COMPARATIVE ANALYSIS OF *SALMONELLA*-INFECTING BACTERIOPHAGES AND CHARACTERIZATION OF BACTERIOPHAGE-HOST INTERACTIONS

by

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Abstract

Numerous outbreaks in North America have been attributed to non-typhoidal *Salmonella enterica*. Bacteriophages (phages), viral bacterial predators, represent agents that could be used for controlling *Salmonella*; yet, relatively little is known about phages and their host interactions. Therefore, the purpose of this thesis was to characterize phages of *Salmonella* on their phenotypic and genomic determinants and phage-host interactions.

Salmonella phages (n=97) were isolated from sites within British Columbia, Canada. Host range analysis revealed diverse patterns of lysis, with several broad host range phages identified. Initial screening demonstrated that phage SI1 exhibited remarkable stability at a variety of pH and temperature values. Artificially- contaminated sprouting alfalfa seeds treated with SI1 resulted in a significant ($p<0.05$) reduction of 38.3±3.0% of viable *S.* Enteritidis following two h of phage treatment, indicating the potential for usage of phage SI1 in food.

Comparative genomic analyses of 45 of the newly isolated phages revealed an abundance of sequence diversity. Genome alignment grouped the phages into 12 clusters with three singletons. Phages within clusters exhibited high genome homology (>98% nucleotide identity), yet between clusters, genomes exhibited a span of diversity (<50% nucleotide identity). Alignment of the major capsid protein also supported the clustering pattern observed with whole genome alignment. We further observed associations between genomic relatedness and the site of isolation, as well as genetic elements related to DNA metabolism and host virulence.

Five mutants of tetracycline-resistant *S.* Agona, resistant to phage SI1 infection, displayed attenuated virulence and antimicrobial resistance. Using a differentiated Caco-2 cell line, Δ95 and Δ96 displayed significantly ($p<0.05$) attenuated invasion compared to the wild-type strain, with Δ96 also exhibiting increased susceptibility to tetracycline. Concordantly,

insertions were observed in *rfaL* involved in lipopolysaccharide biosynthesis, indicating the site of phage attachment. Mutations were observed in a *vgrG* gene involved in type VI secretion. Mutations in the tetracycline resistance cassette were not revealed; it may be hypothesized that altered transcriptional activity could account for the observed tetracycline susceptibility.

Collectively, these findings support the knowledge framework for phage diversity and phage-host interactions that are required for developing phage-based applications for various sectors, including biocontrol, detection and typing.

Lay Summary

Salmonella bacteriophages represent a novel strategy for mitigation of this pathogen in the food supply system. However, to pursue this intervention in the food industry, factors such as biocontrol efficacy and the nature of phage-pathogen interactions must be elucidated. In this study, we investigated the biocontrol capabilities of newly isolated phages to infect their target hosts and observed a high reduction rate of clinically relevant strains of *Salmonella* upon phage treatment. We noted remarkable diversity in our subset of phages and identified novel genes associated with biocontrol efficacy. Lastly, we observed that development of phage resistance in antibiotic-resistant *Salmonella* was associated with an increase in antibiotic sensitivity and diminished disease potential. Together, these data cumulatively add to our understanding of phages and their host interactions, and will further inform intelligent development of phage-based antimicrobials for the food industry.

Preface

A version of Chapter 2 has been published in Frontiers in Microbiology (Fong K, LaBossiere B, Moreno Switt AI, Delaquis P, Goodridge L, Levesque RC, Danyluk MD & Wang S. 2017. Characterization of four novel bacteriophages isolated from British Columbia for control of non-typhoidal *Salmonella in vitro* and on sprouting alfalfa seeds. *Front. Microbiol.* **8:** 2193.) I was responsible for the majority of the work in this chapter and manuscript preparation. LaBossiere B. assisted with phage isolation and the pH and temperature stability assays. All authors helped with reviewing the experimental design and reviewing and editing the manuscript. Goodridge L, Levesque RC, Delaquis P, and Wang S. were responsible for concept formation. Wang, S was the supervisory author.

A version of Chapter 3 has been published in Viruses (Fong K, Tremblay DM, Delaquis P, Goodridge L, Levesque R, Moineau S, Suttle C, & Wang S. 2019. *Salmonella* bacteriophage diversity and host specificity revealed by biological characterization and whole genome sequencing. *Viruses*. 11(9): 854). Tremblay DM and Moineau S sequenced the phage isolates. Delaquis P, Goodridge L, Levesque R and Wang S were responsible for study conceptualization. All authors helped with reviewing the experimental design and reviewing and editing the manuscript. Wang, S was the supervisory author.

I performed the majority of the research described in Chapter 4. Mu, K and Kitts, D provided assistance with the adhesion and invasion assays. Rheault, JG and Levesque, RC were responsible for sequencing and assembly of the mutants. Wang, S and Kitts, D provided feedback on the experimental design and data analysis.

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a_w Water activity
h Hour(s)
CFU Colony forming units

List of Abbreviations

Abi	Abortive infection
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
ARGs	Antimicrobial resistance genes
BC	British Columbia
BHI	Brain-heart-infusion
BIM	Bacteriophage-insensitive mutant
bp	Base pair
CDC	Centers for Disease Control and Prevention
CFIA	Canadian Food Inspection Agency
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
CRISPR	Clustered regular interspaced short palindromic repeats
DMEM	Dulbecco Minimum Essential Medium
dsDNA/RNA	Double-stranded DNA/RNA
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
ICTV	International Committee of the Taxonomy of Viruses
Indel	Insertion/deletion
kb	kilobase
LPS	Lipopolysaccharide
MCP	Major capsid protein
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
min	Minutes
MNP	Multiple nucleotide polymorphism
MOI	Multiplicity of infection
MTase	Methyltransferase
NACMF	National Advisory Committee on Microbiological Criteria for Foods
nt	Nucleotide
OMV	Outer membrane vesicle
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PFU	Plaque forming units
PICI	Phage-inducible chromosome islands
R-M	Restriction-modification
REase	Restriction endonuclease
Sie	Superinfection exclusion
SM	Salt-magnesium
SNP	Single nucleotide polymorphism
SPI	Salmonella pathogenicity island
ssDNA/RNA	Single-stranded DNA/RNA
SUF	Sulfisoxazole

SXT	Sulfamethoxazole/trimethoprim
T3SS	Type 3 secretion system
T6SS	Type 6 secretion system
TA	Toxin-antitoxin
TAE	Tris-acetate EDTA
TEM	Transmission electron microcopy
TET	Tetracycline
tRNA	Transfer ribonucleic acid
TSA	Tryptic soy agar
TSB	Tryptic soy broth
Tukey's HSD	Tukey's Honest Significant Difference
WGS	Whole genome sequencing
WHO	World Health Organization
WT	Wild-type
XLD	Xylose-lysine deoxycholate

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For my family

Chapter 1: Introduction and literature review

1.1 Introduction

The burden of foodborne illness is tremendously vast, with 10% of the global population affected annually, causing 33 million deaths per year (Havelaar et al., 2015). Non-typhoidal *Salmonella enterica* is one of the most common foodborne pathogens worldwide (Lan et al., 2009), and causes the enteric disease known as salmonellosis. Although generally self-limiting, salmonellosis may manifest as nausea, vomiting, diarrhea (Hohmann, 2001) and in severe cases, may progress to reactive arthritis and chronic gastroenteritis (Hoffmann et al., 2015). Globally, it is estimated that 80.3 million cases of salmonellosis arise yearly from contaminated foodstuffs, resulting in over 155,000 deaths (Majowicz et al., 2010). In Canada, non-typhoidal *Salmonella* is estimated to cause 87,500 illnesses annually (Government of Canada, 2016) and ranks third as the causative agent for domestically-acquired foodborne disease (Thomas et al., 2013). Additionally, the economic burden of salmonellosis in Canada amounts to almost $1 billion dollars annually (Todd, 2014).

Numerous North American outbreaks of *Salmonella* associated with a variety of food products (e.g., eggs, chicken products, sprouted vegetables, tahini paste, almonds, melons, cucumbers, etc.) have occurred in recent years (CDC, 2019a). These outbreaks have been attributed to the unique survival capability and adaptability of this pathogen in different niches (Andino & Hanning, 2014), with some serovars being better adapted to certain environments (e.g., low water-activity foods) than others (Fong & Wang, 2016). Although it is well-known that *Salmonella* is commonly associated with poultry and poultry products (Andino & Hanning, 2014), recent large-scale outbreaks in fresh produce (CDC, 2019a). have drawn cause for concern as the traditional methods for produce decontamination are questioned. Importantly, as

fresh produce undergoes minimal processing and is often consumed raw (i.e., negating the effect of an at-home kill step), an alternative strategy of pathogen control is necessary.

Bacteriophages (phages) are viruses of bacteria that have been proposed as a strategy for pathogen mitigation (Goodridge et al., 2018) and have been evaluated for use on a variety of foods, including chicken skin (Goode et al., 2003), cheddar cheese (Modi et al., 2001) and sprouting alfalfa seeds (Fong et al., 2017). Phages offer several desirable attributes that render usefulness in food applications; they are (i) highly specific, usually incapable of crossing species or genus barriers; (ii) kill host prokaryotic cells only; (iii) self-replicating and and self-limiting; and (iv) ubiquitously distributed in nature (Rohwer and Edwards, 2002). Additionally, they have negligible effect on food product quality (Fong et al., 2017). Despite growing evidence, usage as biocontrol agents for *Salmonella* has not been widely adopted, particularly since relatively small pathogen reductions (~1 log CFU/g) have been reported (Kocharunchitt et al., 2009; Pao et al., 2004) and is highly variable across phage, host serovar/strain and study design (Fong et al., 2017; Guenther et al., 2012; Kocharunchitt et al., 2009; Pao et al., 2004). These outcomes may be due to several factors, including the failure to isolate effective phages, a lack of characterization (both phenotypic and genetic) of these phages, and knowledge gaps in our current understanding of the *Salmonella* host(s) in response to phage infection. Therefore, more research is needed in these areas for effective pathogen mitigation and to diminish the economic and health burden of *Salmonella* on society.

1.2 Research overview

The overall objective of this study was to characterize non-typhoidal *Salmonella enterica* phages and their hosts on several phenotypic and genetic determinants, with a particular focus on factors associated with effective biocontrol of this pathogen in the food industry. The research

for this thesis was divided into three parts based on the following three hypotheses.

Hypothesis 1: Phages isolated from British Columbia possess phenotypic determinants important in biocontrol of *Salmonella*.

Hypothesis 1 workflow/objectives:

Salmonella phages were isolated from a variety of environmental sources and investigated on their host ranges. Phages possessing broad host ranges were then assessed on several phenotypic markers, including host range, environmental fitness, morphotype, burst size, lifestyle diversity and biocontrol efficacy.

Hypothesis 2a: *Salmonella* phages isolated in H1 are genetically diverse.

Hypothesis 2b: *Salmonella* phages isolated in H1 possess genetic determinants important for phage biocontrol in food.

Hypothesis 2 workflow/objectives:

Salmonella phages were sequenced and assessed for genetic diversity with phylogenetic and clustering methods. Genetic elements important in the context of food biocontrol were determined.

Hypothesis 3: Bacteriophage-insensitive mutants of antibiotic resistant *Salmonella* are altered in their antibiotic resistance and virulence.

Hypothesis 3 workflow/objectives:

Bacteriophage-insensitive mutants were assayed for their relative resistance to antibiotics of which the parent (phage-sensitive) strain is resistant: sulfisoxazole, sulfamethoxazole/trimethoprim and tetracycline. Additionally, mutants were assessed for their virulence in a Caco-2 cell line. Variant analysis determined genetic mutations associated with these phenotypes.

1.3 Literature review

1.3.1 Overview of non-typhoidal *Salmonella enterica*

1.3.1.1 Microbial characteristics

Members of *Salmonella enterica* are motile Gram-negative, rod-shaped, non-spore-forming facultative anaerobes that are frequently associated with poultry products and foods of animal origin (Andino & Hanning, 2014; Jay et al., 2005). Most members of the Salmonellae ferment lactose, produce hydrogen sulfide, are oxidase negative and catalase positive, allowing for identification with standard biochemical test methods, although recent anomalies with hydrogen sulfide-deficient mutants have been reported (Kovac et al., 2017).

1.3.1.2 Diagnosis

Without proper laboratory testing, *Salmonella* infection is difficult to diagnose due to the ambiguity of symptoms. Often, the symptoms associated with salmonellosis are identical to that of other enteric pathogens and consist of fever, diarrhea and abdominal pain (Hohmann, 2001). For a definitive diagnosis, fecal samples from the patient are tested for the presence of *Salmonella* using a combination of selective agars (Hohmann, 2001), and extensive serotyping may also be used for formal identification (Chiu et al., 2004). The isolate will also be passed on to relevant public health authorities for further characterization, epidemiological surveillance and outbreak monitoring (Government of Canada, 2018).

1.3.1.3 Treatment

Due to increasing antimicrobial resistance, antibiotics are not routinely utilized and only used in cases of severe illness (Centers for Disease Control and Prevention (CDC), 2015). Due to the self-limiting nature of most cases of salmonellosis, oral rehydration is recommended for loss

of fluids from vomiting and diarrhea. In cases of severe diarrhea, intravenous rehydration may be necessary (CDC, 2015).

Besides development of bacterial resistance, antibiotics may increase fecal shedding of *Salmonella*, prolonging the infectivity of these microorganisms (Davies & Davies, 2010). This is due to the broad spectrum activity of these antibiotics, resulting in the impairment of endogenous microbiota which normally "protect" the intestine from harm caused by *Salmonella* (Gopinath et al., 2014). However, antibiotics may be used in one of two scenarios, for: (i) patients who are severely ill (e.g, high fever, severe diarrhea, bacteremia, etc.); and (ii) patients at high risk for additional complications (e.g., infants, people >65 yrs and immunocompromised individuals) (CDC, 2015).

1.3.1.4 Classification of *Salmonella*

The genus *Salmonella* is in the *Enterobacteriaceae* family and comprises two species: *Salmonella bongori* and *Salmonella enterica* (Malorny et al., 2011). *S. bongori* is mainly associated with cold-blooded animals and rarely cases human disease, while *S. enterica* is responsible for the majority of human cases of foodborne illness (Andino & Hanning, 2014). Importantly, *S. enterica* also comprises members causing typhoid fever (e.g., *S. enterica* serovars Typhi and Paratyphi A, B and C), which result in significantly different disease states than foodborne Salmonellae (Hu & Kopecko, 2003).

There are six subspecies within the species *S. enterica*: *enterica, salamae, arizonae, diarizonae, houtenae* and *indica* (Malorny et al., 2011) which are differentiated through biochemical means (Farmer, 1995). Between these subspecies exist >2600 serovars (Andino & Hanning, 2014), which are differentiated by their surface antigens according to the White-Kauffman-Le Minor scheme (Grimont & Weill, 2007). Predominantly, the O-antigen, belonging

to the cell surface lipopolysaccharide (LPS), and the H1 and H2 antigens, belonging to flagellar proteins forming phase 1 and phase 2 flagella, respectively, are assayed with a slide agglutination test based on antibody-antigen interactions (Brenner et al., 2000; Chan et al., 2003). In this assay, visible agglutination occurs upon the antibody interacting with the bacterial antigen, although false-positives can occur due to weak, nonspecific agglutination (Yang et al., 2019). Additionally, some phenotypes of *Salmonella* (e.g., rough, non-motile and mucoid isolates) may lose antigenic expression altogether (Schrader et al., 2008), highlighting the vast limitations of this method. Therefore, higher caliber molecular approaches are often undertaken, or used in conjunction with phenotypic approaches for the accurate subtyping of *Salmonella* (Wattiau et al., 2011).

1.3.1.5 *Salmonella* pathogenicity islands

In *Salmonella*, the primary virulence determinants are encoded on *Salmonella* pathogenicity islands (SPIs), which are 10 to 200 kilobase (kb) regions carrying genes encoding virulence factors, although virulence determinants may also be encoded within mobile genetic elements, such as plasmids (Alcaine et al., 2007; Deng et al., 2015; Mangat et al., 2017). Interestingly, most SPIs are adjacent to tRNA genes and possess a G+C composition different from the rest of the chromosome, suggesting that they were horizontally acquired (Amavisit et al., 2003). All members of *S. enterica* possess conserved and stable SPI-1 and SPI-2, with SPI-2 being purportedly absent from *S. bongori* (Marcus et al., 2000; Nieto et al., 2016). The number of SPIs varies vastly between members of non-typhoidal *Salmonella* (Blondel et al., 2009). Importantly, the presence of one pathogenicity island is sufficient for pathogenic conversion of previously benign microorganisms. Virulence was conferred to a non-pathogenic, laboratory strain of *Escherichia coli* through introduction of a plasmid carrying a pathogenicity island

encoding for Type III secretion (T3S), a mechanism transferring virulence effectors into host eukaryotic cells (McDaniel et al., 1995).

1.3.1.5.1 SPI-1

SPI-1 is the best studied of all SPIs and is 40 kb in length (Marcus et al., 2000). SPI-1 accommodates at least 29 genes, which together encode for a functional Type III secretion system (T3SS-1) (Chan et al., 2003). Upon ingestion of *Salmonella*, the pathogen must survive gastric acidity prior to colonization of the intestinal M cells (Marcus et al., 2000). The T3SS-1 encodes all genes necessary for construction of the "needle-like" apparatus (Figure 1.1), including most of the effector proteins (Lostroh & Lee, 2001). It is believed that T3S is regulated by pH upshift from acidic to alkaline conditions, which reflects passage from the gastric acid to the mildly alkaline pH of the small intestine (Daefler, 1999).

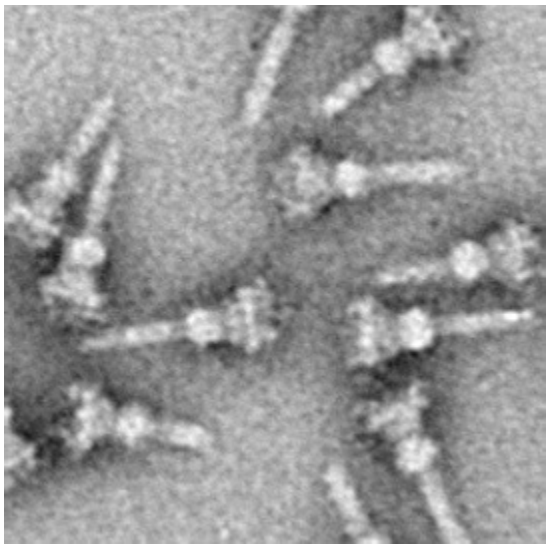

Figure 1.1. Type III secretion system apparatus of *S.* Typhimurium. Image is from Schraidt et al. (2010) and licensed under the Creative Commons Attribution 2.5 Generic license (**https://creativecommons.org/licenses/by/2.5/ca/**).

Structurally, the apparatus is ~50 nm long, spans both the inner and outer membrane of the bacterial cell and acts as a conduit for effector proteins (e.g., SptP, AvrA, Sip family proteins) which disrupt host cell signaling (Burkinshaw & Strynadka, 2014). The effector proteins may go on to interrupt host ubiquitin pathways (which facilitate processes for protein degradation, DNA repair, cell cycle control), modify host cell proteins and alter the host cytoskeleton through actin polymerization (Burkinshaw & Strynadka, 2014).

1.3.1.5.2 SPI-2

In *Salmonella*, SPI-2 also encodes for T3SS (T3SS-2), although it is functionally distinct from the T3SS encoded by SPI-1. Expression of T3SS-2 occurs upon *Salmonella* entry into phagocytic cells, such as macrophages and dendritic cells (Nieto et al., 2016), which are deployed by the host immune system to envelop the infected cell and designate it for removal (Marcus et al., 2000). Interestingly, *Salmonella* is able to survive inside these phagocytes by seeking refuge in a vacuole (termed a "*Salmonella*-containing vacuole") (Waterman & Holden, 2003). Here, it is able to prevent vacuole maturation and fusion with lysozymes (Nieto et al., 2016). The T3SS-2 is thought to be essential for replication of the pathogen in phagocytic cells (Shea et al., 1999), allowing for further persistence and dissemination. It was previously observed that mutations within T3SS-1 were not complemented by T3SS-2, suggesting that these two systems do not share functionality (Marcus et al., 2000). Indeed, T3SS-2 knockout mutants demonstrated attenuated virulence in a mouse model (Grant et al., 2012).

1.3.1.6 Hosts, routes of transmission & serotype-specificity

Overall, *Salmonella* is capable of infecting a range of hosts (i.e., asymptomatic carriers), although certain serovars are better adapted to specific hosts than others (Andino & Hanning, 2014). Serovars Typhi and Paratyphi are highly adapted to humans, causing typhoid fever, and

have no known natural hosts (Brenner et al., 2000). Serotype Choleraesuis, on the other hand, is particularly well-adapted to swine (i.e., pigs, hogs, boars) and causes paratyphoid in these animals (Leekitcharoenphon, 2019). Worldwide, Typhimurium and Enteritidis are the top two zoonotic serovars accounting for the highest disease incidence in humans and are known to colonize the intestines of a wide variety of animals (Ao et al., 2015), including those of domesticated and wild mammals, reptiles, birds and insects (Su et al., 2004). Specifically, *S.* Enteritidis is frequently associated with poultry and shelled eggs, while *S.* Typhimurium is predominantly associated with pig, poultry and bovine meat (European Food Safety Authority (EFSA), 2011). In animal rearing operations, horizontal dissemination of *Salmonella* can occur quickly throughout a flock or herd, while vertical dissemination results in contaminated eggs and progeny (Liljebjelke et al., 2005).

Although the origin of *Salmonella* is animal-associated, in recent years *Salmonella* has been implicated in outbreaks involving non-traditional food vectors, such as low-water activity (a_w) foods (e.g., chia seeds, peanut butter, tahini paste) and produce (e.g., sprouts, cucumbers, papayas) (CDC, 2019a). Contamination of *Salmonella* in these foods is thought to arise either in-field or during processing (Franz & van Bruggen, 2008), and survival of these pathogens in diverse niches has demonstrated serovar and strain-specificity (Fong & Wang, 2016). It was previously demonstrated that *Salmonella* Enteritidis phage type 30 was capable of persisting in an almond orchard over a span of five years (Uesugi et al., 2007), drawing awareness to the risks of contaminated almond-based products (e.g., almond milk, almond butter, etc.).

In an analysis of US outbreak surveillance, it was determined that although serovars Enteritidis and Typhimurium were the most common serovars across all outbreaks, less common serovars tended to be mostly associated with unconventional food products, highlighting limited

survival capabilities in different niches (Snyder & Boktor, 2019). For instance, serovars Newport, Javiana and Saintpaul were the top three serovars accounting for seeded-vegetable linked outbreaks. Furthermore, Newport and Javiana were within the top three serovars for outbreaks associated with fruits and vegetable row crops (Snyder & Boktor, 2019). In a comparative study of five *Salmonella* strains encompassing five serovars, it was revealed that serovar Hartford possessed the greatest persistence across three low-a_w food matrices (peanut oil, peanut shell and chia seeds), while *S.* Typhimurium had the lowest survival (Fong & Wang, 2016). Clearly, these data highlight the need for tailored mitigation strategies regarding high-risk strains potentially present in the food industry, resulting in enhanced consumer risk.

1.3.1.7 Fresh produce-associated outbreaks of *Salmonella*

In general, contamination of agricultural commodities is problematic because some products (e.g., nuts) may be processed further to make other downstream products (e.g., nut butters and pastes), potentially exacerbating the risk of contaminated products being consumed by more people (Fong & Wang, 2016). In fresh produce-related outbreaks, however, a unique threat is present as these foods are often consumed raw and do not undergo further kill steps (e.g., heat) (Fong et al., 2017). Most recently, *S.* Carrau was linked to pre-cut melon, which caused 137 illnesses and 38 hospitalizations (CDC, 2019a; Table 1.1).

The rise of fresh produce-associated outbreaks (CDC, 2019a) may be attributed to several factors, including increased consumption, seasonal changes in processing, and/or enhanced animal agriculture in regions of close proximity to produce growing operations (Lynch et al., 2009). A conventional intervention method used for pathogen mitigation in fresh produce is chemical disinfectants (e.g., hypochlorite, hydrogen peroxide) (National Advisory Committee on Microbiological Criteria for Foods (NACMCF), 1999), but given the number of recent produce-

related outbreaks, questions have been raised regarding their efficacy. Indeed, the effectiveness of chlorine (hypochlorite) diminishes with increasing organic load (Lou et al., 2011; Shen et al., 2013), a problem not unbeknownst to the produce industry (Lou et al., 2011). Irradiation, on the other hand, has also been proposed, which is a process where ionizing energy in the form of gamma photons is emitted by ^{60}Co radioisotopes, machine-generated X-rays or accelerated electrons (Farkas & Mohácsi-Farkas, 2011). This technology has resulted in >two log CFU/g reductions of *Salmonella* in alfalfa seeds (Thayer et al., 2003). However, mixed consumer perceptions towards the utilization of irradiation in foods has hindered its rapid adoption (Brewer & Rojas, 2008; Rollin et al., 2011).

Table 1.1. Fresh produce-related outbreaks within North America between years 2009-2019 inclusive (CDC, 2019; PHAC, 2019).

Year	*Salmonella* serotype	Affected product
2009	*S.* Saintpaul	Alfalfa sprouts
2009	*S.* Montevideo *S.* Newport *S.* Senftenberg	Pistachios
2009	*S.* Cubana	Onion sprouts
2010	*S.* I 4,[5],12:i:-	Alfalfa sprouts
2010	*S.* Newport	Alfalfa sprouts
2011	*S.* Enteritidis	Turkish pine nuts
2011	*S.* Agona	Papayas[a]
2011	*S.* Enteritidis	Alfalfa & spicy sprouts
2011	*S.* Panama	Cantaloupe
2012	*S.* Braenderup	Mangoes
2012	*S.* Typhimurium *S.* Newport	Cantaloupe

2013	*S.* Saintpaul	Cucumbers
2014	*S.* Newport	Cucumbers
2014	*S.* Enteritidis	Bean sprouts
2014	*S.* Braenderup	Nut butter
2015	*S.* Poona	Cucumbers
2016	*S.* Reading *S.* Abony	Alfalfa sprouts
2016	*S.* Montevideo	Pistachios
2016	*S.* Muenchen *S.* Kentucky	Alfalfa sprouts
2017	*S.* Urbana	Maradol papayas[a]
2017	*S.* Newport *S.* Infantis	Maradol papayas[a]
2017	*S.* Anatum	Maradol papayas[a]
2017	*S.* Thompson *S.* Kiambu *S.* Agona *S.* Gaminara	Maradol papayas[a]
2018	*S.* Adelaide	Pre-cut melon
2018	*S.* Montevideo	Raw sprouts
2018	*S.* I 4,[5],12:i:-	Frozen shredded coconut
2019	*S.* Uganda	Papayas[a]
2019	*S.* Carrau	Pre-cut melon

[a] imported from Mexico

1.3.1.8 Outbreak investigation

Pulsed-field gel electrophoresis (PFGE) is considered the gold standard in *Salmonella* outbreak investigations as its discriminatory power allows for separation of closely related

strains (CDC, 2016). Genetic "fingerprints" produced by the electrophoretic motilities of restriction fragments allow public health authorities to distinguish between isolates and identify sources of contamination (Foley et al., 2007). However, PFGE has the potential to result in high numbers of genetic profile clusters produced on the basis of only minor differences, exaggerating the degree of genetic diversity (den Bakker et al., 2014; Wattiau et al., 2011). On the other hand, PFGE may produce indistinguishable or identical profiles in related isolates, necessitating the need for further typing tools to resolve these clusters (Gilpin et al., 2014). With recognition of these limitations and nowadays, with decreased costs associated with other high-throughput technologies, there is currently a push to perform whole genome sequencing (WGS) for isolates involved in outbreaks attributed to food products (CDC, 2016; Didelot et al., 2012).

On the basis of genomic comparison, WGS is a powerful tool that allows for resolution at the level of the nucleotide and the virtual detection of all genetic differences within bacterial isolates (Didelot et al., 2012). Based on its superior genomic resolution and decreased cost, WGS has become a viable option for use in public health diagnostics (Koser et al., 2012). Deng et al. (2015) compared the discriminatory powers of PFGE and whole-genome single-nucleotide polymorphism typing of 52 *S.* Enteritidis isolates, which resulted in the resolution of all outbreak clusters produced by PFGE, providing robust accuracy in the differentiation of closely related isolates. Indeed, WGS is substantially more discriminatory than PFGE, since slight genetic variability between strains does not affect the electrophoretic mobility of restriction fragments (Foley et al., 2007).

1.3.1.9 Antimicrobial-resistant *Salmonella*

1.3.1.9.1 Introduction

Antimicrobial resistance (AMR) constitutes an imminent public health threat worldwide. AMR encompasses resistance to agents of inactivation, including sanitizers, and importantly, antibiotics used to treat disease. There are several classes of antibiotics, each with different cellular targets and modes of action (Table 1.2).

Table 1.2. Modes of action of commonly used antibiotics. Information is cited in Davies & Davies (2010).

Antibiotic class	Example(s)	Cellular target
ß-lactams	Pencillins (ampicillin), cephalosporins (cephamycin), penems (meropenem), monobactams (aztreonam)	Peptidoglycan biosynthesis
Aminoglycosides	Gentamicin, streptomycin, spectinomycin	Protein translation
Glycopeptides	Vancomycin, teicoplanin	Peptidoglycan biosynthesis
Tetracyclines	Minoglycine, tigecycline	Protein translation
Macrolides	Erythromycin, azithromycin	Protein translation
Lincosamides	Clindamycin	Protein translation
Streptogramins	Synercid	Protein translation
Oxazolidinones	Linezolid	Protein translation
Phenicols	Chloramphenicol	Protein translation
Quinolones	Ciprofloxacin	DNA replication
Pyrimidines	Trimethoprim	C_1 metabolism
Sulfonamides	Sulfamethoxazole	C_1 metabolism
Rifamycin	Rifampin	mRNA transcription
Lipopeptides	Daptomycin	Cell membrane
Cationic peptides	Colistin	Cell membrane

In North America, antibiotic resistant *Salmonella* is estimated to account for at least 100,000 infections (CDC, 2013), leading to treatment failures, increased risk of bloodstream infection and increased hospitalization rates (Nair et al., 2018). Strikingly, public health surveillance data has revealed an increase in AMR in Salmonellae from 20%-30% in the early 1990s to almost 70% in some countries since 2000 (Su et al., 2004). In 2017, the World Health Organization (WHO) classified AMR *Salmonella* as a high priority pathogen representing an imminent public health threat (Tacconelli & Magrini, 2017).

AMR *Salmonella* has been associated with a considerable number of outbreaks in North America. In 2017, papayas were contaminated with *S.* Urbana which demonstrated resistance to streptomycin and tetracycline (CDC, 2019). A large scale outbreak of *S.* Poona in cucumbers demonstrated resistance to tetracycline and nalidixic acid in 2015 (CDC, 2015). Most recently, multi-drug resistant (MDR) *S.* Schwarzengrund linked to ground turkey was resistant to ampicillin, gentamicin, streptomycin, sulfisoxazole and tetracycline (CDC, 2019b).

1.3.1.9.2 Mechanisms of antibiotic resistance

Antibiotic resistance is inevitable and represents the expected outcome of selective pressure placed upon bacteria (Von Wintersdorff et al., 2016). Most antibiotics are of bacterial origin, thus, competitor bacteria have evolved unique mechanisms to neutralize the inactivation effects of these molecules. Bacterial resistance to antibiotics may be categorized in different manners; bacteria that inherently possess resistance genes and/or lack drug binding sites (e.g., LPS) possess intrinsic resistance (Davies & Davies, 2010; Olaitan et al., 2014). On the other hand, acquired resistance refers to the acquisition of resistance determinants that previously susceptible bacteria have evolved or acquired (Olaitan et al., 2014).

Bacteria develop resistance according to several mechanisms, accordingly they may be categorized by: (i) modifications of the antibiotic molecule; (ii) prevention in reaching the antibiotic target; (iii) alteration of target sites; and (iv) the induction of global adaptive processes (Munita & Arias, 2016). In general, different antibiotic classes are inactivated through different resistance mechanisms as summarized in Table 1.3.

Table 1.3. General bacterial resistance mechanisms in commonly used antibiotics (Munita & Arias, 2016).

Antibiotic class	General mode(s) of resistance
ß-lactams	Efflux, altered binding site
Aminoglycosides	Antibiotic modification, efflux, altered binding site
Glycopeptides	Global adaptive processes
Tetracyclines	Antibiotic modification, efflux, altered binding site
Macrolides	Antibiotic modification, efflux, altered binding site
Lincosamides	Antibiotic modification, efflux, altered binding site
Streptogramins	Antibiotic modification, efflux, altered binding site
Oxazolidinones	Efflux, altered binding site
Phenicols	Efflux, altered binding site
Quinolones	Antibiotic modification, efflux, altered binding site
Pyrimidines	Efflux, altered binding site
Sulfonamides	Efflux, altered binding site
Rifamycin	Antibiotic modification, efflux, altered binding site
Lipopeptides	Altered binding site
Cationic peptides	Efflux, altered binding site

Both Gram-negative and Gram-positive bacteria may produce enzymes that result in chemical alterations within, or complete destruction of the target antimicrobial molecule. For instance, aminoglycoside resistance is predominantly due to drug modification by aminoglycoside modifying enzymes that modify hydroxyl and amino groups of the aminoglycoside molecule (Ramirez & Tolmasky, 2010). Additionally, chloramphenicol resistance is mediated by the expression of acetyltransferases that facilitate acetylation of this antibiotic, neutralizing its activity (Schwarz et al., 2004). On the other hand, the antibiotic molecule can be destroyed altogether; for example, ß-lactamases destroy the amide bond within the ring of ß-lactam antibiotics (Bush, 2013). More than 340 ß–lactamases have been described, and many of these are plasmid or chromosomally-encoded in *Salmonella* (Hasman et al., 2005). Additionally, the high prevalence of non-typhoidal *Salmonella* carrying genes for extended-spectrum ß-lactamases (i.e., conferring resistance to most third-generation cephalosporins and penicillins, including ampicillin) was previously reported in poultry (Chon et al., 2015).

Antibiotics are prevented in reaching cellular targets through two strategies: decreased membrane permeability and efflux pumps. Bacteria may alter the influx of materials from the external environment to decrease antibiotic uptake and/or limit porin expression, as some hydrophilic antibiotics (e.g., ß-lactams, tetracyclines, some fluoroquinolones) utilize these water-filled channels to traverse the bacterial membrane (Munita & Arias, 2016; Pagès et al., 2008). Efflux pumps extrude antibiotics out of the cell before they can begin to exert toxic effects and are more generalist in nature, affecting a wide range of antimicrobial classes (Munita & Arias, 2016). It has been previously reported that *S.* Typhimurium possesses at least nine multidrug efflux pumps capable of exuding a variety of antimicrobial compounds, highlighting the urgency for novel strategies to battle AMR in *Salmonella* (Nikaido et al., 2008).

Modification of the target site, resulting in decreased affinity for the antibiotic, is a common strategy that has been described in both Gram-positive and Gram-negative bacteria (Munita & Arias, 2016). It was previously observed that multiple nucleotide polymorphisms at nucleotide positions 965 to 967 in the 16S rRNA, the binding site of doxycycline, conferred doxycycline resistance in *Helicobacter pylori* (Nonaka et al., 2005).

AMR through global cell adaptation is arguably the most sophisticated process in terms of drug resistance (Munita & Arias, 2016), as it requires concerted coordination of multiple biochemical systems within the cell. Although not fully understood, in vancomycin-resistant *Staphylococcus aureus*, resistance development is thought to arise through sequential, ordered shifts in genetic markers that form part of regulatory systems controlling cell envelope homeostasis, which is disrupted by vancomycin (Gardete & Tomasz, 2014). Further, it has been shown that mutations in *sigB*, a global stress response regulator, led to enhanced resistance to vancomycin in *S. aureus* (Watanabe et al., 2011).

1.3.1.9.3 Emergence and spread of antimicrobial resistance

In *Salmonella*, ARGs are primarily horizontally transferred via plasmids and class I integrons (Alcaine et al., 2007), although other mobile genetic elements (e.g., bacteriophages) are also vectors for transfer of these genes (Goh, 2016; Goodridge et al., 2018). Plasmids may carry one or more ARGs, and ARGs conferring resistance to a wide variety of antibiotics have been identified (Alcaine et al., 2007). Recently, a hybrid plasmid was identified in *S.* Dublin which carried genes responsible for both virulence and AMR (Mangat et al., 2017). This plasmid encoded a complete *spv* virulence operon and 11 modules conferring antibiotic resistance to six classes of antimicrobials. However, the frequency of conjugation was low, in line with previous observations that virulence plasmids are often vertically inherited (Butaye et al., 2006; Mangat et

al., 2017). In a separate report, Moreno Switt et al. (2012) identified the novel existence of Incl1-IncFIB cointegrated plasmids in *S.* Montevideo which encoded antimicrobial resistance. As these plasmids had not been identified previously in *Salmonella*, it is possible they were horizontally transferred from other related microorganisms (e.g., enterotoxinogenic *E. coli*) (Moreno Switt et al., 2012; Parks & Peters, 2009).

Class 1 integrons are frequently identified in clinically-relevant bacteria, especially *Salmonella* (Deng et al., 2015; Alaine et al., 2007). This particular class of integrons is defined by the presence of an integrase gene, a proximal primary recombination site, a promoter, ethidium bromide-quaternary ammonium compound resistance and sulfonamide resistance genes, and a variable region consisting of a gene cassette containing multiple genes conferring AMR. The variable region is particularly prone to site-specific recombination, enabling the accumulation of diverse ARGs of different origin (Deng et al., 2015). Integrons have been located within plasmids, as part of transposons carried by *Salmonella*, and within *Salmonella* genomic islands that can be mobilized in the presence of conjugative helper plasmids (Deng et al., 2015; Doublet et al., 2005).

Food animal production remains one of the largest contributors to the emergence and dissemination of AMR bacteria, as antibiotics are used to promote growth and prevent and treat infectious disease (McEwan & Fedorka-Cray, 2002) Strikingly, antibiotics used in animal agriculture span the same classes of antibiotics used in humans (McEwan & Fedorka-Cray, 2002). Between 2003-2008, it was shown that the isolation of cetiofur-resistant *S.* Heidelberg from retail chicken products was correlated with increased incidence of human cases of *S.* Heidelberg (Dutil et al., 2010). Concerningly, drug-resistant bacteria have also been recovered from various environmental samples (i.e., during processing) and farms, including antimicrobial-

free animal rearing operations (Alali et al., 2010; Gebreyes et al., 2006; Thakur et al., 2007). Although a withdrawal period (i.e., an approved number of days between the last dose of antibiotic administration and slaughter) is mandatory, the presence of antibiotic residues remaining in the final meat product may lead to the emergence of AMR in the human gut microbiota following consumption (Manyi-Loh, 2018).

1.3.2 Bacteriophages

1.3.2.1 Introduction

Bacteriophages (phages) are viral predators that specifically recognize and infect bacteria (Diaz-Muñoz & Koskella, 2014). Phages are very diverse, assuming a variety of host ranges, morphotypes and nucleic acid compositions (i.e., comprising linear or circular, single-stranded (ss) or double-stranded (ds) DNA or RNA) (Bobay et al., 2012). Genetically, they do not encode any enzymes for replication, thus they are considered obligate parasites as they depend on their bacterial hosts for reproduction (Bobay et al., 2012).

Phages constitute the most abundant biological entities on Earth and are estimated to reduce the global bacterial population by 50% every 48 h (Gilmore, 2012). Their impact on natural processes are enormous as they are key drivers in bacterial evolution (Miller, 2001) and influence global biogeochemical cycles (Suttle, 2007). They were initially discovered in 1915 by William Twort and in 1917, Felix d'Herelle realized their bactericidal properties (Clokie et al., 2011) and proposed the concept of "phage therapy", where phages would be used for the treatment of bacterial infections (Di Lallo et al., 2014). Usage of phages, later, was hindered by the advent of antibiotics in the West, although they continued to be used clinically in Eastern European countries (Clokie et al., 2011). However, with AMR constituting a significant global health threat in the modern era, there is currently a renewed interest in using phages and their

components for therapeutic purposes in several sectors, most notably in the food and health industries. Additionally, the usage of phages in other applications (e.g., typing, detection) has also been explored.

1.3.2.2 Lifestyles

Most commonly, phages assume two general life cycles which serve different purposes and result in different outcomes for both the phage and its host (Díaz-Muñoz & Koskella, 2014; Figure 1.2). There are also alternative lifestyles that have been reported, but are comparatively more uncommon (Cenens et al., 2013). In the first stage of each lifestyle, phages adsorb to a host cell receptor via tail fibers (i.e., the recognition element of the phage). Host receptors include the LPS, flagella, pili, techoic acids and outer membrane proteins (Díaz-Muñoz & Koskella, 2014; Rakhuba et al., 2010). Phages have been shown to adsorb to a variety of surface-associated structures on *Salmonella* (Shin et al., 2012), although the LPS is generally considered the most common receptor in Gram negative bacteria (Rakhuba et al., 2010). Some phages require smooth (S) type LPS (i.e., comprising lipid A, core and O side-chain), while others require rough R type LPS, which lacks the O side-chain but contains both lipid A and the core. Interestingly, some phages can utilize either LPS type for adsorption (Rakhuba et al., 2010).

1.3.2.2.1 Lytic cycle

Following adsorption to the surface of cell, viral nucleic acid is injected into the host bacterial cell, and the host cell "machinery" (e.g., polymerases, helicases, ribosomes, etc.) produces viral proteins encoded in the foreign nucleic acid and begins replicating the viral genetic material (Hobbs & Abedon, 2016). This is followed by self-assembly of the viral proteins and formation of the capsid head. In dsDNA phages, the viral DNA is concatemerized and packaged via a packaging ATPase, also called a terminase, as it effectively cuts the DNA at a

pre-determined length before it is shunted into the capsid (Aksyuk & Rossmann, 2011). Assembly and maturation of the capsid head is then followed by tail assembly, the process of which varies between the different families of dsDNA phage (i.e., *Siphoviridae, Myoviridae, Podoviridae*). Following assembly of virions, the cell will lyse through the production of lytic enzymes (holins and lysins) which kill their hosts and release progeny phage into the environment (Coffey et al., 2010). The lytic cycle will then begin again upon adsorption to a susceptible host (Díaz-Muñoz & Koskella, 2014).

1.3.2.2.2 Lysogenic cycle

The lysogenic cycle also begins after adsorption and injection of nucleic acid into the host cell, but is undertaken exclusively by a specialized phage, known as a temperate phage. It is starkly different from the lytic cycle in that virions are not assembled right away; rather, the genetic material stably integrates into the bacterial genome as a prophage and replicates normally with the host (Díaz-Muñoz & Koskella, 2014). Under certain environmental triggers (e.g, antibiotic stress, ultraviolet light, etc.), the prophage may excise out of the chromosome and initiate a lytic cycle (Bobay et al., 2013). Given that foreign nucleic acid is introduced into the host in lysogeny, the phenotype of the host may be altered (i.e., lysogenic conversion). In fact, temperate phages may carry host-adaptive traits and thus represent a major contributor in shaping bacterial diversity and evolution (Bobay et al., 2013; Howard-Varona et al., 2017). For instance, they have been shown to transfer virulence genes, ARGs and other functional modules into the host cell, thereby altering host behaviour (Penadés et al., 2015; Petty et al., 2006; Von Wintersdorff et al., 2016). In *Salmonella*, prophages are common; a calculated average estimates that there are 2.8 known prophages present per *Salmonella* genome (Casjens & Grose, 2016). However, in an analysis by Motawea et al. (2018), 11,297 discrete prophage sequences were

identified among 1,760 *S. enterica* isolates. Strikingly, one serotype, Kisarawe, was found to possess 15 prophage sequences in its genome (Motawea et al., 2018).

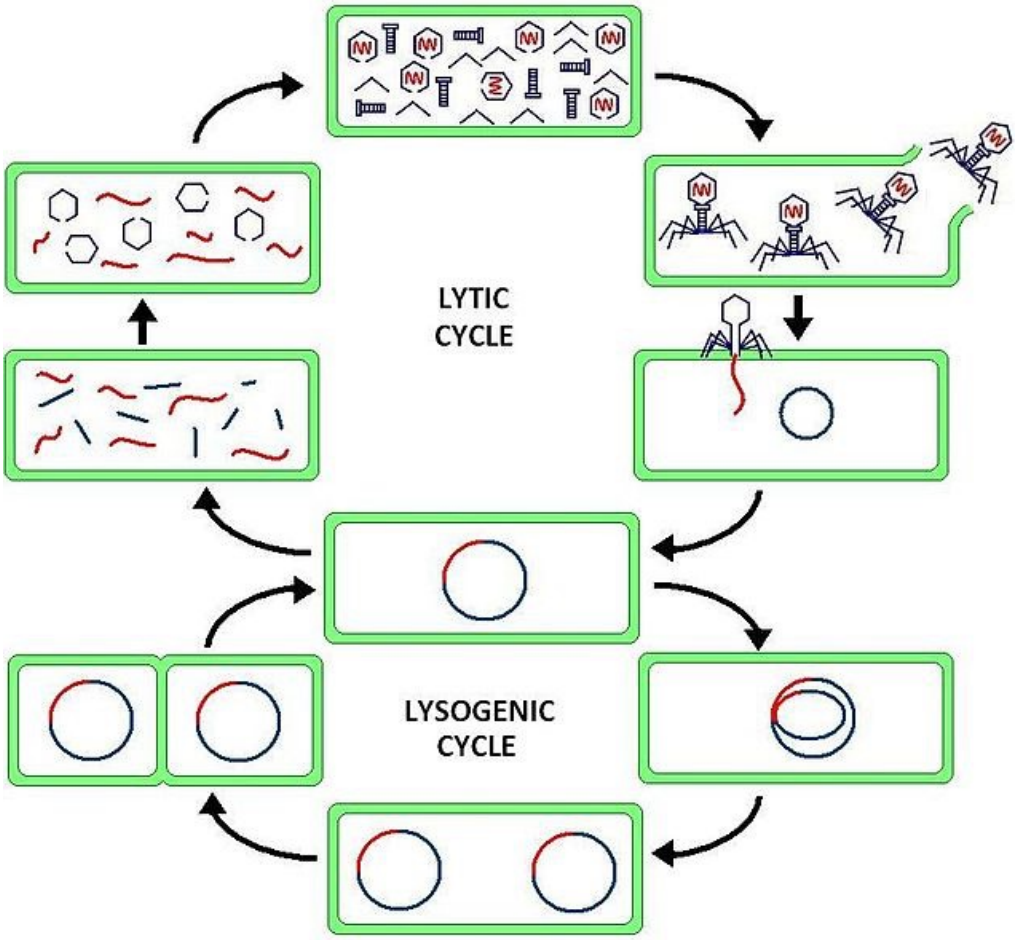

Figure 1.2. Comparison of the lytic and lysogenic cycles of bacteriophages. Green boxes represent the host bacterial cell. Red lines indicate phage nucleic acid; blue lines indicate host nucleic acid. Image is licensed under the Creative Commons Attribution-Share Alike 3.0 Unported license (https://creativecommons.org/licenses/by-sa/3.0/deed.en). Author: Suly12.

Temperate phages carry an integrase gene for integration and excision into and out of the host chromosome. Integrases are enzymes which mediate site-specific recombination of genetic material between two DNA recognition sites: the phage attachment site, *attP* and the bacterial

attachment site *attB*. Each integrase recognizes different sequences, and an integrated phage is flanked by two hybrid sites, *attL* and *attR*, each of which consist of half *attP* and half *attB* sequences, respectively (Groth & Calos, 2004). These then become the substrates for excision out of the host cell, which is induced by various environmental factors (Bobay et al., 2013). Importantly, some prophages have lost the ability to excise out of their host (i.e., cryptic prophages) (Wang & Wood, 2016), leading to the stable retention of genes which are associated with host phenotype conversion (e.g., genes contributing to AMR, virulence, etc.).

1.3.2.2.3 Alternative lifestyles

Some phages, including those infecting *Salmonella*, have alternative lifestyles, namely the carrier state and pseudolysogeny, that are comparatively less well-understood than the classic lytic and lysogenic cycles (Díaz-Muñoz & Koskella, 2014). In the carrier state, a chronic infection is established in the host, and the phage does not integrate into the genome nor induce lysis. Interestingly, a persistent infection is produced where virions are routinely budded from the cell without lysis (Cenens et al., 2013). This has been demonstrated in *E. coli*, where filamentous phages formed 10^{13} viral progeny/mL and slowed down the growth rate of the host (Rakonjac et al., 2011). Remarkably, the carrier state is thought to be associated with host resistance, as the cell does not die and instead, tolerates phage infection (Díaz-Muñoz & Koskella, 2014).

Pseudolysogeny is another phenomenon where the phage does not integrate nor lyse the host. Most commonly, pseudolysogeny is seen in bacteria undergoing starvation and has also been observed in biofilms (Kocharunchitt et al., 2009). It has been thought that in lytic phages, pseudolysogeny may represent a “pause” until conditions become suitable again to initiate lysis (Clokie & Kropinski, 2009). This phenomenon is an important aspect of phage-host interactions

as it represents the likely state of phage in natural environments (since the host is usually lacking nutrients) (Los & Wegrzyn, 2012).

1.3.2.3 Viral taxonomy

1.3.2.3.1 Overview

Initially, phage classification was based primarily on virion morphotype classification through electron microscopy and nucleic acid composition (i.e., dsDNA/RNA or ssDNA/RNA) (Adriaenssens & Brister, 2017). There has since been a renaissance in regards to phage taxonomy and nowadays, both electron microscopy and genome sequencing are used in the assignment of novel phages into their respective families, genera and species (Adriaenssens & Brister, 2017; Nelson, 2004), although problems such as genome rearrangement and high rates of recombination may blur taxonomic boundaries (Nelson, 2004). To date, there are over 9,200 complete viral genomes on NCBI RefSeq (www.ncbi.nlm.nih.gov/refseq/).

Viral taxonomy is currently the responsibility of the International Committee of the Taxonomy of Viruses (ICTV), which outlines categorization and also reviews taxonomic proposals from the scientific community. Within the ICTV, the Bacterial and Archaeal Viruses Subcommittee is responsible for the classification of prokaryotic viruses (i.e., phages) (ICTV, 2019 & Nelson, 2004).

1.3.2.3.2 Taxonomy of the tailed phages

Tailed phages represent approximately 90% of all phages (Eriksson, 2015) and comprise the order *Caudovirales*, which consist solely of dsDNA phage. Within this order are five phage families: *Myoviridae, Siphoviridae, Podoviridae* and *Ackermannviridae* and *Herelleviridiae,* which were recently introduced. *Myoviridae*, *Siphoviridae* and *Podoviridae* are conventionally distinguished by tail morphology; Myoviruses possess long, contractile tails; Siphoviruses

possess long, noncontractile tails, and Podoviruses possess short tails (Figure 1.3; ICTV, 2019). Comparatively, it appears that *Ackermannviridae* and *Herelleviridae* cannot be distinguished from other families on the basis of tail morphology.

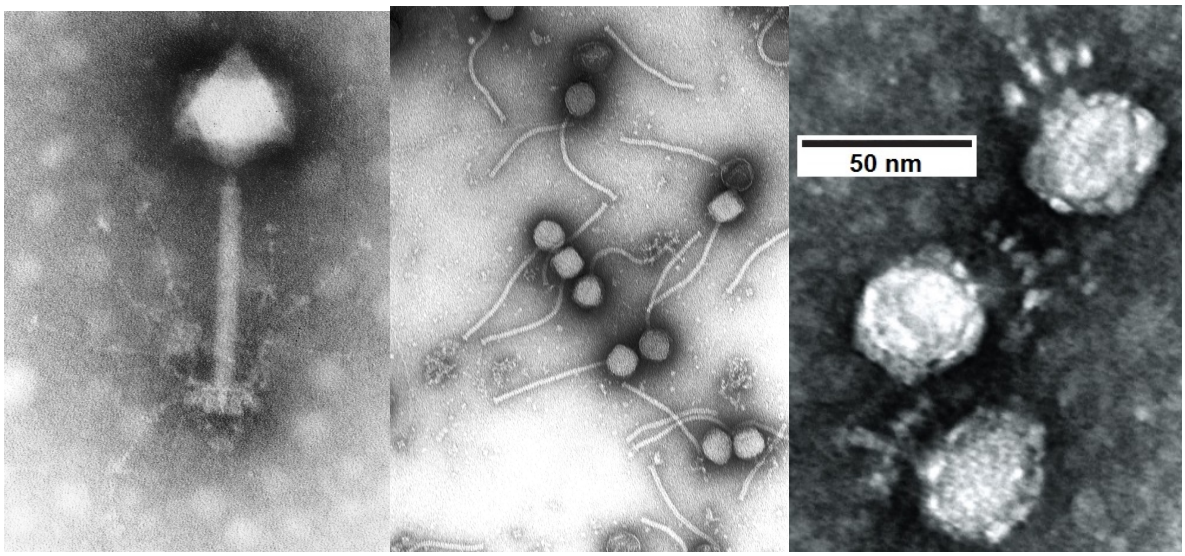

Figure 1.3. Morphotypes of the *Myoviridae, Siphoviridae* and *Podoviridae* families (left to right, respectively). Left; middle: Images licensed under CC BY 2.5 (https://creativecommons.org/licenses/by/2.5/ca/) Author: Ayacop. Right: Image licensed under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/) and obtained from Volozhantsev et al. (2012).

Salmonella phages are represented in all families, however, at present, no *Salmonella* phages have been assigned to the family *Herelleviridae*. Currently, only eight *Salmonella* viruses belong to *Ackermannviridae* and are assigned to the genera *Agtrevirus* and *Kuttervirus* on the basis of genome homology (ICTV, 2019).

DNA-DNA relatedness is commonly used for phage comparison and classification (Kropinski et al., 2018). For ICTV-approved genus demarcation, phages must share >50% nucleotide sequence similarity. Phages within a genus thus have shared characteristics that define the nature of the genus, such as average genome length, number of coding sequences, average

number of tRNAs and presence of benchmark or "signature" genes (Adriaenssens & Brister, 2017). On the other hand, phages belonging to the same species share 95% nucleotide identity, thereby only differing by a maximum of 5%. Each species is currently represented by at least one well-characterized "reference" or "type" species or genome (Adriaenssens & Brister, 2017).

1.3.2.4 Comparative genomics of *Salmonella* phages

Comparative genomics analyses have provided immense insights into phage diversity, evolutionary relationships, phage-host interactions and geographic distribution (Duhaime et al., 2017; Grose & Casjens, 2014; Murphy et al., 2016; Park et al., 2012; Smith et al., 2013; Switt et al., 2013), which has provided a basis for the advent of novel phage therapies in sectors such as human medicine and food biocontrol (Bardy et al., 2016; Fong et al., 2017; Pirnay et al., 2015). In this regard, many authors perform hierarchical clustering on the basis of nucleotide and/or amino acid relatedness and gene conservation, based on pre-determined cut-offs (usually >40% identity or more), which together are useful in assessing shared characteristics, investigating diversity and inferring phylogenetic relationships (Denes et al., 2014; Finke et al., 2017; Grose & Casjens, 2014; Switt et al., 2013). These cluster analyses may also aid with further delineation of relationships based on the conservation of gene content and order (Grose & Casjens, 2014).

Bardina et al. (2016) recently reported on the isolation and genomic characterization of three novel virulent *Salmonella* phages (UAB_Phi20, UAB_Phi78 and UAB_Phi87) with broad host ranges. Through sequence analysis it was revealed that UAB_Phi87 possessed high similarity with *Salmonella* type phage Felix O1 and did not possess unwanted genes that would otherwise hinder its use as an antibacterial agent (i.e., genes encoding for integrase, toxins, etc.) (Bardina et al., 2016). In a separate report, phages (n=83) isolated from sewage were found to possess unique properties, such as the abilities to plaque at low temperature (4ºC) and survive in

the presence of high temperatures, organic solvents and detergents (Jurczak-Kurek et al., 2016). In a study by Mikalová et al. (2017), 15 temperate phages were induced from 40 strains of *S. enterica* and their host ranges compared with 52 pathogenic *Salmonella* isolates. They observed the broad host ranges of phages SEN1 and SEN8, which could be important in terms of biotechnological potential, as certain tools (i.e., involving phage transduction for the delivery of genes of interest) require the integration of genetic material into the host (Mikalová et al., 2017).

In a large-scale comparison of 337 phages infecting members of *Enterobacteriaceae* (Grose & Casjens, 2014), phages were cleanly grouped into 56 clusters based on dot plot analysis of nucleotide and amino acid sequences, which allowed for analysis of sequence similarity, but also mosaic differences and the presence/absence of gene content. For instance, the Felix O1-like cluster (of which the name is derived from the *Salmonella* broad host range phage Felix-O1) comprised phages infecting diverse hosts, including *Salmonella*, *E. coli* and the distantly-related host *Erwinia*. Gene products shared between the phages of this cluster included nucleotide metabolism proteins, phage assembly proteins, NAD biosynthesis proteins, and a number of hypothetical proteins. Together, these reports suggest that cluster analysis is of use in biocontrol, as it allows for the pre-assignment of phages into certain groups based on shared characteristics, such as those with favorable traits (e.g., broad host ranges).

Comparative analysis of phage genomes has also identified the usage of certain conserved core genes as phylogenetic markers for inferences into the whole genome. In microbial communities, the 16S rRNA is routinely used as a benchmark gene to assess biodiversity ((Rajendhran & Gunasekaran, 2011). However, in phages, there is no universal gene that is used to infer phylogeny due to several factors, including the frequent recombination events and the lack of conservation within structural genes (for instance, Podoviruses do not

possess a tail tape measure protein, which is conserved within Myo- and Siphoviruses) (Born et al., 2019). However, taxonomic assignments with a molecular marker can be inferred in selected *groups* of phages (Dwivedi et al., 2012). For instance, the portal vertex protein (*g20*) and major capsid protein (*g23*) are often used to characterize diversity in T4-like Myovirus communities (Zhong et al., 2002; Short & Suttle, 2005; Filee et al., 2005; Wang et al., 2011). In a comparison of 337 *Enterobacteriaceae*-infecting phages, Grose & Casjens (2014) observed the correlation of the major capsid protein (MCP) clustering pattern to that of the whole genome, suggesting it may be used to infer relationships between clusters of phages infecting *Enterobacteriaceae* (Grose & Casjens, 2014). The MCP is a conserved protein that has shown constrained evolution given its functional importance in capsid structure (i.e., representing ~90% of proteins on the capsid head) (Larsen et al., 2008), therefore it is an obvious choice to assess in regards to its functionality as a signature gene. Recently, an MCP polymerase chain reaction (PCR) assay was developed, with MCP-specific primers used to identify members of the FO1-, GJ1-, N4-, SP6-, T4-, T7- and Vi1-like phage groups, of which all phages are strictly lytic (Born et al., 2019). The authors were successful in their cluster assignment of 154 phages infecting members of *S. enterica*, *E. coli* and *Erwinia amylovora*, thereby facilitating the rapid identification of newly isolated phages into groups and the preliminary identification of phage candidates for biocontrol applications (Born et al., 2019).

1.3.2.5 Phage applications in the typing, detection and control of *Salmonella* in food

1.3.2.5.1 Typing

Phage typing of enteric pathogens has traditionally been, and is still currently being used for epidemiological investigation and diagnostics, although overall, it has mostly been replaced by sophisticated molecular DNA fingerprinting methods, such as ribotyping and PFGE

(Chirakadze et al., 2009; Hagens & Loessner, 2008). However, due to its rapid, economical and reproducible technique, it still remains a common diagnostic tool in some situations. For instance, phage typing is the gold standard method for epidemiological surveillance of *S.* Typhimurium (Mohammed, 2017). Multiple *S.* Typhimurium phage typing schemes have been developed, resulting in over 200 definitive phage types (DTs or PTs) (Rabsch et al., 2004). In a comparison of clustered regular interspaced short palindromic repeats (CRISPR) typing and phage typing of *S.* Typhimurium, phage typing was the superior method as it resolved clusters of isolates with identical spacers (Mohammed, 2017).

Phage typing is performed with a specific set of typing phages and phage typing schemes for a specific species or serotype (when typing *Salmonella*, for instance). Fundamentally, phages used in a particular scheme will differ from each other and induce differential patterns of lysis on different groups of bacterial strains. These different lysis patterns give rise to different phage types, to which the bacterial isolates are then given unique identifiers based on this pattern (Chirakadze et al., 2009). In outbreak investigations, PFGE and phage typing are still used in tandem to type outbreak isolates (Ahmed et al., 1995).

1.3.2.5.2 Detection

Given that phages possess remarkable specificity for their hosts, such properties have been utilized for detection of *Salmonella* and other pathogens. Phage production for the purposes of bacterial detection is relatively low-cost and can be easily scaled up for commercial purposes (Petty et al., 2007). Furthermore, the rapid replication and infection cycle of lytic phages mean that rapid detection of pathogens is possible (Schmelcher & Loessner, 2014). Lastly but importantly, phages only multiply in viable (i.e., living) cells, thus the possibility of false

positives is dramatically reduced (unlike in other methods such as PCR, where there is a comparatively high false-positive error rate) (Petty et al., 2007).

It should be noted that phages have been used as indicators for the detection of microorganisms (typically non-pathogens) in various environments. Coliphages (i.e., phages infecting coliform bacteria) have been used as a rapid method to detect and infer coliform abundance and distribution in water, which are themselves indicators of fecal pollution (Gallard-Gongora et al., 2017; Muniesa et al., 2018; Toribio et al., 2019). However, recombinant phage is mostly used for the definitive detection of pathogens (Petty et al., 2007), although the detection potential of native, unmodified phages cannot be understated. There is a broad spectrum of phage-based detection tools today that have been reported and commercialized, and they may be separated into three broad categories based on: (i) detection of components from lysed bacterial cells; (ii) phage amplification; and (iii) detection using phage components (e.g., tail fibers) (Schmelcher & Loessner, 2014).

Phage-mediated host cell lysis results in the release of various cellular components of the target pathogen of interest, including ATP and α- and β-glucosidases, which are then detected. In detection of ATP release, a bioluminescent assay is used where the released ATP is used to drive a reaction converting luciferin into oxyluciferin via luciferase, emitting detectable light (Squirrel et al., 2002). Detection of α- and β-glucosidases leakage from lysed *Bacillus cereus* and *Mycobacterium smegmatis* was also reported via the external addition of substrate (Yemini et al., 2007). On the other hand, phage amplification is comparably more straightforward; an increase in titer is correlated with pathogen presence (Hagens & Loessner, 2007). Genetically modified "reporter" phages have also been engineered, which deliver specific reporter genes (e.g., *lux*-family genes) into the host cell. Upon infection, the gene is expressed and detection of its

product is feasible through measurement of properties such as bioluminescence or fluorescence (Schmelcher & Loessner, 2014). Nowadays, reporter phages represent the most common mode of phage-based detection and have been widely developed for a variety of foodborne pathogens, including *Salmonella* (Thouand et al., 2008).

Utilization of phage components is another possibility in phage-based detection. Because the tail fibers mediate recognition and attachment to the host, a variety of assays have been developed on this basis. For instance, fluorescently labeled phage tail fibers have been harnessed in VIDAS UP technology (i.e., a sandwich ELISA approach) (BioMerieux) for the detection of *Salmonella* (http://www.biomerieux-industry.com/vidasup). Currently, biosensor technology using immobilized phage components as recognition elements also constitutes a fascinating and emerging area of research (Schmelcher & Loessner, 2014). The detection of *Salmonella* in various foods, including tomatoes, milk and spinach has been successful with this approach (Lakshmanan et al., 2007; Li et al., 2010; Park et al., 2013).

1.3.2.5.3 Biocontrol

Phages and their components have been thoroughly investigated for use in numerous food safety applications, and against several target microorganisms, which have led to the commercialization of several approved phage cocktails (i.e., formulations comprising more than one phage) and products (Table 1.4).

Table 1.4. Currently available commercial phage products for food safety applications (Moye et al., 2018).

Company	Phage product	Target microorganism
FINK TEC GmbH (Hamm, Germany)	Secure shield E1	*E. coli*
Intralytix, Inc. (Baltimore,	Ecolicide®(EcolicidePX™)	*E. coli* O157:H7

MD, USA)	EcoShield™	*E. coli* O157:H7
	ListShield™	*L. monocytogenes*
	SalmoFresh™	*Salmonella* spp.
	ShigaShield™ (ShigActive™)	*Shigella* spp.
Micreos Food Safety (Wageningen, Netherlands)	PhageGuard Listex™	*L. monocytogenes*
	PhageGuard S™	*Salmonella* spp.
		E. coli O157:H7
Passport Food Safety Solutions (West Des Moines, IA, USA)	Finalyse®	*E. coli* O157:H7
Phagelux (Shanghai, China)	SalmoPro®	*Salmonella spp.*

Increased awareness of potential use in the food industry has led to numerous published studies investigating their efficacy for control of *Salmonella* at various stages of the food chain (Bardina et al., 2016; Fong et al., 2017; Kocharunchitt et al., 2009; Pao et al., 2004; Sillankorva et al., 2010), as they possess several desirable properties which render them suitable agents for food biocontrol. For instance, they are: (i) self-replicating and self-limiting (i.e., increase and decrease in titer with bacterial infection and decline, respectively); (ii) specific to only their target bacterial hosts (and thus non-toxic in human or animal cells), usually incapable of crossing genus and species barriers; (iii) ubiquitously distributed in nature; and (iv) may be feasibly scaled up for commercial applications (Guenther et al., 2012). An assessment of *E. coli* phage T4 at concentrations of 10^9 PFU/ml in human oral feeding trials are demonstrative of their safety in humans, as there were no adverse effects reported (Bruttin & Brussow, 2005).

The thorough screening and identification of certain characteristics is essential for selection of phage in food production schemes (Kazi & Annapure, 2016). For instance, the phage

should be (i) strictly lytic; (ii) free of genes encoding for AMR and/or virulence; (iii) possess a broad pathogen host range; and (iv) exhibit stability under a variety of storage or treatment-related conditions (e.g., pH up/downshifts, temperature fluctuations, sanitizers), as food is often subject to hurdled interventions (i.e., techniques for pathogen inactivation applied in tandem) (Coffey et al., 2010; Fong et al., 2017; Goodridge et al., 2018). Phages have been used for *Salmonella* biocontrol at all stages of the farm-to-fork continuum with variable success (Fong et al., 2017; Guenther et al., 2012; Kocharunchitt et al., 2009; Lim et al., 2012; Pao et al., 2004; Zinno et al., 2014) and the food industry continues to push for their utilization as they represent "green" antimicrobials which are particularly attractive to consumers. The therapeutic potential of phages in food production (both at the agricultural level (i.e., pre-harvest) and during processing (i.e., post-harvest)) will be discussed herein, with an emphasis on tailed phages infecting *Salmonella*.

1.3.2.5.3.1 Pre-harvest

In pre-harvest applications (i.e., in-field treatment of produce commodities and treatment of food production animals), phages are being used primarily as antimicrobial agents for animal production (Goodridge et al., 2018), although it is also possible that phages be used for pre-harvest treatment of fresh produce commodities. To the best of our knowledge, however, there are no published reports of this strategy. In the case of *Salmonella*, the poultry gastrointestinal tract is a prominent reservoir for these pathogens and poultry are themselves asymptomatic carriers of these zoonotic strains (Nabil et al., 2018). Additionally, swine also represents a reservoir for host-adapted *S.* Typhimurium (Foley et al., 2006). Traditionally, antibiotics and/or vaccination are used for the reduction of *Salmonella* in these animals, but the overuse of antibiotics in commercial rearing operations has contributed to the rise of antimicrobial

resistance (Cloete, 2003). Thus, phages represent a viable option for the reduction of *Salmonella* persisting in these reservoirs, with the added benefit of controlling those strains possessing antibiotic resistance (Clavijo et al., 2019). Alternatively, vaccination has been extensively explored for poultry production, but the high cost has hindered its adoption and chickens have been shown to asymptomatically shed *Salmonella* even after vaccination (Lim et al., 2012).

Phages have been used to alleviate the microbial load of *Salmonella* in poultry according to several reports. Atterbury et al. (2007) isolated 232 *Salmonella* phages from the farm environment (including poultry farms and abattoirs) and wastewater and identified three broad host range phages, which were then individually administered to broiler chickens challenged with several serovars of *Salmonella*. The authors observed a significant reduction of *S.* Enteritidis in the chicken cecum of >4.2 log CFU, while *S.* Typhimurium was reduced by >2.19 log CFU. However, *S.* Hadar was not effectively controlled in the chickens, although the phages demonstrated infectivity towards this serotype *in vitro* (Atterbury et al., 2007). This may be due to the absence of receptor expression on the surface of the bacterial cell under environments encountered in the poultry intestine (Seed, 2015). Lim et al. (2012) also observed effective reduction of pathogen load in three-day old chicks challenged with 5 x 10^7 CFU *S.* Enteritidis upon continuous treatment with 10^9 CFU/g of phage ØCJ07. Notably, *Salmonella* was not detected in 70% of chickens after three weeks of treatment. Horizontal transfer of *Salmonella* has been shown to routinely occur in broiler houses; therefore, the observed eradication of *Salmonella* in these chicks following phage treatment is promising as it may prevent the lateral transfer of this pathogen to other chicks (Lim et al., 2012). In a separate study, high doses of phages (10^{10} PFU) were administered orally to broiler chickens and significant reductions of

Salmonella were observed, although completion eradication could not be achieved (Fiorentin et al., 2005).

Very recently, Clavijo et al. (2019) investigated the effectiveness of a phage cocktail SalmoFREE® for large-scale control of *Salmonella* in a commercial broiler operation, representing the first study to investigate phage reduction of *Salmonella* in commercial rearing operations. SalmoFREE®, a patented cocktail (i.e., mixture) of six lytic phages, was administered in drinking water at 10^8 PFU/mL and *Salmonella* detected from cloacae of challenged chickens. It was observed that *Salmonella* was completely eliminated by day 33 compared to the controls. However, issues such as high rates of chicken mortality due to unforeseen illnesses led to concurrent administration of antibiotics in one of the field trial broiler houses, leading to some broiler houses receiving antibiotic therapy and phage therapy in tandem. However, the results nevertheless highlight the potential of phages for eradication of *Salmonella* in commercial rearing operations, using a feasible method of administration (Clavijo et al., 2019).

Phage control of *S.* Typhimurium in swine has also been evaluated, although to a comparatively lesser extent. In pigs during transport and holding prior to slaughter, *Salmonella* phages were administered via oral gavage which resulted in significant reductions in titers in the ileum, cecum and tonsils (Wall et al., 2010). The authors then investigated phage efficacy when the route of administration was changed by including the phage cocktail in pig feed; it was determined that pigs directly shed significantly less *S.* Typhimurium than those pigs administered the phage cocktail orally (Wall et al., 2010). In another report, significant reductions of *S.* Typhimurium were observed in the rectum of pigs fed with 3×10^9 PFU of a cocktail comprising two lytic phages (Callaway et al., 2011).

1.3.2.5.3.2 Post-harvest/processing

The majority of studies have focused on direct phage application during processing of various foods (i.e., post-harvest and before packaging or storage) (Fong et al., 2017; Guenther et al., 2012; Pao et al., 2004; Spricigo et al., 2013). Thung et al. (2017) investigated the biocontrol efficacy of a lytic *Salmonella* phage SE07 in fruit juice, fresh egg, beef and chicken meat. Following 48 h storage at 4ºC, significant reductions of >2 log CFU/g or ml were observed across all matrices. Additionally, SE07 demonstrated stability between a pH range of 4.0 – 11.0 and infectivity across a range of temperatures (28ºC, 37ºC, 45ºC and 65ºC) (Thung et al., 2017). These values are in accordance with our data, where we also used a single phage isolate SI1 for control of *S.* Enteritidis in sprouting alfalfa seeds and observed ~2.5 log CFU/g reductions of this pathogen (Fong et al., 2017). Guenther et al. (2012) used a broad host range, virulent phage FO1-E2 for control of *S.* Typhimurium in a variety of ready-to-eat foods. A five log CFU/g reduction was observed in turkey deli meat and chocolate, and three log CFU/g reduction was observed on hot dogs and in seafood. Phage treatment in egg yolk resulted in a significant 2.6 log CFU/g decrease after only two days post-treatment. It was hypothesized that the differences in outcome were largely dependent on the food matrix, for instance, the comparatively small reductions observed in egg yolk might be due to reduced diffusion and/or nonhomogeneous distribution of phages in the highly viscous environment (Gnanou Besse et al., 2005). In fact, phage-based applications should be thoroughly validated for use on different food matrices before implementation on a wide-scale (Goodridge & Bisha, 2011).

An interesting study involving the use of temperate phage P22 was used to inactivate *S.* Typhimurium in a variety of food matrices (Zinno et al., 2014). Although the usage of temperate phage is not recommended in food applications, appreciable host inactivation in the order of two

or more log units was demonstrated. In challenge experiments involving a multiplicity of infection (MOI) of 10^8, two to three log reductions (CFU/ml) of *S.* Typhimurium were observed in phage-treated samples following 48 hours of storage at 4ºC. Remarkably, *S.* Typhimurium was not detected in phage-treated whole nor skimmed milk after storage at 4ºC. Together, these data indicate that phage P22 may behave as a virulent phage towards *S.* Typhimurium, although in general, the research community cautions against use of temperate phage in food applications given their ability to transduce genes encoding factors such as antimicrobial resistance and virulence (Goodridge et al., 2018; Guenther et al., 2012; Hagens & Loessner, 2007; Spricigo et al., 2013).

The application of phages for processing of fresh produce represents an attractive alternative to conventional methods, such as chlorine sanitation (NACMF, 1999). Usage of chlorine as a sanitizer has been shown to exhibit decreased efficacy with increasing concentrations of organic matter (e.g., soil) (Fong et al., 2017; Zhou et al., 2015). Furthermore, as fresh produce is minimally processed, phages represent desirable antimicrobials as they do not affect the organoleptic nor quality properties of fresh produce products (Fong et al., 2017).

Leverentz et al. (2001) investigated the biocontrol efficacy of a phage cocktail against *Salmonella* on the surfaces of fresh-cut fruit. The cocktail reduced the pathogen on fresh-cut melon by 3.5 log CFU/g after storage at 5ºC and 10ºC, and by 2.5 log CFU/g after storage at 20ºC. Remarkably, it was also observed that phage treatment was more effective than chemical sanitizers applied concurrently (Leverentz et al., 2001). However, no significant decreases in *Salmonella* were observed on phage-treated apple slices, suggesting that the acidic pH of the apples may have inactivated the phage. It has been demonstrated previously that phage viability is significantly impacted by acidity (Fong et al., 2017; Thung et al., 2017).

Salmonella phage SSP6 was examined for its biocontrol potential of *S.* Oranienburg on experimentally contaminated alfalfa seeds by Kocharunchitt et al. (2008). However, only one log CFU/g of pathogen reduction was observed, which was not significant. The authors hypothesized the existence of a temporary acquired, non-specific phage resistance phenomenon as the permanent development of phage resistance was not observed in *Salmonella* recovered from the sprouts samples (Kocharunchitt et al., 2008).

Recently, a phage cocktail comprising three lytic phages targeting different *Salmonella* host receptors was developed for investigation of biocontrol efficacy on lettuce and cucumber (Bai et al., 2019). Specifically, these phages were shown to adsorb to the flagella, O-antigen and BtuB, an outer membrane protein. It has been shown previously that phage resistance by the host can be mediated by the usage of phages adsorbing to different receptors, and solve the narrow host range of phages used singly for biocontrol (Tanji et al., 2004). Treatment of lettuce resulted in a 4.7-5.5 log CFU/cm^2 reduction of *S.* Typhimurium after 12 h at room temperature storage (25ºC). Similar reductions were reported in cucumber following identical storage conditions; a 4.8-5.8 log CFU/cm^2 reduction of viable *S.* Typhimurium was also demonstrated. The authors also observed a significantly lower frequency in the emergence of bacteriophage-insensitive mutants (BIMs) of *S.* Typhimurium when three-phage cocktails were used as compared to single phages (Bai et al., 2019), consistent with Pereira et al. (2016a).

1.3.2.5.3.3 Phage-derived lysins as antibacterial agents

In the late stages of the infective lytic cycle, phage-encoded lysins begin to accumulate in the cytosol of the host cell, which then rupture the bacterial cell wall and release phage progeny (Young, 1992). In general, ssDNA and ssRNA phages accomplish lysis with a single lysis gene that does not encode for muralytic enzyme activity. Double-stranded DNA phages (i.e., tailed

phages), however, degrade cell wall peptidoglycan with a muralytic enzyme, or lysin (also termed “endolysin”). Structurally the N-terminus comprises the catalytic domain of the enzyme and the C-terminus binds to a specific substrate (usually carbohydrates) found in the cell wall of the host bacterium (Fischetti, 2008). Lysis of the host cell is accomplished with the assistance of a small membrane protein, holin, which exhibits site specificity and is functionally associated with increased membrane permeability (Wang et al., 2000). In tailed dsDNA phages, both the holin and lysin are essential components for host cell lysis and are usually positioned on the same strand in close proximity and transcribed together (Fenton et al., 2010, Fong et al., 2019).

Because of their inherent lysing capabilities, lysins have been investigated as antimicrobial agents (Fischetti, 2008; Horgan et al., 2009; Schmelcher et al., 2012; Yoong et al., 2004). Lysins represent a novel antimicrobial strategy in the war on pathogens and interestingly, kill bacteria incredibly quickly with nanomolar amounts; the application of lysin to *Streptococcus pyogenes* resulted in a reduction of >6 log CFU/ml only seconds after application (Loeffler et al., 2001).

Lysins for the inactivation of Gram-positive bacteria have led to efficient lysis due to direct contact with its peptidoglycan target in the cell wall (Horgan et al., 2009). In Gram-negative bacteria, the outer membrane represents a barrier as it prevents lysin from reaching its active site; therefore, their success has been limited (Fenton et al., 2010). In the food industry, it appears that lysins have limited scope for widespread application as it appears to be predominantly limited to treatment against Gram-positive pathogens. In a study by Gaeng et al. (2000), a phage-derived lysin gene was cloned into recombinant *Lactococcus lactis* that could quantitatively secrete lysins, which in turn rapidly inactivated *Listeria monocytogenes* in the surrounding medium. Secretion of functional lysin was also accomplished in an *L. lactis* strain

used in milk fermentation, raising the possibility of developing functional starter cultures to enhance dairy safety (Gaeng et al., 2000). Additionally, lysin Ply3626, derived from *Clostridium perfringens* phage f3626, has shown promise as an anticlostridial agent in food and poultry feed (Zimmer et al., 2002).

1.3.2.6 Host cell resistance

As is the case with all selective pressures, the development of resistance to phage in bacteria has been documented (Bishop-Lilly et al., 2012; Frampton et al., 2014; Pereira et al., 2016a; Torres-Barceló et al., 2016). Phage resistance of target host pathogens is a problem in the food industry and phage preparations must therefore be carefully formulated to avoid or delay the development of bacterial resistance (Bai et al., 2019); thus, it is of utmost importance that host cell defenses are thoroughly investigated. Additionally, the importance of phage inactivation after food/environment applications has been highlighted, as phage resistance of non-target microflora may also develop, which could lead to problems downstream (e.g., horizontal transfer of phage resistance genes to target pathogens) (Sommer et al., 2019). Often described as a molecular arms race, bacteria have evolved diverse defense strategies to evade phage infection and/or lysis, which may be categorized into "innate" bacterial immunity (e.g., adsorption inhibition, restriction modification, etc.; Figure 1.4) and adaptive

immunity (e.g., CRISPR/Cas9).

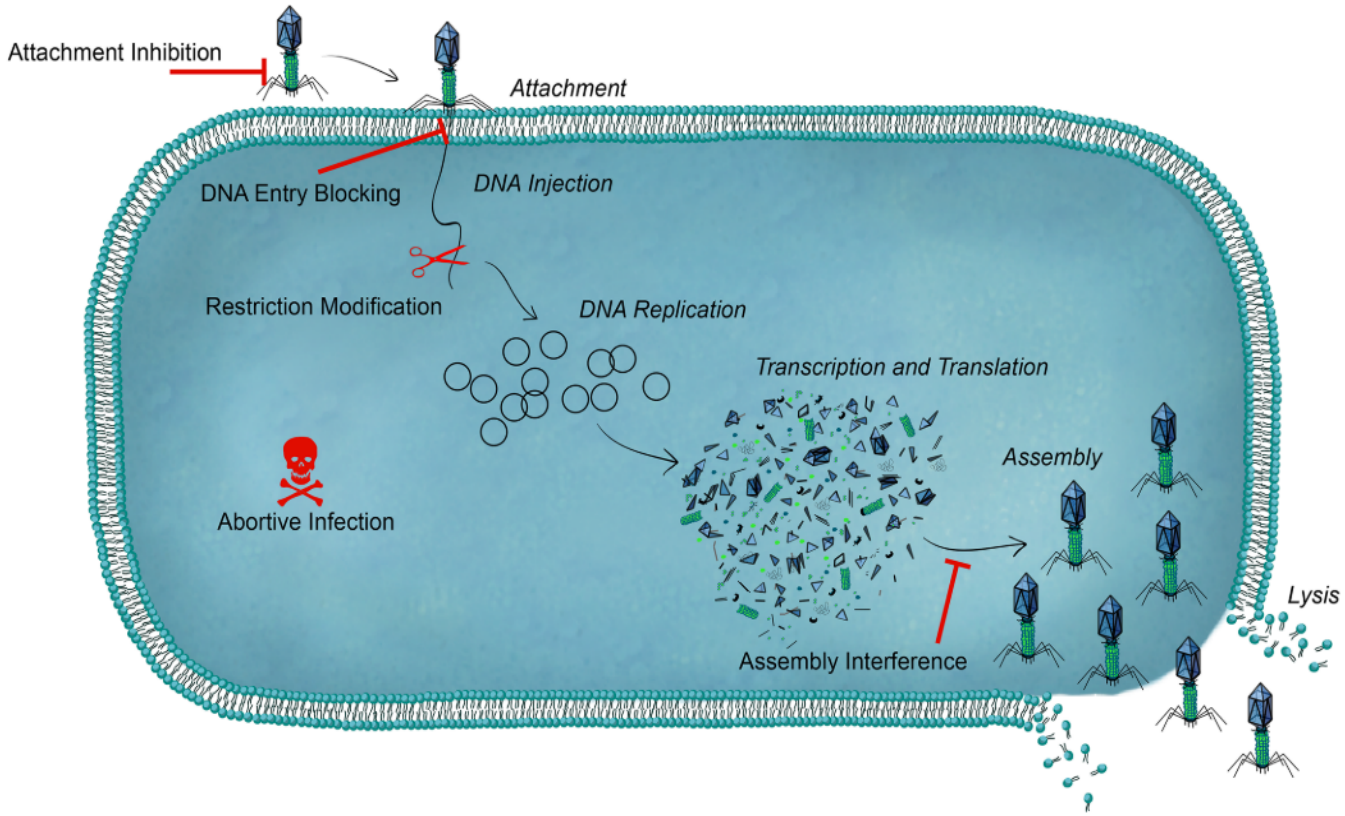

Figure 1.4. An overview of the innate immunity mechanisms of the host bacterial cell against invading phage. The stages of the phage lytic replication cycle are represented in italics. Image is from Seed (2015) and licenced under CC BY 4.0 **(**https://creativecommons.org/licenses/by/4.0/).

1.3.2.6.1 Innate mechanisms of immunity

1.3.2.6.1.1 Prevention of phage attachment

Preventing phage attachment as a means of resistance has been commonly reported in the literature (Labrie et al., 2010; Silva et al., 2016). In the initial stage of the infective cycle, phages adsorb and attach to several receptors on the surface of the bacterial cell, including the LPS, techoic acids, outer membrane proteins, and flagella via the tail fibers (Choi et al., 2013; Li et al., 2019; Rakhuba et al., 2010). Therefore, strategies to prevent phage adsorption commonly include

the modification of receptor structure (Perry et al., 2015) and the concealment of receptors (Scholl et al., 2005).

Pseudomonas aeruginosa PA1 resistant to lytic phage PaP1 was found to possess a large chromosomal deletion of 219.6 kb in length. Deletion of this large genomic fragment resulted in the absence of *galU*, which is involved in O-antigen synthesis, the site of phage adsorption (Figure 1.5; Shuai et al., 2014). A decrease in the expression of LPS O1 antigen also accounted for reduced phage attachment, as seen in *Vibrio cholera* (Seed et al., 2012). More recently, *Salmonella* phage SSU5 adsorption was blocked by the accumulation of mutations (i.e., truncations) in the LPS core of *S.* Typhimurium (Kim et al., 2014). To delay and overcome phage resistance, phage cocktails for use in food must be carefully formulated; ideally phages from different families and utilizing different receptors should be included in these mixtures (Bai et al., 2019; Fong et al., 2019).

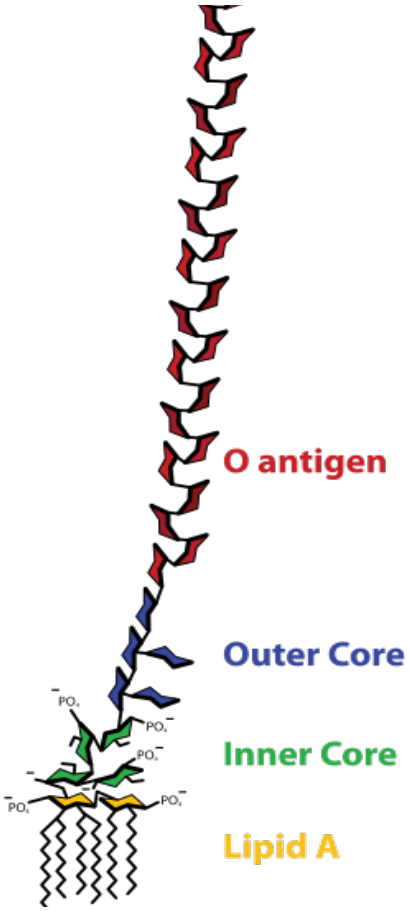

Figure 1.5. Structure of the lipopolysaccharide. Image is licensed under Creative Commons Attribution-Share Alike 3.0 Unported license (https://creativecommons.org/licenses/by-sa/3.0/deed.en). Author: Mike Jones.

Receptors may also be obscured behind a physical barrier, rendering inaccessibility to predator phages. For instance, the K1 capsule of *E. coli* interferes with phage T7 attachment to the LPS (Scholl et al., 2005). Concealment of receptors is also naturally present in certain species of bacteria that are closely associated with biofilms (e.g., *Listeria monocytogenes*), bacterial communities embedded in extrapolymeric substances (Rostøl & Marraffini, 2019). Interestingly, decoys may also be produced by bacteria; phage T4 titers were reduced in the presence of outer membrane vesicle (OMV) production. The budding of OMVs into the environment may serve as

alternative phage attachment sites, leading to the reduction of phages available to attach to cell surface receptors (Manning et al., 2011).

In response to host resistance, phages have also remarkably co-evolved to infect previously resistant bacteria through modification of their recognition elements (i.e., tail fibers) (Samson et al., 2013). Mutant *Bacillus subtilis* phages have been isolated that attach to their previously resistant hosts by acquiring point mutations in the tail fiber-encoding regions of the genome. Interestingly, these phages also had the added impact of evolving a broader host range, expanding to infect non-host *Bacillus* species (Habusha et al., 2018). Additionally, phage λ evolved to target a new receptor OmpF when its native receptor, LamB, was mutated (Chatterjee et al., 2012).

1.3.2.6.1.2 Blockage of DNA entry

Following successful attachment to a surface receptor, the next step in phage infection is injection of DNA into the host cell. Superinfection exclusion (Sie) systems act to block phage DNA injection (Figure 1.4), although they are more specialized resistance mechanisms as they are typically phage encoded (Seed, 2015). Blockage of DNA entry prevents host cell takeover and lysis, and hence the prophage will not be destroyed in the process (Ali et al., 2014). Sie systems are predicted to interact with tail-associated proteins of the phage, such as the tail tape measure protein of *Siphoviridae,* which is involved in channel formation (Bebeacua et al., 2013). In the Salmonellae, the temperate P22 prophage of *S.* Typhimurium encodes two Sie systems, *sieA* and *sieB* (Hofer et al., 1995). At present, there are still many details yet to be elucidated regarding exact mechanistic details. Interestingly, Sie systems also confer a benefit to the surrounding bacterial population, as the infecting phage is rendered non-infectious following ejection of their nucleic acid (Seed, 2015).

1.3.2.6.1.3 Restriction-modification

The host bacterium may encode a restriction-modification (R-M) system that destroys invading DNA. R-M systems are often considered primitive immune systems in bacteria and may be mobilized, spreading phage-resistance mechanisms to previously susceptible bacteria (Kommireddy & Nagaraja, 2013).

R-M systems classically comprise a restriction endonuclease (REase) and a cognate methyltransferase (MTase) (Seed, 2015). The MTase methylates self-DNA (i.e., host DNA) at specific sites, leaving foreign DNA unmodified; while the REase recognizes unmodified DNA and cleaves it into fragments. R-M systems are widely distributed across all members of bacteria and are classified into four types based on subunit composition, sequence recognition, cleavage position, cofactor requirements and substrate specificity (reviewed by Roberts et al., 2003).

Unsurprisingly, phages have also co-evolved along with their resistant hosts to overcome the effects of bacterial R-M systems; a common strategy, the incorporation of modified bases to resist classical R-M systems, has been described. For instance, the substitution of uracil replacing thymine in the phage genome, preventing recognition and cleavage of thymine-containing sites by REases, has been observed in *Bacillus* phages, T-even coliphages and Mu-like phages (Kruger et al., 1983; Warren et al., 1980).

1.3.2.6.1.4 Abortive infection and toxin-antitoxin systems

In contrast to the aforementioned host immunity mechanisms, bacteria may self-sacrifice by self-lysis and/or disruption of its cellular systems to prevent the spread of phage infection. Abortive infection (Abi) leads to injury or death of the infected cell in order to prevent the spread of phage and protect the surrounding clonal population from phage infection (Seed, 2015). Abi systems have been shown to be mobile and are commonly encoded on prophages and plasmids,

and may be accomplished through the disruption of essential cellular processes like translation, transcription and replication. Membrane leakage can also be induced. However, it is not currently understood how phage infection triggers this response in the host (Rostøl & Marraffini, 2019).

Abi systems are diverse and found in a variety of microorganisms, including *Salmonella* (Holguín et al., 2019) although of the Gram negative microbes, it has been very well-characterized in *E. coli* (Bingham et al., 2000). Specifically, upon phage T4 infection, the Lit and PrrC systems are activated in *E. coli* K12 and work to disrupt host translation, thereby halting translation of viral genes. In this system, it is known that the Lit system protease is activated by a peptide of the MCP in T4, a gene that is expressed late in the infective cycle (Bingham et al., 2000).

The toxin-antitoxin (TA) system serves to inhibit the infective cycle of the phage, though it is also involved in stress response, plasmid maintenance and persister cell formation (Rostøl & Marraffini, 2019). TA systems are widespread amongst bacteria and host bacteria may possess more than one TA system; for instance, *E. coli* K12 is predicted to possess at least 37 TA systems (Alawneh et al., 2015). TA systems classically function based on a complex of two proteins; the antitoxin blocks the toxin (usually an endoribonuclease) to prevent cell death, which represents the normal state under favorable environmental conditions. However, upon phage infection, the TA complex separates and the toxin is released, causing cell death and/or temporary growth arrest (Alawneh et al., 2015; Fineran et al., 2009; Holguín et al., 2019). Classically, ribonuclease activity in MazF of the MazF/MazE TA system of *E. coli* K12 is activated upon phage T4 infection, which blocks protein synthesis (Zhang et al., 2005). Phage T4 has been shown to carry an enzyme Alt (ADP-ribosyltransferase) which modifies and inhibits the

activity of MazF, thereby resisting inactivation (Alawneh et al., 2016). Other toxins in the TA systems of *E. coli* (e.g., LsoA, RnlA) have also been shown to be repressed by the *dmd* gene of phage T4 (Otsuka and Yonesaki, 2012).

1.3.2.6.1.5 Assembly interference

Assembly interference of phage progeny is a phage defense mechanism best characterized in *Staphylococcus aureus* (Penadés & Christie, 2015), although it was recently found to be widespread in Gram-negative bacteria (Fillol-Salom et al., 2018).

Phage-inducible chromosome islands (PICIs) are a family of phage-encoded, highly mobile genetic elements that in addition to host defense, also contribute to horizontal gene transfer, host adaptation and virulence (Penadés & Christie, 2015). Typically, PICIs encode for integration and excision genes, factors promoting PICI packaging and dissemination, and a repressor gene that inhibits their expression in the absence of "helper" phage. Additionally, PICIs also commonly encode virulence factors (Rostøl & Marraffini, 2019). These small (~15 kb) genomic islands will excise out of the chromosome and replicate upon infection with specific helper phages, which then disrupt the protein assembly and DNA packaging of helper phage virions. In this system, however, an important distinction is that immature helper phage capsids are filled with PICI DNA rather than phage DNA. The infected cell dies as a consequence of phage infection, but in this defense system, PICIs may be spread to the surrounding clonal population (Seed, 2015). Although the main role of PICIs appear to be the dissemination of genetic material (e.g., virulence genes), they are classified as an anti-phage mechanism due to the interference and disruption of the phage infective cycle (Rostøl & Marraffini, 2019).

1.3.2.6.2 CRISPR-Cas systems

Clustered regularly interspaced short palindromic repeats (CRISPR) systems are classified as adaptive immunity mechanisms rather than innate, as exposure to a previous phage infection has been "memorized" by the cell (Hille et al., 2018). CRISPR-Cas systems have been identified in approximately 50% of sequenced bacteria (Marakova et al., 2010). In an analysis of 606 genomes of *S. enterica* encompassing clinically relevant serovars Typhimurium, Enteritidis, Newport and Heidelberg, 129 unique CRISPR arrays were identified, with *S.* Typhimurium possessing the largest number of different arrays (Shariat et al., 2015).

The genomic loci of CRISPR elements are composed of alternating identical repeats and spacers of 30-40 bp in length (Ishino et al., 1987; Jansen et al., 2002). Importantly, the unique spacer sequences match those sequences found in phage genomes, which was the first indication that it might function as a phage defense system (Mojica et al., 2005). Indeed, invading foreign DNA (e.g., phages, plasmids) is integrated into the host CRISPR array, which thus serves as a memory archive of previous infections (Hille et al., 2018). Adjacent to the CRISPR array is a series of genes encoding Cas proteins. Specifically, these proteins assist with the three stages of adaptive immunity: adaptation, CRISPR RNA (crRNA) maturation and interference (Figure 1.5; Shabbir et al., 2016). In adaptation, the basis of bacterial "memory" is created, where the invading nucleic acid is processed and integrated into the spacer regions of CRISPR array. These "memories" can be retrieved with the CRISPR array, as it is transcribed to produce precursor crRNA (pre-crRNA) that is processed to yield mature crRNAs. Subsequently, the crRNA guides the interference machinery to cleave complementary sequences in the foreign nucleic acids, based on the identical spacer sequences (Hille et al., 2018). Because of the diversity in *cas* genes and interference complexes, the CRISPR-Cas systems have been assigned to two classes (Class 1 and 2) that are further delineated into six types and subtypes (Marakova

et al., 2015). Class 1 Cas-systems are the most widespread systems; and in *Salmonella*, both types of classes have been identified (Shariat et al., 2015).

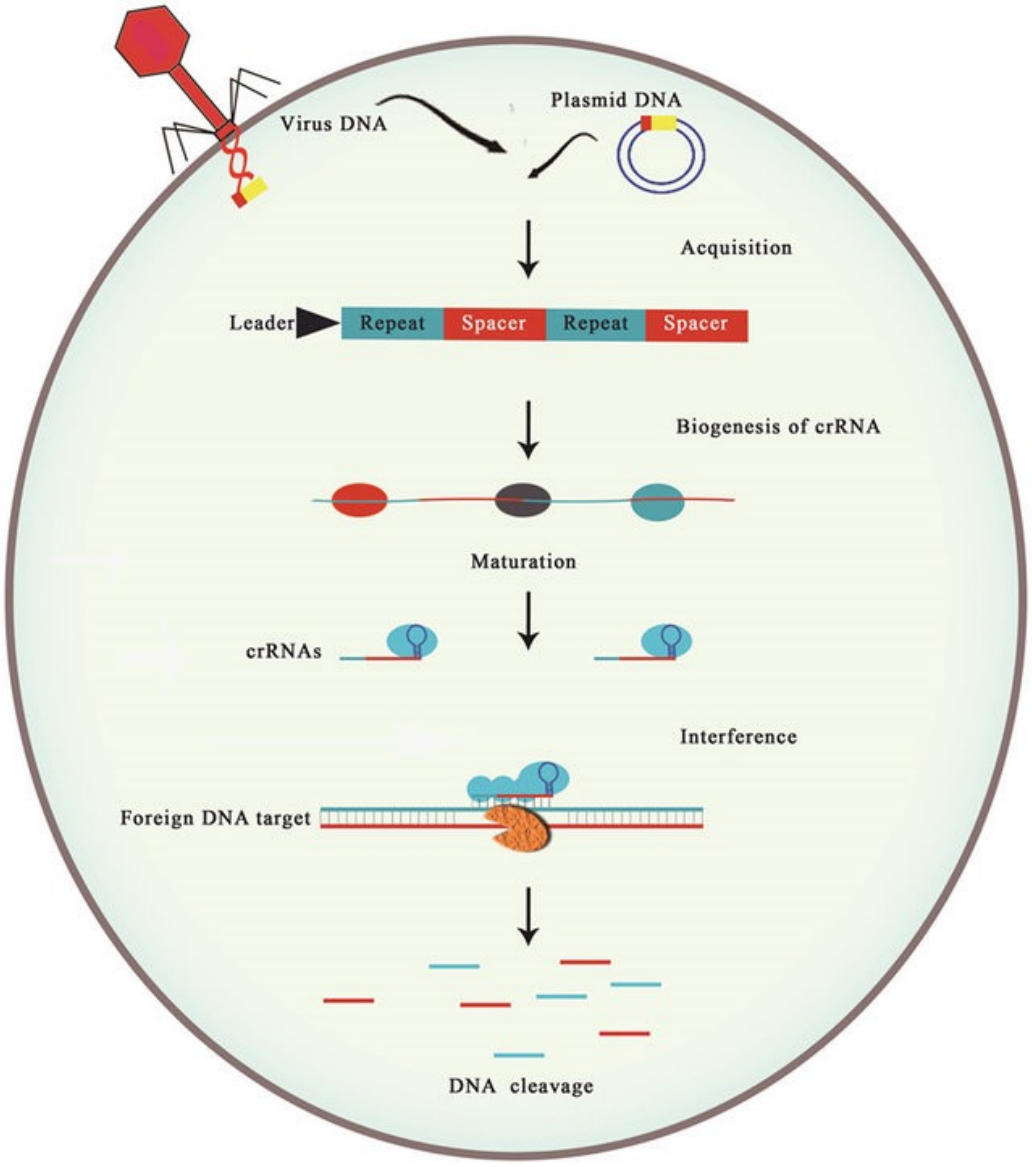

Figure 1.6. The three stages of CRISPR immunity. Image from Shabbir et al., 2016. License: CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

1.3.2.7 Altered host phenotype as a result of phage infection

Development of resistance to infecting phage has been associated with a variety of altered phenotypes in the host (Yang et al., 2019), especially since these induced mutations appear to be nonspontaneous (Léon & Bástias, 2015). Host bacteria have been shown to exhibit altered fitness, most notably growth rate, under phage predation (Ferández et al., 2017). As surviving bacteria have a direct impact on human health, the impacts of phage resistance on host antibiotic susceptibility and virulence will constitute the focus for this section.

Many surface-associated receptors in bacteria have dual roles in both phage attachment and antimicrobial resistance, thus the mutation of these receptors to confer phage resistance is concomitant with a modification in relative antibiotic susceptibility. Phages that attach to antibiotic efflux pumps may additionally select against the expression of the pump, leading to enhanced sensitivity. Chan et al. (2016) recently observed the enhanced susceptibility in multi-drug resistant *Pseudomonas aeruginosa* upon the development of phage resistance. Using a targeted strategy, phage OMKO1 was selected that adsorbed to OprM (outer membrane protein M) of the multidrug efflux systems MexAB and MexXY on the surface of the cell. This resulted in host bacterial selection against expression of OprM, which consequently resulted in decreased expression and function of the efflux systems, leading to enhanced sensitivity of this pathogen to ciprofloxacin, tetracycline, ceftazidime and erythromycin. Remarkably, a 45-fold change in sensitivity to erythromycin was observed (Chan et al., 2016). This phenomenon has also been observed elsewhere. For instance, phage-resistant *E. coli* displayed alterations in TolC, the site of phage attachment, and demonstrated hypersensitivity to novobiocin (German & Misra, 2001). Similarly, the effect of phage may be used to "prime" bacterial cells for antibiotic treatment; Oeschslin et al. (2017) observed that phage and ciprofloxacin delivered in combination to treat *P.*

aeruginosa in rats resulted in a 10,000-fold greater reduction in bacterial load compared to either treatment alone.

The impact of phage resistance on host virulence has also been investigated in detail, although the majority of studies have been conducted in bacteria with direct impacts in human medicine (e.g., *Pseudomonas aeruginosa*). In particular, phages that require virulence factors for attachment may select against the host expression of that particular factor. Phages utilizing LPS for adsorption may induce host resistance by selecting for mutations, such as point mutations and insertion/deletions (indels) in LPS biosynthesis genes, which have implications in human health as the LPS comprises the immunogenic component (Léon & Bastiás, 2015). Additionally, phages targeting bacterial adhesion sites such as adhesion proteins, pili or secretion systems may prevent bacterial attachment and invasion of epithelial cells (Bishop-Lilly et al., 2012; Choi et al., 2013; Kaper et al., 2004). More indirectly, impacts on the transcriptional activity of certain genes in phage-resistant bacteria have also been reported, particularly since the induction of intracellular phage resistance mechanisms interferes with normal cell functioning (Azam & Tanji, 2019). Yang et al. (2019) observed differential expression in a number of genomic loci in phage resistant *Acinetobacter baumanii*. Most notably, transcriptional activity was altered in loci corresponding to secretion systems and antibiotic resistance, with these genes being significantly upregulated (Yang et al., 2019). In a separate study by Fernández et al. (2017), genes related to virulence and adhesion were differentially expressed following low level phage predation of *Streptococcus aureus*; hemolysin genes *hld* were downregulated, whereas *icaA* was upregulated by three-fold. Additionally, transcriptional regulators (*sarZ, lytR, ctsR, vraR, rot, sarV, sigA, sigB*) were differentially expressed to various degrees, which could have profound impacts in all parts of the cell (Fernández et al., 2017). Comparatively, there has been marginal research on

altered host phenotype in phage-resistant *Salmonella*. Nevertheless, as the extent of these genetic trade-offs clearly differ between different host species and potentially different phages, the impacts in phage-resistant *Salmonella* requires further study.

Chapter 2: Investigation of phage-based phenotypic determinants for biocontrol of *Salmonella*

2.1 Introduction

Salmonella serovars have been associated with sprouts-related illnesses, including common serovars (e.g., *S*. Enteritidis, *S*. Typhimurium and *S*. 4,[5],12:i:-) and less common serovars (e.g., *S*. Agona, *S*. Reading, and *S*. Abony) (CDC, 2019). Contamination of seeds used for sprouts production is likely to occur in the field (Food and Drug Administration (FDA), 2015). Once contaminated, sprouting seeds provide an ideal habitat for support and growth of *Salmonella* (Fahey et al., 2006). During the sprouting process, enhanced humidity, warm temperatures, and the release of nutrients from the seed itself, result in the rapid proliferation of *Salmonella* (Fahey et al., 2006); an increase of over 3 log CFU/g of viable *Salmonella* during the sprouting of alfalfa seeds has been reported (Charkoswski et al., 2002). A variety of intervention strategies are available to disinfect sprouts seeds, including the usage of chemical disinfectants (e.g., hypochlorite, calcium hydrogen peroxide), heat, and irradiation (NACMF, 1999). The reliability and consistent implementation of these treatments, however, has been questioned as sprouts-related illnesses continue to rise. In Canada, seed sanitation is not required by law (Canadian Food Inspection Agency (CFIA), 2014), although it is recommended that a seed treatment capable of attaining a minimum three log reduction be considered (CFIA, 2014). However, negative consumer perceptions regarding chemical and physical treatments and its potentially negative impacts on the yield and quality of sprouts may hinder the widespread adoption of such treatments (Kim et al., 2003). Additionally, the efficacy of these treatments has been shown to be highly variable (Montville and Schaffner, 2004). For instance, the reference standard for seed disinfection, 20,000 ppm calcium hypochlorite, has resulted in variable

microbial reductions of 0.51 – 6.90 log CFU/g (Ding et al., 2013). Disinfection with alternative chemicals is also highly variable regarding their microbial kill; electrolyzed oxidizing water previously achieved reductions of 1.66 log CFU/g (Kim et al., 2003), while 5% acetic acid achieved reductions of 2.40 log CFU/g (Lang et al., 2000). Lastly, physical inactivation methods are gaining interest; soaking seeds in hot water at 85°C for 10 s was reported to achieve a 3.0 log CFU/g reduction (Bari et al., 2009). High pressure for 500 MPa for two min similarly achieved a 3.5 log CFU/g reduction (Neetoo and Chen, 2010). However, physical treatments have been shown to inhibit the germination rate and may not be commercially viable methods for disinfection (Bari et al., 2009). Therefore, alternative measures are urgently needed for effective, clean-label decontamination methods, without negatively impacting seed viability.

Bacteriophages (phages) are viral predators of bacteria (Hagens and Loessner, 2007) that have attracted considerable interest as a method for pathogen control on foods. Previous research has evaluated its use on a variety of foodstuffs, including chicken skin (Goode et al., 2003), broccoli and mustard sprout seeds (Pao et al., 2004), fresh-cut produce (Leverentz et al., 2001) and cheddar cheese (Modi et al., 2001). Phages possess several properties that render them suitable for use on food because they are: (i) highly specific, not crossing species or genus barriers; (ii) designed to kill host cells only; (iii) self- replicating and self-limiting; and (iv) ubiquitously distributed in nature (Rohwer and Edwards, 2002). Despite the range of desirable attributes, however, the usage of phages as biocontrol agents for *Salmonella* in sprouts has not been widely adopted, although there have been previous reports of similar efforts (Pao et al., 2004; Kocharunchitt et al., 2009). At an initial density of approximately seven log CFU/g of *S.* Oranienburg, relatively low log reductions of approximately one log CFU/g of *Salmonella* were achieved with phage SSP6 (Kocharunchitt et al., 2009). Additionally, Pao et al. (2004) reported a

1.50 log CFU/g reduction of *Salmonella* upon application of a bacteriophage cocktail on broccoli seeds artificially contaminated with an initial density of 7–7.5 log CFU/g. The relatively low reduced efficacies may have been due to the limited number of effective phages recovered, and/or the failure to adequately characterize phages for this particular purpose. The objectives of this study were to assess a panel of newly isolated *Salmonella* phages from the BC environment on their host ranges, and their infectivity against various *Salmonella* strains *in vitro* and on alfalfa seeds throughout the sprouting process.

2.2 Methods

2.2.1 Bacterial strains and growth conditions

Salmonella strains (n=61) were obtained from various sources, including the International Life Sciences Institute, the *Salmonella* foodborne syst-OMICS database, or were isolated from the Lower Mainland of British Columbia (Table 2.1). Strains were maintained at -80°C in Brain-Heart-Infusion broth (BD/Difco, East Rutherford, NJ, United States) supplemented with 20% glycerol. Working stocks were prepared and maintained on tryptic soy agar (TSA; BD/Difco) at 4°C for a maximum of one month. Fresh overnight liquid cultures were prepared prior to each experiment by inoculating an isolated colony into 10 ml tryptic soy broth (TSB; BD/Difco). Cultures were incubated for 16 h at 37°C with shaking at 170 rpm.

2.2.2 Phage isolation and purification

Bacteriophages were isolated from beach sand (B), poultry environments (housing & feces) (C), sediment (S), cattle feces (F), sewage influent and effluent (E), irrigation water (I), and water tanks in an aquaculture facility (W) in British Columbia, Canada, and as specified in the phage name (Figure 2.1 and Table A1).

Sample enrichment, phage isolation and purification were carried out as described elsewhere (Fong et al., 2017). Briefly, an effort was made to enrich the abundance of phage by mixing 10 g of sample, 90 ml TSB and one ml of a cocktail of seven indicator strains of *Salmonella* (Table 2.1) that were grown for 16 h as described above. Serovars represented by these strains include high-risk types important to food safety and have exhibited high prevalence in human cases of salmonellosis (CDC, 2019; Andino & Hanning, 2014; Ellis et al., 1998). Suspensions were then incubated at 37°C for 22 ± 2 h. The phage-enriched samples were spun at 4,000 x *g* to remove bacteria, and the supernatant passed through a 0.45-µm pore-size polyethersulfone filter membrane (Pall Corporation, Port Washington, NY, United States). Afterwards, 100 µl of filtrate was mixed with 300 µl of each of the indicator *Salmonella* strains (diluted 10-fold after growing for 16 h) and four ml of 0.7% TSA top agar, according to the double-agar overlay method (Adams, 1959). Plates were then incubated at 37°C for 18 ± 2 h for plaque visualization. Plaques were lifted from the agar surface using a truncated sterile pipette tip and re-suspended in 200 µl salt-magnesium (SM) buffer (0.05 M Tris-HCl; 0.1 M NaCl and 0.01 M $MgSO_4$; adjusted to pH 7.5). Suspensions were allowed to rest for at least six h at room temperature. Double agar overlays were then prepared with the suspension as described previously (Adams, 1959). A minimum of three single plaque isolations was performed in series to obtain a clonal phage isolate. Phages were subsequently concentrated by centrifugation and stored at 4°C until further analyses.

2.2.3 Phage host range determination

Prior to host range determination, phage lysates were standardized to a concentration of 10^9 PFU/ml as recommended by Khan Mirzaei and Nilsson (2015). The host ranges of the phages were tested by spotting five µl of lysate, in duplicate, on a lawn of *Salmonella* host cells

grown to 16 h (n = 62, Figure 2.1). Felix-O1 was included in the analysis as a reference as it infects 98.2% of all *Salmonella* strains (Welkos et al., 1974). Plates were incubated at 37◦C for 18 ± 2 h. Zones of clearing were characterized with a scaling system as described by Kutter (2009), where 0 indicated a zone with complete turbidity (no lysis) and +4 indicated a completely clear zone with no turbidity. These values were translated to a heat map (Figure 2.1).

Table 2.1. *Salmonella* strains used for phage isolation and host range determination. Strains indicated with an asterisk (*) indicate its inclusion in the cocktail for phage isolation.

Salmonella strain	Source of isolation
**S*. Enteritidis FSL S5-483[a]	Human
S. Thompson FSL S5-523[a]	Human
**S*. Braenderup FSL S5-373[a]	Human
**S*. Muenchen FSL S5-504[a]	Human
**S*. Montevideo FSL S5-630[a]	Bovine
**S*. Saintpaul FSL S5-649[a]	Human
S. Typhimurium JF-LMFS-001[b]	Irrigation water
**S*. Typhimurium FSL S5-536[a]	Human
S. Javiana FSL S5-406[a]	Human
S. Senftenberg FSL S5-658[a]	Human
S. Mbandaka FSL S5-451[a]	Human
S. Agona FSL S5-517[a]	Human
S. Newport FSL S5-436[a]	Bovine
S. Newport S2[c]	Human
S. Newport S195[c]	Alfalfa seeds
S. Enteritidis S3[c]	Human
S. Enteritidis S187[c]	Leafy greens
S. Canada S30[c]	Chocolate
S. Chingola S32[c]	Seaweed
S. Luciana S43[c]	Cantaloupe

S. Typhimurium S189[c]	Chocolate
S. Heidelberg S191[c]	Cocoa beans
**S*. Thompson S193[c]	Spinach
S. Thompson S194[c]	Feather meal
S. Infantis S198[c]	Pasta
S. Javiana S200[c]	Human
S. Javiana S203[c]	Octopus
S. Saintpaul S204[c]	Chia seeds
S. Saintpaul S205[c]	Shrimp
S. Muenchen S206[c]	Cantaloupe
S. Muenchen S207[c]	Orange juice
S. Agona S213[c]	Mussels
S. Agona S215[c]	Chill tank
S. Oranienburg S216[c]	Canine
S. Hadar S219[c]	Chicken rinse
S. Mbandaka S236[c]	Poultry
S. Mbandaka S238[c]	Pasta
S. Montevideo S239[c]	Thyme
S. Montevideo S241[c]	Poultry
S. Bareilly S258[c]	Human
S. Senftenberg S269[c]	Alfalfa seeds
S. Senftenberg S270[c]	Processed cheese
S. Litchfield S272[c]	Human
S. Litchfield S273[c]	Shrimp
S. Uganda S276[c]	Hog carcass
S. Uganda S277[c]	Ground beef
S. Havana S286[c]	Human
S. Poona S306[c]	Roast beef
S. Poona S307[c]	Cantaloupe
S. Ohio S316[c]	Goat
S. Berta S333[c]	Cheese
S. Liverpool S346[c]	Cantaloupe

S. Rubislaw S348	Horse
S. Typhimurium S441[c]	Environmental
S. Anatum S443[c]	Environmental
S. Typhimurium LT2[d]	Unspecified
4, 5, 12:I:- FSL S5-580[a,e]	Bovine
S. Typhimurium 14028S 1-5[e]	Unspecified
S. Typhimurium var. Copenhagen FSL S5-786[a, f]	Bovine
S. Typhimurium SL1344[g]	Unspecified
S. Schwarzengrund FSL S5-458[a, h]	Human

[a] ILSI North America Collection; [b] Strain isolated from irrigation water in the Lower Mainland, British Columbia, Canada; [c] The *Salmonella* Foodborne Syst-OMICS database (SalFoS) collection; [d] Strain resistant to STR (Lilleengen, 1950); [e] Strain resistant to AMC, AMP, FOX, CHL, STR, SUF, TET; [f] Strain resistant to AMC, AMP, FOX, CRO, CHL, KAN, STR, SUF, TET; [g] Strain resistant to STR (Hoiseth & Stocker, 1981); [h] Strain resistant to AMP, CIP, NAL, SUF, TET, SXT.

2.2.4 Transmission electron microscopy

For selected phages, high titer phage lysates (10^9– 10^{11} PFU/ml) were chosen for transmission electron microscopy (TEM) and prepared for imaging as described previously (Deveau et al., 2006), with modifications. Briefly, one ml of lysate was spun at 4°C for 1.5 h at 21,000 × *g*. The supernatant was subsequently discarded and the last 100 µl were saved. Consequently, one ml of 0.1 M ammonium acetate (Amresco, Solon, OH, United States) was added and the suspension subsequently spun again at 4°C for 1.5 h at 21,000 × *g*. This purification was repeated twice, with the last 100 µl reserved for TEM. For grid preparation, three µl of purified lysates were placed on carbon coated copper grids (Ted Pella, Redding, CA, United States) following glow-discharge. The phage preparations were subsequently negatively stained with 2% phosphotungstic acid (Ted Pella). A Hitachi H-7600 transmission electron microscope was used for acquiring the images at the University of British Columbia Bioimaging Facility. An accelerating voltage of 80 kV was used for imaging.

2.2.5 Determination of phage genome content and restriction enzyme analysis

Prior to nucleic acid extraction, DNase I (Invitrogen, Carlsbad, CA, United States) and RNAse A (Invitrogen) were added to high titer phage lysates (10^9 – 10^{11} PFU/ml) to final concentrations of 10 and 55 µg/ml, respectively, for degradation of host nucleic acid (Merabishvili et al., 2014), followed by incubation at 37°C for 30 min. Phage nucleic acid was then extracted with the PureLink Viral RNA/DNA Mini Kit (Thermo Fisher) as per the manufacturer's instructions. The concentration and quality of the extracted nucleic acid was determined with a Nanodrop spectrophotometer (Thermo Fisher), where an A260/280 ratio of ~1.8 and A260/230 ratio of ~2.0 were considered as pure. Restriction enzyme analysis was conducted to confirm the unique identities of the phages. Nucleic acid was digested with *EcoR*1 (New England Biolabs, Ipswich, MA, United States) according to the manufacturer's instructions. Subsequently, 10 µl volumes of the nucleic acid digests were loaded onto a 1% agarose gel (Amresco) and electrophoresed in 1X Tris-acetate-EDTA (TAE) buffer (Thermo Fisher) at 80 V for approximately one h. Band patterns were visualized using the ChemiDoc MP System (Bio-Rad Laboratories).

2.2.6 Single step growth curves

Single step growth curves were constructed to determine the phage burst sizes and burst times, according to Park et al. (2012), with modifications. Cultures of *S.* Enteritidis were grown for 16 h in TSB (37°C, 170 rpm). One ml of culture was then added to 9 ml of fresh TSB and incubated at 37°C at 170 rpm until an optical density at 600 nm (OD_{600}) of 1.0 (~10^9 CFU/ml; stationary phase) was attained. Phages were then individually added at MOI of 0.01 and allowed to adsorb for five min at room temperature. To remove excess phage particles, the co-culture was spun at 4,000 × *g* at 4°C and the supernatant discarded. The pellets were resuspended in 10 ml of

fresh TSB and incubated at room temperature with gentle agitation. Subsequently, 50 µl aliquots were collected every five min for a total duration of 60 min, immediately serially diluted in SM buffer, and spotted in duplicate on a host agar lawn of *S*. Enteritidis grown to 16 h for titer determination. Plates were incubated at 37°C for 18 ± 2 h for visualization of plaques. This experiment was independently conducted three times for each phage. To calculate the burst sizes of the phages, the initial phage titer was subtracted from the final phage titer.

2.2.7 Lysogeny analysis

Resistant colonies of *S.* Enteritidis in the centers of spot assays (n = five colonies per phage) were selected to test for lysogeny. First, isolated colonies were serially re-streaked five times on TSA to reduce phage carry-over. On the fifth streak, a random colony was selected for PCR to confirm its *Salmonella* identity by using primers specific to *invA*, according to Fong and Wang (2016). Briefly, a single colony was suspended in 200 µl of sterile de-ionized water and lysed in a microwave for two min. PCR detection was then carried out with the TopTaqMaster Mix Kit (Qiagen, Valencia, CA, United States) with primers specific for the *invA* gene (forward: 5'-TCA TGG CAC CGT CAA AGG AAC C-3' and reverse: 5'-GTG AAA TTA TCG CCA CGT TCG GGC AA-3') (Li et al., 2012). PCR cycling conditions were as follows: initial denaturation (three min, 94°C); three-step cycling, including denaturation (30 s, 94°C), annealing (30 s, 56°C), and extension (one min, 72°C); followed by a final extension (10 min, 72°C). Sizes of the PCR products were confirmed with electrophoresis on 2% agarose (Amresco, Solon, OH, United States) with 1X TAE buffer (Thermo Fisher, Waltham, MA, United States). PCR products were visualized using the ChemiDoc MP System (Bio- Rad Laboratories, Hercules, CA, United States). Colonies arising from the fifth streak were cultured to test for phage lysogeny as previously described (Petty et al., 2006). Colonies (n = 10) were suspended in 10 ml

TSB and incubated at 37°C at 170 rpm for 20 h. Cultures were subsequently spun at 4,000 × *g* at 4°C to sediment the bacteria, and the supernatant tested for the spontaneous release of phage particles by spotting 5 µl in duplicate onto prepared *S*. Enteritidis agar overlays. Plates were incubated at 37°C for 18 ± 2 h for visualization of plaques. Supernatants from *S*. Enteritidis infection by Felix-O1, a strictly lytic phage, were used as a negative control.

2.2.8 Temperature and pH stability assay

Phage lysates were diluted in TSB to an initial concentration of ~10^7 PFU/ml and subsequently stored at a range of temperatures (−20, 4, 22, and 37°C) for determination of relative temperature stabilities. Controls (no phage) were included for each temperature. The samples stored at −20°C were prepared in single-use 15 µl aliquots to prevent multiple thawing and freezing events throughout the assay. Temperature stability experiments were conducted three times for each phage. To test pH stability of the phages, phage lysates were diluted to ~10^8 PFU/ml in TSB at varying pH ranges of pH 4.0, 6.0, 8.0, and 10.0 (adjusted with 6 M HCl or 6 M NaOH) and subsequently stored at room temperature for further analyses. Blank controls (no phage) were included for each pH. These pH and temperature ranges were chosen based on previously reported similar assessments (Thung et al., 2017) and reflect the various pH and temperature conditions encountered in produce production chains (Park et al., 2012; Rombouts et al., 2016). pH stability experiments were conducted three times for each phage. Phage titers were assessed on days 2, 4, 8, 10, 14, 16, 20, 25, and 30. Briefly, 10 µl volumes were serially diluted in SM buffer and five µl spotted in duplicate on prepared top agar of *S*. Enteritidis grown to 16 h. Enumeration of plaques was determined after incubation at 37°C for 18 ± 2 h.

2.2.9 Spectrophotometric analysis of phage lysis efficacy

Cultures of *S*. Enteritidis FSL S5-483, *S*. Agona FSL S5-513 and *S*. Typhimurium LMFS-JF-001 were prepared as described previously in section 2.2.1. *S*. Typhimurium LMFS-JF-001 was included in this analysis as it was originally isolated from irrigation water within the Lower Mainland of BC. Following incubation, cultures were spun at 4,000 × *g* and the cell pellets washed three times with fresh TSB. Then, the cultures were loaded into 96-well plates to a final concentration of 5×10^4 CFU/ml and infected with phages SI1, SF1, SS1, and SS4 at MOIs of 1, 10, and 100 PFU/CFU. Plates were placed into a plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, United States) set to 25°C for determination of cell density at OD_{600} every 30 min for 36 h. Each experiment was independently conducted three times.

2.2.10. Phage SI1 biocontrol of *Salmonella* on sprouting alfalfa seeds

The lysate of phage SI1 was tested for its efficacy to control *S*. Enteritidis on germinating alfalfa seed over six days. Six days was chosen as approximately 5–7 days are required for alfalfa seeds to sprout (Kramer and Lim, 2004). Cultures of *S*. Enteritidis grown to 16 h were spun at 4,000 × *g* for 10 min, washed three times with sterile potable water and serially diluted to a final volume of 35 ml of sterile water. The seed was inoculated by drop-wise addition of 15 ml diluted culture to 150 g seed to achieve an initial concentration of approximately log 3.5 CFU/g. Blank controls were processed similarly, but with sterile water only. The seed was placed in a biological safety cabinet at room temperature under continuous air flow for two h. Finally, the seed was transferred to sterile plastic boxes lined with a layer of sterile gauze pad and stored in the dark in a 22°C incubator. Three independent replicate experiments were performed. The lysate of phage SI1 was applied to the seed at 22 h post-inoculation. Briefly, 75 g of inoculated seed was aseptically removed and treated with phage SI1 in 35 ml sterile water to yield an MOI

of approximately 110 PFU/CFU. The seeds were soaked for two h at room temperature with gentle agitation by shaking at 175 rpm. Inoculated seed and controls that received only sterile water were processed in tandem. A two h phage soak was chosen due to (i) the short latent period (25 min) and relatively high burst size (83 phages) of SI1, thereby facilitating approximately four cycles of productive infection; and (ii) the simulation of a logistically feasible decontamination step, performed within a time frame that could be adopted into commercial sprout production practices. Following the soak, the excess fluid was removed by straining through sterile filter paper. Seed samples were then aseptically transferred to sterile plastic boxes lined with a layer of sterile gauze pad. Treated seed was stored in the dark in a 22°C incubator.

The germinating seed was moistened with seven ml of sterile water every 24 h over 6 days. Simultaneously, 10 g of seeds were removed daily and mixed with 100 ml of sterile phosphate buffered saline (PBS; Amresco) in a sterile Whirlpak bag (Nasco, Fort Atkinson, WI, United States). The samples were placed in a Stomacher (Seward, Worthing, West Sussex, United Kingdom) and homogenized for two min at 230 rpm. Subsequently, 100 µl aliquots were serially diluted in PBS and spread over xylose-lysine deoxycholate (XLD; Amresco) agar in duplicate. XLD plates were incubated at 37°C for 22 ± 2 h for enumeration of *Salmonella* (red colonies with black centers). Phage titers were measured by spotting 5 µl in duplicate on TSA seeded with *S*. Enteritidis grown to 16 h. Plates were incubated at 37°C for 18 ± 2 h for enumeration of plaques. To assess the impact of the phage treatment on the final sprout yield, 150 g of seed was artificially contaminated with *S*. Enteritidis and 75 g was withdrawn for phage treatment, as per the procedures described previously. Sprouting seeds were then weighed after 6

days. Three independent replicate experiments were performed, with two technical replicates taken for each measurement.

2.2.11 Statistical analysis

For the pH and heat stability assays, the final titer of the phages after 30 days of treatment was compared to the initial titer at the beginning of treatment with a Student's t-test ($\alpha = 0.05$). To compare the relative susceptibilities of the phages to each treatment, the log decreases in phage titer after 30 days of treatment were calculated (i.e., the difference in log PFU/ml at time zero and after 30 days of treatment). A one-way analysis of variance (ANOVA) was then implemented with a Tukey's Honest Significant Difference post hoc test applied to all significant ANOVA results ($\alpha = 0.05$).

For the sprouts biocontrol assay, the log differences in *Salmonella* counts at each sampling time point between untreated and treated alfalfa seed samples were analyzed with a Student's t-test with a significance level of $\alpha = 0.05$. The differences in weights between the control and treated alfalfa sprouts were also assessed using a Student's t-test ($\alpha = 0.05$).

All statistical analyses were performed using JMP version 11.1.1 (SAS Institute, Inc., Cary, NC, United States). A P-value of ≤ 0.05 was considered statistically significant for all analyses.

2.3 Results & discussion

2.3.1 Isolation of *Salmonella* phages

Overall, 97 *Salmonella* phages were isolated from various environments in BC and exhibited a variety of plaque morphologies. The phages that were isolated were capable of infecting all hosts used in the indicator cocktail (Table A1).

2.3.2 Host ranges of phages

Only 49 phages were included in the host range analysis as the other 48 phages did not reach a titer of 10^9 PFU/ml required for host range analysis with repeated propagation (Fong et al., 2017), suggesting a non-*Salmonella* host or a host that was not available in our collection of strains. Using high titer phages (10^9 PFU/ml), we screened these phage isolates against a panel of 62 *Salmonella* strains to determine their host ranges (Figure 2.1), as lysis of a broad range of *Salmonella* strains is critical for biocontrol applications (Goodridge et al., 2018). Ideally, phage cocktails are formulated from several phages with broad host ranges (Hagens & Loessner, 2007).

The phages exhibited a variety of host ranges (Figure 2.1). Some isolates (e.g., SE21, SE10, SI23) had relatively narrow host ranges, infecting 10, 15 and 16 *Salmonella* strains, respectively; others (e.g., SE13, SE7, SE20) were broader and infected 51, 38, and 35 strains, respectively. Felix-O1 had the broadest host range, infecting 54 of 61 tested strains. Originally isolated in England, Felix-O1 is a virulent phage that infects 98.2% of all *Salmonella* strains and is commonly used in diagnostics and typing (Welkos et al., 1974).

Strains representing serovars Enteritidis and Typhimurium were lysed by most of the phages (Figure 2.1), which is important considering the global association of these serovars with salmonellosis (Andino & Hanning, 2014; CDC, 2019). Further, all of the antibiotic-resistant strains were susceptible to at least one phage, which is noteworthy given the need for new strategies to tackle antibiotic resistance. One *Salmonella* strain, *S*. Rubislaw S348 could not be lysed by the newly isolated phages, which may be due to the lack of a suitable receptor, or other phage-resistance mechanisms.

Some groups of phages exhibited unique host ranges. Phages isolated from irrigation water displayed comparatively limited host ranges, which suggests that the available host

spectrum is narrower than in other sampled environments. For example, phages from sewage exhibited broader host ranges (Figure 2.1), especially SE13, which exhibited the broadest host range of the newly isolated phages. This is consistent with sewage containing an abundance of potential hosts and phages (Parmar et al., 2018). However, phage abundance tends to oscillate with host abundance. The "kill the winner" hypothesis posits that the host that grows most quickly (i.e., the "winner") will be the most susceptible to phage infection, leading to greater diversity of hosts and phage. Phages with broader host range are better adapted to survive environments where host diversity is high, and the abundance of any given genotype is relatively low, or where environmental conditions and bacterial populations change often, for example sewage (Knowles et al., 2016). Sewage water and sludges are good sources of phages with broad host ranges, as are some non-sewage sources (Parmar et al., 2018).

Phages SI1, SF1, SS1 and SS4 were selected for further analysis for several reasons. They exhibited broad host ranges; of the 62 *Salmonella* strains tested, 24 were susceptible to infection by SI1, 23 were susceptible to SS4, 21 were susceptible to SF1 and 20 to SS1, as indicated by a +3 or +4 clearing (Figure 2.1). The similarity between host ranges indicate that these four phages may recognize similar host receptors (Kalatzis et al., 2016). Importantly, *S.* Typhimurium and *S.* Enteritidis, responsible for causing the highest proportions of foodborne salmonellosis (Andino & Hanning, 2015; CDC, 2019), and several strains of AMR *Salmonella* demonstrated susceptibility to these phages, whereas SE13 did not infect *S.* Typhimurium FSL S5-536 included in our study.

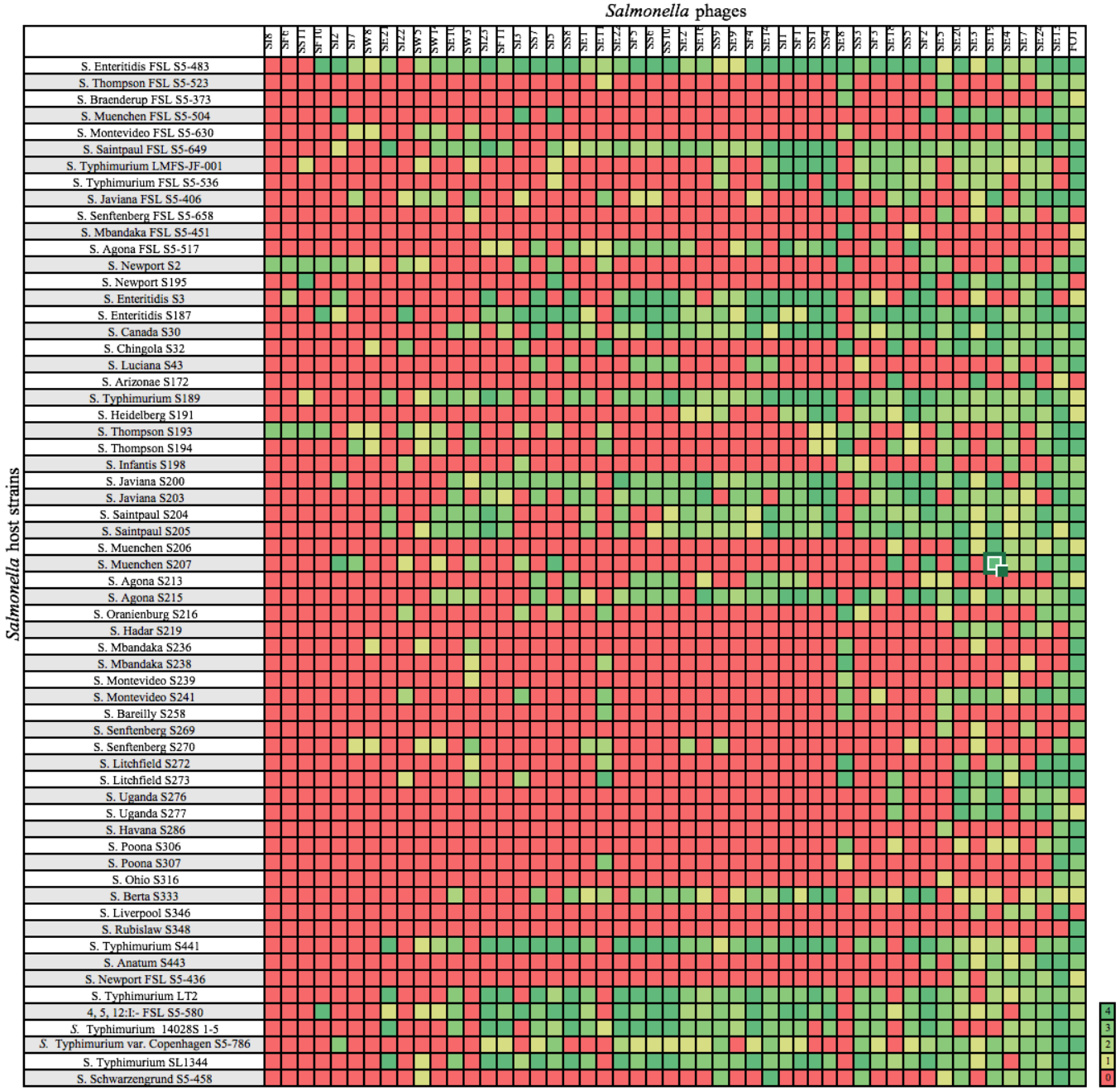

Figure 2.1. Host ranges of newly isolated phages (n=49) against *Salmonella* host strains (n=62). *Salmonella* strains susceptible to phage infection are indicated by a clearing of 1 to 4; 0 = no lysis.

2.3.3 General characterization

2.3.3.1 Phage TEM, burst size, genotyping & lysogeny

SI1, SF1, SS1, and SS4 formed clear plaques on their host, *S.* Enteritidis, although they differed in size. SI1 and SS1 formed 1 mm clear plaques, but SS1 plaques also possessed slightly turbid haloes. Similarly, SS4 and SF1 formed larger plaques of 1.5 mm diameter, but SS4 plaques possessed slightly turbid haloes. It is suggested that halo formation is the result of endolysin secretion upon lysis of host cells (Cornelissen et al., 2012). Structural examination with TEM revealed their distinct morphologies, with all four phages belonging to the family *Siphoviridae* (Figure 2.2), consisting of rigid, non-contractile tails and double stranded DNA. SI1 is 207 ± 5 nm in length with a spherical head and a small appendage structure located at the crown. SF1 is 215 ± 3 nm long, possessing an icosahedral-shaped head. SS1 and SS4 exhibited structural similarity; both with spherical heads and 200 ± 2 nm and 20 ± 5 nm in length, respectively.

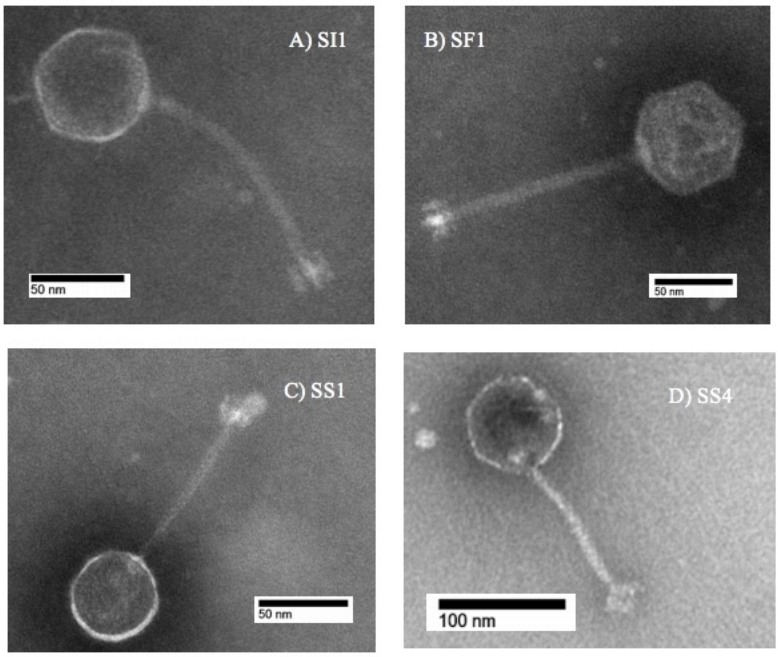

Figure 2.2. Transmission electron microscope images of (A) SI1, (B) SF1, (C) SS1 and (D) SS4.

Subsequent typing of SI1, SF1, SS1, and SS4 was accomplished by restriction enzyme analysis. Digestion with *EcoR*1 yielded four distinct banding patterns, confirming the unique identities of these phages (Figure 2.3). It was found that SI1 has an approximate genome size of 87,000 bp, SF1 with a genome size of 80,800 bp, and SS1 and SS4 with substantially smaller genome sizes of 44,150 and 65,000 bp, respectively.

Some patterns only differed by a few bands, suggesting the conservation of *EcoR*1-specific cutting sites and a familial relationship between the characterized phages. Although Siphoviruses are known to exhibit remarkable mosaicism (Santander et al., 2017), it is expected that several components would exhibit notable similarity as these phages belong to the same family and present similar, though not identical, host ranges. Indeed, *Salmonella* phages fSE1C and fSE4C previously isolated from pickle sauce and ground beef, respectively, were digested with *EcoR*I, *Hind*III, and *Hae*III restriction enzymes and showed very similar banding patterns (Santander et al., 2017). Further analysis revealed a similarity of 43.09% between the genomes, with genes involved in structure, replication, host specificity and DNA metabolism showing remarkable conservation (Santander et al., 2017).

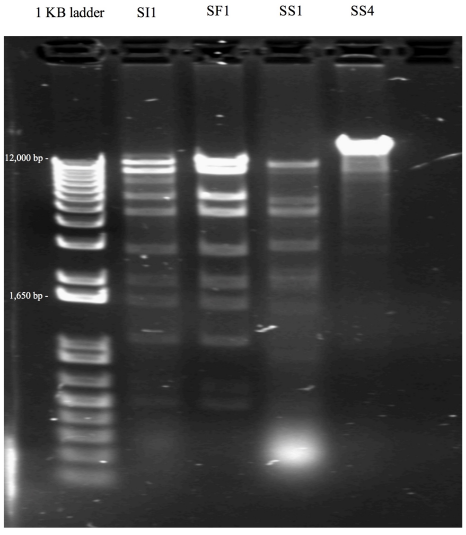

Figure 2.3. Restriction banding patterns of phage upon digestion by endonuclease *EcoR*I.

2.3.3.2 Single step growth curves

Single step growth curves were constructed to determine the infection potential of each phage (Figure 2.4). SI1, SF1, and SS1 possess latency periods of 25 min while SS4 possesses a latency of 30 min. The burst size of SI1 is 83 phages per infected cell, whereas the burst sizes of SF1, SS1, and SS4 are 45, 20, and 31 phages per infected cell. These phage infection parameters are in the range of those observed for *Siphoviridae* phages (Carey-Smith et al., 2006; Silva et al., 2014; Pereira et al., 2016b). For phage therapy in the food industry, it is often desirable to possess short latent periods and high burst sizes (Kalatzis et al., 2016), therefore the infection parameters outlined here demonstrate the potential of the characterized phages, particularly SI1, for use in biocontrol efforts.

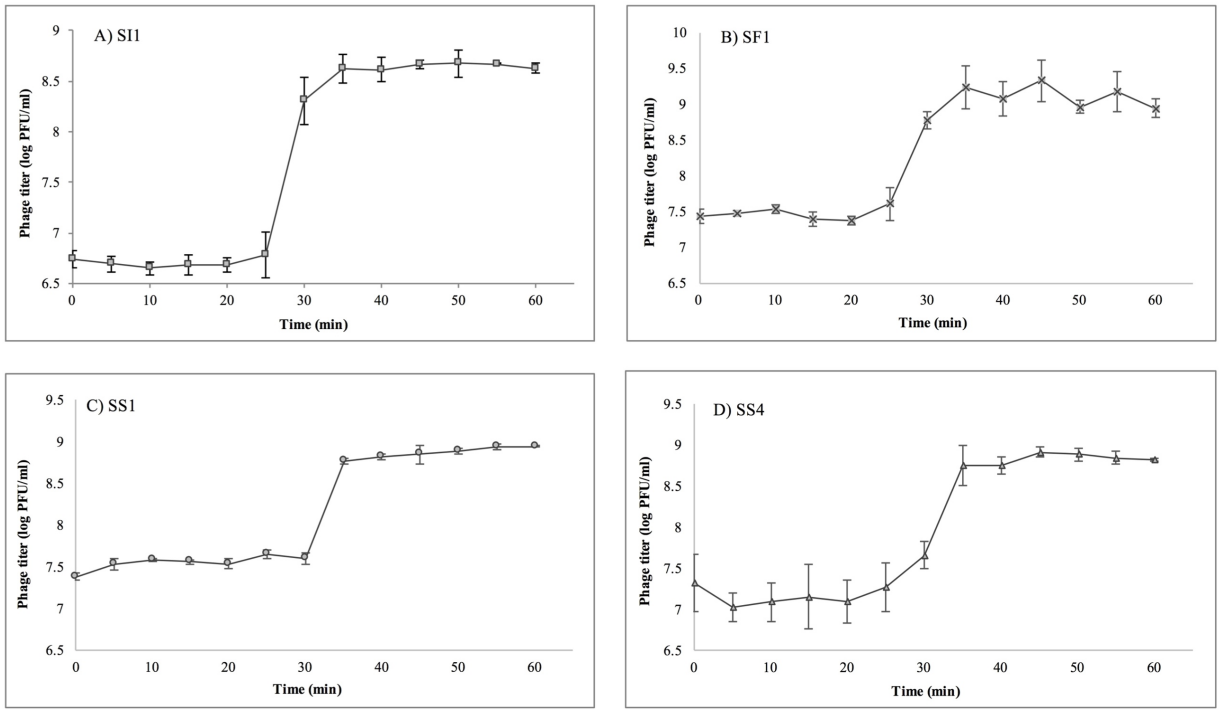

Figure 2.4. Single step growth curves of (A) SI1, (B) SF1, (C) SS1 and (D) SS4. Data shown are the mean of three replicates ± SD.

Phages were tested for harborage of lysogenic elements by culturing phage-resistant colonies of *S.* Enteritidis and testing the supernatant for spontaneous release of phage particles. Absence of lysogenic integration into the host genome is a pre-requisite for phage biocontrol of food (Levin and Bull, 2004). No phage particles were detected upon spot-testing on overlays seeded with *S.* Enteritidis, indicating a strictly lytic life cycle and their suitability for use in phage therapy (Rombouts et al., 2016). Additionally, the production of clear plaques further confirmed their lytic life cycle.

2.3.3.3 pH and temperature stability

At pH = 4, SF1, SS1, and SS4 were reduced to undetectable concentrations (<200 PFU/ml) by day 20 (Figure 2.5). SI1 was reduced to less than 200 PFU/ml by day 30. From day 6 onward, SS1 showed a more rapid decline than SF1 and SS4. These results suggest that of the four phages, SS1 would be the least stable biocontrol agent in acidic conditions, whereas SI1 would be the most stable.

In conditions ranging from pH = 6 – 10, no phage titer decreased by more than 0.544 ± 0.067 log PFU/ml, and only in one instance was titer (SS4 at pH 10) significantly ($p<0.05$) reduced. On average, stability increased as the pH increased from pH 4 to pH 10, but there was some variability between phages. SI1 and SF1 were most stable at pH 10, but SS1 and SS4 were most stable at pH 8. SI1 was significantly ($p<0.05$) less stable than each of SS1, SF1, and SS4 at pH = 8.

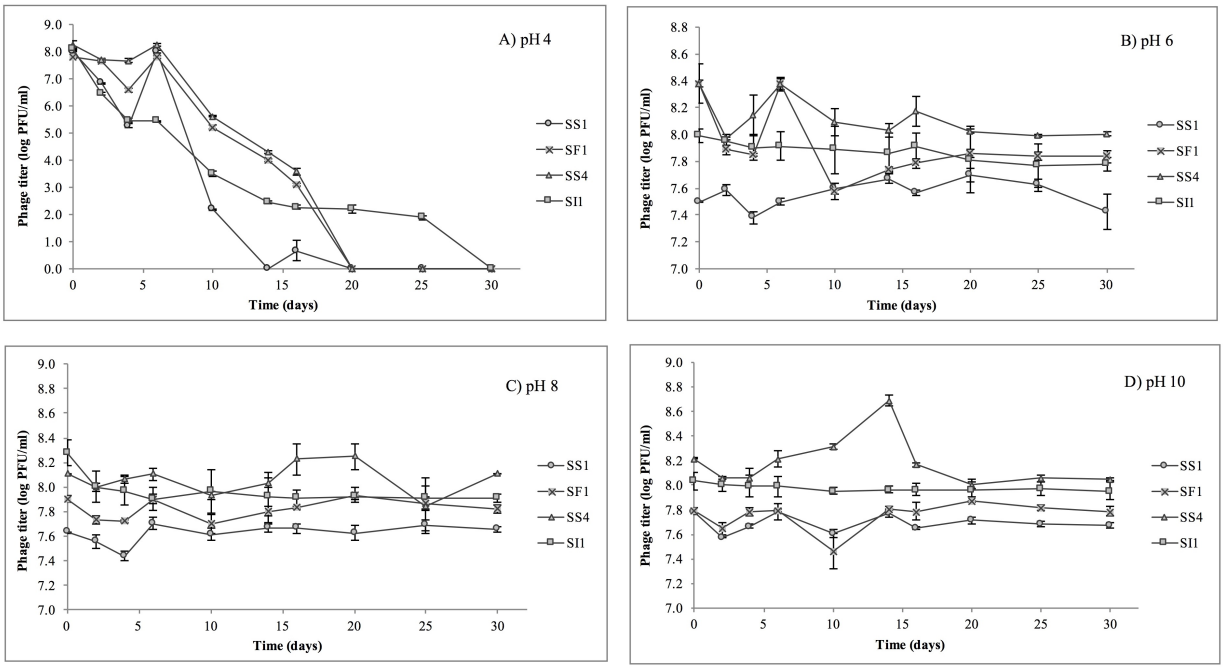

Figure 2.5. Stability of phages SI1, SF1, SS1 and SS4 at (A) pH 4, (B) pH 6, (C) pH 8 and (D) pH 10 over a period of 30 days. Data shown are the mean of three replicates ± SD.

Our results are supported with previous findings of rapid declines in titer at pH 4.2 but only a gradual decline at pH 5.8 (Leverentz et al., 2001). However, Ahiwale et al. (2013) reported instability at pH = 10–12 of *Salmonella* phage. This inconsistency may be due to the structure of individual phages assayed. Having long flexible non-contactile tails, SF1, SS1, SS4, and SI1 belong to the family *Siphoviridae*, whereas phages assayed by Ahiwale et al. (2013) possessed short, stubby, non-contractile tails, representing *Podoviridae*. Similar to our phages, Hamdi et al. (2017) found that Siphovirus SH6 was unstable at pH 2–4 and stable at pH 5–11, whereas Myovirus SH7 was stable at pH 3–11. Differences in isoelectric points (pI) of the phages may also contribute to these differences in stability, particularly at acidic pH, as viral aggregation is common when pH ≤ pI and has previously led to decreases in titer of approximately three log PFU/mL (Langlet et al., 2007).

In the heat stability assay, 9 of 16 phage titers were significantly ($p<0.05$) reduced, but by

no more than 1.0 ± 0.1 log PFU/ml over the range of temperatures tested, suggesting that SS1, SF1, SS4, and SI1 will retain the stability required for use as biocontrol agents at temperatures commonly encountered in produce production chains (Rombouts et al., 2016).

SS1, SF1, and SS4 were significantly ($p<0.05$) more stable at −20, 4, and 22°C than at 37°C (Figure 2.6). From −20 to 22°C, there were no significant ($p<0.05$) differences in stability between SS1, SF1, and SS4, but at 37°C, SF1 was significantly ($p<0.05$) more stable than both SS1 and SS4. SI1 was detected to be most stable ($p<0.05$) at a temperature of 22°C, and significantly ($p<0.05$) more stable than SS1 at this temperature. All four phages were least stable at 37°C, and more stable at −20°C than 4°C. Aside from SI1 being most stable at 22°C, our results agree with previous reports in that Siphovirus stability decreases with an increase in temperature above 20°C (Jepson and March, 2004). Previous work has demonstrated the stability of Podoviruses to be most stable from 4 to 36°C, with highest stability retained at the lower end of the temperature spectrum (Ahiwale et al., 2013). This parallelism between phages of different families suggests that differences in phage tail morphologies may not be a main contributor to the variances in phage stability at different temperatures; however, Thorne and Holt (1971) have reported a negative correlation between temperature and tail contraction, and hence loss of activity, in *Myoviridae* phages. Given the importance of assaying for stability prior to adoption into the commercial market, these results indicate that their environmental stability makes these phages good candidates for use in biocontrol.

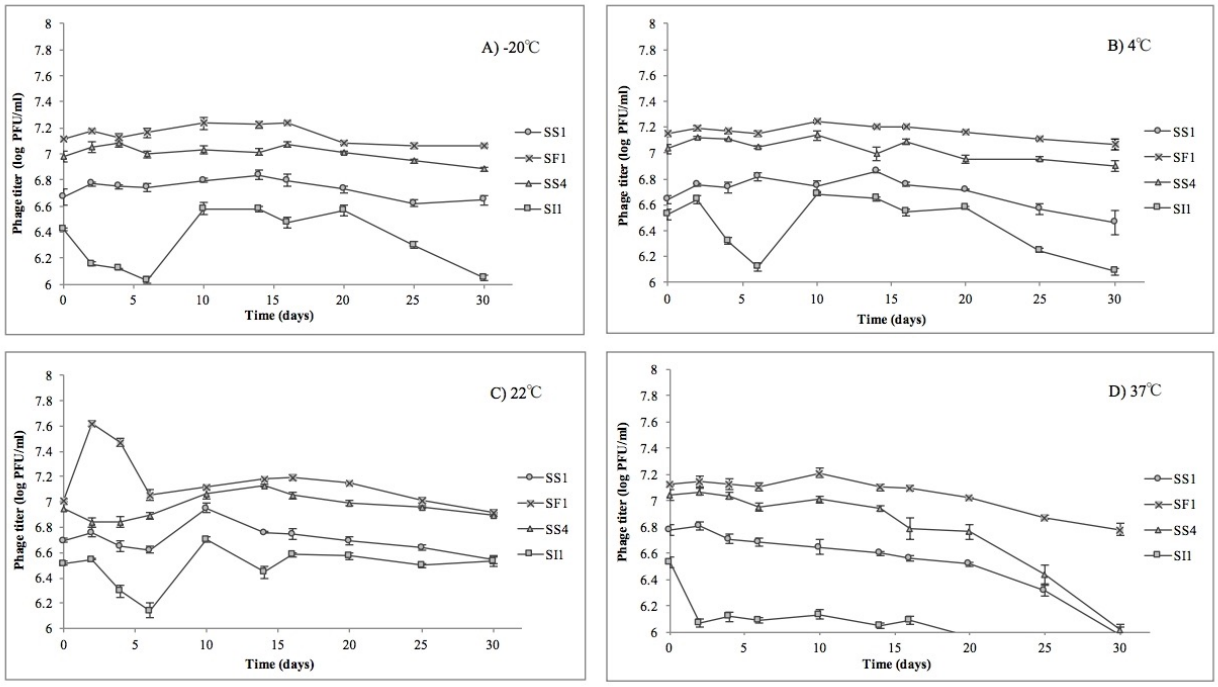

Figure 2.6. Stability of phages SI1, SF1, SS1 and SS4 at (A) -20°C, (B) 4°C, (C) 22°C and (D) 37°C over a period of 30 days. Data shown are the mean of three replicates ± SD.

2.3.3.4 Assessment of *in vitro* phage infectivity in tryptic soy broth

The relative abilities of the four phages to suppress *S.* Enteritidis FSL S5-483, *S.* Agona FSL S5-513 and *S.* Typhimurium LMFS-JF-001 in TSB were assessed at an MOI of 1, 10, and 100 PFU/CFU at 25°C. At MOI = 1, both SI1 and SS4 suppressed the growth of *S.* Enteritidis over a 36 h period. Growth of *S.* Enteritidis was also suppressed by SF1 and SS1, but growth resumed at 13 and 17 h, respectively, after initial infection (Figure 2.7). Growth of *S.* Enteritidis appeared to recover at 31 h following treatment with SS4. An MOI of 10 prolonged the suppression of *S.* Enteritidis to 19 and 25 h when infected with SF1 and SS1, respectively. Further, application of SS4 at an MOI of 10 caused complete inhibition of *S.* Enteritidis growth for 36 h. Finally, phage treatment at an MOI of 100 suppressed the growth of *S.* Enteritidis for the 36 h duration (Figure 2.7).

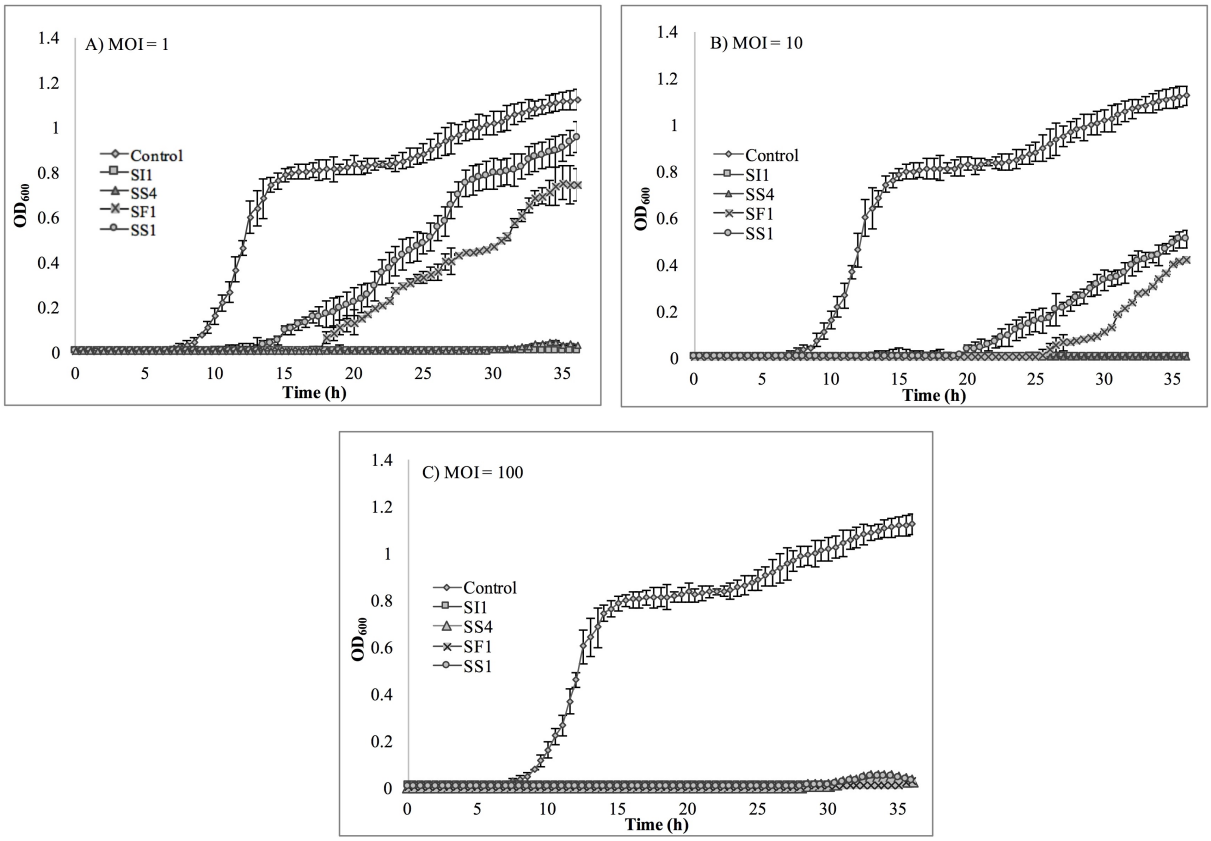

Figure 2.7. *In vitro* analysis of *S.* Enteritidis inhibition by phages SI1, SS4, SF1 and SS1 at (A) MOI=1, (B) MOI=10, (C) MOI=100. Data shown are the mean of three replicates ± SD.

Suppression of *S.* Agona also occurred with all MOIs tested (Figure 2.8), but the extent was not as pronounced as with the host, *S.* Enteritidis. Instead, considerable suppression did not occur until an MOI of 100 was evaluated. At this MOI, it appeared that SS1 was the least effective in controlling *S.* Agona as growth resumed 17 h after the initial infection. In contrast, SI1 and SS4 were the most effective in suppressing growth, although *S.* Agona appeared to recover at 38 and 32 h after infection with these phages, respectively.

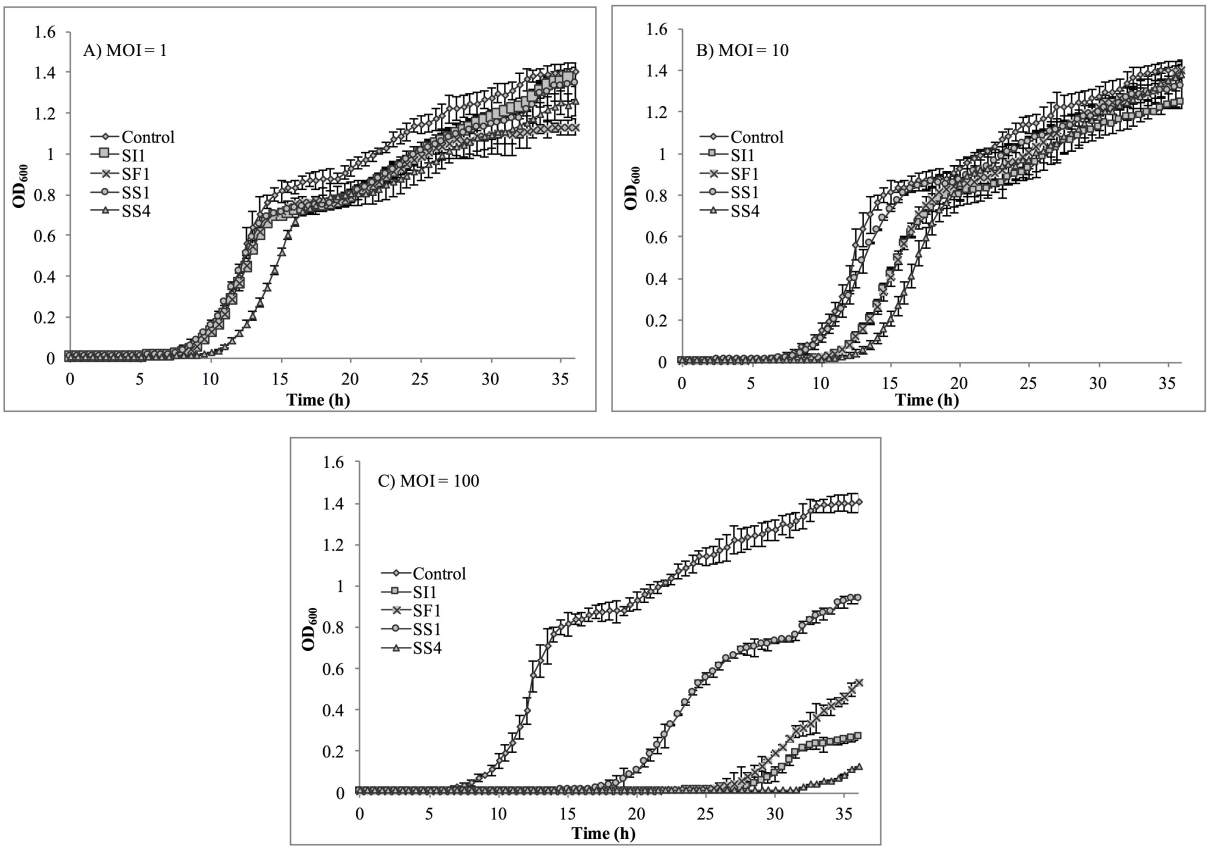

Figure 2.8. *In vitro* analysis of *S.* Agona inhibition by phages SI1, SS4, SF1 and SS1 at (A) MOI=1, (B) MOI=10, (C) MOI=100. Data shown are the mean of three replicates ± SD.

Lastly, *S.* Typhimurium was tested for its susceptibility to the phages in TSB (Figure 2.9). Again, the extent of suppression after phage infection was not as pronounced as with *S.* Enteritidis, but did occur at all MOIs. The most dramatic reduction in growth occurred at MOI 100, although growth was not suppressed entirely for the 36 h duration. At this MOI, SI1 was the most effective in attenuating growth (Figure 2.9).

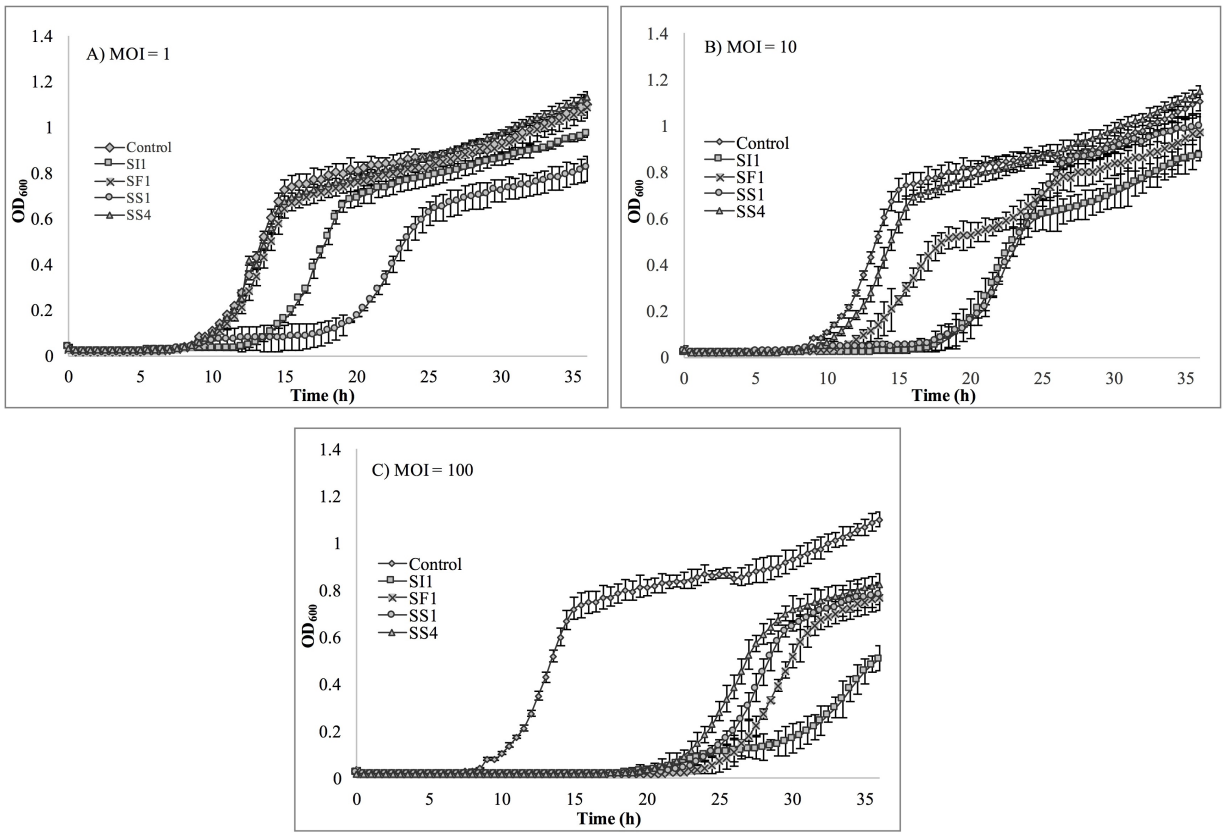

Figure 2.9. *In vitro* analysis of *S.* Typhimurium inhibition by phages SI1, SS4, SF1 and SS1 at (A) MOI=1, (B) MOI=10, (C) MOI=100. Data shown are the mean of three replicates ± SD.

Although not all MOIs were effective in controlling growth of *Salmonella*, nor did all *Salmonella* strains show similar susceptibilities to the phages, it should be noted that phage infection at all MOIs, across all strains, resulted in an extended lag phase [defined as OD_{600} <0.2 (Wang et al., 2009), indicating that the phages had a suppressive effect on *Salmonella*. The ability of the *Salmonella* strains to recover after initial infection is likely due to the emergence of phage-resistant mutants (Guenther et al., 2012). It may be possible to prolong the duration of phage sensitivity by infection with a mixture of phages (Chan et al., 2013), though it was not evaluated in this present study.

With all strains, infection with an MOI of 100 proved to be the most effective and represents the MOI used for many food processing applications (Silva et al., 2014). However, with the phage host, *S.* Enteritidis, growth was completely suppressed at all MOIs with phage SI1, underlining its remarkable efficacy in controlling *S.* Enteritidis *in vitro*. Mechanistically, SI1 may require multiple attachment sites on the bacterial cell membrane for adsorption and/or SI1 receptor sites may be essential for cellular metabolic processes – both of which would contribute to the attenuation of phage resistance by the host (Rakhuba et al., 2010; Kong et al., 2011).

2.3.3.5 Assessment of SI1 to control *Salmonella* on sprouting alfalfa seeds

The ability of SI1 to control *Salmonella* on sprouting alfalfa seeds was assessed. SI1, in particular, was selected for this study as it caused complete inhibition of *S.* Enteritidis in TSB at all tested MOIs and possessed the greatest burst size (approximately 83 phages) and possessed one of the shortest latent periods (25 min) (Figure 2.10). Moreover, *S.* Enteritidis is a serotype previously linked to North American sprout outbreaks (CDC, 2019) and further, has been implicated in the highest number of salmonellosis outbreaks worldwide (Mattick et al., 2001). On the basis of these factors, they were selected for use in this biocontrol study.

Treatment with SI1 (MOI = 100) resulted in a significant ($p<0.05$) 2.51 ± 0.24 log CFU/g reduction of *S.* Enteritidis, two h after treatment (Figure 2.10), corresponding to a decrease of 38.3 ± 3.0% of the initial viable population. This was accompanied by a 1.02 ± 0.33 log PFU/g increase in phage titer (Figure 2.11). In contrast, previous work by Kocharunchitt et al. (2009) reported a one log CFU/g decrease in *S.* Oranienburg populations following application of phage SSP6 onto alfalfa seeds at the beginning of germination. Similarly, a 1.37 log CFU/g reduction of *Salmonella* populations was observed on mustard seeds at 24 h following phage treatment (Pao et al., 2004). Sprout production standards, as set by Health Canada, recommend that sprout

decontamination methods achieve a minimum three log reduction in pathogen counts (CFIA, 2014). Further validation of SI1 infectivity across a range of potential bacterial contaminants and at various stages throughout the sprouting process is therefore required to ensure complete compliance with Health Canada standards, although a >2.5 log CFU/g reduction of *S.* Enteritidis is promising. Additionally, the final weight of the seeds treated with *Salmonella* only (82.40 ± 2.83 CFU/g) was not significantly different ($p>0.05$) than that of the phage-treated sprouts (80.64 ± 1.4 CFU/g), further demonstrating its potential suitability for use in industry.

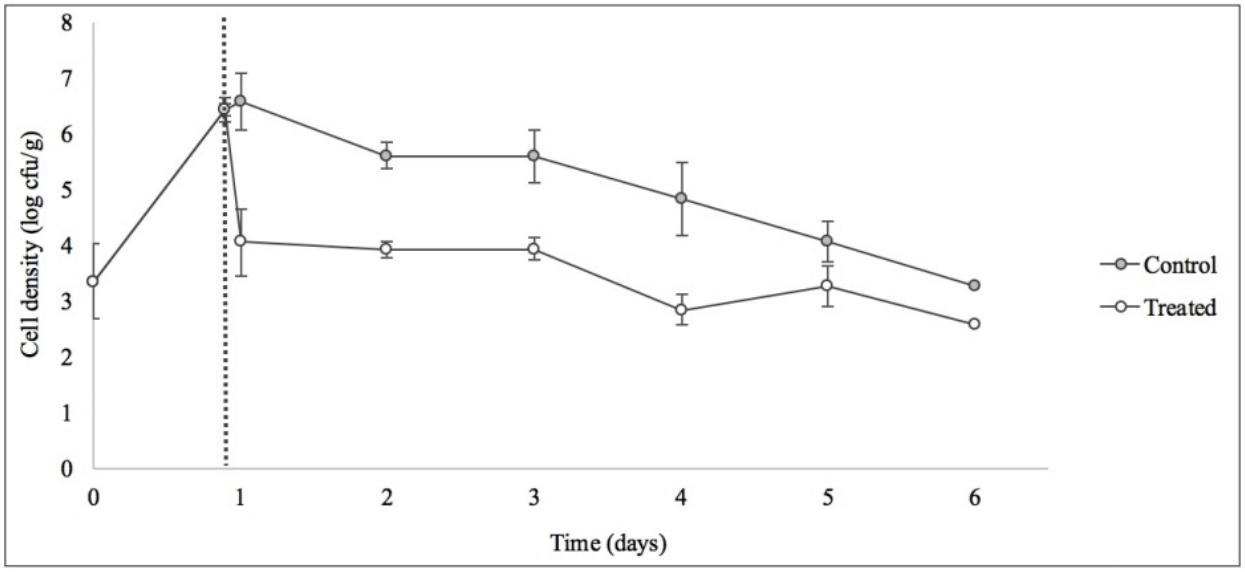

Figure 2.10. Control of *S.* Enteritidis on sprouting alfalfa seeds with phage SI1. Dotted line indicates the phage treatment at 22 h after artificial contamination of the seeds. Data shown are the mean of three replicates ± SD.

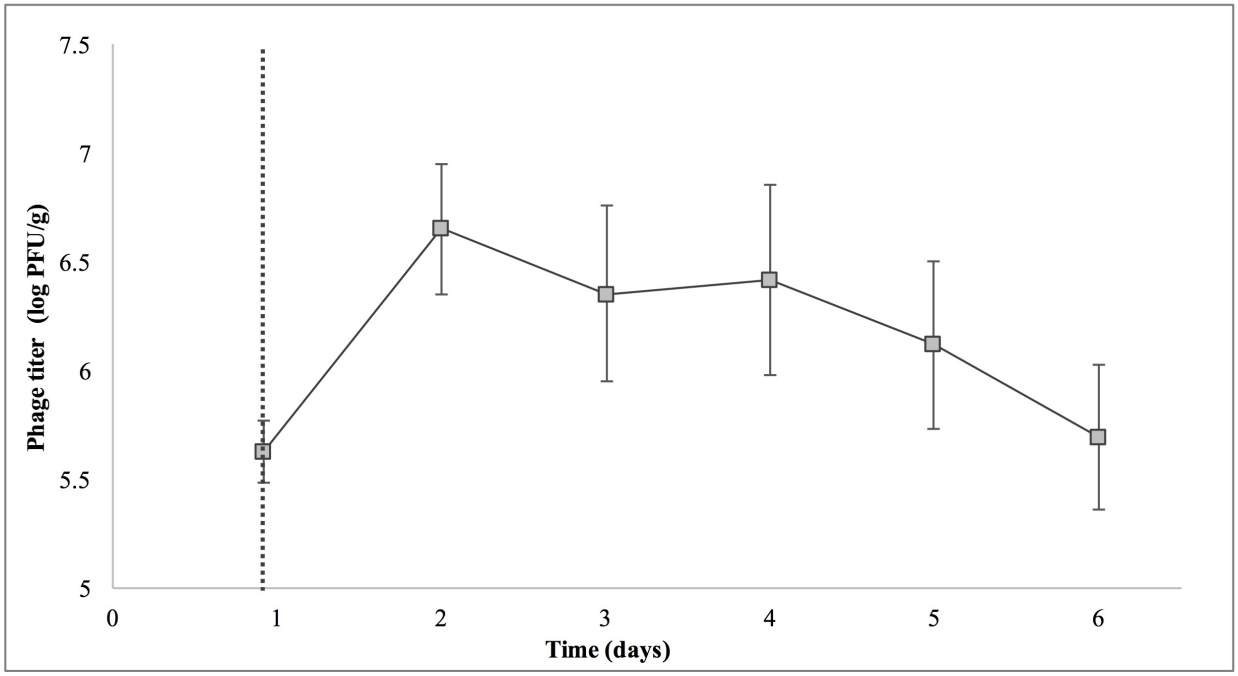

Figure 2.11. Titer of phage SI1 during control of *S.* Enteritidis on alfalfa sprouts. Dotted line indicates the phage treatment at 22 h after artificial contamination of the seeds. Data shown are the mean of three replicates ± SD.

On days 2–6 following phage treatment, *Salmonella* cell densities on treated alfalfa seeds were also reduced, but this was not significant ($p>0.05$) (Figure 2.10). In line with this observation, phage titers increased the day of seed treatment, indicating its replication. However, the initial increase was followed by a stagnation of growth and small decreases in titer (Figure 2.11). Notably, it is presumed that the emergence of phage-resistant *Salmonella* may have contributed to the diminished effectiveness in the days following treatment. Indeed, phage-resistant *Salmonella* has been identified in both *in vitro* systems (Vipra et al., 2013) and foods treated with phage (Kocharunchitt et al., 2009; Guenther et al., 2012). Emergence of bacterial mutants resistant to phage is particularly apparent when MOI values are high, as this enhances the selective pressure to resist infection (Vipra et al., 2013). It has been reported, however, that phage-resistant mutants possess attenuated pathogenicity and diminished fitness (Kong et al., 2011). A possible remedy to control the emergence of such mutants is through the use of a phage

cocktail, which may additionally extend the spectrum of lysis to include other *Salmonella* strains (Chan et al., 2013).

Although the present results are not fully consistent with the data obtained *in vitro*, it is hypothesized that the simplicity of an *in vitro* system represents an ideal scenario for phage infection and multiplication. The nature of a food matrix presents with various complicating factors. For instance, possibilities include biofilm production on alfalfa sprouts (Kocharunchitt et al., 2009), which could hinder phage adsorption (Sutherland et al., 2004); growth of endogenous microbiota naturally present on sprout seeds, which may provide alternative adsorption sites (Ye et al., 2010); or internalization of *Salmonella* into the sprouts itself (Erickson, 2012), rendering them unavailable for phage attack. These factors could account for the diminished efficacy of the phage and also its inconsistent increases in phage titer throughout this assay. It is possible that additional phage treatments throughout the sprouting process, or phage treatment in combination with other treatments (e.g., chlorine or organic acid washes), would further reduce the viable *Salmonella* populations on alfalfa seeds. It should also be noted that the high initial load of *Salmonella* used in this assay is unrepresentative of real world situations, yet is important from a technical perspective to determine the log kill. Ye et al. (2010) reported a six log CFU/ml decrease of *Salmonella* on artificially contaminated mung bean sprouts upon treatment with a combination of six *Salmonella* phages and *Enterobacter asburiae*, a naturally competitive microorganism. Interestingly, this combination treatment was significantly more effective than treatment with phage or *E. asburiae* alone.

2.4 Conclusions

Bacteriophage treatment of produce is an underdeveloped, emerging topic of interest and is currently not used extensively in the produce industry. In this study, four lytic bacteriophages

infecting *Salmonella* were assessed to determine their suitability for biocontrol in alfalfa sprout production. The results revealed that all four phages possessed desirable characteristics for use in biocontrol efforts. Among the phages characterized, SI1 proved to be particularly effective for control of *Salmonella* both *in vitro* and upon application onto sprouting alfalfa seeds. Although promising, future work should also aim to optimize this treatment, such as by incorporating hurdled treatments (i.e., with conventional sanitizers) or designing a multi-phage cocktail. Additionally, phage treatment of other sprouts varieties should be investigated to confirm the potential for use in related produce items.

Chapter 3: *Salmonella* phage diversity revealed by whole genome sequencing

3.1 Introduction

Bacteriophages are the most abundant biological entity on Earth and have been estimated to kill 20 to 25% of microbes daily (Gilmore, 2012; Hendrix, 2002; Suttle, 2017; Turner et al., 2018). Moreover, phages are key contributors to bacterial ecology and evolution through obligate parasitism, using either lytic or temperate life cycles thereby resulting in direct or delayed lysis of bacterial hosts, respectively (Mikalová et al., 2017). Phage-host interactions have contributed vastly to genetic flux through horizontal gene transfer that is responsible for the dissemination and acquisition of important bacterial phenotypes, such as enhanced colonization of the human gut epithelium, antimicrobial resistance and toxin production (Seed, 2015; Turner et al., 2018).

Phage diversity is immense and the global phage gene pool likely represents the greatest biodiversity and largest potential source of novel genes, providing new insights on phage diversity and evolutionary relationships in disparate environments (Clokie et al., 2011; Cortés et al., 2015; Turner et al., 2018). Moreover, this vast diversity is a potential reservoir of antibacterial agents for developing "phage therapies" or "biocontrol" strategies to control bacterial pathogens. Phage-based biocontrol of bacterial pathogens in foods and food processing environments is an attractive alternative to using synthetic antimicrobial agents or physical disinfection treatments that can have harmful effects on humans, animals and plants [Abedon, 2011, Goodridge et al., 2018; Hagens & Loessner, 2007). At least, phages most suited to this purpose should exhibit a broad host range and be free of genes encoding for lysogeny and resistance to antimicrobial agents and/or virulence (Hagens & Loessner, 2007).

Non-typhoidal *Salmonella enterica* is a foodborne pathogen causing high rates of mortality and morbidity worldwide (Majowicz et al., 2010; Scallan et al., 2011). Globally,

bacteria in the genus *Salmonella* cause 93 million enteric infections and 155,000 diarrheal deaths each year [(Majowicz et al., 2010) and although there are animal reservoirs including poultry and swine (Cortés et al., 2015), their presence in other food products such as nuts, produce and ready-to-eat products (CDC, 2018) confirms that it can adapt to diverse environments (Fong et al., 2016).

Comparative genomics approaches have been used to aid in the development of phage-based products targeting several genera including *Acinetobacter*, *Pseudomonas*, *Mycobacterium*, *Lactococcus*, *Vibrio* and *Salmonella* (Turner et al., 2018). These analyses provided insights at genomic and phylogenetic levels (e.g., phage relatedness and the elucidation of novel genetic elements), associations among phage communities across disparate environments, and elucidation of novel phage-host interactions (Finke et al., 2017; Liu et al., 2014; Marinelli et al., 2012; Pope et al., 2015; Switt et al., 2013; Turner et al., 2018). Nevertheless, an in-depth understanding of *Salmonella* phage diversity and phenotype-genotype characteristics is lacking. Therefore, the objective was to perform comparative phenotypic, genomic and phylogenetic analyses of 45 new phage isolates from British Columbia, Canada, that infect non-typhoidal strains of *Salmonella*.

3.2 Methods

3.2.1 *Salmonella* phages

The *Salmonella* phages included in this analysis are listed in Chapter 2 in Figure 2.1.

3.2.2 Phage DNA isolation

Prior to DNA isolation, one ml of the lysates was filtered with a 0.45-µm pore-size cellulose-acetate membrane (Pall Corporation). Subsequently, five µl of both 10X RNAse A (Invitrogen, Carlsbad, CA, United States) and 1X DNAse (Invitrogen) were added to the filtered

lysates for removal of contaminating host nucleic acid. $MgSO_4$ was also added to a final concentration of 10 mM and the suspension was incubated at 37°C for 30 min. Then, 100 µl of lysis solution (2.5% sodium dodecyl-sulfate, 0.25 M EDTA and 0.50 M Tris-HCl (pH 9.0)) was added to the mixture, followed by incubation at 65°C for 30 min. Subsequently, 125 µl of 8 M potassium acetate (Amresco, Solon, OH, United States) was added and the suspensions were placed on ice for 30 min, then spun at 17,000 rpm (27,141 x *g*) for 10 min at 4°C. Afterwards, 500 µl of phenol-chloroform (Amresco) was added, the contents were mixed on a vortex for 1 min, and spun at 14,000 rpm (18,407 x *g*) for 10 min at room temperature. The upper phase containing the DNA was carefully transferred to a clean microcentrifuge tube and an equal volume of isopropanol (Amresco) was added, followed by centrifugation at 17,000 rpm (27,141 x *g*) for 10 min at 4°C. The supernatant was discarded, and the pellet washed 3 times with 70% ethanol and allowed to dry for 15 min. Finally, the pellet was re-suspended in 20 µl Tris-HCl (pH 8.0) and stored at -80°C until analyzed (Moineau et al., 1994).

3.2.3 DNA sequencing and annotation workflow

The DNA library was prepared using the Nextera XT DNA Library Preparation Kit (Illumina, Hayward, CA, United States) according to the manufacturer's instructions and shotgun-sequenced using the Illumina MiSeq platform with the MiSeq Reagent Kit v2 (Illumina). Contigs were assembled *de novo* from the paired-end reads with the Ray assembler version 2.2.0 (Boisvert et al., 2010). Depth of sequencing in the newly isolated phages ranged from 40- to 2564-fold coverage (Table B1).

Open reading frames (ORFs) were identified and annotated with the Rapid Annotation using Subsystems Technology (RAST) pipeline (Aziz et al., 2008). Annotations were also

subsequently verified using the BLASTp algorithm (NCBI), employing an E-value cut-off of 0.01 (Dutilh et al., 2014).

3.2.4 Genomic analysis

Genomic analysis was conducted on the phage genomes to identify phages with desirable characteristics for biocontrol purposes and also to probe the biodiversity of our novel isolates. An *in silico* approach was taken to predict phage morphotypes by comparison with closely related phage genomes using the BLASTp algorithm (NCBI). Genomes that were most closely related (i.e., possessing the highest E-value and >50% query coverage) were chosen to aid in assigning newly sequenced phages to putative families. ARAGORN was used to identify genes encoding putative tRNAs, which employs heuristics and homology comparisons with tRNA consensus sequences for prediction of the tRNA secondary structure (Laslett et al., 2004).

Phylogenetic trees were constructed in MEGA X (Kumar et al., 2018). Nucleotide sequences were aligned using the ClustalW algorithm and the phylogenetic tree constructed using the Maximum-Likelihood method employing 1000 bootstrap replicates. Clusters in the phylogenetic trees were identified using ClusterPicker (Ragonnet-Cronin et al., 2013) with an inter-cluster threshold of 50% nucleotide identity, as has been demonstrated for other phage genomes (Turner et al., 2018). Amino-acid and nucleotide comparisons were conducted for individual phages within clusters using alignment tools such as MEGA X. Visual representations and comparisons of whole genomes were produced using EasyFig (Sullivan et al., 2011).

Phages were annotated using a combination of automatic (i.e., RAST) and manual (NCBI BLASTp) approaches. Phages were classified as putatively temperate when a gene encoding integrase (for integration into the bacterial host chromosome) could be identified; whereas,

phages without this gene were classified as putatively lytic (Sullivan et al., 2011; Switt et al., 2013).

3.3 Results

The genus *Salmonella* comprises a diverse group of microorganisms with well-characterized pan genomes, routes of transmission, pathogenesis and epidemiology (Switt et al., 2013). Despite their potential relevance to the genomic and biological attributes of the genus, there is comparatively little genomic information on *Salmonella* phages (Switt et al., 2013).

3.3.1 General genomic characterization

Genomic characterization was performed to identify phages that possessed particular genomic features that would be pertinent for biocontrol (i.e, rendering them either unable to be used for food biocontrol and/or possessing genes (e.g., tRNA genes, DNA-replication elements) that could potentially constitute an infection/replication advantage in the host). The complete genomes have been deposited into Genbank with accession numbers: MK761195 – MK761199, MK770409 – MK770415, MK972685 – MK972699, MK972700 – MK972717 (Table B2). Putative functions of all ORFs may be accessed through these accessions. Additionally, we describe here distinct patterns of diversity that were revealed through our genomic analysis.

Phages were organized into 12 distinct clusters on the basis of 50% nucleotide similarity (Figure 3.1). There were four genomic singletons (including Felix-O1), which did not cluster into any sub-groups, although they exhibited similarity to previously sequenced phages. For instance, singleton phage SE13 showed ~93% nucleotide sequence identity to *Salmonella* phage BP63 (NC_031250), while SE5, interestingly, was 98% similar to *Erwinia* phage phiEa21-4 (NC_015292) (Table 3.1). Interestingly, phages of Cluster 6 did not show any significant matches to previously isolated phages, indicating the presence of a novel genus or cluster.

Overall, some clusters contained phages with high sequence identity that were isolated from disparate sites (e.g., Clusters 8 and 10). These observations, coupled with the fact that these phages were isolated from proximate regions within British Columbia, suggest that they may be genetically endemic (Switt et al., 2013) and/or transmissible via unknown vectors (e.g., wildlife moving among sites). It has also been suggested that phages within a cluster may share common hosts (Pope et al., 2015) which implies that host populations within a cluster may also be genetically similar (Pope et al., 2015); future metagenomic analyses may verify this hypothesis. We also saw evidence of rich diversity across clusters; phages from sewage were assigned to different clusters (e.g., Clusters 1, 7, 8, 11 and 12) or could not be assigned a specific cluster (Figure 3.1). As wastewater is abundant in nutrients, bacterial hosts, and high rates of horizontal gene transfer, phages isolated from these sites represent a reservoir of novel and diverse genetic materials (Parmar et al., 2018).

A lysogeny module containing a gene encoding integrase was identified in 18 of the 45 phages (Figure 3.1). Although the use of temperate phages is counter-indicated for some biocontrol applications (e.g., in food systems) (Goodridge et al., 2018), phage modification by deletion of lysogeny modules may be considered for other applications, particularly if the phage has a broad host range. It should also be cautioned that in addition to genomic analysis, *in vitro* transduction assays should be carried out to confirm a virulent lifestyle (Fong et al., 2017; Switt et al., 2018).

The MCP was identified and functionally annotated in all phages (Figure 3.2), suggesting that significant divergence of this protein is constrained due to its important conserved role in capsid assembly and maintenance of the viral capsid structure (Deveau et al., 2006). Because it represents a core gene and is thus not known to be horizontally transferred, multiple studies have

assessed the role of MCP as a phylogenetic marker (Adriaenssens, 2014; Casjens & Grose, 2016; Deveau et al., 2006; Finke et al., 2017; Grose et al., 2014), further exacerbated by the fact that there is no benchmark gene used to study phage diversity. Indeed, comparisons of the whole genome and the MCP in our subset of phages show similar clustering patterns (Figures 3.1 and 3.2), potentiating its use as an inference gene for genetic relatedness. For instance, 5/6 of the phages in Cluster 4 in the whole genome dendrogram (Figure 3.1) are also clustered together in Cluster 4 of the MCP dendrogram (Figure 3.2). All phages within Clusters 5 and 6 on both figures are also grouped together similarly. Clusters 8 and 9 of the whole genome dendrogram are also grouped together to form Cluster 7 in the MCP dendrogram. On the basis of the MCP, singletons SE13, SE4 and SE14, as identified in Figure 2, are either grouped together or with other phages (Cluster 4, Figure 3.2), indicating the conservation of this shared core gene. Given the patterns of clustering, these results suggest the MCP may be suitable as a phylogenetic marker for whole genome clustering. Furthermore, determination of the MCP sequence may be useful for pre-assignment of novel phages into groups or clusters which share desirable characteristics for biocontrol (e.g., broad host range, infection efficiency, etc.) (Born et al., 2019; Casjens & Grose, 2016; Grose & Casjens, 2014).

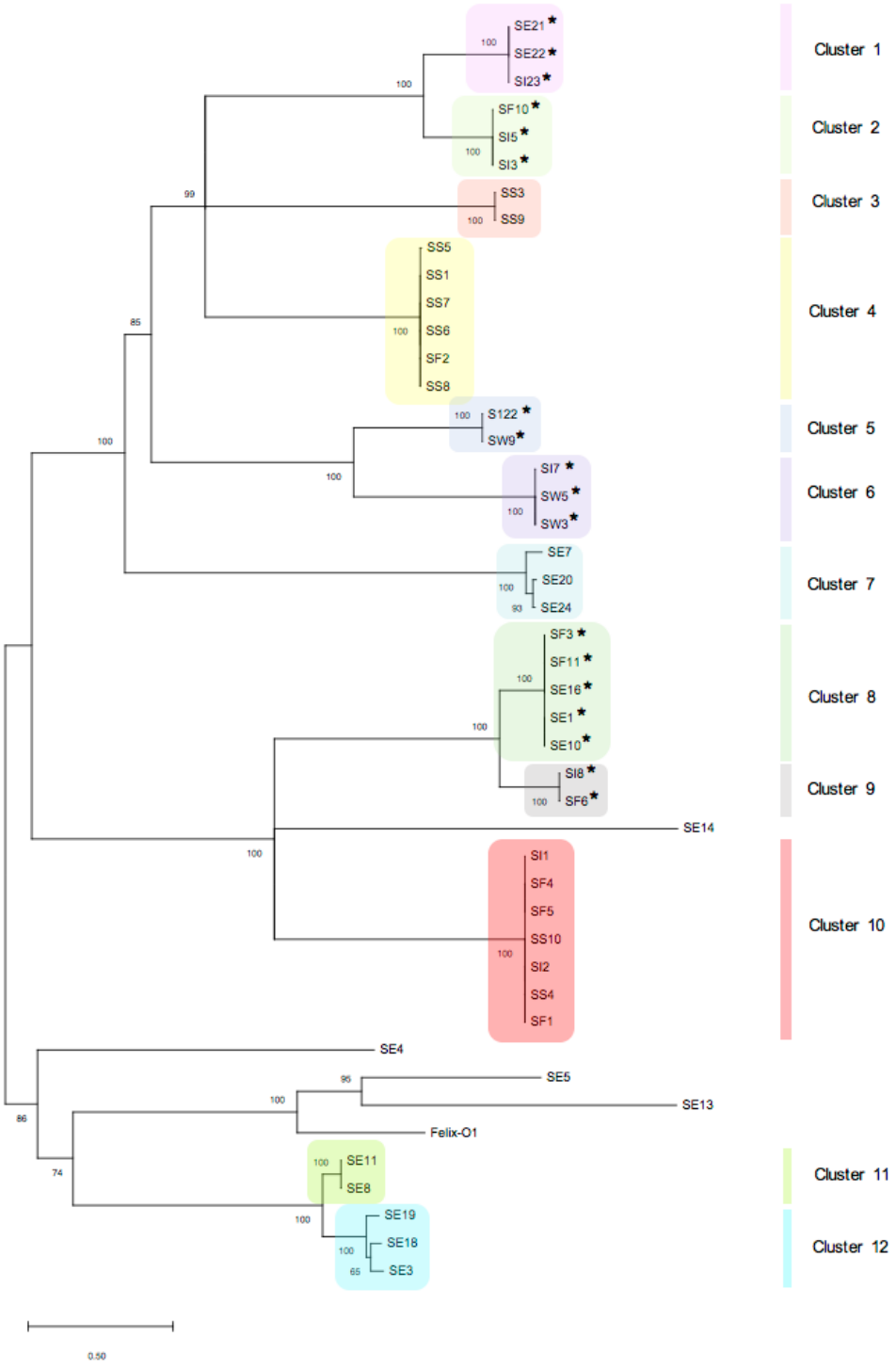

Figure 3.1. Dendrogram of whole genome nucleotide alignment**.** Tree was constructed using the ClustalW alignment and the Maximum-Likelihood method in MEGA X with 1000 bootstrap replicates. Bootstrap percentages are shown next to each node. Scale represents the number of nucleotide substitutions per site. Putatively temperate phages are indicated with an asterisk (*).

Table 3.1. Closest related sequenced phages to newly isolated phages. The closest related phages (possessing highest E-value and >50% query coverage) and their respective genera were determined through nucleotide homology using NCBI BLASTn.

Phage	Cluster	Closest related phage (NCBI best match)	Genus
SE21	1	*Salmonella* phage 103203_sal5	*Lederbergvirus*
SE22	1	*Salmonella* phage 103203_sal5	*Lederbergvirus*
SI23	1	*Salmonella* phage 103203_sal5	*Lederbergvirus*
SF10	2	*Salmonella* phage ST160	*Lederbergvirus*
SI5	2	*Salmonella* phage ST160	*Lederbergvirus*
SI3	2	*Salmonella* phage ST160	*Lederbergvirus*
SS3	3	*Salmonella* phage GG32	*Cvivirinae*
SS9	3	*Salmonella* phage GG32	*Cvivirinae*
SS5	4	*Salmonella* phage vB_SenS_SB3	*Guernseyvirinae*
SS1	4	*Salmonella* phage vB_SenS_SB3	*Guernseyvirinae*
SS7	4	*Salmonella* phage vB_SenS_SB3	*Guernseyvirinae*
SS6	4	*Salmonella* phage vB_SenS_SB3	*Guernseyvirinae*
SF2	4	*Salmonella* phage vB_SenS_SB3	*Guernseyvirinae*
SS8	4	*Salmonella* phage ST3	*Guernseyvirinae*
SI22	5	*Salmonella* phage FSL SP-004	*Peduovirinae*
SW9	5	*Salmonella* phage FSL SP-004	*Peduovirinae*
SI7	6	N/A	N/A
SW5	6	N/A	N/A
SW3	6	N/A	N/A
SE7	7	*Salmonella* phage S147	*Tequintavirus*
SE20	7	*Salmonella* phage Seabear	*Tequintavirus*
SE24	7	*Salmonella* phage S126	*Tequintavirus*
SF3	8	*Salmonella* phage 103203_sal5	*Lederbergvirus*
SF11	8	*Salmonella* phage 103203_sal5	*Lederbergvirus*
SE16	8	*Salmonella* phage 103203_sal5	*Lederbergvirus*
SE1	8	*Salmonella* phage 103203_sal5	*Lederbergvirus*
SE10	8	*Salmonella* phage 103203_sal5	*Lederbergvirus*
SI8	9	*Salmonella* phage ST160	*Lederbergvirus*
SF6	9	*Salmonella* phage SE1	*Lederbergvirus*
SE14	Singleton	*Salmonella* phage S115	*Cvivirinae*
SI1	10	*Salmonella* phage vB_SenS_SB3	*Guernseyvirinae*
SF4	10	*Salmonella* phage vB_SenS_SB3	*Guernseyvirinae*
SF5	10	*Salmonella* phage vB_SenS_SB3	*Guernseyvirinae*
SS10	10	*Salmonella* phage vB_SenS_SB3	*Guernseyvirinae*
SI2	10	*Salmonella* phage vB_SenS_SB3	*Guernseyvirinae*
SS4	10	*Salmonella* phage vB_SenS_SB3	*Guernseyvirinae*
SF1	10	*Salmonella* phage vB_SenS_SB3	*Guernseyvirinae*
SE4	Singleton	*Salmonella* phage ZCSE2	N/A
SE5	Singleton	*Erwinia* phage phiEa21-4	*Ounavirinae*
SE13	Singleton	*Salmonella* phage BP63	N/A
SE11	11	*Salmonella* phage SP01	*Tequintavirus*

SE8	11	*Salmonella* phage SP01	*Tequintavirus*
SE19	12	*Salmonella* phage SP01	*Tequintavirus*
SE18	12	*Salmonella* phage BSP22A	*Tequintavirus*
SE3	12	*Salmonella* phage S147	*Tequintavirus*

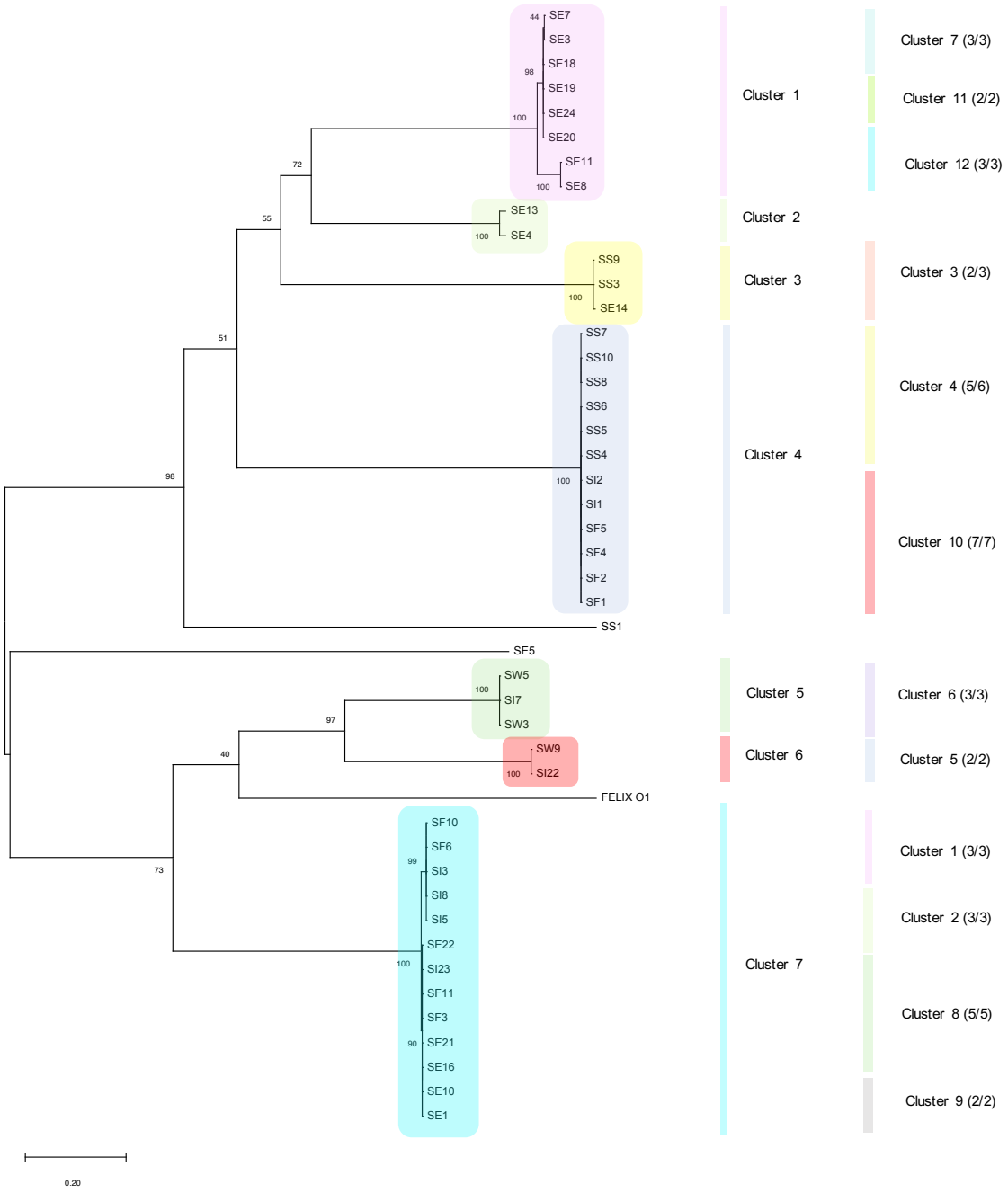

Figure 3.2. Dendrogram of the major capsid protein nucleotide alignment. Tree was constructed using the ClustalW alignment and the Maximum-Likelihood method in MEGA X with 1000 bootstrap replicates. Bootstrap percentages are shown next to each node. Scale represents the number of nucleotide substitutions per site. Clusters from the whole genome dendrogram (represented in the column on the right) are aligned to the clusters of the MCP dendrogram for comparison. The bracketed numbers indicate the number of phages in the whole genome clusters that were also grouped into the same MCP cluster.

3.3.2 Phage classifications

The taxonomic assignments of 45 newly isolated *Salmonella* phages were predicted by *in silico* analysis (Table 3.2) as has been performed by others (Wichels et al., 1998). For those phages that could be assigned a taxonomic rank, 46.7% (21/45) were classified in the family *Siphoviridae*, 28.9% (13/45) and 17.8% (8/45) were assigned to the families *Podoviridae* and *Myoviridae*, respectively, and 6.7% (3/45) could not be assigned to a family. Previously, phages SI1, SF1, SS1 and SS4 were classified as *Siphoviridae*, based on morphology determined by transmission electron microscopy (Fong et al., 2017). The predicted morphotypes also correlated with cluster analysis of the whole genome and the MCP. For example, Cluster 3 of Figure 3.2 solely comprised phages predicted as Myoviruses according to the *in silico* analysis. Additionally, Cluster 4 contained predicted Siphoviruses (Figure 3.2).

Electron microscopy and nucleic acid content have largely provided the basis for taxonomic classification (Adriaenssens & Brister, 2017), with the currently classified viruses exhibiting genomic relatedness in concordance with their morphotypes (Simmonds & Aiewasakun, 2018). The diversity of morphotypes in this collection is important when informing optimal cocktail design. The sensitivity of phage to external factors (e.g., storage, temperature, pH, etc.) varies between morphological families (Bodier-Montagutelli et al., 2017) thus cocktails comprising different phage morphotypes should be considered. Additionally, it has been previously reported that *Salmonella* phages of different morphotypes use different host receptors (Shin et al., 2012) which, when incorporated into a cocktail, diminishes the occurrence of host resistance (Rohde et al., 2018).

Table 3.2. Genotypes and taxonomic assignments predicted from *in silico* analysis of 45 *Salmonella* phage genomes. Asterisks indicate morphotypes which have been confirmed by transmission electron microscopy (Fong et al., 2017). Sources of phages are denoted as follows: sediment (S), cattle feces (F), sewage effluent (E), irrigation water (I), and water tanks from an aquaculture facility (W).

Morphotype classification				tRNA genes										*arnC*			*vriC*			*exoZ*		
Myoviridae	*Siphoviridae*	*Podoviridae*	Unclassified	Phage	Number	Phage	Number	Phage	Number	Phage	Number	Phage	Number	Phage	Cluster	Source	Phage	Cluster	Source	Phage	Cluster	Source
SE4	SE3	SE1	SI7	SE1	0	SF1	0	SI1	0	SS1	0	SW3	0	SE21	1	E	SS3	3	S	SI3	2	I
SE5	SE7	SE10	SW3	SE3	29	SF2	0	SI2	0	SS3	4	SW5	0	SE22	1	I	SS9	3	S	SI5	2	I
SE13	SE8	SE16	SW5	SE4	0	SF3	0	SI3	1	SS4	0	SW9	0	SI23	1	E	SE14	Singleton	E	SF10	2	F
SE14	SE11	SE21		SE5	27	SF4	0	SI5	1	SS5	0			SE16	10	E				SI8	11	I
SI22	SE18	SE22		SE7	29	SF5	0	SI7	0	SS6	0			SF11	10	F				SF6	11	F
SS3	SE19	SF3		SE8	22	SF6	1	SI8	1	SS7	0			SE10	10	E						
SS9	SE20	SF6		SE10	0	SF10	1	SI22	0	SS8	0			SF3	10	F						
SW9	SE24	SF10		SE11	22	SF11	0	SI23	0	SS9	4			SE1	10	E						
	SF1*	SF11		SE13	0					SS10	0											
	SF2	SI3		SE14	4																	
	SF4	SI5		SE16	0																	
	SF5	SI8		SE18	28																	
	SI1*	SI23		SE19	29																	
	SI2			SE20	29																	
	SS1*			SE21	0																	
	SS4*			SE22	0																	
	SS5			SE24	29																	
	SS6																					
	SS7																					
	SS8																					
	SS10																					

3.3.3 Identification of putative phage tRNAs

A range of putative tRNA genes were identified in our phage collection (Table 3.2), with at least one tRNA identified in 36% (16/45) of the phages, consistent with a large-scale comparative analysis of 827 mycobacteriophages, which revealed that 41.4% contained at least one tRNA gene, and that these displayed cluster specificity (Delesalle et al., 2016). The distribution of tRNA-containing phages varied based on the isolation source, with 59% (10/17) of phages from sewage, 25% (2/8) from cattle feces, 38% (3/8) from irrigation water; 22% (2/9) from sediment; and none (0/3) from aquaculture possessing at least one tRNA-encoding gene. The findings suggest that tRNA genes are not uncommon within *Salmonella* phages and may vary geographically. In our study, most phages from sewage had at least one tRNA. As sewage represents a rich source of host diversity, having different tRNAs might enhance phage genome

replication in multiple hosts (Kunisawa, 2000). However, different numbers of phages were recovered from each site; thus, these distribution patterns are preliminary.

It has been proposed that particular tRNA genes benefit phage replication by corresponding to codons used by the phage genome rather than the host (Bailly-Bechet et al., 2007). Accordingly, phages with similar codon usages to that of their hosts will not benefit from retention of tRNA genes and would use that of their host (Kunisawa, 2000). It has also been hypothesized that temperate phages integrate at the position of a host tRNA gene, with the phage tRNA compensating for the interruption in the host tRNA gene (Cheetham & Katz, 1995).

3.3.4 Genomic analysis of SE13

As seen in the Chapter 2, Phage SE13 isolated from sewage possessed the broadest host range of the 45 isolates, lysing nearly all of the AMR strains tested (except *S.* Agona S5-517) and some rare strains (e.g., *S.* Poona S306, S307; uncommonly seen in outbreaks) that were resistant to infection from other phages in our collection, including Felix-O1 (e.g., *S.* Arizonae S172) (Figure 3.1). SE13 also lysed the serovars responsible for the highest rates of infection worldwide, *S.* Enteritidis and *S.* Typhimurium (CDC, 2019). Therefore, based on its broad host range, we sought to provide a genomic analysis of this phage as it represents a good candidate for phage-based biocontrol strategies.

Based on BLASTn, the 52,438 bp genome (G+C = 45.8%) of SE13 (accession: MK770417) revealed 93% identity to *Salmonella* phage BP63, with putative genes involved in structure, host recognition, and metabolism/replication. RAST identified 73 ORFs, suggesting that approximately 9% of the genome is non-coding (Figure 3.3). RAST assigned functions to 13 of 73 ORFs and subsequent verification with NCBI BLASTp further assigned functions to five additional ORFs, including the major capsid protein. The remaining 55 ORFs were classified as

hypothetical. No lysogeny-related modules encoding integrase, nor AMR and/or virulence factors were identified, suggesting it is a good candidate for biocontrol of *Salmonella.*

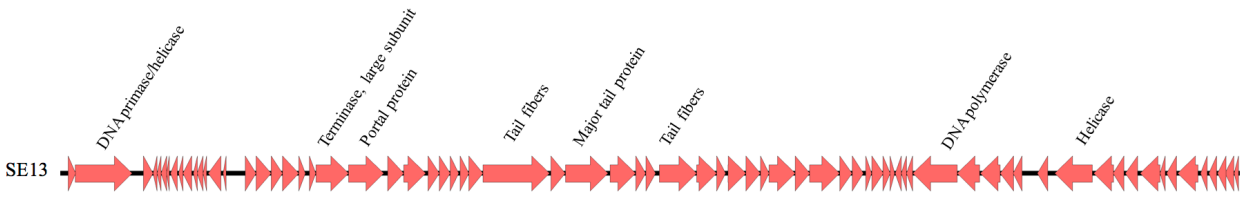

Figure 3.3. Linear whole-genome representation of phage SE13. Large ORFs greater than 1000 bp are indicated. Arrows indicate the direction of transcription.

SE13 possesses synteny, with predicted ORFs encoding genes for structure (major capsid protein, scaffold protein, tail proteins), packaging (large terminase subunit, portal protein), a lysozyme, and a likely cognate holin in close proximity, as has been identified in other phage genomes (Bardina et al., 2016; Kang et al., 2013). Given the broad host range of SE13, the tail fibers are of specific interest. ORFs 31 and 45 encode for putative tail fibers of 986 and 445 amino acids, respectively, and are 93 and 97% similar, respectively, to those of *Salmonella* phage BP63, another broad host range phage that is a component of SalmoPro® (Phagelux, Inc.), a GRAS-certified antimicrobial processing aid for controlling *Salmonella* on foods (PhageLux, 2018). A variety of tail-associated accessory proteins were also clustered together at this locus, including tail-fiber assembly proteins, ORFs 46 and 4. This locus also encoded for tail-associated protein products located on the same strand of the genome, indicating they are likely transcribed together as a module (Weigel & Seitz, 2006).

SE13 also possesses a variety of DNA metabolism-related genes (e.g., thymidylate synthase, deoxycytidylate deaminase, guanylate kinase and nicotinate phosphoribosyltransferase). Due to their disparate positions in the genome, it suggests that these

genes were acquired by separate horizontal gene transfer events with hosts, prophages or other lytic phages during co-infection, particularly since sewage, from which SE13 was isolated, is an environment facilitating a high frequency of horizontal gene transfer and rearrangements (Karkaman et al., 2018).

3.3.5 Genome size, G+C content and identification of DNA metabolism-related genes

Cluster analysis separated the phages into 12 groups (Figure 3.1). ORF prediction using a combination of RAST and BLASTp revealed genetic elements shared among phages in a cluster, as well as distinct genotypes exhibited among clusters. Genome sizes ranged from 30,037 bp to 158,539 bp, and G+C values from 39.2% to 54.4%, in concordance with other phages of *Salmonella* (Mikalová et al., 2017; Switt et al., 2013). We also observed duplicate copies of genes encoding for a variety of replication-related functions in Clusters 3, 7, 11 and 12, which also comprised phages with larger genomes. Duplicate copies may enhance synthesis of proteins involved in replication, and hence increase phage production and evolutionary fitness.

Although the role of these replication modules in large phages remains unclear, it is possible that larger genomes carry accessory genes that are not essential, but which enable more efficient phage replication. Efficient replication may lead to an increased burst size and/or reduced latent period, both of which are desirable when selecting phages for biocontrol purposes (Pereira et al., 2016a). The fact that Clusters 3, 7, 11 and 12 have a substantially different G+C content than that of their hosts suggests that having more genes for self replication may be particularly advantageous (Dupuis & Moineau, 2010; Miller et al., 2003; Switt et al., 2013). For instance, *Salmonella* has a G+C content of 50 to 52% (Switt et al., 2013), whereas, the G+C content of phages in Cluster 3 is ~44%, suggesting the eleven DNA replication elements in these phages may be advantageous. Clusters 7, 11 and 12 also have G+C contents ranging from 39.2%

to 44.7%. Moreover, some clusters with a G+C content similar to that of *Salmonella* (e.g., Clusters 4, 5, 6, 8, 10) have fewer self-replication elements (i.e., ranging from zero to three). Concordantly, *Salmonella* phages isolated from dairy farms in rural New York State with G+C contents differing from that of their hosts also harbored anywhere from 1-12 DNA replication elements (Switt et al., 2013).

Phages with genomes >100,000 bp are represented in Clusters 3, 7, 11, and 12; additionally, SE14, a genomic singleton, possesses a genome of 152,926 bp and 198 ORFs (Figure 3.1). These large genome phages possess accessory genes encoding for proteins involved in phage replication (e.g., DNA polymerase, DNA helicase, DNA primase, replication factor C, sliding clamp loader subunit) that were adjacent (i.e., modular in its arrangement), or separated by a non-related ORF. Further, these genes are positioned on the same strand, implying they are likely to be transcribed together as part of a module (Murphy et al., 2016). Phages of Cluster 3 are represented in Figure 3.4.

These modules were not identified in clusters of phages with smaller genomes (<50,000 bp), nor in genomic singletons SE13 (52,438 bp), SE4 (53, 494 bp) and SE5 (84,567 bp). However, in some of these phages, a small number of replication-related elements were dispersed throughout the genome as well as duplicate copies of select genes encoding for replication-related functions (e.g., DNA ligase, DNA topoisomerase, DNA helicase). Concordantly, duplicate copies of some of these genes were identified in Clusters 3, 7, 11 and 12, representing phages with larger genomes (Figure 3.4).

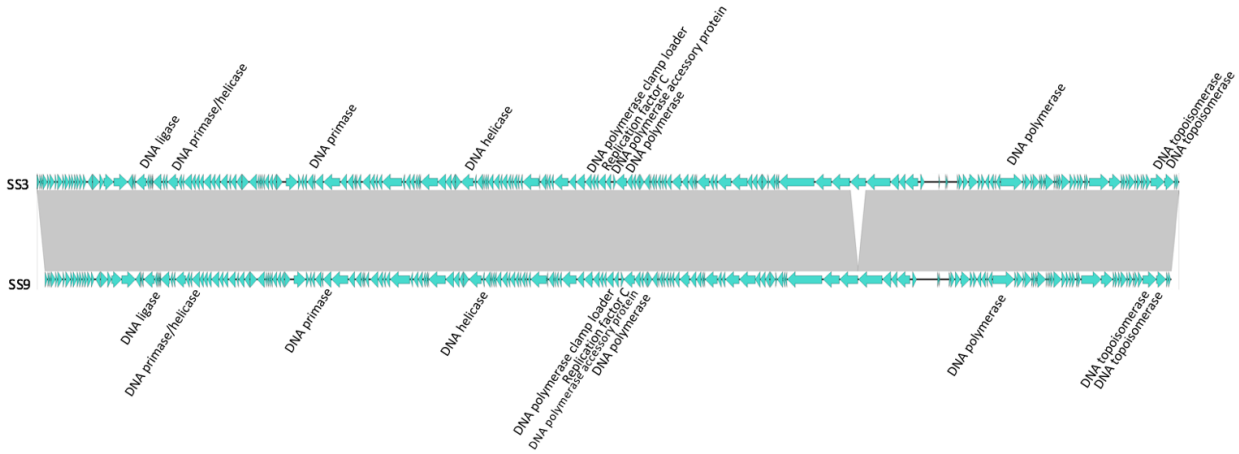

Figure 3.4. Linear whole genome comparison of phages in Cluster 3. ORFs encoding for DNA replication elements are indicated. Grey regions indicate nucleotide homology of >96%. Directions indicate the direction of transcription.

3.3.6 Identification of genes encoding for putative virulence factors

The selection of phages devoid of genetic elements that could pose a risk to human health is critical to biological control applications (Goodridge et al., 2018). Phages can transfer DNA between hosts via transduction (Howard-Varona et al., 2017), which may result in the insertion or deletion of cryptic and/or functional genetic elements, and alter host phenotype (Goh, 2016). These genetic elements may reside in the phage genome for extended durations until a susceptible host is encountered (Howard-Varona et al., 2017).

Some phages harbored one or more copies of a polymyxin resistance protein ArnC of 311 amino acids (Table 3.2), which has also been identified in P22-like viruses 103203_sal5, 146851_sal4, 103203_sal4 and 101962B_sal5, albeit shorter by an amino acid (Paradiso et al., 2016). Naturally synthesized by the bacterium *Bacillus polymyxa*, the polymyxins are a family of last-resort oligopeptide antibiotics used in human medicine that bind to LPS of Gram-negative bacteria, increasing membrane permeability and leakage of intracellular material (Abraham & Kwon, 2009). Alterations in the moieties comprising the LPS may confer resistance to polymyxins. For instance, the synthesis and transfer of 4-amino-L-arabinose to the LPS is carried

out by multiple genes in the *arn* operon (Moskowitz et al., 2012; Abraham & Kwon, 2009), therefore it is unclear if alterations in one gene in this locus would confer resistance to polymyxin. Phage genomes possessing *arnC* grouped into Clusters 1 and 8, which also comprise putatively temperate phages, suggesting a specialized transduction mechanism (Figure 3.1). Further, most phages possessing *arnC* were sourced from sewage (SE21, SE22, SE16, SE10 and SE1), a known reservoir of AMR genes, and "hotspots" of horizontal gene transfer (Karkman, 2018). Gene *arnC* has also been identified in *Salmonella* phages 22 and 34 isolated in India (Karpe et al., 2016). However, the absence of AMR elements in putatively lytic phages highlights their relatively low frequency of generalized transduction, and suitability for biocontrol.

We also identified virulence factors in a small subset of phages (Table 3.2). Virulence factors are naturally found in a broad variety of foodborne pathogens and contribute to enhanced host invasion and environmental fitness (Fong et al., 2016). Cluster 3, comprising phages SS3 and SS9 from sediment, carried a gene encoding an identical large virulence protein VriC of 1,613 amino acids (Table 3.2). SE14, which could not be assigned to a specific cluster, also possessed VriC which possessed 99% amino acid identity to that of the Cluster 3 phages. Homologs of VriC have been found to occur elsewhere, for instance, in *Salmonella* phages SFP10 (99.32% amino acid identity), Sh19 (99.01% amino acid identity) (Parmar et al., 2018) and *E. coli* phage PhaxI (99.13% amino acid identity) (Shahrbabk et al., 2013). Interestingly, *Salmonella* phage 38 appears to possess a truncated form of VriC of 465 amino acids (Karpe et al., 2016). Although phage-encoded, the origin and function of this protein is unclear, therefore it is unknown if homologs possessing near-identical amino acid sequences would possess the same function. Of the newly isolated phages SS3, SS9 and SE14 are not classified as temperate,

suggesting a generalized transduction mechanism. Although the frequency of generalized transduction is quite rare (Howard-Varona et al., 2017), it has been shown to transfer large genome cassettes and pathogenicity islands (Goh, 2016). However, it is unclear if these phages possess a high-transducing frequency, and if *vriC* was transduced into a bacterial host would result in a functional virulence factor.

An exopolysaccharide production protein ExoZ was defined in phages in Clusters 2 and 9. *ExoZ* has been identified in a limited set of phages, including PhWands-1 and PhWands-2 (Moreno Switt et al., 2012). Functionally, this locus encodes virulent effector proteins, as found in clinically-relevant strains of *Pseudomonas aeruginosa* found in cystic fibrosis patients (Mitov et al., 2010). Additionally, some species of *Rhizobium* encode a homolog of ExoZ involved in the acetyl modification of succinoglycan, an exopolymer (York et al., 1998). However, other genes in the *exo* locus are involved in the production of exopolysaccharide (Aird et al., 1991), therefore, if introduced alone it is unlikely to cause phenotypic conversion. Nonetheless, as exopolysaccharide production is involved in biofilm formation (Aird et al., 1991), phages encoding these genes should not be used to control bacterial pathogens, particularly since the rate of transduction is not known.

3.4 Conclusions

Here we described and compared 45 newly isolated *Salmonella* phage isolates on both their basis for biocontrol and biodiversity. Overall, patterns of diversity of the *Salmonella* phages isolated from British Columbia, Canada are complex, although some similarities in the whole genome, MCP sequences and morphotypes occurred among phages isolated from different sites. A novel broad host range phage (SE13) that is genetically distinct from other phage, and which

shows no evidence of virulence-associated genes, represents a promising biocontrol agent against *Salmonella*.

We found several putative virulence genes (e.g., *arnC, vriC, exoZ*) in our phages which have only been reported in a few studies (Karpe et al., 2016; Moreno Switt et al., 2012; Switt et al., 2013). We also saw evidence for a novel association between genome size and DNA metabolism and GC content in our phages, suggesting links between these genes and enhanced phage replication. The carriage of these genetic elements provides insight into the phage-host interactions and, provided they are appropriately assessed on a genetic level, suggests that our phages may be good candidates for future pathogen mitigation strategies.

The advent of high-throughput sequencing has led to an explosion of insight into microbial genomes; although, sequencing of phages has lagged behind that of bacteria, despite their critical roles in bacterial evolution. The characterization of this collection of phages contribute to the limited knowledge surrounding phage diversity and phage-host interactions, and will aid with the development of biocontrol strategies against *Salmonella*.

Chapter 4: Examination of antimicrobial resistance and virulence potential of phage-resistant *Salmonella*

4.1 Introduction

Prior to the development of effective phage-based applications, it is essential that the interaction of the phages with their target hosts be elucidated (Chan et al., 2013). It has been shown experimentally that host bacteria readily develop resistance to phage, particularly at a high MOI (which would likely be the case with using phages for food biocontrol) (Fong et al., 2017; Pereira et al., 2016a), and moreover, reports have documented phage resistance in nature (Azam et al., 2019; Diaz-Munoz, 2014). Although this phenomenon is widespread, there is currently little known about the host phenotype following development of resistance to phage. Therefore, it is important to characterize and elucidate these behaviours and interactions in order to delay or eliminate the develop of resistance, particularly when developing informed phage-based antimicrobials and optimizing their method of application.

Phages have been explored as an alternative treatment for the inactivation of bacteria that possess resistance to antibiotics (Mattila et al., 2015), as it is known that novel antibiotic discovery is not only expensive, but time-consuming, laborious and relies almost exclusively on microbial sources (Donadio et al. 2010; Fernandes & Martens, 2017). Indeed, pharmaceutical companies are withdrawing from this venture due to its unprofitability (Clarke, 2003). Previous reports have demonstrated the successful use of phages in alleviating the load of antimicrobial resistant bacteria in several contexts, such as in human medicine (Mattila et al., 2015; Weber-Dabrowska et al., 2016) and the food industry (Endersen et al., 2014). Unfortunately, the emergence of bacteriophage-insensitive mutants (BIMs) of bacteria has also been reported (Filippov et al. 2011; O'Flynn et al. 2007). BIMs may acquire phage resistance through several

measures, such as restriction-modification, abortive infection, CRISPR/Cas9 mechanisms, and commonly, through mutations in phage receptor sites, thereby preventing phage attachment (Seed, 2015). Additionally, it is known that developing resistance to phage may come with an associated fitness cost to the host, where evolution of an advantageous trait simultaneously reduces performance in another trait. Previous studies have observed slower growth rates (Avrani et al., 2011; Tan & Darby, 2005), decreased virulence (Filippov et al., 2011) and diminished resistance to various antimicrobials (Chan et al., 2016) in BIMs. This fitness cost has been shown to vary across genera and species of bacteria (Ferenci et al., 2015), and there has currently been only nominal work performed in foodborne pathogens, especially *Salmonella*. As a safety consideration, it is critical that the behavior of *Salmonella* BIMs be evaluated, especially in the clinical context. Therefore, the objective of this study was to investigate the fate of phage-resistant AMR *Salmonella* in the context of their said AMR and virulence potential.

4.2 Methods

4.2.1 Bacterial maintenance & growth conditions

A *S.* Agona FSL S5-517 strain of human origin was studied for its AMR upon development of phage resistance as it is resistant to sulfisoxazole (SUF), sulfamethoxazole/trimethoprim (SXT) and tetracycline (TET). Stocks were maintained at -80°C in Brain-Heart-Infusion broth (BD/Difco, East Rutherford, NJ, United States) supplemented with 20% glycerol. Working stocks were prepared on tryptic soy agar (TSA; BD/Difco, East Rutherford, NJ, United States) and maintained at 4°C for a maximum of one month. Prior to experiments, fresh overnight cultures were prepared by inoculating a single colony into five ml tryptic soy broth (TSB; BD/Difco, East Rutherford, NJ, United States). Cultures were incubated for 20 h (stationary phase) at 37°C with gentle shaking at 170 rpm.

4.2.2 Isolation of BIMs of *S.* Agona FSL S5-517

Phage SI1, an obligate lytic phage, was previously isolated and characterized in our laboratory as described in Chapter 2. It was selected in thus study due to strong infectivity against *S.* Agona FSL S5-517 (Fong et al., 2017; also see Chapter 2 (Figure 2.1)). Pure phage lysates were stored at 4°C in SM buffer until further analysis.

Putative BIMs were isolated according to (Pereira et al., 2016a), with modifications. Stationary phase cultures of *S.* Agona were prepared (according to section 2.2.1) and a bacterial lawn made by mixing 300 µl of culture and four ml 0.7% TSA top agar together, then poured onto 1.5% TSA and allowed to set at room temperature. Subsequently, five µl of phage SI1 (~10^9 PFU/ml) was spotted onto the bacterial lawns. Plates were incubated at 37°C for 48 hours to allow for growth of isolated colonies (i.e., putative BIMs) in the centers of lysis zones. Colonies growing in the lysis zones were inoculated into five ml TSB, grown overnight at 37°C for 20 h and streaked onto TSA. Isolated colonies were enumerated after incubation overnight at 37°C. Simultaneously, bacterial lawns were prepared using these overnight cultures and five µl of phage SI1 (10^9 PFU/ml) spotted onto the lawns to confirm bacterial resistance. This subculture process was repeated five times to confirm the absence of a transient phage resistance phenotype (Pereira et al., 2016). Stock solutions of the BIMs were prepared in TSB supplemented with 20% glycerol and stored in -80°C for further analyses.

4.2.3 Minimum-inhibitory concentration assays

BIMs of *S.* Agona were assayed for their resistance to SUF, SXT and TET using a broth microdilution method (Wiegand et al., 2008) and compared to that of the phage-sensitive, wild-type (WT) parent strain . Prior to the MIC assay, resistance of the BIMS to phage SI1 was

confirmed by spotting five µl of phage SI1 (10^9 PFU/ml) onto a bacterial lawn of *S.* Agona, followed by incubation for 18 h at 37°C.

All strains were grown overnight at 37°C in 10 mL TSB for 20 h in triplicate. Cells were harvested by spinning one ml aliquots at 5,000 x g for ten min. Cells were washed three times in phosphate buffered saline (PBS; Amresco, Solon, OH, USA) and resuspended in cation-adjusted Mueller Hinton broth (MHB; Amresco). Aliquots were then transferred in duplicate to 96-well plates containing twofold dilutions of SUF, SXT and TET successively diluted in cation-adjusted MHB (Amresco), to a final concentration of 5 x 10^4 CFU/ml. Plates were subsequently incubated at 37°C for at least 16 h. Growth in the wells, as indicated by turbidity, indicated resistance to the antibiotic. Controls using antibiotic-sensitive *Salmonella* strains were also included. The lowest concentration of each antibiotic which prevented growth of bacteria was deemed the minimum inhibitory concentration (MIC). This assay was performed in triplicate.

4.2.4 Adhesion & invasion assays

4.2.4.1 Caco-2 cell maintenance and differentiation

Caco-2 cells (human colon enterocyte-like; HTB-37, American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco Minimum Essential Medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (Invitrogen, Burlington, ON, Canada) and 100 U/mL penicillin and 100 µg/mL of streptomycin (Sigma) at 37°C in 5% CO_2. Cells were sub-cultured weekly using a 1:10 split ratio with medium changes every two to three days. The passage number used was 20-40.

For cell differentiation, a 24-well tissue culture plate (1.93 cm^2) was seeded with Caco-2 cells at a density of approximately 5 x 10^5 cells/well in DMEM. Cells were allowed to differentiate for 21 days in 5% CO_2, with culture medium changed every two days.

4.2.4.2 Adhesion & gentamicin protection assays

S. Agona strains were grown in triplicate in five ml TSB at 37°C with gentle shaking for 20 h. Bacterial cells were harvested by centrifugation at 5000 x g for 10 min and washed three times with PBS, followed by final resuspension in DMEM without antibiotic supplementation. Prior to the gentamicin protection assay, phage resistance of the BIMs to phage SI1 was confirmed by spotting five µl of phage SI1 (10^9 PFU/ml) onto a bacterial lawn of *S.* Agona, followed by incubation for 18 h at 37°C.

Caco-2 cells were replenished with DMEM without antibiotics 24 hours prior to the invasion assay in order to ensure the viability of invading bacteria. Briefly, seeded cells were washed with PBS and infected with *S.* Agona at a final concentration of 5 x 10^6 CFU/well (MOI=10) in duplicate. Infected Caco-2 cells were then incubated at 5% CO_2 for one h to allow for adhesion and two h to allow for invasion (Bolton et al., 2019; Yamada et al., 2006). The plates were then aspirated and washed three times with 1X PBS to eliminate non-adherent *Salmonella*.

To test for adherence, 500 µl of 0.1% Triton X-100 (Amresco) was added to each well to lyse the Caco-2 cell monolayers and gently mixed by pipetting. Then, 100 µl of the cell suspensions were subsequently serially diluted in PBS and spread-plated onto TSA in duplicate. Plates were incubated at 37°C for enumeration.

In a separate assay to test for invasion, 100 µg/ml of gentamicin (Amresco) was added to each well to eliminate adherent, non-invaded bacterial cells. Plates were incubated at 5% CO_2 at 37°C for two h. Wells were then washed twice with PBS. Subsequently, 500 µl of 0.1% Triton X-100 was added to lyse the Caco-2 cells and gently mixed to release intracellular *Salmonella*. The invading bacteria were immediately serially diluted in PBS and spread-plated onto TSA in

duplicate. Plates were incubated at 37°C for enumeration and colonies counted following 18 ± 2 h of incubation.

4.2.5 DNA preparation, sequencing and variant calling

Genomic DNA was extracted from overnight lysogeny broth cultures (Amresco) at 37°C using the E-Z 96 Tissue DNA Kit (Omega Biotek, Norcross GA, USA) and according to the manufacturer's instructions. Subsequently, 500 ng of genomic DNA was mechanically fragmented for 40 seconds by Covaris M220 (Covaris, Woburn MA, USA) using default settings. Libraries were synthesized using the NEBNext Ultra II DNA library prep kit for Illumina (New England Biolabs, Ipswich MA, USA) according to manufacturer's instructions and were sequenced to obtain 30X of coverage in an Illumina MiSeq 300 bp paired-end run at the Plateforme d'Analyses Génomiques of the Institut de Biologie Intégrative et des Systèmes (Laval University, Quebec, Canada). Each genome was assembled *de novo* with the A5 pipeline version A5-miseq 20140521 (Tritt et al. 2012). Contigs were mapped to a reference genome, *S.* Agona SL483 (Accession: PRJNA20063) using Mauve version 2.4.0 (Darling et al., 2004). Sequence data was then uploaded to the Galaxy platform, where the public server at usegalaxy.org was used for data analysis (Afgan et al., 2018). Adapters were trimmed using the Trimmomatic tool with default parameters (Bolger et al., 2014). Prokka was used for annotation of the reference genome (Seemann, 2014). Sequencing reads of the BIMs were aligned to the WT reference genome using Bowtie2 (Langmead & Salzberg, 2012), followed by local re-alignment using Realigner Target Creator (McKenna et al., 2010). Variants (insertion/deletions (indels)), single nucleotide polymorphisms (SNPs) and multi nucleotide polymorphisms (MNPs)) were called using FreeBayes (Garrison & Marth, 2012). High quality variants were

selected if they possessed a minimum mapping coverage of 8 and a minimum quality score of 20 (90%) (Den Bakker et al., 2014).

4.2.6 Statistical analysis

For the gentamicin protection assay, invasion efficacy was represented as the average number of cells invaded (in CFU) of three biological replicates. Means of the BIMs were compared to that of the WT strain using a one-way ANOVA. Statistical analyses were performed using JMP version 11.1.1 (SAS Institute, Inc., Cary, NC, United States). A *P*-value of ≤0.05 was considered statistically significant.

4.3 Results & discussion

4.3.1 General characterization of *S.* Agona BIMs

Five BIMs of *S.* Agona FSL S5-517 were isolated and designated Δ87, Δ95, Δ96, Δ99 and Δ102. Overall, this assay was performed 200 times, and in many instances we observed a transient, unstable resistance phenomenon that disappeared upon further propagation. Therefore, the rate of resistance development was 2.5% when SI1 was used as the infecting phage.

BIMs did not display marked differences in morphology nor growth rate than that of the parent strain (Figure C1), despite distinct morphologies and changes in growth rate observed in previous studies (Avrani et al., 2011; Tan & Darby, 2005). A range of mutations (including SNPs and indels) were observed in all BIMs, although the number of mutations and the genes mutated varied (Tables C1 to C5 inclusive). Interestingly, some genes and loci that were highly mutable across all BIMs (e.g., *ccm* family genes, *vgrG*, *solA, sufD*, etc), suggesting common mechanisms of cellular modification with phage resistance.

4.3.2 Lipopolysaccharide mutations in BIMs

Two mutants, Δ95 and Δ96, were found to have several identical mutations in *rfaL* and *rfaY*, respectively encoding an O-antigen ligase and LPS core heptose II kinase, proteins involved in LPS synthesis. Variants in these loci comprised SNPs and insertions of varying length, which induced non-synonymous, synonymous and frameshift mutations (Table 4.1). As no other mutations were observed in surface-associated molecules, it seems likely the LPS is the receptor site of phage SI1. *Salmonella* type phage Felix-O1, capable of infecting 99% of Salmonellae, has also been shown to utilize the bacterial LPS for adsorption (Kim et al., 2014), and mutations in loci responsible for LPS synthesis and structure have been previously observed in BIMs of numerous bacteria, including *Salmonella* (Kim et al., 2014), *Yersinia pestis* (Filippov et al., 2011) and *E. coli* (Li et al., 2019). Mutations within *rfaL* disrupt O-antigen biosynthesis and has been previously reported to induce phage resistance of *S.* Typhimurium (Park et al., 2012; Shin et al., 2012) and *E. coli* O157:H7 (Park et al., 2012). Alternatively, deletions within *rfaJ*, *rfaI*, *rfaG*, *rfaF* and *rfaC* disrupted synthesis of the LPS outer core and induced host resistance to *Salmonella* phage SSU5 (Kim et al., 2014). Given the available literature, it is unclear if mutations in *rfaY* would induce a significant change in the LPS core and if this would further result in phage resistance. Therefore, the current data suggests that the O-antigen of the LPS is the site of attachment by phage SI1, although the possibility of outer core specificity cannot be definitively ruled out.

Table 4.1. Mutations in genes involved in lipopolysaccharide synthesis and type VI secretion system of the *S.* Agona BIMs. Mutations presented possess a minimum quality score of 20 and a minimum mapping coverage of 8.

Affected gene	Putative function	Strand	Gene nt position[a]	Strain	Mutation	Sequence [b]	Impact on polypeptide synthesis
rfaL	O-antigen ligase	+	425	WT	3 bp insertion	G------A	Frameshift
				△95		GGATA	
				△96		GGATA	
			429	WT	Substitution	G	None
				△95		A	
				△96		A	
			442	WT	4 bp insertion	T-------A	Frameshift
				△95		TGGGA	
				△96		TGGGA	
rfaY	Lipopolysaccharide core heptose (II) kinase	-	514	WT	23 bp insertion	A---------- A	Frameshift
				△95		AGCGAAGCCCTAAACTTGTTAAAAA	
				△96		AGCGAAGCCCTAAACTTGTTAAAAA	
			538	WT	Substitution	T	Asn --> Lys
				△95		G	
				△96		G	
vgrG1_2	Actin cross-linking toxin, structural tip protein, involved in type VI secretion	+	792	WT	6 bp insertion	T----------	Frameshift
				△87		TCAAGGA	
				△95		TCAAGGA	
				△96		TCAAGGA	
				△99		TCAAGGA	
				△102		TCAAGGA	
			798	WT	Multi-nucleotide substitution	ATTTT	Leu --> Phe Phe --> Gly Tyr --> His
				△87		CGGCC	
				△95		CGGCC	
				△96		CGGCC	
				△99		CGGCC	
				△102		CGGCC	
			810	WT	4 bp insertion	A---------T	Frameshift
				△95		AGAACT	
				△96		AGAACT	
			816	WT	2 bp insertion	T----C	Frameshift
				△95		TCAC	
				△96		TCAC	

			820	WT △95 △96	Substitution	G C C	Gly --> Arg
			823	WT △95 △96	Substitution	G T T	Gly --> Stop codon
			825	WT △95 △96	Substitution	A G G	None
vgrG1_2	Actin cross-linking toxin, structural tip protein, involved in type VI secretion	+	828	WT △95 △96	3 bp deletion	AGGA A------ A------	Frameshift
			831	WT △95 △96	Substitution	C G G	Asp --> Glu
			836	WT △95 △96	Substitution	C A A	Ala --> Glu

[a] relative to wild-type strain
[b] presented in 5’ to 3’ direction

Mutations in genes involved in LPS synthesis were not identified in Δ87, Δ99 nor Δ102, although they demonstrated resistance to phage SI1 (Table 4.1). It may be hypothesized that these strains possess adaptive immunity mechanisms that obscure the correlative relationship between LPS mutations and phage resistance, for instance, CRISPR and/or other adaptive strategies (e.g., restriction-modification, superinfection exclusion, etc.). Therefore, we used CRISPRFinder (Grissa et al., 2007) to identify CRISPR elements in the genomes of the wild-type and derivative strains. Although there were 26 CRISPRs identified (Table C6), the spacer sequences did not possess homology to any known phages, including SI1. It is thus possible that a multitude of other innate immunity mechanisms, including restriction-modification, superinfection exclusion and abortive infection, could play a role in the phage resistance of these strains (Seed, 2015). Future work should aim to identify and functionally characterize known and novel gene cassettes contributing to this phenotype.

4.3.3 Antibiotic sensitivity of BIMs

Compared to the WT strain, BIMs were not altered in their relative sensitivities to SXT nor SUF, exhibiting MIC values of 180/3500 μg/mL (trimethoprim/sulfamethoxazole, respectively) and 2048 μg/mL, respectively (Table 4.2). According to the designated breakpoint values of the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), these MIC values indicate resistance to SXT and SUF (CIPARS, 2016).

Interestingly, Δ96 showed enhanced sensitivity to TET, exhibiting a MIC_{TET} of 76.8 μg/mL compared to an MIC_{TET} of 128 μg/mL as displayed by the other strains. Despite a 60% reduction in MIC, Δ96 is still categorized as TET resistant according to CIPARS (2016). Additionally, it was not known why sensitivity to TET was conferred only to Δ96; the mutations

and phenotypes appear to be spontaneous and/or random, consistent with other studies in phage-resistant bacteria (Yang et al., 2019).

In phage-resistant bacteria, enhanced antibiotic sensitivity may occur if a cellular target represents the recognition site for both antibiotic and phage (Chan et al., 2016). In a targeted approach, phages infecting MDR *Pseudomonas aeruginosa* were isolated that utilized multidrug efflux pumps as receptors (Chan et al., 2016). Phage-resistant mutants of *P. aeruginosa* that possessed mutations in these regions subsequently exhibited enhanced susceptibility to several classes of antibiotics, including ceftazidime, ciprofloxacin, tetracycline and erythromycin, in a genetic trade-off observed as a result of phage infection (Chan et al., 2016).

Table 4.2. Minimum inhibitory concentrations of the *S.* Agona strains. MICs were determined by conducting each test in triplicate.

Strain	MIC (μg/mL)		
	SXT[a]	TET	SUF
Wild type	180/3500	128	2048
△ 87	180/3500	128	2048
△ 95	180/3500	128	2048
△ 96	180/3500	76.8	2048
△ 99	180/3500	128	2048
△102	180/3500	128	2048

[a] Values refer to the MICs of trimethoprim and sulfamethoxazole, respectively.

As previously determined, it is plausible the LPS is the likely receptor site of phage SI1, given the presence of mutations displayed by BIMs in this locus (Table 4.1). However, as

TET specifically binds to regions of the 16S rRNA molecule (Mikula et al., 2014; Pringle et al., Johansson 2007), the direct mechanism behind development of sensitivity is unclear, although genetic tradeoffs as a result of phage resistance have been commonly observed (Ferenci, 2015); it is known that there are cellular costs associated with phage immunity (Seed, 2015; Vale et al., 2015; Vasu & Nagaraja, 2013).

Of the four genes encoding proteins involved in TET resistance (i.e., resistance proteins class B (1), C (2) and TET regulator class A (1)) identified in *S.* Agona FSL S5-517, no observed mutations were sustained as a result of phage resistance (Tables C1 to C5 inclusive). It is possible that alterations in transcriptional activity may account for the observed phenotype. For instance, as a result of phage ∂Abp1 infection, significant ($p<0.05$) downregulation of three antibiotic resistance genes were observed in MDR *A. baumanii* following global transcriptome analysis (Yang et al., 2019). The enhanced expression of efflux pumps in both phage-resistant *Campylobacter jejeuni* and *P. aeruginosa* has also been documented (Blasdel et al., 2019; Sacher et al., 2018), leading to the speculation that efflux pumps and other AMR proteins may possess concerted roles in phage resistance. As lytic phages are currently being considered for control of antimicrobial resistant bacteria, further work into understanding the influence of phage-resistance on ARGs (and particularly, those of AMR *Salmonella*) is clearly warranted.

4.3.4 Adhesion and invasion assays

Adherence represents an important initial step in epithelial cell invasion (Moroni et al., 2006). Rates of adherence of the BIMs were not significantly ($p>0.05$) different than that of the parent strain (Table 4.3). Concordantly, no observed mutations were present in structures involved in adherence (e.g., pili, flagella) (Tables C1 to C5 inclusive). There were, however, a number of mutations observed in various secretion systems across the different BIMs (e.g., Type

IV and Type VI secretion systems). Nevertheless, it appears that the adhesion proteins involved in these secretion systems were not affected (Tables C1 to C5 inclusive). To the best of our knowledge, there are currently no published reports regarding the adhesion properties of phage-resistant *Salmonella*, therefore these results shed light on the phenomena that underlie phage resistance. It is interesting to speculate, however, that adhesion may be affected if adhesion-associated structures (e.g., pili, flagella, etc.) were also used for phage adsorption.

Table 4.3. Caco-2 cell adherence of *S.* Agona strains. The adherence assay was conducted in triplicate and are represented as means ± SD. Means of the BIMs were compared to that of the WT. Different letters indicate significance below α=0.05 (one-way ANOVA).

Strain	Total cells adhered (log CFU ± SD)
WT	6.22 ± 0.23^{A}
△ 87	6.65 ± 0.33^{A}
△ 95	6.28 ± 0.21^{A}
△ 96	6.01 ± 0.19^{A}
△ 99	6.48 ± 0.38^{A}
△ 99	6.56 ± 0.44^{A}

Strains of *S.* Agona displayed varied invasion efficacies (Figure 4.1). Compared to the WT strain, the invasion capacities of Δ87, Δ99 and Δ102 were not significantly affected ($p>0.05$). However, the number of invaded cells of Δ95 and Δ96 showed a decrease of 1.71 ± 0.29 and 1.45 ± 0.31 log CFU, respectively. The attenuated virulence of phage-resistant bacteria has also been identified in other reports; Santander & Robeson (2007) observed that loss of the O-polysaccharide on the LPS of *S.* Enteritidis conferred resistance to phage infection and these

mutants were resultantly avirulent in the nematode *Caenorhabditis elegans*. It is known that *S.* Typhimurium requires an intact O-polysaccharide to trigger programmed cell death in *C. elegans* (Aballay et al., 2001), and loss of this constituent was correlated with loss of virulence. Similarly, the outer core of the LPS is required for entry of *S.* Typhi into epithelial cells (Hoare et al., 2006). Although we also observed mutations in the LPS biosynthesis genes (Table 4.1), we did not use a systemic model of infection, therefore our results are not directly comparable. Mutations in other cellular structures contributing to phage adsorption (e.g., flagella, outer membrane proteins, etc.) have also been associated with attenuated virulence (Feldman et al., 1998; Josenhans & Suerbaum, 2002; Laanto et al., 2012; Yamaoka et al., 2002). Furthermore, it is known that the maintenance of innate and adaptive bacterial immunity (e.g., restriction-modification, CRISPR-Cas systems) have associated cellular costs (Vale et al., 2015; Vasu & Nagaraja, 2013).

Variant analysis revealed several mutations in components involved in the Type VI secretion system (T6SS) of SPI-19 that plays a role in intestinal infection (Table 4.1) (Blondel et al., 2013; Sana et al., 2016). The T6SS has been recently identified in several *Salmonella* serovars and exhibit homology to the contractile components of phages, delivering cytotoxic effector proteins via spikes that enter the host cell (Basler, 2015; Pukatzki et al., 2006; Schroll et al., 2019), although many of the core structural components have not been extensively characterized on a functional level (Ho et al., 2014). Genes encoding Hcp (hemolysin coregulated protein) and three non-identical copies of VgrG (VgrG1_1, VgrG1_2 and VgrG-2), hallmarks of the T6SS (Zong et al., 2019) were identified in *S.* Agona FSL S5-517, together which form the tube and needle-like apparatus of the T6SS (Cascales & Cambillau 2012). Given the dual functionality of VgrG in T6SS structure and cytotoxicity (Bingle et al., 2008; Cascales

& Cambillau, 2012), it is unclear if these genes are individually involved in tip structure, host cytotoxicity, or both. Downstream of the core genes is an *rhs* element that likely encodes a secretion apparatus (Das & Chaudhuri, 2003). Upstream, the *tss* locus was identified, encoding for structural proteins involved in formation of the T6SS baseplate (Ho et al., 2014). Interestingly, the switch in the direction of transcription indicates that different sections of the T6SS apparatus are transcribed and assembled separately (Figure 4.2).

VgrG proteins, in particular, are believed to possess dual functionality as both a cell-puncturing device (Cascales & Cambillau, 2012) and secreted effector protein, resulting in intracellular toxicity, most notably actin modification (Bingle et al., 2008). It has been previously demonstrated that the T6SS of *S.* Gallinarum is required for survival in the infected macrophages of chicks (Blondel et al., 2013). Additionally, *vgrG* deletion mutants of *A. baumanii* were compromised in their adhesion, growth rate and invasion (Wang et al., 2018), as we observed in the present study. Interestingly, mutants also showed enhanced susceptibility to chloramphenicol, demonstrating the dual roles of T6S in both virulence and AMR (Wang et al., 2018).

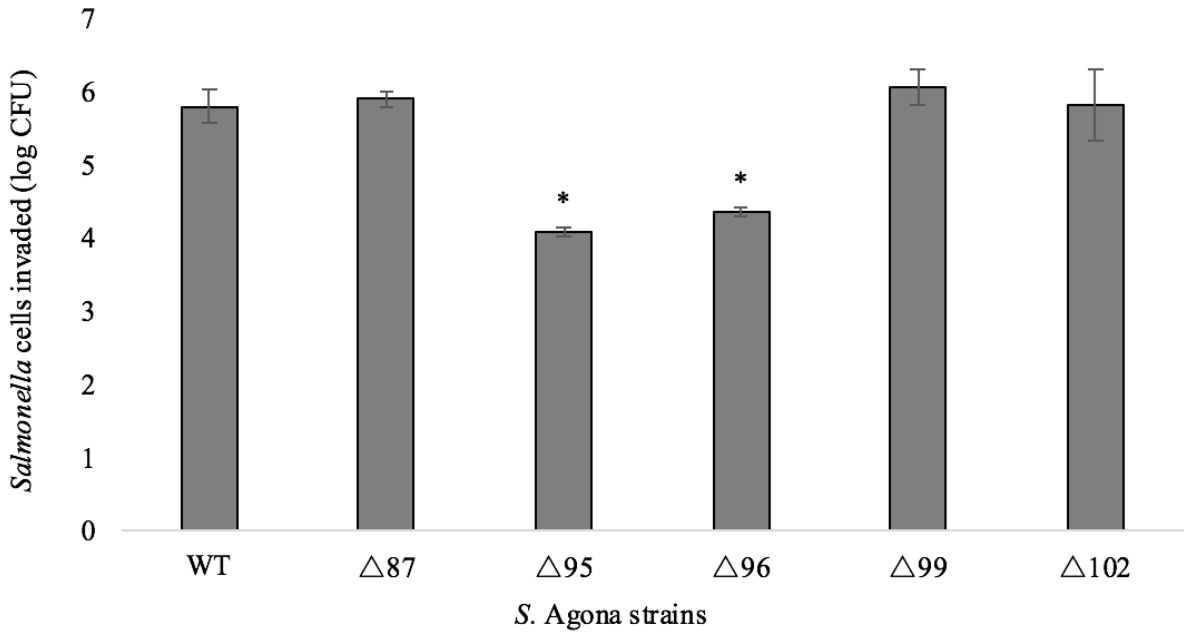

Figure 4.1. Caco-2 cell invasion capacity by the *S.* Agona strains. Error bars indicate the standard deviations of the means of three biological replicates. Means of the BIMs were compared to that of the WT. Asterisks indicate significance below α=0.05 (one-way ANOVA).

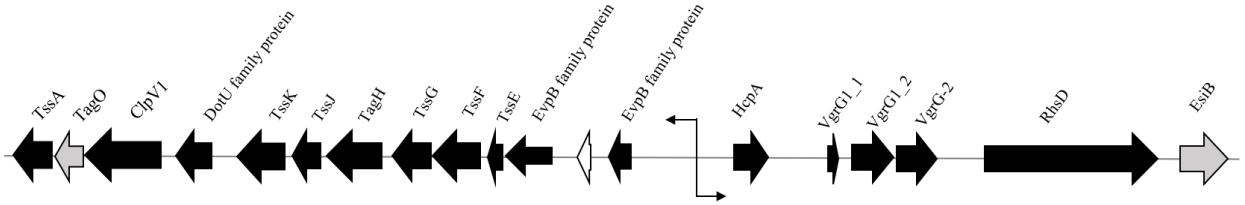

Figure 4.2. Schematic representation of the T6SS gene cluster in *S.* Agona FSL S5-517. Arrows indicate direction of transcription. Black arrows indicate gene products involved in the T6SS. Grey arrows represent gene products of unknown function, but believed to play a role in the T6SS. White arrow indicates a hypothetical protein.

Mutation hotspots, regions where mutations clustered in close proximity, were observed in all BIMs regardless of invasion efficacy, indicating that *vgrG*, and/or regions therein, may be

highly mutable in conditions where phage is present (Table 4.1). In all BIMs, a six-bp insertion induced a frameshift at nucleotide position 792 of *vgrG1_2*, indicating the selective pressure to accumulate indels in multiples of three in an attempt to preserve the reading frame (Danneels et al., 2018; Williams & Wernegreen, 2013). Three downstream polymorphisms also resulted in the conversion of three amino acids (Table 4.1). Interestingly, these mutations did not affect the invasion capacities of Δ87, Δ99 nor Δ102, indicating that these variant sites may preserve the protein structure and/or deleterious effects were minimized due to the indel's close proximity to the 3' end of the gene (951 bp in length) (Williams & Wernegreen, 2013).

Strains Δ95 and Δ96 were shown to be significantly less invasive ($p<0.05$) than the WT strain (Figure 4.1) and we observed a higher number of mutations in the *vgrG* genes than the other BIMs (Table 4.1). Notably, a G to T substitution at nucleotide position 823 induced translation of a stop codon in both BIMs, resulting in a truncated protein and potentially negating any neutral effects of upstream mutations we observed in all BIMs. As mutations were only observed in *vgrG1_2*, it is possible that *vgrG1_1* may partially compensate for loss of invasion efficacy, particularly since the complete loss of virulence was not observed in the present study. However, the extent of this compensation is unknown since the proteins do not share identity and may possess different functions. To our knowledge, this is the first report of mutations in the T6SS as a result of phage resistance in non-typhoidal *Salmonella*.

Differential patterns of gene expression following phage resistance have also been investigated in several studies, although there is a paucity of literature regarding the attenuation of virulence in phage-resistant, non-typhoidal *Salmonella.* In a global transcriptome analysis of phage-resistant *A. baumanii*, the expression of *vgrG* was downregulated by two-fold and a subset of seven other genes involved in Type II, V and VI secretion were also significantly

downregulated (Yang et al., 2019). Separately, other reports have also observed the upregulation of virulence factors as a result of phage resistance (Hosseinidoust et al., 2013, Sistrom et al., 2015). Phage-resistant variants of *P. aeruginosa* PAO1 displayed an upregulation of up to 108.4-fold in key genes involved in Type II, III and VI secretion, which was confirmed with the decreased viability of infected mammalian cells. Given the diverse phenotypes associated with phage resistance, the assessment of *Salmonella* BIMs on their virulence potential, therefore, is clearly warranted, especially when considering human safety in biocontrol applications.

4.4 Conclusions

In considering phages for biocontrol, it is of critical importance to assess the interactions between the phage and the host, particularly since this interaction may determine the success of this novel therapy for such applications. It is known that the host may develop resistance to phage, particularly in environments with continued phage exposure (Oechslin, 2018). Spontaneous mutations in host bacteria affecting fitness, virulence and AMR have been correlated with this phenomenon (Chan et al., 2016; Filippov et al., 2011; Yang et al., 2019).

In assessing the phenotypes of spontaneous BIMs of AMR *S.* Agona, we observed enhanced susceptibility to TET and decreased invasion efficacy in a differentiated Caco-2 cell model. These phenotypes were correlated with mutations in the host LPS, indicating the site of phage attachment, and a plethora of mutations (most notably, truncations in Δ95 and Δ96) in *vgrG1_2*, a core gene of the T6SS. It is important to emphasize that this association should be experimentally confirmed in future studies using targeted genetic approaches (i.e., through functional characterization). Additionally, *in vivo* assays using systemic models of infection will further shed light on the pathogenesis of these BIMs. Although mutations were not observed in host TET resistance genes, it can be speculated from this study that downregulation of AMR

genes following phage resistance may account for this phenotype, as has been observed by others (Yang et al., 2019). If this is the case, variant analysis would not be able to identify this association, however, it is expected that global transcriptomic analysis would elucidate these correlations.

Together, these results indicate altered AMR and virulence phenotypes of phage-resistant *S.* Agona, highlighting the phage-induced genetic tradeoffs in *Salmonella*. These data shed light onto the fate of the host following phage exposure, which is pivotal for research on incorporation of phages into cocktails for food biocontrol. Understanding such phenotypes is critical as it will undoubtedly contribute to the successful development of phage-based approaches in food.

Chapter 5: Conclusions and future directions

5.1 Conclusions

Non-typhoidal *Salmonella enterica* represents an ongoing concern in the food industry. It has the ability to adapt to different environmental niches and conditions, thereby persisting in a variety of products (e.g., fresh produce) and causing numerous outbreaks in North America, contributing to significant economic and health burdens. Given the high proportion of *Salmonella* outbreaks, there has been enhanced attention focused on alternative mitigation strategies for the control of *Salmonella* in foods, particularly foods which are deemed higher-risk to consumers (e.g., fresh produce). A potential intervention strategy which has recently drawn increased awareness is the usage of bacteriophages (phages), viruses that predate bacteria. Phages constitute a unique, but potentially desirable intervention in food, given that they are highly specific to their hosts, exhibit no toxicity to eukaryotic cells, are sustainably sourced and also can be scaled-up for commercial purposes. Additionally, they are ubiquitously distributed in nature (including food systems). However, the sheer number of phages distributed in nature is enormous, and therefore it is of importance to select and characterize phages and their host pathogen interactions in order to intelligently inform and successfully formulate phage preparations for biocontrol of *Salmonella*. Therefore, the objectives of my PhD dissertation were to characterize phages on the basis of phenotype, patterns of genomic diversity, and the behavior of host Salmonellae following phage predation.

Salmonella phages (n=97) were isolated from various regions of British Columbia. Specifically, 45 phages were investigated on their host ranges with 61 strains of non-typhoidal *Salmonella* representing diverse serovars associated with high rates of illness, recent enhanced rates of emergence in foods, and possessing antimicrobial resistance. We observed varied host

ranges; all host strains were susceptible to at least one phage in our collection apart from *S.* Rubislaw. Of these phages, five in particular, SI1, SF1, SS1, SS4 and SE13 were revealed to possess highly desirable characteristics for biocontrol, including broad host ranges and strong infectivity against *Salmonella in vitro*. Phage SI1 was also capable of reducing *S.* Enteritidis on sprouting alfalfa seeds by >2.5 log CFU/g over a two-day period.

Genomes of the 45 newly isolated phages were sequenced and factors contributing to enhanced biocontrol potential were elucidated. Screening of putative genes revealed the carriage of genes encoding integrase, virulence and antimicrobial resistance in some phages, although importantly, SI1, SF1, SS1, SS4 and SE13 were devoid of these genetic elements, indicating their potential for biocontrol. A cluster analysis of the phage genomes revealed an abundance of diversity and also novel associations between genes encoding replication-related functions, phage genome size, and the G+C content association between phage and *Salmonella* host. Generally, a larger genome size and discordant G+C content were correlated with an increased number of genes encoding replication-related functions, which may be advantageous if using these phages as biocontrol agents. Lastly, cluster analysis of the major capsid protein revealed a similar grouping pattern with that of the whole genomes, potentiating its use as a marker for genetic relatedness and potentially allowing for the pre-assignment of phages into groups based on shared genetic properties (i.e., encoding for genes allowing enhanced biocontrol efficacy).

Lastly, we aimed to uncover the mechanisms underlying phage-host interactions. As phage resistance is expected to arise, particularly with continuous, long-term phage treatments, host behavior was elucidated. Using a model system comprising AMR *S.* Agona FSL S5-517 (resistant to TET, SUF and SXT) and phage SI1, we isolated spontaneous, but stable, phage-resistant mutants of *S.* Agona *in vitro*. We observed truncations in lipopolysaccharide synthesis

genes *rfaL* and *rfaY*, indicating that the LPS is the site of SI1 attachment. We also observed enhanced susceptibility to TET and decreased invasion efficacy in a differentiated Caco-2 cell line, indicating the existence of a genetic trade-off associated with development of phage resistance in an enterocyte cell model. The decreased invasion capability in Caco-2 cells was associated with a variety of mutations in the gene encoding VgrG1_2, a protein of the Type VI secretion system. The genetic basis behind increased sensitivity to TET was not identified in our variant analysis. These results enlighten our understanding on phage-host interactions and furthermore elucidate the underlying mechanisms behind phage resistance in the host, which is critical prior to the development of phage therapies.

This work has contributed to the knowledge pool regarding the biocontrol capabilities of *Salmonella* phages, their novel genetic factors and patterns of genetic diversity, and the fate of the host following development of resistance to phage. Such information provides a framework for informing optimal phage therapy against *Salmonella* as well as valuable insight into factors underlying phage success in interventions to control pathogens in the food processing continuum from pre- to post-harvest. Additionally, this work may have further implications in phage-based technologies (e.g., detection, typing) and in sectors that utilize phage-based applications, such as human medicine and diagnostics.

5.2 Future directions

The research in this study provides the basis for a number of future studies. In particular, it would be helpful to individually assess phages isolated from Chapter 2 in their biocontrol efficacy to control a greater variety of *Salmonella* strains and serovars, and using a wider variety of complex food matrices. Data used from this future study should be considered in the formulation of a cocktail, where several phage types are used to target a broad range of non-

typhoidal Salmonellae. Interactions and potential synergistic effects of these phages in a cocktail should be thoroughly elucidated, as well as the optimal mode of storage (e.g., liquid or powder form) and application (e.g., pre-harvest, post-harvest, optimal MOI, etc.).

Patterns of diversity and the elucidation of genetic factors in biocontrol were identified in a subset of newly isolated phages (n=45) in Chapter 3, although other associations remain unclear. It would be interesting to examine the profiles of the hosts (i.e., identities, abundance) with that of the phages recovered from each site. This analysis may have further implications in using phages as predictive tools for the food industry to estimate pathogen identity and abundance. Additionally, a large number of open reading frames encoding for hypothetical proteins were identified in these phages. These novel factors may be important in determining the success of the usage of phages in various therapies, thus functional studies should be undertaken to ascertain the functions of these unknown elements. Lastly, we identified a number of genes encoding self-replication (e.g., DNA metabolism). It would be interesting to correlate the number of genes carried in these phages and their respective burst sizes, latent periods and overall infection capacity in the host, as these may be potential marker genes used to predict outcomes in phage therapy.

A number of unknowns have yet to be elucidated to explain in totality the research presented in Chapter 4. In particular, a range of mutations was identified in various genes of the BIMs which warrant further study (Table C1). For instance, a subset of genes encoding functions involved in cytochrome biosynthesis (i.e., genes within the *ccm* locus) were affected across all BIMs and given the high rate of mutability, their associated phenotypes should be elucidated. Additionally, 3/5 of the *S.* Agona BIMs did not possess truncations in LPS-related genes, the putative binding site of phage SI1. Future work should aim to determine the existence of

intracellular anti-phage mechanisms, such as restriction-modification. As several novel phage resistance mechanisms have been very recently identified (Goldfarb et al., 2015; Ofir et al., 2018) this work may identify previously unknown defense systems which would have significant implications in determining the success of phage-based therapies. Lastly, a global transcriptome analysis would prove useful for identifying altered gene expression patterns as a result of phage resistance in these BIMs. Through a variant analysis we did not identify a genetic basis for increased TET sensitivity, but assessing gene expression of various AMR genes may shed light into this mechanism. Global transcriptomic analysis may additionally elucidate novel gene expression patterns associated with phage resistance and diminished virulence in mammalian cell culture.

Appendix A: Chapter 2 supplemental table

Table A1. Newly isolated *Salmonella* phages from various sites and regions within BC. Site of isolation is reflected in the name of the phage: (B=beach sand; C=chicken environments; E=sewage influent/effluent; F=cattle feces; I=irrigation water; S=sediment; W=water of various sources). Plaque morphology indicates the mean plaque diameter of three independent measurements and the turbidity of the plaque based on a ranking system by Kutter et al. (2009). A +1 indicates extreme turbidity whereas a +4 indicates complete clearing.

	Phage	Site of isolation	BC region of isolation	Indicator *Salmonella* used for isolation	Plaque morphology
1	SB1	Beach sand	Vancouver	Montevideo FSL S5-630	0.8 mm / +2
2	SB2	Beach sand	Vancouver	Montevideo FSL S5-630	0.8 mm / +2
3	SB3	Beach sand	Vancouver	Montevideo FSL S5-630	1 mm / +3
4	SB4	Beach sand	White Rock	Montevideo FSL S5-630	0.9 mm / +2
5	SB5	Beach sand	White Rock	Montevideo FSL S5-630	1 mm / +3
6	SC1	Poultry farm	Abbottsford	Montevideo FSL S5-630	1 mm / +3
7	SC2	Poultry farm	Abbottsford	Thompson FSL S5-523	0.2 mm / +1
8	SC3	Poultry feces	Abbottsford	Montevideo FSL S5-630	1 mm / +3
9	SC4	Poultry feces	Abbottsford	Thompson FSL S5-523	0.2 mm / +1
10	SC5	Poultry feces	Abbottsford	Saintpaul FSL S5-649	3.1 mm / +4
11	SC6	Poultry farm	Abbottsford	Montevideo FSL S5-630	0.3 mm / +3
12	SC7	Poultry farm	Abbottsford	Thompson FSL S5-523	0.4 mm / +2
13	SE1	Sewage effluent	Vancouver	Saintpaul FSL S5-649	4 mm / +2
14	SE2	Sewage effluent	Vancouver	Saintpaul FSL S5-649	3 mm / +3
15	SE3	Sewage effluent	Vancouver	Saintpaul FSL S5-649	1 mm / +3
16	SE4	Sewage effluent	Vancouver	Montevideo FSL S5-630	0.5 mm / +2
17	SE5	Sewage effluent	Vancouver	Braenderup FSL S5-373	0.8 mm / +4
18	SE6	Sewage effluent	Vancouver	Braenderup FSL S5-373	0.2 mm / +2
19	SE7	Sewage effluent	Vancouver	Muenchen FSL S5-504	0.7 mm / +3
20	SE8	Sewage effluent	Vancouver	Thompson FSL S5-523	0.8 mm / +3
21	SE9	Sewage effluent	Vancouver	Saintpaul FSL S5-649	0.5 mm / +3
22	SE10	Sewage effluent	Vancouver	Saintpaul FSL S5-649	0.7 mm / +4
23	SE11	Sewage effluent	Vancouver	Thompson FSL S5-523	0.5 mm / +2
24	SE12	Sewage effluent	Vancouver	Enteritidis FSL S5-483	0.5 mm / +3
25	SE13	Sewage effluent	Vancouver	Enteritidis FSL S5-483	0.6 mm / +4
26	SE14	Sewage effluent	Vancouver	Typhimurium FSL S5-536	1 mm / +4
27	SE15	Sewage influent	Vancouver	Saintpaul FSL S5-649	3 mm / +2
28	SE16	Sewage effluent	Vancouver	Saintpaul FSL S5-649	2.7 mm / +1
29	SE18	Sewage effluent	Vancouver	Typhimurium FSL S5-536	0.5 mm / +3
30	SE19	Sewage influent	Vancouver	Muenchen FSL S5-504	0.5 mm / +4
31	SE20	Sewage effluent	Vancouver	Muenchen FSL S5-504	0.5 mm / +4

32	SE21	Sewage influent	Vancouver	Enteritidis FSL S5-483	2 mm / +2
33	SE22	Sewage effluent	Vancouver	Enteritidis FSL S5-483	2 mm / +2
34	SE23	Sewage influent	Vancouver	Braenderup FSL S5-373	0.25 mm, +2
35	SE24	Sewage influent	Vancouver	Typhimurium FSL S5-536	0.3 mm / +2
36	SF1	Cattle feces	Agassiz	Enteritidis FSL S5-483	1.5 mm / +3
37	SF2	Cattle feces	Agassiz	Enteritidis FSL S5-483	1.1 mm / +2
38	SF3	Cattle feces	Agassiz	Enteritidis FSL S5-483	1.2 mm / +2
39	SF4	Cattle feces	Agassiz	Montevideo FSL S5-630	0.5 mm / +1
40	SF5	Cattle feces	Agassiz	Montevideo FSL S5-630	0.5 mm / +2
41	SF6	#1055 fecal	Agassiz	Montevideo FSL S5-630	0.6 mm / +3
42	SF7	Cattle feces	Agassiz	Montevideo FSL S5-630	0.6 mm / +3
43	SF8	Cattle feces	Agassiz	Montevideo FSL S5-630	0.6 mm / +3
44	SF10	Cattle feces	Agassiz	Montevideo FSL S5-630	0.6 mm / +3
45	SF11	Cattle feces	Invermere	Enteritidis FSL S5-483	2 mm / +2
46	SF12	Cattle feces	Invermere	Typhimurium FSL S5-536	2 mm / +1
47	SI1	Irrigation water	Surrey	Enteritidis FSL S5-483	1 mm / +4
48	SI2	Irrigation water	Abbottsford	Montevideo FSL S5-630	0.8 mm / +3
49	SI3	Irrigation water	Abbottsford	Montevideo FSL S5-630	0.8 mm / +3
50	SI4	Irrigation water	Abbottsford	Montevideo FSL S5-630	0.8 mm / +3
51	SI5	Irrigation water	Abbottsford	Montevideo FSL S5-630	0.8 mm / +3
52	SI6	Irrigation water	Surrey	Montevideo FSL S5-630	0.6 mm / +2
53	SI7	Irrigation water	Surrey	Montevideo FSL S5-630	0.6 mm / +2
54	SI8	Irrigation water	Surrey	Montevideo FSL S5-630	0.6 mm / +2
55	SI9	Irrigation water	Surrey	Thompson FSL S5-523	0.6 mm / +3
56	SI10	Irrigation water	Surrey	Montevideo FSL S5-630	0.7 mm / +3
57	SI11	Irrigation water	Surrey	Montevideo FSL S5-630	0.6 mm / +3
58	SI12	Irrigation water	Surrey	Thompson FSL S5-523	0.7 mm / +3
59	SI13	Irrigation water	Surrey	Montevideo FSL S5-630	0.6 mm / +3
60	SI14	Irrigation water	Surrey	Montevideo S5-630	0.6 mm / +3
61	SI15	Irrigation water	Surrey	Thompson FSL S5-523	0.8 mm / +3
62	SI16	Irrigation water	Surrey	Muenchen FSL S5-504	0.5 mm / +3
63	SI17	Irrigation water	Surrey	Montevideo FSL S5-630	0.6 mm / +3
64	SI18	Irrigation water	Surrey	Montevideo FSL S5-630	0.8 mm, +3
65	SI19	Irrigation water	Surrey	Montevideo FSL S5-630	0.7 mm, +3
66	SI20	Irrigation water	Surrey	Thompson FSL S5-523	0.8 mm, +3
67	SI21	Irrigation water	Surrey	Montevideo FSL S5-630	0.6 mm, +3
68	SI22	Irrigation water	Surrey	Thompson FSL S5-523	0.7 mm, +3
69	SI23	Irrigation water	Invermere	Enteritidis FSL S5-483	3 mm / +4

70	SI24	Irrigation water	Invermere	Typhimurium FSL S5-536	0.7 mm / +1
71	SI25	Irrigation water	Invermere	Thompson FSL S5-523	0.8 mm / +2
72	SI26	Irrigation water	Invermere	Montevideo FSL S5-630	0.7 mm /+2
73	SS1	Sediment	Abbottsford	Enteritidis FSL S5-483	1 mm / +4
74	SS2	Sediment	Abbottsford	Typhimurium FSL S5-536	0.5 mm / +1
75	SS3	Sediment	Abbottsford	Typhimurium FSL S5-536	0.7 mm / +2
76	SS4	Sediment	Abbottsford	Enteritidis FSL S5-483	1.5 mm / +4
77	SS5	Sediment	Abbottsford	Enteritidis FSL S5-483	1.5 mm / +4
78	SS6	Sediment	Surrey	Montevideo FSL S5-630	0.5 mm / +2
79	SS7	Sediment	Surrey	Montevideo FSL S5-630	0.3 mm / +2
80	SS8	Sediment	Surrey	Thompson FSL S5-523	0.5 mm / +1
81	SS9	Sediment	Surrey	Saintpaul FSL S5-649	0.5 mm / +2
82	SS10	Sediment	Surrey	Montevideo FSL S5-630	0.5 mm / +2
83	SS11	Sediment	Abbottsford	Montevideo FSL S5-630	0.3 mm / +2
84	SS12	Sediment	Vancouver	Montevideo FSL S5-630	1 mm / +3
85	SS13	Sediment	Vancouver	Montevideo S5-630	0.9 mm / +2
86	SW1	Aquaculture facility tanks	Langley	Montevideo S5-630	0.6 mm / +4
87	SW3	Aquaculture facility tanks	Langley	Montevideo S5-630	0.7 mm / +4
88	SW4	Aquaculture facility tanks	Langley	Thompson S5-523	0.5 mm / +2
89	SW5	Aquaculture facility tanks	Langley	Montevideo S5-630	0.6 mm / +4
90	SW6	Aquaculture facility tanks	Langley	Thompson S5-523	0.6 mm / +2
91	SW8	Aquaculture facility tanks	Langley	Montevideo S5-630	0.7 mm / +4
92	SW9	Aquaculture facility tanks	Langley	Thompson S5-523	0.4 mm / +2
93	SW10	Aquaculture facility tanks	Langley	Montevideo S5-630	0.7 mm / +4
94	SW11	Aquaculture facility tanks	Langley	Thompson S5-523	0.5 mm / +2
95	SW12	Aquaculture facility tanks	Langley	Montevideo S5-630	0.8 mm / +2
96	SW13	Aquaculture facility tanks	Langley	Montevideo S5-630	0.8 mm / +2
97	SW14	Creek water	Vancouver	Montevideo S5-630	0.7 mm / +2

Appendix B: Chapter 3 supplemental tables

Table B1. Sequencing coverage of newly isolated *Salmonella* phages.

Phage	x coverage
SI1	754
SI22	65
SC5	1156
SC6	124
SE1	299
SE2	552
SE3	444
SE4	585
SE5	288
SE6	192
SE7	117
SE8	168
SE9	387
SE10	531
SE11	301
SE13	1356
SE14	266
SF1	1465
SF3	861
SS1	999
SS3	330
SS4	2564
SS9	65
SS12	85
SW3	272
SW5	40
SW9	324
SE16	1366
SE19	234
SE20	117
SE21	317
SE22	200
SF4	1252
SF5	937
SF6	132
SF10	87

SI2	1597
SI7	59
SS6	872
SS7	955
SS8	1909
SS10	1023
SE18	345
SE21	622
SE24	377
SF2	1014
SF11	1177
SI3	863
SI5	330
SI8	142
SI23	892
SS5	1141

Table B2. Accession numbers of newly isolated *Salmonella* phages.

Accession number	Phage
MK761195	SF4
MK761196	SF5
MK761197	SS4
MK761198	SS10
MK761199	SI2
MK770409	SF1
MK770410	SE3
MK770411	SE13
MK770412	SE5
MK770413	SE4
MK770414	SE16
MK770415	SF11
MK972685	SE10
MK972686	SF3
MK972687	SE1
MK972688	SI8
MK972689	SF6
MK972690	SE14
MK972691	SI1
MK972692	SE21

MK972693	SI23
MK972694	SE22
MK972695	SI5
MK972696	SI3
MK972697	SF10
MK972698	SS3
MK972699	SS9
MK972700	SS1
MK972701	SS6
MK972702	SS5
MK972703	SS7
MK972704	SE19
MK972705	SF2
MK972706	SS8
MK972707	SE24
MK972708	SE7
MK972709	SE20
MK972710	SI22
MK972711	SW9
MK972712	SI7
MK972713	SW5
MK972714	SW3
MK972715	SE11
MK972716	SE8
MK972717	SE18

Appendix C: Chapter 4 supplemental figure and tables

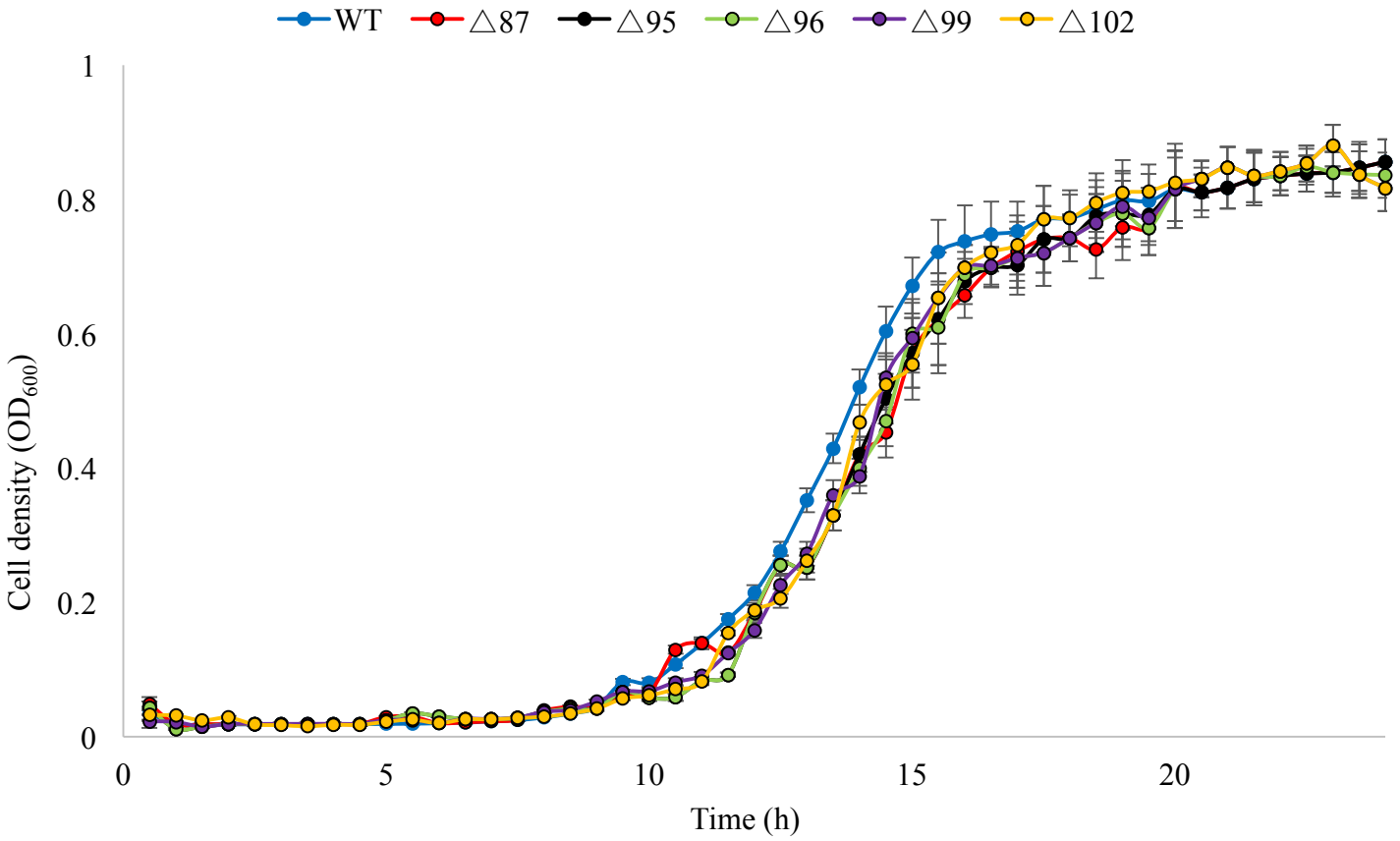

Figure C1. Relative growth rates of the parent and BIM strains of *S.* Agona FSL S5-517. Data shown are the means of three biological replicates ± SD.

Table C1. Mutated genes identified in *S.* Agona BIM Δ87. Mutations shown possess a quality score of >20 and a mapping coverage of >8.

Scaffold	Gene	Strand	Protein/function	Type [a]	Sequence	Alternative sequence [b]	nt Position [c]	Impact on polypeptide
1.1	*ccmA*	-	ATP-binding export protein	SNP	A	G	609	None
1.1	*ccmC*	-	Heme exporter protein	SNP	C	T	240	None
1.1	*ccmC*	-	Heme exporter protein	MNP	TGTCATT CATTAC	**C**GT**A**AT**G** CA**G**TA**A**	521-531	None
1.1	Non-coding region	N/A	Non-coding	Insertion	TAAAAAA	T**GCG**AAA	611308	N/A
1.1	Non-coding	N/A	Non-coding	SNP	T	G	661695	N/A

	region							
2.1	Non-coding region	N/A	Non-coding	SNP	A	T	479242	N/A
3.1	16S rRNA	+	Ribosomal structure	SNP	T	C	468	None
3.1	*orfA*	-	Transposase	Insertion	TCCCGCT GACACCC GTCAG	TCCCGCTG ACACCCGT **TATGGCA TT**CAG	245	Frameshift
4.1	Non-coding region	N/A	Non-coding	SNP	G	T	44	N/A
4.1	Non-coding region	N/A	Non-coding	SNP	T	A	66	N/A
5.1	*ccmH_1*	-	Cytochrome biogenesis protein	SNP	A	C	446	None
6.1	*astA*	-	Arginine N-succinyltransferase	SNP	C	T	736	His → Tyr
6.1	Non-coding region	N/A	Non-coding	MNP	TT	AC	111166	N/A
6.1	Non-coding region	N/A	Non-coding	SNP	T	A	111173	N/A
6.1	Non-coding region	N/A	Non-coding	SNP	C	A	111174	N/A
6.1	Non-coding region	N/A	Non-coding	SNP	A	C	111176	N/A
6.1	Non-coding region	N/A	Non-coding	SNP	T	A	113528	N/A
6.1	Non-coding region	N/A	Non-coding	Insertion	GTGAATG AAGGAT	GTG**T**AATA A**C**GGAT	113529	N/A
6.1	Non-coding region	N/A	Non-coding	Insertion	ACTTA	A**ATATC**TT AT**TTGCA GTGGTGA CCCA**	113545	N/A
6.1	*solA*	+	N-methyl-L-tryptophan oxidase	SNP	T	G	543	None
6.1	*sufD*	-	FeS cluster assembly protein	SNP	G	C	846	Trp → Cys
7.1	*argS*	-	Arginine -tRNA ligase	SNP	G	T	1034	Arg → Leu
7.1	*cmoB*	-	tRNA U34 carboxymethyltransferase	SNP	T	A	241	Ser → Thr
7.1	*narJ*	+	Nitrate reductase molybdenum cofactor assembly chaperone	SNP	G	C	424	Val → Leu

7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	Insertion	TT	T**CAAGGA** T	792	Frameshift
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	MNP	ATTTT	**CGGCC**	798	Leu → Phe Phe → Gly Tyr → His
8.1	*IS256*	-	Transposase	SNP	G	A	75	None
8.1	Non-coding region	N/A	Non-coding	SNP	T	G	50489	N/A
12.1	*purL*	-	Phophoribosylform-ylglycinamidine synthase	SNP	C	A	1428	Asp → Glu
26.1	*impA*	-	Involved in Type IV secretion	Insertion	CTCCC	CTCC**GTA G**C	767	Frameshift
28.1	Non-coding region	N/A	Non-coding	SNP	A	T	26	N/A
29.1	Non-coding region	N/A	Non-coding	SNP	A	G	2251	N/A
30.1	23S rRNA	+	Ribosomal structure	Insertion	TG	T**C**G	505	Frameshift
30.1	23S rRNA	+	Ribosomal structure	SNP	TG	T**A**	506	None
30.1	Non-coding region	N/A	Non-coding	MNP	CGGCG	C**C**G**AT**	1040	N/A
30.1	Non-coding region	N/A	Non-coding	Insertion	AGCGAAC GGG	AGC**CA**GA ACGGG	1045	N/A
30.1	Non-coding region	N/A	Non-coding	Insertion	TGT	T**C**GT	2266	N/A
40.1	16S rRNA	-	Ribosomal structure	MNP	GGAAGTT TT	**A**GAA**C**TTT **C**	1287-1296	None

[a] SNPs clustered in close proximity (i.e., MNPs) are shown on a single line for clarity.
[b] Variants are bolded and underlined. For deletions in the alternative sequence, changes in both the WT and alternative sequence are bolded and underlined.
[c] Positions are shown for mutations occurring in genes. Positions in the scaffold are shown for mutations occurring in non-coding regions.

Table C2. Mutated genes identified in *S.* Agona BIM Δ95. Mutations shown possess a quality score of >20 and a mapping coverage of >8.

Scaffold	Gene	Strand	Protein/function	Type [a]	Sequence	Alternative sequence [b]	nt Position [c]	Impact on polypeptide
1.1	*ccmC*	-	Heme exporter protein	SNP	C	T	246	None
1.1	*ccmC*	-	Heme exporter protein	SNP	G	C	278	Ala → Pro

1.1	*ccmC*	-	Heme exporter protein	SNP	G	A	357	None
1.1	*ccmC*	-	Heme exporter protein	MNP	TGTCATTC ATTAC	**G**GT**T**AT**C** CA**C**TA**T**	522-534	None
1.1	*ccmC*	-	Heme exporter protein	SNP	T	C	540	None
1.1	*ccmC*	-	Heme exporter protein	SNP	G	A	569	None
1.1	*ccmC*	-	Heme exporter protein	SNP	G	A	574	Arg → Gln
1.1	*ccmC*	-	Heme exporter protein	SNP	C	T	588	None
1.1	*ccmD*	-	Heme exporter protein	SNP	G	A	122	Arg → Gln
1.1	*ccmD*	-	Heme exporter protein	SNP	C	G	168	None
1.1	*ccmH_2*	-	Cytochrome biogenesis protein	SNP	G	A	132	None
1.1	Non-coding region	N/A	N/A	SNP	A	C	661695	N/A
1.1	*rfaL*	+	O-antigen ligase	Insertion	GA	G**GAT**A	425	Frameshift
1.1	*rfaL*	+	O-antigen ligase	SNP	G	A	429	None
1.1	*rfaL*	+	O-antigen ligase	Insertion	TA	T**GGG**A	442	Frameshift
1.1	*rfaY*	-	Lipopolysaccharide core heptose (II) kinase	Insertion	AA	A**GCGAAG CCCTAAA CTTGTTA AAA**A	514	Frameshift
1.1	*rfaY*	-	Lipopolysaccharide core heptose (II) kinase	SNP	T	G	538	Asp → Lys
1.1	*rfaY*	-	Lipopolysaccharide core heptose (II) kinase	Insertion	T	T**CAAGGA**	792	Frameshift
3.1	23S rRNA	+	Ribosome structure	Insertion	AAGCGTG TAG	A**GGT**AGC GTG**CAA**T AG	1134	Frameshift
3.1	23S rRNA	+	Ribosome structure	Insertion	GACT	G**AAGACA ACAATTT TC**ACT	1029	Frameshift
3.1	*orfA*	-	Transposase/transposition	Insertion	TG	T**GCAAAG AAT**G	246	Frameshift
4.1	Non-coding region	N/A	N/A	SNP	G	T	44	N/A
4.1	Non-coding region	N/A	N/A	SNP	T	A	66	N/A
6.1	*astA*	-	Arginine N-succinyltransferase	SNP	C	T	736	His → Tyr
6.1	*solA*	+	N-methyl-L-tryptophan oxidase	SNP	T	G	543	None

6.1	*sufD*	-	FeS cluster assembly protein	SNP	G	C	846	Trp → Cys
7.1	*argS*	-	Arginine -tRNA ligase	SNP	G	T	1034	Arg → Leu
7.1	*cmoB*	-	tRNA U34 carboxymethy ltransferase	SNP	T	A	241	Ser → Thr
7.1	*narJ*	+	Nitrate reductase molybdenum cofactor assembly chaperone	SNP	G	C	424	Val → Leu
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	Insertion	TT	T**CAAGGA**T	792	Frameshift
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	MNP	ATTTT	**CGGCC**	798	Leu → Phe Phe → Gly Tyr → His
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	Insertion	AT	A**GAAC**T	810	Frameshift
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	Insertion	TC	T**CA**C	816	Frameshift
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	SNP	G	C	820	Gly → Arg
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	SNP	G	T	823	Gly → Stop codon
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	SNP	A	G	825	None
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	Deletion	A**GGA**	A---	828	Frameshift
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI	SNP	C	G	831	Asp → Glu

			secretion					
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	SNP	C	A	836	Ala → Glu
8.1	*mukB*	+	Chromosome partition protein	MNP	CAAA	**G**AA**G**	1100	Ala → Gly Asn → Ser
12.1	*purL*	-	Phophoribosylformylglycinamidine synthase	SNP	C	A	1428	Asp → Glu
26.1	*impA*	-	Involved in Type IV secretion	Insertion	CTCCC	CTCC**GTAG**C	767	Frameshift
28.1	Non-coding region	N/A	N/A	SNP	A	T	26	N/A
30.1	23S rRNA	+	Ribosome structure	Insertion	TG	T**C**G	505	Frameshift
30.1	Non-coding region	N/A	N/A	Insertion	GCGGCGAGC	GCGGCGA**TAGTA**GC	1039	N/A
34.1	16S rRNA	+	Ribosome structure	Insertion	TC	T**A**C	1477	Frameshift

[a] SNPs clustered in close proximity (i.e., MNPs) are shown on a single line for clarity.
[b] Variants are bolded and underlined. For deletions in the alternative sequence, changes in both the WT and alternative sequence are bolded and underlined.
[c] Positions are shown for mutations occurring in genes. Positions in the scaffold are shown for mutations occurring in non-coding regions.

Table C3. Mutated genes identified in *S.* Agona BIM Δ96. Mutations shown possess a quality score of >20 and a mapping coverage of >8.

Scaffold	Gene	Strand	Protein/function	Type [a]	Sequence	Alternative sequence [b]	nt Position [c]	Impact on polypeptide
1.1	*ccmA*	-	ATP-binding export protein	SNP	A	G	609	None
1.1	*ccmC*	-	Heme exporter protein	SNP	C	T	246	None
1.1	*ccmC*	-	Heme exporter protein	SNP	G	C	278	Ala → Pro
1.1	*ccmC*	-	Heme exporter protein	SNP	G	A	357	None
1.1	*ccmC*	-	Heme exporter protein	MNP	TGTCATTCATTAC	**G**GT**T**AT**C**CA**C**TA**T**	522-534	None
1.1	*ccmC*	-	Heme exporter protein	SNP	C	T	588	None
1.1	*ccmC*	-	Heme exporter protein	SNP	G	A	574	Arg → Gln
1.1	*ccmC*	-	Heme exporter protein	SNP	G	A	569	None
1.1	*ccmD*	-	Heme exporter protein	SNP	G	A	122	Arg → Gln

1.1	*ccmE*	-	Cytochrome biogenesis protein	MNP	TTAC	**C**TA**T**	24	Arg → Trp
1.1	Non-coding region	N/A	N/A	SNP	A	C	661695	N/A
1.1	Non-coding region	N/A	N/A	SNP	A	C	611313	N/A
1.1	*rfaL*	+	O-antigen ligase	Insertion	GA	G**GAT**A	425	Frameshift
1.1	*rfaL*	+	O-antigen ligase	SNP	G	A	429	None
1.1	*rfaL*	+	O-antigen ligase	Insertion	TA	T**GGG**A	442	Frameshift
1.1	*rfaY*	-	Lipopolysaccharide core heptose (II) kinase	Insertion	AA	A**GCGAAGC CCTAAACT TGTTAAAA** A	514	Frameshift
1.1	*rfaY*	-	Lipopolysaccharide core heptose (II) kinase	SNP	T	G	538	Asp → Lys
1.1	*rfaY*	-	Lipopolysaccharide core heptose (II) kinase	Insertion	T	T**CAAGGA**	792	Frameshift
3.1	*orfA*	-	Transposase/transposition	Insertion	TG	T**GCAAAGA AT**G	246	Frameshift
4.1	Non-coding region	N/A	Non-coding	SNP	G	T	44	N/A
4.1	Non-coding region	N/A	Non-coding	SNP	T	A	66	N/A
4.1	Non-coding region	N/A	Non-coding	SNP	C	G	334561	N/A
5.1	*ccmH_1*	-	Cytochrome biogenesis protein	SNP	A	C	446	None
6.1	*astA*	-	Arginine N-succinyltransferase	SNP	C	T	736	His → Tyr
6.1	*solA*	+	N-methyl-L-tryptophan oxidase	SNP	T	G	543	None
6.1	*sufD*	-	FeS cluster assembly protein	SNP	G	C	846	Trp → Cys
7.1	*argS*	-	Arginine -tRNA ligase	SNP	G	T	1034	Arg → Leu
7.1	*cmoB*	-	tRNA U34 carboxymethyltransferase	SNP	T	A	241	Ser → Thr
7.1	*narJ*	+	Nitrate reductase molybdenum cofactor assembly chaperone	SNP	G	C	424	Val → Leu
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved	Insertion	TT	T**CAAGGA**T	792	Frameshift

			in Type VI secretion					
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	MNP	ATTTT	**CGGCC**	798	Leu → Phe Phe → Gly Tyr → His
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	Insertion	AT	A**GAAC**T	810	Frameshift
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	Insertion	TC	T**CA**C	816	Frameshift
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	SNP	G	C	820	Gly → Arg
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	SNP	G	T	823	Gly →Stop codon
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	SNP	A	G	825	None
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	Deletion	A**GGA**	A---	828	Frameshift
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	SNP	C	G	831	Asp → Glu
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	SNP	C	A	836	Ala → Glu
12.1	*purL*	-	Phophoribosylformylglycinamidine synthase	SNP	C	A	1428	Asp → Glu
26.1	*impA*	-	Involved in Type IV secretion	Insertion	CTCCC	CTCC**GTAG**C	767	Frameshift
28.1	Non-coding region	N/A	N/A	SNP	A	T	26	N/A

29.1	Non-coding region	N/A	N/A	Insertion	CAAC	**TATTACGT TTTGGCTG GT**CAAC	3	N/A
29.1	Non-coding region	N/A	N/A	Insertion	GTAC	GTAC**AACC GAGGTGAC TCGTGGCC GTGATGG**	2248	N/A
30.1	Non-coding region	N/A	N/A	Insertion	GCGGCGA GC	GCGGCGA**T AGTA**GC	1039	N/A
34.1	16S rRNA	+	Ribosome structure	Insertion	TC	T**A**C	1477	Frameshift
36.1	Non-coding region	N/A	N/A	Insertion	TGAG	TGAG**ATTC CCCCAGTA**	742	Frameshift

[a] SNPs clustered in close proximity (i.e., MNPs) are shown on a single line for clarity.
[b] Variants are bolded and underlined. For deletions in the alternative sequence, changes in both the WT and alternative sequence are bolded and underlined.
[c] Positions are shown for mutations occurring in genes. Positions in the scaffold are shown for mutations occurring in non-coding regions.

Table C4. Mutated genes identified in *S.* Agona BIM Δ99. Mutations shown possess a quality score of >20 and a mapping coverage of >8.

Scaffold	Gene	Strand	Protein/function	Type [a]	Sequence	Alternative sequence [b]	nt Position [c]	Impact on polypeptide
1.1	*ccmC*	-	Heme exporter protein	SNP	C	T	246	None
1.1	*ccmC*	-	Heme exporter protein	SNP	G	C	278	Ser → Thr
1.1	*ccmC*	-	Heme exporter protein	SNP	G	A	357	None
1.1	*ccmC*	-	Heme exporter protein	SNP	C	T	534	None
1.1	*ccmC*	-	Heme exporter protein	SNP	T	C	540	None
1.1	*ccmC*	-	Heme exporter protein	SNP	G	A	570	Ser → Tyr
1.1	*ccmC*	-	Heme exporter protein	SNP	G	A	575	Arg → Leu
1.1	*ccmC*	-	Heme exporter protein	SNP	C	T	588	None
1.1	*ccmD*	-	Heme exporter protein	SNP	G	A	122	Arg → Gln
1.1	*ccmE*	-	Cytochrome C maturation protein	SNP	C	T	228	None
1.1	*ccmE*	-	Cytochrome C maturation protein	SNP	T	C	249	None
1.1	*ccmE*	-	Cytochrome C maturation protein	SNP	G	T	268	Ala → Ser
1.1	Non-coding region	N/A	N/A	Deletion	G**CCC**C	G**---**C	611298	N/A
1.1	Non-coding region	N/A	N/A	SNP	A	C	611314	N/A

1.1	Non-coding region	N/A	N/A	Insertion	TAGAGAAAC	TAG**CCACGTA**AC	612552	N/A
1.1	Non-coding region	N/A	N/A	Insertion	GGGAGTTCA	GG**ATAATCT**A	612566	N/A
1.1	Non-coding region	N/A	N/A	Insertion	T	G	661695	N/A
3.1	*orfA*	-	Transposase/transposition	Insertion	TG	T**GCAAAGAAT**G	246	Frameshift
4.1	*ycjG*	+	L-Ala-D/L-Glu epimerase	SNP	A	T	296	Gln → Leu
6.1	*astA*	(-)	Arginine N-succinyltransferase	SNP	C	T	736	His → Tyr
6.1	*chbC*	-	Selective sugar transport	SNP	G	A	275	Gly → Asp
6.1	Non-coding region	N/A	N/A	SNP	T	C	111167	N/A
6.1	*solA*	(+)	N-methyl-L-tryptophan oxidase	SNP	T	G	543	None
6.1	*sufD*	(-)	FeS cluster assembly protein	SNP	G	C	846	Trp → Cys
7.1	*argS*	-	Arginine -tRNA ligase	SNP	G	T	1034	Arg → Leu
7.1	*cmoB*	-	tRNA U34 carboxymethyltransferase	SNP	T	A	241	Ser → Thr
7.1	*narJ*	+	Nitrate reductase molybdenum cofactor assembly chaperone	SNP	G	C	424	Val → Leu
7.1	Non-coding region	N/A	N/A	SNP	C	**A**	111169	N/A
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	Insertion	TT	T**CAAGGA**T	792	Frameshift
7.1	*vgrG1_2*	(+)	Tip protein/secreted effector; involved in Type VI secretion	MNP	ATTTT	**CGGCC**	798	Leu → Phe Phe → Gly Tyr → His
8.1	*mukB*	+	Chromosome partition protein	MNP	CAAA	**G**AA**G**	1100	Ala → Gly Asn → Ser
8.1	*nfsA*		Oxygen-insensitive NADPH nitroreductase	SNP	A	G	251	Asn--> Ser
8.1	Non-coding region	N/A	N/A	SNP	T	C	111170	N/A
8.1	*ybjI*	-	5-amino-6-(5-phospho-D-ribitylamino) uracil phosphatase	Deletion	G**CG**G	G--G	718	Frameshift
9.1	Non-coding region	N/A	N/A	SNP	T	C	111172	N/A
10.1	Non-coding	N/A	N/A	SNP	T	G	111173	N/A

	region							
11.1	Non-coding region	N/A	N/A	SNP	T	C	111175	N/A
12.1	Non-coding region	N/A	N/A	SNP	A	G	111176	N/A
13.1	Non-coding region	N/A	N/A	SNP	C	T	111177	N/A
30.1	23S rRNA	+	Ribosome structure	MNP	TCT	T**AG**	842	None
34.1	16S rRNA	+	Ribosome structure	Insertion	TC	T**A**C	729	Frameshift

[a] SNPs clustered in close proximity (i.e., MNPs) are shown on a single line for clarity.
[b] Variants are bolded and underlined. For deletions in the alternative sequence, changes in both the WT and alternative sequence are bolded and underlined.
[c] Positions are shown for mutations occurring in genes. Positions in the scaffold are shown for mutations occurring in non-coding regions.

Table C5. Mutated genes identified in *S.* Agona BIM Δ102. Mutations shown possess a quality score of >20 and a mapping coverage of >8.

Scaffold	Gene	Strand	Protein/function	Type [a]	Sequence	Alternative sequence [b]	nt Position [c]	Impact on polypeptide
1.1	*ccmA*	-	ATP-binding export protein	SNP	A	G	609	None
1.1	*ccmC*	-	Heme exporter protein	SNP	C	T	246	None
1.1	*ccmC*	-	Heme exporter protein	SNP	G	C	278	Ser → Thr
1.1	*ccmC*	-	Heme exporter protein	SNP	G	A	357	None
1.1	*ccmC*	-	Heme exporter protein	SNP	C	T	534	None
1.1	*ccmC*	-	Heme exporter protein	SNP	T	C	540	None
1.1	*ccmC*	-	Heme exporter protein	SNP	G	A	570	Ser → Tyr
1.1	*ccmC*	-	Heme exporter protein	SNP	G	A	575	Arg → Leu
1.1	*ccmC*	-	Heme exporter protein	SNP	C	T	588	None
1.1	Non-coding region	N/A	N/A	Insertion	T	G	661695	N/A
1.1	Non-coding region	N/A	N/A	Insertion	GCTACG	GCGGCCT ATCTCG	612534	N/A
3.1	23S rRNA	+	Ribosome structure	Insertion	GACT	G**AAGACA ACAATTT TC**ACT	1029	Frameshift

3.1	Non-coding region	N/A	N/A	Deletion	TCA**GATT CAGAGT**CCG	TCA---------CCG	2296	Frameshift
4.1	Non-coding region	N/A	Non-coding	SNP	G	T	44	N/A
4.1	Non-coding region	N/A	Non-coding	SNP	T	A	66	N/A
4.1	*rsxC_1*	+	Electron transport complex subunit	SNP	A	G	1911	Thr → Ala
5.1	*ccmH_1*	-	Cytochrome biogenesis protein	SNP	A	C	447	None
6.1	*astA*	-	Arginine N-succinyltransferase	SNP	C	T	736	His → Tyr
6.1	Non-coding region	N/A	Non-coding	MNP	TT	AC	111166	N/A
6.1	*sufD*	-	FeS cluster assembly protein	SNP	G	C	846	Trp → Cys
7.1	*argS*	-	Arginine -tRNA ligase	SNP	G	T	1034	Arg → Leu
7.1	*cmoB*	-	tRNA U34 carboxymethyltransferase	SNP	T	A	241	Ser → Thr
7.1	*narJ*	+	Nitrate reductase molybdenum cofactor assembly chaperone	SNP	G	C	424	Val → Leu
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	Insertion	TT	T**CAAGGA**T	792	Frameshift
7.1	*vgrG1_2*	+	Tip protein/secreted effector; involved in Type VI secretion	MNP	ATTTT	**CGGCC**	798	Leu → Phe Phe → Gly Tyr → His
12.1	*purL*	-	Phophoribosylformylglycinamidine synthase	SNP	C	A	1428	Asp → Glu
26.1	*icmF*	-	Type VI secretion	SNP	T	G	87	None
26.1	*icmF*	-	Type VI secretion	SNP	G	A	78	None
28.1	Non-coding region	N/A	N/A	SNP	A	T	26	N/A
29.1	Non-coding region	N/A	Non-coding	SNP	A	G	2251	N/A
30.1	Non-coding region	N/A	N/A	Insertion	GCGGCGAGC	GCGGCGA**TAGTA**GC	1039	N/A
30.1	Non-coding region	N/A	N/A	SNP	G	C	1052	N/A
34.1	16S rRNA	+	Ribosome structure	Insertion	TC	T**A**C	729	Frameshift

[a] SNPs clustered in close proximity (i.e., MNPs) are shown on a single line for clarity.
[b] Variants are bolded and underlined. For deletions in the alternative sequence, changes in both the WT and alternative sequence are bolded and underlined.
[c] Positions are shown for mutations occurring in genes. Positions in the scaffold are shown for mutations occurring in non-coding regions.

Table C6. CRISPR elements identified in scaffold 2.1 of the parent and BIM strains of *S.* Agona FSL S5-517.

CRISPR start (nt)	CRISPR end (nt)	Spacer	Spacer sequence	Direct repeat consensus sequence
191881	193007	1	TTTTCAGCCCTTGCCGACTGCGGAACGCCCCT	CGGTTTATCCCCGCTGGCGCGGGGAACAC
		2	ATGGATGGCTCATATCCGGTTTCTGTTTCTGC	
		3	GCGAAATAGTGGGGAAAAACCCCTGGTTAACC	
		4	TAGGCCTTGATACCATCGCTCGCACCTCGTCA	
		5	ATATTGATACTGTTCTCAAGCGCCGTTTTAGC	
		6	TGGCGCCAGAAATATTCATGATCATCGGGATT	
		7	TTCATATTCCAGAAAATGCCTGGGTGATGATC	
		8	GTGGTATCTCCTGTCGACCTTGCCGTTAAAAT	
		9	TATAACTGGCGGGTTTTAGTGTCGTTATAAAA	
		10	TCAGGCTTGACGACAGTCTCCAGCCACTCCTG	
		11	GACCGCGCGGGCTATCGTTCAGGACTATTTTT	
		12	TGGAAGATATTGAAAGCGCCCAATCTTCCCAG	
		13	CTCTGTTACACCGTTATGCACAGACCACACAG	
		14	NTTAATGCAAGCGCCATTAACAAGAAAATGAC	
		15	AGCTCCGGCAGGGTCATGTCCCGGCAGGTGCG	
		16	GTTGTGACGAGTTCGTCGTCTTTTTTTTCGCT	
		17	TAGGGAACCTGGCTAAAGTGTCTGAAGGCTAC	
		18	AATTTTTCTGCGGCCAGCGCGGCCAGCGCCTC	
209293	209809	1	CTGCTCAGCGATAACCGGCAGGTTTAGCCGCT	CGGTTTATCCCCGCTGGCGCGGGGAACAC
		2	TTGCCGACCAGATCAATGACAACGCTAACCTT	
		3	CAGATCACAACGGGTGACGTGATTTTTGGAAA	
		4	TCGTTTTTATCTGCATCTCACTCCCCCTTAAC	
		5	GCCTAAGCCGGGATTTGCTTTAGGCCAGTTGG	
		6	AATAAATGGGATGATCCTGTTGTCCGGGATGC	
		7	TCCATCGCTTTTGCCAATCTATCAGACAATTT	
		8	GGCGACTAACGGCGTGCTGACCAGCCAGTCGC	

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