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ORIGINAL ARTICLE

Characterization of Shiga toxin-producing *Escherichia coli* bacteriophages isolated from agricultural environments and examination of their prevalence with bacterial hosts

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Abstract

In the natural environment, ruminant livestock, including cattle, are the main reservoir of the outbreak–causing strains of Shiga toxin-producing *Escherichia coli* (STEC), where bacteriophages sustainably thrive as well. This study focuses on the characterization of STEC-specific bacteriophages isolated from cow manure samples in Maine farms and examines their prevalence with STEC hosts. Phenotypic features of representative isolates were characterized by using a transmission electron microscope. Similarly, host range, one-step growth curve, thermal stability, lytic capability, and genomic analyses were performed to fully characterize selected representative isolates. Results showed that representative bacteriophage isolates belong to *Myoviridae* (S6P10 and S14P12) and *Siphoviridae* (S19). The most prevalent and common bacteriophages (46%) were specific to the O26 serogroup. The farm C sampling site had highly heterogenous bacteriophage populations that were specific to six STEC serogroups. The most prevalent bacteriophage isolate (S1P5, *Escherichia* phage vB_EcoM-S1P5QW) was verified to have a double-stranded DNA genome (166,102 bp) with 266 CDs of which 130 have known functions. The majority of the diverse bacteriophage isolates had strong lytic capabilities and a narrow host range that could withstand selected temperature conditions (−20, 37, and 62°C). Results of bacterial screening showed that STEC host strains were not detected in Farms A, C, and E, but were detected on Farms B and D. In conclusion, the highly-diverse bacteriophage ecology found in cow manure samples may have been an important element in shaping the population of STEC serogroups, specifically in its natural environment, which can provide useful tools for potential antibiotic-free therapeutics and diagnostic technologies.

1 | INTRODUCTION

Bacteriophages are highly abundant (approximately 10^{31}) in nature and can be isolated nearly from everywhere (Callaway et al., 2008; Jurczak-Kurek et al., 2016; Liu et al., 2015; Shahrbabak et al., 2013). As it exhibits a predatory life cycle with bacteria, bacteriophages undermine bacterial host resources for successful infection and multiplication (Hagens & Loessner, 2007; Singh et al., 2012). Additionally,

bacterial hosts express receptor proteins on their cell surfaces allowing bacteriophages to bind and inject ther DNA for cellular control, production, and release of progenies (lytic). During the lysogenic stage, bacteriophage DNA fragments are integrated into the host genome (prophage) and may be replicated along with the host DNA.

Bacteriophages can reside in the gastrointestinal tract (GIT) of mammals as part of the microbial ecosystem (Callaway et al., 2008). The composition of the bacterial ecology systems in the GIT has shown high heterogeneity, especially in ruminants (Mao et al., 2015). Cattle are natural reservoirs of Shiga toxin-producing *Escherichia coli* (STEC), a group of outbreak-causing foodborne pathogens, and serve as its asymptomatic carriers (O'flynn et al., 2004). Approximately 30% of feedlot cattle in North America have been found to shed STEC O157:H7 in their feces (Y. D. Niu et al., 2012). Thus, screening and detection of STEC strains need to be strictly applied on farms and slaughterhouses for appropriate intervention to reduce STEC contamination risks.

Cattle have been reported to shed 10^7 bacteriophages/gram of manure (Callaway et al., 2008; Y. Niu et al., 2009). Some bacteriophages can be highly infective and may possess a wide host range. With this prevalence, bacteriophage-bacterial host(s) relationships present a novel biocontrol potential, especially in reducing the presence of pathogens such as O157:H7, which is shed by ruminants (Wang et al., 2015). This study aimed to characterize isolated STEC-specific bacteriophages from cow manure samples in Maine farms and examine their prevalence with STEC bacterial hosts. Since bacteriophages are natural bacterial predators, they can offer non-antibiotic and chemical-free options to lower the incidence of STEC group contamination in the food and supply network.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and growth conditions

Bacterial strains used were part of the strain collections of the Pathogenic Microbiology Laboratory (University of Maine, Orono, ME), Western and Eastern Regional Research Centers (Albany, CA and Wyndmoor, PA) of the USDA-Agricultural Research Services (ARS), and the US Food and Drug Administration (US FDA), Silver Spring, MD (Table S1). To assess the host range and lytic capabilities of bacteriophages, both STEC and non-STEC strains were tested. In brief, frozen bacterial strains were activated in Brain Heart Infusion (BHI) broth (Neogen) (37°C). The viability of strains was confirmed using appropriate selective media such as MacConkey Agar with Sorbitol (Neogen), xylose lysine deoxycholate (XLD) agar (Neogen), and Palcalm agar (Neogen) on plates.

2.2 | Enrichment and purification of environmentally isolated bacteriophages

Five undisclosed farms in the state of Maine were included in the study. Cow manure samples were randomly collected from various sites around the farms. Fresh manure samples (300 g each) were picked, stored in Whirlpak bags (Fisher Scientific), and transported to the laboratory in iced containers. All samples were stored at −20°C prior to use.

In brief, a sample (10 g or mL) was mixed with modified Tryptic Soy Broth (mTSB) containing 8 mg/L novobiocin and casamino acids (Neogen) (90 mL) before pulsifying (two rounds) at medium speed oscillation (30 s/sample). Homogenized samples were then centrifuged (4000*g*, 15 min) to collect the supernatant, followed by mixing with a cocktail of STEC serogroups (300 µL/strain) and $CaCl_2$ (10 mmol/L). Sample mixtures were then incubated at 37°C for 18 h before adding chloroform (4% v/v, final concentration, 30 min). Centrifugation (4000*g*, 15 min) was performed to collect potential bacteriophages that were suspended in the supernatant.

To identify susceptible STEC host strains, the supernatant was diluted (10,000-fold) and spotted (10 µL) on overnight STEC strains in Tryptic Soy Agar (TSA) (Neogen) plate. Spots that formed after overnight incubation (37°C) confirmed specificity and host susceptibility. Spots were then carefully picked and incubated with the corresponding STEC host (300 µL, overnight) for enrichment; conditions were similar to the previous section.

To purify isolated bacteriophages, a soft agar overlay technique by Kropinski (2009) was conducted (three rounds). Bacteriophage suspension (100 µL) was incubated with STEC hosts (200 µL) and then poured evenly on agar plates (TSA). Plaques were picked for final enrichment. Enriched bacteriophage samples were allowed to pass through a 0.2 µm membrane (Millipore, Billerica, Massachusetts, USA) filter before performing plaque assays. Enriched bacteriophages in TSB were stored at 4°C until further use (Table S2).

2.3 | Physical parameters and morphological characterization

Previously enriched and purified bacteriophage isolates (1 mL) were ultracentrifuged (131,000*g*) for 24 h following the cesium chloride (CsCl) density gradient centrifugation method using Optima MAX-XP (Beckman Coulter Inc.) ultracentrifuge. After the run, a thin whitish band formed and was carefully extracted from the tube. All purified suspensions were kept at −20°C prior to use.

Transmission electron microscopy (TEM) was used to characterize isolated bacteriophages and their morphological features. In brief, purified bacteriophage (1 mL) was ultracentrifuged. Bacteriophage suspensions were dropped (2 µL) onto the copper grids (Formvar film) and negatively stained using uranyl acetate (1.5%, pH 4–4.5), air dried prior to viewing under the TEM (200 kV, Tecnai G2 F20 model FEI).

The bacteriophage suspensions were also diluted in S.M. buffer (pH 7) and analyzed by Litesizer 500 (Anton Paar, Virginia, USA) with Dynamic Laser Light Scattering (DLS) system for the physical parameters such as zeta potential and electrophoretic mobility of the bacteriophage isolates as described elsewhere (Balcão et al., 2022).

2.4 | Host range and lytic capability

Bacteriophages that formed clear zones or plaques were re-tested against non-STEC strains to determine their host range and lytic capability. In brief, overnight non-STEC and generic *E. coli* cultures (100 µL) were individually added to plates with molten TSA (Neogen) and mixed by mild swirling. Once the agar had solidified, spot testing was performed by dropping (10 µL) of bacteriophage stock, and plates were incubated overnight at 37°C.

Furthermore, the activity of representative bacteriophage isolates (S5P8, S5P9, and S6P10) against the bacterial hosts was evaluated. The effects of various multiplicity of infection (MOI) were investigated by monitoring the absorbance (600 nm) of mixed bacteriophage isolates and bacterial host suspensions. In brief, S5P8, S5P9, and S6P10 stocks were diluted until 0 log PFU/ml. Each phage dilution was mixed with an overnight culture of host STEC strain (9 log CFU/ml) and $CaCl_2$ for optical density reading. Measurements were taken every 10 min throughout a 6 h incubation at 37°C (Mangieri et al., 2020). For control, sterile water was added instead of the diluted bacteriophage stocks.

2.5 | Temperature stability

The stability of bacteriophages in different temperatures was evaluated by subjecting representative isolates (S1P2, S6P10, S10P11, S14P12, and S19) to four temperature levels and exposure times: −20°C (24 h), 37°C (40 min), 62°C (40 min), and 95°C (5 min). The procedures were described elsewhere with minor modifications (Jurczak-Kurek et al., 2016). A plaque assay was conducted to measure titer reduction post-exposure, and all plates were incubated overnight at 37°C.

2.6 | One-step growth curve

One-step growth curve experiments were performed as previously reported with minor modifications (Amarillas et al., 2016; Topka et al., 2019; Zhang et al., 2021). The most prevalent bacteriophage isolate (S1P5) was chosen as the representative STEC bacteriophage for the one-step growth curve study. In brief, the host strain STEC O26:H11 HH8 was allowed to grow overnight in TSB at 37°C. The bacteriophage isolate (S1P5) was then added to the overnight culture at a 0.01 MOI and was incubated for 5 mins at room temperature to ensure successful bacteriophage adsorption. The mixture was then centrifuged (10,000*g*) for 5 mins to remove the supernatant and washed three times before resuspending the bacterial pellet in 20 mL TSB. An l00-fold dilution using TSB was also prepared before incubating the sample at 37°C with shaking (90 rpm), a condition which was maintained throughout the experiment. To determine bacteriophage-infected STEC cells, the diluted bacterial culture (10 µL) was mixed with a fresh overnight culture (500 µL) of STEC O26:H11 HH8 and 3 mL 50% molten TSA. After which, the mixture was poured into pre-made TSA plates. During the 60 mins incubation, 1 mL of the sample was collected at a 5 min interval. The sample from each time point was filtered through a 0.22 µM sterile membrane filter for the double-layer plaque assay to determine its latent period. All plates were incubated overnight at 37°C.

2.7 | Molecular and genomic characterization of bacteriophage isolates

From the previous enriched and purified suspensions, the DNA of bacteriophage isolates was extracted using a DNA isolating kit (Norgen, Biotek Corp., ON, Canada) following the manufacturer's instructions for characterization purposes. Conventional PCR was performed to determine the presence of STEC virulence genes, *stx*1 and *stx*2 in all isolated bacteriophages. Specific primers and amplification conditions from previous reports were adapted (Quintela et al., 2015; Table S3).

One representative bacteriophage (S1P5), which was the most common STEC bacteriophage isolate in this study, was further processed for genomic characterization. To construct a DNA library (2 × 250 bp), a TruSeq Nano DNA library preparation kit (Illumina, San Diego, CA) was used before sequencing on the Illumina MiSeq system. Raw reads were trimmed and quality filtered by Trimmomatic (Galazy v0.36.6) at the Q30 threshold and FastQC (Galaxy v0.72), respectively (Bolger et al., 2014; Simons, 2010). SPAdes v3.13.0 on the KBase server was used for De novo assembly of the remaining quality reads (Arkin et al., 2018; Nurk et al., 2013). BLASTn was used to identify the largest contig and then annotated using RAST (Aziz et al., 2008) and Prokka v1.14.5 (Seemann, 2014) pipelines. Uniprot (Consortium, 2015), BLASTp (Altschul et al., 1990), and ORF Finder (Geneious v11.0.4) were used to compare and curate genome annotations. To determine the packaging mechanisms and genome termini, PhageTerm (Garneau et al., 2017) was used, while tRNAs (Lowe & Chan, 2016) were predicted using tRNAscan-SE v2.0 web server. Finally, the presence of antibiotic resistance and virulence genes was determined by ResFinder v3.0 (Joensen et al., 2014) and VirulenceFinder v2.0, respectively. The genome sequence was deposited in GenBank (Accession Number OL956808) (Quintela et al., 2022). This genome sequence was analyzed against a bacteriophage genome that shares the highest average nucleotide identity (96.8%) (*Shigella* phage SHBML-50-1, GenBank Acession Number KX130864.1) using the Mauve tool (Darling et al., 2010) and BLASTn. Circular genome maps were generated and compared using Proksee (Grant et al., 2023).

2.8 | Prevalence and distribution of STEC bacteriophage isolates with STEC hosts

Cow manure samples were screened for the presence of STEC strains following Cooley et al. (2013) with minor modifications. In brief, strains were enriched from fresh samples (1 g) by adding TSB (9 mL)

TABLE 1 STEC bacteriophages isolated from cow manure samples.

	STEC bacteriophage isolates (sources)				
STEC hosts	**Farm A ($n = 5$)**	**Farm B ($n = 4$)**	**Farm C ($n = 1$)**	**Farm D ($n = 10$)**	**Farm E ($n = 2$)**
O26:H11 HH8	+	+	+	+	+
O26:H11 SJ1	–	–	–	–	–
O26:H11 SJ2	–	–	–	–	–
O45:H2 SJ7	–	–	+	–	+
O45:H2 05–6545	–	–	+	–	–
O45:H2 96–3285	–	–	–	–	–
O103:H2 GG7	–	–	–	–	–
O103:H25 SJ11	–	–	+	–	+
O103:H11 SJ12	–	–	–	–	–
O111:H8 EE5	–	–	+	–	–
O111: NM SJ13	–	–	–	–	–
O111:H- 94-0961	–	–	–	–	–
O121:H19 SJ18	–	–	–	–	–
O121:H19 96–1585	–	–	+	–	–
O145: NM SJ23	–	–	–	–	–
O145:H28 07865	–	–	–	–	–
O145:H- 94-0491	–	–	–	–	–
O157:H7 ATCC 12900	–	–	+	–	–

Note: Samples were collected from different farms in Maine. Bacteriophage isolates, sample sources, and STEC strains with successful bacteriophage enrichment are shown in the table. *n*, *sample size*.

TABLE 2 Sources of cow manure samples and other relevant information.

Sample	Animal source	Description/label	Location	Feeding conditions	STEC bacteriophage isolates
Farm A	Cattle	Farm A—Manure 1	Undisclosed	Grain	Negative
		Farm A—Manure 2	Undisclosed	Grain	Negative
		Farm A—Manure 3	Undisclosed	Grain	Positive
		Farm A—Manure 4	Undisclosed	Grain	Positive
		Farm A—Manure 5	Undisclosed	Grain	Positive
Farm B	Cattle	Farm B—Manure 1	Undisclosed	Grain	Negative
		Farm B—Manure 2	Undisclosed	Grain	Positive
		Farm B—Manure 3	Undisclosed	Grain	Positive
		Farm B—Manure 4	Undisclosed	Grain	Negative
Farm C	Cattle	Farm C—Manure 1	Undisclosed	Partial diets	Positive
Farm D	Cattle	Farm D—Manure 1	Undisclosed	Pasture fed	Negative
		Farm D—Manure 2	Undisclosed	Pasture fed	Negative
		Farm D—Manure 3	Undisclosed	Pasture fed	Negative
		Farm D—Manure 4	Undisclosed	Pasture fed	Positive
		Farm D—Manure 5	Undisclosed	Pasture fed	Positive
		Farm D—Manure 6	Undisclosed	Pasture fed	Negative
		Farm D—Manure 7	Undisclosed	Pasture fed	Negative
		Farm D—Manure 8	Undisclosed	Pasture fed	Negative
		Farm D—Manure 9	Undisclosed	Pasture fed	Negative
		Farm D—Manure 10	Undisclosed	Pasture fed	Negative
Farm E	Cattle	Farm E—Manure 1	Fairfield, Maine	Fed with corn for 60 days	Positive
		Farm E—Manure 2	Fairfield, Maine	Fed with corn for 200 days	Positive

(Neogen) and then plated on Washed Blood Agar with novobiocin (Hardy Diagnostics). Then, 1 mL of enrichment was centrifuged (10,000*g*, 2 min) for real-time (R.T.) PCR. Genomic DNA was extracted by boiling (100 µL, 80°C for 5 min, 100°C for 20 min) using PCR thermocycler (Biorad). RT-PCR was conducted by mixing 5 µL extracted DNA (template), 0.3 µM of primer, and 0.2 µM of probes (Cooley et al., 2013). The cycle threshold (C_t) value <27 was considered positive for STEC strains (Table S4).

3 | RESULTS AND DISCUSSION

In this study, all STEC bacteriophages were isolated from cow manure samples. Among the isolates, bacteriophages (S1P1, S1P2, S1P3, S1P4, S1P5, and S1P6) that were specific to STEC O26 were found in all sampling sites suggesting its wide distribution (Table 1). STEC O45 bacteriophages (S4P7, S5P8, and S5P9) and STEC O103-specific bacteriophage (S6P10) were the second most prevalent isolates. In terms of sampling sites, Farm C, where herds were fed with partial diets, had the most diverse bacteriophage isolates as compared to other sites which provided its herd with grain (Farms A and B), pasture (Farm D), and corn (Farm E) (Table 2). The results also imply that non-O157 STEC bacteriophage isolates (STEC O26, O45, and 103-specific bacteriophages) were more abundant in cattle farms as compared to STEC O157 bacteriophage isolates, as suggested by their populations found in cow manure samples. The average pH (7.24 ± 0.14) of the cow manure samples may have also largely contributed to the persistence of STEC bacteriophage isolates which agreed with the previous studies (Nyambe et al., 2016). Bacteriophages are highly stable in the range of pH 5–9 but coagulate at pH 2 and somehow lose viability at pH 3 or lower, and would precipitate within pH 3–4 range though agitation allows its redispersion (Dini & De Urraza, 2010; Jończyk et al., 2011).

3.1 | Morphological features of isolated bacteriophages and zeta potential

The negatively stained bacteriophage isolates in this study were classified under the order *Caudovirales* based on the published parameters in the literature. The morphological dimensions have allowed the classification of *Siphovidirae*, *Myoviridae*, and *Podoviridae* families, all under *Caudovirales* (Hans-W Ackermann, 1998; Jurczak-Kurek et al., 2016). Tailed bacteriophages, either with contractile long and non-contractile or short tails, represent 96% of bacteriophages (Ackermann, 2011; Zinke et al., 2022). Figure 1 shows TEM images of various STEC-specific bacteriophage isolates. S19 (Figure 1a) had a contractile sheathed tail that extended and covered more than half of its tail length (211 nm), possessed an icosahedral head (67 × 60 nm), and with a relatively smaller tail diameter (11.5 nm). S14P12 (Figure 1b) had a larger icosahedral head (107 × 98 nm), a shorter tail length (116 nm) but a larger tail diameter (17.85 nm). Both Hans-W Ackermann (1998) and Jurczak-Kurek et al. (2016) discussed that

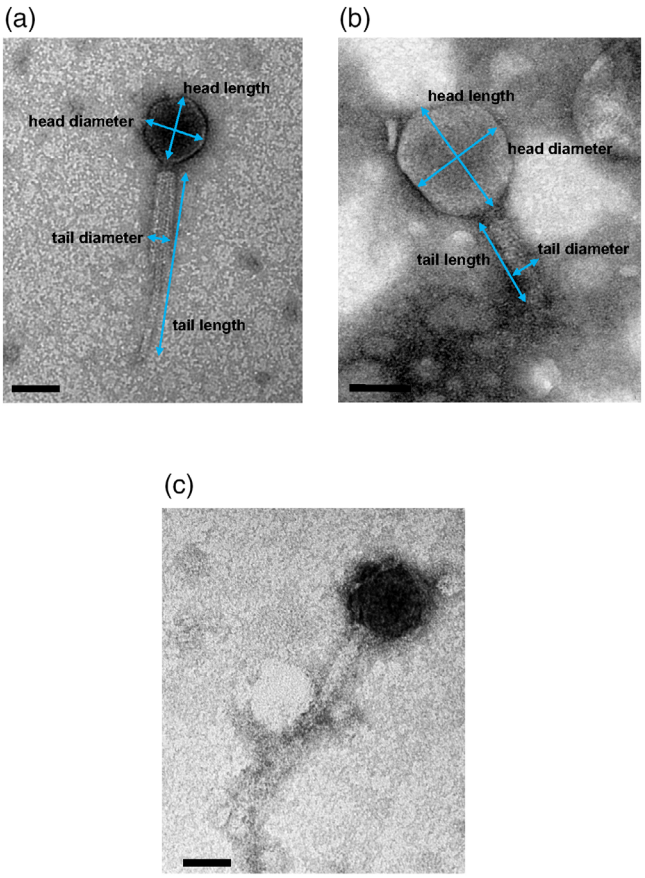

FIGURE 1 TEM images of various STEC-specific bacteriophages isolated from cow manure samples. (a) S19 [O157:H7 bacteriophage], a member of *Siphoviridae*; (b) S14P12 [O121:H19 96–1585 bacteriophage], a member of *Myoviridae*; (c) S6P10 [O103:H25 bacteriophage], a member of *Myoviridae*. Scale bar: 50 nm.

TABLE 3 Zeta potentials and electrophoretic mobility of representative bacteriophages.

STEC bacteriophage isolates	Zeta potential (mV)	Electrophoretic mobility (µm cm V^{-1} s^{-1})
S1P2	−9.8 ± 2.4	−0.7664
S6P10	−9.6 ± 0.9	−0.7485
S10P11	−8.4 ± 2.1	−0.6546
S1412	−11.9 ± 1.5	−0.9296
S19	−8.8 ± 0.9	−0.6835

bacteriophages exhibiting tail lengths <40 nm can be classified as members of *Podoviridae*. However, tails that are longer than 40 nm can be further classified into *Myoviridae* or *Siphovidirae* if the tail diameter is >16 nm and <16 nm, respectively. Based on their morphological features, S19 and S14P12 fall under *Siphoviridae* and *Myoviridae*, respectively. S6P10 (Figure 1c) also belongs to *Siphoviridae* as it exhibited similar morphological features shared by bacteriophages under this family. All long-tailed bacteriophages (i.e., members of *Siphoviridae*

TABLE 4 Results of spot testing of representative STEC bacteriophage isolates against STEC and non-STEC strains.

Bacterial strains	Lysis (plaque formation)—spot testing					
	S1P2	S1P6	S5P9	S10P11	S14P12	S19
O26:H11 HH8	+	+	−	−	+	−
O26:H11 SJ1	−	−	−	−	−	−
O26:H11 SJ2	+	+	−	−	−	−
O26:H2 TB285	+	+	−	−	−	−
O45:H2 SJ7	−	−	−	−	−	−
O45:H2 05–6545	−	−	+	−	−	−
O45:H2 96–3285	−	−	+	−	−	−
O103:H2 GG7	−	−	−	+	−	−
O103:H25 SJ11	+	+	−	−	−	−
O103:H11 SJ12	−	−	−	−	−	−
O111:H8 EE5	−	−	−	−	−	−
O111: NM SJ13	−	−	−	−	−	−
O111:H- 94-0961	−	−	−	−	−	−
O121:H19 SJ18	−	−	−	−	−	−
O121:H19 96–1585	−	−	−	−	+	−
O145: NM SJ23	−	−	−	−	−	−
O145:H28 07865	−	−	−	−	−	−
O145:H- 94-0491	−	−	−	−	−	−
O157:H7 ATCC 12900	+	+	−	−	−	+
Salmonella Agona	−	−	−	−	−	−
Salmonella Anatum	−	−	−	−	−	−
Salmonella Berta	−	−	−	−	−	−
Salmonella Dublin	−	−	−	−	−	−
Salmonella Enteriditis	−	−	−	−	−	−
Salmonella Gallinarum	−	−	−	−	−	−
Salmonella Heidelberg	−	−	−	−	−	−
Salmonella Infantis	−	−	−	−	−	−
Salmonella Javiana	−	−	−	−	−	−
Salmonella Mbandaka	−	−	−	−	−	−
Salmonella Montevideo	−	−	−	−	−	−
Salmonella Newport	−	−	−	−	−	−
Salmonella Oranienburg	−	−	−	−	−	−
Salmonella Saintpaul	−	−	−	−	−	−
Salmonella Senftenberg	−	−	−	−	−	−
Salmonella Thompson	−	−	−	−	−	−
Salmonella Derby 45,340	−	−	−	−	−	−
Salmonella Kentucky 44	−	−	−	−	−	−
Salmonella Muenster MF61976	−	−	−	−	−	−
Salmonella Typhimurium H3379	−	−	−	−	−	−
Vibrio parahaemolyticus 178,028	−	−	−	−	−	−
Enterococcus faecalis	−	−	−	−	−	−
Listeria monocytogenes	−	−	−	−	−	−
E. coli K-12 *MG1655*	−	−	+	−	−	−

and *Myoviridae*) have a large gene (>2 kbp) that encodes for tape measure protein (TMP) which precisely determines tail length (Pell et al., 2009). The isometric elongated head and crisscross-sheathed tail are also prominent morphological features of bacteriophages belonging to *Siphoviridae* and *Myoviridae* (Jurczak-Kurek et al., 2016). During assembly, these preformed tails and heads bind to each other

via the neck proteins. The receptor binding proteins (RBP) in bacteriophages are found on the tail fibers and spikes, which can specifically bind to the receptors located on the bacterial host surfaces (Samson et al., 2013). Receptors may include pilli, flagella, lipopolysaccharides (LPS), outer membrane (O.M.), and teichoic acids (T.A.) (Wang et al., 2015). Receptor localization and its density dictate the specificity of bacteriophage-host adsorption (Rakhuba et al., 2010).

Zeta potential is an electrokinetic characteristic of a layer that surrounds a particle, such as bacteriophages. This potential influences the interaction between molecules in various phases (Robertson et al., 2012). It also measures repulsive forces between particles and serves as an excellent measure of predicting colloidal stability depending on particle interactions (Balcão et al., 2014). Similarly, electrophoretic mobility represents the velocity of particles such as bacteriophages in a solution during measurement, and the negative value indicates its movement in the opposite direction of constant force. The DLS provided reliable measurements of the zeta potential and electrophoretic mobility (Table 3). The zeta potential value of all isolates tested was negative (−10 mV on average) at neutral pH, which was in agreement with the previous reports (Hao et al., 2019; Hosseinidoust et al., 2011; Passaretti et al., 2020) and the average electrophoretic mobility was −0.75 μmcm V^{-1} s^{-1}. Zeta potential measurements provide information about the electrostatic charges on the surface of bacteriophages which is important during its adsorption to the surfaces of host cells and other materials such as nanoparticles (Grygorcewicz et al., 2022; Stachurska et al., 2022). Electrostatic repulsions based on zeta potentials between bacteriophages and their host cells can be reduced or eliminated by applying nano-emulsions, thus enhancing infectivity (Esteban et al., 2016).

3.2 | Stability in various temperatures

Representative STEC bacteriophage isolates (S1P2, S6P10, S10P11, S14P12, and S19) were evaluated in terms of their stability at different temperatures and exposure times, ranging from −20 to 95°C. The bacteriophage isolates survived at −20°C (24 h) and 37°C (40 min). All isolates were sensitive and experienced high titer reduction when exposed to 62°C (40 min), but to some extent, S14P12 was more stable and resistant to this condition with a survival rate of 98.4%. However, a short exposure (5 min) to 95°C of all isolates tested showed very high titer reduction (>6 log PFU/mL), and almost no infective bacteriophage isolates were recovered (Figure S1).

3.3 | Host range and lytic capability

The results of spot tests showed that the six representative bacteriophage isolates could lyse STEC strains with some host overlaps, suggesting a broad host range. None of the isolates tested had lysing activities toward non-STEC strains (Table 4). A typical plaque morphology which was clear and usually associated with virulent or lytic bacteriophages has been observed (1–1.5 mm diameter). Representative bacteriophage isolates, S5P8 and S5P9, were highly infective against O45:H2 as they could inhibit the growth of this host bacterium (MOI = 1), as shown in Figure 2. At time points 130 and 160 min, the bacteriophage-host suspensions were both cleared (OD_{600} = ≤0) as compared to the control (without bacteriophage), which developed a noticeable degree of turbidity (OD_{600} = ≥0.3). These results showed that S5P8 and S5P9 were highly infective and could successfully lyse most of their hosts' cells at MOI = 1 within 2.5 h. S6P10 and host suspension did not achieve complete clearing of reaction volume. At the time point 280 min, there was a significant decrease in turbidity (OD_{600} = ≥0.04) in the S6P10 and host suspension, but it did not achieve complete clearing as previously observed in the other isolates. In terms of host susceptibility, previous studies of Y. D. Niu et al. (2014) showed that the MOI at 0.01 was extremely susceptible, while MOI at 1 was highly susceptible and MOI at 10 was moderately susceptible.

3.4 | One-step growth curve

In the first 15 min, the titer level of S1P5 remained relatively unchanged, then significantly spiked within 50 min before reaching

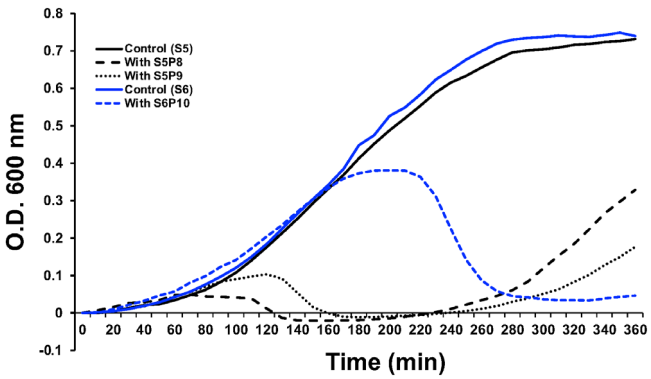

FIGURE 2 Lytic capability of representative bacteriophage isolates (S5P8, S5P9, and S6P10) against O45:H2 at MOI = 1.

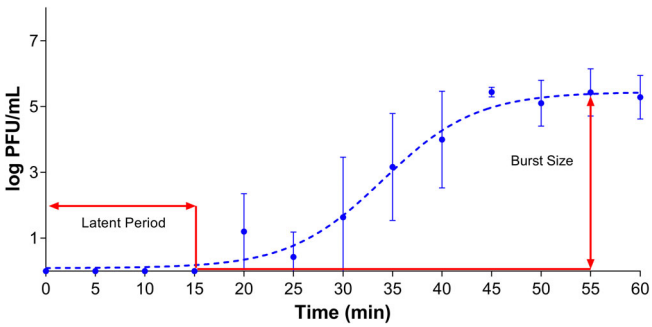

FIGURE 3 The one-step growth curve of S1P5 showed that its latent period was approximately 15 min and a burst size of 88 PFU/infected cells.

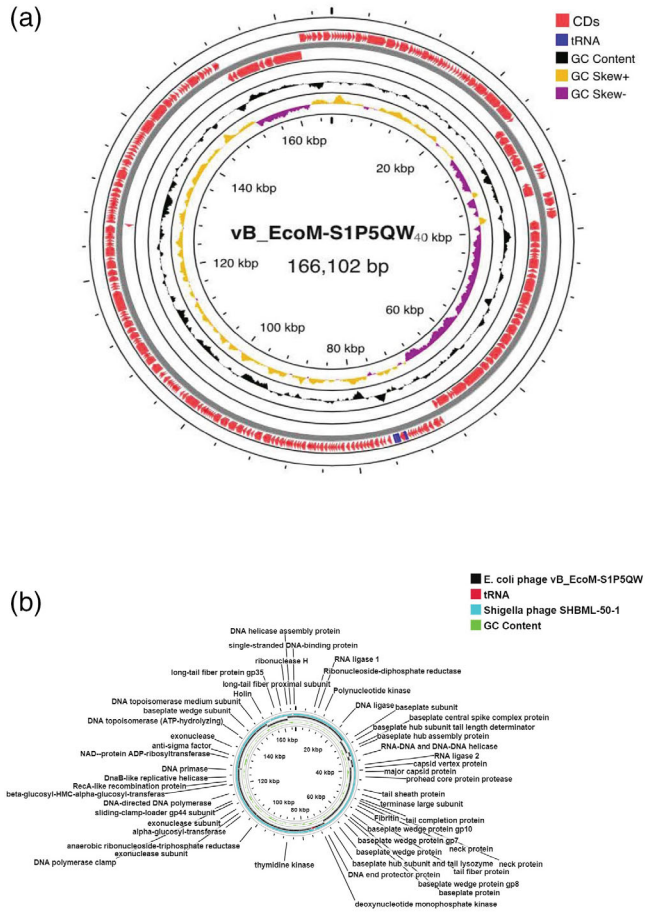

FIGURE 4 Characterization and analysis of S1P5 (*Escherichia* phage vB_EcoM-S1P5QW) genome. (a) Circular genome map of *Escherichia* phage vB_EcoM-S1P5QW using Proksee (Grant et al., 2023). The rings from inside out represent GC skew (yellow and purple), GC content (black), CDs (red), and tRNAs (blue) (b) Whole-genome comparison of *Escherichia* phage vB_EcoM-S1P5QW and the reference phage, *Shigella* phage SHBML-50-1 using blastn and visualization with Proksee (Grant et al., 2023). The CDs with the predicted functions from the phage *Escherichia* phage vB_EcoM-S1P5QW are indicated.

the stationary phase after 55 min with a titer of 5.24 log PFU/mL. The one-step growth curve indicated that S1P5 had a latent period of approximately 15 min (Figure 3) and a burst size of 88 PFU/infected cells.

3.5 | Molecular and genomic characterization of bacteriophage isolates

All STEC bacteriophage isolates were devoid of *stx*1 and/or *stx*2 gene(s) except one isolate, S1P3, which generated a PCR product (*stx*1) (Figure S2).

The representative bacteriophage isolate S1P5 or *Escherichia* phage vB_EcoM-S1P5QW (S1P5) has 166,102 bp genome size with 266 CDs of which 130 have known functions (Figure 4a). At the time of analysis, it shares 96.8% average nucleotide identity with *Shigella* phage SHBML-50-1, its closest relative. More than half of the S1P5QW gene products have the best hits with *Shigella* phage SHBML-50-1 genes, including DNA and RNA polymerases, holin, ligases, major capsid proteins, and hypothetical proteins (Figure 4b).

TABLE 5 Samples screening for the prevalence study of STEC serogroups.

Cow manure sources	STEC screening	
	Washed blood agar with novobiocin	RT-PCR (C_t values ± SD[a])
Farm A	–	Not detected
Farm B	+ colonies	*stx1acd* 20.19 ± 0.66 *stx2f* 19.22 ± 0.81 *stx2ex* 18.99 ± 0.64
Farm C	–	Not detected
Farm D	+ colonies	*stx1acd* 19.33 ± 0.52 *stx2f* 17.66 ± 0.60
Farm E	–	Not detected

Note: STEC strains from various serogroups were screened from different cow manure samples where STEC-specific bacteriophages were previously collected and isolated (Table 1).
[a]C_t values ≤27 was indicative of strong positive reactions and abundance of target DNA.

3.6 | Prevalence and distribution of STEC bacteriophage isolates with STEC hosts

Cow manure samples where bacteriophages were previously isolated also had a follow-up screening for STEC strains (Table 5). All presumptive STEC colonies were tested by RT-PCR, which targeted *stx* variants. Results showed that STEC host strains were not detected on Farms A, C, and E but were detected on Farms B and D.

The gut of healthy cattle is commonly colonized by STEC strains allowing it to spread to the environment via fecal shedding and farm effluent (Fremaux et al., 2008). The prevalence of STEC O157:H7 in cattle fecal shedding has a seasonal pattern that usually peaks during the summer months (Hancock et al., 1997; Y. Niu et al., 2009). In addition, bacteriophage ecology largely influences the level of STEC serogroup that is shed via the fecal route (Wang et al., 2015). Using various STEC screening and detection approaches, this study has shown how the bacteriophage ecology may have played an important role in shaping the population of STEC serogroups, specifically in its natural environment. Though only one sample from Farm C was included in this study, the data suggested that the highly diverse bacteriophage population from this site may have negatively influenced the population of its host to a certain degree, but further evaluations need to be conducted to include other important variables that were beyond the scope of this study. In future studies, additional sampling sites and a higher frequency of cow manure sample collection may

significantly show the correlation that exists between these two microbial populations.

Moreover, commercial companies have been working on various bacteriophage cocktails (Ecolide PX™) specific to foodborne pathogens in cattle, that is, cattle hide and farms that would reduce bacterial counts (Endersen & Coffey, 2020), suggesting its biocontrol capabilities. The highly diverse non-O157 STEC bacteriophage populations, most of which were lytic, exist in the natural environment, offering tools for potential non-antibiotic therapeutics and highly sensitive detection and diagnostic methods. With further studies and optimization efforts, bacteriophage isolates with a broad host range and strong lytic capabilities could be utilized and included in an effective bacteriophage cocktail that can be applied in farms and animals to reduce and control other significant foodborne pathogens. Furthermore, bacteriophage isolates with a narrow host range could be highly useful as diagnostics capture elements for the detection of live foodborne bacteria.

4 | CONCLUSION

The cow manure samples from five Maine farms were processed to isolate and characterize STEC-specific bacteriophages. These isolates were highly infective against the major STEC strains. The most prevalent bacteriophage isolates were specific to O26, and all isolates either belonged to *Myoviridae* and *Siphoviridae* families with icosahedral heads, sheathed tails, and tail fibers. It was found that the Maine cattle farm that fed its herd with partial diets (Farm C) may have influenced the richness and diversity of the isolated bacteriophage populations in addition to other factors such as pH levels and the prevalence of STEC hosts. These diverse bacteriophage isolates had strong lytic capabilities, narrow host range, and could withstand selected temperature conditions (i.e., −20, 37, and 62°C). Moreover, the majority of these isolates did not possess virulence genes (*stx*1 and *stx*2). Future studies need to expand sample collection and increase frequency, as well as the number of host strains used, to ensure that more bacteriophages are collected since bacteriophage isolates are dependent on the available strains for enrichment and further characterization purposes.

AUTHOR CONTRIBUTIONS

Irwin A. Quintela: Conceptualization, experimental design, data collection, and analysis, writing of the manuscript. **Don C. Valentine, Anya Hwang, Tyler Vasse, Kan-Ju Ho:** Data collection and analysis. **Vivian C. H. Wu:** Conceptualization, funding acquisition, review and editing of the manuscript, supervision, and project administration. All authors have given approval for the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests. The authors also disclose that the patents, US Patent No. 10,768,177 and W.O./2020/046712, both comprised some of the bacteriophage isolates described in this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

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