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Article

Genomic and Phenotypic Analysis of *Salmonella enterica* Bacteriophages Identifies Two Novel Phage Species

Sudhakar Bhandare [1,2,†], Opeyemi U. Lawal [3,†], Anna Colavecchio [1], Brigitte Cadieux [1], Yella Zahirovich-Jovich [1], Zeyan Zhong [1], Elizabeth Tompkins [1], Margot Amitrano [1], Irena Kukavica-Ibrulj [4], Brian Boyle [4], Siyun Wang [5], Roger C. Levesque [4], Pascal Delaquis [6], Michelle Danyluk [7] and Lawrence Goodridge [3,*]

[1] Department of Food Science and Agricultural Chemistry, Food Safety and Quality program, McGill University, Montreal, QC, Canada; vetsudhakar@bristolalumni.org.uk (SB); anna.colavecchio@mail.mcgill.ca (AC); brigitte.cadieux@canada.ca (BC); yella.jovich@mcgill.ca (YZ); zeyan.zhong@mail.mcgill.ca (ZZ); tompkins.beth8@gmail.com (ET); mamitrano@wisc.edu (MA)
[2] School of Veterinary Medicine and Science, University of Nottingham, UK; sudhakar.bhandare@nottingham.ac.uk
[3] Canadian Research Institute for Food Safety, Department of Food Science, University of Guelph, Guelph, Ontario, Canada; lawal@uoguelph.ca (OL); goodridl@uoguelph.ca (LG)
[4] Institute for Integrative Systems Biology (IBIS), Laval University, Québec, Canada; kukavica@fmed.ulaval.ca (IKI); Brian.Boyle@ibis.ulaval.ca (BB); rclevesq@ibis.ulaval.ca (RL)
[5] Faculty of Land and Food Systems, University of British Columbia, Vancouver, BC, V6T 1Z4 Canada; siyun.wang@ubc.ca
[6] Agriculture and Agri-Food Canada, Summerland, BC, V0H 1Z0, Canada; pascal.delaquis@canada.ca
[7] Food Science and Human Nutrition Department, University of Florida, USA; mddanyluk@ufl.edu
* Correspondence: goodridl@uoguelph.ca
† Joint First author.

Abstract: Bacteriophages (phages) are potential alternatives to chemical antimicrobials against pathogens of public health significance. Understanding the diversity and host specificity of phages is important for developing effective phage biocontrol approaches. Here, we assessed the host range, morphology, and genetic diversity of eight *Salmonella enterica* phages isolated from a wastewater treatment plant. The host range analysis revealed that six out of eight phages lysed more than 81% of the 43 *Salmonella enterica* isolates tested. The genomic sequences of all phages were determined. Whole genome sequencing data (WGS) data revealed that phage genome sizes ranged from 41 to 114 kb with GC contents between 39.9 and 50.0 %. Two of the phages SB13 and SB28 represent new species *Epseptimavirus SB13* and genera *Macdonaldcampvirus,* respectively as designated by the International Committee for the Taxonomy of Viruses (ICTV) as per genome based taxonomic classification. One phage (SB18) belonged to the *Myoviridae* morphotype while the remaining phages belonged to the *Siphoviridae* morphotype. The gene content analyses showed that none of the phages possessed virulence, toxin, antibiotic resistance, type I-VI toxin-antitoxin modules or lysogeny genes. Three (SB3, SB15 and SB18) out of the eight phages possessed tailspike proteins. Whole genome-based phylogeny of the eight phages with their 113 homologs revealed three clusters A, B, C and seven subclusters (A1, A2, A3, B1, B2, C1 and C2). While cluster C1 phages were predominantly isolated from animal sources, cluster B contained phages from both wastewater and animal sources. The broad host range of these phages highlights their potential use to control the presence of *S. enterica* in foods.

Keywords: *Salmonella*; bacteriophage; diversity; host specificity; comparative phylogenomics; food safety

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1. Introduction

Nontyphoidal *Salmonella* (NTS) is a major cause of foodborne illness and hospitalizations in Canada [1]. Globally, NTS causes approximately 80 million foodborne-related illnesses and 155,000 deaths each year [2]. Non-typhoidal Salmonellosis is caused by the main species of the genus, *Salmonella enterica*, which consists of six subspecies (I, II, IIIa, IIIb, IV, and VI) with subspecies I containing more than 1,500 serotypes with high genetic diversity [3]. In Canada there have been several large national and international outbreaks of Salmonellosis linked to vegetables including whole onions, peaches, frozen corn, and cantaloupes since 2020 [4–7]. These outbreaks have been caused by several *S. enterica* serotypes (Enteritidis, Newport, Soahanina, Sundsvall, Oranienburg), and have highlighted the need for improved approaches to control presence and growth of diverse *S. enterica* in fresh and processed fruits and vegetables, which increasingly contribute to the burden of foodborne disease. Bacteriophages (phages) are increasingly being recognized as natural antimicrobials to reduce the growth and survival of foodborne pathogens (including *Salmonella*) during food production [8–12] because of their ability to kill their host bacteria [13,14], and also due to the fact that phages can exhibit broad host ranges [15] making them useful for controlling diverse bacterial species such as *S. enterica* [16]. The majority of phages described in the scientific literature appear to be generally host specific, infecting a subset of species, strains, or serotypes due to the specificity of their host receptors [17]. While some phages can infect a broad range of bacteria belonging to different serotypes, species, and/or genus [17,18], information on these phages are limited.

While *Salmonella* phages and their genomic sequences have been well documented [17,19–25], the phenotypic and genotypic diversity of *Salmonella enterica* means that there are likely many additional phages with unique features that remain to be characterized. Understanding the biological and genomic characteristics of these phages are essential to the development of phage-based antimicrobial methods to control this foodborne pathogen [13,26]. In this study, we determined the host range spectra, and performed comparative genomic and phylogenetic analyses, as well as morphological characterization of eight *S. enterica* phages isolated from wastewater obtained from the Jean R. Marcotte wastewater treatment plant in Montreal, Québec, Canada.

2. Materials and Methods

2.1. Bacterial Host Strains and Their Growth

Non-typhoidal *S. enterica* isolates (n = 43) representing 30 serotypes, and commonly associated with fresh produce outbreaks were obtained from the *Salmonella* foodborne Syst-OMICS database (SALFOS, Laval University, QC, Canada, https://salfos.ibis.ulaval.ca/) (Figure 1). Frozen stocks of the isolates were maintained at −80 ºC and were revived by streaking on Luria-Bertani (LB) agar plates followed by overnight incubation at 37 ºC. For all experiments, a single colony of a respective isolate from a fresh LB agar plate was inoculated into 10 mL of LB broth and incubated overnight at 37 ºC with shaking at 150 RPM.

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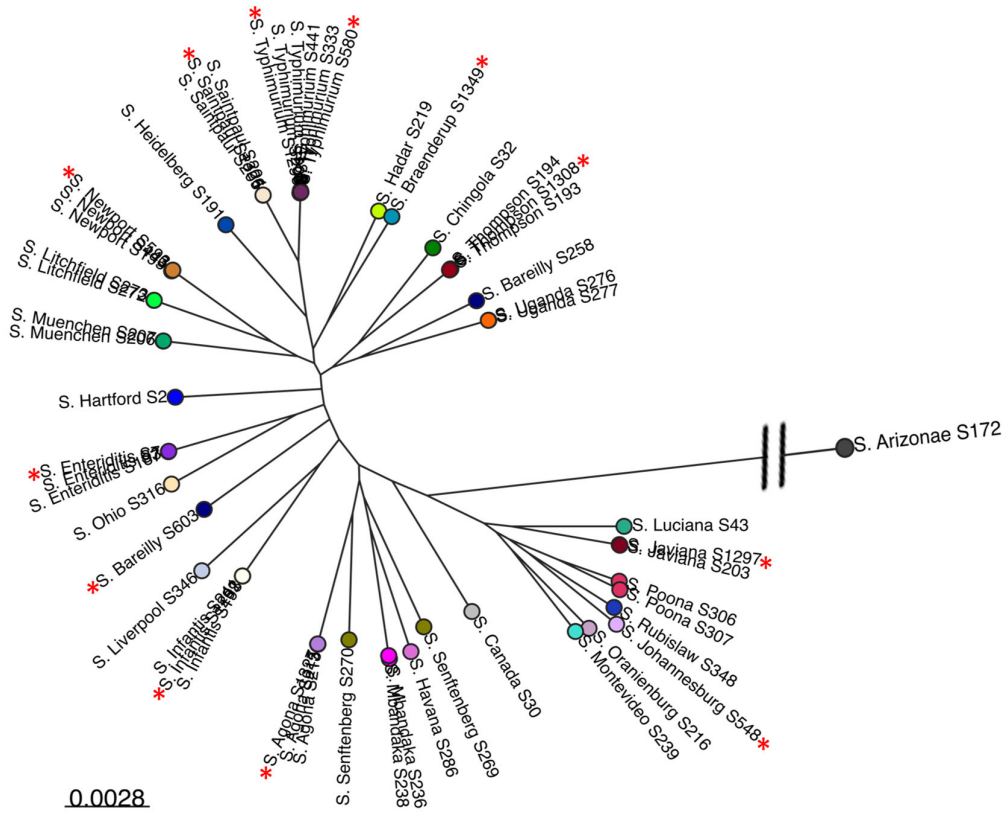

Figure 1. Maximum likelihood tree of 55 *Salmonella* representing 30 serovars used for phage propagation and host specificity in the study. *Salmonella* strains used for propagating the phages in the study are indicated with red star. Core-genome sequence alignment was generated from draft genomes using MAFFT v7.453, and the tree that was bootstrapped with 1000 replicates for node support was constructed using FastTree v2.1.11. The scale bar at the bottom represents nucleotide substitution per site.

2.2. Bacteriophage Isolation and Propagation

Bacteriophage isolation was carried out using pre-treated sewage sludge samples (1L) collected from the Jean-R. Marcotte Wastewater Treatment Plant (WWTP) in Montreal by adopting standard enrichment and agar overlay techniques [27] with a slight modification as described elsewhere [28]. Thirteen *Salmonella* isolates, originally isolated from food plants/fresh produce, and representing thirteen serotypes, were used for phage isolation (Figure 1).

A sewage sample (1 L) was transported to the laboratory on ice, where it was clarified by centrifugation at 10,000 × g for 30 min in 50 mL tubes and filtered through 0.22 µm syringe filter units (Pall Corporation, Port Washington, NY, United States). Thirty milliliters of filtrate was mixed with 9 mL of 5x Rappaport-Vassiliadis (RV), 1 mL of 1 M $CaCl_2$ and 10 mL of an overnight culture of a *Salmonella* isolate, and the suspension was incubated in a 250 mL flask at 37 ºC for 48 h while shaking at 50 RPM. The culture was clarified by centrifugation at 10,000 × g for 10 min and the supernatant was filtered through a 0.22 µm filter to remove bacterial debris. An aliquot (10 µL) of filtrate was spot inoculated on a lawn of the *Salmonella* isolate used for isolation and the plate was incubated at 37 ºC overnight. The next day, the lawns were checked for zones of lysis or individual plaques. Plaque purification was carried out multiple times using a streak plating method, as previously described [29], to obtain a clonal phage isolate. Phage stocks were concentrated by centrifugation (40,000 × g for 2 h at 4 ºC) and stored at 4 ºC until further use. For routine use, phages were propagated by the liquid lysate method [28] where a mid-exponential phase bacterial host culture was infected with phage and incubated overnight. The next day, the phage and the bacterial host culture was centrifuged (10,000

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× g for 10 min) and filtered through a 0.45 μm syringe filter, followed by determination of the phage titre.

2.3. Host Range Profiles

Host ranges for each of the isolated phages were determined using the agar overlay method. Following preparation of agar overlays (as described above), the lawns (prepared for each of the 43 *Salmonella* isolates to be tested) were spot inoculated with aliquots (10 μL) of each phage having titres of 10^8 plaque-forming units (PFUs)/mL. The spots were allowed to dry before incubating overnight at 37 ºC. The scoring for lysis was completed as reported elsewhere [30,31], where 0 indicated no lysis and +3 indicated complete clear lysis.

2.4. Phage DNA Isolation, Sequencing, and Annotation

Genomic DNA from the *Salmonella* phages was extracted using the Wizard DNA Clean-Up system (A7280; Promega) following the modified Promega Wizard method as described by the Center for Phage Technology, Texas A&M University, USA [32]. Extracted DNA was purified by ethanol precipitation [33], and whole genome sequencing (WGS) was performed on an Illumina MiSeq platform with 300-bp paired-end libraries and 30X coverage. Raw sequence reads were assembled using the A5 pipeline [34], and genome annotation was completed using the Bacterial and Viral Bioinformatics Resource Center (formerly PATRIC) [35,36]. Annotations were manually curated, and the coding sequences (CDS) were used to interrogate the NCBI database using BLASTP [37]. An HHpred search of the Pfams database was used to identify conserved protein motifs [38]. To assign a protein to a gene sequence, at least 90% identity was sought in BLASTP searches for protein motifs [39]. Based on the presence or absence of a gene encoding integrase, phages were putatively classified as temperate or virulent, respectively [26].

2.5. Phylogenetic and Comparative Genomic Analyses

The whole-genome alignments of the phages reported in this study and 113 homologs that were extracted from NCBI were generated using MAFFT v7.453 [40]. Maximum likelihood trees were constructed using IQtree v2.2 (https://github.com/Cibiv/IQ-TREE) [41] and visualized using Microreact [42]. Phages were assessed for genes encoding antimicrobial resistance, virulence, and type I-VI toxin-antitoxin modules using CARD [43], VFDB [44,45], and TADB [46], respectively. Likewise, phage genome sequences were screened for presence of tailspike proteins using an in-house manually curated custom database. Whole-genome comparisons and their visual representations were carried out using EasyFig (https://github.com/mjsull/Easyfig) [47].

2.6. Electron Microscopic Imaging of the Phages

Phages were purified for electron microscopy by equilibrium density gradient centrifugation through CsCl at ≈ 22,000 RCF for 24 h in a Beckman Ultra centrifuge (TL100) [48]. Post centrifugation, the residual CsCl was removed from the phage fraction by centrifuging 500 μL of the supernatant fluid through an Amicon Ultra-0.5, 30 kDa MWCO centrifugal filter unit (Millipore Ltd). Following purification, transmission electron microscopy was conducted at the Imaging - Microscopy Platform of the Institute of Integrative Biology and Systems (IBIS), Laval University, Quebec City, Canada.

3. Results

3.1. Phage Isolation and Biological Characterization by Host Range Profile

Eight phages (Table 1) were isolated using *S. enterica* isolates representing serotypes commonly associated with fresh produce outbreaks. The host range profile of the isolated phages was determined using 43 different *Salmonella* isolates from 30 serotypes (Figure 1&2). *Salmonella* isolates representing the top eight plant associated salmonellosis-causing serotypes were given importance due to their frequent implication in produce associated outbreaks, which account for the majority of

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foodborne outbreaks [49–52]. The top eight serotypes were identified as serotypes Newport, Javiana, Enteriditis, Typhimurium, Thompson, Heidelberg, Saintpaul, and Poona (Figure 2). One additional serotype, Litchfield, was also included because it has been implicated in numerous outbreaks associated with melons [53]. The broadest host range was exhibited by phages SB3 and SB6, followed by SB9. Phages SB3 and SB6 lysed 88.3% (n = 38/43) isolates, while phage SB9 lysed 86% (n = 37/43) isolates, phage SB10 lysed 76.7% (n = 33/43), phages SB13 and SB18 lysed 83.7% (n = 36/43) isolates, phage SB28 lysed 81.3% (n = 35/43) and phage SB15 showed the lowest lysis percentage of 67.4% (n = 29/43) isolates. Five of eight (62.5% (SB3, SB6, SB9, SB10, SB13)) phages isolated in this study lysed the top eight serotypes and Litchfield (Figure 2).

Table 1. Summary of *Salmonella enterica* bacteriophages isolated in this study.

Phage	*Salmonella* host strain used for isolation *	Morphotype	New classification	Old classification	GenBank accession number	Genome Length (Kb)	No. of coding genes	No. of tRNA	G+C (%)
SB3	*S. enterica* ser. Enteritidis (S7)	*Siphovirus*	Genus: Jerseyvirus; Species: Jerseyvirus AG11	Genus: Jerseyvirus; Species: *Salmonella* virus AG11	MK578530	41.15	63	0	50.0
SB6	*S. enterica* ser. Javiana (S1297)	*Siphovirus*	Genus: Epseptimavirus	Genus: Tequintavirus	MK809530	112.31	180	16	39.9
SB9	*S. enterica* ser. Saint-Paul (S1326)	*Siphovirus*	Genus: Epseptimavirus	Genus: Tequintavirus	MK867835	113.98	180	20	39.9
SB10	*S. enterica* ser. Typhimurium (S1295)	*Siphovirus*	Genus: Epseptimavirus; Species: Epseptimavirus fuchur	Genus: Tequintavirus; Species: *Salmonella* virus fuchur	MK947458	111.35	183	21	40.1
SB13	*S. enterica* ser. Typhimurium (S580)	*Siphovirus*	Genus: Epseptimavirus;Species: Epseptimavirus SB13.	Genus: Tequintavirus	MK947459	112.51	175	15	39.9
SB15	*S. enterica* ser. Braenderup (S3)	*Siphovirus*	Genus: Jerseyvirus; Species: Jerseyvirus AG11	Genus: Jerseyvirus; Species: *Salmonella* virus AG11	MK759883	41.44	73	0	50.0
SB18	*S. enterica* ser. Infantis (S43)	*Myovirus*	Genus: Ounavirinae; Species: Kolesnikvirus Ea214	Genus: Ounavirinae; Species: Kolesnikvirus	MK759884	85.31	118	17	43.8
SB28	*S. Typhi* ser. T42 DEF 472 (S203)	*Siphovirus*	Genus: Macdonaldcampvirus; Species: Macdonaldcampvirus SB28	Was not classified beyond *Siphoviridae* family	MK947460	45.13	73	0	46.2

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***Salmonella* strain**	**SB3**	**SB6**	**SB9**	**SB10**	**SB13**	**SB15**	**SB18**	**SB28**
S. Hartford S2	1	1	1	1	1	1	3	1
S. Enteriditis S3	2	2	2	2	3	3	0	3
S. Enteriditis S187	3	3	3	3	3	3	1	3
S. Typhimurium S189	2	2	3	2	2	3	3	3
S. Newport S195	2	1	1	1	1	1	2	1
S. Typhimurium S200	2	1	2	2	3	1	1	1
S. Javiana S203	2	2	1	1	3	2	0	1
S. Typhimurium S441	3	1	1	2	3	3	2	1
S. Thompson S193	1	1	2	1	1	1	2	1
S. Thompson S194	2	2	2	3	2	3	3	3
S. Saintpaul S204	2	1	1	3	3	2	1	2
S. Saintpaul S205	3	2	1	3	3	2	1	2
S. Poona S306	1	1	1	1	1	0	1	0
S. Poona S307	3	3	3	3	3	1	1	2
S. Heidelberg S191	1	2	1	1	2	1	2	0
S. Litchfield S272	3	3	3	1	3	1	3	2
S. Litchfield S273	3	3	3	3	3	3	2	2
S. Canada S30	3	1	1	1	3	3	1	1
S. Chingola S32	3	3	2	3	1	3	3	1
S. Luciana S43	1	3	3	1	1	0	1	3
S. Arizonae S172	3	3	3	3	3	3	3	3
S. Infantis S198	1	1	1	0	1	1	3	1
S. Muenchen S206	2	2	1	1	2	0	2	1
S. Muenchen S207	2	2	3	1	3	2	3	2
S. Agona S213	1	1	0	0	1	0	0	0
S. Agona S215	3	1	1	2	2	1	1	1
S. Oranienburg S216	1	2	1	1	1	2	2	1
S. Hadar S219	0	1	0	0	1	0	1	1
S. Mbandaka S236	0	0	0	0	0	0	0	1
S. Mbandaka S238	2	1	1	1	1	0	2	2
S. Montevideo S239	0	0	0	0	0	0	0	0
S. Infantis S241	2	3	3	2	2	1	3	1
S. Bareilly S258	2	0	1	1	0	2	3	1
S. Senftenberg S269	1	2	2	0	0	1	1	2
S. Senftenberg S270	0	0	0	0	1	0	1	0
S. Uganda S276	1	2	1	2	2	0	0	0
S. Uganda S277	2	2	2	2	3	1	1	1
S. Havana S286	3	3	1	0	0	0	2	1
S. Ohio S316	0	1	1	0	0	0	1	1
S. Typhimurium S333	3	1	1	3	3	1	1	2
S. Liverpool S346	2	3	2	1	3	0	1	0
S. Rubislaw S348	1	0	0	0	0	0	0	0
S. Newport S443	2	2	1	1	1	1	1	1
Total strains hit out of 43	38	38	37	33	36	29	36	35
Percentage	88.3	88.3	86	76.7	83.7	67.4	83.7	81.3

Figure 2. Heat map of the lytic spectra of isolated phages with scoring of lysis, where 0 indicates no lysis and 1 to 3 indicates clearing (3 indicates complete clear lysis).

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3.2. Comparative Phylogenomic Analysis of Phages Understudy

To ascertain the suitability of the isolated phages for biocontrol purposes, the phages were sequenced. Raw reads were assembled into draft genomes that were functionally annotated as described above (*see Methods*).

3.2.1. Comparative Gene Content Analysis of Phages under Study

The genome size of the phages ranged from 41 to 114 kb, while their GC contents were between 39.9 to 50.0% (Table 1). Whole genome-based phylogenetic analysis of the eight phages revealed two clusters (cluster A and B) and two singletons (Figure 3). Phages SB28, SB9, SB10 and SB13 were nested together in cluster A, SB15 and SB3 were clustered in cluster B, while SB6 and SB18 were singletons. Homology of phage genomes with previously identified phages were determined using a comparative genomic approach with BlastN. Phages in cluster A were heterogenous in terms of their genome size, number of tRNAs and coding sequences (Table 1). Phages SB9 and SB10 were similar to *Salmonella* phage 116 (accession number NC_048007.1, 99.58% identity, 88% coverage) and *Salmonella* phage fuchur (accession number: NC_048869.1, 97% identity and 96% coverage) that were both isolated from wastewater in Denmark [54] (Figure 4A). Phages SB13 and SB28 respectively had 99% (coverage 92%) and 96% (coverage 79%) nucleotide similarity to *Escherichia* phage Supergirl (accession number: MZ501105.1) that was isolated from a sewage plant in Switzerland (Figure 4B) [55] and *Salmonella* phage E1 (accession number: NC_010495.1) from an unknown source in the United Kingdom (Figure 4C) [56].

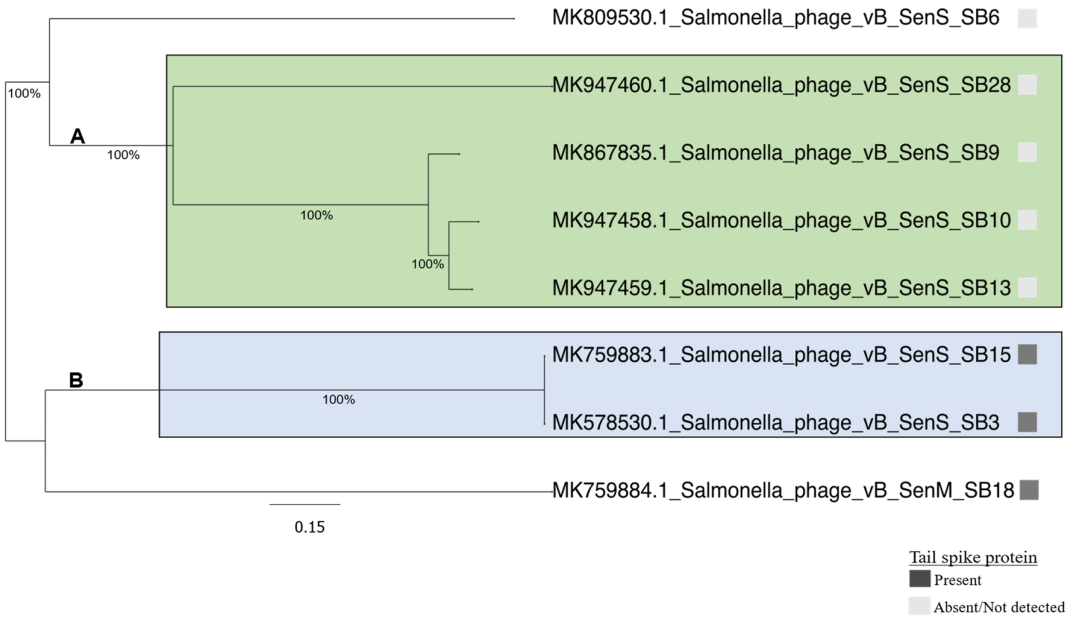

Figure 3. Maximum likelihood phylogenetic tree showing the relatedness of phages under study. The phage genomes alignment was generated using MAFFT v7.453, and the tree that was bootstrapped with 1000 replicates for node support was constructed using IQtree v2.2. The scale bar at the bottom nucleotide substitution per site. Phages in light green rectangle belonged to cluster-A; those within blue rectangle are designated as cluster-B phages, while others were singletons. Grey and white box depicts presence or absence of tail spike proteins in the phages.

Phages SB3 and SB15 (Cluster B) had comparable genome size (41 kB), number of tRNAs and GC content, and shared 99% nucleotide similarity between them, and >95% nucleotide identity (coverage 97%) with *Salmonella* phage vB_SenS_AG11 (accession number NC_041991.1) that was isolated from sewage in Guelph, Canada in 2007 [57,58] (Figure 4D). The singletons: Phage SB6 and SB18 respectively had 99.35 and 98% nucleotide sequence similarity to *Salmonella* phage oselot

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(accession number: NC_048871.1, 95% coverage) recovered from wastewater in Denmark [59], and *Erwinia* phage phiEa21-4 (accession number NC_011811.1, 98% coverage) that was isolated in soil beneath a pear tree with active blight in Canada [60,61] (Figure 4E-F). Collectively, given the broad host ranges of the phages isolated in this study, these results confirm the utility of wastewater as a rich source from which to isolate diverse and broad host range phages [62,63].

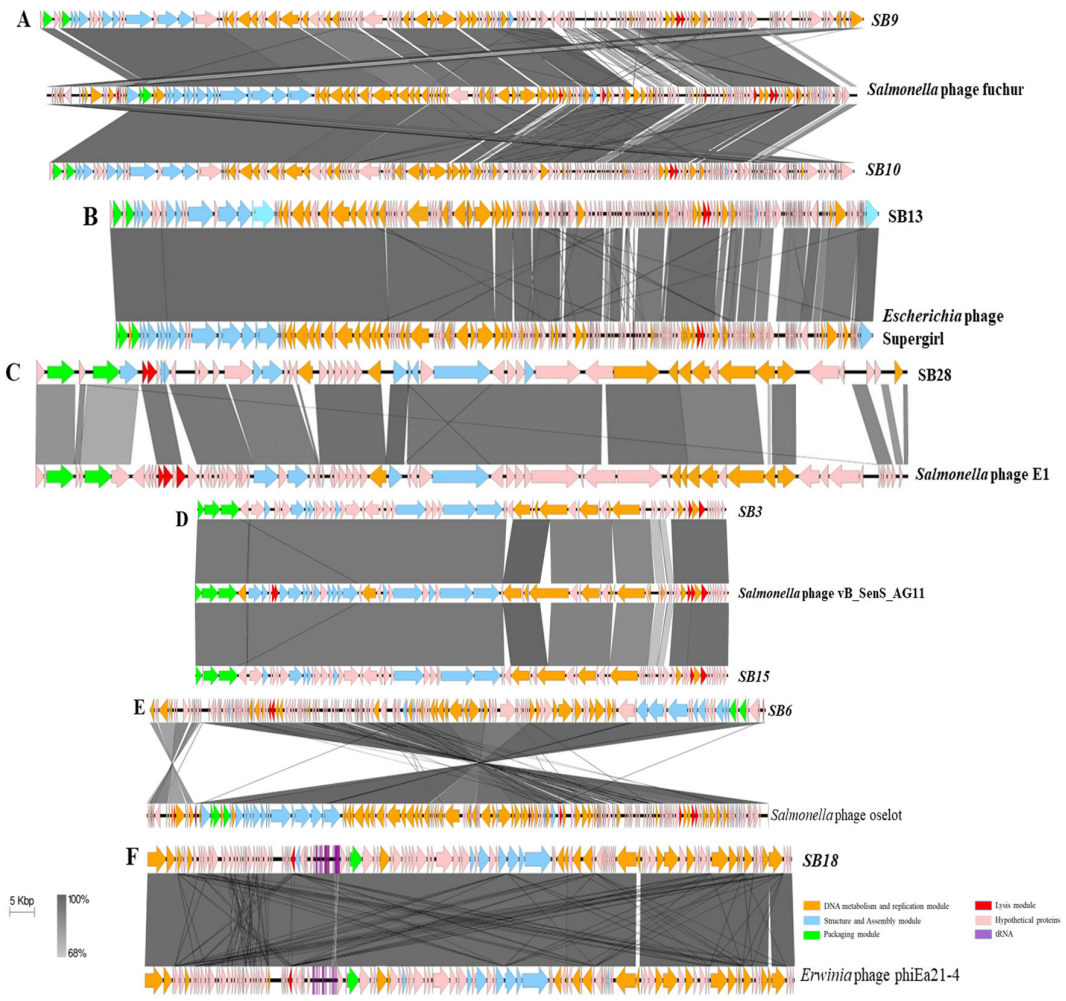

Figure 4. Homology and gene synteny comparison of phages isolated in this study with previously sequenced phages. Blastn comparisons of the phages in this study with their closest references. **(4A)** SB9 and SB10 with *Salmonella* phage fuchur; **(4B)** SB13 with *Escherichia* phage Supergirl;. (**4C**) SB28 with the reference *Salmonella* phage E1; (**4D**) SB3 and SB15 with *Salmonella* phage vB_SenS_AG11; (**4E**) SB6 with *Salmonella* phage oselot and (**4F**) SB18 with *Erwinia* phage phiEa21-4. The direction of arrows indicates the DNA strand direction. Figures were generated using EasyFig.

The genomes of the phages isolated in this study were screened for the presence of lysogeny, antimicrobial resistance, virulence, or toxin-antitoxin related genes. Of note, genes encoding virulence, type I-VI toxin-antitoxin modules, antimicrobial resistance, or lysogeny were not detected in any of the phages. Given the broad host range of the isolated phages, an assessment of the genes encoding bacterial recognition proteins was conducted. Three (SB3, SB15 and SB18) out of the eight phages possessed tailspike proteins (Figure 3). The tailspike proteins from phages SB3 and SB15 were identical (100% nucleotide sequence similarity and coverage) and shared 93% nucleotide sequence similarity with *Salmonella* phage vB_SenS_AG11 tailspike protein (accession number: AF012431.1). Although functional annotation predicted these tailspike proteins to belong to *Salmonella* phage P22-

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like tailspike protein, comparative genomic analysis revealed that they shared only 66% nucleotide similarities with *Salmonella* phage P22 tailspike protein (accession number: NP_059644.1). Phage SB18 had a bulb like structure at the base of the tail typical of phages with tailspike proteins but there were no genes annotated as tailspike protein in its genome. More so, BLASTing phage SB18 genome against a manually curated custom database containing 8077 unduplicated tailspike proteins did not yield any significant hit. However, phage SB18 contained a gene annotated as a baseplate protein that had 97% nucleotide similarity to the baseplate spike protein of *Erwinia* phage phiEa21-4 (accession number: YP_004327040.1).

3.2.2. Assessing the Genetic Relatedness of Phages under Study with Other *Salmonella* Phage Genomes

Phylogenetic analysis of the phages showed high genetic relatedness with other phages. For example, based on genome comparisons with previously identified *Salmonella* phages deposited in public databases, The phages were clustered into three main clusters designated as A, B, which were further grouped into seven subclusters (A1, A2, B1, B2, C1, C2 and C3) (Figure 5). The singleton phages SB6 and SB18 (Figure 3) were placed in different subclusters of cluster C. Phage SB6 clustered with Bacteriophage T5-like in subcluster C1, whereas SB18 was found in subcluster C2 along with *Erwini*a phages phiEa21-4 and *Salmonella* phage ST-3 among others. Phage SB28 with *Salmonella* phage E1 formed a distinct subcluster (A1), while phages SB9, SB10, and SB13 were found in subcluster A2 with other *Salmonella* phages (Figure 5). Conversely, phages SB3 and SB15 were clustered in subcluster B1 together with *Salmonella* phage vB_SenS_AG11. In subcluster A2, phage SB9 was clustered with SB10 and SB13 along with *Salmonella* phages S116, fuchur, and *Escherichia* phage Supergirl, respectively (Figure 5).

Of note, phages that were isolated from animals/animal products were predominantly found in subcluster C1. Relative to other clusters, cluster B contained phages isolated from more diverse sources and were also associated with the presence of tailspike proteins. For the phages with *Salmonella* as host, serotype Typhi was enriched in cluster A **(**Figure 5**)**. Overall, these results reiterate the high genetic diversity that has been observed among *S. enterica* phages [25].

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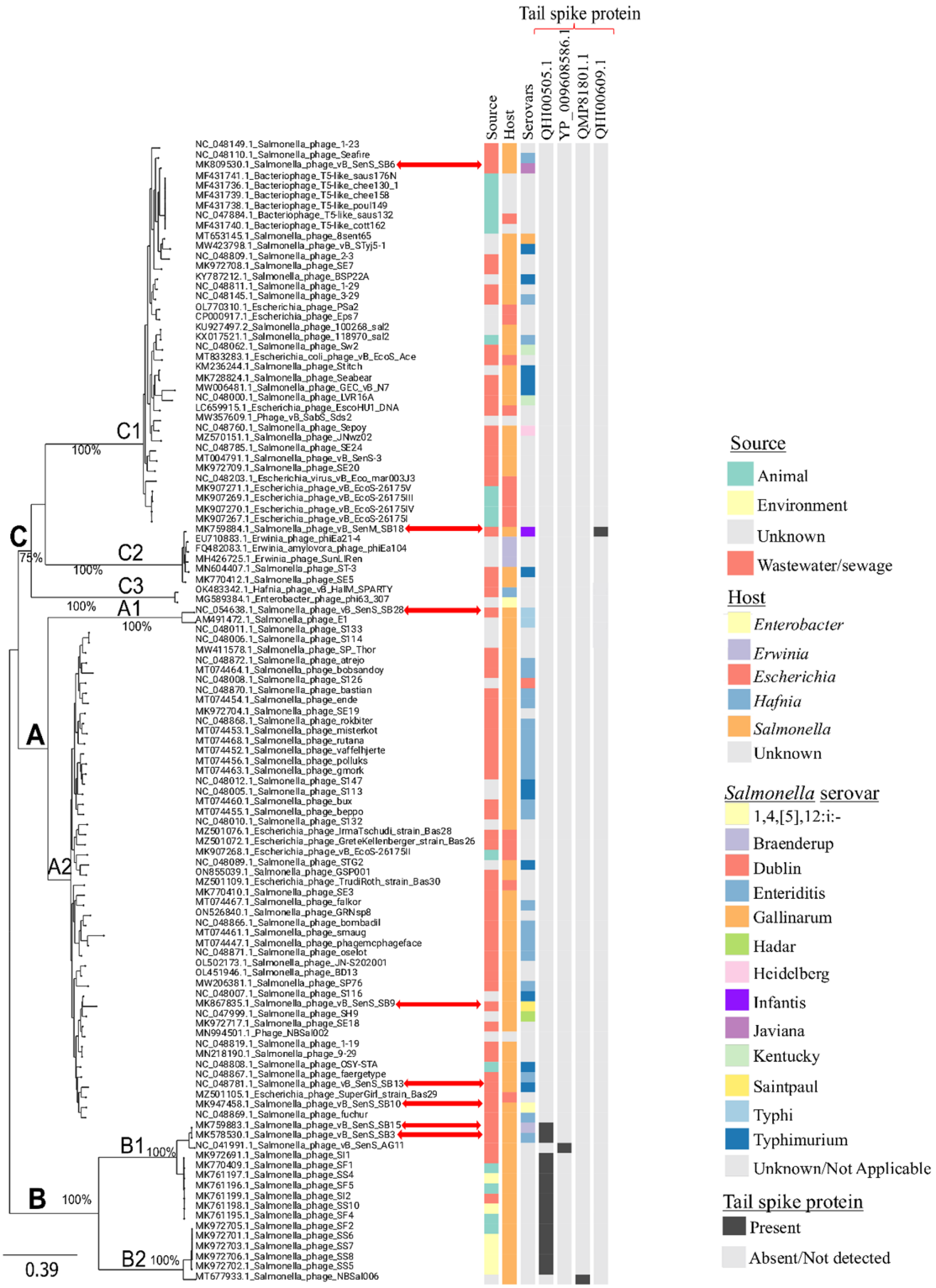

Figure 5. Maximum likelihood phylogenetic tree showing the relatedness of phages under study with previously identified *Salmonella* phages having coverage of ≥80% and nucleotide homology of ≥ 95%. The phage genomes alignment was generated using MAFFT v7.453, and the maximum likelihood tree that was bootstrapped with 1000 replicates for node support was constructed using IQtree v2.2. The first three blocks showed the source, host, and serovars (for *Salmonella* host) of the phages, while the other blocks indicate the presence and absence of tail spike proteins. The scale bar at the bottom indicates nucleotide differences amongst them. The phages under study are indicated with red arrow.

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3.3. Salmonella Phages under Study Are Morphologically and Taxonomically Different

Phage morphological characterization by Transmission Electron Microscopy (TEM) was performed. Based on the TEM imaging, all the phages belonged to the *Siphoviridae* morphotype except SB18, which belonged to the *Myoviridae* morphotype (Table 1). *Siphoviridae* morphotypes have long flexible tails while *Myoviridae* morphotypes are defined by a rigid contractile tail (ICTV [64,65]. Images of phages representative of cluster A, B and C are shown in Figures 6, 7 and 8, respectively.

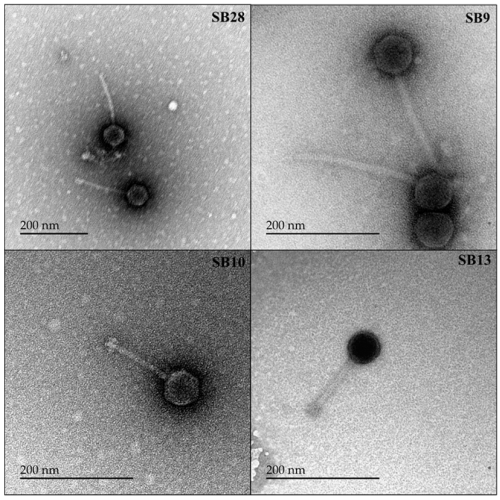

Figure 6. Transmission Electron Microscopy (TEM) images of cluster A phages: subcluster A1: SB28 and subcluster A2: SB9, SB10 and SB13.

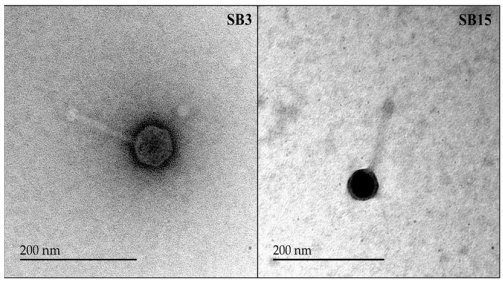

Figure 7. Transmission Electron Microscopy (TEM) images of cluster B phages: SB3 SB15.

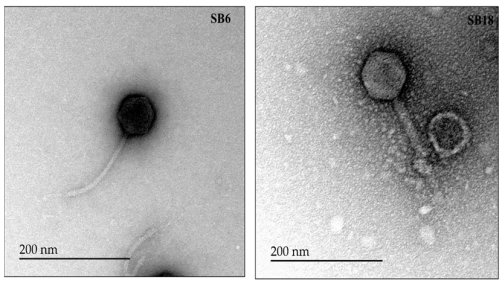

Figure 8. Transmission Electron Microscopy (TEM) images of cluster C phages: subcluster C1: SB6 and subcluster C2: SB18.

In-silico analysis of the isolated phages and their comparison with existing homologs allowed for taxonomical associations. With recent changes to taxonomic classification of phages where major families such as *Siphoviridae*, *Podoviridae*, and *Myoviridae* were abolished by the International Committee on Taxonomy of Viruses (ICTV [64,65] (https://ictv.global/vmr/current). Phylogenomic analysis was employed to classify the phages into classes and genera based on the new ICTV

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classification schema [66]. All the phages were classified into class *Caudoviricetes*. The phage SB6, SB9. SB10 and SB13 were classified as family *Demerecviridae;* subfamily *Markadamsvirinae;* genus *Epseptimavirus* with species level classification only for SB10 and SB13 viz. *Epseptimavirus fuchur* and *Epseptimavirus SB13*, respectively. Phage SB18 belonged to the same family as phages SB6, SB10 and SB13 but was classified as subfamily *Ounavirinae*; genus *Kolesnikvirus*. Phage SB28 represent a novel virus that was recently classified by ICTV as *Macdonaldcampvirus* at genus level and as *Macdonaldcampvirus SB28* at species level. Phages SB3 and SB15, were classified as subfamily *Guernseyvirinae* and *Jerseyvirus AG11* at genus level (https://viralzone.expasy.org/6319).

4. Discussion

Salmonella enterica is a major foodborne pathogen of global importance, and its genomic diversity has been widely studied [67–72]. Subspecies I contains more than 1,500 of the total 2,600+ serotypes in the species and is of most importance with respect to human infections [73]. Many studies have reported on the isolation and characterization of *Salmonella* phages, but these studies report on isolation of phages from only a few of the 1,500 serotypes from subspecies I, with a specific focus on those serotypes most commonly implicated in human disease [74]. In this study, we isolated *Salmonella enterica* phages from wastewater and assessed their diversity and host specificity using a combination of microscopic, biological, and genomic approaches. Phage isolation was conducted on a panel of highly diverse *S. enterica* isolates (Figure 1&2) representing 30 serotypes that are commonly associated with fresh produce outbreaks [75–77]. There are only few reports of phages isolated from many of the serotypes chosen in this study, allowing for an assessment of *Salmonella* phage diversity from food plant associated serotypes.

Wastewater is reported to be a rich source of phages, containing a vast diversity of both temperate and virulent phages that infect a wide array of host bacteria, including *Salmonella* [63,78]. In this study, eight *S. enterica* phages were isolated from the Jean-R. Marcotte WWTP in Montreal, which is the third largest WWTP in North America and provides wastewater treatment for the entire island of Montreal [79]. Montreal (population 4.34 million) is a large urban and diverse city with inhabitants from more than 50 nationalities [80], and a rich history of gastronomy, meaning that a wide variety of North American and ethnic foods are consumed within the city as a whole. Therefore, the size of the Jean-R. Marcotte WWTP and the diversity of wastewater treated there made it an ideal location for isolation of a diverse group of *S. enterica* phages.

Our results indicate that the phages isolated in this study had broad host ranges, as the majority of the *Salmonella* isolates used in this study were lysed by the phages. The broadest host range phages (SB3, SB6 and SB9) lysed more than 85 % of the isolates used for lytic spectra testing, which suggests the presence of one or more conserved receptors used by these phages to infect *S. enterica* and indicates that these phages could be good candidates for phage-based control strategy for reducing microbial contamination of food plant produce. In an earlier study, we demonstrated that phages SB3 and SB6 successfully reduced *S. enterica* populations on lettuce and cantaloupe tissues [12]. Future studies will focus on elucidation of the bacterial receptor/s used by phages SB3, SB6 and SB9 to better assess their potential for use in controlling foodborne contamination due to common and rare *Salmonella* serotypes. In addition, further studies on the potential of other phages isolated in this study to reduce *S. enterica* on food matrices are required to fully assess their biocontrol efficacy.

Whole genome-based phylogeny of the eight phages in this study revealed the uniqueness and high genetic diversity among them and as well as previously sequenced phages. The genome size, GC and gene contents of the phages were heterogenous. Comparative genomic analysis showed that, when compared to 113 phages from public databases, the eight phages from this study were grouped into three different clusters: cluster A (SB28, SB9, SB10 and SB13), cluster B (SB3 and SB15), and cluster C (SB6, SB18) (Figure 5). Phages are known to be heterogenous and have been recognized as one of the major drivers of diversity, evolution and adaption of their hosts in different environmental matrices, including wastewater ([17,19–25,81,82]. *Salmonella* Typhi phages were enriched in cluster A, whereas phages that were isolated from animals/animal products were enriched in cluster C. This

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diversity is reflected in the host and/or source associated clustering of the phages in the phylogeny [20].

Taxonomic classification of phages are based on electron microscopy and whole-genome sequencing [83]. The great majority of the phages sequenced in this study belonged to the *Siphoviridae* morphotype, while one (SB18) belonged to the *Myoviridae* morphotype. Two phages (SB13 and SB28) differed significantly from the previously sequenced phage genomes and represent novel species. Based on this observation, the bacterial and archaeal viruses subcommittee (BAVS) of ICTV has created two new species viz. *Epseptimavirus SB13* and *Macdonaldcampvirus SB28* [84]. Novel genus *Macdonaldcampvirus* has one more species *Macdonaldcampvirus* ViIIE1 attributed to *Salmonella* phage Vi II-E1 (AM491472.1). The knowledge of classification aids in the design of phage cocktails for biocontrol purposes, as phages with different morphotypes use different host receptors [85] and help to overcome phage resistance [86]. Indeed, phage cocktails containing different morphotypes could provide more effective in reducing bacterial loads in food products. In a previous study, the inclusion of phages SB3 and SB6 (*Siphoviridae* morphotype) from this study with three other phages belonging to the *Myoviridae* morphotype in a five phage-cocktail was effective in reducing *Salmonella enterica* on lettuce and cantaloupe flesh sections [12].

One of the safety concerns of using phages as biocontrol in food products is its propensity to harbour and/or facilitate horizontal gene transfer of antimicrobial resistance determinants in the environment [87–89]. In this study, none of the phages carried genes encoding virulence or antimicrobial resistance. Another concern arises from the pharmacological limitations of using phages as antimicrobials. For example, there is a significant size disparity between phage particles and antibiotic and other antimicrobial compounds, with phages being millions of times larger and composed of multiple proteins. This size discrepancy restricts dosing options, diminishes uptake and transportation rates [90]. To address this limitation, interest is increasingly turning to utilizing phage components as antimicrobials. The majority of this work has been conducted using phage lysins that are active against Gram-positive bacteria [91]. These enzymes are not active against Gram-negative bacteria due to the protective nature of the outer-membrane protein. More recently, several groups have demonstrated the antimicrobial effects of phage tail-spike proteins against Gram negative bacteria. Phage tailspike proteins are highly thermostable and protease resistant [92]. They possess carbohydrate depolymerase activity and recognize and cleave components of the lipopolysaccharide (LPS) to position the phage towards a secondary membrane receptor during infection [93]. Ayariga et al. [92] demonstrated that the ε34 phage tail spike protein has enzymatic property as a LPS hydrolase and synergizes with Vero Cell culture supernatant in killing *Salmonella* Newington. Miletic and colleagues [94] expressed the receptor binding domain of the Phage P22 Gp9 tailspike protein in plant tissue (*Nicotiana benthamiana*), and demonstrated that, upon oral administration of lyophilized leaves expressing Gp9 tailspike protein to newly hatched chickens, *Salmonella* concentrations were reduced on average by approximately 0.75 log relative to controls. Other studies led to reduced *Salmonella* motility and colonization [94–96]. In this study, three phages possessed tailspike proteins viz. SB3 (GenBank: QBQ74073.1); SB15 (GenBank: QHI00505.1) and SB18 (GenBank: QHI00609.1). Future work will include isolation and purification of these tailspike proteins, and analysis as potential antimicrobials to control *S. enterica* in foods.

5. Conclusions

In order to study *Salmonella enterica* bacteriophage diversity and host specificity, we isolated and characterized eight bacteriophages using microscopic, biological, and genomic approaches. Biological characterization by host range profile revealed that all eight phages were broad host range phages; genomically none of the phages possessed virulence, toxin, antibiotic resistance or lysogeny genes and we could classify them using their physical/morphological characterization. Phages SB3 and SB6 had been previously identified and proved to be good biocontrol candidates owing to their desirable characteristics. Phages SB9, SB13, SB18 and SB28 had the broadest host range and could be promising candidates for phage-based biocontrol, either alone or in a cocktail. This study reported two new phage species recognised by ICTV i.e *Epseptimavirus SB13* and *Macdonaldcampvirus SB28*.

Most of the phage genomes have a significant number of hypothetical proteins and this lack of understanding or the unknown functions of these proteins could be a limitation to the use of these (and other) phages as biocontrol agents. Nonetheless, our attempt to understand the diversity and host specificity of the isolated phages could contribute constructively to our understanding of phage biology and to better utilize this understanding in the development of biocontrol strategies in controlling *Salmonella* worldwide, in various environmental settings.

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