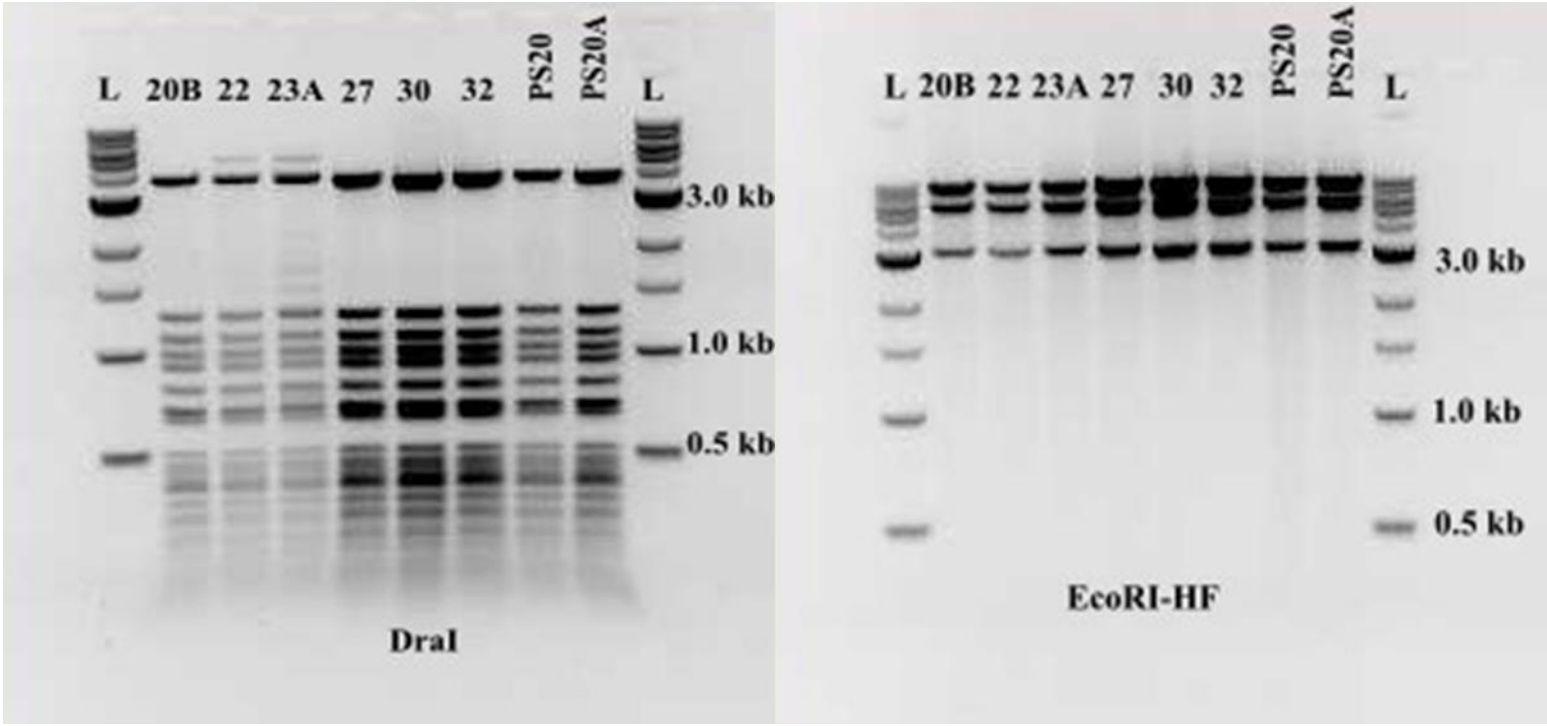

Figure 5. Phage isolates from Kansas swine farm restriction digest results.
All phages isolated from swab enrichments show the same restriction pattern when using either restriction enzyme DraI or EcoRI-HF. “NEB 1kb DNA ladder was used and is denoted by “L”.

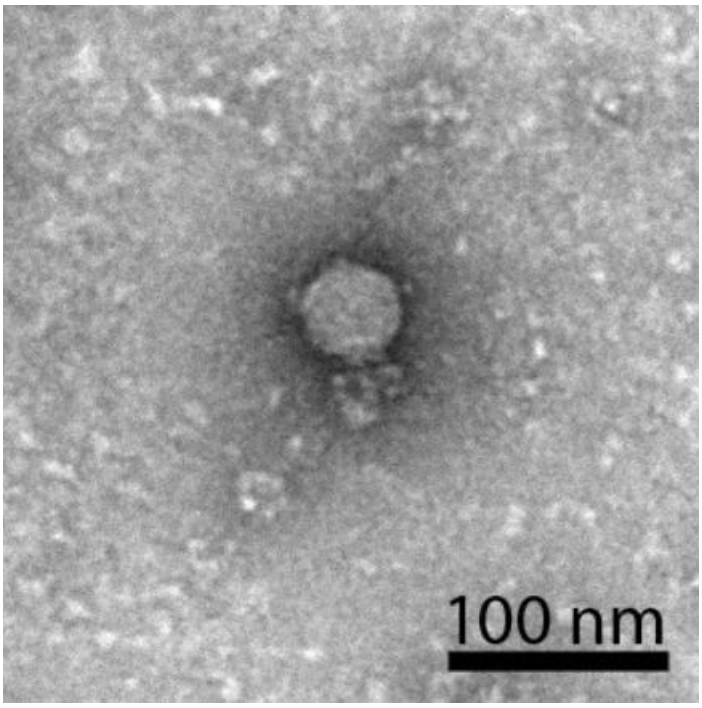

Figure 6. TEM image of phage Portland.
Virions were stained with 2% uranyl acetate and imaged using a JEOL 1200 EX transmission microscope.

Texas swine farm sampling

During preliminary sampling, different types of swine barn environmental samples were collected to determine the presence of *S. aureus* phages. Grain, swine feces and environmental swabs were collected from a Texas swine barn. Swab collection focused on areas in the barn with visible residue, such as dust accumulation on top of water pipes, pen siding and aisleway areas that were not frequently trafficked. These

areas were targeted as most bacteria exist in nature in some form of biofilm, therefore it was assumed that if *S. aureus* may be present in these biofilms, so should *S. aureus* phages (Abedon, 2015). After enrichment, only the environmental swabs taken from the barn were positive for phage and two phages were isolated on human clinical *S. aureus* strains USA300-0114 and NRS255. These two phages were further purified and subcultured so that gDNA could be extracted. Both phages gDNA samples were not susceptible to cutting with either DraI or EcoRI-HF. One of the phages labeled as "TP1" was further characterized by TEM imaging and results showed a *Myoviridae* morphology, therefore the phage was renamed phage Mars Hill (Figure 7).

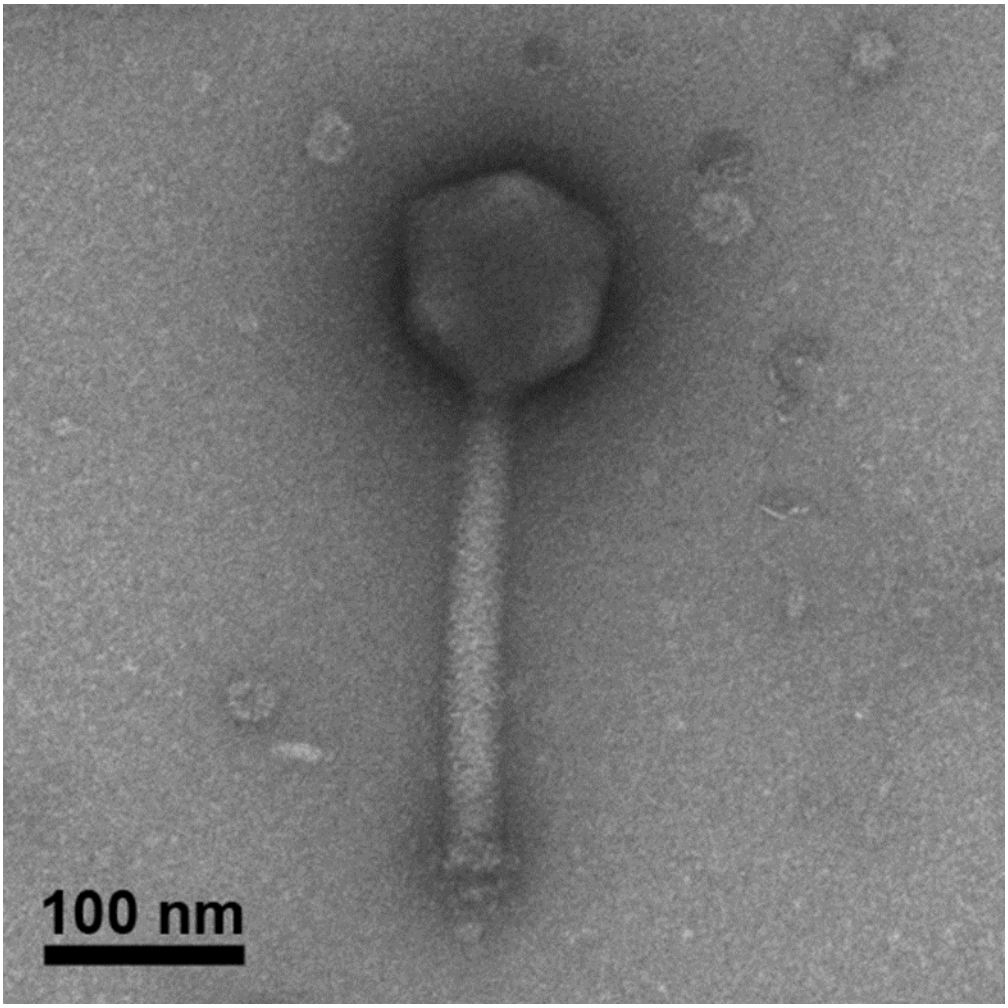

Figure 7. TEM image of Mars Hill.
Virions were stained with 2% uranyl acetate and imaged using a JEOL 1200 EX transmission microscope.

Texas abattoir sampling

Both skin and nasal post-mortem swabs were taken from five hogs at a Texas abattoir. All five skin swabs were positive for *S. aureus* phage while all nasal samples were negative. From these five swab enrichments four phages were isolated, subcultured and gDNA extracted. All four phage isolates displayed the same restriction digest patterns when digested with either DraI or EcoRI-HF (Figure 8). Therefore, one phage isolate which was cultured from the first hog skin sample on USA300-0114 named "RP1" was chosen to move forward with for further characterization. TEM imaging results showed that this phage was of *Myoviridae* morphology and was therefore renamed as phage Maine.

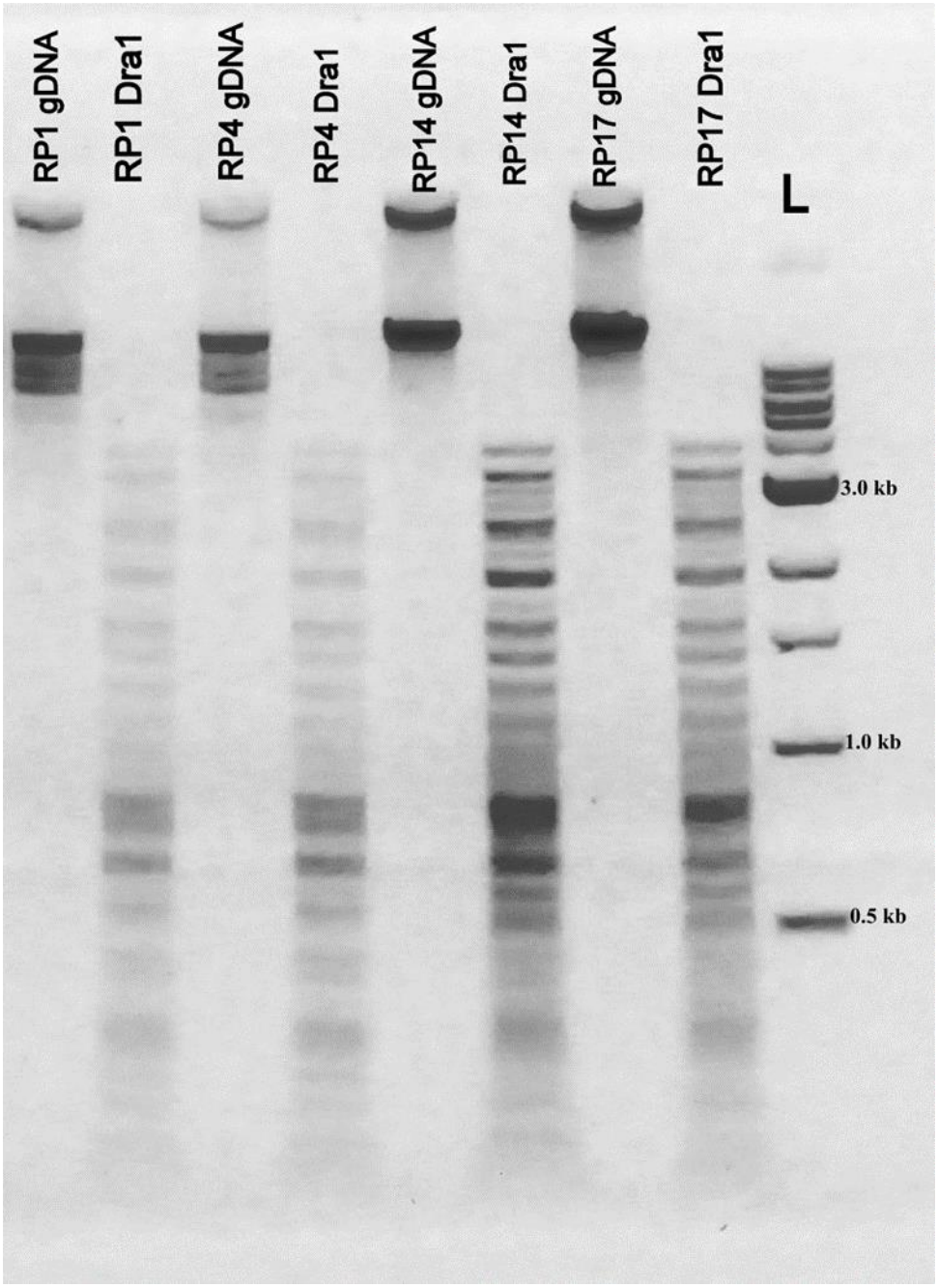

Figure 8. Results of DraI restriction digest.
All four phages isolated from a Texas abattoir show the same digest pattern.

Preliminary host range experiment using the agar overlay method

Phages Mars Hill, Maine and Portland were characterized for their host range against a panel of 20 different human clinical *S. aureus* strains using the agar overlay method (Table 3). Of the three phages, phage Maine displayed the broadest host range with an ability to form plaques on ten different strains; phage Portland displayed the most narrow host range and phage Mars Hill while able to produce a "burn through" phenotype on most bacterial lawns at high concentrations but was not able to produce individual plaques on the majority of strains.

Table 3. Host range of Mars Hill, Maine and Portland.
Phages were tested against a panel of *S. aureus* strains. Phage sensitivity is reported as efficiency of plating (EOP), the ratio of plaques formed on the test strain divided by the number of plaques on the propagation host EOP values. As shown above, the ATCC panel of strains consists of both MRSA and MSSA strains. A '+' symbol indicates confluent lysis formed when a phage was applied at concentrations of 10^9 – 10^6 PFU/ml. No individual plaques were observed in these strains.

Strain No.	Strain Name	ST	CC or PFGE type	SCCmec	Phage sensitivity		
					Mars Hill	Maine	Portland
HFH-30364	NA	NA	USA400	IV	+	+	+
HFH-30522	NA	NA	USA500	IV	+	+	+
NRS70	N315	5	5	II	+	-	-
NRS100	COL	250	8	I	+	+	-
NRS103	Becker	508-SLV	45	NONE	+	+	+
NRS112	MN8	30	30	NONE	+	0.7	0.2
NRS226	HT20020028	1	1	NONE	0.8	+	-
NRS232	HT20020065	22	22	NONE	+	+	+
NRS233	HT20020067	582	15	NONE	+	0.6	8E-04
NRS240	HT20020229	5	5	NONE	+	-	-
NRS242	HT20020238	121	121	NONE	+	0.8	+
NRS253	HT20020354	398	398	NONE	+	+	1
NRS255	HT20020371	80	80	IV	+	+	5E-05
NRS265	HT20020444	88	88	IV	+	0.9	+
NRS348	USA300-0114	8	USA300	IV	1.0	1.0	-
NRS648	CA-347	8	USA300	IV	+	0.5	-
NRS651	CA-409	36	USA200	II	+	0.6	+
NRS653	CA-513	5	USA800	IV	+	0.5	4E-03
NRS662	CO-34	8	USA300	IV	1.0	1.0	+
NRS689	GA-422	72	USA700	IV	0.6	0.2	+

Phage survey of US swine production facilities

A total of 20 swine farms were sampled across the United States (Figure 9). States sampled included: Texas, Wyoming, Nebraska, Minnesota, Iowa, Illinois, Indiana, Michigan, Alabama and North Carolina. Texas had the most sample sites, with six farms total. Iowa, North Carolina, Minnesota, Illinois and Indiana are the top five states for pig

production in the US (USDA, 2017). The majority of barns sampled were considered finishing barns followed by gestation barns. The number of animals residing in the sampled barns ranged from 14 - 12,000 head with an average of 1,721 head per sampled barn. Similarly, there was a large age range of hogs in the barns with newborn piglets being the youngest and one farm having hogs over 4 years of age.

Phage prevalence

Of the 19 farms sampled only once, 12 farms (63%) were positive for phage capable of infecting at least one of the ten *S. aureus* host strains (Table 4a). Environmental samples were enriched against two separate *S. aureus* host panels: one containing four human clinical isolates (including three representatives of major MRSA clades USA300, USA800 and ST80), and one containing six recently collected swine-associated strains (Sun et al., 2015, Otto 2012). A sample was considered positive for *S. aureus* phage if one plaque was isolated on any of the 10 different *S. aureus* enrichment strains. In samples that produced plaques on multiple strains, phage isolates were propagated separately on their respective initial isolation hosts. This methodology was followed as it is impossible to know if one type of observed plaque morphology was indicative of only one phage type in the sample or if there were two different phages with the same plaque morphology. Many different factors can impact plaque morphology such as the adsorption rate, diffusivity, latent period and burst size, therefore phage plaques were propagated on each host in hopes of isolating as diverse a collection of phages as possible (Gallet et al., 2011).

Figure 9. State level locations of swine farms sampled.
Each black number represents how many farms were sampled within a state with a total of 19 farms sampled once and one farm sampled in-depth.

Table 4. 4a Swab results for phage prevalence across the 10 different *S. aureus* hosts used. 4b Farm characteristics.

4a. A "+" sign indicates that the swabs taken from a farm had at least one phage plaque produced on a host lawn after the enrichment process.

		Human Clinical Isolates				Swine Nasal Isolates					
Farm Number	**Farm Location**	USA300-0114	HT20020371	HT20020354	CA-513	PD6	PD10	PD17	PD18	PD19	PD32
Farm 1	TX	+	+	-	-	-	-	-	-	-	-
Farm 2	TX	-	-	-	-	-	-	-	-	-	-
Farm 3	TX	-	+	-	-	-	-	+	+	+	+
Farm 4	TX	+	+	-	-	+	-	+	+	+	+
Farm 5	TX	-	-	-	-	-	-	-	-	-	-
Farm 6	TX	-	-	-	-	-	-	-	-	-	-
Farm 7	MN	-	-	-	-	-	-	+	+	-	+
Farm 8	IL	-	-	-	-	-	-	-	-	-	+
Farm 9	NC	+	+	-	+	+	+	+	+	+	+
Farm 10	IN	-	-	-	-	-	-	-	-	-	-
Farm 11	MN	+	+	-	-	-	-	+	-	-	+
Farm 12	NE	+	+	+	+	+	-	+	+	+	+
Farm 13	NC	-	-	-	-	-	-	-	-	-	-
Farm 14	IA	+	+	-	-	-	-	+	+	-	+
Farm 15	AL	-	+	+	+	-	-	+	+	-	+
Farm 17	MN	-	-	-	-	-	-	+	+	-	-
Farm 18	MI	-	-	-	-	-	-	-	-	-	-
Farm 19	MN	-	-	+	+	-	-	+	+	-	+
Farm 20	IL	-	-	-	-	-	-	-	-	-	-

4b. Farm characteristics recorded from questionnaire results.

Farm Number	State	Animals Residing in Barn	Age Range of Animals	Stage of Production Barn Represents	Type of Operation
Farm 1	TX	120 head	1-6 years	Gestation	Single site farrow to finish
Farm 2	TX	95-100	4-6 months	Finishing	Single site farrow to finish
Farm 3	TX	500	1 parity to 8 parity	Gestation	Gestation
Farm 4	TX	700	gilt to parity 10	Gestation	Gestation
Farm 5	TX	14	2.5+	Cull Barn	Single site farrow to finish
Farm 6	TX	100	210 days	Finishing	Finishing in for the production system
Farm 7	MN	1100	25-26 weeks	Finishing	Finishing in for the production system
Farm 8	IL	1036 sows, 11,000 piglets	1 day to 3+years	Farrowing	Farrowing
Farm 9	NC	200	12mo-36mo	Gestation	Farrowing
Farm 10	IN	2100	26 weeks	Finishing	Contract finisher
Farm 11	MN	826	4 months	Finishing	Finishing in for the production system
Farm 12	NE	6000+	Newborn- 3 years	Farrowing and Gestation	Gilt developer (selected other option)
Farm 13	NC	450	26 weeks	Finishing	Single site farrow to finish
Farm 14	IA	1750	Gestating females	Gestation	Gestation
Farm 15	AL	72	6.5 weeks	Nursery	Research wean to finish
Farm 16	WY	770	1 year +	Gestation	Gestation and farrowing
Farm 17	MN	1000	70-80 days	Finishing	Finishing in for the production system
Farm 18	MI	4182	16-17 weeks	Finishing	Wean to finishing for the production system
Farm 19	MN	1200	14 weeks	Finishing	Contract finisher
Farm 20	IL	1200	6 weeks	Wean to Finish	Contract wean to finish
Farm 16	WY	778	4 years	Gestation	Gestation and farrowing

Of the human-associated strains, the ST80 representative was most susceptible to the phages sampled from swine farms (eight positive samples), with the USA300 strain yielding six positive samples. Of the swine-associated host strains, one of the two ST9 and ST398 representatives were most sensitive, with each strain sensitive to phages sampled from ten sites. Host strains of both human and swine origin were roughly equally susceptible to phages contained in the environmental samples, suggesting that the phages are not "specialized" for swine environments and that there is not a strong distinction between human and swine *S. aureus.* However, very few phages were isolated against PD10, a MRSA ST398 swine strain or PD6, a MSSA ST9 swine strain. Why very few phages were isolated that were able to infect these strains is unclear. However, there are numerous reasons these strains could be less likely to be infected by a phage, such as factors blocking phage adsorption, bacterial defense mechanisms, resident prophages and others that could have caused these strains to be phage-resistant (Seed 2015, Bondy-Denomy et al., 2016).

Additionally, factors such as the season, movement of people and animals, sanitation and many other factors may influence the movement and presence of phages on these farms. It is important to note that only a small number of swabs were collected from a single barn on each farm. Therefore, there may be additional phages that would have been detected should more swabs and or barns have been sampled. Also, because phages are obligate predators of bacteria, the detection and culture of phages from the environment is dependent on the bacterial host strains used. In this study a diverse panel of 10 *S. aureus* strains in two separate enrichment panels was used to attempt to

maximize the number and diversity of phages isolated. These 10 strains represented seven major lineages of *S. aureus*: human clinical isolates of USA300, USA800, ST80 and ST398, and swine-associated strains of ST398, ST9 and ST5. Despite these efforts however, the absence of phage-positive samples does not prove the absence of *S. aureus* phages at a given site. A phage may have been present at numbers too low to be detectable, at discrete sites not captured by swab sampling, or may have been unable to replicate on the host strains used for this study.

Phage isolation and characterization

A total of 71 phage isolates were initially collected from the 20 farm samples with an additional 13 collected from the second sampling of Farm 16. 51 of those phage isolates survived *in vitro* subculturing and could be propagated to titers high enough to provide adequate amounts of gDNA for molecular analysis and sequencing. Five distinct DraI enzyme restriction patterns were observed among these 51 isolated phages, with 4 subgroups further discovered within those groups using EcoRI-HF. Of the 51 phages examined, 20 isolates belonged to group 1, 13 isolates belonged in group 2, 3 were in group 3,10 were in group 4, and 5 were in group 5 (Table 5). The group 1 restriction phenotype is that of undigested phage DNA, indicating that the DNA of these phages cannot be cut by this enzyme. This restriction pattern is the same as observed with phage Mars Hill. However, while lack of DNA digestion is a phenotype, it provides little information or ability to distinguish between phage isolates. Finding so few restriction patterns among the 51 phages was surprising, as the phages were isolated from diverse geographic locations. The lytic phages of *S. aureus* in serogroups D and G

are relatively homogeneous within in their groups and therefore the limited number of restriction patterns reflect this low diversity (Lobocka et al., 2012).

Table 5. Restriction digest of isolated phages.
Total phages isolated for each restriction digest group for both DraI groups and EcoRI-HF subgroups.

Morphology	DraI Restriction Group	EcoRI-HF Subgroups	Number of Phage
Myoviridae	Group 1	2	20
Siphoviridae	Group 2	0	13
Podoviridae	Group 3	0	3
Podoviridae	Group 4	2	10
Podoviridae	Group 5	0	5
		Total	**51**

To further characterize these phages, transmission electron micrographs were taken of 13 of the isolated phages, with TEM images revealing either *Myoviridae*, *Podoviridae* or *Siphoviridae* morphology (Figure 10). Of the 13 phages studied by this method, two were of *Myoviridae*, five were of *Siphoviridae* and six were of *Podoviridae* morphology. In general, restriction digest pattern could be correlated to phage morphology. The two *Myoviridae* observed belonged to restriction group 1, the five *Siphoviridae* to group 2, and five *Podoviridae* were placed in groups 3, 4 or 5, with one *Podoviridae* in group 1 (uncut DNA) (Figure 11). However, the *Podoviridae* with a type 1 pattern may be an error and its DNA preparation and restriction digest pattern would need to be repeated for confirmation. Given the significant portion of a phage genome that is required to encode virion structural proteins, it is extremely unlikely that phages with identical or nearly identical DNA restriction patterns could exhibit different

morphologies. Thus, phages in restriction group 2 are considered to be *Siphoviridae*, and phages in groups 3, 4 and 5 are considered to be *Podoviridae*. Morphological assignment of restriction group 1 phages is more difficult due to the low information contained in this phenotype.

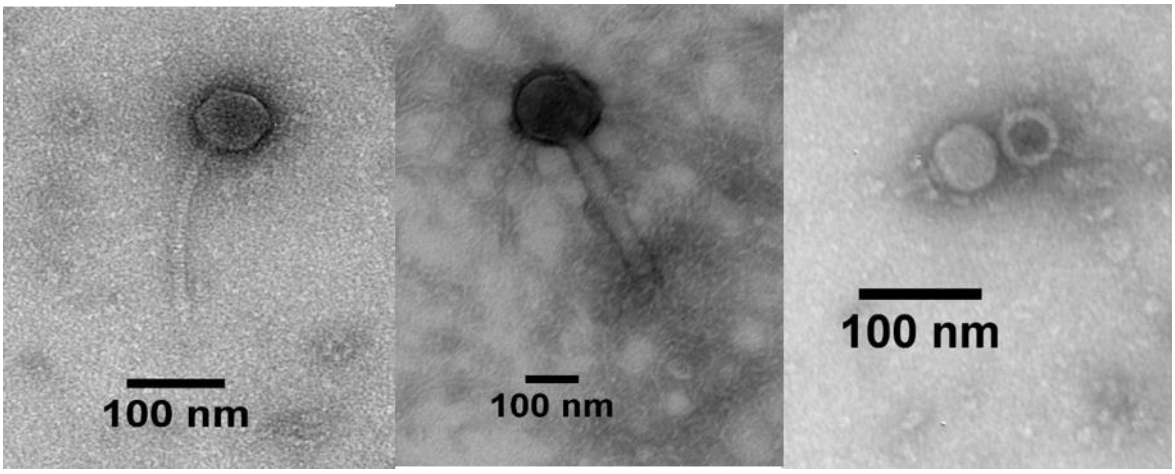

Figure 10. TEM images of all three phage morphologies observed.
From left to right: *Siphoviridae*, *Myoviridae* and *Podoviridae*.

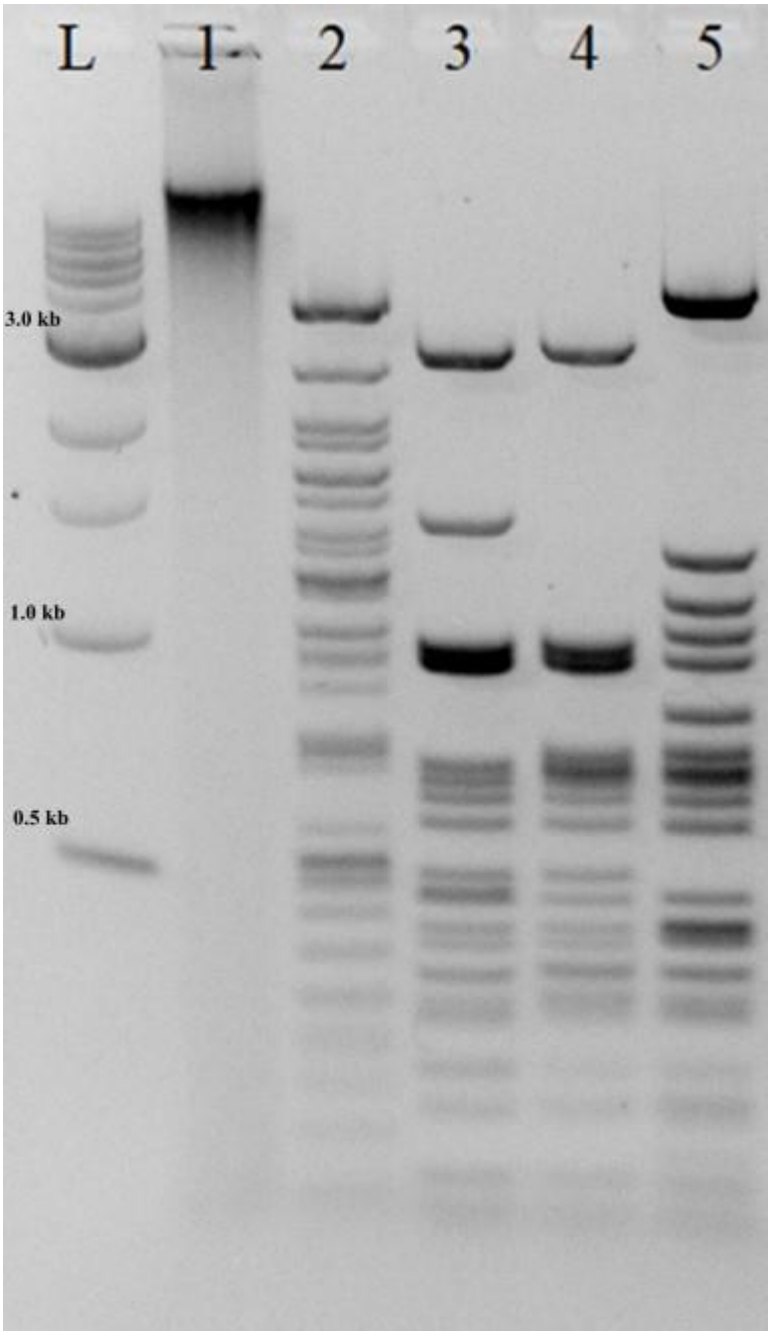

Figure 11. Five DraI restriction digest groups.
These were observed after digesting phage gDNA with enzyme DraI. The "L" represents the New England Biolabs 1 kb ladder.

In-depth sampling of farm 16

One sampling site, Farm 16, was selected for in-depth sampling for phage at two time points to determine the stability and distribution of phage populations at a site over time. Farm 16 was the only farm sampled in this manner, in November of 2016 and then again six months later in May of 2017. In the first in-depth sampling at Farm 16, only one of 30 swabs was positive for phage on one of the six swine *S. aureus* panel strains. At the second sampling, six months later, seven swabs were positive for phage across four of the swine *S. aureus* strains (Table 6). These seven swabs yielded 13 phage isolates, two which were lost during subculturing, nine which have the same restriction pattern and morphology of the single phage isolated from the first sampling and two phage isolates which displayed a group 1 (undigested) restriction pattern. These results suggest the original phage isolated in November 2016 was persistent in the environment over the 6-month sampling interval. Additionally, the movement of both workers and animals around the barns may be a possible explanation of how a new phage moved into the barn. However, more in-depth sampling at various time points across many different locations would be needed to be able to fully comment on the ecology and movement of these phage within swine environments.

Table 6. In-depth sampling results from Farm 16.
While 30 swabs were collected only positive swab locations between the two sampling times are shown. A "+" sign indicates there was at least one plaque formed on the respective strain after the enrichment process.

	11/18/2016 *S. aureus* swine nasal isolates						**5/11/2017** *S. aureus* swine nasal isolates					
Swab	PD6	PD10	PD17	PD18	PD19	PD32	PD6	PD10	PD17	PD18	PD19	PD32
1										+		
4										+		
5									+		+	
10											+	
11				+					+	+		
12												
13									+	+		+
21									+	+		

Microtiter plate assay host range

Fourteen phages were tested at two different concentrations (10^8 and 10^6 PFU/ml) for their ability to effectively infect a panel of 20 different *S. aureus* isolates from both human and swine sources in a liquid plate reader assay (Table 7). Two well-studied *S. aureus* phages, phage K (*Myoviridae*) and phage 44AHJD (*Podoviridae*) were included in this assay to allow for the comparison to novel phages. Additionally, three phages isolated from preliminary experiments, Mars Hill, Maine and Portland were included. The remaining ten phages tested were all isolated from the US swine farm survey. Phage from the survey were selected based on differences in morphology, restriction patterns and difference in farm geographical location. The design of these of experiments allows for the bacterial host to grow and to presumably replicate. This is important as most known phages replicate efficiently in bacteria which are in exponential phase not stationary phase (Adams, 1959). However, there are exceptions,

such as phage T7 which is able to continue to replicate in *Escherichia coli* cells which are in stationary phase (Studier, 1969).

In general, for lytic phages, *Myoviridae* had far broader host ranges than *Podoviridae*. This was expected as *Myoviridae* have been shown to have broad host ranges experimentally and also these phages have larger genomes and therefore are thought to be able to have more genes which are dedicated to combating host defenses (Lobocka et al., 2012). Similarly, only *Myoviridae* were able to infect strains of *Staphylococcus pseudintermedius* and *Staphylococcus epidermidis*. However, this polyvalent ability seems to be very strain-specific and not a broad property of the *S. aureus* phages (Table 8). All *Siphoviridae* tested displayed the narrowest host ranges, which was expected as all reported *S. aureus Siphoviridae* are lysogenic and are either unable to infect most strains or formed stable lysogens that quickly overgrew the culture (Ingmer et al., 2019. At the highest concentration of 10^8 PFU/ml phage, Mars Hill and phage PD17-F16-S-2 displayed the broadest host range with an ability to suppress bacterial growth by 50% or more for 14 of 20 strains tested.

Table 7. Liquid host range results.

14 different phages were tested at two different concentrations over 18 h. Each number represents the percentage of area under the bacterial control curve that each phage was able to suppress. A "100" would mean that the added phage was able to completely suppress the growth of the bacteria. A "0" means that the added phage had no effect on bacterial growth and that the bacteria was able to grow like the control.

		Human Isolates *S. aureus* isolates													Swine *S. aureus* Isolates					
		USA300-0114 (ST8)	NRS103 (ST 508 SLV)	NRS255 (ST80)	NRS253 (ST398)	NRS70 (ST5)	HFH-30364 (ST unknown)	HFH-30522 (ST unknown)	NRS648 (ST8)	NRS653 (ST5)	NRS689 (ST72)	NRS232 (ST22)	N305 (ST unknown)	Xen 36-56 (ST8)	Xen 36-57 (ST8)	PD6 (ST9)	PD10 (ST398)	PD17 (ST398)	PD18 (ST9)	PD19 (ST5)	PD32 (ST9)
Morphology	**Phage (PFU/ml)**																				
myophage	Mars Hill 10^8	81	90	84	8	28	89	68	90	6	92	91	73	93	91	82	5	92	5	8	87
	Mars Hill 10^6	60	11	34	0	4	1	0	0	1	52	0	0	38	42	0	0	0	0	N/A	N/A
myophage	K 10^8	98	4	1	5	98	98	1	98	2	22	1	99	98	98	3	0	60	98	10	97
	K 10^6	93	0	0	0	88	92	0	0	0	2	0	95	93	93	0	0	0	0	0	50
podophage	Portland 10^8	0	1	0	80	0	0	0	0	93	19	0	78	5	5	0	0	78	84	0	86
	Portland 10^6	0	1	0	84	0	0	0	0	73	0	0	60	0	0	1	1	81	78	0	92
podophage	PD32-F19-S-2 10^8	0	0	9	19	0	1	0	0	83	21	1	83	85	82	1	0	75	32	0	89
	PD32-F19-S-2 10^6	0	0	6	1	1	1	0	0	94	3	0	90	2	3	2	1	8	1	0	93
siphophage	PD18-F16-S 10^8	0	2	1	4	1	2	1	0	1	0	0	0	0	2	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	0	0	0	1	0
siphophage	PD17-F11-S 10^8	0	6	1	2	0	0	1	98	1	0	0	0	1	2	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	0	0	0
podophage	PD17-F7-S-2 10^8	0	3	0	84	0	0	0	0	40	4	0	79	0	0	1	0	88	83	0	92
	PD17-F7-S-2 10^6	0	4	0	80	0	0	0	0	0	0	0	54	0	0	1	1	91	78	0	85
podophage	PD17-F4-S-2 10^8	0	5	6	82	0	1	4	0	84	7	0	81	0	1	2	0	85	84	0	87
	PD17-F4-S-2 10^6	0	4	2	86	2	0	1	0	34	2	0	55	0	2	3	1	90	89	2	94
podophage	44AHJD 10^8	10	83	44	61	0	61	0	1	67	68	95	99	80	80	2	1	80	52	0	93
	44AHJD 10^6	2	6	15	6	6	0	0	0	0	10	84	97	87	87	0	0	10	1	4	69
myophage	RP1 10^8	98	62	1	2	0	2	2	99	97	42	2	99	98	98	11	0	87	99	1	97
	RP1 10^6	83	2	0	0	0	0	0	83	65	5	0	96	91	91	0	0	24	0	0	54
podophage	PD32-F19-S-1 10^8	0	1	12	48	0	1	0	0	73	10	0	80	76	79	0	0	56	39	0	81
	PD32-F19-S-1 10^6	0	2	4	14	0	0	1	0	91	0	0	87	1	2	0	0	6	0	0	92
unknown	PD32-F14-S 10^8	0	0	3	83	0	0	0	0	79	21	0	88	20	23	0	0	98	91	0	96
	PD32-F14-S 10^6	0	0	1	26	0	0	0	0	88	0	0	70	0	0	0	0	74	57	0	88
unknown	4018-F19-H 10^8	0	0	1	65	0	0	0	0	86	0	0	81	0	0	0	0	81	76	0	81
	4018-F19-H 10^6	0	0	0	78	0	0	0	0	0	0	0	65	0	0	0	0	80	73	0	93
myophage	PD17-F16-S-2-513 10^8	33	95	49	7	4	89	14	87	7	91	99	86	87	90	77	84	98	92	92	98
	PD17-F16-S-2-513 10^6	18	57	33	0	0	6	1	2	0	77	88	59	69	74	23	0	65	53	11	73

Table 8. Select phage tested at two different concentrations against different *Staphylococcus* species.
Staphylococcus epidermidis and *Staphylococcus pseudintermedius* strains were tested. Each number represents the percentage of area under the bacterial control curve that each phage was able to suppress.

		Other *Staphylococcus* species tested														
Morphology	**Phage (PFU/ml)**	*S. pseudintermedius* 4000	*S. pseudintermedius* 4001	*S. pseudintermedius* 4002	*S. pseudintermedius* 4003	*S. pseudintermedius* 4004	*S. pseudintermedius* 4005	*S. pseudintermedius* 4006	*S. pseudintermedius* 4007	*S. pseudintermedius* 4008	*S. pseudintermedius* 4009	*S. epidermidis* ATCC 12228	*S. epidermidis* NR-45888	*S. epidermidis* NR-45860	*S. epidermidis* HM-118	*S. epidermidis* HM-799
Myophage	Mars Hill 10^8	5	1	0	99	4	99	0	0	2	59	35	0	2	5	62
	Mars Hill 10^6	0	0	0	98	0	97	0	0	0	0	0	0	0	0	0
Myophage	K 10^8	7	7	9	14	13	10	8	5	3	13	0	0	5	15	96
	K 10^6	0	0	0	1	0	0	0	0	0	0	0	0	0	0	55
Myophage	Maine 10^8	2	0	2	99	78	99	0	10	3	3	23	0	0	0	13
	Maine 10^6	0	0	0	33	2	97	0	0	0	0	0	0	0	0	1
Podophage	PD32-F19-S-2 10^8	1	2	2	2	0	0	2	0	0	2	2	0	0	0	0
	PD32-F19-S-2 10^6	0	0	0	1	0	0	0	0	0	1	1	0	0	0	1
Podophage	44AHJD 10^8	0	1	0	1	0	1	0	0	0	1	1	0	0	0	0
	44AHJD 10^6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1

Conclusion

Swine environments are a reservoir for *S. aureus* phages. The types of *S. aureus* phages present in different swine environments across the US appear to be limited as restriction digest results were similar even for samples which were geographically separated. Consistent with this observed low diversity, eight of the 12 phage-positive farms yielded only a single phage type based on DraI restriction digests. This could indicate that a dominant phage group is usually present within a barn, a notion consistent with the persistence of the same phage type in the first and second sampling of Farm 16. This notion was also supported by preliminary sampling results, as each sampling resulted in only one phage type being isolated. However, this would need to be further investigated with additional rounds of in-depth sampling as well as multiple sample sites.

CHAPTER III

GENOMIC ANALYSIS OF *PODOVIRIDAE* ISOLATED FROM SWINE ENVIRONMENTS

Introduction

Historically, *Siphoviridae* phages were the most well-studied of the *S. aureus* phages as they were used for not only *S. aureus* strain typing, but also for their transduction abilities (Lobocka et al., 2012). However, due to their lysogenic nature they are not considered appropriate for phage therapy applications (Gordillo Altamirano and Barr 2019). Recently, more attention is being focused on characterizing the *Myoviridae* and *Podoviridae* of *S. aureus* as these phages are lytic in nature and therefore much better suited to use for phage therapy applications (Leskinen et al., 2017). However, much more research is needed in this area as there are still many genes of unknown function in both types of phages.

Historically, *S. aureus* phages were characterized into serogroups, which were based on the results of phage neutralization by antisera from rabbits which had been injected with specific phage filtrates prior (Roundtree 1949, Rippon 1952, Rippon 1956). Currently, in *S. aureus* phages, there are three different genome size classes seen and each corresponds to a certain morphological group. The three classes of size with their corresponding morphology are: class I: < 20 kb *Podoviridae*, class II: ≈ 40 kb *Siphoviridae* and class III: >125 kb *Myoviridae* (Kwan et al., 2005).

Lytic phages belonging to the morphogenic classification of *Myoviridae* have an icosahedral head with a long contractile tail and possess the largest genomes known in *S.*

aureus phages. These Twort-like phages belong to the *Spounavirinae* subfamily of *Myoviridae* and are members of serogroup D. The canonical member of Twort-like phages for *S. aureus* is phage K, a large, polyvalent myophage (Lobocka et al., 2012)

In contrast, *S. aureus* phages belonging to the *Podoviridae* group have an icosahedral head with a short non-contractile tail and have the smallest genomes. Most *S. aureus Podoviridae* phages show homology to 44AJHD phage and *Bacillus* phage phi29 (Lobocka et al., 2012). Lastly, *S. aureus Siphoviridae* have an icosahedral head but have a non-contractile long tail with a baseplate at the end. These phages have and are in some cases still used to type *S. aureus* strains (Barber and Whitehead 1949, Paul-Satyaseela et al., 2011). All *S. aureus Siphoviridae* characterized to date are temperate, so while useful for the typing of *S. aureus* strains and other applications, they are considered unsuitable for phage therapy (Deghorain et al., 2012).

The earliest reports of *S. aureus Podoviridae* are from papers dating back to the late 1940's and 50's when serology was still being used as the main way to classify *S. aureus* phages as well as *S. aureus* strains. These phages were classified into serological group G (Rountree 1949, Rippon 1956). *S. aureus Podoviridae* belong to the group *Picovirinae* subfamily, which includes not only *S. aureus* phages, but also phages like the well-studied *Bacillus* phage phi29 (Reilly et al., 1977) (Lobocka et al.,2008). Phage phi29 and other *Picovirinae* members are unusual phages in that they have terminal proteins covalently linked to each end of their linear dsDNA (Ito 1978). These terminal proteins are an example of DNA replication initiation that is accomplished by having protein-primed DNA (Meijer et al., 2001). Within this group, the most well

characterized *S. aureus* phages are phages 44AHJD and P68. Phage 44AHJD has a genome of 16,784 bp and a GC content of 29.6%, similarly phage P68 has a genome of 18, 227 bp and GC content of 29.3% (Vybiral et al., 2003). Both phages contain small, 210 bp inverted terminal repeats at the ends of their DNA sequences. Additionally, like phi29, P68 was found to possess terminal proteins and 44AHJD is assumed to have them as well (Vybiral et al., 2003). Currently, 41 phage sequences are reported in GenBank as being 44AHJD-like aka *Rosenblumvirus* (Tax ID:680287), with the majority being isolated on *S. aureus* hosts but with some of the most recently deposited representatives infecting *S. epidermidis* (Culbertson et al., 2019). 22 of those sequences are listed as complete genomes.

In the current study, twenty-two phages isolated in the field survey described in Chapter 2 were sequenced and a comparative genomic analysis of the sequences was conducted. This study provides insight into the genomic diversity of *S. aureus* phages isolated from swine environments. This isolation and further characterization of *S. aureus* phages is the largest single study ever reported for *S. aureus* phages.

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 (Kropinski et al., 2009) 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. After subculturing the phage, parent stocks of each phage were made by the confluent plate lysate method (Adams, 1959). Each phage was propagated on its original isolation host and harvested from the agar overlay with 4-5 mL of lambda diluent (100 mM NaCl, 25 mM Tris-HCl pH 7.4, 8 mM $MgSO_4$, 0.01% w/v gelatin). Phage lysates were vortexed, sterilized by passage through a 0.2 µm syringe filter (Millipore) and stored in the dark at 4 ºC.

Phage genomic DNA extraction

High titer (>10^8 PFU/mL) stocks of each phage were produced as described above and gDNA was then extracted from 10-20 mL of phage stock. To extract gDNA, a nuclease solution (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 5 mM $MgSO_4$ 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 13,000 x g 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 x g for 1 minute to elute the DNA. Extracted gDNA from the phage lysate was then stored at 4 ºC.

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 (Andrews, 2010), and SPAdes 3.5.0 (Bankevich et al., 2012) were then used for read quality control, read trimming, and read assembly, respectively. Preliminary phage relationships were determined by BLASTn against the nr database at NCBI (Camacho et al., 2009). Analyses were performed via CPT Galaxy (https://cpt.tamu/edu/galaxy-pub) (Afgan et al., 2018). Apollo was used for gene annotations (Lee et al., 2013). Genome maps were created using CPT Galaxy's Genome Mapper tool created by Mijalis and Rasche 2013. Track creation of PD32-F19-S-2- this phage was annotated thoroughly and used as an annotation guide and track for the other *Podoviridae*. Phage Portland was annotated by Bonasera et al., 2109, Phage Maine was annotated by Moreland et al., 2019 and Phage Sebago was annotated by Klotz et al., 2019. Genome closure of phage PD32-F19-S-2 was accomplished by the digestion of gDNA with

proteinase K (2 mg/ ml) to remove the terminal proteins this solution was incubated at 37 ºC for one hour and ~1000 bp off of each end was sequenced via PCR. PCR products were sequenced by Sanger sequencing. All other *Podoviridae* genomes were not closed.

Results and Discussion

Overview of sequencing data

Overall, twenty-two isolated phages were sequenced, with four of them having genomes of ~44 kb, seventeen with ~18 kb and one with ~141 kb. Two of these phages, phage Maine and phage Portland were isolated during preliminary sampling (Chapter 2). All other phages were isolated from the phage survey of swine farms across the US. Table 9 indicates what the phage isolates are currently named and what they will be named once deposited into GenBank. The current naming is in the format of host-farm-swine (S) or human panel (H)-number of isolate if there was more than one plaque isolated for each sample. For example, if two plaques were isolated against *S. aureus* strain PD17 from Farm 4, the first pickate would be labeled as PD17-F4-S-1.

Table 9. List of sequenced phages from both preliminary and survey of US swine farm experiments.
Unbolded text indicates what each phage name will be after being deposited into GenBank. Bolded text indicates the phage has already been deposited and what its name is.

Phage Original Name	Phage Genbank Name	Morphology	Genome Size	Source
PD18-F3-S	Springfield	Siphophage	43.93Kb	TX swine barn enviromnetal swabs
PD17-F4-S-1	Paris	Podophage	17.7Kb	TX swine barn enviromnetal swabs
PD17-F9-S	Peru	Podophage	17.71Kb	NC swine barn environmental swabs
PD18-F7-S-1	Penobscot	Podophage	17.95Kb	MN swine barn environmental swabs
PD32-F19-S-1	Poland	Podophage	17.71Kb	MN swine barn environmental swabs
PD17-F7-S-2	Porter	Podophage	17.73Kb	MN swine barn environmental swabs
PD18-F19-S-2	Pownal	Podophage	17.27Kb	MN swine barn environmental swabs
PD18-F15-S-2	Prentiss	Podophage	17.67Kb	AL swine barn environmental swabs
PD32-F7-S-2	Presque Isle	Podophage	17.79Kb	MN swine barn environmental swabs
PD32-F19-S-2	Prospect	Podophage	17.71Kb	MN swine barn environmental swabs
4023-F19-H	Palermo	Podophage	17.82Kb	MN swine barn environmental swabs
PD18-F19-S-1	Palmyra	Podophage	17.71Kb	MN swine barn environmental swabs
PD17-F4-S-2	Parsonsfield	Podophage	17.67Kb	TX swine barn enviromnetal swabs
PD18-F7-S-2	Passadumkeag	Podophage	17.98Kb	MN swine barn environmental swabs
PD17-F15-S-1	Plymouth	Podophage	17.67Kb	AL swine barn environmental swabs
PD17-F7-S-1	Pembroke	Podophage	17.98Kb	MN swine barn environmental swabs
PD32-F4-S-2	Perham	Podophage	17.68Kb	TX swine barn enviromnetal swabs
PD17-F17-S	**Sebago**	Siphophage	43.93Kb	MN swine barn environmental swabs
PD18-F16-S	Skowhegan	Siphophage	43.93Kb	WY swine barn environmental swabs
PD17-F11-S	Sabattus	Siphophage	43.93Kb	MN swine barn environmental swabs
RP1	**Maine**	Myophage	141.56Kb	TX swine abattoir
P4	**Portland**	Podophage	17.82Kb	KS swine barn environmental swabs

Sequenced Siphoviridae

The four ~44 kb genomes sequenced to date are identical with 100% nucleotide identity and are related to known temperate *S. aureus* phage phiETA (AP001553) found in bacterial genome sequences. One of these phages, Sebago, has been deposited into Genbank (MK618716) described in a brief publication (Klotz et al., 2019). Phage Sebago shares ~77% nucleotide identity and 54 genes in common with phage StauST398-3. Phage Stau398-3 was submitted to GenBank in 2013 and was isolated from the genome of an *S. aureus* ST398 isolate collected in France (JQ973847). Out of

the four phages, two were isolated from Minnesota, one from Wyoming and one from Texas. As ST398 is the most common sequence type of *S. aureus* in US swine production, it is not surprising that these phages are most similar to a prophage from that sequence type (Smith et al., 2018). While these phages share ~77% nucleotide identity with phage StauST398-3, they share 100% and 99.91% with two ST398 genomes: ISU926 and RIVM3897 respectively (Bosch et al., 2016, CP017091.1). RIVM3897 is an LA-MRSA isolate obtained from a Dutch patient collected in 2008 (Bosch et al., 2016). ISU926 is also an LA-MRSA and was isolated from a swine nasal swab in the US in 2010 (SAMN04571756). These two strains provide evidence that Sebago-like phages are present in some LA-MRSA from around the world. A possible explanation for this is that these phages provide some benefit to the *S. aureus* host and are therefore very well conserved across multiple ST398 clones (Wang and Wood). However, it is surprising that the Sebago-like phages share 100% nucleotide identity with a portion of the RIVM3897 genome, as lysogenic phages tend to be hotspots for genomic diversity in bacterial genomes and at least some variation at the nucleotide level would be expected (Ramisetty and Sudhakari 2019).

S. aureus Siphoviridae are known to contain and mobilize many different virulence factors in *S. aureus.* For example, *S. aureus Siphoviridae* encode virulence factors such as staphylokinase (*sak*), exfoliative toxin A (*eta*), Panton-Valentine leukocidin (PVL) among others (Goerke et al., 2009). Phage Sebago encodes a putative toxin protein with 100% amino acid identity to proteins simply annotated as “toxin” in multiple *S. aureus* strains. This protein is listed as part of a toxin-antitoxin system in two

S. aureus phages: DW2 (YP_009044997.1) (Keary et al., 2014) and 3MRA (YP_009209304.1) (Santiago-Rodriguez et al.,2015). However, many additional experiments would need to be conducted to verify both the toxin and potential antitoxin system function, as neither of the phages referenced provide molecular evidence for the specific function of this gene. Several classes of these temperate phages are known to be able to transduce DNA between bacterial strains or act as helper phages to mobilize genomic elements resident in some *S. aureus* genomes (Ingmer et al., 2020). The presence of these phages in some samples suggests that these natural processes are occurring in the swine production environment.

Sequenced Myoviridae

Phage Maine was sequenced and has a genome of ~142 kb and has been deposited in GenBank (MN045228) and described in a brief publication (Moreland et al. 2019). Maine is most similar to phage K and JD007 at the nucleotide level with ~83% and 93% similarity respectively. Maine has ~9 kb terminal repeats which was determined by PhageTerm, a tool that helps determine phage termini and packaging mechanisms (Garneau et al., 2017). These repeat regions were expected, as phage K has ~8.5 kb terminal redundancy and this feature is a hallmark of Twort-like phages (Gill, 2014). Maine was the only K-like phage isolated from this study. Why there were so few phages of this type isolated is unclear, however, it may be that K-like phages are not common in swine production environments. Throughout the literature, K-like phages are often isolated from sewage samples and this may be a more appropriate sample source to

isolate K-like phages from (Abatángelo et al., 2017; Synnott et al., 2009; Gutiérrez et al., 2015).

Currently, there are 82 complete genome sequences deposited under *Twortvirinae* in GenBank (Tax ID:2560081). An analysis of 22 of these *Myoviridae* complete genomes found that 70% of the ORFs encoded proteins were of unknown function. However, no antibiotic resistance or virulence were detected in all 22 complete genomes (Cui et al., 2017). Phage Maine has 73% of identified proteins that do not have an assigned function with. Despite these unknowns, Twort-ike phages have been used widely for phage therapy applications as they are lytic and often have broad host ranges (Azam and Tanji 2019).

Sequenced Podoviridae

One *Podoviridae* from preliminary sampling (phage Portland) and sixteen others from the US survey of swine farms (Chapter 2) were sequenced for a total of seventeen *Podoviridae*. The sixteen phages isolated from the survey study originated from five different sample sites (farms 4, 7, 9, 15, and 19). Sequencing revealed low levels of sequence diversity between the isolated *Podoviridae* at both the DNA and protein level, with the most dissimilar genome having 89% identity at the DNA level to all others. Additionally, all *Podoviridae* are highly related to the previously described phages 44AHJD and P68, with the most dissimilar *Podoviridae* still sharing 87% nucleotide identity to P68. The most similar phages in GenBank is phage PSa3 which shares ~88% nucleotide identity with PD32-F19-S-2 (NC_047855.1).

All isolated *Podoviridae* genomes range in size from approximately 17.6 to 18 kb. This makes these phages more similar in genome size to phage P68 (18,227 bp) than 44AHJD (16,784 bp) (Vybiral et al., 2003). All *Podoviridae* regardless of isolation host or geographic location are similar at the nucleotide level for the core protein-coding genes when compared to PD32-F19-S as a reference (Figure 12). PD32-F19-S-2 was chosen as a representative as this phage was one of the first phages of this type to be sequenced and annotated. Both highly conserved genes such as the major capsid the most diverse genes such as "hypothetical gene 8" show that all isolates are similar with no divergence in phylogenetic trees of either gene at the DNA level (Figure 13 and Figure 14). However, phage isolates from farm 7 appear to be the most diverse, as they have multiple hypothetical genes that are not present in phage PD32-F19-S-2. These accessory genes at the beginning and end of each genome are more likely to share no similarity with the other phages (Table 10).

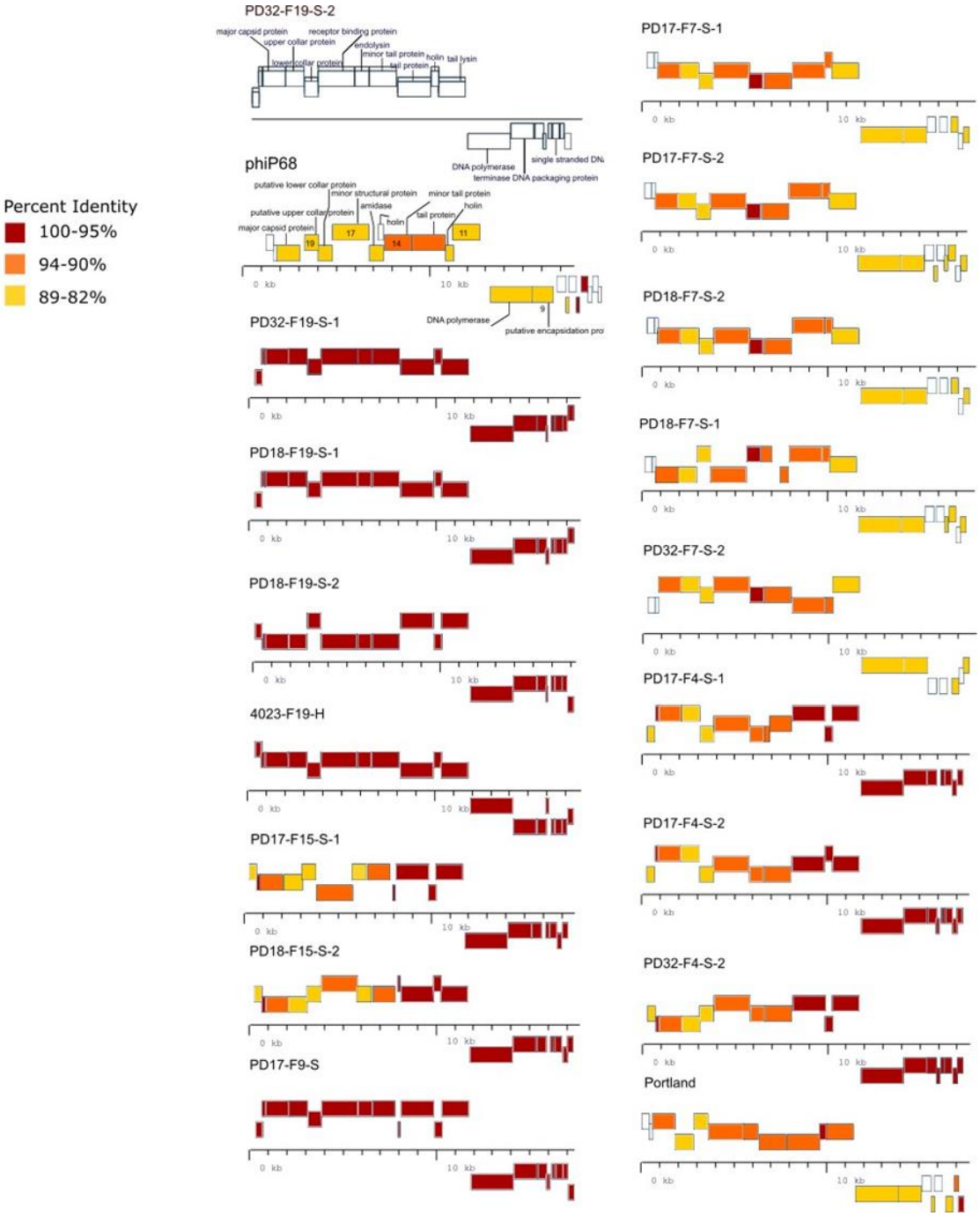

Figure 12. DNA genomic similarity comparison of all *Podoviridae* isolated from preliminary and survey samples.
Phage P68 is included as a canonical reference.

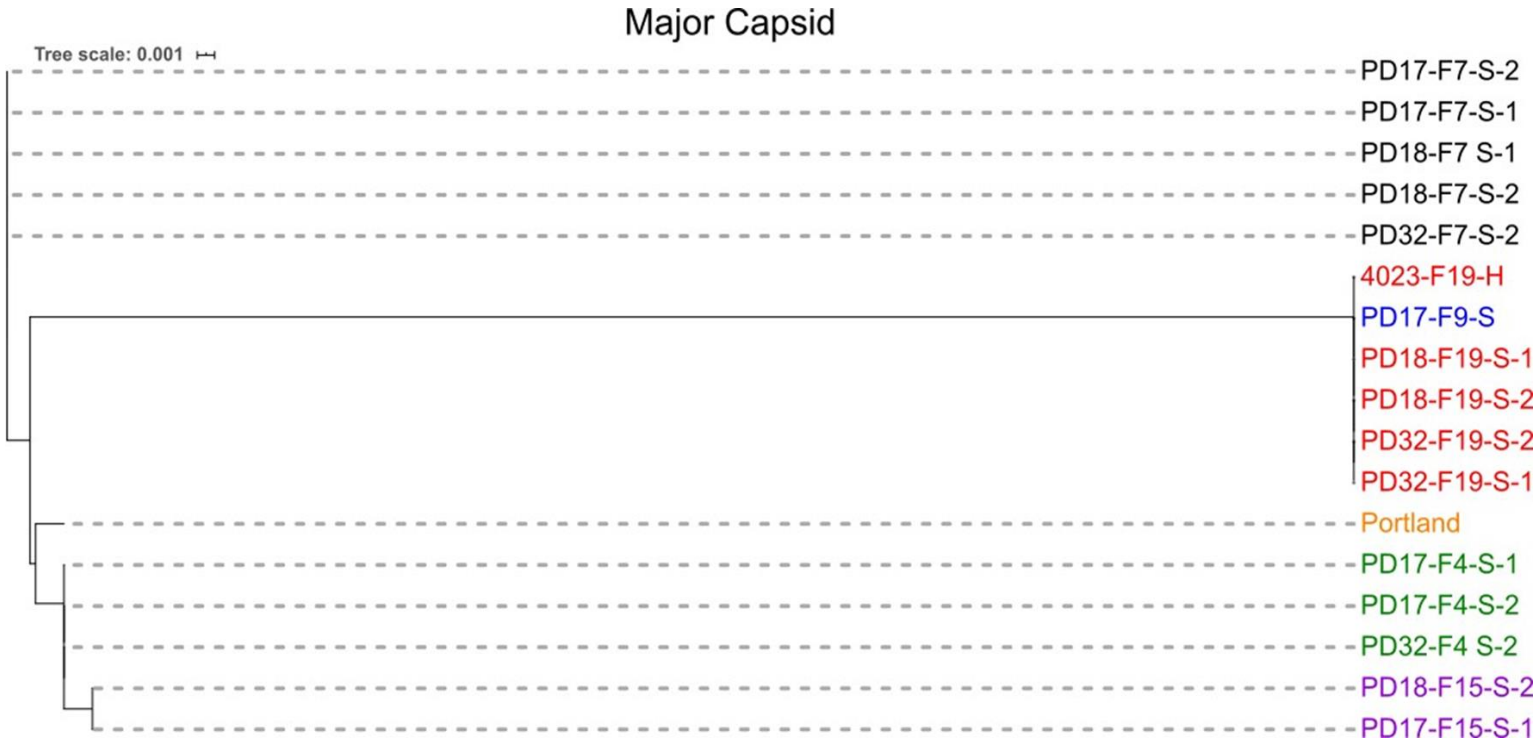

Figure 13. Major capsid gene comparison from all *Podoviridae* at the nucleotide level.
Different colors represent phages isolated from different farms.

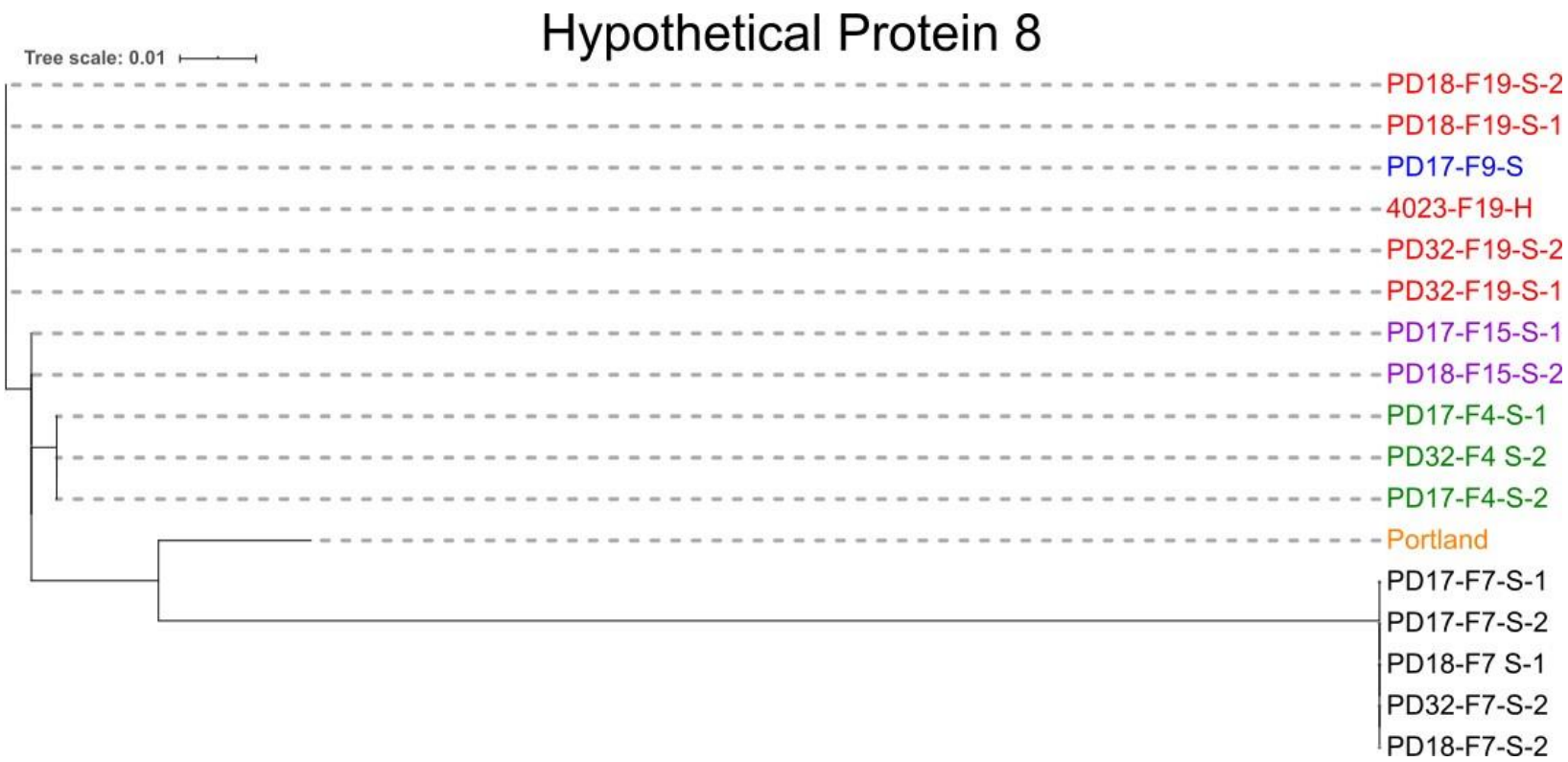

Figure 14. "Hypothetical gene 8" comparison from all *Podoviridae* at the nucleotide level.
Different colors represent phages isolated from different farms.

Gene organization amongst all phages does appear to be modular and follow previous described gene arrangement (Figure 15) Deghorian et al., 2012).However, unlike phage P68 these phages do not contain a second holin gene which can be observed in Figure 12 that is embedded inside the amidase gene of P68 (Takáč et al., 2005). Phage PD32-F19-S-2 contains inverted repeats of 194 bp at both ends of its genome, which is comparable to 44AHJD and P68 which have repeats of 210 bp (Vybiral et al., 2003). Pd32-F19-S-2 gDNA was digested with proteinase K to test for the presence of terminal proteins. Sanger sequencing of the ends of digested and undigested gDNA samples showed that there were an additional 74 bp in the digested gDNA sample. Which specific gene encodes the terminal protein for PD32-F19-S-2 and others is unclear. No single accessory gene is conserved across all the *Podoviridae,* making it difficult to identify this protein (Table 11).

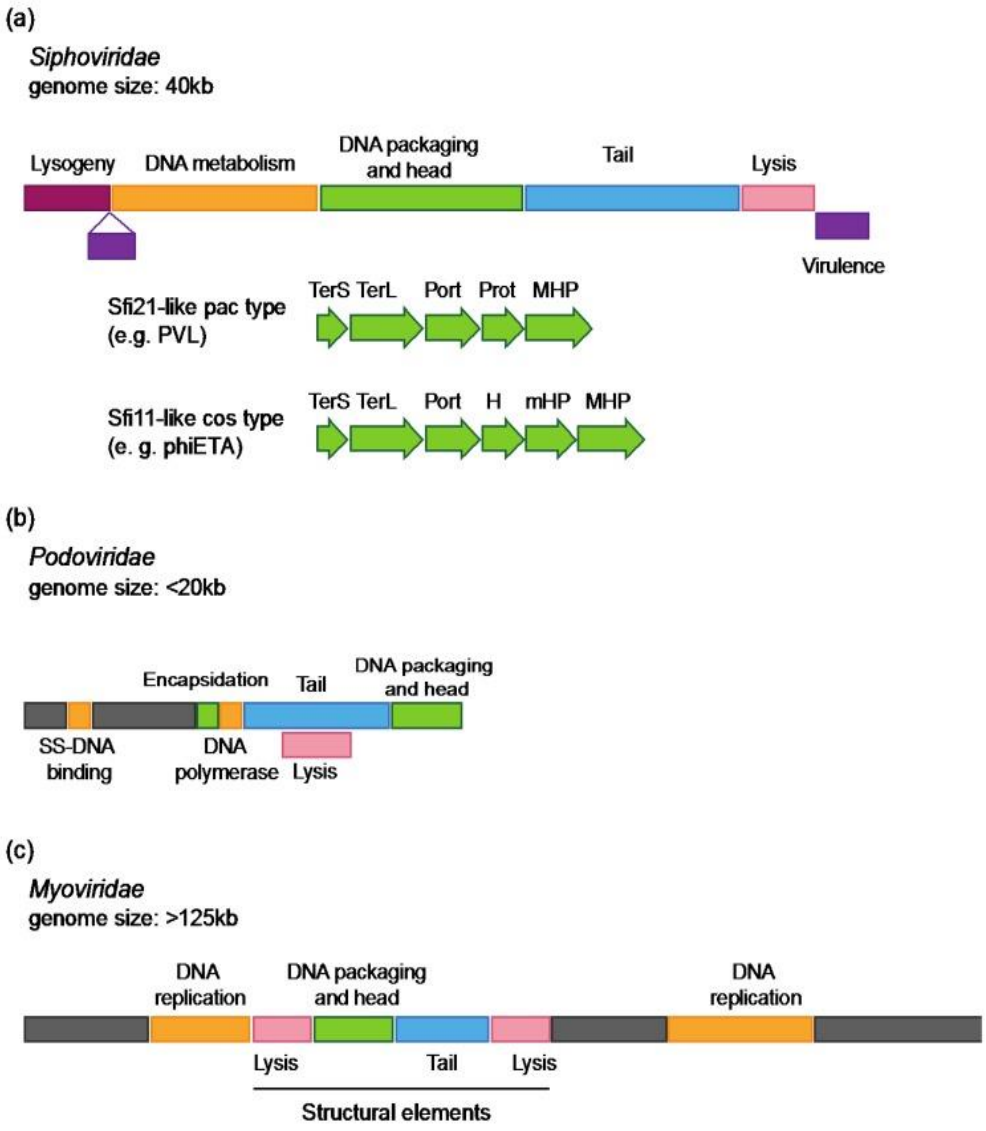

Figure 15. Size of genome and organization for each morphological *S. aureus* phage type. Reprinted from Deghorain and Melderen 2012.

Table 10. Presence and absence of genes in all isolated *Podoviridae* when compared to PD32-F19-S-2 at the DNA level.

		FarmLocation																
		MN	MN	MN	MN	MN	AL	AL	NC	MN	MN	MN	MN	MN	MN	MN	MN	TX
Gene Name	PD32-F19-S-2 proteins	PD32-F19-S-2	PD32-F19-S-1	PD18-F19-S-1	PD18-F19-S-2	4023-F19-H	PD17-F15-S-1	PD18-F15-S-2	PD17-F9-S	PD17-F7-S-1	PD17-F7-S-2	PD18-F7-S-1	PD32-F7-S-2	PD18-F7-S-2	PD17-F4-S-2	PD32-F4-S-2	PD17-F4-S-1	Portland
gp1	Hypothetical 1	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-
gp2	Hypothetical 2	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-
gp3	Major Capsid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
gp4	Upper Collar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
gp5	Lower Collar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
gp6	Receptor Binding Protein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
gp7	Endolysin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
gp8	Minor tail	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
gp9	Tail protein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
gp10	Holin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
gp11	Tail Lysin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
gp12	DNA Polymerase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
gp13	Terminase DNA Packaging Protein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
gp14	Hypothetical 3	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-
gp15	Hypothetical 4	+	+	+	+	+	-	-	+	-	-	-	-	+	+	+	-	+
gp16	Hypothetical 5	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	-
gp17	Single stranded DNA binding protein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
gp18	Hypothetical 6	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+
gp19	Hypothetical 7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Minor tail protein arrangements amongst the *Podoviridae* show four different arrangements (Figure 16). The minor tail protein from PD32-F19-S-2 shares ~89% nucleotide identity with ORF 14 from P68. This gene was identified using structural reconstruction data to be the head fiber of P68. These head fibers are involved in host cell recognition along with the tail fibers and they help position the virion with its tail axis perpendicular to the cell (Figure 17) (Hrebik et al., 2018). Phage genome sequencing was completed after phage host range experiments, however future experiments could be conducted to determine if any of these arrangements of the minor tail protein have an influence on host range. All *Podoviridae* that were chosen for host range (Chapter II), including 44AHJD, have the type 1 arrangement of the minor tail protein and do have some variation in host range. Therefore, while different minor tail protein arrangements may have some influence on host range there are likely other proteins that play a role as well.

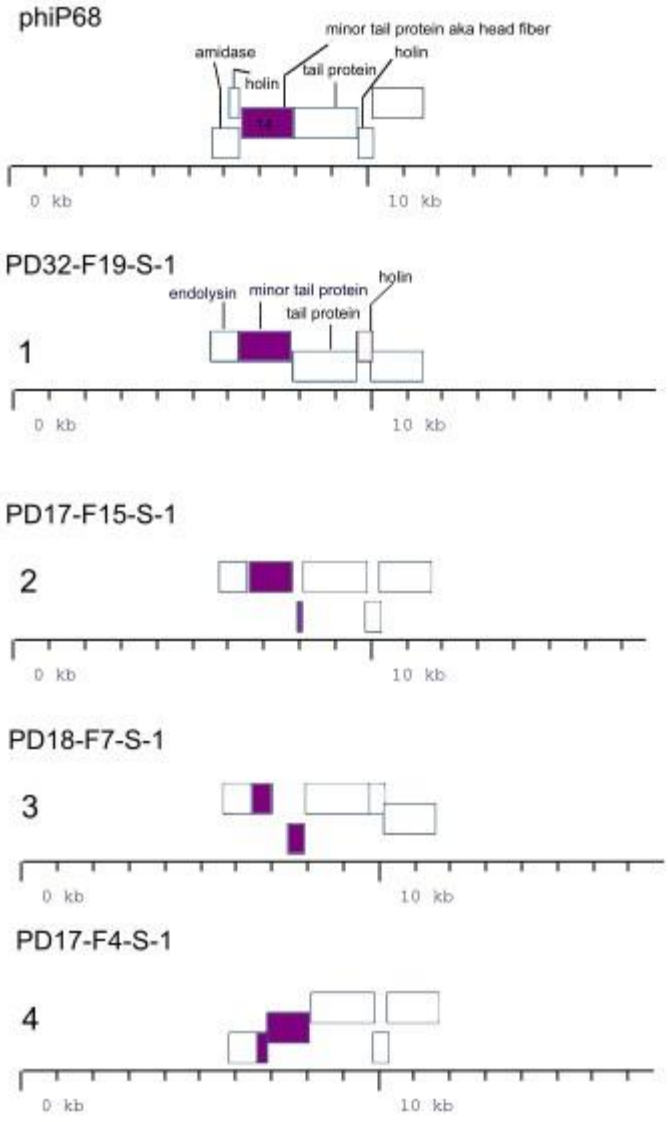

Figure 16. Types of minor tail protein arrangements found in isolated *Podoviridae.*

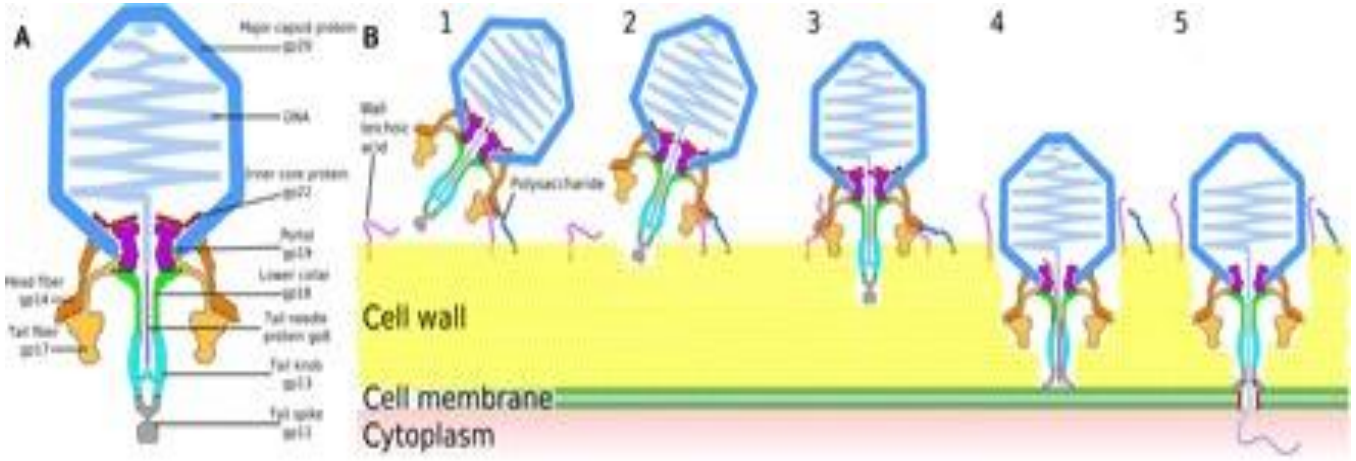

Figure 17. Role of head fibers in *Podoviridae*. Reprinted from Hrebik et al., 2019.
Head fibers (dark orange) help in host cell recognition as well as virion positioning.

Conclusion

Genomes from all three *S. aureus* phage morphotypes, *Myoviridae*, *Siphoviridae* and *Podoviridae* were comparable to well-known *S. aureus* phages. The presence of *Siphoviridae* within the barns indicates that these phages are circulating in the barn environment and may play a role in HGT from one *S. aureus* strain to others in these environments. Additionally, the striking similarity of the three isolated *Siphoviridae* regardless of being isolated from very different geographic locations may be an indication that there is a common *S. aureus* clone in swine production environments where this type of phage is a resident prophage. However, it could also be true that there is some common link between the three different farms, although this seems unlikely.

Interestingly, *Podoviridae* were isolated most frequently and this is contradictory to what was previously thought in that these phages were the rarest of the morphological types to be isolated (Lobocka et al., 2012). These phages are lytic and it is unlikely that they are participating in HGT as they are presumed to possess phi29-like covalently-linked proteins at their genomic termini. Instead, these phages should be considered as an inhouse form of natural biocontrol for *S. aureus*. The overall genomic diversity of between these phages was low, even when compared to well-known phages indicating this low diversity could be a product of the small genome size (~18 kb) these phages possess as there is quite literally less room to acquire new genes. Both ends of each phage genome represent areas where the most diversity is seen. For example, gp1 and gp2 of all phages isolated from farm 7 share no sequence identity with PD32-19-S-2

(Figure 12). Additionally, PD17-F7-S1 and PD32-F7-S-2 only have one of the seven hypothetical proteins in common with PD32-F19-S-2 (Table 11).

Sixteen of the sequenced *Podoviridae* originated from five sites (farms 4, 7, 9, 15, and 19) and analysis shows that phages originating from the same site are more closely related to each other than to phages from other sites (Figure 13 and Figure 14). This suggests an ecological model of phage establishment followed by clonal spread within a site rather than co-colonization by multiple phages. However, this model would have to be verified by further studies that sampled multiple locations over an extended period of time. Additionally, in-depth sampling suggested that over time it is possible for another type of phage to enter the farm, it's unclear if this phage would then outcompete the original phage or if multiple phages would coexist. These results represent the first known systematic survey of phages in swine production as well as the first for *S. aureus* phage. This genomic characterization of phages from swine production environments will allow for further investigation into phenotypic differences such as plaque morphology and host range. For example, the four different variations of minor tail protein arrangements or presence and absence of hypothetical proteins may have an impact on host range (Figure 16 Table 11).

CHAPTER IV

A NEW TYPE OF PHAGE- JUMBO *S. AUREUS* PHAGE

Introduction

Previous investigations of *S. aureus* phages have described only three classes of phages: P68-like *Podoviridae* with genomes of ~18-20 kb, various *Siphoviridae* with genomes of ~45 kb, and large, K-like *Myoviridae* with genomes of ~130 kb with long terminal repeats. Of these types, *Podoviridae* and *Myoviridae* are lytic phage while *Siphoviridae* are lysogenic. (Lobocka et al., 2012; Xia et al., 2014). There is a single report of a novel large *S. aureus Myoviridae* that appears to be distinct from the K-like *Myoviridae*; this phage was named S6 reported by Uchiyama et al., in 2014. While S6 was estimated by Uchiyama et al., 2014 to have a 270 kb genome by pulse-field gel electrophoresis, no genomic sequence for S6 was ever reported. In addition to having a large genome, Uchiyama et al., 2014 identified S6 DNA contains uracil instead of thymine.

Phages that have genomes over 200 kb are classified as jumbo phage (Yuan and Gao, 2017). These phages must have larger virions to contain their large genomes. ~95% of jumbo phages have been isolated from gram-negative hosts, with the only known jumbo phages for gram-positive host being from *Bacillus.* The largest jumbo phage isolated is *Bacillus* phage G, with a genome of 497 kb. Jumbo phages are most frequently isolated from water samples. However, they have also been isolated from other diverse sample types, such as soil, bark and silkworms. Grouping these phages can

difficult, as a large portion of the genome is usually unrelated to other phages in addition to having many proteins of unknown function (Yuan and Gao 2017).

Many phages modify their DNA, eliminate commonly targeted sequences or produce proteins which protect DNA as a means of combatting bacterial host defenses. Bacteria can degrade phage DNA by using restriction modification systems and CRISPR-Cas immune systems (Seed 2015 Samson et al., 2013) Examples of this include phage K which contains no GATC sites in its 140 kb genome as a means of avoiding restriction by a common *S. aureus* restriction modification system, Sau3A, or phage T4 which has glycosylated 5-hydroxymethylcytosine (glc-HMC) which protects the DNA from not only HMC specific nucleases but also CRISPR-Cas9 (Moller et al., 2019, Bryson et al., 2015). Substituting uracil for thymine is also a way of circumventing host defenses. *Bacillus subtilis* phage SPO1 is distantly related to phage K and other Twort-like phages and has hydroxymethyluracil nucleotides in place of thymine. Experiments replacing this base with thymine show that the DNA is much more readily digested with restriction enzymes (Hoet et al., 1992, Stewart et al., 2009). Heavily modified DNA, however, can also present barriers for DNA sequencing methods that are routinely employed for sequencing phage and bacterial genomes. This was true for *Bacillus* phage AR9 which has uracil instead of thymine, Illumina sequencing produced no phage genomic sequence (Lavysh et al., 2016).

The following study seeks to shed light on four novel *S. aureus* jumbo phages isolated from swine environments. This study is important as it is the first report of a novel *S. aureus* phage type based on genomic data.

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 (Kropinski et al., 2009) 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. After subculturing the phage, parent stocks of each phage were made by the confluent plate lysate method (Adams, 1959). Each phage was propagated on its original isolation host and harvested from the agar overlay with 4-5 mL of lambda diluent (100 mM NaCl, 25 mM Tris-HCl pH 7.4, 8 mM $MgSO_4$, 0.01% w/v gelatin). Phage lysates were vortexed, sterilized by passage through a 0.2 µm syringe filter (Millipore) and stored in the dark at 4 ºC.

Phage genomic DNA extraction

High titer (>10^8 PFU/mL) stocks of each phage were produced as described above and gDNA was then extracted from 10-20 mL of phage stock. To extract gDNA, a nuclease solution (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 5

mM $MgSO_4$ 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 13,000 x g 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 x 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’), EcoRI-HF (5’ GAATTC 3’) (New England BioLabs Inc. https://www.neb.com). gDNA samples that showed poor banding patterns or could not be digested by the enzymes listed above were then digested with TaqαI (5’ TCGA 3’). For DraI and EcoRI-HF 300 ng of gDNA from each phage was incubated with each enzyme and CutSmart ® Buffer individually at 37 ºC overnight. For gDNA treated with TaqαI, 300 ng of gDNA was incubated with TaqαI and CutSmart ®

Buffer at 65 °C for two hours. 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 2.5 h.

Illumina Truseq ®

Illumina TruSeq ® Nano low-throughput kit was used to prepare the DNA library. Sequencing of phage DNA was performed by an Illumina MiSeq 250-bp paired-end run with v2 500-cycle chemistry.

Pacific Biosciences

DNA samples were purified using AMPure pb bead prior to following kit methodology. Pacific BioSciences SMRTbell ® Express Template Preparation Kit for libraries >15 kb was attempted twice. All kit methodology was followed with the exception that ligation incubation was conducted overnight. Library preparation was conducted by the Texas A&M Agrilife Genomic and Bioinformatics Service (College Station, TX).

Oxford Nanopore Technologies MinION™

Phage of interest DNA samples were purified using AMPure pb bead prior to following kit methodology. Oxford Nanopore Technologies Rapid Sequencing Kit methodology was followed with DNA samples being run on multiple 106 model flow cells. All flow cell washing methods were followed in between samples including running phage lambda DNA as a positive control to validate cell validity before running sample DNA.

Swift Biosciences™

Library preparation of Mars Hill genomic DNA was conducted using Accel-NGS ® 1S plus DNA library kit and kit methodology was followed. These methods were performed by Dr. Andrew Hillhouse's lab.

Illumina Truseq® PCR-free

Phage DNA was sheared to approximately 350 bp using a Diagenode Bioruptor® Pico. 1 ug of DNA was used to follow the Illumina Truseq ® PCR-free library preparation protocol. Samples were quantified using KAPA qPCR library quantification kit (Roche ©) and diluted to 4nM. Sequencing of phage DNA was performed by an Illumina MiSeq V2 Nano 500 cycle kit. These methods were performed by Dr. Andrew Hillhouse's lab.

FastQC (Andrews, 2010), and SPAdes 3.5.0 (Bankevich et al., 2012) were then used for read quality control, read trimming, and read assembly, respectively. Preliminary phage relationships were determined by BLASTn against the nr database at NCBI (Camacho et al., 2009). Analyses were performed via CPT Galaxy (https://cpt.tamu/edu/galaxy-pub) (Afgan et al., 2018). Apollo was used for gene annotations (Lee et al., 2013).Track creation of phage Mars Hill was annotated thoroughly and used as an annotation guide and track for the other type I phage. Mars Hill termini were determined by using primers to sequence ~1000 bp off each end of the genome. For initial PCR DNA polymerases Phusion U (ThermoFisher), Phusion (Thermofisher) and Taq (New England Biolabs) were tested. Phusion U (ThermoFisher) was used for subsequent PCR. The PCR product was sequenced by Sanger sequencing.

Madawaska and Machias termini were determined using SPAdes (Bankevich et al., 2012) to produce sequences in which the original termini were internal to the genome with >40X coverage. Mapleton remains unclosed. Alignment figures were generated using Progressive Mauve (Darling et al., 2010).

Transduction experiments

The transduction ability of Mars Hill was investigated by Dr. Lichang Sun. Xen 36 and CA-347 were used as donor *S. aureus* strains to recipient strain PD17. Mars Hill was propagated on donor strains using liquid lysate methodology. PD17 was grown in 10 ml TSB supplemented with 5mm $MgCl_2$ at 37 ºC to ~$5x10^8$ CFU/ml. Mars Hill lysate from the either donor strain was then added at an MOI of 0.1 and the culture was incubated for 20 minutes at 30 ºC. The culture was then centrifuged at 13,000 x g for 2 minutes and the cell pellet was resuspended in 1 ml TSB with 50 mM sodium citrate. This culture was then incubated at 37 ºC for an hour, centrifuged at 13,000 x g for 2 minutes and resuspended in 1 ml TSB. Afterwards the culture was centrifuged a final time at centrifuged at 13,000 x g for 2 minutes and resuspended in 10 ml TSB. This culture was plated in 1 ml aliquots onto either TSA plates supplemented with 150 μg/ml kanamycin and incubated overnight at 37 ºC. Transduction efficiency was calculated as the number of transductants (CFU)/ number of phage particles (PFU)

Results and Discussion

Restriction digest

Genomic DNA extracted from *Myoviridae* belonging to restriction group 1 was not able to be digested when using either enzyme EcoRI-HF or DraI. While all other phage types were able to be classified this way, from early on this hinted at the DNA of type I phages being modified in some way that was refractory to the enzymes used. Therefore, enzymes MspI (5' CCGG3') and TaqαI (TaqI) (5' TCGA 3') were used. TaqI was used specifically as it has been shown to cut heavily modified phage DNA such as SPO1 (Huang et al., 1982). Restriction digests using both MspI and TaqI showed extensive cutting of type I phage Mars Hill DNA. These results indicated that the DNA modifications on Mars Hill DNA were most likely on either thymine or adenine nucleotides as MspI, which lacks A or T bases in its recognition sequence, was able to cut the DNA. However, while these two enzymes did cut Mars Hill DNA, there were too many cuts made to allow for easy distinction of different type I phages (Figure 18 lanes 11 and 12). Enzymes ApaI and SmaI also do not cut Mars Hill DNA therefore, these could not be used to help further distinguish type I isolates.

TEM imaging

TEM imaging had already revealed that these phages were of *Myoviridae* morphology (Figure 19). Measurements taken of approximately 10 virions and 6 virions showed that these phages have a mean head size of ~106 nm ± 7 and tail length of ~228 nm ± 7 respectively. However, these measurements would need to be confirmed with additional imaging of other Mars Hill-like phages to provide a more accurate range of

virion size. Imaging taken of jumbo phage S6 is similar with a reported head diameter of 118.1 nm and tail length of 237 nm (Uchiyama et al., 2014). For comparison, phi812 is a Twort-like phage and has a head diameter of 90 nm and a 240 nm tail (Nováček et al., 2016).

Growth conditions of type I phages

Culturing any Type I phage isolate to high titer was performed in liquid culture as plate overlay lysate methods would not yield high titer phage stocks. Additionally, Type I phages were unable to form plaques or propagate in liquid culture at 37 °C, this all culture and enumerations was conducted at 30 °C. This phenomenon was observed when conducting liquid host range experiments, as Mars Hill (formerly known as TP1) was able to effectively kill USA300-0114 cultures at 30 °C but essentially no effect was seen on USA300-0114 culture OD_{550} when incubated at 37 °C (Figure 20). A drop in OD_{550} is observed at 37 °C after treatment with Mars Hill after approximately 10 hours, suggesting that Mars Hill may have some attenuated ability to infect cells at 37 °C (Figure 20). However, these results would need to be further investigated to identify why Mars Hill is able to cause a drop in OD_{550} at 37 °C. This temperature sensitivity between 37 °C and 30 °C was also noted for jumbo phage S6 (Uchiyama et al., 2014).

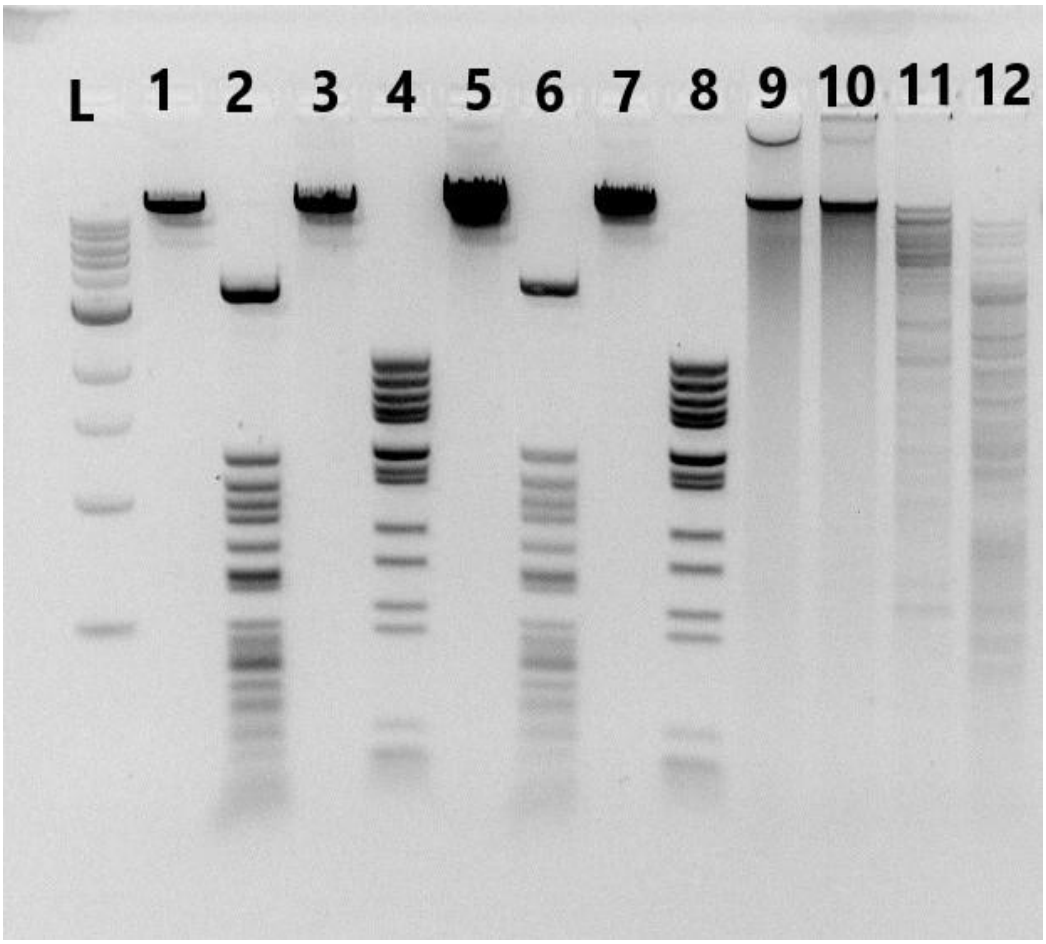

Figure 18. Restriction digest of different types of phages using three different restriction enzymes.
"L" represents New England Biolab's 1 kb DNA ladder. Mars Hill DNA was loaded in the following format: lane 9 undigested gDNA control, lane 10 DraI, lane 11 MspI, lane 12 TaqI. Lanes 1-4 represent the same series for phage Portland and lanes 5-8 represent a Portland-like phage.

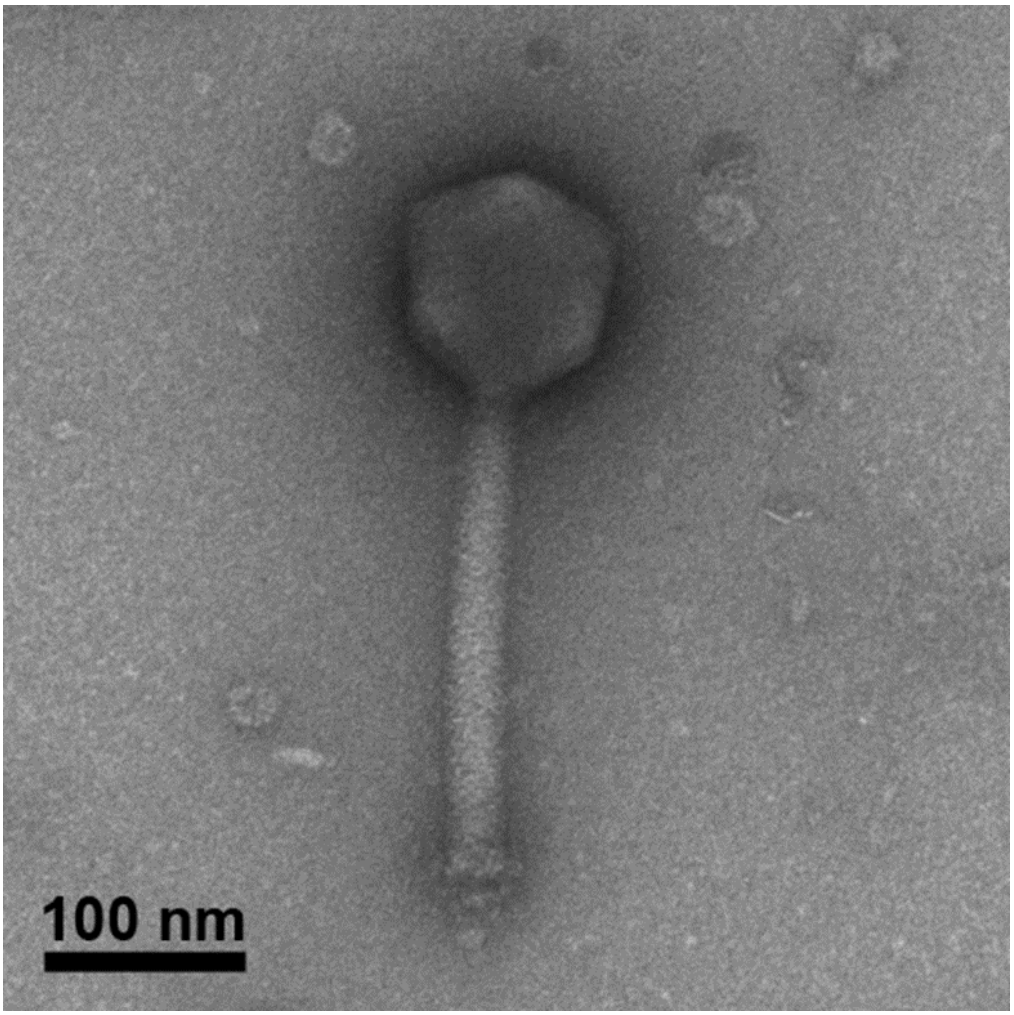

Figure 19. TEM image of Mars Hill.
Virions were stained with 2% uranyl acetate and imaged using a JEOL 1200 EX transmission microscope.

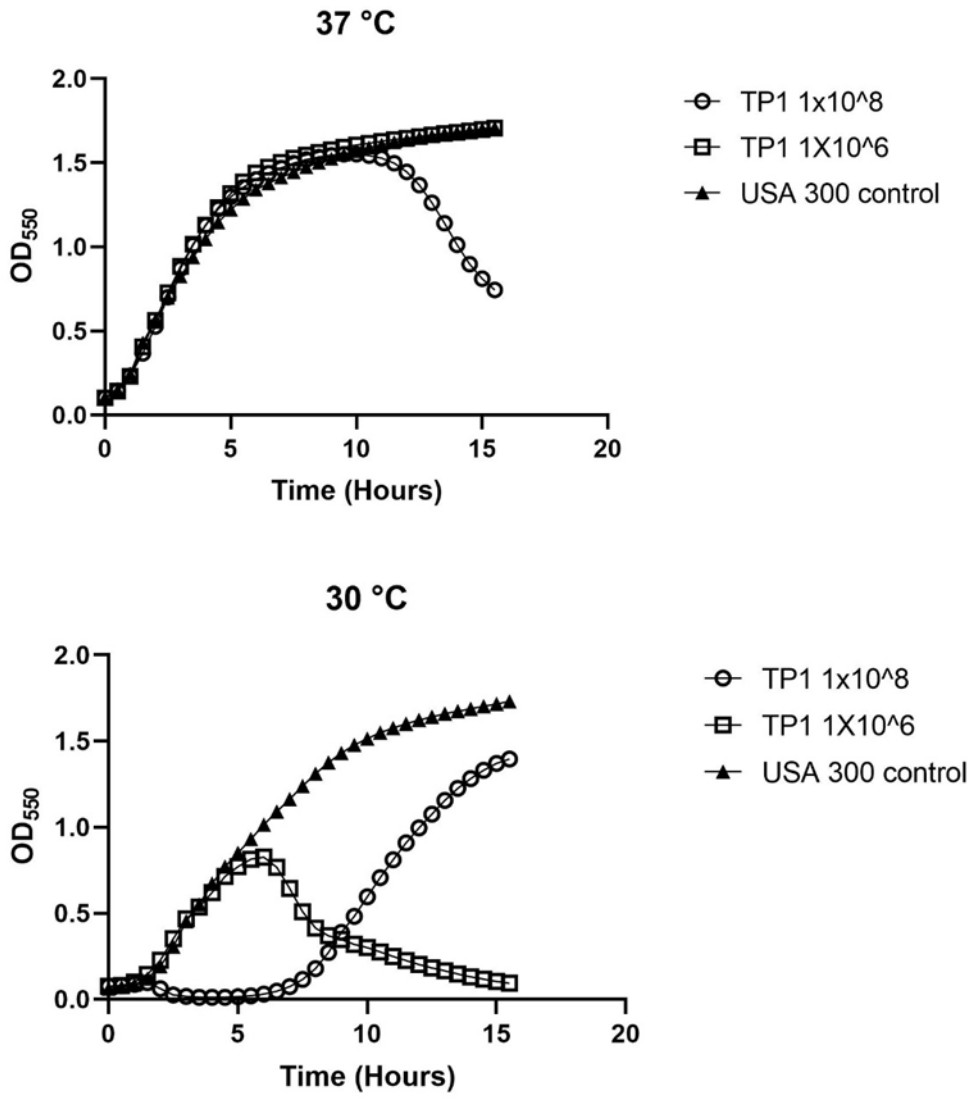

Figure 20. Temperature sensitivity of Mars Hill.
Microtiter 96-well plate host range results of phage Mars Hill (formerly known as TP1) at two different temperatures against host USA300-0114.

Sequencing of type I phages

Type I phage Mars Hill was refractory to sequencing across multiple different platforms. Illumina DNA library preparation using Truseq kits followed by sequencing using Illumina MiSeq 250-bp paired-end V2 500-cycle chemistry was unsuccessful. This workflow was attempted twice with Mars Hill and TP2 isolated during preliminary experiments and then once more two years later again with 4023-F15-H isolated from the US survey of swine environments. While Pacific Biosciences sequencing technology has been used previously to sequence phage with DNA modifications, Mars Hill genomic DNA failed Pacific BioSciences SMRTbell Express Template library preparation kit twice (Klumpp et al., 2014). Additionally, only unusable sequence was obtained when using Oxford Nanopore Technologies Rapid Sequencing Kit and the Accel-NGS® 1S plus DNA library kit from Swift Biosciences™ did not produce a usable library. A genome of Mars Hill was obtained in May of 2019 by using Illumina Truseq PCR-free library preparation and sequencing using an Illumina MiSeq V2 Nano 500 cycle kit.

Failure to obtain a genomic sequence of Mars Hill on multiple sequencing platforms is likely due to this phage having highly modified DNA, most likely a replacement of thymine for uracil as had been reported for the *Bacillus* jumbo phage AR9 and the *S. aureus* jumbo phage S6 (Lavysh et al., 2016) (Uchiyama et al., 2014). This substitution likely inhibits any form of sequencing library preparation that relies on DNA polymerases that are not equipped to handle uracil and therefore no product is produced. Also some companies change chemical formulation of products annually and

therefore there are no guarantees that some of these changes in chemistry also lead to failure to obtain sequence. Phage SPO1 was used as a positive control for the MinION sequencing and only unusable sequence was obtained. SPO1 DNA is comprised of hydroxymethyluracil nucleotides in place of thymine (Stewart et al., 2009). Additionally, traditional cell washing techniques did not work to flush either Mars Hill or SPO1 DNA out of the flow cell. Even after approximately five washes, signal of this DNA was still being registered in the background of a new sample. The specific type of DNA polymerase used in sequencing cartridges by Illumina is proprietary. However, it must be a DNA polymerase that is able to recognize modified DNA as a template for sequencing.

Genomic analysis

By using Illumina Truseq® PCR-free library preparation kit and sequencing using Illumina MiSeq V2 Nano 500 cycle reagents, genomes for four Type I phages were obtained. These phages are Mars Hill, Machias, Mapleton and Madawaska. All phage are "jumbo" phages with an average genome size of 269.7 kb (Table 12). Using a virtual enzyme digestion of Mars Hill gDNA, this phage contains 105 and 769 sites for EcoRI and DraI, respectively. However, as discussed above, no fragmentation was observed when phage genomic DNA was digested with either enzyme.

The closest phages that these jumbo phages are related to are two *Bacillus* phages, AR9 (NC_031039) and vB_BpuM-BpSp (KT895374.1), with all phages being more closely related to vB_BpuM-BpSp (Table 12). Blastn was used to identify the top five that share the most nucleotide identity with Mars Hill. The results from this

workflow show that all of the top five related phages are only weakly related to Mars Hill and that even the most similar, vB_BpuM-BpSp shares only 1.25% percent nucleotide identity with Mars Hill (Table 12).

Table 11. Genomic characteristics of four different jumbo phages.

Phage Original Name	Deposited Name	Host	Genome length (bp)	tRNA	DNA-directed RNA polymerase subunit β copies
TP1	Mars Hill	USA300-0114 (human clinical)	266,637	0	8
4011-F4-H	Madawaska	USA300-0114 (human clinical)	265,500	2	14
PD17-F16-2-S13	Mapleton	PD17 (swine isolate)	271,944	2	13
PD32-F11-S	Machias	PD32 (swine isolate)	274,533	2	14

Table 12. Comparative genomics of Mars Hill.
Percent identity at the nucleotide level for top five related phages for Mars Hill.

	Mars Hill	Terranova	phiSA_BS2	phiSA_BS1	7AX_1	VB_BpuM-BpSp
Mars Hill	100	0.17	0.28	0.29	0.16	1.25
Staph phage Terranova (*S. epidermidis* phage)	0.17	100	10.21	10.39	84.33	0.08
Staph phage phiSA_BS2 (K sized genome size isolated in China)	0.28	10.21	100	93.6	10.11	0
Staph phage phiSA_BS1 (K sized genome isolated in China)	0.29	10.39	93.6	100	10.28	0
Uncultured Caudovirales phage clone 7AX_1 partial genome (*Bacillus* Jumbo phage)	0.16	84.33	10.11	10.28	100	0.06
Bacillus phage VB_BpuM-BpSp (Jumbo phage)	1.25	0.08	0	0	0.06	100

With the genome size of these phages as well as the phenotypic characteristics matching the previously reported *S. aureus* jumbo phage S6 it is probable that these phages are related. However, since there was no genome reported for S6 it is currently not possible to say this for certain (Uchiyama et al., 2014).

Phage S6 DNA was reported to contain uracil instead of thymine in its DNA and therefore it is likely that the four jumbo phages Mars Hill, Machias, Mapleton and Madawaska also contain uracil-containing DNA. The presence of uracil-substituted DNA in these phages is also consistent with the inability to digest this DNA with common restriction enzymes and to obtain DNA sequence by methods commonly used for other organisms. A major obstacle in sequencing was likely the refractory nature of this DNA to PCR using common polymerases such as Taq and Pfu; as shown in Figure 21, Mars Hill genomic DNA could not be amplified by PCR using Taq or Pfu but could be amplified using the polymerase Phusion U, which is able to use uracil-containing DNA templates. The most commonly used library preparation methods for Illumina sequencing contain a PCR step, and the method that ultimately yielded DNA sequence of these phages was a PCR-free library preparation method. Difficulty in sequencing other phages with highly modified DNA has been reported. Phage AR9, which also contains uracil-substituted DNA, was sequenced by Illumina following library amplification using a uracil-compatible DNA polymerase (Lavysh et al., 2016). The *Bacillus* phage CP-51, which contains DNA with hydroxymethyluracil substituted for thymine, was only sequencable by a combination of PacBio and Sanger sequencing (Klumpp et al.,2014).

As seen *in vitro,* the DNA of Mars Hill and other tested *S. aureus* jumbo phages is highly resistant to digestion by common Type II restriction enzymes such as EcoRI and DraI. The substitution of non-standard bases in phage DNA is likely an adaptation that provides broad protection of the phage chromosome from cleavage by restriction systems residing in potential host cells.

The genome of phage Mars Hill is disorganized and does not have distinct gene modules, with genes of similar function scattered all over the ~267 kb genome (Figure 22). This lack of gene organization is common in jumbo phages (Yuan and Gao 2017). Most structural components were identified in the Mars Hill genome such as tail fibers, tail sheath, baseplate wedge, prohead core scaffold and the portal protein.

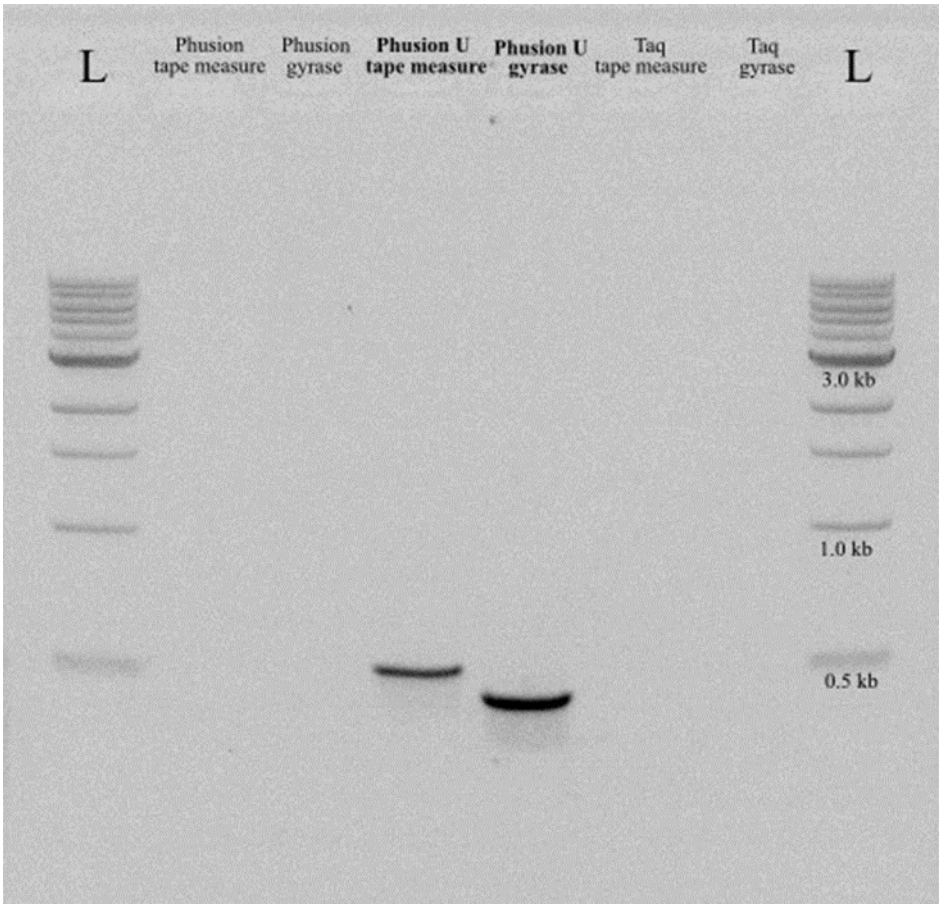

Figure 21. Assessment of DNA polymerases for PCR with Mars Hill DNA.
PCR products from three different polymerases used to amplify a potential tape measure gene and gyrase gene of Mars Hill.

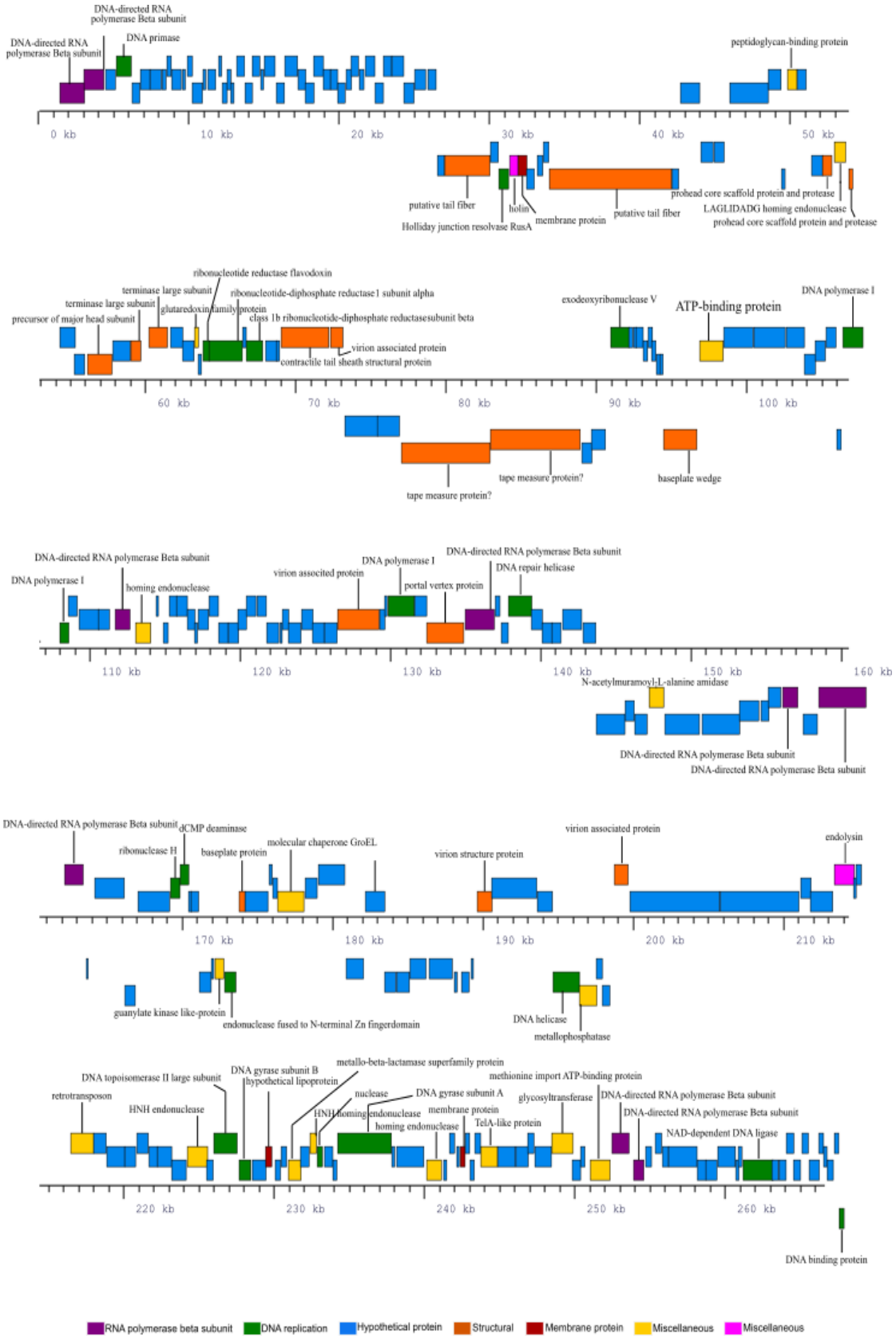

Figure 22. Mars Hill genome map.

However, the major capsid protein was not identified, and two proteins look suitable to be the tape measure proteins. These proteins both contain significant alpha-helical content as well as several predicted transmembrane domains which is indicative of tape measure proteins (Mahony et al., 2016). Structural genes are scattered from approximately 30-200 kb of the Mars Hill genome (Figure 22). All four phages contain dCMP deaminase genes, however, other genes that would add modifications onto uracil such as dUMP hydroxymethylase or HMdUMP kinase were not identified in the genomes of the *S. aureus* jumbo phages (Stewart et al., 2009).

Several jumbo phages encode their own DNA-directed RNA polymerases (Lavysh et al., 2016). All four type I phages have multiple copies and fragments of DNA-directed RNA polymerase β subunit as AR9 does. However, some of these phages have more fragments or copies with Madawaska and Machias having 14, Mapleton 13 and Mars Hill eight (Table 12) (Lavysh et al., 2016). These duplications and fragments are hypothesized to be the results of intron mobility in jumbo phages (Lavysh et al., 2016). Additionally like AR9, a GroEL chaperone protein was identified in the genome of Mars Hill (Lavysh et al., 2016). GroEL has been indicated in helping virion assembly. In T4 a GroEL homolog (gp31) is essential for folding of the major capsid protein and aids in the assembly of phage particles (Linder et al., 1994).

Strangely, there are no identified tRNAs in phage Mars Hill, while all others have two predicted. The presence of tRNAs is positively associated with genome size in phages. The presence of tRNAs in phage genomes is thought to be because the phages use certain codons that are rare in their bacterial hosts and they therefore carry their own

(Bailly-Bechet et al., 2007). For Mars Hill it may be that there were never tRNAs present, the previous tRNAs were lost as there was not enough selection pressure to keep them or that another sequence replaced them (Mira et al., 2001).

An alignment of all four phages shows that genome arrangement is similar, with a few smaller regions that have been rearranged (Figure 23).These regions generally contain hypothetical genes, however, one at ~240,000 bp is a region where a homing endonuclease as well as the DNA gyrase are located at in the Mars Hill. Mars Hill contains four regions with homing endonucleases, which promote gene exchange. Both *Bacillus* phages SPO1 and SP82 encode homing endonucleases (Belfort and Bonocora 2014). In phage T4 this is taken to the extreme with ~11% of the genome encoding homing endonucleases with 15 genes total (Edgell et al., 2010). Similarly, a second alignment using phage vB_BpuM-BpSp shows that only a very small portion of its genome is related to any of the type I phages (Figure 24). This smalls portion is located at ~130,000 bp in which there are hypothetical genes in the Mars Hill genome (Figure 24).

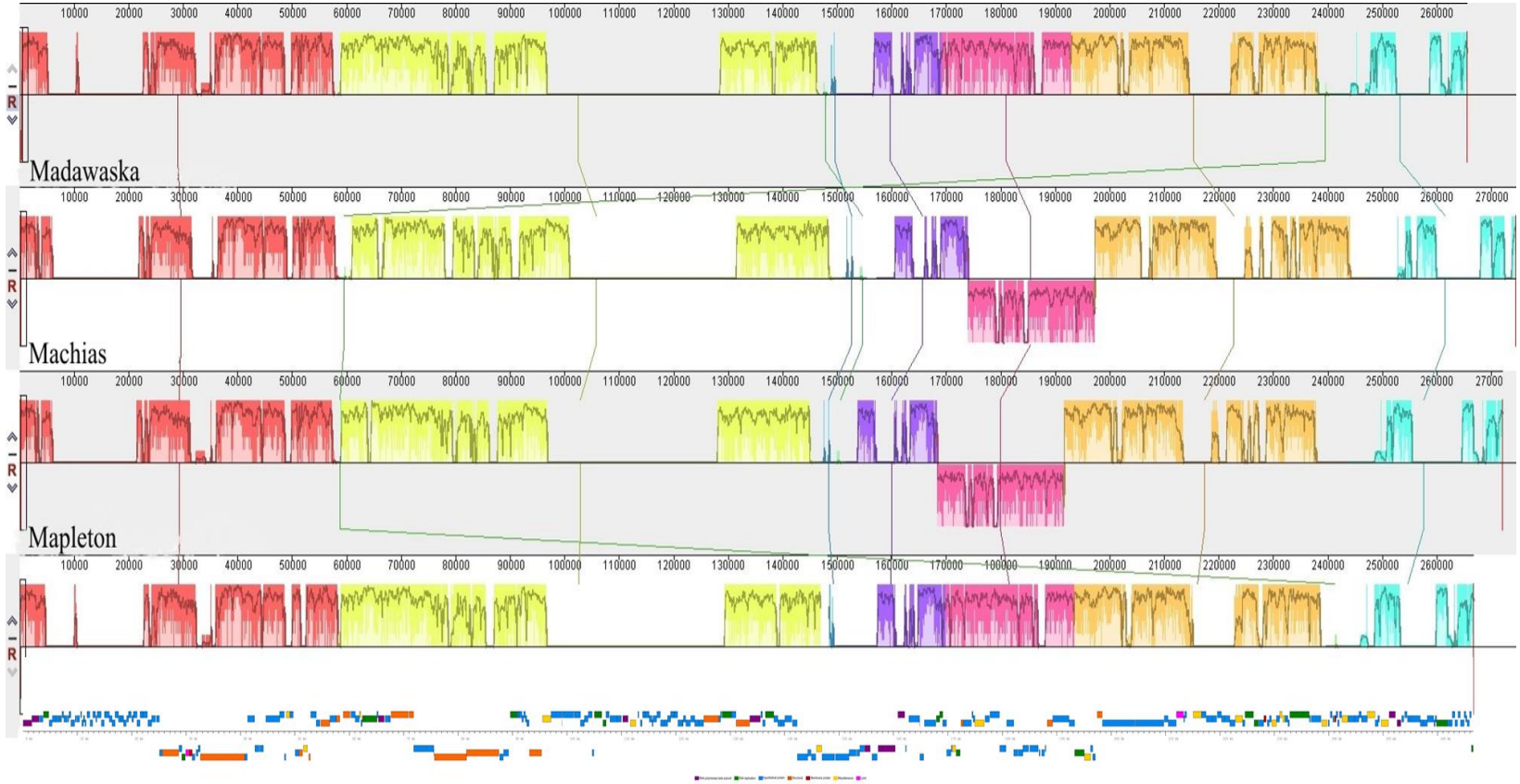

Figure 23. Progressive Mauve alignment of type I phage genomes.
Mars Hill genome map is displayed at the bottom for reference.

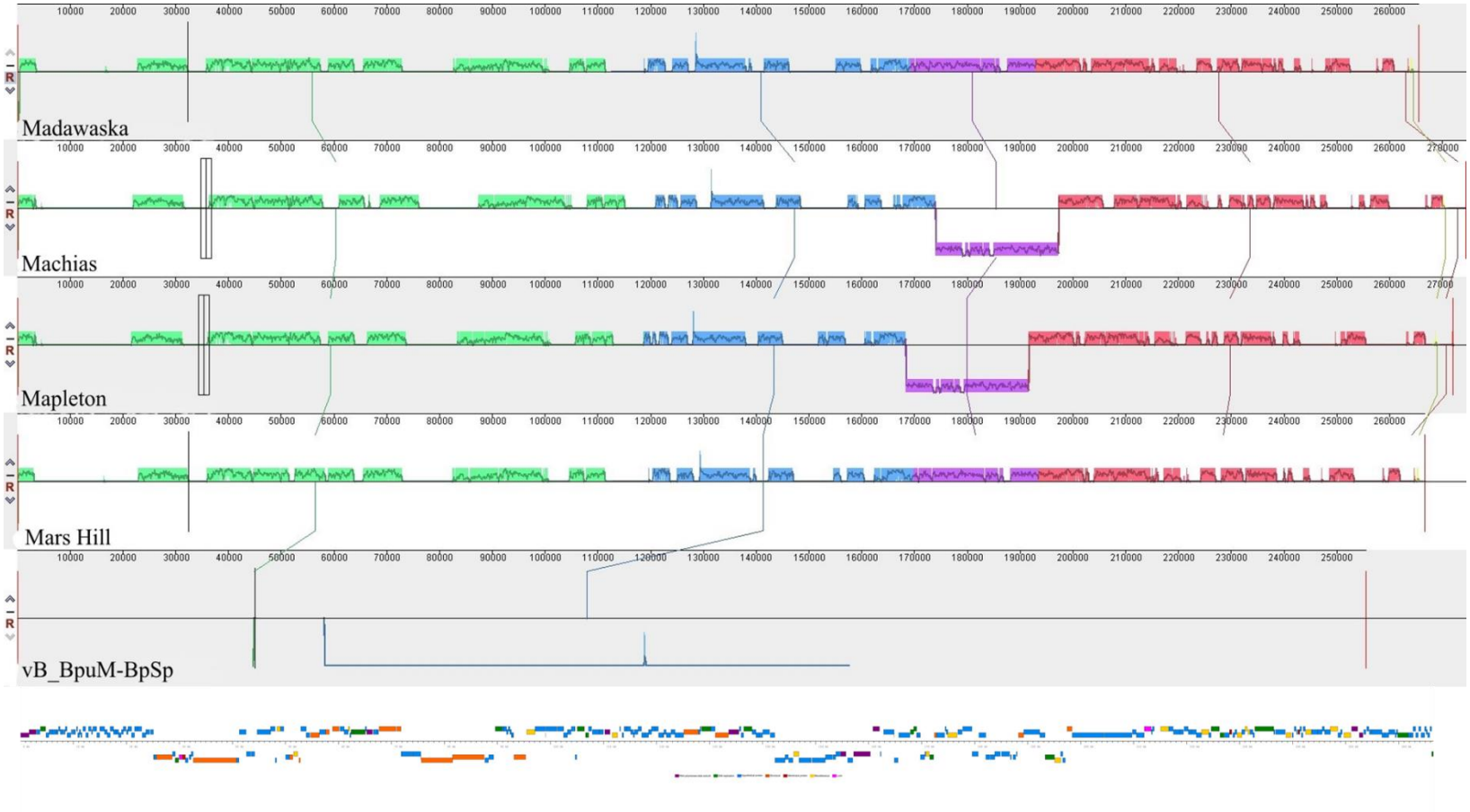

Figure 24. Progressive Mauve alignment of type I phage genomes with *Bacillus* jumbo phage vB_BpuM-BpSp.
Mars Hill genome map is displayed at the bottom for reference

Transduction abilities of type I phages

Phage S6 was reported to transduce a pCU1 plasmid at an efficiency of 10^{-7} transductants per bacteria as well as a plasmid containing the *mecA* gene at a efficiency of $5.2\ 10^{-11} \pm 9.0\ 10^{-11}$ per bacteria into a restriction minus strain of *S. aureus* (RN4220). Transduction of the pCU1 plasmid was also tested for several other species of staphylococcus including *S. epidermidis*, *S. pseudintermedius*, *S. sciuri* and *S. felis*. Transduction efficiency to these species ranged from 10^{-7} to 10^{-10} per bacteria. (Uchiyama et al., 2016). Testing of this phenomenon in Mars Hill was carried out by Dr. Lichang Sun. Initial experiments conducted by Dr. Sun showed that Mars Hill does not adsorb *S. aureus* cells at 37 °C. This result allowed for *S. aureus* cells to recover at 37 °C without the fear that new phage infections would occur. Dr. Sun found that Mars Hill was able to transduce a luciferase plasmid from *S. aureus* strain Xen 36 to a swine nasal isolate PD17 at an efficiency of 1.8×10^{-9} CFU/PFU. This rate is comparable to phi80α, a lysogenic *S. aureus* phage that is used frequently for the transduction of *S. aureus* isolates which is able to transduce plasmids at an efficiency of approximately 10^{-7} to 10^{-9} (Mašlaňová et al., 2016). Mars Hill, phage K and supernatant from *S. aureus* strain Xen 36 were tested for their transduction rate of the chromosomal *mecA* gene was investigated as well, however no transductants were obtained from any treatment. The results of these experiments indicate that type I can transduce plasmids with relatively high efficiency but not chromosomal elements.

Conclusion

Type I restriction digest group phages isolated from swine environments across the US presented unique challenges in both culturing and sequencing. Multiple commonly used sequencing platforms failed to either produce usable libraries or sequence for these phages. Type I phages are of *Myoviridae* morphology and appeared to be similar in size to phage K virions at first glance (Figure 19). However, after sequencing it was revealed that these phages are not K-like and represent a new type of *S. aureus* phage. Genomes were obtained for phages Mars Hill, Machias, Mapleton and Madawaska. All four phages are jumbo phage with genomes well over 200 kb (Yuan and Gao 2017). These phages are only distantly similar to other known phages, with the most similar being *Bacillus* phage vB_BpuM-BpSp (KT895374.1). However, Mars Hill is similar in both morphological and phenotypic characteristics to phage S6 (Uchiyama et al., 2014). All genomes are littered with copies of DNA-directed RNA polymerase β subunit and contain more copies than previously reported in other jumbo phages. Mars Hill, Machias, Mapleton and Madawaska all represent a new type of *S. aureus* phage.

CHAPTER V

CONCLUSION

Swine production environments across the globe provide are a reservoir of *S. aureus* with some harboring MRSA (Dignard and Leibler 2019). This study sampled 20 different swine farms from across the US and found that 63% of swine farms were positive for *S. aureus* phage. This result indicates that in the US these swine production environments also provide a possible reservoir for *S. aureus* phages. From these positive farm samples, 71 phages were isolated, 51 were typed by restriction digest, 13 were imaged using transmission electron microscopy, 9 were characterized for host range and 20 were sequenced.

S. aureus phages isolated in this study were clearly related to previously described phages, except for Mars Hill-like phages, which are a novel class of "jumbo" myoviruses. *Podoviridae* were isolated most frequently and only one K-like myophage was isolated. Limitations of this study include the small number of swabs taken from only one barn on each sampling site. Also, while a wide variety of *S. aureus* strains were used as enrichment hosts, there could have been phages in the environment that did not infect these hosts or were not able to replicate to a detectable limit. Thus the number and type of phage recovered in this work represents the lower bound of phage prevalence and diversity in swine production environments.

Of the virulent phages tested in this study *Podoviridae* displayed the narrowest host ranges and *Myoviridae*, both K-like and Mars Hill-like, displayed the widest range. Both types of *Myoviridae* were also able to infect strains of *S. pseudintermedius* and *S.*

epidermidis although this ability was very strain specific for both *Staphylococcus* species. Several *Siphoviridae* were isolated in this study, indicating that these phages are capable of persisting in the swine barns and potentially mediating horizontal gene transfer. The lack of genomic diversity in these *Siphoviridae* was surprising. The similarity of these phages to previously isolated *S. aureus* strains from swine sources indicates that they are conserved in ST398 swine *S. aureus* isolates.

A seemingly small change in phage isolation incubation temperature had a large impact on this study. *S. aureus* phages were isolated from environmental samples at 30 ºC instead of the traditional 37 ºC in hopes of replicating ambient barn temperature as well as human nasopharynx temperatures which average around 34 ºC (Keck et al., 2000). Additionally, preliminary experimental results showed that plaque formation was optimal at 30 ºC rather than 37 ºC for phage K and other K-like phages. Without this adjustment Mars Hill-like phages would not have been isolated as these phages are temperature sensitive and do not form plaques or lyse their hosts at 37 ºC. These phages would also have been missed should we have attempted a metagenomic study that used standard (i.e., PCR based) library preparation methods on phage from swine environmental samples. This study raises questions about the diversity of phages with modified DNA that are missed by metagenomic studies using traditional library preparations to identity phage sequences.

Mars Hill-like phages are new type of *S. aureus* phage, which share many phenotypic characteristics with phage S6 (Uchiyama et al., 2016). These phages are jumbo phages with genomes of ~269.7 kb. Mars-Hill-like phages possess many

hypothetical genes and identifying even key virion structural proteins has been difficult due to their unrelatedness to other phages. Mars Hill displayed an ability to transduce a luciferase-containing plasmid at an efficiency of 1.8×10^{-9} CFU/PFU which is comparable to commonly used transducing phages for *S. aureus* molecular work. This result indicates that these phages in addition to *Siphoviridae* are also possible vectors for horizontal gene exchange in *S. aureus*. However, Mars Hill was not able to mobilize the chromosomal *mecA* gene and this indicates that chromosomal elements are not highly mobilized by these phages.

A high prevalence of MRSA in swine production environments may pose a threat to worker health, especially for employees involved in activities such as pressure washing and tail docking as these activities generate particle sizes capable of depositing primarily in human upper airways but also the primary and secondary bronchi, terminal bronchi and alveoli (Madsen et al., 2018). LA-MRSA isolates have been found to have a half-life of five days in settled barn dust with an approximate reduction of 99.9% after 66-72 days (Feld et al., 2018). Therefore, vehicles and other movement on the farm may transfer LA-MRSA into or out of these environments. It is possible that the abundant small *Podoviridae* could be used for the biocontrol of *S. aureus* in swine barns. However, *Podoviridae* have narrow host ranges and therefore may not be ideal in a situation where target strains are unknown and have not been tested to see if they are sensitive to these phages. K-like phages could also be used as they do have wide host ranges but should be used with caution as these phages do have the potential to infect other *Staphylococcus* species. This would be especially true for using these phages for

human phage therapy as it is not known what further impacts these phages have on the microbiome. Although Mars-Hill like phages have the broadest host ranges, their ability to transduce plasmid DNA raises concerns for their use in the environment. While the *mecA* gene was not mobilized by Mars Hill in this study, *S. aureus* can carry many different plasmids which carry antibiotic resistance genes, such as vancomycin resistance genes as well as resistance to organic and inorganic ions (Zhu et al., 2008; Malachowa and DeLeo 2010). A previous study showed that nasal decolonization of swine with both K-like and P68-like phages was unsuccessful (Verstappen et al., 2016). Therefore, while these phages may not be suited to administer to workers or animals they could play a role in sanitizing pens or other materials during sanitation processes.

Future directions include investigating the genomic diversity seen in *Podoviridae* genomes that may influence their host ranges, identifying genes of unknown function in sequenced Mars Hill-like phages, sequencing the remaining type I phages, attempting to isolate other Mars-Hill like phages from international swine barn samples and evaluating the potential P68-like *Podoviridae* or K-like *Myoviridae* may have for both disinfecting swine barns of MRSA and phage therapy applications for human *S. aureus* infections. Isolated *Podoviridae* can be 99.9% similar at the DNA level and yet still have different plaque morphology or host range (Chapter 2). Equally puzzling is that these phages each contain only ~20 genes but still have variation in hypothetical genes with unknown function. Phages have been described as a repository of "genetic dark matter" in the biosphere, due to their large numbers, high diversity and high content of genes encoding novel proteins (Hatfull, 2015). Future experiments should include determining which

features of the *Podoviridae* genomes affect host range, such as the different minor tail protein arrangements and identifying the roles of genes of unknown function. There are still 17 more type I phages from this study that could be sequenced and compared to be able to draw more conclusions about this new phage type. The four sequenced Mars Hill-like phages have an average ~270 kb genome containing mostly hypothetical genes, leaving multiple routes for future experiments to be conducted.

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