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# Isolation, identification and characterization of lytic, wide host range bacteriophages from waste effluents against *Salmonella enterica* serovars

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ABSTRACT

The use of bacteriophages is considered as a viable alternative to chemical antimicrobials against foodborne pathogens. The objective of this study was to develop a collection of lytic bacteriophages which will be able to infect different pathogenic *Salmonella enterica* serovars. Phages were isolated from animal feces and sewage samples, purified, characterized morphologically and by DNA fingerprinting, and host ranges were determined. Spot test and efficiency of plaquing (EOP) data indicated that two phages, SEA1 and SEA2 had the broadest host range against *Salmonella* among all isolated phages. SEA2 was highly efficient to infect *S.* Typhimurium DT104 (0.5–1 EOP value). Only phage SSA1 was able to infect *S.* Montevideo. Transmission electron microscopy (TEM) revealed the phages in the collection were mostly (4 out of 6) Siphoviridae, while SEA1 and SEA2 were Myoviridae T4-like phages. SEA1 and SEA2 had the largest genome sizes in the collection, 190 and 170 kb, respectively. Pulsed field gel electrophoresis (PFGE) analysis demonstrated distinct digestion profiles with *Eco*RI for phages SSA1, STD3, STE3 and STF1. However, SEA1 and SEA2 shared a similar restriction enzyme (RE) digestion pattern with same morphotype, but distinct profiles in lysing *Salmonella* strains. These anti-*Salmonella* phages were highly host specific with few exceptions of lytic phages that were able to infect a wide variety of *Salmonella*. These phages have potential for use in applications controlling *Salmonella* on different matrices.



## 1. Introduction

*Salmonella* infections are related to a wide diversity of food products and their source of contamination ranges from human through food processing facilities (Miao & Miller, 1999; Tauxe, Doyle, Kuchenmüller, Schlundt, & Stein, 2010). The estimated annual cost due to foodborne *Salmonella* infections is $2.4 billion in the United States ((USDA-ERS), 2001). According to the most recent report, non-typhoidal *Salmonella* has been identified as the leading cause of foodborne illnesses in USA with estimates of 1 million cases and 378 deaths per year (Scallan et al., 2011). Outbreaks have been associated with multidrug resistant serovars such as *S.* Typhimurium DT104 that often cause serious problems because of the limited treatment options (CDC, 2001). Though preventative measures are available, the recurring outbreaks of *Salmonella* pose an important public health risk that needs great attention and a pursuit for effective antimicrobial alternatives. In this context, bacteriophages have potential as an alternative to antibiotics or other conventional chemical control methods against bacterial pathogens. Bacteriophages are viruses capable of lysing bacteria, and specific lytic phages can kill pathogenic bacteria in their own habitat. Phages are ubiquitous in nature and can often be found in a variety of environments related to their host such as soil, sewage, water, manure, animal and produce farms, as well as different food processing plant effluents.

The application of bacteriophages as a food safety intervention has been recently investigated and a few commercial preparations have been approved and marketed. Bacteriophages are often used in high concentrations to inactivate foodborne pathogens, such as *Escherichia coli* O157:H7, *Salmonella*, *Listeria*, and *Campylobacter* in different foods (Carlton, Noordman, Biswas, De Meester, & Loessner, 2005; Greer, 2005; O'Flynn, Coffey, Fitzgerald, & Ross, 2006; O'Flynn, Ross, Fitzgerald, & Coffey, 2004). Also, in production facilities, phages also have been used to control specific bacteria at pre-harvest and post-harvest stages of food production and storage (Greer, 2005). The Food and Drug Administration (FDA) has

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approved *Listeria* specific phages for use in foods (FDA, 2006) indicating the promising use of bacteriophages in food applications. *Salmonella* specific phages have been isolated and a few lytic wide-activity range phages have been identified. Bielke et al. found only two potent bacteriophages from the isolated pool were able to lyse 7 out of 10 *Salmonella* isolates (Bielke, Higgins, Donoghue, Donoghue, & Hargis, 2007). Flynn et al. described that two lytic phages, st104a and st104b were screened from 100 fecal samples and evaluated as effective therapy to reduce *Salmonella enterica* serovar Typhimurium DT104 in vitro (O'Flynn et al., 2006). However, despite the existing inadequate library of lytic *Salmonella* phages, literature search indicated that there is no currently available phage treatment to control the diverse group of *Salmonella* serovars.

In this study, waste effluents from cattle and swine manure pit and raw sewage from the wastewater treatment plant were screened to isolate a collection of broad spectrum lytic *Salmonella* phages. The selected potent lytic phages were identified morphologically and further characterized to evaluate their lysis efficacy for infecting a wide range of *S. enterica* serovars. This library of lytic phages may be prospective to use with other isolated lytic phages for further food-related applications against *Salmonella*.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

A total of 34 different of *S. enterica* strains belonging to eight serovars were used to serve as host strains and to screen phages from isolation sources (Table 1). All bacterial strains were streaked from the frozen culture stock onto tryptic soy agar (TSA; Neogen Corp., Lansing, MI). Prior to experiments, each strain was grown by picking an isolated colony from TSA plates and inoculating into tryptic soy broth (TSB; Neogen Corp.) and incubated at 37 °C to obtain fresh overnight cultures.

### 2.2. Sample collection

Several waste effluent samples were collected from 1) cattle and swine manure pit from the dairy cattle and swine barns of the University of Minnesota; 2) city sewage treatment plant in Rice Lake City, Wisconsin; and 3) raw sewage sludge, wastewater treatment plant, City of St. Paul. Samples were collected by several visits throughout the spring and summer seasons.

**Table 1**
List of bacterial strains that were used for this study.

| *Salmonella* serovars | Strain ID number | Number of strains | Source of strains |
|---|---|---|---|
| *S.* Agona | Agona FDA | 1 | FDA |
| *S.* Typhimurium | UK-1, 3019907 | 2 | FSML |
| | ATCC 14028, ATCC 700408 | 2 | ATCC |
| | I598, I503, I527, I534, I535, I649, I526, I758, I536, I740, I600 | 11 | VBS |
| | E2009005811 | 1 | MDH |
| *S.* Enteritidis | 2009595, 95657613, I823 | 3 | FSML |
| *S.* Heidelberg | FSIS10 | 1 | FSML |
| *S. Montevideo* | 95573473 | 1 | MDH |
| *S. Newport* | AMO7076, AMO7073, AMO5104, AMO5313, B4442 | 5 | CDC |
| | Newport FDA | 1 | FDA |
| | 2006036 | 1 | FSML |
| *S.* Saintpaul | E2008001236, E2008001177, E2003002913, E2009010674, E2008001358 | 5 | MDH |
| *S.* Tennessee | E200700502 | 1 | MDH |

Abbreviation: FDA, Food and Drug Administration; FSML, Food Safety Microbiology Laboratory, University of Minnesota; ATCC, American Type Culture Collection; VBS, Department of Veterinary and Biomedical Sciences, University of Minnesota; MDH, Minnesota Department of Health; CDC, Centers for Disease Control and Prevention.

### 2.3. Enrichment, isolation, purification and preparation of bacteriophages

All waste samples were collected and centrifuged at 10,000 × *g* for 10 min to remove solid particles and then filtered with 0.45 μm pore syringe filters (Nalgene, Thermo Fisher Scientific Inc. USA). For enrichment, isolation and purification of bacteriophages, modified methods were used from previously published reports (Klieve, 2005; Mullan, 2002; Twest & Kropinski, 2009). *Salmonella* strains were grown overnight at 37 °C in TSB to obtain pure bacterial cultures. 0.1 mL of overnight pure cultures of *Salmonella* strains were inoculated into 10 mL TSB and incubated at 37 °C shaker for 3–4 h to grow exponential phase cultures. Filtered sample supernatant (4.5 mL) was then mixed with 0.5 mL exponential phase bacterial cultures and 0.5 mL 10× concentrated TSB, and incubated at 37 °C for 24–48 h. After incubation, samples were centrifuged at 10,000 × *g* for 10 min, supernatants were filtered with a 0.45 μm filter syringe, and used as enriched phage (EP) samples.

Initially, spot testing was used to isolate the phages. The host bacterial lawn was made by using a tryptone top agar (TTA) (containing per liter: bacteriological agar, 4; tryptone, 10 g; yeast extract, 5 g; NaCl, 7.5 g; Glucose, 10 g; 12 mmol MgSO4; 12 mmol CaCl2) containing host bacterial suspensions that were overlaid on top of TSA agar plates. When the agar overlays were solidified, several EP samples were spotted (1:10, phage:host) onto the lawns in a row and plates were incubated at 37 °C for 18–24 h. After incubation, all plates were examined for positive and clear spot formation. The lysed agar spot was cut and dissolved into phosphate buffer saline (PBS; Neogen, Inc.), centrifuged and filtered again to collect the supernatants. The supernatants were used as spot filtrate.

Plaque assays were conducted to isolate and purify individual phages from spot filtrates. For plaque assay, a series of 10-fold dilutions were made of spot filtrates. Exponential phase host cultures were mixed with dilutions of spot filtrates as host: spot filtrate (2:1) and incubated at 37 °C for 15 min for propagation. These phages and bacterial suspensions were mixed with the TTA agar and poured onto TSA. Plates were incubated at 37 °C for 24 h for plaque formation. To purify the phages, isolated plaques were picked by using a pipette or a wire loop and suspended with PBS. The suspensions were centrifuged and the supernatants were filter sterilized and used as a single phage culture. This purification process of an individual plaque through plaque assay, centrifugation and resuspension was repeated at least 3 times to ensure a pure phage stock.

The high titer phage stocks were prepared by inoculating 1 mL of overnight host bacterial cultures with 100 μl of purified phage stock ($10^5$–$10^6$ PFU $mL^{-1}$) into TSB (with 5 mol $l^{-1}$ $CaCl_2$) and incubated at 37 °C for 18–20 h. When lysis of liquid TSB cultures was visually observed, a few drops of $CHCl_3$ were added for complete lysis of the bacterial cells. The amplified, purified phages were centrifuged at 10,000 × *g* for 10 min to pellet debris and supernatants were filtered to remove bacterial contaminants. The filtered supernatants were stored as high titer ($10^8$–$10^{10}$ PFU $mL^{-1}$) phage stocks at 4 °C and used for the different analysis throughout the study.

### 2.4. Determination of host range

Spot testing was conducted to measure the ability of individual phages to infect different serovars of *Salmonella*. First, bacterial