Engineering Microbiology 3 (2023) 100097

Contents lists available at ScienceDirect

Engineering Microbiology

journal homepage: www.elsevier.com/locate/engmic

Review

The emerging role of recombineering in microbiology

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ARTICLE INFO

Keywords:
Recombineering
Natural products
Viral mutagenesis
Phage genome engineering
Bacterial metabolism

ABSTRACT

Recombineering is a valuable technique for generating recombinant DNA *in vivo*, primarily in bacterial cells, and is based on homologous recombination using phage-encoded homologous recombinases, such as Red$\alpha\beta\gamma$ from the lambda phage and RecET from the Rac prophage. The recombineering technique can efficiently mediate homologous recombination using short homologous arms (~50 bp) and is unlimited by the size of the DNA molecules or positions of restriction sites. In this review, we summarize characteristics of recombinases, mechanism of recombineering, and advances in recombineering for DNA manipulation in *Escherichia coli* and other bacteria. Furthermore, the broad applications of recombineering for mining new bioactive microbial natural products, and for viral mutagenesis, phage genome engineering, and understanding bacterial metabolism are also reviewed.

1. Introduction

Recombineering is a method of homologous recombination (HR) that was developed and used primarily in *Escherichia coli* and is catalyzed by homologous recombinases, such as Red$\alpha\beta\gamma$ from lambda phage and RecET from the Rac prophage [1–5]. In contrast to RecA-dependent HR, the most important advantage of recombineering is that short homologous arms (HA) (only ~50 bp in length) are required to achieve high efficiency of HR. Therefore, linear DNAs, composed of either double-stranded DNAs (dsDNA) simply generated by PCR or restriction digestion or single-stranded DNAs (ssDNA), such as oligonucleotides, can be used as homologous substrates. Secondly, recombineering can quickly and precisely edit target DNA, while not being constrained by the presence of restriction sites and the size of DNA molecules. These advantages make it a very efficient, rapid, simple, and inexpensive molecular tool or molecular technology that has now become the cornerstone for precise genetic engineering.

For recombineering, electrocompetent cells have to be prepared and exogenous DNA with homology arms has to be electroporated into the electrocompetent cells [1]. However, it is difficult to transform exogenous DNA into cells by electroporation for some bacteria which can only be transformed DNA by conjugation or other methods. Thus, this disadvantage of recombineering is unfavorable for broadening its applications. However, this does not affect the emerging role of recombineering in microbiology.

Natural products (NPs) have been invaluable sources of drug discovery for decades. However, it has become increasingly difficult to discover new NPs by traditional approaches, such as bioactivity guided discovery. Bioinformatic approaches have revealed the plethora of NP biosynthetic gene clusters (BCGs) in microorganisms, thereby illuminating their potential for synthesizing structurally and functionally diverse NPs. Since most of these BGCs are silent under standard laboratory conditions, activating silent or cryptic BGCs is essential for discovering new bioactive NPs [6,7], and recombineering can be utilized to activate such BGCs. Recombineering can also be used for reengineering bacterial metabolism for research and industrial purposes.

Although recombineering was initially developed for bacteria, it can also be used to modify viruses. For example, adenoviruses and herpesviruses have been used as vectors for gene therapy and vaccines, and recombineering provides a mechanism for rapid manipulation of these viruses, including the generation of viral infectious clones and viral mutants. Phages are also targets for recombineering. With the decreasing rate of development of new antibiotics, phage therapy has become a promising alternative to antibiotic therapy, and recombineering can enhance the suitability of phages for therapeutic use.

In this review, we summarize the advances in recombineering for DNA manipulation in bacteria with particular emphasis on the applications of recombineering in mining new bioactive microbial NPs, viral mutagenesis and phage genome engineering, and its potential use in bacterial metabolism (Fig. 1).

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https://doi.org/10.1016/j.engmic.2023.100097
Received 20 February 2023; Received in revised form 9 May 2023; Accepted 10 May 2023
Available online 24 May 2023
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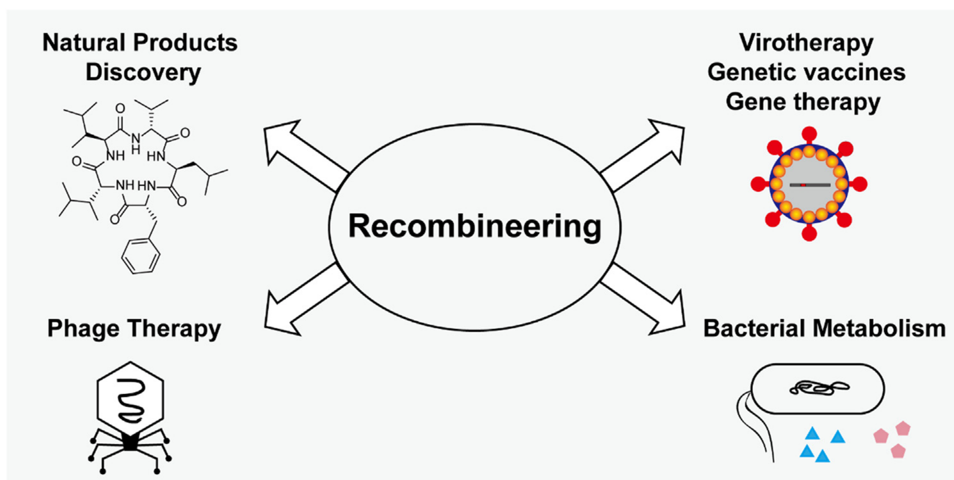

Fig. 1. Overview of the key applications of recombineering. The broad applications of recombineering have been demonstrated in the genome mining of new bioactive microbial natural products, and in viral cloning and mutagenesis, phage genome engineering, and bacterial metabolism.

2. What is recombineering?

2.1. Redα and RecE

Redα and RecE are ATP-independent and Mg^{2+}-dependent enzymes that bind to dsDNA ends and digest dsDNA progressively in the 5′ to 3′ direction, yielding 3′-ended ssDNA overhangs (Fig. 2) [8–11]. Redα forms a toroidal trimer while RecE forms a toroidal tetramer, and each has a tapered channel that is large enough to accommodate dsDNA at the wide end, but only allow passage of ssDNA at the other end [12,13]. Full-length RecE (866 amino acids) is much larger than Redα (226 amino acids) and has an exonuclease domain that encompasses the last 260 C-terminal amino acids. Fu *et al.* showed that full-length RecE along with RecT could efficiently facilitate HR between two linear DNAs, although the function of the N-terminal of RecE is unclear [6].

2.2. Redβ and RecT

Redβ, a 261-amino-acid ssDNA annealing protein (SSAP), catalyzes the annealing of complementary ssDNA produced by Redα to form a DNA-Redβ complex that protects ssDNA from nucleolytic degradation [14]. Redβ forms inactive rings of about 12 subunits without DNA, whereas active rings are composed of 15–18 subunits in the presence of oligonucleotides or ssDNA, and the DNA-Redβ complex forms a left-handed helical filament after the annealing reaction [15,16]. RecT is also an SSAP, similar to Redβ, although RecT can bind to both dsDNA

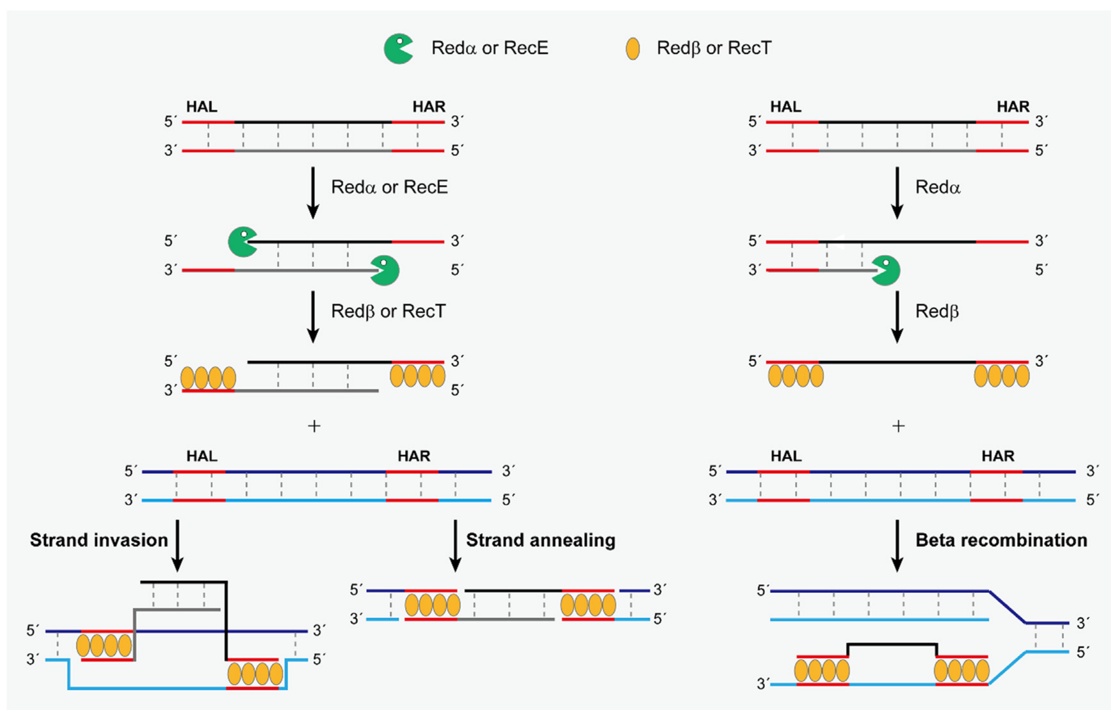

Fig. 2. Models for recombineering. Redα or RecE degrades the dsDNA from the 5′-ends exposing the 3’-ssDNA overhangs, which act as substrates for Redβ or RecT. Redβ or RecT binds to ssDNA to form a protein-DNA complex that protects the ssDNA from degradation. The complex then searches for homologous ssDNA mediated by Redβ or RecT and finally completes HR. LCHR prefers “strand invasion” or “beta recombination” mediated by Redα/Redβ. LLHR prefers “strand annealing” mediated by RecE/RecT. HAL, left homology arm; HAR, right homology arm.

and ssDNA [17]. Recently, researchers found that SSAPs can specifically bind to the C-terminal tail of the host's ssDNA binding protein (SSB), and co-expression of SSAPs and SSBs can remarkably increase recombineering efficiency [18,19].

2.3. Redγ

Redγ comes in two different forms, GamL (16.1 kDa) and GamS (11.4 kDa), which are presumably translated from two different translational start sites [20]. Redγ can prevent the major *E. coli* exonuclease RecBCD from degrading foreign DNAs [21], and although it only naturally exists in lambda phage, Redγ can improve the recombination efficiency of both Redα/Redβ from lambda phage and RecE/RecT from Rac prophage [6,21].

3. Mechanism of recombineering

Firstly, the 5′ to 3′ DNA exonucleases Redα or RecE degrades dsDNA from the 5′-ends, generating 3' ssDNA overhangs, or degrades one strand on short pieces of dsDNA to generate linear ssDNA (Fig. 2). Then, the recombinases Redβ or RecT binds to the ssDNA to form a protein-DNA complex that protects ssDNA from degradation. The protein-DNA complex will look for the complementary ssDNA, mediated by Redβ or RecT, and finally complete HR. Recombineering between a linear DNA molecule and a circular episome/plasmid, both flanked by short HAs, is called linear plus circular homologous recombination (LCHR); recombineering between two linear DNA segments is called linear plus linear homologous recombination (LLHR) [6]. Studies demonstrate that the Red system is more effective in LCHR, whereas RecET is more effective in LLHR [6]. LCHR and LLHR have different mechanisms: LCHR prefers “strand invasion” or “beta recombination”, which is dependent on DNA replication, whereas LLHR prefers “strand annealing”, which is independent of DNA replication [6,22]. However, the mechanism of LLHR is still not clearly understood. LCHR takes place at the replication fork, and 3' ssDNA overhangs complementary to the lagging-strand template are preferentially annealed to the targets [22,23]. The exonuclease activity of Redα prefers a 5′-phosphorylated end to a hydroxylated end, and Redα can asymmetrically digest phosphorylated dsDNA to yield ssDNA. Based on experiments with asymmetric DNA substrates, Red proteins can produce full-length single-stranded intermediates to establish single-stranded heteroduplexes at the replication fork by Redβ to complete the HR. Hence “beta recombination”, a new model for HR, was proposed (Fig. 2) [22].

4. Advances in recombineering for DNA manipulation

Using recombineering, high-copy plasmids, bacterial artificial chromosomes (BACs), and *E. coli* genomes can be precisely manipulated [1,2]. These DNA modifications include deletions, insertions, replacement, point mutations, and gap repair. Direct cloning, DNA assembly, and seamless mutagenesis based on recombineering have been developed.

4.1. Direct DNA cloning based on recombineering

Full-length RecE and RecT have been reported to be more efficient at LLHR than LCHR, and ten BGCs (10–50 kb in length) from *Photorhabdus luminescens* TT01 have been directly cloned using recombineering [6]. Recombineering omits the cumbersome work of traditional construction of genomic DNA libraries and greatly promotes bioprospecting for NPs. Wang *et al.* constructed a series of standard plasmids and developed a fluent protocol for direct cloning and engineering of BGCs [24]. Recently, ExoCET (Exonuclease Combined with RecET recombination), the combination of *in vitro* annealing of T4 DNA polymerase and *in vivo* HR by recombineering, has been developed to directly clone large pieces of DNA [25]. Recombineering provides a powerful tool for the direct cloning of large DNA fragments, such as large BGCs and viral genomes.

4.2. DNA assembly based on recombineering

Two linear DNA fragments can be assembled into a plasmid in one step by RecET-mediated recombineering, and ExoCET can be extended to assemble multiple pieces of DNA. In contrast to transformation-associated recombination (TAR) cloning, which can take at least two weeks for DNA assembly, ExoCET just needs three days. Gibson assembly can only recombine up to twelve DNA fragments at once, whereas ExoCET is able to assemble at least twenty DNA pieces [26]. Song and colleagues refactored the spinosad BGC by joining twelve PCR products with a plasmid in a single reaction using ExoCET [26]. ExoCET multipiece DNA assembly is a broadly applicable tool for reconstructing complex BGCs for diverse aims.

4.3. Seamless mutagenesis based on recombineering combined with CcdB counterselection

In 2014, Wang and colleagues developed an efficient, seamless mutagenesis method by combining recombineering and *ccdB* counterselection [27], which is based on the CcdB/CcdA toxin-antidote system. This mutagenesis method involves two steps. Firstly, a *sm-ccdB* cassette is inserted into the target site in a CcdB-resistant *E. coli gyrA462*$_{\mathrm{Arg\rightarrow Cys}}$ strain or performed with CcdA expression. Secondly, the *sm-ccdB* cassette is replaced by the desired DNA sequence by selecting it against the *ccdB* gene. Recently, Song *et al.* established an improved seamless DNA mutagenesis method called “RedEx” by combining recombineering, *ccdB* counterselection and T4 DNA polymerase-mediated *in vitro* annealing, and this updated method will greatly facilitate combinatorial biosynthesis of NPs [28].

5. Recombineering in other bacteria

Recombineering was originally developed for the genetic engineering of plasmids and BACs in *E. coli*, as well as for the genome modification of *E. coli* [1,2,4,5,20,29,30]. Subsequently, it has been gradually applied, with some modifications, to other Gram-negative bacteria closely related to *E. coli*, such as *Shigella* [31–33], *Yersinia* [34], *Serratia* [35], *Salmonella* [36–38], and so on [39–46]. However, in more distant species, application of the Red system has been limited, and host-specific, phage-derived recombination systems are required. Host-specific recombineering systems have also been established in *Lactobacillus* [47–50], *Mycobacterium* [51], *Pseudomonas* [52–54], *Burkholderia* [55], and some other bacteria [7,56-60] (Table 1).

CRISPR/Cas has initiated a new era for genome engineering. CRISPR/Cas has high efficiency to knockout genes, however, it has low efficiency to knockin genes due to the weak capacity of the native homology-directed repair system in some organisms. Recent years, recombineering combined with CRISPR/Cas has been used to edit genomes of bacteria with high accuracy, such as Cascade-Cas3 combined with *Pseudomonas* phage-encoded homologous recombination [54] and combination of the Red system and CRIPSPR/Cas9 for genome editing in *Klebsiella pneumoniae* [61]. Thus, combination of recombineering and CRISPR/Cas will become a hot trend in the future.

6. Genome mining of new bioactive NPs from microorganisms for drug discovery

The rise of rapid and economical DNA sequencing technologies enabled the discovery of many BGCs in microorganisms, almost tenfold higher than the number of NPs found by traditional methods [62]. However, most of these BGCs are cryptic or silent under laboratory growth conditions. Two types of approach for discovering new bioactive NPs by genome mining have been utilized (Fig. 3) [63], including native host expression and expression in heterologous hosts.

Table 1
Application and development of recombineering in other bacteria.

Species	Proteins				Refs
	5′→3′ exonuclease	ssDNA annealing protein	Host exonuclease inhibitor	ssDNA binding protein	
Shigella flexneri	Redα (phage lambda)	Redβ (phage lambda)	Redγ (phage lambda)		[33]
Yersinia pseudotuberculosis	Redα (phage lambda)	Redβ (phage lambda)	Redγ (phage lambda)		[34]
Serratia marcescens	Redα (phage lambda)	Redβ (phage lambda)	Redγ (phage lambda)		[35]
Salmonella enterica	Redα (phage lambda)	Redβ (phage lambda)	Redγ (phage lambda)		[38]
Pseudomonas aeruginosa	Redα (phage lambda)	Redβ (phage lambda)	Redγ (phage lambda)		[39]
Pantoea ananatis	Redα (phage lambda)	Redβ (phage lambda)	Redγ (phage lambda)		[40]
Vibrio cholerae	Redα (phage lambda)	Redβ (phage lambda)	Redγ (phage lambda)		[41]
Burkholderia cepacia	Redα (phage lambda)	Redβ (phage lambda)	Redγ (phage lambda)		[42]
Burkholderia thailandensis	Redα (phage lambda)	Redβ (phage lambda)	Redγ (phage lambda)		[43]
Burkholderia pseudomallei	Redα (phage lambda)	Redβ (phage lambda)	Redγ (phage lambda)		[43]
Klebsiella pneumoniae	Redα (phage lambda)	Redβ (phage lambda)	Redγ (phage lambda)		[44]
Agrobacterium tumefaciens	Redα (phage lambda)	Redβ (phage lambda)	Redγ (phage lambda)		[45]
Escherichia albertii	Redα (phage lambda)	Redβ (phage lambda)	Redγ (phage lambda)		[46]
Lactobacillus plantarum	Lp_0642 (prophage P1)	Lp_0641 (prophage P1)	Lp_0640 (prophage P1)		[47]
Lactobacillus reuteri		RecTl (*L. reuteri*)			[49]
Lactococcus lactis		RecTl (*L. reuteri*)			[49]
Lactobacillus casei	LCABL_13060 (prophage PLE3)	LCABL_13050 (prophage PLE3)	LCABL_13040 (prophage PLE3)		[50]
Mycobacterium tuberculosis	Gp60 (phage Che9c)	Gp61 (phage Che9c)			[51,99]
Pseudomonas syringae	$RecE_{Psy}$ (*P. syringae*)	$RecT_{Psy}$ (*P. syringae*)			[52]
Pseudomonas aeruginosa	A (phage Ab31)	B (phage Ab31)	Redγ (phage lambda)	S (phage Ab31)	[53]
Pseudomonas putida	A (phage Ab31)	B (phage Ab31)	Redγ (phage lambda)	S (phage Ab31)	[53]
Pseudomonas fluorescens	A (phage Ab31)	B (phage Ab31)	Redγ (phage lambda)	S (phage Ab31)	[53]
Pseudomonas syringae	A (phage Ab31)	B (phage Ab31)	Redγ (phage lambda)	S (phage Ab31)	[53]
Burkholderia glumae	$RecE_{YI23}$ (*B. cordobensis* YI23)	$RecT_{YI23}$ (*B. cordobensis* YI23)			[55]
Burkholderia plantarii	$RecE_{YI23}$ (*B. cordobensis* YI23)	$RecT_{YI23}$ (*B. cordobensis* YI23)			[55]
Photorhabdus luminescens	Pluα (*P. luminescens*)	Pluβ (*P. luminescens*)	Pluγ (*P. luminescens*)		[7]
Xenorhabdus stockiae	Pluα (*P. luminescens*)	Pluβ (*P. luminescens*)	Pluγ (*P. luminescens*)		[7]
Clostridium acetobutylicum		Cpf0939 (*C. perfringens*)			[56]
Bacillus subtilis		GP35 (phage SSP1)			[57]
Schlegelella brevitalea	Redα_{7029} (*S. brevitalea*)	Redβ_{7029} (*S. brevitalea*)			[59]
Agrobacterium tumefaciens	$RecE_{RHI145}$ (*Rhizobium* sp. LC145)	$RecT_{RHI145}$ (*Rhizobium* sp. LC145)	Pluγ (*P. luminescens*)		[60]
Agrobacterium tumefaciens	$RecE_{AGROB6}$ (*A. tumefaciens B6*)	$RecT_{AGROB6}$ (*A. tumefaciens B6*)			[60]
Rhizobium rhizogenes	$RecE_{RHI483}$ (*Rhizobium* sp. Root483D2)	$RecT_{RHI483}$ (*Rhizobium* sp. Root483D2)	Pluγ (*P. luminescens*)		[60]

6.1. Promoter engineering for in situ activation of cryptic BGCs

In native host expression approaches, genetic manipulation (e.g., promoter engineering) is applied to activate NP production (Fig. 3A). In particular, the strategy of promoter engineering has been widely used to activate cryptic BGCs for discovering new NPs [64]. The premise of this approach is that native hosts possess streamlined genetic tools, or a suitable recombineering system can be developed for that host.

With regard to cryptic BGC activation, the importance of promoter elements is undeniable as they are responsible for efficient transcription, which is the first stage of gene expression. Many cryptic BGCs have been activated by a promoter insertion strategy using established recombineering systems in different bacteria. For example, Jia *et al.* activated the 49-kb non-ribosomal peptide synthase (NRPS) BGC *plu2670* by insertion of a tetracycline-inducible promoter (P_{tet}) using the Plu$\alpha\beta\gamma$ system [7]. Promoter engineering enables potentially scalable discovery of new NPs from many other bacteria [55,59,65,66].

6.2. Expression of BGCs in heterologous hosts

When applying genetic engineering in the native host is very difficult, BGCs can be expressed in heterologous hosts (Fig. 3B). There are many reasons for expression of BGCs in heterologous hosts, such as activation of silent BGCs, optimization of production yield, acquisition of new analogs by combinatorial biosynthesis, rational engineering of chassis strains, and elucidation of biosynthesis pathways of NPs.

Direct cloning and genetic engineering of BGCs based on recombineering is an efficient platform for mining new NPs by activating cryptic BGCs in heterologous hosts. Fu *et al.* cloned the *plu3263* NRPS BGC by using full-length RecE direct cloning and expressed two BGCs in *E. coli* to yield luminmides A and B [6]. Many cryptic BGCs have been successfully expressed in heterologous hosts [67–69], and recombineering-assisted heterologous expression greatly facilitates bioprospecting for NPs.

Recombineering is also used to optimize the production yields of NPs. Song *et al.* reported a 328-fold enhancement in spinosad production through heterologous expression in *Streptomyces albus* J1074 [26]. Combinatorial biosynthesis has also provided a user-friendly way to yield new NP analogs. The "RedEx" method developed by Song *et al.* enables seamless DNA mutagenesis of large, multi-modular polyketide BGCs, and they successfully used this method to construct the hybrid spnNEWbusA BGC, which produces the butenyl-spinosyns A and D [28]. Gong *et al.* constructed a *Burkholderia* chassis for the high-yield production of FK228 and new FK228-family (unnatural) natural products [70]. Bian *et al.* expressed epothilones in the heterologous host *Schlegelella brevitalea* DSM7029 [71]. Xu *et al.* identified the chuangxinmycin BGC by heterologous expression and elucidated its biosynthetic pathway [72]. Additionally, Zhong and colleagues elucidated the lipoinitiation process in nonribosomal peptide biosynthesis by (sub)domain swapping or point mutation using recombineering combined with *ccdB* counterselection [73]. Thus, the recombineering-based "RedEx" is a powerful tool for combinatorial biosynthesis to generate new NPs, explore biosynthetic pathways, and uncover the mechanisms of NP biosynthesis.

7. Viral cloning and mutagenesis using recombineering

7.1. Adenovirus library construction

Adenoviruses are non-enveloped, medium-sized (70–100 nm in diameter) viruses with linear dsDNA genomes (26–45 kb) flanked by inverted terminal repeats. Recombinant adenoviruses have been broadly

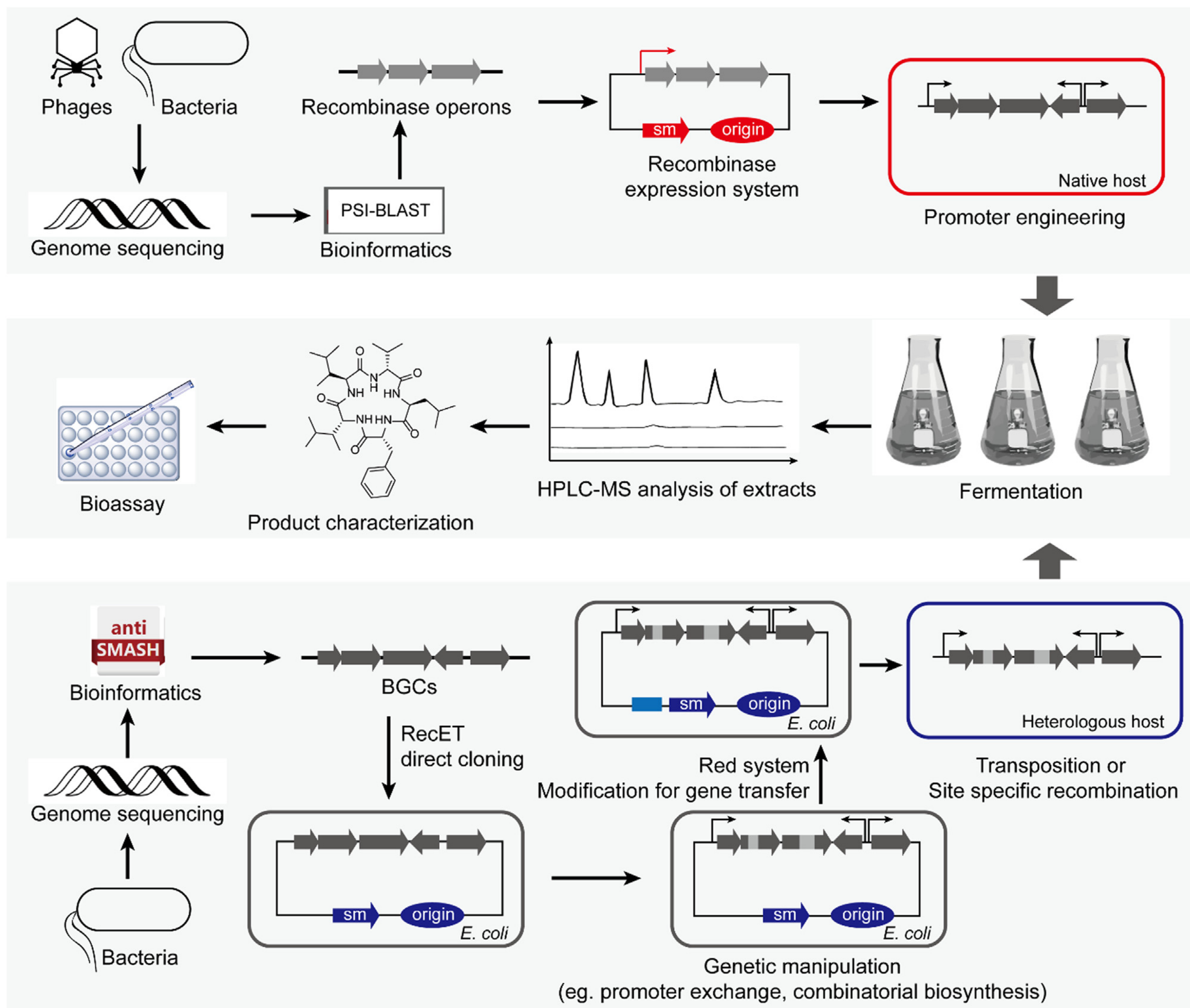

Fig. 3. Strategy for genome mining of cryptic BGCs of microorganisms. For expression of cryptic BGCs in native hosts, the recombineering system is firstly developed by searching for phage or prophage recombinase pairs and optimization of the recombinase expression system. Then, promoter engineering is carried out to activate the cryptic BGCs (top panel). For heterologous expression of cryptic BGCs (bottom panel), the genomes of microorganisms are first sequenced and then analyzed by bioinformatics to predict the BGCs. Then, the BGCs are directly cloned into a vector by recombineering. Next, BGCs are genetically manipulated and transferred into a suitable heterologous host for expression. After fermentation, metabolites are analyzed by HPLC-MS, and further purification, structure elucidation, and bioactivity assay of the products are performed (middle panel). PSI-BLAST and antiSMASH are bioinformatics programs. "sm" in the bottom panel indicates the selectable marker.

developed for oncolytic virotherapy to treat malignant tumors, as gene transfer vectors for gene therapy, and as vaccines against infections [74]. Several strategies have been used to clone adenovirus genomes into vectors, such as conventional cut-and-paste-based cloning strategies, cosmid-based techniques, and traditional HR-based methods [75,76]. However, all these methods are laborious and time-consuming.

Recently, Zhang and colleagues developed an efficient and rapid method to clone intact genomes of adenoviruses via recombineering [74,77] (Fig. 4). Viral genomic DNA isolated from infected cells or clinical samples and the prepared linear vector backbone, flanked by 50 bp HA, are co-electroporated into RecET-expressing *E. coli* GB05-dir. Complete release of the adenoviral genomes from the plasmid backbone is achieved by restriction enzyme digestion, which is the most efficient strategy to rescue recombinant adenovirus. Using this method, an adenovirus library comprising 34 virus types, representing seven adenovirus species, was established.

Using the infectious clones, Red system can be further applied to modify the viral genomes, such as tagging with reporter genes, which can be accomplished with two rounds of LCHR [74]. Thus, this approach provides a rapid and fluent strategy for viral cloning, construction of adenovirus libraries, and mutagenesis for characterization of the recombinant adenoviruses.

7.2. Cloning herpesvirus genomes as BACs

Generation of recombinant viruses to understand their biology and pathogenesis and to develop new vaccines requires fluent and precise genetic manipulation tools. Herpesviruses have relatively large dsDNA genomes (125~240 kb), and since the first reported cloning of a baculovirus genome as a BAC vector [78], BAC technology has become an indispensable method for functional characterization of herpesviruses genomes [79,80].

There are several traditional methods for constructing herpesvirus BACs, such as direct insertion of the BAC vector into the viral genome via HR in virus-permissive cells [81], overlapping cosmids [82], *in vitro* ligation [83], and a sequence-independent *in vitro* transposon-based strat-

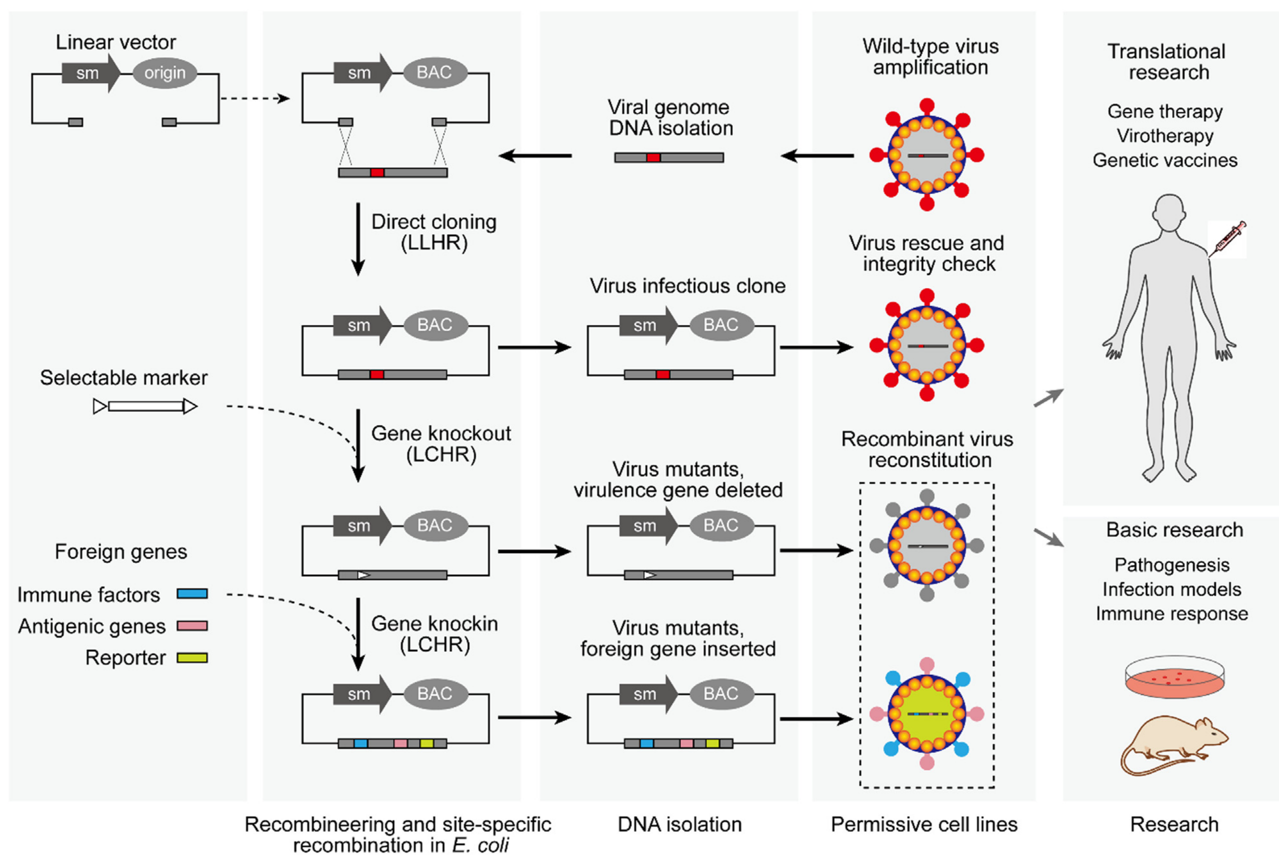

Fig. 4. Generation and mutagenesis of virus infectious clones by recombineering. The process begins with wild-type virus amplification and genomic DNA isolation. The linear cloning vector (for example, p15A-cm for adenovirus or pBAC-cm for herpesvirus), flanked by HAs (50–80 bp), is constructed using either PCR or LLHR. The viral genome DNA and the linear vector are then co-electroporated into a RecET-expressing *E. coli* strain for direct cloning. The resulting infectious clone, which now contains the vector, is validated by restriction enzyme analysis, rescue experiments, and sequencing. Knockout of virulence genes and knockin of foreign genes (such as immune factor genes, antigenic genes, and reporters) are achieved by Red$\alpha\beta$-mediated deletion or insertion combined with counterselection or followed by site-specific recombination. The recombinant virus is rescued for *in vitro* and *in vivo* characterization and further used for basic and translational research. "sm" indicates the selectable marker.

egy [84]. However, removal of the BAC vector sequence from the viral genome is inefficient and time-consuming. Recently, a robust strategy for construction of herpesvirus infectious clones was reported in which the BAC vector is placed at the termini of the linear virus genome via ExoCET (Fig. 4) [85]. For this approach, the isolated viral genomic DNA and the linear BAC vector, flanked by 80 bp HAs at both termini, are incubated *in vitro* for assembly, and then the assembled products are transformed into *E. coli* GB05-dir to obtain the final herpesvirus BACs. Linear viral genome DNA is obtained by cutting off the BAC vector with restriction endonucleases, the recognition sites of which were previously placed at the junction of the linear terminus of the viral genome and the BAC vector backbone. The viral genome is then transfected into mammalian cells to quickly rescue viruses without any further purification procedure. Generation of herpesvirus BACs is straightforward and rapid, requiring only a few days. This direct cloning method can completely bypass the cumbersome purification procedure, which usually requires several passages of the virus.

7.3. Herpesvirus BAC site-specific mutagenesis

Site-specific mutagenesis of the viral BAC DNA can be conducted to functionally analyze each viral gene, insert foreign genes, and delete virulence genes once the viral BAC is generated. However, the large size of BACs precludes the introduction of mutations by conventional enzymatic cleavage and ligation methods. Viral BACs, constructed by incorporating the BAC vector backbone at the viral terminus, are stable in *E. coli* and can be further engineered by the Red system and various site-specific recombinases to accomplish site-specific mutagenesis.

Attenuated recombinant viruses represent a particularly promising vaccine research avenue that could be used to improve existing vaccines and develop new ones [86]. Yuan and colleagues constructed the recombinant attenuated pseudorabies virus (PRV) strain DCD-1 by deleting the virulence genes gE-gI and TK and the dispensable gene gG (Fig. 4) [85]. Briefly, deletion of gE-gI was accomplished by inserting a kanamycin resistance gene flanked by FRT sites using Red$\alpha\beta$-mediated LCHR. Then, the kanamycin resistance gene was removed by Flp recombination, and the systematic expression profiles of loci and exogenous promoters were evaluated by inserting fluorescent reporters. Expression of foreign antigens can stimulate relevant immune responses, and therefore, attenuated recombinant PRVs expressing heterologous antigens can be constructed to develop multivalent PRV vector vaccines [86].

8. Genetic engineering of bacteriophage genomes

Bacteriophages were discovered independently by Twort in 1915 [87] and d'Herelle [88] in 1917 and were then used as early antimicrobial agents. However, with the discovery of penicillin in 1928 and the advent of the antibiotic era, research on phage therapy declined dramatically. In recent decades, widespread use of antibiotics has led to the increased prevalence of multidrug-resistant bacterial infections while the rate of development of traditional small molecule antibiotics has decreased drastically. Thus, the use of bacteriophages as anti-infectives

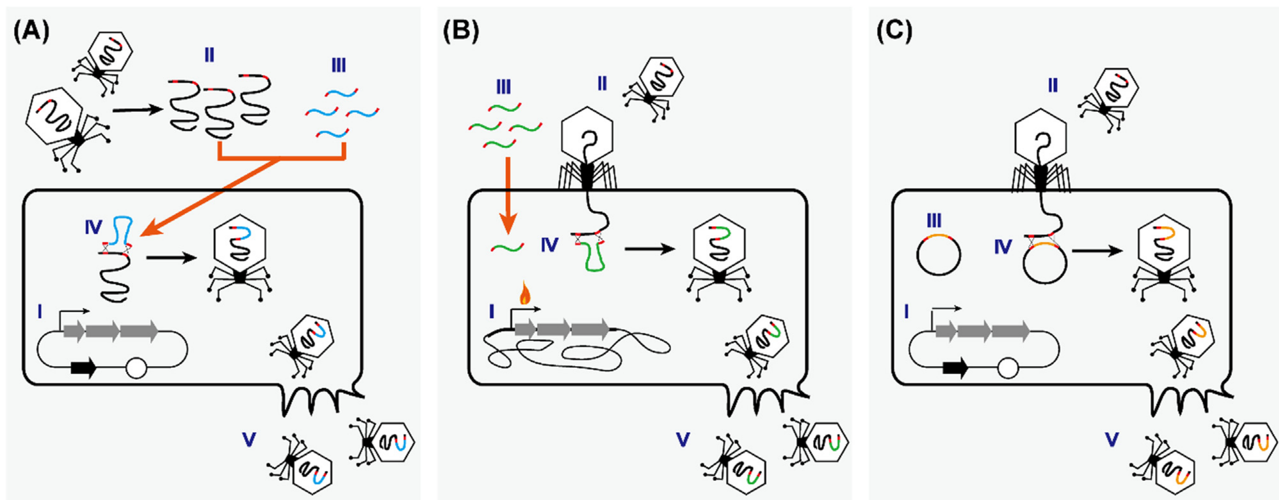

Fig. 5. Engineering of bacteriophage genomes by recombineering. (**A**) Bacteriophage recombineering of electroporated DNAs (BRED). Purified bacteriophage DNA (II) and dsDNA recombineering substrates (III) are co-electroporated into bacterial cells carrying a recombination system (I). Homologous recombination between their HAs (in red) (IV), mediated by recombinases, results in recombinant bacteriophage particles (V, containing DNA fragments in blue). (**B**) *In vivo* recombineering. Bacterial cells carrying a recombinase expression system (I) are infected with the bacteriophage (II) and subsequently transformed with dsDNA or ssDNA (III). Homologous recombination between the bacteriophage genome and the dsDNA/ssDNA (HAs in red) (IV) occurs, and then the recombinant bacteriophage particles (V, containing DNA fragments in green) are recovered. (**C**) Modified *in vivo* recombineering. Bacterial cells carrying a recombinase expression system (I) and a plasmid containing the mutated genes with flanking HAs (III) are infected with the bacteriophage (II). Homologous recombination between the bacteriophage genome and the mutated genes (HA in red) (IV) occurs, and then the recombinant bacteriophage particles (V, containing DNA fragments in orange) are recovered.

has gained renewed interest. However, almost no natural phages can be considered for clinical therapy [89]. Three strategies for engineering of lytic bacteriophage genomes to modulate phage host range, improve phage activity against biofilms, and reduce phage toxicity and immunogenicity have been reported [90].

8.1. Bacteriophage recombineering of electroporated DNA

Bacteriophage recombineering of electroporated DNA (BRED) was initially applied to redesign mycobacteriophages [91,92] and was then expanded to edit other bacteriophages [93,94]. Fig. 5A summarizes the BRED procedure. Firstly, bacteriophage DNA and dsDNA substrate are co-electroporated into electrocompetent, phage-sensitive bacterial cells that express recombinases. The dsDNA substrate contains the inserted DNA segment flanked by HAs, which enable HR to occur between the phage genome and dsDNA substrate. Modified phages can be obtained at high frequencies (10 to 15%) and are easily detected using PCR. However, further purification is needed to isolate the targeted phage mutants after initial PCR screening of plaques containing mutants. BRED can serve as a universally applicable tool for editing bacteriophage genomes in a wide range of taxa.

8.2. In vivo *recombineering*

Although BRED showed high efficiency for engineering bacteriophages, it highly relies on the efficiency of co-transformation of the bacteriophage DNA and dsDNA substrate into the same cell. Thus, it is especially difficult to manipulate in Gram-positive bacteria that show very low transformation efficiencies. Another method, *in vivo* recombineering, was reported using lambda phage as a tool for creating specific changes in *E. coli* phage genomes (Fig. 5B) [95]. Briefly, *E. coli* harboring a defective lambda prophage, which carries the P_L operon controlled by the *cI857* temperature-sensitive repressor, is infected with the bacteriophage to be modified. Following phage infection, the lysogen is induced to express lambda Red recombinases by heating the bacterial culture to 42°C. The ssDNA or dsDNA substrate is electroporated into the cells, and the phage lysates are subsequently recovered and checked for recombinant bacteriophage of which the yield is about 0.5–2%. This method overcomes the transformation limit of BRED to some extent, although it is laborious to screen for the recombinant bacteriophages.

8.3. Modified in vivo recombineering

Wang *et al.* modified the *in vivo* recombineering method to generate *Klebsiella* bacteriophage mutants (Fig. 5C) [96]. The *Klebsiella* host is transformed with two plasmids: one plasmid can be induced to express recombinases, and the second plasmid contains the mutated genes with flanking HAs. HR between the bacteriophage genome and the mutated genes occurs after infection of the host by the target phage, resulting in the generation of the recombinant phages. This study represents the first example of *Klebsiella* phage mutants produced by the *in vivo* recombineering method.

9. Bacterial metabolism

Recombineering is a very efficient and simple tool for generating mutant bacterial strains for applications in industry and agriculture. For example, *Pseudomonas aeruginosa* PAO1 is one of the best producers of rhamnolipids; however, wild-type PAO1 is a human pathogen. To obtain an industrial strain for rhamnolipid production, the *aroA* gene was deleted using recombineering, generating the highly attenuated PAO1*aroA* mutant strain [53], and then, *rhlAB* and *estA* were overexpressed in the PAO1*aroA* mutant to increase rhamnolipid yields. *Pseudomonas protegens* is a plant growth-promoting strain, which can be modified to act as a biocontrol agent and biofertilizer. The *P. protegens* mutants CHA0-Δ*retS-Nif* and Pf-5-Δ*retS-Nif* were constructed via recombineering [97,98], and these multifunctional mutant strains, which have both nitrogen-fixing and bactericidal activities, can be used as biological agents to improve crop yield.

However, most research on bacterial metabolism still uses the traditional RecA-dependent HR method to manipulate gene knockins and knockouts. One critical reason is that no efficient recombineering system has been developed in these bacteria. So, recombineering systems are urgently needed in a broader range of bacteria to facilitate research in bacterial metabolism.

10. Conclusion

Recombineering has been widely used due to its high efficiency and short homology requirements.

In this review, we described the developments and applications of recombineering, including the advances in recombineering for DNA manipulations and the roles for recombineering in the genome mining of new bioactive NPs from microorganisms for drug discovery. We also described how recombineering can be used in the construction of adenovirus libraries, in the direct cloning of herpesvirus genomes as BACs coupled with site-specific mutagenesis for developing vector vaccines, and in the engineering of bacteriophage genomes for the development of phage therapy. We also anticipate that recombineering will facilitate future studies on bacterial metabolism as new recombineering systems are developed and become more widely applied in bacteria.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Given their roles as Editor-in-Chief and Guest Editor, respectively, Dr. Youming Zhang and Dr. Jun Fu had no involvement in the peer review of this article, and had no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Dr. Shengbiao Hu.

Acknowledgement

This work was supported by the National Key R&D Program of China (2019YFA0904000), the National Natural Science Foundation of China (31570094, 81502962, 32170038, 32270088), the Taishan Scholar Program of Shandong Province, the Shandong Provincial Natural Science Foundation of China (ZR2020MC015, ZR2022MC142), and the Fundamental Research Funds of Shandong University (2018GN021).

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