Isolation and characterization of bacteriophages infecting *Salmonella* spp.

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Abstract

Bacteriophages infecting *Salmonella* spp. were isolated from sewage using soft agar overlays containing three *Salmonella* serovars and assessed with regard to their potential to control food-borne salmonellae. Two distinct phages, as defined by plaque morphology, structure and host range, were obtained from a single sample of screened sewage. Phage FGCSSa1 had the broadest host range infecting six of eight *Salmonella* isolates and neither of two *Escherichia coli* isolates. Under optimal growth conditions for *S.* Enteritidis PT160, phage infection resulted in a burst size of 139 PFU but was apparently inactive at a temperature typical of stored foods (5 °C), even at multiplicity of infection values in excess of 10 000. While neither isolate had characteristics that would make them candidates for biocontrol of *Salmonella* spp. in foods, phage FGCSSa1 behaved unusually when grown on two *Salmonella* serotypes at 37 °C in that the addition of phages appeared to retard growth of the host, presumably by the lysis of a fraction of the host cell population.

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Introduction

The potential to use phages as therapeutic agents in controlling human and animal disease has been recognized for some time (Clewley, 2003). More recently the extension of phage biocontrol to food applications has been investigated (Greer, 2005; Hudson *et al*., 2005). There are many points in the food chain at which phages could be applied, each presenting their own challenges. Common to all is the problem of host range, in that for phages to be effective they either need to have a broad host range or they must be used in conjunction with other phages (Kudva *et al*., 1999). Where the host range is too limited phage biocontrol is ineffective (Greer & Dilts, 1990). The use of multiple species in enrichments to isolate broad host range phages has been described (Jensen *et al*., 1998), and in this study we adopted a similar approach to attempt the isolation of broad host range phages infecting *Salmonella* spp., an important food-borne pathogen, by including multiple hosts in agar overlays inoculated with phage-containing sample.

Previous publications have described the inactivation of *Salmonella* spp. on honeydew melon at 5 °C (Leverentz *et al*., 2001) and on chicken skin at 4 °C (Goode *et al*., 2003), in the latter instance at a multiplicity of infection (MOI) of 1. This inactivation is surprising as it occurred beneath the minimum growth temperature of *Salmonella* spp. We sought to isolate new *Salmonella* phages and to examine them for properties which may be useful for biocontrol purposes on refrigerated foods. In addition, interactions with the host under optimum growth conditions were studied.

Materials and methods

Medium used

Lennox L Base (LB) medium (Invitrogen, Carlsbad, CA) was used as a broth or solidified with agar to grow hosts.

Reference cultures and phage stocks

Most of the cultures used were obtained from the New Zealand Reference Culture Collection, or the Enteric Reference Laboratory, ESR, Kenepuru Science Centre: NZRM352 *S.* Enteritidis PT 4, NZRM3484 *S.* Enteritidis PT 9a, ERL031500 *S.* Infantis, NZRM 383 *S.* Menston, NZRM423 *S.* Saintpaul, NZRM1891 *S.* Typhimurium PT 160 and NZRM 480 *Escherichia coli*. In addition the University of Canterbury provided *S.* Typhimurium LT2 and *E. coli* RR1.

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Agar layer (overlay) method

Base plates, poured with 20–30 mL of medium, used agar at a concentration of 1.5%, whereas soft agar overlays were at 0.7% (Adams, 1959). Soft agar was stored in 3 mL volumes, melted when required, and tempered to 48.5 °C before use. Exponential phase host (3–4 h culture) and bacteriophages were mixed in each overlay before pouring onto dried base plates. The soft agar was swirled to produce a uniform top layer and incubated at 37 °C for 24 h before inspection for plaque formation.

Isolation of phages infecting *Salmonella*

Sewage samples were either diluted (one in 10) in SM buffer [0.05 M TRIS, 0.1 M NaCl, 0.008 M $MgSO_4$, 0.01% (weight in volume) gelatin pH 7.5] and incubated at 4 °C overnight, or used directly before centrifugation at 1600 ***g*** for 25 min and filtration through a disposable 0.22 μm pore-size filter (Millipex GP, Millipore, Cork, Ireland). A volume (100 μL) of the filtrate was added to a prewarmed soft agar overlay to which 100 μL of exponential phase cultures of three *Salmonella* isolates (*S.* Typhimurium PT160, *Salmonella* LT2 and *S.* Infantis) were added, and the agar poured as an overlay. Plates were incubated as described above. Plaques were purified by serial dilution and plating to soft agar overlays according to methods in Adams (1959).

Preparation of high titre phages stocks

Purified phages were diluted serially in SM buffer to give a concentration that would provide confluent lysis of the host in a soft-agar overlay plate. For each dilution three to five plates were overlaid and, after incubation, plates with almost confluent lysis chosen. To recover phages SM (5 mL) was added to each plate and left at room temperature for at least 1 h, and the plates swirled regularly. The liquid was decanted into centrifuge tubes containing 20 mL of SM (Adams, 1959), and the soft-agar overlay layer added to the tube after scraping from the base plate. The contents were vortex-mixed before shaking for 30 min, and the overlay removed by centrifugation at 1300 ***g*** for 10 min. The supernatant was then filtered (0.22 μm) and 0.2% chloroform added before storage at 4 °C. The titre of the stock was determined by the overlay method.

Preparation of phages for transmission electron microscopy

To produce phages for electron microscopy high titre stocks were prepared in SM buffer as described above. The liquid was decanted into tubes, centrifuged at 12 000 ***g*** for 10 min. The supernatant was decanted into another tube and centrifuged at 49 500 ***g*** for 90 min. The supernatant was discarded and the pellet was gently resuspended in 0.5 mL SM buffer which was filtered (0.22 μm) and stored at 4 °C until required. A drop of phage stock was applied to a 200-mesh grid and left for 1 or 2 min at which time the excess liquid was drawn off with filter paper and the grid given time to air dry. A drop of 2%-phosphotungstic acid (aqueous), buffered to pH 6.5, was applied for 15 or 25 s to stain the phages, excess liquid was drawn off with filter paper and the grid air dried. The grid was then loaded into the electron microscope (Hitachi H-600) and phages examined at × 100 000–260 000 magnification. Q1

Host range determination

Ten-fold dilutions of high titre stocks were prepared in SM broth. Exponential phase suspensions of host strains were prepared as described above. Overlays were inoculated with 100 μL host and poured on a base plate previously marked in a grid to allow identification of each inoculum. Once the overlay was gelled and dried, 10 μL of the 10^{-2}, 10^{-4}, 10^{-6} phage dilutions were spotted on the overlay. The plates were incubated at 37 °C, and examined for plaques after 18–24 h. In addition, exponential phase cultures of the host strains (Table 1) were prepared as described, and overlays inoculated with a host and 100 μL of a serial dilution of the phage stocks. The plates were incubated at 37 °C and plaques enumerated for each host/phage combination.

Single-step growth curve and growth under optimal conditions

A standard protocol (Carlson & Miller, 1994) was adapted for use with the *Salmonella* phage, except that the duration of the experiment was increased to 85 min. Calculations were performed as described for phage T4 (Carlson & Miller, 1994). The number of phages produced from each infected centre, the burst size, was derived from applying a sigmoid curve of best fit.

Growth curves were produced with the host *S.* Saintpaul at 37 and 42 °C, and on *S.* Typhimurium PT160 at 37 °C in the presence of phage FGCSSa1. LB was inoculated with the host and phage at a MOI values in the range 0.3–3, and growth of the host monitored periodically by measuring the optical density at 650 nm using a Shimadzu UV mini 1240 (Shimadzu Corporation, Kyoto, Japan) until stationary phase was reached.

Host inactivation at low temperatures

To assess the potential of phage FGCSSa1 to act as a biocontrol agent in chilled foods experiments were performed *in vitro* to provide information on host lysis at pH 5.5–7.0 and at 5 °C. A range of MOI values was used as calculated from phage and host numbers measured at the beginning of the experiment. In many cases MOI values in

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Table 1. Host range of *Salmonella* phages FGCSSa1 and FGCSSa2

Host	FGCSSa1 Spot test	FGCSSa1 Titre (PFU mL^{-1})	FGCSSa2 Spot test	FGCSSa2 Titre (PFU mL^{-1})
S. Typhimurium PT150 NZRM 1891	+	5.4×10^9	+	2.0×10^{10}
S. Typhimurium LT2	+	6.2×10^9	+	1.8×10^{10}
S. Typhimurium PT 12A	+	6.3×10^9	+	1.7×10^{10}
S. Infantis ERL 03150	–	NA	–	NA
S. Menston NZRM 383	–	NA	–	NA
S. Saintpaul NZRM 423	+	3.6×10^9	–	NA
S. Enteritidis PT4 NZRM 352	+	5.5×10^9	–	NA
S. Enteritidis PT 9a NZRM 3484	(+)	3.1×10^9	–	NA
Escherichia coli NZRM 480	–	–	–	NA
Escherichia coli RR1	–	–	–	NA

+, clear plaques; (+), opaque plaques; –, no plaques.
NA, not applicable as no lysis in spot test; PFU, plaque-forming units.

excess of 10 000, and up to 87 000, were used to investigate potential inactivation by lysis from without (Tarahovsky *et al.*, 1994). Host and phage counts were performed as described above.

Results and discussion

Phage isolation

To attempt the isolation of phages with broad host ranges, mixed cultures of three different salmonellae was inoculated with screened raw sewage samples prepared as described above and eight phages were isolated from three samples collected on different days. Preliminary results indicated that none of these phages were able to from plaques on *S.* Infantis, whereas they all formed plaques on *S.* Enteritidis PT4 and PT9a, and *S.* Saintpaul.

When propagated, one turbid plaque on one lawn yielded two distinct plaque types, a small plaque-forming isolate FGCSSa1, and a large plaque-forming isolate FGCSSa2. These two phages were selected for further characterization on the basis of differences in plaque morphology.

Electron microscopy

The two phages isolated were morphologically distinct and appear to be similar to *Salmonella* phages isolated previously. Phage FGCSSa1 was assigned to the family *Myoviridae* because of the presence of a tail with a contractile sheath (Fig. 1). Phage FGCSSa2 was assigned to the family *Siphoviridae* as indicated by the presence of a long tail and the absence of a contractile sheath (Fig. 2). Both phages had isometric heads. FGCSSa1 had a mean head diameter of 107 nm, a tail length of 123 nm, and a tail diameter of 20 nm.

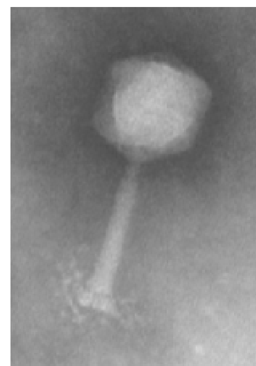

Fig. 1. Electron micrograph of *Salmonella* phage FGCSSa1. Dimensions are presented in the results.

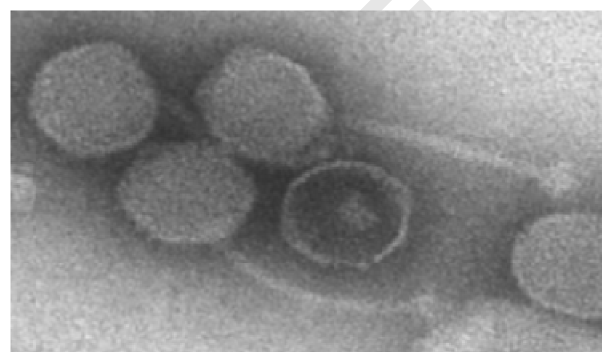

Fig. 2. Electron micrograph of *Salmonella* phage FGCSSa2. Dimensions are presented in the results.

FGCSSa2 had smaller dimensions with a mean head diameter of 66 nm, tail length of 112 nm and a tail diameter of 9 nm.

The morphology of phage FGCSSa1 is similar to *Salmonella* phage Vi I (H.-W. Ackermann, pers. commun., 2004; Ackermann *et al.*, 1970; Demczuk *et al.*, 2004). Phage FGCSSa2 is morphologically similar to *Salmonella* paratyphi B phage Jersey (H.-W. Ackermann, pers. commun., 2004; Demczuk *et al.*, 2004).

Host range

FGCSSa1 was lytic on six of eight *Salmonella* hosts but did not infect the *Escherichia coli* isolates tested (Table 1). The phage that produced a large plaque, FGCSSa2, was lytic on only three of the eight *Salmonella* hosts, two of which were present in the overlay used for isolation, and did not form plaques on either *E. coli* isolate tested. The opaque plaques on the mixed host lawn occurred because neither phage lysed *S.* Infantis. On single hosts the plaques were clear. Where lysis occurred, the plating efficiency was very similar on each host.

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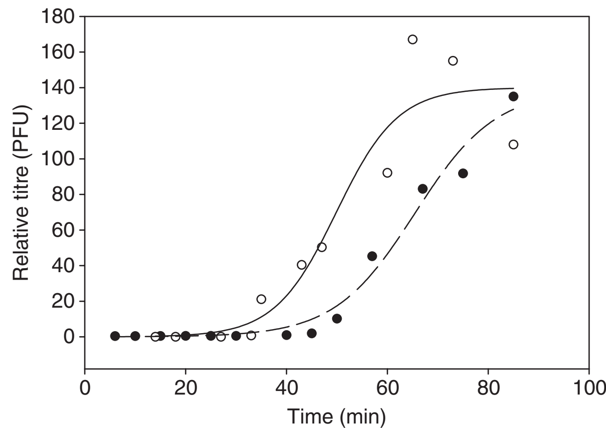

Fig. 3. Single step growth of *Salmonella* phage FGCSSa1 with host *S.* Typhimurium PT 160 at 37 °C, pH 7.0. ●, Free phages; ○, phage liberated using chloroform. The lines represent the sigmoid curve of best fit for free phage ($R^2 = 0.97$), and phage liberated by chloroform treatment ($R^2 = 0.91$).

The proportion of potential hosts lysed by the two phages was similar to that reported for phage isolates ViI and Jersey in that phages Jersey and FGCSSa2 have more restricted host ranges than FGCSSa1 and ViI (Demczuk *et al.*, 2004).

Single step growth curve

The burst size for FGCSSa1 was 139 PFU (± 13) and, under the conditions used, the latent period was 50 min (± 5 min) (Fig. 3). The eclipse time was 38 min (± 9 min). The proportion of phages adsorbed was 99% within 4 min. The latent period and burst size differed from that previously reported for phage ViI. The significance of this difference is unclear because no details of culture conditions were provided in that report (Cerquetti & Hooke, 1993). The burst size produced was not unusual, and is of the same order as that observed for phage T4 growing on *E. coli* (Hadas *et al.*, 1997). The latent period was long compared with an *E. coli* phage incubated under conditions approaching the optimum for the host (Ellis & Delbrück, 1939) where the latent period was 30 min, and for T4 the latent period was as short as 18 min increasing to 35 min under suboptimal conditions (Hadas *et al.*, 1997). It was, however, similar to that for a phage infecting *E. coli* O157:H7 (Goodridge *et al.*, 2003). Both burst size and latent period are influenced by host, medium and temperature of incubation (Guttman *et al.*, 2005) and specific growth rate (Hadas *et al.*, 1997).

Growth at 37 and 42 °C

While phage FGCSSa1 showed a typical single step growth curve with *S.* Typhimurium PT160 (Fig. 3) and *S.* Saintpaul (data not shown) the kinetics of inactivation at 37 and 42 °C were unusual. When FGCSSa1 was grown on two salmonellae there was incomplete lysis of the host culture at both 42 and 37 °C (typical data are shown in Fig. 4). Instead there was a consistent retardation of the growth rate in the presence of the phage at both temperatures. For example, during the course of the experiments where *S.* Typhimurium PT160 was the host (Fig. 4) the titre of phages rose from 1.2×10^7 to 2.9×10^9 PFU mL^{-1} at an MOI 0.3, and 1.0×10^8 to 2.6×10^9 PFU mL^{-1} at an MOI of 2.5. Simultaneously, the number of host cells increased, as shown in Fig. 4, to reach a final concentration of 8.4×10^8 CFU mL^{-1}.

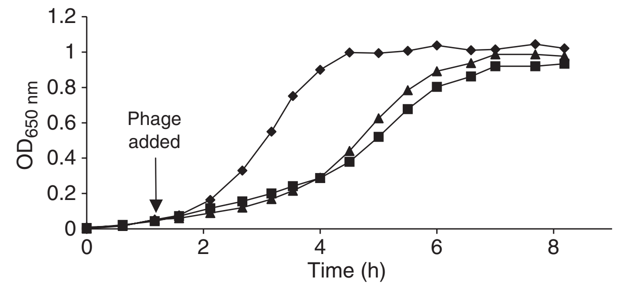

Fig. 4. Growth of *S.* Typhimurium PT160 at 37 °C in the presence (■ multiplicity of infection (MOI) = 0.3, ▲ MOI = 2.5) or absence (◆) of phage FGCSSa1. An optical density of 1 was equivalent to 8.4×10^8 CFU mL^{-1} host cells under these conditions.

Retardation in the growth of salmonellae in the presence of the phage was seen in all three experiments using *S.* Saintpaul at 37 °C, it occurred at 37 and 42 °C, at pH 7 and 5.8, and was similar, and perhaps more pronounced, for *S.* Typhimurium PT160 infected at two MOI values (Fig. 4). In total, 10 curves showing similar results have been produced. A possible explanation of the observed data is that only a subpopulation of the host is susceptible to phage infection. This has been shown for the *E. coli* phage Mu^{L}, where 15% of the population was considered to be resistant to phage lysis (Fischer *et al.*, 2004). A very similar set of curves to the ones presented here was produced for this mutant and its host (Mizoguchi *et al.*, 2003). However, the hypothesis of a limited host subpopulation susceptible to the phage needs to be confirmed for this host/phage system.

As the phage produced conventional plaques on both hosts at 37 °C, there may be a difference in behaviour on solid and liquid media. This work needs to be extended to food matrices to determine which pattern of lysis would occur there.

Phage/host interactions at low temperatures

Phage FGCSSa1 was chosen for investigation of low temperature kinetics because of its broader host range. Experiments conducted at 5 °C and pH 7.0 in LB broth failed to demonstrate lysis of *S.* Typhimurium PT160 even at MOI values in excess of 10 000 with incubation continued up to 50 h. Similarly, extended incubation up to 120 h at this

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temperature at an MOI approaching 10^3 at pH 5.8 showed no significant changes in numbers of either host or FGCSSa1. These concentrations of phage might be expected to lyse hosts even without the liberation of progeny phages, a phenomenon called lysis from without (Tarahovsky *et al*., 1994). It is possible that some of the inactivation reported on foods is also due to lysis from without, or occurs during enumeration when the host is incubated at its optimum growth temperature after attachment of the phage to the cell at refrigeration temperatures.

Conclusions

The observations concerning the incomplete lysis of the host culture are unusual; to the authors' knowledge this is the first description of such a phenomenon in *Salmonella* phages and there has only been one other report that notes similar activity in an *Escherichia coli* O157 phage (Fischer *et al*., 2004). Other reports present data which may show the same effect (e.g. Ripp & Miller, 1997; Loc Carrillo *et al*., 2005) and some descriptions of pseudolysogeny might account for the observations (Ackermann & DuBow, 1987) while others may not (Ripp & Miller, 1997). As was noted by Fischer *et al*. (2004) this coexistence needs to be understood to allow the successful application of phages as biocontrol agents.

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References

Ackermann H-W, Berthiaume L & Kasatiya SS (1970) Ultrastructure of Vi pahges I to IV of *Salmonella typhi*. *Can J Microbiol* **16**: 411–413.

Ackermann H-W & DuBow MS (1987) *Viruses of Prokaryotes. Volume 1. General Properties of Bacteriophages*, CRC Press, Baton Rouge, FL.

Adams MH (1959) *Bacteriophages*, Interscience Publishers, Inc., New York.

Carlson K & Miller ES (1994) *Molecular Biology of Bacteriophage T* (Karam JD, ed) American Society for Microbiology, Washington, DC.

Cerquetti MC & Hooke AM (1993) Vi I typing phage for generalized transduction of *Salmonella typhi*. *J Bacteriol* **175**: 5294–5296.

Clewley JP (2003) The day of the phage. *Comm Dis Pub Health* **6**: 260–262.

Demczuk W, Ahmed R & Ackerman H-W (2004) Morphology of *Salmonella enterica* serovar Heidelberg typing phages. *Can J Microbiol* **50**: 873–875.

Ellis EL & Delbrück M (1939) The growth of bacteriophage. *J Gen Physiol* **22**: 365–384.

Fischer CR, Yoichi M, Unno H & Tanji Y (2004) The coexistence of *Escherichia coli* serotype O157:H7 and its specific bacteriophage in continuous culture. *FEMS Microbiol Lett* **241**: 171–177.

Goode D, Allen VM & Barrow PA (2003) Reduction of experimental *Salmonella* and *Campylobacter* contamination of chicken skin by application of lytic bacteriophage. *Appl Environ Microbiol* **69**: 5032–5036.

Goodridge L, Gallaccio A & Griffiths MW (2003) Morphological, host range, and genetic characterization of two coliphages. *Appl Environ Microbiol* **69**: 5364–5371.

Greer GG (2005) Bacteriophage control of foodborne bacteria. *J Food Prot* **68**: 1102–1111.

Greer GG & Dilts BD (1990) Inability of a bacteriophage pool to control beef spoilage. *Int J Food Microbiol* **10**: 331–342.

Guttman B, Raya R & Kutter E (2005) *Bacteriophages: Biology and Applications* (Kutter E & Sulakvelidze A, eds), pp. 29–66. CRC Press, Boca Raton, FL.

Hadas H, Einav M, Fishov I & Zaritsky A (1997) Bacteriophage T4 development depends on the physiology of its host. *Microbiology* **143**: 179–185.

Hudson JA, Billington C, Carey-Smith G & Greening G (2005) Bacteriophages as biocontrol agents in food. *J Food Prot* **68**: 426–437.

Jensen EC, Schrader HS, Rieland B, Thompson TL, Lee KW, Nickerson KW & Kokjohn TA (1998) Prevalence of broad host-range lytic bacteriophage of *Sphaerotilus natans*, *Escherichia coli*, and *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **64**: 575–580.

Kudva IT, Jelacic S, Tarr PI, Youderian P & Hodve CJ (1999) Biocontrol of *Escherichia coli* O157 with O157-specific bacteriophages. *Appl Environ Microbiol* **65**: 3767–3773.

Leverentz B, Conway WS, Alavid1ze Z, Janisiewicz WJ, Fuchs Y, Camp MJ, Chighladze E & Sulakvelidze A (2001) Examination of bacteriophage as a biocontrol method for *Salmonella* on fresh-cut fruit: a model study. *J Food Prot* **64**: 1116–1121.

Loc Carrillo CM, Atterbury RJ, El-Shibiny A, Connerton PL, Dillon E, Scott A & Connerton IF (2005) Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens. *Appl Environ Microbiol* **71**: 6554–6563.

Mizoguchi K, Morita M, Fischer CR, Yoichi M, Tanji Y & Unno H (2003) Coevolution of bacteriophage PP01 and *Escherichia coli* O157: H7 in continuous culture. *Appl Environ Microbiol* **69**: 170–176.

Ripp S & Miller RV (1997) The role of pseudolysogeny in bacteriophage–host interactions in a natural freshwater environment. *Microbiology* **143**: 2065–2070.

Tarahovsky YS, Ivanitsky GR & Khusainov AA (1994) Lysis of *Escherichia coli* cells induced by bacteriophage T4. *FEMS Microbiol Lett* **122**: 195–200.

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