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An Improved Recombineering Approach by Adding RecA to λ Red Recombination

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Abstract

Recombineering is the use of homologous recombination in *Escherichia coli* for DNA engineering. Of several approaches, use of the λ phage Red operon is emerging as the most reliable and flexible. The Red operon includes three components: Redα, a 5' to 3' exonuclease, Redβ, an annealing protein, and Redγ, an inhibitor of the major *E. coli* exonuclease and recombination complex, RecBCD. Most *E. coli* cloning hosts are *recA* deficient to eliminate recombination and therefore enhance the stability of cloned DNAs. However, loss of RecA also impairs general cellular integrity. Here we report that transient RecA co-expression enhances the total number of successful recombinations in bacterial artificial chromosomes (BACs), mostly because the *E. coli* host is more able to survive the stresses of DNA transformation procedures. We combined this practical improvement with the advantages of a temperature-sensitive version of the low copy pSC101 plasmid to develop a protocol that is convenient and more efficient than any recombineering procedure, for use of either double- or single-stranded DNA, published to date.

Index Entries: Recombineering; Red/ET; recA; λ red; counter selection; BAC.

1. Introduction

Bacterial artificial chromosomes (BACs) are valuable resources for gene expression and functional studies *(1,2)* because they can carry DNA fragments large enough to encompass most intact eukaryotic genes, and many gene clusters, with all the regulatory elements. However, the large size of BACs precludes DNA engineering using conventional cloning methods. To address the need for a DNA engineering methodology for BACs, in vivo homologous recombination techniques, collectively termed recombineering or Red/ET recombination, have been established.

Thus far, in *Escherichia coli* two main alternatives, RecA-dependent homologous recombination *(3–5)* and Red/ET recombination *(6–12)* have been developed. RecA-dependent engineering typically requires the prior construction of a purpose-specific targeting vector in a plasmid with a conditional origin of replication. This dedicated vector should also include relatively long homology regions (>500 bp), the *recA* gene, a selectable marker, and a counterselection mechanism. The Red/ET recombination is based on the expression, in *trans*, of either the *red* operon of λ phage or the analogous *recE*/*recT* genes from Rac prophage. It is proficient with homology arms as short as 40–50 bp, which permits the use of polymerase chain reaction (PCR)-amplified fragments generated from oligonucleotides synthesized to include the short homology arms *(13–15)*.

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Previously, we described two plasmids, pBAD-ETg and pBAD-gba, and variations for transient expression of RecE/RecT or Redα/Redβ induced by L-arabinose *(6,8,16)*. Expression of Redγ was included to inhibit RecBCD *(17)*. By placing the phage recombination genes in an operon on a high copy plasmid under the glucose repressed, L-arabinose induced, BAD promoter, three practical advantages for recombineering were achieved.

First, the tightly regulated BAD promoter permits tight regulation of the recombinogenic window. The use of homologous recombination for DNA engineering of cloned sequences entails a certain risk because restoration of the capacity for homologous recombination provokes the rate of undesired intramolecular recombination in cloned DNA sequences. Tight regulation of the recombinogenic window, by limiting the time of expression of the recombination proteins, minimizes this risk.

Second, high recombination efficiency was achieved as a result of high levels of phage protein expression from the high copy ColE1-derived plasmids.

Third, use of a plasmid-based expression platform permitted the simple transfer of recombineering potential into new *E. coli* hosts. This is particularly advantageous for BAC engineering because the host cells containing a BAC can be transformed with the expression plasmid. In contrast, recombinogenic hosts like DY380 *(10,18)* or YZ2000 *(7)* carry the phage proteins on the *E. coli* chromosome. Use of these hosts for BAC engineering requires their transformation with the BAC. This is less convenient than plasmid transformation and also requires a step to check that the BAC has not been rearranged by the transformation procedure.

The advantages of the pBAD-ETg and pBAD-gba recombineering approach included a disadvantage. After recombineering, the plasmids contaminate BAC DNA preparations. Here we report a solution to this disadvantage while retaining the advantages. In this new system, Red/ET recombination is conferred by a low copy, temperature-sensitive, plasmid that carries a *redγ*/*redβ*/*redα*/*recA* operon placed under an L-arabi-nose-inducible promoter. More recombinant colonies are recovered when RecA is expressed because it facilitates cell survival after transformation. We compare the new system with existing Red/ET systems.

2. Materials and Methods
2.1. Construction of Plasmids

pBAD-gba *(16)* was generated by PCR of the red operon and cloning into pBAD24 *(19)* between *Nco*I and *Hin*dIII sites. A PCR-amplified *recA* gene from JC8679 was integrated into the Red operon in pBAD-gba by Red/ET recombination to generate pBAD-gbaA. The pSC101 origin and *repA* gene were copied by PCR using pMAK-705 *(20)* as the template. The tetracycline gene flanked by homology arms to the *pSC101 ori + repA* cassette was obtained by PCR of pBR332, and the two fragments were recombined in YZ2000 cells *(7)* to generate pSC101-tet. The pSC101-BAD-gbaA (tet) was then generated by replacing the CoLE1 ori plus *amp* gene in pBAD-gbaA with pSC101-repA-tet cassette. To build the *rpsL-neo* plasmid, first, *Tn5-neo* from pJP5603 *(21)* was recombined with a *pSC101 ori + repA* cassette to form pSC101-neo by Red/ET recombination, then a wild-type *rpsL* allele was copied by PCR from *E. coli* DH5α using primers that added a ribosome-binding site, and then integrated in front of *Tn5-neo*. Further details of the plasmids as well as supporting information are available at http://www.genebridges.com.

2.2. Preparation of Donor DNA Fragments

Standard reaction conditions and *Taq* polymerase were used to amplify all PCR products used here. For all primers listed later, nucleotides in italics are homology arms to the targeted sequence, whereas those in standard type are the PCR primer regions. Additional sequences included between these regions are shown in bold type (here *Xho*I sites). The Tn5-neo cassette used in the experiments of **Table 1** was amplified from pR6K-Tn5-neo (**Fig. 1**) with primers 5'-*TAGAACGGAGTAACCTCGGTGTGCGGTTGTATGCCTGCTGTGGATTGCTG*TGGACAGCAAGCGAACCGGAATTGC-3' and 5'-*TACCGAGCTC*

Table 1
Comparison of Recombination Efficiencies Conferred by pBAD-gba, pBAD-gbaA, pSC101-BAD-gbaA, and DY380

Plasmid/strain	pBAD-gba	pBAD-gbaA	DY380	pSC101-BAD-gbaA
A. DSBR assay				
Km	4.9×10^5	23.2×10^5	1.6×10^5	14.7×10^5
Cm	2.4×10^8	1.9×10^8	2.1×10^8	3.0×10^8
Percent efficiency	0.20	1.22	0.08	0.49
B. pACYC184 transformation				
− L-arabinose	8.9×10^8	8.9×10^8	2.1×10^8	9.2×10^8
+L-arabinose	1.2×10^8	4.5×10^8	0.8×10^8	8.7×10^8
C. ss oligo repair assay				
Km	4.8×10^6	15.0×10^6	0.5×10^6	8.6×10^6
Cm	2.4×10^8	2.4×10^8	2.9×10^8	2.1×10^8
Percent efficiency	2.00	6.25	0.17	4.10
D. DSBR assay with chemical-competent cells				
Km	420	3100	25	2200
Cm	4.6×10^7	4.1×10^7	4.2×10^7	4.4×10^7
Percent efficiency	9.1×10^{-4}	75.6×10^{-4}	0.6×10^{-4}	50×10^{-4}

All cells contained a BAC carrying approx 150 kb mouse genomic DNA. (**A**) Evaluation of DSBR efficiency using a *Tn5-neo* PCR product. Cells, either containing the plasmids as indicated or DY380, were plated onto kanamycin (Km) or chloramphenicol (Cm) plates to score homologous recombinants and total number of surviving cells. (**B**) Transformation efficiencies of the competent cell preparations, either induced with L-arabinose or not, were measured by electroporation of pACYC184 and plating onto kanamycin plates. (**C**) Evaluation of single-stranded oligonucleotide recombination efficiencies using repair of a defective neo gene and selection for restoration of kanamycin resistance. This defective neo gene had been integrated into the same mouse genomic BAC. (**D**) The same assay as in (**A**) except chemically competent, not electrocompetent, cells were used. Results show a representative experiment. All experiments have been repeated at least three times with equivalent results.
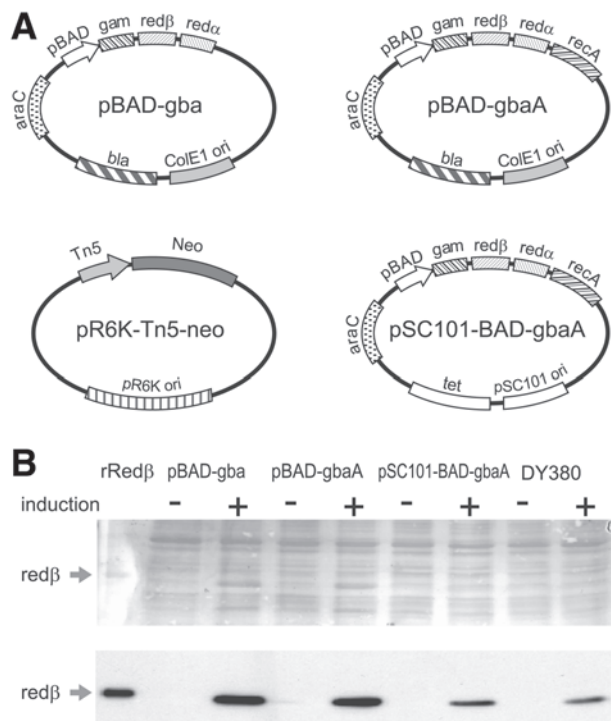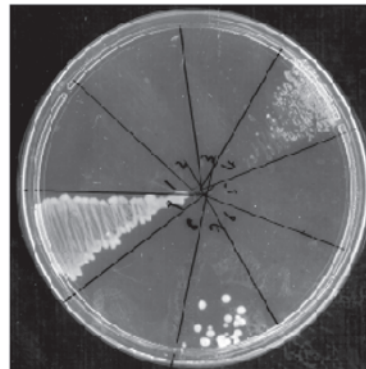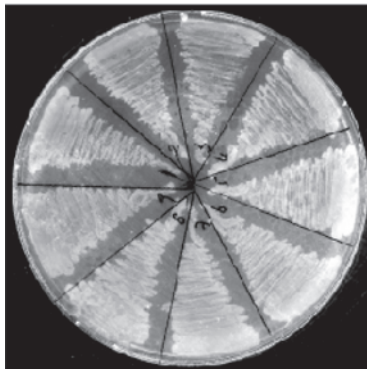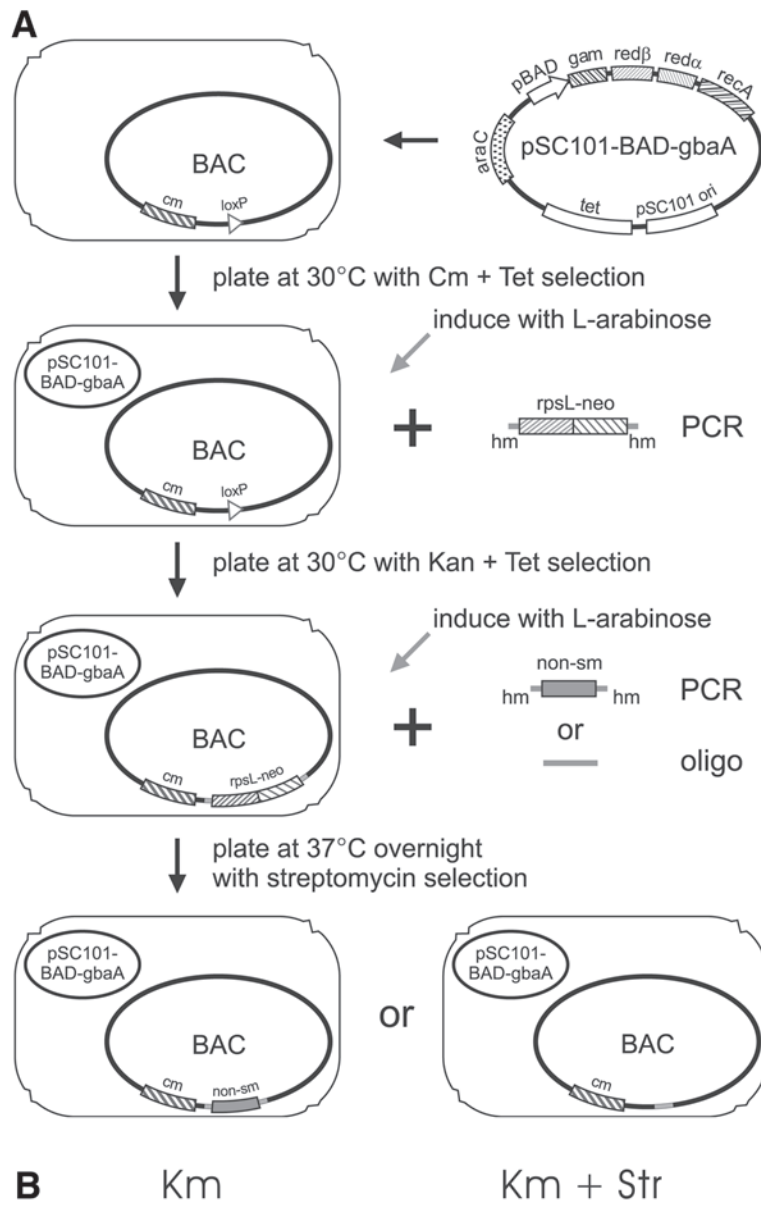

Fig. 1. (**A**) Schematic presentation of the plasmids used. pBAD-gba and pBAD-gbaA are based on pBAD24. The *gam*/*red*β/*red*α cassette, as well as the *gam*/*red*β/*red*α/*recA* cassette, were placed downstream of the L-arabinose-inducible BAD promoter P$_{BAD}$. Both plasmids carry the CoLE1 replication origin and an ampicillin-resistance gene. The pSC101-BAD-gbaA carries a temperature-sensitive pSC101 replication origin/repA region from pMAK705 and a tetracycline-resistance gene, as well as the BAD-*gam*/*red*β/*red*α/*recA* operon. The pR6K-Tn5-neo is based on a minimal version of pJP5603 by deletion of the nonessential regions using *Tn5-neo*, Red/ET recombination, and Km selection. (**B**) Western blot analysis of Redβ in the HS996 competent cells harboring pBAD-gba, pBAD-gbaA, and pSC101-BAD-gbaA with/without L-arabinose induction, as well as DY380 strain with/without 42°C heat induction. Cells (2.5×10^8) were subjected to SDS-PAGE, followed by immunoblot transfer, stained with Ponceau S (top) and incubated with anti-redβ polyconal serum (bottom). Redβ is indicated by the arrows. Purified Redβ (50 ng) was loaded in the lane at the left (rRedβ).
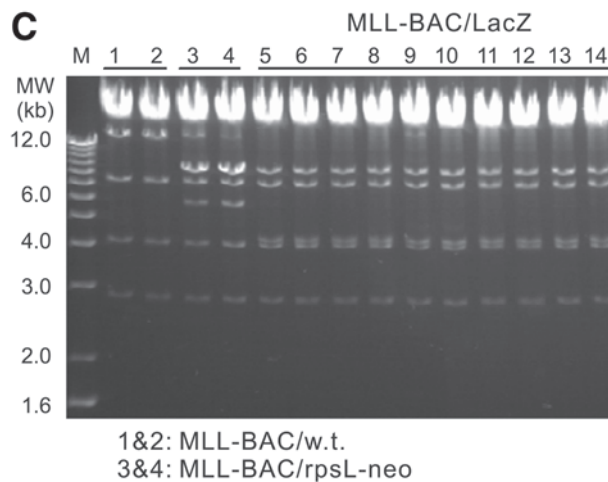
Fig. 2. Modification of a BAC containing the mouse *mll* gene by two rounds of Red/ET recombineering. **(A, opposite page)** Schematic presentation of the strategy. The BAC was left in its original *E. coli* HS996 host. A 593-bp fragment containing the loxP site was deleted after Red/ET recombination. These cells were transformed with pSC101-BAD-gbaA with selection for tetracycline resistance and culture at 30°C. Expression of the gbaA operon was induced with L-arabinose shortly before preparation of electrocompetent cells and transformation with an *rpsL-neo* PCR product. Integration by homologous recombination into the BAC was selected by acquisition of Km resistance. The cells were also subject to selection with tetracycline and cultured at 30°C to retain pSC101-BAD-gbaA. After a second round of L-arabinose induction and preparation of electrocompetent cells, the cells were electroporated with a nonselected marker (non-sm) PCR product like the *lacZ* gene in this article. Selection for homologous recombination was exerted by counterselection with streptomycin to eliminate the *rpsL* gene. **(B, opposite page)** The streptomycin-sensitive phenotype imposed by expression of *rpsL* was tested by streaking colonies onto sectors of plates containing antibiotics. Eight colonies (no. 1–8) were picked from the Km (15 µg/mL) plus chloramphenicol (15 µg/mL) after insertion of *rpsL-neo*. As control, cells containing the *mll* BAC and a Km resistance plasmid, pGK-neo, were also streaked as no. 9 on the plates. All these colonies were first streaked on the plate with Km (15 µg/mL) alone, and then streaked on the plate with Km (15 µg/mL) plus streptomycin (50 µg/mL). **(C)** *Xho*I digestion analysis of the modified *mll* BAC clones. Lanes 1 and 2 are two isolates of the original *mll* BAC; lanes 3 and 4, insertion of *rpsL-neo* into the *mll* BAC reduced the 13-kb band to 5.6 kb; lanes 5–14, replacement of *rpsL-neo* with the *lacZ* PCR product reduced the 5.6-kb band to 4.2 kb.

*GAATTCGCCTATAGTGAGTCGTATTACAA TTCACTGGCC***CTCGAG**TCAGAAGAA C TCG TCAAGAAGGCG-3'. The 1.4-kb rpsL-neo fragment used for deleting the loxP site in an Mll-BAC (**Fig. 2**) was amplified from the newly created pRpsL-neo plasmid with primers 5'-*TGACATGTCGTCGTAACCTGTAGAACGGAG TAACCTCGGTGTGCGGTTGT***CTCGAG**GGC CTGGTGATGATGGCGGGATCG-3' and 5'-*TACCGAGCTCGAATTCGCCCTATAGTGAGT CGTATTACAATTCACTGGCC*TCAGAAGAA CTCGTCAAGAAGGCG-3'. The 3.2-kb LacZ

fragment used for replacing the rpsL-neo cassette was amplified from pSVpaX1 *(22)* with primers 5'-*TGACATGTCGTCGTAACCTGTAGAACGG AGTAACCTCGGTGTGCGGTTG*ATGGTCGCG AGTAGCTTGGCAC-3' and 5'-*TACCGAGCT CGAATTCGCCCTATAGTGAGTCGTATTACAA TTCACTGGCC***CTCGAG**CTTCACAAAGATC CCCCCTGCC-3'.

The 100-nucleotide single-stranded oligonucleotide used for repair of the kanamycin (Km) resistance gene is, with the repair site underlined, GCCAGGCTCAAGGCGCGCATGCCCGACG

GCGAGGATCTCGTCGTGACCCATGGCGA
TGCCTGCTTGCCGAATATCATGGTGGAAA
ATGGCCGCTTTTCTG.

2.3. Preparation of Competent Cells and Transformation

Before performing Red/ET recombination, a conventional transformation method was used to transform pBAD-gba, pBAD-gbaA, and pSC101-BAD-gbaA into BAC host cells. For electro-transformation used for Red/ET recombination, overnight cultures containing both the BAC and Red/ET plasmid were diluted 50-fold (600 μL into 30 mL for max-preparation, 30 μL into 1.5 mL for min-preparation) in LB medium, grown to an $OD_{600} = 0.2$–0.3 at 30°C for pSC101-BAD-gbaA and 37°C for pBAD-gba and pBAD-gbaA, L-arabinose was added to 0.1% and cells were grown to an $OD_{600} = 0.35$–0.4. Cells were then centrifuged for 5 min at 6000g for maxi-preparation and 0.5 min at 12,000g for mini-preparation at 2°C. The pellet was resuspended in ice-cold 10% glycerol and centrifuged again. After that, cells were resuspended in 100 μL of ice-cold 10% glycerol for max-preparation and 30 μL for min-preparation, 0.2 μg of PCR product was added into the competent cells and electroporated. Electroporation was performed using ice-cold cuvets and an Eppendorf electroporator 2510 set at 1350 V. One milliliter of LB medium was added after electroporation. The cells were incubated at 37°C for 70 min with shaking and spread on appropriate antibiotic plates. Chemical competent cells for Red/ET recombination were prepared by rubidium chloride *(23)*. The culture conditions and L-arabinose induction were the same as for electrocompetent cells. For DY380, the competent cells were prepared according to the references *(10,18)*.

2.4. Identification of the Modified BACs

Colonies were randomly picked after overnight incubation, cultured in 2.0 mL of LB medium containing corresponding antibiotics at 37°C for more than 10 h, and BAC DNA was extracted according to the procedures of the kit used (Qiagen). BAC DNA was then digested with appropriate enzymes and run with 0.8% agarose at 50 V.

3. Results

3.1. Transient Co-Expression of RecA Promotes the Yield of Red/ET Recombination

Several lines of evidence show that the expression of RecA neither contributes to, nor detracts from, the molecular mechanism of Red/ET recombination *(6,10,16,24–26)*. We previously noted that a *recA* proficient and *recBC* deficient strain yielded more colonies of correct recombinants than the equivalent *recArecBC* strain. This effect was attributable to improved transformation efficiency *(6)*. Consequently, we reasoned that transient co-expression of RecA with the Red proteins, particularly the RecBC inhibitor, Redγ, may be practically beneficial. To test this idea, the *recA* gene was cloned into the plasmid pBAD-gba to create pBAD-gbaA (**Fig. 1A**). These plasmids were introduced into HS996 host strain carrying a mouse genomic BAC clone. Expression of the recombination proteins was induced shortly before preparation of competent cells for electroporation with a *Tn5-neo* PCR product flanked with 50-bp homology arms to target a site in a mouse genomic BAC. After recombination, 20 colonies from each group were analyzed by suitable restriction enzyme digestion, and all of them were correct recombinants without any sign of unwanted intramolecular rearrangements (data not shown). This concords with our, and other investigators, experience that the ratio of homologous to illegitimate recombination under various conditions of Red/ET protein expression is extremely favorable and we conclude that total Km-resistant colony numbers record Red/ET recombination efficiency in these experiments. Co-expression of RecA promoted colony numbers four- to fivefold (**Table 1A**).

To evaluate whether the effect of RecA expression was because of an improvement in transformation efficiency, competent cells were prepared before or after L-arabinose induction and transformed with pACYC184 followed by selection for Km resistance (**Table 1B**). Two effects were observed. First, L-arabinose induction decreased transformation efficiency. Second, expression of RecA improved transformation efficiency about fourfold (**Table 1B**; compare L-arabinose induc-

tions of pBAD-gba and pBAD-gbaA). Hence the major contribution of RecA to improved yield of resistant colonies was the result of improved transformation efficiency.

3.2. Comparison of Chromosomal vs Plasmid-Borne Red Operon Expression

Because the overall purpose of our experiments was to determine the most efficient conditions for Red/ET recombineering, the experiments of **Table 1** also included parallel experiments with DY380. This strain is also proficient for Red/ET recombination; however, the Red operon is expressed from a λ prophage integrated into the chromosome and is controlled by the temperature-sensitive λ cI repressor *(18)*. Hence the Red operon genes, γ, β, α, are expressed from a chromosomal, not plasmid, site after heat, not L-arabinose, induction shortly before competent cells are prepared for the recombineering step. Recombineering with DY380 was less efficient than with either pBAD-gba or pBAD-gbaA, in large part because of differences in transformation efficiencies between the two host strains (HS996 and DY380).

3.3. pSC101 Origin-Based Red Operon Expression Vector

In addition to lower transformation efficiencies, a second disadvantage of chromosomally encoded Red/ET expression systems, such as DY380 or YZ2000, *(7)* is that the target BAC needs to be transformed into this host for recombineering. In contrast, a plasmid-based Red/ET expression platform can be transformed into hosts containing the BAC. However, after recombination of the BAC, it can be difficult to eliminate the high copy pBAD-gba for purification of BAC DNA. We, therefore, cloned the BAD-gbaA operon into the low copy plasmid pMAK (**Fig. 1A**). This pSC101-derived plasmid presents a significant convenience because it replicates only at low temperatures (30°C) and can be eliminated by culture at 37°C *(20)*. Using pSC101-BAD-gbaA as the Red/ET expression plasmid and a protocol to eliminate the plasmid after temperature shift, the yield of recombinants was significantly better than pBAD-gba and DY380 (**Table 1**). Therefore pSC101-BAD-gbaA combines recombineering efficiency

with convenience of processing and BAC DNA preparation.

Analysis of Redβ protein expression from these four configurations showed that, as expected, expression from the high copy plasmids was the highest and expression from the low copy plasmid pSC101-BAD-gbaA was about twofold higher than from the chromosomal site in DY380 (**Fig. 1B**). Taken together with the differences of transformation efficiencies between HS996 and DY380 (**Table 1B**), pSC101-BAD-gbaA combines the advantages of convenience and efficiency with the previously established advantages in a simplified experimental procedure.

3.4. Oligonucleotide-Directed Mutagenesis

The high efficiencies of Red/ET recombination permit the direct use of oligonucleotides for mutagenesis and DNA engineering *(16,25–27)*. Whereas Red/ET recombination with double-stranded DNA occurs by a double strand break repair (DSBR) mechanism *(24)*, recombination mediated by single-stranded oligonucleotides (ss oligos) appears to use a different mechanism *(16,25,26,28)*. Therefore we evaluated the new expression plasmids in a mutagenic test with an ss oligo. In this test, a 4-bp mutation in the Km resistance gene (*neo*) was repaired using a 100-nucleotide ss oligo. This assay has been described *(16)* except, in this experiment, the mutant neo gene was inserted into a 150-kb mouse genomic BAC (data not shown). Correction of the mutation by incorporation of the ss oligo restores Km resistance. Plating cells after electroporation of the ss oligo onto chloramphenicol (Cm) plates scored total colonies carrying the BAC. Plating on Km plates scored recombinants. As with the DSBR assay, pBAD-gbaA delivered more recombinants than pBAD-gba and pSC101-BAD-gbaA also worked well (**Table 1C**). Therefore the efficiency and convenience of pSC101-BAD-gbaA is also applicable to engineering with ss oligos.

3.5. RecA Improves Red/ET Recombination Efficiency When Using Chemically Competent Cells

All Red/ET recombination methods so far have relied on electroporation. Yields of recombinants

from cells made competent by chemical methods have proven to be extremely low. Because chemical competent cells offer certain advantages for automation, we tested whether the benefit of RecA co-expression was also evident in chemically competent cells. As shown in **Table 1**, this is indeed the case. Although yields are low when compared to electroporation, the improvement delivered by co-expression of RecA significantly improves the feasibility of use of chemically competent cells and automated pipetting processes.

3.6. Application of pSC101-BAD-gbaA to a Two-Step BAC Engineering Exercise

To validate the convenience of pSC101-BAD-gbaA for recombineering, we applied it to the engineering of a BAC carrying the *mll* gene from mice *(29)*. All BACs carry a loxP site in the vector. The presence of this loxP site can be a problem if the BAC is used for transgenesis in the mouse because it will complicate later use of Cre recombinase-mediated conditional strategies. Therefore one purpose of the experiment was to remove the loxP site. A second purpose was to evaluate the stability of the cloned mouse genomic DNA through two rounds of recombineering. The strategy is diagrammed in **Fig. 2A**. First, the host carrying the BAC was transformed with pSC101-BAD-gbaA. Shortly before harvesting to make electrocompetent cells, expression of the gbaA operon was induced by L-arabinose and culture at 37°C. Then a PCR fragment carrying a Tn5 promoter, *rpsL-neo* gene flanked by 50-bp homology arms to the BAC vector was introduced. The cells were cultured at 37°C for 70 min, followed by plating with Km and tetracycline selection and incubation at 30°C overnight. The tetracycline selection and culture at 30°C was to retain pSC101-BAD-gbaA for a second round of Red/ET recombination. The next day, eight single colonies were picked to tubes and cultured under selection at 30°C for 5 h. Half the culture was then inoculated into more medium for culture at 37°C overnight, elimination of pSC101-BAD-gbaA and BAC DNA preparation. The other half was used in the test of **Fig. 2A** and to prepare new electrocompetent cells as previously, followed by

introduction of a PCR fragment that carried the *lacZ* gene flanked by the same homology arms. Streptomycin counterselection was exerted to eliminate the *rpsL* gene by Red/ET recombination, and cells were cultured at 37°C to eliminate pSC101-BAD-gbaA. As can be seen in **Fig. 2B**, sensitivity to streptomycin conveyed by the *rpsL* gene was satisfactory. Examination of the BACs by restriction analysis showed that all colonies had been engineered correctly with no sign of unwanted rearrangements (**Fig. 2C**). Furthermore, pSC101-BAD-gbaA was efficiently eliminated and all counterselected colonies expressed *lacZ* (data not shown). Therefore we have developed a robust and efficient recombineering protocol based on the conveniences offered by the temperature sensitivity of this pSC101.

4. Discussion

We present an improved recombineering system based on Red/ET recombination that concurrently makes use of RecA to promote the total number of surviving colonies.

As for all cloned DNAs, BACs are maintained in a *recA* strain to avoid rearrangements caused by constitutive expression of the RecA protein. However, RecA plays other roles in the maintenance of cellular integrity that may have a practical benefit. Our results demonstrate that transient expression of RecA, together with Red proteins in the *recA* BAC host cells, increases the number of recombinant products after transformation either by electroporation or chemical transformation. The mechanism for RecA facilitation of transformation or cell survival is not clear. Because RecBCD is inhibited by Redγ, we do not believe that this effect is caused by recombination functions such as repair of chromosomal damage. Possibly RecA may be acting to induce the SOS response and delay septation *(30)*.

The modified system based on the moderate copy and temperature-sensitive plasmid pSC101-BAD-gbaA combines several advantages.

1. It delivers portability to move Red recombination into new hosts. When the host harbors the BAC to be engineered, this presents a significant advantage over the use of strains like

DY380 or YZ2000, which require transformation with the BAC followed by the need to confirm that the BAC has not been damaged. Notably, Court and co-workers *(31)*, who developed DY380, have recently developed a portable system to address these disadvantages.

2. It delivers tight regulation of the recombinogenic window by use of the glucose repression and arabinose induction properties of the BAD promoter.

3. pSC101-BAD-gbaA can be easily eliminated from the host by temperature shift, or retained for multiple rounds of recombineering. Hence, it is easy to purify the recombinant product DNA free of the expression plasmid.

4. It delivers high levels of colonies containing correct recombinants. Whereas this level is not as high as when the gbaA operon is expressed from a high copy plasmid, it is higher than either pBAD-gba or DY380.

So far, recombineering has been performed in electrocompetent cells. Chemically competent cells have not been used because of low efficiency. Although further improvements will be useful, the facilitation of Red recombination by co-expression of RecA permits the exploration of automated applications that are not possible with electroporation.

Previously, we used a *sacB-neo* fusion gene for two-step selection and counterselection with Red/ET recombination *(32)*. However, this cassette was difficult to amplify by PCR because of its large size (approx 4 kb), and sucrose counterselection is often leaky. Use of the wild-type *rpsL* allele, which can restore the dominant streptomycin-sensitive phenotype in a streptomycin-resistant background *(33)*, is more satisfactory. This *rpsL-neo* gene is only 1.3 kb and is easier to amplify by PCR. Second, unlike toxicity selection with sucrose in SacB-mediated counterselection, selection for resistance to streptomycin is more routinely successful.

Because Red/ET recombination requires only short homology regions, usually 50 bp, the homology arms can be made in synthetic oligonucleotides that are attached to cassettes containing antibiotic resistance genes by PCR *(6)*. Therefore,

each round of recombineering can be rapid. Usually, the template for the PCR reaction is a plasmid. However, such plasmids have been a common source of "carry-over" background because any residual, intact, template plasmid present with the electroporated PCR product will also produce colonies upon selection for the encoded antibiotic resistance gene (here *Tn5-neo* for Km resistance). Several ways to eliminate this background have been used, including digestion of the PCR reaction with Dpn1, multiple cleavage of the PCR template with restriction enzymes that do not cut within the amplified region, or gel purification of the PCR product. Here we eliminated this source of background by cloning the *Tn5-neo* cassette into an R6K origin plasmid (**Fig. 1A**). Plasmids based on the R6K origin require expression of the Pir protein for replication *(34)*, because virtually all *E. coli* hosts lack *pir*, use of this plasmid as the PCR template totally eliminates the background. This improvement in the methodology also simplifies the overall protocol because no extra steps are needed to eliminate carry over background.

We have modified dozens of BACs, cosmids, and other targets including the *E. coli* chromosome with the pSC101-BAD-gbaA system. For recombineering based on selection for the integration of an antibiotic resistance gene, the probability of the correct recombination is routinely more than 85% of resistant colonies. This concords with our, and other investigators, experience that the ratio of homologous to illegitimate recombination with Red/ET is very favorable. Ideally, recombineering accomplishes a seamless change not associated with the nearby presence of a selectable gene. This can be accomplished by either counterselection or, for small changes, unselected oligonucleotide mutagenesis followed by physical screening methods. For counterselection steps, the ratio of correctly engineered to unwanted products is highly variable. Often the desired product is found after counterselection in most of the resistant colonies. Occasionally, the desired product is outnumbered by deletion products. This relates to deletion of the counterselectable gene by recombination between internal repeats lying on either side of the counterselectable gene. For

recombineering with oligonuclueotides without selection, efficiencies ranging from 0.1 to 10% of colonies have been obtained. Although certain aspects of this variability have been defined *(28)*, recombineering with oligonucleotides requires molecular screening, which can be tedious. Further work to explore recombineering options using counterselection or oligonucleotide-directed mutagenesis is now required for improvements in efficiencies and user-friendly applicability. The pSC101-BAD-gbaA system is a further improvement for fluent recombineering.

Acknowledgments

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