Conceptual Framework and Advances in CRISPR-Cas Based and BRED Phage Engineering

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Abstract— The increased proliferation of multi-antibiotic resistant bacterial pathogens necessitates the development of alternate and supportive therapies to antibiotics. Being natural predators of bacteria, bacteriophages have garnered recent attention for their untapped potential to mitigate the public health problem. Progress in synthetic biology has led to the genetic engineering of phages to ameliorate their efficacy and precision via methods such as genome editing, modification of tail fibers, and treatment with chemical mutagens. This review will elucidate two promising genetic engineering strategies, CRISPR-Cas and Bacteriophage Recombineering of Electroporated DNA (BRED), as well as the advances and potential future developments in the field.

Keywords— Bacteriophages, Genetic Engineering, CRISPR-Cas systems, BRED Phage Engineering

I. INTRODUCTION

With an upward of 10^{31} phage particles in the biosphere [1], bacteriophages constitute the most ubiquitous organisms on Earth. The highly evolved nanomachines target bacterial host cell walls, inducing the host to undergo autolysis and release progeny virions. With the emergence of antibiotic-resistant pathogens and the low rate of new antibiotic discovery [2], there is a growing interest for the generation of phage variants for prophylactic and therapeutic applications, underscored by upcoming randomized clinical trials such as NCT03808103 [3,4].

Over the past 10 years, various in vitro and in vivo synthetic technologies have been applied to construct recombinant phages with evolutionarily refined host specificity [5]. Phage host range has been extended previously by methods such as the modification of the receptor binding domains [RBDs] found in phage tail fiber protein complexes. For instance, the fd filamentous phage infected E. coli bearing F pili, while filamentous IKe infected E. coli bearing N or I pili. The fusion of the RBD of IKe gene 3 protein [pIII] to the N terminus of the fd pIII extended its specificity [6]. However, despite the expanded host range in the modified phages, the inoculated bacteria could develop phage resistance through pathways including receptor mutation and abortive infection systems[7]. Other methods earlier used such as random mutagenesis by the use of inducing agents including UV and chemicals such as hydroxylamine and N-methyl-N'-nitro-N-nitrosoguanidine require extensive screening to distinguish a mutant of interest from the large volume of phages generated [8]. Therefore, approaches involving the creation of targeted phages through homologous recombination have been increasingly applied, in which the sequence that is to be inserted is cloned into a vector with flanking regions that are matching downstream and upstream elements of the genome sequence of the bacteriophage, followed by a selection process. Although, as phage lysis destroys the bacterial host, no generic approach such as the antibiotic resistance marker for bacteria works as efficiently for phage selection, prompting the development of strategies such as BRED and the use of CRISPR-Cas. BRED increases the effectiveness of homologous recombination by reducing the number of phages to be screened to identify the phage mutant of interest whereas CRISPR-Cas allows counter selection against the bacteriophages that have not been modified and enriched for the required mutant [9].

II. CRISPR-CAS PHAGE ENGINEERING

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and their associated genes (cas) form an adaptive immune system found in 95% Archaebacteria and 48% Eubacteria that protect microbial cells from DNA invasion. CRISPR loci consist of several repeated sequences containing around 30 base pairs which are separated by similar length 'spacer' sequences, often similar to phage genome segments [10]. Small CRISPR RNAs (crRNAs) are formed after processing the single large transcript in a CRISPR locus in the repeat regions. After they are complexed with cas proteins, the crRNA/Cas complexes break apart foreign DNA molecules at sites that bear complementarity to the crRNAs, providing resistance against phages [11]. CRISPR systems are presently separated into two classes, six types, and thirty-three subtypes based upon cas gene composition and mechanism differences. The Class I systems, namely

Types I, II and IV, encode multisubunit effector complexes, while Class II systems, namely Types II, V, VI, depend upon a single subunit to kill nucleic acid invaders [12]. Type I and II are frequently used for genetic engineering, providing a specific and robust selection of recombinant DNA, with Type I systems encoding for the Cas3 protein and Type II systems encoding for the Cas9 protein [13].

One instance of the use of the type I-E CRISPR-Cas system was to enhance the engineering of the T7 phage genome. Homologous recombination was used to delete the nonessential T7 gene or gene 1.7 by propagating the T7 phage in a bacterial host containing plasmid carrying regions of homology to downstream and upstream regions of gene 1.7. The resultant phage population consisted of wild-type as well as recombinant phages lacking gene 1.7. To enrich the desired phages, a CRISPR-Cas based counterselection system was used. In it, the lysate of the mutant phage was plated on host bacteria carrying 3 plasmids which encoded the components that are required for the activity of the CRISPR-Cas system: the cas3 degradation machinery, the targeting cascade complex, and the CRISPR spacers (flanking sequences in the CRISPR-Cas system) targeting gene 1.7. It resulted in the selective cleavage of the wild-type phage genomes, containing phage gene 1.7, but not of the recombinant phage genomes, lacking phage gene 1.7, which thus prevented replication of the former phages and enrichment for the latter, enabling isolation of desired recombinants [14]. CRISPR-Cas 3 targeting does not have a sequence-dependent activity and results in bacterial death regardless of the gene targeted [15].

The Type II CRISPR-Cas system originating from bacteria such as Streptococcus thermophilus and Streptococcus pyogenes has been utilized to select for Streptococcus phage 2972 recombinants that underwent small and large DNA deletions, gene replacements, and point mutations. In the report, orf33 from the genome of phage 2972 was replaced with a methyltransferase gene of the type II restriction/modification system LlaDCHI from L. lactis, showing that the CRISPR-Cas engineering system could be utilized to introduce a functional methyltransferase gene into phage genome [16]. Both Type I and Type II CRISPR-Cas systems require a DNA repeat-spacer and individual additional requirements. The I CRISPR-Cas system needs a Cas3 enzyme in addition to a five protein complex referred to as cascade, consisting of CasA, CasB, CasC, CasD, and CasE. The Type II CRISPR-Cas requires trans-activating CRISPR RNA and Cas9 endonuclease [17, 18, 19].

III. BRED PHAGE ENGINEERING

For a few prototype phages such as λ, T4, and T7, mutational mapping and mutant isolation by recombination have been described. The efficiency of recombination has been ameliorated by co-opting the natural recombination systems of temperate phages by a method termed as recombineering. It is enabled by bacteriophage-derived recombination proteins and has the advantage of utilizing DNA substrates with short regions of homology. The method was developed in E. coli by the use of phage λ Red recombination proteins, Exo and Beta, which expeditiously advanced homologous recombination between homologous targets in the chromosome of the bacteria and linear DNA substrates [21, 22, 23]. The RecE and RecT proteins of the Rac prophage operate in a similar manner and as thus used for mutant construction, allowing mutagenesis of lytically replicating phages as well as mutagenesis of lysogenic phages through prophage recombineering [24, 25, 26, 27, 28]. The identification of RecE/RecT homologs in phage Che9c led Marinelli et al. to apply the BRED technique for the first time to modify mycobacteriophages [20].

The recombineering substrates used for BRED include the phage DNA and the double-stranded DNA (dsDNA), composed of the DNA segment to be inserted along with regions of homology to the loci closely downstream and upstream of the region of the bacteriophage genome to be modified. BRED involves coelectroporating the dsDNA and phage DNA into electrocompetent bacterial cells that carry a plasmid that encode proteins encouraging high levels of homologous recombination, such as the RecE/RecT-like proteins [29, 30]. The bacteria are recovered, mixed with the wild-type variant and plated following electroporation, after which the plates are checked for the presence of bacteriophage plaques. PCR is then used for screening individual plaques, indicative of the lysis of bacterial cells, for the desired mutated phage genome [31]. The in vitro method is utilized for the making of unmarked deletions of non-essential and essential genes, point mutations and nonsense mutations, the insertion of gene tags, and the accurate addition of foreign genes [20]. Recombination efficiency has been significantly improved as desired bacteriophages have been acquired at high frequencies [10-15%], enabling the identification of mutants with only 2 rounds of a small number of PCR reactions [32].

However, BRED depends on the compatibility of the bacteriophage to be modified with the recombinases and on a high electroporation efficiency, which is difficult to reach for phages that have large genomes.

IV. ADVANCES

Phage therapy, prevalent since the early 1900s [33], is the administration of bacteriophages directly to a patient with the intention of lysing the bacterial pathogen causing infection [34]. Bacteriophages have several advantages over traditional chemical antibiotics as they are bactericidal agents with low inherent toxicity, minimal disruption of normal flora bacteria, biofilm clearance, and single-dose potential. However, natural phages have the disadvantage of specificity since they have a narrow spectrum of action and the causative bacteria must be identified in advance [35]. Phage engineering enables the modification of phages to overcome such obstacles.

The CRISPR-Cas system has been widely used to not only target desired mutant phages but also to modify the phages in vivo, with the Type I CRISPR system used to modify phages of bacteriophage T7 [14] and lytic phage ICP1 [36]. Phages altered with the Type II system include phage P2 of Lactococcus lactis, phiKpS2 of Klebsiella pneumoniae, and phages T7, T4, T2, and KF1 of Escherichia coli [37-40]. Type III systems have been used to alter phages of Staphylococcus epidermidis and Staphylococcus aureus [41].

BRED has been applied to genetically alter mycobacteriophages and coliphages. In the genetic engineering of lytic phages of M. smegmatis, E. coli, and Salmonella enterica, it provided a recovery efficiency of recombinant phage of ca. 20%, allowing for their detection by PCR plaque screening [20, 42, 43, 44]. A recent compassionate use case using BRED phage engineering describes the first use of engineered phage to treat a severe disseminated Mycobacterium abcessus infection in a 15-year-old cystic fibrosis patient. BRED was utilized to engineer a lytic derivative of phage ZoeJ and precisely remove its repressor gene 45 [45], which effectively killed the pathogen. Intravenous phage treatment with a three-phage cocktail was administered every twelve hours for thirty-two weeks, associated with significant clinical improvement. The bacteriophages could be detected in the patient's serum a day after initiation of therapy, reaching a titer of $\geq$ 109 PFU/ml, they were undetectable after 6 days [46]. Although the study was uncontrolled, the results indicated that prolonged intravenous treatment of a genetically engineered phage is possible without harm to the patient, while producing high therapeutic phage levels in the patient's serum, suggesting the multiplication of phages in vivo. The report also indicates that intravenous phage therapy in a human patient does not prompt the production of antibodies that have the potential to affect its therapeutic efficacy.

Clinical trials are crucial to evaluate the efficacy of phage treatment, as phage therapy has only occasionally been applied by doctors for compassionate treatment, with around twenty-five reports of compassionate bacteriophage therapy cases published over the last twenty years. While most cases were successful, reports used different bacteriophages in varied amounts for different conditions, underscoring the necessity for phage therapy to be systematically evaluated. The first clinical trial of CRISPR-enhanced bacteriophage therapy (NCT04191148) was successfully completed in February 2021. The study LBx-1001, a multi-center randomized, double-blind and placebo-controlled study, tested LBP-EC01, a CRISPR-Cas3-enhanced phage precision medicine product that targets Escherichia coli causing urinary tract infections. According to Locus Biosciences, the study resulted in a decrease in the level of E. coli bacteria in the bladder of the patients in addition to no drug-related adverse effects, proving the potential and safety of the precision-based approach.

To facilitate precise phage genome engineering, Wetzel et Al. combined the BRED and CRISPR-Cas approach to describe CRISPY-BRED. The technique uses the Streptococcus thermophilus CRISPR-Cas9 system, taking advantage of the inactive and active Cas proteins that are described for gene silencing and genome editing in Mycobacterium, and phage-derived recombination proteins. A CRISPR-Cas9 plasmid is created encoding a single guide RNA [sgRNA] corresponding to the BuzzLyseyear gene 43 to be deleted. After the introduction of the plasmid into M. smegmatis mc2155, the BuzzLyseyear genomic DNA and a 500 bp synthetic DNA consisting of the sequences upstream and downstream gene 43 are co-electroporated into M. smegmatis mc2155 carrying the recombineering plasmid pJV138, followed with plating of the progeny with M. smegmatis mc2 155psgRNA cells in the presence of kanamycin and PCR screening. This recombineering approach with CRISPR-mediated counter selection makes the recovery of recombinants simpler compared to BRED and is expected to have applicability to bacteriophages of several other bacterial hosts [47].

V.   OUTLOOK AND CONCLUSIONS

Unless the global issue of anti-microbial resistance (AMR) is surmounted, it has been estimated that, by 2050, ten million people will die each year from AMR infections, with the ESKAPE (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species) pathogens at the heart of the crisis [48]. Phage therapy promises to be a part of the multidimensional strategies for treating drug-resistant pathogens. In particular, phages with AMR-targeting CRISPR systems could lead to changes in the microbial genetic landscape through the deletion of AMR genes in specific targeted bacteria. While their use at present as monotherapy is not very likely in cases of serious illness, the application versatility of phages, such as their combination with certain antibiotics and with other phages forming phage cocktails with a collectively greater antibacterial spectrum of activity, provides a significant advantage. Apart from anti-microbial therapy, phages have been previously used for drug delivery systems, imaging, tissue scaffolds, and diagnostics. Phage VLP vaccines have shown great efficiency in animal models, leading some to enter clinical trials [49,50]. Although phages allow a high-precision approach for therapy, the use of designer phages is still in its infancy, in part due to the tremendous diversity of phages. So far, phage engineering has only involved a minuscule percentage of the existing phage types. For example, the M13 phage has been widely used for material science applications even though phages of other morphologies might further enhance practical applications of phages in the field [51].

CRISPR-Cas and BRED edited bacteriophages can revolutionize bacteriophage therapy, although real-world application techniques would have to be developed. Strategies such as the inactivation of phages to prevent their

propagation outside the lab, for example, by modification of the bacteriophage genome by removal of essential protein genes, followed by their addition in trans in production hosts, can help tackle the issue. The application of machine learning and artificial intelligence advancements, such as the provision of insights into probable genetic elements encoding host rang and the prediction of phage-host interactions [52], could increase the efficacy of the techniques as well.

With rapid advances in the biotechnological toolbox for genetic engineering, modified phages still require close evaluation due to their influence on genome evolution, bacterial community dynamics, and ecosystem biogeochemistry [53]. Although its widespread use would prove to be a challenging process, with rapid advances in the field and a non-prohibitive regulatory framework for the application of phage therapy, its potential utility, in combination with CRISPR-Cas and BRED techniques, could by large outreach those from a solely clinical standpoint.

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