Investigation of flagellotropic phage interactions with their motile host bacteria

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Biological Sciences

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May 12, 2021
Blacksburg, VA

Keywords: phage, receptors, Agrobacterium, Salmonella

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ABSTRACT

Bacteriophages cohabit with their bacterial hosts and shape microbial communities. To initiate infection, phages use bacterial components as receptors to recognize and attach to hosts. Flagellotropic phages utilize bacterial flagella as receptors. Studies focused on uncovering mechanistic details of flagellotropic phage infection are lacking. As the number of phage-based applications grows, it is important to understand these details to predict the potential outcomes of phage therapy. To this end, we studied two flagellotropic phages: Agrobacterium phage 7-7-1 and bacteriophage χ. Phage 7-7-1 infects Agrobacterium spp., while bacteriophage χ infects Salmonella and Escherichia coli.

Chapter 1 consists of a literature review. Chapter 2 addresses factors underlying phage-bacteria coexistence. We document the emergence of a sector-shaped lysis pattern following co-inoculation of phage χ and one of its Salmonella hosts on swim plates. We propose that this pattern serves as a reporter for balanced phage-bacteria coexistence. Using a combined experimental and mathematical modelling approach, we discovered that variations to intrinsic factors (i.e., bacterial motility, phage adsorption) skews the pattern towards either bacterial or phage predominance. Thus, this computational model may be used to predict phage therapy application outcomes.

Chapter 3 details the identification of cell surface receptors essential for phage 7-7-1 infection using a transposon mutagenesis approach. We identified three Agrobacterium sp. H13-3 genes involved in phage 7-7-1 infection. Using mass spectrometry and other analyses, we determined that the LPS profiles of strains lacking these genes varied compared to the wild type.
Thus, LPS is a secondary cell surface receptor for phage 7-7-1. Chapter 4 focuses on the discovery of phage encoded receptor binding proteins (RBPs) in *Agrobacterium* phage 7-7-1. Using an RBP screen, we discovered three candidate RBPs. We learned that our top candidate, Gp4, inhibits the growth of *Agrobacterium* sp. H13-3 cells in a motility and glycan dependent manner. Because of its bacteriostatic activities, this protein is a promising candidate for therapeutic use. Overall, the described works contribute to a deepened understanding of flagellotropic phage infection and the factors influencing their coexistence with motile bacteria. These works will contribute towards the development of phage therapies using whole phage or their components.
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GENERAL AUDIENCE ABSTRACT

Bacteriophages, or phages for short, are the natural killers of bacteria. Like antibiotics, they can also be used as medicines to treat bacterial infections. Their attack on bacteria begins by recognizing specific parts of the bacterial cell and attaching to them. These parts are called receptors. To use phages as medicines it is important to understand how they recognize and kill bacteria. This information is helpful when deciding which phage should be given to treat a bacterial infection and to predict the outcomes of these treatments. In this work, we focused on two phages to answer different questions. Both phages use long helical thread-like structures, called flagella, as receptors. Flagella help the bacteria to move through the environment and reach new areas with more nutrients.

One of these flagella-dependent phages, called phage 7-7-1, infects plant pathogens that cause tumor-like growth in plants. We found that this phage uses two very different host cell components during infection and identified one of the phage proteins that interacts with these receptors. This protein prevents the growth of the plant pathogen, which makes it a promising candidate for therapeutic use. We also investigated how another bacterial virus, bacteriophage $\chi$, is spread throughout the environment and co-exists with its motile bacterial host. We built a computational model that can predict how altering different variables affects phage-bacteria coexistence. With additional research, this model will be a useful tool for predicting the outcomes following phage treatment.
DEDICATION

Para mi familia, especialmente mis padres sin los que nunca hubiera tenido la oportunidad de realizar mis sueños y los que me enseñaron como trabajar y enfocarme.
ACKNOWLEDGEMENTS

Thank you to my family and all of those who believed in me throughout this journey. If I could thank you all as you deserve, I would easily need another dissertation length document so I will do my best to sum up my appreciation here in no particular order.

To the entire Nicola lab, especially Dr. Anthony Nicola and Dr. Darin Weed for taking a chance on me, sparking my love for virology, and for patiently answering my endless questions. A special thanks to Sue Pritchard for guiding me through pipetting 101 and all of our lengthy chats in the lab. And also to Dr. Becca Weed for the delicious food, good talks, and being a friend.

To Dr. William B, Davis and Dr. Mary Sanchez-Lanier for mentoring me, believing in my abilities, and pushing me to reach for opportunities I thought were out of my reach.

To my McNair family at WSU, especially Dr. Raymond Herrera and Dr. Mary Crowell, who expected the best and pushed me to excel. I could not have gotten here without you and the support (financial and emotional) that I got from McNair.

To the First Scholars program at WSU, especially Alicia Shrestha and Eva Navarijo, who always were there to listen though the struggles and moments of joy.

To the Scharf lab, where I have met some of the brightest and best people. Ben, thank you for showing me to enjoy the moment. Hardik, thank you for helping me ask the right questions. Rafael, thank you for showing me that caring and maintenance is an important part of science too. Katie, thank you for being Supergirl and setting an example of what a female scientist should be. Karl, thank you for providing balance to the lab and for always stopping to smell the flowers. Alfred, thank you for your positive energy and encouragements. And to Nate, thank you for always being there to crack a joke, establishing phage corner with me, and being a good friend.

To the various undergrads that have worked for me who all taught me something and helped me better develop as a mentor.

Catherine Freed, thank you for being a good friend, your positive disposition, and for the numerous paint nights/shopping trips that helped keep me grounded these last couple of years.

To Manisha, Holly, and Bidisha thank you for the brunches, the fun wine outings, and together building a strong group of women in microbiology.

To Dr. Scharf, for always pushing me to do better, answering my not-so-smart questions, listening, and overall showing me what type of mentor I want to be. I will never be able to express just how much I have learned from you.

To Earl, for challenging me and helping me see what I could not. You are a pleasant relief from the normal.

To Dr. Liz Zamora, without you my time at WSU would not have been the same. Thank you for helping me to become more in tune with my emotions, listening to my whining, and inspiring me to be a better scientist.

To Chey and Grizelda, I cannot even begin to tell you how much you mean to me. I am lucky to have met you in Gannon-Gold and for all the great memories. Your presents, conversations, and constant belief in me pushed me through this journey more than you can know.

To my sisters, who are chingonas, my prime examples of strength and will tell me the truth even when I do not want to hear it.

To my brother, who inspires me every day to work hard and enjoy what I do.
Gracias a mis padres por todos sus sacrificios. Mama tu me has enseñado como luchar contra la adversidad y por eso he podido salir adelante. Papa, gracias por siempre recordarme que aunque estes desacuerdo al final de el dia tengo que tomar mis propias decisions, aunque a veces esas decisiones me lleven lejos de ti.

And last but certainly not least, thank you to my husband Tim Arapov, for listening to my griping, being the voice of reason (sometimes), challenging me, and loving me when I am not easy to love.
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Chapter 1: Introduction

Bacteriophages, the viruses that infect bacteria, cohabit with their hosts in various environments. Because of their bactericidal activities, phages shape microbial communities and drive their evolution. Moreover, they are the most abundant biological particles on planet Earth (1). Bacteriophages are diverse in structure and are grouped based on their genomic content (DNA or RNA) and morphology. Tailed phages are part of the Caudovirales order, which is comprised of 3 families: the Myoviridae, Siphoviridae, and Podoviridae. Myoviruses have contractile tails while siphoviruses have long, flexible tails. Conversely, podoviruses have short non-contractile tails (1-4) (Figure 1.1 B). The genome sizes of bacteriophages are also variable with some, referred to as jumbo phages, containing genomes larger than 200 kb (5).

Despite bacteriophage diversity, the general infection scheme is highly conserved. Lytic phage infection can be summarized into four parts: entry, viral replication, virion production and assembly, followed by virion egress. During viral entry, phage virions interact with host cell receptors, which are native components of a bacterial cell, to initiate infection. Receptor binding allows the phage to gain access to the cell surface and facilitates the ejection of viral genetic material from the virion capsid and injection into the host cell cytoplasm. Then, the host cell machinery is hijacked to replicate, transcribe, and translate the viral genome. Following translation, proteins are assembled to generate new viral particles and the phage induces host cell lysis. This process frees phage progeny and enables colonization of more host cells (3, 4, 6). For lysogenic phages there is an extra step following DNA injection into the host cell that involves integration into the host cell chromosome generating a prophage. However, during a process
called induction, the prophage is excised from the bacterial genome and the infection cycle continues as described above for lytic phages (4) (Figure 1.1 A).

Without phage engagement with host cell receptors during viral entry, infection is not possible. Moreover, this step of infection is a critical determinant of phage host range as not all bacterial cells have the receptors required for recognition. The type of receptor used during phage infection varies, but the most common examples are membrane proteins, efflux pumps, lipopolysaccharides (LPS), teichoic acids, and capsular polysaccharide (CPS) (3, 7-9).

Flagellotrophic phages use bacterial flagella, helical threadlike structures that drive movement, as initial receptors. Studies centered on this type of phage are limited. As more phage-based applications emerge to combat bacterial infections in different settings, it is critical to understand different types of phage-host interactions to 1) select the best phages for these applications and 2) understand the possible tradeoffs resulting from use of phage. To this end, we focused our studies on two different models for flagellotrophic phage-host interactions: Agrobacterium phage 7-7-1 infecting Agrobacterium spp. and bacteriophage χ targeting Salmonella enterica and Escherichia coli. Resistance to flagellotrophic phages typically occurs via the alteration or abolishment of flagellar function. Since motility is an important virulence factor, phage therapies involving flagellotrophic phages may result in bacterial attenuation, highlighting the promise for flagellotrophic phages in these therapies. By studying the two systems mentioned above we aimed to build a larger understanding of flagellotrophic phage interactions with their hosts. We paid special attention to detailing interactions taking place between virions and host cell receptors during viral entry.

**Phage-flagella interactions: a critical step during flagellotrophic phage infection**
Flagellotropic phages attach to host flagellar filaments to initiate infection. Although the modes of interactions with flagellar filaments differ, reliance on rotation for efficient and, in some cases, successful infection is conserved (10-13). Using these filaments for recognition of host cells may confer specific advantages including an increased surface area for encountering hosts, providing additional specificity for discriminating between host and non-hosts, and ensuring that host cells are metabolically ripe for infection. Regardless of the reasons underlying these interactions, flagellotropic phages rely on flagella as initial binding targets (10-12, 14-18). Flagellar filament structure and composition varies depending on the bacterium in question. Regardless of differences in flagella, some of the components that form this structure are highly conserved amongst diverse bacterial species (19-23).

**Flagellar rotation and structure in different bacterial systems**

The flagellation of bacteria varies in flagellar number and their positioning on the cell. Flagella can be distributed along different locations on the cell surface. The flagellation can be polar, with one or more flagella located on the poles of the cell, or peritrichous, with flagella present all over the cell body (24, 25). Some bacteria also contain endo-flagella which are located inside the bacterial cell (21, 24, 25). Polar flagella can be further described as monotrichous (single flagellum on one end of the cell), amphitrichous (filament on opposite ends of the cell), or lophotrichous (multiple flagella stemming from one spot) (24, 25). There is also a newly proposed mode of flagellation called rototrichous, where a single flagellum on one side of the cell is positioned longitudinally, and its motor rotates the filament around the host cell body (26). In addition to variances in flagellation patterns, there are differences in the flagella structure. The movement of bacterial flagella is driven by a rotary motor embedded in the cell
membrane and driven by ion motive force (20, 27). In *Salmonella enterica* and *Escherichia coli*, the flagellar motors at the base of the rotate bidirectionally and can switch this rotation from a counterclockwise (CCW) to clockwise (CW) direction. CCW rotation results in bundling of the peritrichous flagellar filaments and smooth swimming called a run. The switching from CCW to CW rotation induces a tumble due to the disruption of the flagellar bundle and reorientation of the cell body. In the absence of attractants, this switching between swimming and tumbling occurs randomly and is called the random walk. However, when an attractant is present, the tumbling instances are reduced and the runs sustained for a longer time. This is called the biased random walk and ultimately results in chemotaxis or movement in response to chemical stimuli present in the environment (19, 21, 22, 28). In contrast, flagellar motors of *Agrobacterium* sp. H13-3 and *Sinorhizobium meliloti* do not switch direction and rotate CW exclusively. Tumbling occurs when the rotation speeds of the flagellar filaments are decreased asynchronously, resulting in the bundle breaking apart (29, 30). Despite these differences, the flagellar structure of the four species is similar. Extensive structural and molecular studies done on *E. coli* and *S. enterica* have culminated in the construction of a model. A flagellum is made up of a basal body, hook, and filament. The basal body functions as a rotary motor powered by proton motive force across the cell membrane. It has a stator and a rotor component. (19, 22, 23). The stator is made up of the MotA and MotB protein complexes that surround the rotor at the flagellar base and anchor it to the cell wall. The MotA/B complexes form a channel through which protons flow to fuel the rotation of the motor (31). In *Agrobacterium* sp. H13-3 and *S. meliloti*, flagellar rotation also involves MotC and MotE (Figure 1.2). MotC forms a complex with MotB to enable proton flow through the MotA/B channels. MotE is a chaperone for MotC that aids in correct folding and transport of MotC while en route to MotB (32).
The rotor consists of the C-ring, MS-ring, and the rod. In addition, the L- and the P-ring function as bushings to hold the rod in place (31). FlgI forms the P-ring which is found in the bacterial peptidoglycan. The L-ring is composed of FlgH and resides in the outer membrane. The C-ring resides in the cytoplasm and is made up of FliM, FliN, and FliG. The C-ring interacts with the stator units for torque generation. Interaction of FliM with the phosphorylated and activated chemotactic response regulator CheY-P controls the direction of rotation in *E. coli* or the rotational speed in *Agrobacterium* sp. H13-3 and *S. meliloti*. The MS ring is constituted by FliF and serves as an anchor to the cytoplasmic membrane. FlgE makes up the hook, which serves as the flexible connector between the rod and the filament (19, 21, 23). It is important to note that FliK is the main regulator of hook length (33).

The filament portion of the flagellum is made up of flagellins, which vary in type and number based on the bacterial species (21, 23). In *Salmonella enterica*, the flagellar filaments are made up of FliC and FljB, which are expressed alternately so that only one flagellin comprises the filament in a given cell at a given time (34). In *Agrobacterium* sp. H13-3, the FlaA, FlaB, and FlaD subunits make up the flagellar filament. Of the three, FlaA is the primary flagellin required for proper flagellar structure. In addition to FlaA, one of the secondary flagellins is essential for motility (21, 35, 36) (Figure 1.2).

*Agrobacterium phage 7-7-1 and its host, Agrobacterium sp. H13-3*

Our primary model for examining flagellotrophic phage interactions is *Agrobacterium* phage 7-7-1. This phage infects *Agrobacterium* spp., a genus comprised of gram-negative bacteria most well-known for causing crown gall disease in various plants (36-41). These tumor inducing pathogens cause irreparable changes to host plants, resulting in reduced crop yields and
plant growth. There is currently no treatment for getting rid of these infections, and growers instead must manage infections by discarding diseased plants, sanitizing tools, and avoiding planting in areas where recent infections were seen (40). The annual economic impacts of these infections are burdensome and in the state of California are estimated to be $23 million annually (41). Using phage to kill pathogenic *Agrobacterium* spp. is a promising solution to this pressing problem.

The first identified host for phage 7-7-1 was *Agrobacterium* sp. H13-3, a flagellated, soil-dwelling bacterium. It was initially isolated from the rhizosphere of *Lupinus luteus* in Hungary (42) and has also been found to reside in the rhizosphere of switchgrass in Kellogg Biological Station in Hickory Corners, Michigan via metagenomic data (43). Although it lacks a Ti plasmid, which renders it non-pathogenic, it is closely related to *Agrobacterium tumefaciens*. Additionally, this bacterium has been used as a model for studying motility and flagellar structure in *Alphaproteobacteria*, which differ from the canonical *E. coli* and *Salmonella* paradigms (44-47).

*Agrobacterium* sp. H13-3 has a genome size of 5.57 Mb containing a 2.82 Mb circular chromosome, 2.15 Mb linear chromosome, and a 0.6 Mb accessory plasmid (47). This bacterium moves through the soil environment by using its peritrichous flagella that rotate exclusively clockwise (30, 35). Phage 7-7-1 uses these rotating flagellar filaments to initiate infection. This phage has a 69 kb dsDNA genome and belongs to the *Myoviridae* family. Its structure consists of a contractile tail, a hexagonal head, collar, tail sheath, and 5 bushy tail fibers 16 nm in length (38) (See Figure 1.3). Phage 7-7-1 is exclusively a lytic phage and produces 120 particles per infected cell during its 80-minute replication cycle (37, 48).
Bacteriophage $\chi$ and its *Salmonella* host

Our other model for flagellotropic phage infection involved bacteriophage $\chi$, which infects *Enterobacteriaceae* like *Salmonella enterica* and *Escherichia coli*. For our studies, we specifically examined interactions between this phage and *Salmonella enterica* serovar Typhimurium 14028s. This bacterium contains a 4.8 Mb circular chromosome and a 0.9 Mb plasmid (49). *Salmonella* belonging to the Typhimurium serovar are pathogenic causing gastroenteritis in humans. The most common presentations of illness include abdominal pain, fever, and severe diarrhea (50). In the United States alone there are roughly 100,000 *Salmonella* infections annually that are antibiotic resistant (51), pushing the need for alternative methods of treating these infections, such as using bacteriophages. Bacteriophage $\chi$ has a 59 kb dsDNA genome and is a member of the *Siphoviridae* family. It targets *Salmonella enterica* serovar Typhimurium 14028s by hijacking its bidirectionally rotating flagellar filaments to initiate infection (11). The virion is made up of an icosahedral head, a long non-contractile tail and a single tail fiber 220 nm in length (52) (Figure 1.4). Phage $\chi$ is a lytic phage with a latent period of 60 minutes and a production of 200 particles per cell (53).

Modifications to flagella and rotation affect host cell infection by phage 7-7-1 and bacteriophage $\chi$

Phage 7-7-1 requires the presence of rotating flagella for successful infection (36, 37). If the flagellar filaments are structurally intact, but not rotating due to the deletion of *motA*, the phage does not infect. Flagellar rotation speeds also directly affect virion production. For example, modifications to MotA that result in faster flagellar rotation yield higher number of virions produced over time. Meanwhile, slower flagellar rotation results in lower numbers of
phage progeny. Flagellar structure is also an important determinant of phage 7-7-1 infection. Alterations to flagellins that result in straight, truncated flagella reduce overall infection (36). Rotation and flagellar structure also impact infection by bacteriophage $\chi$. Flagellar rotation is required for infection by this phage as exemplified by resistance to phage in strains that have paralyzed flagella. Deletions of flagellin genes that result in the absence of a flagellar filament has the same effect. Bacterial mutants that have polyhooks (i.e., extended hook length) but no flagellar filaments are still sensitive to infection by $\chi$, but to a lesser extent than wild type. Moreover, the polymorphism of flagellar filaments is also an important determinant for $\chi$ infection. Flagellar filaments are made up of 11 protofilaments. In Salmonella and E. coli, these protofilaments have two confirmations, referred to as L or R. Different combinations of these protofilaments result in polymorphs with altered filament surface structures that change helical parameters such as handedness and pitch (28, 30, 46). In S. Typhimurium polymorphic flagellar mutants that contain a higher fraction of R-protofilaments have helical grooves with a smaller pitch and are $\chi$ phage resistant (28). It is hypothesized that this is due to the inability of the $\chi$ tail fiber to fit into these tighter grooves (54). Additionally, the direction of flagellar rotation plays an important role. Mutants with flagella that have a bias towards CCW rotation are more sensitive to $\chi$ phage infection. However, mutants with flagella that only rotate in the CW direction are $\chi$ resistant. This has led to the hypothesis that CW rotation pulls phage particles towards the cell surface (54).

**Phage translocation down the flagellar filament**

Following flagellar attachment, flagellotrophic phages like phage 7-7-1 and $\chi$ phage must make their way down to the cell surface to continue infection. $\chi$ phage is proposed to move down
the flagellar filament analogous to the way a nut moves down a bolt, using its tail fiber to adhere to the grooves in the host’s flagellar filament (54). The mechanism used by phage 7-7-1 to traverse the length of the flagellar filament is likely different than that of χ phage. As previously mentioned, the flagellar motor of Agrobacterium sp. H13-3 rotates unidirectionally, while the motors of Salmonella and E. coli switches rotation direction (29, 30). Moreover, the filament surface structures of enterobacteria are different from that of Agrobacterium spp. Salmonella and E. coli have plain flagella, which switch helicity (left-handed to right-handed) and have a smooth surface structure. Meanwhile, Agrobacterium sp. H13-3 has complex filaments that do not switch handedness and have a coarser surface structure (55, 56). The filament surface structure is an important determinant of phage infection for χ as described above. This is likely also true for phage 7-7-1 given that modifications to the overall flagellar filament shape, i.e., production of straight flagellar filaments that still contain helical ridges and grooves permit phage infection (35, 36). Furthermore, the tail fibers in these phages are vastly different from one another. χ phage has one long tail fiber, while phage 7-7-1 has 5 bushy tail fibers(11, 38, 52). Given the variations in host flagellar motor rotation and filament surface structures in addition to differences in the tail fibers on virions, the mechanisms used for phage translocation down the flagellar filament are likely different for these two phages.

**Objectives of this work**

The growing number of phage applications calls for a need in understanding diverse phage interactions with their bacterial hosts. Flagellotropic phages were discovered in the 1930s (53), but have not been extensively studied. To design well-Informed phage therapies and truly understand the tradeoffs resulting from these therapies, it is imperative that we characterize
diverse interactions between phages and their hosts, especially those which are currently understudied. Thus, we set out to build a model for flagellotrophic phage infection by focusing our investigations on two phages with different host ranges.

In chapter 2 we explored the factors important for phage dispersal by motile bacteria using a hybrid experimental and computational modelling approach. We propose that a novel, sector-shaped lysis pattern is representative of a balanced co-existence between bacteriophage $\chi$ and *Salmonella enterica* serovar Typhimurium 14028s. We determined that intrinsic factors are important in maintaining the phage-host balance. Ultimately, we created a model that, with more work, can serve as a useful tool to predict the outcomes of phage therapy applications. In chapter 3, we focus on identifying essential cell surface receptors used by *Agrobacterium* phage 7-7-1. We identified three genes involved in phage infection using transposon mutagenesis and determined that the gene products were involved in lipopolysaccharide (LPS) biosynthesis. Thus, phage 7-7-1 uses LPS as a secondary cell surface receptor. In chapter 4 we focused on discovering phage encoded receptor binding proteins (RBPs) in phage 7-7-1. Our investigation resulted in the identification of 3 putative RBPs. Our most promising candidate, Gp4, was examined in more detail, and we uncovered that it inhibits the growth of *Agrobacterium* sp. H13-3 cells in a motility and glycan dependent manner. This work highlights the potential of this protein for therapeutic use, although more work is required to determine the mechanism underlying the observed host cell growth inhibition. Altogether, these studies widen our understanding about flagellotrophic phage interactions with other cell surface receptors following flagellar filament translocation, how these phages are dispersed by and co-exist with their motile hosts, and which phage proteins are responsible for mediating interactions with receptors.
REFERENCES

Figure 1.1. Summary of bacteriophage life cycles and diversity. A) General infection scheme for bacteriophages and B) diversity of phage particles. Figure obtained with permission from Ofir and Sorek 2018 (4).
Figure 1.2. Modified flagellar structure for *Agrobacterium* sp. H13-3. Gene functions were deduced from the closely related *Sinorhizobium meliloti*. Figure provided by and used with permission from Dr. Birgit Scharf.
Figure 1.3. The structure of flagellotropic phage 7-7-1  A) as illustrated by EM. B) Sketch of phage 7-7-1 structure including its hexagonal head, collar, contractile tail, and tail fibers. Image used under CC BY 2.0 from Kropinski et al., 2012 (38).
Figure 1.4. Electron micrograph of bacteriophage \( \chi \) illustrating its hexagonal head, long flexible tail, and single tail fiber. Portion of figure used with permission from Schade et al., 1967 (11).
Chapter 2: Formation of phage lysis patterns and implications on co-propagation of phages and motile host bacteria

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Running title: Lysis pattern and phage-bacteria co-propagation

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Attribution: XL performed computational experiments and analyzed this data. FG conducted the quantification of phage and bacteria in the swim plates. FG and NE performed the various swim plate experiments. Manuscript was written by XL, FG, NE, BES, and JC.
ABSTRACT

Coexistence of bacteriophages, or phages, and their host bacteria plays an important role in maintaining the microbial communities. In natural environments with limited nutrients, motile bacteria can actively migrate towards locations of richer resources. Although phages are not motile themselves, they can infect motile bacterial hosts and spread in space via the hosts. Therefore, in a migrating microbial community coexistence of bacteria and phages implies their co-propagation in space. Here, we combine an experimental approach and mathematical modeling to explore how phages and their motile host bacteria coexist and co-propagate. When lytic phages encountered motile host bacteria in our experimental set up, a sector-shaped lysis zone formed. Our mathematical model indicates that local nutrient depletion and the resulting inhibition of proliferation and motility of bacteria and phages are the key to formation of the observed lysis pattern. The model further reveals the straight radial boundaries in the lysis pattern as a tell-tale sign for coexistence and co-propagation of bacteria and phages. Emergence of such a pattern, albeit insensitive to extrinsic factors, requires a balance between intrinsic biological properties of phages and bacteria, which likely results from co-evolution of phages and bacteria.
INTRODUCTION

Viruses that specifically target bacteria, bacteriophages or phages, are critical components of the microbial world. They are found in almost every natural environment, including soil, waters, oceans, and bodies of macroorganisms (e.g., human guts) (1-3). Furthermore, they are the most abundant organisms in the biosphere (2). Through their interactions with bacteria, phages constantly regulate the ecology, evolution, and physiology of microbial communities (1, 2). Because of their antimicrobial activity, the application of phages in food processing, agriculture, and medicine has exploded in recent years (4-6). Development of these applications benefits from fundamental knowledge about how phages interact with bacteria in a microbial community and how they are dispersed in their microenvironment.

As obligate parasites of bacteria, phages must coexist with their hosts at the population level (1). This coexistence, however, appears rather inconceivable because phages have a huge proliferative advantage over bacteria. The generation cycles of phage and bacteria fall in comparable time frames, with the phage latent period and bacterial division cycle both on the order of an hour (7). But in each generation cycle a bacterium produces two daughter cells, while one phage produces ~100 new phage particles. Thus, it would follow that phages would quickly outnumber and annihilate the host bacterial population (8, 9). However, phages and bacteria have coexisted in natural environments for eons. Recent theoretical and experimental studies demonstrated that the evolutionary arms race could maintain coexistence of phages with host bacteria (10-13). Coevolution could drive a phenotypic and genotypic diversity in the ability of phages to attack the bacteria and the ability of bacteria to resist the attacks, thereby maintaining the balance between phages and host bacteria (10, 14-16). However, for a successful evolutionary arms race, phages and bacteria need to coexist at least over the time scale required
for the emergence of beneficial mutations (8, 9). It is therefore critical to understand the population dynamics of phage-bacteria systems and conditions for their coexistence below the evolutionary time scale.

Previous studies on coexistence of phages and bacteria mostly focused on well-mixed, nearly homeostatic systems, such as cultures grown in chemostats (17-23). Naturally occurring systems of phages and bacteria, however, often do not satisfy the conditions found under these defined laboratory settings. Firstly, natural systems typically do not offer a constant environment. Unlike chemostats, where steady levels of nutrients and waste are maintained, natural systems often experience sporadic deposition and replenishing of resources, and fluctuations in other conditions. Secondly, natural systems usually exhibit spatial heterogeneity to various degrees. The spatial inhomogeneity can significantly impact dynamical coexistence in the phage-bacteria systems (8, 9, 24-26).

A critical spatial process in the phage-bacteria system is the migration of bacteria and phages. Many motile bacteria can migrate towards nutrient-enriched areas via chemotaxis. Phages themselves are not motile, so their dispersal relies on either passive diffusion or transport by their hosts. However, diffusion is very inefficient for covering long distances. In addition, diffusion of phage particles is typically reduced by higher bacterial densities and increased viscosities due to bacterial exopolysaccharide production in biofilms (27-29). Therefore, spatial dispersal of phages mostly relies on infection of and transportation by their motile host bacteria. It is poorly understood how phages and bacteria in a constantly migrating microbial community achieve coexistence, which implies their co-propagation in space.

In this work we explored the co-propagation of phages and motile bacteria using a simple experimental design, in which phages and bacteria were co-inoculated in a soft agar nutrient
medium (30) (Fig 1.1a). The low agar concentration enabled motile bacteria to swim through the matrix, which, in combination with bacterial growth, resulted in the formation of visible “swim rings” (30). Inoculation of bacteria and phages in separate locations allowed the experimental setup to mirror realistic scenarios, in which expanding bacterial populations encounter phages in a spatial domain. The described experiment generated a highly reproducible sector-shaped lysis pattern. This pattern cannot be explained by any previous mathematical models describing phage plaque formation (31-36), which inevitably produce circular patterns (S1). Here we constructed a new mathematical model for the spatial dynamics of phages and bacteria, which reproduced the observed lysis pattern and revealed local nutrient depletion as the key to formation of the lysis pattern. Moreover, our model revealed that the sector-shaped lysis pattern with straight radial boundaries requires a balance between intrinsic biological properties of phages and bacteria but does not depend on extrinsic factors. Such a pattern was further shown to be a tell-tale sign for extended spatial co-propagation of phages and bacteria, implying dependence of co-propagation on intrinsic balance between phages and bacteria. This is the first time that a sector-shaped lysis pattern has been reported in phage-bacteria systems. Our study of this phenomenon via an integrated modeling and experimental approach provides critical insights into naturally occurring dispersal and cohabitation of phages infecting motile bacteria.

MATERIALS AND METHODS

**Bacterial strains and phages.** The strains of bacteria and phages used are listed in Table 2.1.

**Media and growth conditions.** *Salmonella enterica* serovar Typhimurium 14028s was grown in MSB at 37 °C. MSB is a modified LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) supplemented with 2 mM MgSO₄ and 2 mM CaCl₂. *Escherichia coli* strains were grown at 30 °C in T-broth containing 1% tryptone and 0.5% NaCl at 30 °C.
Construction of mutant strains. The protocol for lambda-Red genetic engineering (58, 59) was followed to make *S. Typhimurium* mutant lacking *tar*.

**Phage drop assay.** Swim plates containing MSB medium (for *S. Typhimurium*) or T-broth (for *E. coli*) and 0.3% bacto agar were inoculated with 2.5 μl of a stationary phase bacterial culture in the center of the plate along with 2.5 μl of phage suspension (MOI = 25.4) at a 1 cm distance from the inoculation point. A 2.5 μl spot of 0.85% saline was placed at the same distance from the bacterial inoculation point, opposite from the phage suspension, as a control. Plates were incubated at 37 °C (*S. Typhimurium*) or 30 °C (*E. coli*) for 14 hours. All plates were imaged using the Epson Perfection V370 scanner. Phage drop assays with slight modifications were conducted to test different variables. For the rod-shaped inoculations, sealed glass capillaries of different lengths were immersed in phage suspension and pressed against the soft agar at a distance of 0.5 cm from the bacterial inoculation point. To test the effect of varying inoculation distances, phage suspensions were inoculated at 0.5, 1.0, 1.5, 2.0, and 2.5 cm from the bacterial inoculation point. For altered nutrient concentration experiments, the initial concentrations of tryptone and yeast extract were adjusted to be 0.05, 0.1, 0.5, 0.75, or 2.0 times of the regular nutrient concentration, which is referred to as a concentration of 1. To evaluate the effect of phage number, the initial phage stock was serially diluted ten-fold and then spotted on the plate. In experiments conducted with λ phage, the swim plates were supplemented with 10 mM MgSO₄ and 0.2% maltose.

**Phage titer.** Serial dilutions of the phage stock were made and 100 μl of each dilution was added to host bacterial cells with an OD₆₀₀ of 1.0. Bacteria-phage mixtures were incubated for 10 min at room temperature. Each mix received 4 ml of pre-heated 0.5% soft agar and was then overlaid on LB plates. Plates were incubated at 37 °C for 4-6 h. The titer of the phage stock was
determined by counting the plaques on the plate that yielded between 20 to 200 plaque forming units and multiplying the number by the dilution factor.

**χ phage preparation.** Dilutions of phage suspensions mixed with bacteria were plated to achieve confluent lysis as described in the phage titer protocol using 0.35% agar for the overlay. Following formation of plaques, 5 ml of TM buffer (20 mM Tris/HCl (pH=7.5), 10 mM MgSO4) was added to each plate and incubated on a shaking platform at 4 °C for a minimum of 6 h. The soft agar/buffer mixture was collected, pooled, and bacteria were lysed by adding chloroform to at final concentration of 0.02%. Samples were mixed vigorously for 1 min, transferred to glass tubes, and centrifuged at 10,000 x g for 15 min at room temperature. The supernatant was passed through a 0.45 μm filter and NaCl was added to a final concentration of 4%. The protocol of phage preparation was followed as described in (60). The final phage stock was stored in TM buffer at 4 °C.

**Bacteria and phage quantifications.** Phage drop assays were conducted as described above. At the 14-hour end point, different areas of the plate were sampled by taking agar plugs using a 10 ml syringe barrel with plunger. Each agar plug was placed in 1 ml of 0.85% saline and incubated at room temperature for 10 min with shaking to allow even mixture of the agar. Serial dilutions of each sample were plated on LB agar plates and incubated at 37 °C overnight. For phage quantifications, 100 μl of chloroform was added to each sample. The number of phage particles present in each sample was quantified as described in the phage titer protocol. Densities reported correspond to plaque forming units (for phage) or colony forming units (for bacteria). To compare with model results, the volume densities were converted to area densities, based on 0.5 cm thickness in the agar, i.e., area density (cm⁻²) = volume density (CFU/cm³ or PFU/cm³) × 0.5 cm.
Model setup. We constructed a mean-field diffusion-drift-reaction model for the bacteria-phage system. Our model includes four variables: density of susceptible bacteria $B(x, t)$, density of infected bacteria $L(x, t)$, density of phages $P(x, t)$, and nutrient concentration $n(x, t)$. The motility and chemotaxis of bacteria are represented by the Keller-Segel type diffusion and advective terms widely used in the literature (61, 62). The equations governing the spatiotemporal dynamics of bacteria, phages and nutrient read as Eqs. (1) ~ (4).

Susceptible bacteria:

$$\frac{\partial B}{\partial t} = D_{B_{\text{max}}} \nabla \left[ \left( \frac{n}{n + K_p} \right)^2 \nabla B \right] - D_{B_{\text{max}}} \beta_p \nabla \left( \frac{K_p}{(n + K_p)^2} B \nabla n \right) - \frac{n}{B + L + K_b} BP$$  

Infected bacteria:

$$\frac{\partial L}{\partial t} = D_{L_{\text{max}}} \nabla \left[ \left( \frac{n}{n + K_p} \right)^2 \nabla L \right] - D_{L_{\text{max}}} \beta_p \nabla \left( \frac{K_p}{(n + K_p)^2} L \nabla n \right) + \frac{n}{B + L + K_b} BP$$

Phages:

$$\frac{\partial P}{\partial t} = D_p \nabla^2 P - \frac{K_b}{B + L + K_b} (L + B) P + \frac{\beta k_l L n}{B + L + K_b}$$

Nutrient:

$$\frac{\partial n}{\partial t} = D_n \nabla^2 n - \lambda \gamma_{\text{max}} \frac{n}{n + K_n (B + L)}$$

Eqs. (1) ~ (4) incorporate the following model assumptions.
(1) The division rate of susceptible bacteria follows the Monod rate law (63).

(2) Division of the infected bacteria is neglected because they are likely lysed before dividing. But they consume nutrients at the same rate as susceptible bacteria (changing this rate does not affect the qualitative behavior of the model).

(3) The phage adsorption rate decreases with increasing bacterial density. This is how New Assumption (ii) in Results is implemented.

(4) Multi-adsorption is considered, i.e., phages can be adsorbed onto bacteria that are already infected.

(5) Because phage assembly requires energy, we assume that the lysis period elongates as nutrient level decreases. This is how New Assumption (i) in Results is implemented.

(6) Because bacterial motility requires energy, it depends on nutrient level. This dependence is reflected by the fraction containing $K_e$ in both the diffusion and chemotaxis terms. This applies to both susceptible and infected bacteria.

The variables and parameters of the model are summarized in Table 2.2.

RESULTS

We designed a series of quantitative experiments based on our previously described phage drop assay (37), which allow a spatially propagating bacterial population to encounter phages. *Salmonella enterica* serovar Typhimurium 14028s and $\chi$ phage were inoculated 1 cm apart (Fig 2.1a) on 0.3% agar plate containing bacterial growth medium (30, 38). As the bacterial population grew, nutrients were consumed. Due to the low agar concentration, bacteria swam through the matrix and followed the self-generated nutrient gradient via chemotaxis, causing spreading of the bacterial population and the appearance of a swim ring. As the bacterial swim ring expanded, it reached the phage inoculation point. The phages then infected the bacteria and
generated a lysis area with low bacterial density in the swim ring (Fig 2.1b). This experiment gave rise to an intriguing sector-shaped lysis pattern (Fig 2.1b). Most strikingly, as the bacterial swim ring expanded, the radial boundaries of the lysis area stayed unchanged behind the expanding front, resulting in a frozen or immobilized lysis pattern (Fig 2.1b, S10). Once the spreading of the swim ring stopped at the plate wall, the lysis pattern persisted for at least 48 hours.

To understand the formation of this lysis pattern, we constructed a mean-field partial differential equation (PDE) model for the phage-bacteria system (Eqs.(1) ~ (4)). Like the previous phage plaque models (31-36), our model depicts the basic processes underlying the proliferation and propagation of phages and bacteria. Namely, the bacteria consume nutrients, divide, and move up the nutrient gradient via chemotaxis-directed swimming motility. Once infected by phages, the bacterium is lysed after a latent period, and releases new phage progeny. Note that the run-and-tumble mechanism of bacterial chemotaxis results in a biased random walk of the bacterial cells up the nutrient gradient. The random walk is expressed in the model as the cell diffusion terms and the bias as the cell drift terms (Eqs.(1) and (2)). The diffusion of bacteria characterizes the overall effect of active motility and random tumbling of bacteria. Therefore, diffusion and motility of bacteria will be used interchangeably in the rest of this work. In addition, we incorporated the following new assumptions about phage-bacteria interactions in the model, which are critical elements for generating the sector-shaped lysis pattern (S1).

(i) Nutrient deficiency inhibits phage replication (Fig 2.1c). Because phage replication in the host bacteria requires energy, it is likely reduced at low nutrient levels.

(ii) High bacterial density inhibits phage production (Fig 2.1c). Inhibition of phage attack by quorum sensing signals has been documented in various bacteria, including E. coli (39),
Vibrio (40, 41), and Pseudomonas (42), and could be a widespread phenomenon. This inhibition stems from reduction of phage receptors (in E. coli and Vibrio) or other mechanisms. High bacterial density, which induces production of the quorum sensing signal, could hence decrease phage production.

Our model reproduces the lysis pattern observed in the phage drop assay (Fig 2.1b, S1 Movie) and quantitatively matches the bacterial and phage density profiles throughout different areas of the agar plate (Fig 2.1d). Both experimental and modeling results displayed the highest bacterial density at the inoculation point (area 4), followed by areas outside the lysis sector (areas 1 and 3), along the radial boundaries of the lysis pattern (area 5), in the middle of the lysis pattern (area 2), and the lowest at the outer edge of the lysis sector (area 6). The predicted phage densities also matched the experimental results, i.e., highest in the middle of the lysis sector and lowest at the edge of the swim ring near the wall of the plate (Fig 2.1d). It should be noted that the lysis area was not entirely void of bacteria. In both experimental and modeling results, a low density of bacteria remained within the lysis area. In the model, nearly all bacteria in this area are infected bacteria (Fig 2.1e, f). In reality, this subpopulation could also include phage-resistant bacteria, which has not been encompassed in our current model.

The model further reveals local nutrient depletion as the key reason for the lysis pattern to immobilize behind the expanding front of the bacterial swim ring. According to the simulation results, as the swim ring expands, nutrients are depleted within the ring (Fig 2.1h). Nutrient depletion inhibits both phage production (Fig 2.1i) and bacterial motility (Fig 2.1j-m). Note that phages rely on the infection of motile bacteria to propagate in space, because the passive diffusion of phage particles \(D_p \sim 1 \mu m^2 h^{-1}\) is negligible compared to the “active” diffusion of bacteria resulting from run-and-tumble \(D_R \sim 10^5 \mu m^2 h^{-1}\). Therefore, inhibition of bacterial
motility, especially motility of the infected bacteria (Fig 2.1l, m), also hinders spatial propagation of phages. Together, the inhibition of phage production and propagation due to local nutrient depletion and reduction of bacterial motility results in immobilization of the lysis pattern at the interior of the bacterial swim ring. The lysis pattern only actively grows at the expanding front of the swim ring, where nutrient supply from the unoccupied periphery can support active phage production and propagation (Fig 2.1i-m).

**The lysis pattern reflects radial projection of phage initiation zone**

Interestingly, the angle of the lysis sector decreased in the experiment when phages were inoculated further away from the bacterial inoculation point and vice versa (Fig 2.2a). This observation was successfully reproduced and explained by our model (Fig 2.2a). The lysis patterns in these cases approximately reflect the radial projection from the bacterial inoculation point over an approximately 0.7 cm circle centered at the phage inoculation point (Fig 2.2a, cartoon). This circle roughly corresponds to the model-predicted area that is occupied by phages when nutrients initially get depleted at the phage inoculation point (Fig 2.2a, 3rd and 4th rows, nutrient depleted to 5% of initial level). We hereby term this area the “phage initiation zone”, which marks the initialization of the steady expansion of the lysis pattern. Specifically, after the phage initiation zone is established, the phage and bacterial densities at the expanding front remain at a steady level throughout the rest of the pattern formation (S2).

Our model further predicts how the phage initiation zone and the projected lysis pattern rely on additional factors, which have all been confirmed by experiments (Fig 2.2). Firstly, the phage initiation zone is predicted to encompass the original phage inoculation area (Fig 2.2a, b). In the corresponding experiments, when phages were inoculated with rods that were significantly longer than the size of the phage initiation zone during point inoculation, the phage initiation
zone became dominated by the rod size, and the lysis pattern roughly reflected the radial projection of the phage inoculation area (Fig 2.2b). Secondly, the size of the phage initiation zone is predicted to remain roughly the same and result in similar angles of the lysis sector, despite changes in total nutrient concentration (Fig 2.2c). This prediction was also validated by experiment (Fig 2.2c). Thirdly, the phage initiation zone is predicted to enlarge and result in larger angles in the lysis sector, as the initial phage particle number increases; again, this was validated by experiment (Fig 2.2d). This result can be understood in that lowering the initial phage number reduces the number of phages being produced by the time nutrients get depleted at the phage inoculation point and shrinks the phage initiation zone. When the initial phage number is too low, phages fail to establish the initiation zone and subsequently the lysis sector (Fig 2.2d, $P_0 = 1.5 \times 10^2$). The model also predicts that the initial bacteria number does not affect the lysis pattern (S3). Overall, our modeling and experimental results show that the lysis pattern maintains straight radial boundaries (Fig 2.2) despite changes in the extrinsic factors tested above, i.e., distance between inoculation point, size of inoculation area, overall nutrient level, and initial phage/bacteria number.

**Competition between phages and bacteria determines shape of lysis pattern**

Although the straight radial boundaries of the lysis pattern are maintained under various external conditions like nutrient level and initial inoculation, our model predicts a significant change in the lysis pattern when intrinsic biological parameters are altered. In the simulation results, promoting the proliferative efficiency of phages (by increasing phage adsorption rate, phage burst size or lysis rate of infected bacteria) causes the lysis pattern to flare out, and decreasing phage proliferation causes the lysis pattern to close up (Fig 2.3, horizontal axes). Meanwhile, promoting bacterial proliferation causes the lysis pattern to curve inward and close
up, and vice versa (Fig 2.3, vertical axes). To understand this result, note that both bacterial and phage proliferation depend on nutrient (expressed as increasing functions of local nutrient concentration in the model, see Materials and Methods). As previously shown, proliferation of bacteria causes nutrient to be depleted inside the bacterial swim ring (Fig 2.1h). Therefore, the bacterial proliferation rate at the expanding front determines how fast nutrients get depleted locally. This time further determines angular spreading of phages along the expanding front of the swim ring, because phage proliferation only thrives before local nutrient depletion. Therefore, either stronger phage proliferation or a weaker bacterial proliferation (causing slower nutrient consumption) allows phages to spread in an accelerated fashion as the swim ring expands, resulting in a flared-out lysis pattern. Vice versa, a weaker phage proliferation relative to bacterial proliferation results in a lysis pattern with edges closing inwards. The sensitivity of the predicted lysis pattern to phage and bacterial proliferation suggests that the experimentally observed lysis pattern requires a balance between the proliferation of the bacterial and phage strains tested.

**Bacterial motility and chemotaxis affects the lysis pattern**

We next used the model to investigate how bacterial motility and chemotaxis influence the shape of the lysis pattern. Bacterial motility is reflected by the bacterial diffusion coefficient in the model. A higher cell speed corresponds to a larger diffusion coefficient (43). Expectedly, a larger diffusion coefficient causes faster expansion of the bacterial swim ring in the model (Fig 2.4a). The chemotactic efficiency, on the other hand, characterizes the bias of bacterial diffusion. Chemotaxis promotes the directed motility of bacteria in the radial direction due to the nutrient gradient formed by bacterial nutrient consumption (Fig 2.1h). Consistently, the model predicts
that higher chemotactic efficiency expedites expansion of the bacterial swim ring (Fig 2.4a), because the moving cells at the expanding front can follow the nutrient gradient more efficiently. The effects of bacterial motility and chemotactic efficiency on the lysis pattern, however, are predicted to be exactly opposite to each other. According to the simulation results, increasing the bacterial diffusion coefficient or decreasing the chemotactic efficiency causes the pattern to flare out (Fig 2.4a). Vice versa, decreasing the bacterial diffusion coefficient or increasing chemotactic efficiency causes the pattern to close up (Fig 2.4a). The model generated this result because increasing bacterial diffusion coefficient, i.e., increasing bacterial motility, promotes mixing of infected and susceptible bacteria (Fig 2.4b, top row). Such mixing is critical for spatial propagation of phages and angular expansion of the lysis pattern, because phages cannot move on their own and rely on infected bacteria to spread in space. In contrast, enhancing chemotaxis inhibits such mixing, because it effectively promotes parallel motion of the bacteria along the radial direction towards the high-nutrient area outside the swim ring (Fig 2.4b, bottom row). Taken together, bacterial motility and chemotaxis need to be in balance to generate a lysis pattern with straight radial boundaries. Collectively, these findings and those from the model in the previous section indicate that bacterial motility and chemotaxis are required to be in balance with bacterial and phage proliferation rate to generate straight radial boundaries in the lysis pattern (S4).

To test these model predictions, we performed the phage drop assay with strains of *S. Typhimurium* 14028s containing deletions in two chemoreceptor encoding genes, *tar* and *tsr*. Strains with deletions in *tar* or *tsr* did not significantly change the lysis pattern, whereas the strain containing a deletion of both genes produced a moderate flare-out of the lysis pattern (S5).
This result is qualitatively consistent with the model prediction that weaker chemotactic efficiency causes a pattern with a wider angle (Fig 2.4a).

We further tuned bacterial motility through varying the agar density. We only experimented with lower agar densities, because higher agar densities are known to switch the mode of bacterial motility to surface swarming, and the results would not be comparable to those obtained from soft agar. In our experiments softer agar indeed increased bacterial motility, as the bacterial swim ring took a shorter time to reach the edge of the plate (7 h in 0.2%, 9 h 0.25% agar and 14 h in 0.3% agar). However, we found similar sector-shaped lysis patterns regardless of the agar density (S6a). To understand why higher bacterial motility in softer agar did not change the lysis pattern, it is important to note that lowering the agar density also increases the passive diffusion of small molecules such as nutrients (44, 45). In our model, when bacterial and nutrient diffusion coefficients are proportionally varied, the sector-shaped lysis pattern is indeed maintained (S6b, diagonal from bottom left to top right). The subtle increase of the angle in the pattern in softer agar was also reproduced by the model (S6b, diagonal from bottom left to top right). It is worth noting that agar density per se is an extrinsic factor. Although agar density affects bacterial motility, an intrinsic property, the effect of the latter on the lysis pattern is canceled by the accompanying changes in nutrient diffusion. These experimental and modeling results further confirm our conclusion above that the lysis pattern is insensitive to extrinsic factors.

**Straight radial boundary of lysis pattern is a telltale sign for extended co-propagation**

A closer look at the model results reveals that the straight lysis pattern boundaries in the lysis pattern implies co-propagation of bacteria and phages over extended periods (Fig 2.5). Unlike the sector-shaped pattern with straight radial boundaries, a flared-out or closed-up lysis
pattern indicates that one species would outcompete the other during the co-propagation (Fig 2.5a). For example, the result at the upper right corner of Fig 5a shows a case where phages encircle bacteria and block their further propagation in space. Vice versa, the result at the lower left corner of Fig 2.5a shows the opposite case where phage propagation is blocked by bacteria. For the less extreme flared-out or closed-up lysis patterns (e.g., Fig 2.5a, the last column on the 2nd row), one species would eventually encircle and block the other, if the simulation had been run on a larger spatial domain that allow further spatial expansion.

Remarkably, similar sector-shaped lysis patterns with straight radial boundaries were observed in different bacteria-phage pairs (Fig 2.5b). First, we performed the phage drop assay using \textit{E. coli} and phage \( \chi \) and found a similar sector-shaped lysis pattern (Fig 2.5b, first row). Since \( \chi \) is a bacterial flagella-dependent phage (46-48), we further examined whether infection of motile \textit{E. coli} by a non-flagellotrophic phage, \( \lambda \), would generate a similar lysis pattern. Phage \( \lambda \) was chosen because of an overlapping bacterial host range with \( \chi \). Interestingly, similar sector-shaped lysis patterns were observed independent of the utilized phage type (Fig 2.5b). Combined with the model findings above and earlier results on sensitivity of the pattern to intrinsic parameters, these highly similar sector-shaped lysis patterns indicated that both \textit{Salmonella} and \textit{E. coli} achieved intrinsic biological balance with both phages, \( \lambda \) and \( \chi \).

**DISCUSSION**

In this work, we combined experiments and modeling of lysis pattern formation to investigate the coexistence and co-propagation of phages and bacteria in space. Our experimental setting has strong implications for realistic scenarios, where an expanding bacterial population encounters phages and mediates their dispersal. We observed the formation of an asymmetric, sector-shaped lysis pattern, which cannot be explained by previous models for lysis pattern
formation in phage-bacteria systems. Our new mathematical model successfully reproduced the experimental observation and revealed the importance of nutrient depletion in maintaining the geometric asymmetry initialized in the system. Specifically, local nutrient depletion inhibits phage production and propagation behind the expanding front of the bacterial swim ring, thus immobilizing the lysis pattern. Without the immobilization effect, the lysis pattern would lose asymmetry and eventually reduce to a circle, as predicted by the previous models for phage plaque formation (31-36).

Most importantly, straight radial boundaries of the lysis pattern present a tell-tale sign that a phage-bacteria system is capable of co-propagation over extended period (Fig 2.5a). Therefore, the shape of the lysis pattern can serve as a reporter of co-propagation. The model further demonstrated that a straightly expanding pattern requires balance among bacterial proliferation efficiency, phage proliferation efficiency, bacterial motility and bacterial chemotaxis (Figs 2.3, 2.4 and S4). The balance of biological properties keeps angular expansion of the lysis pattern in pace with its radial expansion and creates straight radial boundaries in the evolving lysis pattern. In contrast, the straight boundary is insensitive to extrinsic factors, including nutrient levels, initial phage/bacterial numbers, agar density, and temperature (Fig 2.2, S3, S6, S8 and S9). Together, these findings suggest that balance of intrinsic factors supports robust co-propagation of phages and bacteria regardless of variations in the environment or initial conditions. Interestingly, we experimentally discovered similar sectorial lysis patterns with straight radial boundaries in two different enteric bacterial species paired with two different phages (Fig 2.5b). These lysis patterns implied intrinsic biological balance between the phages and their bacterial hosts. This phenomenon suggests that natural pairs of bacteria and phages could have shaped their biological properties to allow robust spatial coexistence and co-
propagation, likely as a result of coevolution. In the future, we will perform the phage drop assay on other phage-bacteria pairs to examine whether this conclusion may be generalized universally.

The model predictions on extrinsic factors were verified by our experiments (Fig 2.2, S6). It was much more complicated, though, to vary the intrinsic biological properties in a controlled fashion. We are relegating these experimental testing to future work, which will also provide feedback for model refinement. For example, the predicted lysis pattern only changed significantly in the model when chemotactic efficiency was increased from the default value (Fig 2.4a), whereas a flared-out lysis pattern occurred experimentally in a strain lacking two major chemoreceptors (S5). This quantitative discrepancy indicates that the chemotaxis term and/or parameters in the model should be modified in the future.

Our study specifically underscores the importance of co-propagation of phages and bacteria, i.e., their coexistence in the context of a migrating microbial community. We found that co-propagation requires not only a balance between the proliferative efficiency of phages and bacteria, but also between their ability to spread in space (autonomous spreading of bacteria vs. bacteria-mediated spreading of phages). The requirement of balanced proliferative efficiency is known to create the selective pressure that drives the evolutionary arms race between phages and bacteria in their ability to attack and resist attack (10, 12, 14-16). Similarly, the requirement of balanced ability to spread in space could also create a selective pressure to drive an arms race in evolving stronger ability to spread in space (Fig 2.5a, dark shaded staircase). For example, the emergence of flagellotropic phages, i.e., phages specifically targeting actively rotating bacterial flagella (49), could reflect an evolved strategy for phages to improve their ability to propagate in space. Interestingly, our model predicts a significant impact of chemotactic efficiency on co-
propagation of phages and bacteria (Fig 2.4 and S9d). Therefore, bacteria could, in principle, evolve higher chemotactic efficiency as a counterattack on phage infection. In the future we will examine whether the arms race between phages and bacteria indeed affect the diversity in genes regulating bacterial chemotaxis.

The sector-shaped lysis pattern in our phage-bacteria system is reminiscent of the sector-shaped bacterial patterns formed in range expansion experiments with two bacterial strains (50-53). Similar to our system, many range expansion experiments demonstrated the important role of spatial factors in coexistence of interacting species in expanding microbial communities, e.g., (50, 51). However, the underlying process driving pattern formation in the bacterial range expansion experiments is different from that in our phage-bacteria system. In the range expansion experiments, the bacterial patterns result from random genetic drift in the otherwise co-inoculated, well-mixed populations at the expanding front of the bacterial population. The stochastic nature of the genetic drift process causes random fluctuations in the number, size and boundary shape of the patterns. In contrast, the lysis patterns in our system rely not on random genetic shift, but on establishment of the phage initiation zone (Fig 2.2) as the bacterial population migrates past the phage inoculation loci. The phage initiation zone is established upon a large number of phage particles, with diminishing stochasticity. Hence, our lysis patterns are smooth. Likely for a similar reason, a smooth pattern was observed in a range expansion experiment of two bacterial strains inoculated with a distance apart (54). Interestingly, although the two engineered bacterial strains in (54) play the roles of predator and prey, respectively, representing similar inter-species interactions as in our phage-bacteria system, the pattern did not exhibit straight radial boundaries and the predator strain won eventually. This difference
highlights the capability of extended co-propagation as an evolved property of systems of naturally co-existing species.

Our current work exploited the simplest possible experimental and model setup to understand how phages and bacteria coexist and co-propagate in space, using lytic phages and uniform initial nutrient concentration. In the future, we will modify our experimental and model parameters to investigate additional factors, such as lysogeny and non-uniform nutrient distribution, on the spatial ecodynamics of phage and bacteria. We will also incorporate coevolution between phages and bacteria into our model and experimental set up, to investigate the long-term co-propagation under the effect of evolution. Findings from this work have strong implications for dispersal of phages in microbial communities and lay the groundwork for future applications, such as phage therapy. In the future, we hope to create a model that will aid successful selection and engineering of phages for targeted applications by providing information on phage dispersal and interaction with host bacteria in the corresponding environment. Last but not least, the principles revealed in this work about co-propagation of motile hosts and passive pathogens could be broadly applicable to general host-pathogen communities.

ACKNOWLEDGEMENTS

We thank Kelly T. Hughes for the gift of \( \chi \) phage, Rüdiger Schmitt for \( \lambda \) phage, Howard C. Berg for providing us with pKD46 and various \( E. coli \) strains, Rasika M. Harshey for the gift of the \( Salmonella \) strains, and Elizabeth Denson for creating the \( Salmonella \) Typhimurium strain lacking the \( tar \) gene. We thank Dilara Long for contributing to simulations of an earlier version of the model and William Mather for providing the python script for automatic imaging.
REFERENCES


Table 2.1. Biological materials used in this study

<table>
<thead>
<tr>
<th>Species/strains/plasmids</th>
<th>Relevant characteristics</th>
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<td><em>tar&lt;sup&gt;-&lt;/sup&gt;</em>&lt;br&gt;<em>tsr&lt;sup&gt;-&lt;/sup&gt;</em>&lt;br&gt;<strong>Tc</strong>&lt;br&gt;(−86 from ATG of <em>fliY</em>)</td>
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<td><em>fliY</em>5221::Tn10dTc (&lt;br&gt;(−86 from ATG of <em>fliY</em>)</td>
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Table 2.2. Parameters of mathematical model.

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<td>Maximum diffusion coefficient of bacterial population (effective diffusion coefficient when bacteria assume maximum motility)</td>
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<td>Estimated from cell velocity and tumbling frequency [43, 64]</td>
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<td>$n_0$</td>
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<td>$R$</td>
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*Fitting to experimentally observed expansion rate of bacterial swim ring
Figure 2.1. Sector-shaped lysis patterns emerge due to nutrient depletion. (a) Schematic of experimental procedure. Bacteria, phages, and a saline control were spotted on swim plates and incubated for 14 h, which resulted in the development of the sector-shaped lysis pattern. (b) Experimental and model results of the lysis pattern over time. (c) Interactions between key processes in the model. Pointed arrows: positive influences. Blunt arrows: negative influences. (d) Quantitative comparison of bacterial and phage densities between experiment (yellow bars)
and model (red bars). In the experiment, areas labeled by yellow numbers were sampled for phage and bacteria quantifications (see Methods). Corresponding areas in the model are labeled by red numbers. (e) Simulated density of total bacteria. The dashed green outline of the bacteria-dense area is superimposed on (f-m) for reference. (f) Simulated density of infected bacteria. (g) Simulated density of phages. (h) Simulated nutrient concentration (per unit area). Right: Orange curve shows the nutrient concentration profile along the axis of symmetry of the lysis pattern (blue dashed line in left panel). (i) Simulated phage production rate. Active phage production only happens at the outer edge of the lysis area. (j) Simulated spatial flux of total bacteria. (k) Intensity of bacterial spatial flux (~ length of arrow in (j)). Bacteria are motile only at the outer edge of the swim ring. (l) Simulated spatial flux of infected bacteria. (m) Intensity of spatial flux of infected bacteria (~ length of arrow in (l)). Infected bacteria are only motile at the outer edge of the lysis area. The spatial flux shown in (j-m) represents the sum of diffusion flux and chemotaxis flux. Length of the arrow is proportional to magnitude of the spatial flux. (e-m) present snapshots of model simulation at 10 h, an intermediate time at which the colony expansion and pattern formation progress steadily.
Figure 2.2 The lysis pattern reflects the radial projection of the phage initiation zone and is insensitive to extrinsic factors. Model and experimental results with (a) phages inoculated at different distances from the bacterial inoculation point, (b) phages inoculated with rods of different lengths, (c) different initial nutrient levels, and (d) different initial phage number. In (a-d), the phage initiation zones represent model-predicted areas occupied by phages when the nutrient level at the center of the phage inoculation area drops below a certain threshold (set at 5% of the initial nutrient level in model simulations). Cartoons summarize scenarios leading to

Figure 2.3. Competition between bacterial and phage proliferation determines the shape of lysis patterns. Simulated lysis patterns with various bacterial growth rate constants versus (a) phage adsorption rate constants, (b) phage burst sizes, and (c) phage-induced lysis rate constants.
Figure 2.4. Effects of bacterial motility and chemotaxis on the shape of lysis patterns. (a) Simulated lysis patterns with various bacterial diffusion coefficients and chemotactic efficiencies. The diffusion coefficient in the model reflects the efficiency of bacterial motility. (b) Illustration of how bacterial diffusion and chemotaxis affect the evolution of lysis patterns. The cartoon illustrates a hypothetical history of expansion and mixing of two bacterial patches that would occur along the expanding front of the swim ring. Dark and light grey patch starts with infected and susceptible bacteria, respectively. Because nutrient is depleted behind the expanding front, bacterial expansion along the radial axis only occurs in the outward direction. Large bacterial diffusion promotes expansion equally in all directions, which enhances mixing between the infected and susceptible bacteria and leads to more effective phage propagation and
flare-out of the lysis pattern (top row). In contrast, large chemotactic efficiency promotes expansion only against the nutrient gradient (radial direction), which effectively parallelizes bacterial motion, reduces their mixing in the angular direction, and causes the lysis pattern to close up (bottom row).

Figure 2.5. Co-propagation between phage and bacteria is reflected by the straight radial boundary of lysis pattern. (a) Lysis patterns and corresponding spatial patterns of phages with various bacterial diffusion coefficients and phage adsorption rate constants. Red/grey gradient in the background illustrates the relative rate at which phages encircle bacteria (red) or bacteria encircle phages (grey). Red shade in simulated patterns: density of phages. Grey shade in
simulated patterns: density of bacteria. Grey shadowed staircase: potential trajectory of evolutionary arms race on which phages and bacteria maintain balance with each other. Refer to S7 Fig for clearer, separate view of the lysis patterns and phage patterns. (b) Experimental results from pairs of different bacterial species and their cognate phages.
Chapter 3: More than rotating flagella: LPS as a secondary receptor for flagellotrophic phage 7-7-1

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Running title: Receptors of Agrobacterium sp. H13-3 for phage 7-7-1

Key words: complex flagellar filaments, glucose epimerase, glycosyltransferase, flagellar motor, motility

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ABSTRACT

Bacteriophage 7-7-1, a member of the Myoviridae family, infects the soil bacterium Agrobacterium sp. H13-3. Infection requires attachment to actively rotating bacterial flagellar filaments, with flagellar number, length, and rotation speed being important determinants for infection efficiency. To identify secondary receptor(s) on the cell surface, we isolated motile, phage-resistant Agrobacterium sp. H13-3 transposon mutants. Transposon insertion sites were pinpointed using arbitrary-primed polymerase chain reaction and bioinformatics analyses. Three genes were recognized, whose corresponding proteins had the following computationally predicted functions: AGROH133_07337, a glycosyl transferase, AGROH133_13050, a UDP-glucose 4-epimerase, and AGROH133_08824, an integral cytoplasmic membrane protein. The first two gene products are part of the lipopolysaccharide (LPS) synthesis pathway, while the latter is predicted to be a relatively small (13.4 kDa) cytosolic membrane protein with up to four transmembrane helices. Phenotypes of transposon mutants were verified by complementation and site-directed mutagenesis. Additional characterization of motile, phage resistant mutants is also described. Given these findings, we propose a model for Agrobacterium sp. H13-3 infection by bacteriophage 7-7-1 where the phage initially attaches to the flagellar filament and is propelled down towards the cell surface by clockwise flagellar rotation. The phage then attaches to and degrades the LPS to reach the outer membrane, and ejects its DNA into the host using its syringe-like contractile tail. We hypothesize that the integral membrane protein plays an important role in events following viral DNA ejection or in LPS processing and/or deployment. The proposed two-step attachment mechanism may be conserved among other flagellotrophic phages infecting Gram-negative bacteria.
INTRODUCTION

Bacteriophages are part of all microbial ecosystems and thus influence evolution and population dynamics (1, 2). Estimates have placed the total global phage count at roughly $10^{31}$ phage particles, making them the most abundant organisms on Earth (3, 4). Phage populations are particularly high in a diverse range of soil types and locations. For example, there are approximately $10^8$-$10^9$ phage particles per gram in the rhizosphere and bulk soil (5, 6). Although phage populations are diverse in terms of morphology, genetic material, host range, and life cycles, those belonging to the tailed phage order, *Caudovirales*, are the most numerous. These viruses contain large dsDNA genomes (7-9). Despite their abundance and diversity, the ecological implications of phage presence and infection within soil microbial communities is not well studied. Unraveling the biology of phage-host relationships is key to understanding microbial systems, their abundance, and evolution.

Regardless of viral classification, accurate host cell recognition is imperative for successful infection and replication. Infection of bacterial cells by phages is governed by the presence of bacterial cell surface receptors such as lipopolysaccharides (LPS) and membrane proteins (10-12). Many large DNA phages of the *Caudovirales* order, such as P22 or T4, that infect Gram-negative hosts, bind to the lipopolysaccharide layer through their tailspikes (*Podoviridae*) or tail fibers (*Siphoviridae* and *Myoviridae*) located at the end of the phage tail (13). This first attachment to LPS is specific, but reversible. The tail associated complexes possess receptor-destroying enzymatic activity, resulting in the cleavage of LPS O-antigen repeats. The specificity of tail-spike associated hydrolases is a determinant of host range. Degradation of LPS allows the phage to pass through the outer LPS region gaining access to the
secondary receptor on the cell surface or directly to the membrane. This second interaction
results in irreversible attachment. *Myoviridae* phages, like T4, possess long, contractile tails that
pierce the outer membrane of the host for ejection of viral DNA (13). The membrane-puncturing
device disrupts the intermembrane peptidoglycan layer via lysozyme activity. The phage tail can
directly penetrate the cytoplasmic membrane to eject its DNA or, alternatively, interacts with a
bacterial cytoplasmic inner membrane protein to transfer phage DNA into the cytoplasm (14-17).
Phages with short tails, like the podovirus P22, are not able to use their tails to reach the inner
membrane. Instead they rely on interactions with secondary receptors on the cell surface to
trigger release of viral proteins before genome ejection, which are hypothesized to aid in
protection and transport of the DNA to and through the inner membrane. The process of DNA
translocation is the least understood step in bacteriophage infection (9, 18-20).

Bacterial pili and flagella are targets of phages in the *Caudovirales* order (21). Flagellotropic phages, which use the bacterial flagellum as a host receptor for attachment, remain
poorly characterized. The mere presence of flagella is not sufficient for infection. Flagellar
rotation has been shown to be a requirement for infection, resulting in the proposal of a “nut and
bolt” mechanism for phage translocation along the flagellar filament to the cell surface (21, 22).
The family of flagellotropic phages is represented by \( \Phi \) of *Escherichia coli* and *Serratia
marcescens*, \( \Phi \)CB13 and \( \Phi \)CbK of *Caulobacter crescentus*, PBS1 and SP3 of *Bacillus subtilis*,
\( \Phi \)AT1 of *Erwinia carotovora*, F341 of *Campylobacter jejuni*, \( \Phi \) OT8 of *Serratia* sp. ATCC
39006 and *Pantoea agglomerans*, and 7-7-1 of *Agrobacterium* sp. H13-3 (21, 23-32). It is
speculated that infection by flagellotropic phages is species-specific due to the unique surface
structures of bacterial flagellar filaments. For example, phage 7-7-1 only infects *Agrobacterium*
sp. H13-3 and not closely related *Rhizobiaceae* species (33).
The lytic phage 7-7-1 was initially isolated from a compost soil in Germany (34) and is a member of the *Myoviridae* family. This phage family consists of a head, collar, and a contractile tail, with *E. coli* phage T4 representing the most-well studied example (35). Phage 7-7-1 shows relatively little overall DNA sequence similarity to other phages (36). It has a hexagonal head with a diameter of 68 nm and a 135 nm long contractile tail that is connected to bushy tail fibers (23, 34, 37). Its eclipse period takes about 60 minutes and phage propagation is completed after 80 minutes with a burst size of 120 particles per bacterial cell (23, 38). The host of phage 7-7-1, *Agrobacterium* sp. H13-3, formerly known as *Rhizobium lupini* H13-3 (39), is classified as a non-pathogenic member of *Agrobacterium* based on genome structure and phylogenetic analyses (40). The closely related, non-host *Agrobacterium tumefaciens* is well known as the causative agent for crown gall induction in plants, which places an economic burden on orchards and vineyards worldwide (41).

The clockwise-rotating *Agrobacterium* sp. H13-3 flagellum is classified as complex and consists of three flagellin proteins, the primary flagellin FlaA which is essential for motility and the secondary flagellins FlaB and FlaD which have minor functions in motility (42, 47). The role of individual flagellins in flagellar structure and motility is paralleled by their importance for phage infection. The absence of minor flagellins does not or only moderately affects infection, while the lack of the major flagellin FlaA severely reduces infection efficiency. A mutant lacking all three flagellin genes is phage resistant (33). The speed of flagellar rotation also correlates with the efficiency of phage infection; a mutant with faster rotating flagella is infected more effectively, while slower flagellar rotation significantly reduced the efficiency of infection. Furthermore, strains that are non-motile are resistant to phage infection (33). We hypothesize that phage 7-7-1 interacts with bacterial cell surface receptors after it has traversed down the
flagellar filament and reached the cell surface. However, such cell surface receptors have not yet been identified. In this study, we used transposon mutagenesis combined with a screen for motile, phage-resistant *Agrobacterium* sp. H13-3 mutants and investigated genetic variations underlying resistance. Bioinformatics analysis of transposon insertion sites identified that two genes related to lipopolysaccharide biosynthesis along with an integral inner membrane protein of unknown function are essential for infection by phage 7-7-1. These new findings, together with previous results, culminated in the development of an infection model for phage 7-7-1.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** Derivatives of *E. coli* K-12 and *Agrobacterium* sp. H13-3 (81) strains, and the plasmids used are listed in Table 1.

**Media and growth conditions.** *E. coli* strains were grown in LB at 37°C (82). *Agrobacterium* sp. H13-3 was grown in TYC (0.5% tryptone, 0.3% yeast extract, 0.087% CaCl₂ x 2H₂O [pH 7.0]) or NY (0.8% nutrient broth, 0.3% yeast extract) as indicated, at 30°C (83). The following antibiotics were used at the indicated final concentrations in LB, NY, or TYC media: for *E. coli*, ampicillin at 100 µg ml⁻¹, chloramphenicol at 30 µg ml⁻¹, kanamycin at 50 µg ml⁻¹, tetracycline at 10 µg ml⁻¹; for *Agrobacterium* sp. H13-3 in TYC, neomycin at 120 µg ml⁻¹ (12 µg ml⁻¹ in liquid medium), tetracycline at 10 µg ml⁻¹ (2.5 µg ml⁻¹ in liquid medium), streptomycin at 600 µg ml⁻¹.

**Phage preparation.** *Agrobacterium* sp. H13-3 was grown in NY at 30°C to an OD₆₀₀ of 0.6. After ensuring that bacteria were motile, the culture was diluted into 200 ml NY to a final OD₆₀₀ of 0.03. The bacteria were infected with phage 7-7-1 at an M.O.I of 0.005 and shaken for 24 h at 30°C. The resulting phage-containing lysate was adjusted to 4% NaCl, left on ice for 30 min, and
centrifuged at 10,000 g for 30 min at 4°C. Polyethylene glycol 8,000 was added to the supernatant resulting in a final concentration of 10% (w/v) and incubated for 16 h at 4°C. Phage particles were sedimented at 15,000 g for 30 min at 4°C and suspended in 2 ml TM buffer (20 mM Tris/HCl [pH=7.5], 10 mM MgSO₄). The concentrated phage suspension was then layered on a 10-50% (w/v) iodixanol (OptiPrep™, Accurate Chemical and Scientific Corporation, Westbury, NY, USA) gradient and centrifuged at 200,000 g for 2 h at 15°C, using an SW-41 Ti rotor. A visible band approximately 20 mm from the bottom of the gradient was extracted with an 18-gauge syringe and dialyzed against TM buffer at 4°C with two buffer changes. Following this protocol, phage titer ranged from 10¹¹-10¹² plaque forming units (PFUs) ml⁻¹. The final phage stock was stored in TM buffer at 4°C.

**Infectivity assays.** Cultures were grown in NY from single colonies at 30°C for 18-26 h until they reached an OD₆₀₀ of 0.3. For each strain, two cultures were adjusted to an OD₆₀₀ of 0.03 with NY, one was incubated with bacteriophage 7-7-1 at an MOI of 1.0, and the second was incubated as a control in a shaking incubator at 220 rpm and 30°C. OD₆₀₀ readings were recorded after 24 h and each experiment was run in quadruplicates.

**Transposon mutagenesis and selection of phage-resistant mutants.** Mobilization of plasmid pJG110 from *E. coli* DH5α to *Agrobacterium* sp. H13-3 RU12/001 was accomplished by triparental mating using *E. coli* DH5α harboring pRK600 as a helper strain essentially as described by Griffitts & Long (2008)(84). Each strain was scraped from freshly streaked TYC or LB agar plates and suspended as dense suspensions in 200 µl LB. One hundred µl of recipient strain and 5 µl of helper and donor strain each were mixed, with 100 µl of the mixed suspension spread on an LB agar plate and incubated at 30°C for 20 h. The bacterial lawn was scraped off the plate,
suspended in 3 ml LB containing 10% glycerol and stored at -80°C. A 0.5 ml aliquot of the triparental mating mix was diluted into TYC containing streptomycin and neomycin to an OD<sub>600</sub> of 0.1. The culture was grown for 6 h at 30°C to an OD<sub>600</sub> of 0.2. Motility of cells was confirmed through light microscopic examination. Phage was added to an MOI of 1.0 and the culture was shaken for 16 h at 30°C. Cells were sedimented by centrifugation, suspended in 3 ml LB containing 10% glycerol, and stored at -80°C. Transposants were selected by plating serial dilutions on TYC agar plates containing streptomycin and neomycin and incubation for 3 days at 30°C. Individual colonies were spotted on Bromfield swim plates and incubated 24 h at 30°C. Colonies forming a swim ring were spotted on TYC agar plates containing streptomycin and neomycin and on Bromfield swim plates as a second screening.

**Chromosomal DNA preparation.** *Agrobacterium* sp. H13-3 DNA was isolated from 4 ml TYC cultures grown for 36 h at 30°C. Cells were sedimented by centrifugation, suspended in 500 μl 25 mM EDTA, pH 8.0, and mixed with 400 μl 20% (w/v) sucrose in 1 mM EDTA, 10 mM Tris/HCl, pH 8.0. After incubation for 30 min at -30°C, 10 μl 10 mg/ml RNase and 10 μl 100 mg/ml lysozyme were added to the thawed cell suspension and incubated for 45 min at 37°C. One hundred fifty μl 5% sodium lauroyl sarcosinate and 4 μl 100 mg/ml proteinase K were added, followed by an overnight incubation at 50°C. After addition of 100 μl 3 M sodium acetate, pH 5.2, the mixture was extracted with an equal volume of phenol twice and with 24:1 chloroform:isoamyl alcohol once. Chromosomal DNA was precipitated by the addition of an equal volume of isopropanol and sedimented by centrifugation. The DNA pellet was washed with 70% ethanol and suspended in 100 μl H₂O.

**Genetic manipulations.** Transposon insertion sites were identified by arbitrary PCR (85) essentially as described by Griffitts & Long (2008) (84). Bacterial template DNA was used at a
concentration of 0.2 ng/µl with oligonucleotide TSP1: GTTTACTTTGCAGGCTTCCCAAC and ARB1A: GCCACGCGTCGACTAGTACNNNNNNNNACGCC or ARB1B: GCCACGCGTCGACTAGTACNNNNNNNNNTGCCG in a first-round reaction with Taq Polymerase (NEB), under the following conditions: 94°C for 3 min; six cycles of 94°C for 20 sec, 33°C for 20 sec, 70°C for 1 min; and 30 cycles of 94°C for 20 sec, 43°C for 20 sec, 70°C for 1 min. In a second round, primers TSP2: AGCTGGCAATTCCGGTTCGCTTG and ARB2: GCCACGCGTCGACTAGTAC were used to amplify from a 30-fold dilution of first round reaction, under the following cycling conditions: 94°C for 3 min; 30 cycles of 94°C for 20 sec, 52°C for 20 sec, 70°C for 1.5 min. PCR products were sequenced using primer TSP2. Plasmid DNA was purified with Wizard Plus SV Miniprep system (Promega) and PCR products were purified from agarose gels using a Wizard SV Gel and PCR Clean-Up System (Promega). Deletion constructs were generated using the overlap extension PCR method described by Higuchi (86). These constructs were cloned into the mobilizable suicide vector pK18mobsacB, which was then used to transform E. coli S17-1, and conjugally transferred to Agrobacterium sp. H13-3 by filter mating according to the method of Simon et al. (87). Allelic replacement was achieved by sequential selections on neomycin and 10% sucrose as described previously (88). Confirmation of allelic replacement and elimination of the vector was obtained by gene-specific primer PCR and DNA sequencing. The broad-host range plasmid pBBR1MCS-3 was utilized to complement transposon-insertion strains. Broad-host range plasmids were used to transform E. coli S17-1 and then transferred conjugally to Agrobacterium sp. H13-3 by streptomycin-tetracycline or streptomycin-neomycin double selection (89).

**Motility assays.** Swim plates containing Bromfield medium and 0.3% Bacto Agar were inoculated with 3 µl droplets of the test culture and incubated at 30°C for 4 days. Motile cell samples were
observed with a Nikon Eclipse E600 phase-contrast microscope.

**Lipopolysaccharide purification and analysis.** Rough and smooth lipopolysaccharides (LPS) were essentially purified according to Johnson and coworkers (90). Motile bacteria were harvested from a 1 liter TYC culture at an OD₆₀₀ of 0.3 via centrifugation at 10,000 x g for 15 min at 4°C. Cells were washed with 1 ml of phosphate-buffered saline (100 mM NaCl, 80 mM Na₂HPO₄, 20 mM NaH₂PO₄, pH 7.), centrifuged as mentioned above, and suspended in 25 ml of 50 mM Na₃PO₄, 5 mM EDTA, 0.05% (w/v) NaN₃. Suspensions were blended using a Waring blender at top speed for 1-2 min. and stirred for 16 h at 4°C in the presence of 0.1g of hen egg white lysozyme. The suspensions were further incubated for 20 min at 37°C and blended at top speed for 3 min.

After the volume was adjusted to 100 ml using 20 mM MgCl₂, bovine pancreas ribonuclease and deoxyribonuclease were added to reach a final concentration of 1-2 µg/ml, and samples were incubated for 10 min at 37°C, followed by a 10 min incubation at 60°C. After addition of proteinase K to a final concentration of 25 µg/ml, samples were incubated for 1 h at 60°C. The samples were then heated to 65-70°C and an equal volume of 95% phenol was added. After 15 min of vigorous stirring, extracts were cooled to 15°C. Separation was achieved by centrifugation at 18,000 x g for 15 min at 4°C. The top aqueous phase was removed and transferred to a new tube. An equal volume of water was added and extraction with phenol was repeated. The aqueous phases were pooled and dialyzed against 4 l of H₂O until phenol odor was no longer recognized (approx. 3-4 changes). LPS was then lyophilized and suspended in H₂O to reach a concentration of 5 mg/ml. To disperse aggregates and remove particulate matter suspensions were heated to 65°C and centrifuged at 12,000 x g for 10 min at 4°C. Samples underwent ultracentrifugation at 105,000 x g for 3 h at 4°C. The centrifugation step was repeated until the content of protein and nucleic acid in supernatants was less than 1%. The supernatant was lyophilized and used as LPS stock. Twenty microliter
Laemmli sample loading buffer containing 5% (v/v) β-mercaptoethanol were added and samples incubated for 5 min at 100°C. Separation and staining of LPS in polyacrylamide gels were performed as described (91, 92). After separating 2 µl LPS in a 15% SDS acrylamide gel at 20 mA, the gel was incubated in fixation solution (40% ethanol, 5% acetic acid). The fixation solution was replaced with oxidation solution (40% ethanol, 5% acetic acid, 0.7% periodic acid), and the gel was incubated on a rotary shaker at 40 rpm for 5 min. After washing three times with 1000 ml distilled H₂O at 50 rpm for 15 min, the gel was incubated at 70 rpm for 10 min in staining solution (19 mM NaOH, 197 mM NH₄OH, 0.67% AgNO₃) and washed three times with 1000 ml distilled H₂O at 40 rpm for 15 min. To develop the silver stain, the gel was incubated in developer solution (0.005% (w/v) citric acid, 0.05% (v/v) formaldehyde) at 45 rpm for 2-5 min, then washed three times with 1000 ml distilled H₂O for 15 min before documentation.

**Polysaccharide staining.** TYC plates were supplemented with either Congo Red or Calcofluor White stain to final concentrations of 150 µg/ml or 20 µg/ml, respectively. Overnight cultures were diluted with TYC medium to reach an OD₆₀₀ of 0.3, and 5 µl of each were spotted onto each plate. Plates were then incubated for 4 days at 30°C and imaged.

**MALDI-TOF mass spectrometry.** Purified LPS samples were analyzed, essentially as described for whole cell analyses (63), using 2,5-dihydroxybenzoic acid (DHB) as matrix. Samples were prepared in sandwich format and analyzed with an ABSciex 4800 Proteomics Analyzer using the reflectron in negative ion mode.

**Adsorption assays.** Overnight bacterial cultures in TYC were diluted with TYC to an OD₆₀₀ of 0.3 and a final volume of 10 ml. Cells were evaluated for motility through light microscopic examination. Phage was added at an MOI of 0.01, and cultures were incubated for 10 min at 30°C to allow for adsorption to occur. As a control, TYC medium was mock treated with the same
amount of phage as described above. A 1-ml aliquot of infected cultures and the control was centrifuged at 15,000 x g for 3 min at 4°C. Serial dilutions of each supernatant were mixed with 100 μl of Agrobacterium sp. H13-3, incubated for 5 min at room temperature, and mixed with 4 ml of molten TYC containing 0.5% agar. This mixture was layered onto TYC plates and incubated overnight at 30°C to enable plaque formation. The adsorption percentages were calculated using the following formula: \[1-(\text{phage titer of supernatant after cells were removed/\text{phage titer of control reaction mixture without bacterial cells}})\] x 100.

RESULTS

Transposon mutagenesis of Agrobacterium sp. H13-3 and identification of phage-resistant, motile transposon mutants.

We hypothesized that phage 7-7-1 infection required binding to secondary receptors on the cell surface of Agrobacterium sp. H13-3 following interactions with the primary receptors, flagella. We therefore developed a scheme to identify Agrobacterium sp. H13-3 mutants that are both phage-resistant and motile. First, we created a pool of mutants upon Tn5 transposon delivery to strain RU12/001 by conjugation. A liquid TYC culture containing streptomycin and neomycin (selecting for Agrobacterium sp. H13-3 mutants containing transposon insertion) was inoculated from the conjugation pool and grown until cells became motile. Next, phage-resistant mutants in this culture were selected by continuous growth in the presence of phage. Transposon-containing Agrobacterium sp. H13-3 cells were further selected by plating on TYC agar plates with streptomycin and neomycin. To screen for motile, phage-resistant mutants, 1,000 colonies were spotted on Bromfield swim plates. Since more than 40 genes are required to produce a functional flagellum (40, 43), the vast majority of the mutants were non-motile. However, we identified 17 motile, phage-resistant mutants in this screen (Table 3.1). Using
arbitrary primed polymerase chain reaction (PCR), we obtained flanking sequences from all transposon insertion sites in our mutant set. The insertions resided in two genes, AGROH133_07337 (07337), annotated as a glycosyl transferase and AGROH133_08824 (08824), whose gene product is predicted to be an integral membrane protein (Table 2). For 07337, eight insertion mutants were identified with six different insertion sites, while for 08824, nine insertion mutants with five different insertion sites were obtained. A second round of screening on swim plates was performed and 16 motile mutants were identified out of a total of 800. From these mutants, seven were 07337 insertional mutants, eight were 08824 mutants, and one mutant, PR18, had a transposon insertion in AGROH133_13050, coding for a UDP-glucose 4-epimerase (Table 3.2). In conclusion, we identified three Agrobacterium sp. H13-3 genes potentially important for mediating sensitivity to phage 7-7-1.

**Verification of the phage-resistant phenotype by complementation and in-frame deletions.**

To verify that disruption of these three genes by the transposon mediated phage resistance, we performed two independent experiments. First, we chose P13 and P222 as representative transposon insertion mutants of genes 07337 and 08824, respectively, along with PR18, the 13050 transposon mutant, and conjugationally transferred derivatives of the broad-host range plasmid pBBR1MCS-3 (56), expressing the respective genes to generate complemented strains (Table 3.1). Secondly, we constructed unmarked in-frame deletion strains for each gene by allelic replacement (Table 3.1). Next, we analyzed whether the resulting strains either restored sensitivity to phage (complemented strains) or gained resistance to phage (deletion strains). We performed infectivity assays by adding phage at an MOI (multiplicity of infection) of 1.0 to motile Agrobacterium sp. H13-3 NY cultures and allowing growth for 24 h before the OD_{600} was measured. Fig. 3.1 displays the results for all strains in comparison to the wild-type strain (RU12/001) and a non-flagellated
mutant (RU12/006). Wild-type cultures in the absence of phage grew to an average OD$_{600}$ of 1.80, but no growth was observed in the presence of phage. Growth of the non-flagellated strain, the transposon mutants, and the strains deleted for 07337 (RU12/015), 08824 (RU12/016), and 13050 (RU12/017) was unaffected by the addition of phage. In contrast, transposon-insertion strains carrying complementation plasmids behaved like wild type and displayed no growth in the presence of phage. Transposon mutant PR18 (Tn insertion in the epimerase gene) grew to a statistically significantly lower OD$_{600}$ than wild type (p-value <0.05), but growth of the corresponding in-frame deletion strain RU12/017 was indistinguishable from wild type. Therefore, the absence of gene 13050 was not the cause of the slight growth defect, but possibly a secondary mutation or polar effects of the transposon insertion. In summary, this portion of our work verified that the disruption of genes 03773, 08824, and 13050 caused the phage-resistant phenotype.

**Motility of complemented transposon mutants and deletion strains.**

All phage resistant mutants of *Agrobacterium* sp. H13-3 described thus far in the literature are non-motile (23, 33). To demonstrate that motility is not contributing to the phage resistance of the identified transposon mutants, we quantified the motility of representative strains on Bromfield swim plates. After 4 days of incubation at 30°C, the wild type (RU12/001) formed a swim ring with a diameter of approximately 55 mm, whereas the non-motile strain RU12/006 only grew in the inoculation point (Fig. 3.2). Swim ring diameters of transposon insertion and deletion strains were indistinguishable from that of wild type, supporting the claim that motility is not a factor in conveying phage-resistance of strains defective in genes 03773, 08824, and 13050.

**Putative functions of AGROH133_07337, AGROH133_13050, and AGROH133_08824.**

AGROH133_07337 codes for a glycosyltransferase belonging to family 25 (GT25; PF01755) (45). This family of glycosyltransferases is populated by proteins that are solely
involved in lipopolysaccharide (LPS) biosynthesis, catalyzing the transfer of various sugar moieties from activated donor molecules to specific acceptors on the LPS chain during its biosynthesis (46). AGROH133_13050 encodes a UDP-glucose 4-epimerase (EC: 5.1.3.2), which carries out the reversible epimerization of UDP-glucose to UDP-galactose, the cognate substrate for galactosyltransferases. In analogy to orthologs from other Gram-negative bacteria, this gene can be named \textit{galE} (47-49) and may also be directly involved in LPS biosynthesis. AGROH133_08824 codes for a 128-aa hypothetical protein, which is predicted to be an integral cytoplasmic membrane protein with up to four transmembrane helices (Fig. S1) (50). The gene appears to be monocistronic and located upstream of a gene cluster encoding the five subunits of an F-type proton-transporting ATPase (40). The gene synteny is conserved in all sequenced bacteria that carry this gene (40, 51-57).

**Characterization of cell surface profiles of deletion strains**

Since two of the three identified genes encode proteins involved in LPS synthesis, we examined the effect of their deletions on various cell surface properties. First, we purified LPS from the deletion and wild-type strains and analyzed their compositions by SDS-PAGE and silver staining. However, we were unable to identify differences in LPS composition between deletion mutants and the wild type (Fig. S2). Next, we evaluated the effect of gene deletions on sensitivity to sodium chloride, a non-ionic detergent (TritonX-100), and a bile acid (cholic acid), because changes in the LPS profile can result in increased sensitivity to these compounds (58, 59). In these assays, serial dilutions of the wild type and each deletion strain were spotted on TY plates supplemented with 3 M NaCl or TYC plates containing 0.5% TritonX-100, or 0.5% cholic acid. After incubation for two days at 30°C, we were unable to see significant differences between the control and experimental plates regardless of the treatment (Fig. S3). We next assessed possible
differences in polysaccharide profiles by spotting the deletion and wild type strains on TYC plates containing Congo Red or Calcofluor White (60, 61). After four days of incubation at 30°C, slight differences between the deletion and wild-type strains were apparent on both types of plates. On plates containing Calcofluor White, RU12/015 and RU12/016 fluoresced to similar intensities as the wild-type strain. Meanwhile, RU12/017 appeared duller (Fig. 3.3, top panel), indicating that the approximate number of (1 → 3) and (1 → 4) β-glucopyranosyl units in the polysaccharide of RU12/017 was different (62). On Congo Red containing plates, RU12/017 clearly had an increased pigment uptake, while pigment uptake in RU12/015 and RU12/016 appeared to be slightly above that of the wild-type strain but less than that of the aforementioned mutant strains (Fig. 3.3, bottom panel). This result is suggestive of an enhanced incorporation of (1 → 4) α-glucopyranoside units into the polysaccharide of the deletion mutants, with the most significant increase in RU12/017 (62). It is important to note that in both assays, RU12/017 exhibited a rough colony phenotype while RU12/015 and RU12/016 were just as mucoid in appearance as the wild type. Nonetheless, the results demonstrate that the dye-binding properties and hence extracellular matrices (60) of all three deletion strains are different than that of RU12/001.

As a final means to characterize the surface properties of the four strains, the LPS isolates from the deletion and wild-type strains were submitted to MALDI-TOF mass spectrometry (63). While the wild-type strain exhibited a repetitive set of ions with a Δm/z of 176, which is generally attributed to hexuronic acids or methylhexoses, the deletion mutants did not (Fig. 3.4). Since the full structures of LPS from Agrobacterium sp. H13-3 as well as the ionization efficiencies of the LPS isolates are not known, it is not possible to assign structures to the observed spectra. However, it is relatively clear that the deletion strains are more similar to one another than to the wild type strain.
**Adsorption of phage 7-7-1 to deletion strains.**

As an indirect measure of alteration of cell surface structures, we quantified the adsorption of phage 7-7-1 to wild type and the deletion strains. Phage 7-7-1 was added at an MOI of 0.01 to motile *Agrobacterium* sp. H13-3 cultures and incubated for 10 minutes to allow adsorption to occur. An equivalent volume of medium was mock treated with phage. Cell culture supernatants were assayed for plaque forming units (PFUs) using plaque overlay assays and the percentage of adsorption was calculated by normalizing to the medium control. There were no differences in adsorption of phage 7-7-1 to the wild type or the deletion mutant strains (Fig. 3.5). Although the average adsorption value for RU12/016 was lower than that for the other deletion strains, this result was not statistically significant (p-value >0.05). The percent adsorption of phage to a strain that has paralyzed flagella, RU12/012, was the lowest of all the tested strains (Fig. 3.5), indicating that the mere presence of flagella is not sufficient to mediate the levels of adsorption seen with wild type. We conclude that adsorption of phage 7-7-1 is not affected by the differences in cell surface structures of RU12/015, RU12/016, and RU12/017, but is affected by lack of motility or absence of flagella.

**DISCUSSION**

The recognition of specific host surface receptors is essential for successful bacteriophage infection. Flagellotrophic phages use the bacterial flagellum for initial binding and its rotation to traverse down to the cell surface near the distal end of the flagellar filament (21, 22, 27), but further steps in the infection mechanism are poorly understood. The *C. crescentus* phages ФСВ13 and ФСbК first make reversible contact with the bacterial flagellum before they irreversibly bind to the secondary surface receptor, the pilus portal. A rotating *C. crescentus* flagellum is not a prerequisite for infection, but it facilitates the positioning of phages in...
proximity to the pilus insertion sites and thus increases the probability of interaction (21). In contrast, a rotating bacterial flagellum is an absolute requirement for infection of *Agrobacterium* sp. H13-3 by bacteriophage 7-7-1 (33). However, the nature of a secondary phage receptor(s) on the *Agrobacterium* sp. H13-3 cell surface that mediate irreversible binding to the host is unknown.

In this study, Tn5 insertion mutants of *Agrobacterium* sp. H13-3 were selected for resistance to bacteriophage 7-7-1. Most of these mutants were non-motile, confirming that motility is essential for infection. However, approximately 2% of the phage-resistant mutants were motile and exhibited wild-type behavior on swim plates (Fig. 3.2). Insertions were located in three genes, AGROH133_07337, 08824, and 13050 (Table 3.2), with AGROH133_07337 and 13050 encoding enzymes that are part of the LPS biosynthetic pathway. Although more than 50 genes are involved in LPS synthesis (64), we only identified two genes in our transposon mutagenesis screen that function in this role. This may be attributed to the essential nature of genes involved in the early biosynthetic steps and the deleterious effects of some mutations which affect the later steps (64). Furthermore, there is extensive evidence linking LPS mutations in other Gram-negative bacteria to defects in motility or complete abolishment of motility. These pleiotropic effects have been reported for *E. coli*, *S. typhimurium*, *Mesorhizobium loti*, *Rhizobium leguminosarum*, and *Rhizobium tropici* (65-69).

Modifications to the LPS core, especially the inner core, have been shown to reduce motility, alter production of flagella, or eliminate flagellar synthesis in *E. coli* and *S. typhimurium* (67, 69, 70). It is important to highlight that infection of *E. coli* by flagellotropic phage $\chi$ is affected by LPS mutations because of the effects these mutations have on motility
Therefore, using motility as a selection mechanism, we could only identify mutants with LPS deficiencies that do not affect motility.

There were few discernable alterations between the LPS from wild type versus that of the deletion mutants as analyzed via silver stain after SDS gel-electrophoresis (Fig. S2). Additionally, mutants were just as sensitive as the wild type to 0.3 M NaCl, 0.5% Triton X-100, and 0.5% cholic acid supplemented in rich medium agar plates (Fig. S3), which goes against distinctive features of LPS mutants described in the literature (58, 59). A possible explanation for this observation is that these LPS modifications do not result in increased permeability to these compounds.

The subtle differences in polysaccharide profiles illustrated by Congo Red and Calcofluor White staining (Fig. 3.3) alongside unchanged phage 7-7-1 adsorption values for the wild type and deletion strains (Fig. 3.5) provide ample evidence that the extracellular region of the mutants can still promote phage binding. These results initially led us to hypothesize that the modifications to LPS were minor. However, using a MALDI-TOF protocol targeting the Lipid A component, we observed a clear difference between LPS isolates from the wild type and mutant strains, with loss of an apparent repeat structure (Fig. 3.4). Adsorption to these mutant strains remained the same as to the wild type (Fig. 3.5) despite the loss of this repeat structure probably because motility remained unaffected (Fig. 3.2). Lower levels of phage 7-7-1 adsorption only occurred when binding to strains lacking flagella (RU12/006) or with paralyzed flagella (RU12/012) was evaluated (Fig. 3.5). Similar levels of adsorption to strains with paralyzed flagella have been documented for flagellotrophic phage χ (25). This suggests that motility is not only important for infection by flagella-targeting phages but also for adsorption to their bacterial host. It is worth mentioning that strains PR18 and RU12/017 lacking the epimerase both had dryer colony
morphologies as compared to the other strains, which became more evident after storage of plates at 4°C, indicating an involvement of the 13050 gene product in extracellular polysaccharide (EPS) production. This is highly plausible considering the epimerase reaction occurs well before bifurcation of the LPS and EPS pathways. This observation is also consistent with the results gained from spotting the strains on Congo Red and Calcofluor White containing TYC plates (Fig. 3.3).

Although the structure of *Agrobacterium* sp. H13-3 LPS is unknown, closely related *Agrobacterium* and *Rhizobiaceae* species use galactose as a building block (72). Hence 13050 (galE) may provide the activated galactose moiety for use by the GT25 glycosyltransferase (AGROH133_07337). The O-specific polysaccharide (antigen) represents the most variable part of the LPS, partially due to the large monosaccharide variety, which results in an enormous structural diversity. Since changes in LPS due to loss of AGROH133_07337 or 13050 function caused phage resistance, we conclude that a specific region of LPS contains the phage receptor. Our results suggest that galactose is an important part of the phage receptor structure. This conclusion is supported by the fact that the *E. coli* C lysing phage ΦX174 does not adsorb to LPS lacking the core terminal galactose residue (73). In addition to the primary flagellar receptor mediating host specificity, the variability in the LPS structure of *Rhizobiaceae* adds an extra layer of specificity for phage 7-7-1 adsorption. This may help explain the narrow host range of 7-7-1, which is restricted to *Agrobacterium* sp. H13-3 (33). The exact structure of the LPS receptor unit remains to be elucidated.

The third gene identified to be essential for *Agrobacterium* sp. H13-3 infection by phage 7-7-1, AGROH133_08824, encodes a small, hypothetical integral cytoplasmic membrane protein consisting of up to four transmembrane helices. The function of the gene product in infection is
unknown, but we hypothesize that it is involved in events following ejection of viral DNA into
the host cell cytoplasm or in a step during LPS biosynthesis and transport from the inner
membrane to the outer membrane. LPS assembly and translocation are intricate, ATP dependent
processes. In *E. coli* and *Salmonella typhimurium*, an inner membrane spanning protein, MsbA,
and the LptB2FGC protein complex play central roles in LPS transport which are energized by
ATP hydrolysis (74-76). MsbA flips newly synthesized LPS from the cytoplasm to the
periplasmic inner membrane leaflet, where LPS interacts with LptB2FGC (59). Binding of LPS
by LptC initiates its transport along the LptCAD protein bridge connecting the inner and outer
membranes (77). At the end of the protein bridge, the translocon LptDE exports LPS and inserts
it in the outer membrane (60). AGROH133_08824 may serve as an accessory protein to one of
the two processes at the inner membrane executed by MsbA or LptB2FGC. Interestingly, the
AGROH133_08824 gene is located upstream of genes predicted to code for the five subunits of
an F-type proton-transporting ATPase. Both the presence of this gene and the gene synteny is
conserved in various *Agrobacterium* and *Rhizobium* species (Fig. S1) indicating that the function
of AGROH133_08824 may be important in these systems. Investigation of the role of this gene
in phage infection is a subject of future studies.

We have created the working model depicted in Fig. 6 drawing from our current and past
findings on phage 7-7-1 infection of *Agrobacterium* sp. H13-3. To paint a more complete
picture, we have also utilized findings from studies centered around LPS targeting phages and
phages that use inner membrane proteins during infection to further inform our model. We
propose the following: to initiate infection phage 7-7-1 attaches to the flagellar filament and uses
its clockwise rotation to move towards the cell surface (Fig. 3.6A). Next, the phage’s tail fibers
bind to the secondary receptor, which in this system is likely a particular region of the O-specific
polysaccharide containing galactose. As has been shown for other phages that utilize LPS as a
receptor, phage 7-7-1 enzymatically cleaves specific sugars on the polysaccharide chain after
binding, resulting in access to the outer membrane. The degrading enzyme is likely located at the
phage tail end, as was first described for P22 (78, 79). Subsequently, the phage tail punctures the
outer membrane and degrades the peptidoglycan layer; this peptidoglycan degrading activity has
been demonstrated in other phages (35, 79, 80). The viral DNA is then ejected into the host cell
(Fig. 3.6B). We currently have two hypotheses regarding the role of the inner membrane protein
in phage 7-7-1 infection. This protein may interact with the phage tail to trigger its anchoring
into the host’s inner and outer membranes prior to ejection of viral DNA or in direct viral DNA
transport as has been demonstrated in other systems (14-17 Fig. 6C1). Alternatively, the integral
membrane protein could function in some part of LPS assembly and transport to the outer
membrane (Fig. 3.6C2). It remains to be seen if the host specificity of phage 7-7-1 is dictated by
these outer membrane modifications. Moreover, future studies will investigate whether other
flagellotrophic phages, such as the E. coli phage χ, use LPS as a secondary receptor.

ACKNOWLEDGEMENTS

This study was supported by NSF grant MCB-1253234 to B.E.S., the McNair Achievement
Program of Washington State University, the Multicultural Academic Opportunities Program of
Virginia Tech, the Ford Foundation Predoctoral Fellowship and the Virginia Tech Cunningham
Fellowship awarded to F.G. We thank Joel Griffitts for the gift of plasmids pJG110 and pRK600,
and members of the Scharf lab for critical reading of the manuscript.

REFERENCES


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<td>Km⁺; 568-bp XbaI/SalI fragment containing an AGROH133_07337 in-frame deletion cloned into pK18mobsacB</td>
<td>This study</td>
</tr>
<tr>
<td>pBS431</td>
<td>Tc⁺; 387-bp KpnI/XbaI fragment containing AGROH133_08824 cloned into pBBR1-MCS3</td>
<td>This study</td>
</tr>
<tr>
<td>pBS432</td>
<td>Tc⁺; 813-bp KpnI/XbaI fragment containing AGROH133_07337 cloned into pBBR1-MCS3</td>
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</tr>
<tr>
<td>pBS461</td>
<td>Tc⁺; 984-bp KpnI/XbaI fragment containing AGROH133_13050 cloned into pBBR1-MCS3</td>
<td>This study</td>
</tr>
<tr>
<td>pBS462</td>
<td>Km⁺; 526-bp XbaI/HindIII fragment containing an AGROH133_13050 in-frame deletion cloned into pK18mobsacB</td>
<td>This study</td>
</tr>
<tr>
<td>pK18mobsacB</td>
<td>Km⁺; lacZ mob sacB</td>
<td>(94)</td>
</tr>
<tr>
<td>pJG110</td>
<td>Ap⁺, Km⁺; Tn5-110; Transposon delivery vector</td>
<td>(84)</td>
</tr>
<tr>
<td>pRK600</td>
<td>Cm⁺; colE1 oriV; RP4tra⁺ RP4oriT; helper in triparental matings</td>
<td>(95)</td>
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</table>

a Nomenclature according to Bachmann (96) and Novick et al. (97).
b Numbers in parenthesis give nucleotide position of Transposon insertion.
Table 3.2. Transposon mutants of AGROH133_07337, AGROH133_08824, and AGROH133_13050.

<table>
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<th>Insertion site gene</th>
<th>Same insertion site as</th>
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<td>147</td>
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<td>AGROH133_08824</td>
<td>[2589377-2589763]</td>
<td>387 bps (gene length)</td>
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<td>984 bps (gene length)</td>
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<td>PR18</td>
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Figure 3.1 Optical density of cultures grown for 24 h at 30°C in the absence and presence of phage 7-7-1. Motile cultures were adjusted to an OD$_{600}$ of 0.03 and phage 7-7-1 was added to an MOI of 1.0. Black and grey bars depict cultures grown in the absence and presence of phage, respectively. Each bar represents the average of four independent experiments and error bars represent standard deviations (student’s $t$ test for RU12/001 and PR18, $P < 0.05$).
Figure 3.2. Motility behavior of mutants compared to *Agrobacterium* sp. H13-3 wild type (RU12/001). Tested strains were transferred by micropipette (3 μl) onto Bromfield swim plates and incubated at 30°C for 4 days. The swim ring diameter reflects the motility of a given strain. Each bar represents the average of four independent experiments and error bars represent standard deviations.
Figure 3.3. Calcofluor White and Congo Red staining of mutant and wild-type polysaccharides in vivo. Strains were spotted on TYC agar supplemented with Congo Red or Calcofluor White and incubated at 30°C for 4 days. Images are representative of 3 independent experiments conducted in triplicate.
Figure 3.4. MALDI-TOF analysis of the lipopolysaccharides isolated from the wild type and three mutant strains.
Figure 3.5. Adsorption of phage 7-7-1 to wild type and mutant strains. Phage 7-7-1 was added to cultures at an MOI of 0.01 and incubated at 30°C for 10 min. The number of free phage particles in the cleared supernatant was determined in a plaque assay. Adsorption values were calculated by normalizing to the amount of free phage present in the medium control. Each bar represents results from 3 independent experiments conducted in duplicate and error bars represent standard deviations (student’s t test for RU12/016, P > 0.05).
Figure 3.6. Proposed model of infection by bacteriophage 7-7-1. (A) Phage 7-7-1 attaches to the flagellum (primary receptor) of *Agrobacterium* sp. H13-3 and is propelled to the base by clockwise flagellar rotation. (B) The tail fibers of 7-7-1 bind to a specific region of the O-specific polysaccharide (secondary receptor) and the polysaccharide chain is enzymatically cleaved. The phage tail punctures through the outer membrane, degrades the peptidoglycan layer, and initiates ejection of viral DNA. (C1) The integral 4-transmembrane protein AGROH133_08824 facilitates infection in an unknown fashion after viral DNA ejection or (C2) serves as an accessory protein during initial LPS biosynthesis and/or its transport to the outer membrane.
Chapter 4: Identification of Receptor Binding Proteins in Flagellotropic Agrobacterium Phage 7-7-1

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Running title: RBPs in flagellotropic phage 7-7-1

Key words: Depolymerase, flagella, growth inhibition, host, LPS, phage, RBP

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ABSTRACT

The rapid discovery of new and diverse bacteriophages has driven the innovation of approaches aimed at detailing interactions with their bacterial hosts. Previous studies on receptor binding proteins (RBPs) have largely relied on their identification in silico and are based on similarities to well-characterized systems. Thus, novel phage RBPs unlike those currently annotated in genomic and proteomic databases remain largely undiscovered. In this study, we employed a screen to identify RBPs in flagellotropic Agrobacterium phage 7-7-1. Flagellotropic phages utilize bacterial flagella as receptors. The screen identified three candidate RBPs. Homology modelling predicted that Gp4 is a trimeric, tail associated protein with a central β-barrel, while the functions of Gp102 and Gp44 are less obvious. Studies with purified Gp41-247 confirmed its ability to bind and interact with host cells, highlighting the robustness of the RBP screen. We also discovered that Gp41-247 inhibits the growth of host cells in a motility and lipopolysaccharide (LPS) dependent fashion. The FlaGrab protein found in the Campylobacter jejuni phage NCTC 12673 exhibits a similar bacteriostatic effect that is also reliant on functional flagellar filaments and glycans. Hence, our results indicate the involvement of FlaGrab-like proteins in flagellotropic phages that interact with rotating flagellar filaments and host glycans to inhibit host cell growth, which presents an impactful and intriguing focus for future studies.
INTRODUCTION

Bacteriophage centered research and applications have boomed in recent years due to increased numbers of antibiotic resistant bacterial infections and the growing need for antibiotic alternatives (1-3). Consequently, phage discovery initiatives have increased to build both public and private phage repositories, which resulted in approximately a ten-fold increase in phage genomes deposited in publicly available databases in a span of 20 years (3). Detailed investigations of phage infection mechanisms have also increased, albeit at a much slower pace. These efforts are hindered by two conflicting factors: 1) the need for rapid discovery of phages with specific host ranges for phage therapy applications versus 2) the time-consuming experimental characterization of phage gene product function. Thus, phage characterization with the end goal of rapidly producing phage therapies largely focuses on determining host range, whole phage genome sequencing, morphological characterization, measurements of killing efficiencies in host planktonic or biofilm states, and, less commonly, identification of cell surface receptors (1, 2, 4, 5).

A focus on interactions taking place during viral entry (i.e., phage binding and interactions with receptors) can yield a plethora of information, including the underlying determinants of host range, potential mechanisms of phage resistance, and evolutionary tradeoffs following phage treatment. Methods for rapid detection of phage binding to host cells and identification of receptors have been developed and improved upon to make the approaches high throughput (5-7). However, phage-host interactions during entry are typically investigated unilaterally, largely focusing on the bacterial hosts. Such studies have identified outer membrane proteins, efflux pumps, capsular polysaccharide (CPS), lipopolysaccharide (LPS), pili, and flagella as receptors used by bacteriophages (8-14). Inarguably, a holistic understanding of the
mechanisms underlying phage entry entails equally detailed analyses of phage components involved in interacting with bacterial receptors. Phage encoded receptor binding proteins (RBPs) mediate recognition and attachment to host cells during viral entry. The study of RBPs presents a promising avenue for identifying new antimicrobial candidates, engineering of phages to overcome bacterial resistance to phage, and informing the development of new therapeutic approaches and tools to overcome pathogen detection limits (15-19).

Although RBPs have different receptor targets, they possess similarities. These proteins are typically resistant to proteases and thermostable. Additionally, they often assemble in trimeric states and feature conserved β-sheets in their central domains (16, 20-22). Classically, the N-terminal regions are responsible for binding to other virion components (i.e., baseplates) while the C-terminal regions interact with receptors on host cells (23). Examples of RBPs include tail fibers, tail spikes, and head fibers (9, 10, 20, 24). Phages that use polysaccharides as receptors carry proteins with the ability to enzymatically degrade these sugar chains. They are broadly defined as depolymerases and target glycan-containing bacterial components on host cell surfaces such as LPS, EPS, and CPS via their central domains (1, 2, 4, 24, 25). The most well-studied depolymerase is Gp9 from Salmonella phage P22, which specifically targets LPS by cleaving bonds between rhamnose and galactose in the O-antigen (15, 20, 26). Purified Gp9 has been shown to reduce Salmonella loads in chickens (15). The promise for depolymerase-based therapies has also been demonstrated in other systems highlighting their potential for use as therapeutics or adjuvants. Furthermore, their efficacy in degrading biofilms has sparked an increase in the number of CPS- or LPS-specific depolymerase structures available in the last decade (16, 20, 21, 27-39).
Identification of RBPs has been largely conducted via bioinformatics analyses, which are dependent on sequence homology to well-characterized RBPs and/or genetic synteny (40). This approach is problematic because it excludes the identification of RBPs that diverge largely from those in well-studied systems. Often, RBPs remain unidentified in phage genomes or are instead annotated vaguely as “tail protein” or “baseplate protein”. Although such designations aid in narrowing down the search for RBPs within one genome, the experimental verification of RBP identity and function is required. This typically involves cloning, expression, and purification of the candidates. Inarguably, this is time consuming and labor intensive with no guarantee of identifying an RBP. The need for more rapid methods allowing the identification of novel RBPs has not gone unnoticed and one method to address this issue has already been developed (22).

The focus of this work was to identify RBPs in *Agrobacterium* phage 7-7-1. The phage’s host range is limited to some members of gram-negative *Agrobacterium* spp., which are most well-known for being the causative agents of crown gall disease (23, 41). (37, 38). Phage 7-7-1 is a flagellotropic or flagella-dependent member of the *Myoviridae* family (23, 41, 42). This class of phages begin infection by interacting and binding to the flagellar filaments of their bacterial hosts (12, 40-47). Flagella are used by bacteria to move throughout their environments and are powered by a motor located at the flagellar base that relies on proton-motive force (48). In *Alphaproteobacteria*, like *Agrobacterium* spp., the flagellar motors only rotate clockwise and any alteration in swimming direction is achieved by asynchronously modifying rotational speed of individual flagellar motors (49, 50). Recognition of flagella may confer another level of host specificity for these phages and also provides a means to reach the cell surface, subsequently aiding interactions with other cell surface components. The mechanisms underlying the
translocation of phages on flagellar filaments continues to be an open question and seems to lack a universal mechanism (12, 43, 44, 51).

We have previously shown that infection by phage 7-7-1 is negatively affected by reductions in flagellar length and number and, most notably, that flagellar rotation is required for infection (42). We also discovered that LPS is an essential secondary cell surface receptor (13). Considering the different mechanisms employed by flagella-dependent phages during infection, we hypothesize that RBPs in these systems would be different than those currently described. To date, there has been no identification of an RBP in a flagellotropic phage. In this work, we set out to pinpoint RBPs in *Agrobacterium* phage 7-7-1. Ultimately, information gained from these studies will contribute to a model that delineates flagella-dependent phage entry mechanisms and informs possible protein-based therapeutics against *Agrobacterium* spp.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** *E. coli* and *Agrobacterium* sp. H13-3 (52) strains, their derivatives, and the plasmids used are listed in Table 4.1.

**Media and growth conditions.** *Agrobacterium* sp. H13-3 strains were grown in TYC (0.5% tryptone, 0.3% yeast extract, 0.087% CaCl₂ x 2 H₂O [pH 7.0] or NY (0.8% nutrient broth, 0.3% yeast extract) at 30°C. *E. coli* strains were grown in Lysogeny Broth (LB) (53) at 37°C. For *E. coli*, kanamycin was used at a concentration of 50 μg ml⁻¹ and chloramphenicol at 30 μg ml⁻¹ in LB. Streptomycin and neomycin were used at 600 μg ml⁻¹ and 120 μg ml⁻¹, respectively, in TYC or NY when culturing *Agrobacterium* sp. H13-3.

**Phage propagation and purification.** The protocol for propagating and purifying phage was followed as previously described (13, 42). Briefly, 200 ml NY cultures of *Agrobacterium* sp. H13-3 cells at an OD₆₀₀ of 0.03 were infected with phage at an MOI of 0.005 and incubated with
shaking at 30°C for 24 hours. NaCl was added to a final concentration of 4%, left on ice for 30 min, and centrifuged at 10000 x g for 30 minutes at 4°C. Precipitation was accomplished by adding polyethylene glycol 8000 to the supernatant (10% w/v) and further incubation at 4°C for 16 hours. Phage particles were sedimented by centrifugation, suspended in 2 ml of TM buffer (20 mM Tris-HCl pH 7.5, 10 mM MgSO₄), and overlaid on a 10 to 50% (w/v) iodixanol (OptiPrep; Accurate Chemical and Scientific Corporation, Westbury, NY) gradient. Following centrifugation at 200,000 x g for 2 hours at 15°C a blue-white band containing virions was extracted with an 18-gauge syringe and dialyzed against TM buffer at 4°C. Phage was titered via the standard plaque assay. The phage titer ranged from 10¹⁰ to 10¹² PFU ml⁻¹. Phage stocks were stored long term at 4°C.

**Phage 7-7-1 DNA library construction.** Phage DNA was extracted using the phenol-chloroform method. Briefly, 150 μl 5% sodium lauroyl sarcosinate and 4 μl proteinase K (100 mg/ml) were added to 1 ml of phage stock (1.87 x 10¹¹ PFU). The mixture was incubated at 50°C for 2 hours and 100 μl of 3 M sodium acetate (pH 5.2) was added to the mixture. One volume of phenol:chloroform:isoamyl (25:24:1) was added, after which the sample was vortexed vigourously and centrifuged for 5 minutes at 14,000 x g at room temperature. The aqueous phase containing DNA was removed and placed in a new microcentrifuge tube and the phenol-based separation was repeated two more times. DNA was precipitated by adding an equal volume of isopropanol followed by centrifugation. The DNA containing pellet was washed with 70% ethanol and resuspended in 50 μl of nuclease-free water. Following purification, the phage DNA was randomly fragmented via nebulization. In brief, 5-25 μg of phage DNA was diluted in 750 μl of shearing buffer (10 mM Tris, 1 mM EDTA, 10% glycerol, pH 8.0). Random fragmentation of the phage DNA was achieved by nebulizing under 10 psi for 20 seconds,
generating fragments from 1-4 kb. The desired range of fragment sizes was determined by analyzing the distribution of gene sizes in the phage 7-7-1 genome. Precipitation of DNA was conducted by adding 0.3 M sodium acetate (pH 4.8), 4 μl of 20 mg/ml of mussel glycogen (Sigma Aldrich), 700 μl of isopropanol and incubating on dry ice for 15 minutes or at -20°C overnight. The precipitated DNA was then centrifuged at 12,000 x g for 15 minutes at room temperature, washed with 800 μl of 80% ethanol, and centrifuged again as before. Once the pellet was dry, it was resuspended in nuclease-free water. The DNA fragments were blunt end repaired with T4 DNA polymerase (NEB) following the manufacturer’s protocol. The blunt end repaired fragments were precipitated as described above and mixed with dephosphorylated and EcoRV digested pET30a in preparation for ligation. The ligation reaction was set up in accordance to the manufacturer’s instructions using 50 ng of phage DNA and 20 ng of the vector. Transformation of E. coli TOP10 cells with the ligation mixture was achieved using heat shock. The presence of phage DNA fragments was evaluated by purifying plasmids and conducting single digestion reactions using EcoRV. We observed that 7 of 10 colonies contained pET30a with phage fragment inserts. Following confirmation, colonies were pooled and the plasmid DNA was isolated using the Wizard Plus SV Miniprep system (Promega). BL21(DE3) cells were then transformed with this library of phage DNA. This process was repeated until we obtained the target number of transformants required for 1.7x coverage of the phage 7-7-1 genome. This number was determined by the Clarke-Carbon equation: 

\[ N = \frac{\ln (1-P)}{\ln (1-f)} \]

where P is the probability that a fragment will be present in the library and F is the average fragment size/genome size in bp.

**RBP Screen.** This protocol was followed as previously described (22) with some modifications. In summary, E. coli BL21 (DE3) cells harboring the phage 7-7-1 genomic library were grown on
nitrocellulose membranes. The membranes were then placed colony side up on LB supplemented with kanamycin and 0.4 mM β-D-1 thiogalactopyranoside (IPTG) and incubated overnight at 30°C to allow for protein expression. Following induction, the membranes were removed from the agar, placed in empty petri dishes, and freeze/thawed five times at 5-minute intervals. Circular, pre-cut Whatman filter paper was saturated with the commercially available bacterial protein extraction reagent (BPER, Thermo Scientific) supplemented with 1 x HALT™ protease inhibitor (Thermo Scientific), 3 mg of lysozyme, and DNase I (1 U/ml), and protein containing membranes were placed on top of the filter paper for 1 hour at room temperature. The membranes were washed one time with Phosphate Buffered Saline (100 mM NaCl, 80 mM Na₂HPO₄, 20 mM NaH₂PO₄ pH 7.4) with 0.5% Tween 20 (PBST) for 5 minutes, then blocked in PBST containing 5% skim milk for 1 hour. Following blocking, membranes were washed with PBST three times for 5-10 minutes. Remaining colony debris was removed by lifting with a Kimwipe. Membranes were UV-irradiated for 15 minutes and then incubated in 500 mM NaCl at room temperature overnight on a slowly rotating platform. Following 3 washes with PBST as described above, a suspension containing 10⁷ CFU/ml in blocking solution was added to the membranes for 1 hour at room temperature with gentle shaking. Membranes were washed 3 times with PBST and excess liquid removed by flicking the membranes. The membranes were then placed on TYC plates supplemented with streptomycin, incubated at 30°C overnight, and then at room temperature for a maximum of 24 hours. Host cell growth was compared to the original position of colonies on the master plate (from which the colonies were lifted). As a verification, these colonies were subjected to a second round of screening. Transformants capable of producing protein that bound host cells were cultured in LB at 37°C overnight and
plasmids isolated using the Wizard Plus SV Miniprep system (Promega). Finally, the plasmids were sequenced using the oligonucleotides T7 terminator primer (GCTAGTTATTGCTCAGCGG) or T7 promoter primer (TAATACGACTCACTATAGGG) to uncover the sequence of the phage genome fragment. For the confirmatory RBP screen, the same protocol was essentially followed except that the initial transformants contained the plasmids pBS1218 to pBS1228 (Table 1). For visualization, membranes were stained with amido black stain 10b (0.25% amido black, 50% methanol, and 10% acetic acid) for 5 minutes and destained twice for 10 minutes with destaining solution (45% methanol and 10% acetic acid).

**Genetic construction of recombinant plasmids and bacterial strains.** Phage 7-7-1 DNA was isolated as described above. Plasmid DNA was isolated using the Wizard Plus SV Miniprep system (Promega) while PCR products were purified from 1% TAE agarose gels using the Wizard SV gel and PCR clean-up kit (Promega). These amplicons were cloned into pET30a using restriction enzymes to obtain fusions with N- or C- terminal 6x histidine tags. Following Sanger sequencing to confirm the constructs, *E. coli* strains BL21(DE3) or Lemo 21(DE3) were transformed with appropriate plasmids using heat shock in preparation for protein expression. Allelic replacement was used to generate the *Agrobacterium* sp. H13-3 ΔfliK deletion strain as described previously (13, 42).

**Recombinant protein expression and purification.** Cells were grown at 37°C in LB plus the appropriate antibiotic with shaking (225 rpm) until an OD₆₀₀ of 0.6-0.8 was reached. IPTG was added to a final concentration of 0.4 mM and cultures grown at 30°C for 5 hours. Lemo21 (DE3) cells were used and 250 μM rhamnose was added to the LB medium before induction. Cells were harvested by centrifugation and suspended in binding buffer (20 mM NaPi, 500 mM NaCl, 20 mM imidazole, pH 7.4) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF). Lysis
was achieved by passing the cell suspension 3 times through a French pressure cell at 20,000 lbs/in² (SLM Aminco, Silver Spring, MD). Soluble and insoluble fractions were separated via centrifugation at 72,600 x g at 12°C for 45 minutes. The soluble lysates were filtered using a 0.2 μm polyethersulfone (PES) membrane syringe filter and loaded onto a 5 ml nickel-nitrilotriacetic acid (Ni-NTA) column (GE Healthcare Life Sciences) using fast-performance liquid chromatography (FPLC; ÄKTA Go; Cytiva Life Sciences). The protein was eluted in a linear gradient comprised of binding buffer and elution buffer (20 mM NaPi, 500 mM NaCl, 400 mM Imidazole, pH 7.4). Protein containing fractions were pooled and concentrated using an Amicon concentrator system and regenerated cellulose membranes (10 kDa MWCO; Millipore, Billerica, MA). The protein was dialyzed into PBS (100 mM NaCl, 80 mM Na2HPO4, 20 mM NaH2PO4, pH 7.2). Visual analysis of purified protein was conducted via SDS-PAGE. Protein concentrations were evaluated by generating a standard curve using bovine serum albumin and Bradford reagent (Bio-rad) as described by the manufacturer and deducing the concentration of the target protein by mapping to the curve. Protein yields ranged from 0.02 mg/ml to 1 mg/ml from 4 liters of expression cultures.

**Liquid clearance assay.** Stationary phase *Agrobacterium* sp. H13-3 cells were diluted to an OD$_{600}$ of 0.03 in 4 ml of TYC containing streptomycin, and 0.6 μg/ml of purified Gp41-247 was added to the culture. Density of the cultures was determined by measuring the OD$_{600}$ following 24 hours of incubation at 30°C. Images of samples were taken with a Nikon D3400 camera.

**Growth curve experiments.** Stationary phase cultures of *Agrobacterium* sp. H13-3 in TYC with streptomycin were diluted to an OD$_{600}$=0.03, and 200 μl were deposited in wells of clear flat bottom 96-well plates. Purified Gp41-247 was added to each well at a concentration of 0.6 μg/ml and plates were sealed with AeraSeal™ breathable sealing film (Excel Scientific). Plates were
incubated in an INFORS HT Multitron at 30°C for 48 hours with shaking at 225 rpm. Cell
densities were determined via OD\textsubscript{600} at different time points using a BioTek Cytation 5 plate
reader. As a positive control, phage 7-7-1 was added to at an MOI=1. Equal volumes of PBS
were added as negative controls.

**Lawn clearance experiments.** Bacterial strains were seeded in TYC top agar (0.5% agar) and
overlaid on TYC nutrient plates. Following solidification of agar at room temperature, 10 μl of buffer, purified protein, or 10^7 PFU of phage were spotted on the agar. Spots were allowed to dry completely at room temperature and then incubated at 30°C overnight. Images of plates were captured using a Nikon D3400 camera.

**RESULTS**

**Construction of the randomly fragmented phage 7-7-1 DNA library and identification of candidate RBPs.**

An updated structural analysis of *Agrobacterium* phage 7-7-1 virions revealed the
presence of head and tail filaments in addition to previously reported tail fibers (personal
communication, Dr. Ariane Briegel, Leiden University). This finding indicates the possibility for
the presence of multiple RBPs within phage 7-7-1 virions. However, the annotated genome
contains only one gene that is implicated to have RBP function: 7-7-1\_00102 (23). We
hypothesize that multiple RBP encoding genes were not identified bioinformatically because of
their potential novel features. Therefore, we set out to discover RBPs in phage 7-7-1 using a
previously developed method (22). This involved generating a randomly fragmented genomic
DNA library of phage 7-7-1 and screening this library for candidates using an RBP identification
assay (Figure 4.1). We screened 10,000 of these colonies and identified 13 clones capable of
producing gene products that bound *Agrobacterium* sp. H13-3 cells. Upon purifying the plasmids
and sequencing their inserts, we used the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to evaluate the identity of the phage genome fragments and the SnapGene software (from Insightful Science; available at snapgene.com) to analyze open reading frames (ORFs). We identified 6 unique fragments, encompassing 5 genes (Table 4.2; Figure 4.2, left). By considering the predicted ORFs and predominantly encompassed genes per fragment, we further deduced which genes are more likely to code for RBPs. For example, the FG19-1 fragment is 753 bp in length and covers 7-7-1_0003 and 7-7-1_0004. Only 16 bp map to 7-7-1_0003, while 741 bp encompass 7-7-1_0004 (note that the reading frames are overlapping (Table 4.2, Figure 4.2). Moreover, the other unique fragment mapping to this genomic region only encompasses 7-7-1_0004 (FG15, FG28; Table 4.2). Thus, we conclude that the gene responsible for producing protein capable of binding host cells is 7-7-1_0004. In a similar fashion, we pinpointed 7-7-1_00044 as the gene encoding a candidate RBP. One unique fragment encompassed only 7-7-1_00044 (FG5, F6) while the other fragment maps to 7-7-1_00044 and 7-7-1_00045 (FG42). Although the predicted 125 bp ORF for FG42 only covers 12 bp of 7-7-1_00044, we deduced that this gene is likely the RBP encoding gene because of its recurrence in the screen. Meanwhile, fragments FG9, FG10, FG26, and FG44 only contain one gene, 7-7-1_000102. Ultimately, we concluded that the genes responsible for the observed binding of host cells are 7-7-1_0004, 7-7-1_00044, 7-7-1_000102, 7-7-1_00044.

**Predicted functions of Gp4, Gp102, and Gp44.**

We then used BLASTP (54), Phure2 (55), and SWISS-MODEL (56-58) to gain insight on possible protein functions and predicted structures of the gene products. According to BLASTP, Gp4 (7-7-1_0004) and Gp102 (7-7-1_000102) are predicted to be associated with the phage tail, while Gp44 (7-7-1_00044) is a hypothetical protein (Table 4.3). Next, we used the
Phyre2 server and SWISS-MODEL to generate protein models based on homology and gain further insight on potential function. The results generated using all three tools coincide. In BLASTP, Gp4 is denoted as a tail biosynthetic protein for its similarity to a Mu-like prophage tail protein in *Bradyrhizobium* sp. BTAi1 (YP_001242396.1). The top result with both Phyre2 and SWISS-MODEL is the phage MuSo2 tail protein from *Shewanella oneidensis* (PDB ID 3CDD). The Phyre2 generated model has 80% coverage, 21% sequence identity, and a confidence value of 100 (Table 4.3, top row). Using SWISS-MODEL, we generated a homotrimeric homology model for Gp4 (Figure 3) using the prophage MusSo2 tail protein as a template. The global model quality estimate (GMQE) value is 0.46 and the sequence identity is 20%. The model encompasses 374 of 454 amino acids (82%), only lacking the last 80 amino acid residues. One noticeable feature in this model is the central β-barrel structure (Figure 4.3A, top half of model; 4.3B, right half of model), which is a hallmark feature of depolymerases (16, 21, 22, 26, 30). Thus, we hypothesize that Gp4 may be the LPS-targeting depolymerase for this phage.

We were not able to generate any high confidence models for Gp102 or Gp44 using Phyre2 or SWISS-MODEL. Either the coverage was very low with a high confidence value or the coverage was high with lower confidence (Table 4.3, middle and bottom rows). Gp102 is designated as a putative tail fiber protein in BLASTP, due to its similarity to the large tail fiber proximal subunit in Enterobacteria phage JSE (YP_002922323.1). However, the top Phyre 2 template is the hydrolase XylC from *Thermoanaerobacterium saccharolyticum* JW/SL-YS485. The percent coverage is 9% with a 36% sequence identity and 96.3 confidence value. Gp44 was listed as a hypothetical protein in BLASTP. Using Phyre2, the top template is an aldolase from *Kordia algicida* OT-1 with a 37% coverage, 14% sequence identity and a confidence value of
Similar results were obtained when using SWISS-MODEL (data not shown). In conclusion, we present convincing evidence that Gp4 is associated with the phage tail but do not have straightforward insight into the predicted functions of Gp102 and Gp44.

**Confirmation of initial RBP screen and identification of functional plasmid constructs.**

To verify our previous findings and to create a strategy for subsequent analyses using purified proteins, we conducted a second RBP screen. This time, our starting *E. coli* populations contained defined plasmid constructs rather than the randomly fragmented phage 7-7-1 genome library. Specifically, plasmids contained the full length or truncated versions (reflecting the fragments of the genes identified in the initial screen) of the RBP encoding genes N- or C-terminally fused to a 6x histidine tag. Since the results from the initial screen only implicated fragments of the RBPs, through this approach we also sought to evaluate whether the full-length versions are capable of binding host cells and, thus, which versions to use for subsequent approaches. *E. coli* cells containing vector pET30a were included as negative control and representative plasmids eliciting host binding from the initial screen as positive controls. To account for variations in binding, membranes containing proteins from *E. coli* cells with defined plasmid constructs were set up in duplicate. We developed a scoring system to better describe the observed levels of host cell binding. A schematic summarizing our observations and scoring is included in Figure 4.4 and Table 4.4, respectively.

To visualize binding, we stained the host cell containing membranes with the amido black 10b stain, which pigmented areas on the membrane containing both protein and host cells (Figure 4.4b). A score of +++ reflects high levels of host cell binding in areas containing protein. Meanwhile, a ++ score represents host cell binding in some of the areas imbedded with protein. Finally, a score of + denotes non-specific binding as illustrated by bacterial growth throughout
the membrane in few or no distinct spots. Results from stained membranes containing full length or truncated versions of Gp4 and controls are included in Figure 4.4b as a representative example of our scoring system.

During the confirmation screen for plasmids containing 7-7-1_0004, we observed the most host cell binding for *E. coli* BL21(DE3) with pBS1218. This plasmid encodes a fragment of Gp4, encompassing amino acids 1-247 (Gp41-247), N-terminally fused to a 6x histidine tag. For plasmids containing 7-7-1_000102, *E. coli* BL21 (DE3) with pBS1221 elicited the most host cell binding in comparison to the positive and negative controls. This plasmid encodes the full-length version of Gp102, C-terminally fused to a 6x histidine tag. Finally, *E. coli* BL21 (DE3) with pBS1224, encoding Gp44, C-terminally fused to a 6x histidine tag, was responsible for evoking the most host cell binding (Table 4.4). In summary, the second RBP screen confirmed that Gp4, Gp102, and Gp44 bound host cells. For the remaining part of this study, we focused on characterizing interactions between Gp4 and *Agrobacterium* sp. H 13-3 host cells. This RBP candidate occurred with the highest incidence in the initial RBP screen and *in silico* analyses permitted the hypothesis that it is an LPS-targeting RBP of phage 7-7-1. Thus, we decided to investigate its activity in more detail.

**Purified Gp4 inhibits the growth of host cells in liquid cultures.**

We isolated Gp41-247 with an N-terminal 6x histidine tag, produced from *E. coli* Lemo21 (DE3) cells transformed with pBS1218 (Table 4.1), via Ni-NTA affinity chromatography. Plasmid pBS1218 was selected because it elicited the highest level of host cell binding during our confirmatory RBP screen described above. The Lemo21(DE3) expression strain was chosen, because it allowed us to increase the proportion of soluble Gp41-247 as has been documented for other proteins (59). We then added the purified protein to growing bacterial cultures of wild-type
*Agrobacterium* sp. H13-3. We observed a dramatic growth inhibition in liquid cultures as indicated by a 7-fold reduction in liquid culture turbidity following addition of Gp41-247 compared to the control culture (Figure 4.5). The average OD₆₀₀ for cultures receiving Gp41-247 was 0.2 while for control cultures the average OD₆₀₀ was 1.4. Meanwhile the density of cultures treated with phage 7-7-1 only reached an OD₆₀₀ of 0.05. Visually, the clearance resulting from the addition of Gp41-247 resembled that of control cultures receiving phage 7-7-1 (MOI=1) (Figure 4.5A). However, this effect was not as pronounced, because the density of Gp41-247 containing cultures was significantly higher than that of cultures that received phage (Figure 4.5B; student’s *t* test, *P* < 0.05). We noticed a sedimentation of debris at the bottom of test tubes that received Gp41-247 that was not observed with the other treatments (Figure 4.5A, left tube). The identity of this debris and reasons underlying its presence are the subject of future investigations.

To determine the effect of this protein on host cell growth, we generated growth curves of cultures in 96-well plates following addition of Gp41-247, PBS, or phage 7-7-1 at an MOI=1. We measured the density of these cultures at 0, 17, 19, 21, 26, 29, 42, and 48 hours of growth. Treatment with Gp41-247 results in growth stagnation at an OD₆₀₀ of 0.27-0.28 from 26 to 29 hours, followed by a slight decrease to an OD₆₀₀ of 0.21 after 48 hours of growth (Figure 4.6). The cultures that received PBS increased in growth for 29 hours to a maximum OD₆₀₀ of 0.40 and then experienced a decrease in growth at 42 and 48 hours to an OD₆₀₀ of 0.35. Meanwhile, the cultures that received no additive continued to increase for 42 hours to a maximum OD₆₀₀ of 0.40 after which the growth plateaued. Compared to cultures that did not receive any additive, cultures with added Gp41-247 exhibited nearly a two-fold decrease in growth after 48 hours (student’s *t* test; *P* < 0.005). In comparison, cultures that received PBS grew to an OD₆₀₀ that was
1.5 times higher than that of the Gp41-247 treated cultures following 48 hours of growth (student’s \( t \) test; \( P < 0.005 \)). Cells that received phage 7-7-1 sustained an OD\(_{600}\) below 0.01 up until 29 hours and steadily increased to meet the density of the Gp41-247 treated cultures at 42 and 48 hours; this is likely due to the emergence of resistance bacteria. Altogether, these results established that the liquid clearing effect is likely due to a bacteriostatic effect of Gp41-247.

**Lawn clearance effects of purified Gp4.**

To gain an insight on factors that mediate the observed bacteriostatic effect and to potentially identify the receptor targets for this RBP, we evaluated the ability of purified Gp41-247 to clear lawns of wild-type and phage-resistant mutant *Agrobacterium* sp. H13-3 strains. This was achieved by seeding bacteria grown under motile conditions (OD\(_{600}\) of 0.3) in top agar, overlayed on TYC plates containing streptomycin, which were then spotted with serial dilutions of Gp41-247 on the solidified top agar. Equal volumes of buffer and phage 7-7-1 were spotted as controls on the same plates. Following overnight incubation at 30°C, we observed clearance of wild-type *Agrobacterium* sp. H13-3 cells in a dose-dependent manner on locations where Gp41-247 dilutions were spotted. There was no evident clearing in areas that received PBS, while spots of phage 7-7-1 produced uniform clearing (Figure 4.7; Table 4.5). Next, we evaluated whether Gp41-247 could clear lawns of motility mutants that lack flagella (RU12/006) or possess non-rotating flagella (RU12/012 and RU12/023). These strains are resistant to clearance by Gp41-247. This suggests that the activity of Gp4 is dependent on flagellar-mediated motility. We also conducted the same experiment on strains containing LPS mutations (RU12/015, RU12/016, RU12/017) and observed the same resistance phenotypes to Gp41-247. It is important to note that the motility of the LPS mutants does not differ from that of the wild-type strain (Table 4.5) (13).
Thus, these results indicate that the bacteriostatic activity of Gp41-247 is dependent on actively rotating flagella and native LPS.

DISCUSSION

Host recognition by bacteriophages is an important and essential step in initiation of infection. Initial interactions are mediated by phage encoded RBPs. Flagellotrophic phages first bind to the bacterial flagellar filament and, in some cases, interact with secondary cell surface receptors such as LPS, efflux pumps, and CPS (13, 14, 40, 47). RBPs in non-flagellotrophic phages have been identified, resulting in a wealth of knowledge surrounding common features of these proteins including resistance to proteolysis, thermostability, and in some cases the ability to degrade glycans (16, 20, 21, 24, 28-39, 60). In myoviruses and siphoviruses, RBPs typically come in the form of tail fibers while in podoviruses tail spikes are more common RBPs (9, 10). However, some myoviruses also contain tail fibers and tail spikes (21). In addition, phages can possess head fibers that interact with host cell components (12, 61). The identification of these RBPs has largely relied on in silico analyses based on sequence and structure homology or genomic location (40). However, for phages infecting non-model organisms, this approach can be less fruitful. In flagellotrophic phages, the identification of RBPs has primarily involved the detection of head or tail filaments using electron microscopy (EM). Examples include the single long tail filament of phage χ, the head filament of φCbK, and three helical tail filaments of phage PBS1 (12, 43, 46). These filamentous RBPs were imaged as they were in close proximity of flagella, which is indicative of interaction. However, there are no published studies that confirm physical interaction, molecular binding mechanisms, or the genetic identity of these flagellotrophic phage RBPs.
It is not unusual for phages to contain multiple RBPs as has been shown with myovirus CBA120. This phage has 4 tail spikes, each with different LPS targets that confer different host specificities allowing for a broader host range (60). An updated structural analysis of phage 7-7-1 virions using cryogenic EM (cryo-EM) revealed the presence of head fibers and tail filaments in addition to previously observed bushy, short tail fibers (personal communication, Dr. Ariane Briegel, University of Leiden) (23). Thus, these appendages are prime RBP candidates, although interactions between these filaments and flagella are not as readily discernable in EM studies as for phage χ, φCbK or PBS1 (12, 43, 46). Initially, it was hypothesized that the tail fibers were responsible for mediating interactions between phage 7-7-1 and host flagella (41). Although a ‘nut and bolt’ model for translocation of bacteriophage χ and other flagella-dependent phages has been proposed, it becomes increasingly clear that there is no uniform mode of phage translocation along the flagellar filament (12, 45, 51). This is not only due to the differences in phage appendages (i.e., head vs. tail fibers), but also because of the differing modes of flagellar rotation and filament structure. In both, *E. coli* and *C. crescentus*, the flagellar motors exhibit bidirectional rotation to switch between swimming and tumbling behaviors as the cell moves through the environment in response to chemotactic stimuli (12, 62). Meanwhile, in *Agrobacterium* spp. and *Sinorhizobium meliloti*, the motors only exhibit rotation in the clockwise direction, instead asynchronously slowing down rotational speed to switch between swimming and tumbling (49, 50). Due to the differences in flagellar rotation and filament structure, the modes for phage translocation down the flagellar filament are likely different for phage 7-7-1 than those that have been documented for phage χ and φCbK.

This hypothesis is further compounded by the differences in virion structure. Phage 7-7-1 possesses head filaments, tail fibers, and tail filaments. The phage 7-7-1 head fibers appear
structurally different than the single, flexible, long head filament in phage φCbK, which wraps around the flagellar filament like a lasso allowing for translocation to the cell surface (12, 45). Phage 7-7-1 also has multiple head filaments. Aside from differences in numbers, the kinked appearance of its head fibers more closely resembles those documented for the Bacillus subtilis infecting phage φ29. This tailed phage has 55 head fibers protruding from its capsid. Deletion of the head fibers in phage φ29 does not impede infection but does influence virion positioning on the cell surface (61). Thus, head fibers seem to play auxiliary roles that enhance binding of virions and thus likely confer advantages in increasing the number of infection events (12, 61).

The phage 7-7-1 head filaments may be functioning in a similar fashion. Tail appendages, such as the long, flexible tail filaments of χ and PBS1 wrap around their host flagellar filaments (43, 46). These phages lack tail fibers resembling the short, splayed tail fibers of phage 7-7-1. The presence of both the long tail filaments and short tail fibers further indicates that the mechanisms for interacting with host receptors are likely different for phage 7-7-1.

Because of the structural differences described above and the lack of identification of RBPs in phage 7-7-1 (aside from the annotation of 7-7-1_000102 as a putative tail fiber), we decided to search for RBPs in this system. We screened a randomly fragmented genome library of phage 7-7-1 for RBP candidates using an in vivo host cell binding assay (22) (Figure 4.1). We identified 6 unique gene fragments producing proteins responsible for binding Agrobacterium sp. H13-3 cells (Figure 4.2). The results implicated 3 genes encoding RBPs: 7-7-1_0004 (Gp4), 7-7-1_000102 (Gp102), and 7-7-1_00044 (Gp44) (Table 4.2). Fragments containing 7-7-1_0004 had the highest rate of occurrence (6 fragments), followed by 7-7-1_000102 (4 fragments) and 7-7-1_00044 (3 fragments). A second screening with defined plasmid constructs confirmed the initial results (Table 4.4). In silico protein homology searches using Phyre2 implicated Gp4 as a tail
associated protein, while the predicted functions of Gp102 and Gp44 were of a hydrolase and aldolase, respectively (Table 4.3). Although we were unable to produce high confidence structural models for Gp102 and Gp44, we did generate a model of Gp4 using SWISS-MODEL (Figure 4.3). This model was built using the structure of prophage MuSo2 tail protein from S. oneidensis as a template. Most notably, the model predicts the association of Gp4 in a homotrimeric state and the formation of a central β-barrel structure. Phage-derived depolymerases targeting LPS or CPS commonly possess β-sheets in their central domains, which contain the catalytic site responsible for enzymatic degradation of glycans and frequently form homotrimers (9, 20, 21, 26, 35). We hypothesize that Gp4 functions as a depolymerase degrading the secondary cell surface receptor for phage 7-7-1: LPS. For these reasons, we focused on the further analysis of Gp4.

The second RBP binding screen identified the Gp4 fragment comprising amino acid residues 1-247 with an N-terminal 6x-His tag to efficiently bind host cells (Table 4.4). Therefore, Gp41-247 was purified using Ni-NTA affinity chromatography, and its effect on growing liquid cultures was evaluated. Interestingly, we discovered that Gp41-247 inhibits the growth of liquid host cultures (Figures 4.5 and 4.6). Similar lawn clearance effects have been documented and demonstrated to be bacteriostatic not bactericidal (56, 57). Our present data do not address if the observed growth inhibition is due to host cell lysis, which is the subject of future work. Further investigations of mutant Agrobacterium sp. H13-3 strains indicated that growth inhibition is dependent on intact flagellation and flagellar rotation as well as wild type surface glycans (Figure 4.7). There is precedent for a phage-encoded protein that interacts with host flagella (64). The FlaGrab protein of phage NCTC 12673 inhibits growth of Campylobacter jejuni and interacts with the flagellar filament via specific flagellar glycans (47, 63). This protein reduces
overall motility of the host and alters the transcription of energy metabolism pathways. FlaGrab was initially thought to function as an RBP, but its absence in virions indicates that it may instead function as an effector protein (63). Similarly to Gp4 (Figures 4.5 and 4.6), flagellar rotation is also required for its interaction. CPS is an additional receptor for phage NCTC 12673 and mutations to CPS result in phage resistance. However, FlaGrab is still able to clear lawns of CPS mutants, indicating that its activity is not related to CPS hydrolysis. Meanwhile, Gp4 is not able to clear lawns of non-motile mutants that are either lacking flagella or possess non-rotating flagella, as well as LPS mutants, indicating interactions with flagella and LPS (Figure 4.7). Thus, although FlaGrab and Gp4 possess some mechanistic similarities, there are also distinct differences in their mode of action.

The mechanism underlying the observed growth inhibition by FlaGrab was not determined. However, a mechanosensing hypothesis was proposed, in which FlaGrab binding to flagella reduces motility by causing flagellar stiffness. The cell then compensates by increasing proton flow to power flagellar rotation, which disrupts the electron transport chain and alters transcription of energy metabolism pathways. Although the proposed mechanosensing hypothesis could arguably occur in response to any protein that binds to flagella, FlaGrab relies on the specific recognition of flagellar glycans. Thus, the proposed response would only occur if this protein is also able to use flagellar glycans for the recognition of and binding to flagella.

Currently, our data do not warrant the establishment of a working hypothesis for the mechanism underlying Gp4-mediated growth inhibition of *Agrobacterium* sp. H13-3. However, we can conclude that the growth inhibition is dependent on functional flagella and native LPS. The presence of phage proteins that are reliant on functional flagellar filaments and presence of specific glycans in two different flagellotrophic phages is exciting. The existence of these
similarly functioning proteins indicates the possibility that FlaGrab-like proteins play an important and conserved role in flagellotropic phage infection processes. Uncovering the molecular mechanisms behind these interactions will aid in a more comprehensive model for flagella-dependent phage infections.
REFERENCES


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Table 4.2. Mapping of DNA fragments to phage 7-7-1 genome.

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| FG5               | 29376…29560 | 2 | 185/351 (44) | 29379…29555 | N | 2
| FG6               | 29376…29560 |
| FG42              | 29553…29745 | 14/351 (44) | 9553…29690 | N |

122
Table 4.3. RBP candidate protein homology.

<table>
<thead>
<tr>
<th>Protein</th>
<th>BLASTP Function</th>
<th>Phyre2</th>
<th></th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Top template</td>
<td>Amino acids aligned</td>
<td>% coverage</td>
</tr>
<tr>
<td>Gp4 (454 AA)</td>
<td>Tail biosynthetic protein</td>
<td></td>
<td>Prophage MuSo2 tail protein from <em>Shewanella oneidensis</em></td>
<td>2-366</td>
<td>80</td>
</tr>
<tr>
<td>Gp102 (587 AA)</td>
<td>Putative tail fiber</td>
<td></td>
<td>Hydrolase XylC from <em>Thermoanaerobacterium saccharolyticum</em> JW/SL-YS485</td>
<td>192-248</td>
<td>9</td>
</tr>
<tr>
<td>Gp44 (116 AA)</td>
<td>Hypothetical protein</td>
<td></td>
<td>Aldolase from <em>Kordia algicida</em> OT-1</td>
<td>65-109</td>
<td>37</td>
</tr>
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Table 4.4. Confirmatory RBP screen binding analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Plasmids</th>
<th>Protein expressed</th>
<th>Binding$^c$</th>
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<tbody>
<tr>
<td>7-7-1_0004</td>
<td>pBS1218</td>
<td>6xHis-Gp41-247</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>pBS1219</td>
<td>Gp41-247-6xHis</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>pBS1222</td>
<td>6xHis-Gp4</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>pBS1228</td>
<td>Gp4-6xHis</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>FG19-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pET30a</td>
<td>Gp41-246-6xHis$^a$</td>
<td>++</td>
</tr>
<tr>
<td>7-7-1_000102</td>
<td>pBS1220</td>
<td>6xHis-Gp102</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>pBS1221</td>
<td>Gp102-6xHis</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>pBS1227</td>
<td>Gp102363-436-6xHis</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>FG9</td>
<td>6xHis-Gp102363-436</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>pET30a</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>7-7-1_00044</td>
<td>pBS1223</td>
<td>6xHis-Gp44</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>pBS1224</td>
<td>Gp44-6xHis</td>
<td>+++</td>
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<td></td>
<td>pBS1225</td>
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<td></td>
<td>pBS1226</td>
<td>Gp4453-115-6xHis</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>FG5</td>
<td>6xHis-Gp4453-114$^b$</td>
<td>+++</td>
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<tr>
<td></td>
<td>pET30a</td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

$^a$ Fragment ended with two nucleotides that were not in frame; therefore, pBS1218 and pBS1219 include an extra nucleotide to generate a fragment with an additional native codon.

$^b$ Fragment ended with two nucleotides that were not in frame; therefore, pBS1225 and pBS1226 include an extra nucleotide to generate a fragment with an additional native codon.

$^c$ (+++) denotes specific binding in areas containing protein with no background growth, (++) indicates mostly specific binding with some background growth, and (+) represents nonspecific bacterial growth throughout the membrane (background).
Table 4.5. *Agrobacterium* sp. H13-3 strain genotypes and resulting flagellar and motility associated properties.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Flagellar or motility phenotype</th>
<th>Other phenotypes</th>
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<tr>
<td>RU12/001</td>
<td>Wild type</td>
<td>Normal</td>
<td>n/a</td>
</tr>
<tr>
<td>RU12/006</td>
<td>ΔflaA ΔflaB ΔflaD</td>
<td>No flagella</td>
<td>n/a</td>
</tr>
<tr>
<td>RU12/012</td>
<td>ΔmotA</td>
<td>Non-motile</td>
<td>n/a</td>
</tr>
<tr>
<td>RU12/015</td>
<td>ΔAGROH133_08824</td>
<td>Normal motility</td>
<td>Mutant LPS</td>
</tr>
<tr>
<td>RU12/016</td>
<td>ΔAGROH133_07337</td>
<td>Normal motility</td>
<td>Mutant LPS</td>
</tr>
<tr>
<td>RU12/017</td>
<td>ΔAGROH133_13050</td>
<td>Normal motility</td>
<td>Mutant LPS</td>
</tr>
<tr>
<td>RU12/023</td>
<td>ΔfliK</td>
<td>Non-motile; abnormal hook</td>
<td>n/a</td>
</tr>
</tbody>
</table>
Figure 4.1. Illustration of RBP screen method. Transformants containing the phage genome library were lifted onto nitrocellulose membranes and protein expression was induced by placing on IPTG containing nutrient agar. Cells were lysed, membranes blocked and washed prior to the addition of motile host cell bacteria. The membranes were washed to remove unbound host cells and then incubated at 30°C to allow for host cell growth. Transformants containing RBP encoding gene fragments were identified by comparing areas of host cell growth on membranes with original locations on master plate. Plasmids were isolated and sequenced. In silico analysis of sequences was used to identify genes coding for RBPs and predicting ORFs.
Figure 4.2. Fragment mapping to phage 7-7-1 genome. (A) Illustration of unique fragments in reference to the genome and (B) the predicted ORFs as determined by the SnapGene software.
Figure 4.3. Predicted protein model of Gp4. A SWISS-MODEL generated protein model for Gp4 with the absolute quality estimates (QMEAN) colorimetrically displayed. The prophage MuSo2 tail protein from *Shewanella oneidensis* (PDB ID 3CDD) was used as a template. The global model quality estimate (GMQE) was 0.46 while the sequence identity was 20.24%. The model encompassed 374/454 amino acids. N, N terminus; C, C terminus.
Figure 4.4. RBP confirmation screen summary. (A) Transformants with known plasmid constructs were lifted onto nitrocellulose membranes and subjected to the RBP identification assay. Levels of host cell binding were phenotypically assessed compared to the negative controls (empty pET30a containing membranes). Binding was graded as follows: (+++) specific binding in all or most areas containing protein, (++) mostly specific binding with some areas
containing protein, and (+) mostly nonspecific bacterial growth throughout the membrane. (B) Results following staining of membranes containing full length or truncated versions of Gp4 as well as positive and negative controls.
Figure 4.5. Effect of purified Gp4 on liquid cultures. Liquid cultures of *Agrobacterium* sp. H13-3 (OD$_{600}$ = 0.03) received purified 40 μl Gp4 (0.06 μg/μl), PBS, or phage 7-7-1 (MOI=1) and incubated at 30°C for 24 hours. Representative images of the cultures are show in (A) while the final densities are illustrated in (B). Data reflects 3 biological replicates conducted in triplicate. The error bars represent standard deviations for each data set (student’s *t* test for Gp4 and phage 7-7-1 treatments, P < 0.05).
Figure 4.6. Growth curves for bacterial cultures under various conditions. *Agrobacterium* sp. H13-3 (OD$_{600}$= 0.03) cells were deposited in 96-well plates and received 2 μl of Gp4$_{1-247}$ (0.06 μg/μl), PBS, phage 7-7-1 (MOI=1), or no additive. Growth of the cultures was measured via OD$_{600}$ at 17, 19, 21, 27, 29, 42, and 48 hours using a plate reader. Data are representative of 4 independent experiments conducted in quadruplicate and error bars reflective of standard deviation. Student’s $t$ test for Gp4$_{1-247}$ and PBS or phage 7-7-1 at 42 and 48 hours, P < 0.005.
Figure 4.7. Lawn clearance experiments with Gp41-247. TYC plates overlaid with TYC top agar (0.5% agar) containing wild-type and various phage resistant mutant strains received 10 μl spots of PBS, different concentrations of Gp41-247, or phage 7-7-1 (10^7 PFU). Following overnight incubation at 30°C the plates were assessed for spots of clearance and imaged. The genotypes and resulting phenotypes for each strain are listed in Table 4.5.
Chapter 5: Final Discussion

Bacteriophages were initially discovered in 1915 by Frederick Twort and later in 1917 by Felix d’Herelle (1, 2). Since then, phages have served as tools to understand fundamental principles of biology, including the confirmation that DNA is responsible for carrying genetic information and discovery of restriction enzymes. Additionally, phages have been used to combat bacterial infections. In fact, initial phage therapies date back to 1919 and have continued, largely in Eastern Europe, until the present day. In the last decade, there has been a resurgence of phage therapy in response to growing numbers of antibiotic resistant bacterial infections (1, 2).

As for any other treatment or therapeutic, detailed analysis of modes of action, efficacy, and safety, are needed. Phage therapy requires that a phage with the correct host range is identified before it can be propagated and prepared for administration to patients or other applications. In the United States, phage therapy is predominantly used as an emergency treatment for antibiotic resistant infections (2, 3). However, phages are also used to surface sterilize surfaces in food processing and as additives in deli meats, cheeses, and produce. These applications prevent the contamination of foods with bacterial pathogens (4, 5). The biggest obstacle to using phage therapy is the availability of ready-to-use phages with predetermined host ranges. Thus, the initial step in phage therapy is typically phage discovery followed by characterization of their host range. In some cases, like when a patient is dying from a bacterial infection and urgently needs phage therapy, there is a time constraint that makes it difficult to holistically characterize newly discovered phages prior to patient administration. In response, the establishment of phage repositories to broaden access to phages with defined host ranges has been the focus of several initiatives including Phage Directory (6) and the Center for Innovative Phage Applications at the University of California San Diego (7). While this is a step in the right
direction, we also need to build models that are representative of the molecular interactions taking place between different phage-host pairs. Building such models can allow us to make predictions about the outcomes of phage therapies for phages that use the same host cell receptors and have conserved infection mechanisms without having to generate this data on the bench every time. This approach necessitates the study of phages that employ diverse methods for infecting their hosts.

Flagellotrophic phages are largely understudied despite the first discovery of flagella-dependent phage χ by Felix d’Herelle himself in 1930 (8). Classically, studies on this phage type focused on interactions with flagellar filaments, which uncovered that deflagellation, disruptions to flagellar rotation and structure result in phage resistance (9-13). However, studies centered on the downstream interactions between flagellotrophic phages and their hosts are lacking. In chapter 2, we highlighted important factors underlying the co-existence of flagella-dependent phage χ and one of its Salmonella hosts. Through this examination, we determined that intrinsic factors, such as phage adsorption rates and bacterial division rates, are important for a balanced phage-bacteria co-existence. Modifications to these factors pushes either phages or bacteria to dominate. Although this finding was not surprising, it underlined the importance of knowing and accounting for these factors to predict outcomes of interactions between different phage and bacteria pairs. Without this information, building reliable models that accurately predict the outcomes of phage applications is not possible. With more research, we propose to build a more robust model that can be used as a tool to inform the selection of phages for different applications. Having such a model will circumvent the need to systematically test these outcomes given that the proper information for phages and their cognate hosts will be available or can be
easily derived. Thus, we focused the remainder of our work on generating molecular knowledge on flagellotropic phage infection mechanisms to uncover more information on intrinsic factors.

In chapter 3, we focused on detailing the interactions between *Agrobacterium* phage 7-7-1 and one of its hosts, *Agrobacterium* sp. H13-3. By screening for transposon mutants that were motile and phage resistant, we showed that transposon insertions in three genes are responsible for the resistance to phage 7-7-1. Two of the genes, AGROH133_08824 and AGROH133_07337, encode a glycosyl transferase and an epimerase, respectively, that function in LPS biosynthesis. Meanwhile the third gene, AGROH133_13050, has no predicted function. Transposon strains that contained the genes complemented *in trans* regained sensitivity to phage, while strains with targeted, in-frame deletions of each gene were resistant. Furthermore, the motility of these strains did not differ from that of the wild-type stain indicating that the phage resistance phenotype is not due to a polar effect on motility. Further analysis of the deletion strains yielded no hallmark phenotypes of LPS mutants (i.e., increased sensitivities to detergents and salts). Through mass spectrometry analysis of LPS isolated from each of the deletion strains and the wild type, we determined that the ion spectra of LPS from each mutant strain is different than the wild type. This indicated that LPS is used as a secondary receptor for phage 7-7-1 following interactions with the flagellar filament. Since the LPS for *Agrobacterium* sp. H13-3 is not characterized, we could not ascertain what the exact binding site within the LPS is used by phage 7-7-1. Thus, we looked to known LPS structures of closely related *Agrobacterium* species for clues. Members of the *Rhizobiaceae* family, like *Agrobacterium* spp., commonly utilize galactose as a common building block (14). The mass spectrometry data indicated that the deletion strains lack repeating methylhexoses or hexuronic acids. Therefore, it is possible that
galactose is the sugar moiety targeted by phage 7-7-1. However, this hypothesis needs to be tested to truly determine the exact binding site within the LPS.

Other flagellotropic phages like bacteriophage χ and phage NCTC 12673 also use secondary cell surface receptors following interactions with flagellar filaments. Bacteriophage χ targets efflux pumps, while phage NCTC 12673 uses CPS (15, 16). Use of additional cell surface receptors may confer an extra layer of specificity for these phages, with the different receptor targets contributing to their varying host ranges. Targeting a receptor or receptor component that is more widespread may result in a broader host range while targeting a specific glycan, which is not present in surface structures of many bacteria, may confer a narrow host range.

Knowing the identities of receptors used by phages also allows us to make some predictions about possible tradeoffs stemming from phage resistance. For instance, in multidrug resistant (MDR) *Pseudomonas aeruginosa* PAO1 and PA14, resistance to phage OMK01 results in regained sensitivity to antibiotics. This phage uses the MexAB-OprM and/or MexXY-OprM efflux pump systems as receptors. Thus, phage resistance results in an evolutionary trade-off that alters efflux pump function. This indicates a benefit of using phages in combination with antibiotics to combat MDR *P. aeruginosa* infections (3, 17). In the presence of flagellotropic phages, bacteria that are non-motile or have other mutations that affect flagellar function and structure dominate the population (9-11, 13). Although this confers phage resistance, motility provides an evolutionarily fitness advantage. In the case of *Agrobacterium* spp., a loss in motility impacts the bacterial cell’s ability to move towards host plant roots and successfully infect these plants. Acquiring phage resistance at the cost of altering LPS can also be detrimental. For example, there are documented effects of mutations in core LPS that result in severe motility defects, which would affect infection by flagellotropic phages (18-21). Additionally, the LPS
core region is an important factor mediating attachment to roots and subsequent host plant colonization in a variety of soil bacteria, including Rhizobium leguminosarum and Azospirillum spp. (22, 23). Although the role of LPS in Agrobacterium pathogenesis is less clear, there is some evidence for involvement in plant cell binding and subsequent colonization (14, 24). While phage resistance may be thought of as a negative outcome of phage therapy, in some cases it can be beneficial leading to evolutionary trade-offs that are detrimental to pathogenic bacteria. Attenuation or reduction of bacterial pathogenicity following phage treatment is a desirable outcome for the design of standalone phage or combined phage-antibiotic therapies (3, 25). The effect of the LPS mutations that confer phage resistance as described in chapter 3 and their overall effect on Agrobacterium spp. pathogenesis is an avenue for future research.

A holistic evaluation of phage-host interactions is an essential part of designing phage therapies. Such an approach can also uncover additional therapeutic options, such as the use of phage proteins like depolymerases or holin-like proteins to kill bacterial cells (26-28). In chapter 4, we searched for phage 7-7-1 encoded RBP candidates with the larger goal of identifying phage proteins that interact with host cell receptors and have therapeutic potential against Agrobacterium spp. Using an RBP screen, we identified 3 RBP candidates in phage 7-7-1: Gp4, Gp102, and Gp44. Based on protein homology analysis, Gp4 is likely associated with the phage tail, while the function of the other two proteins is less clear. Because of the predicted trimerization and β-barrel structure, which are hallmarks of phage-associated depolymerases (26, 29-33), we envisioned that Gp4 is the LPS-targeting depolymerase in phage 7-7-1. Thus, we focused our study on unravelling the function of Gp4. We discovered that this protein has bacteriostatic effects and that its activity is dependent on flagellar function and presence of native LPS. Flagellotropic phage NCTC 12673, which infects Campylobacter jejuni, possesses a
protein that exhibits similar features. The FlaGrab protein is also able to inhibit the growth of host cells in a mode that is reliant on flagellar function and specific glycosylation patterns on the flagellar filament (16, 34, 35). Although the mechanism of action underlying the bacteriostatic activities of FlaGrab is unknown, the similarities between it and Gp4 are exciting and indicate that FlaGrab-like proteins may be a conserved part of flagellotropic phage infection. The investigation of the underlying mechanism for the bacteriostatic effects of Gp4 is the subject of future work. Furthermore, we are interested in exploring the utilization of Gp4 as a therapeutic and/or preventative measure against crown gall disease caused by \textit{Agrobacterium} spp. In other systems, the application of phage proteins with enzymatic activities targeting surface glycans has been promising in treating bacterial infections and even biofilms (29, 36-39). Thus, using phage components that typically function in overcoming the host cell barriers for infection present an alluring avenue for developing new standalone therapeutics or adjuvants.

The described works contribute to a deepened understanding of flagellotropic phage infection and the factors influencing phage-bacteria coexistence in these systems. Our studies reiterate the importance of exploring the molecular interactions during the early stages of phage infection. Not only do these interactions allow us to predict whether certain properties tip the scales towards phage or bacterial proliferation, but also to make broader conclusions about the evolutionary tradeoffs stemming from phage resistance. Notably, we built a more comprehensive model for the infection of \textit{Agrobacterium} sp. H13-3 by phage 7-7-1 and highlight the use of LPS as an additional, essential cell surface receptor in this system. We discovered the identity of a phage protein, Gp4, which relies on host flagellar motility and glycans to inhibit host cell growth. The observed bacteriostatic effect warrants further investigations of the therapeutic promise of Gp4 and the underlying mechanisms contributing to host cell growth inhibition.
These works also open more avenues for future work including determining the effects of the observed LPS mutations documented in chapter 3 on bacterial pathogenesis and exploring the roles of the other two candidate RBPs identified in chapter 4 in phage 7-7-1 infection. Using predictive computational models in conjunction with our knowledge of the molecular interactions between different phage types and host cells, we can build powerful predictive tools. This will allow us to design well-informed and robust phage therapies quickly, ultimately helping us combat difficult to treat or previously untreatable bacterial infections.

REFERENCES


Supplementary 1. Both direct and indirect negative dependences of phage proliferation on bacterial density are necessary for generating straight radial boundaries in the lysis pattern. (a) Simulated lysis pattern formation with both Assumptions (i) and (ii). Same results as Fig. 1b, second row. (b) Simulated lysis pattern formation without Assumption (i), but with Assumption (ii). (c) Simulated lysis pattern formation without Assumption (ii), but with Assumption (i). (d) Simulated lysis pattern formation without both Assumptions. As described in Results, Assumption (i) states that nutrient deficiency inhibits phage replication, and Assumption (ii) states that high bacterial density inhibits phage production.
Supplementary 2. Model results show steady bacteria and phage densities at the expanding front after the phage initiation zone emerges. (a) Lysis patterns over time. Blue dashed line: cutline over which the density profiles are plotted in (b). (b) Density profiles of susceptible bacteria over the cutline at the labeled times. (c) Density profiles of phages over the cutline at the labeled times.
Supplementary 3. Simulation results with various numbers of inoculated bacteria.
Supplementary 4. Lysis pattern with straight radial boundaries requires balance between intrinsic properties of bacteria and phage. Simulated lysis patterns with (a) various phage adsorption rate constants and bacterial diffusion coefficients, (b) various bacterial division rate constants and bacterial diffusion coefficients, (c) various phage adsorption rate constants and chemotactic efficiencies, and (d) various bacterial division rate constants and chemotactic efficiencies.
Exp.

Deletion: ΔTar  Deletion: ΔTsr  Deletion: ΔTar ΔTsr

Supplementary 5. Experimental results of S. Typhimurium strains lacking chemoreceptors.
Supplementary 6. Lysis patterns with different bacterial motility. (a) Experimental results with different agar densities. Plates were incubated until bacterial swim rings reached the edge of the plate (7 h for 0.2%, 9 h for 0.25%, and 14 h for 0.3% agar), and the images were taken at the end of the experiments. (b) Simulated lysis patterns with various bacterial diffusion coefficients and nutrient diffusion efficiencies. The bacterial diffusion coefficient in the model reflects the efficiency of bacterial motility.
Supplementary 7. Co-propagation between phage and bacteria is reflected by the straight radial boundary of lysis pattern. (a) Lysis patterns and (b) corresponding spatial patterns of phages with various bacterial diffusion coefficients and phage adsorption rate constants. Superposition of (a) and (b) gives Fig 5a in the main text. Grey shadowed staircase: potential trajectory of evolutionary arms race on which phages and bacteria maintain balance with each other.
Supplementary 8. Effect of temperature on lysis pattern. *Salmonella enterica* serovar Typhimurium 14028s was incubated with χ phage at 37°C (temperature used for all other experiments except where otherwise indicated), 30°C, or room temperature (RT). Plates were incubated until the bacterial swim rings reached the edge of the plate (14 h at 37°C, 22.5 h at 30°C, 38 h at RT). Although there are slight differences in lysis angle, the overall shape of the pattern remains largely the same.
Supplementary 9. The shape of lysis area depends on the competition between phages and bacteria, but not the initial nutrient level. Simulated lysis patterns with various initial nutrient levels and (a) bacterial division rate constants, (b) phage adsorption rate constants, (c) bacterial diffusion coefficients, (d) chemotactic efficiencies.
Supplementary 10. Time evolution of the lysis pattern in experiment and model. Each frame displays corresponding time points in experiment vs. model. Total time 14 h. Color bar represents density of bacteria (cm$^2$) in model.
Appendix B: Supplementary figures for Chapter 3

A. Sequence with differences highlighted along with similar proteins in other Agrobacterium and Rhizobium species.

>tr|F0L3E4|F0L3E4_AGRSH Uncharacterized protein OS=Agrobacterium sp. (strain H13-3) OX=861208 GN=AGROH133_08824 PE=4 SV=1
MEFYFPAEFGEQLAFGAAVSVVIGLFFMFAPGATLRAFGLQAIGERRDGYAALVRSSLAGFYLGALGAALLAQPMLVA
YLAFGAAFGLSVFGGILSILSDGATVRNFILLVHVLLSALSLSYVFGLV

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</tr>
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</table>

B. RaptorX-generated structures. Similar structures were found with other modeling programs.

Supplementary 2. SDS-PAGE of isolated lipopolysaccharides from different bacterial strains.

Equal volumes (2 μl) of each sample were loaded in the following lanes: 1, wild-type strain; 2, RU12/015; 3, RU12/016, 4, RU12/017. The gel was visualized using silver staining.
Supplementary 3. Growth of wild type and phage resistant mutants on media containing 0.3 M NaCl (B), 0.5% TritonX-100 detergent (D), or 0.5% cholic acid (F). Growth on media controls supplemented with water instead of 0.3M NaCl (A), 0.5% Triton-X100 (C), or 0.5% cholic acid (E) are also depicted. Overnight cultures were adjusted to an OD$_{600}$ of 0.3 and evaluated for motility. Serial 1:10 dilutions of each culture were made and 5 µl were spotted on the plates.
Images were taken after at least 2 days of incubation at 30°C. These images are representative of 3 independent experiments per condition conducted in duplicate.