Suppression of Salmonella Growth by Wild-Type and Large-Plaque Variants of Bacteriophage Felix O1 in Liquid Culture and on Chicken Frankfurters

JEAN M. WHICHARD,† NAMALWAR SRIRANGANATHAN, AND F. WILLIAM PIERNER*

Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, Virginia 24061-0342, USA

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ABSTRACT

The bacteriophage Felix O1, a member of Myoviridae, is specific for, and possesses a broad host range within, the genus Salmonella. This work explores a Felix O1 phage-based intervention for Salmonella enterica serotype Typhimurium DT104 that is potentially applicable at several stages of animal production and processing. A variant of Felix O1 was obtained that produces a larger, clearer plaque phenotype (LP) on Salmonella Typhi than wild-type Felix O1 (WT) does, not unlike r mutants of phage T4. LP exhibited slightly more extensive overall suppression of Salmonella Typhi in brain heart infusion (BHI) broth, as ascertained on the basis of culture turbidity (optical density at 600 nm). Both phage variants suppressed log phase BHI broth cultures containing $8.2 \times 10^6$ CFU of Salmonella Typhimurium DT104 per ml. A PFU/CFU ratio of 1.0 was effective for WT and LP, whereas increasing the PFU/CFU ratio to 5.0 did not increase suppression. Untreated Salmonella-contaminated frankfurters were compared with treated samples (PFU/CFU ratio, $1.9 \times 10^4$) to test WT and LP for their ability to suppress Salmonella growth on chicken frankfurters contaminated with 300 CFU of Salmonella Typhimurium DT104. Suppression levels of 1.8 and 2.1 log units were achieved with WT and LP, respectively ($P = 0.0001$), but no difference was found between the performances of the two variants ($P = 0.5088$).

In the interest of enhancing the safety of the food supply, researchers continue to pursue novel phage-based interventions for the reduction of bacterial hazards in meats and other foods (6, 15, 16, 20, 21). Some recent studies have addressed Salmonella contamination of ready-to-eat foods such as fruit and cheese (15, 16), whereas others have approached Salmonella contamination of poultry products by way of preharvest feeding trials involving mixtures of several phages (21). Phage cocktails can be useful in reducing the emergence of phage-resistant subpopulations and in maintaining efficacy against many different serotypes (15, 21). Phage host range is another potential consideration in the design of a versatile intervention.

Bacteriophage Felix O1 (also called phage O1, O1, or O-1) is a member of the A1 group of the Myoviridae (2). This bacteriophage was first referenced by Felix and Callow (5) and was the anti-O phage used in the original scheme for the identification and typing of Salmonella Typhi. Felix O1 is fairly unique among Salmonella bacteriophages by virtue of its broad host range within, and its specificity for, the genus. Of 15 serogroups tested in one study, all but 2 included members that could be productively infected by this phage (11). Furthermore, Kallings (11) found that in general, other gram-negative enteric bacteria are resistant to lysis by Felix O1. Generally speaking, Felix O1 infects smooth strains of Salmonella, whereas many rough isolates are resistant to it (10, 17). Smooth strains are typically more virulent than rough ones. However, susceptibility to Felix O1 is determined by the steric availability of the terminal N-acetylglucosamine in the lipopolysaccharide core, and smooth strains resistant to phage O1 have been identified (10, 17).

Salmonella Typhimurium DT104 is an important pathogen that is frequently resistant to several drugs and has been isolated from several food animal production systems, including poultry (4). Foods of animal origin are considered an important source of human disease caused by this pathogen (4). Felix O1 productively infects a poultry field isolate of Salmonella Typhimurium DT104, and the titer of a given phage suspension propagated on this organism is comparable to ($<1.0$ log lower than) that obtained on Salmonella Typhi phage type Tannanarive, the strain used for propagating phage O1 in this study. Because of Salmonella Typhimurium DT104’s susceptibility to Felix O1, we thought that this phage might be useful in a biological intervention for this important human pathogen.

Although chemical mutagens such as hydroxylamine can be used to produce mutant phages (21), mutations may also arise spontaneously. In an unrelated experiment, serial in vivo passage of Felix O1 wild-type (WT) in mice resulted in the fortuitous isolation of a variant of Felix O1 that produces a larger, clearer plaque on Salmonella Typhi

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*Author for correspondence. Tel: 540-231-4529; Fax: 540-231-3426; E-mail: pierson@vt.edu.†Present address: U.S. Department of Health and Human Services, Centers for Disease Control, National Center for Infectious Diseases, Division of Bacterial and Mycotic Diseases, Foodborne and Diarrheal Diseases Branch, National Antimicrobial Resistance Monitoring System, Mail Stop G29, 1600 Clifton Road, Atlanta, GA 30333, USA.
than that produced by WT. We have designated this variant LP (large plaque). The LP variant exhibits the same plaque phenotype as \( r \) (rapid-lysis) mutants of phage T4 do (9). T4 particles mutant in \( r \) genes are free of the mechanism known as lysis inhibition, a delay of host cell lysis that occurs when the multiplicity of infection is high or when superinfection by other phages occurs (11). It is possible that LP is an \( r \) mutant of some kind, potentially free of the lysis inhibition mechanism. An ideal phage-based anti-

Salmonella intervention agent would have predictable infection and lysis properties regardless of the PFU/CFU ratio. A rapid-lysis variant might be more desirable for a food-processing application, since there may be little practical control over the actual PFU/CFU ratio and the presence of superinfecting phage particles in a processing setting.

The experiments performed in this study included initial observations of the behavior of Felix O1 in liquid Salmonella culture and an applied component addressing the efficacy of this phage in the suppression of Salmonella growth on a further-processed poultry product. In addition to testing the efficacy of Felix O1 WT for the suppression of Salmonella growth, we studied the comparative efficacies of WT and LP in a broth system as well as on chicken frankfurters. This study was designed to exploit Felix O1 for its bactericidal abilities and to determine the comparative performances of WT and LP variants in the suppression of Salmonella growth on a homogeneous poultry product.

**MATERIALS AND METHODS**

**Bacteriophages and bacterial strains.** For the experiments, Felix O1 WT was amplified from a clonal derivative of a stock obtained from Dr. Hans Ackermann, Université Laval, Quebec, Canada. The LP variant was obtained fortuitously in an unrelated experiment that involved in vivo serial passage of WT in mice. The methods of Merrill et al. (19) were used with modification. Briefly, mice were injected intraperitoneally (i.p.) with a high-titer stock of Felix O1, and phage was propagated from plasma and spleens collected at 24 h for use in the subsequent i.p. injection. The LP variant was isolated from the spleen of a mouse after the eighth passage. WT and LP were propagated on Trypticase soy agar (Difco, Becton-Dickinson, Sparks, Md.) containing 0.5 mM CaCl_2 (TSA-CaCl_2) with a soft agar overlay (3); Salmonella Typhi phage type Tannanarie was used as the bacterial host. Phage suspensions were prepared by harvesting 30 Felix O1 plaques from propagation plates, eluting them in 3 ml of tryptone peptone (TP) broth (Difco), and carrying out chloroform treatment. The titers of the suspensions were determined by counting plaques from serial 10-fold dilutions of suspensions applied to quadrants of TSA-CaCl_2 on which 100 \( \mu \)l of a 0.5 McFarland suspension of Salmonella Typhi had been spread. Plates were incubated overnight at 37°C. The concentration of each suspension was adjusted by dilution with the appropriate volume of TP broth. The Salmonella Typhimurium DT104 used in these experiments was a turkey isolate obtained in Virginia. The phage type was determined at the National Veterinary Services Laboratories in Ames, Iowa. This strain was stored at \(-80^\circ\)C in LB containing 20% glycerol. Following subculture of the freezer stock onto TSA-CaCl_2, the isolate was grown in brain heart infusion (BHI) broth (Difco) for use in the spectrophotometric comparison of Felix O1 WT and Felix O1 LP and for use in the frankfurter contamination and treatment experiments.

**TABLE 1. Group assignments for frankfurter experiments**

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculum</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.85% NaCl</td>
<td>TP broth</td>
</tr>
<tr>
<td>2</td>
<td>Salmonella Typhimurium DT104</td>
<td>Felix O1 WT</td>
</tr>
<tr>
<td>3</td>
<td>Salmonella Typhimurium DT104</td>
<td>Felix O1 WT</td>
</tr>
<tr>
<td>4</td>
<td>0.85% NaCl</td>
<td>Felix O1 WT</td>
</tr>
<tr>
<td>5</td>
<td>Salmonella Typhimurium DT104</td>
<td>Felix O1 LP</td>
</tr>
<tr>
<td>6</td>
<td>0.85% NaCl</td>
<td>Felix O1 LP</td>
</tr>
</tbody>
</table>

*Frankfurters were given either a 0.85% NaCl sham inoculum or approximately 300 CFU of Salmonella Typhimurium DT104 in a volume of 250 \( \mu l \).

*Frankfurters were treated immediately after inoculation with either a TP broth sham or 5.25 \( \times 10^8 \) PFU of Felix O1 WT or LP in a volume of 250 \( \mu l \).

**Spectrophotometric comparison of Felix O1 WT and Felix O1 LP.** Suspensions of WT and LP were prepared as previously described. The day before the experiment, a single colony of Salmonella Typhi or Salmonella Typhimurium DT104 was used to inoculate a tube of BHI broth, which was grown overnight at 37°C without shaking. The overnight culture was diluted 1,000-fold in prewarmed BHI broth and was grown for 3 h in a 36°C water bath with reciprocal shaking (at 200 cycles per min). The 3-h culture was then diluted 20-fold in prewarmed BHI broth, and 5-ml aliquots were dispensed into glass tubes. One milliliter of adjusted phage suspension or TP broth control was added to designated tubes. Tubes were shaken in a reciprocal water bath at 36°C (at 200 cycles per min), and the optical density of samples at 600 nm OD_600 was determined with a Bausch & Lomb Spectronic 20 (Rochester, N.Y.) at 20-min intervals for 4 h. The concentration of bacteria just prior to phage infection was determined so that the actual PFU/CFU ratio could be determined. In each case, data shown represent one repetition of the experiment with a sample size of 2, 3, or 5 for each phage variant.

**Frankfurter contamination and treatment.** Although chicken frankfurters are infrequently associated with Salmonella contamination (6), they were chosen because their homogeneous nature facilitated reproducible results in our applied efficacy studies. Chicken frankfurters from a single lot were purchased from a local grocery store. Frankfurters were removed from packages such that the majority of the liquid in the package was left behind. Cylindrical samples (ca. 10 g each) were excised from frankfurters excluding the ends (five from each), and the samples were placed in separate stomacher bags, which were held closed and refrigerated until the samples were used. These weighed, bagged samples were used within 4 days. Samples were randomly allocated to one of six treatments in a randomized complete block design in which the experimental day was the block (Table 1). Ten blocks of the experiment were performed. On each experimental day, frankfurters were held at room temperature for 30 min to 1 h before they were used.

Briefly, on each experimental day, a culture of Salmonella Typhimurium DT104 was prepared by diluting an overnight BHI broth culture prepared from a single colony 1,000-fold in prewarmed BHI broth and shaking at 36°C in a reciprocal shaker (at 200 cycles per min) for 3 h. The 3-h culture was diluted for use with sterile 0.85% NaCl at room temperature such that each sample would receive approximately 300 CFU in a total inoculum volume of 250 \( \mu l \) (groups 2, 3, and 5). Groups that were not inoculated with Salmonella (groups 1, 4, and 6) were given 250 \( \mu l \) of 0.85% NaCl. Phage suspensions were diluted with TP broth.
such that each treated sample would receive approximately $5.25 \times 10^6$ PFU in a total volume of 250 μl. Achievement of the target bacterial and phage concentrations would mean a PFU/CFU ratio of approximately $1.75 \times 10^4$ for groups 3 and 5. Frankfurters that were not treated with phage were instead given 250 μl of TP broth. In each case, the liquids were added directly to the noncut surface of the frankfurter sample, and the samples were oriented in their stomacher bags such that they would remain in contact with the liquids that were applied. After treatment, the stomacher bags were closed with clips and the samples were incubated at 22°C for 24 h. The frankfurters were then cooled rapidly on ice and homogenized in a Stomacher Lab-Blender 80 (Tekmar, Cincinnati, Ohio). The resulting suspension was immediately strained through two layers of cheesecloth and held on ice pending titration of *Salmonella* and/or phage. Serial dilutions of strained suspensions were plated on XLT4 agar (Difco), and black-centered non-lactose-fermenting colonies that resulted were used to calculate the concentration of *Salmonella* per gram of frankfurter. Phage counts were similarly determined by treating strained suspensions with several drops of chloroform and plating serial dilutions on quadrants of TSA-CaCl$_2$ plates that had been spread with 100 μl of a 0.5 McFarland suspension of *Salmonella Typhimurium* phage type Tannanarive.

Statistical analysis of suppression of BHI broth cultures. The GLM procedure (SAS System, Version 8.0, SAS Institute, Cary, N.C.) was used to compare the suppression of *Salmonella* growth in BHI broth achieved by WT with that achieved by LP. Formulas for expected curves were generated, and the significance of overall differences in means between phage variants was determined.

Statistical analysis of suppression of bacterial growth on poultry frankfurters. Results from a pilot study were used to estimate the sample size necessary in a randomized complete block design to ensure the detection of a 1.0-log difference between the performance of WT and that of LP in the suppression of *Salmonella* growth on frankfurters (power ≥ 80%, α = 0.05, sigma = 0.659; PASS, Version 6.0, NCSS, Kaysville, Utah). This sample size calculation led to the estimate that 15 blocks would be necessary to test the difference in performance between the two variants. That is, 15 samples would be necessary to detect a 0.5-log difference in performance between phage variants. Log-transformed colony counts for treatment groups 2, 3, and 5 were used as response variables, and responses for each treatment across blocks were compared by one-way analysis of variance and Tukey’s post hoc comparison of least-squares means (SAS MIXED Procedure, Version 8.0, SAS Institute). The standard error of the mean for each group was corrected on the basis of the pooled mean square error. After 10 blocks were completed, the experiment was terminated because it was apparent that there was no difference between the performance of WT and that of LP. Residual plots were used to assess the adequacy of the model and the appropriateness of the significance tests performed. Data from the 10 blocks were also used to conduct a retrospective power analysis. This analysis determined the sensitivity of the assay in terms of the smallest difference in performance that could be detected between the two phage variants by the described methods (two-sample t test, PASS, Version 6.0, NCSS).

**RESULTS**

Comparative *Salmonella Typhi* suppression by WT and LP in broth. Spectrophotometric measurements of WT- and LP-infected broth cultures were used to characterize the behavior of WT and LP in liquid culture (7, 14). Figure 1 shows the OD$_{600}$ of *Salmonella Typhi* BHI broth cultures over time following treatment with WT or LP at a PFU/CFU ratio of 0.14. The subtle but reproducible difference in the OD$_{600}$ achieved by LP and that achieved by WT was typical for experiments involving *Salmonella Typhi*, although the biological significance of this difference is unknown. An overall difference of means was found between WT- and LP-treated groups ($P = 0.0001$).

Suppression of *Salmonella Typhimurium* DT104 in broth at various PFU/CFU ratios. The optimal PFU/CFU ratio for culture suppression of *Salmonella Typhimurium* DT104 was determined for WT (Fig. 2A) and LP (Fig. 2B) in broth, with OD$_{600}$ being used as a measure of viability. PFU/CFU ratios of 1.2 were effective in suppressing *Salmonella Typhimurium* DT104 in BHI broth for both of the phage variants tested. A higher PFU/CFU ratio (6.1) was no better than a PFU/CFU ratio of 1.2 for the suppression of culture turbidity for either phage variant.
Comparative *Salmonella* Typhimurium DT104 suppression by WT and LP in broth. The performance of WT on *Salmonella* Typhimurium DT104 was compared with that of LP with PFU/CFU ratios close to 1.0 (Fig. 3). No difference in overall means was found between WT and LP treatments (*P* = 0.9902).

Performance of WT and LP on poultry frankfurters. Although there was no difference between the performance of Felix O1 WT and that of Felix O1 LP on *Salmonella* Typhimurium DT104 in broth, LP was still included in subsequent applied experiments. Pilot studies showed that PFU/CFU ratios higher than those found to be necessary for the broth experiments were necessary to affect *Salmonella* growth on frankfurters. PFU/CFU ratios of ≥100 were able to suppress growth, but the lowest effective frankfurter PFU/CFU ratio was not determined. Figure 4 depicts the abilities of Felix O1 WT and Felix O1 LP to suppress the growth of *Salmonella* Typhimurium DT104 on poultry frankfurters on the basis of 24-h *Salmonella* concentrations for groups 2, 3, and 5. Significantly lower numbers of *Salmonella* were recovered from groups treated with either WT (group 3) or LP (group 5) than from the *Salmonella*-inoculated frankfurters in group 2 (*P* < 0.0001). However, no difference in 24-h *Salmonella* concentrations was found between WT- and LP-treated groups (*P* < 0.5088). An uninoculated control was included in every block. No naturally occurring salmonellae were isolated from these control frankfurters (group 1).

**DISCUSSION**

Both the WT and the LP variants of phage Felix O1 show promise as anti- *Salmonella* food applications. The conditions used in the applied aspect of this study were extreme in terms of temperature abuse and initial bacterial inoculum. The temperatures chosen were designed to test the limits of this phage-based intervention to ascertain whether either variant showed any promise under conditions that put the bacterial component at a growth advan-

FIGURE 2. Salmonella Typhimurium DT104 suppression in broth by WT (A) and LP (B). Log phase Salmonella Typhimurium DT104 was treated with Felix O1 variants in BHI broth at the PFU/CFU ratios indicated. Datum points represent the mean OD₆₀₀ values for two samples. The initial bacterial concentration was 8.2 × 10⁶ CFU/ml.

FIGURE 3. WT and LP suppression of *Salmonella* Typhimurium DT104 in BHI broth. OD₆₀₀ was determined at 20-min intervals following treatment of log phase BHI cultures of *Salmonella* Typhimurium DT104 (9.0 × 10⁶ CFU/ml at 0 min). The PFU/CFU ratios were 1.1 and 0.96 for WT and LP, respectively. Datum points represent means ± standard errors of the mean for five samples.

FIGURE 4. WT and LP suppression of *Salmonella* Typhimurium DT104 on poultry frankfurters. The initial bacterial concentration was 8.0 × 10⁷ CFU/ml. The PFU/CFU ratios were 0.12 and 0.96 for WT and LP, respectively. Datum points represent means ± standard errors of the mean for five samples.
FIGURE 4. Suppression of *Salmonella Typhimurium* DT104 in chicken frankfurter samples by WT and LP. A random complete block design in which the experimental day was the block was used to test the comparative suppression of *Salmonella* growth on poultry frankfurter samples. Approximately 300 CFU of log phase *Salmonella Typhimurium* DT104 was added to randomly allocated frankfurter samples, followed by immediate treatment with TP broth placebo (*Salmonella* control), with WT, or with LP at a PFU/CFU ratio of $1.9 \times 10^4$. The log-transformed concentration of *Salmonella* per gram of frankfurter after 24 h of incubation at $22^\circ C$ is reported as the mean ± the standard error of the mean for 10 samples.

tage. Subsequent studies are indicated to assess the performance of these phages under more reasonable abuse temperatures, such as $10^8^\circ C$ (8). Poultry products contaminated with *Salmonella* might contain as few as 10 CFU (23). It was expected that a lower starting inoculum level might adversely affect the reproducibility of this assay and might necessitate many more samples to determine the overall and comparative efficacies of phage treatment. The relatively high inoculum level (300 CFU) was designed to maximize the chance of finding any existent measurable difference between WT and LP with a reasonably small number of samples. Studies addressing lower levels of contamination are indicated in the continued study of this potential application.

Both variants effected approximately 2-log$_{10}$ suppression of *Salmonella* growth. Considering the conditions, it is no surprise that significant growth still occurred (Fig. 4). It would be necessary to test phage performance under lower temperature conditions at a lower level of contamination on a raw poultry vehicle more likely to be naturally contaminated with *Salmonella* to assess the utility of WT or LP in a practical application.

A cocktail of several phages might be necessary to achieve acceptable suppression of pathogens and prevent the emergence of resistant subpopulations (8, 15, 16, 20, 21). It is not known whether the growth that occurred in the face of WT and LP treatment is due to resistant sub-

culations. If this were found to be a problem, the use of additional phages would be warranted (15).

Although frankfurters are not necessarily a frequent culprit in foodborne *Salmonella* infections (6), their homogeneity offers an advantage in experiments addressing minor differences in the efficacies of phage variants. A retrospective power analysis involving data from the current experiment showed that had the phage variants performed differently, as little as a 0.5-log difference in bacterial suppression would have been detectable (two-sample $t$ test, Power = 90%, $\alpha = 0.05$, $\sigma_1 = \sigma_2 = 0.38$). Although the criterion for biologically important differences might well be much higher than 0.5 log, the ability to detect more minor differences in performance might still be important both in guiding the choice of phages and testing various enhancements for the application of an intervention. The 0.5-log difference criterion was used as the lower difference limit likely to have any measurable impact on the success of a phage-based food intervention. With a relatively small sample size, this assay is more than adequately sensitive to statistically significant differences that would be biologically important in designing an application.

The preliminary study of LP has not revealed the genetic reason for the LP phenotype, and it remains to be seen whether LP is a rapid-lysing mutant. Several genes, including the $r$ (rapid lysis) genes, can produce a rapid-lysing mutant in T4 (18). DNA sequence comparison of WT and LP did not show a mutation in the putative $r$II$^B$ gene (data not shown), but mutations in several other genes can produce a rapid-lysing phenotype. Rapid-lysing mutants would not be subject to lysis inhibition, the delay of host cell lysis that can occur when the phage concentration is high (9). Therefore, rapid-lysing mutants might offer an advantage in a phage-based food intervention in which the PFU/CFU ratio might be very high. Rapid-lysing mutants of T4 produce far fewer progeny than their wild-type counterparts as determined in single-step infections (9). Further work is necessary to determine whether there are burst size differences for WT and LP. Phage counts were obtained from the frankfurter assays and were found to be less reproducible than bacterial counts (data not shown). The determination of these differences in phage yield for the frankfurter model may necessitate a different eluent to help reduce experimental error within groups (12).

Phages have been found to occur naturally in meats and even in further-processed foods (13). Nevertheless, the safety and prudence of adding large quantities of biological entities such as phage to human foods should be explored carefully. Even though phage infection may be specific for a bacterial host, the infection of bacteria can have ramifications for eukaryotic systems, including those of humans (22). Some phages are able to transfer virulence properties among bacteria. Phage-mediated virulence has been demonstrated for diseases such as cholera, in which genetic transfer by a temperate phage can transform a normally non-pathogenic organism into one that can cause disease (22). Felix O1 is most likely a lytic phage rather than a temperate one. If this is the case, all cells infected by phage O1 are killed, thus precluding the opportunity for increased viru-
lence. Our laboratory has recently determined the entire DNA sequence of the Felix O1 genome (GenBank accession no. AF320576) in order to screen for genes that could increase *Salmonella* virulence. No obvious virulence genes have been found through homology searches. However, more extensive studies to test the ability of Felix O1 to transfer genes among *Salmonella* should be performed.

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REFERENCES