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Isolation of *Salmonella* Bacteriophages from Swine Effluent Lagoons

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ABSTRACT

Bacteriophages (phages) associated with *Salmonella* were collected from nine swine manure lagoons in Mississippi. Phages were isolated by an enrichment protocol or directly from effluent. For enrichment, chloroform-treated samples were filtered (0.22 μm) and selectively enriched by adding a cocktail of *Salmonella* strains in trypticase soy broth. After overnight incubation at 35°C, chloroform was added and samples stored at 5°C. Enriched samples were tested by double agar layer (DAL) plaque assay against individual *Salmonella* isolates. Phage titers of 2.9×10^8 to 2.1×10^9 plaque forming units (pfu) per mL were produced, but estimation of phage titers in lagoons was not possible. For direct isolation, effluent was clarified by centrifugation, filtered (0.22 μm), and used in DAL plaque assays to select single-plaque isolates for 15 *Salmonella* strains. Plaque counts varied among *Salmonella* strains and lagoons. The most sensitive strain for direct phage recovery was ATCC 13311. Phage titers estimated by direct isolation with ATCC 13311 ranged among lagoons from 12 to 148 pfu per mL. In limited host range tests, 66 isolates recovered by the enrichment protocol produced plaques only on Enteritidis and Typhimurium strains of *Salmonella* and none produced plaques on lagoon isolates of *Citrobacter*, *Escherichia*, *Proteus*, *Providencia*, or *Serratia*. Electron microscopy (EM) showed purified enrichment isolates had *Podoviridae* morphology (tailless 50-nm icosahedral heads with tail spikes). Electron microscopy of clarified concentrated effluent showed 5.5:1 tailless to tailed phages. The isolated phages have potential as typing reagents, specific indicators, and biocontrol agents of *Salmonella*.

BACTERIOPHAGES (phages) are natural viral pathogens of bacteria. Phages exist wherever bacteria occur, and share a common ecology with their respective bacterial hosts (Goyal et al., 1987). A recent review suggests that phage or phage-like genomic sequences may account for the major genomic differences between members of bacterial species (Brussow et al., 2004). Considered to be the most abundant entities in the biosphere, phages with total numbers estimated from 10^{30} to 10^{32} (Kutter and Sulakvelidze, 2005) may be more resistant than their host bacteria to environmental changes and may persist in the absence of host bacteria (Duran et al., 2002). Phages of bacterial pathogens of animals are shed in animal feces and may be isolated from soil and aqueous environments (Calvo et al., 1981; Hurst, 1997). They are cultured in host bacteria by conventional microbiological methods and are identified and classified primarily by their morphology in electron microscopy (EM) and bacterial host ranges (Ackerman and Nguyen, 1983).

Virulent phages cause bacterial host cell lysis and not only function to control bacterial populations, but can be used as indicators of bacterial (fecal) contamination (Miller et al., 1998; Tanji et al., 2003) and as tools for identifying (typing) specific bacterial strains (Brenner et al., 1999; Leclerc et al., 2000; Sinton et al., 1998; Welkos et al., 1974). Typing phages have been employed to classify many human bacterial pathogens, including *Salmonella* (Anderson et al., 1977; Farmer et al., 1975; Kuhn et al., 2002a, 2002b). Virulent phages also have potential as specific biological control agents for bacterial pathogens in human, animal, and plant diseases (Alisky et al., 1998; Borah et al., 2000; Kudva et al., 1999; Lederberg, 1996; Leverentz et al., 2001; Lorch, 1999; Schuch et al., 2002). Recent reviews testify to a renaissance in the study of the potential beneficial uses of bacteriophages as biocontrol agents in food (Greer, 2005; Hudson et al., 2005), for phage therapy (Brussow, 2005), and for wastewater treatment (Withey et al., 2005). Lytic phages have been shown to reduce experimental *Salmonella* contamination in chickens (Toro et al., 2005) and on chicken skin (Goode et al., 2003), sprout seeds (Pao et al., 2004), and fresh-cut fruit (Leverentz et al., 2001). Phages have been used to remediate colibacillosis caused by *E. coli* in broiler chickens (Huff et al., 2002a, 2002b, 2005), and against poultry related strains of *Clostridium perfringens* (Siragusa et al., 2004). Specificity among virulent phages offers promise for targeted biological intervention and environmental remediation in animal rearing and manure storage facilities before disposal, transport, or processing of manures contaminated with specific human bacterial pathogens (Mueller, 1980).

The intimate role played by phages in *Salmonella* ecology may complicate their potential uses in biocontrol. *Salmonella* phages are believed to play a critical role in *Salmonella* evolution and to mediate horizontal transfer of virulence genes among *Salmonella* strains (Baumler, 1997; Porwollik and McClelland, 2003; Rabsch et al., 2002). About 95% of natural strains of *S.* Typhimurium, for example, have been reported to contain temperate phages as prophages (Schicklmaier et al., 1998), which may prevent super-infection by similar phages (Porwollik and McClelland, 2003). Cocktails

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Abbreviations: DAL, double agar layer; EM, electron microscopy; pfu, plaque forming units; TSA, trypticase soy agar; TSB, trypticase soy broth.

comprising multiple phages and specificities may be required to overcome this potential problem.

The objectives of the present study were to collect and partially characterize *Salmonella* phages associated with swine manure. Interestingly, the first member of the genus *Salmonella* was isolated from swine (Salmon and Smith, 1885). The present study describes the methodology for collection, isolation, and preliminary characterization of *Salmonella* phages from swine manure lagoons as part of a search for phages which might prove useful for *Salmonella* indicators or biocontrol agents.

MATERIALS AND METHODS

Collection and Isolation of Phages

Salmonella Host Cultures

Salmonella strains used as phage hosts were obtained from the American Type Culture Collection (Table 1). Isolates were not necessarily selected for their association with swine, but rather were chosen to include type strains of *S.* Typhimurium and Enteritidis, three DT104 isolates, and a small but diverse panel of isolates recommended for use in testing food sanitation processes. Two of the *S.* Typhimurium isolates (R37 and R47) were unusual in that they were negative for H_2S production in our laboratory tests (McLaughlin and Balaa, 2006). Freeze-dried primary cultures were reconstituted in trypticase soy broth (TSB) overnight at 35°C, then stored in 1- to 2-mL aliquots at −70°C. Secondary cultures were prepared from thawed primary cultures, grown in TSB overnight at 35°C, and inoculated on slants of brain heart infusion agar (BHI). The BHI slant cultures were grown overnight at 35°C, then sealed and stored in the dark at 5°C. Subcultures were produced weekly, or biweekly as needed, from stored BHI slant cultures inoculated to TSB, grown overnight at 35°C, and streaked on blood agar (trypticase soy agar, TSA, + 5% sheep red blood cells). Blood agar plates were checked for purity and uniformity and working cultures prepared by transfer of single isolated colonies to TSB.

Collection of Lagoon Effluent

Samples were collected from two anaerobic swine lagoons located in Lowndes County in east-central Mississippi in September 2002, April 2003, and February 2004. Samples of about 7 mL each were drawn from a depth of about 16 cm using 30-cm-long sterile disposable transfer pipettes and a tree pruning tool modified to hold the pipette and squeeze the pipette bulb with a pull of the rope. The handle of the modified pruning tool was telescopically extended to allow collection of samples to approximately 3 m in from the edge of the lagoon. A new pipette was used for each sample and effluent was dispensed into sterile 15-mL plastic culture tubes. A hand-pumped vacuum device on an extendable handle was used for collection of larger sample volumes of 150 to 200 mL. A 250-mL sterile collection bottle mounted on a PVC float at one end of a telescoping painter's pole was filled by applying a vacuum. The pump was connected through plastic tubing by luer lock fittings to a sterile disposable 10-mL pipette fixed to draw effluent from a uniform depth of 21 cm. The collection pipette, tubing, and bottle were changed between samples and a different sanitized float was used for each lagoon. A 0.22-μm inline filter protected the vacuum lines and pump, which remained attached to the extendable pole. Samples were collected using this device in May 2004 from the two Lowndes County lagoons and from seven similar lagoons located in Clay County, MS. The pump device was also used to collect three 200-mL samples from one of the Lowndes County lagoons in August 2004, for EM studies of total lagoon phages. All samples were held on ice in a cooler, returned to the laboratory, and held at 5°C for processing within a few hours of collection.

Enrichment Protocol

Effluent samples were treated with chloroform and stored at 4°C overnight to allow larger suspended sediments to settle out. These crudely clarified samples were then passed through 0.22-μm syringe filters. Several filter changes were needed to complete the filtration of each 10- to 15-mL of sample. Lytic phages were selectively enriched by mixing filtered effluent with double strength trypticase soy broth inoculated with a mixture containing serotypes Typhimurium (ATCC 43971 and ATCC 14028) and Enteritidis (ATCC 13076). After overnight incubation at 35°C, chloroform was added and samples were stored at 5°C. Enriched samples were tested by double agar layer (DAL) plaque assay against individual *Salmonella* isolates. Phage titers of 2.9×10^8 to 2.1×10^9 plaque forming units (pfu) per mL were produced. For isolation of phages, enriched samples were diluted in sterile saline in 10-fold series and used in DAL plaque tests at appropriate dilutions to yield separated individual plaques suitable for single-plaque transfer.

Table 1. *Salmonella* isolates (*S. enterica* subsp. *enterica*) used in the present study.

			Published antigenic formulas	
R number†	ATCC‡ number	Serovar (phage type)	ATCC	Popoff and Le Minor (1997)
R04	14028	Typhimurium	4,5,12:i:1,2	1,4,[5],12:i:1,2
R05	13076	Enteritidis§	1,9,12:g,m:-	1,9,12:[f],g,m,[p]:[1,7]
R21	43971¶	Typhimurium	4,5,12:i:1,2	1,4,[5],12:i:1,2
R36	13311	Typhimurium	4,5,12:i:1,2	1,4,[5],12:i:1,2
R37	700730	Typhimurium		1,4,[5],12:i:1,2
R38	BAA-189	Typhimurium (DT104)		1,4,[5],12:i:1,2
R39	BAA-190	Typhimurium (DT104)		1,4,[5],12:i:1,2
R40	BAA-191	Typhimurium (DT104)		1,4,[5],12:i:1,2
R41	BAA-215	Typhimurium	4,5,12:i:1,2	1,4,[5],12:i:1,2
R42	BAA-707	Agona		1,4,[5],12:f,g,s:[1,2]
R43	BAA-708	Enteritidis		1,9,12:[f],g,m,[p]:[1,7]
R44	BAA-709	Michigan		17:l,v:1,5
R45	BAA-710	Montevideo		6,7,14:g,m,[p],s:[1,2,7]
R46	BAA-711	Gaminara		16:d:1,7
R47	BAA-712	Typhimurium		1,4,[5],12:i:1,2

† Laboratory reference number for the present study.
‡ American Type Culture Collection.
§ Type strain.
¶ Type species.

Direct Isolation Protocol

Effluent samples were clarified by centrifugation (10 000 × *g* 30 min at 5°C), passed through 0.22-μm filters, and used in DAL plaque assays against the host isolates listed in Table 1. Single-plaque phage isolates were recovered.

Phage Lysate Production

Selected bacteriophages were increased by inoculation of log-phase broth cultures of the respective host strain and production of bacteriophage lysates in sufficient volume for additional characterization and analysis. Lysates were clarified by centrifugation (10 000 × *g* 15 min at 5°C), decanted, and filtered through 0.45-μm cellulose acetate filters into sterile glass vials, treated with chloroform (to 2.5%), and stored at 5°C (Adams, 1959).

Double Agar Layer (DAL) Plaque Methods

Phages were detected and enumerated by mixing 100 μL of test sample in aqueous media with 100 μL of fresh log-phase *Salmonella* host culture in TSB into 5.0 mL of soft TSA (0.75% agar) melted and held at 45°C in a water bath. Test suspensions were mixed by vortexing and dispensed uniformly over the surface of 20 mL of hard TSA in 96-mm-diameter plates. Soft agar overlays were allowed to harden at room temperature then plates were inverted and incubated overnight at 35°C. Plaques (pfu) were counted or individually subcultured as appropriate to the enumeration or isolation protocol (Adams, 1959).

Host Range Tests

Phage isolates recovered by the enrichment protocol were tested against each of the three *Salmonella* strains used in the enrichment and against strains of other bacterial species which were isolated from the same lagoon samples from which the phages were isolated. Bacterial hosts were *Salmonella* isolates R04, R05, and R21 representing serotypes Enteritidis and Typhimurium and lagoon isolates of *Citrobacter youngae*, *Escherichia coli*, *E. fergusonii*, *Proteus mirabilis*, *Providencia rettgeri*, and *Serratia marcescens*. Phage lysates were transferred into separate wells of 96-well sterile culture plates. A 48-pin replicator was used to transfer 5-μL droplets of the phage lysates from the 96-well plate to the surface of freshly prepared DAL TSA plates containing top agars inoculated from fresh TSB cultures of the respective bacterial test hosts. The stainless steel tips of the replicator were flame-sterilized and air-cooled between transfers. Inoculated plates were allowed to air-dry for a few minutes in a biological safety cabinet, then plates were inverted and incubated overnight at 35°C. Plaque formation, indicating a susceptible host for the respective phage, was assessed after 12 h.

Electron Microscopy

Phage lysates from 12 enrichment protocol isolates were stained with uranyl acetate and examined in a transmission electron microscope to determine the morphologies of these phages. Additional samples of nonselected, clarified, and concentrated lagoon effluent were also examined for the presence of phages. These "whole" lagoon samples were clarified by centrifugation (15 000 × *g* 30 min 5°C followed by 27 000 × *g* 30 min 5°C). Clarified effluent was either treated with chloroform to 2.5% and filtered (0.45 μm followed by 0.22 μm) (CCF), or filtered (0.45 μm followed by 0.22 μm) without chloroform treatment (CF). Portions of the CF and CCF effluents were subsequently treated with PEG (7% PEG 8000 with 0.5 *M* NaCl) to concentrate phages. The PEG was added and mixed thoroughly and effluents held at 5°C 1 h, then PEG precipitates were collected by centrifugation (27 000 × *g* 30 min at 5°C), resuspended in sterile distilled water, and stored at 5°C. Preparations from the above clarified, filtered, and PEG-concentrated effluent (CF/PEG) and clarified, chloroform-treated, filtered, and PEG-concentrated effluent (CCF/PEG) were examined by electron microscopy.

RESULTS AND DISCUSSION

Collection and Isolation of Phages

Sixty-six single-plaque phage cultures were isolated in 2002 and 2003 representing both Lowndes County lagoons and all three *Salmonella* hosts used in the enrichment protocol (Table 2). Sixty-six additional single-plaque phage cultures were isolated in 2004 by the direct protocol, which showed *Salmonella* isolates R36 (ATCC 13311) and R47 (BAA-712) to be more susceptible to effluent phages than the other strains (Table 3). In 2004 phages were replicated from all *Salmonella* host serotypes except Gaminara (R46). Phage titers among the lagoons were estimated by the direct isolation protocol using phage recovery data from R36 tests, which ranged from 12 to 148 pfu per lagoon. Data from this isolate were used because of the relatively greater susceptibility of R36 and its positive response in picking up phages across all seven Clay County lagoons (Table 3). By comparison, R47, the other *Salmonella* isolate to show positive phage tests from all seven Clay County lagoons, detected from 1 to 46 pfu per plate (Table 3). Although only a few *Salmonella* serovars were used in these tests, their relative ranking for incidence of *Salmonella* phages, which may indicate the relative incidence of the respective *Salmonella* serovars, was Typhimurium > Michigan > Enteritidis > Agona > Mondivideo. No phages were picked up by Gaminara. This ranking is interesting, but may not be representative of the actual serotype distributions in the lagoons, given the facts that considerable variation in phage detection was noted among the Typhimurium isolates and that other serovars were represented by only one or two isolates. For such a comparison to be meaningful and to examine the use of phages as surrogate indicators, this approach should be tested in future research using a much larger panel of serovars in studies comparing concurrent *Salmonella* and phage isolations.

Host Range Tests

Phage isolates from the enrichment protocol produced plaques on each of the three *Salmonella* strains used in the enrichment, but not against strains of other bacterial species isolated from the same lagoons. Bacterial hosts were *Salmonella* isolates R04, R05, and R21 representing serotypes Enteritidis and Typhimurium and nonhosts were lagoon isolates of *C. youngae*, *E. coli*, *E. fergusonii*, *P. mirabilis*, *P. rettgeri*, and *S. marcescens* (Table 4). It should be noted that some of the phage generated by the enrichment protocol may

Table 2. Host range reactions of enriched phage isolates with *Salmonella* isolates and lysate titers with enriching strains.

	Isolation host‡ titer (−log 10)			Test host reactions§											
Phage†	**R04**	**R05**	**R21**	**R36**	**R37**	**R38**	**R39**	**R40**	**R41**	**R42**	**R43**	**R44**	**R45**	**R46**	**R47**
***1* (EM)¶**	***3c***	**5c**	**4c**	+t	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−
2	***5c***	**4c**	**3c**	+c	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−/+ tc
3	***3c***	**4c**	**4c**	+c	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−
4	***4c***	**5c**	**4c**	+c	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−/+ tc
5	***4c***	**5c**	**5c**	+c	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−/+ tc
***6* (EM)**	**4c**	***5c***	**4c**	±t	+c	+c	±c	−/+tc	+c	±t	+c	−	−	−	−
7	**7c**	***4c***	**4c**	+t	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−
8	**2**	***3***	**2**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**
9	**4c**	***3c***	**3c**	±t	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−
10	**4**	***4***	**3**	±	+	+	+	−	+	±	+	−	−	−	−
***11* (EM)**	**5c**	**5c**	***4c***	+t	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−/+ tc
12	**6c**	**4c**	***4c***	+t	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−
13	**4c**	**4c**	***4c***	+c	+c	+c	+c	±tc	+c	+t	+c	−	−	−	−
14	**4c**	**4c**	***4c***	±c	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−
15	**3c**	**2c**	***3c***	±c	±c	+c	±nd	−	±c	−t	±c	−	−	−	−
16 (EM)	***5c***	**5c**	**3c**	+t	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−/+ tc
17	***5c***	**5c**	**4c**	t	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−
18	***5c***	**5c**	**4c**	+c	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−/+ tc
19	***4c***	**4c**	**3c**	±t	+c	+c	+c	−/+tc	+c	±t	+c	−	−	−	−
20	***4c***	**4c**	**3c**	+c	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−
21 (EM)	**3t**	***4c***	**3c**	±	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−
22	**4t**	***3c***	**3c**	±c	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−
23	**4c**	***2c***	**3c**	±t	+c	+c	±c	±tc	+c	−t	+c	−	−	−	−
24	**4t**	***4c***	**3c**	t	+c	+t	+c	±tc	+c	−/t+	+c	−	−	−	−
25	**4c**	***4c***	**3c**	±t	+c	+c	±c	−/+tc	+c	−/t+	+c	−	−	−	−
26	**5t**	**5c**	***4c***	+c	+c	+c	+c	±tc	+c	±tc	+c	−	−	−	−/+ tc
27 (EM)	**3t**	**3c**	***3c***	±t	±c	+c	+c	±tc	+c	−/+t	+c	−	−	−	−
28	**5t**	**4c**	***4c***	+c	+c	+c	+c	+tc	+c	±t	+c	−	−	−	−/+ tc
29	**5t**	**3c**	***3c***	±t	+c	+c	+t	−/+tc	+c	±t	+c	−	−	−	−
30	**8t**	**5c**	***4c***	+t	+c	+c	+c	±tc	+c	±tc	+c	−	−	−	−/+ tc
***31* (EM)**	***6t***	**4c**	**4c**	+t	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−/+ tc
32	***5t***	**5c**	**4c**	+t	+c	+c	+c	±tc	+c	±tc	+c	−	−	−	−
33	***6t***	**6c**	**4c**	+t	+c	+c	+c	±tc	+c	±tc	+c	−	−	−	−
34	***6t***	**5c**	**4c**	+t	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−
35	***5t***	**5c**	**4c**	+t	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−
36	***5t***	**6c**	**4c**	+t	+c	+c	+t	±tc	+c	±t	+c	−	−	−	−
37	***5c***	**6c**	**4c**	+t	+c	+c	+t	±tc	+c	±tc	+c	−	−	−	−
38	***6c***	**4c**	**4c**	+t	+c	+c	+t	±tc	+c	±tc	+c	−	−	−	−
39	***5c***	**4c**	**4c**	+t	+c	+c	+t	±tc	+c	±tc	+c	−	−	−	−
40	***7c***	**5c**	**4c**	+t	+c	+c	+t	±tc	+c	±t	+c	−	−	−	−
41	***5c***	**4c**	**4c**	+t	+c	+c	+t	±tc	+c	±t	+c	−	−	−	−
42 (EM)	***7c***	**6c**	**4c**	+t	+c	+c	+c	−/+tc	+c	±t	+c	−	−	−	−
***43* (EM)**	**5t**	***5c***	**4t**	±t	+c	+t	+c	±tc	+c	±t	+c	−	−	−	−
44	**4t**	***5c***	**4t**	+t	+c	+t	+c	±tc	+c	±t	+c	−	−	−	−
45	**5c**	***6c***	**4t**	±t	+c	+t	+c	±tc	+c	±t	+c	−	−	−	−
46	**4t**	***6c***	**3t**	+t	+c	+t	+	±tc	+c	±t	+c	−	−	−	−
47	**5t**	***4c***	**3t**	±t	+c	+t	+	±tc	+c	±t	+c	−	−	−	−
48	**5t**	***4c***	**4t**	±t	+c	+t	+	±tc	+c	±t	+c	−	−	−	−
49	**4c**	***4c***	**4c**	+t	+c	+c	+c	−/+tc	+c	±t	+c	−	−	−	−
50	**4c**	***4c***	**3c**	+c	+c	+c	+c	−/+tc	+c	±t	+c	−	−	−	−
51	**5t**	***4c***	**4t**	±t	+c	+c	+t	−/+	+c	±t	+c	−	−	−	−
52	**4c**	***5***	**4c**	±	+c	+c	+t	−/+	+c	±t	+c	−	−	−	−
53	**4**	***5***	**4**	+	+	+	+	−/+	+	±	+c	−	−	−	−
54 (EM)	**5**	***5***	**4**	±	+	+	+	−/+	+	±	+	−	−	−	−
***55* (EM)**	**5**	**4**	***4***	+	+	+	+	−/+	+	±	+	−	−	−	−
56	**5c**	**4c**	***5c***	+t	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−
57	**6c**	**6c**	***5c***	+t	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−
58	**5t**	**5c**	***5t***	+t	+c	+c	+t	±tc	+c	±t	+c	−	−	−	−
59	**7c**	**5c**	***4c***	+t	+c	+c	+t	±tc	+c	±tc	+c	−	−	−	−
60	**6c**	**5c**	***4c***	+t	+c	+c	+c	±tc	+c	±t	+c	−	−	−	−
61	**7**	**6**	***4***	+	+	+	+	±	+	±	+	−	−	−	−
62	**7**	**6**	***6***	+	+	+	+	±	+	±	+	−	−	−	−
63	**6**	**6**	***4***	+	+	+	+	−/+	+	±	+	−	−	−	−
64	**5**	**6**	***4***	+	+	+	+	±	+	±	+	−	−	−	−
65	**6**	**5**	***4***	+	+	+	+	±	+	±	+	−	−	−	−
66 (EM)	**9**	**6**	***4***	+	+	+	+	±	+	±	+	−	−	−	−

† Isolates in italic type originated from one lagoon and isolates in non-italic type from a second.
‡ Italic type indicates the host from which the phage was originally isolated by single plaque culture.
§ A + sign indicates plaque formation; ± indicates small or diffuse plaque; −/+ indicates a very small plaque; − indicates no plaque formation; c indicates a clear plaque; t indicates a turbid plaque; tc indicates a turbid plaque with a clear center; nd indicates "no description" due to apparent loss of infectivity in storage following isolation.
¶ Isolates examined by electron microscopy.

Table 3. Numbers of phage plaques† on *Salmonella* strains inoculated with samples collected from seven swine effluent lagoons in western Clay County, Mississippi, May 2004.

	Lagoon							
Salmonella isolate	1	2	3	4	5	6	7	Total
R04 Typhimurium				1	1			2
R05 Enteritidis		1				4	1	6
R21 Typhimurium		11				5	4	20
R36 Typhimurium	68	12	118	111	115	142	148	714
R37 Typhimurium				2	1		1	4
R38 Typhimurium	1	3	1				2	7
R39 Typhimurium		1		3			2	6
R40 Typhimurium			1			4		5
R41 Typhimurium			3					3
R42 Agona		1	1					2
R43 Enteritidis		3	1				1	5
R44 Michigan	1	6	5		1		2	15
R45 Montevideo							1	1
R46 Gaminara								0
R47 Typhimurium	31	1	46	29	25	35	24	191
Total	101	39	176	146	143	190	186	981

† Total plaque forming units per isolate and lagoon.

have been induced temperate phages from the host bacterial isolates used for enrichment and not necessarily wild-types from the effluent lagoons. It is also possible that enrichment could have encouraged recombinations between the wild-type lagoon phages and prophages of the enriching hosts. Genetic analysis of enriching hosts for the presence of prophage sequences followed by genetic analysis of enriched phage products would be needed to identify such recombinants.

Table 4. Hosts (underlined) and nonhosts of 66 enriched lagoon phage isolates.

Isolate†	Bacterial identity
L003	*Citrobacter youngae*
L030	*Citrobacter youngae*
L046	*Citrobacter youngae*
L009	*Escherichia coli*
L010	*Escherichia coli*
L012	*Escherichia coli*
L015	*Escherichia coli*
L022	*Escherichia coli*
L024	*Escherichia coli*
L025	*Escherichia coli*
L026	*Escherichia coli*
L028	*Escherichia coli*
L029	*Escherichia coli*
L043	*Escherichia coli*
L044	*Escherichia coli*
L048	*Escherichia coli*
L049	*Escherichia coli*
L052	*Escherichia coli*
L001	*Escherichia fergusonii*
L037	*Proteus mirabilis*
L002	*Providencia rettgeri*
L005	*Providencia rettgeri*
L013	*Salmonella enterica* subsp. *arizonae*
L014	*Salmonella enterica* subsp. *arizonae*
L033	*Salmonella enterica* subsp. *arizonae*
L045	*Salmonella enterica* subsp. *arizonae*
L047	*Salmonella enterica* subsp. *arizonae*
R05	*Salmonella enterica* subsp. *enterica* serovar Enteritidis
R04	*Salmonella enterica* subsp. *enterica* serovar Typhimurium
R21	*Salmonella enterica* subsp. *enterica* serovar Typhimurium
L035	*Salmonella* spp.
L008	*Serratia marcescens*

† L (lagoon) isolates were selected from the same swine effluent lagoons from which the 66 phages were isolated. None of the 66 phages infected any of the L isolates. All of the 66 phages infected all three of the R isolates of *Salmonella*, which had been used in the enrichment and selection process from which the 66 phages were collected.

Electron Microscopy

Phage lysates from 12 enrichment protocol isolates all contained identical appearing *Podoviridae* phages with tailless icosahedral heads about 50 nm across and tail spikes (Fig. 1). Samples of clarified concentrated "whole" effluents contained both tailed and *Podoviridae* phages (Fig. 2), but particle counts showed more tailless than tailed phages. Total numbers of tailless and tailed phages counted on 11 grids were 61 (range = 1 to 10) and 11 (range = 0 to 4), respectively, from clarified chloroform-treated samples after filtration and PEG concentration. Total numbers of tailless and tailed phages counted on 10 grids from clarified samples after filtration and PEG concentration without chloroform treatment were 18 (range = 0 to 9) and 0, respectively. These results demonstrated that other phage morphology types were present in the lagoons, but *Podoviridae* phages like the *Salmonella* isolates predominated. Use of chloroform in the isolation protocols may have influenced, or perhaps skewed the results, as many phages are inactivated by chloroform treatment. In the samples examined here, however, filtration without chloroform treatment significantly reduced the grid counts of tailless phages and eliminated tailed phages.

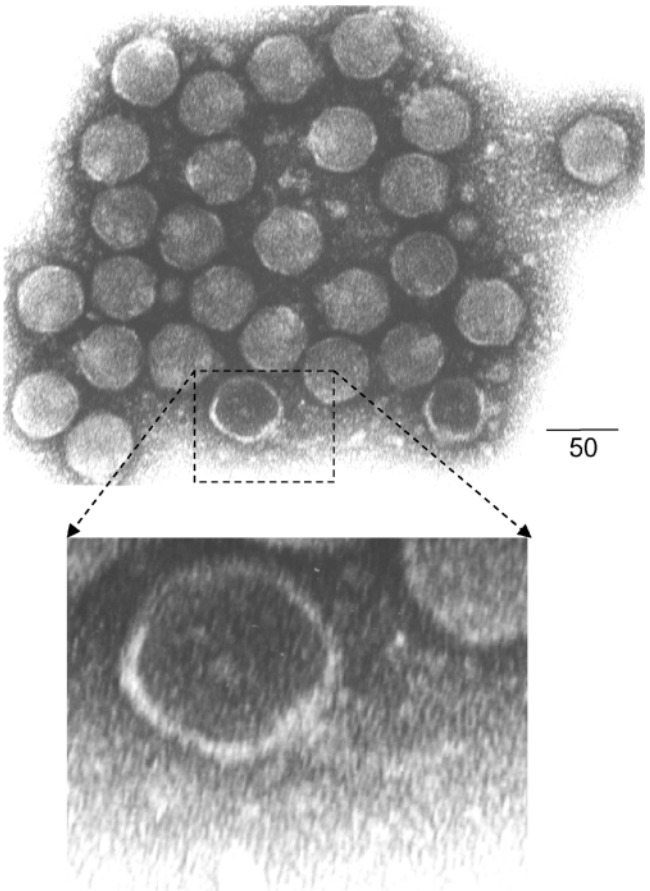

Fig. 1. Electron micrograph and enlargement of the Podoviridae phages typically found among the enriched *Salmonella* phages in this study. Bar = 50 nm.

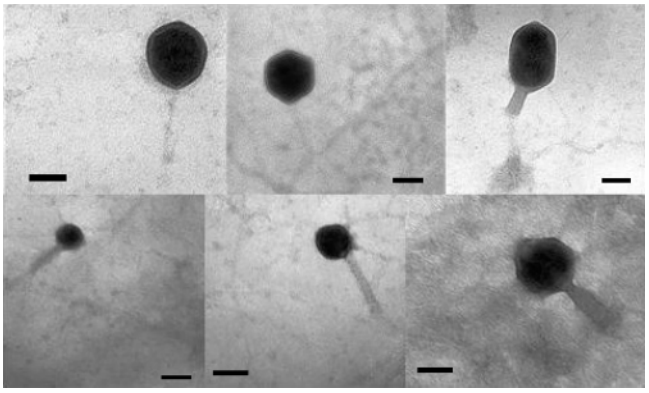

Fig. 2. Bacteriophages found in electron microscopic observations of effluent from swine lagoons. Bars = 50 nm.

Future research with these phages should complete their characterization, determine their relationships to each other, and produce tools, such as specific antibodies or nucleic acid probes for these comparisons. Such tools could also be applied in tracking the phages in the environment and assessing their ecological relationships with *Salmonella*. Further characterization and development of the phages will facilitate their use as typing reagents, specific indicators, and biocontrol agents of *Salmonella*.

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