Bacteriophage therapy of *Escherichia coli* O157:H7 experimental infection in mice

Running head: bacteriophage therapy

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Abstract

The study describes a phage lytic for *Escherichia coli* O157:H7. The phage was isolated from bovine manure. Upon selection in vivo, the phage acquired the capacity to persist in the circulatory system of mice for at least 38 days. The phage ($10^8$ pfu/mouse) sterilizes in 48 h mice infected with a lethal dose of *Escherichia coli* O157:H7 ($10^7$ cfu/mouse) and can save about 60% of the bacteremic mice. About 40% of the mice die, presumably as a consequence of the massive release of Shiga toxins that follows the rapid lysis of bacteria by the phage.

Keywords: Phage therapy; *Escherichia coli* O157:H7; in vivo phage selection; phage persistence in blood
Introduction

*Escherichia coli* serotype O157:H7 is a Shiga toxin-producing strain. In humans this pathogen can cause hemorrhagic colitis and potentially life-threatening sequelae, such as haemolytic uremic syndrome [1]. Healthy cattle are a major reservoir of *E. coli* O157:H7 [2]. Most food borne outbreaks have been traced to contaminated ground beef and raw milk [2]. Contaminated fruits, vegetables and water represent additional vehicles of infection [3]. Originally responsible of large outbreaks in North and South America, Europe, Japan and Australia [3, 4], *E. coli* O157:H7 epidemics have also occurred recently in developing countries [3]. This pathogen is a global public health concern.

At present the treatment of *E. coli* O157:H7 infection is only supportive. The use of antibiotics is in fact contraindicated [5]. Antibiotics can induce expression of the Shiga toxin gene carried by a prophage present on the bacterial chromosome [1, 6, 7]. Toxin-binding resins [6], recombinant bacteria expressing the receptor of the Gb₃ toxin [5] and a vaccine against the *E. coli* O157:H7 adhesion molecule intimin [8] represent possible future therapies.

This paper explores the capacity of phage to kill in vivo *E. coli* O157:H7. The destructive effect of phage on their hosts has been known since phage discovery. However, limited knowledge of phage biology and the advent of antibiotics [9] affected adversely the use of phage as antibacterial agents. Recent breakthroughs in the genetic manipulation of phage [10] and rigorous studies conducted in animals [11] have resurrected phage therapy. In this study we describe how the capacity of phage for rapid evolution can be exploited to surmount some of the limitations that phage display as therapeutic agents. An *E. coli* O157:H7 specific phage was isolated from bovine manure and manipulated by selection to persist in the circulatory system of an experimental animal. The phage thus obtained prevented death in about 60% of mice treated with a lethal dose of *E coli* O157:H7. Two
reports [12, 13] have already described the isolation of phage lysing *E. coli* O157:H7. Both reports exploited phage for the control of *E. coli* O157:H7 in foods.
METHODS

_Bacterial strains._  *E. coli* strains (Table 1) were grown from single colonies in LB medium at 37°C to an absorbance at 600 nm of 1 (equivalent approximately to $10^9$ cfu/ml). Cultures were centrifuged ($10^4$ x g for 10 min) and the pellet suspended in Ringer solution (Oxoid, Milan, Italy) at $10^9$ cfu/ml. Appropriate dilutions from this preparation were made in Ringer solution and used for in vivo and in vitro experiments. VT1 and VT2 toxin genes were detected by PCR [14].

_Mice._ Experiments were carried out on female Balb/c mice (8-10 weeks old) at the animal facility of the University of Naples. Phage ($\phi W$ and $\phi D$) were expanded on the non pathogenic *E. coli* strain 35218 and, before inoculation, were purified [15]. Phage and bacteria (*E. coli* strains 35150 or 35218) were inoculated intraperitoneally. Animal experiments were approved by the Animal Care Committee of the University of Naples.

_Phage isolation._ Fifty ml of fluid bovine manure were centrifuged at $10^4$ x g for 10 min. The supernatant was filtered through a 0.22 μm membrane (Millipore, Molsheim, France) and then treated with 10% chloroform. One ml of the chloroform free supernatant was diluted ten fold with LB broth (Oxoid, Milan, Italy) containing the bacterial host ($10^7$ cfu of *E. coli* 35150 or *E. coli* 35218) and incubated for 5 h at 37°C. One ml of the lysate was treated again with chloroform, mixed with soft agar and poured on agar plates containing host cells ($10^6$ cfu). Single plaques were suspended in 3 ml of LB medium containing the bacterial host ($10^6$ cfu) and incubated at 37°C overnight. Following treatment with chloroform, the lysate was added to one litre of LB broth infected with 10 ml of an overnight bacterial culture. The phage thus isolated was denominated $\phi W$ (W for wild).

_Phage selection for persistence in the circulatory system of mice._ First, the serial passage procedure [10] was used. Briefly, phage $\phi W$ ($10^8$ pfu/mouse) were inoculated into 3
mice. A blood sample was collected from each mouse 8, 12 or 24 h after inoculation; the samples of plasma (about 500 μl) were treated with chloroform and the phage expanded in bacteria. The phage persisting in the blood for progressively longer time were injected into a new group of 3 mice. This procedure proved unsatisfactory. The following protocol was therefore tried out.

Four mice were concurrently injected with phage \( \phi W \) \((10^8 \text{ pfu/mouse})\) and \( E. coli \) 35218 \((10^9 \text{ cfu/mouse})\). The phage were injected into one side of the abdomen and the bacteria into the other side. Another group of 4 mice was injected with phage \( \phi W \) \((10^8 \text{ pfu/mouse})\) and, at 12 h intervals, with 4 individual doses of \( E. coli \) 35218 \((2.5 \times 10^8 \text{ cfu/dose})\). Blood samples were collected periodically from the mice. The samples of plasma were treated with chloroform, serially diluted in LB broth and the phage titre determined. The phage isolated by this procedure was denominated \( \phi D \).

**Phage persistence in vivo.** Phage \( \phi W \) and \( \phi D \) were inoculated \((10^8 \text{ pfu/mouse})\) into separate groups of mice \((3 \text{ mice/group})\). The animals were sacrificed 6, 12 or 24 h after inoculation and the phage titre in the blood, liver and spleen was determined.

**Antibacterial activity of phage \( \phi W \) and \( \phi D \) in vivo.** Two groups of 4 mice each were inoculated with phage \( \phi W \) or \( \phi D \), respectively \((10^8 \text{ pfu/mouse})\). Immediately after mice were infected with \( E. coli \) 35150 \((10^7 \text{ cfu/mouse})\). The number of bacteria present 12, 24, 48 h post-infection in the feces, liver, spleen, and kidney was recorded. Organs and feces were weighed, homogenized, diluted with phosphate buffered saline (PBS) and plated. \( E. coli \) 35150 colonies were selected on sorbitol MacConkey agar plates [16].

One group of 32 mice was inoculated with \( E. coli \) 35150 only \((10^7 \text{ cfu/mouse})\); another group of 32 mice was inoculated with \( E. coli \) 35150 \((10^7 \text{ cfu/ml})\) and, immediately after, with \( \phi D \) \((10^8 \text{ pfu/mouse})\); a third group of 5 mice was inoculated only with the phage
(10^8 pfu/mouse). The mortality rate between mice treated with phage and those not treated was compared by the Student t test using the SPSS package.

**Phage characterization.** E. coli 35150 in exponential growth phase (10^7 cfu in 50 μl of sterile saline) were incubated with purified phage (10^7 pfu). Lysis was interrupted at timed intervals (10 sec, 1 min, 5 min) by the addition of glutaraldehyde and bacteria adsorbed onto 100 mesh carbon support films (Formovar, Chicago, USA). Purified phage were adsorbed directly onto support films. Samples were stained with uranyl acetate pH 4.5. Electron micrographs were taken with a transmission microscope (Philips EM 2085) equipped with a Megaview II camera (SIS, Münster, Germany) at 10^5 magnifications.

The gene coding for the phage major structural protein (msp) was detected as described [17]; phage DNA was analyzed using the Random Amplified Polymorphic DNA (RAPD) technique described by Malorny et al. [17] and the primers reported by Labrie and Moineau [18].
RESULTS

Toxicity of E. coli strains 35150 and 35218 in mice. Three groups of mice (4 mice/group) were infected with $10^6$, $10^7$, and $10^8$ cfu of *E. coli* strain 35150, respectively. While $10^6$ cfu did not kill any of the mice, $10^7$ and $10^8$ cfu killed all the mice, in 3-4 days. Ten more animals were infected with $10^7$ cfu of *E. coli* 35150; 9 died within the above time interval and 1 survived. The dose of $10^7$ bacteria was adopted as the minimum lethal dose for in vivo experiments.

Two more groups of mice (4 mice/group) were infected with *E. coli* strain 35218. One group received 4 doses of bacteria at 12 h intervals ($2.5 \times 10^8$ cfu/dose). Another group received 1 dose of $10^9$ cfu. During the next 4 weeks, both groups of mice did not show any apparent sign of disease.

Phage selection for persistence in the blood. The phage $\phi W$ was injected into 3 Balb/c mice ($10^8$ pfu/mouse). A blood sample was collected from each mouse 8, 12, or 24 h after the injection. The phage persisting longer in the blood was amplified in *E. coli* 35218 and transferred into a new group of 3 mice. The phage isolated after 5 passages remained in the blood of mice for 3 days and its titre was approximately $10^4$ pfu/ml. These results seemed unsatisfactory and the experiment was therefore interrupted.

Expansion in vivo of phage population on *E. coli* 35218 (the non pathogenic strain) was expected to favour the emergence of rare phage particles with the required characteristic (i.e. long persistence in blood). To test this hypothesis, 4 mice were injected with the phage $\phi W$ ($10^8$ pfu/mouse) and immediately after with a single dose of *E. coli* 35218 ($10^9$ cfu/mouse). Another group of 4 animals was injected with phage $\phi W$ ($10^8$ pfu/mouse) and, at 12 h intervals, with 4 doses of *E. coli* 35218 (individual dose: $2.5 \times 10^8$ cfu/mouse). Administration of the bacteria allowed the isolation of a phage strain that persisted in the
blood for 38 days. Bacteria were more effective when administered at 12 h intervals (Table 2).

This phage strain was expanded in vitro from a single plaque and denominated φD.

**Stability of φD.** The phage φD was injected into 3 Balb/c mice (10^8 pfu/mouse) and the phage titre monitored periodically. Blood samples collected from the mice 32 days after inoculation showed a titre of 10^8 to 10^9 pfu/ml, confirming the data reported in Table 2. The experiment established that the capacity acquired by φD in the course of the selection to persist in the circulatory system was stable.

**The fate of φW and φD in vivo.** Four mice were injected with 10^8 pfu of φW and another 4 with 10^8 pfu of φD. The phage particles present in the spleen, liver, and blood were counted 6, 24, and 48 h after inoculation. The results (Table 3) demonstrate that φD remains in the circulation for more than 1 month, while φW is rapidly trapped inside the spleen and liver (and possibly other organs). The mice maintained in vivo φD without displaying visible long term negative effects. LB broth was inoculated with feces (1 g) collected from mice inoculated with φD and non inoculated. The number of colonies varied in both groups of mice from 10^{11} to 10^{12}. Thus, multiplication of φD in vivo (Table 3) apparently did not cause substantial quantitative alterations of the natural microflora.

**Phage φD and φW share a common ancestry.** RAPD analysis of the wild-type (φW) and mutant (φD) phage strains revealed that they have similar – though not identical – patterns (Fig 3). In addition, the 2 strains display the same host range (Table 1) and both have the major structural protein gene (msp), unique to the phage family of the Syphoviridae (Fig 1). These data, taken together, demonstrate that φD is not a mere contaminant, but a derivative of φW. The data demonstrate also that φD, in the course of selection, has acquired new properties, at the level of DNA (an unique RAPD profile) and at the phenotypic level.
(prolonged permanence in the mouse circulation, and, as shown in the next section, higher virulence in vivo for their prokaryotic host).

**Characterization of \( \Phi D \).** The phage \( \Phi D \) lyses *E. coli* strains producing the toxins VT1 and/or VT2 as well as non enterohemorrhagic strains, such as *E. coli* 35218 (Table 1). Further properties of \( \Phi D \) are presence of the msp) gene (Fig 1), isometric head and contractile tail (Fig 2). Thus, \( \Phi D \) – conforming to the majority of phage [19, 20] - displays a chimeric origin, with ultrastructural features (contractile tail) of the Myoviridae [21] and molecular features (presence of the msp gene) of the Syphoviridae [18].

The phage collected 5 days after the first inoculation of mice with \( \Phi W \) and *E. coli* strain 35218 displayed the RAPD profile characteristic of \( \Phi D \). This phage sample was poured on soft agar and 10 individual plaques were amplified and tested by RAPD. All phage samples displayed the same profile. Thus, \( \Phi D \), not only emerged rapidly, but immediately become the dominant (and possibly the exclusive) phage strain present in the mouse circulation.

**Antibacterial activity of phage \( \Phi W \) and \( \Phi D \) in vivo.** Three groups of mice (3 mice/group) were injected with *E. coli* 35150 alone, with *E. coli* 35150 and \( \Phi W \), with *E. coli* 35150 and \( \Phi D \), respectively. The 3 groups of mice received the same number of phage particles (\( 10^8 \) pfu/mouse) and bacteria (\( 10^7 \) cfu/mouse). One mouse from each group was sacrificed at 24 h intervals and the number of bacteria infecting the liver, spleen, kidneys, and the feces was counted. Preliminary experiments showed that the liver and kidneys are the organs most heavily colonized by *E. coli* 35150. The results (Table 4) demonstrate that \( \Phi D \) is by far more efficient than \( \Phi W \) in killing the bacteria (48 h after inoculation with \( \Phi D \), *E. coli* 35150 became undetectable in the feces).

Attempts to save mice infected with *E. coli* 35150 were then carried out. Based on the results reported above, only phage \( \Phi D \) was used for this purpose. The phage (\( 10^8 \) pfu/mouse)
saved 60% of the infected mice. Survival curves of phage-treated and untreated mice were significantly different (P < 0.05) (Fig 4). During the observation period (4 weeks), the saved animals remained healthy and *E. coli* 35150 was not detected in the feces. Heating φD at 80°C for 20 min [21] however destroyed completely its protective ability. The evidence that φD must be alive to be effective indicates that the phage produces the protective effect by killing the bacteria, rather than inducing a non-specific immune response.
DISCUSSION

We used mice to examine the potential of phage therapy for *E. coli* O157:H7 infection. Application of phage therapy seemed particularly appropriate in circumstances (*E. coli* O157:H7 infection) where the usefulness of antibiotics is questioned. Initiation of this project also stemmed from the positive results phage therapy displayed against several *E. coli* pathogenic strains [11, 22].

Phage reproduce very rapidly inside the host and often a single dose is sufficient to counteract infection [23, 24, 25, 22]. Phage normally do not damage the natural bacterial flora, a characteristic particularly important in the case of enteric infections [9]. Even more significant is the evidence that phage [23] and phage lysins [26, 27, 28] are active against antibiotic-resistant bacteria.

However, if phage offer special opportunities, they also pose special problems. A major problem is caused by the immune system, that rapidly recognizes phage and clears them from the circulatory system [10, 29]. We solved this problem by exploiting the extraordinary diversity and abundance of phage [30, 19, 20] and the following capacity to respond rapidly to selection. First, we isolated from manure a phage lytic to pathogenic strains of *E. coli* (O157:H7 strains) as well as to non pathogenic strains (strain 35218). Given the huge size and diversity of phage population, the search for a phage with the proper host range was not difficult. Then we injected mice with the wild phage (ϕW) and, concurrently, with the *E. coli* strain 35218. In the presence of a bacterial host – sensitive to ϕW and non-pathogenic for the mouse – the phage multiplied to a high number without damaging the animal. From the greatly expanded phage population, mutants exhibiting the trait of interest (long persistence in the circulatory system) rapidly emerged. The procedure permitted the isolation in a few days of a phage mutant (ϕD) that persisted in the mouse circulatory system for more
than one month (Table 2). The approach, based on conventional breeding, is no less rapid or
powerful than methods based on genetic engineering. If of general applicability, this
procedure can improve substantially the curative potential of phage, extending it to chronic
bacterial infections and to the prophylaxis of bacterial infections.

The selection method adopted in this study provided the best results when bacteria
were administered over several days, rather than in one dose (Table 2). Presumably,
administration of bacteria in one dose helped mainly the propagation of wild phage particles,
many of which were subsequently purged in the liver and spleen. The administration of
bacteria in multiple doses allowed bacteria to remain in the circulatory system beyond the
time necessary for mutants to appear and propagate exponentially. These results demonstrate
that the presence of the bacterial host can help the phage with the appropriate characteristic to
emerge. The procedure does not require serial passages. This characteristic is also important
because it avoids loss of genetic variability while phage selection takes place. This loss
inevitably occurs in the case of selection by serial passages [10], where each passage becomes
a bottleneck for the mutant population size. Finally, the selection method described here may
be useful as an alternative, when selection based on serial passages is unsuccessful [31].

Selection for phage persistence in the circulatory system of mice correlated with
increased virulence of $\phi$D for its bacterial host. We did not investigate the nature of this
positive correlation. The most plausible suggestion that we can propose is that genetic drift or
hitchhiking determined the linkage between the two traits and their simultaneous evolution
[32].

Phage $\phi$D became rapidly dominant over $\phi$W. Five days after inoculation of mice with
$\phi$W and *E. coli* strain 35218, $\phi$D was the only circulating phage. From the HIV virus (the
only virus for which data on the relative fitness in vivo are available) we know that small
differences in fitness between strains – as small as 1% to 2% [33] – are sufficient to confer a
mutant significant replicative advantage. Very likely, the selective advantage of \(\phi D\) on the ancestral strain \(\phi W\) is represented by its capacity to evade the innate immune system.

The phage \(\phi D\) can kill \(E. coli\) O157:H7 in vivo very efficiently: within 48 h from inoculation, \(\phi D\) (\(10^8\) pfu/mouse) can achieve sterilization of the mice inoculated with \(E. coli\) O157:H7 (\(10^7\) cfu/mouse) (Table 4). However, \(\phi D\) can save from death only about 60% of the mice infected with the same number of bacteria (Fig 4). Thus, if the criterion of validation of therapy is sterilization of the animal, the therapy is highly effective; if the criterion is the percentage of animals saved from death, then phage therapy is only partially effective.

Following inoculation of \(E. coli\) O157:H7 and \(\phi D\), mice remain healthy for 24 to 48 h; in the next 48 to 72 h, about 40% of treated animals develop the symptoms (paresis, convulsions, cachexia and death) seen in mice treated with Shiga toxins [34, 35]. In these cases, failure of therapy cannot be attributed to the emergence of phage-resistant bacteria, nor to an inadequate distribution of phage in the tissues of the animal: no colonies of \(E. coli\) O157:H7 could in fact be isolated from mice infected with \(E. coli\) O157:H7 and \(\phi D\) (Table 4). A more likely explanation is that death is caused by the release of large quantities of Shiga exotoxins (and possibly endotoxin) following the rapid lysis of a large number of bacteria by the phage. This interpretation is reinforced by the evidence that the Shiga toxin gene is inducible by phage mediated lysis [36].

If we attribute correctly the partial failure of the therapy to the fast killing activity of \(\phi D\), then the next property we need to add to this phage is the capacity to kill the bacterial cell, leaving it intact. Non-replicating phage have already been obtained by genetic engineering and shown to be effective bactericidal agents [20, 37]. After Hagens and Blasi [38], we plan to insert a mutant holin gene or a restriction endonuclease gene into \(\phi D\). Given the extraordinary abundance of phage in nature, we will also attempt to isolate directly from the environment a phage with the property to kill, but not lyse, the target cell.
A serious concern about phage therapy is that lytic phage may facilitate the transfer of toxic genes from pathogenic bacteria to the commensal flora [37, 39, 22]. A non replicative form of $\phi$D therefore would be therapeutically more effective and, at the same time, safe with respect to the potential release of virulence genes.

In conclusion, this study provides tangible evidence of the potential of phage therapy, but also of its limitations. Phage can be modulated profoundly: by selection, as shown here, or by genetic engineering methods, as shown elsewhere [38, 40]. In this respect, phage represent a real alternative to chemical antibiotics [41]. At the same time, the phage-mediated release of toxins (endotoxin and exotoxins) is not a phenomenon restricted to the *E. coli* O157:H7 infection model described here; it occurs frequently in the course of phage therapy [42, 38, 39] and requires an urgent solution.
Table 1. *Escherichia coli* strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Sensitivity to</th>
<th>Verotoxin genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\phi W$</td>
<td>$\phi D$</td>
</tr>
<tr>
<td>10732$^a$</td>
<td>O1:K1:H7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10852$^a$</td>
<td>O7:K1:H7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>35218$^b$</td>
<td>NK$^c$</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>25922$^b$</td>
<td>NK$^c$</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8739$^b$</td>
<td>NK$^c$</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10798$^a$</td>
<td>O18:K5:H7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10799$^a$</td>
<td>O18:K5:H7</td>
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<td>+</td>
</tr>
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<td>10800$^a$</td>
<td>O18:K1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13526$^a$</td>
<td>O157</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8579$^a$</td>
<td>O157:H7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>35150$^b$</td>
<td>O157:H7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12900$^b$</td>
<td>O157:H7</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

$^a$ DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GMBH
$^b$ ATCC, American Type Culture Collection, USA
$^c$ NK, not known
Table 2. Persistence of phage φD in the circulation

<table>
<thead>
<tr>
<th>Phage</th>
<th>Bacterial dose (CFU/mouse)</th>
<th>Number of doses</th>
<th>Phage titer (PFU/ml)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>φD</td>
<td>10(^9)</td>
<td>1</td>
<td>4 x 10(^8) ± 1.2 x 10(^8)(^b)</td>
</tr>
<tr>
<td>φD</td>
<td>2.5 x 10(^8)</td>
<td>4</td>
<td>7 x 10(^9) ± 2.3 x 10(^9)(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Measured 38 days post phage injection.

\(^b\) Means ± standard deviation of titres of three mice.
Table 3. Distribution of phage $\phi_W$ and $\phi_D$ in vivo$^a$

<table>
<thead>
<tr>
<th>Phage</th>
<th>Time, h</th>
<th>Titer (PFU/gr)</th>
<th>Titer (PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>Spleen</td>
</tr>
<tr>
<td>$\phi_W$</td>
<td>6</td>
<td>$3.3 \times 10^7 \pm 1.1 \times 10^7$</td>
<td>$3.0 \times 10^7 \pm 2.6 \times 10^7$</td>
</tr>
<tr>
<td>$\phi_D$</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$\phi_W$</td>
<td>24</td>
<td>$3.6 \times 10^6 \pm 1.5 \times 10^6$</td>
<td>$3.3 \times 10^6 \pm 1.5 \times 10^6$</td>
</tr>
<tr>
<td>$\phi_D$</td>
<td>24</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$\phi_W$</td>
<td>48</td>
<td>$3.4 \times 10^8 \pm 1.2 \times 10^8c$</td>
<td>$3.9 \times 10^8 \pm 1.3 \times 10^8c$</td>
</tr>
<tr>
<td>$\phi_D$</td>
<td>48</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ Values are means ± standard deviation of three replicas.

$^b$ Phage number inoculated at time 0: $10^8$ pfu/mouse.

$^c$ Phage replicate in vivo, presumably infecting *E.coli* bacteria present in the intestinal tract.
Table 4. Antibacterial activity of phage \( \Phi W \) and \( \Phi D \) in vivo\(^a\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time, h</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Feces</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E. coli )</td>
<td>6</td>
<td>( 3.7 \times 10^8 )</td>
<td>( 3.5 \times 10^8 )</td>
<td>( 4.2 \times 10^8 )</td>
<td>( 4.5 \times 10^8 )</td>
</tr>
<tr>
<td>O157:H7(^b)</td>
<td></td>
<td>( \pm 1.2 \times 10^8 )</td>
<td>( \pm 1.2 \times 10^8 )</td>
<td>( \pm 1.7 \times 10^8 )</td>
<td>( \pm 2.4 \times 10^8 )</td>
</tr>
<tr>
<td>( E. coli )</td>
<td>6</td>
<td>( 7.3 \times 10^7 )</td>
<td>( 1.7 \times 10^7 )</td>
<td>( 4.2 \times 10^7 )</td>
<td>( 2.8 \times 10^7 )</td>
</tr>
<tr>
<td>O157:H7 + ( \Phi W )(^c)</td>
<td></td>
<td>( \pm 2.1 \times 10^7 )</td>
<td>( \pm 0.6 \times 10^7 )</td>
<td>( \pm 1.7 \times 10^7 )</td>
<td>( \pm 1.5 \times 10^7 )</td>
</tr>
<tr>
<td>( E. coli )</td>
<td>6</td>
<td>( 2.7 \times 10^6 )</td>
<td>( 3.3 \times 10^6 )</td>
<td>( 4.7 \times 10^6 )</td>
<td>( 4.2 \times 10^7 )</td>
</tr>
<tr>
<td>O157:H7 + ( \Phi D )(^c)</td>
<td></td>
<td>( \pm 2.1 \times 10^6 )</td>
<td>( \pm 1.5 \times 10^6 )</td>
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<td>( \pm 1.9 \times 10^6 )</td>
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<td>( E. coli )</td>
<td>24</td>
<td>( 2.6 \times 10^8 )</td>
<td>( 3.7 \times 10^8 )</td>
<td>( 3.5 \times 10^9 )</td>
<td>( 4.1 \times 10^9 )</td>
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<tr>
<td>O157:H7(^b)</td>
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<td>( \pm 1.5 \times 10^8 )</td>
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<td>( \pm 1.3 \times 10^9 )</td>
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<tr>
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<tr>
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<tr>
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<td>( 3.0 \times 10^2 )</td>
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<tr>
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<td>( \pm 1.5 \times 10^2 )</td>
<td>( \pm 3.0 \times 10^2 )</td>
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<tr>
<td>( E. coli )</td>
<td>48</td>
<td>( 5.3 \times 10^8 )</td>
<td>( 4.2 \times 10^8 )</td>
<td>( 7.2 \times 10^9 )</td>
<td>( 6.0 \times 10^9 )</td>
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<tr>
<td>O157:H7(^b)</td>
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<td>( \pm 2.5 \times 10^8 )</td>
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<td>( \pm 2.5 \times 10^9 )</td>
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<td>( E. coli )</td>
<td>48</td>
<td>( 4.0 \times 10^4 )</td>
<td>( 2.0 \times 10^4 )</td>
<td>( 7.0 \times 10^5 )</td>
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<td>( \pm 2.0 \times 10^5 )</td>
<td>( \pm 0.9 \times 10^5 )</td>
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</table>

\(^a\)Values are means ± standard deviation of three replicas.

\(^b\)10^7 cfu/mouse.

\(^c\)10^8 pfu/mouse.
FIGURES

Fig. 1
Fig. 4
FIGURE LEGENDS

Fig. 1. Presence of the major structure protein gene in the phage $\phi$W (lane a), $\phi$D (lane b), and phage $\Phi$7 (lanes c and d); phage $\Phi$7 was included as positive control [18]. M= 1 kb ladder. PCR fragments obtained using specific primers [18]. Fragments were separated on agarose gel and stained with ethidium bromide.

Fig. 2. Electron micrograph of phage $\phi$D stained with uranyl acetate. (A) Phage display isometric head and contractile tail. (B) Phage particle adhering to the bacterial surface. Magnification: $x$ $10^5$

Fig. 3. Phage $\phi$W (lanes: a, c, e, h) and $\phi$D (lanes: b, d, f, i) analyzed with 4 different random primers. M = 1 kb ladder. g = empty lane. Phages are similar, but not identical.

Fig. 4. Efficacy of phage therapy against $E. coli$ O157:H7. (A) Survival curve of mice (n=32) infected with $10^7$ cfu of $E. coli$ O157:H7, but not treated with $\phi$D. (B) Survival curve of mice (n=32) infected with $10^7$ cfu of $E. coli$ O157:H7 and immediately after treated with $10^8$ pfu of $\phi$D. Curves are significantly different (P <0.05). Mice treated with $10^8$ pfu of $\phi$D (n=5) remained healthy.
REFERENCES


