Comparing Methods to Genetically Engineer Bacteriophage and Increase Host Range

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ABSTRACT

Introduction:

Antibacterial resistance is an emerging problem in military medicine. Disruptions to the health care systems in war-torn countries that result from ongoing conflict can potentially exacerbate this problem and increase the risk to U.S. forces in the deployed environment. Therefore, novel therapies are needed to mitigate the impact of these potentially devastating infections on military operations. Bacteriophages are viruses that infect and kill bacteria. They can be delivered as therapeutic agents and offer a promising alternative to traditional antibiotic chemotherapy. There are several potential benefits to their use, including high specificity and comparative ease of use in the field setting. However, the process of engineering phages for military medical applications can be a laborious and time-consuming endeavor. This review examines available techniques and compares their efficacy.

Materials and Methods:

This review evaluates the scientific literature on the development and application of four methods of bacteriophage genome engineering and their consideration in the context of military applications. Preffered Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were followed for a systematic review of available literature that met criteria for analysis and inclusion. The research completed for this review article originated from the United States Military Academy's library "Scout" search engine, which compiles results from 254 available databases (including PubMed, Google Scholar, and SciFinder). Particular attention was focused on identifying useful mechanistic insight into the nature of the engineering technique, the ease of use, and the applicability of the technique to countering the problem of antimicrobial resistance in the military setting.

Results:

A total of 52 studies were identified that met inclusion criteria following PRISMA guidelines. The bioengineering techniques analyzed included homologous recombination (12 articles), in vivo recombineering (9 articles), bacteriophage recombineering of electroporated DNA (7 articles), and the CRISPR-Cas system (10 articles). Rates of success and fidelity varied across each platform, and comparative benefits and drawbacks are considered.

Conclusions:

Each of the phage engineering techniques addressed herein varies in amount of effort and overall success rate. CRISPR-Cas-facilitated modification of phage genomes presents a highly efficient method that does not require a lengthy purification and screening process. It therefore appears to be the method best suited for military medical applications.

INTRODUCTION

Bacterial infections remain a leading cause of illness throughout the world, and the rise of antibiotic resistance poses a significant threat to human health. In the USA, approximately 2 million people are infected with multidrug-resistant (MDR) bacteria per year, causing greater than 23,000 deaths.[1] In war-torn regions of the world without a reliable health care system, these numbers will undoubtedly be higher, suggesting that bacterial infections will be a significant persistent risk for deployed troops in austere environments. The global decrease in the efficacy of traditional antibiotic treatments requires the development of new strategies, and bacteriophage (phage) offers a promising therapeutic solution. Phages are viruses that selectively infect and kill bacteria and, since their discovery in 1915, have been successfully administered as remedial treatments.[2] However, the discovery of penicillin in 1928 and the subsequent "antibiotic age" that followed largely supplanted phage research efforts.[3] More recently, as antibiotic resistance has flourished in bacteria, coupled with the continued decline in novel antimicrobial discovery, treatment options against clinically and militarily relevant bacteria have become very limited.

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The use of phages within the context of military medicine was dramatically illustrated in 2018 in which a 30-year-old victim of a suicide bombing at the Brussels airport with a MDR *Klebsiella pneumoniae* infection was treated with a combination of pre-adapted phages and conventional antibiotics.[4] It was found that this combination was successful and led to an improved condition, a resolution of symptoms, and an absence of bacterial growth from bone fragments that were recovered post-treatment. The phage that was used in this case was previously isolated from a sewage water sample from Tbilisi, Georgia, and showed broad host range and required 15 rounds of co-evolution to reduce the incidence of bacterial phage resistance. The phage was applied several months after the bombing and after multiple rounds of antibiotic treatment had been attempted. It is reasonable to suspect that if phage therapy had been applied in a preventive manner immediately following the bombing, then the long course of the infection may have been prevented. This may be especially true if multiple types of phages could have been applied that had been pre-adapted to the circulating bacterial pathogens in the location in which the bombing had occurred. However, this approach will require a combination of medical intelligence, environmental surveillance, and phage preparation.

There are two primary benefits to phage therapy when compared to conventional therapy regimens, such as antibiotic treatment. First, phages are capable of self-replication at the infection site, reducing the cost of production of antibacterial therapy compared to antibiotics and helping to relieve the strain on the military health care budget caused by excess antimicrobial production.[2,5] Second, because of the specificity of phages to their host strain, complications that result from off-target kill of beneficial, commensal bacteria following antibiotic treatment can be largely mitigated. This is especially true if phage therapy is used as a first-line treatment for bacterial infections.[6] In addition, phages can also penetrate bacterial biofilms when antibiotics cannot.[7,8] Phage therapy is not without limitations, however. Single-phage treatment often results in phage resistance within the bacterial host. Clinical applications have required the development of multiphage cocktails, accompanied by frequent laboratory-based screenings to match a specific phage with susceptible bacterial hosts.[9] In addition, phage infection leads to bacterial lysis, which may have adverse effects on the patient because of the release of lipopolysaccharide or endotoxins that can elicit an adverse immune response.[9] Although successful application of phages against biofilms has been well demonstrated, the density of the extracellular matrix can severely impact the diffusion of phages within.[10,11] With renewed interest in phage research and the recent success of phage therapy in numerous clinical settings, efforts to enhance phage utility aim to mitigate these shortcomings.

Genetic engineering of phages has aimed to overcome many of the limitations mentioned above, to enable phage therapeutics to become a more readily accessible treatment option against MDR infections in both clinical and military settings. Common modifications have included the removal of undesirable traits in phage genomes, including genes that cause lysogeny or mobile genetic elements associated with virulence and drug resistance.[12] Dedrick et al. recently demonstrated this approach by designing bioengineered phage cocktails to treat a cystic fibrosis patient with a recalcitrant MDR *Mycobacterium abscessus* infection.[13] The bacterial recognition components of phages, namely the structural tail fibers, have also been engineered to overcome resistance to phage treatment. A phage designed to express a chimeric tail fiber recognizing two distinct components of the *Escherichia coli* cell wall was shown to control resistance and prevent mutations from occurring.[14] The current review article examines the most common techniques currently employed in phage bioengineering strategies. These synthetic approaches include homologous recombination (HR), in vivo recombineering (IVR), bacteriophage recombineering of electroporated DNA (BRED), and CRISPR-based phage genome editing. Although recent literature has provided excellent discussion of these topics, we aim to explore these techniques in the context of military applications and the unique challenges these pose when considering benefit to the warfighter.[15–17]

RESULTS

Homologous Recombination

Homologous recombination is a naturally occurring type of genetic recombination that results from the collocation of nucleic acid molecules that have similar or identical sequences. It is an enzyme-driven process that results in a physical exchange of materials between adjacent nucleic acid strands. Homologous recombination is involved in the mechanism of horizontal gene transfer, the genetic exchange system that is responsible for the spread of antibiotic resistance in bacteria.[18] The machinery of HR can be intentionally modified and utilized in phage genome engineering to produce progeny phage particles with desired genomic sequences. However, the low frequency of recombination and lengthy screening process of progeny phages poses a rate-limiting barrier to the efficiency and precision of this method in bacteriophage engineering.[19]

Homologous recombination in bacteria occurs via the RecBCD pathway for the induction of double-stranded DNA breaks and the RecF pathway for the induction of single-stranded breaks.[20] When the RecBCD protein binds to the double-stranded break, a helicase unwinds the DNA strands and continues unwinding them until a specific nucleotide sequence known as the Chi site is encountered.[21] This signals RecA proteins to load onto the newly single-stranded DNA to search for matching sequences on the homologous chromosome. When found, strand invasion occurs, forming a D-loop, which is then cut to form a cross-strand structure known as a Holliday junction.[21] Other proteins, such as RuvABC and

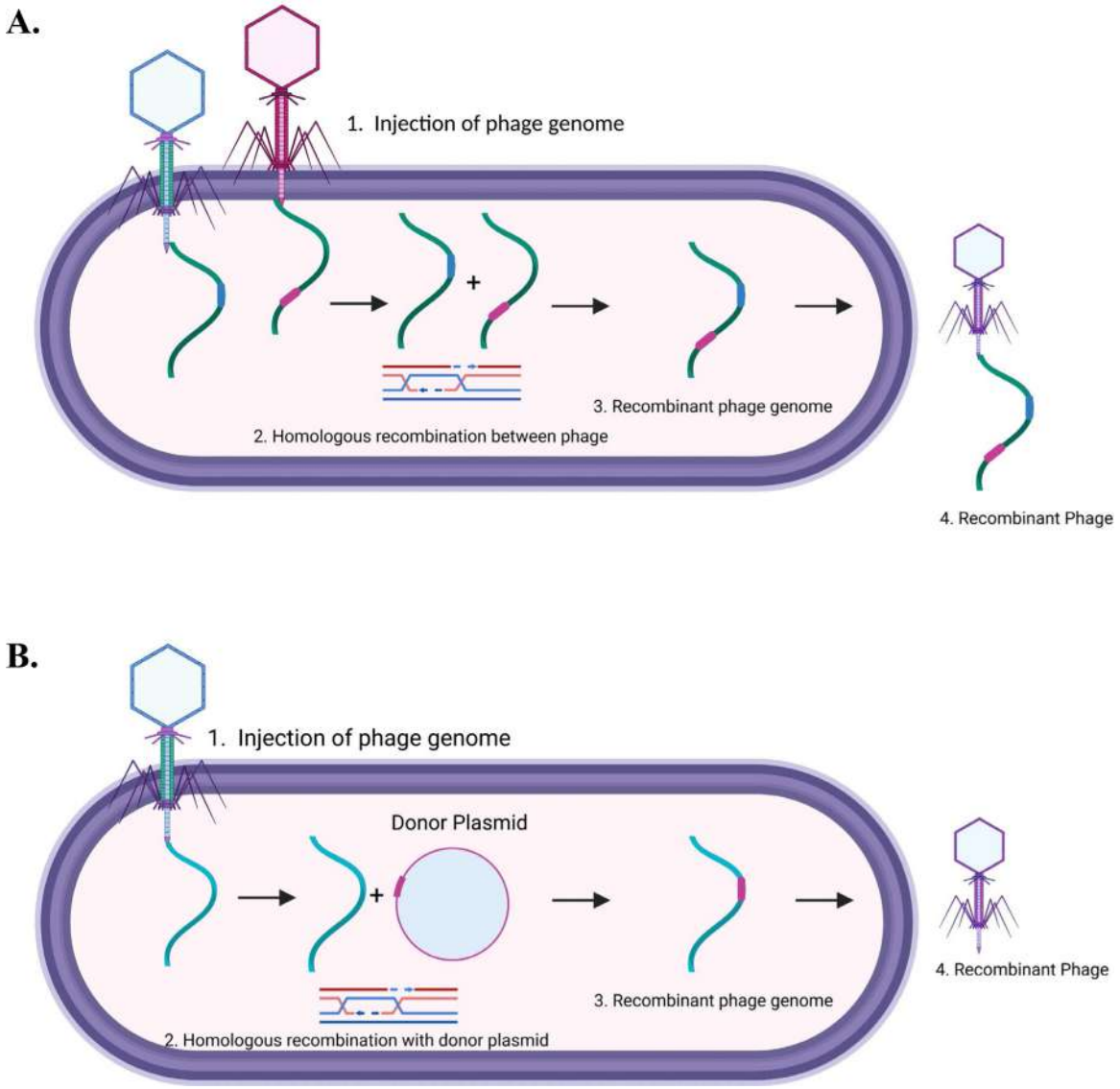

FIGURE 1. Schematic of traditional homologous recombination–based techniques. (A) Phage cross technique where two parent phages inject their genomes and homologous recombination occurs. (B) Donor-plasmid technique where one parental phage injects their genome into the host cell and recombination occurs with an engineered plasmid containing the desired sequence.

RecG, are then recruited to resolve this structure resulting in the production of two recombinant DNA molecules.[22]

Double-stranded DNA breaks are repaired by either non-homologous end joining (NHEJ) or homology-directed repair (HDR).[23] The HDR mechanism has been found to be the most effective means of the introduction of successful and precise genetic modifications into the phage genome during phage engineering. In order to increase the likelihood of the occurrence of HDR rather than NHEJ, fusion proteins can be developed with HDR effectors that can be used to localize at double-stranded breaks, significantly reducing the probability of NHEJ events.[23] Additionally, it has been shown that two specific fusion proteins known as the Cas9-CtIP fusion and the MS2-CtIP fusion tended to increase the ratio of HDR to NHEJ events by a factor of 14.9, indicating that it may be possible to use similar fusion proteins for modified phage production.[23] This is because the proportion of HDR to NHEJ events is an important consideration in increasing the precision of genomic engineering as well as in increasing the likelihood of desired recombination events in bacteriophage modification.

The earliest phage genome engineering experiments focused on HR-based techniques. Two methods were initially employed in these efforts. The first technique is known as phage cross, where two distinct phage particles with desired genomic sequences are used to simultaneously infect a host cell (Fig. 1A). Homologous recombination between the two naturally occurs during phage genome replication, and genetic

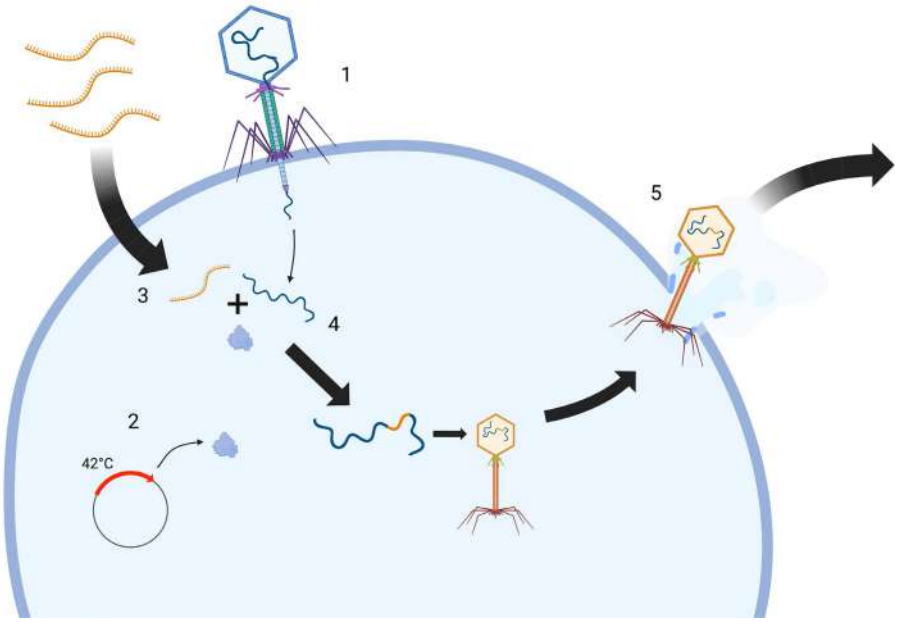

FIGURE 2. Schematic of in vivo recombineering. (1) Bacterial cells containing a target phage genome and plasmid containing the pL operon are produced by transformation and infection. (2) The pL operon under a temperature-sensitive repressor is activated by heating. (3) The bacterial cells are electroporated with ssDNA containing the gene of interest. (4) Recombination occurs via the Exo and Beta proteins encoded by the pL operon. (5) Recombinant phages lyse the cell and are released, recovered, and screened.

information is exchanged. This results in the formation of recombinant phage particles, and those containing the desired genomic sequences can be selected for. The second technique utilizes a donor plasmid with a homologous sequence to the target phage (Fig. 1B). After the desired sequence, plasmid is constructed and transformed into a cell and the target is then used to infect that cell. Homologous recombination occurs between the phage genome and the donor plasmid, resulting in a set of recombinant phages carrying the desired mutation.[23] More recent application of HR with phages infecting *Bacillus cereus* demonstrated that structural phage components can be modified to broadened host range recognition, providing greater control over contamination in dairy products.[24]

HR-based techniques are often inefficient, and they are plagued by certain rate-limiting steps.[19,23] In the phage cross technique, complete specification of the genome modification is not possible and the genomes of the progeny phages depend completely on the genome sequences of the parental phages and the laws of probability. In addition, the frequencies of recombination are often low and the screening process for selecting the desired phage is time consuming.[23] Specific modifications are able to be made with the donor plasmid technique; however, the screening processes are time consuming and the frequencies of recombination are quite low.[23] In addition, it is difficult to ensure that homology-directed recombination will occur rather than NHEJ.[20] Overall, HR techniques have demonstrated that phage genome modification is possible in the laboratory setting for research purposes but they are generally not amenable to rapid large-scale production of therapeutically useful phages.

In Vivo Recombineering

In vivo recombineering is another method of phage genome engineering in which genetic recombination is carried out within a bacterial cell (Fig. 2). This system contains two key proteins known as Exo and Beta that perform a double-stranded break repair resembling the process of HR.[25,26] Specifically, the Exo protein is an exonuclease that degrades double-stranded DNA adjacent to a double-stranded break to generate a region of ssDNA. The Beta protein functions to catalyze the binding of a substrate DNA molecule with a desired mutation to the newly generated ssDNA. These two proteins can be harnessed to perform phage engineering by supplying a bacterial cell with the phage λ recombination machinery on a plasmid, a modified gene of interest, and a phage genome to be altered. This has been demonstrated in *E coli*, using the inducible operon *pL* under control of the temperature-sensitive repressor *cI857*.[27,28] DNA molecules containing mutations of interest were produced through PCR and introduced into the bacterial cells via electroporation, and the cells were infected with target phages. At 42 °C, the operon is active, resulting in transcription of the Beta and Exo genes and recombination occurred with the PCR product and the phage genome. Although up to 13% of recovered phages were the products of recombination, only 2% were free of unwanted mutations.[28] Single-stranded DNA oligos were found to be most successful when the length is approximately 70 base pairs and the desired alterations are in the middle of the strand. It was also shown that both complementary versions of the oligomers were equally efficient in producing recombination events.[28] For dsDNA substrates, it has been found that 50 base

pair strands were most efficient in producing recombination events.[29]

Although the IVR technique has been successfully used to generate recombinant bacteriophages, it also has several limitations. Most importantly, the low yield of recombinant phages containing desired mutations and the potential for undesired mutations implies that this method is a low-efficiency technique.[28,29] In addition, this procedure requires a method for distinguishing between the desired recombinant phages and phages with undesired mutation and these screening steps appear to be rate-limiting to producing therapeutically useful quantities of phages.

Bacteriophage Recombineering of Electroporated DNA

Bacteriophage recombineering of electroporated DNA is a way to construct bacteriophage particles with targeted mutations. Bacteriophage recombineering of electroporated DNA can be used to perform deletions, point mutations, and nonsense mutations in both essential and nonessential genes.[30] Application of this method has been demonstrated against *Yersinia pseudotuberculosis* and *Salmonella enterica*, pathogens that cause food-borne illnesses and are known military hazards.[31] Procedurally, a phage DNA template and a targeting substrate, with short homology to the target, are co-electroporated into bacterial cells, then plated to allow for plaque formation (Fig. 3A). The resulting plaques are derived from individual cells that have taken up phage DNA and converted it into infectious lysing particles, and the desired mutants can be identified by PCR analysis (Fig. 3B). This engineering method typically produces an average of 1 plaque out of 20 with phages containing desired mutations mixed with the wild type. It thus requires many repetitive steps of purification, PCR, and replating to obtain the desired mutant phage in a purified form.[32]

Although the BRED methodology includes time-consuming rounds of multiple purification steps, PCR analysis, and replating, its application may be applicable in the military medical research setting. The ability to target and remove undesirable traits in phages is especially useful when developing potential therapies. In addition, BRED allows for reporter genes to be inserted at precise genomic locations, creating the potential for designing diagnostic phage applications to identify pathogens.[31] Thus, BRED offers the prospect of moving beyond the genomic description of novel genes and genomes and allows phage biology to be accessible to functional genomics and phage engineering. This method may also enable a systems-wide characterization of bacteriophages and an increased understanding of their molecular circuitry in a more cohesive and global manner.[32]

CRISPR/Cas Systems

CRISPR RNAs and Cas nucleases serve as a part of the bacterial adaptive immune system, allowing for the detection and destruction of phages and other foreign genetic material and preventing the deleterious effects of infection.[33] This system has been harnessed as a powerful genome editing tool, which has recently expanded to direct engineering of phages themselves (Supplemental Fig. S1). CRISPR-Cas engineering uses a specified guide RNA homologous to the region on the phage to be modified. The Cas nuclease then recognizes the guide RNA and cleaves at the homologous region on the phage genome. The site is then repaired using a defined template containing the mutation of interest. It has been found that double-stranded DNA breaks in phage DNA caused by CRISPR can accelerate HR and that CRISPR systems can negatively select phages with mutations of interest by targeting and degrading the genomes of wild-type phages. Most phage engineering protocols rely on type I and type II (also known as CRISPR-Cas9) systems because of their relative simplicity and ease of use in comparison to other systems.[33,34] This has proven to be a highly effective methodology, with up to 99% efficiency rate.[35] The ability of the CRISPR-Cas system to select against wild-type phages is greatly simplified compared to the other techniques described above. Thus, the CRISPR-Cas system removes one of the major rate-limiting steps involved in phage engineering.

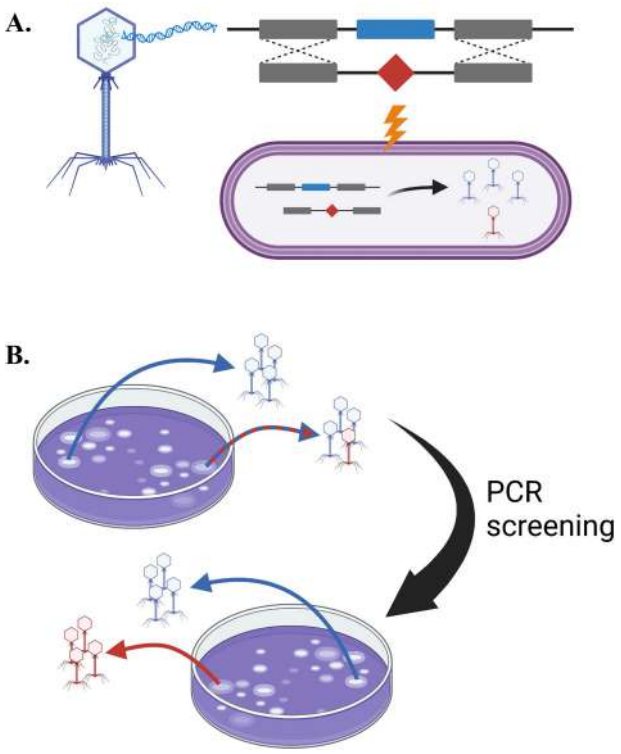

FIGURE 3. Bacteriophage recombineering of electroporated DNA overview. (A.) Cells of a recombineering proficient bacteria are made electrocompetent and co-electroporated with bacteriophage DNA and a recombination substrate. (B.) Mixed transformants are identified via plaque assay and verified by PCR screening of flanked ends. Successful recombinants are isolated from subsequent plaques assays of purified, recombineered phages.

TABLE I. Summary of Attributes for Each of the Phage Engineering Methodologies Discussed in This Article

Methodology	Mechanism	Limitations	Accuracy	Adaptability	Scalability
Homologous recombination	Enzyme-driven exchange of nucleic acid sequences (naturally occurring).	Long screening time.	Low frequency of recombination dependent upon probability.	Intentional modifications can be performed in the laboratory setting.	Lengthy screening time limits scalability.
In vivo Recombineering	Double-stranded break repair in bacterial cells using artificially introduced enzymes.	Low yield of recombinant phages containing desired mutations.	Low accuracy because of inability to control rate of desired mutation.	Can successfully generate recombinant bacteriophage in the laboratory setting.	Low frequency of desired mutation limits scalability.
Bacteriophage recombineering of electroporated DNA (BRED)	Phage DNA template and target sequence artificially introduced into bacterial cells.	Rate-limiting step is purification and analysis which impacts scalability.	Laboratory studies indicate 1 plaque out of 20 containing desired mutations.	Has been adapted to *Salmonella* spp. and *Yersinia* spp.; can directly target desirable traits.	Requires many steps of purification and analysis. Limited scalability.
CRISPR/Cas9	Recognition of region of interest by engineered guide RNA followed by cleavage and repair.	Potential for off-targeting effects and the introduction of undesired mutations.	This method has a demonstrated 99% efficiency rate in the laboratory setting.	Simplified methods have been developed increasing adaptability and speed.	CRISPR/Cas9 has been adapted for scalable manufacturing.

CRISPR-Cas9 systems have also been used to successfully modify specific characteristics of phages, including the structural tail fibers, which serve as the host recognition and anchoring portion of the virus. Work conducted by Hoshiga et al. used the CRISPR-Cas system to alter T2 phage infectivity toward *E coli* by changing genome segments coding for short- and long-tail fibers.[36] In this experiment, two distinct phage strains were employed: One which had been previously shown to infect *E coli* and one which had no previously documented infectious capability. Template DNA from each phage containing portions known to encode for tail fibers was transformed into host bacteria and then infected with T2 phages to produce recombinant phages. A CRISPR-Cas system was then used to target wild-type phage DNA, resulting in a rapid production of recombinant phages with an increased infectivity rate. These results demonstrate the unparalleled ability of the CRISPR-Cas system to select for phages with desired characteristics and indicate that this method may be useful in the development of therapeutic phages for military applications. Additionally, the CRISPR-Cas10 system has been shown to effectively edit staphylococcal phages, utilizing endogenous HR events in the bacterial host.[37] The methodology has been applied to remove virulent phage genes and broaden host infectivity.[37] Further, CRISPR-Cas12a has been employed in combination with IVR to efficiently modify both low- and high-copy number plasmids with greater reduced mutagenicity, as compared to recombineering alone.[38]

DISCUSSION

Although the precise nature of the future battlefield is unpredictable, biological factors will be just as important as technological factors for the success or failure of military operations. It has been estimated that approximately 1.2×1030 bacterial cells are present on Earth, with the majority living as members of multi-species biofilms.[39] It is therefore an inescapable fact that military personnel will be exposed to a wide variety of bacterial species during training, transportation, and deployment. The majority will be harmless commensals, but the few pathogenic strains can be devastating to personnel deployed in a combat zone. It has been established that bacterial pathogens in war wounds are typically acquired at the time of injury.[40] Therefore, limiting or preventing initial colonization at the time of injury would be an ideal way to reduce the burden of wound infection in the operational environment. Phages can be adapted for this purpose; however, the specific nature of phage activity would require knowledge of the pathogens most likely to be encountered in the operational environment coupled with a supply of pre-adapted phages capable of lysing them. With the continued rise of MDR in pathogens, as well as the dangers posed by both emerging and engineered biothreats, the military medical community must take special interest in efficient and safe strategies to develop and deploy countermeasures.[41] As the U.S. Military advances into new theaters and continues to operate in austere environments, quick and easily reproducible techniques for responding to biothreats must be explored. Phage engineering stands as a viable alternative and supplement to current antibacterial techniques. To be useful, phage engineering techniques must be efficient, cost-effective, and ready to apply to the challenges created by new bacterial strains and engineered biothreats. The various attributes of the methodologies described in this article are summarized in Table I.

Phage preparation involves several rounds of scale-up procedures in order to achieve the necessary high titer and volume required for therapeutic use. An important consideration is

the removal of bacterial byproducts that can be released from lysed cells, which can pass through common filtration methods used in phage enrichment. Endotoxins in particular can elicit an adverse immune reaction called a cytokine storm, and their removal is vital for safe clinical use of phage preparations.[42] Phages themselves may also trigger a humoral response, as seen with the development of antibodies against phage-structural proteins in a murine model by oral administration, in addition to the abundance of phage-neutralizing antibodies present in humans from natural exposure.[43,44] Studies of phage therapies used in humans indicate minimized effects of immune response against phages in relation to positive clinical outcomes.[45] Despite the promising future of phage therapy, the only currently available option for clinical phage applications in the USA is in compassionate care scenarios. Although the FDA has yet to publish approval standards, several clinical trials have been approved to further study the safety and efficacy of phage usage to treat chronic infections.[46,47]

Homologous recombination using only the natural recombination system leads to low recombination rates, requiring massive screening efforts to recover engineered mutants with the desired modification. Most of the alternatives also lead to low mutant yields, with BRED resulting in a 2% yield that is difficult to detect and must be plated multiple times to recover desired isolates.[32] Similarly, IVR yields relatively low success, with ~0.5 to 2% recombination rate and requires large and extended clean-up and purification efforts.[2] The most efficient phage editing methods analyzed were the ones relying on CRISPR-Cas systems, which can achieve an editing rate exceeding 99%.[35] The tunable nature of CRISPR-Cas also allows for diverse applications. It has been employed on *Staphylococcus aureus* phages to broaden host-range recognition via tail fiber modification.[48] Additionally, a temperate phage of *S aureus* was engineered to carry a CRISPR-Cas system to remove virulence factors from the bacteria, resulting in significantly reduced toxin production.[48] Further, engineered phage-reporter systems have garnered commercial interest as rapid, sensitive, and high-throughput detection/diagnostics tools.[49]

The development of a thermostable phage attached to bandages or wound dressings that can be deployed to the field environment presents another exciting application that would facilitate the prevention of infection and lessen the burden of bacterial pathogenesis on military personnel. Phage have been isolated from sources with the ability to remain active at temperatures ranging from 55 °C to 70 °C, and some phages are able to maintain stable at temperatures exceeding 100 °C.[50] In addition, it has been found that the phage can be adapted to higher temperature and to increased host range by chemical-mediated mutagenesis. A recent study found that the phage can be quickly adapted by exposure to sodium pyrophosphate followed by selection.[51] Sodium pyrophosphate is a chelating agent which induces random mutations in the phage genome by distorting the phage head. During contingency operations, random mutagenesis may be a viable means of quickly adapting a specific phage or group of phages to the most encountered strains of bacteria in the operational environment. These phages can then be attached to the bandage or dressing material. It has been found that phages can be covalently attached to nanofibers in a dressing in such a way as to orient the tails of the phage toward the exterior environment facilitating the interaction of the phage with local bacterial pathogens.[52]

CONCLUSION

Although each of the currently described genome modification procedures can produce genetically modified phages, the effort required to collect a significant yield of phage particles incorporating desired genetic changes varies greatly between methods. Indeed, most of the methods that were evaluated in this study depend a great deal on individual technique, a lengthy screening process, and serendipity. Most appear to have low success rates. This evaluation has determined that the use of the CRISPR-Cas system has the greatest potential to produce modified phages with the targeted introduction of useful changes to their phenotypes and genotypes. It is therefore recommended that the CRISPR system be explored to produce therapeutic phages for military applications so that they can be rapidly designed, produced, and fielded to serve the needs of U.S. forces in the deployed environment.

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Supplementary material

Supplementary material is available at *Military Medicine* online.

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CONFLICT OF INTEREST STATEMENT

None declared.

DATA AVAILABILITY

The data that support the findings of this study are all available on request from the corresponding author.

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