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A *Yersinia pestis*-specific, lytic phage preparation significantly reduces viable *Y. pestis* on various hard surfaces experimentally contaminated with the bacterium

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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 (YpP-G, Y, R and YpsP-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 ϕA1122. 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. 10^9, 10^8 and 10^7 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. 10^6 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*.

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

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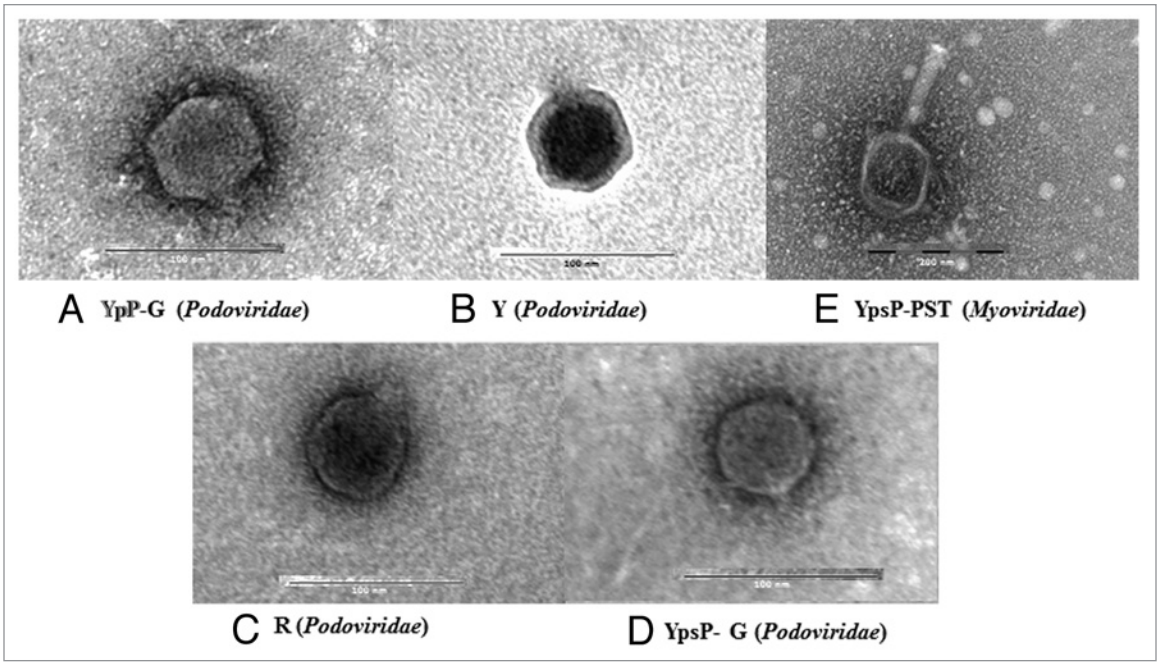

Figure 1. Electron micrographs of five *Y. pestis* phages.

other 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 *phago* meaning to *eat* or to *devour*).[5] 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 (Meipharianí A, personal communication). However, with the exception of a few recent studies,[7-10] 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 10^9 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 species[14]). 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. 10^4 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. 10^9 PFU/mL.

In order to validate their specificity, the phages (at a concentration of ca. 10^9 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 4 of the 5 *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, 2 of the 6 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 publication[20] 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 YpsP-G) 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. 10^9 PFU/mL) that included YpP-G, Y, R and YpsP-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

Table 1. 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
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	+	+	+	+	+	+
C14735	+	+	+	+	+	+
C1522	+	+	+	-	-	+
C2614	+	+	+	-	+	+
C2944	+	+	+	-	+	+
C1045	+	+	+	-	+	+
C790	+	+	+	-	+	+
771G	+	+	+	-	+	+
1390G	+	+	+	-	+	+
1391G	+	+	+	+	+	+
1393G	+	+	+	-	+	+
2095G	+	+	+	+	+	+
3072G	+	+	+	-	+	+
3757G	+	+	+	-	+	+
3769G	+	+	+	-	+	+
8786G	+	+	+	+	+	+
8787G	+	+	+	+	+	+
8906G	+	+	+	+	+	+
NR 15	+	+	+	-	+	+

Table 1. Lytic activity of diluted phage preparations (approximately ca. 100 PFU/per spot) against 59 strains of *Y. pestis*

NR 16	+	+	+	-	+	+
NR 17	+	+	+	-	+	+
NR 18	+	+	+	-	+	+
NR 20	+	+	+	-	+	+
NR 635	+	+	+	-	+	+
NR 636	+	+	+	-	+	+
NR 637	+	+	+	-	+	+
NR 638	+	+	+	+	+	+
NR 639	+	+	+	-	+	+
NR 640	+	+	+	-	+	+
NR 641	+	+	+	-	+	+
NR 642	+	+	+	-	+	+

were no 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.[8,21,22] 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-sequence and assemble 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 genomic sequences 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,[23] Y was > 99% identical to ϕA1122 (with a query coverage of 99%), and YpP-G was > 99% identical to the previously sequenced phage Yepe2,[24] 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[25] 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^{-4}), 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} [27]. 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. 10^9 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 2. Lytic activity of diluted phage preparations (containing ca. 10^9 PFU/mL) against 38 non-*Y. pestis* strains

Strains	YpsP-G	Y	R	YpsP-PST	YpP- G	φA1122
E. coli AH237-3	-	-	-	-	-	-
E. coli AH 1444	-	-	-	-	-	-
E. coli AH 1383	-	-	-	-	-	-
E. coli AH2941–1	-	-	-	-	-	-
E. coli ATCC 35401	+	+	+	+	-	+
Klebsiella pneumoniae env 1	-	-	-	-	-	-
Klebsiella pneumoniae env 7	-	-	-	-	-	-
Klebsiella pneumoniae env 10	-	-	-	-	-	-
Klebsiella pneumoniae env 11	-	-	-	-	-	-
Klebsiella pneumoniae env 17	+	+	+	+	-	+
Salmonella Enteritidis 250/23	-	-	-	-	-	-
Salmonella Enteritidis 253/26	-	-	-	-	-	-
Salmonella Enteritidis 261/34	-	-	-	-	-	-
Salmonella Enteritidis 270/43	-	-	-	-	-	-
Salmonella Enteritidis 274/47	-	-	-	-	-	-
Shigella sonnei ATCC 9290	-	-	-	-	-	-
Shigella sonnei S43	+	+	+	+	+	+
Shigella sonnei S44	+	+	+	+	+	+
Shigella sonnei S45	+	+	+	+	+	+
Shigella sonnei S46	+	+	+	+	+	+
Vibrio cholerae env	-	-	-	-	-	-
Vibrio cholerae O395	-	-	-	-	-	-
Vibrio cholerae N16961	-	-	-	-	-	-
Vibrio cholerae O139	-	-	-	-	-	-
Vibrio cholerae non O1	-	-	-	-	-	-
Listeria monocytogenes env 1	-	-	-	-	-	-
Listeria monocytogenes env 3	-	-	-	-	-	-
Listeria monocytogenes env 6	-	-	-	-	-	-
Listeria monocytogenes env 7	-	-	-	-	-	-
Listeria monocytogenes env 11	-	-	-	-	-	-
Yersinia pseudotuberculosis ATCC 23207	-	-	-	+	-	-
Yersinia pseudotuberculosis NR4371	+	+	+	+	-	-
Yersinia pseudotuberculosis NR4372	-	-	-	+	-	-
Yersinia pseudotuberculosis NR4373	+	-	+	+	-	-
Yersinia pseudotuberculosis NR4374	+	-	+	+	-	-
Yersinia pseudotuberculosis NR4375	-	-	-	+	-	-
Yersinia pseudotuberculosis NR4380	+	-	+	+	-	-
Yersinia pseudotuberculosis NR804	+	+	+	+	-	-

assay, the observed decontamination would be 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 *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 *Y. pestis* recovered from the PBS and neutralizer control groups. If the concentrations of viable *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 *Y. pestis*-contaminated matrices and the YPP-100 phages—and, therefore, the data accurately represent the *Y. pestis* killed during 5 min of exposure to YPP-100. On the other hand, the observation that the number

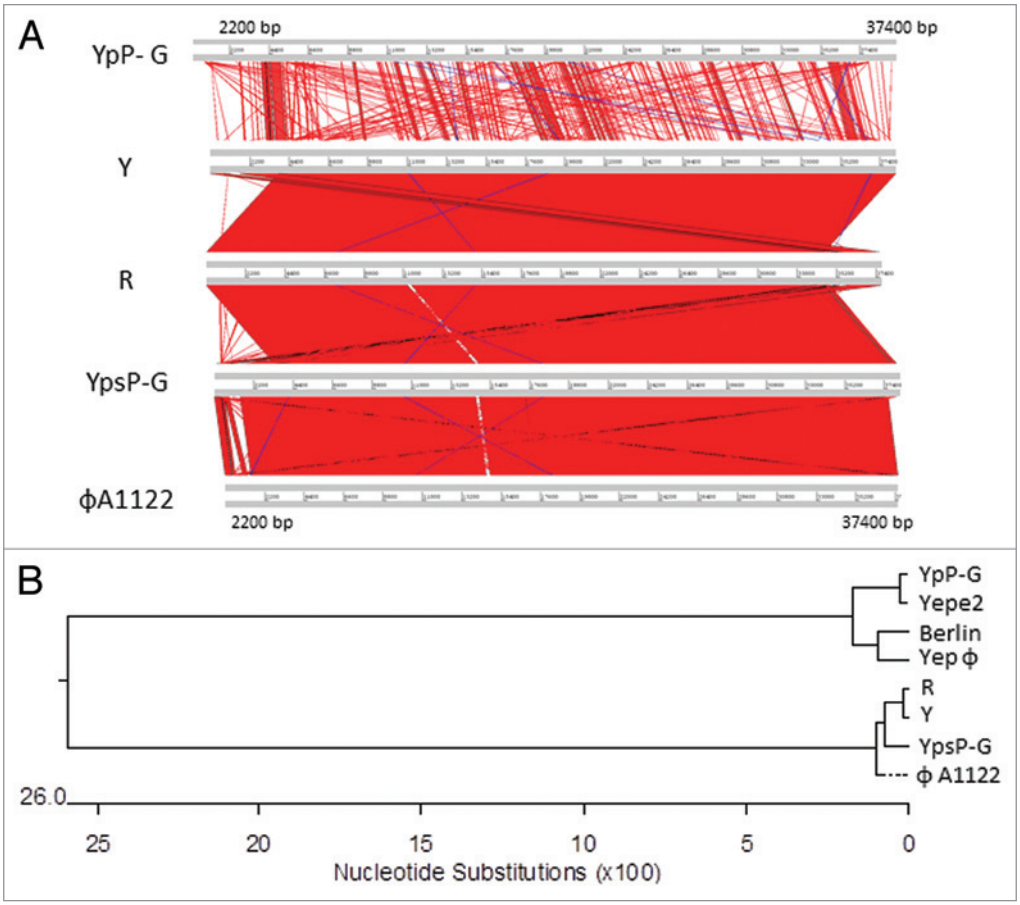

Figure 2. (**A**) Genome comparison of 5 *Y. pestis* lytic phages using Artemis comparison tool. Red lines indicate direct match and blue lines indicate reverse complimentary match; (**B**) Weighted phylogenetic tree of the eight *Y. pestis* phages.

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[8] was only 94% even when they used a much more concentrated phage cocktail (10^8 PFU/mL). The higher-then-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.[8,9,21,29-32] In addition, at least one recent publication[33] has suggested that bacteriophages could be used to remove bacterial biofilms from

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 Tsertsvadze (National Center for Disease Control and Public Health). ϕA1122,[23] 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. 10^9 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,[14] and the remaining 13 *Y. pestis* strains were obtained from BEI Resources. Also, our "hard surface decontamination" studies of YPP-100 utilized a mixture of three genetically unrelated strains of *Y. pestis* (CO92, KIM and 1670G). Mid-log phase

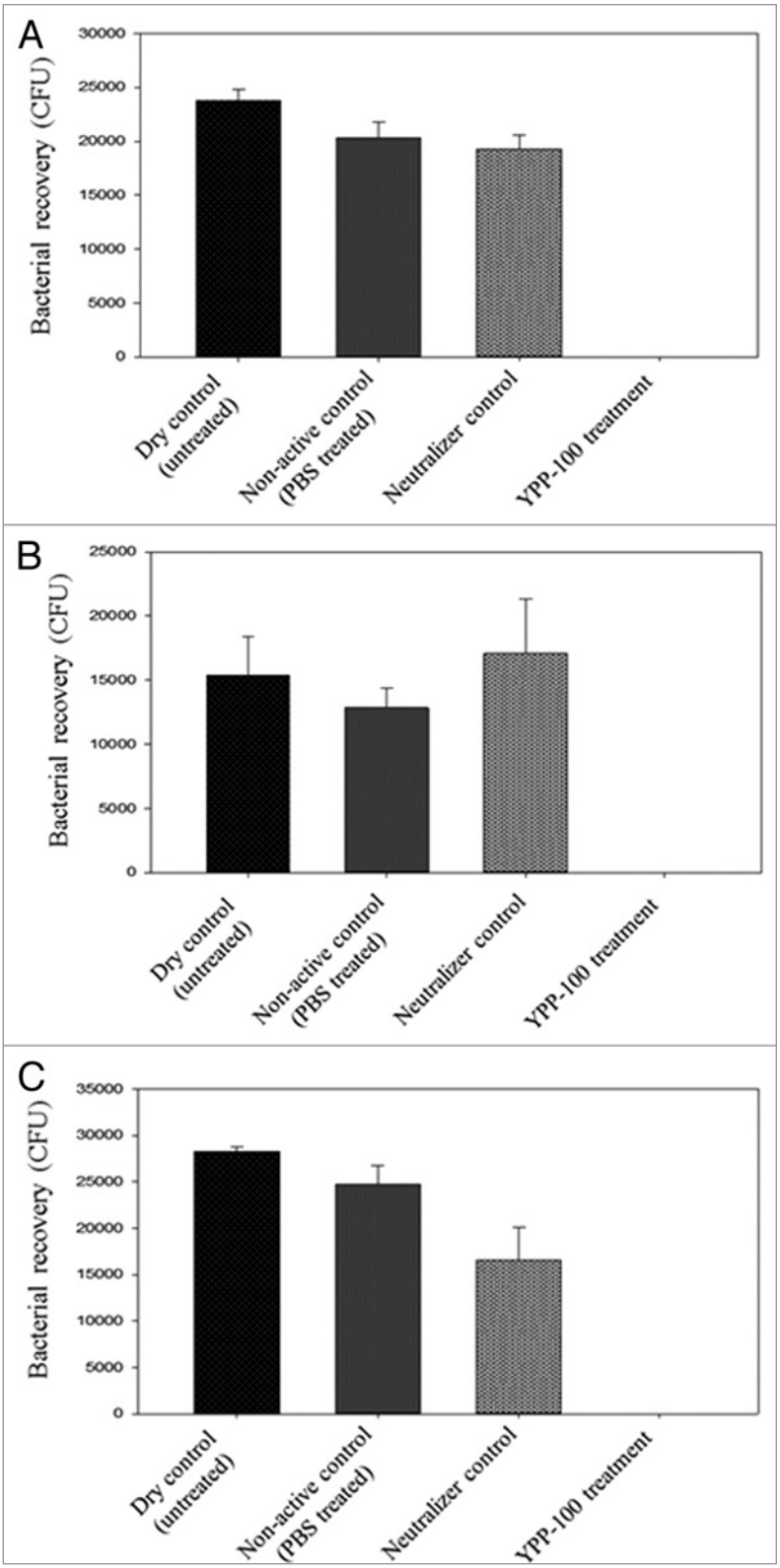

Figure 3. Decontamination of *Y. pestis*-contaminated hard surfaces treated with YPP-100 containing ca. 10^9 PFU/mL, (**A**) gypsum board, (**B**) glass and (**C**) stainless steel.

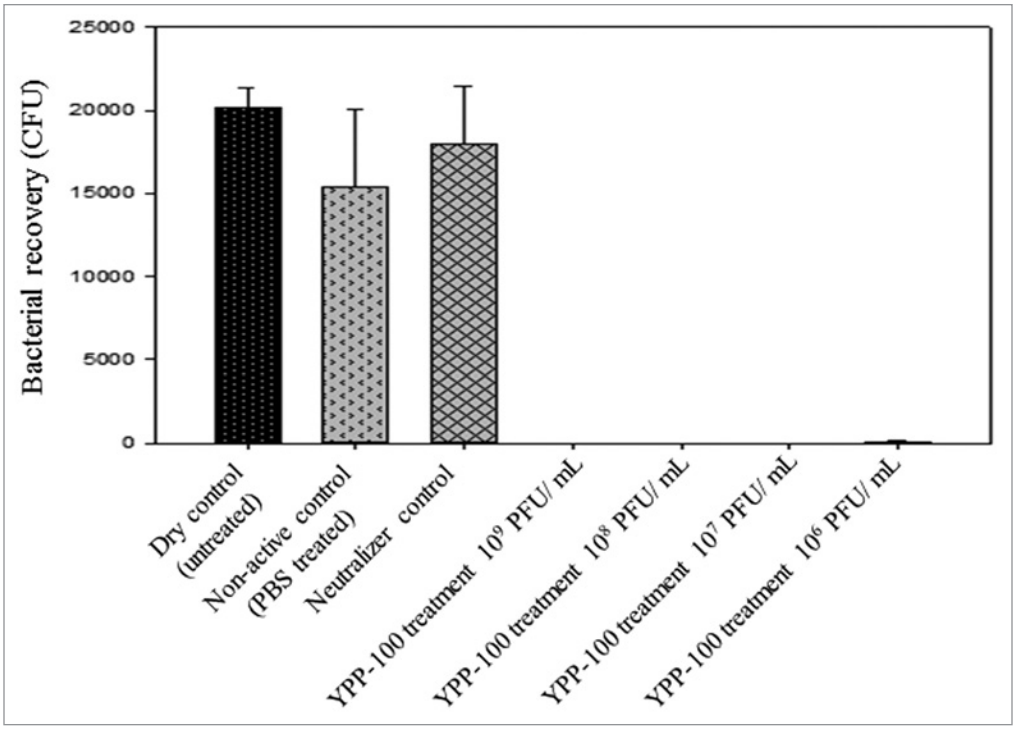

Figure 4. Effect of YPP-100s phage concentration on reducing *Y. pestis* contamination of stainless steel surfaces.

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 > 90X (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 MegAline 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 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 gypsum 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 "nonactive 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. 10^8 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 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 remove unattached 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. 10^8, 10^7 and 10^6 PFU/mL) were used to treat the matrices.

The neutralizer test also was done as described in the above-cited study.[36] 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 BHI 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.

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