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Development of a broad-spectrum *Salmonella* phage cocktail containing Viunalike and Jerseylike viruses isolated from Thailand

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ABSTRACT

Salmonella is one of the most common agents of foodborne disease worldwide. As natural alternatives to traditional antimicrobial agents, bacteriophages (phages) are emerging as highly effective biocontrol agents against *Salmonella* and other foodborne bacteria. Due to the high diversity within the *Salmonella* genus and emergence of drug resistant strains, improved efforts are necessary to find broad range and strictly lytic *Salmonella* phages for use in food biocontrol. Here, we describe the isolation and characterization of two *Salmonella* phages: ST-W77 isolated on *S*. Typhimurium and SE-W109 isolated on *S*. Enteritidis with extraordinary *Salmonella* specificity. Whole genome sequencing identified ST-W77 as a Myovirus within the *Viunalikevirus* genus and SE-W109 as a Siphovirus within the *Jerseylikevirus* genus. Infectivity studies using a panel of *S*. Typhimurium cell wall mutants revealed both phages require the lipopolysaccharide O-antigen, with SE-W109 also recognizing the flagella, during infection of *Salmonella*. A combination of both phages was capable of prolonged (one-week) antibacterial activity when added to milk or chicken meat contaminated with *Salmonella*. Due to their broad host ranges, strictly lytic lifestyles and lack of lysogeny-related genes or virulence genes in their genomes, ST-W77 and SE-W109 are ideal phages for further development as *Salmonella* biocontrol agents for food production.

1. Introduction

Salmonella enterica is a ubiquitous, enteropathogenic bacterial species that represents one of the most socioeconomically important pathogens affecting global food producers and public health systems (Oh and Park, 2017). In the United States alone, non-typhoidal *Salmonella* bacteria cause an estimated 1.2 million illnesses, 26,500 hospitalizations and 420 deaths every year with contaminated food as the major source (CDC, 2019). In the European Union, there were 91,662 confirmed human salmonellosis cases in 2017 resulting in 16,796 hospitalizations and 156 reported deaths (EFSA, 2018). The two most common serovars in all human cases are *S*. Enteritidis and *S*. Typhimurium. Critically, antibiotic resistance strains of *S*. Enteritidis and *S*. Typhimurium (Su et al., 2004) and other *Salmonella* serovars are becoming more prevalent (Divek et al., 2018), for example, the multi-drug resistant *S*. Typhimurium strain DT104 has evolved and disseminated rapidly across the globe (Wang et al., 2019). *Salmonella* contaminations can occur at any point during food manufacturing; however, most human infections occur through consumption of raw or undercooked foods and from cross contamination during food preparation (El-Sharkawy et al., 2017; Omwandho and Kubota, 2010). The most contaminated food production sectors for *Salmonella* are egg, meat and dairy products, for which several biocontrol strategies have been suggested, including bacteriophages (phages) (Coughlan et al., 2016; O'Sullivan et al., 2019; Oh and Park, 2017).

Phages are viruses that specifically infect bacteria and represent the most abundant type of organism in the biosphere (Clokie et al., 2011). Owing to their inherent ability to infect bacteria with species or even

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strain level specificity, phages can be used to selectively kill pathogenic bacteria while leaving other bacteria unaffected. Phages are already used for diagnostic and remedial applications in food processing (Moye et al., 2018), biotechnology (Harada et al., 2018) and medicine, i.e., phage therapy (Gordillo Altamirano and Barr, 2019). The successful application of phages to control foodborne bacterial contaminations has been widely reported (Bigot et al., 2011; Carlton et al., 2005; Grant et al., 2016; Guenther et al., 2012; Higgins et al., 2005; Spricigo et al., 2013; Sukumaran et al., 2015; Thanki et al., 2019), with the first phage-based product approved by the Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA) in 2006 to target *Listeria monocytogenes* contaminations on ready-to-eat (RTE) food products (Coughlan et al., 2016). The FDA and USDA have also approved phage-based products for targeting foodborne *E. coli, Salmonella* and *Shigella* (Moye et al., 2018). As biocontrol agents, phages must meet certain criteria, such as a broad host range (infecting the desired species or genera), be able to withstand the food processing environment including food physiochemical conditions, and contain no known genomic information associated with pathogenicity or potentially allergenic properties (Endersen et al., 2014). In addition, temperate phages should be excluded as biocontrol agents as they are typically less effective at killing than virulent phages and can integrate into their hosts leading to potential transfer of virulence genes or antibiotic resistance genes that pose the risk of generating pathogenic strains (Hagens and Loessner, 2010; Moye et al., 2018). Finally, due to the diversity of *Salmonella* strains, phages isolated in one country may not necessarily efficiently lyse bacteria dominant in other countries, so additional *Salmonella* phage with useful properties are still needed.

In this study, we describe the isolation and characterization of *Salmonella* phages from Thailand with the aim of developing phage biocontrol agents. Two strictly lytic phages, ST-W77 and SE-W109, were selected due to their broad *Salmonella* host ranges for further characterization and genome sequencing, and were combined into a two-component cocktail that was effective at inhibiting *Salmonella* growth in milk and contaminated chicken meat.

2. Materials and methods

2.1. Bacterial cultures, media and growth conditions

All strains used in this study are listed in Supplementary Table 2. The strains were cultured at 37 °C in Trypticase Soy Broth (TSB) and agar (TSA) (Titan Biotech, Delhi, India). As required, *S.* Typhimurium mutant strains (Marti et al., 2013) were supplemented with antibiotics, Chloramphenicol (25 μg/mL) or Kanamycin (200 μg/mL for TSB, 50 μg/mL for TSA).

2.2. Mitomycin C induction to identify prophage-free Salmonella strains for phage isolation

To identify non-lysogenic (prophage-free) *S.* Enteritidis and *S.* Typhimurium hosts, *Salmonella* isolates (Supplementary Table 1) were screened using mitomycin C induction as described (Clokie and Kropinski, 2009). In short, 50 μL of an overnight *Salmonella* culture was added to 5 mL of TSB media and grown until an initial absorbance at OD_{600} of 0.2 (mid-log phase). The culture was then incubated at 37 °C for 30 min before adding mitomycin C (Sigma-Aldrich, Missouri, USA) to a final concentration of 0.2 μg/mL. The absorbance at OD_{600} was measured over 6–8 h (or until a decrease of the optimal density is observed). At regular time points, 500 μL of the sample was collected, spun down to remove residual bacteria and assessed for phage induction by spot assay against each of the 87 *Salmonella* strains (data not shown). For any non-plaque formers additional double-layer plaque assays were performed as an alternative method to identify phages (Clokie and Kropinski, 2009).

2.3. Sample collections, bacteriophages isolation and propagation

Phages were isolated from chicken dropping and sewage samples using standard spot and double-layer agar assays as previously described (Phothaworn et al., 2019). As phages can be inactive by prolonged exposure to UV light (Clokie et al., 2011), all sewage samples were collected from a depth of 30 cm or less from open sewers protected from UV (e.g., covered by aquatic weeds). This is the area where appropriate oxygen tension supports the existence of bacterial species, and thus an ideal zone for phage isolation (Liu et al., 2018; Ravva and Sarreal, 2014). Briefly, 2 g of fecal sample was mixed in SM buffer (50 mM Tris-HCl, 100 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatin, pH 7.5) and then centrifuged at 4500×*g* for 10 min at 4 °C. Sewage samples were processed with a phage enrichment step. Each of the sample was filtered through a 0.45 μm membrane filter (GE Healthcare Life Sciences, Marlborough, USA), then mixed with 200 mL of 2X TSB, then 400 μL of exponential growth phase of *Salmonella* strains (4–5 different isolates) were added, and the mixture was incubated at room temperature for 48 h with shaking. Thereafter, the mixture was centrifuged at 6500×*g* for 30 min at 4 °C. The supernatant from the chicken dropping and sewage samples was filtered through a 0.45 μm membrane filter, and 10 μL of filtrate was spotted onto a bacterial lawn containing 100 μL of a mid-log phase *S.* Typhimurium strain 273 (*S.* Tm 273) or *S.* Enteritidis strain 37 (*S.* E 37) culture mixed with 3 mL soft agar, and incubated overnight at 37 °C. Samples producing plaques (zones of localized lysis) were confirmed to contain phages using the double agar overlay plaque technique. A single plaque was randomly picked from each spot and purified a minimum of three times by repeated double agar overlays against the same isolation host.

Larger stocks of single phage isolates were generated as follows: Ten soft agar overlays presenting confluent lysis were produced for each phage and the crude phage lysate was collected by washing the plates with a total of 15 mL SM buffer for a minimum of 5 h. Bacterial cells were removed by centrifugation (6500×*g*, 30 min, 4 °C) and filtration (0.45 μm) before the phage was pelleted by centrifugation (20,000×*g*, 45 min, 4 °C). The supernatant was discarded and the phage pellet gently resuspended with 1 mL SM buffer. The phage suspension was centrifuged (1800×*g*, 15 min), filtered (0.45 μm) and finally stored at 4 °C.

2.4. PCR screening to exclude Chi-like phages

We previously identified Chi-like, temperate phages as a predominant type isolated from Thai chicken farms (Phothaworn et al., 2019). To exclude these temperate, and therefore non-applicable, phages from our study a PCR screen was performed. DNA from phage isolates were amplified using forward (5′-TTCAGACCCACGGATGGTTG-3′) and reverse (5′-AGAAAGCGGCTACAACACGA-3′) primers for the gene encoding major capsid protein E from the Chi-like *Salmonella* phage SPN19 (GenBank Accession no: NC_019417.1). Phage isolates that gave a positive PCR amplicon of 511-bp in length were excluded from further investigations.

2.5. Characterization of the selected phages

2.5.1. Phage host range analysis and identification of phage receptors

To assess the lytic spectrum of the isolated phages against *Salmonella* spp. and other bacteria, spot assays were performed as a screening test on lawns of target bacterial strains including various serovars of *Salmonella enterica* and other Gram-negative and Gram-positive bacteria. Positive lysis samples were confirmed by double-layer plaque assay (Clokie and Kropinski, 2009). Efficiency of plating (E.O.P.) experiments were performed against cell wall component null mutants of *S.* Typhimurium (Table 3). In short, the same PFU/mL of each phage was plated on the mutant strains and the wildtype strain. The difference in PFUs is reported as a percentage difference compared to the wildtype strain. The

experiment was performed in triplicate as mean values with standard deviation.

2.5.2. Phage morphology examination by transmission electron microscope (TEM)

A high titer of phage particles were negatively stained for 30 s with 2% uranyl acetate on QUANTIFOIL®-carbon-coated copper grids (Electron Microscopy Sciences, Hatfield, PA, USA) and observed at 100 kV with a Phillips CM12 scope equipped with a Gatan Orius 1K camera (Gatan, Pleasanton, CA, USA).

2.5.3. Thermal and salt stability tests

To test thermal stability, 200 μL of phage suspension (~10^{11}-10^{12} PFU/mL) was incubated at different temperatures (25, 40, 50, 60, 70, 80 and 90 °C) for 1 h in a thermocycler. To test salt stability, NaCl (0, 1, 2 and 3%) was added to 200 μL of phage suspension for 1 h. Remaining viable phages from both treatments were titered by double-layer plaque assay (Clokie and Kropinski, 2009). The experiment was performed in triplicate, and the data is presented as mean values with standard deviation.

2.5.4. Phage genomic DNA sequencing

Phage genomic DNA was prepared by phenol-chloroform extraction as previously described (Sambrook et al., 2001). Genome sequencing was carried out using 314v2 chips on Ion Torrent PGM (Thermo Fisher Scientific, Massachusetts, USA), Torrent Suite version 4.4.3. The sequences were assembled using MEGAHIT software version 1.2.1 (Li et al., 2016). Both, the Prokka version 1.12 (Seemann, 2014) and Rapid Annotation using Subsystem Technology (RAST) pipeline (McNair et al., 2018) were used to annotate the sequences. The phage genome map was generated and visualized using Artemis version 17.0.1 (Carver et al., 2012). Completed genome sequences of *Salmonella* phages ST-W77 and SE-W109 are available from the GenBank database under accession numbers KX765865 and KX649889, respectively.

2.6. Application of phage cocktail treatment in pasteurized milk

To test the efficiency of phage cocktail against *Salmonella* cultures, a 1:1 combined cocktail of ST-W77 and SE-W109 was added to 30 mL of pasteurized milk containing 10^3 CFU each of *S*. Enteritidis strain 54436 (Amp^R) and *S*. Typhimurium strain 54437 (Amp^R) at MOIs equivalent to 10^3, 10^5 and 10^7 and then incubated at room temperature. SM buffer alone was used as a phage-free control. At defined time points (6 h; 1–7 days) the number of *Salmonella* cells was determined by plating on TSA-Amp agar. The experiment was performed in triplicate as the mean values with standard deviation.

2.7. Application of phage cocktail treatment on poultry

Individual chicken breasts were purchased from a local supermarket and tested for natural *Salmonella* contaminants using conventional culture-based techniques (ISO 6579–1: 2017) before being aseptically cut into 25 g pieces. 500 μL of *S*. Enteritidis strain 54436 (Amp^R) and *S*. Typhimurium strain 54437 (Amp^R) (10^3 CFU/mL each) were pipetted onto 25 g pieces of chicken meat and kept at 4 °C for 1 h. 10 μL of a high titer ST-W77 and SE-W109 cocktail (10^{12} PFU/mL each) was applied to the center of a 10 × 10 cm piece of plastic wrap and spread to the edges, resulting in ~10^8 PFU/cm^2. The phage-treated plastic wrap was wrapped around the artificially contaminated chicken pieces and stored at 4 °C. Viable *Salmonella* and phage titers were enumerated at indicated time points (5 min, 15 min, 30 min, 1 h, 24 h and 7 days) by colony plating on TSA-Amp agar and double-layer agar assay, respectively. The experiment was performed in triplicate (three pieces per time point) with results shown as the mean values with standard deviation.

2.8. Statistical analysis

Data values from multiple measurements were averaged, and standard deviations calculated. All of statistic data were analyzed using GraphPad Prism software (San Diego, California, USA). The One-Way ANOVA test was used to analyze the effects of thermal and salt on phage viability in Fig. 2.

3. Results and discussion

3.1. Isolation of broad range Salmonella phages

Salmonella phages were isolated from 285 environmental samples, comprising 158 chicken dropping samples and 127 canal and sewer samples collected from various areas in Bangkok, using soft agar overlay assays and clinical isolates of *S*. Typhimurium strain 273 (*S*. Tm 273) or *S*. Enteritidis strain 37 (*S*. E 37). Thai canals and sewer discharge were sampled due to the high prevalence for *Salmonella* spp. (and therefore *Salmonella* phages) reported in these environments (Yajima and Koottatep, 2010).

To avoid mixed phage populations within individual plaques due to possible prophage induction, *S*. Tm 273 and *S*. E 37, as well as 85 other *Salmonella* strains, were screened for lysogeny by inducing putative prophages using mitomycin C. While we realize that not all prophages are mitomycin C inducible, this quick screening approach allowed us to remove a large proportion of lysogens from the pool of potential propagation hosts. Nevertheless, for future largescale production, all suitable propagation strains should be whole genome sequenced, screened by PCR for prophage elements (Martín et al., 2006), or tested with alternative inducers such as UV, antibiotic (fluoroquinolones) and reactive oxygen species (ROS) prior to use (Clokie and Kropinski, 2009).

In total, 41 clinical isolates and 46 poultry farm isolates of *S*. Enteritidis and *S*. Typhimurium were treated with mitomycin C as previously described (Clokie and Kropinski, 2009). Overall, 61% (25/41) of clinical isolates, including *S*. Tm 273 and *S*. E 37, were negative for prophage induction by mitomycin C and therefore suitable as phage isolation hosts (Table 1). Conversely, the majority of *Salmonella* farm isolates (67.4%; 31/46) were positive for prophage induction, suggesting a higher prevalence of lysogenic *Salmonella* strains from the sampled chicken farms compared to the clinic. After identifying *S*. Tm 273 and *S*. E 37 as prophage-free strains they were used for phage isolation.

Plaque formation was observed for 68.1% of all environmental samples (194/285) when *S*. Tm 273 or *S*. E 37 were used as isolation hosts. From each of the 194 plaque-positive plates, a single plaque was randomly picked and propagated three more times against the isolation host, ensuring each phage isolate consisted of a single phage population with no cross contamination from neighboring phage plaques. Eighty-two of the phage isolates formed clear plaques and were selected for further characterization to identify strictly lytic phages for biocontrol of *Salmonella* in food. Previously, we reported on the predominance of flagellotropic, Chi-like *Salmonella* phages across geographically dispersed poultry farms in Thailand (Phothaworn et al., 2019), with two representative phages from this study, STm101 and STm118 (GenBank Accession no: KX765862 and KX765863, respectively) identified as temperate phages.

To remove possible temperate phages from being further

Table 1
Mitomycin C (MMC) induction of bacteriophages from *Salmonella* bacterial strains including those isolated from commercial farm and clinical samples.

Sources of sample	No. of samples	MMC induction of phages	
		% Positive	% Negative
Clinical samples	41	39.0 (16/41)	61.0 (25/41)
Poultry farm samples	46	67.4 (31/46)	32.6 (15/46)
Total	**87**	**54.0 (47/87)**	**46.0 (40/87)**

investigated, we aimed to exclude any Chi-like phages on the assumption they are likely to be temperate phages. Using PCR screening, we tested all 82 clear-forming plaque isolates for the presence of the gene encoding major capsid protein E from the Chi-like *Salmonella* phage SPN19 (GenBank Accession no: NC_019417.1). Overall, 34.1% (28/82) of the phages were positive and therefore excluded from further characterization. Interestingly, all 28 of Chi-like phages identified were isolated from poultry farm samples, supporting our previous observation that Chi-like phages are a predominant phage type found in Thai poultry farms (Phothaworn et al., 2019). The remaining 54 phages selected for further investigation were all isolated from sewage samples where the high amount of human-derived bacteria within the environment potentially leads to a greater diversity of phages compared to poultry farms.

3.2. Selection of Salmonella phages ST-W77 and SE-W109 for biocontrol application

Besides conforming to a strictly lytic lifestyle (determined by genome sequencing, as described below), all phages used for biocontrol should demonstrate a broad host range against a specific pathogenic species. The host ranges of the remaining 54 phages were tested against 30 different *Salmonella* isolates belonging to five serovars of *Salmonella enterica* that represent the most severe foodborne serovars of *Salmonella*: *S.* Enteritidis (n = 10), *S.* Typhimurium (n = 5), *S.* Virchow (n = 5), *S.* Hadar (n = 5) and *S.* Choleraesuis (n = 5). Only two phages, designated ST-W77 (isolated using *S.* Tm 273) and SE-W109 (isolated using *S.* E 37), were capable of lysing all 30 *Salmonella* strains (Supplementary Table 3). Both phages were selected for a more complete host range assessment against a second, larger library of 97 *Salmonella* strains (to give a total of 127 *Salmonella* strains tested) from 22 different serovars, as well as 58 non-*Salmonella* control strains (Table 2). Remarkably, SE-W109 was capable of infecting all 127 *Salmonella* strains tested and none of the control strains. Notably, the *Salmonella* strains tested included 95 commercial farm isolates, 32 clinical isolates, and 47 strains featuring antibiotic resistance, therefore making SE-W109 a highly effective anti-*Salmonella* phage for further biocontrol development. Although not infecting all strains, phage ST-W77 also demonstrated an impressive host spectrum, capable of infecting 82.7% (105/127) of *Salmonella* strains, including 55.3% (26/47) of antibiotic-resistant isolates, and none of the control strains. Interestingly, the clear lysis zones produced by SE-W109 presented a larger radius than those observed for ST-W77 (Fig. 1A), suggesting either a higher efficiency of bacterial lysis or rate of diffusion for SE-W109 progeny phages. Overall, we concluded that as individual phages or in combination (i.e., a phage cocktail), ST-W77 and SE-W109 have great potential to be further developed as broad and *Salmonella*-specific biocontrol agents.

3.3. TEM and one-step phage growth curves of ST-W77 and SE-W109

Transmission electron microscopy (TEM) identified ST-W77 as a Myovirus with an isometric capsid (78 ± 2 nm) and a long contractile tail (132 ± 2 nm) connected to a branched baseplate resembling that of various members of the *Viunalikevirus* genus, including *E. coli* phage CBA120 (Adriaenssens et al., 2012) (Fig. 1B). On the other hand, SE-W109 was identified as a Siphovirus with a smaller, icosahedral head (62 ± 2 nm) and non-contractile tail connected to a less extensive baseplate structure (124 ± 2 nm) (Fig. 1E).

3.4. Phages stability under different temperatures and NaCl concentrations

Phages typically present high physiochemical stability against environmental influences, such as pH, temperature, salinity and common disinfectants making them effective and resilient biocontrol agents for use in different food matrices (Sommer et al., 2019). We decided to test the ability of phages ST-W77 and SE-W019 to retain infectivity after incubation under different temperatures and salinity conditions. To measure temperature resilience, 200 μL of high titer ST-W77 (3.0×10^{11} PFU/mL) and SE-W109 (1.0×10^{11} PFU/mL) were incubated for 1 h at varying temperatures (25–90 °C), replicating temperatures the phages could be exposed to during food processing, with remaining viable phage particles enumerated by soft agar overlay assay (Fig. 2A and B). Both phages tolerated temperatures up to 40 °C without any loss of infectivity; however, viable particle numbers began decreasing above 50 °C with approximately 50% of viable phages lost after incubation at 70 °C. As expected with extreme temperatures, no viable phages remained after incubation at 80 °C or 90 °C. Nevertheless, the result suggest that both phages were stable and retained infectivity when stored at ambient temperatures.

We then tested the stability of both phages in varying salinity by exposing them to salt concentrations typically used for chicken meat preservation (0–3% NaCl) for 1 h at room temperature and measuring the remaining viable phage particles. No loss in infectivity was observed for either phage (Fig. 2B and C). Finally, we monitored the stability of both phage preparations when stored in SM buffer (50 mM Tris-HCl, 100 mM NaCl, 8 mM MgSO4, pH 7.5) at typical storage temperatures of 4 °C and room temperature over 30 days by measuring viable phage numbers every ten days in triplicate. No drop in phage viability was observed at either temperature for the Siphovirus SE-W109 over the 30 days (Fig. 2F). In contrast, the titer of ST-W77 remained stable for ten days, but then dropped significantly between day 10–20 from 2.6×10^{12} to 1.3×10^{7} PFU/mL and 3.5×10^{7} PFU/mL when stored at room temperature or 4 °C, respectively, with viable phage numbers remaining at these levels until day 30 (Fig. 2E). While our limited investigation on

Table 2
Host range analysis of phages ST-W77 and SE-W109 against 127 isolates of *Salmonella* spp. and 58 isolates of other foodborne bacterial pathogens. A complete list of strains is provided in Supplementary Table 2.

	No. of isolates tested	No. of isolates lysed (%)	
		ST-W77	SE-W109
Salmonella serovar			
S. Enteritidis	32	32/32 (100%)	32/32 (100%)
S. Typhimurium	20	20/20 (100%)	20/20 (100%)
S. Virchow	15	15/15 (100%)	15/15 (100%)
S. Hadar	12	5/12 (41.7%)	12/12 (100%)
S. Choleraesuis	10	10/10 (100%)	10/10 (100%)
S. Agona	4	3/4 (75%)	4/4 (100%)
S. Albany	4	4/4 (100%)	4/4 (100%)
S. Anatum	4	4/4 (100%)	4/4 (100%)
S. I 4, [5],12:i:	4	3/4 (75.0%)	4/4 (100%)
S. Give	3	0/3 (0%)	3/3 (100%)
S. Mbandaka	3	0/3 (0%)	3/3 (100%)
S. Altona	3	3/3 (100%)	3/3 (100%)
S. Corvallis	2	0/2 (0%)	2/2 (100%)
S. Chester	2	2/2 (100%)	2/2 (100%)
S. Stanley	2	2/2 (100%)	2/2 (100%)
S. Bovismorbificans	1	0/1 (0%)	1/1 (100%)
S. Kentucky	1	0/1 (0%)	1/1 (100%)
S. Panama	1	1/1 (100%)	1/1 (100%)
S. Saintpaul	1	0/1 (0%)	1/1 (100%)
S. Schwarzengrund	1	0/1 (0%)	1/1 (100%)
S. Singapore	1	0/1 (0%)	1/1 (100%)
S. Weltevreden	1	1/1 (100%)	1/1 (100%)
Total *Salmonella*	**127**	**105/127 (82.7%)**	**127/127 (100%)**
Non-*Salmonella* strains			
Escherichia coli	15	0/15 (0%)	0/15 (0%)
Shigella spp.	15	0/15 (0%)	0/15 (0%)
Vibrio parahaemolyticus	11	0/11 (0%)	0/11 (0%)
Listeria monocytogenes	12	0/12 (0%)	0/12 (0%)
Staphylococcus aureus	5	0/5 (0%)	0/5 (0%)
Total non-*Salmonella*	**58**	**0/58 (0%)**	**0/58 (0%)**

Table 3
Receptor identification of phages ST-W77 and SE-W109 by efficiency of plating (EOP %) against *S.* Typhimurium cell surface receptor mutants.

S. Typhimurium	Other designations and features	% Efficiency of plating (EOP)		Reference
		ST-W77	SE-W109	
Parental strain	DB1755	100	100	Marti et al. (2013)
Δ*waaL*	DB7155, Δ*waaL*: Kan^r; enzyme for ligation of the O-Polysaccharide (O-PS) to the lipid A-core.	0	0	Marti (2013)
ΔLPS	DB7155 ΔLPS::Cm^r; all LPS synthesis genes for regions distal to KDO residues deleted.	0	0	Marti et al. (2013)
Δ*flgK*	SL1344_CH502, Δ*flgK*::Kan^r; hook-filament junction protein	86.8 ± 1.3	0.2 ± 0.1	Choi et al. (2013)
Δ*ompA*	DB7155, Δ*ompA*::Kan^r; outer membrane protein A	94.3 ± 3.1	95.1 ± 3.3	Marti et al. (2013)
Δ*ompC*	DB7155, Δ*ompC*::Kan^r; outer membrane protein C	95.0 ± 1.0	94.8 ± 1.5	Marti et al. (2013)
Δ*ompX*	DB7155, Δ*ompX*::Kan^r; outer membrane protein X	95.4 ± 1.6	94.1 ± 3.4	Marti et al. (2013)
Δ*btuB*	DB7155, Δ*btuB*::Kan^r; TonB-dependent vitamin B12 transporter	97.2 ± 0.9	95.2 ± 3.1	Marti et al. (2013)
Δ*fadL*	DB7155, Δ*fadL*::Kan^r; long-chain fatty acids transporter	97.6 ± 0.9	92.0 ± 1.7	Marti et al. (2013)
Δ*tsx*	DB7155, Δ*tsx*::Kan^r; nucleoside-specific outer membrane channel	95.0 ± 0.9	90.6 ± 3.1	Marti et al. (2013)
Δ*tonB*	DB7155, Δ*tonB*::Kan^r; active transport regulator protein TonB	91.0 ± 1.0	86.4 ± 2.3	Marti et al. (2013)

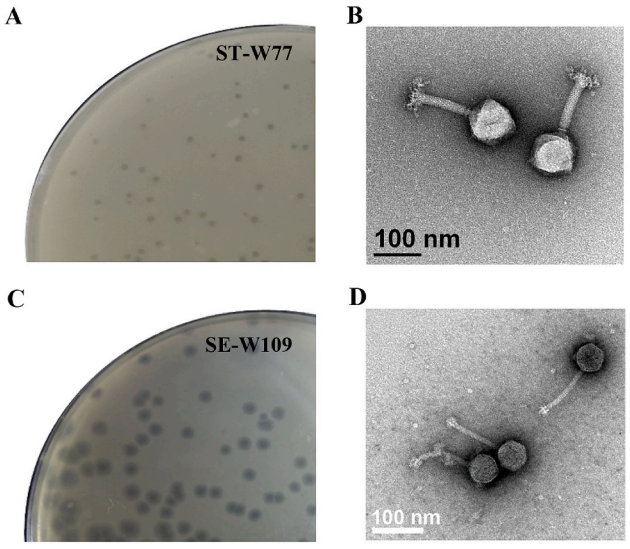

Fig. 1. Plaque and phage morphology of *Salmonella* phages ST-W77 and SE-W109. ST-W77 (A) and SE-W109 (C) present clear plaques of different sizes on their propagation hosts *S.* Tm 273 and *S.* E 37, respectively. TEM analysis confirmed ST-W77 (B) as a member of the *Viunalikevirus* genus within the *Myoviridae* family with a contractile tail and extensive baseplate complex and SE-W109 (D) as a member of the *Siphoviridae* family with a long non-contractile tail.

external and chemical factors affecting phage stability suggests that phage SE-W109 is more stable than ST-W77, we realize that application of these phages as biocontrol agents necessitates more extensive investigations with alternative buffer systems (e.g., pH, salts, temperature) to find an optimal storage condition (Jonczyk et al., 2011).

3.5. Genome analysis identifies ST-W77 and SE-W109 as virulent phages

The use of strictly lytic (virulent) phages is essential for biocontrol applications in food due to the potential risks (e.g., transduction of virulence factors) associated with temperate phages (Mahony et al., 2011; Philipson et al., 2018). In order to screen for integrase genes, as well as potential genes encoding virulence factors, toxins, or antimicrobial resistance, we performed whole genome sequencing of both ST-W77 and SE-W109 genomes. The genome of ST-W77 is 157,789-bp with a G + C content of 44.5%. It consists of 217 predicted ORFs and five tRNA genes. Prokka (Seemann, 2014) and RAST (Aziz et al., 2008) analyses identified 29 ORFs encoding structural proteins, 38 ORFs encoding DNA replication/repair/transcription proteins, 4 ORFs encoding packaging/host lysis proteins, 9 ORFs encoding miscellaneous proteins and 132 ORFs encoding hypothetical proteins (Fig. 3A and Supplementary Table 4). Genome analysis classified ST-W77 as a member of the *Viunalikevirus* genus within the *Myoviridae* family. The ST-W77 genome shares 98% and 96.6% nucleotide sequence similarity with Viunalike viruses *Salmonella* phage vB_SalM_SJ_3 (GenBank Accession no: KJ174318) (Zhang et al., 2014) and CBA120 (GenBank Accession no: NC_016570.1) (Adriaenssens et al., 2012; Kutter et al., 2011).

The genome of SE-W109 was shorter at 42,148-bp with a G + C content of 49.6%. It consists of 62 predicted ORFs and no tRNAs. Prokka (Seemann, 2014) and RAST (Aziz et al., 2008) analyses identified 11 ORFs encoding structural proteins, 8 ORFs encoding DNA replication/repair/transcription proteins, 10 ORFs encoding packaging/host lysis proteins, 2 ORFs encoding miscellaneous proteins and 31 ORFs encoding hypothetical proteins (Fig. 3B and Supplementary Table 5). Phage SE-W109 was classified as a member of the *Jerseylikevirus* genus within the *Siphoviridae* family. The SE-W109 genome shares the highest nucleotide sequence similarity (94.0%) with another Jerseylike-virus *Salmonella* phage SE2 (GenBank Accession no: NC_016763) (Tiwari et al., 2012). Importantly, no integrases, virulence factors or antimicrobial resistance genes were identified in either phage genome. Finally, the combination of phages with difference biological properties can enhance the efficiency of a phage cocktail (Chan and Abedon, 2012), as such it is important to note that ST-W77 (as a Viunalike virus) and SE-W109 (as a Jerseylike virus) are located in different clusters for *Salmonella* phages according to the phylogenic tree generated by Switt et al. (2015).

3.6. Receptor identification for Salmonella phages ST-W77 and SE-W109

To identify the bacterial receptor(s) used by phages ST-W77 and SE-W109, we tested the ability of both phages to infect and lyse (assessed by plaque formation) ten strains of *S.* Typhimurium deficient for various cell wall components proposed as phage receptors (Table 3). Plaque formation of both phages was not affected by removal of the outer membrane proteins (>90.6% EOP), with only a slight drop in plaque formation observed for SE-W109 against the *S.* Tm Δ*tonB* strain (86.4%). However, the significant drop in plating efficiency observed for SE-W109 (EOP of 0.2% ± 0.1) against the flagella-deficient strain (Δ*flgK*) implies the flagella is a receptor for SE-W109 binding. Conversely, ST-W77 was not affected by the lack of flagella, which only saw a slight drop in the EOP to 86.8% against this strain. Interestingly, both phages failed to form any plaques when plated on lawns of *S.* Typhimurium deficient in lipopolysaccharide (ΔLPS). LPS is a key virulence factor of *Salmonella,* which is composed of a phospholipid anchor (Lipid A) attached to a conserved saccharidic core connected to a repetitive polysaccharide structure of high variability and length called the O-antigen. The O-antigen is a major determinant of *Salmonella* serovars and serves as the primary receptor for many phages and their tailspike proteins. Furthermore, no plaques were formed by either phage on *S.*

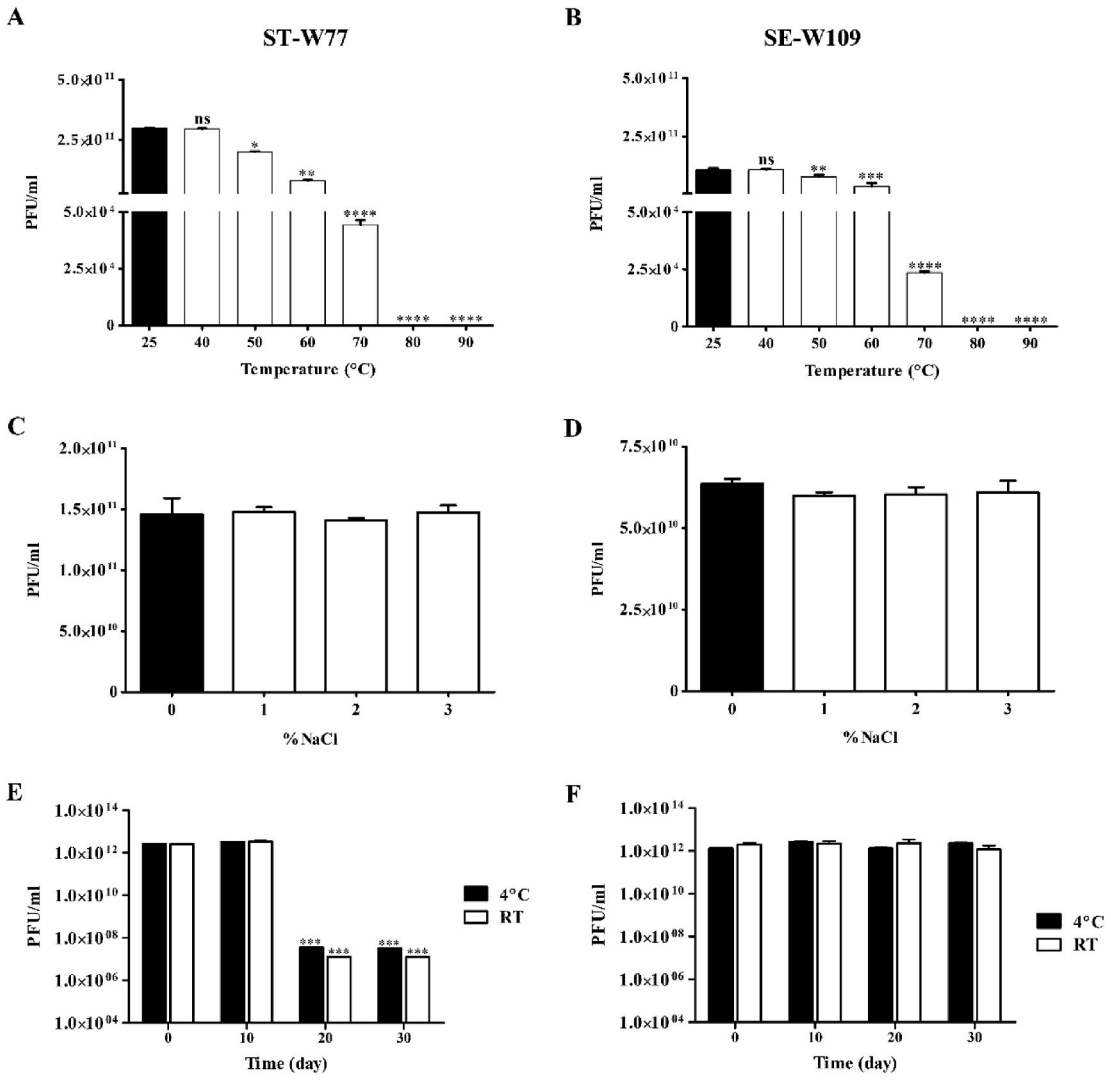

Fig. 2. Tolerance of *Salmonella* phages ST-W77 and SE-W109 to temperature and salt. The viability of both phages was determined using double-layer plaque assay after storage for 1 h in temperatures ranging from 25 to 90 °C (**A** and **B**), after storage for 1 h in the presence of 1–3% (w/v) of NaCl solution (**C** and **D**), and after 30 days storage in SM buffer at 4 °C or room temperature (**E** and **F**). All experiments were performed in triplicate and reported as mean values and SD.

Typhimurium lacking the O-antigen (*ΔwaaL;* deficient for the WaaL mediator of O-antigen ligation to the LPS core), meaning both phages must recognize the *Salmonella* O-antigen as a receptor (Broeker and Barbirz, 2017).

Interestingly, ST-W77 (Fig. 1B) and other Viunalike viruses feature extensive decorations on their baseplates formed by protruding tailspike (TSP1-4) complexes encoded by four consecutive genes within the structural regions of these phages (Adriaenssens et al., 2012). *Escherichia* phage CBA120 (Kutter et al., 2011) is the best-characterized Viunalike virus, for which all four tailspikes have been structurally resolved and their roles in host recognition and strain-specific O-antigen binding and degradation have been determined (Chen et al., 2014; Plattner et al., 2019). Phage ST-W77 shares 96.6% sequence similarity with CBA120 (GenBank Accession no: NC_016570.1) and features four homologous TSPs within its structural region (Fig. 3B) that must also mediate the O-antigen specificity observed for ST-W77.

Bioinformatics analyses identified Gp62 of phage SE-W109 as a putative tailspike protein sharing 73% and 61% sequence similarity with the structurally resolved tailspikes of the *Salmonella* Podovirus P22 (Steinbacher et al., 1994) and *Salmonella* Myovirus Det7 (Walter et al., 2008), respectively, which also recognize and degrade specific O-antigen repeats via intrinsic endorhamnosidase activity during phage infection (Steinbacher et al., 1996; Walter et al., 2008). Due to the extensive host range observed for phage SE-W109 (Table 2) as well as its ability to recognize two receptors – the flagella and O-antigen of *Salmonella* (Table 3) – it would be of great interest to explore the potential of SE-W109 Gp62 and other putative RBPs within its genome as bio-probes for foodborne *Salmonella* detection, such as previously described for other phage RBPs (Denyes et al., 2017; He et al., 2018; Javed et al., 2013; Schmidt et al., 2016; Singh et al., 2011).

3.7. Application of a ST-W77 and SE-W109 cocktail to control *Salmonella* in food

We next investigated the anti-*Salmonella* capacity of a combined ST-W77 and SE-W109 cocktail to control *S*. Typhimurium and *S*. Enteritidis contaminated milk and chicken meat. Varying titers of the combined cocktail was added to 30 mL of milk artificially contaminated with 10^3 CFU of both *Salmonella* strains to test three different multiplicities of infection (MOI; the ratio of phages to bacteria). When high titers of phage were applied, with an MOI of 10^5 or 10^7 equivalent to the addition of 10^8 and 10^{10} PFU, respectively, no viable *Salmonella* could be recovered by direct plating after 6 h corresponding to a reduction of approximately 3 logs (Fig. 4A). Furthermore, no viable cells could be

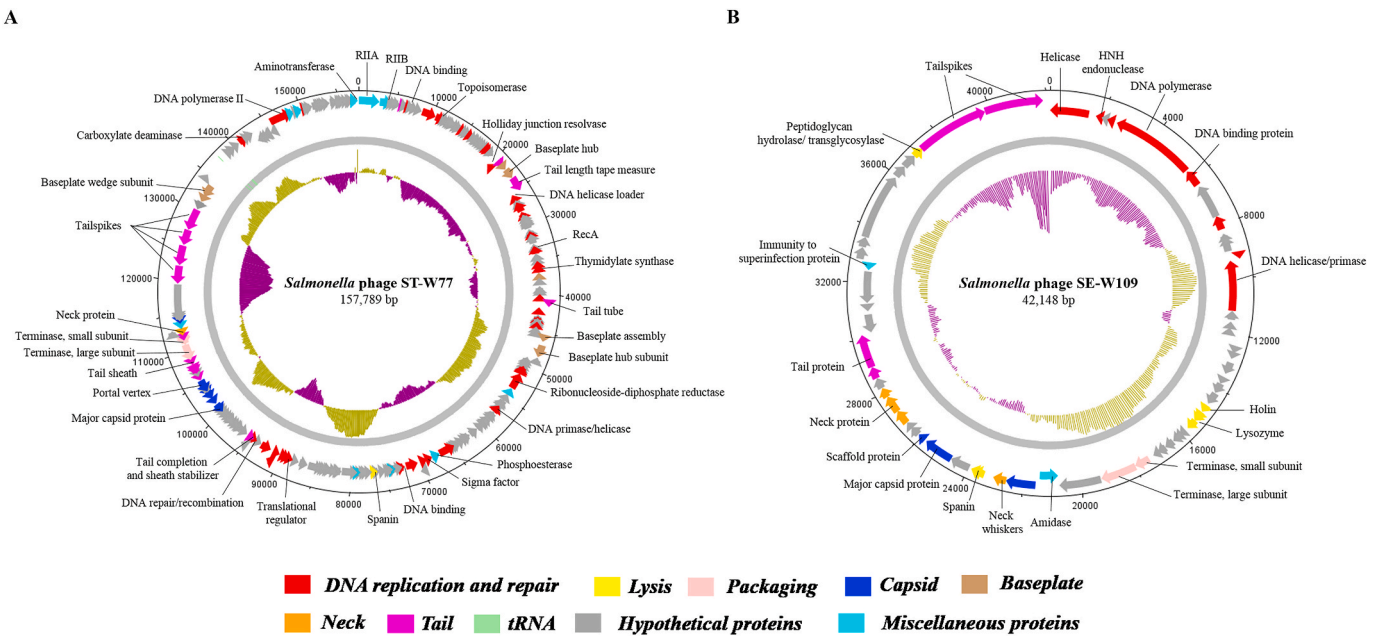

Fig. 3. Circular genome maps of phages ST-W77 (A) and SE-W109 (B). Genomes are annotated and colored based on predicted molecular function with inner plots presenting the GC content across the genomes (yellow above average, purple below average). Figure generated using Artemis tool (version 17.0.1). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

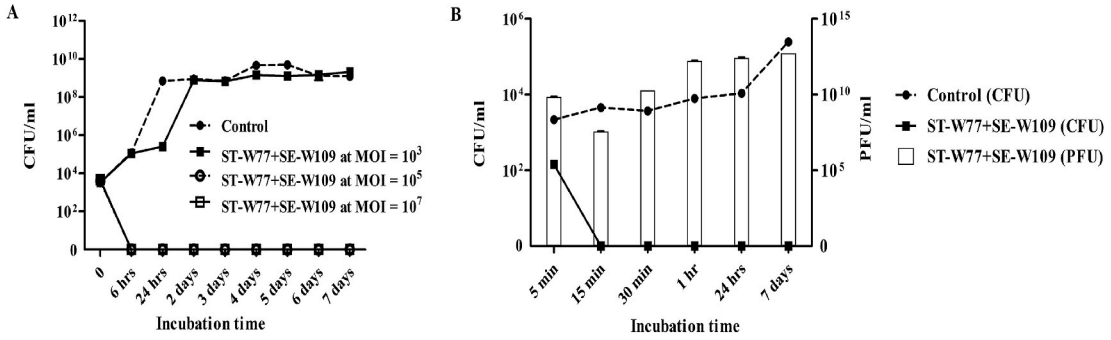

Fig. 4. Efficiency of the ST-W77 and SE-W109 phage cocktail against *Salmonella* contaminated food. **A)** Milk (30 mL) spiked with 10^3 CFU of *S.* Typhimurium and *S.* Enteritidis was treated with different MOIs of the phage cocktail as indicated. Viable cell numbers were quantified at indicated time points by direct plating. SM buffer alone was used as a control. **B)** Chicken meat was spiked with *S.* Enteritidis and *S.* Typhimurium (10^3 CFU each/25 g chicken), wrapped in plastic coated with 10^8 PFU/cm^2 (or SM buffer alone as a control) and incubated at 4 °C. Viable cell numbers and phage titers were determined at indicated time. All experiments were performed in triplicate and reported as mean values and SD.

detected after the full week of treatment, indicating prolonged biocontrol of *Salmonella* by both treatments. Conversely, when treated with the lowest MOI of 10^3 (10^6 PFU added) there was a slight decrease in bacterial growth after 24 h, however, no difference could be observed when compared to the negative control (no phages added), with a sharp increase in bacterial count between 24 and 48 h.

We next investigated the ability of the phage cocktail to eradicate *Salmonella* from the surface of chicken meat. To allow identification against background microflora, drug-resistant (Amp^R) indicator strains of *S.* Typhimurium and *S.* Enteritidis were used to contaminate 25 g pieces of meat. The meat was then wrapped with plastic wrap pre-coated with ~10^8 PFU/cm^2 and stored at 4 °C. For 7 days, the viable *Salmonella* numbers and overall phage titer (ST-W77 and SE-W109 combined) were determined by washing the chicken pieces and directly plating the supernatant (CFU determination) or performing soft agar overlays (PFU determination). As shown in Fig. 4B, no *Salmonella* could be detected between 15 min after wrapping up to the final sampling on day 7. In contrast, viable *Salmonella* were present and continuously grew in the chicken breast sample after treatment with SM buffer alone (control group). Interestingly, while the titer of phages (PFU/mL) appeared to drop after 15 min of treatment, phage titer was higher than the initial titer of the cocktail used after just 1 h and remained at ~10^{12} PFU/mL for the whole 7 days. Potentially, phage amplification had occurred from infecting and killing the contaminating *Salmonella*.

Overall, the immediate and prolonged eradication of *Salmonella* growth in milk by the cocktail closely resembled previous observations of *Salmonella* phage control in foods, for instance, the use of phage FO1-E2 alone (Guenther et al., 2012) or in combination with phage S16 (Marti et al., 2013) provided similar eradication of *Salmonella* from chocolate milk when applied at an MOI of 10^5 for 6 days. Interestingly, other studies using lower MOIs have reported reductions in *Salmonella* numbers, but not complete eradication as observed here. For instance, a cocktail of *Salmonella* phages BSPM4, BSP22A and BSP101 applied at an MOI of 10^4 to *S.* Typhimurium contaminated iceberg lettuce or cucumber could accomplish 4.7–5.5 and 4.8–5.8 log CFU/cm^2 reductions, respectively, but did not completely eliminate *Salmonella* (Bai et al., 2019). Similarly, a cocktail of phages SP-1 and SP-3 at an MOI of 10^3 was capable of reducing *S.* Enteritidis numbers on chicken cuts but did not

completely eliminate the containing bacteria (Augustine and Bhat, 2015). In summary, ST-W77 and SE-W109 are broad range *Salmonella* phages with great potential to be developed further as biocontrol agents for food production and preservation. The phage cocktail showed complete elimination of *Salmonella* from milk and chicken meat when a sufficiently high concentration of phage was added, i.e., an MOI of 10^5 or above, equivalent to other phage biocontrol studies. Nevertheless, it will be of interest to see if this phage cocktail remains active against *Salmonella* for longer than the seven days investigated here as residual and undetected *Salmonella* could always develop resistance to both phages during prolonged storage.

Author contributions

Conceptualization, SK; Funding acquisition, SK; Investigation, PP, MD, RS, JL, MI, JK; Validation, PP, EK, MD; Writing - original draft, PP; Writing - review & editing, MD, EG and SK.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2020.103586.

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