

Characterization of *Salmonella* Bacteriophages Isolated from Swine Lagoon Effluent

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Abstract Four *Salmonella* bacteriophages that had been originally isolated from swine manure lagoons were characterized and compared to each other and to well-known *Salmonella* phages P22 and Felix 01. Host ranges of the lagoon phages were similar to each other in spot tests on reference strains of *Salmonella*, but differed slightly from each other on a panel of *Salmonella* lagoon strains. In single-step growth at 35°C the lagoon phages had latent periods of 15 to 20 min and burst sizes from 100 to 230. The lagoon phages and P22 were purified by cesium chloride (CsCl) gradient centrifugation and used to produce specific antisera and DNA. The lagoon phages were indistinguishable from each other but distinct from P22 and Felix 01 in immunodiffusion and infectivity neutralization tests and in restriction digest analysis.

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Introduction

Bacteriophages are common in aqueous environments [6] and, because of their host specificity, have been exploited in a variety of practical applications. Phages have been used as indicators of bacteria and bacterial (manure) contamination [17, 18, 23] and as typing reagents [3, 10, 33]. Phages have been used recently for targeted biological control of human, animal, and plant bacterial diseases [2, 4, 15, 16, 19, 28], but their potential in these areas remains largely unexplored. Phages have been applied in food safety to reduce *Salmonella* in chickens [31], on chicken skin [11], sprout seeds [27], and fresh-cut fruit [19], and in cheese production [25]. The U.S. Food and Drug Administration recently approved phage treatment to control *Listeria* on meat and poultry products (http://www.intralytix.com/Intral_News_PR081906.htm). Phages have been proposed as biocontrol agents in food safety [12, 14], phage therapy [5, 29, 30], and wastewater treatment [34].

Salmonella-specific phages recently isolated from swine manure lagoons have been proposed for *Salmonella* biocontrol [22]. Characterization of their unique biological properties is prerequisite to exploring that potential [7]. The objectives of the research reported here were to extend the characterization of selected *Salmonella* swine lagoon phages and to compare these lagoon isolates with each other and with the well-characterized *Salmonella* phages, P22 and Felix 01.

Materials and Methods

Salmonella Cultures

Reference *Salmonella* isolates were obtained from the American Type Culture Collection (ATCC). These included

S. enterica subsp. *enterica* (ex Kauffman and Edwards) Le Minor and Popoff serovar Typhimurium isolates ATCC 14028, 43971, 13311, and 19585, referred to here as R04, R21, R36, and R48, respectively. Swine lagoon isolates of *Salmonella* (S isolates listed in Table 1) were collected in May 2004 from five lagoons in Clay County, Mississippi, and identified based on cultural characteristics, API profiles (bioMérieux Vitek, Inc., Hazelwood, MO), and PCRs with *Salmonella*-specific (*inv A*) primers.

Phage Isolates

Four *Salmonella*-specific phages were obtained from a collection of phages isolated from two swine lagoons in Lowndes County, Mississippi, in September 2002 [22]. Listed as phages 1, 11, 16, and 26 in the earlier work [22], the phages are referred to here with the additional prefix “P” (for phage) and “R” (according to the respective *Salmonella* reference host in which they were isolated and propagated) as PR04-1, PR04-16, PR21-11, and PR21-26. These phages were selected because they included one phage from each of the two lagoons for each of the isolation hosts, R04 and R21. Isolates of the well-characterized *Salmonella* reference phages, P22 and Felix 01, were obtained from the ATCC and Felix d’Herelle Collection (Laval University, Quebec, Canada), respectively. P22 was propagated in R48 and Felix 01 in R36.

Phage Lysate Production

Phage lysates were prepared by inoculation of exponential growth phase broth cultures of the appropriate *Salmonella*

host strain as described [21]. Lysates were clarified by centrifugation (5000g, 10 min at 5°C), decanted, and filtered through 0.45- μ m cellulose acetate filters into sterile glass or polypropylene vials. The filtered lysates were treated with chloroform (to a 2.5% final concentration), stored at 5°C, and titered by double-agar-layer plaque assay [1]. Lysates were diluted in SM buffer (0.01 M MgSO₄; 0.1 M NaCl; 0.05 M Tris, pH 7.5) to appropriate levels for each experiment.

Plaque Assay and Spot Test

Phages were detected and enumerated by mixing 0.1 ml of test sample in aqueous medium with 0.1 ml of fresh exponential growth phase *Salmonella* host culture in TSB into 5.0 ml of soft TSA (0.75% agar), which was previously melted and tempered 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 (1.5% agar) 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. Plaque forming units (pfu) were counted 4–15 h later [1]. Unless indicated otherwise, all plaque assays used the respective *Salmonella* propagation hosts as lawns for the different phages.

Spot tests were conducted as described [21]. Briefly, phage lysates diluted in SM were transferred from 96-well microplates with a 48-pin replicator and spotted onto the surface of freshly prepared *Salmonella* host lawns in TSA plates. Plates were incubated overnight at 35°C and observed for plaque formation.

Table 1 Spot test responses of *Salmonella* reference strains (R) and lagoon isolates (S) to *Salmonella* phages

Phage ^b	Titer (pfu/ml)	Spot test reactions of <i>Salmonella</i> isolates ^a							
		R04 6704752 ^c	R21 6704752	S1 4704752	S8 6704552	S16 6704752	S21 6705552	S23 6705752	S24 6714752
PR04-1	9.3×10^9	‡	‡	±	—	—	—	—	—
PR04-16	1.8×10^{10}	‡	‡	±	—	—	—	—	—
PR21-11	9.3×10^9	‡	‡	±	—	±	—	—	±
PR21-26	1.4×10^{10}	‡	‡	±	—	—	—	—	—
P22	2.0×10^8	+	‡	+	—	—	—	—	—
Felix 01	5.4×10^8	—	—	±	—	—	—	—	—
Control	TSB	—	—	—	—	—	—	—	—

^a R04, ATCC 14028; R21, ATCC 43971; S isolates, representative *Salmonella* isolates from swine lagoon effluent. Spot test reactions: (‡) large clear spot 2–3 times the diameter of the inoculated area; (+) clear spot >1 but <2 times the diameter of the inoculated area; (±) small clear spot(s) within the inoculated area; (—) no spot formation

^b Three replicate samples of each phage lysate inoculum in TSB were spotted on test lawns with a stainless-steel pin replicator and plates were incubated overnight at 35°C

^c Biochemical profile, bioMérieux Vitek, Inc., Hazelwood, MO

Single-Step Growth

Single-step growth procedures were followed as described [8], using only the respective isolation/propagation host strains: PR04-1 and PR04-16 in R04 and for PR21-11 and PR21-26 in R21. Turbid exponential growth phase *Salmonella* cultures were inoculated with phage and aliquots were diluted for incubation and subsequent phage assay. All incubations were at 35°C. Samples taken at different times postinfection were tested for phage using the respective host strain. Duplicate test samples were plated for each time interval and data means were analyzed and plotted using Excel (Microsoft Corp., Redmond, WA). Remnants of the original undiluted phage-inoculated turbid *Salmonella* host cell cultures were held at 35°C for observation of phage clearing.

Phage Purification

Phages were inoculated from stocks into fresh TSB *Salmonella* host cultures in exponential growth phase at 35°C. Inoculated cultures were observed for clearing, which signaled phage lysis. Cleared cultures were treated with chloroform to a 2.5% final volume, shaken vigorously to mix, and held overnight at 4°C. Supernatants containing phages were decanted and phages were precipitated with PEG (7% PEG 8000 and 0.03 M NaCl, final concentrations) for 4–5 h at 5°C. Phage precipitates were collected by centrifugation (5000g, 30 min, 5°C), resuspended in SM, and centrifuged to equilibrium overnight in CsCl ($d = 1.75$). Phage was removed from CsCl gradients by puncturing the side walls of the centrifuge tubes and withdrawing the single phage band with a 26-gauge hypodermic needle and 3-ml syringe. Preparations were cleared of CsCl by membrane filter centrifugation and repeated washing with SM or by exhaustive dialysis in SM. The UV absorbance profiles were determined for each purified and concentrated phage prep and phage nucleoprotein concentration estimates calculated ($1 \text{ mg/ml} = 10_{A_{260\text{nm}}}$). Purified phage stocks were stored at 5°C.

Antiserum Production

Phage-specific polyclonal antisera were produced in young adult female New Zealand White rabbits following a series of intramuscular and subcutaneous immunization injections with phages PR04-1, PR04-16, PR21-11, PR21-26, and P22. Purified phage in SM was periodically emulsified and injected with an equal volume of Freund's adjuvant over the course of several months (complete adjuvant was used for initial injections only and incomplete adjuvant for

subsequent injections). Immune sera were collected, separated from cellular fractions by centrifugation, mixed with an equal volume of glycerol, and stored at -15°C . Respective preimmune sera were similarly processed and stored.

Serological Tests

Phage antisera from each serum collection were titered by microprecipitin tests in 96-well plates. Relationships among the phages were determined by immunodiffusion tests in 0.8% (w/v) agarose gels prepared with 20 ml of gel suspension in the bottom of a standard 96-mm-diameter petri plate. Reactant wells 6 mm in diameter and 4 mm apart were cut in the gel using a No. 2 cork borer, and the agarose plugs removed. Purified phage preparations of PR04-1, PR04-16, PR21-11, and PR21-26 were placed in central wells and surrounded by the respective homologous and heterologous antisera in the peripheral wells. Formation of precipitin lines was recorded after 2–3 days of incubation in a moist chamber at room temperature. Antibody neutralization of phage infectivity was conducted by mixing 0.1 ml of antiserum and 0.1 ml of phage stock in 0.8 ml of PBS and incubating the mixture for 2.5 h at 35°C. Phage and antiserum mixtures were tested for phage infectivity on host R21 by plaque assay as described above.

Restriction Analysis

Phage DNA was isolated and purified from CsCl-purified lysates using the Qiagen Lambda Mini Kit (Qiagen, Valencia, CA). Purified DNAs were digested individually with *EcoRI* and *HindIII* and analyzed by agarose gel electrophoresis alongside commercially available markers (Fermentas Inc., Glen Burnie, MD).

Results and Discussion

Plaque Assay and Spot Test

The time required for plaque formation and the size and morphology of the plaques formed by the lagoon phages were different from P22 and Felix 01 on R21 host lawns. Within 3–4 h the lagoon phages formed large (>1-mm-diameter) clear plaques, but P22 formed only a few small (<1-mm-diameter) plaques during this time and Felix 01 showed no visible plaques after 4 h. After 15 h, the lagoon phage plaques were 2–3 mm in diameter, P22 plaques were about 1 mm in diameter and appeared as turbid circular spots with clear edges, while the Felix 01 plaques were tiny and easily overlooked in casual observation.

In the spot test, the lagoon phages were indistinguishable from each other in R04 and R21 (Table 1). Relatively large spots (confluent lysis) were visible within 4 h of spotting. Phage P22 also grew on these host strains, but P22 growth on R04 was slower and spots were smaller than in R21. The lagoon phages and P22 also grew similarly on lagoon isolate S1, the only *Salmonella* lagoon strain to support growth of Felix 01, considered a specific reagent for *Salmonella* because it lyses a very high percentage of strains [17, 18]. With the exception of PR21-11, which grew poorly on lagoon host strains S16 and S24, none of the phages grew on the other lagoon isolates of *Salmonella*. Long term coculture of phages and their host bacteria, both in the laboratory and in nature, can result in the generation or selection of phage-resistant bacterial strains [9, 24, 26]. Serial selection of phages for inclusion in biocontrol cocktails has been proposed to overcome the development of phage resistance [32]. Resistance to phages, however, may come at the cost of reduced fitness for the bacteria. Phage-resistant host populations may be less competitive than their phage-sensitive predecessors and thus show population decline as a secondary effect and not from direct phage lysis [13, 20]. This phenomenon may be important in selecting phages for biocontrol of *Salmonella* in lagoons.

Single-Step Growth Curves

Growth of the four lagoon phages was similar, but not identical, owing in part to differences between host R04 (Figs. 1A and B) and host R21 (Figs. 1C and D). Latent periods were 20 min or less for all four phages and all growth was completed by 45 min. Burst sizes ranged from 100 for PR04-1 to 230 for PR21-11. Average burst size was greater in R21 than R04. Multiplicity of infection (MOI) ranged from 0.01 to 0.02. Additionally, all of the remnant phage-inoculated turbid *Salmonella* cultures (from which the dilutions had been made for the single-step growth experiments) were observed to clear between 45 and 50 min after inoculation. These observations were consistent with the plaque assay results (relatively rapid formation of plaques that continued to enlarge with time) and demonstrated that the lagoon phages have the potential to rapidly reduce *Salmonella* populations.

Serological Tests

Antisera specific to all four lagoon phages formed continuous precipitin lines in agarose gel immunodiffusion tests with the purified lagoon phages. Although the intensity of precipitin lines varied with the specific antiserum and

Fig. 1 Single-step growth of *Salmonella* phages in their respective hosts at 35°C: (□) spontaneously released phage; (◇) phage released by chloroform lysis. (A) Phage PR04-1 with *Salmonella* host isolate ATCC 14028, MOI = 0.014; (B) phage PR04-16 with *Salmonella* host isolate ATCC 14028, MOI = 0.017; (C) phage PR21-11 with *Salmonella* host isolate ATCC 43971, MOI = 0.011; (D) phage PR21-26 with *Salmonella* host isolate ATCC 43971, MOI = 0.022

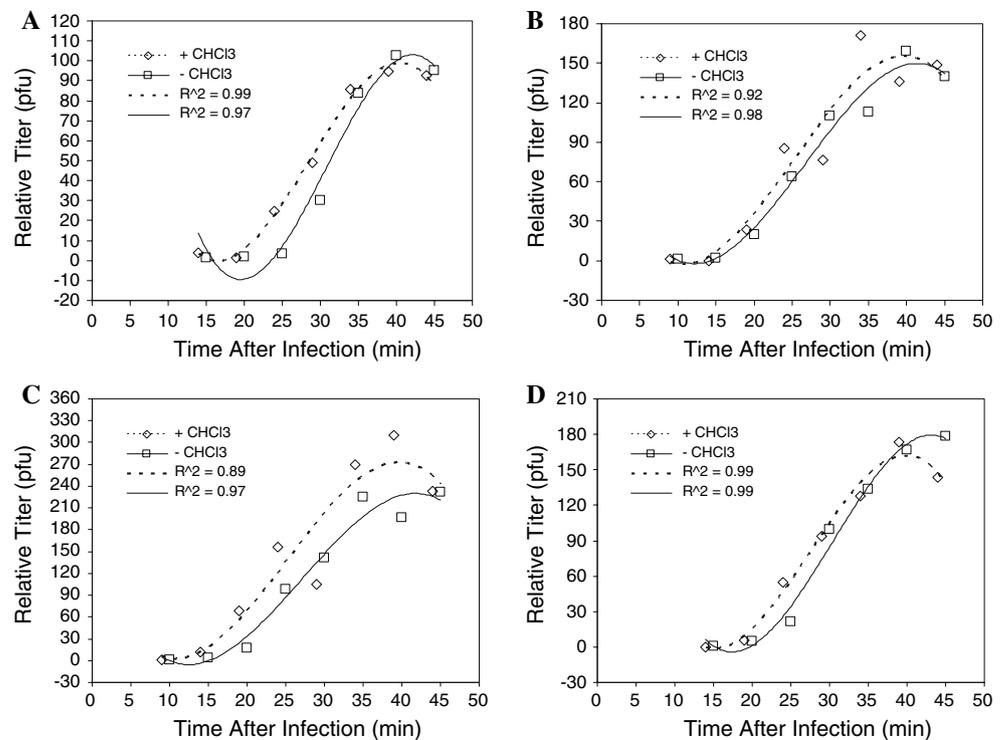


Table 2 Infectivity neutralization of *Salmonella* phages by *Salmonella* phage antisera

Phage	Phage plaque count (pfu/plate) ^a						
	Antiphage serum					Pre- immune	PBS
	aPR04-1	aPR04-16	aPR21-11	aPR21-26	aP22	serum controls	buffer control
PR04-1	0	0	0	0	110	94	72
PR04-16	0	0	0	0	81	81	73
PR21-11	0	0	0	0	105	86	62
PR21-26	0	0	0	0	63	98	61
P22	112	132	84	134	0	108	125
Felix 01	190	190	145	149	167	175	116

^a Means from two replicate plates

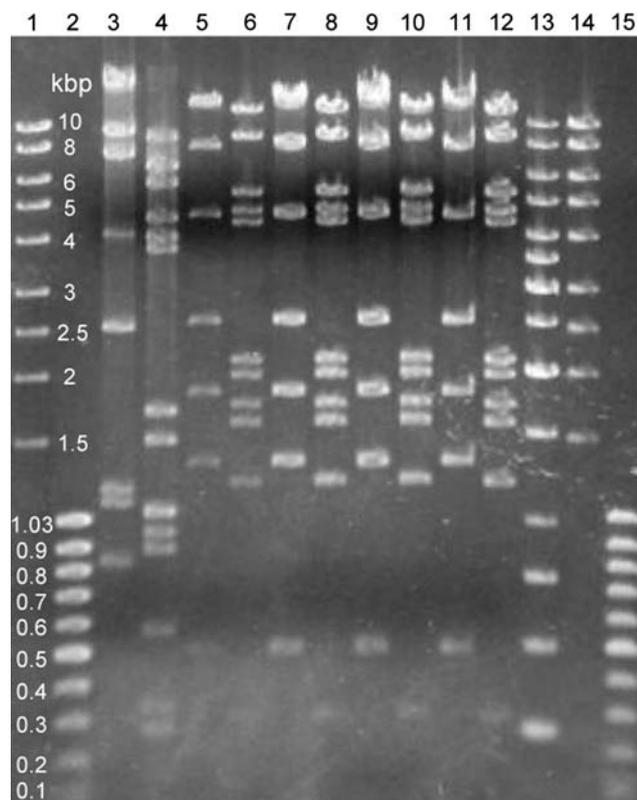


Fig. 2 Restriction enzyme digests of *Salmonella* phage DNA. Lanes 1 and 14, Fermentas high-range marker; Lanes 2 and 15, Fermentas low-range marker; Lane 3, P22 *EcoRI* digest; Lane 4, P22 *HindIII* digest; Lane 5, PR04-1 *EcoRI* digest; Lane 6, PR04-1 *HindIII* digest; Lane 7, PR04-16 *EcoRI* digest; Lane 8, PR04-16 *HindIII* digest; Lane 9, PR21-11 *EcoRI* digest; Lane 10, PR21-11 *HindIII* digest; Lane 11, PR21-26 *EcoRI* digest; Lane 12, PR21-26 *HindIII* digest; Lane 13, Fermentas 1-kb marker

phage titers, there were no qualitative differences among the four antisera and the four phages; cross-reactivity was observed in each case. Antiserum to P22 did not react with the lagoon phages, nor did the lagoon phage antiserum react with P22. Earlier work by McLaughlin et al. [22] showed that the lagoon phages belonged to the same family

as P22 (Podoviridae), and the results reported here show that the lagoon phages are different from P22.

Results of infectivity neutralization tests with the anti-phage antisera confirmed these observations. Treatment with antiserum to any of the four lagoon phages completely inactivated all of the lagoon phages but did not affect P22 or Felix 01 (Table 2). In the reciprocal test combinations, antiserum to P22 completely eliminated infectivity in P22 but had no effect on the lagoon phages or Felix 01. Treatment with preimmune serum did not affect phage infectivity.

Restriction Analysis

Respective restriction digest profiles of the four lagoon phages were indistinguishable from each other but distinct from P22 (Fig. 2). These results are consistent with those of the plaque assays, spot tests, and serological tests.

Lytic *Salmonella* lagoon phages PR04-1, PR04-16, PR21-11, and PR21-26 exhibited characteristics favorable for use in biocontrol, specifically rapid lysis of and reduction of *Salmonella* populations and burst sizes that yielded at least two log₁₀ increases in progeny phage. A characteristic unfavorable for use in *Salmonella* biocontrol, at least in these lagoon environments, was failure to lyse a panel of *Salmonella* lagoon isolates (Table 1). Based on the results of host range tests, serological tests, restriction analysis, growth curves, and plaque formation and morphology, we conclude that the four lagoon phages characterized in this study are isolates or strains of the same phage, but distinct from *Salmonella* phages P22 and Felix 01.

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