PHAGE RESISTANCE IN LISTERIA MONOCYTOGENES: GENETIC AND PHYSIOLOGICAL REFUGE

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PHAGE RESISTANCE IN LISTERIA MONOCYTOGENES: GENETIC AND PHYSIOLOGICAL REFUGE

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Listeria-infecting phages are currently being used to control and detect the important foodborne pathogen *Listeria monocytogenes*; however, little is known about mutations that confer phage resistance to *L. monocytogenes*, which are known to be selected for by virulent phages, or how environmental conditions affect the susceptibility of *L. monocytogenes* to phage infection. In the studies presented here we (i) use whole genome sequencing to identify specific mutations that confer phage resistance to *L. monocytogenes* and (ii) examine how temperature affects phage susceptibility to phage infection. We found that *Listeria* phages selected for mutations in a handful of genes that affect wall teichoic acid structure, and that these mutations conferred phage resistance by inhibiting adsorption of phages to the cell surface. We also showed that temperature significantly affects *L. monocytogenes'* susceptibility to phage infection by affecting both the plaquing efficiencies and adsorption efficiencies of *Listeria* phages. Together, this dissertation reveals specific examples of how *L. monocytogenes* can gain genetic refuge from phage infection (resistance by mutation) and has shown clearly that *L. monocytogenes* can gain physiological refuge from phage infection (transient resistance).

BIOGRAPHICAL SKETCH

Thomas (Tom) Denes was born on November 29, 1986 in New Haven, CT as the younger of two children to Douglas Denes and Julia Downs. Tom graduated from The Evergreen State College with a Bachelors of Science in 2009. He spent the next two years researching phage infection dynamics in *Escherichia coli* under the supervision of Drs. Elizabeth Kutter and Andrew Brabban. Tom started as a doctoral student at Cornell University in Comparative Biomedical Sciences in 2011. Outside of the lab, Tom is an outdoor enthusiast who loves to travel to new places and explore the natural world.

To my parents and brother

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It should also be noted that Martin Wiedmann is a scientific advisor for and has financial interests in Sample6, a company using phage-based technology to detect bacterial pathogens.

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PREFACE

This dissertation examines phage resistance in *L. monocytogenes* that is caused by mutations in the host cell's genome and changes to the physiological state of the host cell by environmental conditions. The former can be described as *Listeria* gaining genetic refuge from phage infection, and the latter can be described as the host cell gaining physiological refuge from phage infection. The introduction for this dissertation consists of a published review article (1) where we discuss the implications of bacterial foodborne pathogens gaining physiological refuge from phage-infection to the food-safety industry, which uses phages for the control and detection of bacterial foodborne pathogens. The second chapter consists of a published research article (2) where we explore how *L. monocytogenes* gains genetic refuge from phage infection. The third chapter consists of a published research article where we show that *L. monocytogenes* can gain physiological refuge from phage infection (3).

1. **Denes T**, **Wiedmann M**. 2014. Environmental responses and phage susceptibility in foodborne pathogens: implications for improving applications in food safety. Curr Opin Biotech **26**:45–49.
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CHAPTER 1

ENVIRONMENTAL RESPONSES AND PHAGE SUSCEPTIBILITY IN FOODBORNE PATHOGENS: IMPLICATIONS FOR IMPROVING APPLICATIONS IN FOOD SAFETY

Authors

Thomas Denes and Martin Wiedmann.

Source

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Abstract

Bacterial foodborne pathogens can rapidly respond to changes in their environment, granting them the ability to survive under a broad range of conditions. In doing so, they undergo physiological alterations that can influence the efficacy of detection and interventions used in the food industry. As bacteriophage-based applications in food safety are gaining traction, it is crucial that we consider the effect the environment can have on phage–host interactions. This review aims to bridge knowledge of the responses of bacterial foodborne pathogens to changing

environmental conditions with our understanding of phage–host interactions. An improved understanding of these intersections will aid in the development of bacteriophage-based products for the detection, biocontrol and biosanitation of foodborne pathogens.

Introduction

Bacterial foodborne pathogens can survive and replicate under a broad range of environmental conditions. To adapt to a changing environment they must first sense and then respond to the change; the response typically affects gene expression and leads to changes in cell physiology that favor growth or survival under the new conditions. These changes in physiological state can affect the cell's susceptibility to antimicrobials or sanitizing agents, and can interfere with detection systems. As bacteriophage-based control and sanitization products are now on the market for use in the food industry, and with phage-based detection products in development, it is necessary to consider the effect environmental conditions can have on phage–host interactions to maximize the efficacy of these new tools.

Bacteriophages are the viruses of bacteria. They are found in nearly every environment on Earth and are estimated to globally outnumber prokaryotes by ten to one (1, 2). Shortly after their co-discovery by Frederick Twort and Felix d'Herelle in the early 20th century, they were harnessed for their therapeutic potential. However, after the discovery of broad-spectrum antibiotics, phage applications were soon largely forgotten in the U.S. and in Western Europe until interest was renewed by the rise of antibiotic resistant pathogens. The development of phage-based products is now

moving rapidly in the food safety sector. In 2006, a Listeria phage biocontrol product was approved by the USFDA for use in ready-to-eat food products (3). The product consists of a 'cocktail' of lytic bacteriophages that can be applied directly to food to reduce or prevent contamination by Listeria monocytogenes. Several other phage-based control applications are also used by the food industry, and a number of new bacteriophage-based products, including detection systems, are under development. There are several current review articles that cover phage-based applications in the food industry (4) with emphasis on biocontrol (5-7) and detection applications (8).

In this review we focus on how phage–host interactions are affected by environmental responses that alter the physiological state of bacterial foodborne pathogens. Host physiology can affect phage–host interactions through changing (i) the host cell's susceptibility to phage infection and/or (ii) the productivity of a phage infection (Figure 1.1). We define 'susceptibility' as the likelihood of a cell being lysed or lysogenized after collision with a phage, and 'productivity' as a measure of phage produced per infection over the time until lysis. It has long been established that host cell physiology affects the productivity of phage infection (9); however, it is not clear to what extent changes in environmental conditions modulate host cell susceptibility to phage infection.

Bacteria rapidly respond to their environment

Bacterial foodborne pathogens not only have to survive in a food matrix, but most must also survive in the human host where they may then establish an infection. To do this, the cell utilizes mechanisms of signal transduction to sense changes in the

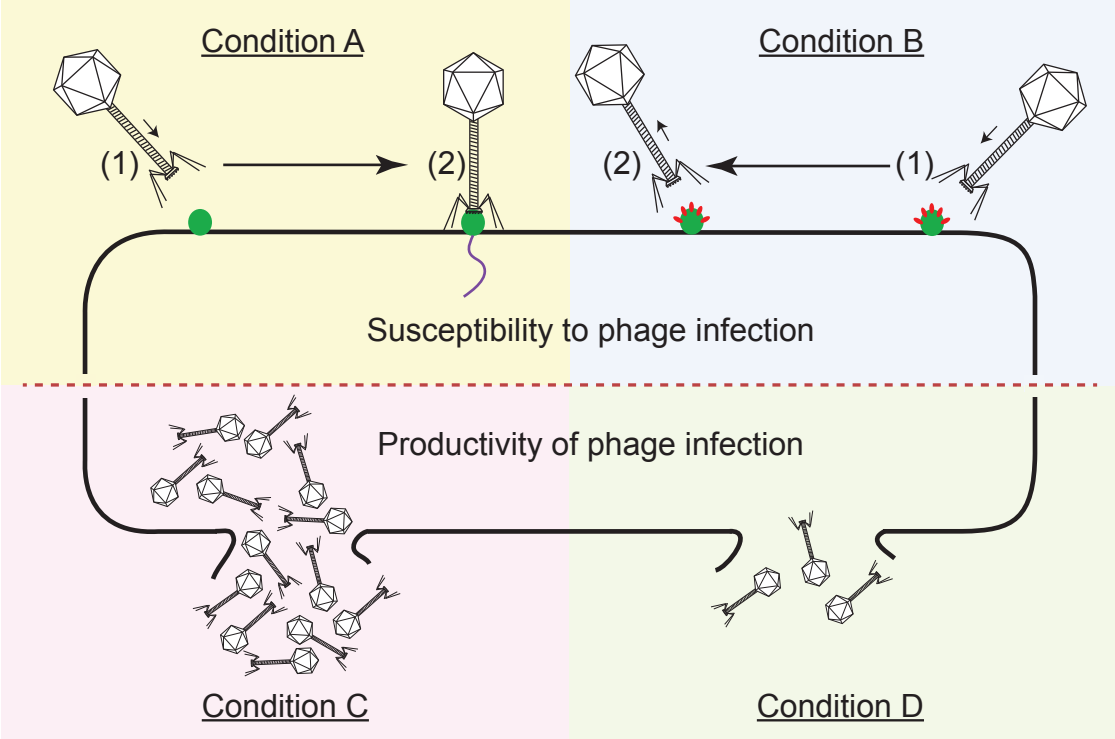

Figure 1.1 Susceptibility to phage infection and/or productivity of phage infection can be affected by environmental conditions. The dotted red line separates a hypothetical host-cell that is conditionally susceptible to phage infection (with higher susceptibility under condition A as compared to condition B) from an infected cell that produces more or less progeny phage under different conditions (with the cell supporting production of more progeny under condition C as compared to condition D). Green ovals represent phage receptors, and red ovals represent structural modifications.

extracellular environment and initiate a cellular response; such mechanisms include (i) one-component systems, which are a single protein fusion of an environment-sensing input domain and a cell-response-initiating output domain (10), (ii) two-component systems, which have input and output domains that are encoded by separate genes, (iii) extracytoplasmic functioning (ECF) sigma factors, which resemble two-component systems, except that stimulation of the input protein leads to uncoupling and activation of a sigma factor (11), and (iv) the more complex chemotaxis systems which enable bacteria to move across a chemical gradient (12). Bacterial cells can also respond to some environmental changes through internal sensing mechanisms such as thiol-based redox switches and temperature-dependent mRNA structures, both of which can affect gene expression (13, 14). Changes in the environment that require the rapid induction of large sets of genes often lead to a cellular response involving activation of alternative sigma factors. Once activated, alternative sigma factors can associate with RNA polymerase and redirect transcription to sets of genes that share a common promoter sequence (15). After altering global gene expression, the cellular response ultimately leads to physiological changes that enhance the cell's growth or survival under the new conditions.

Environmental conditions impact control strategies and detection of foodborne pathogens

Environmental conditions, and their effect on cell physiology, can impact the efficacy of bacterial foodborne pathogen detection and control strategies. Detection assays that involve reactions with the surface of the pathogen, for example, antibody-

based systems, have been shown to vary in efficacy when *L. monocytogenes*, *Salmonella enterica*, and *Escherichia coli* are exposed to different conditions (16). For example, *E. coli* pre-exposed to low (3.5) or high (9) pH had been shown to produce a much smaller signal when detected with a bead-based immunoassay (17). Similarly, the efficacies of some disinfectants are affected by growth conditions that influence surface charge and hydrophobicity of L. monocytogenes (18). The bactericidal activities of a chlorine sanitizer and a 2% lactic acid solution were significantly reduced when treating sliced, versus unsliced, apples contaminated with *E. coli* and *Salmonella* (19).

Environmental conditions and host state physiology can affect susceptibility to phage infection

It is well established that the structure and composition of the bacterial cell surface is in a constant state of flux (20, 21). The first step of phage infection requires phage recognition of a specific surface receptor; however, it is not well understood how changes in host physiology may affect the cell's susceptibility to phage infection. As an example, components of wall teichoic acids (WTA) and acetyl groups in peptidoglycan are common phage recep- tors amongst Gram-positive organisms (22) and both composition and structure of WTA and peptidoglycan are known to be affected by a changing environment (23). Although the impact of this conditional regulation on the cell's susceptibility to phage remains to be defined for Gram-positive species, condition-dependent expression of surface proteins that function as phage receptors in Gram-negative organisms has been shown to affect the cell's

susceptibility to phage infection. For example, when E. coli is exposed to the quorum-sensing signal N-acyl-L-homoserine (AHL), the phage λ receptor, LamB, is down-regulated, resulting in a decreased phage adsorption rate and increased chance of survival during co-incubation with virulent λ. Additionally, phage χ, which adsorbs to the flagellum, was shown to bind less efficiently to AHL exposed E. coli (24). Genes involved in cellular regulation have also been linked to phage susceptibility. A Vibrio cholera strain that is resistant to phage JSF9 became susceptible when transposon insertion disrupted adenylate cyclase or cyclic AMP (cAMP) receptor protein encoding genes. JSF9 was shown to adsorb to the transposon insertion mutant but not the WT strain, indicating cAMP signaling was likely regulat- ing the cell surface in such a way as to prevent JSF9 from binding its receptor. As cAMP is an important signaling molecule among prokaryotes (25), these observations raise the question of whether cAMP may also be regulating phage susceptibility in other organisms.

After adsorption of phage to the host cell, there are still many mechanisms by which the cell can resist phage infection; examples include CRISPR-Cas mediated resistance, abortive infections, and restriction modification (26). One type II restriction-modification system, found in L. monocytogenes epidemic clone II strains, was shown to be efficiently expressed at growth temperatures of 25 °C and below but not at higher temperatures (27). The strains harboring this restriction-modification system were broadly resistant to phage infection at 25 °C, but susceptible at higher temperatures (28).

Environmental conditions and host state physiology affects productivity of phage infection

Productivity of phage infection has been shown to correlate with host growth rate; for example, E. coli phage T4 production is higher under conditions that promote faster growth for the host. T4 adsorption rate was also shown to increase under these conditions; however, this increase correlated with an increase in the surface area of the host cell (29) and can thus be attributed to the greater likelihood of phage–host collision. Also, starvation of phage-infected E. coli has been shown to inhibit phage development for T4, lytic l, and P1; phage production could be restored by reintroduction of a carbon source into the media (30, 31). Pseudo- monas-infecting phages ACQ and UT1 were able to proceed through the infection cycle when infecting stationary phase cells, but showed a reduced burst size and extended latent period as compared to infection of log phase cells; however, phage BLB was unable to reproduce in stationary phase cells (32). A more recent study evaluated the frequency of attached phages and the abundance of intracellular phages among bacterioplankton that were sorted into different physiological groups. The authors found no effect of host physiological state on adsorption; however, there was significantly more phage production found amongst cells with high nucleic acid content (indicative of log phase growth) than amongst those with low nucleic acid content or compromised membranes (33).

Conclusions

Bacteriophage-based applications hold great promise in food safety

applications; however, selecting phages for use in products on the basis of their infective potential under laboratory conditions may not be an ideal approach. Bacterial foodborne pathogens in food matrices and the food processing environment will rarely be found in exponential growth, and will often be severely stressed or injured. As these conditions are known to affect the structure and composition of the cell's surface, it is important to understand how food-associated environmental conditions may impact the adsorption efficiency of phages used in a product. Adsorption of phage to target is an essential step for any phage-based application; it is therefore crucial that phage selected for use in products efficiently bind to their target under all relevant conditions.

We must also consider how host physiological state can affect the productivity of phage infection. Translational reporter-based phage detection systems are especially dependent on a productive infection following adsorption; the greater the expression of the reporter molecule, the greater the assay's detection limit. A productive infection can also benefit phage-based biocontrol products, particularly in cases where replication of the phage in the environment is necessary for adequate dissemination, for example, biocontrols that target biofilms (34).

It may be impractical to evaluate candidate phages under all the conditions under which a product needs to function, but with some basic understanding of a phage's biology one can make useful predictions. For example, if it is known that a phage infects *L. monocytogenes* using a receptor located on the flagellum, as does PBS1 in *Bacillus subtilis* (35), that phage will likely perform poorly at 37 °C as expression of flagellar motility genes is repressed at this temperature (36). Similarly, if an *E. coli*

phage binds to TolC, it is likely that this phage would perform best in nutrient rich environments where TolC expression is highest (37). For phages that adsorb to either lipopolysaccharides (LPS) or wall teichoic acids (WTA), it is important to consider how composition and structure of these surface features can vary depending on the physiological state of the cell. For example we know the extracellular concentration of Mg^{2+} can influence LPS modification in Gram-negative bacteria (38), and cell envelope stress leads to an increased rate of D-alanine incorporation in WTA amongst Gram-positive species (39, 40).

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CHAPTER 2

SELECTION AND CHARACTERIZATION OF PHAGE-RESISTANT MUTANT STRAINS OF *LISTERIA MONOCYTOGENES* REVEAL HOST GENES LINKED TO PHAGE ADSORPTION

Authors

Thomas Denes, Henk C. den Bakker, Jeffrey I. Tokman, Claudia Guldimann, and Martin Wiedmann.

Source

Abstract

Listeria-infecting phages are readily isolated from *Listeria*-containing environments, yet little is known about the selective forces they exert on their host. Here, we identified that two virulent phages, LP-048 and LP-125, adsorb to the surface of *Listeria monocytogenes* strain 10403S through different mechanisms. We isolated and sequenced, using whole genome sequencing, 69 spontaneous mutant strains of 10403S that were resistant to either one or both phages. Mutations from 56 phage-resistant mutant strains with only a single mutation mapped to 10 genes representing five loci on the 10403S chromosome. An additional 12 mutant strains

showed two mutations, and one mutant strain showed three mutations. Two of the loci, containing seven of the genes, accumulated the majority (n=64) of the mutations. A representative mutant strain for each of the 10 genes was shown to resist phage infection through mechanisms of adsorption inhibition. Complementation of mutant strains with the associated wild-type allele was able to rescue phage susceptibility for six out of the 10 representative mutant strains. Wheat germ agglutinin, which specifically binds to N-acetylglucosamine, bound to 10403S and mutant strains resistant to LP-048, but did not bind to mutant strains resistant to only LP-125. We conclude that mutant strains resistant to only LP-125 lack terminal N-acetylglucosamine in their wall teichoic acid (WTA), whereas mutant strains resistant to both phages have disruptive mutations in their rhamnose biosynthesis operon, but still possess N-acetylglucosamine in their WTA.

Introduction

Virulent phages have been shown to present a tremendous selective pressure on their bacterial host populations. Not only is phage predation a major driver of bacterial diversification (1, 2), but it may also select for hypermutators, which could increase the frequency of mutations in bacterial populations (3, 4). Whereas bacteria are limited to one cell division per generation, a single phage-infected cell can produce a burst ranging from less than five to over 1,000 progeny phages in a similar period of time (5-7). Phages consequently have the capability to rapidly outgrow their bacterial hosts and can significantly reduce or eliminate susceptible bacteria in the local environment (8, 9). Therefore, the potential for bacterial strains to persist in an

environment containing lytic phages may be contingent upon that strain accumulating spontaneous mutations that grant resistance to phage infection (10). These phage-resistant mutant strains most typically resist phage infection through mechanisms of adsorption inhibition, i.e., alterations of the cell surface that affect phage attachment (11). However, one study reported that nearly all phage-resistant mutant strains of *Streptococcus thermophilus* had acquired CRISPR spacers that matched invading phage genomes (12); these S. thermophiles mutant strains would be expected to resist phage infection after the adsorption step. Phage-resistant mutant strains that resist infection through mechanisms of adsorption inhibition have been well-characterized at the genomic level for Gram-negative bacteria (13-16); however, fewer studies address the genetics of adsorption inhibiting phage-resistant mutant strains of Gram-positive bacteria (17).

Listeria monocytogenes is a Gram-positive bacterial foodborne pathogen that causes the potentially severe disease listeriosis (18). In the U.S., an annual 1,445 hospitalizations and 255 deaths are attributed to *L. monocytogenes* (19), with an estimated negative economic impact at over $2.5 billion (20). One strategy that is being explored to control *L. monocytogenes* in food and food processing environments is to exploit lytic phages as agents to kill off contaminant Listeria (21-23). However, it has been shown that *Listeria* populations treated with phages can give rise to phage-resistant mutant strains that can grow in the presence of the applied phages (24, 25). To our knowledge, no study to-date has characterized these *Listeria* mutant strains beyond determining their sensitivity to phage infection.

Listeria phages have been readily isolated from environmental sources, including dairy silage (26, 27), sewage effluent (28), sheep feces (29), and food processing plants (24, 30, 31). Currently characterized *Listeria* phages are all members of the order Caudovirales, i.e. tailed phages, and can be organized into evolutionarily conserved groups based on morphology and genome composition (32). The host ranges of *Listeria* phages have been shown to often correspond to host serotype (26, 31, 33). For example, A118 has been reported as a predominantly serotype 1/2-infecting phage, and A500 has been reported as a predominantly serotype 4b-infecting phage (33). Differences between the serotypes of *Listeria* can be attributed to the composition of wall teichoic acids (WTA; cell surface polysaccharides); serotype 1/2 strains are decorated with terminal rhamnose and N-acetylglucosamine (GlcNAc) residues, whereas 4b strains are decorated with terminal glucose and galactose residues (34). *Listeria* phages that have been evaluated for use as biocontrol agents nearly all belong to the genus Twortlikevirus of the family Myoviridae (25, 29, 35). Two *Listeria*-infecting twortlikeviruses, LP-048 and LP-125, share a very high nucleotide identity (~97% average nucleotide identity across 93% of their genomes) (32) and display broad, yet different, host ranges against a panel of *L. monocytogenes* isolates representing a diversity of lineages and serotypes (26). As reported here, follow-up characterization of these two *Listeria* phages revealed very different rates of adsorption. We hypothesized that these closely related phages attach to their hosts through different mechanisms. Thus, we selected and characterized, using whole genome sequencing, L. monocytogenes mutant strains resistant to phages LP-048

and/or LP-125 to identify different absorption mechanisms and phage-host interactions that are associated with these two twortlikeviruses.

Materials and methods

Growth conditions. Bacterial strains were grown overnight (16 ± 2 h) on BHI (Becton, Dickinson and Company, Franklin Lakes, NJ) agar at 37ºC or in BHI broth with aeration (210 RPM) at the indicated temperature. Strains were stored at -80ºC in BHI broth containing 15% glycerol. Strains used in this study can be found in Table 1 and Supplemental Table 1.

Phage lysates were prepared as previously described (26) and stored in the dark at 4ºC. Phage enumeration was conducted after serial dilution with SM buffer (100 mM NaCl, 8 mM MgSO4•7H20, 0.002% [w/v] gelatin, and 50 mM Tris-Cl adjusted to a pH of 7.5) followed by a double agar overlay plaque assay (36) using modified LB MOPS (LB medium buffered with 50 mM MOPS at a pH of 7.6) as previously described (26). Briefly, agar overlays were made with 0.7% (w/v) LB MOPS agar supplemented to give final concentrations of 0.1% (w/v) glucose, and 10mM of each $MgCl_2$ and $CaCl_2$; agar underlays were made with 1.5% (w/v) LB MOPS also supplemented with glucose and salts. Plated phage samples were incubated at 30ºC for 16 ± 2 h. Phages used in this study can be found in Table 2.1.

One-step growth experiments. In order to determine the growth kinetics of phages LP-048 and LP-125, standard one-step growth experiments were performed (37). A 5-mL liquid culture of *L. monocytogenes* was grown in LB MOPS to an OD_{600} of 0.1 and then supplemented with 50 µl of each 1M $CaCl_2$ and 1M $MgCl_2$. Following

Table 2.1 Strains, phages, and phage susceptibility.

[a]Specific location of the mutations in the phage resistant mutant strains listed can be found in Supplemental Table 2.1.

[b]Strong lysis (++), weak lysis (+), or no lysis (-) was observed between the indicated strain and phage over three replicate experiments. Only minor variation was observed between replicates.

[c]WGA (wheat germ agglutinin) binding (+), or lack of binding (-), was determined as shown in Fig. 4. Not all strains were tested (indicated by NT)

Strain or phage	Description[a]	Phage used to select mutant	Phage sensitivity[b]		WGA binding[c]
			LP-048	LP-125	
L. monocytogenes strains					
10403S	Lineage II, serotype 1/2a		++	++	+
FSL D4-0014	10403S, nonsense mutation in LMRG_00541	LP-125	++	−	−
FSL D4-0161	FSL D4-0014::pTD01 (pPL2::LMRG_00541)		++	++	+
FSL D4-0119	10403S, nonsense mutation in LMRG_00542	LP-048	−	−	+
FSL D4-0156	FSL D4-0119::pTD02 (pPL2::LMRG_00542)		++	++	NT
FSL D4-0118	10403S, nonsense mutation in LMRG_00543	LP-048	−	−	+
FSL D4-0160	FSL D4-0118::pTD11 (pPL2::LMRG_00543 with LMRG_00542 promoter)		−	−	NT
FSL D4-0126	10403S, nonsense mutation in LMRG_00545	LP-048	−	−	+
FSL D4-0155	FSL D4-0126::pTD08 (pPL2::LMRG_00545 with LMRG_00542 promoter)		+	++	NT
FSL D4-0028	10403S, missense mutation in LMRG_00546 (amino acid change of Thr to Ile)	LP-048	−	++	+
FSL D4-0158	FSL D4-0028::pTD09 (pPL2::LMRG_00546 with LMRG_00542 promoter)		++	++	NT
FSL D4-0082	10403S, missense mutation in LMRG_01009 (amino acid change of Pro to Gln)	LP-125	++	−	−
FSL D4-0159	FSL D4-0082::pTD10 (pPL2::LMRG_01009 with LMRG_01010 promoter)		++	−	−
FSL D4-0057	10403S, missense mutation in LMRG_01319 (amino acid change of Asn to Thr)	LP-125	++	−	−
FSL D4-0153	FSL D4-0057::pTD03 (pPL2::LMRG_01319)		++	−	−
FSL D4-0068	10403S, nonsense mutation in LMRG_01697	LP-125	++	−	−
FSL D4-0154	FSL D4-0068::pTD05 (pPL2::LMRG_01697)		++	++	+
FSL D4-0065	10403S, nonsense mutation in LMRG_01698	LP-125	++	−	−
FSL D4-0162	FSL D4-0065::pTD06 (pPL2::LMRG_01698 operon)		++	++	+
FSL D4-0087	10403S, missense mutation in LMRG_01709 (amino acid change of Ile to Met)	LP-125	++	−	−
FSL D4-0163	FSL D4-0087::pTD07 (pPL2::LMRG_01709)		++	−	−
FSL R9-0915	Serotype 7		−	−	−
Phages					
LP-048	Twortlike *Listeria* phage, shown to infect serotype 1/2, 4a, 4b, and 4c strains (24, 26, 32)				
LP-125	Twortlike *Listeria* phage, shown to infect serotype 1/2, 3a, 3b, 4a, and 4b strains (24, 26, 32)				

that, $1x10^8$ PFU of the appropriate phage was added to the culture (MOI of ~0.1). The infected culture was incubated in a water bath at 30ºC with aeration. At each time point, two samples were taken; one 100 µl sample was transferred into a tube containing several drops of chloroform and the other sample was immediately diluted and enumerated (yielding the concentration of infected cells and free viable phages), using 10403S as the titering host. At the end of the growth experiment chloroformed phage samples were enumerated (yielding the total concentration of viable phage particles in the sample, including intracellular phages). The average burst size was calculated by dividing the average concentration of infected cells and free viable phages from the three time points following the first step of lysis (time points 90 min, 100 min, and 110 min for LP-048 and time points 80 min, 90 min, and 100 min for LP-0125) by the average concentration of infected cells and free viable phage from the first three time points post-infection (as described by Hyman et al. (38)).

Isolation of phage-resistant mutant strains. Individual colonies of *L. monocytogenes* 10403S were used to inoculate BHI broth. The liquid cultures were incubated overnight at 30ºC and then each culture was diluted 1:100 into 5 mL of fresh BHI and further incubated until an OD_{600} of 0.85 was reached. Following that, 50 µl of filter-sterilized 1M $CaCl_2$ and 1M $MgCl_2$, and $1x10^8$ PFU of phage were added to each culture. After an additional incubation of 24 h, one sample from each infected culture was plated on BHI agar. A single colony was isolated from each plate, those confirmed to resist phage infection by spot assay (described below) were stored as “phage-resistant mutant strains.” All phage-resistant mutant strains were subsequently grown directly from freezer stocks as liquid cultures in order to reduce

the number of cell divisions and thus the likelihood of a mutation reverting a phage resistant phenotype prior to an experiment.

DNA extraction, sequencing, and bioinformatics. DNA was extracted from *Listeria* using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany). The manufacturer's recommended protocol for DNA extraction from Gram-positive bacteria was followed with the addition of an RNase treatment. After incubation with Proteinase K and prior to addition of buffer AL, 4 µl of RNase A (100 mg/ml; Qiagen) was added to each sample, followed by incubation at 37ºC for 10 min. Genomic DNA was submitted to the Cornell University Life Science Core Laboratory Facilities where library preparation and DNA sequencing was performed. A Nextera XT DNA Sample Preparation Kit (Illumina Inc., San Diego, CA) was used to prepare the library, and 100 base-pair reads were obtained by sequencing of the library on the Illumina HiSeq 2500 platform. Single nucleotide polymorphisms (SNPs) were called using both a reference based and de novo variant detection method. For the reference based method, reads were mapped against the genome sequence of *L. monocytogenes* 10403S (GenBank accession NC_017544.1) with BWA version 0.7.3 (39) using the BWA-MEM algorithm. SNPs were called using VarScan version 2.3.4 (40). Only SNPs with a minimal coverage of 50% of the genome-wide average coverage (GAC), a minimal variant coverage of 50% of the GAC, a minimum alternative variant frequency of 95%, and a p value ≤ 0.01 were considered for further analyses. Cortex_var version 1.0.5.14 (41, 42) was used for the de novo variant detection (both SNP and insertion/deletion events) as outlined by Den Bakker et al. (43).

L. monocytogenes 10403S is not known to harbor any plasmids; de novo assembly of sequencing reads obtained from this study further confirmed this. The raw sequencing reads generated in this study have been deposited in the Sequencing Read Archive with the following BioProject ID: PRJNA261154.

Strain construction. Integration plasmids for complementing phage-resistant mutant strains were constructed by cloning the desired WT open reading frame (ORF), and the desired promoter and 5' untranslated region (UTR) into the multiple cloning site of pPL2 (44). Constructs with promoters and 5'UTRs fused to a downstream ORF were created using splicing by overlap extension (SOE) PCR (45). PCRs for cloning were carried out using Q5 DNA polymerase (New England BioLabs, Ipswich, MA). PCRs for Sanger sequencing were carried out with GoTaq Flexi DNA polymerase obtained from Promega (Madison, WI). Restriction enzymes (BamHI, SalI, and NotI) and ligase (T4 DNA Ligase) used for cloning were obtained from New England BioLabs. Plasmid constructs were first replicated in NEB 5-alpha *E. coli* (New England BioLabs), then extracted with a Plasmid Midi Kit (Qiagen) and confirmed by Sanger sequencing at the Cornell University Life Science Core Laboratory Facilities (Ithaca, NY). The constructs were then transferred into *L. monocytogenes* by either conjugation with *E. coli* SM10 and selection of streptomycin and chloramphenicol resistant colonies or by electroporation (46). Constructed strains are shown in Table 1.

Spot tests and adsorption assays. Spot tests of both LP-048 and LP-125 were conducted, as three independent replicates, on bacterial strains to determine the strains susceptibility to phage-infection. Five μl of phage lysate at 1 x 10^8 PFU/mL was

spotted in duplicate, on duplicate lawns, and then incubated at 30ºC for 16 ± 2 h. Spots were then evaluated for strong lysis (++), some lysis (+), or no lysis (-).

Adsorption of LP-048 and LP-125 to bacteria was determined by enumeration of viable phages that fail to adsorb to the test bacteria after co-incubation. Fifty µl volumes of bacterial culture grown at 30ºC for 16 h (OD_{600} values ranged from 1.4 to 1.7) were transferred into centrifuge tubes containing 912 µL BHI broth, 20 µL of phage lysate at $1x10^9$ PFU/mL, 9 µL of 1M $CaCl_2$, and 9 µL of 1M $MgCl_2$ (salts were added immediately prior to the addition of bacteria). The bacteria and phage mixtures were incubated for 15 min at 30ºC with aeration. Following that, bacteria and any adsorbed phages were sedimented by centrifugation at 17,000 g for 1 min in an Eppendorf Micro centrifuge 5417C (Hamburg, Germany). The supernatants were then filtered through 0.2 µm SFCA syringe filters (Thermo Fisher Scientific, Waltham, MA). Viable phages left in the filtrates were enumerated. The % adsorption was defined as the loss of phages (%) from each sample, after co-incubation with bacteria, centrifugation, and filtration, as compared to the sample with the greatest concentration of that respective phage remaining in the filtrate; these samples with the highest concentration were set as 0% adsorption for the respective phage in the respective replicate experiment (these samples were not always the BHI control). One-way analysis of variance (ANOVA) was used to analyze the effect of strain (WT 10403S, Phage-resistant mutant strains, FSL R9-0915, and the BHI control were included in the analysis) on phage adsorption, and a Dunnett post-hoc test ($\alpha = 0.05$) was used to identify significant differences in adsorption % between WT 10403S and the mutant strains and controls. In order to identify if the complemented mutants

showed partially restored phage adsorption, a *t-test* (assuming unequal variances; α = 0.05) was performed between each mutant strain (that showed significantly different phage adsorption from WT) and the respective complemented mutant. All statistical analyses of phage adsorption were performed separately for LP-048 and LP-125 with JMP statistical software (JMP11, SAS Institute, Inc., Cary, NC).

Wheat germ agglutinin binding assay. A wheat germ agglutinin (WGA) Alexa Fluor® 488 conjugate (Life Technologies, Carlsbad, CA) was used to detect the binding of WGA to *Listeria*. To fix cells, 17 µL of 16% (w/v), methanol-free, formaldehyde solution (Thermo Scientific) was added to 50 µL of an overnight *Listeria* culture, followed by incubation at room temperature for 15 min. Cells were then sedimented by centrifugation at 2,655 g for 5 min in an Eppendorf Microcentrifuge 5417C and resuspended in 100 µL of phosphate-buffered saline (PBS). The suspension was then mixed with 1 µL of WGA- Alexa Fluor® 488 conjugate (1mg/mL) and incubated for 15 min at room temperature. The samples were then sedimented again (same conditions) and resuspended in 100 µL of PBS. Bacterial cells were then mounted on glass slides and imaged on a confocal laser scanning microscope (Carl Zeiss, Peabody, MA).

Results

One-step growth curves reveal different adsorption rates for LP-048 and LP-125. To determine differences in infection kinetics of LP-048 and LP-125, one step growth curves were performed on the serotype 1/2a *L. monocytogenes* strain 10403S (Figure 2.1). The most striking difference between the two phages was

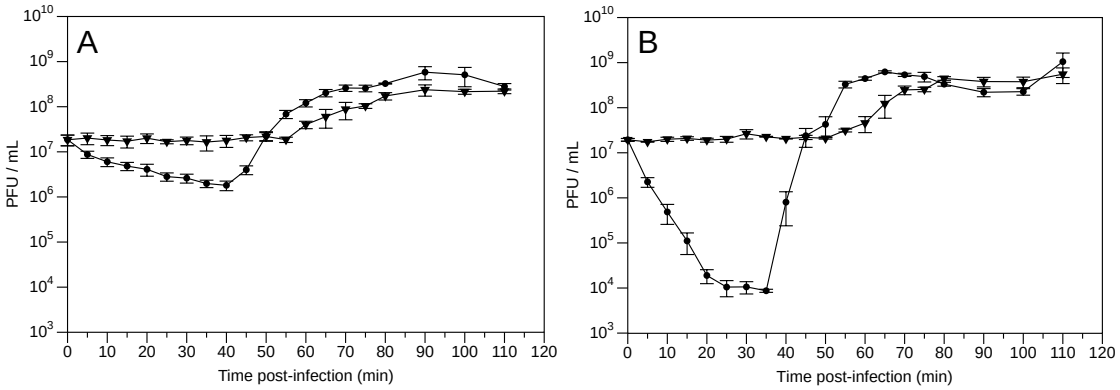


Figure 2.1 One-step growth experiments of *Listeria* phages (A) LP-048 and (B) LP-125. Triangles are values for samples that were directly plated, representing the cumulative concentration of infected host cells and unadsorbed viable phages. Circles are values for samples that were treated with chloroform prior to plating, representing the total concentration of viable phages (including intracellular phage). Time point 0 values represent the theoretical input of phage, which was calculated by averaging values from the first three time points of directly plated samples. Initial drop in titer of chloroformed samples indicate adsorption of phage to bacteria. All values are the arithmetic mean of three independent experiments and error bars display the standard error.

observed in their adsorption rates. Whereas, after 20 min of co-incubation with host bacteria 78.2% (2.2% standard error [SE]) of LP-048 adsorbed to the host bacteria (Fig. 1a), 99.9% (0.0% SE) of LP-125 adsorbed to the host in the same period of time (Figure 2.1b); this indicates a less efficient adsorption of LP-048 to 10403S under these conditions. Possible explanations for this difference could be different concentrations of the available receptors for the two phages or differences in the affinity of the receptors for the two phages; these explanations are consistent with observations of phage lambda adsorption under varying receptor concentrations and receptor affinities (47). The eclipse period, defined as the period of time taken for the first viable phage particles to mature post-infection, was between 40 and 45 min for LP-048 and 35 to 40 min for LP-125. The latent period, defined as the time taken for the infected cell to lyse post-infection, was between 55 and 60 min for LP-048 and between 50 and 55 min for LP-125. The average burst size, defined as the average number of phage particles produced per infected cell, was 13.6 (SE 3.1) for LP-048 and 21.3 (SE 4.5) for LP-125.

Whole genome sequencing of phage-resistant mutant strains reveals host-genes essential for phage infection. Phage-resistant mutant strains derived from *L. monocytogenes* 10403S were selected for by either confrontation with LP-048 or LP-125. Out of a total of 110 confrontations, 95 resulted in the isolation of phage-resistant mutant strains. These mutant strains were later screened by spot assay, which confirmed them as true phage-resistant mutant strains. Sixty-nine phage-resistant mutant strains, as well as the parent strain 10403S, were sequenced on the Illumina HiSeq platform. Mutations that were detected in sequenced strains were mapped

against the 10403S reference genome (Figure 2.2a). A total of 83 mutations were identified; three mutations were each found in two separate mutant strains (see supplemental Table 2.1). Therefore, 80 unique mutations were identified. Fifty-six mutant strains showed a single mutation, 12 mutant strains showed two mutations, and one mutant strain showed three mutations. Mutations from phage-resistant mutant strains with only a single mutation were surmised to be the mutations most likely to cause a phage-resistant phenotype; these were termed “mutations of interest” (shown in red in Figure 2.2). Out of the 80 unique mutations identified, 67 were found in 10 genes, located in five chromosomal loci (see Table 2.2 for distribution of these mutations among the 10 genes); mutations from all 56 mutant strains with a single mutation were found in these five loci. In addition to these 67 mutations, 13 other mutations were found outside these five loci. All 13 of these mutations were found in mutant strains that contained more than one mutation and were thus not further characterized. These 13 mutations included five synonymous substitutions (shown in green in Figure 2.2a) and 7 other mutations (shown in blue in Figure 2.2a) as well as one SNP found in an intergenic region flanked by LMRG_01577 and LMRG_01588 (shown in green in Figure 2.2a; see Supplemental Table 2.1 for details on these mutations).

A representative mutant strain for each of the ten genes containing “mutations of interest” was selected for further characterization; if possible the mutant strain containing the most upstream nonsense mutation in the gene of interest was selected as representative (see Table 2.2). When these 10 representative mutant strains were characterized for phage susceptibility by spot assays, all mutant strains were found to

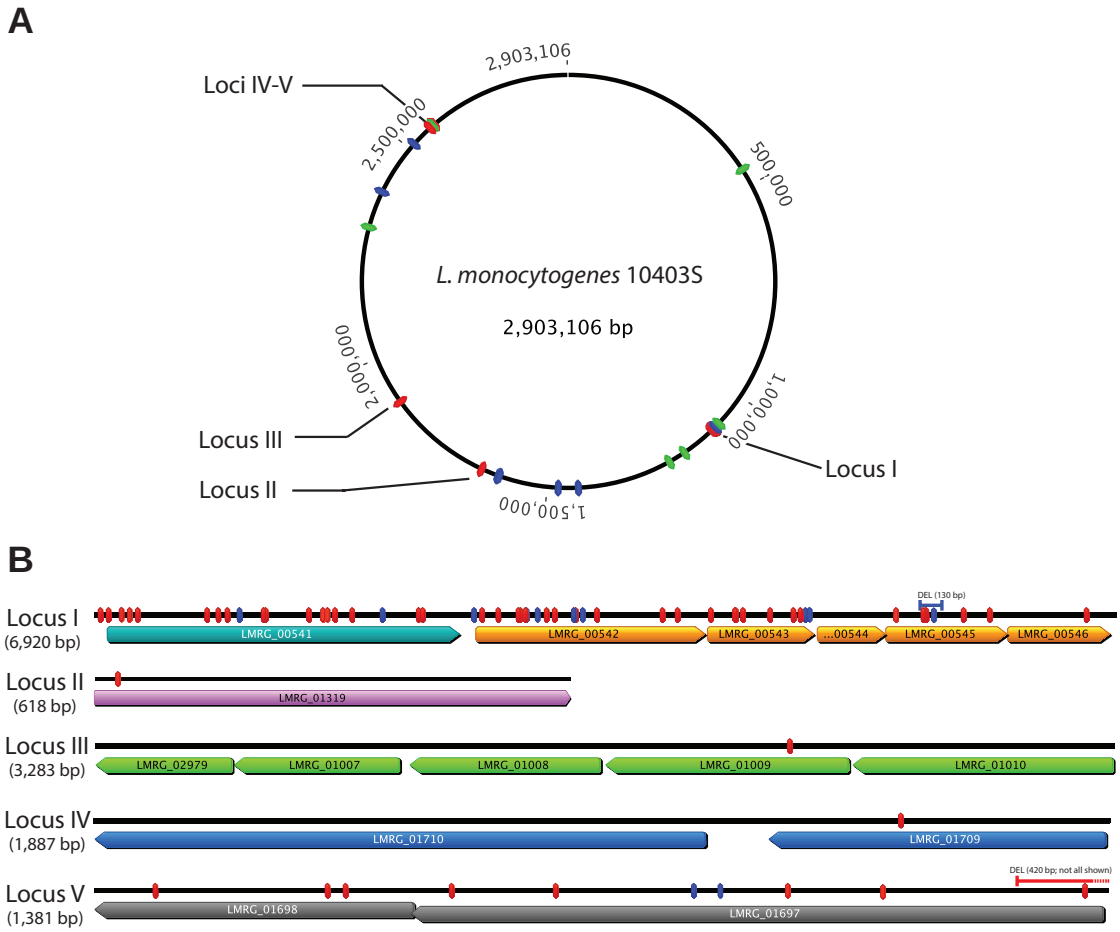


Figure 2.3 Mutations from phage-resistant mutant strains mapped against (A) the 10403S reference genome and (B) key loci on the 10403S chromosome. Individual spontaneous mutations are identified as colored ovals. Red ovals represent mutations in strains with only a single mutation. Blue ovals represent nonsense mutations, missense mutations, frameshift mutations, or mutations in regulatory DNA sequences, from mutant strains with more than one mutation. Green ovals represent synonymous mutations (no change in amino acid sequence) or a SNP in a featureless intergenic region from a mutant strain with more than one mutation.

TABLE 2.2 Genes with mutations in phage-resistant *L. monocytogenes*

				No. of unique mutations[c]						
					Mutation type[d]				Selected by:	
Gene[a]	EGDe homolog	Locus[b]	Function	Total	FS	MS	NS	O	LP-048	LP-125
LMRG_00541	lmo1079	I	putative membrane protein	19	8	0	8	3	0	19
LMRG_00542	lmo1080	I	putative glycosyltransferase (GT-A type)	16	2	9	4	1	13	3
LMRG_00543	lmo1081	I	glucose-1-phosphate thymidylyltransferase (RmlA)	9	3	4	2	0	7	2
LMRG_00545	lmo1083	I	dTDP-glucose 4,6-dehydratase (RmlB)	8	0	6	1	1	6	2
LMRG_00546	lmo1084	I	dTDP-4-dehydrorhamnose reductase (RmlD)	1	0	1	0	0	1	0
LMRG_01319	lmo1647	II	1-acyl-sn-glycerol-3-phosphate acyltransferase	1	0	1	0	0	0	1
LMRG_01009	lmo1862	III	putative lipase/acylhydrolase	1	0	1	0	0	0	1
LMRG_01709	lmo2538	IV	uracil phosphoribosyltransferase	1	0	1	0	1	0	1
LMRG_01697	lmo2550	V	putative glycosyltransferase (family 2)	8	3	3	1	1	0	8
LMRG_01698	lmo2549	V	cell wall teichoic acid glycosylation protein (GtcA-like)	3	0	1	2	0	0	3

[a]Only 10 genes of interest are listed here. A full list of all genes with mutations is included in Supplemental Table 1.

[b]Locus designation in this study (see Fig. 2).

[c]Number of unique mutations found in this study that are present in the listed genes. See Supplemental Table 1 for mutation-specific details.

[d]Frameshift (FS), missense (MS), nonsense (NS), other (O; large insertion or deletion, mutation in promoter, or mutation in 5' UTR).

be resistant to either one or both phages (Table 2.1). Sanger sequencing confirmed the mutations of interest in all 10 mutant strains. Additionally, each representative mutant strain was complemented *in trans* with the wild-type (WT) allele of the respective gene in which the mutation is located; phage susceptibility was at least partially restored in six out of 10 of the complemented mutants (as detailed below).

Two loci accumulated a majority of the unique mutations found in the strains sequenced in this study (64/80). One of these two loci (Locus I) contains six genes, five of which accumulated a total of 53 unique mutations (Figure 2.2b; Table 2.2). LMRG_00541, the first gene in the locus, encodes a putative membrane protein and makes up a one-gene operon that accumulated 19 unique mutations identified here (Table 2.2); all 19 mutations in LMRG_00541 were selected for in the presence of LP-125. Eight of the mutations in LMRG_00541 were single base-pair deletion frameshift mutations, four of which were likely phase variants as the deletions occurred in homopolymeric tracts (≥6 bp in length) of adenine or thymine. One of these putative phase variants, at base position 1,095,105, was found in two sequenced strains (see Supplemental Table 2.1). Another putative phase variant that was also found in two sequenced strains was located between the -10 and -35 promoter signals of LMRG_00541 (Supplemental Table 2.1). This mutation was also a single nucleotide deletion in a homopolymeric tract. This deletion reduced the gap between promoter signals from 17 nucleotides to 16 nucleotides, which could affect transcription of the operon. The representative mutant strain for LMRG_00541, FSL D4-0014, showed resistance to LP-125 but remained susceptible to LP-048; complementation of the

mutation *in trans* with the WT allele of LMRG_00541 restored susceptibility of the mutant strain to LP-125 (Table 2.1).

The second operon in Locus I (Figure 2.2b) contains five genes, four of which accumulated a total of 34 unique mutations identified here (Table 2.2). LMRG_00542 encodes a putative GT-A type glycosyltransferase and accumulated a total of 16 unique mutations, 13 of which were selected for by LP-048. LMRG_00543, LMRG_00545, and LMRG_00546 encode rhamnose biosynthesis enzymes and together accumulated a total of 18 unique mutations, 14 of which were selected for by LP-048 (Table 2.2). One of the mutations, in LMRG_00542 (strain FSL D4-0114), is a likely phase variant as it is a single base-pair deletion in a seven base-pair homopolymeric tract of adenine residues (see supplemental Table 2.1). Whereas the LMRG_00542, LMRG_00543, and LMRG-00545 mutant strains were all shown to be resistant to both LP-048 and LP-125 by spot assay, the LMRG_00546 mutant strain was only resistant to LP-048. Complementation with the respective WT alleles restored phage susceptibility for the LMRG_00542, LMRG_00545, and LMRG_00546 mutant strains (Table 2.1), but not for the LMRG_00543 mutant strain. Failure to successfully complement the LMRG_00543 mutation could be due to a polar effect.

The other locus that accumulated a considerable number of mutations (n= 11), Locus V, represents a two-gene operon that includes LMRG_01697, which encodes a putative glycosyltransferase, and LMRG_01698, which encodes a GtcA-like wall teichoic acid (WTA) glycosylation protein. One frameshift mutation, in LMRG_01697, was identified as a putative phase variant (strain D4-0093); this

mutation consisted of an insertion of two nucleotides ("ta") that extended a dinucleotide tandem repeat from four to five repeats. Another mutation in this locus was identified as a large deletion (431 bp) starting at the end of the flanking gene LMRG_01696 (encoding transcription termination factor Rho) and ending in the beginning of LMRG_01697. Representative mutant strains of both LMRG_01697 and LMRG_01698 were found to be resistant to only LP-125 by spot assay; complementation of mutations in both representative mutant strains with the respective WT alleles restored phage susceptibility (Table 2.1).

Three mutations of interest each mapped to a different locus on the chromosome (Loci II, III, and IV; Figure 2.2), all of these mutations were non-synonymous substitutions (see Table 2.1 for the amino acid substitutions). The three genes with these mutations were LMRG_01009, which encodes a putative lipase/acylhydrolase, LMRG_01319, which encodes a 1-acyl-sn-glycerol-3-phosphate acyltransferase, and LMRG_01709, which encodes a uracil phosphoribosyltransferase (Table 2.2). All three mutant strains were found to be resistant to only LP-125 by spot assay. Complementation of the mutations found in these mutant strains with the respective WT alleles failed to restore phage susceptibility (Table 2.1).

Phage-resistant mutant strains of *L. monocytogenes* resist phage by adsorption inhibition. To determine if LP-048 and LP-125 could adsorb to the phage-resistant mutant strains isolated in this study, adsorption assays were performed for the parent strain, and each representative mutant strain and their respective complemented strains (see Figure 2.3). After a 15 min co-incubation, over 95% of both LP-048 and LP-125 adsorb to WT 10403S; for the phage-resistant mutant strains,

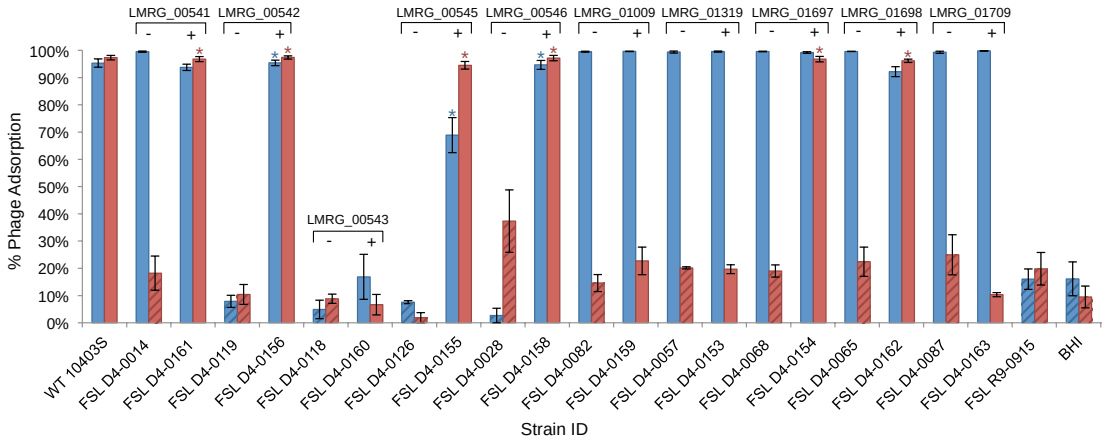


Figure 2.3 Adsorption of phages LP-048 (blue) and LP-125 (red) to the *L. monocytogenes* parent strain 10403S, phage-resistant mutant strains, and complemented mutant strains. Phage-resistant mutant strains and their respective complemented mutants are adjacent to one another, and brackets above the bars are labeled with the affected gene. A (-) sign under the brackets indicates the mutant strain and a (+) sign indicates the complemented mutant strain. Stripes on the mutant strain and negative control strain bars indicate a significant difference in adsorption % from WT 10403S ($p < 0.0001$). An asterisk (*) above a complemented mutant strain bar indicates the complemented mutant showed a significant increase of adsorption % as compared to the phage-resistant mutant strain (only tested for phage-resistant mutant strain and phage combinations that were significantly different from WT 10403S). Values shown are the arithmetic mean of three independent experiments, and error bars display the standard error.

adsorption was severely reduced (Figure 2.3). For example, mutant strains resistant to both LP-048 and LP-125, which have mutations in LMRG_00542, LMRG_00543, and LMRG_00545, showed ≤ 10% adsorption for both phages (Figure 2.3). FSL D4-0028, which has a mutation in LMRG_00546 and is resistant to only LP-048, showed 2.7% (2.7 % SE) adsorption of LP-48 and 37.3% (11.5% SE) adsorption of LP-125. Mutant strains resistant to only LP-125 all showed ≤ 25% adsorption of LP-125 and ≥ 99% adsorption of LP-048 (Figure 2.3). The six complemented mutants that showed restored phage susceptibility by spot assay, with mutations in LMRG_00541, LMRG_0542, LMRG_0545, LMRG_0546, LMRG_01697, and LMRG_01698, also showed restoration of phage adsorption; although FSL D4-0155, which was complemented with a WT LMRG_00545 allele, showed that LP-048 adsorption was only restored to 68.9% (6.5% SE; Figure 2.3).

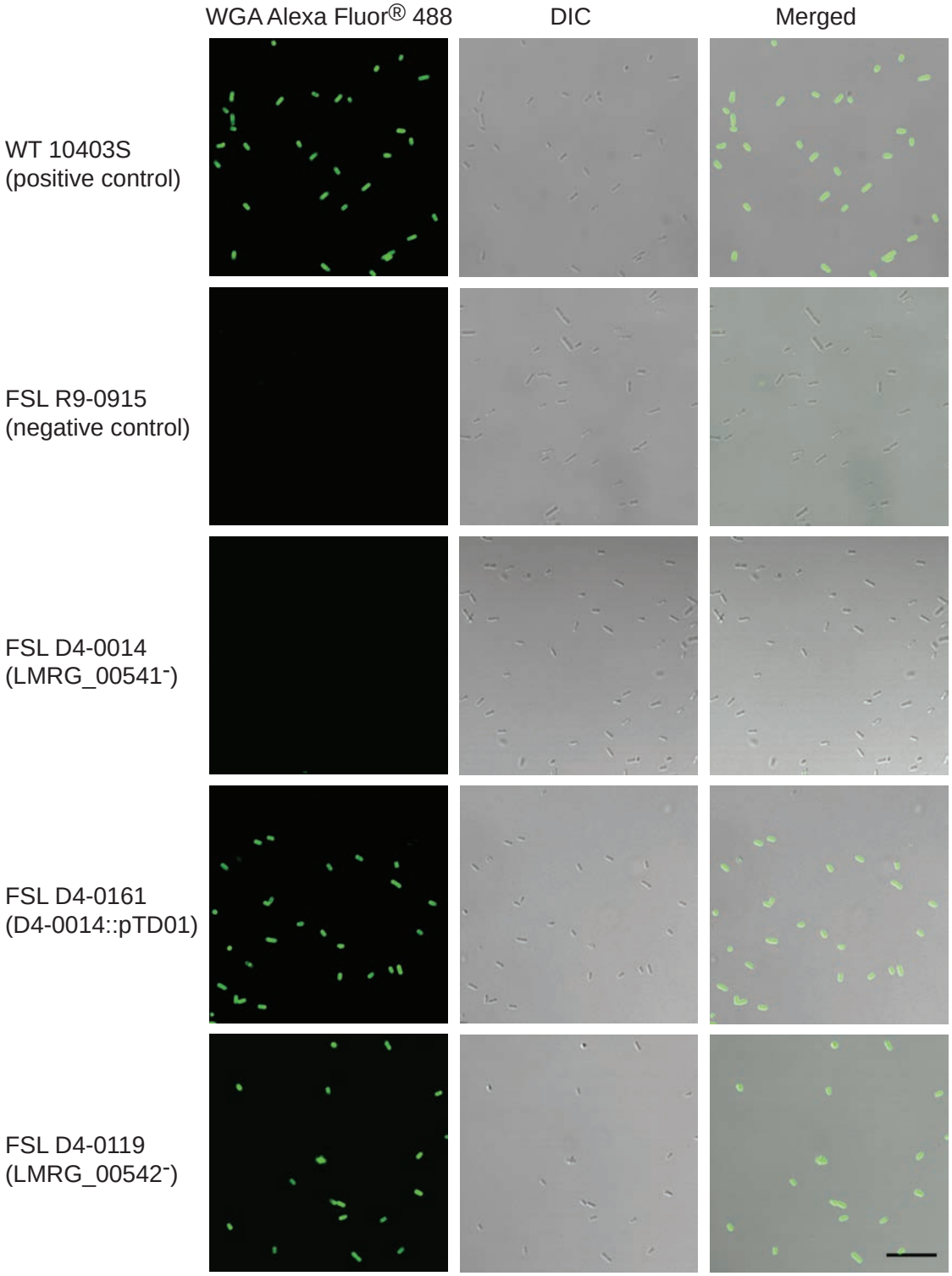
Phage-resistant mutant strains resistant to LP-125 and susceptible to LP-048 lack terminal N-acetylglucosamine in their wall teichoic acid. As LMRG_01697 and LMRG_01698 (lmo2549 and lmo2550 in EGDe) have been previously shown to be necessary for decoration of wall teichoic acid (WTA) with N-acetylglucosamine (GlcNAc) residues (48), we tested the representative phage-resistant mutant strains isolated in this study for the presence of terminal GlcNAc in their WTA. To do this, we determined whether the lectin wheat germ agglutinin (WGA), which specifically binds to GlcNAc, could bind to selected *L. monocytogenes* mutant strains that showed phage resistance. The serotype 7 *L.* monocytogenes strain FSL R9-0915, which was shown to resist infection to both LP-048 and LP-125 by spot assay (see Table 2.1) and was adsorption deficient for both phages (Figure 2.3), was

included as a negative control; a serotype 7 strain was selected as the negative control as they lack any substituents in their WTA polyol phosphate chains (34). As expected, we observed WGA binding to the *L. monocytogenes* parent strain 10403S (see Figure 2.4 and Table 2.1), but not to FSL R9-0915 or the representative LMRG_01697 and LMRG_01698 mutant strains. All six representative mutant strains resistant to LP-125 and susceptible to LP-048 (including the representative LMRG_01697 and LMRG_01698 mutant strains) did not bind WGA (Table 2.1; see Figure 2.4 for example). Mutants complemented with the LMRG_00541, LMRG_01697, and LMRG_01698 WT alleles all showed restoration of WGA binding (Table 2.1, Figure 2.4), whereas the mutants complemented with the LMRG_01009, LMRG_01319, and LMRG_01709 WT alleles did not show restoration of WGA binding, consistent with a lack of restoration of phage sensitivity in these mutant (Table 2.1, Figure 2.3). All four tested representative mutant strains that were resistant to LP-048 did bind WGA, suggesting that they still possess terminal GlcNAc in their WTA (Table 2.1, Figure 2.4).

Discussion

In this study, we fully sequenced an unprecedented 69 phage-resistant mutant strains of *L. monocytogenes* followed by further genetic and phenotypic characterization of selected mutant strains. Our data show that (i) mutations of interest accumulated primarily in two chromosomal loci, and in a total of ten genes; (ii) six genes were conclusively linked to phage adsorption, including three genes conclusively shown to contribute to WTA decoration; and (iii) evidence of phase

Figure 2.4 Binding of wheat germ agglutinin (WGA) Alexa Fluor® 488 conjugate and bacterial cells observed by laser scanning microscopy. Fluorescent images are displayed in column 1 and differential interference contrast (DIC) microscopy images of the same field are shown in column 2. The merged images (column 3) show that either all or none of the cells fluoresce. The black scale bar in the lower-right-hand corner represents a distance of 10 µm. Images are shown for selected strains, all 10 mutant strains and their respective complemented strains were tested.


WGA Alexa Fluor® 488
DIC
Merged
WT 10403S
(positive control)
FSL R9-0915
(negative control)
FSL D4-0014
(LMRG_00541-)
FSL D4-0161
(D4-0014::pTD01)
FSL D4-0119
(LMRG_00542-)

variation was found in three of the genes linked to phage adsorption. Overall, our results provide insight into phage-resistant mutant strains of *L. monocytogenes* and improve our understanding of the evolution of this important pathogen. Our results also will guide future studies needed to further assess the benefits and consequences of using phages as biocontrol agents.

Mutations associated with phage-resistance were found primarily in two loci, which contain key genes linked to phage adsorption. Phage-resistant mutant strains in many different phage-host systems have been shown to resist phage infection through mechanisms of adsorption inhibition (11, 14, 49), as opposed to mechanisms that inhibit phage DNA entry, replication, or escape (50). Similarly, we found that phage-resistant mutant strains of *L. monocytogenes* showed significant reduction in the adsorption of one or both phages used in this study. N-acetylglucosamine (GlcNAc) has been previously characterized as a phage receptor for *L. monocytogenes* (33, 51-53); consistent with these previous observations, we found eight and three mutations, respectively, in the genes LMRG_01697 and LMRG_01698, both of which have been shown to be necessary for glycosylation of terminal GlcNAc in the wall teichoic acid (WTA) of *L. monocytogenes* (54). However, a majority of mutations (n=53) were found in a region not previously linked to phage susceptibility (Locus I). Interestingly, genes within the two operons of Locus I were associated with different phage-resistance phenotypes. A nonsense mutation in LMRG_00541 was shown to affect LP-125 adsorption and glycosylation of WTA with terminal GlcNAc (as supported by WGA binding experiments), and was shown not to affect LP-048 adsorption; the same phenotype was observed in the

LMRG_01697 and LMRG_01698 mutant strains. Mutations in genes from the neighboring operon (i.e., LMRG_00542, LMRG_00543, LMRG_00545 and LMRG_00546; the LMRG_00542 operon hereafter) were all shown to affect both LP-125 and LP-048 adsorption, and did not affect glycosylation of WTA with GlcNAc. Interestingly, the missense mutation found in LMRG_00546 did not affect resistance to LP-125 in spot assay experiments; this mutation also had a lesser effect on the adsorption of LP-125 than other mutations in the operon. The LMRG_00542 operon encodes orthologs of well characterized proteins with a role in rhamnose biosynthesis, such as RmlA (glucose-1-phosphate thymidylyltransferase; orthologous to LMRG_00543) (55), RmlB (dTDP-glucose 4,6-dehydratase; orthologous to LMRG_00545) (56), and RmlD (dTDP-4-dehydrorhamnose reductase; orthologous to LMRG_00546) (57); all these are enzymes essential for the conversion of glucose-1-phosphate to dTDP-L-rhamnose (58).While Zhang et al. (59) proposed this operon was responsible for the *Listeria* serovar-specific biosynthesis of the sugar-nucleotide precursor needed for rhamnose decoration of WTA, and Den Bakker et al. (60) identified it as belonging to a putative O-antigen determining cluster, phenotypic studies are still needed to confirm that these enzymes contribute to rhamnose decoration of WTA. Our data that mutations in the rhamnose biosynthesis operon cause phage resistance is consistent though with previous observations that rhamnose inhibited *Listeria* phage A118 adsorption (33), and with a study by Habann et al. (53), which showed the A511 receptor-binding protein binds to N-acetylglucosamine and suggested it also binds to rhamnose. Although we did not restore phage susceptibility by complementing the nonsense mutation in LMRG_00543 (which encodes the RmlA

ortholog), this failure to complement was most likely due to the mutation causing a polar effect on downstream genes LMRG_00545 and LMRG_00546 (which we conclusively linked to phage susceptibility and adsorption through complementation experiments). The nonsense mutation early in LMRG_00543 would likely leave a considerable length of mRNA free of ribosomes (>800 bp), such an effect could increase the probability of Rho-dependent transcriptional termination (61). Alternatively, but considerably less likely, the construct used to complement the mutation may not have expressed WT LMRG_00543 as intended, or rhamnose biosynthesis may not be necessary for phage susceptibility.

The three phage-resistant mutant strains that contained the missense "mutations of interest" that were not successfully complemented (LMRG_01009, LMRG_01319, and LMRG_01709) were all resistant to LP-125 and lacked terminal GlcNAc in their WTA. It is possible that phage susceptibility could not be restored by complementation for these mutant strains because their respective mutations had a dominant effect over the respective WT allele; it is also possible that these mutations are not responsible for the observed phage resistance phenotype and that resistance is due to polar effects or other, non-identified, mutations. However, lmo2537 (the LMRG_01710 homolog in 10403S) encodes UDP-*N*- acetylglucosamine 2-epimerase, which is a precursor of the teichoic acid linkage unit (62, 63). This suggests LMRG_01709 may have a direct effect on the composition of WTA and phage resistance, as LMRG_01709 is part of the same operon as LMRG_01710; however, we cannot definitively exclude a polar effect on LMRG_01710. Future experiments to

further characterize these three mutant strains will be necessary to confirm and understand specific functional links between these mutations and phage adsorption.

The five synonymous mutations identified in this study were not further characterized, as they were not the sole mutations found in their respective phage-resistant mutant strains. Although less likely, these mutations may still play a role in phage resistance, as synonymous mutations can affect cellular processes (e.g., translation efficiency due to codon biases, or mRNA structures) (64); future studies on these mutant strains will be needed to address this.

None of the mutations identified here mapped to either of the CRISPR systems present in 10403S (CRISPR-II or RliB-CRISPR) (65), leading us to conclude that phage-resistant mutant strains from this study did not develop CRISPR-mediated phage resistance. As our de novo assembly based genome sequence analyses did identify a 60 bp insertion and two large deletions (130 bp and 420 bp), we are confident that our methodology would have detected the acquisition of a new CRISPR spacer in any of the phage-resistant mutant strains sequenced in this study.

Three genes conclusively linked to phage adsorption show evidence for phase variation. Phase variation is a mechanism that has been shown to provide transient resistance to phage-infection for Gram-negative bacteria (14, 66). We identified seven unique putative phase variant mutations in this study, two of which were each found in two separate mutant strains. All of the putative phase variants we identified would be generated by the general mechanism of slipped-strand mispairing (67), as opposed to other mechanisms of phase variation such as DNA rearrangement or gene conversion (68). Six of the putative phase variants were single nucleotide

deletions in adenine or thymine homopolymeric tracts (one in the LMRG_00541 promoter, four in LMRG_00541 and one in LMRG_00542). These mutations are very similar to the phase variants found in phage-resistant *Vibrio cholerae* (14), where single nucleotide deletions in poly(A) tracts were identified in O1 antigen biosynthesis genes. One of the putative phase variants identified here extended a TA dinucleotide tandem repeat in LMRG_01697 from four to five repeats. A similar phase variation, which involved the loss or gain of a single repeat within a tetranucleotide tandem repeat, was found in *Staphylococcus aureus icaG*, which is linked to production of the polysaccharide adhesin β-1-6-linked N-acetylglucosamine (69); phage resistance phenotypes of this phase variation was not evaluated though. Previously, Orsi et al. (70) identified phase variation within a homopolymeric tract of seven adenine residues found within the internalin A gene (*inlA*) of *L. monocytogenes*. Based on the data reported by Orsi et al. (70) on the frequency of phase variation, we hypothesize that the putative phase variants identified here are much more likely than other frameshift mutations to revert to a WT genotype (restoring the full open reading frame). If so, such mutations could provide a transient genotypic escape for *L. monocytogenes* from phage predation; after the selective pressure of phage-infection passes, any mutant strains that reverted back to the WT genotype would have the opportunity to outcompete the phage-resistant mutant strains.

Identification of distinct mutations in phage-resistant mutant strains of *L. monocytogenes* provides initial insight into the types of phage-resistant mutant strains that may emerge after exposure to or treatment with *Listeria* phages. Whole genome sequencing (WGS) has recently been used to identify mutations in

phage-resistant mutants of *Escherichia coli* (13), *Vibrio cholerae* (14, 71), *Bacillus anthracis* (49), and *Synechococcus* (15), demonstrating the power of next generation sequencing in improving our understanding of phage-host interactions. In this study, WGS of 69 mutant strains enabled us to observe parallel evolution of phage resistance in *L. monocytogenes* 10403S under the selective pressure of lytic phages, as evidenced by mutations repeatedly and independently occurring in the same genes and causing the same phenotypic effects. As WTA have been shown to be associated with virulence functions (72), including one study that showed a teichoic acid biosynthesis gene is essential for virulence of *L. monocytogenes* in mice (63), further studies to determine how the mutations found in this study affect virulence, as well as fitness under a variety of different environmental and stress conditions will be valuable. Additional studies should also examine the frequency of occurrence and stability of the mutations identified here across different environmental conditions. Together, these types of studies will facilitate better assessment of the safety of using *Listeria* phage products for control applications and will grant insight on how phages may drive the evolution and pathogenicity of *L. monocytogenes*. Information on conditional expression of genes linked here to phage adsorption (73) could also provide insight on whether *L. monocytogenes* is capable of escaping phage predation by gaining physiological refuge (where a bacterium becomes transiently resistant to phage infection). Transient phage resistance has been reported for *E. coli*; in one study phage resistance was induced under maltose deficient conditions (74), while in another study phage resistance was induced by the quorum-sensing signal *N*-acyl-L-homoserine lactone (75). Additionally, phage-resistant mutant strains identified in this study may

prove to be useful as hosts for isolating new phages that adsorb to different surface features and as screening tools to identify *Listeria* phages that use the same receptors as LP-048 and LP-125.

Conclusions. Under the selective pressure of virulent phages, strains of *L. monocytogenes* harboring spontaneous mutations that grant phage-resistance will survive and outcompete the susceptible parental strains. Phage-resistant mutant strains of *L. monocytogenes* from this study were all shown to resist phage-infection through mechanisms of adsorption inhibition. Post-adsorption mechanisms of phage-resistance, such as CRISPR-mediated phage immunity, were not found in any of the phage-resistant mutant strains; however, several of the mutations found were identified as putative phase variants, suggesting that phase variation may be an important genetic mechanism for the survival of *L. monocytogenes* under phage-mediated selective pressure.

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Supplemental Table 2.1 Mutations identified in phage-resistant mutant strains

[a]Mutations found at or between nucleotide positions 1,092,522 and 1,100,531 are in Locus I (see Table 2.2). Mutations found at nucleotide positions 1,655,229, 1,893,070, and 2,569,423 are in loci II, III, and IV, respectively. Mutations found at or between nucleotide positions 2,579,547 and 2,580,821 are in Locus V.

[b]Ribosomal binding site (RBS)

Nucleotide Position[a]	Feature	Mutation Type	WT	Mutant	Strain(s)	Phage Selection
463,573	LMRG_00133	Synonomous	C	T	FSL D4-0023	LP-048
1,080,104	LMRG_00533	Synonymous	C	T	FSL D4-0135	LP-048
1,090,381	LMRG_00539	Missense	T	A	FSL D4-0115	LP-048
1,092,522	Promoter (LMRG_00541)	Deletion	AT	A	FSL D4-0058, FSL D4-0076	LP-125
1,092,585	LMRG_00541	Frameshift	GA	G	FSL D4-0055	LP-125
1,092,693	LMRG_00541	Nonsense	G	A	FSL D4-0001	LP-125
1,092,758	LMRG_00541	Nonsense	G	T	FSL D4-0014	LP-125
1,092,823	LMRG_00541	Nonsense	G	A	FSL D4-0010	LP-125
1,093,392	LMRG_00541	Insertion (60 bp)			FSL D4-0084	LP-125
1,093,480	LMRG_00541	Frameshift	CG	C	FSL D4-0080	LP-125
1,093,551	LMRG_00541	Frameshift	GA	G	FSL D4-0052	LP-125
1,093,651	LMRG_00541	Frameshift	CA	C	FSL D4-0094	LP-125
1,093,848	LMRG_00541	Nonsense	T	A	FSL D4-0005	LP-125
1,093,859	LMRG_00541	Frameshift	GT	G	FSL D4-0092	LP-125
1,094,214	LMRG_00541	Nonsense	T	A	FSL D4-0073	LP-125
1,094,330	LMRG_00541	Nonsense	C	T	FSL D4-0081	LP-125
1,094,356	LMRG_00541	Nonsense	A	T	FSL D4-0056	LP-125
1,094,429	LMRG_00541	Frameshift	GA	G	FSL D4-0002	LP-125
1,094,564	LMRG_00541	Nonsense	G	T	FSL D4-0096	LP-125
1,094,814	LMRG_00541	Frameshift	CA	C	FSL D4-0066	LP-125
1,095,105	LMRG_00541	Frameshift	TA	T	FSL D4-0071, FSL D4-0083	LP-125
1,095,136	LMRG_00541	Frameshift	TGA	TT	FSL D4-0095	LP-125
1,095,555	RBS[b] (LMRG_00542)	SNP	G	A	FSL D4-0089	LP-125
1,095,626	LMRG_00542	Missense	G	A	FSL D4-0079	LP-125
1,095,756	LMRG_00542	Frameshift	AT	A	FSL D4-0113	LP-048
1,095,917	LMRG_00542	Missense	C	A	FSL D4-0139	LP-048
1,095,933	LMRG_00542	Nonsense	T	A	FSL D4-0119	LP-048
1,095,971	LMRG_00542	Missense	G	A	FSL D4-0141	LP-048
1,095,980	LMRG_00542	Missense	T	A	FSL D4-0129	LP-048
1,096,070	LMRG_00542	Missense	T	G	FSL D4-0129	LP-048
1,096,148	LMRG_00542	Nonsense	G	A	FSL D4-0054	LP-125
1,096,215	LMRG_00542	Nonsense	T	G	FSL D4-0142	LP-048
1,096,367	LMRG_00542	Missense	G	C	FSL D4-0115	LP-048
1,096,379	LMRG_00542	Missense	G	A	FSL D4-0133	LP-048
1,096,438	LMRG_00542	Missense	G	A	FSL D4-0023	LP-048
1,096,556	LMRG_00542	Frameshift	TA	T	FSL D4-0114	LP-048
1,097,083	LMRG_00542	Missense	A	C	FSL D4-0128	LP-048
1,097,209	LMRG_00542	Nonsense	G	T	FSL D4-0026	LP-048
1,097,480	LMRG_00543	Nonsense	G	T	FSL D4-0118	LP-048
1,097,675	LMRG_00543	Missense	T	C	FSL D4-0121	LP-048
1,097,687	LMRG_00543	Missense	C	A	FSL D4-0136	LP-048
1,097,737	LMRG_00543	Frameshift	TA	T	FSL D4-0063	LP-125
1,097,964	LMRG_00543	Missense	G	A	FSL D4-0049	LP-125
1,098,147	LMRG_00543	Missense	C	T	FSL D4-0021	LP-048
1,098,206	LMRG_00543	Frameshift	GA	G	FSL D4-0127	LP-048
1,098,248	LMRG_00543	Nonsense	G	T	FSL D4-0022	LP-048
1,098,283	LMRG_00543	Frameshift	TG	T	FSL D4-0132	LP-048
1,098,984	LMRG_00545	Missense	T	A	FSL D4-0117	LP-048
1,099,197	LMRG_00545	Deletion (130 bp)			FSL D4-0116	LP-048
1,099,206	LMRG_00545	Missense	G	A	FSL D4-0075	LP-125
1,099,218	LMRG_00545	Missense	T	C	FSL D4-0138	LP-048
1,099,230	LMRG_00545	Missense	C	A	FSL D4-0046	LP-125
1,099,283	LMRG_00545	Missense	G	T	FSL D4-0135	LP-048
1,099,530	LMRG_00545	Missense	A	C	FSL D4-0029	LP-048
1,099,748	LMRG_00545	Nonsense	A	T	FSL D4-0126	LP-048
1,100,531	LMRG_00546	Missense	C	T	FSL D4-0028	LP-048
1,178,945	LMRG_00640	Synonomous	G	A	FSL D4-0094	LP-125
1,219,965	LMRG_00680	Synonomous	C	T	FSL D4-0058	LP-125
1,433,686	Promoter	Deletion	TA	T	FSL D4-0090	LP-125
1,478,073	LMRG_00938	Missense	T	A	FSL D4-0066	LP-125
1,612,263	LMRG_01359	Missense	G	C	FSL D4-0022	LP-048
1,615,806	Promoter	Deletion	CA	C	FSL D4-0094	LP-125
1,655,229	LMRG_01319	Missense	A	C	FSL D4-0057	LP-125
1,893,070	LMRG_01009	Missense	G	T	FSL D4-0082	LP-125
2,302,986	Intergenic	SNP	T	C	FSL D4-0132	LP-048
2,388,650	LMRG_01479	Missense	G	A	FSL D4-0116	LP-048
2,517,595	LMRG_01760	Missense	C	T	FSL D4-0089	LP-125
2,569,423	LMRG_01709	Missense	A	C	FSL D4-0087	LP-125
2,577,269	LMRG_01701	Synonomous	T	C	FSL D4-0067	LP-125
2,579,547	LMRG_01698	Nonsense	C	T	FSL D4-0077	LP-125
2,579,784	LMRG_01698	Nonsense	C	T	FSL D4-0065	LP-125
2,579,808	LMRG_01698	Missense	G	A	FSL D4-0007, FSL D4-0060	LP-125
2,579,952	LMRG_01697	Frameshift	GT	G	FSL D4-0072	LP-125
2,580,096	LMRG_01697	Frameshift	C	CTA	FSL D4-0093	LP-125
2,580,285	LMRG_01697	Missense	C	T	FSL D4-0090	LP-125
2,580,320	LMRG_01697	Frameshift	GT	G	FSL D4-0067	LP-125
2,580,414	LMRG_01697	Nonsense	G	A	FSL D4-0068	LP-125
2,580,546	LMRG_01697	Missense	G	A	FSL D4-0059	LP-125
2,580,735	LMRG_01697	Deletion (421 bp)			FSL D4-0070	LP-125
2,580,821	LMRG_01697	Missense	G	T	FSL D4-0069	LP-125

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CHAPTER 3

TEMPERATURE SIGNIFICANTLY AFFECTS THE PLAQUING AND ADSORPTION EFFICIENCIES OF *LISTERIA* PHAGES

Authors

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Source

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Abstract

Listeria-infecting phages are currently being used to control and detect the important foodborne pathogen *Listeria monocytogenes*; however, the influence of environmental conditions on the interactions between *L. monocytogenes* and its phages has not been explored in depth. Here, we examined the infective potential of four *Listeria* phages (two each from the P70-like and P100-like phages of *Listeria*) against five strains of *L. monocytogenes* (representing serotypes 1/2a, 1/2b, 4a, and 4b) grown under a range of temperatures (7ºC – 37°C). We show that the plaquing efficiencies for all four phages were significantly affected by temperature. Interestingly, no plaques were observed for any of the four phages at 37°C. Adsorption assays

performed with the P100-like phages, LP-048 and LP-125, showed that LP-048 had a severely reduced adsorption efficiency against susceptible strains at 37°C as compared to 30°C, suggesting that there is considerably less accessible rhamnose (LP-048's putative phage receptor) on the host at 37°C than at 30°C. LP-125 adsorbed to host cells at 37ºC, indicating that the inability for LP-125 to plaque at 37°C is not due to adsorption inhibition. LP-048 showed significantly higher adsorption efficiency against a mutant strain lacking N-acetylglucosamine in its wall teichoic acids than the parental strain at both 30°C and 37°C, suggesting that N-acetylglucosamine competes with rhamnose for glycosylation sites on the wall teichoic acids. The data presented here clearly shows that *L. monocytogenes* can gain physiological refuge from phage infection, which should be carefully considered for both the design and implementation of phage-based control and detection applications.

Introduction

Listeria monocytogenes is the foodborne pathogen that causes listeriosis, which has a mortality rate of 20 to 30% (1) and causes an estimated 255 deaths per year in the U.S. It has been estimated that the total annual cost of *L. monocytogenes* in the U.S. is ~$2.5 billion (2, 3). A recent meta-analysis on the global burden of listeriosis found that there is an estimated 23,150 human incidents of listeriosis every year (4). Outbreaks caused by *L. monocytogenes* are a major concern for the food industry; between 1998 and 2008 there have been 24 confirmed outbreaks of listeriosis in the U.S., which resulted in a total of 359 illnesses, 215 hospitalizations and 38 deaths (5).

Since 2006, lytic bacteriophages (or "phages") have been used according to national or regional regulations as biocontrol agents that specifically target *L. monocytogenes.* Studies that have evaluated the efficacy of phage-based biocontrol of *L. monocytogenes* have shown promise for this listeriocidal application(6); however, several obstacles still need to be overcome to improve the long-term efficacy of phage-based biocontrol of *L. monocytogenes.* For example, the influence of environmental conditions on the susceptibility of *L. monocytogenes* to phage infection has not been explored in depth (7). A study by Kim et al. showed that typically phage-sensitive epidemic clone II strains of *L. monocytogenes* were broadly resistant to phage infection at temperatures below 30ºC (8). A follow-up study found that the temperature-dependent phage resistance was due to a restriction modification system that was expressed at lower temperatures (9); however, it is not known if other strains have similar temperature dependent mechanisms of phage-resistance.

Here, we examined how temperature affects the infection potential of *Listeria*-infecting phages against a representative panel of *L. monocytogenes* strains. Our goal was to advance our understanding of how environmental conditions affect phage-susceptibility of *Listeria* by focusing on the key condition, temperature. Further, this study was designed to provide knowledge that can improve the implementation and design of phage-based biocontrol and detection applications.

Materials and Methods

Phages and Bacterial strains.

Two phages each from the obligate lytic phage groups P100-like phages (phages LP-048 and LP-125) and P70-like phages (LP-026 and LP-037) were selected for use in this study (10) [Table 3.1]. We also included *Listeria* phage A511 (Table 3.1) so that we could make comparisons to other studies. The *L. monocytogenes* strains used in this study were selected to represent a diversity of lineages (Lineage I, II, and IV) and serotypes (1/2a, 1/2b, 4a, and 4b) [Table 3.1]. The P100 phages used in this study were propagated on Mack and the P70-like phages used in this study were propagated on FSL J1-208 (also the isolation host) as previously described (11). The plasmid cured strain of FSL J1-208, designated FSL B2-294, was also included in this study to test whether the plasmid is linked to phage susceptibility (Table 3.1). The strains 541-NM, 542-NM, and ΔdltA (Table 1) were used in this study because the changes to their surface structures are known to affect (12), or may affect, phage adsorption. Strains 541-NM-C and 542-NM-C were included (Table 3.1) to show that complementation of the mutations in 541-NM and 542-NM with the wild-type alleles of LMRG_00541 and LMRG_00542, respectively, rescues the wild-type phenotype.

Efficiency of plaquing assays

Bacterial lawns were prepared using a double agar overlay method (13) using modified LB MOPS media (at a pH of 7.4) as previously described (11). A 30 µl aliquot of *L. monocytogenes* culture grown overnight (16 ± 2 hrs) in BHI broth (shaking at 210 RPM) was used to seed each lawn. Immediately after bacterial lawns were poured the plates were acclimated for 60 to 90 min at the experimental temperatures before phage dilutions were spotted on the acclimated lawn. Phage

Table 3.1 Phages and bacterial strains used in study.

Strain or Phage	Alternative ID*	Description	Source or reference
L. monocytogenes strains			
10403S		Lineage II, Serotype 1/2a	(39)
Mack		Lineage II, Serotype 1/2a	(40)
FSL J1-175	J1-175	Lineage I, Serotype 1/2b	(41, 42)
F2365		Lineage I, Serotype 4b	(43, 44)
FSL J1-208	J1-208	Lineage IV, Serotype 4a	(19)
FSL B2-294	B2-294	J1-208 cured of pLMIV	(19)
FSL D4-0014	541-NM	Nonsense mutation (NM) in LMRG_00541; 10403S background; GlcNAc^{-}†	(12)
FSL D4-0161	541-NM-C	541-NM complemented (C) with WT allele of LMRG_00541	(12)
FSL D4-0119	542-NM	Nonsense mutation (NM) in LMRG_00542; Rha^{-}‡	(12)
FSL D4-0156	542-NM-C	542-NM complemented (C) with WT allele of LMRG_00542	(12)
Hel-867	ΔdltA	Deletion mutation (Δ) of LMRG_02073 (*dltA*)	Gift from H. Marquis
Phages			
LP-026		P70-like phage	(10, 11)
LP-037		P70-like phage	(10, 11)
LP-048		P100-like phage	(10, 11)
LP-0125		P100-like phage	(10, 11)
A511		P100-like phage	(45)

*Strain ID used in the manuscript text and figures.
†The wall teichoic acids of the indicated strain lack N-acetylglucosamine (GlcNAc).
‡The wall teichoic acids of the indicated strain lack rhamnose (Rha).

dilutions that were spotted on plated ranged in concentration from $2x10^2$ to $3x10^9$ PFU/mL. Immediately after spotting, the plates were incubated at the experimental temperatures. Temperatures were selected to represent conditions phage-based products would be expected to function under; for example, 6.5ºC is a temperature that some refrigerated foods may be held at, and is similar to the 7ºC temperature used by Kang et al. in *Listeria* growth studies on cold-smoked salmon (14). Plates incubated at 21 ± 1°C, 25 ± 1°C, 30 ± 2°C, and 37 ± 1°C were incubated for 16 ± 2 hrs before enumeration. Plates incubated at 12 ± 1°C and 16.5ºC ± 1.5°C were incubated for 40 ± 2 hrs before enumeration. Plates incubated at 6.5 ± 1.5°C were incubated for 7 days before enumeration. Longer incubation times at lower temperatures were needed to allow for visible plaques to form. Only visible plaques were counted; faint turbidity without plaque formation was scored as zero.

Adsorption assays.

LP-048 and LP-125 adsorption assays were performed by following a modified version of a previously described procedure (12). Modifications were made to adapt the procedure to compare adsorption at different experimental temperatures (30ºC vs. 37ºC). Briefly, overnight cultures (incubated for 16±2 hrs) were grown in BHI broth at the experimental temperatures with shaking at 210 RPM and then diluted 1/100 into fresh BHI broth. The diluted cultures were then incubated at their respective experimental temperature (30ºC or 37ºC) with shaking at 210 RPM until they reached an OD_{600} of 0.4. Then, 200 µl volumes of cultures (at an OD_{600} value of 0.4) were transferred into microcentrifuge tubes containing 762 µl BHI broth, 20 µl phage lysate

at 1×10^9 PFU/mL, 9 µl of 1M $CaCl_2$, and 9 µl of 1M $MgCl_2$ (salts were added immediately prior to the addition of bacteria). The bacteria and phage mixture was then incubated for 15 min at either 30°C or 37°C with aeration.

Statistical analyses

All statistical analyses were carried out in R (V.3.0.3)(15). Data handling was performed using reshape (16). Linear models were constructed using the lm command from the base package. Pairwise comparisons at all factor levels were made using lsmeans with Tukey multiple testing correction (17). The cutoff for significance was set at $\alpha = 0.05$.

Models for efficiency of plaquing experiments and models for adsorption assay experiments had the factors strain, temperature, and biological replicate, as well as the 2-way interaction between strain and temperature. Relative efficiency of plaquing values were the model responses for plaquing experiments, whereas the $\log_{10}$ reductions of PFU in the supernatant were the model responses for adsorption assays.

BLAST searches

BLAST searches (18) were conducted against the *L. monocytogenes* 10403S (GenBank accession no. NC_017544.1), F2365 (GenBank accession no. NC_002973.6), and J1-208 (GenBank accession no. NZ_CM001469.1) genomes with the protein query sequences for the LmoH7 restriction-modification system (GenBank accession nos. EAL08857.1 and EAL08858.1). We defined our cutoff for homology as 25% shared sequence identity with ≥50% query coverage.

Data and script availability

The raw data and R scripts used in this study can be found in the supplementary materials of the original published article (doi: *10.3389/fmicb.2016.00631*).

Results

Spot assays reveal that temperature affects phage infection of L. monocytogenes

Initial spot testing of phages A511, LP-125, and LP-048 revealed no visible plaques on Mack or F2365 at 37°C, although faint clearings were observed where the most concentrated phage solutions were spotted (Figure 3.1). All three phages formed visible plaques on Mack at 30ºC (Figure 3.1); however, only LP-125 and A511 formed visible plaques on F2365 at 30ºC. The efficiency of plaquing (EOP) of LP-125 and A511 at 30ºC was lower on F2365 than on Mack (Figure 3.1).

Efficiency of plaquing of P100-like phages is affected by growth temperature

Quantitative plaquing assay data collected at six different temperatures with five different host strains and two different P100-like phages (LP-048 and LP-125) were used to statistically evaluate the effects of host and temperature on plaquing. For LP-048, plaquing efficiency was significantly affected by both strain ($p<0.001$) and temperature ($p<0.001$); there was also a significant interaction between strain and temperature ($p<0.001$; see Supplemental File 1). LP-048 formed visible plaques on 10403S, Mack, and J1-175 at all temperatures except 37°C, but did not form visible

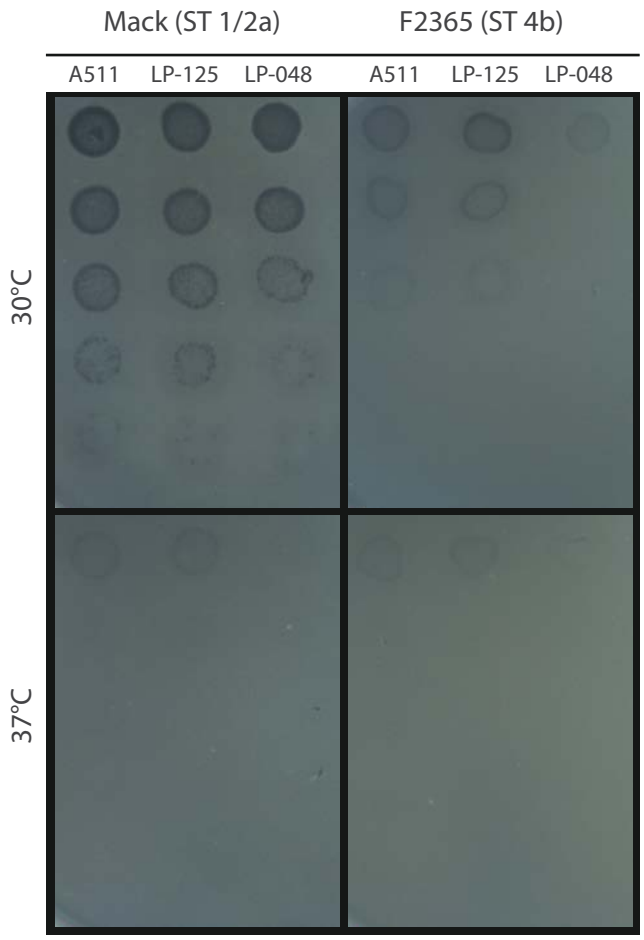


Figure 3.1 *Spot assays of phage dilutions at 30°C and 37°C.* Dilutions of phages A511, LP-125, and LP-048 were spotted on lawns of Mack and F2365, which were pre- and post-incubated at the temperature indicated in the figure. Phage dilutions decrease from top (most concentrated; ~2 x 10^7 PFU/mL) to bottom of the figure (least concentrated; ~2 x 10^3 PFU/mL). Each dilution was 1/10.

plaques on F2365 and J1-208 at any temperature (Figure 3.2a). Effects of temperature on plaque formation were similar for 10403S and Mack; LP-048 showed a trend of having a higher EOP at lower temperatures (Figure 3.2a) with numerically largest EOPs at 12°C and significantly lower EOPs at higher temperatures for both host strains (Figure 3.2a). Interestingly, temperature dependent patterns of plaque formation on the host strain J1-175 were different; on this host strain, the EOP values of LP-048 were significantly lower at 12ºC and 21ºC as compared to the EOP values at 6.5ºC, 25ºC and 30ºC (Figure 3.2a). This may suggest there is a mechanism of phage-resistance in J1-175 (e.g., a restriction modification system) that is only expressed at temperatures between 12°C and 21°C. This would be consistent with the previously described temperature-dependent restriction modification system found in epidemic clone II strains of *L. monocytogenes*, which is only active at temperatures below 30ºC (9).

For LP-125, plaquing efficiency was significantly affected by both strain ($p<0.001$) and temperature ($p<0.001$); there was also a significant interaction between strain and temperature ($p<0.001$; see Supplemental File 1). LP-125 formed visible plaques on 10403S, Mack, and F2365 at all temperatures except 37°C, but did not form visible plaques on J1-175 and J1-208 at any temperature (Figure 3.2b). Effects of temperature on plaque formation were similar for 10403S and Mack; LP-125 showed a trend of having a higher EOP at lower temperatures (Figure 3.2b) with numerically largest EOPs at 12 °C and significantly lower EOPs at 30ºC for both host strains (Figure 3.2b). Interestingly, temperature dependent differences in plaque formation were more pronounced on F2365; on this host strain the EOP was

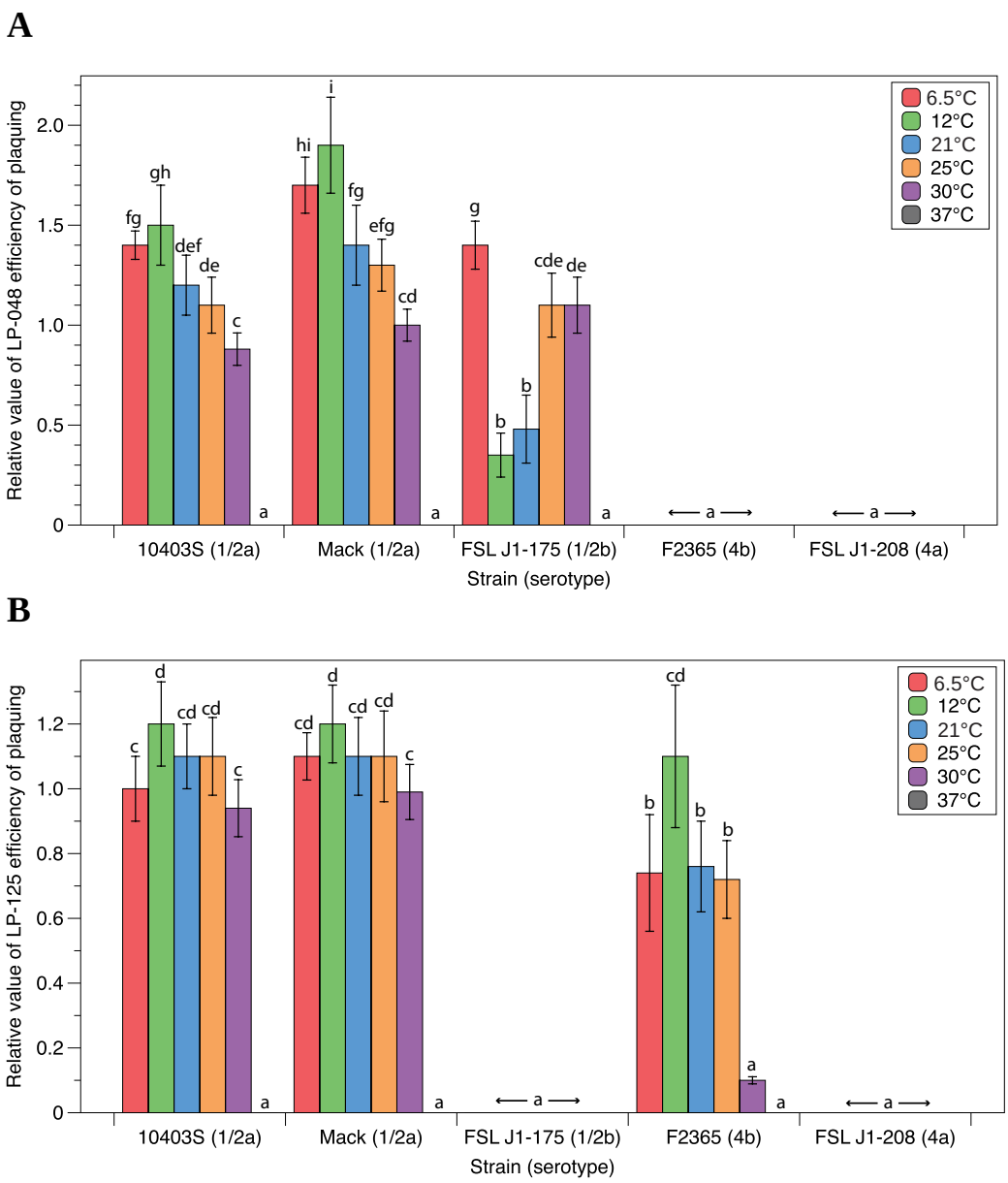


Figure 3.2 *Plaquing efficiencies of P100-like phages at varying growth temperatures*. The relative number of visible plaques under different strain and temperature conditions were compared for phages (A) LP-048 and (B) LP-125. All values are relative to the phages isolation conditions (strain Mack at 30°C). Bars that do not share any letters represent values that are significantly different; for example, a bar marked with d, e, and f is significantly different from a bar marked with g and h. Three biological replicates were conducted. Error bars represent the standard error.

significantly higher at 12ºC (as compared to all other temperatures), and the EOP was significantly lower at 30ºC than at temperatures between 6.5ºC and 25ºC (Figure 3.2b).

Efficiency of plaquing of P70-like phages is affected by growth temperature

Quantitative plaquing assay data collected at six different temperatures with three different host strains and two different P70-like phages (LP-026 and LP-037) were used to statistically evaluate the effects of host and temperature on plaquing. Host strains Mack, J1-175, and F2365 were not used because P70-like phages have a narrow host range as compared to P100-like phages (11). For LP-026, plaquing efficiency was significantly affected by both strain ($p<0.001$) and temperature ($p<0.001$; see Supplemental File 1). LP-026 formed visible plaques on J1-208, B2-294, and 10403S at all tested temperatures except at 37ºC (Figure 3.3a). Effects of temperature on plaquing were similar for J1-208 and B2-294; LP-026 showed a general trend of infecting at a higher EOP at lower temperatures, with the numerically highest EOP at 16ºC for both strains, and significantly lower EOPs at 30ºC than at 16ºC for B2-294 (Figure 3.3a). On strain 10403S, the EOP of LP-026 was numerically highest at 16ºC; however, no significant differences were observed between the tested temperatures (Figure 3.3a).

For LP-037, plaquing efficiency was significantly affected by both strain ($p<0.001$) and temperature ($p<0.001$); there was also a significant interaction

between strain and temperature ($p<0.001$; see Supplemental File 1). LP-037 formed visible plaques on J1-208, B2-294, and 10403S at all tested temperatures except for

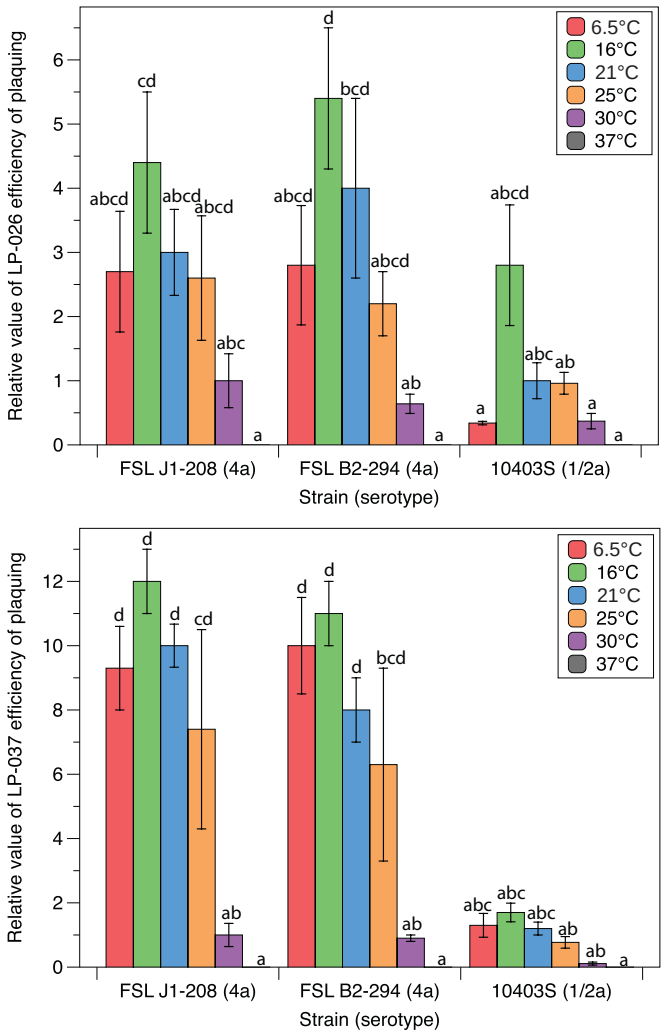


Figure 3.3 *Plaquing efficiencies of P70-like phages at varying growth temperatures*. The relative number of visible plaques under different strain and temperature conditions were compared for phages (A) LP-026 and (B) LP-037. All values are relative to the phages isolation conditions (strain J1-208 at 30°C). Bars that do not share any letters represent values that are significantly different; for example, a bar

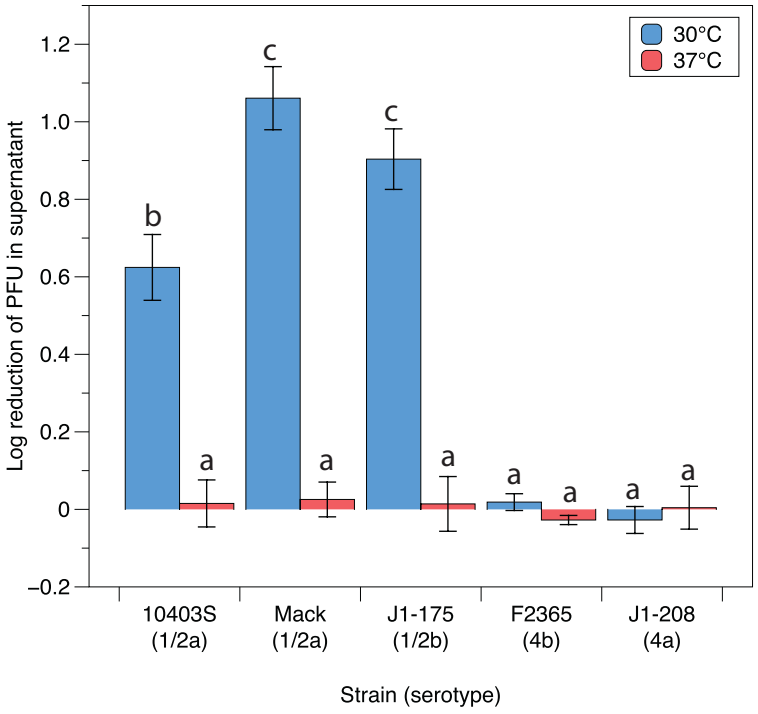
marked with a, b, and c is significantly different from a bar marked with d. Three biological replicates were conducted. Error bars represent the standard error.

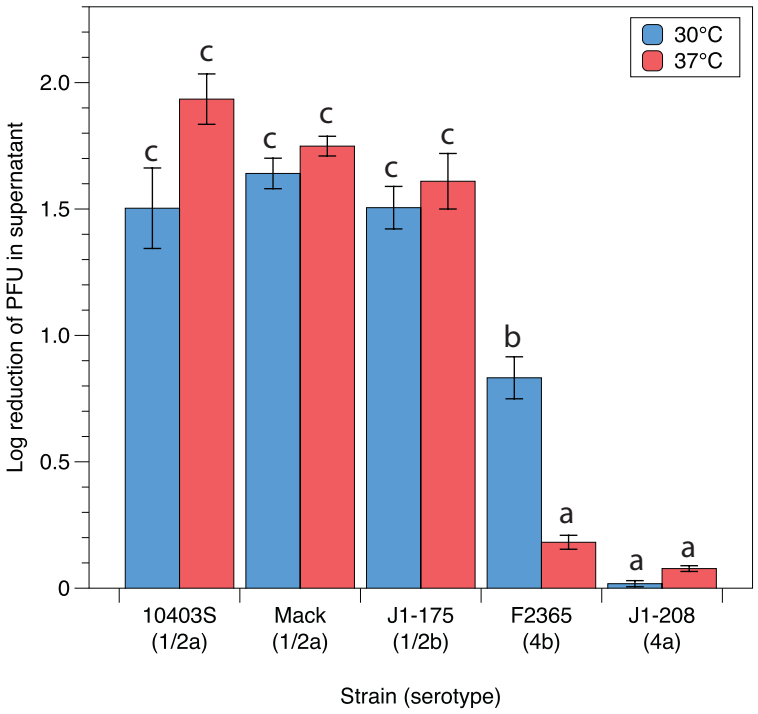
37ºC (Figure 3.3b). Effects of temperature on plaquing were similar for J1-208 and B2-294; LP-037 showed a general trend of infecting at a higher EOP at lower temperatures, with the numerically highest EOP at 16ºC, and significantly lower EOPs at 30ºC than at 16ºC for each strain (Figure 3.3b). On strain 10403S, the EOP of LP-037 was numerically highest at 16ºC; however, no significant differences were observed between the tested temperatures (Figure 3.3b). Neither LP-026 nor LP-037 showed any significant differences in EOP between J1-208 and the plasmid cured strain of J1-208, B2-294 (Figure 3.3). This suggests that the plasmid harbored by J1-208, which is unique amongst *L. monocytogenes* in gene content (19), does not have an effect on the efficiency of plaquing of the P70-like phages.

Adsorption efficiencies of LP-048 and LP-125 are affected by temperature

As no visible plaque formation was observed at 37ºC, we set out to determine whether adsorption was inhibited at this temperature. For these experiments, we used the P100-like phages LP-125 and LP-048 as we have previously identified their putative binding receptors (12). The adsorption assays showed, based on the $\log_{10}$ reduction of free phage after co-incubation with bacteria, that LP-048 had significantly lower adsorption efficiencies at 37ºC than at 30ºC (0.01 to 0.03 vs. 0.62 to 1.1 log reduction) on the strains observed to be susceptible to LP-048 (10403s, Mack, and J1-175) [Figure 3.4a]. We did not observe LP-048 adsorption at 30ºC or 37ºC on the

Figure 3.4 *Adsorption efficiencies of P100-like phages (A) LP-048 and (B) LP-125 at 30°C and 37°C.* Values shown are the $\log_{10}$ reduction of free phage in the supernatant of each co-incubation between phage and host. The $\log_{10}$ reduction is calculated by subtracting the log-transformed number of phage left in in the supernatant from the log-transformed number of phage in the negative control (BHI). Lower values represent less efficient adsorption. Bars that do not share any letters represent values that are significantly different; for example, a bar marked with b is significantly different from a bar marked with c. Three biological replicates were conducted. Error bars represent the standard error. Adsorption efficiencies of P100-like phages (A) LP-048 and (B) LP-125 at 30°C and 37°C. Values shown are the $\log_{10}$ reduction of free phage in the supernatant of each co-incubation between phage and host. The $\log_{10}$ reduction is calculated by subtracting the log-transformed number of phage left in in the supernatant from the log-transformed number of phage in the negative control (BHI). Lower values represent less efficient adsorption. Bars that do not share any letters represent values that are significantly different; for example, a bar marked with b is significantly different from a bar marked with c. Three biological replicates were conducted. Error bars represent the standard error.


30°C
37°C
Log reduction of PFU in supernatant
1.2
1.0
0.8
0.6
0.4
0.2
0
−0.2
b
a
c
a
c
a
a
a
a
a
10403S (1/2a)
Mack (1/2a)
J1-175 (1/2b)
F2365 (4b)
J1-208 (4a)
Strain (serotype)


30°C
37°C
Log reduction of PFU in supernatant
2.0
1.5
1.0
0.5
0
c
c
c
c
c
c
b
a
a
a
10403S (1/2a)
Mack (1/2a)
J1-175 (1/2b)
F2365 (4b)
J1-208 (4a)
Strain (serotype)

strains where LP-048 plaquing was not seen (J1-208 and F2365) [Figure 3.4a]. Interestingly, LP-125 showed nearly the same adsorption efficiencies at both 30ºC and 37ºC on the serotype 1/2 strains 10403s, Mack, and J1-175; however, the adsorption efficiency of LP-125 on strain F2365 was significantly lower at 37ºC than at 30ºC (0.18 vs. 0.83 log reduction) [Figure 3.4b]. It should also be noted that LP-125 did adsorb to the strain J1-175 (Figure 3.4b), despite not forming any visible plaques on the strain (Figure 3.2b). This suggests that a phage resistance mechanism other than adsorption inhibition is preventing LP-125 from successfully infecting strain J1-175. Interestingly, this resistance mechanism is not effective against LP-048, which shares a high nucleotide similarity to LP-125 (10).

Adsorption efficiency of LP-048 is increased in a mutant strain lacking GlcNAc as a wall teichoic acid sugar

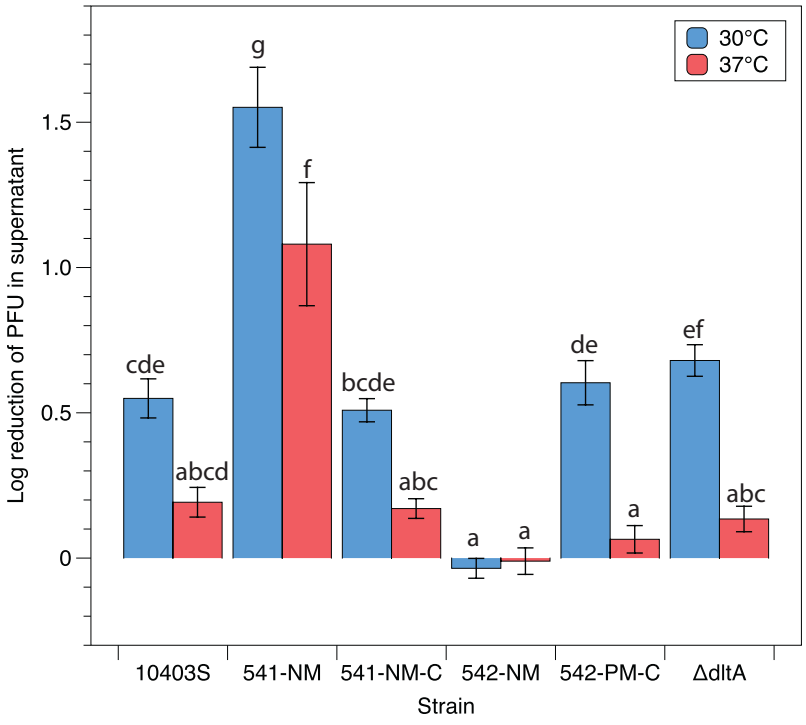
LP-048 requires rhamnose biosynthesis genes in its host for adsorption (12), these genes have since been confirmed to be essential for rhamnosylation of wall teichoic acids (WTA) (20). We thus formulated two competing hypotheses: (i) that down-regulation of rhamnose expression on the WTA is responsible for the reduced adsorption efficiency of LP-048 observed at 37ºC, or (ii) that up-regulation of GlcNAc expression on the WTA is responsible for the reduced adsorption efficiency of LP-048 observed at 37ºC (as GlcNAc likely competes for the same sites on the polyol phosphate backbone of the WTA). We show here that LP-048 still adsorbs more efficiently against the mutant strain without GlcNAc in its WTA (541-NM) at 30ºC

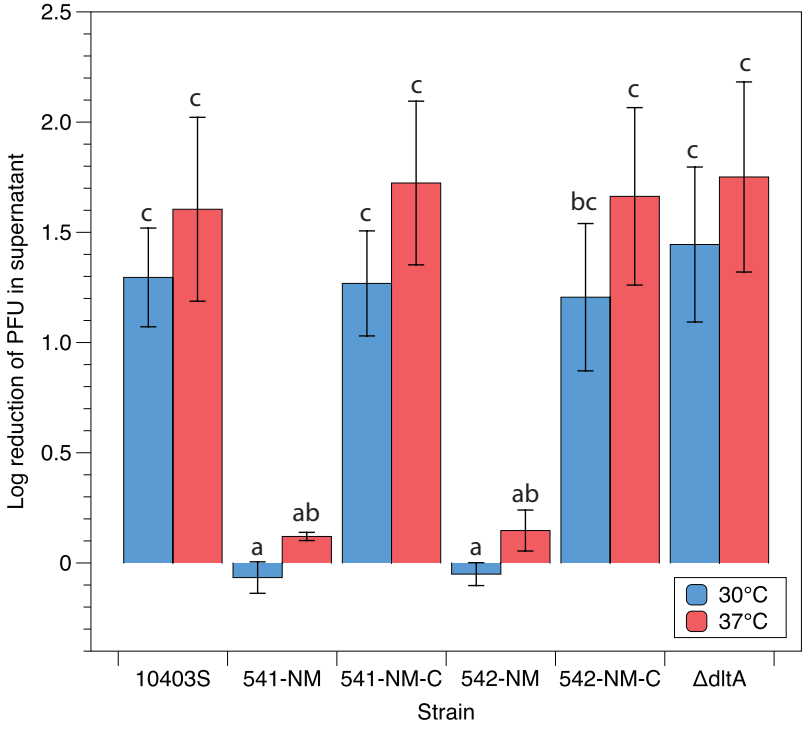
than at 37ºC (no significant difference of the effect of temperature between 10403S and 541-NM). This supports the first hypothesis that rhamnosylation of WTA is likely being directly downregulated at 37ºC. We also show that LP-048 does adsorb significantly more efficiently against the 541-NM mutant than against the parental strain at both 30ºC (1.55 vs. 0.55 log reduction) and 37ºC (1.08 vs. 0.19 log reduction) [Figure 3.5a]. This suggests that there is competition between rhamnose and N-acetylglucosamine glycosylation sites on the WTA (discussed further below); however our data do not support that up-regulation of GlcNAc expression on the WTA is responsible for the reduced adsorption efficiency of LP-048 at 37ºC. Consistent with our previous results (12), LP-048 and LP-125 showed severely reduced adsorption to the rhamnose deficient WTA mutant, 542-NM, and LP-125 showed severely reduced adsorption to 541-NM (Figure 3.5). Complementation of 541-NM and 542-NM with their respective wild-type alleles (i.e., LMRG_00541 and LMRG_00542) restored the strains' observed phenotypes to those of the respective parental strain (10403S) [Figure 3.5]. We also tested adsorption of phages LP-048 and LP-125 at 30ºC and 37ºC against the strain ΔdltA, which is incapable of incorporating D-alanine onto its teichoic acids; no significant differences were observed between ΔdltA and its parental strain (Figure 3.5).

No homologous genes to LmoH7 were found in the host strains used in this study

Our observation that LP-125 does still adsorb at 37°C suggests that there is an adsorption-independent mechanism of phage resistance that is preventing plaque formation at 37°C. We performed BLAST searches against the genomes of strains

Figure 3.5 *Adsorption efficiencies of P100-like phages (A) LP-048 and (B) LP-125 on teichoic acid component mutants*. Values shown are the $\log_{10}$ reduction of free phage in the supernatant of each co-incubation between phage and host. The $\log_{10}$ reduction is calculated by subtracting the log-transformed number of phage left in in the supernatant from the log-transformed number of phage in the negative control (BHI). We also tested the ΔdltA strain complemented with the WT allele of *dltA*; however, no significant differences from either the ΔdltA strain or WT 10403S were observed. Lower values represent less efficient adsorption. Bars that do not share any letters represent values that are significantly different; for example, a bar marked with c, d, and e is significantly different from a bar marked with f. Three biological replicates were conducted. Error bars represent the standard error.


30°C
37°C
Log reduction of PFU in supernatant
1.5
1.0
0.5
0
g
f
cde
abcd
bcde
abc
a
a
de
a
ef
abc
10403S
541-NM
541-NM-C
542-NM
542-PM-C
ΔdltA
Strain


Log reduction of PFU in supernatant
2.5
2.0
1.5
1.0
0.5
0
c
c
c
a
ab
c
c
a
ab
bc
c
c
c
30°C
37°C
10403S
541-NM
541-NM-C
542-NM
542-NM-C
ΔdltA
Strain

10403S, J1-208, and F2365 (the host strains with available genome sequences) to see if any genes homologous to the temperature dependent LmoH7 restriction-modification system found in epidemic clone II strains of *L. monocytogenes* (9) were present. No homology was found, which suggests that the adsorption-independent mechanism of phage resistance we have observed in this study is different from that caused by LmoH7.

Discussion

In this study, we evaluated the effect of temperature on the infection process of four *Listeria* phages, representing two distinct phage groups. We showed that (i) efficiency of plaquing is significantly affected by temperature, most notably that no plaques were observed under any phage-host combination at 37º, that (ii) adsorption efficiency is affected by temperature in specific cases, but does not explain the general lack of observed plaque formation at 37ºC, and that (iii) there is competition between rhamnose and GlcNAc glycosylation of the WTA at both 30°C and 37°C (which affects phage adsorption). Our data clearly showed that *L. monocytogenes* can gain physiological refuge from phage infection (i.e., transient resistance), which should be carefully considered when selecting combinations of phages for use in biocontrol or detection applications (7). These data are practically important as phage-based applications for *Listeria* may need to function under a range of temperatures; for example, biocontrol tools used in food safety will likely need to work at both refrigeration and ambient temperatures, and detection assays may be expected to

function under standard laboratory growth temperatures for *Listeria* (e.g., 30ºC and 37ºC).

Temperature affects the efficiency of plaquing of P100-like phages and P70-like phages

Here, we showed that the efficiencies of plaquing (EOP) of phages infecting *L. monocytogenes* are affected by temperature; these results are consistent with those found in other phage-host systems. For example, Sanders and Klaenhammer found that the EOP of the *Streptococcus* phage Φ18 was significantly affected by temperature, and specifically, showed that the growth temperature of the bacteria prior to infection had more of an effect on EOP than the incubation temperature post-infection (21). In another study, McConnell and Wright showed that *Salmonella* phage Felix-01 showed a higher EOP on a non-sensitive strains at incubation temperatures of 23ºC and 30ºC than at 34°C, 38°C, and 42°C (22). However, Ellis and Delbruck (23)did not find any significant differenced in the EOP of an *Escherichia* phage under different growth temperatures (37ºC, 24ºC, and 10ºC); although, unlike in the experiments here and by Sanders and Klaenhammer (21), Ellis and Delbruck did not acclimate their host bacteria to the experimental temperature prior to exposure to the phage.

Previously, Kim and Kathariou showed that *L. monocytogenes* Epidemic Clone II strains were resistant to phage infection at temperatures below 37°C and that phages A511 and 20422-1 were active on other *L. monocytogenes* strains (including serotype 1/2a, 1/2b, 1/2c, and 4b strains) at 37ºC (8). In contrast, A511 did not form visible

plaques at 37°C under the specific conditions used in our study (Figure 3.1). In a follow-up study, Kim et al. found that a restriction modification system was expressed at temperatures below 37ºC, and that deleting this restriction modification system restored phage susceptibility at 25ºC (9). In this study, we showed a very different pattern of temperature affecting the plaquing efficiency of *L. monocytogenes.* None of the phages tested in this study formed visible plaques at 37ºC, and all of the phages showed the highest EOP at temperatures under 21ºC. These results were consistent with much earlier observations that 37ºC incubation temperature had a deactivating effect on temperate *Listeria* phages (24). One possible explanation for the differences observed between our study and the study by Kim et al. could be that the phages we used were all isolated from silage in New York State (USA), whereas A511 and 20422-1 were isolated from sewage and a turkey processing plant, respectively. Therefore, the phages from silage may have adapted to cooler conditions than phages A511 and 20422-1 (silage is typically stored outdoors, and New York is a temperate climate); however, as we observed here that A511 showed the same plaquing phenotype as LP-125 against Mack and F2365 at 30°C and 37°C (i.e., no plaquing was observed at 37°C), future studies will be needed to test this.

Adsorption inhibition may prevent plaquing of LP-048, but not of LP-125, at 37ºC

Previously, both LP-048 and LP-125 were shown to have severe adsorption defects to *L. monocytogenes* strains with deleterious mutations in genes that are essential for rhamnosylation of WTA, which suggested that both phages use rhamnose as a phage receptor. However, LP-125 was shown to also require terminal N-

acetylglucosamine (GlcNAc) in the WTA for successful host adsorption (12). Thus, GlcNAc and rhamnose are likely the phage receptors for LP-125 on serotype 1/2a strains, which is consistent with phages A511 (25) and P35 (26), both of which bind to GlcNAc and rhamnose. However, it should be noted that conclusions from Habann et al. (25)differ from those from a prior study that identified peptidoglycan as A511's receptor (27); this inconsistency is presumably due to limitations in the methodology used by Wendlinger et al., such as relying on cell fractions, that might have different structures exposed than those found on the surface of intact cells, to assess phage adsorption. Our observation that LP-048 has a severely reduced adsorption efficiency to *L. monocytogenes* at 37ºC suggests that rhamnose expression in the wall teichoic acids is down regulated at 37ºC. However, as LP-125 is still able to bind to *L. monocytogenes* at 37°C with similar efficiency as at 30°C, there is likely still sufficient rhamnose present at 37°C to facilitate LP-125 binding to GlcNAc residues. Furthermore, our observation that LP-125 does still adsorb at 37°C suggests that there is an adsorption-independent mechanism of phage resistance that is preventing plaque formation at 37°C. Whereas we found no homologous genes to the previously-described temperature dependent restriction modification system LmoH7 (9), it is possible that a restriction-modification system not homologous to LmoH7 is present in the strains tested in this study. Alternatively, there could be an unrelated temperature dependent phage-defense system such as an abortive-infection system, CRISPR-Cas system, or super-infection exclusion system (28).

We also showed that LP-125 could adsorb to the serotype 4b strain F2365 at 30°C, although significantly less efficiently than to serotype 1/2 strains. This is likely

because serotype 4b strains do not possess rhamnose or terminal GlcNAc in their WTA. Instead, the WTA of serotype 4b strains are decorated with terminal glucose and galactose (29, 30), whereas the WTA of serotype 4a strains are only decorated with terminal galactose (31). As LP-125 binds to serotype 4b strain F2365 but not to the serotype 4a strain J1-208, we hypothesize that glucose could be the phage receptor for LP-125 on serotype 4b strains. Interestingly, we also showed that LP-125 has a significantly reduced adsorption efficiency on F2365 at 37°C, as compared to 30°C. This suggests that the surface receptor used by LP-125 on the serotype 4b strain F2365 is either down regulated or less accessible at 37°C.

There is likely competition between rhamnose and GlcNAc glycosylation of the WTA at both 30°C and 37°C

Our observation that the mutant strain lacking GlcNAc in its WTA (541-NM) allowed for significantly greater adsorption of LP-048 at both 30°C and 37°C suggests that there is competition between the glycosylation of the WTA with rhamnose and GlcNAc. Both glycosyl groups are linked to the WTA from the same hydroxyl sites on the polyol phosphate backbone (30). The mutant strain lacking GlcNAc WTA decorations would have more hydroxyl groups in the WTA available for rhamnosylation, which would explain the increased adsorption of LP-048 against this mutant strain. Given that we still see a significant difference in adsorption of LP-048 against 541-NM between 30°C and 37°C, we can rule out that the effect of temperature on LP-048 adsorption is solely due to differential regulation of GlcNAc glycosylation of the WTA; down regulation of GlcNAc at 37°C is not increasing

rhamnosylation of WTA at 37°C. It is much more likely that rhamnosylation of the WTA is directly regulated in response to temperature. Although outside of the scope of this study, further research will be needed to elucidate the mechanisms putatively regulating the glycosyl decorations of *Listeria's* WTA.

L. monocytogenes can gain physiological refuge from phage infection

Physiological refuge can be defined as the ability of a phage-susceptible bacterium to gain transient resistance to phage-infection. The concept was postulated by Lenski to account for the stability of virulent phage and host populations (32). Previous studies have provided evidence that bacterial cells can take physiological refuge from phage-infection. For example, a number of studies have shown that adsorption rates of *E. coli* phages are affected by the physiological state of the host (33-36). The susceptibility of the Fermicutes *Lactococcus* (37) and *Lactobacillus* (38) to phage infection has also been shown to be affected by the physiological state of the host. A specific temperature dependent restriction-modification system has been shown to be present in a clonal group of *L. monocytogenes* strains, which demonstrated a specific case of *L. monocytogenes* taking physiological refuge (9). However, we have now shown that temperature generally affects *L. monocytogenes'* susceptibility to phage infection through both mechanisms of adsorption inhibition as well as unidentified internal (i.e., post-adsorption) mechanisms of phage resistance. The data presented here clearly shows that *L. monocytogenes* can gain physiological refuge from phage infection, which should be carefully considered for both the design and implementation of phage-based control and detection applications. These data also

offer insight on the evolutionary dynamics of *L. monocytogenes* and its phages, which can help in our understanding of the diversity and transmission of this important foodborne pathogen.

Author Contributions

TD and JT designed the experiments. MW provided reagents. JT performed the experiments. DK and TD performed the data analyses. TD prepared the figures. TD, JT, DK, and MW interpreted the data. TD and JT wrote the manuscript with valuable feedback from MW and DK.

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CHAPTER 4

CONCLUSIONS

In this dissertation, we (i) identified specific mutations that conferred phage resistance to *L. monocytogenes* and (ii) determined that temperature significantly affects the phage susceptibility of *L. monocytogenes*.

In chapter 2, we performed whole genome sequencing on phage-resistant mutants of *L. monocytogenes* 10403S to identify the specific mutations that conferred phage resistance. We found that a majority of the mutations occurred in genes that likely affected wall teichoic acid structure. We showed that the adsorption efficiency of *Listeria* phages against the phage-resistant mutants was significantly reduced. We also showed that the mutations that only conferred resistance to LP-0125 lacked terminal N-acetylglucosamine in the wall teichoic acids, while the mutations that conferred resistance to both LP-048 and LP-125 putatively lacked rhamnose in their wall teichoic acids. Since the publication of this chapter as a peer-reviewed research article (1), another group confirmed that the genes we identified as affecting resistance to both LP-048 and LP-125 are necessary for rhamnosylation of wall teichoic acids in *L. monocytogenes* (2). One of our most interesting findings was that several of the mutations were putative phase variants. This suggests that phase variation might be a mechanism that allows *L. monocytogenes* to more rapidly develop phage-resistance, and that there would be a much greater likelihood of these mutations reverting to the wildtype genotype.

In chapter 3, we show that *Listeria* phages are overall less efficient at warmer temperatures. This was particularly interesting as 30ºC and 37 ºC are standard growth conditions for *Listeria*, and most *Listeria* phages are routinely isolated and propagated at 30 ºC. The *Listeria* phages used in this study were not able to infect *L. monocytogenes* at 37ºC (3). We also showed that temperature likely affects the surface composition of *L. monocytogenes*, as phage adsorption was affected by temperature for some phage/strain combinations. Overall, this study conclusively shows that *L. monocytogenes* is able to gain physiological refuge from phage infection, and that this needs to be considered by those using phage as biocontrols and detection tools for *L. monocytogenes*.

The work presented here furthers our understanding of the interactions between *Listeria* and its bacteriophages. Given that *Listeria* phages are currently used for control and detection applications for *L. monocytogenes*, this knowledge can be leveraged to assess the efficacy and practicality of phage-based applications and to improve the design of future phage-based products. This knowledge also helps further our understanding of the evolution and ecological dynamics of *L. monocytogenes,* as *Listeria* phages are known to be present in the environments that harbor *Listeria* (4). Future studies can build on this knowledge by determining the fitness costs of mutations that confer phage-resistance to *L. monocytogenes*; for example, do these mutations affect the virulence of *L. monocytogenes*? Future studies can also seek to identify specific mechanisms of how temperature affects phage-susceptibility; for example, what is the mechanism that grants phage-resistance to *L. monocytogenes* at 37ºC?

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