PHENOTYPIC CHARACTERIZATION AND COMPARATIVE GENOMICS ANALYSIS OF LISTERIAPHAGES

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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August 2012

PHENOTYPIC CHARACTERIZATION AND COMPARATIVE GENOMICS ANALYSIS OF *LISTERIA* PHAGES

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Listeria monocytogenes is a foodborne pathogen that is widespread in natural and urban environments. *L. monocytogenes* is also commonly found in food processing facilities where specific strains or subtypes have been shown to persist over time. Listeriaphages are phages that can specifically infect *L. monocytogenes* and other *Listeria* spp. Phages have been approved for use as a biocontrol agent for this pathogen; however knowledge on its efficacy against diverse *L. monocytogenes*, particularly from food associated environments, is still limited. We developed a collection of diverse phages for further studies which included (i) phenotypic and genotypic characterization of listeriaphages isolated from dairy farms, (ii) evaluation of phage susceptibility and development of phage cocktails against persistent subtypes from food-associated environments, and (iii) whole genome sequencing and comparative genomics of selected phages. Over 100 listeriaphages were isolated from silage samples collected over the course of 1.5 years on two dairy farms. Initial phenotypic and genotypic characterization of phages from this collection revealed considerable host range (9 lysis groups) and genomic diversity (genome sizes of 25–140 kb). Among the 9 major *Listeria* serotypes used to determine the host range, the serotype 3c strain was found to be highly resistant to phages while serotype 4 strains

were the most susceptible to phages. Variation in phage susceptibility (4.6–95.4%) was observed among different persistent isolates of multiple ribotypes from a food processing facility. While phage cocktails could temporally reduce the bacterial populations of some subtypes, others were unaffected by phage treatment. *L. monocytogenes* isolates also rapidly developed phage-resistance characteristics in laboratory challenge studies. Whole genome sequencing of 10 listeriaphages revealed considerable genomic diversity of listeriaphages on dairy farms. The phage genome sizes could be classified into 3 ranges: small (36–38 kb; n=3), mid-sized (64–67 kb; n=4), and large (133–135 kb; n=3). All genomes were found to be organized into 3 functional modules: (i) DNA packaging and structural proteins; (ii) cell lysis; (iii) DNA replication, modification, and metabolism. Genomes of six newly sequenced phages appear to resemble three previously described listeriaphages. However, four phages showed no sequence homology to any bacteriophages in the NCBI databases, suggesting they are novel listeriaphages. Our data provide valuable information for further development of effective and suitable phage-based biocontrol agents and other applications.

BIOGRAPHICAL SKETCH

Kitiya Vongkamjan is from Maha sarakham, Thailand. Kitiya went to Canada in 2003 to attend the university after finishing high school at Miss Porter's School in Farmington, CT. Kitiya graduated from Guelph University (Ontario) with honors in Food Science in 2007. Kitiya worked as a work-study student at the Canadian Research Institute for Food Safety (CRIFS) during her junior and senior years. She had a great opportunity to work with colleagues who were the experts in the food microbiology-related field and she later decided to continue her graduate study focusing on food microbiology. Kitiya then joined the department of Food Science at Cornell University as a Ph.D. student in August 2007 under the supervision of Dr. Martin Wiedmann. Kitiya was involved in a number of leaderships and extracurricular activities at school and national levels. She joined the Cornell Food Science team for the Danisco product development competition and for the IFT Developing Solution for Developing Countries competition in 2008-09. Kitiya was a co-chair for the IFT Foundation Fun Run in 2009-10 to raise money for scholarships for students in Food Science across the country. Kitiya was also a student representative in the program of Infection and Pathobiology of the Cornell College of Veterinary Medicine. Kitiya received IFT foundation scholarships, poster awards, a number of travel grants to attend several conferences such as the American Society for Microbiology, IFT, and the International Symposium on Problems of Listeriosis *XVII*. During her 4^{th} year, Kitiya had an opportunity to intern with Danisco-DuPont under the BioProcess Development team in the Cultures Division in France, and the Food Protection group

in Denmark for 6 months. Kitiya is a member of American Society for Microbiology; IFT International, Food Microbiology, Muscle Foods, and Biotechnology Divisions; International Association for Food Protection (IAFP), The Phi Tau Sigma, and Cornell Thai Student Association (CTA).

ACKNOWLEDGMENTS

I would like to express my sincerest thanks to Dr. Martin Wiedmann for giving me the opportunity to work in his research group, and for all of his support and mentorship throughout my degree. He has been a positive influence in my academic and professional goals. I would also like to thank Dr. Kathryn Boor for her support and advice over the years, and to Dr. Loren Tauer who served as my committee member in AEM. I would like to thank my former and current "people" at the Food Safety Lab. I cannot put everyone's names here –Matt S., Lorraine, PJ, Travis, Laura, Steve, Tom D. Thanks for making my life in the lab much easier. I would not be able to complete my research without all of you. Thanks for allowing me to discuss about anything, research related or personal issues…and this is how you guys have helped me get through things. I would like to express my special thanks to Dr. Andrea Moreno who has been of great help and support on my Q-A-B exams as well as my research. I would like to thank our lab technicians, postdoc, and food science staff- Esther, Sherry, Maureen, Barbara, Dr. Henk den Bakker, and Janette for their prompt response in getting anything I requested for my research.

My parents, they are the biggest engines of my life. None of my accomplishments would have been possible without them. I will never thank them enough. Thanks to both of my lovely brothers, my grandma, and my aunts for their supports for a decade while I am in school.

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LIST OF ABBREVIATIONS

L. monocytogenes	*Listeria monocytogenes*
L. ivanovii	*Listeria ivanovii*
spp.	species
CFU	colony forming unit
PFU	plaque forming unit
TNTC	too numerous to count
MOI	multiplicity of infection
LMPM	*L. monocytogenes* plating medium
BHI	Brain Heart Infusion
LEB	*Listeria* enrichment broth
PBS	Phosphate Buffer Saline
PFGE	Pulsed Field Gel Electrophoresis
RTE	ready-to-eat
RTD	routine test dilution
TEM	transmission electron microscopy
GRAS	generally recognized as safe
ORF	open reading frame

CHAPTER ONE

Introduction

L. monocytogenes is a Gram-positive, facultative intracellular foodborne pathogen that causes listeriosis, a rare but severe disease as indicated by a mortality rate between 20-30% (Vazquez-Boland et al., 2001). According to the US CDC, about 1,600 human listeriosis cases, including 255 deaths occur annually in the US. Of the vast majority of human listeriosis cases, 99% appears to be caused by consumption of foods contaminated with *L. monocytogenes* (Mead et al., 1999). On average from 1998-2008, 2.4 outbreaks per year were reported to the CDC. It has been shown that medical costs and productivity losses due to listeriosis are estimated to be $2.3 billion a year in the US (Ivanek et al., 2005). Foodborne listeriosis cases have been shown to be decreased since FoodNet began the active surveillance for laboratory-confirmed of listeriosis cases in 1996. Although FoodNet surveillance preliminary data for 2009 show a modest increase in the incidence of listeriosis, the incidence of listeriosis continues to be substantially lower than at the start of FoodNet surveillance in 1996 (Anonymous, 2010).

L. monocytogenes is widely distributed in nature and has been isolated from a variety of environmental sources, e.g. water, soil, silage, plant vegetation, and food processing plants (Beuchat, 1996; Fenlon, 1999; Fenlon, 1985; Gianfranceschi et al., 2003; Lyautey et al., 2007; Ho et al., 2007). A number of studies have reported a high

prevalence of *L. monocytogenes* in dairy farm environments (Borucki et al., 2005; Fenlon et al., 1995; Fox et al., 2009; Husu, 1990; Skovgaard and Morgen, 1988). Silage (i.e., fermented plant material that is commonly used as feed for ruminants), if spoiled or not fermented properly, has often been found to contain *L. monocytogenes* (Fenlon et al., 1996; Arimi et al., 1997), including at high numbers (>10^7 CFU/g silage) (Wiedmann, et al. 1999) and appears to be the most important source of *L. monocytogenes* responsible for listeriosis cases and outbreaks in ruminants (Borucki et al., 2005; Fenlon et al., 1996). The high prevalence of *L. monocytogenes* on dairy farms not only suggests that these environments may represent a major reservoir for *L. monocytogenes* (Ivanek et al., 2006), but also suggests that silage may be a superior source for listeriaphage isolation.

L. monocytogenes has also been commonly found in various types of food processing facilities (e.g., meat, poultry, dairy, and seafood processing plants) (Autio et al. 2002; Eifert et al., 2005; Lappi et al., 2004; Ojeniyi et al., 1996). *L. monocytogenes* have ability to grow over a wide range of temperatures (Farber and Peterkin, 1991), including refrigeration temperatures (<4°C to 5°C) (Walker et al., 2008), and pHs (Farber and Peterkin, 1991; Phan-Thanh and Montagne, 1998). These characteristics allow this pathogen to survive under stressful environmental conditions, and thus it is difficult to control this pathogen in the environment, particularly in food processing environments. In the seafood industry, specifically in smoked-fish processing plants, the major source of *L. monocytogenes* in RTE seafood products is cross-contamination in the processing plant environments (Norton et al., 2001; Hoffman et al., 2003; Hu et al., 2006; Rørvik et al, 1995; Thimothe et al., 2004).

Previous studies have shown that specific strains or subtypes could persist in these environments for months to more than a decade (Lappi et al., 2004; Orsi et al., 2008; Williams et al., 2011).

In the recent years (2008-2011), this pathogen has become an important public health problem in the US due to recurrent multistate outbreaks of listeriosis in a variety of foods (e.g., dairy products, meats, processed ready-to-eat (RTE) foods, fruits, and vegetables) (CDC, 2011). The most recent multistate outbreak of listeriosis was linked to cantaloupes which led to over 30 deaths, resulting in the deadliest foodborne illness outbreak in the US since 1924 (CDC, 2011). For decades, *L. monocytogenes* has been an important issue for the economy of the US food industry as demonstrated by a number of recalls of processed food products in the US. The annual cost of these recalls has been estimated as high as $1.2 to $2.4 billion in the US (Ivanek et al., 2006).

Listeriaphages are viruses that can specifically infect and lyse *Listeria* spp. However, at present, no listeriaphages with the ability to infect *L. grayi* have been found (Loessner and Rees, 2005). Listeriaphages have been successfully isolated from diverse sources, e.g., silage, water, sewage, soil, milk, cheese, as well as from lysogenic *L. monocytogenes* strains (Hodgson, 2000; Loessner et al., 1991). Overall, listeriaphages have been isolated from three different methods: (i) from environmental samples contaminated with *Listeria* spp., (ii) from *L. monocytogenes* lysogenic strains, and (iii) from listeriaphage collections around the world (Hodgson, 2000). Listeriaphages are well adapted to their hosts, and most of them can complete the lytic cycles between 10 and 37°C, except for some temperature-sensitive phages that can

only multiply at 25°C and below (Hodgson, 2000). Comparative genomic analysis has shown that listeriaphages have dsDNA genomes with size ranging from ca. 36 kb to ca. 131 kb, with G+C content of ca. 34.8 mol % to ca. 40.8 mol % (Dorscht et al., 2009; Klumpp et al., 2008). The functional modules of listeriaphage genome are organized into three clusters (Dorscht et al., 2009): (i) left cluster represents "late genes", which are transcribed rightward and encoding structural proteins, DNA packaging, and lysis system, (ii) gene cluster in the middle which is transcribed mostly in the opposite direction and encoding the lysogeny functions (integrase and repressor), and the prophage attachment and integration locus *attP*, and (iii) right cluster represents "early genes", which are transcribed rightward (similar to the late genes) and encoding products required for the early stage of phage reproduction (products for replication, recombination, and modification of the phage DNA). In addition, in virulent phages (strictly lytic), the lysogeny control functions are absent, therefore, the organization of the phage genomes will show only the early and late genes modules (Dorscht et al., 2009).

Listeriaphage has been studied and investigated its effectiveness as a biocontrol agent for *L. monocytogenes* in a variety of foods (e.g., hot dogs, soft cheese, and salmon fillet) (Carlton et al., 2005; Guenther et al., 2009; Leverentz et al., 2004; Soni and Nannapaneni, 2010). In addition, GRAS (generally recognized as safe) status has been granted to the listeriaphage P100, a virulent, wide-host-range phage with the ability to infect multiple serotypes and species of *Listeria* (Anonymous, 2007). While use of bacteriophage is recognized as an alternative strategy for controlling *L. monocytogenes* in raw and RTE foods (Anonymous, 2012), information

on the phage susceptibility of diverse *L. monocytogenes* strains that have been recovered from and persisted in food processing plant environments is still limited.

An improved understanding of listeriaphage ecology and diversity, including in primary production environments, is critically needed. In this study, we used dairy farms as a model system in a longitudinal study to (i) gain a better understanding of the ecology and diversity of listeriaphages in farm environments and in silage in particular; and (ii) further develop listeriaphage collections. In addition to development of a diverse phage collection, we also evaluated phage susceptibility (individual phages, phage cocktails, and a commercial phage product) of diverse persistent subtypes from food-associated environments, and whole genome sequencing and comparative genomic analysis of selected phages were performed to better understand genomic diversity and relationships of phages obtained from dairy farms.

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CHAPTER TWO

Listeriaphages are abundant and reveal considerable host range and genomic diversity in silage samples collected on dairy farms

ABSTRACT

As the foodborne pathogen *Listeria monocytogenes* is common in dairy farm environments, it is likely that phages infecting this bacterium ("listeriaphages") are abundant on dairy farms. To better understand the ecology and diversity of listeriaphages on dairy farms and to develop a diverse phage collection for further studies, silage samples collected on two dairy farms were screened for *L. monocytogenes* and listeriaphages. While only 4.5% of silage samples tested positive for *L. monocytogenes*, 47.8% of samples were positive for listeriaphages, containing up to >1.5 x 10^4 PFU/g. Host range characterization of the 114 phage isolates obtained, with a reference set of 13 *L. monocytogenes* strains representing the 9 major serotypes and four lineages, revealed considerable host range diversity; phage isolates were classified into 9 lysis groups. While one serotype 3c strain was not lysed by any phage isolates, serotype 4 strains were highly susceptible to phages and were lysed by 63.2 to 88.6% of phages tested. Overall, 12.3% of phage isolates showed narrow host range (lysing 1 to 5 strains), while 28.9% of phages represented broad host range (lysing ≥11 strains). Genome sizes of the phage isolates were estimated to range from approx. 26 to 140 kb. The extensive host range and genomic diversity of phages

observed here suggests an important role of phages in the ecology of *L. monocytogenes* on dairy farms. In addition, the phage collection developed here has the potential to facilitate further development of phage-based biocontrol strategies (e.g., in silage) and other phage-based tools.

INTRODUCTION

Listeria monocytogenes is a Gram-positive pathogenic bacterium that can cause a severe foodborne disease, listeriosis, in humans and farm ruminants. *L. monocytogenes* is considered ubiquitous in nature and has been isolated from a variety of environmental sources, e.g. water, soil, silage, plant vegetation, and food processing plants (5, 18, 19, 24, 30, 43). A number of studies have reported a high prevalence of *L. monocytogenes* in dairy farm environments (7, 20, 22, 34, 56). In addition, a previous study has found a considerably higher prevalence of *L. monocytogenes* in dairy farm environments than in urban and natural environments (46). Ruminants, including cattle, sheep, and goats, are not only often fecal shedders of *L. monocytogenes*, but are also hosts in which *L. monocytogenes* can cause a severe disease (42). Silage (i.e., fermented plant material that is commonly used as feed for ruminants), if spoiled or improperly fermented, has often been found to contain *L. monocytogenes* (3, 21), including at high numbers (>10^7 CFU/g silage) (63). Spoiled silage has also been reported to be the most important source of *L. monocytogenes* responsible for listeriosis cases and outbreaks in ruminants (7, 21). The high prevalence of *L. monocytogenes* on dairy farms and particularly in silage not only suggests that these environments may represent a major reservoir for *L.*

monocytogenes, but also suggests that silage may be a superior source for listeriaphage isolation (35).

Bacteriophages infecting *L. monocytogenes* and other *Listeria* spp. have been isolated from diverse sources (e.g., sewage, silage, water, food processing plant environments) as well as from lysogenic *L. monocytogenes* strains (31, 37, 41). Listeriaphages isolated from different sources have also previously been evaluated for host range diversity. For example, Loessner and Busse (41) observed 16 different lysis patterns, which could be classified into four lysis groups, among 16 listeriaphages isolated from sewage or lysogenic strains. While most *L. monocytogenes* serotype 1/2a and 4b strains were lysed by at least one of these phages, the majority of serotype 3a, 3b, and 3c strains were resistant to all phages. In another study, Hodgson (31) found that 6/59 phages represented broad host range, exhibiting ability to lyse all four strains of serotype 1/2 and all eleven strains of serotype 4b tested. Similarly, Kim et al. (37) reported that 9/12 listeriaphages isolated from two turkey processing plants were characterized as broad host range phages, exhibiting ability to lyse the majority of *L. monocytogenes* serotype 1/2a strains (16/26) and 4b strains (38/39). A number of listeriaphages from these and other studies have been well characterized, including by genome sequencing (12, 38, 38, 66), and have been developed for biocontrol and other applications, such as phage A511 (28, 29) and P100 (12, 29, 52). While listeriaphages A006, A500, B025, P35, and P40 have been characterized as members of the *Siphoviridae*, phages B054, A511, and P100 are members of the *Myoviridae*; all of them have double-stranded DNA genomes (12, 16, 38).

Recent studies suggest potential uses of listeriaphage as a biocontrol agent for *L. monocytogenes* in a variety of ready-to-eat (RTE) foods (12, 29, 32, 39, 40, 52). Some studies have also suggested the suitability of phages in controlling foodborne pathogens at the preharvest level and reducing shedding in animals (10, 11, 54). Only one study, by Kim et al. (37), has evaluated phage diversity in food processing plant environments; a better understanding of ecology and diversity of listeriaphage, including in primary food production environments, is thus still needed. Further establishment of diverse phages collections will also facilitate the development, improvement, and evaluation of listeriaphage based-biocontrol strategies. In this study, we used dairy farms as a model system in a longitudinal study to (i) gain a better understanding of the ecology and diversity of listeriaphages in farm environments, particularly in silage; and (ii) further develop listeriaphage collections.

MATERIALS AND METHODS

Sample collection. A total of 134 silage samples were collected from silage bunkers of two dairy farms in New York State between October 2007 and July 2009. The two dairy farms were selected based on owner's willingness to allow for frequent sample collection. No information on previous prevalence of *Listeria* spp. or bacteriophages was available for these farms. For farm 1, two preliminary sampling visits were completed in 10/2007 and 01/2008, with 19 samples collected (Table 2.1). Phage recovery results for the preliminary visits are not reported here as these collected samples were used to optimize phage isolation procedures. Subsequently, 62 samples were collected on farm 1 over seven sampling visits, one visit in 04/2008, followed by

Table 2.1. Recovery of *Listeria* spp., *L. monocytogenes*, and listeriaphages from silage samples collected.

Farm visit # (Sample collection date [mo/yr])	No. of silage samples tested	No. of samples positive for:			
		Listeria spp.[a]	*L. monocytogenes* (No. of isolates)	No. of samples that yielded plaques (no. of phage isolates)	
				Direct isolation	Enrichment method
Farm 1 - Preliminary sampling[b]					
3 (10/2007)	9	2	1 (2)	n/a	n/a
4 (01/2008)	10	2	3 (5)	n/a	n/a
Total	19	4	4 (7)	n/a	n/a
Farm 1					
5 (04/2008)	10	7	0	4 (7)	4 (6)
6 (08/2008)	10	0	0	4 (7)	3 (7)
7 (09/2008)	10	0	0	0	5 (0)[c]
8 (10/2008)	7	5	0	1 (2)	1 (0)[c]
9 (11/2008)	8	3	0	3 (3)	4 (6)
10 (12/2008)	9	2	0	1 (2)	2 (3)
11 (01/2009)	8	1	0	2 (2)	4 (4)
Total	62	18	0	15 (23)[d]	23 (26)[d]
Farm 2					
1 (02/2009)	10	2	0	3 (6)	5 (7)
2 (03/2009)	9	3	0	4 (6)	4 (7)
3 (04/2009)	10	0	0	3 (3)	1 (1)
4 (05/2009)	9	3	0	4 (7)	5 (7)
5 (06/2009)	7	6	2 (2)	2 (4)	3 (5)
6 (07/2009)	8	0	0	2 (5)	4 (7)
Total	53	14	2 (2)	18 (31)[e]	22 (34)[e]

Table 2.1. (Continued)

[a] *Listeria* spp. refers to *Listeria* spp. other than *L. monocytogenes*.

[b] Preliminary sampling visits 1 to 4 were used to collect samples for optimizing phage isolation procedures; results for phage isolation from these preliminary efforts are not reported. Visits 3 and 4 also included silage samples that were tested for *Listeria* spp. and *L. monocytogenes*; results were reported here as *L. monocytogenes* isolates were only obtained during visits 3 and 4 to farm 1.

[c] Positive samples yielded no phages that could be propagated.

[d] For farm 1, 12 samples were positive after enrichment only, while 4 samples were positive only by direct isolation and 11 samples were positive by both methods

[e] For farm 2, 10 samples were positive after enrichment only, while 6 samples were positive only by direct isolation and 12 samples were positive by both methods.

6 monthly sampling visits between 08/2008 and 01/2009. For farm 2, a total of 53 samples were collected during 6 monthly visits (Table 2.1). At each sampling visit, seven to ten silage samples were collected from silage bunkers into a sterile Whirl-Pak bag (Nasco, Modesto, CA). Only silage samples with a pH > 5.5 were collected as a pH at this level indicates improperly fermented silage, increasing the likelihood of *Listeria* spp. and listeriaphage isolation. Silage samples used for isolation here showed pH values of 6 to 6.5. Silage samples were processed for *Listeria* spp. and listeriaphage isolation within 6 h of collection; samples were thus not refrigerated upon collection.

Isolation of *L. monocytogenes*. Each silage sample (10 g) was transferred to a sterile Whirl-Pak bag and mixed with 90 ml of *Listeria* enrichment broth (LEB; Difco, Becton Dickinson, Sparks, MD). After 24 h and 48 h of incubation at 30°C, 50 µl of the enrichment was streaked onto Oxford plating medium (Difco, Becton Dickinson, Sparks, MD), followed by incubation at 30°C for 48 h. For each sample, up to four *Listeria*-like colonies were substreaked onto *L. monocytogenes* plating medium (LMPM; R-F Laboratories, Downers Grove, Il). Plates were incubated at 37°C for 48 h. On LMPM, *L. monocytogenes* and *L. ivanovii* appear as blue colonies, indicating phospholipase activity, while other *Listeria* spp. appear as white colonies (49). Blue colonies on LMPM plates were further characterized, as detailed below, to classify them to species and subtypes, while samples with white colonies representing *Listeria*-like characteristics were classified as positive for *Listeria* spp. other than *L. monocytogenes*.

Automated *EcoR*I ribotyping. Isolated blue colonies from LMPM were substreaked onto Brain Heart Infusion (BHI; Difco, Becton Dickinson, Sparks, MD) agar plates for characterization by an automated *EcoR*I ribotyping using the RiboPrinter® System (Dupont Qualicon™, Wilmington, DE). The RiboPrinter® software classifies ribotype patterns into DuPont IDs (e.g., DUP-1043) and a given DuPont ID can contain more than one distinct ribotype pattern (i.e., patterns that differ by a single weak band within a given DuPont ID). Different patterns within a given DuPont ID were designated with an additional letter (e.g., DUP-1043A and DUP-1043B).

Pulsed Field Gel Electrophoresis (PFGE) analysis. *L. monocytogenes* isolates were also characterized using the standard CDC *L. monocytogenes* PulseNet PFGE protocol (25, 26) with two restriction enzymes (*Apa*I and *Asc*I). PFGE was performed using the Bio-Rad™ CHEF Mapper electrophoresis unit. Images of PFGE patterns were acquired using the Bio-Rad™ Gel Doc software version 1.1 and analyzed using BioNumerics Software version 4.2 (Applied Maths, Sint-Martens-Latem, Belgium).

Bacterial strains and cultures for listeriaphage isolation. Four *L. monocytogenes* strains, representing serotypes 1/2a, 1/2b, 4a, and 4b, were consistently used as hosts for listeriaphage isolation and enrichment (Table 2.2). These serotypes include the most common *L. monocytogenes* serotypes and have been commonly used for listeriaphage isolation in other studies (31, 37, 41). While inclusion of *L. monocytogenes* isolates found on either farm would potentially improve detection of phages on a specific farm, this approach would have affected our ability to compare isolation frequency or levels of phages between farms without bias.

Table 2.2. *L. monocytogenes* strains used for listeriaphage isolation and phage host range determination.

L. monocytogenes strain (previous ID)[a]	Lineage	Source	Serotype	Ribotype	Reference(s)
FSL J1-175*	I	Water	1/2b	DUP-1042A	(4)
FSL J1-169	I	Human	3b	DUP-1052A	(23, 27)
FSL J1-049	I	Human	3c	DUP-1042C	(23, 65)
FSL R2-574 (F2365)*	I	Food	4b	DUP-1038B	(45)
FSL F6-367 (MACK)*	II	Lab strain	1/2a	DUP-1030A	(31)
FSL C1-115	II	Human	3a	DUP-1039C	(23, 27)
FSL J1-094	II	Human	1/2c	116-1501-S-4	(6, 23)
FSL F2-695	IIIA	Human	4a	DUP-1061A	(50)
FSL F2-501	IIIA	Human	4b	DUP-18606	(50)
FSL J2-071	IIIA	Animal	4c	DUP-1061A	(48, 50)
FSL W1-110	IIIC	Unknown	4b	DUP-1055	(15, 23)
FSL J1-208*	IV	Animal	4a	DUP-10142	(50)
FSL J1-158	IV	Animal	4b	DUP-10142	(15, 23)

[a] *L. monocytogenes* used as host strains for listeriaphage isolation are indicated with *; strains FSL J2-071, FSL J1-208, and FSL J1-158 were isolated from ruminants with clinical listeriosis symptoms.

Host strains were stored at -80°C in BHI broth with 15% glycerol and were streaked onto BHI agar plates before use. An overnight broth culture of each host strain was prepared by inoculating an isolated colony from a BHI agar plate into 5 ml of LB MOPS (LB medium buffered with 50 mM MOPS, pH 7.6). Cultures were incubated for 18 h at 30°C, with shaking at 220 rpm, to reach an OD_{600} of 0.5–0.6 (approx. 1 x 10^9 CFU/ml).

Isolation of listeriaphages. Listeriaphage isolation was performed, on the same samples used for *L. monocytogenes* isolation, following two methods: (i) direct phage isolation, and (ii) phage isolation after enrichment. Phage isolation after enrichment was used to isolate phages that may be present at low levels, while direct isolation facilitated isolation of phages with distinct plaque morphologies and allowed for phage quantification.

For direct phage isolation, silage samples (10 g) were mixed with 90 ml of Phosphate Buffered Saline (PBS), pH 7.4, in a sterile Whirl-Pak bag with a filtered screen (Nasco), followed by a manual homogenization. Each sample (approx. 90 ml) was then filtered through a 0.45-µm bottle-top filter, followed by filtration of a 1-ml aliquot through a 0.2-µm syringe filter. While we appreciate that recovery of some large phages may be jeopardized when using a 0.2-µm filter, this pore size has been used by others to isolate listeriaphages (37, 41). Filtrates from a 0.2-µm filter were used for phage isolation using the double-layer plate method (41), with minor modifications. Briefly, an overlay was prepared by mixing 300 µl of a 1:10 dilution of an overnight culture of a host strain (approx. 3 x 10^7 CFU/ml) with 100 µl of the sample filtrate and 4 ml of the soft agar, 0.7% LB MOPS/Glu/Salts agarose (LB

medium buffered with 50 mM MOPS, pH 7.6; 10 mM each $MgCl_2$ and $CaCl_2$) (31). This overlay mixture was poured onto a freshly prepared bottom agar plate (1.5% LB MOPS/Glu/Salts agarose). For each filtrate, this double-layer isolation was performed separately with each of the four host strains. Overlay plates were incubated at 30ºC for 24 h, followed by phage purification as detailed below.

For phage isolation after enrichment, 10 g of silage was transferred to a sterile Whirl-Pak bag with a filtered screen and mixed with 90 ml of LB MOPS, followed by addition of a 1 ml of the mixed overnight cultures of the four host strains (Table 2.2). The mixed cultures were prepared with 250 µl of each overnight host grown as described in the above section, thus containing approx. 2.5×10^8 CFU of each host strain. The sample enrichment was incubated at 30°C for 24 h. An aliquot (100 µl) of each sample enrichment was used for sequential filtration and phage isolation as detailed above.

Phage purification and preparation of high-titer phage lysate stock. One representative of each plaque morphology present on a given plate was used for phage purification. An isolated plaque was picked with a sterile Pasteur pipette and suspended in 100 µl of PBS. Four 10-fold serial dilutions of the plaque-PBS suspension were used to prepare overlay plates as described above, using the appropriate host strain. After incubation for 24 h at 30ºC, the overlay plate yielding the lowest number of isolated plaques was used for two more phage purification passages. An isolated plaque from the third passage was used to prepare three overlay plates. After 24 h incubation at 30ºC, 5 ml of PBS was used to harvest the overlay, followed by addition of chloroform to a final concentration of 2% (vol/vol),

centrifugation at 5500 rpm (rotor JA-17, Beckman Instruments, Palo Alto, CA) for 15 min, and filtration of the supernatant using a 0.2-µm syringe filter. While we appreciate that some phages may be sensitive to chloroform, phage titers sufficient for our experiments were obtained with this approach. Titers for each phage were determined by a spot test using the respective host strain used for phage growth. Eight 10-fold serial dilutions of the phage lysate (10 µl each) were spotted onto the host lawn, followed by incubation at room temperature for 24 h. Phage titers were also used to determine the routine test dilution (RTD), which was defined as the highest dilution that just fails to give confluent lysis. Phage lysate stocks were stored at 4ºC.

Listeriaphage host range determination. Spot tests of the 114 phages isolated here were performed, as two independent replicates, on 13 *L. monocytogenes* reference strains (see Table 2.2 for details on strains). These strains were chosen to represent the 9 most common serotypes as well as all four currently recognized *L. monocytogenes* lineages. Lawns for each reference strain were prepared as described above and spot tests were performed with 10 µl of phage lysates adjusted to a 100x RTD, representing approx. 1 x 10^5 to 5 x 10^6 PFU/ml (Table S2.1 in suppl. materials). Absence of bacterial inhibitory effects caused by high-titer phage suspensions was confirmed in the serial dilution spot tests detailed above. After 24 h of incubation at room temperature, each spot on the lawn was evaluated for lysis (+) or no lysis (-). Lysis was defined as occurrence of multiple single plaques or turbid or confluent lysis at a spot.

Phage lysis profiles on the 13 host strains were used to identify clusters of phages with similar host ranges. For this analysis, a spot test was considered as lysis if plaquing

was observed in at least one replicate. Hierarchical clustering was performed using Ward's method and binary distance in the R software (version 2.14.0; R Development Core Team, Vienna, Austria [http://www.R-project.org]). Clusters with a reference approximately unbiased (AU) value of >45% were assigned a cluster designation (e.g., cluster A).

Listeriaphage genome size determination. At least 25% of phage isolates obtained from each visit to a given farm (and at least one phage isolate from each visit) were selected for genome size determination. To the extent possible, phage isolates were selected to represent multiple isolation hosts. DNA extraction was performed using phage lysates prepared as described above, except that SM (NaCl-$MgSO_4$) buffer, pH 7.4, was used for phage harvest. Phages were precipitated using Polyethylene glycol 8000, in the presence of 1 M NaCl, followed by resuspension in SM buffer. DNase I (Promega BioScience, San Luis, Obispo, CA) (5 μg/ml final concentration) and RNase A (Sigma) (30 μg/ml final concentration) were added to digest nucleic acids from lysed bacterial cells. After addition of EDTA to a final concentration of 20 mM, phage DNA was purified using digestion with proteinase K (0.2 mg/ml) and SDS (0.5%), followed by extraction with phenol/chloroform, and ethanol precipitation. Genome sizes were then estimated using PFGE as previously described (33, 57). Briefly, the gel was run for 22 h in 1X TBE buffer (pH 8.0), at a 0.5 s to 5 s switch time, 6 V/cm, and an angle of 120°. Size standards of 8−48 kb and a λ PFGE marker (both Bio-Rad, Hercules, CA) were used to facilitate estimation of genome sizes, which was performed using BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium).

Transmission Electron Microscopy (TEM). TEM characterization was performed on seven selected phage isolates representing various genome sizes and lysis groups. Grids were prepared by placing 5 µl of phage lysate (>10^7 PFU/ml), prepared as described above, onto 200-mesh Formar-carbon-coated copper grids. After adsorption for 5 s, excess phage lysate was removed with a filter paper. Grids containing phage lysate were stained for 5 s with 5 µl of three different stains: 2% uranyl acetate, 2% sodium phosphotungstate, and 2% ammonium molybdate. Images were acquired with a Tecnai T-12 TWIN TEM (FEI, Hillsboro, OR).

Statistical Analysis. To estimate odd ratios for phage susceptibility of (i) serotype 4 and non-serotype 4 strains, and (ii) strains of different lineages, logistic regression was performed using a generalized linear model. The final model was then used to predict prevalence of phage susceptibility for strains with different characteristics, including 95% confidence intervals. All statistical analyses were performed using the R software (version 2.14.0; R Development Core Team, Vienna, Austria [http://www.R-project.org]).

RESULTS

Despite infrequent isolation of *L. monocytogenes*, listeriaphages are commonly isolated from silage samples collected on dairy farms. Among the 134 silage samples collected on two dairy farms (81 and 53 samples from farms 1 and 2, respectively), four samples from farm 1 and two samples from farm 2 were positive for *L. monocytogenes*. For farm 1, seven *L. monocytogenes* isolates obtained from 4 different samples collected during the two preliminary visits (10/2007 and 01/2008;

Table 2.1) were further characterized. These seven isolates represented four different PFGE types as well as three different ribotypes (DUP-1039C, DUP-1045A, DUP-1042C; see Figure S2.1 in suppl. materials), and were classified into lineage I (3 isolates) and II (4 isolates). The two *L. monocytogenes* isolates from farm 2 (Table 2.1) represented the same PFGE type as well as the same ribotype (DUP-1052A), and both were classified into lineage I (Figure S2.1). In addition, *Listeria* spp. other than *L. monocytogenes* were also isolated from a number of silage samples (Table 2.1). Excluding the 19 samples collected during the two preliminary sampling visits to farm 1, a total 115 silage samples (62 and 53 samples from farms 1 and 2, respectively) were screened for listeriaphages. Of these, 55 samples were positive for phages and 114 listeriaphage isolates were recovered, using four *L. monocytogenes* hosts and two phage isolation methods (i.e., direct isolation and isolation after enrichment). For farm 1, 27/62 samples were positive for phages, yielding 49 phage isolates (Table 2.1); 12 samples were positive after enrichment only, while 4 samples were positive only by direct isolation and 11 samples were positive by both methods. For this farm, 23 and 26 of the 49 phage isolates were obtained from direct isolation and isolation after enrichment, respectively. For farm 2, 28/53 samples were positive for phages, yielding 65 phage isolates (Table 2.1); 10 samples were positive after enrichment only, while 6 samples were positive only by direct isolation and 12 samples were positive by both methods. For this farm, 31 and 34 of the 65 phage isolates were obtained from direct isolation and isolation after enrichment, respectively. A possible explanation for detection of phages, in some samples, by direct isolation but not by enrichment would be either (i) degradation of phages during enrichment (e.g., due to proteases or

nucleases present in the enrichment or produced by bacteria, other than the host strains), or (ii) entry into a lysogenic cycle during enrichment.

The direct phage isolation method also allowed for enumeration of listeriaphages present in a given sample with a detection limit of 1.0×10^2 PFU/g (Table 2.3). Phage levels in 15 samples from farm 1 that were positive by direct isolation ranged from 1.0×10^2 to 1.5×10^4 PFU/g, with two samples showing phage levels that were "too numerous to count (TNTC)" on at least one host strain (Table 2.3). For farm 2, phage levels in 18 samples that were positive by direct isolation ranged from 1.0×10^2 to 1.2×10^4 PFU/g, with eight samples showing phage levels that were TNTC on at least one host strain. Due to variations in plaque sizes, TNTC could represent between 100 and 200 plaques per plate, therefore TNTC is estimated to represent $>2.0 \times 10^4$ PFU/g in our study.

Listeriaphages isolated here represent a wide diversity of host range characteristics. Host range determination of all 114 phage isolates, with 13 diverse *L. monocytogenes* reference strains (Table 2.2), classified these phage isolates into 56 different lysis profiles. Clustering analysis classified these lysis profiles into 9 distinct lysis groups (Figure 2.1 and Table S2.2 in suppl. materials). Each lysis group included between 4 (lysis group G) and 27 (lysis group I) phage isolates. While most lysis groups were comprised of similar numbers of phage isolates from each farm, three groups (E, G, and H) included phage isolates predominantly from farm 2, and group I included phage isolates predominantly from farm 1 (Table S2.2). Among the 9 lysis groups, two groups (E and F; representing about 28.9% of phages tested) demonstrated broad host range, exhibiting ability to lyse 11 to 12 of the 13 reference

strains. Only 5/49 phage isolates from farm 1, but 28/65 phage isolates from farm 2 fell into these two broad host range groups (Table S2.2). Two lysis groups (A and C), representing a narrow host range phages with ability to lyse 1 to 5 strains, included 12.3% of the 114 phages characterized. The majority of the 114 phages (58.7%) showed ability to lyse between 6 and 10 of the reference strains tested and were classified into five lysis groups (B, D, and G–I).

Most listeriaphages lyse all serotype 4 strains as well as the serotype 1/2a strain Mack. Among the 13 reference strains, seven strains representing serotypes 4a (n=2), 4b (n=4), and 4c (n=1) were lysed by 63.2 to 88.6% of the 114 phages (Table 2.4). Among the "non-serotype 4" strains, only the serotype 1/2a strain Mack was also lysed by a large proportion of phage isolates (74.6%), while the other serotype 1/2b, 1/2c, 3a, and 3b strains were lysed by 22.8 to 40.4% of phage isolates. The serotype 3c strain FSL J1-049 was not lysed by any phage isolates (Table 2.4). Hierarchical clustering of these reference strains based on similarities in phage susceptibility was consistent with these findings. The seven serotype 4 strains and the serotype 1/2a strain Mack were classified into the same major cluster (X), while the serotype 3c strain FSL J1-049, which was highly resistant to all phages, was classified into its own cluster (Z; see Figure 2.1). The other serotype 1/2b, 1/2c, 3a, and 3b strains were grouped into cluster Y (Figure 2.1). Overall prevalence of phage susceptibility was 51.9% (95% CI: 35.5–67.8) among the non-serotype 4 strains and 88.9% (95% CI: 80.3–94.0) among the serotype 4 strains (Table 2.4), indicating a significant difference in phage susceptibility among these two groups ($p<0.001$).

Table 2.3. Enumeration of listeriaphages for samples positive by direct phage isolation.

Sample collection date (mo/yr)	Sample	Enumeration[a] (PFU/g) of listeriaphages on host strain (serotype)			
		J1-175 (1/2b)	F2365 (4b)	MACK (1/2a)	J1-208 (4a)
Farm 1					
04/2008	H-S5-S31D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	4.0×10^2
	H-S5-S32D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	2.0×10^2	5.3×10^3
	H-S5-S39D	$<1.0 \times 10^2$	4.3×10^3	$<1.0 \times 10^2$	1.2×10^3
	H-S5-S40D	$<1.0 \times 10^2$	$>2.0 \times 10^4$	$<1.0 \times 10^2$	$>2.0 \times 10^4$
08/2008	H-S6-S44D	$<1.0 \times 10^2$	2.0×10^2	$<1.0 \times 10^2$	2.0×10^2
	H-S6-S46D	$<1.0 \times 10^2$	5.0×10^3	1.0×10^3	$>2.0 \times 10^4$
	H-S6-S47D	$<1.0 \times 10^2$	3.0×10^2	1.0×10^2	1.0×10^3
	H-S6-S50D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	4.0×10^2
09/2008	None	None	None	None	None
10/2008	H-S8-S64D[b]	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	(i) 7.0×10^2 (ii) 2.6×10^3
11/2008	H-S9-S68D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	2.5×10^3
	H-S9-S72D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	1.1×10^3
	H-S9-S73D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	1.2×10^3
12/2008	H-S10-S80D	$<1.0 \times 10^2$	1.0×10^2	$<1.0 \times 10^2$	2.0×10^2
01/2009	H-S11-S85D	$<1.0 \times 10^2$	1.0×10^2	$<1.0 \times 10^2$	$<1.0 \times 10^2$
	H-S11-S90D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	1.5×10^4
Farm 2					
02/2009	A-S1-S1D	$<1.0 \times 10^2$	6.0×10^2	6.0×10^2	8.0×10^2
	A-S1-S8D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	1.2×10^3
	A-S1-S10D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$>2.0 \times 10^4$	1.0×10^4
03/2009	A-S2-S15D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$>2.0 \times 10^4$
	A-S2-S16D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$>2.0 \times 10^4$
	A-S2-S17D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	1.2×10^4
	A-S2-S18D	$<1.0 \times 10^2$	1.0×10^2	1.0×10^2	$>2.0 \times 10^4$
04/2009	A-S3-S22D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	1.0×10^2
	A-S3-S23D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$>2.0 \times 10^4$
	A-S3-S24D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$>2.0 \times 10^4$
05/2009	A-S4-S30D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	1.0×10^2
	A-S4-S31D	$<1.0 \times 10^2$	4×10^2	1.2×10^3	3.2×10^3
	A-S4-S34D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	2.0×10^2	$<1.0 \times 10^2$
	A-S4-S36D	$<1.0 \times 10^2$	1.0×10^2	$<1.0 \times 10^2$	1.0×10^2
06/2009	A-S5-S42D	$<1.0 \times 10^2$	3.0×10^3	3.8×10^3	$>2.0 \times 10^4$
	A-S5-S43D	$<1.0 \times 10^2$	$<1.0 \times 10^2$	1.0×10^2	$<1.0 \times 10^2$
07/2009	A-S6-S47D	$<1.0 \times 10^2$	3.0×10^2	1.0×10^2	$<1.0 \times 10^2$
	A-S6-S48D	$<1.0 \times 10^2$	8.0×10^2	3.3×10^3	$>2.0 \times 10^4$

[a] Samples that did not yield plaques on a given host were reported as $<1.0 \times 10^2$ PFU/g of silage, the detection limit of the method used.

Table 2.3. (Continued).

Due to variations in plaque sizes, presence of 100-200 plaques typically represented the cut-off for countable plaque numbers; samples that yielded too numerous plaques to be counted were thus reported as $>2.0 \times 10^4$ PFU/g of silage.

[b] This sample showed two plaque morphologies, number of PFU/g was reported for each type of plaque morphology, indicated as (i) and (ii).

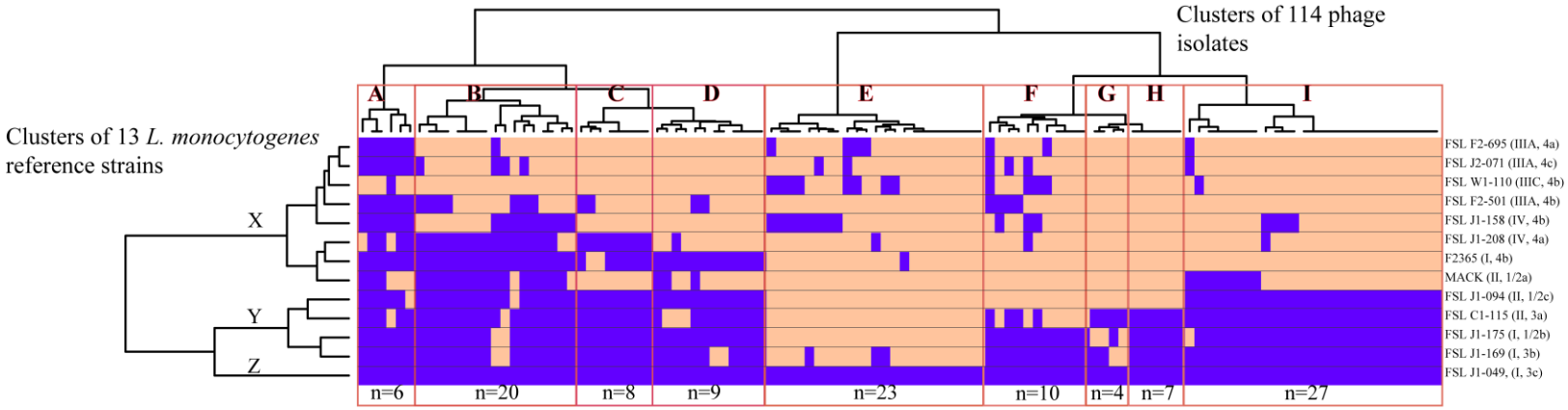


Figure 2.1. Heatmap and hierarchical clustering of lysis profiles from the host range determination of 114 listeriaphages. Beige represents lysis (+) and blue represents no lysis (-) on a given strain. Phage isolates are shown on the horizontal axis; clusters are designated (A to I) above the figure based on similarities of the lysis profiles using Ward's method and binary distance in the R software with a reference approximately unbiased (AU) value of >45%. Host strains are shown on the vertical axis; clusters are designated (X to Z) on the left of the figure based on similarities in susceptibility to phages.

Strains of lineages III and IV were lysed by a large proportion of phage isolates (77.2–88.6%; see Table 2.4). This is consistent with the fact that all strains from these two lineages represent serotype 4. Lineage I strains showed considerable diversity regarding phage susceptibility, representing the range from 0 to 63.2% (Table 2.4). Overall, prevalence of phage susceptibility was higher among strains in lineage III (98.5%; 95% CI: 75.2–89.2) and IV (83.3%; 95% CI: 75.2–89.2) as compared to those in lineage I (68.1%; 95% CI: 47.4–83.4) and II (25.4%; 95% CI: 12.3–45.3). Consistent with the high phage susceptibility of serotype 4 of lineage III and IV strains, the majority of phages were isolated on the lineage IV serotype 4a host strain FSL J1-208 (60/114 phages) and the lineage I serotype 4b host strain F2365 (25/114 phage isolates) (Table S1 and Figure S2.2 in suppl. materials).

Listeriaphages markedly differ in genome sizes, indicating genetic diversity of phages on dairy farms. Phage genome sizes were determined, for at least one phage isolate per visit to a given farm, to initially characterize the genetic diversity of the phages isolated here. Among 72 phage isolates tested (30 and 42 from farms 1 and 2, respectively), ten (four from farm 1, and six from farm 2) did not yield a clear band (or bands) after PFGE analysis, even though OD_{260} measurements suggested presence of appropriate amounts of nucleic acid to yield a detectable band. These 10 phage isolates represented 4 different lysis groups. While further analysis on a 0.7% agarose gel showed a nucleic smear, suggesting a single stranded RNA or DNA genome, additional experiments will be needed to characterize the genome of these isolates. The other 62 phage isolates showed a genome size range from 26 to 140 kb (Table 2.5). One phage from farm 2, classified into lysis group F, initially showed three bands

Table 2.4. Susceptibility of *L. monocytogenes* reference strains to listeriaphages.

L. monocytogenes Strain	Serotype (Lineage)	No. of phage lysis groups[a] lysing specific strain (%)	No. of phages lysing specific strain (%)	% Prevalence of phage susceptibility[b] (95% CI)
Non-serotype 4 strains				51.9 (35.5–67.8)
MACK	1/2a (II)	8 (89)	85 (74.6)	
FSL J1-175	1/2b (I)	4 (44)	29 (25.4)	
FSL J1-094	1/2c (II)	6 (67)	46 (40.4)	
FSL C1-115	3a (II)	6 (67)	35 (30.7)	
FSL J1-169	3b (I)	4 (44)	26 (22.8)	
FSL J1-049	3c (I)	0	0	
Serotype 4 strains				88.9 (80.3–94.0)
FSL F2-695	4a (IIIA)	8 (89)	101 (88.6)	
FSL J1-208	4a (IV)	9 (100)	101 (88.6)	
F2365	4b (I)	8 (89)	72 (63.2)	
FSL F2-501	4b (IIIA)	8 (89)	93 (81.6)	
FSL J1-158	4b (IV)	8 (89)	84 (73.7)	
FSL W1-110	4b (IIIC)	7 (78)	88 (77.2)	
FSL J2-071	4c (IIIA)	8 (89)	99 (86.8)	

[a] See Table S2.2 for details on the 9 lysis groups. A phage lysis group was classified as lysing a reference strain if any phages in a given lysis group showed lysis on a given host strain.

[b] Prevalence of phage susceptibility ($p<0.001$) among reference strains that were classified into (i) non-serotype 4 strains and (ii) serotype 4 strains.

of approx. 41, 83, and 115 kb; PFGE analysis of this phage DNA after heating at 75°C for 15 min showed a single band at 40 kb, indicating presence of cohesive ends that facilitated genome multimerization. For 35 phage isolates, PFGE analysis revealed two bands of similar size. The size difference of these two bands was approx. 3–6 kb. Twenty-three phage isolates from farm 1 showed the "two-band" pattern with sizes of 58–64 kb for the small band and 63–68 kb for the large band; for farm 2, 12 phage isolates showing the "two-band" patterns with sizes of 57–63 kb for the small band and 61–68 kb for the large band. These phages represented seven lysis groups (A–F, and I). Although all phage lysates were prepared after purification for three passages, selected phage isolates with these two-band patterns were re-purified, but still maintained the same patterns. PFGE after heat treatment at 72ºC for 15 min (performed for selected phages) also yielded the same patterns, suggesting that secondary structures (or presence of cohesive ends) may not be responsible for the observed two-band patterns. While both bands typically showed different DNA concentrations, there was no consistent pattern such that either the larger or smaller band was always at a higher concentration (see Table S2.3 in suppl. materials). Full genome sequencing of four phage isolates from different lysis groups with these banding patterns (unpublished data) allowed for assembly into a single genome of a size nearly the same as the larger band, suggesting the presence of a single phage. While a variety of packaging mechanism may explain these two chromosome variants, phages with these patterns might be "headful packaging" phages which contain genomes that are terminally redundant and circularly permuted as observed in phages P1, P22, and T4 (14, 58, 60, 64). Assembly of two capsid variants with different sizes

Table 2.5. Genome size diversity of selected listeriaphages[a]

Phage lysis group	Genome size[b] (kb) of representative phage isolates from each farm [visit no.[c]] {phage family[d]}	
	Farm 1	Farm 2
A	61/65 [6] 31 [9]	57/61 [4] {Sipho} 58/63 [5]
B	62/65 [5] {Sipho} 61/66 [6] 58/63 [8] 60/66 [10] 61/65 [11]	66; 60/65 [1] 65 [2] 58/63 [3] 57/62 [4] 60/65 [5] 60/63 [6]
C	64/68 [6] 62/67 [10]	63 [1] 61/65; 63/68 [2]
D	62/67 [6] 61/66; 63/68 [11]	62; 63; 60/65 [1] 61/65 [2] 68 [6]
E	N/D	97; 119 [1] 140 [5] 59/63; 70; 117; 127 {Myo}; 131; 132; 134; 135; 136 [6]
F	61/65 [5] 59/63; 61/67 [9]	121 [1] 64 [2] 41/83/115 [4]
G	None	123 [4]
H	123 [9]	32 [2] {Myo} 26 [4]
I	59/63 [5] 33 {Myo}; 60/64; 61/65 [6] 58/64 [8] {Sipho} 62/66 [9] 61/65 [10]	32 [2]

Table 2.5. (Continued).

[a] At least 25% of phage isolates obtained from each visit to a given farm were selected for genome size estimation; for each visit at least one isolate was characterized. To the extent possible, phage isolates were selected to represent multiple isolation hosts.

[b] Phage genome sizes were estimated by PFGE analysis and size estimation using the BioNumerics software. 'None' indicates no phage isolate was classified into this lysis group; 'N/D' indicates genome size determination was not performed with phage isolates of this lysis group. For some phage isolates, two bands of similar sizes were observed by PFGE analysis and the estimated sizes for both bands are indicated (e.g., 60/65 kb).

[c] See Table 2.1 for details on farm sampling visits.

[d] TEM characterization was performed on seven selected phage isolates representing various genome sizes and different lysis groups. Phage family classification is indicated in {}; 'Myo' represents *Myoviridae*, and 'Sipho' represents *Siphoviridae*.

could lead to packaging of two distinct chromosome lengths which could then result in variants of two sizes. This is consistent with data on T4, which has been shown to form a petite variant, which could be more or less common than the full-size capsid, e.g., depending on time after infection (17).

Overall, all nine lysis groups included phages with various genome sizes (Table 2.5). Genome size diversity was also observed among phages in a given lysis profile from the same farm. For example, phages in lysis group F from farm 2 revealed three distinct genome sizes (Table 2.5). These findings suggest that phages exhibiting similar host ranges, even among phages from the same farm, still show considerable genetic diversity. Genome sizes of phages from farm 1 ranged from approx. 31 kb (one phage of lysis group A) to 123 kb (one phage of lysis group H). For farm 2, the smallest phage genome size was approx. 26 kb (one phage of lysis group H), while 12/42 phage isolates, classified into 3 lysis groups (E–G), showed large genome sizes with the range of 97 to 140 kb (Table 2.5).

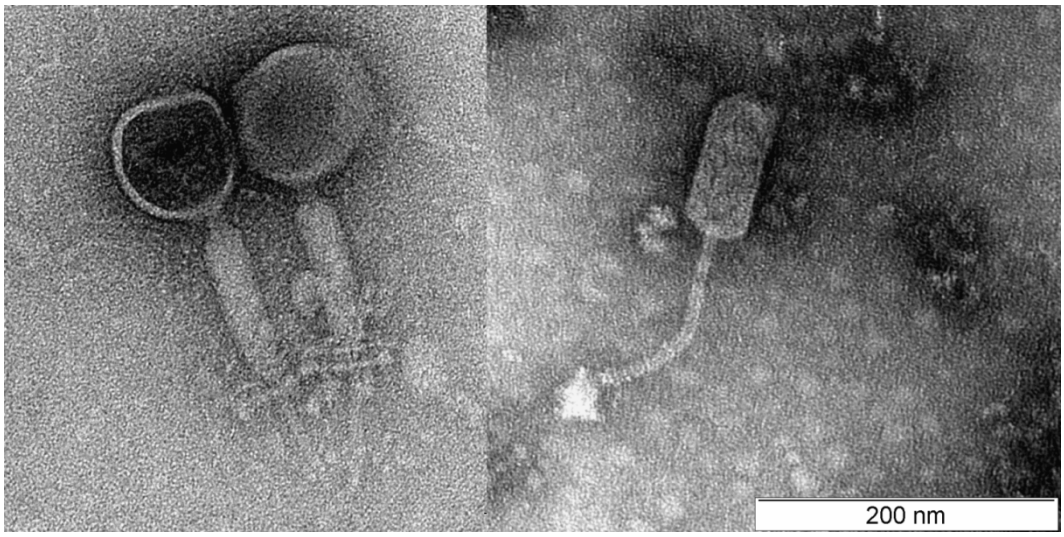
Combined analysis of phage genome size and lysis patterns of phages from a given farm also provided preliminary evidence of phage persistence. For example, for farm 1, phages representing genomes of the “two-band” patterns (approx. 60 and 65 kb), classified into lysis group I, were isolated from samples collected during five visits to farm 1 (Table 2.5). For farm 2, phages that grouped into lysis group B and showed these two-band patterns were also isolated over multiple visits (Table 2.5). While data on genome size and host range patterns indicate re-isolation of the same or similar phages from a given farm over time, analysis of these phages (e.g., restriction

fragment length polymorphism (RFLP) analysis) is needed to assess persistence of specific phages on these two farms.

TEM shows that selected listeriaphages represent two families of tailed phage. Seven selected phages (four and three phage isolates from farm 1 and 2, respectively) were characterized by TEM. Phage images allowed for classification of these phages as tailed phages of two phage families, *Myoviridae* and *Siphoviridae* (Table 2.5 and Figure 2.2). Three phage isolates, representing lysis groups E, H, and I, exhibited morphotype A1 (binary symmetry with contractile tail) and were thus classified in the *Myoviridae* family (1, 2) (Table 2.5). Four phage isolates, representing lysis groups A (n=1), B (n=1), and I (n=2), exhibited morphotype B3 (binary symmetry with a long, noncontractile tail) and were thus classified in the *Siphoviridae* family (1, 2) (Table 2.5).

DISCUSSION

In this study we used dairy farms as a model system to develop a better understanding of the ecology and diversity of listeriaphages, with a focus on silage, which is well established to support growth of *L. monocytogenes* to high levels and to be a source associated with animal listeriosis. Our data specifically demonstrate that (i) listeriaphages are abundant in silage available on dairy farms, (ii) *L. monocytogenes* lineage III and IV and serotype 4 strains are highly susceptible to phages, and (iii) except for a largely conserved ability to lyse serotype 4 strains, listeriaphages show considerable host range and genome size diversity. The diverse phage collection described here also represents a promising resource for further development of



(A) (B)

Figure 2.2. TEM images of selected listeriaphages. (A) listeriaphage LP-124 (lysis group E), obtained from farm 2 on host strain F2365 (serotype 4b). This phage exhibits morphotype A1 with long contractile tail; this phage is thus classified into the *Myoviridae* family. (B) listeriaphage LP-010 (lysis group B), obtained from farm 1 on host strain FSL J1-208 (serotype 4a). This phage exhibits morphotype B3 with long non-contractile tail; this phage is thus classified into the *Siphoviridae* family. Size bar applies to both panel A and B.

listeriaphages as a biocontrol agent (e.g., to control *L. monocytogenes* in silage) and other phage-based applications as well as for further genomic studies of listeriaphages.

Listeriaphages are abundant in farm environments and may be persist over time. While phages are in general well known to be the most abundant entities in the environment (8, 9, 51), the relative abundance of species-specific phages (e.g., listeriaphages) in different environments is less well studied. In our study, listeriaphages were isolated from the majority of silage samples with some samples representing phage levels of $>1.5 \times 10^4$ PFU/g of silage. Interestingly, a high prevalence of phages infecting *L. monocytogenes* was observed despite the fact that the majority of silage samples were not positive for *L. monocytogenes*, possibly suggesting that *L. monocytogenes* were eliminated by phages present. On the other hand, as *Listeria* spp. other than *L. monocytogenes* were isolated from a number of silage samples, other *Listeria* spp. may be hosts that facilitated replication of these phages. In addition, it is possible that members of other closely related Gram-positive bacterial genera could serve as natural hosts of listeriaphages as supported by the finding that some *Staphylococcus aureus* phages had been shown to facilitate horizontal transfer of DNA into *Listeria* (13). Further phage host range characterization with other potential hosts, particularly *Listeria* spp. isolates, would be needed to better understand whether hosts other than *L. monocytogenes* could facilitate propagation of phages isolated here.

While silage samples have previously been used to isolate listeriaphages for further characterization (31) and while it is well known that poorly fermented silage is commonly contaminated with high levels of *L. monocytogenes* (18, 19, 22, 30),

prevalence and levels of listeriaphages in silage have not previously been reported. In one previous study that reported listeriaphage prevalence among samples collected from two turkey processing plants, 12 listeriaphage isolates were obtained from 8 out of 113 samples tested (37). The high prevalence of listeriaphages observed in silage samples here, not only suggests that improperly fermented silages, and possibly dairy farm environments in general, are good substrates for listeriaphage isolation, but also suggests that phage mediated horizontal gene transfer in *L. monocytogenes* may be particularly frequent in these environments. This hypothesis is consistent with the previous finding that lineage III and IV *L. monocytogenes* strains, which are highly susceptible to phages (see below) and most common among ruminants, also show high level of horizontal gene transfer (44, 47, 50).

Based on phage lysis and genome size patterns, our data also provide preliminary evidence that listeriaphages persist in dairy farm environments over time. While no other studies have reported or investigated listeriaphage persistence in natural environments, some studies provide evidence for persistence of other phages in different environments (e.g., marine environment, dairy processing plants, and slaughter facility) (33, 36, 53, 61). For example, Rousseau and Moineau (53) reported that two lactococcal phages, isolated from the same cheese factory over 14 months apart, showed not only the same lysis pattern (determined using 30 *L. lactis* strains), but also showed "100% identical genomes", suggesting persistence of these phages in this facility for more than a year.

***L. monocytogenes* lineage III and IV strains (serotypes 4a, 4b, and 4c) are highly susceptible to phages and represent superior hosts for phage isolation.** Host range

determination of the 114 phage isolates showed that *L. monocytogenes* lineage III and IV strains (all represent serotypes 4a, 4b, and 4c) as well as the only lineage I serotype 4b strain included in our host strain set were lysed by the majority of our phage isolates. These observations are consistent with a number of previous studies (31, 37, 41, 59), including a report by Loessner and Busse (41), who reported that most serotype 4 strains (96%) were sensitive to at least 1 of the 16 phages tested. By comparison, Kim et al. (37) found that serotype 4b strains were typically sensitive to most phages isolated from the turkey processing plants. Somewhat contradictory to our findings, Shen et al. (55) reported that 5/8 *L. monocytogenes* isolates, classified into serogroup 4b based on PFGE typing, showed resistance to listeriaphage cocktail consisting of 6 phages. Our study also showed that the serotype 1/2a strain Mack (classified into lineage II) was lysed by most phages. This finding is consistent with the study by Kim et al. (37) that found the majority of serotype 1/2a strains (16/26) to be sensitive to most phages tested. A study by Rossi et al. (52) also showed that a serotype 1/2a strain spiked in Brazilian fresh sausage samples was sensitive to listeriaphage P100.

The findings that the one serotype 3c strain evaluated was resistant to all phages tested here and that the serotype 3a and 3b host strains were resistant to a considerable number of phages are consistent with previous report by Loessner and Busse (41) that serotype 3a, 3b, and 3c strains were typically untypable by phage typing, due to their resistance to all 16 phages tested. Kim et al. (37) also found that all 3 isolates from turkey processing plants representing serotypes 3c or 1/2c were not lysed by phage A511 and two broad host range listeriaphages obtained from the same environment.

Moreover, Shen et al. (55) found that 11/51 *L. monocytogenes* isolates classified based on PFGE typing, into serogroup 3b or 1/2b, were resistant to a listeriaphage cocktail. While specific mechanisms of phage resistance for serotype 3 and 1/2c strains remain unknown, cell wall teichoic acids (TA) and glucosamine in particular have been shown to be receptors for listeriaphages and absence or alteration of this TA substituent can convey phage resistance [e.g., Wendlinger et al. (62)].

Overall, our data not only provide further evidence that, on a population basis, *L. monocytogenes* serotypes differ in phage resistance, but also suggest that selection of *L. monocytogenes* strain(s) as hosts for phage isolation can considerably affect phage isolation frequency. Lineages III and IV and serotype 4b strains, as well as the serotype 1/2a strain Mack are likely to facilitate better phage recovery and thus are highly recommended as hosts for phage isolation. In addition, use of strains with serotypes that are typically resistant to phages as hosts for phage isolation will facilitate isolation of phages that may be able to lyse these strains and thus can be important for biocontrol and other applications.

Except for a largely conserved ability to lyse serotype 4 strains, listeriaphages show considerable host range and genome size diversity. Host range determination of the 114 phage isolates showed that these phage isolates could be classified into 9 lysis groups. Lysis groups E and F, which included broad host range phages with ability to lyse 11 to 12 strains, accounted for 28.9% of the 114 phages. By comparison, Loessner and Busse (41) found that only 3/16 phages characterized in their study were classified into the broad host range phage group, whereas most phages in this collection represented narrow host range (lysis of 9 to 21 out of 57 strains).

Interestingly, all broad host range phages in their study (41) were isolated from environmental samples (i.e., sewage). Similarly, all 6 broad host range phages reported by Hodgson (31) were isolated from sewage and silage samples. However, a study by Kim et al. (37) reported that the majority of phages (i.e., 9/12) from the turkey processing plants were classified in the broad host range group with ability to lyse all 27 *L. monocytogenes* strains and 4/5 *Listeria* spp. tested. Differences in sources of phages and protocols, including host strains used for enrichment and phage isolation, may contribute to the differences in host ranges observed among the phages from these studies.

While a considerable number of listeriaphages (>400 phages) have previously been isolated and characterized, genome sizes of <20 listeriaphages have been determined using PFGE analysis or genome sequencing (12, 16, 31, 38). The majority of previously reported listeriaphages showed genome sizes with a range of 35.6 kb (phage P40; accession no. EU855793) to 48.2 kb (phage B054; accession no. DQ003640). While no previous listeriaphage genome between 50 and 130 kb has been reported, two *Myoviridae*-family listeriaphages showed large genome sizes, of 131.4 kb (phage P100; accession no. DQ004855) and 137.6 kb (phage A511; accession no. DQ003638). By comparison, the 72 phages whose genome sizes were determined here showed genome sizes ranging from approx. 26 to 140 kb, including several phages with genome sizes of between 55 and 70 kb. A number of phages isolated in the current study thus show genome sizes that have not been previously reported among listeriaphages, suggesting that these are novel listeriaphages. However, additional

analyses, including, for example, genome sequencing, are still needed to assess similarities among phages in our collection and previously described phages. The phage collection developed here will provide opportunities for further studies on the genomics and biology of listeriaphages, in addition to providing a potential initiation of further development of phage-based biocontrol strategies (e.g., control of *L. monocytogenes* in silage) and other applications. However, additional comprehensive characterization of these phages is necessary for identification of specific phages appropriate for these applications. For example, full genome sequencing is particularly needed to confirm that phages to be used as a biocontrol agent do not carry antibiotic resistance or putative virulence genes.

ACKNOWLEDGEMENTS

The project was supported by USDA-CSREES Hatch Funds (NYC-143445) and the Royal Thai Government Fellowship (to KV). We thank Richard Calendar of UC Berkeley for providing a *L. monocytogenes* strain, and for his helpful suggestions on the phage work. We also thank Matthew Stasiewicz and Pajau Vangay for their help on statistical analysis.

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CHAPTER THREE

Listeriaphage susceptibility of persistent *Listeria monocytogenes* subtypes isolated from a smoked fish processing plant

ABSTRACT

Contamination of Ready-To-Eat foods with *Listeria monocytogenes* can typically be traced back to post-processing contamination from environmental sources; contamination is often linked to subtypes that persist in food associated environments. Although phage-based biocontrol strategies have been proposed for controlling this pathogen, information on the efficacy of phage treatments against diverse *L. monocytogenes* subtypes from food associated environments is still limited. We identified subtypes that were repeatedly found ("persistent") in a smoked fish processing facility by using *Eco*RI ribotyping data for isolates obtained in 1998–2009. PFGE analysis of 141 isolates (9 ribotypes) confirmed persistence for up to 11 years. Characterization of selected isolates representing persistent subtypes showed a wide range of susceptibility to a panel of 28 phages, ranging from 4.6% (ribotype DUP-1043A) to 95.4% (ribotype DUP-1044A). In challenge studies using phage cocktails and a commercial phage product, one isolate (ribotype DUP-1043A) was not affected by any treatments. In phage susceptible isolates, a reduction in *L. monocytogenes* counts of up to 4 log units was observed within 8 h after treatments, but subsequent re-growth occurred. Survivor isolates obtained after 24 h of treatment showed decreased susceptibility to individual phages included in the phage cocktail, suggesting rapid emergence of resistant subtypes.

1. Introduction

The foodborne pathogen *L. monocytogenes* is widely distributed in nature and has been isolated from the environment of various types of food processing facilities (e.g., meat, poultry, dairy, and seafood processing facilities) (Autio et al., 2002; Eifert et al., 2005; Lappi et al., 2004; Ojeniyi et al., 1996) as well as retail establishments (Sauders et al., 2004). Contamination of Ready-To-Eat (RTE) foods usually occurs at the post-processing stage, with food processing environment representing the key source of *L. monocytogenes* that contaminates RTE foods (Kathariou, 2002; Kornacki and Gurtler, 2007; Tompkin, 2002). In many cases, contamination of food with *L. monocytogenes* can be linked to strains that were repeatedly found ("persistent") in a food processing facility or other environment (e.g., at retail). Specific *L. monocytogenes* subtypes have also been shown to persist in food associated environments for months to more than a decade (Lappi et al., 2004; Orsi et al., 2008; Williams et al., 2011).

Listeriaphages (*Listeria*-specific bacteriophages) have been studied and evaluated for their efficacy as a biocontrol agent for *L. monocytogenes* in a variety of foods (e.g., hot dogs, soft cheese, and salmon fillet) (Carlton et al., 2005; Guenther et al., 2009; Leverentz et al., 2004; Soni and Nannapaneni, 2010). In addition, GRAS (generally recognized as safe) status has been granted to the listeriaphage P100, a virulent, broad-host-range phage with the ability to infect multiple serotypes and species of *Listeria* (Anonymous, 2007). While use of bacteriophage has been proposed as an alternative strategy for controlling *L. monocytogenes* in raw and RTE foods (Anonymous, 2012), some concerns have been raised about routine application of

phage treatment in foods (EFSA, 2009). Specifically, previous studies have shown that phages could not completely eliminate *L. monocytogenes* populations in different food matrices and that subsequent re-growth of *L. monocytogenes* occurred (Guenther et al., 2009; Bigot et al., 2011; Leverentz et al., 2003; Soni et al., 2009), raising concerns about emergence of phage resistant populations during or after treatment. Other concerns about the presence of phage-resistant strains of *L. monocytogenes* have also been raised (EFSA, 2009; Ferreira et al., 2011). Kim et al. (2008) evaluated for phage resistance among *L. monocytogenes* isolated from turkey processing plants, including some apparent persistent strains, and found that some isolates in their study were resistant to all phages tested. In a similar study, Ferreira et al. (2011) evaluated for resistance against 26 phages among the 41 isolates obtained from sausages and environments associated with sausage production and distribution, and could identify one isolate that was resistant to all 26 phages as well as a number of isolates that showed weak lysis (or resistance) with all phages tested in the study. While these studies provide some initial characterization of phage resistance patterns among *L. monocytogenes* isolates from food associated sources, further comprehensive data are still needed on (i) susceptibility of diverse *L. monocytogenes* strains which have been recovered from and persisted in food processing plant environments to individual phages as well as phage cocktails (mixture of several phages), and (ii) emergence of phage resistance after treatment with phages.

In the current study, we thus identified persistent subtypes in a smoked fish processing facility using *Eco*RI ribotyping data for isolates recovered from 1998–2009. Selected isolates representing these persistent subtypes were used to (i) further

characterize by Pulsed Field Gel Electrophoresis (PFGE) analysis to confirm persistence; (ii) evaluate phage susceptibility of selected isolates against phages, phage cocktails, and a commercial phage product; and (iii) evaluate survivor isolates recovered after 24 h from phage challenge experiments for resistance against previously challenged phages.

2. Materials and methods

2.1. *L. monocytogenes* isolates

Most *L. monocytogenes* isolates selected for characterization in this study had previously been isolated from environmental, raw fish, and finished product samples from a single smoked fish processing facility. These isolates were recovered from 1998–2004 (Lappi et al., 2004; Hoffman et al., 2003; Hu et al., 2006; Norton et al., 2001; Thimothe et al., 2004), and all of these isolates were previously characterized by automated *Eco*RI ribotyping. Isolates have been stored at -80°C in Brain Heart Infusion (BHI; Difco, Becton Dickinson, Sparks, MD) broth with 15% glycerol and were streaked onto BHI agar plates followed by incubation at 37°C for 24 h before use.

2.2. Sample collection

In addition to analysis of previously reported isolates, sample collection from the same processing facility was performed in two sampling periods for *Listeria* isolation and characterization (Table 3.1): (i) 10/2007 to 09/2008 and (ii) 10/2008 to 10/2009. In the first sampling period, a total of 226 samples representing a variety of

environmental, raw fish, and finished product samples were collected and tested for *L. monocytogenes* (Figure S3.1). In the second sampling period, 11 drain locations were sampled 12 times throughout approx. one year. A total of 132 environmental samples collected from this period were tested for *L. monocytogenes* as well as listeriaphages. Samples from environmental sites (e.g., drains and floors), food contact surfaces (e.g., slicing machine), and non-food contact surfaces (e.g., pellet jack) were collected using sterile sponges, essentially as described previously (Lappi et al., 2004). Bags containing sponges were shipped overnight on ice to the laboratory and processed within 24 h of sample collection.

2.3. *L. monocytogenes* isolation

For samples collected during the first sampling period (10/2007 to 09/2008), *Listeria* enrichment was performed by transferring each sample sponge into a sterile Whirl-Pak bag containing 90 ml of *Listeria* enrichment broth (LEB; Difco, Becton Dickinson, Sparks, MD), followed by homogenization in a stomacher for 60 s. After incubation at 30°C for 24 and 48 h, a 50-µl aliquot was streaked onto Oxford plating medium (Difco, Becton Dickinson, Sparks, MD) and plates were incubated at 30°C for 48 h. For each sample that showed *Listeria*-like colonies on Oxford, four *Listeria*-like colonies (or fewer if less than four colonies were obtained) were substreaked onto *L. monocytogenes* plating medium (LMPM; R-F Laboratories, Downers Grove, Il), which was subsequently incubated at 37°C for 48 h. On LMPM, *L. monocytogenes* and *L. ivanovii* appear as blue colonies (indicating phospholipase activity), while other *Listeria* spp. appear as white colonies (Restaino et al., 1999). Blue colonies on LMPM

Table 3.1. Recovery of *Listeria* spp., *L. monocytogenes*, and listeriaphages from samples collected from a smoked fish processing facility between Nov. 2007 and Nov. 2009.

Sampling visit (date, m/d/y)	No. of samples tested	No. of samples positive for			No. of *L. monocytogenes* isolates characterized as specific ribotype (DUP-)							
		Listeria spp.[a]	*LM*	Listeria phages[b]	1039 C	1040 A	1043 A	1045 B	1052 A	1062 A	1062 C	Other[c]
Frist sampling period												
1 (10/10/07)	35	0	9	n/a	1	5					3	
2 (10/17/07)	5	0	2	n/a		2						
3 (10/18/07)	8	1	3	n/a	1	2						
4 (10/31/07)	42	2	10	n/a		9			1			
5 (11/14/07)	42	5	15	n/a	3	1	5	1	5			
6 (12/5/07)	48	3	15	n/a	8	2	1		1	1		2
7 (2/4/08)	5	0	0	n/a								
8 (5/20/08)	12	0	0	n/a								
9 (5/21/08)	7	0	0	n/a								
10 (9/16/08)	22	0	2	n/a		2						

Table 3.1. (Continued).

Second sampling period												
11 (10/28/08)	11	2	2	2	1	1						
12 (11/25/08)	11	2	2	0	1		1					
13 (01/13/09)	11	2	3	1	2		2					2
14 (02/25/09)	11	4	3	0	1		1			1		
15 (03/25/09)	11	4	2	0	1		1					
16 (04/28/09)	11	5	2	0			1					1
17 (05/27/09)	11	0	2	0	1							1
18 (06/23/09)	11	3	1	0			1					
19 (07/29/09)	11	5	0	0								
20 (08/25/09)	11	3	1	0	1							
21 (09/29/09)	11	6	2	0	1				1			
22 (10/26/09)	11	4	4	0	2					1		1
Total	358	51 (14.2%)	80 (22.3%)	3 (2.3%)	24	24	13	1	8	3	3	7

Table 3.1. (Continued).

[a] *Listeria* spp. excludes *L. monocytogenes* (*LM*).

[b] Collected samples from the first sampling period (i.e., visit 1 to 10) were not used for listeriaphage isolation, therefore, results this period are indicated as “n/a”.

[c] Other ribotype patterns included DUP-18042 (n=1); DUP-1451S2 (n=2); DUP-1042A (n=2); DUP-1030A (n=1); DUP-1053A (n=1).

plates were thus further characterized, as detailed below, to classify them into species and subtype.

The protocol for *L. monocytogenes* isolation from samples collected in the second sampling period (10/2008 to 10/2009) required some modifications as each sample sponge was used for both *L. monocytogenes* and listeriaphage isolation. Briefly, a given sponge was added to a sterile Whirl-Pak bag with a filtered screen containing 10 ml of Phosphate Buffer Saline (PBS), pH 7.4. This sample was manually homogenized and then a 5 ml aliquot from this sample was transferred to a new sterile Whirl-Pak bag without a filtered screen, which was used for *Listeria* enrichment and isolation following the protocol detailed above. The Whirl-Pak bag containing the sponge and the remaining PBS was used for listeriaphage enrichment and isolation as detailed below.

2.4. *L. monocytogenes* characterization

2.4.1. Automated *Eco*RI ribotyping

At least one *L. monocytogenes* isolate from each sample that was positive for *L. monocytogenes* was selected for ribotyping. Isolated positive blue colonies recovered from LMPM were substreaked onto BHI agar, followed by incubation at 37°C for 24 h. An isolated colony from BHI plate was used for characterization by automated ribotyping using the *Eco*RI enzyme and the RiboPrinter® System (Dupont Qualicon™, Wilmington, DE), following to the manufacturer's instruction. While the RiboPrinter® software classifies ribotype patterns into DuPont IDs (e.g., DUP-1042), a given DUP ID can contain more than one distinct ribotype pattern (i.e., patterns differ

by a single weak band within a given DuPont ID); distinct patterns within a given DUP ID were designated with an additional letter (e.g., DUP-1043A and DUP-1043B).

2.4.2. Pulsed Field Gel Electrophoresis (PFGE) analysis

Ribotype data for both previously reported isolates and isolates obtained as part of the study reported here, were analyzed together to select isolates for PFGE analysis. Initial analysis identified 9 different *Eco*RI ribotypes that were represented by ≥ 10 isolates among 1,849 samples collected from this facility (Table 3.2). For each ribotype, at least two isolates recovered from the first and the last sampling visits of each sampling period were selected for characterization by PFGE. If available, additional isolates for each ribotype were selected conveniently to represent different sampling sites and sampling types (e.g., samples from door handle, apron, floor, raw salmon, or RTE products). Overall, 141 isolates were selected for PFGE characterization in our study (Table 3.3).

PFGE was performed using the standard CDC PulseNet PFGE protocol for *L. monocytogenes* (Graves and Swaminathan, 2006; Graves and Swaminathan, 2001), with two restriction enzymes (i.e., *Apa*I and *Asc*I). PFGE gels were run on the Bio-Rad CHEF Mapper electrophoresis unit. PFGE images were acquired using the Bio-Rad Gel Doc software version 1.1. Patterns were normalized and further analyzed with BioNumerics Software (Applied Maths, Sint-Martens-Latem, Belgium) using unweighted pairs group matching, a Dice correlation coefficient with a tolerance of 1.5%, and an optimization of 1.5%. *Asc*I and *Apa*I patterns were initially analyzed

separately; unique patterns were assigned separate Cornell University (CU) numbers (e.g., CU-121) for both restriction patterns. PFGE profile was designated by using the CU number of *Asc*I PFGE pattern, followed by the CU number of *Apa*I PFGE pattern. For example, if the *Asc*I PFGE pattern is CU-19 and the *Apa*I PFGE pattern is CU-22, the PFGE profile was designated as CU-19, 22.

2.5. Listeriaphage enrichment and isolation

For phage enrichment, 95 ml of LB MOPS (LB medium buffered with MOPS, final conc. 50 mM, pH 7.6) was added to a bag containing a sponge and the remaining PBS as detailed above. The mixture was homogenized for 60 s with a stomacher, followed by addition of a 1 ml mixture (250 µl of overnight culture for each of the four host strains; Table S3.1). These strains were selected to represent the 4 most common *L. monocytogenes* serotypes (1/2a, 1/2b, 4a, and 4b), which have been previously used for phage isolation (Kim et al., 2008; Hodgson, 2000; Loessner and Busse, 1990). Each host was grown overnight in 5 ml of LB MOPS at 30°C (with shaking at 220 rpm) to reach an OD_{600} of 0.5–0.6 (approx. 10^9 CFU/ml). The phage enrichment was incubated at 30°C for 24 h, followed by initial filtration through a 0.45 µm filter and subsequent filtration of a 1 ml aliquot through a 0.2 µm filter. Phages were than isolated separately with each of the four host strains, using an overlay method as previously detailed by Ferreira et al. (2011), and followed by phage purification as also detailed by Ferreira et al. (2011). Phage stocks were stored at 4°C.

Table 3.2. Common *L. monocytogenes* ribotypes isolated, between 1998 and 2009, from samples collected in the smoked fish processing facility studied here.

Ribotype (DUP-)	No. of *L. monocytogenes* isolates representing specific ribotype in each sampling year or period[a]							Total no. of isolates [no. of years this ribotype persisted]
	1998	2000	2001	2002	2004	2007/08	2008/09	
1027A	0	7	1	3	0	0	0	10 [2]
1042B	1	6	3	1	0	0	0	11 [4]
1044A	7	5	1	7	0	0	0	20 [4]
1042C	1	6	0	7	1	0	0	12 [6]
1043A	0	19	18	9	2	6	7	61 [9]
1045B	2	12	2	0	0	1	0	15 [10]
1039C	23	52	9	6	0	13	11	97 [11]
1052A	1	13	7	10	1	7	1	39 [11]
1062[b]	7	17	5	1	1	4	2	33 [11]
Total no. of samples tested (total sampling visits)	229 (5)	256 (16)		524 (22)[c]	482 (5)	226 (10)	132 (12)	1849 (70)

[a] Data for 1998, 2000, 2001-2002, and 2004 were previously published (Lappi et al., 2004; Hoffman et al., 2003; Hu et al., 2006; Norton et al., 2001; Thimothe et al., 2004). Samples tested included environmental samples, fish in process, and raw fish.

[b] Isolates reported as DUP-1062, DUP-1062A, and DUP-1062C were grouped together and are listed here as "DUP-1062".

[c] Sampling visits in 2001 and 2002 were reported in a single manuscript (Lappi et al., 2004).

Table 3.3. Summary of PFGE profiles of *L. monocytogenes* isolates representative of persistent ribotypes and selection of isolates for evaluation of phage susceptibility.

Ribotype (DUP-)	PFGE profile[a]	[Total no. of isolates characterized] No. of isolates representing specific PFGE profile	No. of band(s) different between PFGE profiles[b] (*Asc*I pattern, *Apa*I pattern)	*L. monocytogenes* isolates selected for phage testing[d]					
				First sampling period		Middle sampling period		Last sampling period	
				Isolate ID (FSL-)	Isolation date	Isolate ID (FSL-)	Isolation date	Isolate ID (FSL-)	Isolation date
1027A		[11]							
	CU-58, 99	8	0	H1-038	02/2000	L3-043	02/2002	L4-162	10/2002
	CU-58, 98	3	0, 2	H1-050	02/2000	H1-163	03/2000	T1-227	06/2001
1039C		[13]							
	CU-182, 173	5	0	H1-003*	03/2000	L4-396	12/2002	V1-009	10/2008
	CU-182, 174	2	0, 2	N1-449	09/1998	-	-	H1-486	07/2000
	CU-182, 172	1	0, 1	T1-061	03/2001	-	-	-	-
	CU-80, 173	1	>3	-	-	-	-	-	-
	CU-118, 218	1	>3	-	-	-	-	-	-
	CU-182, 233	1	>3	-	-	-	-	-	-
	CU-180, 231	1	>3	-	-	-	-	-	-
	CU-81, 219	1	>3	-	-	-	-	-	-
1042B		[12]							
	CU-55, 98	6	0	H1-099	03/2000	H1-412	07/2000	T1-384	05/2001
	CU-55, 99	2	0, 2	H1-174	03/2000	-	-	H1-406	08/2000
	CU-200, 227	1	>3	-	-	-	-	-	-
	CU-182, 172	1	>3	-	-	-	-	-	-
	CU-159, 95	1	>3	-	-	-	-	-	-
	CU-241, 51	1	>3	-	-	-	-	-	-

Table 3.3. (Continued).

1042C		[13]							
	CU-18, 22	7	0	H1-178	03/2000	H1-459	08/2000	L3-397	05/2002
	CU-156, 5	2	>3	-	-	-	-	-	-
	CU-5, 5	2	>3	-	-	-	-	-	-
	CU-287, 5	2	>3	-	-	-	-	-	-
1043A		[26]							
	CU-35, 248	19	0	H1-006*	02/2000	R6-819	11/2007	V1-098	06/2009
	CU-35, 289	4	0, 1	T1-930	11/2001	H6-154	06/2004	R6-836	11/2007
	CU-35, 291	1	0, 2	R6-850	11/2007	-	-	-	-
	CU-289, 99	1	>3	-	-	-	-	-	-
	CU-9, 240	1	>3	-	-	-	-	-	-
1044A		[16]							
	CU-258, 67	12	0	N1-114	08/1998	T2-083	12/2001	L4-412	12/2002
	CU-258, 69	1	0, 1	H1-139	03/2000	-	-	-	-
	CU-259, 67	3	2, 0	N1-052	08/1998	N1-061	08/1998	H1-490	08/2000
1045B		[16]							
	CU-200, 227	11	0	N1-315	08/1998	H1-426	08/2000	T1-269	07/2001
	CU-199, 228	1	>3	-	-	-	-	-	-
	CU-167, 173	1	>3	-	-	-	-	-	-
	CU-280, 222	1	>3	-	-	-	-	-	-
	CU-286, 175	1	>3	-	-	-	-	-	-
	CU-175, 212	1	>3	-	-	-	-	-	-
1052A		[13]							
	CU-40, 248	4 (i)[c]	0	N1-350	10/1998	H1-470	08/2000	T1-127	04/2001
	CU-42, 253	4 (ii)[c]	3, 2	R6-740	10/2007	R6-913	12/2007	V1-119	09/2009
	CU-42, 246	2	(i) >3; (ii) 0, 1	T2-075	12/2001	-	-	L3-055	02/2002
	CU-8, 96	2	(i) >3; (ii) >3	-	-	-	-	-	-
	CU-167, 173	1	>3	-	-	-	-	-	-
1062A		[21]							

Table 3.3. (Continued).

CU-107, 140	12	0	N1-053*	08/1998	H6-175	06/2004	V1-142	10/2009
CU-100, 140	2	2, 0	T1-261	07/2001	T1-938	11/2001	-	-
CU-270, 126	3	>3	-	-	-	-	-	-
CU-195, 159	3	>3	-	-	-	-	-	-
CU-182, 173	1	>3	-	-	-	-	-	-

[a] Only the most common PFGE profiles and those representing their closely related PFGE profiles (band differences <3) are shown in Figure 3.2. Profiles were designated by using the CU numbers (CU-*Asc*I pattern, *Apa*I pattern).

[b] No. of band(s) different between each observed PFGE profile and the most common PFGE profile. Differences between profiles included the comparison of both *Asc*I and *Apa*I patterns. The most common PFGE profiles refer to unique PFGE profiles that were frequently observed among these representative isolates characterized. In this table, the most common PFGE profiles are listed as the first profile under a given ribotype. ">3" indicates that no. of bands different is >3 for either *Asc*I pattern or *Apa*I pattern or both.

[c] These two PFGE profiles tied for the most common PFGE profile observed in representative *L. monocytogenes* isolates of the ribotype DUP-1052A.

[d] For a spot test, isolates were selected among those that represented the most common PFGE profiles and the profiles that show less than 3-band different (closely related profiles). In addition, three isolates indicated with (*) were selected for phage cocktail treatments.

2.6. Evaluation of phage susceptibility of persistent *L. monocytogenes* subtypes

Of the 114 isolates that were characterized by PFGE, 50 isolates were selected for evaluation of susceptibility against a panel of 28 phages. Briefly, within a given *Eco*RI ribotype, 3 isolates were selected among isolates that represented the predominant PFGE profile ("the most common PFGE profile") to include 3 major sampling periods (Table 3.3): (i) the earliest sampling visit and (ii) the latest sampling visit, as well as (iii) a sampling visit representing a date approx. centered between the earliest and the latest sampling visit that yielded this common PFGE profile ("middle sampling period"). Within a given ribotype, additional isolates representing all PFGE profiles closely related to the most common PFGE profiles (i.e., profiles that differed by ≤3 bands from the most common PFGE profile for either *Asc*I or *Apa*I pattern (Tenover et al., 1995)) were also tested. While these additional isolates were selected to include one isolate from each of the three sampling periods if available, isolates with PFGE profiles closely related to the most common profile were often available for only 1 or 2 sampling periods. For example (Table 3.3), among the 5 isolates of the ribotype DUP-1039C representing the most common PFGE profile, 3 were selected to include (i) the earliest sampling period (i.e., 03/2000); (ii) the latest sampling period (i.e., 10/2008); and (iii) the middle sampling period (i.e., 12/2002). Another closely related PFGE profile was identified in only 2 isolates, these two additional isolates were also selected represent only (i) the earliest sampling period (i.e., 09/1998), and (ii) the latest sampling period (i.e., 07/2000).

The 50 isolates selected as detailed above were tested for susceptibility to 28 listeriaphages (Table S3.2), including (i) 23 phages previously isolated from three

dairy farms, representing diverse host ranges, and (ii) 5 phages recovered from this food processing facility. A spot test was performed by spotting 3 µl of each phage, representing a titer range of 1×10^6 to 5×10^8 PFU/µl, on a lawn of each isolate as previously described (Ferreira et al., 2011). Absence of bacterial inhibitory effects caused by high-titer phage suspensions was confirmed in the serial dilution spot tests previously described by (Loessner and Busse, 1990). After incubation for 24 to 48 h, phage lysis present on the lawn was scored either (+) for phage lysis or (-) for no lysis. Lysis was defined as multiple single plaques or turbid or confluent lysis. Three independent replicates of the spot test were performed for each isolate; the overall result for a given phage-*L. monocytogenes* isolate pair was considered lysis (+) if plaquing was observed in at least 2 replicates.

2.7. Treatments of persistent *L. monocytogenes* isolates with phage cocktails and a commercial phage product

Based on ribotyping and PFGE results, we identified three persistent subtypes, defined as identical ribotype/PFGE profile combinations, that were isolated over the longest times. These included (i) ribotype DUP-1039C, PFGE profile CU-182, 173 (isolated over 8 years), (ii) ribotype DUP-1062A, PFGE profile CU-107, 140 (isolated over 11 years), and (iii) ribotype DUP-1043A, PFGE profile CU-35, 248 (isolated over 9 years) (Table 3.3). For each of these three persistent subtypes, a single isolate from the earliest isolation date (i.e., isolates FSL H1-003; FSL N1-053; FSL H1-006) was selected for phage challenge experiments aimed at evaluating the efficacy of

phage cocktail treatment. The lab strain Mack (ribotype DUP-1030A) (Hodgson, 2000), was included in the experiments as a control strain.

Phage cocktails included three phages (combined with the same PFU/ml for each phage), selected based on their ability to lyse all or the highest number of the isolates of a given ribotype tested with a spot test. Specifically, isolates FSL H1-003, FSL N1-053, and Mack were challenged with a phage cocktail that included phages LP-039, LP-040, and LP-048; while the isolate FSL H1-006 was challenged with a unique phage cocktail that included phages LP-030, LP-038, and LP-125. The phages included in a given phage cocktail were selected to include phages from various sources and phages with various genome sizes (Table S3.2). Challenge experiments with these phage cocktails were conducted using an overnight culture of each *L. monocytogenes* isolate, grown to an OD_{600} of 0.5–0.6 (approx. 10^9 CFU/ml) at 30°C, as detailed above. To yield a measurable *L. monocytogenes* counts after the phage treatments, initial inoculum levels of 10^5 CFU/ml and 10^6 CFU/ml were used. Phage challenge experiments were performed in a volume of 10 ml at MOI (multiplicity of infection) levels of 1, 10, and 100. All treatments were adjusted to the same final volume with SM buffer, including $CaCl_2$ and $MgCl_2$ to a final conc. of 10 mM. For comparison, we also performed challenge experiments of these 4 strains with the commercial phage product (which has been approved for use by the USDA and FDA), following the same procedures detailed above. The commercial phage product was diluted in SM buffer to allow for challenge experiments at the 3 MOI levels. For the mock-treated controls, SM buffer was used instead of the phage cocktails or the commercial phage product. All treatments and controls were incubated at 30°C with

aeration (shaking at 220 rpm). Samples were taken every 4 h for 24 h and a 50-µl aliquot from each treatment and control was plated, using a spiral plater, onto BHI agar in duplicate for the enumeration of surviving *L. monocytogenes* (after incubation of BHI plates at 37°C for 18 to 24 h).

An isolated *L. monocytogenes* colony recovered after 24 h of the phage and the mock treatments was collected to (i) perform PFGE analysis to confirm the strain identity (following the protocols detailed above); and (ii) re-evaluate phage susceptibility against the individual phages that were included in the phage cocktails or the commercial phage product using the spot test protocol detailed above).

2.8. Statistical analysis

Logistic regression was used to estimate the percent likelihood of phage susceptibility of randomly selected isolates within each of the nine ribotypes that were repeatedly found in this facility; 95% confidence intervals for susceptibility were calculated from parameters estimates. Analysis was performed with JMP Software (version 9.0; SAS Institute Inc., Cary, NC).

3. Results

3.1. Isolation of *L. monocytogenes* and listeriaphages

Overall, 80 of 358 samples tested (22.3%) were positive for *L. monocytogenes* and 51 samples (14.2%) were positive for other *Listeria* spp. (Table 3.1). Among the 226 samples from the first sampling period (visits 1 to 10), 56 were positive for *L.*

monocytogenes (24.8%); and among the 132 samples from the second sampling period (visits 11 to 22), 24 were positive for *L. monocytogenes* (18.2%) (Table 3.1).

Among the 132 samples tested for listeriaphages in the second sampling period, only 3 samples (2.3%) were listeriaphage-positive (Table 3.1). These three samples yielded 5 phage isolates as each of two samples yielded plaques on two different host strains (Table S3.2).

3.2. Characterization of representative *L. monocytogenes* isolates by the automated *Eco*RI ribotyping

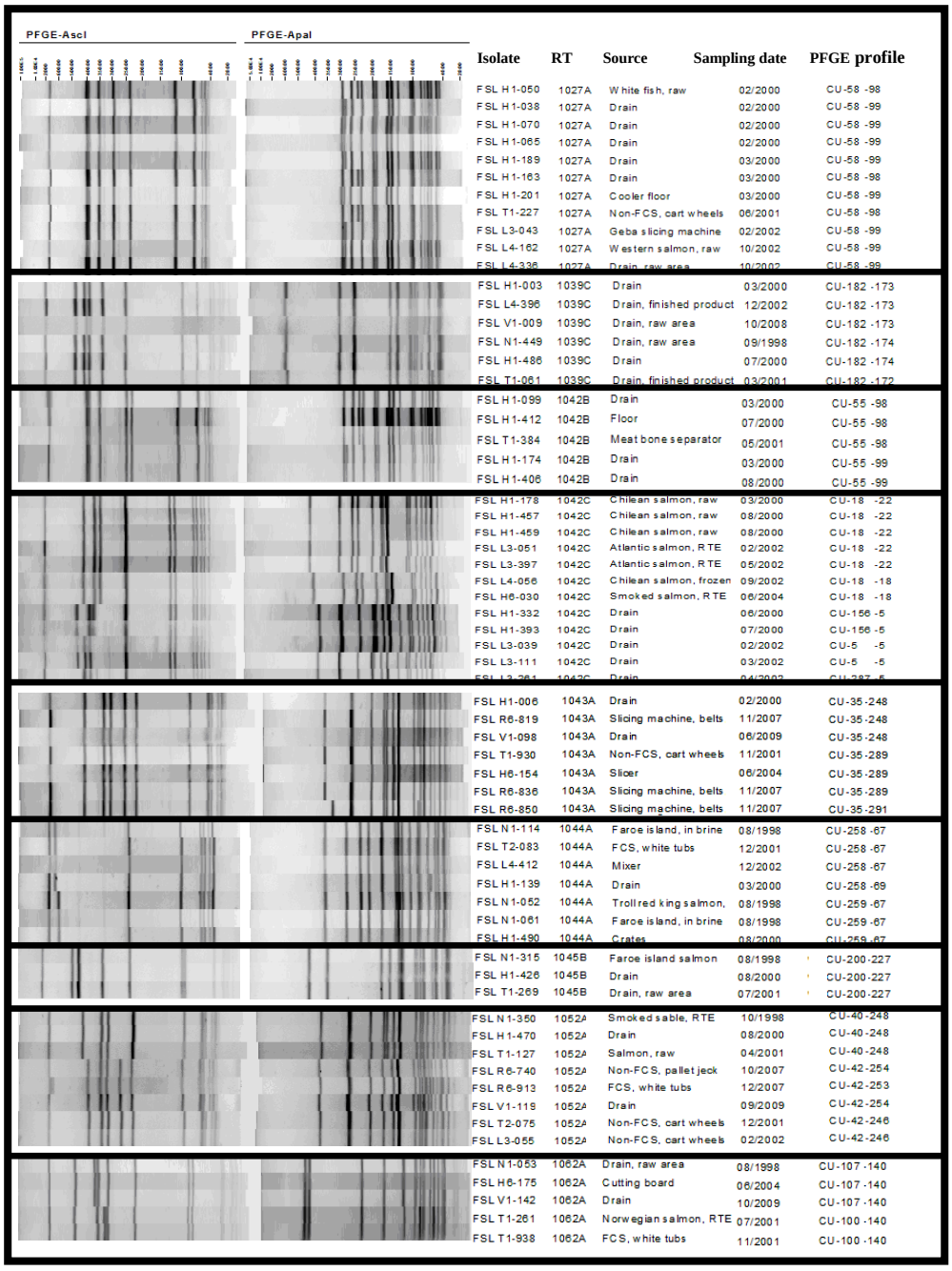
For each of the 80 *L. monocytogenes*-positive samples identified in the study reported here, one *L. monocytogenes* isolate was conveniently selected for *Eco*RI ribotyping. For two samples collected during visit 13, one and two additional isolates were characterized by *Eco*RI ribotyping as the isolate from each sample initially yielded a ribotype that had been rarely found in this facility; this approached was used to determine whether these samples may have included isolates representing multiple ribotypes. Overall, 12 ribotype patterns were observed among the 83 isolates characterized here (Table 3.1). The majority of these isolates represented ribotypes, DUP-1039C (n=24) and DUP-1040A (n=24); the other 10 ribotype patterns were observed in 1 to 13 isolates (Table 3.1). A number of environmental samples collected from food contact surfaces as well as 3 raw fish samples collected during visits 1 to 10 yielded isolates that represented ribotype DUP-1040A (Figure S3.1).

3.3. Identification of ribotypes isolated over multiple sampling periods between 1998 and 2009

To identify subtypes of *L. monocytogenes* that appear to have persisted in the food processing facility studied here, ribotyping data for *L. monocytogenes* isolated during the 5 previous studies from this facility in 1998–2004 (Lappi et al., 2004; Hoffman et al., 2003; Hu et al., 2006; Norton et al., 2001; Thimothe et al., 2004) were evaluated together with the ribotyping data from the current study (2007–2009). We identified 9 *Eco*RI ribotypes that were recovered ≥ 10 times from environmental samples collected in this facility; all of these ribotypes were identified over at least three sampling periods/years (Table 3.2). Specific ribotypes were isolated in this facility over approx. 2 years (i.e., DUP-1027A) to 11 years (i.e., DUP-1039C, DUP-1052A, and DUP-1062).

3.4. Characterization of representative *L. monocytogenes* isolates by PFGE

Overall, *Asc*I and *Apa*I PFGE of 141 *L. monocytogenes* isolates yielded 31 and 30 patterns, respectively, resulting in 44 PFGE profiles based on both restriction enzymes (Figure 3.1 and Table 3.3). Within a given ribotype, 2 to 8 different PFGE profiles were identified. For most ribotypes, at least one PFGE profile was clearly the most common PFGE profile among the isolates characterized. For example, 8 of 11 isolates with ribotype DUP-1027A were classified into PFGE profile CU-58, 99. On the other hand, among the 13 isolates with ribotype DUP-1052A, we found two most common PFGE profiles (CU-40, 248 and CU-42, 253) with each profile represented



Isolate	RT	Source	Sampling date	PFGE profile
FSL H1-050	1027A	White fish, raw	02/2000	CU-58 -98
FSL H1-038	1027A	Drain	02/2000	CU-58 -99
FSL H1-070	1027A	Drain	02/2000	CU-58 -99
FSL H1-065	1027A	Drain	02/2000	CU-58 -99
FSL H1-189	1027A	Drain	03/2000	CU-58 -99
FSL H1-163	1027A	Drain	03/2000	CU-58 -98
FSL H1-201	1027A	Cooler floor	03/2000	CU-58 -99
FSL T1-227	1027A	Non-FCS, cart wheels	06/2001	CU-58 -98
FSL L3-043	1027A	Geba slicing machine	02/2002	CU-58 -99
FSL L4-162	1027A	Western salmon, raw	10/2002	CU-58 -99
FSL L4-336	1027A	Drain, raw area	10/2002	CU-58 -99
FSL H1-003	1039C	Drain	03/2000	CU-182 -173
FSL L4-396	1039C	Drain, finished product	12/2002	CU-182 -173
FSL V1-009	1039C	Drain, raw area	10/2008	CU-182 -173
FSL N1-449	1039C	Drain, raw area	09/1998	CU-182 -174
FSL H1-486	1039C	Drain	07/2000	CU-182 -174
FSL T1-081	1039C	Drain, finished product	03/2001	CU-182 -172
FSL H1-099	1042B	Drain	03/2000	CU-55 -98
FSL H1-412	1042B	Floor	07/2000	CU-55 -98
FSL T1-384	1042B	Meat bone separator	05/2001	CU-55 -98
FSL H1-174	1042B	Drain	03/2000	CU-55 -99
FSL H1-406	1042B	Drain	08/2000	CU-55 -99
FSL H1-178	1042C	Chilean salmon, raw	03/2000	CU-18 -22
FSL H1-457	1042C	Chilean salmon, raw	08/2000	CU-18 -22
FSL H1-459	1042C	Chilean salmon, raw	08/2000	CU-18 -22
FSL L3-051	1042C	Atlantic salmon, RTE	02/2002	CU-18 -22
FSL L3-397	1042C	Atlantic salmon, RTE	05/2002	CU-18 -22
FSL L4-056	1042C	Chilean salmon, frozen	09/2002	CU-18 -18
FSL H6-030	1042C	Smoked salmon, RTE	06/2004	CU-18 -18
FSL H1-332	1042C	Drain	06/2000	CU-156 -5
FSL H1-393	1042C	Drain	07/2000	CU-156 -5
FSL L3-039	1042C	Drain	02/2002	CU-5 -5
FSL L3-111	1042C	Drain	03/2002	CU-5 -5
FSL L3-261	1042C	Drain	04/2002	CU-287 -5
FSL H1-006	1043A	Drain	02/2000	CU-35 -248
FSL R6-819	1043A	Slicing machine, belts	11/2007	CU-35 -248
FSL V1-098	1043A	Drain	06/2009	CU-35 -248
FSL T1-930	1043A	Non-FCS, cart wheels	11/2001	CU-35 -289
FSL H6-154	1043A	Slicer	06/2004	CU-35 -289
FSL R6-836	1043A	Slicing machine, belts	11/2007	CU-35 -289
FSL R6-850	1043A	Slicing machine, belts	11/2007	CU-35 -291
FSL N1-114	1044A	Faroe island, in brine	08/1998	CU-258 -67
FSL T2-083	1044A	FCS, white tubs	12/2001	CU-258 -67
FSL L4-412	1044A	Mixer	12/2002	CU-258 -67
FSL H1-139	1044A	Drain	03/2000	CU-258 -69
FSL N1-052	1044A	Troll red king salmon,	08/1998	CU-259 -67
FSL N1-061	1044A	Faroe island, in brine	08/1998	CU-259 -67
FSL H1-490	1044A	Crates	08/2000	CU-259 -67
FSL N1-315	1045B	Faroe island salmon	08/1998	CU-200 -227
FSL H1-426	1045B	Drain	08/2000	CU-200 -227
FSL T1-269	1045B	Drain, raw area	07/2001	CU-200 -227
FSL N1-350	1052A	Smoked sable, RTE	10/1998	CU-40 -248
FSL H1-470	1052A	Drain	08/2000	CU-40 -248
FSL T1-127	1052A	Salmon, raw	04/2001	CU-40 -248
FSL R6-740	1052A	Non-FCS, pallet jeck	10/2007	CU-42 -254
FSL R6-913	1052A	FCS, white tubs	12/2007	CU-42 -253
FSL V1-119	1052A	Drain	09/2009	CU-42 -254
FSL T2-075	1052A	Non-FCS, cart wheels	12/2001	CU-42 -246
FSL L3-055	1052A	Non-FCS, cart wheels	02/2002	CU-42 -246
FSL N1-053	1062A	Drain, raw area	08/1998	CU-107 -140
FSL H6-175	1062A	Cutting board	06/2004	CU-107 -140
FSL V1-142	1062A	Drain	10/2009	CU-107 -140
FSL T1-261	1062A	Norwegian salmon, RTE	07/2001	CU-100 -140
FSL T1-938	1062A	FCS, white tubs	11/2001	CU-100 -140

Figure 3.1. *Asc*I and *Apa*I patterns of the predominant PFGE profiles, and the closely related PFGE profiles within each ribotype, that were observed among *L. monocytogenes* isolates repeatedly recovered from a smoked fish processing facility between 1998 and 2009. PFGE profiles were assigned CU numbers (CU-*Asc*I pattern, *Apa*I pattern).

by 4 isolates. These two PFGE profiles differed by 3 bands in the *Asc*I pattern and two bands in the *Apa*I pattern.

Within most ribotypes, some isolates with different PFGE profiles could be considered closely related to the most common PFGE profile. Relatedness is based on the "3-band rule" (Tenover et al., 1995), which proposes that isolates that differ by ≤3 bands in a PFGE pattern with a given enzyme can be considered closely related if they are linked epidemiologically. For example, while 8 isolates with ribotype DUP-1027A were classified as CU-58, 99, 3 isolates with this ribotype were classified into profile CU-58, 98; these two profiles showed the same *Asc*I pattern, but differed by 2 bands in the *Apa*I pattern (Figure 3.1 and Table 3.3), suggesting closely related PFGE profiles. On the other hand, characterization of 13 isolates with ribotype DUP-1042C identified two distinct types of PFGE profiles. Among these isolates, 7 isolates represented the most common PFGE profile (CU-18, 22) while 6 other isolates representing 3 different PFGE profiles that differed by >3 bands from CU-18, 22 in each *Asc*I and *Apa*I patterns (Table 3.3). Relative to each other, these 6 isolates had the same *Apa*I pattern but differed by ≤3 bands in their *Asc*I pattern. Overall, these data suggest that the ribotype 1042C isolates characterized here represent two distinct clonal groups.

Overall, PFGE analysis confirmed that genetically similar *L. monocytogenes* isolates were recovered from multiple visits, providing further evidence for *L. monocytogenes* persistence, in this facility, for 1 to 11 years (Figure 3.1). For example, we identified 12 isolates with ribotype DUP-1062A, PFGE profile CU-107, 140, including samples collected in 08/1998 (the first recovery) and 10/2009 (the latest recovery), indicating persistence over at least 11 years (Table 3.3). We also identified

5 isolates with ribotype DUP-1039C, PFGE profile CU-182, 173; this subtypes was recovered from samples collected in 03/2000 (first recovery) and 10/2008 (latest recovery), suggesting persistence over 8 years.

3.5. Evaluation of phage susceptibility in *L. monocytogenes* isolates of persistent subtypes

Overall, 50 *L. monocytogenes* isolates (3–7 isolates per ribotype; Table 3.3) were selected for a spot test with 28 selected listeriaphages (Table S3.2). For 7 ribotypes, all isolates tested were lysed by multiple phages, with 2 to 23phages lysing all isolates of a given ribotype (Table 3.4). On the other hand, even though several phages could lyse some isolates with ribotypes DUP-1043A and DUP-1042B, not a single phage was capable of lysing all isolates of either of these ribotypes. For example, 23 of 28 phages (82%) lysed all 7 ribotype DUP-1044A isolates, while the other 5 phages (18%) lysed between 4 and 6 isolates with this ribotype (Table S3.4). Only 5 phages (18%) lysed all 6 isolates with ribotype DUP-1039C, one of the most common ribotypes in this food processing facility; 9 phages did not lyse any of these isolates.

To further characterize phage susceptibility of *L. monocytogenes* isolates grouped into one of the nine ribotypes repeatedly found in this facility, we also calculated the likelihood that a randomly selected isolate within a given ribotype is lysed by a randomly selected phage from our set of 28 phages. Phage susceptibility calculated with this method ranged from 4.6 % (for isolates with ribotype DUP-1043A) to 95.4% (for isolates with ribotype DUP-1044A, Table 3.4).

Table 3.4. Phage susceptibility of the 9 most common persistent ribotypes.

Ribotype, DUP- (no. of *L. monocytogenes* isolates tested[a])	No. of phages[b] capable of lysing all isolates of a given ribotype tested (%)	No. phages that could not lyse any isolates of a given ribotype tested (%)	Likelihood (%) of phage susceptibility for isolates within ribotype (95% CI)[c]
1043A (n=7)	0 (0.0)	23 (82.1)	4.6 (2.1–8.9)
1045B (n=3)	9 (32.1)	17 (60.7)	35.7 (23.8–49.4)
1042C (n=3)	7 (25.0)	15 (53.6)	36.9 (24.8–50.6)
1052A (n=8)	2 (7.1)	10 (35.7)	42.4 (32.5–52.9)
1042B (n=5)	0 (0.0)	6 (21.4)	45.0 (33.6–56.8)
1062A (n=5)	5 (17.9)	3 (10.7)	45.0 (41.4–48.7)
1039C (n=6)	5 (17.9)	9 (32.1)	45.8 (34.9–57.1)
1027A (n=6)	5 (17.9)	7 (25.0)	54.2 (42.9–65.1)
1044A (n=7)	23 (82.1)	0 (0.0)	95.4 (91.1–97.9)

[a] For a spot test, isolates were selected among those that represented the most common PFGE profiles and the closely related PFGE profiles to the common ones (see Table 3.3).

[b] 28 selected phages (Table S3.2) isolated from dairy farms and this food processing facility, representing diverse host ranges and genome sizes, were used in a spot test with *L. monocytogenes* isolates.

[c]This represents the likelihood that a randomly selected isolate within a given ribotype is lysed by a randomly selected phage.

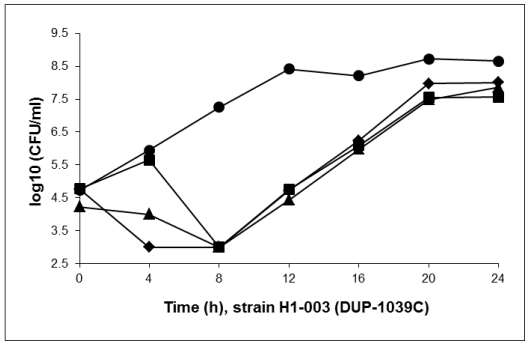
3.6. Phage cocktail treatment against *L. monocytogenes* isolates from a smoked fish processing facility

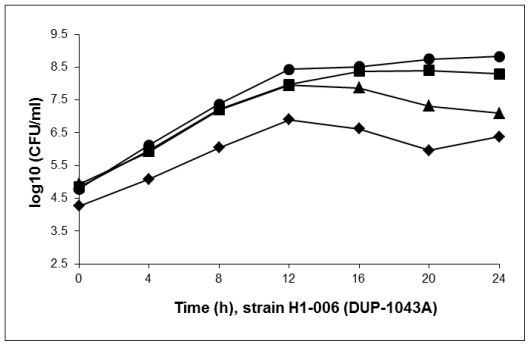
To evaluate the efficacy of phage cocktails to control persistent *L. monocytogenes***,** we tested the susceptibility of three isolates representing ribotype/PFGE type combinations that were isolated over the longest times (8 to 11 years) in this facility (Table 3.2). Each phage cocktail was made of 3 phages that were able to lyse the highest number to all of isolates of a given persistent ribotype tested. The cocktail that was used to treat isolates with ribotypes DUP-1062A (FSL N1-053) and DUP-1039C (FSL H1-003) contained 3 phages that each could lyse all isolates tested within a given ribotype. It was not possible to select a phage cocktail with 3 diverse phages that each could effectively lyse all isolates with ribotype DUP-1043A (FSL H1-006) as isolates with this ribotype were resistant to most phages (4.6% phage susceptibility; Table 3.4). Each of the 3 phages selected for this cocktail could lyse only 2 of the 6 isolates with this ribotype in the spot test experiments.

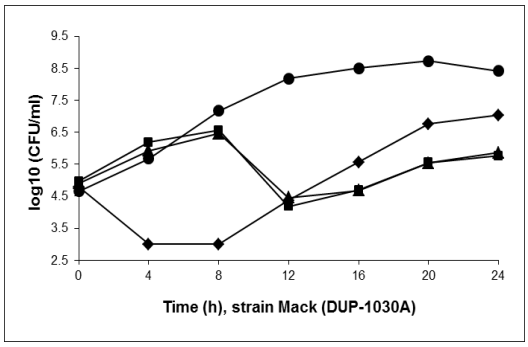
Overall, phage cocktail challenge experiments showed initial killing of isolates FSL H1-003, FSL N1-053, and Mack at both initial inoculum levels tested (10^5 and 10^6 CFU/ml) more rapidly with treatments at MOI 10 and 100 as compared to treatment at MOI 1 (Figure 3.2). After 4 to 8 h of phage cocktail treatment, *L. monocytogenes* numbers typically showed approx. 3 to 4 log lower counts as compared to the mock-treated control. The control strain Mack showed less noticeable killing as compared to persistent isolates FSL H1-003 and FSL N1-053. After 8 to 12 h, these 3 isolates showed re-growth in virtually all treatments (expect for Mack with initial inoculum level of 10^6 CFU/ml and treated with MOI 1).

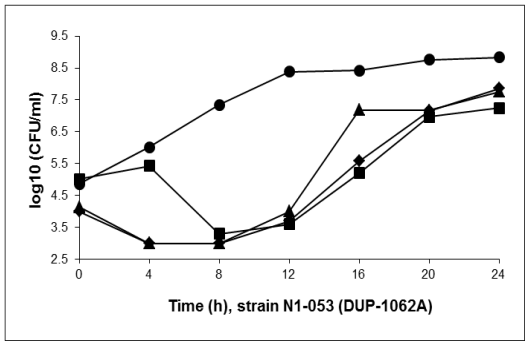
Figure 3.2. Effects of phage cocktail treatment (3 MOIs) of 3 persistent *L. monocytogenes* strains and a lab strain (Mack). Symbols: (●), mock-treated control; (■), MOI 1; (▲), MOI 10; (♦), MOI 100. Initial *L. monocytogenes* inoculum levels: (A) 10^5 CFU/ml, and (B) 10^6 CFU/ml.

A.

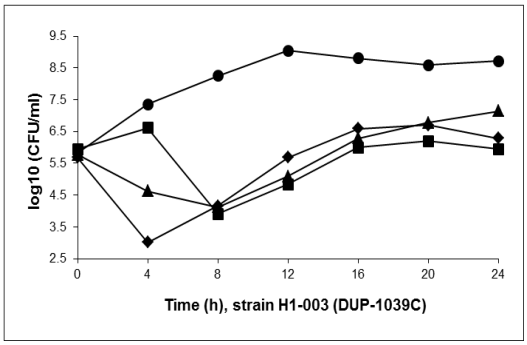

log10 (CFU/ml)
Time (h), strain H1-003 (DUP-1039C)

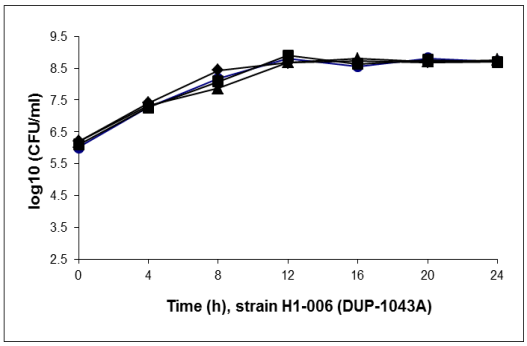

log10 (CFU/ml)
Time (h), strain H1-006 (DUP-1043A)

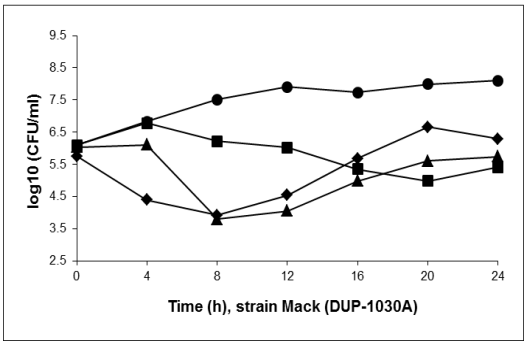

log10 (CFU/ml)
Time (h), strain Mack (DUP-1030A)


log10 (CFU/ml)
Time (h), strain N1-053 (DUP-1062A)

B.


log10 (CFU/ml)
Time (h), strain H1-003 (DUP-1039C)


log10 (CFU/ml)
Time (h), strain H1-006 (DUP-1043A)


log10 (CFU/ml)
Time (h), strain Mack (DUP-1030A)

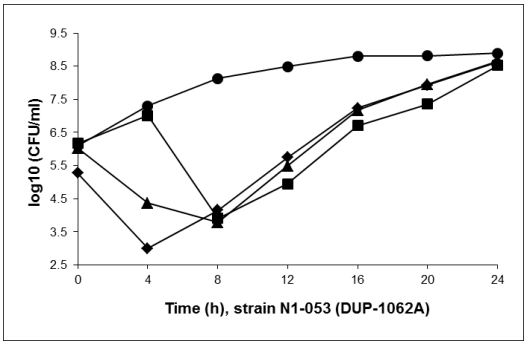
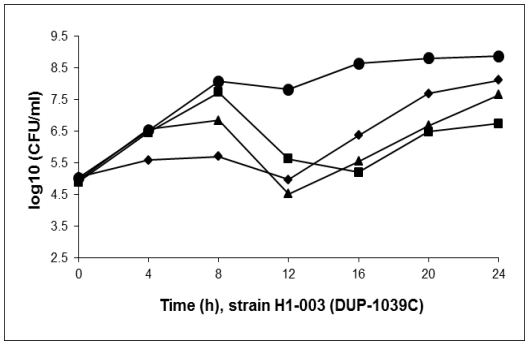
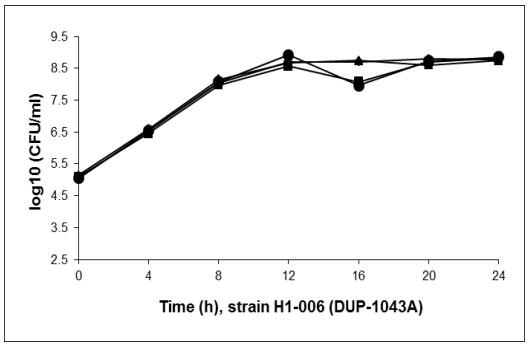
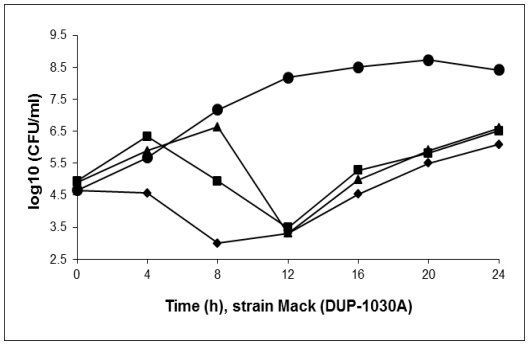

log10 (CFU/ml)
Time (h), strain N1-053 (DUP-1062A)

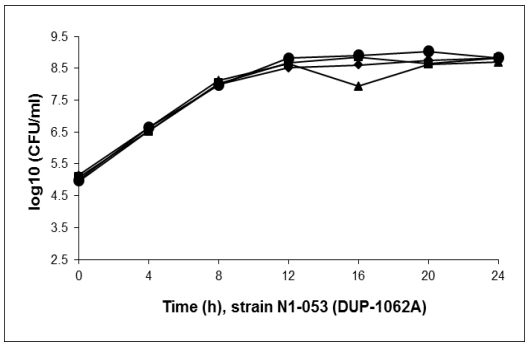
Figure 3.3. Effects of commercial phage product treatment (3 MOIs) of 3 persistent *L. monocytogenes* strains and a lab strain (Mack). Symbols: (●), mock-treated control; (■), MOI 1; (▲), MOI 10; (♦), MOI 100. Initial *L. monocytogenes* inoculum levels: (A) 10^5 CFU/ml, and (B) 10^6 CFU/ml.

A.

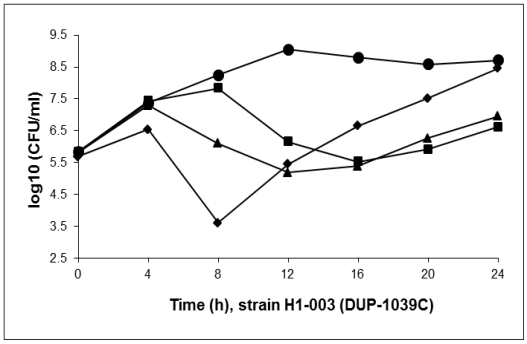

log10 (CFU/ml)
Time (h), strain H1-003 (DUP-1039C)

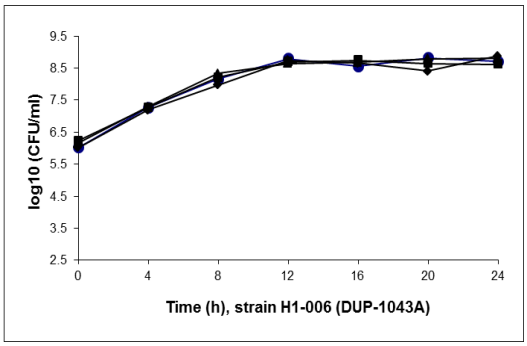

log10 (CFU/ml)
Time (h), strain H1-006 (DUP-1043A)

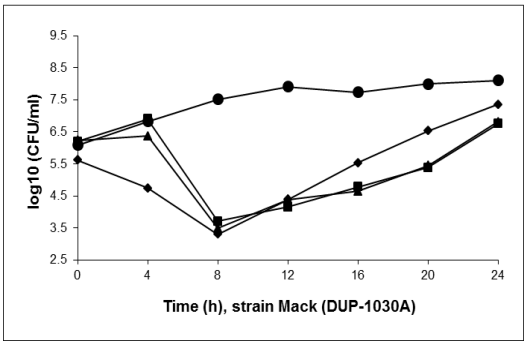

log10 (CFU/ml)
Time (h), strain Mack (DUP-1030A)

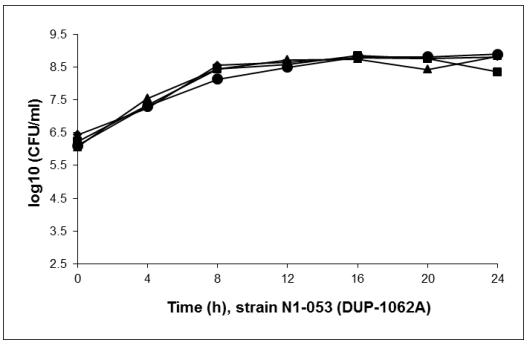

log10 (CFU/ml)
Time (h), strain N1-053 (DUP-1062A)

B.


log10 (CFU/ml)
Time (h), strain H1-003 (DUP-1039C)


log10 (CFU/ml)
Time (h), strain H1-006 (DUP-1043A)


log10 (CFU/ml)
Time (h), strain Mack (DUP-1030A)


log10 (CFU/ml)
Time (h), strain N1-053 (DUP-1062A)

Overall, phage treatments showed 0.2–2.8 log lower counts after 24 h as compared to the mock-treated control (Figure 3.2 and Table S3.3). Another isolate FSL H1-006, approx. 1.7–2.5 log lower counts at 24 h were observed in treatments at MOI 10 and 100 with initial inoculum level of 10^5 CFU/ml as compared to the mock-treated control. On the other hand, no reduction of *L. monocytogenes* populations was observed in all treatments with initial inoculum level of 10^6 CFU/ml (Figure 3.2 and Table S3.3).

Treatments with a commercial phage product showed virtually no reduction of *L. monocytogenes* counts for isolates FSL H1-006 and FSL N1-053 with both initial inoculum levels and all 3 MOIs (Figure 3.3). Isolates FSL H1-003 and Mack showed clear reduction of bacterial counts after treatments with this commercial phage product (with both initial inoculum levels and all 3 MOIs), with maximum reductions after 8 or 12 h of treatment (Figure 3.3). Similar to treatment with our phage cocktails, both isolates showed re-growth after 8 to 12 h of treatment and bacterial counts after 24 h showed between 0.3 and 2.3 log lower counts as compare to the mock-treated control (Figure 3.3 and Table S3.3).

Nine surviving isolates recovered from challenge studies of the 3 persistent isolates with either of our phage cocktails, *L. monocytogenes* isolates recovered after 24 h of treatment (one isolate for each MOI, inoculum level of 10^5 CFU/ml) were confirmed to have the same PFGE pattern as the isolate used to prepare the inoculum (Figure S3.2). These surviving isolates were also re-evaluated for susceptibility to (i) individual phages used in the phage cocktails and (ii) a commercial phage product, as appropriate to their previous treatments only. For FSL H1-003 and FSL N1-053, all

Table 3.5. Phage susceptibility of persistent *L. monocytogenes* isolates recovered after 24 h of phage treatment at different MOIs.

Isolate (ribotype, DUP-)	Treatment with phage cocktail[b] or a commercial phage product which isolate was recovered from[a]	Post-treatment susceptibility to listeriaphage[c]			
		A	B	C	A commercial phage product
Isolates treated with phage cocktail containing LP-039 (phage A), LP-040 (phage B), and LP-048 (phage C)					
H1-003 (1039C)	Mock	+	+	+	+
	1	-	-	-	-
	10	-	-	-	-
	100	-	-	-	-
N1-053 (1062A)	Mock	+	+	+	+
	1	-	-	-	-
	10	-	-	-	-
	100	-	-	-	-
MACK (1030A)	Mock	+	+	+	+
	1	-	-	+	-
	10	-	-	+	-
	100	-	-	+	-
Isolates treated with phage cocktail containing LP-030 (phage A), LP-038 (phage B), and LP-125 (phage C)					
H1-006 (1043A)	Mock	-	+	+	+
	1	-	+	-	+
	10	-	+	-	+
	100	-	+	-	+

[a] Isolates tested for phage susceptibility included (i) an isolate recovered from the Mock treated control at t=24 h, (ii) isolates recovered, at t=24 h, after treatment with the phage cocktail at MOIs of 1, 10, and 100, and (iii) isolates recovered, at t=24 h, after treatment with a commercial phage product at MOIs of 1, 10, and 100.

Table 3.5. (Continued).

[b] Different phage cocktails were used for treatment of (i) FSL H1-003, FSL N1-053, and Mack (phages LP-039, LP-040, and LP-048) and (ii) FSL H1-006 (LP-030, LP-038, and LP-125).

[c] Isolates recovered from treatment with the phage cocktails were tested against phages A, B, and C, while isolates recovered from treatment with a commercial phage product were tested against only the commercial indicates lysis and (-) indicates no lysis.

isolates recovered after phage treatment were resistant to each phage in phage cocktail as well as a commercial phage product (Table 3.5). For FSL H1-006 and Mack, the isolates recovered after phage treatment showed resistance to two of the three phages in our custom phage cocktail as well as a commercial phage product. Importantly, plaques of phage LP-038 as well as a commercial phage product on the lawn of the isolate FSL H1-006 showed only turbid, consistent with the observation that isolate FSL H1-006 showed limited or no reduction of bacterial numbers during phage treatment (Figure 3.2).

4. Discussion

Isolation and characterization of *L. monocytogenes* isolated from a smoked-fish processing facility in the current study (2007–2009), along with data collected in the same facility in 1998–2004 (Lappi et al., 2004; Hoffman et al., 2003; Hu et al., 2006; Norton et al., 2001; Thimothe et al., 2004), allowed us to identify a number of subtypes (based on both *Eco*RI ribotyping and PFGE) that appear to have persisted in this facility for up to 11 years. Evaluation of selected *L. monocytogenes* isolates, representing different persistent subtypes, for susceptibility against individual listeriaphages and phage cocktails showed that (i) persistent subtypes include both phage susceptible and phage resistant *L. monocytogenes* isolates; and (ii) while phage cocktails appear to temporally reduce *L. monocytogenes* populations, isolates can rapidly develop a phage-resistance characteristic in laboratory challenge studies.

4.1. Within a given food processing facility, a number of distinct *L. monocytogenes* subtypes can persist over prolonged times (>10 years).

Initial analysis of the *Eco*RI ribotyping data for *L. monocytogenes* isolates obtained over 11 years in a single facility through previous studies (Lappi et al., 2004; Hoffman et al., 2003; Hu et al., 2006; Norton et al., 2001; Thimothe et al., 2004) and the current study reported here, identified 9 ribotype that were repeatedly found over the time from 2 to 11 years. While one cannot exclude re-introduction of isolates (e.g., from the surrounding environment) with these ribotypes, however, since Good Manufacturing Practices (GMPs) are in place to control this re-introduction issue, our data could particularly suggest persistence of these subtypes in this food processing facility. Long-term persistence of *L. monocytogenes* has been reported in the environments of food processing facilities manufacturing a range of products, including dairy products (Kabuki et al., 2004; Unnerstad et al., 1996), meat products (Williams et al., 2011; Ferreira et al., 2011; Senczek et al., 2000), poultry products (Rørvik et al., 2003), and seafood products (Lappi et al., 2004; Hoffman et al., 2003; Rorvik et al., 2000) as well as in retail environments (Sauders et al., 2004). In addition, full genome sequencing of multiple isolates has supported persistence for > 10 years of a specific *L. monocytogenes* strains in a food processing facility (Orsi et al., 2008).

We also used PFGE to further characterize isolates with the same ribotype that were repeatedly recovered over time in an effort to independently confirm persistence. PFGE in all cases confirmed persistence, as supported by identification of the most common PFGE profile among isolates that were repeatedly recovered over time. For 7

ribotypes, we also identified at least some isolates that appear to be unrelated to the most common PFGE profile, as supported by PFGE patterns that differed by >3 bands from the most common profile. This finding is consistent with the well supported notion that 2-enzyme PFGE usually provides more subtype discrimination of *L. monocytogenes* as compared to *EcoR*I ribotyping (Fugett et al., 2007). Interestingly, in a number of cases we found that multiple isolates, which we initially identified as persistent, represented closely related PFGE profiles within a given ribotype. Consistent with (Ferreira et al., 2011), data suggest that some persistent *L. monocytogenes* strains may diversify during prolonged times (e.g., through acquisition or replacement of prophages; for example, see Orsi et al. [2008]). Overall, the combination of ribotyping and PFGE data allowed for robust identification of persistent strains, providing a unique data set for characterization of phage susceptibility among food-associated strains as discussed below.

4.2. Persistent *L. monocytogenes* subtypes include both highly phage susceptible and phage resistant *L. monocytogenes* isolates.

A number of studies have also reported the ability of phages or phage cocktails to reduce individual *L. monocytogenes* strains inoculated into foods, including catfish fillets, hot dogs, soft cheese (Carlton et al., 2005; Guenther et al., 2009; Soni et al., 2009). Nevertheless, limited peer reviewed data are available on phage susceptibility of diverse *L. monocytogenes* strains and subtypes associated with foods and food processing environments. As it is well established that a considerable proportion of *L.*

monocytogenes contamination in RTE foods can be linked to persistence of *L. monocytogenes* in food processing facilities, it is particularly relevant to characterize phage resistance among these persistent *L. monocytogenes* strains in food-associated environments. At present, only two previous studies (Ferreira et al., 2011; Kim et al., 2008) evaluated phage susceptibility in multiple *L. monocytogenes* strains that were repeatedly isolated from food processing environments. Another study by Carlton et al. (2005) evaluated the effectiveness of the listeriaphage P100 on the surface of soft cheese that was spiked with a strain of *L. monocytogenes* found to persist in the production equipment of a dairy plant.

Initial characterization, by a spot test, showed considerable variation in phage susceptibility among isolates representing 9 ribotypes found to persist in a food processing facility for up to 10 years. While one subtype (DUP-1044A) was found to be susceptible to most phages tested, two subtypes (DUP-1043A and DUP-1042B) were resistant to most phages. Kim et al. (2008) evaluated phage susceptibility among different *L. monocytogenes* isolates from turkey food processing plants, including some that represented persistent subtypes, and found considerable variation in susceptibility, with a number of isolates showing resistant to all three phages tested. A previous study by Ferreira et al. (2011) similarly observed that 8 of 19 persistent isolates were resistant to or showed only weak lysis with all 26 phages tested in the study. While susceptibility of different serotypes varies among studies, a number of studies suggest that serotype 4b isolates are typically more likely to be susceptible to phages (for example, Kim et al. [2008]), while serotypes 3c isolates appear to often be resistant to many phages (Loessner and Busse, 1990). While mechanisms responsible

for variations in phage susceptibility in *L. monocytogenes* are not well elucidated, several phage-resistance mechanisms have been identified in different organisms; for example, inhibition of phage adsorption or phage DNA injection, restriction and modification systems in the hosts, abortive infection (Labrie et al., 2010).

Overall, our data not only support considerable variation in phage susceptibility among *L. monocytogenes* strains and isolates, but also show that phage resistant strains are commonly found among *L. monocytogenes* isolates that have persisted in food associated environments. A number of previous studies used *L. monocytogenes* representing serotypes 1/2a or/and 4b, which are typically highly susceptible to phages (Kim et al., 2008; Loessner and Busse, 1990), in challenge experiments to evaluate the ability of phages to reduce *L. monocytogenes* populations inoculated into foods (Guenther et al., 2009; Leverentz et al., 2004; Soni and Nannapaneni, 2010; Leverentz et al., 2003; Soni et al., 2009; Rossi et al., 2010). Although these previous experiments support as proof of principle the capability of phages in reducing *L. monocytogenes* loads in foods under certain conditions (Soni et al., 2009; Rossi et al., 2010), the presence of phage resistant *L. monocytogenes* isolates in food associated environments indicates that phage treatment may fail to eliminate some strains that are likely to naturally contaminate foods.

4.3. While phage cocktails appear to temporally reduce *L. monocytogenes* populations, isolates can rapidly develop a phage-resistance characteristic in laboratory challenge studies.

In addition to presence of phage resistant *L. monocytogenes* subtypes, rapid emergence and/or selection of phage resistant mutants may lead to a concern associated with use of phages to control *L. monocytogenes*. Consistent with this concern, our data show that even with susceptible *L. monocytogenes* strains, where phage cocktails showed considerable reduction of *L. monocytogenes* within the first 8 to 12 h of treatment, subsequent re-growth of *L. monocytogenes* was observed in most treatments. A similar pattern was observed by Bigot et al. (2011) who reported a 4-log-unit reduction in *L. monocytogenes* counts after 5 h of the phage treatment (at 30°C in the broth model) with subsequent re-growth to the same level as that of the phage-free control after 24 h. In addition, a number of studies also reported evidence of *L. monocytogenes* re-growth in foods treated with phages (Guenther et al., 2009; Bigot et al., 2011; Leverentz et al., 2003; Soni et al., 2009). On the other hand, Soni and Nannapaneni (2010) reported no evidence for *L. monocytogenes* re-growth over 48 h at 30°C, based on optical density (OD_{630}) monitoring of *L. monocytogenes* co-cultured with listeriaphage P100. This may reflect limited sensitivity of OD-based measurements as compared to bacterial enumeration or that re-growth after phage treatment may be less likely for certain phage-host combinations used in various studies. Reduction in phage titers over time could be one possible explanation for the re-growth of *L. monocytogenes* in some phage challenge experiments. Soni et al. (2009) reported a decrease in phage population on phage-treated catfish fillet of about 2 $\log_{10}$ PFU over a 10-day storage period, and hypothesized that this could be responsible for the regrowth of *L. monocytogenes* on the fillet. However, in our study phage titers remained unchanged or slightly increased over 24 h in the broth challenge

experiments (data not shown), suggesting that decreased phage titers are not responsible for the re-growth patterns we observed here.

Interestingly, characterization of *L. monocytogenes* isolates recovered after 24 h of phage cocktail treatment showed diminished susceptibility of these isolates to individual phages used in the phage cocktails, suggesting that emergence or selection for phage-resistant *L. monocytogenes* strains may be responsible for the re-growth patterns observed. Similar to our findings, O'Flynn et al. (2004) reported re-growth of *E. coli* O157:H7 after an initial 5-log reduction of *E. coli* O157:H7 counts within 5 h of treatment with a phage cocktail using a broth model. These surviving isolates recovered from these experiments were also found to be the bacteriophage-insensitive mutants (BIMs) with reduced susceptibility to each phage in the phage cocktail. Recovery of phage-resistant bacterial cells was also observed after cell lysis of *S. aureus* challenged with phages (Synnott et al., 2009). On the other hand, several studies that showed re-growth of *L. monocytogenes*, in inoculated foods, after treatment with listeriaphages P100 and/or A511 (Carlton et al., 2005; Guenther et al., 2009) did not find evidence for emergence or selection of phage-resistant *L. monocytogenes*. Some of these authors hypothesized that the re-growth was caused by surviving cells which remained uninfected due to immobilized phage particles in the food matrices that were unable to diffuse to reach theses target cells. While different mechanism may be responsible for *L. monocytogenes* re-growth in different model systems (e.g., broths versus inoculated foods) and with different phages and host strains, our data clearly support that phage resistant *L. monocytogenes* can rapidly emerge under certain conditions. We appreciate that emergence of phage resistant *L.*

monocytogenes after phage treatment in broth at 30°C does not necessarily reflect conditions encountered in foods stored at refrigeration temperatures. Further studies on emergence of phage resistant *L. monocytogenes* after different phage treatment conditions will be necessary.

5. Conclusions

Identification of *L. monocytogenes* strains that have persisted in food associated environments provides relevant set of strains which should be used for evaluation of treatments aimed at reducing or controlling *L. monocytogenes* in food processing plant environments and contaminated foods. Using *L. monocytogenes* isolates representing diverse subtypes that have persisted in a food processing facility, we not only found evidence that some of the subtypes may be resistant to the majority of phages, leading to the potential treatment failures, but we also found, at least under some conditions, rapid emergence of phage resistant *L. monocytogenes* clones. Our data are consistent with a recent EFSA report (EFSA, 2009) whose conclusions included that (i) “bacteriophage insensitive mutants might exist among the populations of target bacteria”; and that (ii) the “frequency of these mutations and their consequences are likely to vary according to the bacteriophage, the conditions of its application and the target bacteria”.

Future efforts for research and development are thus clearly needed before application of phage-based biocontrol for *L. monocytogenes* become widely used. Key research needs include, but are not limited to (i) resistance to phages and phage cocktails of *L. monocytogenes* subtypes that are commonly found in food processing

plant environments, with a focus on isolates that have persisted in a given facility (as these isolates are particularly likely to contaminate foods and may be more likely to be phage resistant, facilitating persistence); (ii) mechanisms of phage-resistance in *L. monocytogenes*; and (iii) frequency and mechanisms of emergence of phage resistant *L. monocytogenes* mutants.

Acknowledgements

The project was supported by Hatch Funds of the Cooperative State Research, Education, and Extension Service, U.S. Department of Agriculture (USDA-CSREES, NYC-143445) and the Royal Thai Government Fellowship (to KV).

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CHAPTER FOUR

Genomic diversity of *Listeria* phages isolated from farm environments

ABSTRACT

Listeria phages have been reported to have a high prevalence on dairy farm environments. To obtain information on genome characteristics and diversity of *Listeria* phage isolated from dairy farms, genomes of 10 *Listeria* phages, representing different host ranges, were sequenced on the Illumina platform. Newly sequenced phages showed genome sizes that could be classified into 3 ranges; small (36-38 kb; n=3), mid-sized (64-67 kb; n=4), and large (133-135 kb; n=3). Of the three small-sized phages, one phage contained a lysogeny control module suggesting this phage has a lysogenic lifecycle. All other phages sequenced in this study lack this lysogeny control module, suggesting these phages have lytic lifecycle. The lysogenic phage showed similarity to *Listeria* phage PSA, while the other small-genome size phages show similarity to *Listeria* phage P35. Three large-genome phages showed high amino acid sequence similarity to one another and to the genome of *Listeria* phages A511 and P100. Genomes of the four mid-sized phages were highly similar, but did not show similarity to any previously described *Listeria* phages, suggesting these are novel *Listeria* phages. The genomes of these phages appear to have a considerable mosaic origin; with many genes showing similarity to genes found in *Firmicute*-specific phages. Our findings show considerable genomic diversity of *Listeria* phages on dairy farms. Genomes of the newly described *Listeria* phages here feature a high degree of

mosaicism, which may have arisen from horizontal transfer of specific genes or functional modules during the evolution of these *Listeria* phages.

INTRODUCTION

Listeria monocytogenes is an important foodborne pathogen responsible for severe infections, listeriosis, in both animals and humans (14, 29). Previous studies have also shown that phages play an important role in the evolution and virulence of many pathogens (for review see (4)), however, knowledge on the roles of *Listeria* phages contributing to their *Listeria* hosts' pathogenicity and biology is still limited. The focus of *Listeria* phage research has been previously studied on host range (19, 25), ability to transduce (16), and genomic structure and gene functions (5, 11, 20, 26, 42).

Currently available genomes of *Listeria* phages show the range in size of 35.6 to 134.4 kb (available at www.ncbi.com; (11)). Molecular and *in silico* analyses by Dorscht et al. (11) showed that *Listeria* phage genomes, particularly temperate phages, can be found in several phylogenetic clades and display a conserved genome organization. Dorscht et al. (11) also showed that some phages contained regions in different parts of their genomes that showed high homology to proteins encoded by various non-*Listeria* hosts such as *Enterococcus faecalis* V583 (32), *Staphylococcus* phages 77 and 3A (21).

Horizontal gene transfer (HGT) is a major cause of mosaic genomes and genomic diversity in bacteriophages (15). A phage genome is considered mosaic when a genome contains regions of obvious sequence similarity to closely related phages,

while regions are interspersed with segments that are apparently unrelated (15). Phages are known to exhibit a mosaic relationship with phages infecting the same or different host species (10, 28). Mosaic relationship can occur at the level of genetic module, at the level of the gene, and within genes (30). Mosaicism is not uniform across phages; it can found across phages of different genome sizes, phages with variations in genome organization or mechanisms in DNA packaging and replication (15). Some phages have genes encoding proteins that enhance recombination between phage genomes, which can result in speeding up their evolution or improving their competitive fitness (4, 28).

In this paper we present a comparative genomic analysis of 10 phages to probe the genomic diversity of *Listeria* phages, as well as the evolutionary mosaic relationships among *Listeria* phages and other closely related bacteriophages. We performed our analysis on phages isolated from dairy farms. As farm environments display a high abundance and diversity of *Listeria* hosts (31), they provide a good source for a wide diversity of *Listeria* phages.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. The 10 *Listeria* phages sequenced in this study were isolated from silage samples collected on 2 dairy farms in New York State between 08/2008 and 07/2009 (Table 1). Phages were isolated using three *L. monocytogenes* host strains; FSL J1-208 (lineage IV, serotype 4a), F2365 (lineage I, serotype 4b), and Mack (lineage II, serotype 1/2a). The 10 *Listeria* phages here exhibited diverse host ranges observed as determined by a spot test on 13 *L.*

monocytogenes isolates representing the nine most common serotypes as well as the four phylogenetic lineages (Vongkamjan et al., submitted, 2012). Genome sizes of the 10 phages have been estimated by Pulsed Field Gel Electrophoresis (PFGE) and three phages sequenced in this study (LP-037, LP-110, LP-124) were characterized by Transmission Electron Microscopy (TEM) in a previous study (Vongkamjan et al., submitted, 2012).

Preparation of phage lysate and phage DNA extraction. Phage lysates and genomic DNA were prepared according to the following protocol. An isolated plaque from the third purification passage was picked with a sterile Pasteur pipette and suspended in 100 µl of SM buffer (100 mM NaCl, 8 mM $MgSO_4$ and 50 mM Tris-HCl, pH 7.4). A 10-fold dilution of the plaque-SM suspension was used to prepare three overlay plates by mixing 100 µl of this diluted suspension with 300 µl of a 1:10 dilution of an overnight culture (approx. 10^8 CFU/ml) of an appropriate host strain (the strain which was used for phage isolation). The overlay plates were incubated at 30°C for 24 h. After 24 h 5 ml of SM buffer was added to each plate and the overlay was scraped into a 25-ml centrifuge tube with a sterile cell scraper (Becton Dickinson, Sparks, MD). Chloroform was added to the overlay to reach a final concentration of 2% (vol/vol), and the overlay-chloroform mixture was centrifuged at 4200 x *g* for 15 min. The supernatant was subsequently filtered using a 0.2 µm syringe filter. The resulting phage lysate was then used for DNA extraction. Polyethylene glycol 8000 in the presence of 1 M NaCl was added to the phage lysate to purify and concentrate the phages. Removal of bacterial nucleic acid contamination was performed using DNase I (Promega BioScience, San Luis, Obispo, CA) (5 µg/ml final concentration) and

RNase A (Sigma) (30 μg/ml final concentration). After 30 min of incubation at room temperature, the digestion reactions were inhibited by addition of EDTA to a final concentration of 20 mM. Subsequently proteinase K (0.2 mg/ml) and SDS (0.5%) were added to break down phage capsids and particles. Phage DNA was subsequently extracted using a standard phenol/chloroform protocol, followed by an ethanol precipitation step (34).

Phage genome sequencing, annotation, and analysis. Library preparation and DNA sequencing was performed at the Cornell University Life Science Core Laboratory Facilities, using the Illumina GA II sequencing platform (Illumina Inc., San Diego, CA). Thirty-six base pair reads were assembled by *de novo* using the Velvet (41). Assemblies consisting of one contig were obtained for three phages. For the remaining seven phages with multiple contig pseudogenomes were created as detailed below. Mauve genome alignment software (9) was used to visualize genome similarities between phages sequenced in this study and previously described *Listeria* phages. Contigs of phage genome assemblies consisting of multiple contigs were ordered according to their alignment to finished genome sequences of *Listeria* phages with high sequence homology to the genome to be ordered. To create a pseudogenome, the ordered contigs were concatenated and 5 Ns were inserted between the 3' end of the first contig and the 5' of the following contig to designate the start and stop of the original contigs. All sequences were submitted to the RAST (available at http://rast.nmpdr.org/) genome annotation service (3) for automatic annotation. Additionally tRNAs were predicted using tRNAscan-SE 1.21 (27). Further homology searches of nucleotide and predicted amino acid sequences manual annotation were

performed through NCBI databases (http://www.ncbi.nlm.nih.gov) using the BLAST algorithms (2). Sequence alignments were performed with CLUSTALW version 2.0 (22). Phage genome maps were drawn using Easyfig (37).

Large terminase subunit analysis. Comparative analysis of the large terminase (TerL) subunit can predict in which different functional classes these terminases fall and thus predicting the putative packaging strategies of phages encoding certain terminase (6). To infer the putative DNA packaging strategies of individual phages, a phylogenetic analysis of the large terminase subunit was performed. Amino acid sequences of the large terminase subunit of the newly sequenced phages and AA sequences of the previously sequenced phages with known packaging strategies were aligned using MAFFT version v6.846b (17) using the EINSI algorithm. This algorithm aligns conserved domains among sequences while aligning divergent regions only between closely related entrees. This alignment was used to infer a maximum likelihood (ML) tree using RAxML version 7.30 (36) using a BLOSUM62 plus Gamma model of amino acid evolution. Hundred rapid bootstrap replicates were performed to assess the robustness of the individual clades.

Extended pan-genome analysis. To infer to what extend the environmental phages sequenced in this study attribute new gene families to the pan-genome of *L. monocytogenes*, we used the binomial mixture model for pan-genome size estimation of Snipen et al (35) to estimate the size of the *L. monocytogenes* pan-genome with and without the phages sequenced in this study. To infer the *L. monocytogenes* pan-genome, we used high quality draft and finished genome sequences representing all major phylogenetic lineages; 10403S (Genbank accession CP002002.1), 08-5923

(Genbank accession CP001604.1), EGD-e (Genbank accession AL591824.1), Finland 1998 (Genbank accession CP002004.1), FSL R2-561 (Genbank accession CP002003.1), J0161 (Genbank accession CP002001.1), 08-5578 (Genbank accession NC_013766.1), F2365 (Genbank accession NC_002973.6), CLIP 80459 (Genbank accession FM242711.1), Scott A (Genbank accession AFGI00000000.1), FSL J1-208 (Genbank accession CM001469.1) and HCC23 (Genbank accession NC_011660.1). Addition of the phage genomes to the analysis would decrease the core genome to a number far below the expected core genome of *L. monocytogenes*, we created artificial genomes consisting of genes of *L. monocytogenes* FSL J1-208 and the individual phage genomes. The pan-genome size estimate of the *L. monocytogenes* and the environmental phages was based on the *L. monocytogenes* genomes mentioned above and the 'artificial genomes'.

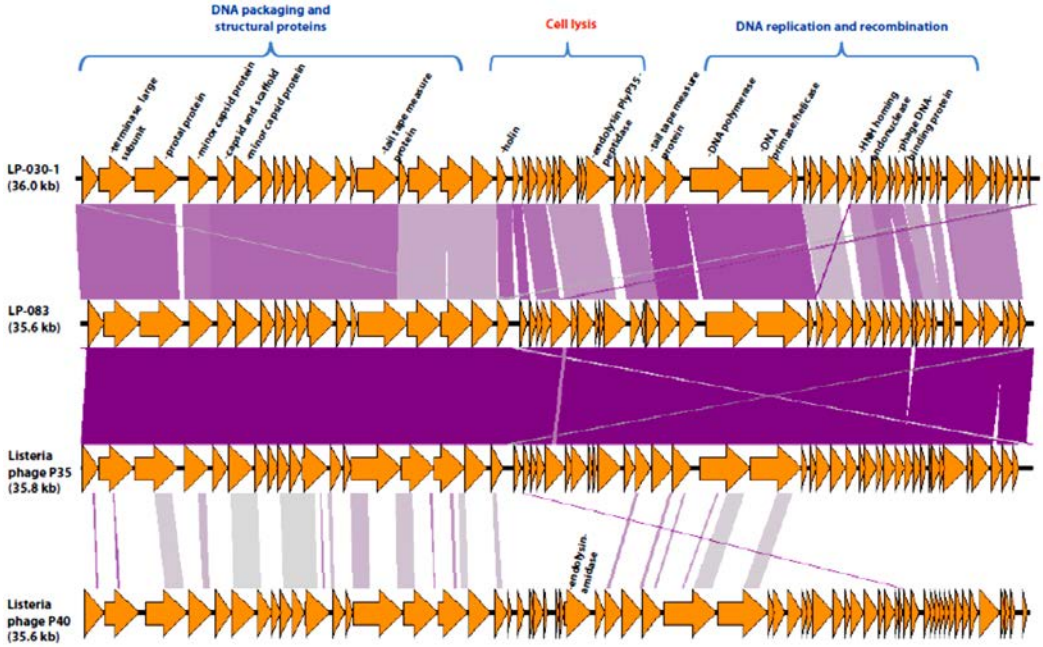
RESULTS

Genomes of *Listeria* phages feature diversity. Genomes of the 10 *Listeria* phages could be classified into 3 major size ranges (Table 1): (i) small genomes: 36-38kb [n=3]; (ii) mid-sized genomes: 64-67kb [n=4]; (iii) large genomes: 133-135kb [n=3]. The number of predicted ORFs correlated with genome sizes for all phages. Small-genome phages contained 57-67 predicted ORFs. Mid-sized and large genome -phages showed about 110-114 and 171-181 predicted ORFs, respectively. Previous characterization by Electron Microscopy revealed that phages LP-037, LP-110 were classified into the *Siphoviridae* family, while the large-genome phage LP-124, was classified into the *Myoviridae* family (Vongkamjan et al., submitted, 2012). Among

the newly sequenced phages, 8 phages showed average G+C contents of 35 to 37%, which is slightly lower than those observed in most *L. monocytogenes* strains available in Genbank as of July 26, 2012 (38 to 39%). Phage LP-030-1 and LP-083 and showed an average G+C content of 39.8 and 40.9%, respectively. tRNA genes were not found in *Siphoviridae*-family phages, however, 17 tRNAs were found in all 3 *Myoviridae*-family phages.

Genomes of phages LP-030-1 and LP-083 are similar to the previously described *Listeria* phage P35 and their genome organization is highly conserved. Sequence analysis of two phages with the smallest genomes observed in this study, LP-030-1 and LP-083, revealed that these genomes resembled genome of the previously described *Listeria* phage P35 (11). Their genome sizes and numbers of predicted ORFs were found to be nearly identical (Figure 4.1A). To predict the life cycle, we conducted a Blast search for sequence homology and conserve domain of the lysogeny control (i.e, integrase, repressor, cro repressor, or cro-like repressor) (11, 26, 42); the analysis revealed no sequence similarity of any of these genes in their genomes, indicating a lytic life style of these phages. Overall, the three lytic phages shared an identical genome organization and sequence similarity > 80% at the amino acid level for all functional modules (Figure 4.1A). All ORFs on these genomes were transcribed rightwards and could be divided into 3 major functions. The first module (ORFs 1-18), located at the left-arm of the genome map, comprises genes encoding proteins responsible for DNA packaging (e.g., TerL) as well as structural proteins, including head and tail morphogenesis. The second module, located in the middle of the genomes (ORFs 19-29), comprises “cell lysis” genes encoding a putative holin and

A.



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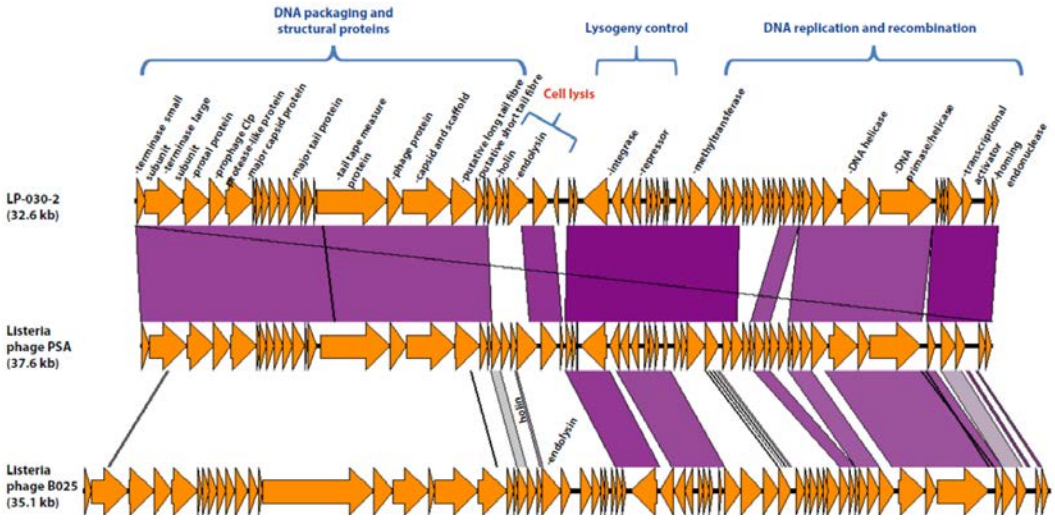

Figure 4.1. Genome map alignments of small-genome *Listeria* phages (<40 kb). Gene products with amino acid sequence identities are linked by color-shaded areas as follows: grey, 25 to 40% identity; purple, ≥ 55%. Putative functions of selected genes under specific cluster modules are indicated here.

endolysin. These genes were also clustered together with multiple small ORFs with unknown function. The last module at the right-arm of the genome map includes genes encoding a relative large number of proteins involving in DNA processing (replication, recombination, and modification) which is considered the "early" functions. Among ORFs in this early gene cluster, several genes revealed high sequence similarity not only to those of phage P35, but also *Listeria* phages P40 (11), *Bacillus subtilis* phage SPP1 (NC_004166.2), and *Enterococcus faecalis* V583 (32). Putative holins of phages LP-030-1 and LP-083 were classified in a phage_holin_4 family which also showed high sequence homology to that of *Listeria* phages P35 and P40, *Lactococcus* phage 1358 (12) and *Streptococcus* phage Cp-1 (33). Putative endolysins in phages LP-030-1 (ORF 29) and LP-083 (ORF 28) displayed moderate (60% AA identities) and high (99% AA identities) homology to the endolysin of phage P35, PlyP35.

Genome of phage LP-030-2 shows high similarity to the previously described *Listeria* phage PSA, while the cell lysis module is diverse among these phages. Although the genome of the sequenced phage LP-030-2 is slightly smaller than that of the previously described phage PSA (42), both phages shared sequence similarity at the amino acid level of approximately 80% of their genomes. Among the predicted 67 ORFs, only 25 were found to be homologous to proteins with a functional assignment, while 42 ORFs encode proteins without known functions. Overall, both LP-030-2 and PSA showed a similar genome organization displaying defined modules of the early and late genes similar to those described in lytic phages above. However, an additional lysogeny control module, comprised of genes encoding an integrase and a repressor

for the lysogeny functions was also observed in both genomes. The lysogeny control was located in between the cell lysis cluster and the early gene cluster (DNA replication and DNA modification). In addition, genes in this lysogeny control module (ORFs 33-39) were found to be oriented in the opposite direction to the other three functional modules (Figure 4.1B). Having ability to encode both integrase and repressor suggests that these phages could have both lytic and temperate life cycles. While genes in most functional modules showed homology to previously genes in Genbank, genes in the cell lysis module and several ORFs coding for proteins with unknown function in the DNA replication and modification module (Figure 4.1B) showed no similarity to genes in the public databases. The endolysin (ORF 31; 308 aa) of phage LP-030-2 is not only different from that of phage PSA (ORF 31, 314 aa), it is also unique due to its protein structure, which consists of a peptidoglycan recognition protein (PGRP) domain and is classified in a class of N-acetylmuramoyl-L-alanine amidase (24). An endolysin with this domain has not been previously described in any *Listeria* phages, however, this N-acetylmuramoyl-L-alanine amidase was found to be homologous to *Xly* of *L. monocytogenes* HPB2262 and *L. innocua* Clip11262. Moreover, a homolog of this gene has been also found in many *Bacillus* phages or *Bacillus* spp. (13). Interestingly, the holin protein in this phage contains a phage_holin_5 family domain (PF06946), which is different to that in phage PSA, but also found in phages A118 and A500. Interestingly, the temperate *Listeria* phage B025 revealed most regions of homology in the lysogeny control (e.g., integrase and repressor proteins), part of the DNA replication module, and the terminase large subunit. This may suggest that these temperate phages are likely to have (i) identical

attP/attB sites; $tRNA^{Arg}$ has been identified as *attB* integration site in phages PSA and B025 (11, 42); (ii) identical DNA packaging strategy or genome structure; genomes of phages PSA and B025 have been identified to have a complementary single-stranded overlapping (cohesive) ends (11, 42). However, most proteins for head and tail morphogenesis, and the endolysin in the cell lysis cluster of B025 and phages LP-030-2 and PSA showed only limited sequence homology, indicating that phage B025 may exhibit different morphology than the other two phages. This finding also suggests that endolysins are not conserved in these phages.

Genomes of the three sequenced *Myoviridae*-family *Listeria* phages show high sequence homology to *Listeria* phages A511 and P100. Alignment of genome sequences showed that the three genomes of LP-048, LP-124 and LP-125 are very similar to each other, with a sequence similarity at the amino acid level of approximately 90% across the predicted ORFs in their genomes. These genomes are organized into two major functional modules: (i) a DNA packaging and structural protein module, and (ii) a DNA replication, metabolism, and repair module (Figure 4.2). In addition to the two defined functional modules (early and late genes), genomes also contain a cluster at the left-arm of the genome map which comprises of mostly genes encoding proteins with no obvious function. Genes producing serine/threonine protein phosphatase (ORF 42), protein RtcB (ORF 66), and 17 tRNAs were also located in this cluster. Interestingly, RtcB was found in this cluster which is likely to act as RNA ligase that mediates the joining of broken tRNA-like stem-loop structures in case of tRNA damage as this has been previously proposed as a component of an

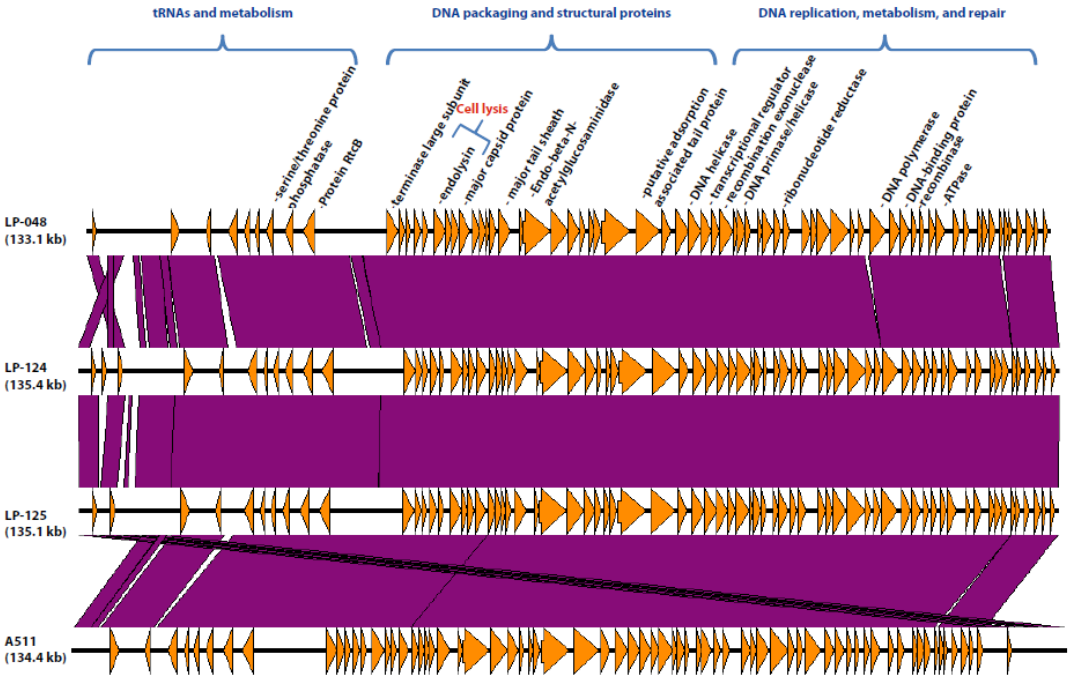

Figure 4.2. Genome map alignments of *Listeria* phages that display large genomes (>130 kb). Gene products with amino acid sequence identities ≥ 90% are linked by purple-shaded areas. Putative functions of selected genes under specific cluster modules are indicated here.

Escherichia coli RNA repair operon (38). Overall, these genomes displayed the gene orders and defined modules similar to genome of phage A511 (Figure 4.2), while only a cluster at the left-arm was found in the opposite end of the genome of phage P100. All 17 tRNA genes found in these phages were homologous to those of phages P100 and A511.

The first major module following the left-arm gene cluster starts at ca. 44 kb-region, comprising DNA packaging and structural gene cluster. While the putative small terminase gene could not be identified, the putative large terminase genes (ORFs 82-83) were located at the beginning of this gene cluster, followed by structural genes encoding for, for example, major capsid (ORF 95), tail sheath (ORF 102), and adsorption associated tail (ORF 115). The cell lysis cluster in these genomes was not identified in a separate module, such as the cell lysis module found in phages LP-030-1, LP-083, and LP-030-2. However, the endolysin gene (ORF 88) was found to be embedded in the DNA packing and structural gene cluster. Putative endolysins of the three phages displayed significant homology to that of phages A511 and P100 (Ply100), while a putative holin was not found in the cell lysis cluster of these genomes. Interestingly, two large ORFs (106-107) located near the genes encoding structural proteins were identified to encode putative tail lysins and one was found to be an endolysin of another class, endo-beta-*N*-acetylglucosaminidase (glucosaminidase) (24). This putative tail lysin showed high homology to the putative tail lysin of *Enterococcus* phage phiEF24C (39) and *Lactobacillus* phage Lb338-1 (1), as well as the ORF56 encoding an enzyme of phage K which is identified as phage tail-associated muralytic enzyme (TAME) (40).

The second functional module comprises a gene cluster encoding proteins responsible for DNA replication, metabolism, repair, and modification. This gene cluster is flanked by a helicase gene (ORF 120) and ATPase gene (ORF 166). Predicted gene products required for DNA replication and repair observed in this module include the following proteins: helicase (ORFs 120), transcription regulator (ORF 121), exonuclease subunits (ORFs 123-124), primase (ORF 126), DNA polymerase (ORFs 146-147), DNA binding protein (ORF 149), recombinase (ORF 150), DNA repair exonuclease (ORF 157), an integration host factor (ORF 162), and ATPase (ORF 166). Enzymes involved in nucleotide metabolism such as ribonucleotide reductase (ORFs 132-135), and ribose-phosphate pyrophosphokinase (ORF 141). A few other ORFs encode proteins that could facilitate the posttranslational modification such as flavodoxin and thioredoxin (ORFs 137-138) proteins. In addition, a lysogeny control module was not observed in these genomes, thus indicating a lytic life cycle of these phages. On an overall scale, genomes of these three sequenced phages contain a large region with a significant homology to (i) other known *Listeria* phages A511 and P100; (ii) other *Myoviridae* phages infecting *Firmicutes*, i.e., *S. aureus* phage K and *Lactococcus* phage LP65.

Novel lytic *Listeria* phages of the *Siphoviridae* reveal a genome size of about 65-67 kb and their genomes are organized into two major modules similar to phage A511. Genomes of four phages show the approximate size of 65-67 kb and similar numbers of predicted ORFs. These genomes revealed approximately 80% sequence identity at the nucleotide level (Figure 4.3). Of about 110-116 predicted ORFs, only 16-18 encoded proteins with high sequence similarity to proteins of known function

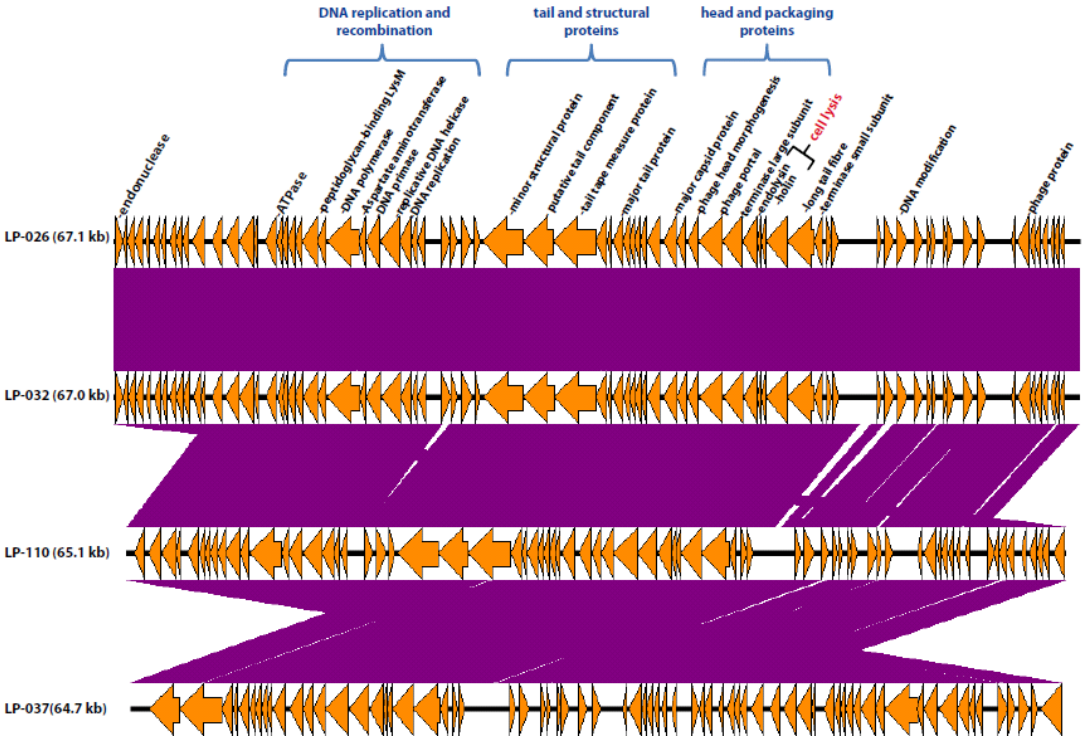

Figure 4.3. Genome map alignments of novel *Listeria* phages. Genomes of these phages show the range of 65-67 kb in sizes. Gene products with amino acid sequence identities ≥ 80% are linked by purple-shaded areas. Putative functions of selected genes under specific cluster modules are indicated here.

from the database. However, a number of proteins show similarities to proteins involved particularly in the head and tail morphogenesis in a variety of phages such as *Staphylococcus* phage SAP6, *Bacillus* phage SPP1, and *Brochothrix* phage NF5 (18). Their terminase large subunits are also homologous to that of *Staphylococcus* phages SAP6 and phiNM, suggesting that they may have similar DNA packaging strategy, known as a headful packaging mechanism, producing a collection of circularly permuted, terminally redundant DNA molecules. In addition, some of the hypothetical proteins show homology to phage and prophage proteins of unknown function with DUF 1642 and DUF 2479 domains. These domains are commonly identified in *Listeria* spp. and various phages (e.g., *Staphylococcus* phage 187, *Listeria* phage PSA, *Brochothrix* phage NF5, and *Enterococcus* phage phiEF24C).

Based on the TEM characterization of LP-110, these phages are classified as members of the *Siphoviridae* family, featuring a binary (A1) head (55 nm) and a long tail (156 nm).

We found that their structural proteins showed no sequence homology to those of *Listeria* phages, suggesting a likely cause of the morphology difference between these phages and other previously described *Listeria* phages. Overall, their genomes are organized into two major modules as in the three lytic *Myoviridae*-family phages described above. The first defined module following this cluster comprises genes encoding proteins for DNA packaging, head and tail morphogenesis, and is flanked by genes coding the terminase small subunit and minor structural protein. Similar to the three lytic phages, the cell lysis cluster in these phages (holin and endolysin genes) is also embedded in this module. A putative holin is identified with a domain of the

phage_phi LC3 family (TIGR01598) which is a homolog of a holin protein in *Listeria* phages A006 and B025 as well as *Brochothrix* phage NF5, and *LysA* in *Lactococcus* phage phiLC3 (23). An endolysin identified in these phages contain a domain that is classified into a class of L-alanyl-D-glutamate peptidase, the same class as PlyA006, PlyA500, PlyP35, and PlyP100 proteins of *Listeria* phages, but not identical to any of these. The second functional module includes genes for DNA replication, modification, and metabolism. A number of proteins in this cluster display homology to proteins of not only different phages, but also different bacterial species.

Overall, the genome organization is conserved across the four phages. Although some regions of the two major modules as well as the cell lysis gene cluster of these phages shared homology to several *Listeria* phages on the NCBI databases, these genomes did not resemble any *Listeria* phages currently in the public database. In addition, the range of their genome sizes has not been identified in previously described *Listeria* phages. These findings suggest that this set of *Listeria* phages reported here may represent novel lytic *Listeria* phages of a family of *Siphoviridae*. In addition, among the four phages, only phage LP-110 was isolated from a different farm from others, suggesting a wider geographical distribution of these phages. Interestingly, while three phages from the same origin exhibited relatively broad host range, phage LP-110 could not lyse other reference *L. monocytogenes* strains tested besides its host strain, indicating that any differences between their genomes may be responsible for the host range diversity among these phages.

Large terminase subunit analysis. The *Listeria* phages described in this study cluster into three clades with separate functional identities (Figure 4.4). One clade

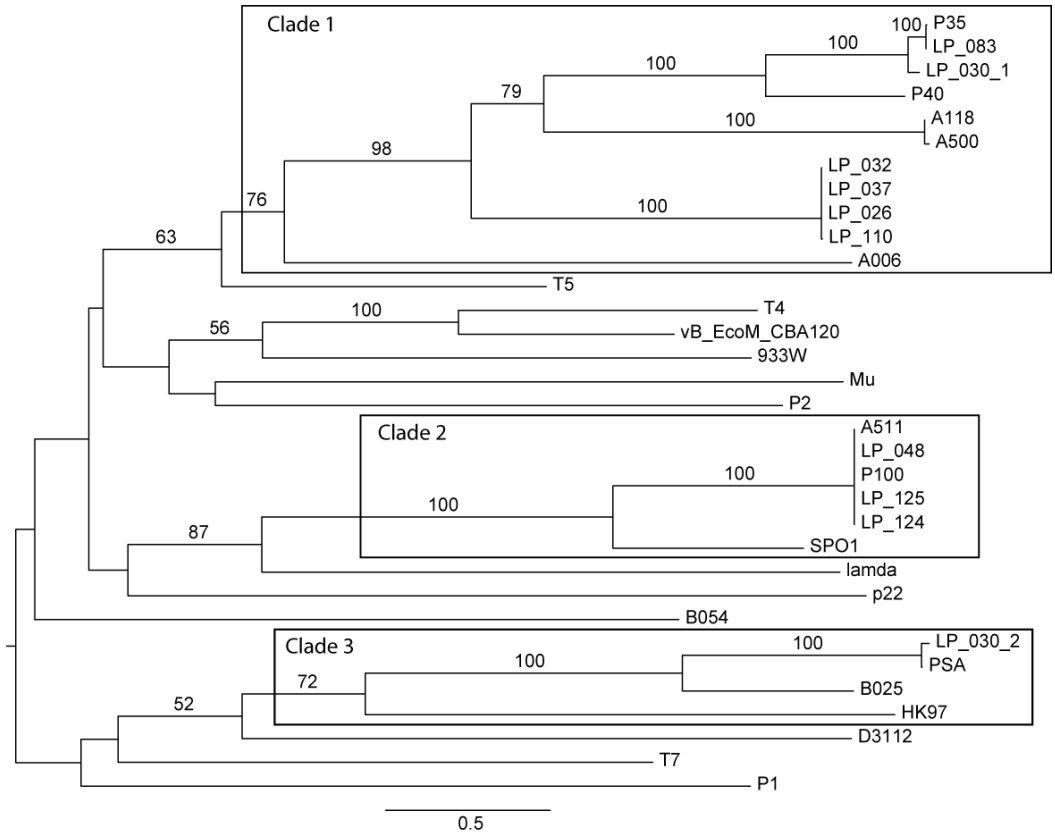

Figure 4.4. Maximum likelihood tree based on analysis of the amino acid sequences of the large terminase subunit. Maximum likelihood bootstrap values >50 are indicated above the branches. Three moderately to well-supported clades could be recognized for the *Listeria* phages sequenced in this study; clade 1, a clade consisting of circularly permuted and terminally redundant phages, clade 2, a clade consisting of phage with long exact terminal repeats and a non-permuted genome, and clade 3, a clade consisting of phages with phages with single stranded cohesive ends. See Table S1 for additional information on the other phages used in this analysis.

with strong support (bootstrap >90) branches further into two subclades, also with strong support; the first subclade contained phages LP-083, LP-030-1 and the previously described circularly permuted and terminally redundant phages P35 and P40 (11), the second subclade consisted of the novel phages LP-032, LP-037, LP-026, and LP-110. Additionally, the circularly permuted and terminally redundant phages A118 and A500 also cluster within the greater clade, suggesting these phages follow a headful DNA packaging strategy, a characteristic of phages with circularly permuted genomes with random terminal redundancy (11, 26). The second clade identified by TerL analysis included LP-048, LP-124, and LP-125 which cluster with strong support to SPO1, a *Bacillis subtilus* phage with long exact terminal repeats and a non-permuted genome, as well as to the SPO1-like listeriaphage A511 (20). The third clade with strong support contained LP-030-2 and the previously described phages PSA and B025, two phages with single stranded cohesive ends on their chromosomes (11, 42). There is also moderate support (bootstrap >70) for this clade clustering with HK97, a model *Escherichia coli* phage with cohesive ends (7).

Environmental phages do not contribute a large number of genes to the pan-genome. Estimates of the size of the *Listeria monocytogenes* pan-genome with or without the genes of the phages sequenced in this study were 8,043 genes and 7,429 genes, respectively. This result indicates that these phages contribute limited number of rare/new genes to the pan-genome.

DISCUSSION

In the current study, we sequenced and analyzed 10 genomes of *Listeria* phages that were isolated from farm environments in New York State, to obtain information on genome organization, gene functions, genetic diversity, and relatedness to previously sequenced phages. These analysis revealed (i) a novel group of *Listeria* phages characterized by a genome size of approximately 65-67 kb, (ii) three groups of phages with genome characteristics similar to previously sequenced phages, and (iii) a limited number contribution of new genes by these environmental phages to the pan-genome of *Listeria monocytogenes*.

Whole genome sequencing reveals a novel group of *Listeria* phages with a genome size of approximately 65-67 kb. In this study we found one group of four *Listeria*-phages with genome sizes between 64,747 bp and 67,115 bp, which showed high (between 96 and 99 % average nucleotide identity) sequence similarity to each other, but only showed partial (i.e. one or two genes) similarity with other *Listeria*-phages and *Firmicute*-associated phages. This partial similarity suggests a mosaic origin of these phages, a phenomenon commonly encountered in bacteriophages (15). Phylogenetic analysis of the large terminase subunit indicates these phages have a circularly permuted and terminally redundant DNA packaging strategy similar to *Listeria*-phages P40, P35, A118 and A500. Electron Microscopy of at least one of the phages (i.e., LP-110) indicates that this novel group of putatively lytic phages belongs to the family of the *Siphoviridae*. The discovery of a group of novel *Listeria*-phages demonstrates that even within a small number of phage genome sequences, novel phages can be discovered. More effort to sequence *Listeria* phages from different

environments will reveal more novel phages and give us a better understanding of *Listeria* phage diversity.

Two groups of phages resemble previously sequenced phages. While mid-sized phages represent a novel group of *Listeria* phages, some of the small and large sized phages show a remarkable resemblance to previously sequenced phages. Phages LP-030-1 and LP-083 showed a high sequence similarity, both on the amino acid and nucleotide level, to phage P35 (11). Phage P35 is a phage isolated from the same geographical area (New York State) and same environment (silage) as phages LP-030-1 and LP-083 (16), which may explain the high sequence homology of this phage to some of the phages sequenced in this study. Overall we found the similarity in the genomic organization and homologous gene content of (i) phages LP-030-2 and PSA; and (ii) the large sized phages A511 as well as P100 and phages LP-048, LP-124, and LP-125. These findingsis remarkable because of the previously sequenced phages were isolated from sewage in central Europe within the last two decades (5, 20). The similarity between European phages and North American phages may either suggest a relative genomic conservedness of these phages. This would imply that while genomic mosaicism can be clearly observed in most of these phages, the rate of recombination responsible for this mosaicism is not high enough to cause observable recombination events after the divergence of European and North American host populations. Alternatively, it may indicate rapid dispersal of both phage and host populations, a scenario suggested for the major clonal groups of *L. monocytogenes* (8).

Environmental phages potentially contribute a limited number of new genes to the pan-genome of *Listeria monocytogenes*. To infer the contribution of

environmental phage genomes to the pan-genome of *L. monocytogenes*, we estimated the size of the pan-genome with and without the environmental phages sequenced in this study. Addition of the 10 phages resulted in a limited (8.3%) increase in the pan-genome, suggesting only limited contribution of environmental phages of potential new genes to the pan genome of the host. It should be noted, however, that only a small sample of phages was included in this study, and the genomes sequenced here represent only a limited selection of the diversity of the *Listeria* phages encountered in the environment. Moreover, the method of isolation of these phages is biased towards a lytic phenotype of the phages and may therefore give an even more unbalanced view of the diversity and thus the contribution of environmental phages to the pan-genome.

This study demonstrates that the sequencing of the genomes of a limited number of *Listeria* phages from a limited geographical region can increase our understanding on the diversity, evolution and putatively the biogeography of these phages. In particular the discovery of a group of novel *Listeria* phages shows that our understanding of these important drivers of the ecology and evolution of *L. monocytogenes* is far from complete.

ACKNOWLEDGEMENTS

The project was supported by Hatch Funds of the Cooperative State Research, Education, and Extension Service, U.S. Department of Agriculture (USDA-CSREES, NYC-143445) and the Royal Thai Government Fellowship (to KV).

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CHAPTER FIVE

Conclusions

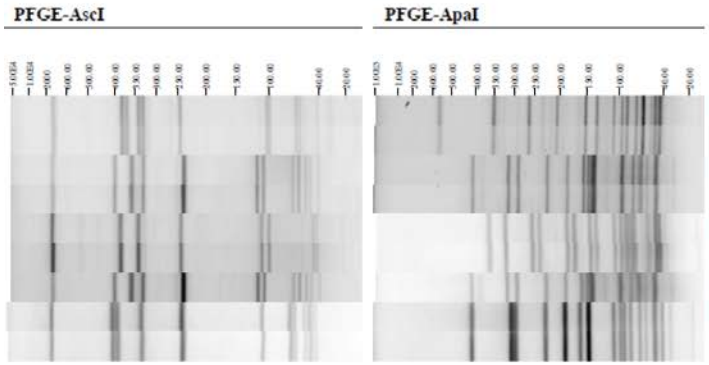
Listeria monocytogenes is an important foodborne pathogen responsible for severe infections, listeriosis, in both animals and humans (Mead et al., 1999; Farber and Peterkin, 1991). The most recent multistate outbreak of listeriosis occurred in 2011 was linked to cantaloupes which led to over 30 deaths, resulting in the deadliest foodborne illness outbreak in the US since 1924 (CDC, 2011). Development of control measures for this pathogen has become a high priority. A phage-based biocontrol agent for *L. monocytogenes* has been approved for use in a variety of RTE foods. However, knowledge of its efficacy against various strains of this pathogen present in food-associated environments has been limited. In this study, we developed a collection of diverse phages which is a valuable tool for further studies by (i) isolating and characterizing *Listeria* spp. and listeriaphages from dairy farms, (ii) evaluating phage susceptibility on persistent isolates and developing phage cocktails against persistent subtypes, and (iii) characterizing the genomic diversity and relationships of selected phages obtained from dairy farms.

Over 100 listeriaphages were isolated from silage samples collected on two dairy farms. Phenotypic and genotypic characterization of phages from this collection revealed considerable host range (9 lysis groups) and genomic diversity (genome sizes of 25–140 kb). Among the 9 major *Listeria* serotypes used in the host range

determination, serotype 3c strain was found to be highly resistant to phages while serotype 4 strains were the most susceptible to phages. Phage evaluation data in this study show variations (4.6–95.4%) in phage susceptibility among various persistent subtypes in food-associated environments, suggesting an importance to develop effective and suitable phage cocktails for specific subtypes. Results from phage cocktail treatments revealed that *L. monocytogenes* populations were temporally reduced for only 8 h after treatment initiation, while some subtypes were not affected by the treatment. Surviving isolates recovered after 24 h of treatment showed decreased susceptibility to individual phages included in the phage cocktail, suggesting rapid emergence of resistant subtypes. Our findings here also indicate that the use of phage cocktails may not always be an appropriate strategy for controlling pathogens. Whole genome sequencing and comparative genomic analysis of 10 selected phages revealed high genomic diversity of *Listeria* phages from dairy farms. We also found that four newly sequenced phages here are novel listeriaphages. Nine phages showed a lytic life cycle while only one phage had a lysogeny control present in its genome. Genomes of three newly sequenced phages appeared to resemble the previously described listeriaphages A511 and P100, despite differences in isolation origins, suggesting geographical distribution of this phage family (*Myoviridae*).

Overall, our diverse phage collection, phage susceptibility data, and information from the comparative genomic analysis of phages will provide a promising tool for further development of phage-related applications, e.g., phage-based biocontrol and phage-encoded lytic enzyme agents for *L. monocytogenes*, and diagnostic tools for foodborne pathogens, to reduce incidence of foodborne disease.

Appendix One



Isolate ID	Ribotype	Lineage	*SigB* Allelic type	Sampling date	CU Pattern No_AscI	CU Pattern No_ApaI
FSL V1-001	DUP-1039C	II	57	10/2007	NYCU.GX6A16.0184	NYCU.GX6A12.0129
FSL V1-002	DUP-1039C	II	57	10/2007	NYCU.GX6A16.0184	NYCU.GX6A12.0129
FSL V1-003	DUP-1042C	I	58	01/2008	NYCU.GX6A16.0018	NYCU.GX6A12.0242
FSL V1-004	DUP-1042C	I	58	01/2008	NYCU.GX6A16.0018	NYCU.GX6A12.0242
FSL V1-005	DUP-1045A	II	57	01/2008	NYCU.GX6A16.0185	NYCU.GX6A12.0206
FSL V1-006	DUP-1045A	II	57	01/2008	NYCU.GX6A16.0185	NYCU.GX6A12.0206
FSL V1-007	DUP-1042C	I	58	01/2008	NYCU.GX6A16.0018	NYCU.GX6A12.0242
FSL V1-117	DUP-1052A	I	63	06/2009	NYCU.GX6A16.0011	NYCU.GX6A12.0282
FSL V1-118	DUP-1052A	I	63	06/2009	NYCU.GX6A16.0011	NYCU.GX6A12.0282

Figure S2.1. PFGE types, sigB allelic types, and ribotype patterns of *L. monocytogenes* isolates obtained from two dairy farms.

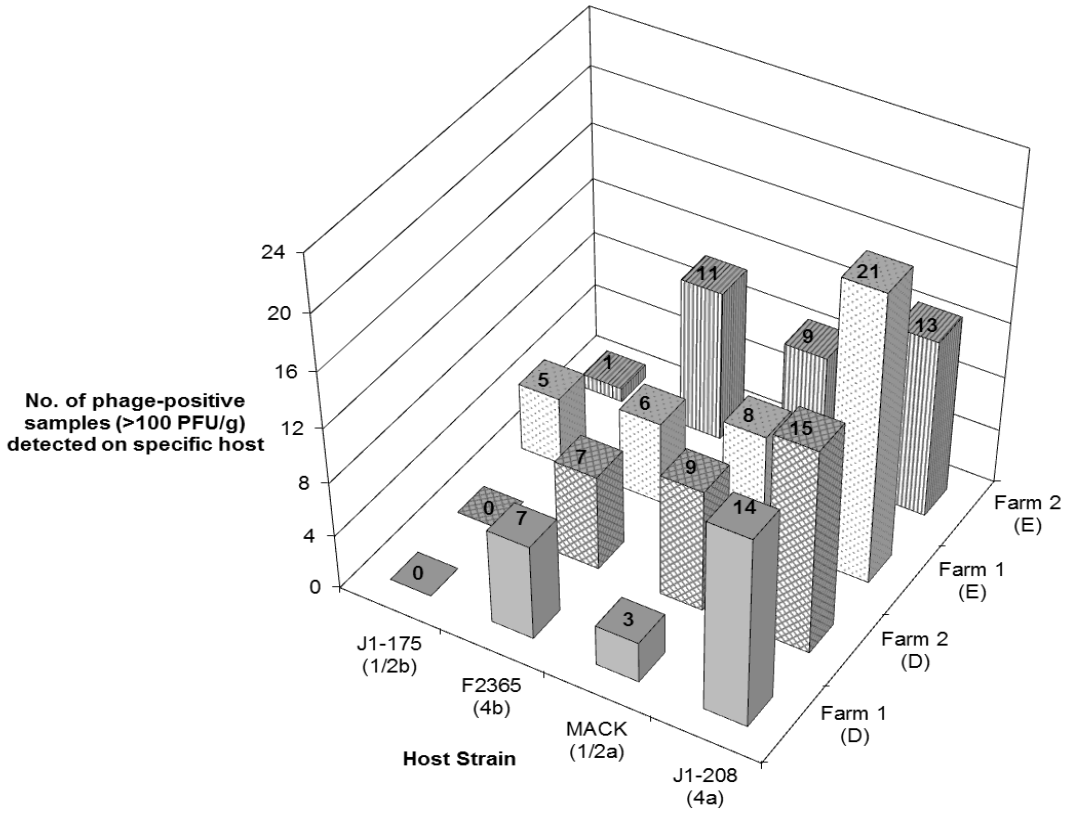


Figure S2.2. Number of phage-positive samples, among silage samples collected on two dairy farms, detected on the four *L. monocytogenes* hosts by (i) direct phage isolation (D); and (ii) isolation after phage enrichment method (E).

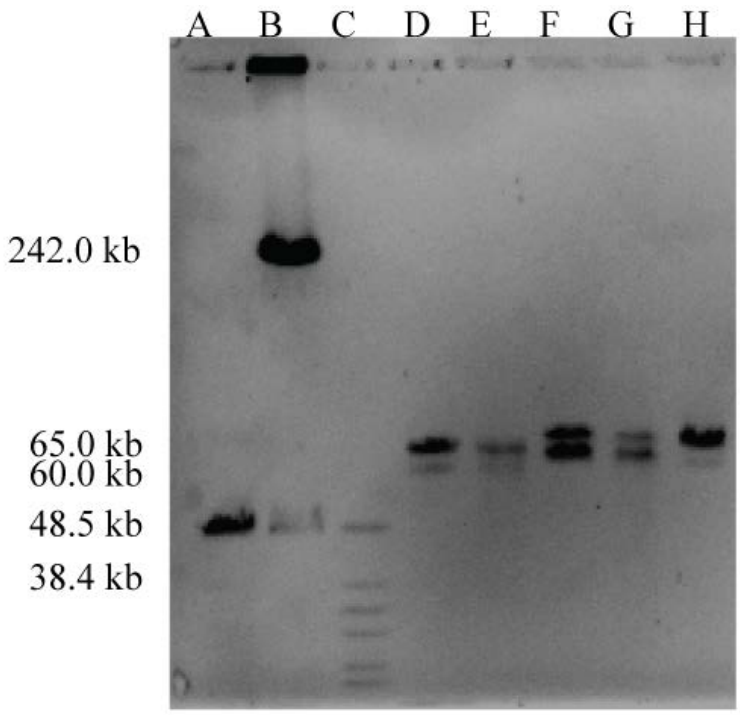
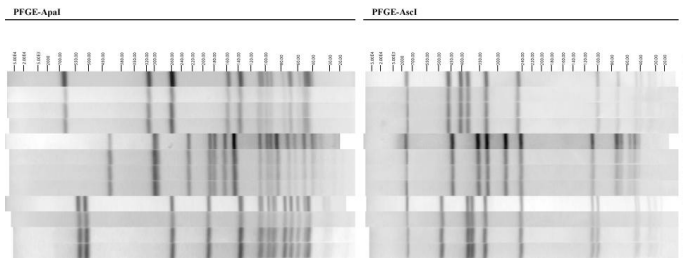


Figure S2.3. PFGE image of DNA of selected listeriaphages that showed two bands. Size markers: (A), lambda; (B), marker range 48kb-1Mb; (C), marker range 8-48kb. Phage DNA: (D) and (F), LP-026 and LP-036 without heat treatment; (E) and (G); LP-026 and LP-036 with heat treatment; (H), LP-037 without heat treatment.

Figure S3.1. Ribotype pattern profile of *L. monocytogenes* isolated from samples collected from a variety of sampling sites in a smoked fish processing facility as well as raw product samples from Nov. 2007 to Nov. 2009; see Table 3 for details of sampling visits. (—) indicates that collected samples were tested negative for *L. monocytogenes*. "blank" in this table indicates that no sample was collected from these sites. Ribotype patterns that were frequently observed (> 2 sampling visits) during this sampling period were highlighted. Note that environmental samples used for isolation of *L. monocytogenes* and *Listeria* spp. during visit no. 11 to 22 were only obtained from drains 1 to 11.

Sample type/sampling site	Ribotype (DUP-) of representative *L. monocytogenes* isolates recovered from samples collected at visit no.																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Raw Product Samples																						
Meat	1040A 1062C		1040A				—															
Skin	1062C																					
Drains																						
Drain 1					1039C 1052A	1039C					—	1039C	1039C	1039C	—	—	—	—	—	—	—	1039C
Drain 2					1052A	1040A					—	—	—	1062A	—	—	—	—	—	—	—	—
Drain 3					1043A	1043A					—	—	—	—	—	1043A	1039C	1043A	—	—	1052A	—
Drain 4	1039C	1040A			—	1039C					—	—	—	—	—	—	—	—	—	—	—	1062A
Drain 5		1040A			1052A	1053A					—	—	1042A	—	—	—	—	—	—	—	—	—
Drain 6					1043A	—					—	—	—	—	—	—	—	—	—	—	—	—
Drain 7					1040A	—					1040A	—	—	—	—	—	—	—	—	1039C	1039C	1039C
Drain 8					—	1040A					—	—	—	—	1039C	—	—	—	—	—	—	1030A
Drain 9					1052A						1039C	1043A	1043A	1043A	1043A	1451S2	1451S2	—	—	—	—	—
Drain 10											—	—	—	—	—	—	—	—	—	—	—	—
Drain 11											—	—	—	—	—	—	—	—	—	—	—	—
Drain 8A					—																	
Drain 10A					—																	
Drain 13A					—																	
Drain 14A					—																	
Drain 15A					1039C																	
Drain 16A					1043A																	
Drain 18A					1052A																	
Drain 19A					1039C																	
Drain 20A					—																	
Drain 21A					—																	
Drain 22A						—																
Drain 25A						1039C																
Drain A					1043A	1039C																
Drain B					1043A	18042 1062A																
Microwave oven drain				—																		
Wet room drain				—																		
Floors/Walls																						
Wet room floor	—																					
Colonial floor	—			—		1039C																
Colonial walls				—																		
Floor - slicing dept.				—						—												
Floor - oven room					—																	
Floor - salad dept.										—												

Sample type/sampling site	Ribotype (DUP-) of representative *L. monocytogenes* isolates recovered from samples collected at visit no.																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Food Contact Surfaces																						
Truck (empty screens)	—																					
Skinning machine (incoming belt)	—							—	—													
Skinning machine (outcoming belt)	—			—		—				—												
Slicing machine (incoming belt)		—				—	—	—	—													
Slicing machine (outcoming belt)	1040A	—	1040A		—	—	—															
Slicing machine (knife)	—	—	—																			
Slicing machine (plastic scraper)				1040A																		
Slicing machine (pin belt)				1040A	—	—		—		1040A												
Slicing machine (accumulating roller)				1040A																		
Slicing machine (controls)				1040A		—																
Slicing machine (outcoming belts & plastic scraper)				1040A																		
Slicing machine (other food contact parts)						—																
Table (trimming)	—			—		—		—	—	—												
Salad mixer (drum)										—												
Salad mixer (belt)										—												
Betcher knife	—									—												
Bone separator										—												
Scale remover				—																		
Gloves - slicing dept.	1040A																					
Gloves - skinning dept.	—									1040A												
Gloves - filet layout						—																
Paddle dip					—																	
Non-Food Contact Surfaces																						
Cart handles - slicing dept.			1039C																			
Cart wheels - slicing dept.										—												
Pallet jack - wet room				1052A																		
Pallet jack - finished product area				—																		
Plastic totes/tubes				1040A	1045B	1052A																
Handles				—																		
Conveyor										—												
Tank drains - raw area			—																			
Soacking hoses - raw area			—			—																
Dumpster - raw area						1039C																
Forklift forks - wet room				—																		
Forklift wheels - wet room				—																		
Microwave handles				—																		
Plastic apron					—																	
Employee's shoes - slicing dept.										—												



Isolate ID	Phage treatment which isolate obtained from	*Asc*I pattern number	*Apa*I pattern number
FSL H1-003	Orignial	CU-182	CU-173
FSL V1-214	MOI 1	CU-182	CU-173
FSL V1-218	MOI 10	CU-182	CU-173
FSL V1-222	MOI 100	CU-182	CU-173
FSL H1-006	Orignial	CU-35	CU-248
FSL V1-230	MOI 1	CU-35	CU-248
FSL V1-234	MOI 10	CU-35	CU-248
FSL V1-238	MOI 100	CU-35	CU-248
FSL N1-053	Orignial	CU-107	CU-140
FSL V1-246	MOI 1	CU-107	CU-140
FSL V1-250	MOI 10	CU-107	CU-140
FSL V1-254	MOI 100	CU-107	CU-140

Figure S3.2. *Asc*I and *Apa*I patterns of (i) original *L. monocytogenes* isolates (prior to phage treatment); and (ii) *L. monocytogenes* isolates recovered after 24 h of phage cocktail treatment for MOIs 1, 10, and 100.

Appendix Two

Table S2.1. Summary of listeriaphage isolates in the current study.

No.	Host	Phage	Farm	Plaque Morphology	Genome size	(kb) [farm visit #]	100x RTD (PFU/ml)
1	J1-208	LP-009	Farm1	0.5 mm, clear, circular			7.0E+05
2	J1-208	LP-010	Farm1	0.5 mm, clear, circular	61.8/65.2	62/65 [5]	2.7E+05
3	J1-208	LP-011	Farm1	0.5 mm, clear, circular			1.2E+06
4	J1-208	LP-012	Farm1	1.5 mm, large, hazy, circular			3.2E+06
5	J1-208	LP-013	Farm1	0.5 mm, clear, circular	59.1/63.4	59/63 [5]	3.0E+06
6	J1-208	LP-014	Farm1	0.5 mm, clear, circular			2.1E+05
7	J1-208	LP-015	Farm1	0.5 mm, clear, circular			4.2E+06
8	J1-208	LP-016	Farm1	0.5 mm, clear/ a bit hazy, quite circular	no band	no band	5.0E+05
9	J1-175	LP-017	Farm1	0.5 mm, clear, circular			4.3E+06
10	MACK	LP-018	Farm1	0.5 mm, clear/ a bit hazy, quite circular			2.7E+06
11	MACK	LP-019	Farm1	0.5 mm, clear/ a bit hazy, quite circular	60.6/64.8	61/65 [5]	4.2E+06
12	F2365	LP-020	Farm1	1.5 mm, large, hazy, circular			1.5E+05
13	F2365	LP-021	Farm1	0.3-0.5 mm, clear/ a bit hazy, circular	no band	no band	1.5E+05
14	J1-208	LP-022	Farm1	0.5 mm, clear, circular	64.2/67.9	64/68 [6]	8.0E+05
15	J1-208	LP-023	Farm1	0.3 mm, clear, circular	61.0/66.2	61/66 [6]	3.3E+06
16	F2365	LP-024	Farm1	1.5 mm, large, hazy, circular			3.8E+05
17	MACK	LP-025	Farm1	0.5 mm, clear/ a bit hazy, quite circular	60.6/64.6	61/65 [6]	1.9E+05
18	J1-208	LP-026	Farm1	0.3 mm, clear, circular	61.3/66.3	61/66 [6]	2.6E+05
19	F2365	LP-027	Farm1	1.5 mm, large, hazy, circular			1.1E+06
20	MACK	LP-028	Farm1	0.5 mm, clear/ a bit hazy, quite circular			1.0E+05
21	J1-208	LP-029	Farm1	0.3 mm, clear, circular			4.8E+06
22	F2365	LP-030	Farm1	1.5 mm, large, hazy, circular	32.6	33 [6]	1.7E+06
23	MACK	LP-031	Farm1	0.3-0.5 mm, clear/ a bit hazy, circular	59.7/64.4	60/64 [6]	4.1E+05
24	J1-208	LP-032	Farm1	0.3 mm, clear, circular	62.4/67.3	62/67 [6]	3.3E+05
25	F2365	LP-033	Farm1	1.5 mm, large, hazy, circular			3.8E+05
26	MACK	LP-034	Farm1	0.3 mm, clear/a bit hazy, circular			1.5E+05
27	J1-208	LP-035	Farm1	0.5 mm, clear, circular	60.6/64.9	61/65 [6]	5.2E+05
28	J1-208	LP-036	Farm1	0.3 mm, clear/a bit hazy, circular	57.9/63.3	58/63 [8]	2.4E+06
29	J1-208	LP-037	Farm1	1.5 mm, large, clear/a bit hazy, circular	57.7/63.9	58/64 [8]	3.1E+06
30	J1-208	LP-041	Farm1	0.3 mm, clear/a bit hazy, circular			2.0E+06
31	J1-208	LP-042	Farm1	0.3 mm, clear/a bit hazy, circular			2.0E+05
32	J1-208	LP-043	Farm1	0.3 mm, clear/a bit hazy, circular			5.3E+06
33	J1-208	LP-044	Farm1	1.5 mm, large, clear/a bit hazy, circular	61.1/66.9	61/67 [9]	7.0E+05
34	J1-208	LP-045	Farm1	0.3 mm, clear/a bit hazy, circular	58.6/62.9	59/63 [9]	6.3E+05
35	J1-208	LP-046	Farm1	0.3 mm, clear/a bit hazy, circular	61.8/65.9	62/66 [9]	3.8E+06
36	MACK	LP-047	Farm1	0.3 mm, clear/a bit hazy, circular			2.2E+06
37	MACK	LP-048	Farm1	0.3 mm, clear/a bit hazy, circular	122.8	123 [9]	1.0E+05
38	MACK	LP-049	Farm1	1.5 mm, large, clear/a bit hazy, circular	31.2	31 [9]	9.0E+04
39	J1-208	LP-050	Farm1	0.3 mm, clear/a bit hazy, circular	60.3/65.5	60/66 [10]	2.0E+05
40	J1-208	LP-051	Farm1	0.3 mm, clear/a bit hazy, circular	62.1/67.4	62/67 [10]	1.9E+06
41	J1-208	LP-052	Farm1	0.3 mm, clear/a bit hazy, circular	60.7/65.1	61/65 [10]	1.2E+06
42	F2365	LP-053	Farm1	1.5 mm, large, clear/a bit hazy, circular			1.7E+06
43	F2365	LP-054	Farm1	1.5 mm, large, clear/a bit hazy, circular	no band	no band	2.9E+06
44	F2365	LP-057	Farm1	1.5 mm, large, clear/a bit hazy, circular	no band	no band	1.8E+06
45	J1-208	LP-058	Farm1	0.3 mm, clear/a bit hazy, circular	61.2/65.7	61/66 [11]	3.8E+05
46	J1-208	LP-059	Farm1	0.3 mm, clear/a bit hazy, circular	60.6/64.7	61/65 [11]	5.1E+05
47	J1-208	LP-060	Farm1	0.3 mm, clear/a bit hazy, circular	62.8/67.8	63/68 [11]	3.6E+06
48	J1-208	LP-061	Farm1	0.3 mm, clear/a bit hazy, circular	60.9/64.9	61/65 [11]	4.0E+05
49	J1-208	LP-062	Farm1	0.3 mm, clear/a bit hazy, circular	61.2/66.3	61/66 [11]	5.8E+06
50	F2365	LP-063	Farm2	0.1 mm, pinprick, clear, circular	no band 2x	no band	2.7E+06
51	MACK	LP-064	Farm2	1.5 mm, large, clear, circular	97.0	97 [1]	1.6E+06
52	J1-208	LP-065	Farm2	1.5 mm, large, clear, circular	62.6	63 [1]	4.4E+05
53	MACK	LP-066	Farm2	1.5 mm, large, clear, circular	no band	no band	2.9E+05
54	J1-208	LP-067	Farm2	1.5 mm, large, clear, circular	61.7	62 [1]	3.6E+05
55	F2365	LP-068	Farm2	0.1 mm, pinprick, clear, circular	118.6	119 [1]	8.7E+05
56	MACK	LP-069	Farm2	0.3-0.5 mm, clear/ a bit hazy, circular			2.2E+05
57	J1-208	LP-070	Farm2	0.3-0.5 mm, clear/ a bit hazy, circular	63.2	63 [1]	2.3E+06
58	J1-208	LP-071	Farm2	0.3-0.5 mm, clear/ a bit hazy, circular	66.1	66 [1]	4.5E+06
59	J1-208	LP-072	Farm2	1.5 mm, large, clear, circular	60.1/64.9	60/65 [1]	2.6E+06
60	J1-208	LP-073	Farm2	0.3-0.5 mm, clear/ a bit hazy, circular	60.7/65.6	61/66 [1]	3.6E+06
61	F2365	LP-074	Farm2	0.3-0.5 mm, clear/ a bit hazy, circular			4.0E+06
62	MACK	LP-075	Farm2	0.3-0.5 mm, clear/ a bit hazy, circular	120.8	121 [1]	6.0E+05
63	F2365	LP-076	Farm2	0.5 mm, clear, circular	no band 3x	no band	1.4E+06
64	MACK	LP-077	Farm2	0.3-0.5 mm, clear/ a bit hazy, circular	no band	no band	1.9E+06
65	J1-208	LP-078	Farm2	1.5 mm, large, clear, circular			2.0E+06
66	J1-208	LP-079	Farm2	1.5 mm, large, clear, circular			3.5E+06
67	J1-208	LP-080	Farm2	1.5 mm, large, clear, circular	61.1/65.1	61/65 [2]	1.3E+06
68	J1-208	LP-081	Farm2	1.5 mm, large, clear, circular	61.2/65.0	61/65 [2]	3.4E+06
69	J1-208	LP-082	Farm2	1.5 mm, large, clear, circular	63.9	64 [2]	2.1E+06

Table S2.1.(Continued).

No.	Host	Phage	Farm	Plaque Morphology	Genome size	Genome size[a] (kb) [farm visit	100x RTD (PFU/ml)
70	MACK	LP-083	Farm2	0.3-0.5 mm, clear/ a bit hazy, circular	32.0	32 [2]	2.0E+05
71	J1-208	LP-084	Farm2	0.3-0.5 mm, clear/ a bit hazy, circular			4.6E+06
72	F2365	LP-085	Farm2	1.5 mm, large, clear/a bit hazy, circular	31.7	32 [2]	9.0E+05
73	J1-208	LP-086	Farm2	0.3-0.5 mm, clear/ a bit hazy, circular	64.9	65 [2]	2.1E+05
74	J1-208	LP-087	Farm2	1.5 mm, large, clear, circular	62.8/67.7	63/68 [2]	3.5E+05
75	J1-175	LP-088	Farm2	0.3-0.5 mm, clear/ a bit hazy, circular	no band	no band	2.0E+05
76	J1-208	LP-089	Farm2	0.5 mm, clear, circular			8.0E+05
77	J1-208	LP-090	Farm2	0.5 mm, clear, circular	58.0/63.3	58/63 [3]	2.7E+06
78	J1-208	LP-091	Farm2	0.5 mm, clear, circular			3.6E+05
79	J1-208	LP-092	Farm2	0.5 mm, clear, circular			2.2E+06
80	F2365	LP-093	Farm2	0.3-0.5 mm, clear/ a bit hazy, circular	no band 9/01/09	no band	1.4E+06
81	F2365	LP-094	Farm2	1.5 mm, large, clear/a bit hazy, circular	25.8	26 [4]	1.3E+06
82	F2365	LP-095	Farm2	0.3-0.5 mm, clear/ a bit hazy, circular	123.3	123 [4]	1.6E+06
83	F2365	LP-096	Farm2	0.3-0.5 mm, clear/ a bit hazy, circular			1.3E+06
84	MACK	LP-097	Farm2	0.5 mm, clear, circular			1.3E+06
85	MACK	LP-098	Farm2	0.5 mm, clear, circular			1.7E+06
86	MACK	LP-099	Farm2	0.5 mm, clear, circular			1.6E+06
87	MACK	LP-100	Farm2	0.5 mm, clear, circular			4.5E+06
88	MACK	LP-101	Farm2	1.5 mm, large, clear, circular	40.7/83.4/115.1	41/83/115 [4]	8.5E+05
89	MACK	LP-102	Farm2	0.5 mm, clear, circular			2.5E+05
90	MACK	LP-103	Farm2	0.5 mm, clear, circular			9.0E+04
91	MACK	LP-104	Farm2	0.5 mm, clear, circular			7.0E+05
92	F2365	LP-105	Farm2	0.3-0.5 mm, clear/ a bit hazy, circular	139.6	140 [5]	2.7E+05
93	F2365	LP-106	Farm2	0.3-0.5 mm, clear/ a bit hazy, circular			2.6E+05
94	F2365	LP-107	Farm2	0.3-0.5 mm, clear/ a bit hazy, circular			2.8E+06
95	J1-208	LP-108	Farm2	0.5 mm, clear, circular			1.9E+06
96	J1-208	LP-109	Farm2	0.5 mm, clear, circular			1.8E+06
97	J1-208	LP-110	Farm2	0.5 mm, clear, circular	56.5/61.1	57/61 [4]	3.2E+05
98	J1-208	LP-111	Farm2	0.5 mm, clear, circular			3.4E+05
99	J1-208	LP-112	Farm2	1.5 mm, large, clear, circular	56.6/61.5	57/62 [4]	1.1E+06
100	J1-208	LP-113	Farm2	0.5 mm, clear, circular	57.8/62.7	58/63 [5]	1.7E+06
101	J1-208	LP-114	Farm2	0.5 mm, clear, circular			2.9E+05
102	J1-208	LP-115	Farm2	0.5 mm, clear, circular	59.8/65.0	60/65 [5]	3.0E+05
103	F2365	LP-116	Farm2	0.3 mm, clear, circular	130.9	131 [6]	6.0E+05
104	MACK	LP-117	Farm2	0.5 mm, clear, circular	117.0	117 [6]	2.7E+05
105	J1-208	LP-118	Farm2	0.5 mm, clear, circular	68.2	68 [6]	2.5E+05
106	F2365	LP-119	Farm2	0.5 mm, clear, circular	136.2	136 [6]	3.5E+05
107	MACK	LP-120	Farm2	0.5 mm, clear, circular	134.0	134 [6]	7.0E+05
108	J1-208	LP-121	Farm2	0.5 mm, clear, circular	58.6/62.7	59/63 [6]	1.2E+06
109	J1-208	LP-122	Farm2	0.5 mm, clear, circular	59.4/63.4	60/63 [6]	3.3E+05
110	MACK	LP-123	Farm2	0.5 mm, clear, circular	131.8	132 [6]	3.9E+06
111	F2365	LP-124	Farm2	0.5 mm, clear, circular	127.0	127 [6]	9.4E+05
112	MACK	LP-125	Farm2	0.5 mm, clear, circular	135.0	135 [6]	2.6E+06
113	J1-208	LP-126	Farm2	0.5 mm, clear, circular	69.6	70 [6]	2.9E+05
114	F2365	LP-127	Farm2	0.3 mm, clear, circular			3.3E+06

Table S2.2. Lysis groups for listeriaphages isolated from two dairy farms.

Phage lysis group	No. of phage isolates from each farm (visit no.[a])	Lysis pattern of *L. monocytogenes* reference strain (serotype)[b]												Phage lysis pattern description
		Mack (1/2a)	J1-175 (1/2b)	J1-094 (1/2c)	C1-115 (3a)	J1-169 (3b)	J1-049 (3c)	F2-695 (4a)	J1-208 (4a)	F2365 (4b)	F2-501 (4b)	J1-158 (4b)	W1-110 (4b)	J2-071 (4c)	
A	F1: 2 (6, 9) F2: 4 (3-5)	+/-	-	+/-	+/-	-	-	-	+/-	-	-	+/-	-	-	Lysis on specific hosts: 1/2a, 1/2c, 3a, 4a, and 4b strains
B	F1: 10 (5, 6, 8, 10, 11) F2: 10 (1- 6)	+/-	-	-	+/-	+/-	-	+	+	+/-	+/-	+/-	+	+	No lysis of 1/2b, 1/2c and 3c strains
C	F1: 3 (6, 9, 10) F2: 5 (1, 2, 5)	-	-	-	-	-	-	+	+	-	+/-	-	+	+/-	Lysis on specific hosts: mostly 4a, 4b, and 4c strains
D	F1: 4 (6, 11) F2: 5 (1, 2, 6)	+/-	+/-	+/-	+/-	+/-	-	+/-	+	-	+/-	+/-	-	+/-	No lysis of only 3c and 4b strains
E	F1: 1 (5) F2: 22 (1, 4-6)	+	+	+	+	+/-	-	+/-	+/-	+/-	+	+/-	+/-	+/-	Broad host range.

Table S2.2. (Continued).

F	F1: 4 (5, 9) F2: 6 (1, 2, 4)	+	+/-	+	-	+/-	-	+	+	+	+	+	+	+	Broad host range. No lysis of only 3a and 3c strains
G	F1: 0 F2: 4 (4, 5)	+	-	+	+/-	-	-	+/-	+/-	+	-	+	+/-	+/-	Lysis on mostly 4a, 4b, and 4c strains
H	F1: 1 (9) F2: 6 (2, 4, 5)	+	-	+	+/-	-	-	+/-	+/-	+	+	+	+/-	+/-	Lysis on mostly 4a, 4b, and 4c strains
I	F1: 24 (5, 6, 8-11) F2: 3 (2, 4)	+/-	+/-	-	-	-	-	+	+	+	+	+	+	+	Lysis on mostly 4a, 4b, and 4c strains

[a] "F1" and "F2" indicate farm 1 and farm 2, respectively; see Table 1 for details on farm visits and phage recovery results from each farm visit.

[b] (-) indicates no lysis of a given strain by any phages in the lysis group; (+)indicates lysis of a given strain by phages in the lysis group; (+/-) indicates that while some phages in the lysis group showed lysis on a given strain, others did not. Heatmap and clustering analysis classifying phage lysis groups are shown in Fig. 1.

Table S3.1. *L. monocytogenes* strains used as hosts for listeriaphage isolation

L. monocytogenes strain (alias)	Lineage	Source	Serotype	Ribotype
FSL J1-175	I	Water	1/2b	DUP-1042A
FSL R2-574 (F2365)	I	Food	4b	DUP-1038B
FSL F6-367 (Mack)	II	Lab strain	1/2a	DUP-1030A
FSL J1-208	IV	Animal	4a	DUP-10142

Table S3.2. Set of 28 phages used in the spot test[a]

No.	Phage	Farm	Visit#/source	Host	Plaque Morphology	Phage Titer (pfu/ml)
1	LP-022	Farm1	6/Bunker7Silage	J1-208	0.5 mm, clear, circular	2.1E+08
2	LP-025	Farm1	6/Bunker6Silage	MACK	0.5 mm, clear/ a bit hazy, quite circular	1.9E+06
3	LP-026	Farm1	6/Bunker6Silage	J1-208	0.3 mm, clear, circular	2.6E+08
4	LP-030	Farm1	6/Bunker6Silage	F2365	1.5 mm, large, hazy, circular	1.7E+06
5	LP-031	Farm1	6/Bunker6Silage	MACK	0.3-0.5 mm, clear/ a bit hazy, circular	4.1E+06
6	LP-037	Farm1	8/Bunker4Silage	J1-208	1.5 mm, large, clear/a bit hazy, circular	3.1E+08
7	LP-048	Farm1	9/Bunker7Silage	MACK	0.3 mm, clear/a bit hazy, circular	1.0E+06
8	LP-054	Farm1	10/Bunker5Silage	F2365	1.5 mm, large, clear/a bit hazy, circular	2.9E+06
9	LP-070	Farm2	1/Bunker1Silage	J1-208	0.3-0.5 mm, clear/ a bit hazy, circular	2.3E+08
10	LP-082	Farm2	2/Bunker3Silage	J1-208	1.5 mm, large, clear, circular	2.1E+08
11	LP-083	Farm2	2/Bunker3Silage	MACK	0.3-0.5 mm, clear/ a bit hazy, circular	2.0E+07
12	LP-085	Farm2	2/Bunker3Silage	F2365	1.5 mm, large, clear/a bit hazy, circular	3.2E+08
13	LP-086	Farm2	2/Bunker3Silage	J1-208	0.3-0.5 mm, clear/ a bit hazy, circular	2.1E+08
14	LP-101	Farm2	4/Bunker2Silage	MACK	1.5 mm, large, clear, circular	1.0E+06
15	LP-105	Farm2	5/Bunker1Silage	F2365	0.3-0.5 mm, clear/ a bit hazy, circular	2.7E+06
16	LP-110	Farm2	4/Bunker2Silage	J1-208	0.5 mm, clear, circular	3.2E+08
17	LP-124	Farm2	6/Bunker3Silage	F2365	0.5 mm, clear, circular	2.9E+06
18	LP-125	Farm2	6/Bunker3Silage	MACK	0.5 mm, clear, circular	2.6E+07
19	LP-128	Farm3	2/Silage	J1-208	0.5 mm, clear, circular	1.0E+06
20	LP-129	Farm3	2/Silage	J1-208	0.5 mm, clear, circular	1.0E+06
21	LP-141	Farm3	1/Silage	J1-208	0.3 mm, clear, circular	1.0E+06
22	LP-143	Farm3	1/Silage	F2365	1.5 mm, large, clear, circular	1.7E+06
23	LP-177	Farm3	6/Silage	F2365	0.5 mm, clear, circular	1.0E+06
24	LP-038	Plant	1/Drain #3	MACK	0.5 mm, clear, circular	1.0E+06
25	LP-039	Plant	1/Drain #9	MACK	0.3-0.5 mm, clear/ a bit hazy, circular	1.1E+06
26	LP-040	Plant	1/Drain #9	J1-175	0.5 mm, clear, circular	1.1E+06
27	LP-055	Plant	3/Drain #9	MACK	0.5 mm, clear, circular	2.0E+07
28	LP-056	Plant	3/Drain #9	J1-175	0.5 mm, clear, circular	1.0E+06

[a] Details on phages no. 1-18 are reported by Vongkamjan et al. (submitted, 2012). Phages no. 24-28 were isolated in the current study.

Table S3.3. Reduction of L. monocytogenes in the phage challenge experiments.

Strain	Time after treatment (h)	Reduction of *L.monocytogenes* in each treatment as compared to the mock-infected control [log (cfu/ml)]											
		Inoculum level of 10^5 cfu/ml						Inoculum level of 10^6 cfu/ml					
		Commercial phage product			Phage cocktail			Commercial phage product			Phage cocktail		
		MOI 1	MOI 10	MOI 100	MOI 1	MOI 10	MOI 100	MOI 1	MOI 10	MOI 100	MOI 1	MOI 10	MOI 100
H1-003	0	0.13	0.09	-0.04	-0.04	0.51	-0.03	-0.01	0.02	0.13	-0.11	0.06	0.14
H1-003	4	0.06	-0.04	0.93	0.30	1.95	2.95	-0.07	0.07	0.82	0.75	2.74	4.35
H1-003	8	0.35	1.23	2.38	4.26	4.26	4.26	0.42	2.14	4.63	4.33	4.12	4.09
H1-003	12	2.18	3.29	2.83	3.66	3.98	3.69	2.89	3.85	3.58	4.20	3.95	3.35
H1-003	16	3.43	3.09	2.27	2.11	2.22	1.96	3.27	3.41	2.15	2.80	2.51	2.21
H1-003	20	2.32	2.12	1.11	1.17	1.24	0.75	2.66	2.32	1.06	2.38	1.80	1.89
H1-003	24	2.12	1.21	0.75	1.09	0.79	0.64	2.09	1.76	0.25	2.76	1.56	2.42
H1-006	0	-0.04	-0.09	0.00	-0.06	-0.15	0.52	-0.21	-0.14	-0.01	-0.09	-0.18	-0.19
H1-006	4	0.08	-0.06	0.02	0.15	0.20	1.04	0.00	-0.02	0.08	0.00	-0.06	-0.13
H1-006	8	0.11	-0.07	0.02	0.15	0.17	1.34	-0.04	-0.17	0.19	0.08	0.30	-0.27
H1-006	12	0.35	0.26	0.21	0.46	0.48	1.53	0.10	0.15	0.05	-0.10	0.11	0.12
H1-006	16	-0.12	-0.78	-0.74	0.14	0.65	1.90	-0.19	-0.12	-0.10	-0.08	-0.24	-0.17
H1-006	20	0.04	0.13	-0.06	0.34	1.43	2.77	0.16	0.02	0.41	0.06	0.10	0.13
H1-006	24	0.03	0.11	0.08	0.54	1.74	2.45	0.09	-0.10	-0.17	0.01	-0.05	0.02
N1-053	0	-0.08	-0.14	0.04	-0.16	0.72	0.86	-0.13	0.04	-0.33	-0.07	0.08	0.82
N1-053	4	0.12	0.01	0.12	0.59	3.01	3.01	-0.02	-0.24	0.06	0.29	2.93	4.30
N1-053	8	-0.05	-0.15	-0.01	4.04	4.34	4.34	-0.32	-0.31	-0.42	4.22	4.34	3.97
N1-053	12	0.15	0.16	0.30	4.78	4.38	4.68	-0.10	-0.23	-0.17	3.54	3.00	2.75
N1-053	16	0.04	0.96	0.30	3.21	1.23	2.83	-0.03	0.06	0.02	2.10	1.64	1.58
N1-053	20	0.39	0.41	0.28	1.79	1.59	1.60	0.06	0.39	0.05	1.46	0.86	0.89
N1-053	24	0.01	0.14	-0.01	1.59	1.08	0.98	0.54	0.06	0.08	0.37	0.23	0.26
Mack	0	-0.28	-0.24	0.00	-0.31	-0.24	-0.12	-0.10	-0.14	0.47	-0.01	0.06	0.34
Mack	4	-0.66	-0.21	1.11	-0.50	-0.25	2.68	-0.08	0.46	2.09	0.05	0.72	2.44
Mack	8	2.22	0.53	4.16	0.61	0.71	4.16	3.81	4.03	4.21	1.31	3.73	3.60
Mack	12	4.68	4.86	4.86	3.98	3.71	3.80	3.75	3.51	3.51	1.89	3.85	3.36
Mack	16	3.22	3.52	3.97	3.81	3.82	2.93	2.95	3.09	2.21	2.39	2.76	2.05
Mack	20	2.91	2.82	3.22	3.17	3.18	1.97	2.61	2.55	1.47	3.02	2.39	1.33
Mack	24	1.90	1.82	2.33	2.65	2.54	1.38	1.35	1.30	0.75	2.70	2.36	1.81

Table S3.4. Phage susceptibility[a] of persistent isolates recovered from a food processing facility.

Isolate	Ribotype (DUP-)	LP-022	LP-025	LP-026	LP-030	LP-031	LP-037	LP-038	LP-039	LP-040	LP-048	LP-054	LP-055	LP-056	LP-070	LP-082	LP-083	LP-085	LP-086	LP-101	LP-105	LP-110	LP-124	LP-125	LP-128	LP-129	LP-141	LP-143	LP-177
H1-038	1027A	0	0	0	0	0	1	1	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
H1-050	1027A	1	0	1	0	0	1	0	1	1	1	0	1	1	1	1	0	0	0	1	1	1	1	1	1	1	0	0	1
H1-163	1027A	1	0	1	0	0	1	1	1	1	1	0	1	1	1	1	0	1	0	1	1	0	1	1	1	1	0	0	1
L3-043	1027A	1	0	1	0	0	1	1	1	1	1	0	0	1	1	0	0	0	0	0	1	0	1	1	0	0	0	0	1
L4-162	1027A	0	0	0	0	0	1	1	1	1	0	0	0	0	1	0	0	0	0	0	1	1	1	1	0	1	0	0	1
T1-227	1027A	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	0	1
Total no. of isolates that phages lysed		4	0	4	0	1	6	5	6	6	5	0	4	5	6	3	0	2	0	3	5	3	5	5	3	4	0	0	6
No. of phages that lysed the majority of isolates		1	0	1	0	0	1	1	1	1	1	0	1	1	1	0	0	0	0	0	1	0	1	1	0	1	0	0	1
No. of phages that lysed all isolates tested		0	0	0	0	0	1	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Isolate	**Ribotype (DUP-)**	**LP-022**	**LP-025**	**LP-026**	**LP-030**	**LP-031**	**LP-037**	**LP-038**	**LP-039**	**LP-040**	**LP-048**	**LP-054**	**LP-055**	**LP-056**	**LP-070**	**LP-082**	**LP-083**	**LP-085**	**LP-086**	**LP-101**	**LP-105**	**LP-110**	**LP-124**	**LP-125**	**LP-128**	**LP-129**	**LP-141**	**LP-143**	**LP-177**
H1-003	1039C	0	0	0	0	0	1	1	1	1	1	0	1	1	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1
H1-486	1039C	1	0	0	0	0	1	1	1	1	1	0	1	1	1	1	0	1	0	1	1	0	1	1	0	0	1	0	1
L4-396	1039C	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	0	1	1	1	0	0	1	1	0	0	0	0	1
N1-449	1039C	0	0	0	0	0	0	0	1	1	1	0	1	1	0	1	0	1	0	1	0	0	0	0	0	1	0	0	0
T1-061	1039C	0	0	0	0	0	1	1	1	1	1	0	1	1	1	1	0	1	0	1	1	0	1	1	0	0	1	0	1
V1-009	1039C	0	0	1	0	0	1	1	1	1	1	0	1	1	0	0	0	0	1	1	1	0	1	1	0	0	0	0	0
Total no. of isolates that phages lysed		1	0	1	0	0	4	4	6	6	6	0	6	6	2	3	0	4	2	5	4	0	5	5	0	0	3	0	4
No. of phages that lysed the majority of isolates		0	0	0	0	0	1	1	1	1	1	0	1	1	0	0	0	1	0	1	1	0	1	1	0	0	0	0	1
No. of phages that lysed all isolates tested		0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Isolate	**Ribotype (DUP-)**	**LP-022**	**LP-025**	**LP-026**	**LP-030**	**LP-031**	**LP-037**	**LP-038**	**LP-039**	**LP-040**	**LP-048**	**LP-054**	**LP-055**	**LP-056**	**LP-070**	**LP-082**	**LP-083**	**LP-085**	**LP-086**	**LP-101**	**LP-105**	**LP-110**	**LP-124**	**LP-125**	**LP-128**	**LP-129**	**LP-141**	**LP-143**	**LP-177**
H1-099	1042B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H1-174	1042B	1	0	1	0	0	1	0	1	1	1	0	1	1	1	1	0	1	0	1	1	0	1	1	1	1	0	0	1
H1-406	1042B	1	0	1	0	0	1	1	1	1	1	0	1	1	0	1	0	1	1	1	1	0	1	1	0	1	0	0	1
H1-412	1042B	1	0	1	0	0	1	0	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1
T1-384	1042B	1	0	1	1	0	1	1	1	1	1	0	1	1	1	0	0	0	0	1	0	1	1	0	0	0	0	0	1
Total no. of isolates that phages lysed		4	0	4	1	0	4	2	4	4	4	0	4	4	3	2	0	2	1	2	3	0	3	3	2	2	0	1	4
No. of phages that lysed the majority of isolates		1	0	1	0	0	1	0	1	1	1	0	1	1	1	0	0	0	0	0	1	0	1	1	0	0	0	0	1
No. of phages that lysed all isolates tested		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Isolate	**Ribotype (DUP-)**	**LP-022**	**LP-025**	**LP-026**	**LP-030**	**LP-031**	**LP-037**	**LP-038**	**LP-039**	**LP-040**	**LP-048**	**LP-054**	**LP-055**	**LP-056**	**LP-070**	**LP-082**	**LP-083**	**LP-085**	**LP-086**	**LP-101**	**LP-105**	**LP-110**	**LP-124**	**LP-125**	**LP-128**	**LP-129**	**LP-141**	**LP-143**	**LP-177**
H1-178	1042C	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1
H1-459	1042C	1	0	1	0	0	1	0	1	1	0	0	1	1	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1
H6-030	1042C	1	0	1	0	0	1	0	1	1	0	0	1	1	0	0	0	0	0	0	1	0	1	1	1	1	0	0	1
Total no. of isolates that phages lysed		2	0	3	0	0	3	0	3	2	0	0	2	2	0	0	0	0	0	0	3	0	3	3	1	1	0	0	3
No. of phages that lysed the majority of isolates		1	0	1	0	0	1	0	1	1	0	0	1	1	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1
No. of phages that lysed all isolates tested		0	0	1	0	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1
Isolate	**Ribotype (DUP-)**	**LP-022**	**LP-025**	**LP-026**	**LP-030**	**LP-031**	**LP-037**	**LP-038**	**LP-039**	**LP-040**	**LP-048**	**LP-054**	**LP-055**	**LP-056**	**LP-070**	**LP-082**	**LP-083**	**LP-085**	**LP-086**	**LP-101**	**LP-105**	**LP-110**	**LP-124**	**LP-125**	**LP-128**	**LP-129**	**LP-141**	**LP-143**	**LP-177**
H6-154	1043A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
R6-819	1043A	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
R6-836	1043A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
R6-850	1043A	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
T1-930	1043A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V1-098	1043A	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total no. of isolates that phages lysed		0	0	0	2	0	0	2	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0
No. of phages that lysed the majority of isolates		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
No. of phages that lysed all isolates tested		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Isolate	**Ribotype (DUP-)**	**LP-022**	**LP-025**	**LP-026**	**LP-030**	**LP-031**	**LP-037**	**LP-038**	**LP-039**	**LP-040**	**LP-048**	**LP-054**	**LP-055**	**LP-056**	**LP-070**	**LP-082**	**LP-083**	**LP-085**	**LP-086**	**LP-101**	**LP-105**	**LP-110**	**LP-124**	**LP-125**	**LP-128**	**LP-129**	**LP-141**	**LP-143**	**LP-177**
H1-139	1044A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H1-490	1044A	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L4-412	1044A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
N1-052	1044A	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
N1-061	1044A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
N1-114	1044A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	1
T2-083	1044A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	0	1
Total no. of isolates that phages lysed		7	7	7	7	5	7	7	7	7	7	7	7	7	7	7	7	7	6	5	7	6	7	7	7	7	7	4	7
No. of phages that lysed the majority of isolates		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
No. of phages that lysed all isolates tested		1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	0	1
Isolate	**Ribotype (DUP-)**	**LP-022**	**LP-025**	**LP-026**	**LP-030**	**LP-031**	**LP-037**	**LP-038**	**LP-039**	**LP-040**	**LP-048**	**LP-054**	**LP-055**	**LP-056**	**LP-070**	**LP-082**	**LP-083**	**LP-085**	**LP-086**	**LP-101**	**LP-105**	**LP-110**	**LP-124**	**LP-125**	**LP-128**	**LP-129**	**LP-141**	**LP-143**	**LP-177**
H1-426	1045B	0	0	1	0	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1
N1-315	1045B	0	0	1	0	0	1	0	1	1	0	0	1	1	1	0	0	0	0	0	1	0	1	1	0	0	0	0	1
T1-269	1045B	0	0	1	0	0	1	0	1	1	0	0	0	1	1	0	0	0	0	0	1	0	1	1	0	0	0	0	1
Total no. of isolates that phages lysed		0	0	3	0	0	3	0	3	3	0	0	1	3	2	0	0	0	0	0	3	0	3	3	0	0	0	0	3
No. of phages that lysed the majority of isolates		0	0	1	0	0	1	0	1	1	0	0	0	1	1	0	0	0	0	0	1	0	1	1	0	0	0	0	1
No. of phages that lysed all isolates tested		0	0	1	0	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1
Isolate	**Ribotype (DUP-)**	**LP-022**	**LP-025**	**LP-026**	**LP-030**	**LP-031**	**LP-037**	**LP-038**	**LP-039**	**LP-040**	**LP-048**	**LP-054**	**LP-055**	**LP-056**	**LP-070**	**LP-082**	**LP-083**	**LP-085**	**LP-086**	**LP-101**	**LP-105**	**LP-110**	**LP-124**	**LP-125**	**LP-128**	**LP-129**	**LP-141**	**LP-143**	**LP-177**
H1-470	1052A	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
L3-055	1052A	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	1	1	0	1	1	0	1	1	1	1	0	0	1
N1-350	1052A	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
R6-740	1052A	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	1	1	1	0	1	1	1	1	0	0	1
R6-913	1052A	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	1	1	1	0	1	1	1	1	0	0	1
T1-127	1052A	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T2-075	1052A	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	1
V1-119	1052A	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	1	1	1	0	1	1	1	1	0	0	1
Total no. of isolates that phages lysed		0	0	0	0	0	5	8	5	5	8	0	5	5	0	5	2	5	4	5	6	0	5	7	5	5	0	0	5
No. of phages that lysed the majority of isolates		0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0	1	1	0	1	1	1	1	0	0	1
No. of phages that lysed all isolates tested		0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Isolate	**Ribotype (DUP-)**	**LP-022**	**LP-025**	**LP-026**	**LP-030**	**LP-031**	**LP-037**	**LP-038**	**LP-039**	**LP-040**	**LP-048**	**LP-054**	**LP-055**	**LP-056**	**LP-070**	**LP-082**	**LP-083**	**LP-085**	**LP-086**	**LP-101**	**LP-105**	**LP-110**	**LP-124**	**LP-125**	**LP-128**	**LP-129**	**LP-141**	**LP-143**	**LP-177**
H6-175	1062A	0	0	0	1	0	0	0	1	1	1	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
N1-053	1062A	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T1-261	1062A	1	0	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
T1-938	1062A	1	0	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
V1-142	1062A	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total no. of isolates that phages lysed		2	0	2	1	0	2	2	5	5	5	0	5	5	2	2	2	3	2	2	1	2	2	2	2	2	2	2	2
No. of phages that lysed the majority of isolates		0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
No. of phages that lysed all isolates tested		0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a]Phage susceptibility detected by a spot test performed in 3 independent replicates. "1" represents lysis from at least two replicates; "0" represents no lysis.

Table S4.1. Information of additional phages used in the terminase large subunit analysis.

Phage	GenBank Accession No.	DNA Packaging Strategy
T4	NP_049776.1	Circularly Permuted, Terminally Redundant
Lambda	NP_040581.1	Cohesive Ends
P100	AAY53308.1	Direct Terminal Repeats
SPO1	YP_002300330.1	Direct Terminal Repeats
A511	YP_001468454.1	Direct Terminal Repeats
P40	YP_002261418.1	Circularly Permuted, Terminally Redundant
P35	YP_001468786.1	Circularly Permuted, Terminally Redundant
A500	YP_001468388.1	Circularly Permuted, Terminally Redundant
A118	NP_469451.1	Circularly Permuted, Terminally Redundant
B025	YP_001468641.1	Cohesive Ends
A006	YP_001468842.1	Circularly Permuted, Terminally Redundant
PSA	CAC85558.1	Cohesive Ends
B054	AAY53107.1	Circularly Permuted, Terminally Redundant
Mu	NP_050632.1	Host Ends
T7	AAP34063.2	Direct Terminal Repeats
T5	AAU05290.1	Direct Terminal Repeats
HK97	NP_037698.1	Cohesive Ends
P2	NP_046758.1	Cohesive Ends
P22	YP_063734.1	Circularly Permuted, Terminally Redundant
D3112	NP_938233.1	Host Ends
933W	NP_049511.1	Circularly Permuted, Terminally Redundant
P1	YP_006576.1	Circularly Permuted, Terminally Redundant
vB_EcoM_CBA120	YP_004957856.1	Circularly Permuted, Terminally Redundant