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Short communication

Selection of polyvalent bacteriophages infecting *Salmonella enterica* serovar Choleraesuis

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ABSTRACT

Background: Ideally, bacteriophages of pathogenic bacterial hosts should be polyvalent to be able to replicate in an alternative nonpathogenic bacterium. Thus, accidental infection by the original host can be avoided when bacteriophage lysates are used in biocontrol protocols.
Results: From 15 wastewater samples, collected at different sites in the V Region in Chile, we selected three bacteriophages (FC, FP, and FQ) capable of productively infecting *Salmonella enterica* serovar Choleraesuis. By transmission electron microscopy (TEM) observation, the bacteriophages were found to belong to the order Caudoviridae. Molecular analyses indicated that FC, FP, and FQ contained double-stranded DNA genomes, of sizes similar to bacteriophage P22, and distinct recognition sites for the restriction endonucleases HaeIII and HindIII. Assays of host range revealed that the bacteriophages were polyvalent and thus capable of infecting different strains of *Escherichia coli* and other serovars of *Salmonella*.
Conclusion: We have isolated new bacteriophages of the serovar Choleraesuis with various potential applications in relation to this pathogenic bacterium.

1. Introduction

First described in the beginning of the last century, polyvalent bacteriophages are capable of productively infecting more than one host [1]. These bacteriophages were reported for members of Enterobacteriaceae [2]. The authors obtained isolates infecting *Escherichia coli*, *Klebsiella pneumoniae*, and *Aerobacter aerogenes*.

Among polyvalent bacteriophages, those active against *Salmonella enterica* are promising as they can be isolated and used as alternative or complementary biocontrol agents against this pathogen [3,4]. In this respect, bacteriophages that can replicate in *Salmonella* and nonpathogenic strains of *E. coli* are particularly useful. Bacteriophage production in *E. coli* would be safer than propagation in the original pathogenic host as these bacteriophage preparations may accidently deliver the pathogen to the host's cells. However, studies related to this topic are scarce. Notably, Bielke et al. [5] tested the lytic activity of wide-host-range bacteriophages on *Salmonella* strains for reducing *Salmonella* counts in poultry products [6].

More recently, three bacteriophages isolated as part of the European project Phaghevet-P were tested for polyvalency. Of these bacteriophages, phi PVP-SE exhibited a lytic effect on different *Salmonella* serovars and two nonpathogenic *E. coli* strains [7]. In addition, phiKP26, another polyvalent bacteriophage, was found to infect *Salmonella* and *E. coli* [8].

Recently, Leon et al. [9] found that the classic *E. coli* lysogenic bacteriophage P1 could naturally infect and proliferate in *S. enterica* serovar Choleraesuis, in contrast to other serovars of *Salmonella*, which are not naturally susceptible to P1. These results indicate that this bacterium shares certain features with the original host of P1: *E. coli*. Thus, we hypothesized that the selection of bacteriophages active against serovar Choleraesuis could yield isolates capable of infecting *E. coli*.

In this study, for the first time, we report the isolation of bacteriophages using *S. enterica* serovar Choleraesuis as a selective host. The three DNA bacteriophages obtained, belonging to the order Caudoviridae based on morphology, were found to be polyvalent and capable of infecting different strains of *E. coli* and other serotypes of *S. enterica*.

2. Materials and methods

2.1. Isolation and purification of lytic bacteriophages against serovar Choleraesuis VAL 201

2.1.1. Sampling

Wastewater samples were collected from several estuaries in the V Region of Chile: San Antonio, El Tabo, Concón, Higuerillas, 2 Norte, Quintero, Loma Larga I, Loma Larga II, Cartagena, Algarrobo, and Caleta

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Portales. They were collected in sterile glass bottles, transported to the laboratory in a cooler, and maintained at 4°C until processing the next day.

2.1.2. Bacterial growth

A rifampicin-resistant (Rif^r) mutant (VAL 201) of a strain of *S. enterica* serovar Choleraesuis — originally isolated from a diseased pig and obtained from Dr. Roy Curtiss III (Arizona State University) — was used for the phage enrichment experiments and assays of viral activity. VAL 201 was routinely cultured in LB broth or agar [10] at 37°C. Rifampicin was added at a concentration of 100 μg/mL.

2.1.3. Bacteriophage enrichment

To each flask containing 20 mL of LB broth and Rif, 5 mL of different wastewater samples and 1 mL of an overnight (o/n) culture of VAL 201 were added. After 24 h of incubation while being shaken (200 rpm), 1-mL portions of each enrichment culture were centrifuged at 13,400 × g for 10 min. The supernatants were transferred to fresh tubes, to each of which 50 μL of $CHCl_3$ was added.

To determine the presence of lytic phage, lawns of VAL 201 were seeded with 1-μL samples from the enrichment cultures. These were then incubated for 24 h to detect clear zones of lysis. From these, individual purified phage plaques were obtained by understreaking.

2.1.4. Bacteriophage amplification and purification of viral particles

Liquid phage lysates were obtained by inoculating exponentially growing cultures of VAL 201 with individual phage plaques. The shaken cultures were monitored based on the OD_{550} values until a minimum was reached. From these lysates, bacteriophage virions were purified by the polyethylene glycol method as described by Sambrook et al. [11] for the purification of bacteriophage λ.

2.2. Phage characterization

2.2.1. Transmission electron microscopy

The pure phage preparations (20 μL, 10^{11} pfu/mL) were diluted (1:1) in Milli-Q (MQ) water, and the samples were negatively stained as performed by Goodridge et al. [12] using 300 MESH copper grids coated with FORMVAR. The samples were examined under a Zeiss EM-109 transmission electron microscope at magnification ranging from 50,000× to 140,000× at 50 KV. They were photographed using a Timax 100 film. Determinations were made at the Electronic Microscopy Unit of the Institute of Biomedical Sciences at the University of Chile.

2.3. Molecular characterization

2.3.1. Extraction of viral genomic material and restriction with nucleases

The purified phage suspensions (1 mL, 10^{11} pfu/mL) were used to isolate bacteriophage DNA by a mini-preparation protocol reported by Kaiser et al. [13] but using proteinase K (20 mg/mL, GibcoBRL) instead of pronase. The phage DNA was precipitated with ethanol and finally stored in TE buffer at -20°C until use [11]. To analyze the genetic material of the different bacteriophages, digestion with DNase I, and with the restriction endonucleases EcoRI, BamHI, and HaeIII, was performed according to the enzyme manufacturer's instructions (Fermentas). The DNA digests with restriction enzymes were resolved by 6% polyacrylamide gel electrophoresis (TAE buffer), and the fragments were detected under ultraviolet (UV) light as described by Sambrook et al. [11]. Whole phage DNA and digests with DNase I were analyzed on a 1% agarose gel in TAE buffer and visualized under UV light, as described previously [11].

2.4. Host range and lytic activity

To determine the host range of the bacteriophages, duplicate plaque assays [14] were performed using different strains of *E. coli* and *Salmonella* inoculated (10^7 cfu·mL^{-1}) in 3 mL of soft LB agar (0.7%) overlaid on a regular LB agar plate. The phage preparations (10^9 pfu/mL) were applied as 1-μL inocula on top of the seeded plates. Lytic plaque formation (+) or absence of plaques (-) was examined after 24 h of incubation at 37°C. To determine the decay curve of VAL 201, an o/n culture of the bacterial strain was used to start (1:25 dilution) fresh cultures of VAL 201. After 1 h of incubation at 37°C while being shaken at 200 rpm, the experimental cultures were inoculated with the different studied phages at a multiplicity of infection (MOI) of 1. The control culture was left uninoculated. Samples were collected every 30 min, and the OD_{550} value was measured in triplicate until a minimum was reached in the phage-infected cultures.

3. Results

3.1. Selection of lytic bacteriophages

Of the 15 samples studied, 13 displayed phages active against VAL 201, which formed either clear or turbid plaques. From the phage producing a clear plaque (1 mm in diameter), we consistently propagated three phages, denoted as FC, FP, and FQ reaching titers of 2.07 × 10^{12} pfu/mL, 5.5 × 10^{11} pfu/mL, and 4.1 × 10^{11} pfu/mL, respectively.

3.2. Bacteriophage morphology

The electron microscopic images of the studied bacteriophages are shown in Fig. 1. These phages were composed of a head and a tail without an envelope, measuring 166 nm (FC), 220 nm (FP), and 152 nm (FQ) in length. The heads were isometric and hexagonal in shape with icosahedral symmetry and a diameter of 62 nm (FC), 82 nm (FP), and 64 nm (FQ).

The main morphological difference between the three phages was their tails: FC and FQ exhibited a long, thick, rigid structure with helical symmetry. The FC tail was 104 nm in length and 14 nm in width; FQ was 88 nm in length and 14 nm in width. Unlike FC and FQ, FP showed a long, thin, flexible tail of helical symmetry, measuring 138 nm in length and 7 nm in width. None of these phages showed the presence of a neck, a base plate, spikes, or fibers.

3.3. Characterization of viral genomic material

First, we tested whether the genomic material of FC, FP, and FQ was DNA. Upon treating the phage genomic material with DNase I, the nucleic acids of all three phages were found to be sensitive to this nuclease, indicating DNA as the phage genomic material. Furthermore, upon agarose gel electrophoresis, the DNA genomes of FC, FP, and FQ were found to have similar molecular mass, approximately equivalent to that of the 43.5-kbp genome of the temperate bacteriophage P22. These results are shown in Fig. 2.

In addition, we tested the sensitivity of bacteriophage DNA genomes to the restriction endonucleases BamHI, EcoRI, HaeIII, and HindIII to determine the differences between FC, FP, and FQ. We found that EcoRI discriminated between the three phages and BamHI between FP and the other two phages. Restriction with HaeIII and HindIII led to the formation of multiple fragments in the three viral genomes. The restriction patterns, shown in Fig. 3, helped clearly differentiate the genomes of the three phages under study. Moreover, restriction with the tested endonucleases indicated double-stranded DNA as the genomic material of the three phages.

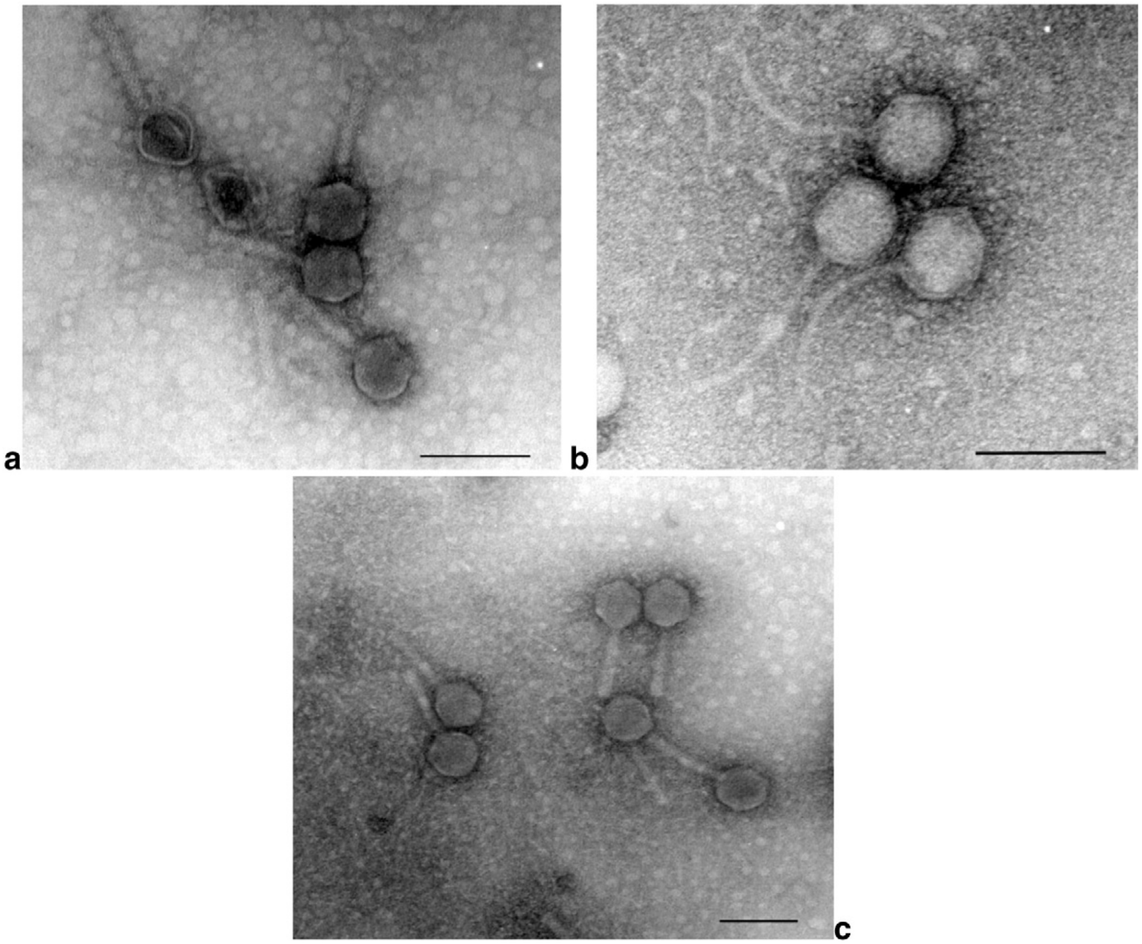

Fig. 1. Electron micrographs at 140,000× of (a) FC and (b) FP, and 85,000× of (c) FQ. Bar = 100 nm.

3.4. Host range of bacteriophages

The plaque assays of the FC, FQ, and FP phages were conducted using different strains of *E. coli* and *Salmonella*. These phages were found to be polyvalent, capable of infecting many of the strains tested in our experiments, *via* clear plaque formation. These results are shown in Table 1. The FP bacteriophage showed the widest host range. Furthermore, we observed that bacteriophage-insensitive mutants arose at a frequency of about 10-^{7}.

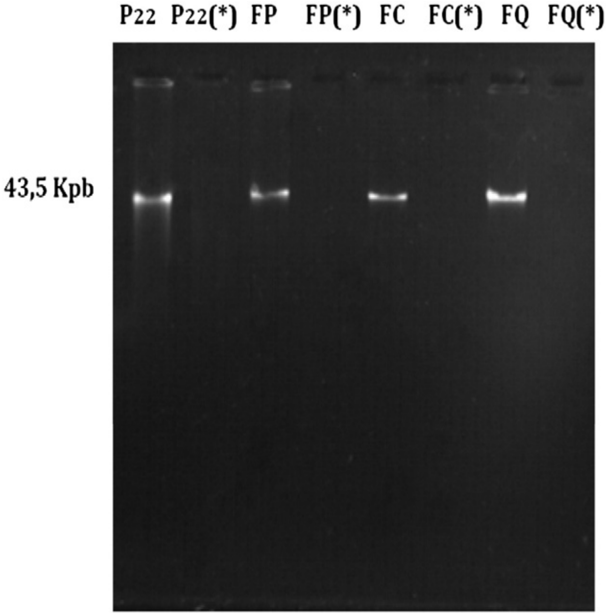

Fig. 2. Agarose (1%) gel electrophoresis of bacteriophage nucleic acids. The molecular mass marker is DNA of P22 (43.5 kpb). (*): genomic material treated with DNase I.

3.5. In vitro VAL 201 decay caused by FC, FP, and FQ

To assess the infective activity of our phage isolates against the serovar Choleraesuis, we determined the corresponding bacterial decay curves. FC, FP, and FQ were added separately to exponentially growing cultures (37°C) of serovar Choleraesuis strain VAL 201 using a MOI of 1. The resulting decay was followed by a decrease in optical density. The results are shown in Fig. 4.

4. Discussion

In this study, we aimed to isolate and characterize polyvalent bacteriophages that can infect both serovar Choleraesuis and *E. coli*. We obtained the FC, FP, and FQ phages, which were found to contain double-stranded DNA genomes. This finding is consistent with the high prevalence of this type of phage in nature [15].

Furthermore, analyses involving transmission electron microscopy (TEM) micrographs and phage DNA restriction patterns allowed us to distinguish between the three phages more precisely. In fact, the FP bacteriophage could be clearly differentiated from FC and FQ, because of its long, flexible tail, whereas the latter shared similar morphology.

The electrophoretic analyses of bacteriophage DNA indicated that all three phages had genomes of approximately the same size

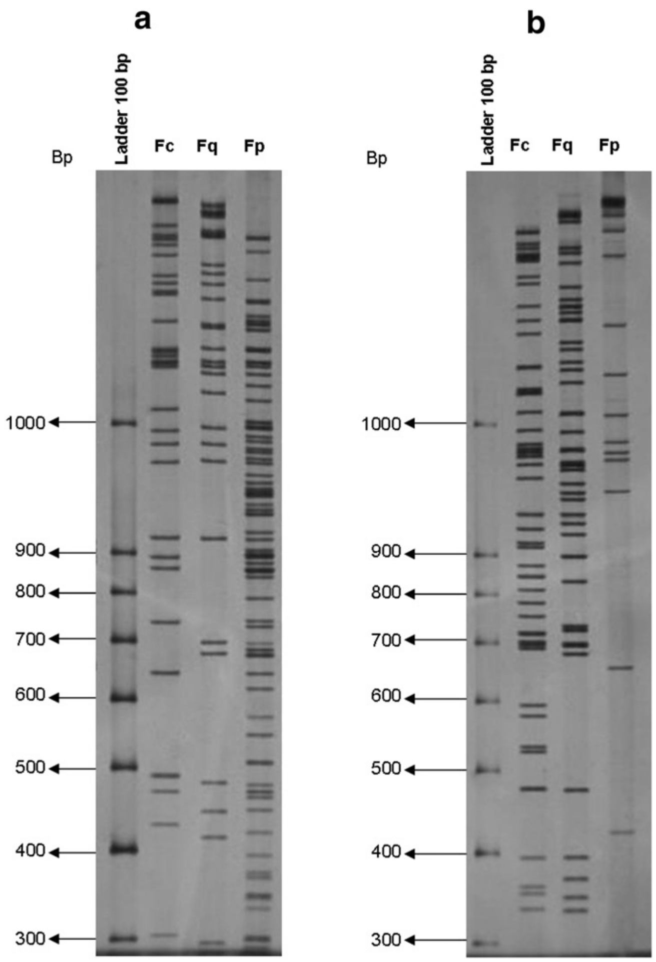

Fig. 3. Polyacrylamide (6%) gel electrophoresis of bacteriophage nucleic acids. The markers used are 1-kb and 100-bp Promega. The genomic material was treated with (a) HaeIII and (b) HindIII endonucleases.

(43.5 kbp). However, the analysis with restriction endonucleases showed differences between FC and FQ, which otherwise appear highly similar. Again, consistent with morphological data, DNA analyses showed that FP was clearly distinct from FC and FQ.

Based on these analyses, we then examined the International Committee on Taxonomy of Viruses (ICTV) criteria for the taxonomic classification of viruses [16]. Based on these data, we suggest that FC, FP, and FQ be classified in the order Caudovirales, characterized by bacterial viruses with double-stranded DNA in non-enveloped capsids, icosahedral heads, and rigid/flexible helical tails.

Table 1
Host range of FC, FP, and FQ.

Host	FC	FQ	FP
Escherichia coli Sw	-	-	-
Escherichia coli B	-	-	+
Escherichia coli C	+	+	+
Escherichia coli K12	-	-	+
Salmonella Choleraesuis (VAL 201)	+	+	+
Salmonella Enteritidis13076	-	-	-
Salmonella Enteritidis PT4	-	-	-
Salmonella Typhimurium	-	-	-
Salmonella Agona	-	-	-
Salmonella Typhi	-	-	+
Salmonella Pullorum	-	-	-
Salmonella Paratyphi *B*	-	-	+

(+): lytic plaque; (-): no plaque.

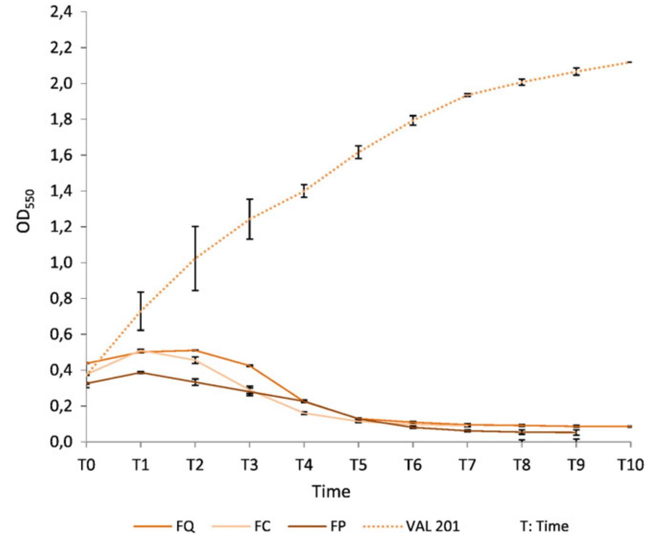

Fig. 4. Bacterial decay curves of VAL 201 with phages FQ, FC, and FP. Time intervals (T) of 30 min. The dotted line represents growth of a non-infected VAL 201 culture.

FC, FQ, and FP can be further distinguished by their tail morphology. We suggest that FC and FQ be classified under Myoviridae and FP under Siphoviridae [16].

It is well known that phages are host specific in their lytic activity, which depends on the presence of particular bacterial cell receptors and other factors that control the ability of bacteriophages to multiply in their hosts [17]. However, others [5,18] have argued that not all phages are host specific; moreover, polyvalent phages that are capable of proliferating in different bacterial genera have also been found. The latter were found in this study, as FC, FP, and FQ were capable of productively infecting strains of *E. coli* and *S. enterica*. This suggests the presence of common recognition sites for the three phages, at least in *E. coli* C and serovar Choleraesuis. Furthermore, this is also consistent with the close phylogenetic link between these two bacteria [19]. In addition, due to their polyvalent nature, of C, FP, and FQ can be propagated in the nonpathogenic host *E. coli* C, precluding potential risks involved in the use of bacteriophage preparations [7].

In summary, we have isolated and characterized new bacteriophages of the serovar Choleraesuis, which can be applied in various ways [20] in relation to this pathogenic bacterium.

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