Introduction

The contamination of hard surfaces with pathogenic bacteria is a significant problem in many industries (e.g., in the food industry, where food processing equipment frequently becomes contaminated with foodborne bacteria) and civilian settings (e.g., ranging from hospital rooms to airplane interiors), and it would be of particular concern if “class A” agents were involved; i.e., pathogenic bacteria that can readily spread person-to-person and cause high mortality, public panic and social disruption (www.bt.cdc.gov/agent/agentlist-category.asp#a). Among the four bacterial species (Yersinia pestis, Bacillus anthracis, Francisella tularensis and Clostridium botulinum) currently classified as class A agents, Y. pestis (the etiologic agent of “plague”) is particularly significant because ca. 200 million deaths have been attributed to plague throughout recorded history. Also, Y. pestis is highly contagious and can spread rapidly person-to-person via the aerosol route, which makes containment difficult,1 and many strains have the ability to survive in various environmental niches where they can endure harsh conditions and persist for many years.2,3 Therefore, if such strains contaminate environments/surfaces to which humans are exposed, the public health consequences could be devastating. The problem is further exacerbated by the fact that decontamination of buildings, air handling systems, equipment and personnel contaminated with Y. pestis presents considerable challenges, particularly when time is of the essence to restore critical assets to functionality and traditional disinfection techniques may themselves damage or imperil those assets.

In this context, although chemical sanitizers can be effective in decontaminating various surfaces, they may be corrosive, which limits or sometimes completely rules out their use for certain materials and/or equipment.4 Thus, novel, so-called ‘Green’ approaches that are safe, environmentally friendly and effective are needed to deal with contamination by Y. pestis and other

Keywords: bacteriophage, phage, Yersinia pestis, surface decontamination

Five Y. pestis bacteriophages obtained from various sources were characterized to determine their biological properties, including their taxonomic classification, host range and genomic diversity. Four of the phages (Yp p-G, Y, R and Yps p-G) belong to the Podoviridae family, and the fifth phage (YpsP-PST) belongs to the Myoviridae family, of the order Caudovirales comprising of double-stranded DNA phages. The genomes of the four Podoviridae phages were fully sequenced and found to be almost identical to each other and to those of two previously characterized Y. pestis phages Yepe2 and ϕ A1122. However, despite their genomic homogeneity, they varied in their ability to lyse Y. pestis and Y. pseudotuberculosis strains. The five phages were combined to yield a “phage cocktail” (tentatively designated “YPP-100”) capable of lysing the 59 Y. pestis strains in our collection. YPP-100 was examined for its ability to decontaminate three different hard surfaces (glass, gypsum board and stainless steel) experimentally contaminated with a mixture of three genetically diverse Y. pestis strains CO92, KIM and 1670G. Five minutes of exposure to YPP-100 preparations containing phage concentrations of ca. 10⁹, 10⁸ and 10⁷ pFU/mL completely eliminated all viable Y. pestis cells from all three surfaces, but a few viable cells were recovered from the stainless steel coupons treated with YPP-100 diluted to contain ca. 10⁶ pFU/mL. However, even that highly diluted preparation significantly (p = < 0.05) reduced Y. pestis levels by ≥ 99.97%. Our data support the idea that Y. pestis phages may be useful for decontaminating various hard surfaces naturally- or intentionally-contaminated with Y. pestis.
class A bacterial pathogens and lytic bacteriophages, may be one such approach.

Bacteriophages (phages), or viruses that kill bacteria, were first identified in the early part of the 20th century by Frederick Twort and Felix D’Herelle, who called them bacteriophages or bacteria-eaters (from the Greek phage meaning to eat or to devour). Because of their remarkable antibacterial activity, phages were used to treat bacterial diseases of humans and agriculturally important animals almost immediately after their discovery. However, with the advent of antibiotics, therapeutic applications of phages were all but forgotten in the West, although they continued to be used to prevent and treat bacterial infections of humans in the former Soviet Union and some Eastern European countries (for a review, see ref. 6). In the former Soviet Union, phage preparations were also used to decontaminate hospital rooms contaminated with pathogenic bacteria (Mehipariani A, personal communication). However, with the exception of a few recent studies, rigorous scientific data demonstrating the efficacy of phage treatment for reducing bacterial contamination of inanimate, hard surfaces are sparse. Furthermore, to the best of our knowledge, such studies have not been performed for any of the bacteria currently classified as class A pathogens of high bioterrorism importance; e.g., Y. pestis. Hence, in this communication, we present the results of a ‘proof-of-concept’ study aimed at characterizing a cocktail of phages possessing potent lytic activity against Y. pestis, and determining its ability to decontaminate various hard surfaces experimentally contaminated with Y. pestis.

Results and Discussion

Bacteriophage taxonomy. Four of the five Yersinia phages (YpP-G, Y, R and YpsP-G) had capsids possessing icosahedral symmetry and a mean diameter of about 50 to 60 nm, had a short (5- to 10-nm-long) tail (Fig. 1), and were classified as members of the Podoviridae family of double-stranded DNA bacteriophages of the order Caudovirales. The fifth phage (YpsP-PST) had a capsid with icosahedral symmetry and a width and length of ca. 90 nm and 120 nm, respectively, and it had a long tail with tail fibers (Fig. 1). It was classified as a member of the Myoviridae family of double-stranded DNA phages of the order Caudovirales. Phage Y and R have been previously characterized by Knapp and Zwillenberg 1964;11 they belong to the Podoviridae family of double-stranded DNA phages. Myoviridae and Podoviridae phages are usually strongly lytic, and they are increasingly being used in various commercial phage cocktails, including those (1) cleared by the FDA and USDA for food safety applications (e.g., ListShield™; 21 CFR §172.785) and (2) used in the United States, during the first Phase I human clinical trial of a multivalent phage preparation for treating bacterial infections of wounds.12,13

Sensitivity of Y. pestis strains to the phages. The ability of the phages in our collection to lyse Y. pestis was examined by screening 59 strains of Y. pestis for their sensitivity to five phages by spot test assay at 28°C. The φA1122 phage (used by the CDC as a diagnostic agent for Y. pestis) was included, as the sixth, reference phage during our screening. All 59 strains were cleared by all six phages at a concentration of 10⁹ PFU/mL (data not shown) by spot test assay. The Y. pestis strains in our collection were isolated from various sources (rodents, fleas, soil, etc.) in various countries (the United States, Iran, Georgia, Azerbaijan, Armenia, etc.), and during a time period of more than three decades.14 Also, based on the analyses of their genomes they were found to be fairly heterogeneous (in relative terms, given the fact that Y. pestis is a genetically very homogeneous species14). The observation that
our phages killed 100% of the Y. pestis strains we tested suggests that they have a broad host range and are well suited for lysing Y. pestis strains, irrespective of the strains’ origin and geographic distributions.

Sensitivity testing was also performed with significantly more dilute phage preparations containing ca. 10^3 PFU/mL (Table 1), which favors identifying the most potent bacteriophages. Some of the phages in our collection did not lyse some of the Y. pestis strains at lower concentration even though they lysed at higher concentrations; e.g., YpP-G did not produce a clear spot against Y. pestis strain 1853G when used at a concentration of ca. 10^4 PFU/mL, but it produced a clear spot with the same strain when used at a concentration of ca. 10^3 PFU/mL.

In order to validate their specificity, the phages (at a concentration of ca. 10^3 PFU/mL) were also used to screen a collection of non-Y. pestis strains by spot test assay (Table 2). Lytic phages are known to be highly specific for their hosts; i.e., they usually only lyse strains or a subgroup of strains within the same species or some very closely-related species.15–19 Our results are in general agreement with that previously well-documented property of phages. For example, none of the six Y. pestis phages lysed strains of Listeria monocytogenes, a Gram-positive bacterium that is genetically distinct from Y. pestis. Also, cross-genera lytic activity was not observed against the V. cholerae and Salmonella Enteritidis strains we tested. However, some of the phages lysed some strains of other genera. Strains of Shigella sonnei were particularly sensitive; e.g., all of the six Y. pestis phages lysed four of the five S. sonnei strains we tested. Also, surprisingly (because Y. pseudotuberculosis and Y. pestis are very closely-related species), the Y. pestis phages cross-lysed fewer Y. pseudotuberculosis strains than they did S. sonnei strains. For example, two of the six phages (ϕA1122 and YpP-G) did not lyse any of the eight Y. pseudotuberculosis strains we examined, and the remaining four phages lysed only a few of the Y. pseudotuberculosis strains. These results are in agreement with those of an earlier publication20 which reported that a diagnostic phage for Y. pestis was active against several serotypes of Shigella, such as S. dysenteriae 1 and 9, S. flexneri 2a, S. boydii 1 and 6 and S. sonnei. A possible explanation for this phenomenon is that Shigella spp and Y. pestis share a common receptor, which enables Y. pestis phages to attach to and lyse Shigella strains possessing that receptor, and vice-versa. Additional studies are needed to address this hypothesis; however, for immediate practical purposes, this potential for cross-genera lytic activity must be kept in mind when using Y. pestis phages for diagnostic purposes. Under the conditions examined, one phage (YpP-G), of the two phages (YpP-G and YpS-P) that are components of Y. pestis diagnostic phage preparations produced in the former Soviet Union was the most specific to Y. pestis; i.e., it only lysed 4 of the 38 non-Y. pestis strains we tested. Its specificity was closely followed by the CDC’s diagnostic phage ϕA1122, which lysed 6 of the 38 non-Y. pestis strains. Neither phage lysed any of the eight Y. pseudotuberculosis strains at 25°C (Table 2).

Sensitivity testing was repeated for YPP-100 (ca. 10^4 PFU/mL) that included YpP-G, Y, R and YpS-PST phages. When YPP-100 was tested against each of the 59 Y. pestis strains in our collection, it lysed 100% of them, which suggests that there were no

| Strains | YpS-P-G | Y | R | YpS-PST | YpP-G | ϕA1122 |
|---------|---------|---|---|---------|-------|--------|
| 1392G   | +       | + | + | -       | -     | +      |
| 1412G   | +       | + | + | -       | -     | +      |
| 1413G   | +       | + | + | +       | -     | +      |
| 1670G   | +       | + | + | -       | +     | -      |
| 1851G   | +       | + | + | +       | -     | +      |
| 1852G   | +       | + | + | +       | -     | +      |
| 1853G   | +       | + | + | +       | -     | +      |
| 1952G   | +       | + | + | -       | +     | +      |
| 1953G   | +       | + | + | +       | +     | -      |
| 1954G   | +       | + | + | -       | +     | +      |
| 3064G   | +       | + | + | -       | -     | +      |
| 3065G   | +       | + | + | +       | -     | +      |
| 3066G   | +       | + | + | +       | +     | -      |
| 3067G   | +       | + | + | -       | -     | +      |
| 3073G   | +       | + | + | -       | -     | +      |
| 3082G   | +       | + | + | +       | +     | +      |
| 3083G   | +       | + | + | +       | -     | +      |
| 3758G   | +       | + | + | +       | +     | -      |
| 3768G   | +       | + | + | +       | +     | -      |
| 3770G   | +       | + | + | +       | +     | -      |
| 8788G   | +       | + | + | +       | -     | +      |
| 8789G   | +       | + | + | +       | -     | +      |
| 8790G   | +       | + | + | +       | -     | +      |
| 8791G   | +       | + | + | +       | -     | +      |
| 8792G   | +       | + | + | +       | +     | +      |
| 8793G   | +       | + | + | +       | +     | +      |
| 8794G   | +       | + | + | +       | +     | +      |
| 8907G   | +       | + | + | +       | +     | +      |
| 8908G   | +       | + | + | +       | +     | +      |
| C1473S  | +       | + | + | +       | +     | +      |
| C1522   | +       | + | - | +       | -     | +      |
| C2614   | +       | + | - | +       | -     | +      |
| C2944   | +       | + | - | +       | +     | -      |
| C104S   | +       | + | - | +       | +     | -      |
| C790    | +       | - | + | +       | +     | -      |
| 771G    | +       | - | + | +       | +     | -      |
| 1390G   | +       | - | + | +       | +     | -      |
| 1391G   | +       | + | + | +       | +     | -      |
| 1393G   | +       | + | + | +       | +     | +      |
| 1952G   | +       | + | + | -       | -     | +      |
| 2095G   | +       | + | + | +       | -     | +      |
| 3072G   | +       | + | + | -       | +     | -      |
| 3757G   | +       | + | + | +       | -     | +      |
| 3769G   | +       | + | + | -       | +     | -      |
| 8786G   | +       | + | + | +       | +     | -      |
| 8787G   | +       | + | + | +       | -     | +      |
| 8906G   | +       | + | + | +       | -     | +      |
| NR 15   | +       | + | + | -       | +     | -      |
interactions among component monophages that deleteriously affected the host range of YPP-100. Phage preparations composed of two or more phages are generally considered to be best suited for many practical applications, including the treatment of foods that may be contaminated with foodborne bacterial pathogens, and for treating bacterial diseases of humans and domesticated livestock. Two reasons have been proposed for that idea: (1) phage cocktails are likely to have a broader host range than do single phage-containing preparations, and (2) if two or more phages lyse the same bacterial strain after attaching to different surface receptors, that redundancy reduces the risk of bacterial resistance emerging against the phage cocktail, thus improving its long-term efficacy. All of our subsequent efficacy studies were performed with the YPP-100 phage cocktail, as described below.

**Genome composition.** Four of the five phages were sequenced to an average depth of 94, 1341, 1043 and 330 for Y, YpsP-G, R and YpP-G, respectively. Despite several attempts, we were unable to fully-assemble and sequence YpsP-PST’s genome because of intrinsic issues with its DNA. Based on the small fragments of available sequence data for YpsP-PST (data not shown), the phage appears to be closely related to Enterobacteria phage RB14 and Enterobacteria phage AR1, which are T4-like phages. The genomic sequences of the four phages were compared, by the BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST/), to previously sequenced phage genomes available in GenBank. YpsP-G and R were 99% identical (with a query coverage of 97%) to the previously sequenced *Y. pestis* diagnostic phage φA1122. Y was > 99% identical to φA1122 (with a query coverage of 99%), and YpP-G was > 99% identical to the previously sequenced phage Yepe2, with a genome coverage of 99%. Furthermore, comparative analyses performed with the Artemis comparison tool (ACT) revealed that three of the four phages we sequenced (Y, R and YpsP-G) are almost identical to each other, and that the fourth phage (YpP-G) is also related to them (Fig. 2A). Also, the phylogenetic tree of the four phages (Fig. 2B) supports their very high relatedness.

The genome sizes and G+C contents of the four sequenced phages were as follows: (1) YpsP-G: a 38.23-kb genome with a GC content of 48.22%, (2) Y: a 37.71-kb genome with a GC content of 48.35%, (3) R: a 38.28-kb genome with a GC content of 48.34% and (4) YpP-G: a 39.41-kb genome with a GC content of 47.24%. For comparison, φA1122 has a 37.55-kb genome with a GC content of 48.32%. As mentioned above, all of our *Y. pestis* phages are very similar to each other; therefore, the small variations among them probably are due to minor genetic rearrangements in their genomes. However, those minor variations resulted in some differences in their host ranges. For example, although YpsP-G was 99% similar to φA1122, four coding sequences in YpsP-G were not found in φA1122. Those codons encoded four hypothetical proteins and hypothetical 6.3 protein were different from the codon that is present in φA1122 by having ten additional amino acids. Also, the tail fibers and host specificities of the two phages differed; e.g., YpsP-G lysed several *Y. pseudotuberculosis* strains, but φA1122 did not lyse any of them (Table 2). Phage tail fibers are known to be responsible for phage-host bacteria recognition which could explain the differences in their host ranges.

Genomic analyses of the four phages enabled us to determine their genetic relatedness and provided us with some basic information about their genome sizes and G+C contents. Another important consideration was to determine whether any of the phages contained any “undesirable” genes identified by the Food and Drug Administration (FDA) (40 CFR § 725.421), because any phages found to contain such genes should be excluded from commercial phage preparations developed for food safety-enhancing, surface decontamination and human clinical applications. None of our four fully-sequenced phages contain undesirable genes (at an estimated cut-off e-value of ≤ 10⁻⁵), thus making them well-suited for inclusion in phage preparations that may be further developed and evaluated for various practical applications. An e-value of 0 indicates a perfect match/absolute identity between the test sequence and a sequence in the database; however, in practice, significant matches are considered to be those with e-values ≤ 10⁻⁵. The cut-off e-value for our analysis was one order of magnitude more stringent at ≤ 10⁻⁴, which provided strong assurance that undesirable genes were not missed.

**Surface decontamination by YPP-100.** For the proof-of-concept, “surface decontamination” studies we used three matrices (glass coverslips, gypsum board and stainless steel coupons) to mimic some of the hard surfaces likely to be encountered in real-life settings. The details of the experimental design are presented in Materials and Methods. Briefly, after contaminating the surfaces with a mixture of three genetically-distinct strains of *Y. pestis*, the matrices were treated with undiluted YPP-100 containing ca. 10⁹ PFU/mL, stored at room temperature for 5 min. The free phages (i.e., phages unattached to bacteria adhering to the matrices’ surfaces) were removed by washing with PBS, and the concentrations of viable *Y. pestis* remaining on the surfaces were determined. The YPP-100 treatment completely eliminated *Y. pestis* from all three matrices; i.e., viable *Y. pestis* were not recovered from any of the YPP-100-treated groups (Fig. 3A–C). The sensitivity of the assay was one *Y. pestis* cell/20 mL of PBS (see Materials and Methods); thus, even if we assume that some viable bacteria remaining on the surfaces were not detected by our

| Table 1 (continued). Lytic activity of diluted phage preparations (approximately ca. 100 PFU per spot) against 59 strains of *Y. pestis* |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Strains | YpsP-G | Y | R | YpsP-PST | YpP-G | φA1122 |
| NR 16 | + | + | + | - | + | + |
| NR 17 | + | + | + | - | + | + |
| NR 18 | + | + | + | - | + | + |
| NR 20 | + | + | + | - | + | + |
| NR 635 | + | + | + | - | + | + |
| NR 636 | + | + | + | - | + | + |
| NR 637 | + | + | + | - | + | + |
| NR 638 | + | + | + | - | + | + |
| NR 639 | + | + | + | - | + | + |
| NR 640 | + | + | + | - | + | + |
| NR 641 | + | + | + | - | + | + |
| NR 642 | + | + | + | - | + | + |
the concentrations of viable \textit{Y. pestis} recovered from those two control groups were not significantly different, it would indicate that the filtration step effectively removed phage unattached to bacteria from the Nalgene filters after the specified contact time (i.e., 5 min in our experiments) between the \textit{Y. pestis}-contaminated matrices and the YPP-100 phages—and, therefore, the data accurately represent the \textit{Y. pestis} killed during 5 min of exposure to YPP-100. On the other hand, the observation that the number of viable \textit{Y. pestis} cells decreased by at least 99.99% and would be statistically significant (p $\leq$ 0.05).

A separate control group (the “neutralizer control”) was used to verify that the reduction in the number of viable \textit{Y. pestis} cells resulted from a 5 min interaction with YPP-100 rather than interaction with YPP-100 during 24 to 48 h incubation of the filters on BHI agar. The design of the neutralizer control study is described, in detail, in Materials and Methods. The idea behind the neutralizer control was to compare the levels of viable \textit{Y. pestis} recovered from the PBS and neutralizer control groups. If the concentrations of viable \textit{Y. pestis} recovered from those two control groups were not significantly different, it would indicate that the filtration step effectively removed phage unattached to bacteria from the Nalgene filters after the specified contact time (i.e., 5 min in our experiments) between the \textit{Y. pestis}-contaminated matrices and the YPP-100 phages—and, therefore, the data accurately represent the \textit{Y. pestis} killed during 5 min of exposure to YPP-100. On the other hand, the observation that the number of viable \textit{Y. pestis} decreased by at least 99.99% and would be statistically significant (p $\leq$ 0.05).

### Table 2. Lytic activity of diluted phage preparations (containing ca. 10^9 PFU/mL) against 38 non-\textit{Y. pestis} strains

| Strains                        | YpsP-G | Y | R | YpsP-PST | YpsP- G | ϕA1122 |
|--------------------------------|--------|---|---|----------|---------|--------|
| \textit{E. coli} AH237-3       | -      | - | - | -        | -       | -      |
| \textit{E. coli} AH 1444       | -      | - | - | -        | -       | -      |
| \textit{E. coli} AH 1383       | -      | - | - | -        | -       | -      |
| \textit{E. coli} AH2941-1      | -      | - | - | -        | -       | -      |
| \textit{E. coli} ATCC 35401    | +      | + | + | +        | -       | +      |
| \textit{Klebsiella pneumoniae} env 1 | -      | - | - | -        | -       | -      |
| \textit{Klebsiella pneumoniae} env 7 | -      | - | - | -        | -       | -      |
| \textit{Klebsiella pneumoniae} env 10 | -      | - | - | -        | -       | -      |
| \textit{Klebsiella pneumoniae} env 11 | -      | - | - | -        | -       | -      |
| \textit{Klebsiella pneumoniae} env 17 | +      | + | + | +        | -       | +      |
| \textit{Salmonella} Enteritidis 250/23 | -      | - | - | -        | -       | -      |
| \textit{Salmonella} Enteritidis 253/26 | -      | - | - | -        | -       | -      |
| \textit{Salmonella} Enteritidis 261/34 | -      | - | - | -        | -       | -      |
| \textit{Salmonella} Enteritidis 270/43 | -      | - | - | -        | -       | -      |
| \textit{Salmonella} Enteritidis 274/47 | -      | - | - | -        | -       | -      |
| \textit{Shigella} sonnei ATCC 9290 | -      | - | - | -        | -       | -      |
| \textit{Shigella} sonnei 543   | +      | + | + | +        | +       | +      |
| \textit{Shigella} sonnei 544   | +      | + | + | +        | +       | +      |
| \textit{Shigella} sonnei 545   | +      | + | + | +        | +       | +      |
| \textit{Shigella} sonnei 546   | +      | + | + | +        | +       | +      |
| \textit{Vibrio cholerae} env   | -      | - | - | -        | -       | -      |
| \textit{Vibrio cholerae} O395  | -      | - | - | -        | -       | -      |
| \textit{Vibrio cholerae} N16961 | -      | - | - | -        | -       | -      |
| \textit{Vibrio cholerae} O139  | -      | - | - | -        | -       | -      |
| \textit{Vibrio cholerae} non O1 | -      | - | - | -        | -       | -      |
| \textit{Listeria monocytogenes} env 1 | -      | - | - | -        | -       | -      |
| \textit{Listeria monocytogenes} env 3 | -      | - | - | -        | -       | -      |
| \textit{Listeria monocytogenes} env 6 | -      | - | - | -        | -       | -      |
| \textit{Listeria monocytogenes} env 7 | -      | - | - | -        | -       | -      |
| \textit{Listeria monocytogenes} env 11 | -      | - | - | -        | -       | -      |
| \textit{Yersinia pseudotuberculosis} ATCC 23207 | -      | - | - | +        | -       | -      |
| \textit{Yersinia pseudotuberculosis} NR4371 | +      | + | + | +        | -       | -      |
| \textit{Yersinia pseudotuberculosis} NR4372 | -      | - | - | -        | +       | -      |
| \textit{Yersinia pseudotuberculosis} NR4373 | +      | - | - | +        | -       | -      |
| \textit{Yersinia pseudotuberculosis} NR4374 | +      | - | - | +        | -       | -      |
| \textit{Yersinia pseudotuberculosis} NR4375 | -      | - | - | +        | -       | -      |
| \textit{Yersinia pseudotuberculosis} NR4380 | +      | - | + | +        | -       | -      |
| \textit{Yersinia pseudotuberculosis} NR804 | +      | + | + | +        | -       | -      |
of *Y. pestis* recovered from the PBS “non-active control” group was significantly more than that recovered from the neutralizer control would suggest that the filtration step did not remove free phages after contact between the contaminated matrices and YPP-100. In our study, the concentrations of viable *Y. pestis* recovered from the PBS and neutralizer control groups were not significantly different ($p \geq 0.05$) from one another (Fig. 3). This observation suggests that the filtration step employed during our neutralizer control testing effectively removed most of the free phages from the filters, at least to a level that did not significantly impact the outcome of the testing. Therefore, we believe that the data presented in Figure 3 accurately reflect *Y. pestis* acquiring phages in YPP-100 during 5 min contact time resulting in subsequent killing *Y. pestis*.

Subsequent studies examined the ability of three 10-fold, serial dilutions (containing ca. $10^8$, $10^7$ and $10^6$ PFU/mL) of YPP-100 to decontaminate stainless steel coupons experimentally contaminated with *Y. pestis* (Fig. 4). Treatment with the two least-diluted preparations ($10^8$ and $10^7$ PFU/mL) eradicated *Y. pestis* from the coupons, but a small number of viable cells were recovered from the coupons treated with YPP-100 diluted to contain $10^6$ PFU/mL. However, even treatment with that most-diluted preparation significantly reduced the viable *Y. pestis* levels of the coupons by 99.97% ($p \leq 0.05$). This is an outstanding efficacy which, though, is difficult to explain by simple “phages lyse their targeted bacteria” mechanism because, based on the density of phages (determined in PFU/mL) and the bacteria we used during our studies, we cannot mathematically explain the observed dramatic reduction in recoverable *Y. pestis* cells from the hard surfaces. Also, for comparison, in similar experiments with *E. coli* O157:H7 the percentage of reduction observed by Abuladze et al. 2008 was only 94% even when they used a much more concentrated phage cocktail ($10^8$ PFU/mL). The higher-than-expected efficacy in surface decontamination we observed with YPP-100 may be due to various factors, including YPP-100 having a higher killing titer compared with its plaque titer. The outcome of the treatment was highly reproducible which suggest that the data are robust; the mechanisms responsible for the better then expected efficacy (including whether or not higher killing titer was responsible for the observed outcome) will need to be evaluated in subsequent studies.

Phage preparations have been used to decontaminate pathogenic bacteria-contaminated hospital rooms in the former Soviet Union, and several recent studies have demonstrated the ability of phage cocktails to reduce significantly the contamination of food and hard surfaces by foodborne bacterial pathogens. In addition, at least one recent publication has suggested that bacteriophages could be used to remove bacterial biofilms from

![Figure 2](image_url)

**Figure 2.** (A) Genome comparison of 5 *Y. pestis* lytic phages using Artemis comparison tool. Red lines indicate direct match and blue lines indicate reverse complimentary match; (B) Weighted phylogenetic tree of the eight *Y. pestis* phages.
food processing environments. To the best of our knowledge, however, the present communication is the first report that describes the ability of *Y. pestis*-specific phages to eliminate *Y. pestis* contamination from various hard surfaces, which may have some significant practical implications. For example, the corrosive nature of many chemical sanitizers often precludes their use on certain materials and/or equipment; whereas, YPP-100 is a noncorrosive aqueous preparation suitable for application to hydrophilic/nonhydrophobic surfaces. In a very different, but somewhat related context, the same or similar phage preparation may also be useful for preventing and treating plague, especially if the infecting strains are resistant to multiple antibiotics or have epitopes that are different from those against which current vaccine development efforts are focusing.

**Materials and Methods**

**Bacteriophages.** Our collection of *Y. pestis* phages included 6 bacteriophages (Y, R, YpsP-PST, YpP-G, YpsP-G and ϕA1122), three of which (Y, R and YpsP-PST) were obtained from the American Type Culture Collection (ATCC): ATCC 23053-B1, ATCC 23208-B1 and ATCC 23207-B1, respectively. Two phages (YpP-G and YpsP-G) were components of diagnostic phage preparations produced in the former Soviet Union and used to identify *Y. pestis*, and they were kindly supplied by Dr. Nikoloz Tsartsvadze (National Center for Disease Control and Public Health). ϕA1122, the plague-diagnostic phage used at the US Centers for Disease Control and Prevention, was used as a reference phage in our host range studies characterizing the phages’ lytic activity against *Y. pestis* and non-*Y. pestis* strains. Each phage had a distinct restriction fragment length polymorphism (RFLP) profile (data not shown). After propagating the phages in their appropriate *Y. pestis* or *Y. pseudotuberculosis* host strains growing in brain heart infusion (BHI) broth, the bacterial debris were removed by centrifugation and membrane filtration, and phage stocks were stored (-20°C) in BHI broth supplemented with 15% (v/v) glycerol. YPP-100 was prepared by mixing five phages (YpsP-G, Y, YpP-G, R and YpsP-PST) in equal concentrations, to yield a phage cocktail with a mean titer of ca. 10⁹ PFU/mL. The preparation was stored refrigerated (2 to 4°C) until used.

**Bacterial strains.** All experiments with viable *Y. pestis* were performed in a BSL-3 laboratory at the EPI-UF. A total of 59 *Y. pestis* strains and 38 non-*Y. pestis* strains were used in our studies. Forty-six of the *Y. pestis* strains were described in our previous publication, and the remaining 13 *Y. pestis* strains were obtained from BEI Resources. Also, our “hard

![Figure 3. Decontamination of *Y. pestis*-contaminated hard surfaces treated with YPP-100 containing ca. 10⁹ PFU/mL. (A) gypsum board, (B) glass and (C) stainless steel.](image-url)
surface decontamination” studies of YPP-100 utilized a mixture of three genetically unrelated strains of Y. pestis (CO92, KIM and 1670G). Mid-log phase cultures of each strain were obtained by incubating (28 ± 2°C, 48 ± 2 h) BHI agar plate streaked with a specimen of a frozen stock culture, followed by incubating (28 ± 2°C, 18 h, shaking at 200 rpm) BHI broth (10 ml) inoculated with a well-isolated colony from the BHI agar plate. A uniform suspension of the bacterial preparation needed to contaminate the surfaces of the hard matrices was obtained by (1) vortexing (15 sec) a mixture (15 ml, total volume) of the three mid-log phase cultures in a conical tube (50-mL capacity), and (2) adjusting the suspension (with PBS) to contain ca. 10^7 CFU/mL. The bacterial suspension was used immediately to contaminate the hard matrices, or it was placed on ice for not more than 10 min before being used.

Electron microscopy. Phage particles were negatively stained with 1% uranyl acetate and examined with a Hitachi H-7000 transmission electron microscope. Their taxonomic assignments were made according to the phage classification scheme developed by Ackermann and Berthiaume.34

Host range determinations. The ability of the six Y. pestis phages in our collection to lyse 59 Y. pestis strains was determined by the classical spot test assay,35 using phage stocks diluted to contain ca. 10^9 PFU/mL and 10^4 PFU/mL. Briefly, soft agar “lawns” of the “test bacteria” were incubated (24 h, 28°C) after spotting with aliquots (10 µL) of the diluted phage preparations. The results were recorded as “+” if a clear spot was observed, and as “−” if clear spot was not observed. The assays were performed three times with 10^9 PFU/mL and two times with 10^4 PFU/mL. Also, the six Y. pestis phages’ specificity; i.e., their inability to lyse strains other than those of Y. pestis, was determined with a collection of 38 non-Y. pestis strains and YPP-100 containing ca. 10^9 PFU/mL. Those test strains included 5 strains of Escherichia coli, Klebsiella pneumonia, Salmonella enterica serotype Enteritidis, Shigella sonnei, Vibrio cholerae and Listeria monocytogenes and 8 strains of Y. pseudotuberculosis. The reproducibility of the results was confirmed by performing the host range determinations three times.

Genome sequencing and bioinformatics analyses. The genome sequences of YpsP-G, Y, R and YpP-G were determined (at the NMRC) by pyrosequencing technology, with Roch/454 Life Sciences GS20 Sequencer. Purified DNA from phage specimens was sequenced to an average depth of coverage of > 90× (reads per base). The sequence fragments were assembled into large contigs, and the gaps in the sequences were filled by PCR amplification and Sanger sequencing. The completed genomes were annotated with Rapid Annotation using Subsystem Technology (RAST), and they were compared with one another by the Artemis comparison tool (www.sanger.ac.uk/resources/software/act/). The nucleotide sequences of the phage genomes were aligned using Lasergene MegAlign software, and a phylogenetic tree was drawn using the DNASTAR program (DNASTAR, Inc.). The phage sequences have been deposited with the GenBank, under accession numbers JQ965703: YpsP-G, JQ965700: Y, JQ965701: R and JQ965702: YpP-G.

General design of the studies utilizing Y. pestis-contaminated hard surfaces. The studies were conducted essentially as described previously for E. coli O157:H7-specific phages.8 The ability of YPP-100 to reduce or eliminate Y. pestis contamination of hard surfaces was examined using three hard, inanimate surfaces (all

---

**Figure 4.** Effect of YPP-100s phage concentration on reducing Y. pestis contamination of stainless steel surfaces.
ca. 25 × 25 mm in size): glass coverslips, gypsum board (drywall board) slips and stainless steel coupons. The stainless steel coupons and glass coverslips were sterilized in an oven for ≥ 30 min and allowed to cool before use, and the gyspum board slips were autoclaved for 90 min with a 20 min drying cycle. Before each study, all of the matrices were pretreated with 5% (wt/vol) skim milk to “dirty” their surfaces, in order to mimic real-life settings where surfaces are often covered with dried organic matter.

Three experimental groups were examined during our studies of each of the Y. pestis-contaminated matrices, and all tests were done in triplicates. The matrices in all three groups were contaminated with Y. pestis by pipetting aliquots (10 μL) of the bacterial culture containing 10^7 PFU/mL onto their surfaces and spreading them using pipet tip, before allowing the inoculums to dry at room temperature for 15 to 25 min, or until visibly dry. The matrices in Group 1 were not treated with anything and served as the “dry control,” the matrices in Group 2 were treated with PBS (0.1 mL/matrix) and were designated the “non-active control,” and each matrix in Group 3 was treated with ca. 0.1 mL of YPP-100 containing ca. 10^9 PFU/mL, resulting in a final phage concentration of ca. 10^6 PFU/matrix. After storage (5 min at room temperature), the excess PBS and YPP-100 was removed from the matrices in Groups 2 and 3, respectively, by holding them vertically for 20–30 sec and allowing the excess liquid to drain into a disinfectant solution. Subsequently, the test and control matrices were mixed gently (30 sec) in separate conical tubes (50-mL capacity) containing peptone water (20 mL), three 10-fold serial dilutions (10^−1, 10^−2 and 10^−3) were prepared with peptone water, the undiluted and diluted mixtures were immediately passed through separate membrane filters (0.45-μm pore-size), and each filter was washed with PBS (20 mL) to unattach phages. The washed filters were placed upside down on BHI agar in separate Petri dishes, incubated (28°C, 24 to 48 h), and the number of recovered Y. pestis was enumerated by counting the colonies that grew on the filters. Since the entire 20-mL volumes of peptone water were filtered through the Nalgene membrane filters, the resulting counts represented the total CFU recovered from each of the tested hard surfaces; i.e., the test’s detection limit was 1 cell per 20 mL. The phage dilution experiments were performed as described above, except (1) only steel coupons were used, and (2) three 10-fold dilutions of YPP-100 (containing ca. 10^6, 10^5 and 10^4 PFU/mL) were used to treat the matrices.

The neutralizer test also was done as described in the above-cited study. Briefly, after 5 min of contact time with YPP-100 and removing the excess YPP-100 as described above, each of the phage-treated matrices was mixed (30 sec) gently in separate conical tubes (50-mL capacity) containing peptone water (20 mL), the mixtures were passed through membrane filters (0.45-μm pore-size) and the filters were washed with PBS (20 mL). The washed filters were placed in separate conical tubes containing peptone water (20 mL) and the Y. pestis-contaminated matrices from the neutralizer control group were added to the appropriate tubes. After the tubes were vortexed (30 sec) gently, three 10-fold serial dilutions (10^−1, 10^−2 and 10^−3) of the mixtures were passed through a new set of membrane filters (0.45-μm pore-size) designated the “neutralizer filters.” After the neutralizer filters were washed with PBS (20 mL), each of them was placed upside down on BH agar in a separate Petri dish, and the number of recovered Y. pestis was enumerated by counting the colonies that grew on the filters after incubation (28°C, 24 to 48 h).

**Statistical analyses.** Statistical analyses were performed using online statistical tools available at VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html). One-way analysis of variance (ANOVA) and Tukey Honestly Significant Difference (HSD) tests were used to determine whether the observed differences in Y. pestis recoveries from the YPP-100-treated and PBS-treated hard surfaces were statistically significant. Similar tests were performed for the number of viable bacteria in the neutralization treatment and carrier treatment. p values of < 0.05 were considered statistically significant.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Dr. Nikoloz Tsutsadze for providing the YpP-G and YpsP-G phages, and Dr. Arnold Kreger for editorial assistance. Amiram Meiharian is acknowledged for his discussions about using phages to decontaminate hospital rooms in the former Soviet Union. A.S. holds an equity stake in Intralytix, Inc., a Maryland corporation that develops therapeutic phage preparations. The US. Environmental Protection Agency, through its Office of Research and Development, partially funded (contract #EP-C-08-031, to A.S.) and collaborated in the research described in this publication. It has been subject to an EPA administrative review but does not necessarily reflect the views of the Agency. No official endorsement should be inferred. EPA does not endorse the purchase or sale of any commercial products or services. The sequencing work was supported by Defense Threat Reduction Agency of the Department of Defense under contract TMTI-IB06RSQ002 to NMRC. The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the US. Government.

**References**

1. Perry RD, Ferretheron JD. Yersinia pestis—etiologic agent of plague. Clin Microbiol Rev 1997; 10:35-66; PMID:893658.
2. Christie AB, Chen TH, Elberg SS. Plague in camels 1970-1972. J Appl Microbiol 1977; 42:409-15; PMID:85409.
3. Mollker HH. Experimental preservation of plague in soil. Bull Soc Pathol Exot Filiales 1963; 56:1168-82; http://dx.doi.org/10.1093/infdis/141.6.724.
4. Courchesne NM, Parisien A, Lan CQ. Production and application of bacteriophage and bacteriophage-encoded lysins. Recent Pat Biotechnol 2009; 3:37-45; PMID:19149721; http://dx.doi.org/10.2174/187220809787172678.
5. Duckworth DH. “Who discovered bacteriophage?” J Infect Dis 1980; 141:724-6; PMID:7391614; http://dx.doi.org/10.1111/j.1537-2717.1980.tb11329.x.
6. Sulakvelidze A, Kutter E. Bacteriophage therapy. J Appl Microbiol 2005; 99:449-59; PMID:16108766; http://dx.doi.org/10.1111/j.1365-2672.2005.02699.x.
7. Sharma M, Ryu JH, Beuchat LR. Inactivation of Escherichia coli O157:H7 in biofilm on stainless steel by treatment with an alkaline cleaner and a bacteriophage. J Appl Microbiol 2005; 99:449-59; PMID:16108766; http://dx.doi.org/10.1111/j.1365-2672.2005.02699.x.
8. Abduladze T, Li M, Menetrey MM, Dean T, Senecal A, Sulakvelidze A. Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by Escherichia coli O157:H7. Appl Environ Microbiol 2008; 74:6230-8; PMID:18723645; http://dx.doi.org/10.1128/AEM.01465-08.
10. Hibma AM, Jassim SA, Griffiths MW. Infection and removal of L-forms of Listeria monocytogenes with bred bacteriophage. Int J Food Microbiol 1997; 34:197-207; PMID:9093566; http://dx.doi.org/10.1016/S0168-1606(96)00190-7.

11. Knapp W, Zwillenberg LO. Morphological Differences between Pasteurella-Bacteriophages. (Brief Report). Arch Gesamte Virusforsch 1964; 14:563-6; PMID:14184630; http://dx.doi.org/10.1007/BF01555868.

12. Sulakvelidze A, Panenterack G. Industrial and regulatory issues in bacteriophage applications in food production and processing. In: Šabo PM, Griffiths MW, eds. Bacteriophages in the control of food- and waterborne pathogens. Washington, DC: ASM Press, 2010:297 - 326.

13. Rhoads DD, Wolcott RD, Kuskowski MA, Wolcott BM, Ward LS, Sulakvelidze A. Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. J Wound Care 2009; 18:237-8, 240-3; PMID:19661847.

14. Rakhuba DV, Kolomiets EI, Dey ES, Novik GI. Differences between Pasteurella-Bacteriophages. (Brief Report). Arch Gesamte Virusforsch 1964; 14:563-6; PMID:14184630; http://dx.doi.org/10.1007/BF01555868.

15. Vinga I, Baptista C, Auzat I, Petipas I, Lurz R, Tavares P, et al. Role of bacteriophage SP1 tail spike protein gp21 on host cell receptor binding and trigger of phage DNA ejection. Mol Microbiol 2012; 85:289-309; PMID:22171743; http://dx.doi.org/10.1111/j.1365-2958.2011.07931.x.

16. Hooton SP, Timms AR, Rowell J, Wilson R, Connerton IF. Salmonella Typhimurium-specific bacteriophage ΦSH19 and the origins of species specificity in the Y01-like phage family. Virul J 2011; 8:498; PMID:22047448; http://dx.doi.org/10.1186/1473-422X-8-498.

17. Otter JA, Kearns AM, French GL, Ellington MJ. Pantox-Valentine leukocidin-encoding bacteriophage and gene sequence variation in community-associated methicillin-resistant Staphylococcus aureus. Clin Microbiol Infect 2010; 16:68-73; PMID:19709067; http://dx.doi.org/10.1111/j.1469-0691.2009.02925.x.

18. Sutherland JW. Highly specific bacteriophage-associated polysaccharide hydrolyses for Klebsiella aerogenes type 8. J Gen Microbiol 1976; 94:211-6; PMID:932688; http://dx.doi.org/10.1099/00221287-94-1-211.

19. Rakhuba DV, Kolomiets EI, Dey ES, Novik GI. Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell. Pol J Microbiol 2010; 59:145-53; PMID:21035576.

20. Nunes MP, Sarrasuna I. Bacteriophage specificity in the identification of Yersinia pestis as compared with other enterobacteria. Rev Bras Pesqui Med Biol 1978; 11:359-63; PMID:375327.

21. Viazis S, Akhtar M, Feirtag J, Diaz-Gonzalez F. Reduction of Escherichia coli O157:H7 viability on leafy green vegetables by treatment with a bacteriophage mixture and trans-cinnamaldehyde. Food Microbiol 2011; 28:149-57; PMID:21056787; http://dx.doi.org/10.1016/j.fm.2010.09.009.

22. Brussow H. Phage therapy: the Escherichia coli experience. Microbiology 2005; 151:2133-40; PMID:16000704; http://dx.doi.org/10.1099/mic.0.27849-0.

23. Garcia E, Elliott JM, Ramanculov E, Chain PS, Chu MC, Molineux IJ. The genome sequence of Yersinia pestis bacteriophage phiA1122 reveals an intimate histology with the coliphage T3 and T7 genomes. J Bacteriol 2003; 185:5248-62; PMID:12923098; http://dx.doi.org/10.1128/JB.185.17.5248-5262.2003.

24. Savalia D, Severinov K, Molineux I. Genomic sequences and analysis of several T7-like bacteriophages. In Press.

25. Steven AC, Tusz BL, Maizel JV, Unser M, Parry MC, Molineux IJ. The genome sequence of Yersinia pestis biovars and analysis of several T7-like bacteriophages. In Press.

26. Boyd E. Bacteriophages and bacterial virulence. In: Kutter E, Sulakvelidze A, eds. Bacteriophages: Biology and Applications. Boca Raton, FL: CRC Press, 2003:223-65.

27. Miller ES, Heidelberg JF, Eisen JA, Nelson WC, Durkin BJ, et al. Complete genome sequence of the broad-host-range vibriophage KVP40: comparative genomics of a T4-related bacteriophage. J Bacteriol 2003; 185:5220-33; PMID:12923095; http://dx.doi.org/10.1128/JB.185.17.5220-5233.2003.