Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2011, Article ID 971296, 10 pages
doi:10.1155/2011/971296

Review Article

Bacterial Artificial Chromosome Mutagenesis Using Recombineering

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Received 2 August 2010; Accepted 21 October 2010

Academic Editor: Masamitsu Yamaguchi

Gene expression from bacterial artificial chromosome (BAC) clones has been demonstrated to facilitate physiologically relevant levels compared to viral and nonviral cDNA vectors. BACs are large enough to transfer intact genes in their native chromosomal setting together with flanking regulatory elements to provide all the signals for correct spatiotemporal gene expression. Until recently, the use of BACs for functional studies has been limited because their large size has inherently presented a major obstacle for introducing modifications using conventional genetic engineering strategies. The development of *in vivo* homologous recombination strategies based on recombineering in *E. coli* has helped resolve this problem by enabling facile engineering of high molecular weight BAC DNA without dependence on suitably placed restriction enzymes or cloning steps. These techniques have considerably expanded the possibilities for studying functional genetics using BACs *in vitro* and *in vivo*.

1. Introduction

The information generated from the Genome Projects will be of the greatest value if it can be converted into functional data, particularly if this increases our understanding of normal gene function and allows strategies to be developed for prevention and treatment of human disease. Likewise, the output of genetic variants of human disease uncovered by the International HapMap Project [1] requires effective tools to accurately translate this growing knowledge to model systems for functional studies.

Bacterial artificial chromosomes, or BACs, are fertility-(F-) factor-based plasmid vectors that replicate stably in low copy number [2, 3]. Because of their large insert capacity, BACs can carry complete genes containing flanking distant regulatory DNA that provide signals for correct spatio-temporal gene expression [4–9]. As the average size of the protein-coding genes annotated in Human Genome Project is 27 kb [10], most of the genes are well within the cloning capacity of BAC vectors. Thus, BACs carrying full-length genes in their natural chromosomal setting is becoming an attractive resource for studying genome structure and function, representing an exciting alternative to conventional vector systems.

However, until about a decade ago, the large size of BACs has presented major hurdle for their precise manipulation to introduce specific changes such as mutations, reporter genes, and markers for functional studies in the mammalian environment [11–13]. To address this problem, a number of techniques were developed in the late 1990s that were based on homologous recombination pathways, which meant they were not limited by the size of a BAC, permitting much more flexible engineering compared to conventional genetic engineering using restriction enzymes or site-specific recombination methods. One of these is recombineering (recombination-mediated genetic engineering [14]), which was adapted from bacteriophage where the recombination genes were carefully delineated and moved to mobile plasmid systems that were transferable to host *E. coli* strains.

Recombineering technology has gradually evolved into a powerful new approach to studying genetic function using genes in their natural genomic context that were widely

Table 1: Comparing conventional BAC modification strategies with recombineering.

	Conventional strategies	Recombineering
BAC Modification Strategies	Homologous recombination: RecBCD and RecF pathways	RecE pathway: RecE, RecT (+Gam)
	Site-specific integration: Integrases of different pathways (Cre, Flp)	Lambda Red pathway: Exo, Beta, Gam
Flexibility	Require pre-engineered or existing specific sites: Chi sites, recombinase attachment sites	Sequence independent, but need prior knowledge of target sequence
Homology requirement	Long homology of ~1 kb is required for efficient homologous recombination	As short as 40 bp of homology is sufficient
Versatility	May require specific strain backgrounds to work	Mobile systems can be easily transferred to and adapted for use in a wide range of strains and species
Efficiency and facileness	Low efficiency for homologous recombination	At least 50-fold higher than traditional homologous recombination
	Labourious and lengthy	Can be done in days

available from clones in BAC libraries [15]. Furthermore recombineering have been further improved by combining with other genetic engineering methods such as site-specific integration systems for more sophisticated BAC manipulations [16–19]. This paper will focus on the rise of recombineering as the major homologous recombination-mediated approach for BAC modifications, as well as its novel applications and future prospects in the art of genetic tinkering.

2. BAC Modifications Using Recombineering

Recombineering is defined as homologous recombination mediated by phage-based recombination systems, which include the Rac prophage-derived RecE pathway and also the *E. coli* λ phage Red pathway (Table 1) (reviewed by Copeland et al. [14]). The RecE pathway principally involves RecE and RecT proteins, while the λ Red pathway is mediated by its Exo and Beta proteins (reviewed by Court et al. [20]).

The key feature of recombineering strategies is that it is independent of *E. coli* endogenous homologous recombination functions, and it therefore, can be transferred using mobile plasmids into hosts that are recombination deficient (e.g., *RecA* background) to introduce recombination proficiency transiently [21–24]. Transient systems that expose the target DNA for only a short time to the recombination enzymes provide the added benefit of facilitating the stable modification of DNA substrates that are traditionally prone to rearrangements due to recombination, for example, BACs containing repetitive DNA [25].

Recombineering can be used for many modes of BAC mutagenesis as well as cloning, based on the design of the targeting substrate (Figure 1). The target site can be either on a resident plasmid, chromosomal region, or even exogenous source of DNA [26], while the incoming targeting substrate can either be a linear dsDNA [21, 22] or single-stranded oligonucleotides [27, 28]. As the required length of shared homology for efficient recombineering is typically only 40–50 bp [22], targeting substrates can be easily produced by standard PCR procedures or oligo synthesis.

The ability of recombineering to mediate recombination with open-ended linear dsDNA is made possible by the additional expression of λ Gamma (Gam) protein to the Red [21, 29] and RecE pathways [22, 23]. Gam inhibits the exonuclease activity of RecBCD and spares linear dsDNA from degradation, allowing it to recombine to its target [30, 31]. With coordinated control of Gam expression, BACs can be modified in RecBCD+ host strains with linear dsDNA while avoiding the deleterious effects of RecBCD null mutation and constitutive Gam expression on cell viability [23, 31, 32]. On the other hand, recombineering with single-stranded oligos requires only the function of Beta [27] or RecT [33] and is only slightly affected by RecBCD in the absence of Gam expression [27].

By incorporating homologous sequences at the two ends of the targeting substrate to correspond to sequences flanking a target site, recombination at the two homologous sites can effectively insert the targeting substrate in place of the target site (Figure 1(a)). This is a straightforward one-step strategy to delete a gene of interest while introducing a foreign sequence, usually a selectable antibiotic marker gene or a transgene of interest. If no native DNA needs to be removed, new sequences can also be introduced by designing the flanking homology arms of the targeting substrate to correspond to the target site without any gap in between (Figure 1(b)). Operational sites such as antibiotic marker and recombinase recognition sites (*loxP,* FRT, etc.) can be integrated this way with minimal disruption to the target DNA.

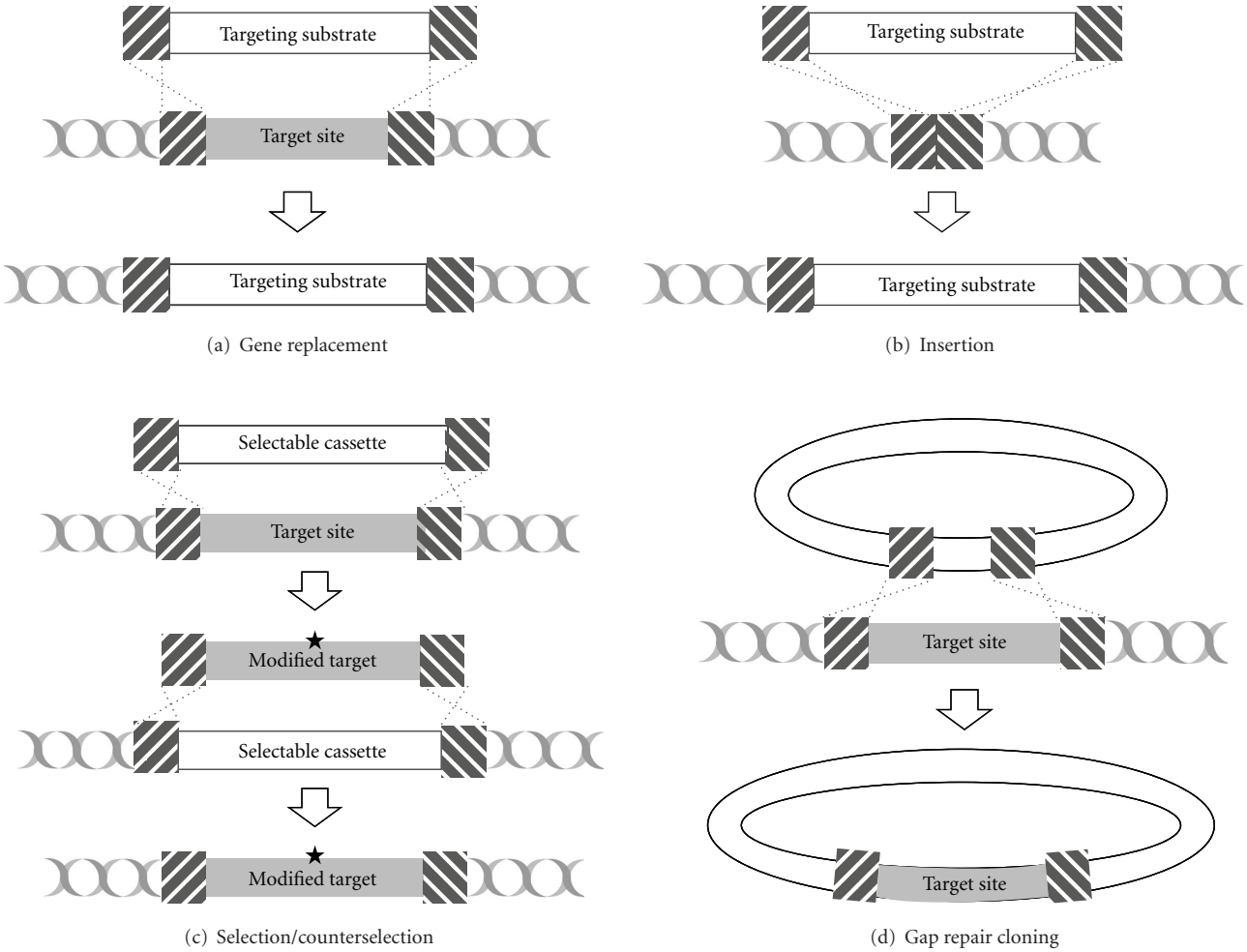

Figure 1: General applications of recombineering in BAC modifications. Recombineering is applicable in various mutagenesis strategies, depending on the design and nature of the targeting substrate and target site; see text for more details. Stippled boxes denote homologous sequences for recombination. (a) Gene replacement. Recombineering can be employed to replace a target site with any sequence of interest. (b) Insertion. DNA can also be introduced by recombineering without removing any of the existing sequence. (c) Selection/counterselection. Recombineering can mediate subtle modifications such as nucleotide substitutions via two rounds of recombinations, by first introducing a selectable cassette followed by replacement of the cassette with the modified version of the target site. (d) Gap repair cloning. A target site of interest can be cloned from a fragment or plasmid into a linearised vector *in vivo* by recombineering, through recombination between the ends of the cloning vector and target site.

Besides the removal, insertion, or replacement of large segments, subtle modifications with no operational sequence introduced can be achieved by the selection/counter-selection strategy [22] (Figure 1(c)). This two-round recombineering strategy first involves the replacement of the target site with a selectable cassette, of which the presence can be selected for and against. The cassette is then subsequently replaced by a modified version of the target site, and the correct recombinants are recovered by selecting against the selectable cassette. Popular options of counter-selection markers include *sacB* [22], *rpsL* [34], I-*Sce*I endonuclease [35], as well as markers that can both be selectable for and against including *galK* [36], *thyA* [37], and *tolC* [38]. Subtle mutations such as nucleotide substitution can also be generated in single-step recombineering with ss-oligo as targeting substrate; however, screening of colonies will be required to detect correct recombinants [24].

One remarkable use of recombineering is the effective gap repair cloning of any target site of interest, which is similar to transformation-associated recombination (TAR) cloning in recombination-proficient yeast [39]. This is done by constructing a cloning vector with homology arms corresponding to the target site, which is joined to form circular closed plasmid following recombination (Figure 1(d)). The recombinant plasmid vector can then be easily retrieved with plasmid purification protocols. This *in vivo* cloning application is a powerful method as it can facilitate retrieval of a target site from various origins, including linear dsDNA cassette, resident plasmid, chromosome, and even genes from a complex mixture of total genomic DNA [26].

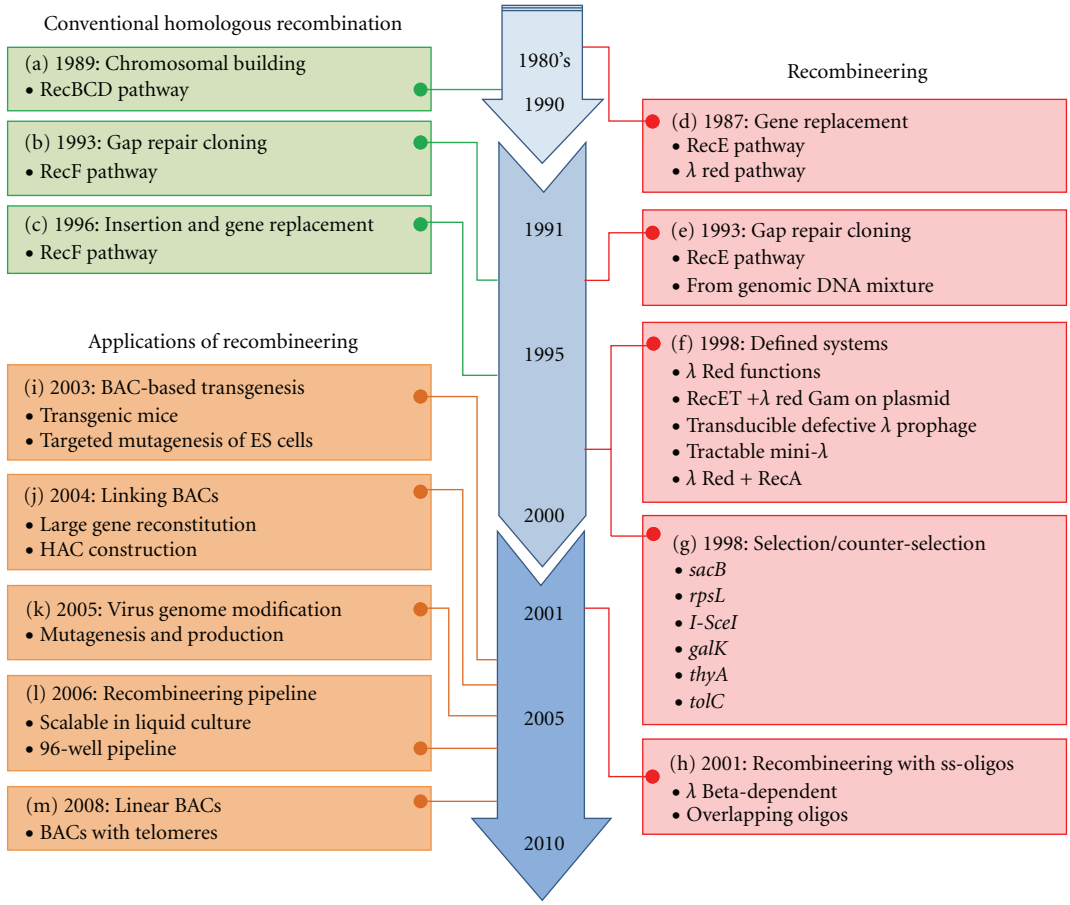

Figure 2: Evolution of recombineering as a major modification strategy for BACs. The evolution of homologous recombination over time since the 1980s is depicted in green (conventional homologous recombination) and red boxes (recombineering), respectively, while major applications of recombineering are depicted in orange boxes. Conventional homologous recombination was the main approach for BAC modifications between 1980s and early 1990s ((a)–(c)), alongside early recombineering techniques mediated by *E. coli* strains carrying activated λ or Rac recombination functions ((d)-(e)). Following the establishment of defined recombineering systems in late 1990's (f), recombineering emerged as the favourable method over conventional homologous recombination, and technologies for recombineering rapidly expanded in the next few years ((g)-(h)). Recombineering has, thereafter, been adopted for use in a wide range of biological applications ((i)–(m)). See text for details.

However, due to the difficulty of rescuing large genes from total DNA, so far, this technique has not been used to directly clone genomic fragments into BAC clones.

3. Emergence of Recombineering as an Ideal BAC Modification Technique

To deal with the large size that is inherent of BACs, several novel methods were tested based on site-specific recombination systems, including the P1-derived Cre-*lox* [40, 41], the baker's yeast *Saccharomyces cerevisiae* Flp-FRT machineries [42], as well as traditional homologous recombination methods, but they have mostly had limited success (Table 1). First, they require the ready presence or introduction of strategically placed recombination sites on both the donor and recipient DNA molecules, which will be left as "footprints" in the modified constructs [43, 44]. Additionally, the effects of pseudoattachment sites in a system, if any, must be investigated ahead in order to eliminate retargeting to unwanted sites [19]. Furthermore, the reversible reactions of some integrases (e.g., Cre [45] and FLP [46]) must be taken into consideration as the frequency of correct recombination could be reduced. Nevertheless the precision, and in some cases reversibility, offered by site-specific integration systems are very useful properties for flexible genetic engineering. Hence, recombineering and site-specific integration systems can complement each other in a synergistic manner as recombineering can accurately position integrase attachment sites in any location of choice [47].

In *E. coli*, the 5′G-C-T-G-G-T-G-G 3′octamer, known as Chi (crossover hotspot instigator), is a hotspot that stimulates recombination close to this sequence via the recBCD pathway [48]. A Chi-based strategy was used to target fusion of the green fluorescent protein reporter gene into a zebrafish *GATA-2* BAC, in which the authors used a non-replicative targeting linear construct containing properly oriented Chi sites located near each end to stimulate homologous recombination [6]. The resulting *GATA-2* promoter

GFP-fusion BAC displayed the correct expression pattern of the reporter gene in developing skin EVL cells, neurons, and circulating blood cells [6]. Although this strategy simplified the targeting and identification of recombinant BACs, like site-specific integration systems, its major limitation is the time-consuming step of generating targeting DNA flanked by Chi sites (Table 1).

BACs are generally maintained in the *E. coli* host strain DH10B because it has many suitable qualities, including permissive for cloning of methylated eukaryotic DNA, enhanced transformation of very large DNA, high transformation efficiency, and the ability to yield high-quality DNA during DNA isolation [3, 49]. However, because this host is recombination deficient (recA-), exploiting its intrinsic homologous recombination mechanism for BAC manipulation had been challenging. Early homologous recombination strategies for BACs were thus developed in other *E. coli* mutant strains. One of the earliest methods using the RecBCD pathway of *E. coli* was described by O'Connor et al. [50] (Figure 2(a)). The authors used a combination of complex steps of homologous and site-specific recombination in *E. coli* to recombine overlapping regions of large genomic fragments carrying the *Drosophila ultrabithorax (ubx)* gene, a technique they called "chromosomal building" [50] (Figure 2(a)).

Approximately a decade later, Messerle et al. [51] used this rather tedious technique to mutagenize the mouse cytomegalovirus (mCMV) genome, contained in a 230 kb BAC and to generate virus mutants. Soon after, this technique was extended into *E. coli* DH10B by transiently supplying the *recA* gene [52]. The *lacZ* gene was fused in-frame with the promoter of the *RU49* gene located on a 131 kb BAC and shown to correctly express in a spatio-temporal fashion in mice [52].

The recombination potential of the RecF pathway that operates in *recBC sbcBC* mutant strains has also been applied to construct plasmids by *in vivo* cloning a DNA fragment into a linearised vector via the gap repair approach [53, 54] (Figure 2(b)). Similarly, the endogenous RecF pathway of *E. coli* BJ5183 was used to clone and mutagenize the complete adenovirus genome, simplifying the process of generating adenovirus variants [55, 56] (Figure 2(c)). Curiously, low-efficiency gap repair activity was reported in the RecA- *E. coli* strain DH5α but the pathway(s) mediating the homologous recombination was not explained and this result has not been verified by other publications since [57].

These studies highlighted the exciting use of endogenous homologous recombination pathways directly in *E. coli* to generate precise gene modifications, but technical limitations exist for these strategies (Table 1). Firstly, the high levels of background rearrangements observed in some cases [6, 51, 52] was a concern. Moreover, traditional recombination technologies typically require long homologous sequences of about 1 kb for efficient recombination rate [54], which is too long to accurately synthesise with standard PCR procedures. As these RecA-dependent strategies only work in strains with specific mutant backgrounds (e.g., *recBC sbcB* for RecF pathway [53] and *recA* for transient RecA [52]), they are either not transferable to general strains or BACs will need to be shuttled from the specific strain out after modification.

It became clear that although it was possible to engineer changes to large BACs using various established conventional strategies in *E. coli*, a straightforward, efficient, and stable BAC manipulation technique in *E. coli* that leaves no modification footprints behind would be more favourable. The quest for such a tool resulted in development of recombineering technology [14]. Recombineering is advantageous over conventional BAC modification strategies in several aspects (Table 1). Most importantly, recombineering facilitates the introduction of almost unlimited modifications to BACs with very high efficiency compared to traditional homologous recombination [21, 22]. Virtually, any position on a BAC can be targeted as long as its sequence is known and the required homology size (~40 bp) [22] is short enough to be synthesised in vitro. In addition to its tremendous target flexibility, the development of mobile recombineering systems [21–23, 29], where the recombination function can be introduced into any host via a plasmid, greatly enhances its scope of application in various bacterial strains (Figure 2(f)). The simplicity of recombineering strategies also translates to technical ease, while more sophisticated recombineering strategies can introduce clean mutagenesis without any footprints as site-specific recombination does.

4. Evolution of Recombineering Technologies

Early research that led to the development of recombineering (Figure 2) can be traced back to the exploitation of *E. coli* mutant strains that carry functional phage recombination mechanisms derived from lambda and the RecE/RecT genes from the Rac prophage (Figures 2(d)-2(e)) [58, 59]. However, it was not until the advent of regulatable and defined recombineering systems in late 1990s (Figure 2(f)) that the technologies and potential of recombineering became well established.

4.1. Gene Replacement/Insertion/Deletion. *E. coli* K12 strains carrying the defective Rac prophage [58] has been utilised for mutagenesis of *E. coli* chromosomes and plasmids since the 1980s. The recombination proficiency in this strain was eventually discovered to be attributed to *sbcA* mutation which causes overexpression of the RecE pathway [60], leading to recombination activity that phenotypically suppresses the *recBC* mutations [61]. In 1987, Kiel et al. constructed mutants of K12 strains by exchanging the target chromosomal segment with a mutant gene carried by an incoming plasmid, utilising the RecE pathway endogenous to K12 [62] (Figure 2(d)). About a decade later, the potential of recombineering for gene replacement was tapped into by inducible expression of λ Red genes [21] and RecET [22] (Figure 2(f)). Soon after, the λ Red-mediated recombineering was used to construct a series of chromosomal gene replacement strains, using PCR cassettes as targeting substrates [63, 64].

4.2. Gap Repair Cloning. Similarly the gap-repair ability of a *recBC sbcA* strain, where the RecE pathway operates, was used to directly clone PCR products *in vivo* by Oliner et al.

[59] (Figure 2(e)). Kawaguchi and Kuramitsu [65] followed by adapting this approach to describe a cloning method in a *recBC sbcA* strain that they designated "homologous ligation", with homology length as short as 20 bp. The power of recombineering in gap repair cloning was eventually demonstrated by Zhang et al. [26] (Figure 2(e)), who conducted highly efficient *in vivo* subcloning in *E. coli* using a concept similar to TAR cloning in yeast (see above) [39].

4.3. Defined Recombineering Systems. The milestone in recombineering development is the establishment of defined recombineering systems, which consist of RecE/RecT or λ Red Exo/Beta under inducible promoters for tight expression regulation (Figure 2(f)). Despite not being involved in the actual recombination reaction, λ Red Gam is incorporated into these systems to facilitate the use of open-ended linear dsDNA as targeting substrates by protecting them from recBCD degradation [21–23] (Figure 2(f)). These plasmid-based systems permit tight regulation of the recombineering genes, thereby limiting unwanted recombination events, and also allow transfer of the systems for use in various strains and even species (Table 1).

As the λ prophage carries all the necessary Red genes for recombineering, a system based on defective λ prophage was developed [64] (Figure 2(f)). Among the key features of this system is the temperature-sensitive regulation of Red genes based on cI repressor and also its mobility to other *E. coli* strains by P1 cotransduction. However the elimination of this defective prophage-based system requires additional effort, as a specific recombination reaction to replace the prophage with selectable cassette is necessary [64].

Because of the incompatibility of the defective prophage system with certain BACs [24], a tractable mini-λ system was subsequently developed [24] (Figure 2(f)). This mini-λ is deleted for lethal genes and can be easily excised out via λ attachment sites. The reported recombination efficiency was so high that direct screening of recombinant clones without selection was feasible [24]. The frequency obtained was 1 in 61 for direct nucleotide substitution by ss-oligo recombineering as confirmed by sequencing [24].

Next, a unique hybrid recombineering system that brings together RecA and Red-mediated homologous recombination techniques was developed [66] (Figure 2(f)), following the establishment of RecA-mediated recombination method in neuroscience research [67, 68]. In this method, the RecA-dependent technique was integrated with the λ Red recombineering for seamless modification of BACs. This hybrid recombineering utilizes Red recombineering to insert ("pop-in") a DNA sequence of interest alongside marker genes and *RecA* on a selectable cassette, followed by RecA expression from the cassette to excise ("pop-out") vector sequences [66].

In another work, RecA was incorporated into a different λ Red recombineering system but not for its recombination function [69] (Figure 2(f)). Instead, transient RecA expression was induced to increase survival rate of transformants, leading to about 4- to 5-fold improvement in transformation efficiency and consequently up to 8-fold increase in recombination frequency [69]. While not experimentally proven yet, transient expression of RecA might be beneficial to RecET recombineering as it does to λ Red recombineering.

4.4. Selection/Counterselection Mutagenesis. Although the one-step recombineering strategy for mutagenesis is easy and straight forward, often operational sequences like antibiotic resistance gene will be left behind. In order to create "clean" mutation without the potential disruptive effects of integrated operational sequences, a counterselection strategy is employed (see Figure 1(c)). While antibiotic resistance genes are generally the choice for selection markers, the option for counter selection is constantly being explored (Figure 2(g)). One of the earliest counter-selection genes used in recombineering is *sacB* [22] (Figure 2(g)), which enables counterselection by sucrose addition. Spontaneous mutations in *sacB* that ablate sucrose sensitivity do arise during the course of recombination, however [22], so careful screening of recombinant clones is necessary for the confirmation of desired genotype. Another counter-selection marker used is the wild-type *rpsL*+ allele (Figure 2(g)), which was first used by Imam et al. [34] for PAC modifications in streptomycin-resistant *rpsL*− DH10B. However, rearrangements on the clones were observed, warranting the need for additional recombinant screening. One novel counterselection strategy is the use of the highly specific I-*Sce*I endonuclease [35] (Figure 2(g)), by expressing it to cleave the 18 bp I-*Sce*I recognition site present only in nonrecombinant clones. Unfortunately, the observation of high false positives, due to background deletion of I-*Sce*I gene and recircularisation events, required extra effort in ensuring high I-*Sce*I expression level and *in vitro* linearisation of recombinant BACs before electroporation [35].

Compared to counter-selection markers that need to be used in conjunction with another selection marker, marker genes that serve the dual purpose of selection and counter-selection are more convenient to use. One such example is *galK* (Figure 2(g)), which is adapted for use in recombineering by Warming et al. in Δ*galK* strains [36]. By having only one marker gene in the selectable cassette, selection/counter-selection is easily accomplished by simply alternating the substrate (galactose for selection, 2-deoxy-galactose for counter-selection) during the two-step recombineering [36]. Nevertheless, low level of spontaneous deletions spanning *galK* was observed although they were concluded to be due to background reaction in DH10B [36].

Another example of dual-selectable marker gene for use in recombineering is *thyA* [37] (Figure 2(g)), which can be selected for in the absence of thymine, and against by the addition of thymine and trimethoprim in minimal growth media. As this selection method must be performed in *thyA* null mutant background, it might be restricted for use in general strains or require additional *thyA* knockout step for adaptive use. Nevertheless, it is a highly efficient approach that gives over 90% of accurate selection frequency with low levels of backgrounds up to 0.08% [37]. More recently, the *tolC* gene which encodes an outer membrane protein (Figure 2(g)) was adapted for selection/counter-selection purpose in recombineering [38]. This technique takes a similar approach to *thyA* and *galK* selection, as a

Δ*tolC* background is required for the mutant *tolC* gene to be selectable by SDS addition and counterselectable by Colicin E1 [38]. Again, spontaneous background mutational events were observed for Colicin E1 selection, warranting the need to screen potential recombinant colonies [38].

4.5. Recombineering with Single-Stranded Oligos. The exciting potential of recombineering was expanded further when single-stranded oligonucleotides as short as 30 nt were found to effectively mediate recombinering (Figure 2(h)). Furthermore, this method only requires the Beta [27] or RecT function [70], with only slight dependence on Gam expression [27]. Interestingly, comparable efficiencies between RecT and λ Beta in mediating ss-oligos recombineering have been demonstrated in some work [70], while RecT protein has been observed to be slightly less efficient than λ Beta in other [33]. Recombineering is invariably more efficient when the targeting oligos correspond to the lagging strand in relation to the replication origin, a phenomenon termed 'strand bias' [27, 70]. This is thought to reflect the more abundant availability of exposed ssDNA for Beta or RecT binding at the lagging strand during replication [27]. Recombineering by ss-oligos was taken a step further by using oligos that overlap as little 6 bp to create dsDNA substrates with single-stranded overhangs [28] (Figure 2(h)). While only Beta is needed for efficient recombineering of dsDNA with 3′ overhangs, a surprising observation was that when Exo was coexpressed a higher recombination rate of dsDNA with 5′ overhangs was obtained [28].

5. Applications of Recombineering

5.1. BAC-Based Transgenesis. Recombineering has been an invaluable tool in mouse transgenesis [14]. Because of the large insert capacity of BACs, the introduced transgenes are less sensitive to positional effects due to inclusion of regulatory sequences in the insert [7] and hence effective in building disease model systems with expression profile reflecting actual pathogenesis. Besides introducing transgene, BACs can also be applied in targeted mutagenesis in the mouse genome. One excellent demonstration of this application is the engineering of homozygous knockouts in mouse embryonic stem (ES) cells [71] (Figure 2(i)). Two knockout BAC constructs that targeted the same gene were introduced to mouse ES cells in two rounds of injections, with targeting efficiency ranging from 7% to as high as 28% [71]. More recently, this method was successfully adapted to create homozygous knockouts in human ES cells (Figure 2(i)), expanding the applications of transgenesis BACs from mouse to human model systems [72].

5.2. Linking BACs. The accuracy of recombineering in manipulating very large DNA molecules makes it a practical option for joining large DNA fragments to construct big artificial chromosomes (Figure 2(j)). This application was first demonstrated in the fusion of two separate but overlapping BACs, typically to reconstitute large genes that span more than one clone into a single recombinant BAC clone [19, 66, 73] (Figure 2(j)). Following the success of joining related BACs, Kotzamanis et al. retrofitted a ~70 kb-alphoid DNA into a human BAC clones via the λ Red recombineering system in an attempt to build human artificial chromosomes (HACs) (Figure 2(j)). After transfection, the resulting fusion minichromosome contained a functional centromere, and the subcloned gene was expressed correctly [16]. Although an additional step is needed to assemble a "linker" plasmid to join the two targets by providing homology sequences, it is a small price to pay for a universal method to link two or more unrelated BAC clones [16].

5.3. Virus Mutagenesis and Production. One notable use of homologous recombination is its successful applications in manipulation of clinically important viruses (Figure 2(k)). As virus genomes can reach over 200 kb in size (e.g., the genome size of cytomegalovirus is 235 kb), modification of large virus genomes in its entirety poses technical challenges. However, with the high efficiency of recombineering in manipulating large DNA plasmids, virus genomes can be subcloned into an appropriate vector and essentially undergo engineering as BACs would. Recombinant viruses have been constructed using conventional homologous recombination technologies [51, 55, 56, 74], but overall with lower efficiency than recombineering. From vaccinia virus [75], herpes virus [76], to baculovirus [77] (Figure 2(k)), mutagenesis and production of these viruses were greatly facilitated by recombineering technologies, as the virus genomes can be directly modified on a BAC vector, which can subsequently serve as the production host once it is transfected into a suitable cell host for virus reconstitution. This strategy saves tremendous time and effort, as clonal viruses can be produced without tedious plaque purification procedure [75].

5.4. Recombineering Pipeline. An important breakthrough in recombineering application came about when a scalable, liquid, culture-based recombineering pipeline was developed in 2006 for *Caenorhabditis elegans* [17] (Figure 2(l)). In just four days, this λ Red-mediated recombineering pipeline could facilitate EGFP tagging and subcloning of multiple BAC clones simultaneously [17]. Efficiency of the pipeline was further enhanced when Poser et al. increased the throughput to 96-well format [78] (Figure 2(l)). Coining the technology as "BAC TransgeneOmics", this improved pipeline enables high-throughput transgenesis method such as protein tagging in any model system, including mammalian cell cultures [78]. Very recently, a specialised recombineering pipeline was developed for generation of specific constructs targeting conditional alleles, through a series of recombineering reactions involving replacement, gap repair, and insertions [47].

5.5. Recombineering Linear BACs. One novel application of recombineering developed recently is its use to introduce structural on top of sequential changes to BACs [79] (Figure 2(m)). By integrating the telomeric sequences *tos* of the linear prophage N15 into a large 100 kb BAC construct followed by appropriate expression of the phage's telomerase TelN, the BAC was able to stably replicate as a linear episome

with resistance to RecBCD exonuclease attack *in vivo* in *E. coli* [79] (Figure 2(m)). After transfer into HeLa cells, this linear BAC produced accurately spliced β-globin mRNA, suggesting that this conformation may potentially be useful as a vector for mammalian gene expression or assembly of artificial chromosomes [79].

6. Conclusions and Perspectives

BAC homologous recombination technologies have benefited the research community in ways unimaginable only decades before. From microbiology, virology to human genetics, neuroscience, and proteomics, the power to clone and manipulate large pieces of intact genetic information with high fidelity has enabled researchers to design and conduct insightful studies in their respective fields.

With recombineering being increasingly applied in creative ways, the only limiting factor for the potential of recombineering is our imagination. As functional homologues of recombineering enzymes are being discovered in a broader range of bacteria and their phages [33], more novel and improved recombineering technologies could be on the horizon. The use of recombineering functions in non-*E. coli* species (e.g., *Vibrio cholera* [18], *Yersinia pseudotuberculosis* [80], and *Pseudomonas aeruginosa* [81]), as well as the successful transfers of phage DNA-modifying enzymes (e.g., PhiC31 [82] and cre-*loxP* [83]) from traditional hosts (*E. coli*) to mammalian cells are encouraging signs that their adaptation for use in eukaryotic systems is not too far from becoming reality.

Acknowledgment

The authors would like to apologise to researchers whose work are not cited due to space constraint. Q. Chen is a Ph.D. student supported by Higher Degree by Research (HDR) Scholarship from Monash University.

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