Highly efficient deletion method for the engineering of plasmid DNA with single-stranded oligonucleotides

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The λ phage Red recombination system has been used to modify plasmid, bacterial artificial chromosome (BAC), and chromosomal DNA in a highly precise and versatile manner. Linear double-stranded DNA fragments or synthetic single-stranded oligonucleotides (SSOs) with short flanking homologies (<50 bp) to the target loci can be used as substrates to direct changes, including point mutations, insertions, and deletions. In attempts to explore mechanistic bases under this recombination process, we and others have previously identified factors that influence SSO-mediated single base substitutions. In this report, we focus our study on SSO-mediated deletion on plasmids. We found that SSOs as short as 63 bp were sufficient to mediate deletion as long as 2 kb with efficiency higher than 1%. Strand bias was consistently observed, and SSOs with sequences identical to the nascent lagging strand during replication always resulted in higher efficiency. Unlike SSO-mediated single nucleotide substitution, homology on each side of SSO flanking the fragment to be deleted was important for successful deletion, and abolishing the host methyl-directed mismatch repair (MMR) system did not lead to detectable changes in deletion efficiency. Finally, we showed that by optimizing its design, SSO-mediated deletion was efficient enough to make it possible to manipulate plasmids without selectable markers.

INTRODUCTION

The ability to direct site-specific modifications on DNA sequence has numerous advantages and applications, especially during the post-genomic era, in which manipulation of large pieces of DNA has become a routine task. The exploitation of phage recombination function serves as an alternative methodology to achieve these purposes and has allowed researchers to overcome limitations endowed by conventional cloning strategy using restriction enzymes and ligase.

Subsequent to its seminal report, recombinogenic engineering using the phage recombination proteins has been well received by numerous laboratories and is documented by the vast amount of literature (1), which also illustrates its versatility for general cloning purposes. Moreover, development of these systems has provided methodologies to suit individual needs. These

methods are based either on the *rac*-encoded RecET system or the bacteriophage- λ Red recombination system (2–6). The RecET-based homologous recombination is mediated by the RecE and RecT proteins, while the Red-based homologous recombination is mediated by three λ -recombination proteins, *exo* (α), *bet* (β), and *gamma* (γ), collectively known as the Red proteins. PCR-generated targeting cassettes have been used to generate deletions and insertions using both systems (2–4). In addition, using the λ Red system, it was shown that synthetic single-stranded oligonucleotides (SSOs) may also be used as substrates to generate sequence-specific alterations on DNA molecules with even higher efficiencies. At certain positions on the chromosome, >1% of the SSO-treated cells may be recombinant, making it possible to screen for mutants without using a selectable marker (7–9).

Although the exact mechanism of Red-mediated recombination remains elusive, we and others have studied factors affecting efficiency of SSO-mediated single base substitutions, which include length of the oligonucleotide, single-base mismatch that SSO harbors, and its orientation in relation to DNA replication machinery (7,10–12). Besides single nucleotide substitutions, the ability to delete specific DNA sequences from a template is another desired protocol. In an attempt to further advance the utility of phage recombination, we undertook a study to examine factors that are important for deletion of DNA sequences on plasmid DNA. We found that SSO with sequence corresponding to the nascent lagging strand of replication always directed higher efficiency during a deletion process. Importantly, balanced homology of SSO with sequence flanking the fragment to be deleted is required. Additionally, methyl-directed mismatch repair (MMR) is not involved in this process. Finally, we further demonstrated that SSO-mediated deletion of plasmid DNA can be achieved without the use of a selectable marker.

MATERIALS AND METHODS

Strains of Bacteria

A modified DH10B strain, called DY380, harboring the *red* genes under the tight control of the temperature sensitive λ -*cI857* repressor has been generated (13). Incubation of DY380 cells at 42°C results in the inactivation of the temperature-sensitive λ repressor and, in turn, allows expression of the Red proteins that catalyze recombination. DY380 Δ *mutS* was generated using recombineering as described previously (11). Genotypes of all bacteria used in this study are listed in Table 1.

Reporter Plasmids and Oligonucleotides

To generate the insertion mutant reporters (~2-kb insertion), a PCR-amplified *frt*-kanamycin-*frt* cassette

using oligonucleotides kCMF and kCMB with homology to the sequence of the chloramphenicol acetyltransferase (*CAT*) gene was recombined into the *CAT* gene carried on pBlue-scriptIISK(+) plasmid and pBlue-scriptIISK(+) plasmid to give p(+)-mCMKan and p(-)-mCMKan, respectively. One base pair of the *CAT* gene was also deleted during insertion. Subsequent removal of the kanamycin gene using the 294-Flp strain (courtesy of Francis Stewart, The European Molecular Biology Laboratory, Heidelberg, Germany) results in two additional reporter plasmids, p(+)-mCMftr and p(-)-mCMftr, both of which carry a ~100-bp insertion in the *CAT* coding sequence. Plasmid used for introduction of deletion mutation, pDB220, was described by Bell et al. (14). All oligonucleotides (Proligo Singapore, Singapore) used in this study are listed in Table 2.

Preparation of Recombination-competent Cells and Transformation

Recombination-competent cells were prepared according to Yu et al. (3). Briefly, 1 mL overnight cultures inoculated from a single colony were diluted 50-fold in LB medium (Invitrogen Hong Kong Ltd., Tsuen Wan, Hong Kong) and grown to $A_{600} = 0.4$. Expression of Red proteins was then induced by shaking the cultures at 42°C for 15 min. The cells were quickly chilled on ice and washed with ice-cold sterile water (three times with 50 mL water). The cell pellets were then resuspended in 1 mL sterile water, and an 80-μL aliquot of recombinogenic bacterial cells were used for each transformation by electroporation at 1.8 kV, 25 mF using the *Escherichia coli* Pulser (Bio-Rad Pacific Ltd., Quarry Bay, Hong Kong). The cells were then incubated in 1 mL LB media at 32°C for 1.5 h

Figure 1. Two sets of deletion reporters. (A–D) The solid black arrows indicate the orientation of the genes, while the solid gray arrows specify the direction of transcription (under the control of the Tn3 promoters). The gray arc indicates the 2 kb or 100 bp insertion in the mutated chloramphenicol acetyltransferase (*CAT*) gene. The open arrows inside the plasmid denote the direction of replication from the ColE1 replication origin. Replication and transcription of *CAT* gene are of different orientation in p(+) plasmids and are of same orientation in p(-) plasmids. (E) Sequences of single-stranded oligonucleotide (SSO) 89C and 89G are shown as examples. Depending on the plasmid used, the sequence of SSO may be identical to the lagging or leading strand of replication.

with shaking, before spreading onto LB agar plates (Invitrogen, Hong Kong Ltd.) supplemented with appropriate antibiotics. Ampicillin, kanamycin, and chloramphenicol (Sigma) were supplemented at concentrations of 50, 25, and 6.25 μg/mL, respectively.

Calculation of Efficiency of Recombination

Recombination-competent DY380 cells were transformed by electroporation with the correction SSO together with the plasmid reporter. Aliquots of the transformed bacterial cells were spread onto LB plates supplemented with either ampicillin or chloramphenicol, which were then incubated overnight at 32°C. Recombination between the SSO and the target DNA bearing the insertions was indicated by the appearance of chloramphenicol-resistant (*cm^r*) colonies, with the efficiency of the recombination

calculated by dividing the number of *cm^r* colonies by the number of the ampicillin-resistant (*amp^r*) colonies.

RESULTS AND DISCUSSION

The *CAT* deletion reporter was constructed to contain a ColE1 replication origin, an *amp^r* gene, and a mutant *CAT* gene under the control of a constitutive promoter (Tn3). Two individual *CAT* reporters, p(+)-mCMftr and p(-)-mCMftr (Figure 1, C and D), were used to assay the efficiencies of SSO-mediated deletion. The *CAT* function is abolished due to a ~100-bp insertion in its coding sequence to replace one original base pair. The two plasmids were identical, except that the *CAT* reporter gene cassettes were oriented in opposite directions relative to the direction of replication. To assay the likelihood of a larger deletion event, p(+)-mCMKan and p(-)-mCMKan

Table 1. Genotypes of Bacteria Used in the Study

Strain	Genotype
DH10B	<i>F⁻ mcrA Δ (mrr-hsdRMS-mcrBC) φ80dlacZ Δ M15 Δ lacX74 deoR recA1 endA1 araD139 Δ (ara, leu) 7649 galU galK rspL nupG</i>
DY380	DH10B [<i>λc1857 (cro-bioA) < tet</i>] ^a
DY380Δ <i>mutS</i>	DY380 [<i>mutS < kan</i>] ^b

^a*cro-bioA < tet* indicates substitution of *cro-bioA* with *tet*.
^b*mutS < kan* indicates substitution of *mutS* gene with *kan*.

Figure 2. Efficiency of plasmid DNA deletion mediated by single-stranded oligonucleotides (SSOs) of different lengths. (A–D) For each diagram, the y-axis represents efficiency of SSO-mediated deletion of the fragment inserted into chloramphenicol acetyltransferase (*CAT*) gene (for calculation, see the Materials and Methods section). The x-axis represents the length of SSO used. Two complementary SSOs of each length were used. The black box represents the efficiency generated by using SSOs with sequences identical to the nontemplate strand, while the black triangle represents those identical to the template strand. Each spot represents results of at least three independent experiments and plotted as mean $\pm$ SEM.

(Figure 1, A and B) were constructed, and the *CAT* function was similarly abolished due to a ~ 2 -kb insertion in its coding sequence to replace one original base pair. The two reporters differ in their orientation relative to the unidirectional replication origin. Cells transformed with either of these reporters are resistant to ampicillin, but remain sensitive to chloramphenicol.

Pairs of complementary SSOs of different lengths were designed to delete the sequence inserted in the *CAT* reporter gene and reintroduce the 1 bp deleted during generation of the reporter, restoring the function of *CAT* (Figure 1E). The base pair introduced was a silent mutation of the original base pair, so that we could differentiate real deletion events from contaminations. All shorter SSOs are exact nested subsets of the longer ones. The sequences of SSO 23C/37C/63C/89C are identical to the nontemplate strand of the *CAT* gene or the *CAT* messenger RNA (mRNA), and the sequences of 23G/37G/63G/89G are identical to the template strand. Their sequences are also identical to either the nascent

Table 2. Oligonucleotides Used in the Study

SSO	Sequence	Use/Function
kCMF	5'-ATACGGAATTCGGATGAGCATTATCATCAGGCGGGCAAGAATGT GAATAAAGGCCGACGATTCTGCAACCCTATGCTACTCCG-3'	Forward primer to insert Kan ^r sequence into <i>CAT</i> allele
kCMB	5'-CAGCTGGATATTACGCGCTTTTAAAGACCGTAAAGAAAAATAA GCACAAGTTTTACGCTCCCGCGGATTGTCTACTCAGGAG-3'	Reverse primer to insert Kan ^r sequence into <i>CAT</i> allele
89C	5'-ACGGCCTTTTAAAGACCGTAAAGAAAAATAAGCACAAGTTTAC CCGGCCTTTATTCACATTCTTGCCGCGCTGATGAATGCTCATCC-3'	Nontemplate SSO to delete the insertion in the m <i>CAT</i> gene
89G	5'-GGATGAGCATTATCATCAGGCGGGCAAGAATGTGAATAAAGGCCG GGTAAACTTGTGCTTATTTTCTTTACGGTCTTTAAAAAGGCCGT-3'	Template SSO to delete the insertion in the m <i>CAT</i> gene
89C-1	5'-CTCAATGTACCTATAACCAGACCGTTCAGCTGGATATTACGGCC TTTTTAAAGACCGTAAAGAAAAATAAGCACAAGTTTACCGGCC-3'	Nontemplate SSO to delete the insertion in the m <i>CAT</i> gene
89G-1	5'-TCACCAGCTACCGTCTTTTATTGCCATACGGAATTCGGATGAG CATTATCATCAGGCGGGCAAGAATGTGAATAAAGGCCGGTAAAAC-3'	Template SSO to delete the insertion in the m <i>CAT</i> gene
89C-2	5'-GTTTTACCGGCCCTTTATTCACATTCTTGCCGCGCTGATGAATGCT CATCCGGAATTCGGATGGAATGAAAGACGGTGAGCTGGTGA-3'	Nontemplate SSO to delete the insertion in the m <i>CAT</i> gene
89G-2	5'-GGCCGGGTAAAACCTTGTGCTTATTTTCTTTACGGTCTTTAAAAAG GCCGTAATATCCAGCTGAACGGTCTGGTTATAGGTACATTGAG-3'	Template SSO to delete the insertion in the m <i>CAT</i> gene
13A	5'-CCCGGCCCGCAGGAGAACACCTTCCCCAAGGGCGAGCCGGATCTGAAGAAGT CCAGCAAGAACAAGCCACACGTCAAGCGACCCATGAACGCCTTCATGGT-3'	Lagging-strand SSO to delete the 105 bp dimerization domain of gSox9
13B	5'-ACCATGAAGGCGTTCATGGGTCGCTTGACGTGTGGCTTGTCTTCTGCTGGAC TTCTTCAGATCCGGCTCGCCCTTGGGGAAGGTGTTCTCTGGGGCCGGG-3'	Leading-strand SSO to delete the 105 bp dimerization domain of gSox9
SeIF	5'-CAAGGGCGAGCCGGATCTG-3'	Detection of Sox9 deletion mutant
SeIR	5'-GACCAAGACCCAGGATG-3'	Detection of Sox9 deletion mutant

CAT, chloramphenicol acetyltransferase; SSO, single-stranded oligonucleotide.

leading or lagging strand, depending on their relative orientation to the direction of plasmid replication.

Several factors that could possibly influence the efficiency of deletion were investigated in this study. We first tested whether the length of SSO could proportionally affect the efficiency of deletion by using SSOs of four different lengths: 23, 37, 63, and 89 bp. The efficiency was extremely low when the length of SSO was 23 or 37 bp, but rose when the length was increased to 63 or 89 bp. It is possible that a minimum length of homology might be needed by β for efficient searching and pairing of the SSO to its target. Interestingly, we obtained a steady increase in deletion efficiency when using SSOs with sequences identical to the template strand during transcription of the reporter (23C/37C/63C/89C). On the other hand, a plateau was reproducibly observed when SSOs with sequences identical to the nontemplate strand (23G/37G/63G/89G) were used, with the highest efficiency achieved by the SSO of 63 bp (Figure 2). We could not find a sound explanation for this phenomenon yet, but this might simply reflect the difference in base composition of two sets of SSOs.

By comparing efficiencies obtained using these two different sets of SSOs, we also observed that a strand bias existed and that the efficiency was always higher when SSOs with sequences identical to the lagging strand were used, regardless of if they were identical to the template or nontemplate strand. This was particularly obvious when SSOs of 89 bp were used. This observation was consistent with the current anneal-integration model, in which the SSO might anneal to its target during the replication process. Through lagging strand synthesis, more single-stranded regions are revealed, and this creates a higher chance for incorporation of the SSO (8). Our results, together with recent similar findings by Thomason et al. (12), indicate that the pairing of the SSO to its target during deletion still occurs during DNA replication.

To evaluate the importance of homology that the SSO carries flanking the plasmid sequence to be deleted, we tested the efficiency of deletion using three pairs of SSOs of identical lengths. 89C and 89G have balanced homology (44 and 44 bp) flanking the sequence to be deleted, 89C-1 and 89G-1 have 82 bp homology on the 5' side and

6 bp homology on the 3' side, while 89C-2 and 89G-2 have 6 bp homology on the 3' side and 82 bp on the 5' side. Compared with that achieved using an SSO with balanced homology (89C or 89G), the efficiency decreased dramatically to background levels when either SSO with imbalanced homology (89C-1, 89G-1, 89C-2, or 89G-2) was used. This phenomenon was also repeatable using any of the four deletion reporter plasmids (Figure 3). In contrast, during SSO-mediated single base substitution in mammalian cells, the homologous sequence that the SSO carries on the 5' side of the mutation has been shown to be more important than that on the 3' side (15). Similarly in the *E. coli* Red system, the homology on the 5' side of the mutation was also more important during SSO-mediated single base substitution than that on the 3' side (unpublished data). It is apparent from this observation that SSO-mediated single base substitution and deletion use different mechanisms. For the point mutation, the importance of 5' homology may reflect a single homology searching step. As long as the 5' side is annealed to the target, the 3' homology is just one base aside and could be easily paired. However, for deletion, homology on two sides is far apart, and we speculate that another round of searching for homology, which occurs sequentially or simultaneously, might be necessary for pairing of the 3' side homology, thus making the 3' homology equally important.

Besides the nature of the SSO, we went on to study the effect of MMR on deletion efficiency. MMR has been demonstrated to have great impact on the efficiency of SSO-mediated single base substitution, during which it dominates over the strand bias imposed by the orientation of SSO in relation to the direction of replication and favors the SSO bearing the mismatch that is poorly recognized by the system (7). We repeated our experiments using SSOs of 89 bp in a MMR-defective strain DY380 Δ mutS. However, no significant difference in efficiency could be identified between wild-type and MMR defective cells, and the strand bias we observed before remained unchanged (Figure 4). This result was not surprising and could be

Figure 3. Efficiency of plasmid DNA deletion mediated by single-stranded oligonucleotides (SSOs) of different polarity. (A) Three pairs of SSOs and their orientation to the fragment to be deleted are shown. The number of base pairs of homology on either side of the fragment to be deleted was listed besides each pair of SSOs as a format of 5' homology plus 3' homology. (B and C) For each diagram, the y-axis represents efficiency of SSO-mediated deletion of the fragment inserted into chloramphenicol acetyltransferase (*CAT*) gene (for calculation, see the Materials and Methods section). The x-axis represents the SSO used. Each column represents results of at least three independent experiments plotted as mean $\pm$ SEM.

Figure 4. Efficiency of single-stranded oligonucleotide (SSO)-mediated deletion in wild-type and mutS-defective cells. (A and B) For each diagram, the y-axis represents the efficiency of SSO-mediated deletion of the fragment inserted into chloramphenicol acetyltransferase (CAT) gene (for calculation, see the Materials and Methods section). The x-axis represents the length of SSO used. Two complementary SSOs were used. The white bar represents the efficiency generated by using SSO 89C, while the gray bar represents the efficiency generated by using SSO 89G. Each column represents results of at least three independent experiments plotted as mean $\pm$ SEM.

explained by the failure of the MMR system to detect the 100-bp or 2-kb loop generated by the pairing of the SSO to its target, as the MMR system could only recognize point mutations and 2–4 mismatches (16). It is thus not necessary to perform the deletion in mismatch-deficient cells to pursue higher deletion efficiency.

To test whether the efficiency of deletion was sufficiently high to delete any sequence on the plasmid without using any selectable marker, we designed two SSOs to introduce a 105-bp deletion in a 12 kb plasmid pDB220 (14), which contained the mouse Sox9 genomic DNA cloned into the pBlue-script vector. These two SSOs, 13A and 13B, were 100 bp in length, with 50 bp on each side homologous to the sequences flanking the 105-bp region to be deleted. The sequence of 13A was identical to the lagging strand of plasmid replication, while 13B was identical to the leading strand sequence (Figure 5A). SSO 13A or 13B were co-electroporated with pDB220 into recombinogenic competent cells. The electroporated cells were then plated on LB plates supplemented with ampicillin and incubated overnight at 32°C. Individual colonies were picked from each plate and subjected to PCR analysis with the primers Self and SelR. Deletion mutants could be

easily distinguished from the wild-type plasmid by comparing the sizes of the PCR products (Figure 5B). When SSO 13A was used, an average of 3 to 5 deletion mutants could be identified by PCR from every 100 colonies examined. The deletion mutant was purified, and the precise deletion of 105 bp on the plasmid was confirmed by sequencing. When SSO-13B was used, no deletion mutants were detected among over 400 colonies examined.

Conclusion

In conclusion, a lagging SSO with balanced homology was an ideal molecule to mediate deletion of plasmid DNA. The efficiency was sufficiently high that the deletion was not limited to reporter genes, and the clone containing the deletion event could be easily identified using PCR. This highly efficient SSO-mediated deletion of plasmid DNA could therefore bring increased convenience to molecular cloning, which is not limited to the deletion of plasmid DNA of variable lengths, but also extends to the insertion of DNA sequences. Limited by the length of synthetic SSOs, which is usually shorter than 150 bp, SSO-mediated insertion of long sequences becomes impossible, although there are reports that this could be achieved by using multiple overlapping SSOs (17). Using the Red system, insertion of long DNA fragments could be achieved through recombineering together with an antibiotic selectable marker, which could be subsequently removed by Cre/Flp recombinase if the selectable marker is flanked by two loxPs/frts. However, Cre/Flp recombinase would inevitably leave the unwanted loxP/frt sequence on the plasmid DNA, which might cause disruption of coding or regulatory sequences. Although this problem could be resolved by performing recombineering twice and by using negative selection (thyA and galK) (18,19), such systems require specialized culture medium and reagents. Instead, using SSO-mediated deletion, removal of a selectable marker or an unwanted loxP/frt sequence would also be seamless and efficient. Besides functioning in *E. coli*, we recently discovered that SSO could

Figure 5. Single-stranded oligonucleotide (SSO)-mediated 105-bp deletion without using selectable markers. (A) The orientation of SSO 13A and 13B to the 105-bp fragment to be deleted is shown. 13A is identical in sequence to the lagging strand of replication, while 13B is identical to the leading strand. Self and SelR located outside the sequence covered by 13A or 13B. (B) Colony PCR results using Self and SelR as primers. Representative results with different *Escherichia coli* colonies as templates were shown: lane M, 1-kb molecular weight marker (1-kb plus ladder; Invitrogen, Hong Kong); lanes 1, 2, and 3, colonies containing wild-type plasmid, a mixture of wild-type and deletion mutant plasmids, and deletion mutant plasmid, respectively, were used as templates; lane 4, PCR negative control.

also mediate deletion in a mammalian genome (20), and this could potentially extend the usages of SSOs in manipulating the mammalian genome as well.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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