Engineered Phagemids for Nonlytic, Targeted Antibacterial Therapies

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S *Supporting Information*
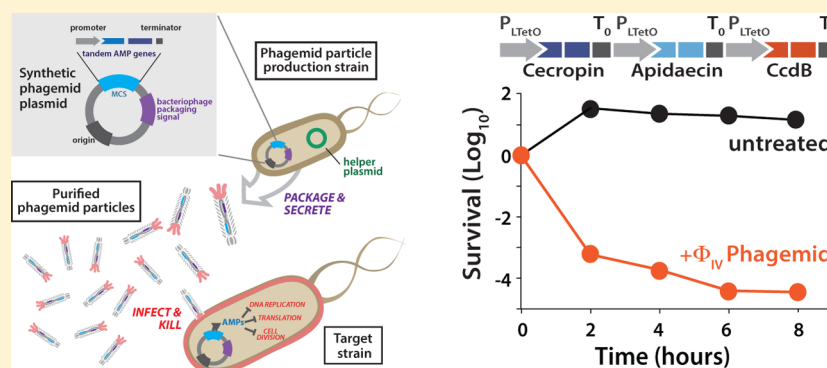
ABSTRACT: The increasing incidence of antibiotic-resistant bacterial infections is creating a global public health threat. Because conventional antibiotic drug discovery has failed to keep pace with the rise of resistance, a growing need exists to develop novel antibacterial methodologies. Replication-competent bacteriophages have been utilized in a limited fashion to treat bacterial infections. However, this approach can result in the release of harmful endotoxins, leading to untoward side effects. Here, we engineer bacterial phagemids to express antimicrobial peptides (AMPs) and protein toxins that disrupt intracellular processes, leading to rapid, nonlytic bacterial death. We show that this approach is highly modular, enabling one to readily alter the number and type of AMPs and toxins encoded by the phagemids. Furthermore, we demonstrate the effectiveness of engineered phagemids in an in vivo murine peritonitis infection model. This work shows that targeted, engineered phagemid therapy can serve as a viable, nonantibiotic means to treat bacterial infections, while avoiding the health issues inherent to lytic and replicative bacteriophage use.

KEYWORDS: Antimicrobial peptides, phagemids, targeted bacterial killing, bacterial toxins, synthetic gene networks, bacteriophage resistance

Antibiotic-resistant bacterial infections are an increasing concern in clinical and nonclinical settings.[1] Current first-line treatments rely upon the administration of small-molecule antibiotics to induce bacterial cell death. It is widely acknowledged that these broad-spectrum treatments disrupt the patient's normal microflora, allowing resistant bacteria and fungal pathogens to take advantage of vacated niches.[2] Therefore, a renewed interest has emerged for the use of targeted bacteriophage therapy to combat bacterial infections. Bacteriophages offer several distinct advantages over traditional antibiotic treatment, including high bacterial target specificity and reduced collateral damage to the host microbiota. They also have the potential to deliver synthetic gene networks, which can be designed to disrupt bacterial structures and processes through the expression of antibacterial or sensitizing genes.[3] Historically, phage therapy has relied upon lytic bacteriophages, which cause bacterial cell death through the rupture of the bacterial cell membrane. However, bacterial lysis results in the release of expressed proteins and endotoxins into the surrounding environment.[4] This in turn can lead to detrimental side effects, ranging from diarrhea to sepsis and even death.
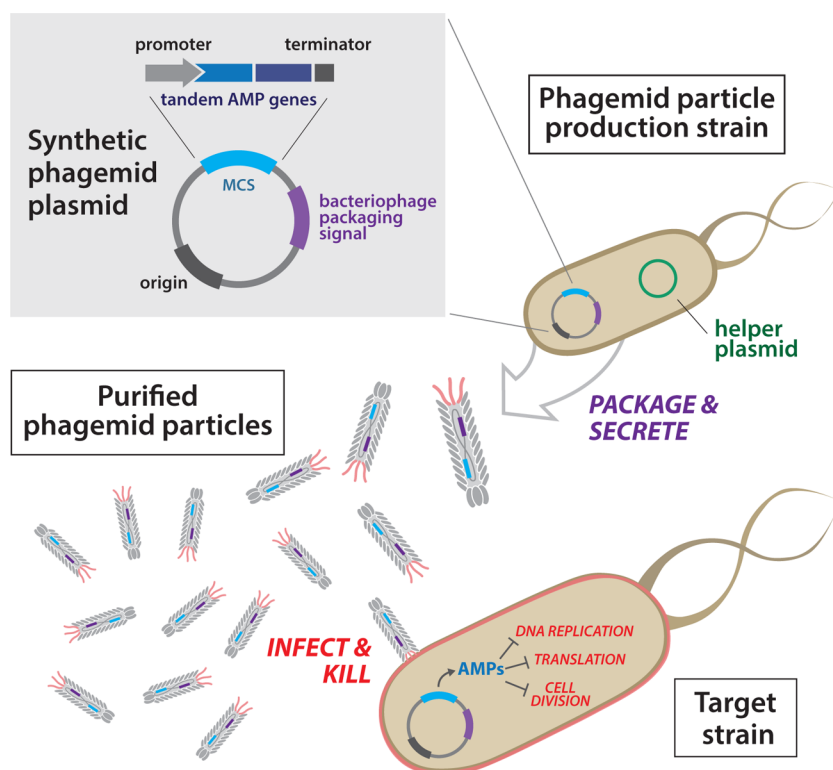
Figure 1. Overview of antibacterial phagemid construction. Phagemid plasmids, which carry an engineered antibacterial network, bacteriophage-packaging signal, and high-copy origin of replication, are first transformed into a production strain harboring a helper plasmid. Next, secreted phagemid particles are isolated from the production strain and purified. Resulting engineered phagemid particles are then used to infect target bacteria, causing expression of antibacterial proteins, which inhibit intracellular processes and cause nonlytic bacterial death.

Previous efforts have attempted to overcome this by using lysogenic bacteriophages that, in contrast to their lytic counterparts, secrete mature bacteriophage particles without causing cellular lysis. Although lysogenic bacteriophages have been engineered to serve as adjuvants to antibiotics by overexpressing sensitizing proteins,[5] their dependence upon antibiotics makes them inherently nonlethal and ineffective on their own. Most importantly, lysogenic bacteriophage genomes become less reliable over time due to fluctuations in genome copy number as they become packaged into viral particles. Repeated infection—reinfection cycles can also render infected bacteria resistant to further bacteriophage infection as the target cell tries to escape superinfection,[6] reducing the effect of repeated treatment. These limitations diminish the effectiveness of bacteriophage therapies as a viable treatment strategy.

In the present study, we engineered a modular bacterial phagemid system, which expresses a variety of nonlytic antimicrobial peptides (AMPs) and toxin proteins, to address the rising need for nontraditional, antibacterial treatment solutions. Phagemids, which employ bacteriophage proteins but selectively package a synthetic plasmid, provide a marked benefit over standard bacteriophage therapies, lytic, and lysogenic alike. This system limits the serious side effects linked to lytic bacteriophage approaches and improves upon lysogenic therapies by allowing for the direct delivery of specific high-copy plasmids to target cells in a single round of infection, ensuring consistent network expression levels and long-term stability (Supporting Information Figure 1).

Our phagemid system relies upon the expression of two plasmids: the first plasmid carries a bacteriophage-packaging signal and the desired antibacterial gene network, while the second plasmid contains a phagemid helper system,[7] which generates the bacteriophage proteins required for particle assembly but is not packaged itself. Together, these plasmids produce bacteriophage particles that selectively package an engineered plasmid harboring a synthetic gene network and a stable origin of replication rather than the bacteriophage genome. This approach allows for sustained network expression and reduces the formation of bacteriophage resistance by avoiding bacteriophage particle replication and superinfection in target cells.

We designed this phagemid system to be highly modular by employing the previously developed plug-and-play cloning platform developed by Litcofsky et al.[8] This platform employs a high-copy plasmid that contains a large multiple cloning site (MCS) into which we inserted a range of engineered antibacterial networks along with the F1 origin of replication, which serves as the packaging signal for the M13 bacteriophage.[9] The fully constructed phagemid plasmid was then transformed into a production strain, carrying the M13 cP phagemid helper plasmid. Phagemid particles produced by this strain were then used for single-round infection of the target *Escherichia coli* (*E. coli*) cells, leading to nonlytic bacterial cell death (Figure 1).

In order to initially screen AMPs that cause nonlytic bacterial cell death, we developed a test network expressing two tandem copies of the antibacterial gene of interest under regulation of the tetR-repressed P_{LtetO} promoter. Importantly, this design allows for activation of antibacterial networks in target wildtype (WT) strains, but represses these networks in tetR (Tet repressor protein) expressing test and production strains. Synthetic design of the ribosome binding site[10] (RBS) for each
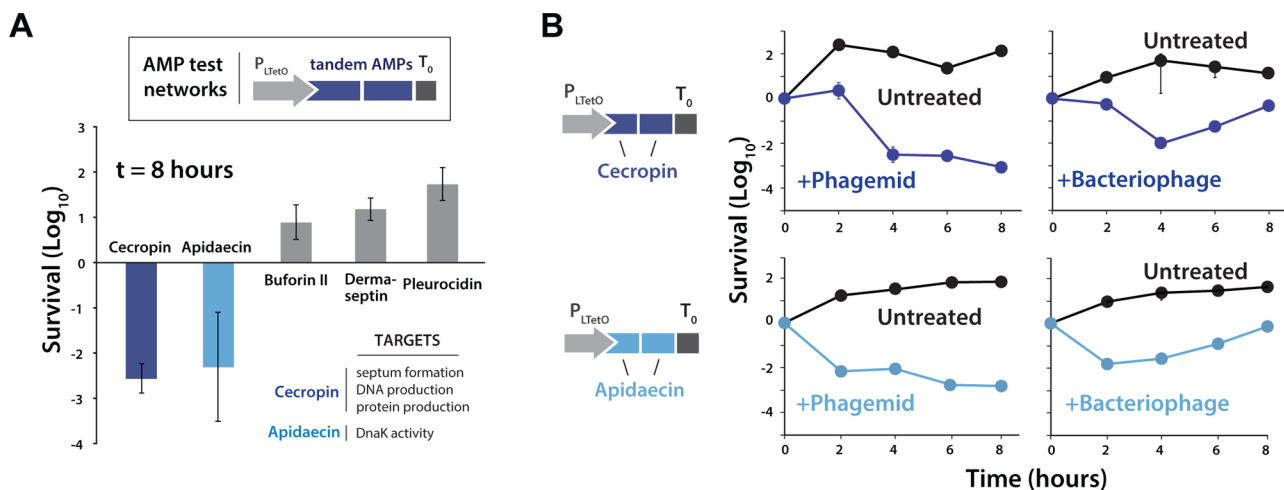
Figure 2. Testing antibacterial peptide candidates in test and phagemid networks. (A) Five antibacterial networks, expressing cecropin, apidaecin, buforin II, dermaseptin, or pleurocidin genes, were cloned into the testing strain, mgpro, and tested for their ability to cause bacterial cell death following induction with 100 ng/mL anhydrotetracycline (aTc), which relieves the inhibition of the P_{LtetO} promoter by tetR. (B) Antibacterial networks expressing either cecropin or apidaecin networks were introduced into phagemid and bacteriophage plasmids, and the resulting particles were tested against EMG2 *E. coli* for their ability to cause nonlytic bacterial death.
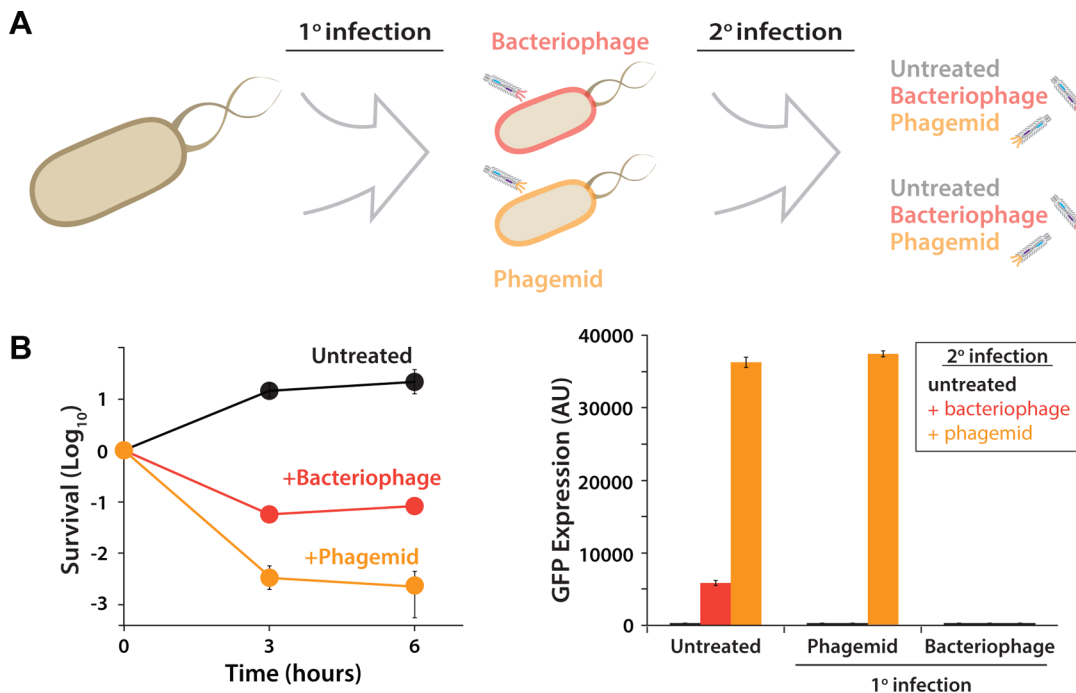

Figure 3. Bacteriophage resistance determination for phagemid and bacteriophage therapies. (A) EMG2 *E. coli* were infected with either bacteriophage or phagemid particles carrying the cecropin antibacterial network. Six hours post infection these cells were monitored for network functionality and diluted overnight. The following day a secondary infection of either bacteriophage or phagemid particles carrying a GFP expression network was administered. These cells, including an uninfected control, were then monitored for GFP expression 6 h post infection. (B) Infection data from the primary infection with phagemid or bacteriophage particles carrying the cecropin network showed expected bacterial death. Reinfection data from the secondary infection with phagemid or bacteriophage particles carrying a GFP network show bacteriophage resistance formation in samples exposed to bacteriophage but not phagemid particles.

antibacterial gene allowed for the independent tuning of expression levels. Five AMPs, cecropin PR-39,[11] apidaecin Ia,[12] buforin II,[13] dermaseptin,[14] and pleurocidin[15] were selected for their reported ability to rapidly disrupt intracellular processes and induce nonlytic bacterial death[16] (Supporting Information Table 1). Together with their P_{LtetO} promoter, synthetic RBS, and terminator, these AMP genes were transformed into the test strain, mgpro, and their efficacy for bacterial death was measured upon anhydrotetracycline (aTc) induced expression

(Figure 2A). We found that cecropin PR-39 (cecropin) and apidaecin Ia (apidaecin) were effective at inducing bacterial cell death; cecropin inhibits septum formation as well as DNA and protein production, while apidaecin inhibits the enzyme DnaK and binds to bacterial lipopolysaccharide. The remaining AMPs tested did not induce bacterial cell death, possibly due to post-translational modifications or microenvironmental differences with previous studies that our growth conditions did not
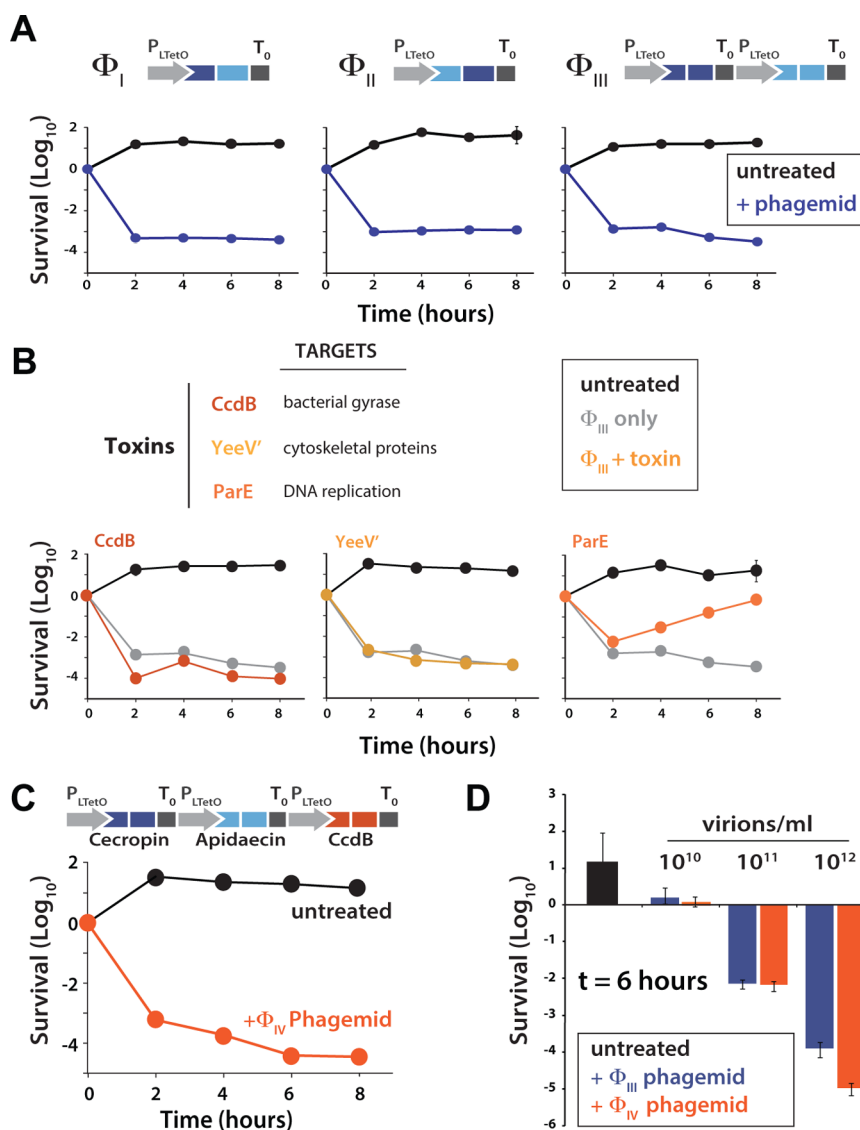
Figure 4. Modulation of AMP networks and enhancement by toxin networks. (A) Phagemids carrying synthetic networks that express combinations of cecropin and apidaecin antibacterial peptides were tested against EMG2 *E. coli* for their ability to cause bacterial death. These phagemid networks were designated ϕ_I, ϕ_{II}, and ϕ_{III}, respectively. (B) Three toxin networks, expressing ccdB, yeeV′, or parE genes were designed under regulation of the P_{LTetO} promoter. These networks were cloned into the MCS of the ϕ_{III} plasmid, and purified particles were screened against EMG2 *E. coli* for possible synergy with cecropin and apidaecin networks. Purified phagemid particles were tested alongside ϕ_{III} particles for induction of bacterial death. (C) An overexpressing ccdB network was cloned into the MCS of the ϕ_{III} plasmid. Purified phagemid particles were screened against EMG2 *E. coli* for their ability to cause nonlytic bacterial death. (D) Various final concentrations of ϕ_{III} and ϕ_{IV} phagemid particles were tested against target EMG2 bacteria. Bacterial cell viability was assessed 6 h post infection.

replicate. On the basis of these results, we decided to use cecropin and apidaecin for subsequent experiments.

To examine the efficacy of cecropin and apidaecin as antibacterial therapeutics, we introduced these AMP expression networks into the phagemid system. After transforming the system into the phagemid production strain (DH5αpro) carrying the M13 helper plasmid, we collected the purified phagemid particles and screened them against the target bacterial strain. Additionally, we introduced these antibacterial networks into the M13 bacteriophage system in order to compare their antibacterial effects. We found that treatment with phagemid particles harboring networks expressing cecropin or apidaecin reduced bacterial cell viability by 2−3 orders of magnitude (Figure 2B). This effect persisted for several hours post infection. In contrast, target cells infected with bacteriophage particles carrying the same AMP expression

networks failed to produce the same level of bacterial death. Although initial killing was observed, the cell population recovered after approximately 4 h of infection, likely due to inconsistent network expression.

In addition to the phagemid's ability to sustain bacterial death, we tested whether this approach induced resistance in target cells at a level comparable to that seen with bacteriophage therapy.[17] Target bacteria were first infected with either phagemid or bacteriophage particles expressing the cecropin AMP network and then subsequently reinfected with phagemid or bacteriophage particles expressing a GFP network (Figure 3A). Bacteria first infected with bacteriophage particles expressing the cecropin network were found to be resistant to a repeated bacteriophage infection with a GFP-expressing network (Figure 3B). In contrast, bacteria first infected with phagemid particles carrying the cecropin network maintained

their ability to be reinfected with a second round of GFP-expressing phagemid particles. These results highlight both the unreliability of bacteriophage therapy and the increased risk of resistance formation, which are avoided or reduced through the use of phagemids.

After distinguishing phagemids as a superior therapy option over bacteriophages, we next studied the modular nature of the phagemid system. Since cecropin and apidaecin target distinct intracellular processes, we hypothesized that simultaneous targeting could potentially increase phagemid-induced bacterial cell death. To test this hypothesis, we created three combination networks expressing both AMPs together (Figure 4A). Two of these networks (designated ϕ_I and ϕ_{II}) expressed a single copy of each AMP in varying order, while a third network (designated ϕ_{III}) expressed two copies of each AMP with a P_{LtetO} promoter driving each set. The combined phagemid networks had an enhanced effect upon the target bacteria with the ϕ_{III} network producing a 3.5 log reduction in bacterial cell viability.

To potentially increase the antibacterial efficacy of the phagemid treatment, we next chose to evaluate the effects of three bacterial toxins, Ccdb, YeeV′, and ParE, by introducing networks expressing the toxin genes into the modular ϕ_{III} phagemid platform. The first toxin, CcdB, is a topoisomerase inhibitor that interferes with DNA gyrase and results in the breakdown of bacterial DNA,[18−20] leading to cell death. YeeV is a toxin that inhibits cellular division by targeting two cytoskeletal proteins, FtsZ and MreB;[21] however, this dual inhibition causes cells to balloon and lyse, which is undesirable for our purposes. Sole inhibition of FtsZ can be accomplished by expression of a modified version of the YeeV protein truncated at the C terminus by 52 residues (designated YeeV′), which results in filamented cells that do not lyse.[21] The last toxin, ParE, acts by halting the F1* formation from both chromosomal and plasmid DNA replication origins by inhibiting bacterial gyrase, causing filamentation and cell death.[22] Infection of target cells with phagemid particles carrying the combined AMP-CcdB network resulted in increased bacterial cell death, leading to a 4.0 log reduction in bacterial cell viability within the first 2 h (Figure 4B). The addition of yeeV′ resulted in bacterial cell death comparable to that of ϕ_{III} alone, while the addition of parE led to reduced killing, possibly due to direct interaction with our phagemid plasmid's ability to replicate.

With these results, a final synthetic network employing the most productive toxin was generated. This construct, designated ϕ_{IV}, overexpressed ccdB through tandem gene expression in order to maximize its antibacterial effect and was combined with the ϕ_{III} AMP network. Purified phagemid particles were then tested against the target bacteria for induced bacterial cell death (Figure 4C). Expression of ϕ_{IV} provided almost an order of magnitude of increased bacterial cell death compared to ϕ_{III} alone. This difference was further enhanced by increasing the virion particle dose, resulting in over 5.0 log reduction in bacterial survival after 6 h of exposure to the ϕ_{IV} network (Figure 4D). Taken altogether, our screen identified that the combined phagemid-based expression of two copies of cecropin PR-39, apidaecin Ia, and ccdB genes resulted in robust killing of target E. coli bacteria.

We next tested the in vivo efficacy of our system by employing a murine model for E. coli peritonitis.[23] Seven-week-old C57Bl/6 female mice were administered 106 colony forming units (CFUs) of the target bacteria via intraperitoneal

(IP) injection. After 1 h, mice were treated with IP injections of phagemids expressing the ϕ_{IV} antibacterial-toxin network (Figure 5A). Mice were either not treated, injected with
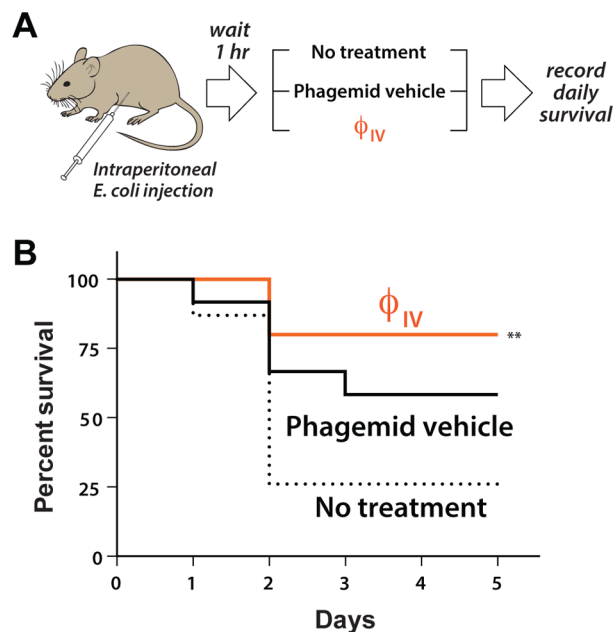

Figure 5. Murine peritonitis infection model with ϕ_{IV} expressing phagemid particles. (A) Overview of murine model for bacterial peritoneal infection with EMG2 E. coli bacteria and subsequent phagemid treatment. (B) Survival data for murine infection model with phagemid treatments and controls. Mice were divided into three groups: untreated ($n = 22$), vehicle only ($n = 12$), and ϕ_{IV} ($n = 10$). Data were obtained from two separate experiments and significance was determined by a Mantel−Cox test.

phagemid particles that do not express any genes (vehicle), or injected with phagemid particles containing the ϕ_{IV} network (Figure 5B). Mice given the ϕ_{IV} phagemid treatment had an average survival rate of 80% over the course of the experiment, compared to 27% survival in the untreated group, an increase that was statistically significant ($p = 0.003$). The vehicle group experienced an improved survival of 58%, which was not statistically significant when compared to the untreated group ($p = 0.08$). This effect was not observed in vitro (Supporting Information Figure 2), suggesting that the increased survival was due to interactions with the host. A survival advantage provided by phage particles has been previously reported[24] and may be associated with type I interferon and other proinflammatory gene induction upon exposure to phage capsid proteins. This proinflammatory effect is beneficial for treatment of bacterial infections, as phagemid particles prime the host immune system against unwanted bacteria. Taken together, our results show that engineered phagemids can effectively target and produce highly effective nonlytic killing of E. coli in vitro and in vivo without the use of antibiotics.

In this work, we developed a synthetic biology platform to produce nonlytic, bacterial cellular death without reliance upon traditional antibiotics. By designing and applying phagemid constructs containing selected AMPs, alone or in combination with bacterial toxins, we were able to develop an approach that achieved over a 5.0 log reduction in bacterial cell viability in vitro and resulted in over 80% survival in a virulent mouse model of peritonitis. While the approach presented here relies

upon the well-characterized M13 bacteriophage for phagemid production, similar systems can be produced using alternative bacteriophage systems to expand the repertoire of targetable bacteria. The modular nature of this system allows for the replacement and addition of individual components or whole networks within the engineered phagemid for the targeting of specific bacteria. When a packaging signal becomes characterized for a desired bacteriophage, it could be swapped with the F1 origin of replication in our synthetic antibacterial phagemid plasmid and cloned into a production strain that expresses the proper phage proteins. This enables production of both targeted and broad-spectrum antibacterial treatments, depending upon bacteriophage selection. While some toxins tested here had little effect on the target *E. coli* strain, the selected toxins have a broad-range activity across many bacterial species.[18−22,25] Additionally, because our choices for antibacterial peptides are broad spectrum,[11,12] this system should provide a plug-and-play therapeutic that can be readily modified to suit its target and will therefore function in many target bacteria. Because of the stable nature of phagemids and decreased likelihood of resistance formation through superinfection, our system provides a marked advantage for targeting bacterial infections over current bacteriophage techniques.

With recent discoveries highlighting the benefits and importance of healthy bacterial microbiomes[26,27] as well as the rapid rise in antibiotic resistance, targeted therapies such as this, which do not rely on antibiotics, could provide an invaluable tool for treating bacterial infections and reducing the prevalence of antibiotic-resistant bacterial strains without producing collateral damage in the commensal bacterial population. The decreasing cost of DNA synthesis technologies over the past decade[28] and low cost of phagemid production should allow this platform to provide inexpensive and effective therapy options for bacterial infections in settings such as hospitals, where growing bacterial resistances and healthcare costs are becoming more of a problem.[29]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The experimental details and supplementary figures. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nanolett.5b01943.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

■ ABBREVIATIONS

AMP, antimicrobial peptide; CFU, colony forming unit; GFP, green fluorescent protein; IP, intraperitoneal; MCS, multiple cloning site; RBS, ribosomal binding site; WT, wildtype

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