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Control of *Salmonella* Newport on cherry tomato using a cocktail of lytic bacteriophages

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ABSTRACT

Bacteriophages have been envisioned as tools to control a variety of foodborne pathogenic bacteria. *Salmonella* is a foodborne pathogen that is a threat to public health around the world. Contaminated tomatoes have been associated with several *Salmonella* outbreaks. Hence, the objective of this work was to identify and characterize different lytic bacteriophages against *Salmonella* Newport, as one of top ten *Salmonella* serovars associated with human salmonellosis in North America, and then apply these phages to enhance the safety of cherry tomatoes. Four lytic phages against *Salmonella* Newport were selected based on their ability to lyse a majority of the 26 screened *Salmonella* serovars. The selected phages belong to *Myoviridae* (vB_SnwM_CGG4-1, vB_SnwM_CGG4-2) and *Siphoviridae* (vB_SnwM_CGG3-1, vB_SnwM_CGG3-2) families. They were found to be stable at different temperatures and pH, have latent periods ranging from 53 to 65 min and burst sizes from 92 to 177. In addition, the two *Myoviridae* phages have a lower frequency of developing bacteriophage insensitive mutants when compared with the *Siphoviridae* phages. No significant change in virulence gene expression was observed in the developed bacteriophage insensitive mutants when compared to the parental phage sensitive strain. Furthermore, the vB_SnwM_CGG4-1 genome revealed no homology to virulence or lysogenic genes. A phage cocktail was used to control the growth of *S.* Newport in broth medium and on contaminated cherry tomato. Complete inhibition of bacterial growth in broth medium was observed at 25 °C for 24 h. In addition, a 4.5 $\log_{10}$ unit reduction in the bacterial count was observed when applying the phage cocktail onto contaminated tomatoes stored at 22 °C for 3 days. These findings suggest that the isolated phages can be used for biocontrol of *S.* Newport to improve the safety of ready-to-eat (RTE) produce.

1. Introduction

Salmonella is a major global health concern and one of the leading causes of foodborne illnesses and mortality worldwide with an estimated 93 million gastroenteritis cases and 155,000 deaths being reported each year (Hoffmann and Scallan, 2017; Majowicz et al., 2010; Scallan et al., 2015; Thomas et al., 2013). There are estimates of 87,500 illnesses, 925 hospitalizations and 17 deaths caused by *Salmonella* every year in Canada (Thomas et al., 2015). *Salmonella enterica* subsp. *enterica* serovar Newport (*S.* Newport) is one of the most important non-typhoidal *Salmonella* serovars that has been associated with several major outbreaks due to consumption of contaminated foods of animal and plant origin including, but not limited to, ground beef, poultry, alfalfa sprouts, tomatoes, soft cheese and cucumber (CDC, 2002; Espié et al., 2005; Kirk et al., 2004; Sangal et al., 2010; Shariat et al., 2013; Sivapalasingam et al., 2003; Ward et al., 2002). In the USA, it has been reported to be among the top three *Salmonella* serovars associated with foodborne outbreaks since 1970 (CDC, 2013). The recent annual summary from the National Enteric Surveillance program (NESP) also ranked *S.* Newport among the top ten common *S. enterica* serovars accounting for human salmonellosis cases reported in Canada in 2015 (http://publications.gc.ca/site/eng/432850/publication.html).

Interestingly, a multidrug-resistant *S.* Newport strain was associated with a multistate outbreak in the USA due to the consumption of

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ground beef with 42 confirmed cases (Schneider et al., 2011). In 2002, an outbreak of *S.* Newport that caused illness in 510 patients in 26 states of the USA was attributed to tomatoes (Greene et al., 2007). The outbreak strain was traced back to the eastern shore of Virginia, where it was isolated from pond water used to irrigate tomato fields. Another similar study showed the predominance of *S.* Newport (56.8%) in samples collected from ten irrigation ponds in produce farms over a 2-year period (Li et al., 2014). Sixteen out of the isolated twenty nine *S.* Newport strains exhibited resistance to a wide range of antibiotics from ampicillin to ceftriaxone, a third generation cephalosporin. Furthermore, it was found that multi-drug resistance (MDR) and drug susceptibility of *S.* Newport strains can persist in manure-amended soil incubated at ambient temperature for up to 332 days (You et al., 2006).

Despite the many attempts to develop effective technologies to combat microbial contamination, food safety is still a challenge because of food market globalization and spread of antibiotic resistance (Dijksterhuis and Samson, 2006). Many efforts have been made to search for new effective, safe, and sustainable antimicrobial agents to improve food safety. Bacteriophages (phages) have emerged as a promising natural and green technology for food preservation and safety (Anany et al., 2014; Goodridge and Bisha, 2011; Hagens and Loessner, 2010; Moye et al., 2018; Schmelcher and Loessner, 2014). Phages are bacterial viruses that infect bacterial cells with high specificity (Brovko et al., 2012). Virulent (lytic) phages are considered as attractive candidates to be used as antimicrobial agents in the food industry as they are able to replicate exponentially in the presence of susceptible bacteria and eliminate target bacteria regardless of their antimicrobial resistance profiles (Moye et al., 2018). Moreover, phages are seen as environmentally friendly and could be considered as a natural alternative to chemical preservatives (Goodridge and Bisha, 2011). The use of bacteriophages to control the growth of diverse pathogens has been reported in various food commodities including, but not limited to, chicken (Atterbury et al., 2003; Fiorentin et al., 2005; Goode et al., 2003; Higgins et al., 2005), pig skin (Hooton et al., 2011), cooked and raw beef (Bigwood et al., 2008), cheddar cheese made from raw and pasteurized milk (Modi et al., 2001) and fresh produce, including sprouts (Pao et al., 2006; Ye et al., 2010), lettuce (Sharma et al., 2009), and freshly cut apple and honeydew slices (Leverentz et al., 2001). Currently, a number of commercially available phage products (e.g. SalmoFresh™, ListShield™ and PhagheGuard S™) intended for food applications have been granted Generally Recognized as Safe (GRAS) designation by the FDA (Moye et al., 2018). Most of the published results so far strongly support the notion that phages can be added to our arsenal of tools to combat foodborne pathogens and enhance food safety throughout the food supply chain. However, further research is required to tackle more phage/host/food product combinations and provide more characterized phages showing a biocontrol potential for food application. Based on the need for improved *S.* Newport control in food products, and the growing track-record of phages as antibacterial/food safety agents, in this research we evaluate the efficacy of newly isolated and characterized specific lytic phages to control *S.* Newport on cherry tomatoes to mitigate the risk of the associated outbreaks, beginning with phage isolation and cocktail development.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) were obtained from Oxoid, Canada. Semisolid overlay (TSBss) media was prepared by adding agar to TSB to a concentration of 0.5% w/v. All of these media were used to culture the bacterial host and to isolate and propagate their phages. *Salmonella* spp. strains were selected from the Canadian Research Institute for Food Safety (CRIFS) Culture Collection at the University of Guelph (Table 1). *Salmonella* Newport C 389 was used for phage isolation and propagation. Pure cultures were obtained from −80 °C frozen stocks and maintained at 4 °C.

Table 1
Salmonella serovars that were used for phage isolation and propagation from CRIFS culture collection. *S.* Newport C389 was used for phage isolation and propagation. All strains were involved in the host range study.

Salmonella enterica Serovars	CRIFS culture collection number	*Salmonella enterica* Serovars	CRIFS culture collection number
S. Enteritidis	C 417	*S.* Agona	C 386
S. Typhimurium	C 1077	*S.* Bredeney	C 388
S. Typhimurium	C 391	*S.* Poona	C 390
S. Heidelberg	C 434	*S.* Choleraesuis	C 402
S. Thompson	C 412	*S.* Johannesburg	C 403
S. Newport	C 389	*S. Urbana*	C 404
S. Braenderup	C 410	*S.* Haardt	C 406
S. Hadar	C 405	*S.* Tennessee	C 408
S. Oranienburg	C 4008	*S.* Ohio	C 409
S. Saintpaul	C 433	*S.* Mbandaka	C 411
S. Reading	C 383	*S.* Sendai	C 414
S. Brandenburg	C 384	*S.* Berta	C 416
S. Indiana	C 385	*S.* Bredeney	C 432

2.2. Enrichment and isolation of bacteriophage

For isolation of the phages, four sewage water samples collected from the Guelph Wastewater Treatment Plant (WWTP) (Guelph, ON, Canada) were examined for the presence of phages. Ten milliliter of each sample were enriched in an equivalent volume of TSB medium and 100 µl of an overnight culture of *S.* Newport. The mixture was incubated for 16 h with shaking (150 rpm) at 37 °C. After incubation, the suspensions were centrifuged at 7000 × *g* for 20 min at 4 °C (Beckman Avanti J-20 XPI, Beckman Coulter Inc., Mississauga, ON, Canada). The supernatant was then transferred to another tube and was filtered through a 0.45 µm sterile disposable filter (Mandel Scientific, Guelph, ON, Canada) prior to storage at 4 °C. Detection of phage activity was performed using the spot test technique (Sambrook et al., 1989). Phage activity was detected in the enriched samples by spotting 10 µl from each previously prepared supernatant onto the overlay containing 100 µl of exponentially growing bacterial culture, which was allowed to dry for 20 min before incubation for 16 h at 37 °C. After incubation, the plates were investigated for the presence of lysis zones. These zones were then removed from the TSA plates by cutting the soft layer from the plate using sterile pipette tips and placing them separately into 300 µl of CM phage buffer (0.735 g/l $CaCl_2 \cdot 2\,H_2O$: 2.5 g/l $MgSO_4 \cdot 7\,H_2O$; 0.05 g/l gelatin; 6 ml/l 1 M Tris buffer; pH 7.2). The tubes were held at room temperature overnight to let the phage particles diffuse out of the soft agar.

2.3. Purification of phages

The isolated phages were purified using the soft agar overlay method (single plaque assay) (Sambrook et al., 1989). Plaques of varying sizes and morphologies were picked from the overlay plates incubated at 37 °C for 16–20 h and placed separately in 300 µl CM phage buffer. The tubes were kept at room temperature overnight to allow the phage particles to diffuse into the buffer. Selection of individual plaques from the soft agar was repeated three successive times. The phage suspensions were stored at 4 °C prior to propagation.

2.4. Propagation of bacteriophage

Phage propagation was performed using the soft agar overlay method (Sambrook et al., 1989). Volumes of 100 µl of the phage suspension were mixed with approximately 10^8 CFU/ml of *S.* Newport and incubated for 15 min at ambient temperature to allow attachment. Following this step, 4 ml of molten TSB containing 0.5% agar were

added, mixed and poured onto TSA plates. After solidification, the plates were incubated at 37 °C for 16–20 h. Phage particles were collected by adding 3 ml of CM phage buffer to each plate and the top layer of soft agar from all plates was scraped off using sterile spreaders. The collected overlay was transferred into 50 ml tubes. The remaining agar and phages were washed from the plates with an additional l ml of the buffer for each plate and added to the collected overlay. The tubes were placed on ice for 30 min. The mixture was vortexed gently (Vortex-Genie-2; Scientific Industries, Inc., Bohemia, NY) and centrifuged at 7000 ×*g* for 20 min at 4 °C. The supernatant was filtered through a 0.45 μm membrane filter. The phage lysate was stored at 4 °C after determination of its titre (PFU/ml) as previously described by Sambrook et al., 1989.

2.5. Characterization of selected Salmonella phages

2.5.1. Transmission electron microscopy

The four isolated *Salmonella* phages were examined by transmission electron microscopy (TEM). To prepare the phages for TEM, 1 ml of high titre phage stocks ($\geq 10^{10}$ PFU/ml) was centrifuged at 16,000 ×*g* for 1 h at 4 °C (Microfuge R centrifuge, Beckman Coulter Inc.) and washed twice with CM buffer. The supernatant was discarded and the pellet was gently re-suspended in 20 μl of CM buffer. Five microliters of the re-suspended phages were applied onto 300-mesh copper grids coated with formvar and allowed to stand for 2 min. The excess liquid was drawn off by filter paper and the remaining phages were negatively stained with 2% (w/v) uranyl acetate for 30s and then the excess liquid was drawn off again by blotting with filter paper (Ackermann, 2012). Finally, the samples were examined in a LEO 912AB electron microscope (Energy filtered TEM, EFTEM, LEO 912ab model operated at 100 kV, Zeiss, Germany).

2.5.2. Host range study

The host ranges were assessed for four isolated phages by measuring the optical density (OD) of the tested bacterium in the presence of phage using the Bioscreen C Microbiology Plate Reader (Labsystems, Helsinki, Finland) as previously described (Anany et al., 2011).

The experimental parameters for all of the experiments were the following: single, wide band (wb) wavelength; 25 °C incubation temperature; five-minute preheating time; kinetic measurement; measurement time 24 h; reading every 20 min and agitation at a medium intensity for 10 s before measurements. Fifty microliters of the phage lysate were transferred to separate wells of the sterile honeycomb plates of the Bioscreen C reader (Fisher Scientific, Mississauga, ON), and then each of the wells was inoculated with 150 μl of the diluted overnight host bacterial culture (around 10^3 CFU/ml). The multiplicity of infection (MOI) was around 3×10^4. The control wells contained phage only, TSB, or bacteria plus 50 μl of CM. All samples were tested in triplicate. The OD data were analyzed using the Bioscreen C data processing software version 5.26 (Labsystems, Helsinki, Finland) to determine the detection time (time required for each test well to increase by 0.3 OD units). Detection times (h: min) were converted to decimal values, averaged, and the mean control detection time was subtracted from all control data for each isolate tested and expressed as detection time difference (DT diff). Instead of having positive and negative results and based on this time difference, the lytic activity of the phages were designated as; (N): in which phage did not cause any delay in bacterial growth and the growth curve was similar to that of the control; (D): phage caused a delay in growth by < 5 h; (D+): phage caused a delay in growth by five or more hours; and (C): in which phage caused complete inhibition of the growth during the 24 h test period.

2.5.3. Stability of S. Newport phages at different temperatures and pH values

The pH stability of the four isolated phages was tested as follows: l00 μl of the phage lysate of about 10^{10} PFU/ml were added to 900 μl of buffer solutions at different pH values (5.0, 7.0, and 9.0), and then incubated at 37 °C for 1 h, 3 h, 5 h, 24 h, and 1 week. Each treatment was performed in triplicate. After incubation, ten-fold serial dilutions for each treatment were made and l00 μl of each were mixed with l00 μl of overnight bacterial culture, incubated for 15 min at 37 °C, then 4 ml of overlay media were added to the mixture and spread over TSA plates. Plates were incubated at 37 °C and the phage titre was determined the next day. The average of the triplicate counts was taken and the $\log_{10}$ unit reduction in the phage titre was calculated. For the temperature treatments, 1 ml of four phage lysates of around10^{10} PFU/ml was incubated at different temperatures (−25 °C, 4 °C, 22 °C, 37 °C, 45° or 55 °C) for one day, one week, or two weeks. Each temperature treatment was performed in triplicate and the average of triplicate counts was calculated.

2.5.4. One-step growth curve

One-step growth curve experiments were used to determine burst sizes and latent periods for the selected phages as described by (Kropinski, 2018). Phage of around 1×10^5 PFU/ml was added to its host bacterium 1×10^7 CFU/ml to achieve MOI of around 0.01 and incubated in a water bath at 37 °C for 5 to 25 min. Then, 1 ml was removed and added to l00 μl of chloroform and mixed. One hundred microliters of this mixture were added to 4 ml of overlay medium containing approximately 10^8 CFU/ml of the host bacterium and poured onto TSA plates to determine the degree of adsorption of phage to bacterial cells. After 5.5 min, the original mixture of phage and bacterial suspension was serially diluted into fresh TSB (10^{-2}, 10^{-3}, 10^{-4} dilutions) and the three tubes corresponding to the three different dilutions were incubated in a water bath at 37 °C. Beginning from 7 min after addition of phage to its host and at 5 min intervals for 180 min, the diluted suspensions were plated by mixing 100 μl of the suspension with 4 ml of overlay medium containing 10^8 CFU/ml of indicator bacterium, and pouring the mixture onto a TSA plate. The number of plaques was determined after 24 h of incubation at 37 °C. The relative burst size was determined using the equation:

$$\text{Relative burst size} = \frac{\text{final titre-initial titre}}{\text{initial titre}}$$

The relative burst size at different times was plotted against time to determine the latent period and burst size.

2.5.5. Determination of frequency of bacteriophage insensitive mutant (BIM) development

The frequency of bacteriophage insensitive mutant (BIM) development was determined for four isolated phages as described previously (O'Flaherty et al., 2005; O'Flynn et al., 2004). Phage lysate was mixed with bacterial host culture at an MOI of 10 and incubated for 15 min at 37 °C. Then, 4 ml of overlay agar medium were added and poured onto TSA plates. The plates were incubated for 6–20 h at 37 °C. Both pure phage suspensions and a cocktail of phages were used. Any developing colonies were counted, and the BIM frequency (number of surviving colonies divided by the original bacterial concentration) was calculated. All experiments were performed in triplicate.

2.6. Virulence gene expression in bacteriophage insensitive mutants by real-time quantitative PCR (RT-qPCR)

The BIMs of *S.* Newport were subcultured three times and tested for their phage sensitivity to make sure that they are phage resistance. Then they were grown in Luria Bertani (LB) (Difco) broth for 16 h and diluted (1:100) in 10 ml fresh LB medium. Sample tubes were shaken at 250 rpm at 37 °C for 4 h, until an OD_{600} of 0.5 was reached. Cells were harvested by centrifuging at 5000 ×*g* for 3 min at room temperature using a Beckman JLA-16.250 centrifuge and immediately processed for total RNA extraction.

2.6.1. RNA extraction and cDNA synthesis

The bacterial cell pellets were resuspended in a solution containing 10 µl of proteinase K (Qiagen, Toronto, ON, Canada), 200 µl of Tris-EDTA buffer (Sigma Aldrich, Oakville, ON, Canada), 60 µl of 20 mg/ml lysozyme (Sigma, Oakville, ON, Canada), and incubated at 37 °C for 1 h with shaking at 450 rpm. RNA was extracted using the RNeasy minikit (Qiagen) according to the manufacturer's instructions. After RNA extraction, residual DNA elimination and RNA cleanup was performed in each sample using DNase I (Roche Applied Science, Laval, Quebec, Canada) and Cleanup kit (Qiagen) as described previously (Mundi et al., 2013). The purified RNA was used immediately for reverse transcription (RT) using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Burlington, ON, Canada) according to the manufacturer's instructions. For each sample, a control without reverse transcriptase was included to confirm the absence of contaminating DNA. Synthesis of cDNA was performed in a Mastercycler Gradient Thermocycler (Eppendorf Scientific, Westbury, NY, USA) under the following conditions: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min, and hold at 4 °C. The cDNA was stored at −20 °C until further analysis.

2.6.2. RT-qPCR

The primers selected for the amplification of the virulence genes in *S.* Newport are shown in Table 2. The real-time PCR assay was carried out in a ViiA™ 7 detection system (Applied Biosystems, Carlsbad, CA, USA) in a 20-µl volume reaction mixture containing 1.5 µl of template cDNA, 1 µl of each of the primers (final concentration of 400 nM), 10 µl of SYBR Select PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), and 6.5 µl of DNase/RNase-free deionized water. Quantitative real-time PCR for the four selected virulence genes (*hilA*, *ssrB*, *invA* and *sopD*) of *S.* Newport was performed and the gene encoding 16s rRNA was used as the reference gene. The amplification conditions were: an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 54 °C for 30 s, and 72 °C for 45 s. All PCR reactions were done in triplicate and the average cycle threshold (Ct) values were calculated. The virulence gene expression in bacteriophage sensitive bacterial strains was compared to resistant strains. Real-time PCR amplification efficiencies and relative expression ratios were calculated as described previously (Pfaffl, 2001). The following equations were used:

$$\Delta Ct = Ct \text{ of reference gene} - Ct \text{ of target (virulence) gene}$$

$$\Delta\Delta Ct = \Delta Ct \text{ of Sample} - \Delta Ct \text{ of Control}$$

$$\text{Relative expression level} = 2^{\Delta\Delta Ct}$$

2.7. Phage DNA isolation and purification

DNA extraction from purified CGG4-1 phage was performed using Norgen's Phage DNA Isolation Kit, according to the instructions of the manufacturer (Norgen Biotek Corp. Thorold, ON, Canada). High titre phage suspensions were incubated at room temperature for 15 min with 20 units of DNase I per 5 ml phage suspension, followed by thermal inactivation of DNase I (75 °C for 5 min). The resulting suspension was incubated with 500 µl of Lysis Buffer and 4 µl of 20 mg/ml proteinase K for 30 min at 55 °C and subsequently 65 °C for 15 min. After addition of 320 µl of isopropanol, phage DNA was purified from the treated suspension using column chromatography.

DNA concentration was determined by sample absorbance at 260 nm (DU640 spectrophotometer; Beckman Coulter).

2.8. Sequencing and genome analysis of CGG4-1 phage

CGG4-1 phage was selected for sequencing based on its broad host range pattern and strong lytic activity compared to the other isolated phages. CGG4-1 phage DNA was isolated as described in the preceding section. Isolated DNA was sequenced by 454 pyrosequencing at the Genome Quebec Innovation Centre (Montreal, QC, Canada). Annotation was done using MyRast (Aziz et al., 2008; Brettin et al., 2015; Overbeek et al., 2013) and Geneious (Kearse et al., 2012). Ribosome binding sites were determined visually (Shine and Dalgarno, 1974). Transcription terminators were identified using ARNold (Gautheret and Lambert, 2001). Genes encoding tRNAs were confirmed using tRNAScan-SE v.1.21 (Lowe and Chan, 2016). The amino acid sequences of the open reading frames (ORFs) were screened against the National Center for Biotechnology Information (NCBI) using BLSATp to find similar, characterized, proteins (Gish, 1993). Conserved domains were checked using Pfam (Finn et al., 2015) and InterProScan (Jones et al., 2014). Transmembrane domains were determined using TMHMM (Krogh et al., 2001) and Phobius (Käll et al., 2004). Selected proteins were compared in silico with those of related phages using EMBOSS Matcher (http://www.ebi.ac.uk/Tools/psa/emboss_matcher/). A genome map was prepared using CGView (Stothard and Wishart, 2004), aligning the genome of CGG4-1 with those of phages T4, S16 and STML-198. The annotated genome sequence for the *Salmonella* Newport vB_SnwM_CGG4-1 was submitted to the NCBI nucleotide database under accession number KU867307.

2.9. Bacteria challenge experiments in broth medium and on cherry tomatoes

2.9.1. Effect of Salmonella phage cocktail on the growth of S. Newport in TSB

The efficacy of a phage cocktail composed of four lytic *S.* Newport specific phages to control their host was assessed in TSB. An overnight culture of *S.* Newport was diluted to 10^3 CFU/ml. Phage cocktail was mixed with the bacterial suspension to obtain MOIs of 10^3 and 10^5 and incubated for 24 h at 25 °C. Samples were collected at 1, 3, 5, 7 and 24 h and serially diluted in sterile 0.85% saline to count the surviving bacterial cells. Each dilution was plated onto TSA plates in duplicate and incubated for 24 h at 37 °C. Colonies were counted to determine the bacterial $\log_{10}$ reduction in each sample.

Table 2
Primers used in this study.

Gene	Gene function	Primer sequence	References
16s rRNA	Housekeeping gene	F: 5′-CAAGTCATCATGGCCCTTAC-3′ R: 5′-CGGACTACGACGCACTTTAT-3′	Asakura et al. (2012)
ssrB	Effector protein	F: 5′-TGGTTTACACAGCTACCAA-3′ R: 5′-GGTCAATGTAACGCTTGTTT-3′	Sharma (2014)
hilA	A transcriptional regulator for SPI-1	F: 5′-TGTCGGAAGATAAAGAGCAT-3′ R: 5′-AAGGAAGTATCGCCAATGTA-3′	Sharma (2014)
sopD	A transcriptional regulator for SPI-1	F: 5′-ATTAATGCCGGTAACTTTGA-3′ R: 5′-CTCTGAAAACGGTGAATAGC-3′	Sharma (2014)
invA	Invasion of the host cells	F: 5′-GAAATTATCGCCACGTTCGGGCAA-3′ R: 5′-TCATCGCACCGTCAAAGGAACC-3′	Rahn et al. (1992)

2.9.2. Effect of Salmonella phage cocktail on the growth of S. Newport on cherry tomato

A phage cocktail composed of four lytic *S.* Newport specific phages isolated in this study (CGG3-1, CGG3-2, CGG4-1, and CGG4-2) was applied to cherry tomatoes artificially contaminated by *S.* Newport. Tomato fruits were purchased on the first day of the experiment from a local grocery store and initially screened for contamination with *Salmonella* spp. using specific medium XLT4 and Modified Brilliant green agar medium (Oxoid, Fisher Scientific). Approximately 10 g of tomato fruit per sample were washed three times with sterile deionized water and left to dry. The tomato fruits were then immersed in 10^3 CFU/ml bacterial culture prepared in 0.85% saline and left for 1 h to allow bacteria to adsorb to the fruit. Excess bacterial culture was discarded and the tomatoes were left to dry for 15 min. The artificially contaminated tomato fruits were treated with 10 ml of phage cocktail (10^6 or 10^8 PFU/ml) and left for 1 h to allow attachment of phage to the surface of the fruit. Excess phage solution was discarded and tomato was again left to dry for 15 min. Phage buffer was added to the untreated fruits (contaminated - untreated). Negative controls were treated with 0.85% saline and phage buffer instead of the bacterial and phage suspensions (not contaminated – untreated). Treated, untreated, negative control tomato fruits were placed in separate stomacher bags and stored 0, 1, 2, 3, and 4 days at room temperature (25 °C). Triplicate samples were prepared for each treatment and sampling time. At each sampling time, the tomato samples were transferred to a Whirl-Pak bag (Nasco, Fort Atkinson, WI) containing 10 ml of 0.85% saline solution and the sample was stomached for 1 min at 200 rpm (Seward Blender Stomacher 400 circulator, Seward Laboratory Systems Inc., NY). The liquid portion was transferred to a sterile centrifuge tube and serially diluted in sterile 0.85% saline solution. Aliquots of 100 µl were plated in duplicate on Modified Brilliant green agar plates and incubated at 37 °C for 24 h. Colonies were counted and used to calculate the bacterial count (CFU/g) in each sample.

All experiments were carried out in triplicates. Results are reported as means of the triplicates ± standard deviation.

3. Results

3.1. Isolation and characterization of S. Newport phages

Four phages producing clear lytic plaques were isolated and purified from sewage water collected from Guelph Wastewater Treatment Plant (WWTP) using *S.* Newport as permissive and enrichment host. The phages are designated as CGG3-1, CGG3-2, CGG4-1, and CGG4-2. The selected phages were subjected to a set of characterization experiments including morphology, host range pattern, stability at different pH and temperature, One step growth curve and development of BIMs. These are crucial criteria to identify potential phages for biocontrol application.

3.1.1. Morphology

The four selected *S.* Newport phages were examined by TEM to determine their mophotypes. All the examined phages have icosahedral heads and contractile or non-contractile tails. These phages essentially represent four different morphotypes (Fig. 1). *Salmonella* phages CGG3-1 and CGG3-2 are members of the *Siphoviridae* family as determined by the presence of a long, flexible tail and the absence of a contractile sheath, whereas phages CGG4-1 and CGG4-2 have contractile tails and therefore belong to the family *Myoviridae*. The respective head diameters and tail lengths of phages CGG3-1, CGG3-2, CGG4-1, and CGG4-2 were determined (Table 4).

3.1.2. Host range analysis

Host range pattern was determined for each of the selected phages by turbidimetric measurement over time using a Bioscreen C plate reader. Different *Salmonella* serovars associated with previous foodborne outbreaks were used in this experiment. *Salmonella* phage CGG3-1 infected 16 strains and completely inhibited the growth of 8 out of the 26 tested *Salmonella* serovars. However, the other siphovirus, CGG3-2, had the narrowest host range of the tested *Salmonella* phages as it infected only six *Salmonella* serovars and caused a complete growth inhibition for only one serovar. On the other hand, the myophages, CGG4-1 and CGG4-2, were able to infect all the examined 26 *Salmonella* strains to varying degrees. Phage CGG4-1 caused complete growth inhibition of 19 serovars, while phage CGG4-2 caused complete growth inhibition of 15 serovars (Table 3).

3.1.3. Stability of Salmonella phages at different pH and temperature

The four isolated phages were stored in buffers of different pH values (5.0, 7.0, and 9.0) and temperatures (−25 °C, 4 °C, 22 °C, 37 °C, 45°and 55 °C). Then the log reductions (PFU/ml) in phage count were determined by the overlay technique. The four *Salmonella* phages were not affected by storage at the tested pH values for up to 24 h (Fig. 2). On the other hand, there was no reduction in the titre of any of the phages when stored at −25 °C, 4 °C, 22 °C or 37 °C. However, the stability at 45 °C and 55 °C was variable among the phages. CGG3–1 and CGG3–-2 phages were more stable than CGG4-1 and CGG4-2 phages at 55 °C, with approximately 1 $\log_{10}$ reduction in phage titres after 24 h while more than five $\log_{10}$ reduction in CGG4-1 and CGG4-2 phage titre was observed. At the same temperature, there was 5 $\log_{10}$ reduction in both CGG3-1 and CGG3-2 count after one week, and seven and 8 $\log_{10}$ reduction, respectively, after two weeks. No lytic activity was detectable in the CGG4-1 and CGG4-2 phage suspensions after one week of incubation at 55 °C. At 45 °C there was approximately 1 $\log_{10}$ reduction in CGG3-1 and CGG3-2 phage count after one week, and 4 and 3 $\log_{10}$ reduction, respectively, when the incubation time was two weeks. Incubation for 24 h at 45 °C resulted in 1 $\log_{10}$ reduction in CGG4-1 phage titre, with no apparent effect observed on CGG4-2 phage. After one week of incubation at 45 °C, CGG4–1 phage was reduced in titre by 3 $\log_{10}$ units and CGG4-2 was reduced in titre by about 1 $\log_{10}$ PFU/ml. After incubation for two weeks at 45 °C, CGG4-1 phage count was reduced by approximately 4 $\log_{10}$ units while CGG4-2 phage count was reduced by about 1.5 $\log_{10}$ units (Fig. 3).

3.1.4. One-step growth curve

Burst sizes and latent periods were determined for the four *Salmonella* phages (Fig. 4). The *Siphoviridae* phages (CGG3-1 and CGG3-2) have lower burst sizes (100 and 92) with latent periods of 59 and 65 min, respectively, compared to CGG4-1 and CGG4-2, which have burst sizes of 177 and 149 PFU/ml and latent periods of 53 and 58 min, respectively.

3.1.5. Bacteriophage insensitive mutants (BIM)

The frequency of *S.* Newport BIM development against the selected phages was determined after incubation with individual phages and a cocktail of the four phages at 37 °C for 18 h (Table 5). *S.* Newport was able to develop resistant mutants against individual phages as well as against the phage cocktail. CGG3–2 and CGG3–1 caused a remarkably higher frequency of BIMs compared to the other *Salmonella* phages. Interestingly, CGG 4–1 and CGG 4–2 were associated with BIM frequencies of 2.66×10^{-10} and 1.06×10^{-9}, respectively. Using a cocktail of the four phages resulted in a BIM frequency of 5.5×10^{-9}.

3.2. Virulence genes expression of bacteriophage insensitive mutants

The expression of virulence genes in *Salmonella* Newport BIMs was investigated and the original phage sensitive strains were used as a control for comparison. A minor increase in the expression (1.14, 1.45, 1.72, and 1.24-fold change) of the four virulence genes (*hilA*, *ssrB*, *invA* and *sopD*, respectively) in *S.* Newport BIM strain as compared to the original sensitive strain was observed (Table 6).

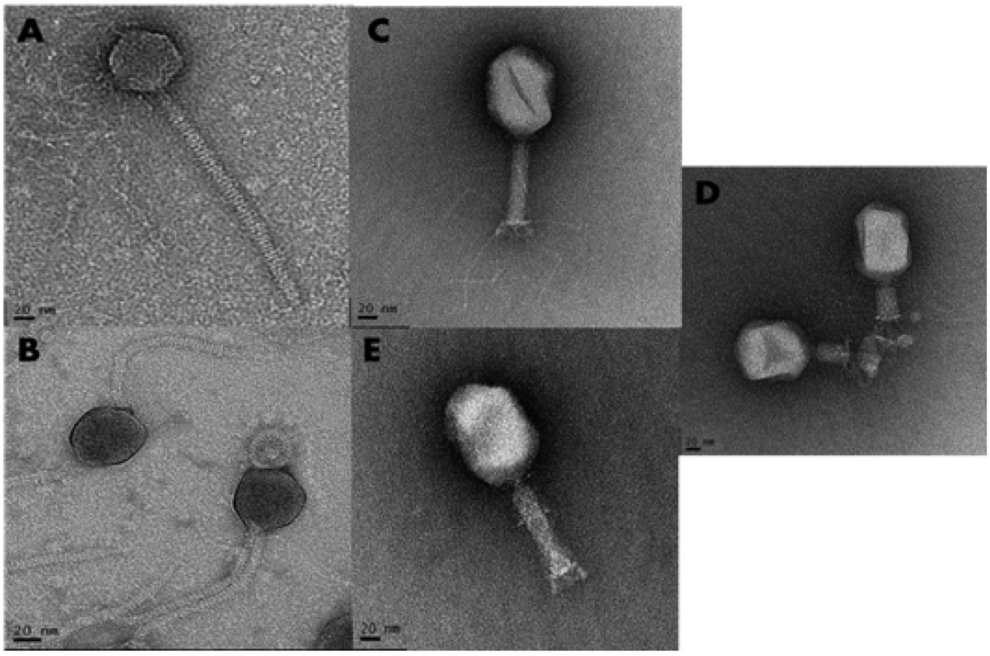

Fig. 1. Electro-micrograph represents the morphology of four *Salmonella* Newport phages using TEM and showed that (A) CGG 3-1 phage and (B) CGG 3-2 are related to *Siphoviridae*. (C) CGG 4-1 phage, (D) CGG 4-1 phage with contractile tail and (E) CGG 4-2 phage are related to *Myoviridae*.

Table 3
Host range pattern of the selected *Salmonella* phages using Bioscreen C.

Salmonella serovars	CGG3-1	CGG3-2	CGG4-1	CGG4-2
S. Enteritidis	D	N	C	D
S. Typhimurium 1077	N	N	C	C
S. Typhimurium 391	D	N	C	C
S. Heidelberg	N	N	C	D+
S. Thompson	N	N	C	C
S. Newport	D+	D+	C	C
S. Braenderup	D	N	D+	C
S. Hadar	D+	D+	C	C
S. Oranienburg	D	N	D	D
S. Saintpaul	D	N	C	C
S. Reading	D	N	D+	D+
S. Brandenburg	N	N	D+	D+
S. Indiana	N	N	C	C
S. Agona	N	N	D+	D+
S. Bredeney	D	N	C	D+
S. Choloraesuis	D	N	C	D
S. Johannesburg	D	D	C	D+
S. Urbana	D	D	C	C
S. Haardt	C	C	C	C
S. Tennessee	D+	N	D+	C
S. Ohio	D	N	D	D
S. Mbandaka	N	N	C	C
S. Sendai	D	D	C	C
S. Berta	D	N	C	C
S. Bredeney	D	N	C	D+
S. Poona	D	D	C	C

C: complete lysis of the bacterial growth.
D+: > 5 h delay in bacterial growth to reach log phase compared to control (i.e. Detection time difference > 5 h).
D: < 5 h delay in bacterial growth to reach log phase compared to control (i.e. Detection time difference < 5 h).
N: no effect of phage on bacterial growth.

3.3. General features of CGG4-1 genome

The whole genome of vB_SnwM_CGG4-1 was sequenced and revealed a circularly permuted, linear dsDNA genome with 159,878 bp and a GC content of 36.9% (Fig. 5). A total of 259 ORFs were identified in the CGG4-1 genome. Characterized orthologues were found for 121

Table 4
Dimensions of the isolated phages.

Phage	Head diameter (nm)	Tail length (nm)	Family
CGG3-1	76.03 ± 1	211.54 ± 2	*Siphoviridae*
CGG3-2	66.47 ± 1	203.44 ± 2	*Siphoviridae*
CGG4-1	104.07 ± 0.5	110.21 ± 3	*Myoviridae*
CGG42	88.02 ± 2	94.44 ± 2	*Myoviridae*

Table 5
Bacteriophage insensitive mutant development against *Salmonella* phages.

Phage	BIM frequency[a]
CGG3-1	$7.88^{a}10^{-5}$
CGG3-2	$1.95^{a}10^{-4}$
CGG4-1	$2.66^{a}10^{-10}$
CGG4-2	$1.06^{a}10^{-9}$
Cocktail	$5.5^{a}10^{-9}$

[a] BIM frequency = number of surviving colonies/original bacterial titre).

Table 6
Expression ratio of the virulence genes in *Salmonella* Newport bacteriophage resistant strains normalized to the expression of housekeeping genes and compared to the bacteriophage sensitive parental strains.

Virulence genes	Expression ratio[a]
hilA	1.14 ± 0.46
ssrB	1.45 ± 0.37
invA	1.72 ± 0.66
sopD	1.24 ± 0.44

[a] Values more than +2 are considered significant up-regulation. The expression ratio is normalized to the housekeeping gene; *16s rRNA* in *S*. Newport. The experiments were performed in triplicate.

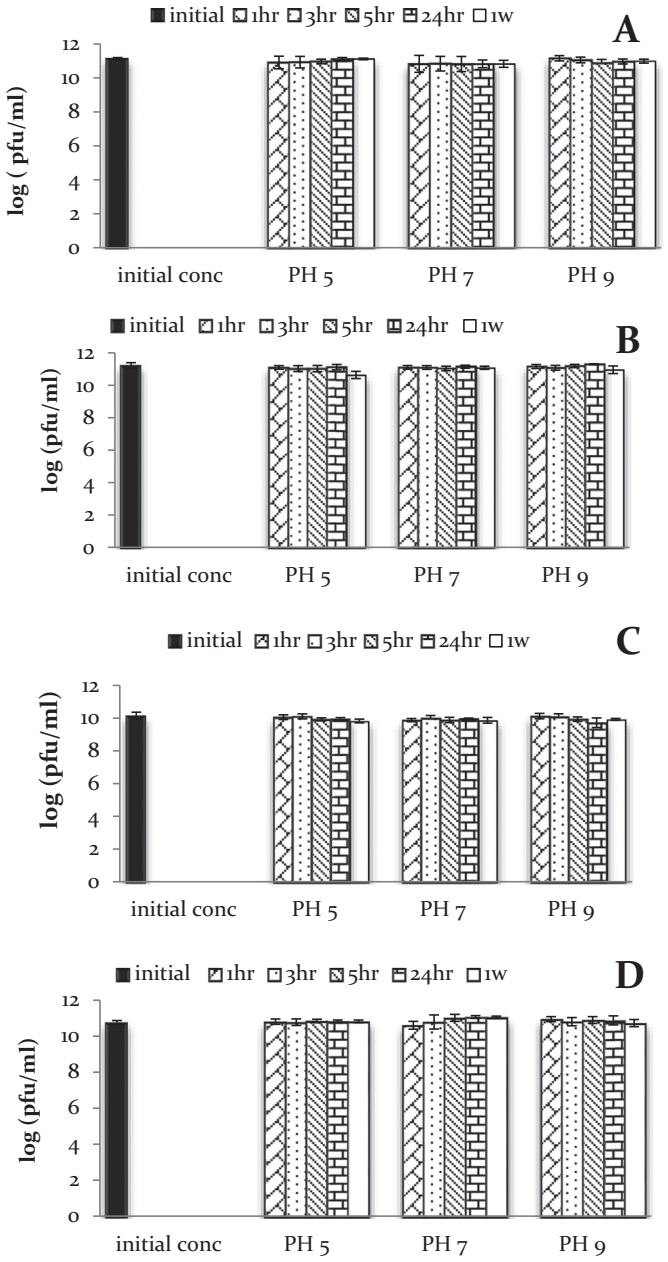

Fig. 2. Stability of four *Salmonella* phages stored at different pH values (A) CGG3-1, (B) CGG3-2, (C) CGG4-1 and (D) CGG4-2.

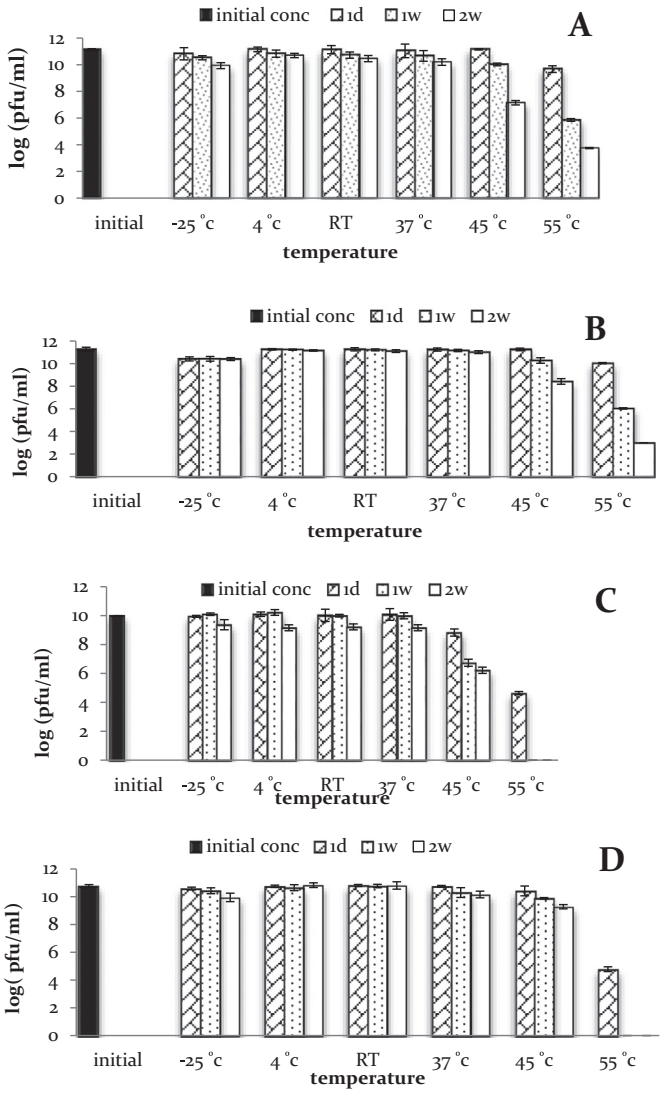

Fig. 3. Stability of four *Salmonella* phages stored at different temperatures (A) CGG3-1, (B) CGG3-2, (C) CGG4-1and (D) CGG4-2.

of the genes and 138 encode hypothetical proteins. The general features of putative ORFs of CGG4-1 are shown in Supplementary File 1. The genome of CGG4-1 is organized in functional modules, including packaging and morphogenesis, nucleic acid metabolism, DNA replication, tRNAs, and lysis cassette encoding modules. No lysogenic, virulence and/or pathogenicity associated genes were identified in the genome.

3.3.1. Packaging and morphogenesis homologs

Sequence-based analysis identified genes predicted to be involved in morphogenesis and packaging of the phage. Forty-seven of the genes that encode putative orthologues of characterized proteins are either similar to structural proteins or proteins that are involved in morphogenesis. Among predicted structural protein orthologues are major capsid protein (ORF 159), small outer capsid protein (Soc; ORF 023), highly antigenic outer capsid protein (Hoc) I and II (ORFs_167 and _169, respectively), baseplate hub subunits (ORF 179, ORF 181), baseplate wedge subunit proteins (ORF 176), long tail fiber proteins, including homologs of T4 gp34, gp35, gp36, gp37, and gp38 (ORF 234 to ORF 238), and sheath formation proteins (ORFs 151, 148, 132). Other loci are homologous to genes that are known to encode head assembly chaperones (ORFs 033, 197), tail formation chaperone (ORF 128), protease inhibitors (ORFs 170 and 065), and terminase (ORFs 149 and 150).

3.3.2. Nucleotide metabolism and DNA replication

Several genes encoding predicted orthologues of proteins directly involved in nucleotide metabolism were identified in the CGG4-1 genome. The identified ORFs include exonuclease A (ORF 12), dTMP thymidylate synthase (ORF 219), ribonucleotide reductase B subunit (ORF 212), ribonucleotide reductase A subunit (ORF 214) and thymidine kinase (ORF 99). While genes involved in DNA replication include DNA ligase (ORF 187), RNA ligase 2 (ORF 162) and RNA ligase A (ORF 210), a primase/helicase (ORF 32 and 72), DNA polymerase (ORF 40) and DNA helicase (ORFs 14, 172 and 173), DNA helicase loader (ORF 230), DNA endonuclease (ORF 275), DNA topisomerase II medium subunit (ORF 5 and 251), double stranded DNA binding protein (ORF 232), recombination, repair, and ssDNA binding protein (ORF 175), and DNA end protector protein (ORF 132). Homologs of genes encoding proteins that act as hydrogen donors for ribonucleotide synthesis in T4

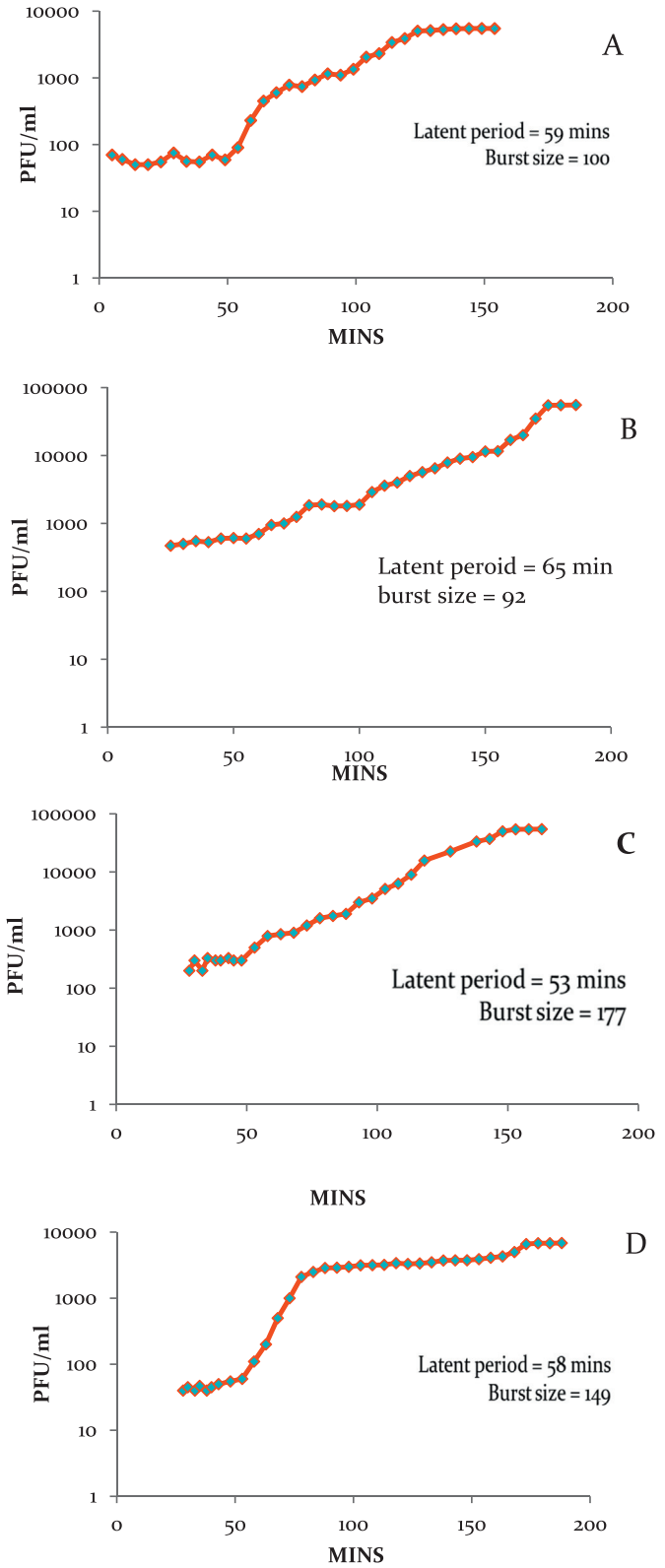

Fig. 4. One step growth curves showing latent period and burst size values of *Salmonella* phages (A) CGG3-1, (B) CGG3-2, (C) CGG4-1 and (D) CGG4-2.

were also identified, including glutaredoxin (ORF 061) and thioredoxin (ORFs 072 and 087). Putative orthologues of ribonucleotide reductase A and B (ORFs214 and 212, respectively) and thymidine kinase (ORF 099) were also identified. Additionally, the genome of CGG4-1 encodes putative dCMP hydroxymethylase, β-glucosyl-transferase, and a β-glucosyl-HMC-α-glucosyl-transferase (ORFs 37, 36, and 35, respectively). The CGG4-1 genome also contains three copies of sequences homologous to homing endonuclease; genes 171, 213 and 218 and (SegD homing endonucleases) gene 159.

3.3.3. Regulatory sequences

Several genes predicted to encode transcriptional regulators were identified including an anti-sigma factor (ORF 016), a decoy of sigma32 (ORF 021), a sigma factor for late transcription (ORF 055), an anti-sigma 70 protein (ORF 240), and an ADP-ribosyltransferase (ORF 185). An activator of middle period transcription (ORF 248) and a late promoter transcription accessory protein (ORF 231) were also identified. Several genes homologous to T4 genes encoding products that play a role in regulating translation were also identified, including a site-specific RNA endonuclease (ORF 107) and RNA ligases (ORFs 162 and 210).

3.3.4. tRNA coding genes

The CGG4-1 genome possesses three tRNA genes with anticodons for three amino acids, namely, methionine, glutamine and arginine (anticodons CAT, TTG and TCT, respectively). The three tRNA genes lie between the 57,785 to 58,581 bp region of the genome.

3.3.5. Lysis

One gene product was determined in genome to be a holin encoded by gene 239, which is a homolog to coliphage T4 holin lysis mediator. As for the lysin, ORFs 114 and 115 are predicted to encode lysozymes. The genome of phage CGG4-1 also contains genes predicted to have a role in lysis inhibition (Paddison et al., 1998), including *rIIA* (ORF 1), *rIIB* (ORF 259), *rI* (ORF 97), and *rIII* (ORF 196).

3.4. Application of bacteriophage as a biocontrol agent against S. Newport

First, the efficacy of the phage cocktail to control the growth of S. Newport in broth medium was investigated. The addition of phage cocktail at MOI of 10^3 the target bacterial suspension in TSB resulted in inhibition of *S.* Newport (below detection limit, < 1 $\log_{10}$ CFU/ml) for up to 7 h. However, the count increased gradually to 10^5 CFU/ml after 24 h at 25 °C (Fig. 6). Conversely, obvious reductions and inhibition of bacterial growth (below detection limit, < 1 $\log_{10}$ CFU/ml) achieved after the addition of phage cocktail at MOI of 10^5 after 1, 3, 5, 7 and 24 h incubation at 25 °C compared to control counts (Fig. 6).

Next, the efficacy of the phage cocktail for controlling *S.* Newport on artificially contaminated cherry tomato fruits was assessed. Treated tomato fruits were incubated at room temperature (25 °C). The count of the surviving bacterial cells was determined at regular intervals and compared to the untreated samples. Fig. 7A and B show the effect of phage cocktail at MOIs of 10^3 and 10^5, respectively, on growth. Phage addition at an MOI of 10^3 resulted in around 2 $\log_{10}$ reduction in the *S.* Newport count after 1, 2, 3 and 4 days when compared to the untreated tomato fruits (Fig.7 A). At MOI of 10^5, 3 $\log_{10}$ reduction of the *S.* Newport count was observed after 1 day, while after 2, 3, and 4 days, the reduction in bacterial count was approximately 4.4 $\log_{10}$ CFU/g (Fig. 7B).

4. Discussion

Bacteriophages have been envisioned as a promising green technology to control different foodborne pathogenic bacteria (Moye et al., 2018). Careful selection of the candidate phages is the first step in the process and is essential for their successful application to enhance food safety. In this study, four phages with different plaque morphologies targeting the foodborne pathogenic bacterium *Salmonella* Newport were isolated from sewage. Two of the isolated phages belong to the family *Myoviridae* and the other two belong to the family *Siphoviridae*. The two myoviruses possessed broad host ranges and were able to infect 100%

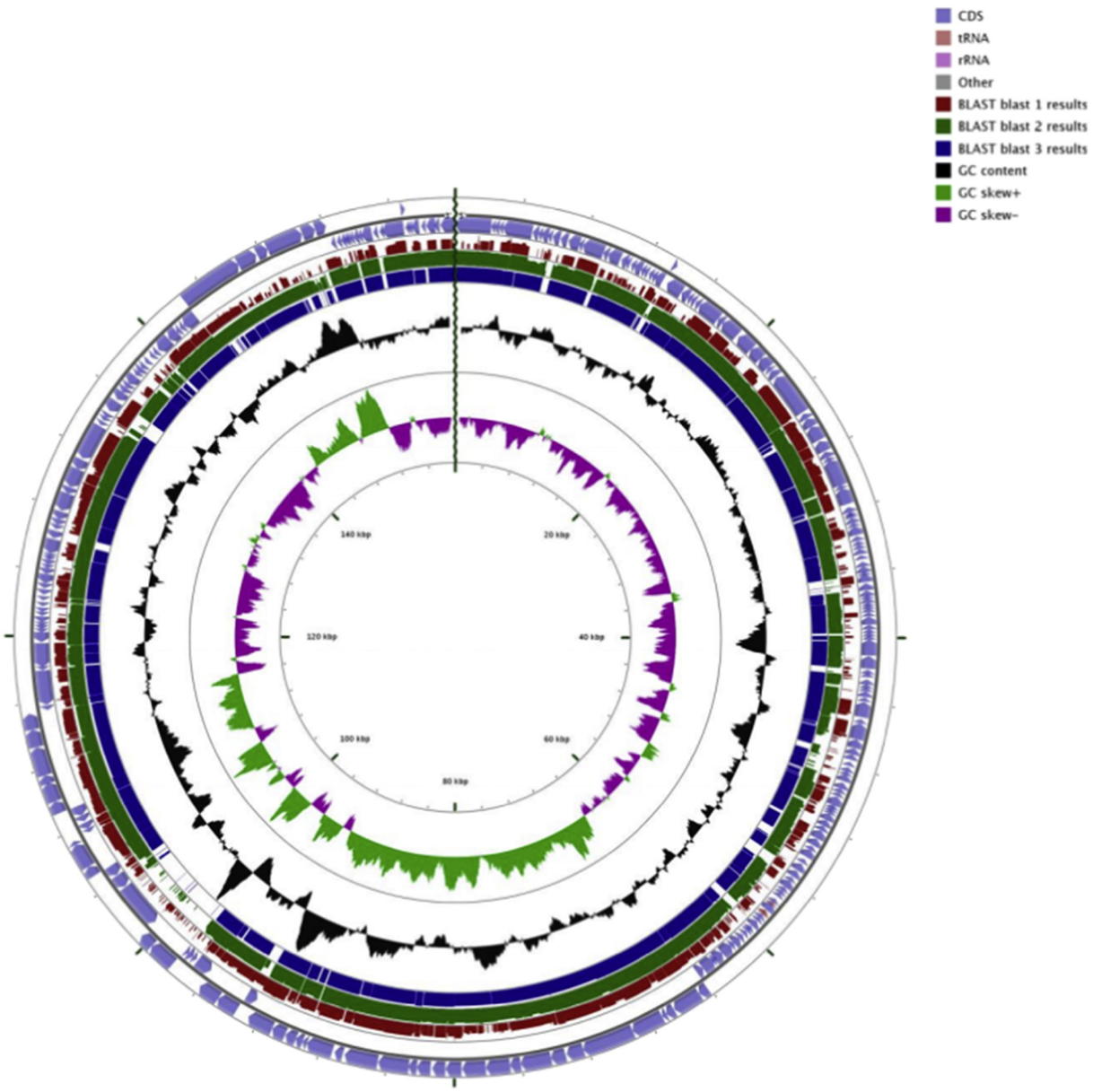

Fig. 5. Genetic and physical map of phage CGG4-1 aligned with T4 (red), S16 (green), and STML-198 (purple) prepared using Blast X analysis. Accession number KU867307. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of the tested *Salmonella* serovars while one of the two isolated siphoviruses was able to infect 25% of the tested serovars. Using a microtitre plate-based approach to determine the host range pattern of the isolated phages not only provides a high throughput assay to screen large number of bacterial strains but also delivers information on the level of the bacterial growth inhibition due to phage infection (Anany et al., 2011; Xie et al., 2018). The differences in susceptibility of the tested strains to the different phages may be due to development of resistance against these phages through a variety of possible mechanisms, including abortive infection, variation in membrane phage receptors (adsorption blocking), and/or a restriction endonuclease modification system (Petty et al., 2007; Pires et al., 2016). Host range analysis is one of the most important criteria for selecting lytic phages intended to be used for biocontrol applications. Felix 01 is an example of a broad host range *Salmonella* phage; it lyses 96.5% of *Salmonella* serovars (Whichard et al., 2003). The limitation of a narrow host range could be overcome using phage cocktails (Goodridge and Bisha, 2011). Hence, a cocktail of the four isolated phages was used in this study.

As stability at different environmental conditions increases the applicability of phages intended to be used as biocontrol tools throughout the food supply chain, the isolated *S.* Newport phages in this study were incubated at different pH values and temperatures. All phages tolerated the tested acidic and alkaline conditions for up to 24 h without a significant reduction in their titres. An optimum pH range from 6 to 8 was reported for long term storage of most phages while some phages such as T2 phage lost 50% of its infectivity after two weeks of storage at pH from 5 to 9 (Jończyk et al., 2011). On the other hand, there was no significant reduction in the titre of the four isolated phages in this study when stored at −25 °C, 4 °C, RT, or 37 °C for 2 weeks. However, a significant reduction in phage activity was noticed when phages were kept at 45 °C and 55 °C and the two *Siphoviridae* phages showed higher thermostablity than the two *Myoviridae* phages and maintained infectivity for up to two weeks at these two temperatures. Anany (2010) reported < 0.5 log reduction in *Salmonella* siphovirus AG11 counts when it was stored at 42 °C for 24 h.

One-step growth curves for the four *Salmonella* phages showed that

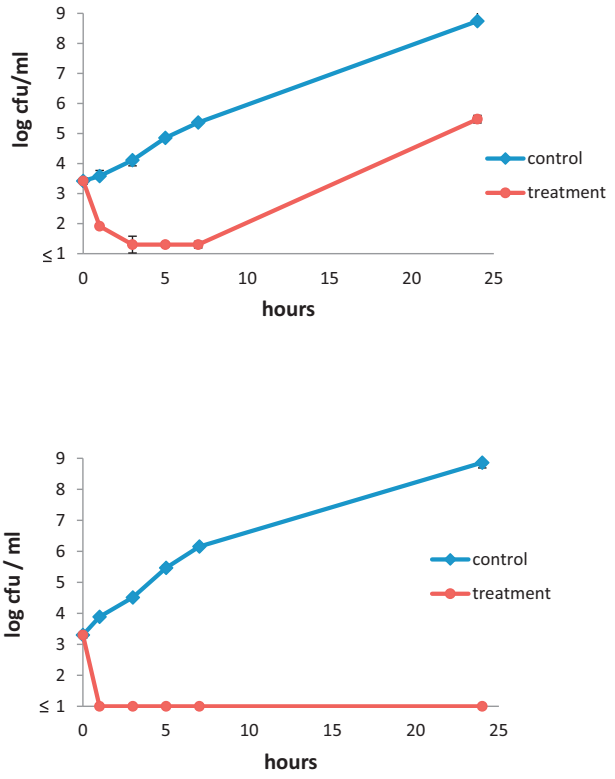

Fig. 6. Biocontrol of *Salmonella* Newport growth in TSB using *Salmonella* phage cocktail added at two different MOIs. (A) MOI = 10^3 and (B) MOI = 10^5.

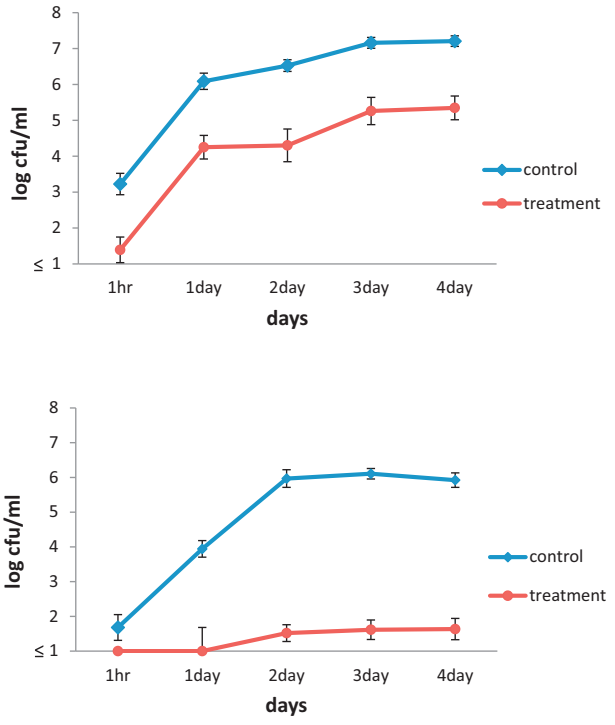

Fig. 7. Biocontrol of *Salmonella* Newport growth on fresh cherry tomato fruits using *Salmonella* phage cocktail added at two different MOIs (A) MOI = 10^3 and (B) MOI = 10^5.

the two *Siphoviridae* phages have lower burst sizes with longer latent period than the two phages that belong to *Myoviridae*. It has been suggested that phages with short latent periods can infect a greater number of bacterial cells in less time compared to those possessing a longer latent period, which makes the former better suited to biocontrol (Abedon, 1989; Lenski and Levin, 1985).

Investigating the development of bacteriophage insensitive mutants (BIMs) of *Salmonella* Newport revealed a low frequency of emergence of resistant mutants against CGG4-1 and CGG4-2 and a higher frequency of BIMs against CGG3-1 and CGG3-2. Similar results were obtained in a previous study where two *Salmonella* lytic siphoviruses, st104a and st104b, exhibited greater BIM frequency than the myovirus FelixO1 (O'Flynn et al., 2006). The low frequency of BIM emergence in the presence of phages CGG4-1 and CGG4-2 suggests that these phages have the ability to overcome different host resistance mechanisms (Labrie et al., 2010), making them attractive candidates for biocontrol applications. One of the concerns of using phages as a biocontrol/therapeutic tool for pathogenic bacteria is the development of phage resistant strains (Coffey et al., 2010; García et al., 2008; Maura and Debarbieux, 2011).

Interestingly, it was reported that the development of bacteriophage resistance can be associated with reduction in the virulence fitness of parental bacterial pathogenic strains (Anany, 2010; León and Bastías, 2015). Hence, we aimed in this study to investigate the potential consequences of phage resistance development on virulence gene expression in *S.* Newport in comparison with the parental phage sensitive strain. The increase in virulence gene expression in the selected BIMs of *S.* Newport at 37 °C was below the value (two or more fold change) at which the gene expression should be considered as significant (Amiri-Jami et al., 2015; Delcenserie et al., 2012; Mundi et al., 2013). These results might be attributed to the target genes involved in this experiment, which mostly encode type three secretion systems in *Salmonella* and are usually not surface structures that act as phage receptors (Jerse et al., 1990; Lu and Henning, 1994). The lack of significant increase in virulence gene expression in the *S.* Newport BIMs leads to no concern about using these specific phages as antimicrobials to enhance food safety. However, a more holistic and non-biased gene expression study is required to draw a general conclusion about the influence of the development of phage resistance mechanisms on bacterial virulence. The recent advances in RNA-sequencing technology can provide a non-biased gene expression or whole transcriptome analysis for a better comprehension of the development of bacteriophage resistant mutants and its associated secondary cost on host virulence (Leskinen et al., 2016).

Having the broadest host range and strongest lytic activity of the four phages that were isolated, CGG4-1 phage was selected for genome sequencing. CGG4-1 is a T4-like phage with a circularly permuted linear dsDNA genome. The majority of the sequenced phages isolated have linear double stranded DNA genomes and belong to the order *Caudovirales* (Hatfull and Hendrix, 2011). The CGG4-1 genome shares 79% and 80% overall nucleotide similarity to *Salmonella* phages STML-198 and S16, revealing it to be a new member of the *S16virus* genus (Marti et al., 2013). In phage T4, the receptor binding domain is found at the distal end of gp37, which forms the distal half of the distal tail fiber (LTF) (Kutter et al., 2005). In the case of phage S16, gp38 serves as the receptor binding protein and was shown to specifically bind to OmpC. The genome of CGG4-1 encodes a putative gp38 that shares 70% sequence identity with the S16 gp38. CGG4-1 may therefore also infect its host via OmpC or related surface proteins. As with S16, in silico analysis showed that CGG4-1 encodes dCMP hydroxymethylase, β-glucosyl-transferase, and a β-glucosyl-HMC-α-glucosyl-transferase. As reasoned by Marti et al. (2013), the presence of these genes may account for its broad host range by encoding gene products responsible for producing modified DNA bases, which makes the DNA incapable of being cleaved by many restriction endonucleases. The annotated genes of CGG4-1 phage genome did not show homology to any available lysogenic and virulence gene sequences in the NCBI database. This is an important criterion for phages intended to be used in biocontrol and therapeutic applications (Hagens and Loessner, 2010; Monk et al., 2010).

Due to the public health risk posed by *Salmonella* spp., lytic phages specific to *Salmonella* spp. have been proposed as an alternative strategy to mitigate its risk in different environments and food matrices (Atterbury, 2009; Coffey et al., 2010; Goodridge and Bisha, 2011; Hagens and Loessner, 2010; Moye et al., 2018). The results of the bacterial challenge experiments in this study indicate the suitability and effectiveness of using a phage cocktail to reduce growth of *S.* Newport in artificially contaminated cherry tomatoes. A high density of phage particles was used to ensure sufficiently rapid contact and infection of the targeted bacterial cells. Treating contaminated cherry tomato fruits with phage cocktail at MOI of 10^5 caused > 4 $\log_{10}$ reduction in the bacterial count when compared with the untreated samples after 4 days of incubation at room temperature (25 °C). Similar results were obtained in other studies that evaluated the efficacy of applying lytic bacteriophages to reduce *Salmonella* spp. growth in various food products (Heyse et al., 2015; Kang et al., 2013; Leverentz et al., 2001; Spricigo et al., 2013; Whichard et al., 2003). For instance, dipping chicken breasts in *Salmonella* phage cocktail at MOI of 10^3 resulted in 2.2 and 0.9 $\log_{10}$ CFU/g reduction of *S.* Typhimurium and *S.* Enteritidis, respectively (Spricigo et al., 2013). Furthermore, *Salmonella* phage cocktail consisting of PA13076 and PC2184 phages applied at MOI of 10^4 in three different food products (pasteurized whole milk, chicken breast and Chinese cabbage) contaminated with *S.* Enteritidis caused a significant reduction in bacterial counts of up to 4 $\log_{10}$ CFU/g in all tested foods (Bao et al., 2015). Interestingly, it was found that using single *Salmonella* phage, P22 to control *S.* Typhimurium growth on artificially contaminated tomato fruits at MOI of 10^2 caused only 0.7 $\log_{10}$ CFU/tomato reduction in the bacterial count after storage at 20 °C for 7 days while 3.02 $\log_{10}$ CFU/tomato reduction was observed over the same period at 10 °C (López-Cuevas et al., 2016). The growth of *S.* Newport on cucumber was approximately 1.8 log lower than control when the artificially inoculated cucumbers were treated with *Salmonella*-specific lytic phages at MOI of 10^5, and analyzed immediately after treatment. On days 1 and 4 of storage at 10 °C, the phage treated cucumbers had 1.48 and 0.77 log CFU lower counts, respectively, compared to the controls (Sharma et al., 2017). The use of phages for biocontrol and therapy is generally thought to be safe (Abedon et al., 2011). However, it is important to subject candidate phages to genomic analysis to test for the presence of antibiotic resistance and virulence-associated genes, as well as potential immune reactive allergens (Bardina et al., 2016). Therefore, in addition to CGG4-1, which was not found to encode antibiotic resistance or virulence-associated genes, the other phages used in this study should also be sequenced before this cocktail can be applied in the food industry.

This work demonstrates the efficacy of lytic *Salmonella* phages to control the growth of *S.* Newport on cherry tomato. The demonstrated characteristics of isolated phages in this study have shown that they have the potential to mitigate the risk of *Salmonella* throughout the produce supply chain to enhance the safety of ready-to-eat produce.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.01.003.

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