

[15] Recombineering: *In Vivo* Genetic Engineering in *E. coli*, *S. enterica*, and Beyond

By JAMES A. SAWITZKE, LYNN C. THOMASON, NINA COSTANTINO,
MIKHAIL BUBUNENKO, SIMANTI DATTA, and DONALD L. COURT

Abstract

“Recombineering,” *in vivo* genetic engineering with short DNA homologies, is changing how constructs are made. The methods are simple, precise, efficient, rapid, and inexpensive. Complicated genetic constructs that can be difficult or even impossible to make with *in vitro* genetic engineering can be created in days with recombineering. DNA molecules that are too large to manipulate with classical techniques are amenable to recombineering. This technology utilizes the phage λ homologous recombination functions, proteins that can efficiently catalyze recombination between short homologies. Recombineering can be accomplished with linear PCR products or even single-stranded oligos. In this chapter we discuss methods of and ways to use recombineering.

Introduction

What Is Recombineering?

In vivo genetic engineering using the bacteriophage lambda (λ) recombination proteins and short DNA homologies has been termed “recombineering” (recombination-mediated genetic engineering) (Ellis *et al.*, 2001) and is the subject of this chapter.

Genetic engineering has been instrumental in revolutionizing studies in molecular biology for over 30 years since the discovery of restriction enzymes. *Escherichia coli* has been the standard host used to recover the products of this *in vitro* genetic engineering. Since the late 1990s, however, new *in vivo* technologies have emerged that greatly simplify, accelerate, and expand genetic engineering in *E. coli*, *Salmonella enterica*, and other organisms. Now, within a week a researcher can modify any nucleotide(s) of choice in almost any manner. Further, these genetic engineering technologies do not rely on *in vitro* reactions carried out by restriction enzymes and DNA ligase. Instead, they utilize the bacteriophage λ homologous recombination proteins collectively called “Red” to directly modify DNA within a bacterial cell. Importantly, the Red proteins require only 50 bases

of homology to catalyze efficient recombination. These homologies are small enough that they can be provided by synthetic oligonucleotides.

Red Proteins and Properties

Homologous recombination is the process whereby segments of DNA are exchanged between two DNA molecules through regions of identical DNA sequence, the end result being new combinations of genetic material. Generalized recombination catalyzed by the *E. coli* recombination proteins occurs when there are about 100 base pairs of homology for exchange and becomes more efficient with longer homologies (Shen and Huang, 1986; Watt *et al.*, 1985).

Normally, linear DNA introduced into *E. coli* is degraded by the powerful RecBCD nuclease. Although *in vivo* genetic engineering systems have been previously attempted (for a review of other systems, see Court *et al.*, 2002), none have been fully satisfactory. In contrast, the Red proteins of phage λ and the RecET proteins of the cryptic *rac* prophage have properties that allow recombination of a linear, modifying DNA containing short (50 bp) homologies with appropriate target sequences, thereby allowing rapid and efficient genetic engineering (Muyrers *et al.*, 1999, 2000; Yu *et al.*, 2000; Zhang *et al.*, 1998, 2000). Other similar systems will also undoubtedly be developed (Poteete, 2001; Poteete and Fenton, 1993; Vellani and Myers, 2003); however, in this review we concentrate on the λ Red system, Exo, Beta, and Gam.

The λ Gam protein inhibits the RecBCD and SbcCD nuclease activities, preserving linear DNA and thereby allowing it to be used as a substrate for recombination (Chalker *et al.*, 1988; Gibson *et al.*, 1992; Karu *et al.*, 1975; Kulkarni and Stahl, 1989; Murphy, 1991). Linear DNA is required for Red-mediated recombination (Stahl *et al.*, 1985; Thaler *et al.*, 1987a, 1987b). This can be either a linear double-strand DNA (dsDNA) generated by PCR or a short single-stranded DNA (ssDNA) oligonucleotide (oligo) carrying homology to the target (Court *et al.*, 2002).

The λ Exo protein, a dsDNA-dependent exonuclease, processes linear dsDNA. Exo requires a dsDNA end to bind and remains bound to one strand while degrading the other in a 5'-3' direction (Carter and Radding, 1971; Cassuto and Radding, 1971; Cassuto *et al.*, 1971). This results in dsDNA with a 3' ssDNA overhang, the substrate required for the Beta protein to bind. Exo is required only for recombineering with dsDNA substrates (Ellis *et al.*, 2001; Yu *et al.*, 2000).

λ Beta is a ssDNA-binding protein that can promote the annealing of complementary DNA strands. Beta can bind stably to ssDNA greater than 35 nucleotides (Mythili *et al.*, 1996) and protect the DNA from single-strand

nuclease attack (Karakousis *et al.*, 1998; Muniyappa and Radding, 1986). Beta is the only known λ function required for recombineering with ssDNA oligos (Ellis *et al.*, 2001).

Recombineering with linear dsDNA requires all three Red proteins. Gam is needed to protect the linear substrate. Since Beta and Exo form a complex (Radding *et al.*, 1971), it is reasonable to suggest that as Exo degrades a chain of dsDNA, Beta binds to the newly formed ssDNA (Karakousis *et al.*, 1998; Li *et al.*, 1998). However, Beta alone is sufficient for recombination with ssDNA substrates (Ellis *et al.*, 2001; Yu *et al.*, 2000).

Expression of Red Proteins from a Defective Prophage

The Red proteins are encoded by the *gam*, *bet*, and *exo* genes located next to each other in the *pL* operon of λ . The timing and level of expression of these genes is of critical importance for the highest recombineering efficiencies. Prolonged expression of Gam can lead to plasmid instability (Murphy, 1991; Silberstein and Cohen, 1987; Silberstein *et al.*, 1990) and toxic effects to the cell (Friedman and Hays, 1986; Sergueev *et al.*, 2001). Inappropriate expression of Exo and Beta can lead to unwanted rearrangements, which is especially problematic in working with eukaryotic DNA cloned into BACs.

For ease of movement between strains, several labs have cloned various combinations of the Red genes on plasmids under the control of heterologous promoters (Datsenko and Wanner, 2000; Muyrers *et al.*, 1999, 2000; Zhang *et al.*, 1998, 2000). Although these systems have been effectively used for recombineering, plasmid-borne systems can be prone to inappropriate expression problems. More recently developed plasmid systems have abrogated some of these problems (see “Prophage-Containing Recombineering Plasmids” section).

Our laboratory has developed and utilized a λ prophage for expression of the Red genes (Court *et al.*, 2002; Ellis *et al.*, 2001; Yu *et al.*, 2000). The prophage is defective in that it has been deleted for the lysis, DNA replication, and structural genes of the phage, but retains the critical features of transcriptional control and importantly, the Red functions (Fig. 1A). With this prophage, the Red genes are expressed from the *pL* operon under the control of the temperature-sensitive repressor, CI857. Thus, when the cells are at a low temperature (<37 °C), the CI857 repressor is active and there is no expression of the Red genes except in a rare subpopulation of spontaneously induced cells. After a brief temperature upshift to 42 °C, the CI857 repressor denatures, allowing transcription from *pL* and thereby Red expression. Upon shifting back to low temperature, CI857 renatures and again completely blocks transcription of *pL*. Thus, Red functions are

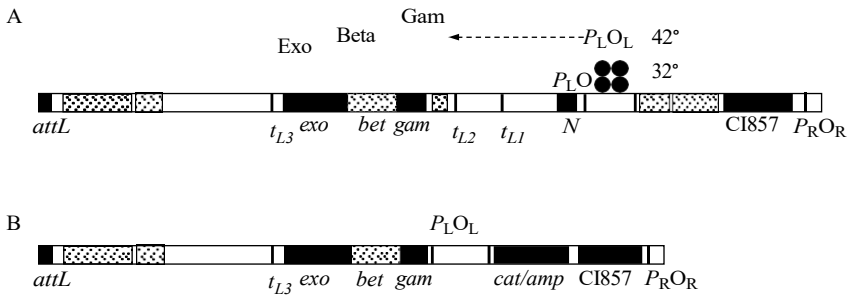


FIG. 1. Diagram of the defective prophage used for recombineering. (A) Standard defective prophage originally described in Yu *et al.* (2000). The *red* genes—*exo*, *bet*, and *gam*—are under control of the temperature-sensitive repressor, CI857. Transcription of the *red* genes (beyond t_{L1}) requires the N protein. (B) The minimal prophage as described in Datta *et al.* (2006). Transcriptional terminators t_{L1} and t_{L2} as well as the N gene have been deleted. The minimal prophage is no longer dependent on N protein but still is regulated by CI857. In both cases, at temperatures less than 34 °C, CI repressor (filled circles) binds the operators and prevents transcription of the p_L operon. At 42 °C, the temperature-sensitive CI857 repressor denatures and thus allows transcription of the *red* genes.

available for a short but sufficient time to recombine the sequences of interest and then they are removed to minimize extraneous events. Gam is extremely toxic to cells, but this short pulse of expression does not interfere with cell viability (Sergueev *et al.*, 2001).

In this review, we focus on using recombineering to manipulate DNA on the bacterial chromosome, plasmids, or phage. However, recombineering is just as useful to modify BACs containing DNA from other organisms for functional genomic studies (Copeland *et al.*, 2001; Lee *et al.*, 2001; Muyrers *et al.*, 1999; Swaminathan *et al.*, 2001; Warming *et al.*, 2005). Discussion of the mechanism(s) of recombineering can be found elsewhere (Costantino and Court, 2003; Court *et al.*, 2002; Ellis *et al.*, 2001).

Methods

Standard Recombineering Protocol

The steps for executing the standard recombineering protocol in *E. coli* or *S. enterica* include: (1) preparation of electrocompetent cells that contain the λ recombination proteins needed for recombineering, (2) transformation of those cells with the DNA substrate using electroporation, (3) outgrowth, (4) selection or screening for the chosen genetic change, (5) confirmation of the genetic alteration, and (6) elimination of the λ stuff.

The following protocol outlines the procedure that we have found to produce the most consistent results. Some parameters have been optimized while others have not (Yu *et al.*, 2000, 2003). Any deviation from this protocol may produce less than satisfactory results, but modifications may prove necessary in other organisms.

Preparation of Electrocompetent and Recombineering-Proficient Cells

The first step is to produce cells that are competent for both the uptake of DNA and for recombineering. With our standard prophage expression system where the cells contain the *l red* genes under CI857 control, a 5-ml overnight culture is grown in Luria broth (LB) at 30 to 32 °C. This culture is then diluted at least 70-fold (0.5 ml of overnight culture into 35 ml of fresh LB) and grown in a 125-ml baffed flask with shaking (200 rpm) at 32 °C until the OD₆₀₀ is 0.4 to 0.5. Fifteen milliliters of culture are then rapidly shifted to 42 °C and incubated with shaking (200 rpm) for 15 min to induce production of the Red proteins. The rest of the cells remain at 32 °C (the uninduced control). After 15 min, all flasks are placed in an ice-water bath and swirled to rapidly cool them. Flasks are swirled intermittently in the ice bath for 5 to 10 min until the cultures are completely chilled. The cells are pelleted by centrifugation at 4600 *g* (6700 rpm in a Sorvall SA-600 rotor) for 7 min in a 4 centrifuge. The supernatant is decanted or aspirated, and the cells are gently suspended with 1 milliliter of ice-cold sterile distilled H₂O using a large disposable pipette tip or gentle shaking. A vortex must not be used for this or subsequent steps as cells in H₂O are fragile. After the cells are suspended, an additional 30 ml of ice-cold sterile distilled water is added to each tube and gently rocked to mix before pelleting again at 4600 *g* for 7 min. The pellet will be very loose and great care must be taken not to lose the cells while decanting the supernatant. Again the pellet is gently suspended with 1-ml ice-cold distilled H₂O. The cells are then transferred to a chilled microfuge tube and pelleted in a 4 microfuge for 30 sec at maximum speed. Finally, each preparation of cells is suspended in 200 μ l of ice-cold distilled H₂O and kept on ice until electroporation. This should be enough cells for four or five electroporations. We always use freshly prepared electrocompetent cells for the highest efficiencies, but cells can be frozen at -80 °C in 12% glycerol for future recombineering, albeit at a lower efficiency (Yu *et al.*, 2000).

Transformation by Electroporation

Once the cells are competent, the DNA substrate is introduced by electroporation. We use the standard conditions recommended for *E. coli* and *Salmonella* in a Bio-Rad electroporator: 1.8 kV with 0.1-cm cuvettes that have been chilled on ice. Other conditions have not been tested

thoroughly by our laboratory. We typically mix 100 to 300 ng of salt-free PCR product (see [preparation of linear DNA](#)) or 50 to 100 ng of salt-free ssDNA (oligos) with 50 μ l of electro-competent cells. They can be mixed in either a cold microfuge tube and then moved to the cuvette, or mixed directly in the electroporation cuvette with similar results. Important controls include induced cells with no DNA and uninduced cells with DNA. Optimal electroporations give a time constant of more than 5.0 msec. Lower time constants may produce recombinants but at a lower efficiency, and may reduce total cell viability. Immediately after electroporation, 1 ml of LB is added to the electroporation cuvette, and cells are transferred to a sterile culture tube. Subsequent steps depend on the specifics of the desired recombination event.

Outgrowth

Once LB has been added to the electroporated cells, a minimum 30-min incubation at 32 is necessary to allow their recovery from electroporation. Several outgrowth options are available; the appropriate one depends on the type of recombinants generated and the method being used to identify recombinants. In general, the options are to dilute and spread the dilutions on agar plates after the 30-min outgrowth, or to incubate longer and grow the electroporation mixture in LB before dilution and plating. In the first case, each electroporated cell is plated before significant cell division occurs, and in the second case, the electroporated cells grow and divide before plating.

At the time of recombination, there are several replicating copies of the bacterial chromosome (four to eight), but recombination is restricted in most instances to one of these and, in the case of oligonucleotide recombinants, to one strand of one copy ([Costantino and Court, 2003](#)). Thus, during further growth of these cells (either on plates or in LB), the DNA copies present at recombination segregate from one another, separating recombinant from unrecombined DNA copies. If cells are spread on agar before outgrowth, recombinant colonies that form will be a mixture of recombinant and parental cells. If sufficient time is allowed for outgrowth in liquid culture, each colony will be relatively pure, but the frequency of recombinant colonies will be reduced by the outgrowth and segregation process. This dilution effect could be as much as 4- to 16-fold for *E. coli* growing in LB because of the multiple replication forks and DNA copies present at the time of electroporation and recombination ([Sergueev et al., 2002](#)).

Outgrowth before plating is critical for finding recombinants in certain situations. For example, when a drug-resistance cassette is used for targeting, recombinants are selected in the presence of the drug. In this situation, the recombinant cassette must be expressed before the cell carrying it is

challenged with the drug. Usually 2 to 3 h of outgrowth in the absence of drug selection are required for sufficient expression. As a different example, when a gene that makes a conditionally toxic product to the cell is targeted for replacement by recombination, then complete segregation should be allowed so that only a pure recombinant cell (i.e., one that does not contain the toxic gene) remains (to avoid toxicity on selection). Examples of this are the counter-selected genes such as *sacB*, *galK*, and *thyA*, which will be described later. In this case, a longer outgrowth in liquid media should be allowed to generate recombinant cells free of the gene and its toxic product.

Plating cells soon after electroporation reduces the number of colonies that need to be screened when nonselective procedures are used to find recombination. Once recombinant colonies are found, however, the recombinant cells within the colony must be purified away from the parental segregants, which are also present.

Selection or Screening for Mutants

When cells are ready for dilution and plating, tenfold stepwise dilutions should be made in TMG, minimal salts, or similar osmotically balanced medium (Arber *et al.*, 1983; Sambrook and Russell, 2001). Luria broth may be used for dilutions if selection is for a drug resistance. The appropriate dilution and plates to use for selecting/screening for recombinants depends on the specifics of the recombineering being performed. In initial experiments, a wide range of dilutions should be plated for both selection of recombinants and determination of cell viability. For example, if a PCR product was used to insert a drug cassette, then we optimally see 10^3 to 10^4 recombinants per 10^8 viable cells (Table I). If, however, an oligo (ssDNA)

TABLE I
A COMPARISON OF RECOMBINEERING EFFICIENCIES WITH VARIOUS SUBSTRATES

Strain	Number of Recombinants/ 10^8 Viable Cells			
	dsDNA ^b	Oligo Repair with Lagging Strand ^a		
		T/C ^c	C/C	Multibase mismatch ^d
Wild-type	10^4	10^5	10^7	10^7
<i>mutS</i>	10^4	10^7	10^7	10^7

^aUsing the leading strand, recombination is up to 30-fold reduced as compared to the lagging strand.

^bFor example, replacing the *galK* gene with a drug cassette.

^cOr any mispair other than a C/C.

^dFour or more mismatches in a row.

is used for recombineering a point mutation, the frequency of recombination is routinely 10^5 per 10^8 viable cells, and under some conditions may be as high as 25% of the total viable cells (Table I) (Costantino and Court, 2003). In the strains that we use, we find 10^7 to 10^8 viable cells per milliliter after electroporation and a 2-h outgrowth. In some strains we see up to a 10-fold reduction in viability after electroporation. It is important to verify total viable cells to ensure there are enough cells to isolate recombinants. To determine the total cells that survive electroporation, dilutions are plated nonselectively on L plates and incubated at 34 °C.

If a high level of recombination is expected ($>10^5/10^8$ viable), cells can be plated nonselectively on L plates and recombinants screened for by checking individual colonies for the desired phenotype or genotype. For example, if ssDNA was used to recombineer a new restriction site into a gene, a diagnostic PCR fragment followed by restriction analysis can be used to identify the recombinant colonies. Single base changes can also be detected by the mismatch amplification assay-PCR (MAMA-PCR) method (Cha *et al.*, 1992; Swaminathan *et al.*, 2001). Another method to screen for nonselected recombinants is colony hybridization of cells (L. C. Thomason, *et al.*, unpublished results, 2005b). For this method, the sequence inserted by recombineering must be unique to the recombinant so it can be used as a probe. Finally, in some cases, it is possible to detect recombinants directly on nonselective plates. For example, if the recombinant produces altered colony morphology or a slow-growth phenotype, these can be detected directly by looking for that minority class of colonies (Thomason and Sawitzke, unpublished results).

As an alternative to screening nonselected colonies, a two-step selective protocol can be used to modify a region of interest. First, the targeted region is replaced by a dual selection cassette such as *cat-sacB* (see “[Selection/Counter-Selection for Gene mutation, Replacement, and Fusion](#)” section), then an oligo (or PCR product) containing the mutations can be introduced in the second step. With this method, there is selection for both steps so that no screening is required. This protocol is useful for making numerous site-specific mutations in a region of interest.

Confirming Mutations

Candidate recombinants must be purified by streaking out for single colonies on the appropriate plates before further testing. Once recombinant candidates have been purified, the desired changes can be confirmed by PCR analysis, restriction analysis, and DNA sequencing. Sequence analysis will also confirm that no extraneous changes were made. It is known that inadvertent changes can arise because of errors introduced during oligosynthesis (Oppenheim *et al.*, 2004).

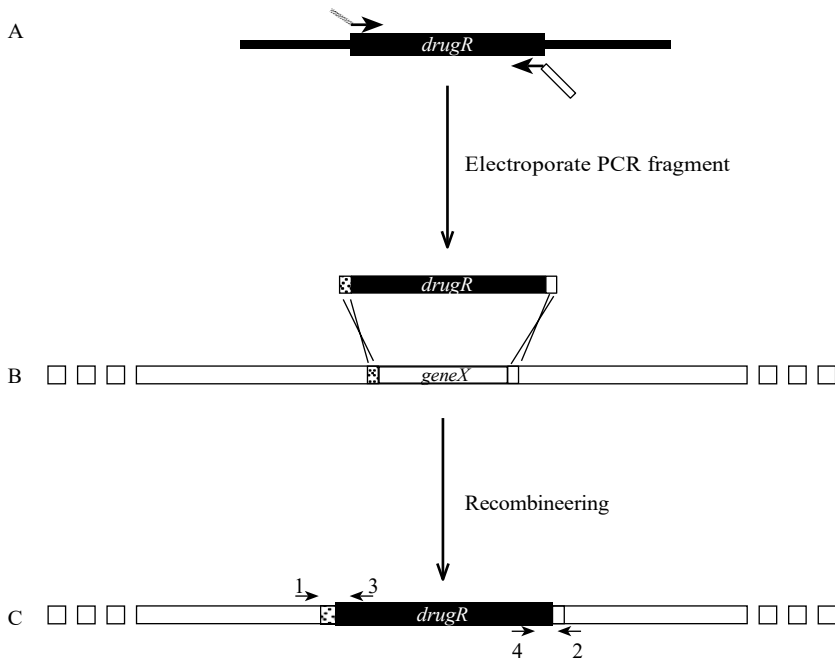


FIG. 2. Using recombineering to replace a gene with a drug-resistance cassette. (A) A pair of hybrid primers that contain at their 5' end, 50 bases of homology to the intended target, and at their 3' end, sequence for priming a template for a drug-resistance (*drugR*) cassette (Table III). PCR using these primers and the proper template produces the linear substrate with the *drugR* flanked by 50-bp homologies. The primer design determines precisely where the drug cassette will insert. In this example, we fully replace "*geneX*" with a drug cassette using homologies that flank *geneX*. (B) The *drugR* fragment is electroporated into Red-induced cells where recombineering takes place. (C) Drug-resistant clones are checked for gene replacement by PCR analysis. PCR using primers 1 and 3, 2 and 4, and 1 and 2 should yield products of predicted sizes.

For an antibiotic cassette or other insertion, PCR can be used to confirm its location. Two primers, internal to the insertion, should be designed pointing out towards each end of the insert to be paired with primers flanking the site of insertion (see Fig. 2C and legend). Predicted fragments from all the various primer pairs should be checked (Yu *et al.*, 2000). Sequencing can be done to fully verify all junctions if necessary.

Elimination of the l Stuff

After recombineering, in many cases it is desirable or necessary to remove the *red* (and other *l*) genes. This may be accomplished in several ways, and the choice depends on the details of the experiment and which recombineering system is being used. In general, the *red* genes can be removed from the

strain in which the recombineering took place, or alternatively, the newly constructed recombinant can be moved to a clean genetic background. For genetic experiments, the latter is usually preferable, especially if a mismatch-repair mutant strain was used.

If the altered DNA resides on a plasmid or BAC, then the newly made construct will often be moved away from the recombineering genes during the course of the protocol by plasmid isolation and re-transformation into a nonrecombineering host (see “[Recombineering on a Plasmid](#)” section, and [Warming *et al.*, 2005](#)).

If the new construct resides in the chromosome and has a selectable phenotype (e.g., drug resistance or auxotrophy), generalized transduction using phage P1 (P22 in *S. enterica*) can be used to move it to a clean genetic environment, away from the recombineering strain. Using generalized transduction to move a point mutation on the chromosome, especially one without a selectable or easy-to-screen phenotype, can be difficult to accomplish. In such cases, it may be necessary or at least easier to remove the recombineering system from that strain. If the defective prophage was used for recombineering, then it can be removed either by generalized transduction (e.g., use linked *nadA::Tn10*) or by recombineering a PCR fragment of the wild-type *attB bio* region made from a nonlysogen to replace the prophage ([Yu *et al.*, 2000](#)). You can select for growth on minimal medium without biotin at 42 °C since the prophage makes the strain temperature sensitive and a biotin auxotroph.

Some of the prophage-containing recombineering plasmids have a temperature-sensitive origin of replication ([Table II](#)), and a temperature shift will encourage loss of the plasmid ([Datta *et al.*, 2006](#)). Plasmid loss is accomplished by diluting an overnight cell culture containing the temperature-sensitive plasmid 1000-fold in LB and growing at 37 °C for more than 4 h. Dilutions are then plated on L plates at 32 °C. After this regimen, nearly 100% of tested colonies have lost the plasmid.

TABLE II RED-
PRODUCING PLASMIDS

Plasmid ^a	Origin	Drug resistance
pSIM5	pSC101 <i>repA</i> ^{ts}	Chloramphenicol
pSIM6	pSC101 <i>repA</i> ^{ts}	Ampicillin
pSIM7	pBBR1	Chloramphenicol
pSIM8	pBBR1	Ampicillin
pSIM9	pRK2 <i>trfA</i> ^{ts}	Chloramphenicol
pSIM18	pSC101 <i>repA</i> ^{ts}	Hygromycin
pSIM19	pSC101 <i>repA</i> ^{ts}	Spectinomycin

^aPlasmids are further described in [Datta *et al.*, 2006](#).

Preparation of Linear DNA for Recombineering

Linear DNA that is either single- or double-stranded is needed for recombineering. Whether you should use ss- or ds-DNA depends on the details of the construct being made.

Oligo Design for ssDNA Recombineering

For ssDNA recombineering, we order salt-free oligos with no further purification. In some cases, gel purification can be used to reduce unwanted base deletion mutations introduced during oligo synthesis (Oppenheim *et al.*, 2004). If there is a selection for function, then most of these unwanted mutations in the oligo will be selected against. The oligo is reconstituted at a concentration of 1 nmol/ μ l in Tris EDTA(TE) and stored at -20°C . Multiple freeze/thaw cycles are avoided by making working stock aliquots at a final concentration of 10 pmol/ μ l in dH_2O . Use 0.5 μ l of this working stock for 50 μ l of electro-competent cells. We use 70 base oligos for recombineering. Base changes should be centered in the oligo as much as possible, although anywhere within the “middle” 20 bases of a 70-base oligo give similar frequencies of recombinants (Costantino and Court, unpublished results).

For a given target, there are two complementary ssDNA oligos, either one of which can be used for recombineering. One corresponds to the DNA strand that is replicated as the “leading strand” and the other to the “lagging strand.” The lagging strand oligo corresponds in sequence to Okazaki fragments. The efficiency of recombination is up to 30-fold higher with the oligo that corresponds to the lagging strand (Costantino and Court, 2003; Ellis *et al.*, 2001). These data help support the model that Beta anneals the ssDNA oligo at the DNA replication fork (Court *et al.*, 2002; Ellis *et al.*, 2001). Thus, for ssDNA recombineering, the oligo of choice is the one that corresponds to the lagging strand sequence.

Preparing Linear dsDNA

If linear dsDNA is the substrate for recombineering, PCR is normally used to generate this substrate. We use standard reaction conditions with a high-fidelity PCR kit. Each 70 base salt-free primer contains two parts (Yu *et al.*, 2000)—the 5' ends contain the 50 bases of homology to the target, whereas the 3' end of the oligo primes the DNA to be inserted (Yu *et al.*, 2000). Thus, the precise join point of the final recombinant product is defined by the oligo design (Fig. 2). When creating deletions, gene replacements, or fusion proteins with recombineering, it is important to keep polarity in mind. An out-of-frame replacement can potentially eliminate expression of downstream genes causing unintended phenotypes.

The PCR-generated targeting DNA often contains a drug-resistance marker flanked by homology sequences, but it can contain any sequence

that can be selected or screened for. Recombineering to insert or remove a large heterology is less efficient than creating a single base change (Table I), so a direct selection or a two-step selection/counter-selection (see below) should be used when possible. Table III details the primers we use for amplifying drug cassettes with their promoters and transcription terminators. They have been chosen to allow efficient PCR synthesis, and ultimately, expression of the drug cassette. The PCR products are purified with a commercially available PCR cleanup kit before recombineering.

The method used for PCR amplification can have dramatic effects on the experimental results. Often the template for the PCR is a plasmid from which drug-resistance and other cassettes are amplified. It is important to use the least amount of plasmid DNA possible for the reaction. Template plasmid DNA still present during electroporation will give rise to drug-resistant colonies because transformation of supercoiled plasmid is very efficient. Plasmid DNA can be greatly reduced after the PCR reaction by digesting with DpnI, which cuts methylated DNA but not the unmethylated PCR products. Transformation of uninduced cells with the linear vector mix will give an estimate of the amount of uncut plasmid template still present in the preparation. This is an important control.

Because of the problems caused in getting rid of plasmid DNA, non-plasmid templates may be preferred. For example, cassettes already cloned into the bacterial chromosome can be amplified. Alternatively, PCR amplified cassettes can be maintained as stock DNA templates for subsequent amplification. Care must be taken if the template for PCR is also a PCR product, since serial amplifications will cause mutations to accumulate in the PCR products, thus resulting in problems. We have seen the *sacB* gene become less sensitive to sucrose as a result of repeated amplifications (Thomason, unpublished results). Therefore, make a stock template once from an original source. Once it is used up, make a new stock from the original source.

Maximizing Recombination

Methyl-directed mismatch repair (MMR) reduces recombination frequencies (Costantino and Court, 2003). The MMR system recognizes and repairs base pair mismatches and small (1 to 3 bp) deletions, but not larger heterologies. In the absence of MMR activity, recombination frequencies can be increased. The frequency of recombineering to insert or remove a large heterology is not affected by mismatch repair.

Methyl-Directed Mismatch Repair Mutants

In *E. coli*, the MMR system includes, among other functions, MutH, MutL, MutS, the UvrD helicase, and the Dam methylase. Cells containing

TABLE III
PRIMER PAIRS FOR AMPLIFYING CASSETTES

Drug cassette	Potential template sources ^a	Primer pair
Ampicillin	pBR322 (New England Biolabs) and derivatives	5 ⁰ CATTCAAATATGTATCCGCTC 5 ⁰ AGAGTTGGTAGCTCTTGATC
Kanamycin	pBBR1MCS-2 (Kovach <i>et al.</i> , 1994), <i>Tn5</i> (Ahmed and Podemski, 1995) Note: this is not the same kanamycin gene as in <i>Tn903</i> .	5 ⁰ TATGGACAGCAAGCGAACCG 5 ⁰ TCAGAAGAAGCTCGTCAAGAAG
Chloramphenicol	pACYC184 (New England Biolabs)	5 ⁰ TGTGACGGAAGATCACTTCG 5 ⁰ ACCAGCAATAGACATAAGCG
Tetracycline	<i>Tn10</i> (Hillen and Schollmeier, 1983) Note: this is not the same tetracycline gene as in pBR322 or pACYC184	5 ⁰ CAAGAGGGTCATTATATTTCCG 5 ⁰ ACTCGACATCTTGGTTACCG
Spectinomycin	pBBR1MCS-5 (Kovach <i>et al.</i> , 1994), DH5 PRO (Clontech)	5 ⁰ ACCGTGGAACGGATGAAGGC 5 ⁰ AGGGCTTATTATGCACGCTTAA
<i>cat-sacB</i> cassette	pK04/pEL04 (Lee <i>et al.</i> , 2001)	5 ⁰ TGTGACGGAAGATCACTTCG 5 ⁰ ATCAAAGGGAAAACGTCCATAT
PCR fragment to remove prophage	<i>E. coli</i>	5 ⁰ GAGGTACCAGCGCGGTTTGATC 5 ⁰ CTCGGTCTTAATCGACAGCAAC

^a We often grow an overnight of cells containing the desired drug-resistance template in the chromosome; 2 l of this overnight is an excellent template for PCR. We have listed some commonly found sources of these sequences, but others may be suitable. As multiple versions of drug-resistance cassettes are available (as noted above), caution must be used to be certain that these primers will prime your template. Notes: All primers included in this table are designed so that the PCR product will contain a promoter (if appropriate) for the drug-resistance gene. All cassettes except for the kanamycin gene also contain a transcription terminator. We are currently engineering a terminator for the kanamycin cassette. Using other priming oligos that are not shown here, a PCR product can be generated to replace a gene from its start to stop codons with a drug-resistance gene from its start to stop codons, thus producing the drug-resistant recombinant with the gene's native regulation.

a mutation that eliminates any of these functions exhibit increased levels of recombination with ssDNA, given that the recombinants are no longer removed by the MMR system (Costantino and Court, 2003; Li *et al.*, 2003). More than a 100-fold increase in recombination can be achieved by eliminating the MMR system when changing a single base (Table I). This increase allows up to 25% of the cells surviving electroporation to become recombinants when a lagging strand oligo is used, making screening for recombinants easy. The drawback to this method is that MMR-deficient strains are mutagenic, causing the frequency of extraneous mutations to be increased.

C/C Mismatch

With careful design, high levels of recombineering can be achieved in strains that are wild-type (WT) for mismatch repair (Costantino and Court, 2003). This is possible because some mismatches are poorly corrected by the MMR system. The hierarchy of repair from poorest to most efficiently repaired is C/C < A/G, T/C, T/T < G/G, A/A, A/C, G/T (Dohet *et al.*, 1986; Su *et al.*, 1988). If the recombining oligo creates a C/C mismatch when annealed to the target sequence, this mismatch is not recognized by the MMR system and is not repaired. In practical terms, this means that any G can be efficiently changed to a C. In fact, a C/C mispair within 6 bp upstream or downstream of a second desired change prevents the second change from being repaired (N. Costantino and D. Court, unpublished results). Thus, generating C/C mismatches allows high levels of recombineering at many positions without the negative side effect of the strain being mutagenic.

Other Means of Maximizing Recombination

Another method to evade the MMR system while recombineering is to design the oligo with multiple adjacent base changes. With careful design the additional changes can introduce or remove a restriction site that will aid confirmation. Using this trick, a single point mutation can be made in two steps with high levels of recombination in both steps (Yang and Sharan, 2003). With the first event, four to six changes are made that cover the mutational site of interest. Next, a second oligo recombination event can be used to change the sequence back to WT except for the desired point mutation.

Finally, the MMR system can be inhibited temporarily by a dominant negative allele of the *mutS* gene (Haber and Walker, 1991) or by addition of 2-aminopurine (2-AP) (Costantino and Court, 2003). Incubation of cells for 3 h with 75 μ g/ml 2-AP increased the level of recombination, but not to that obtained with the complete absence of mismatch repair. Thus, 2-AP

can be used to increase recombination frequencies with limited general mutagenesis of the cells.

Genetic Manipulations

Several other useful genetic tricks are available that facilitate the manipulation of DNA with recombineering. With this toolkit, nearly any construct can be made efficiently and seamlessly.

Selection/Counter-Selection for Gene Mutation, Replacement, and Fusion

Another two-step protocol is frequently used to make changes for which there is no selection. This method is useful to make a protein fusion that has no obvious phenotype, to mutagenize a region, or alter a specific base and leave no other changes. In the first step, dual selection cassettes containing both selectable and counter-selectable markers are recombineered into the target location. At this first step, selection is used to insert the markers near a base or region to be changed. In the second step, counter-selection is used to replace the dual selection cassette with the final DNA construct.

We routinely use the *cat-sacB* cassette (Ellis *et al.*, 2001; Thomason *et al.*, 2005a) with an initial selection for chloramphenicol resistance in the first round of recombineering, and a final selection *sacB* in the second round. The *sacB* gene makes *E. coli* sensitive to sucrose; thus, plates containing sucrose (see “Media”) can be used to select against cells containing this gene (Gay *et al.*, 1985). After insertion of the cassette by recombineering and selection for chloramphenicol-resistant recombinants, several isolates should be purified and tested for sucrose sensitivity. We have found instances when the expression of the *sacB* cassette is affected by its orientation at the target (L. C. Thomason *et al.*, unpublished results). Thus, at some loci, both orientations may need to be tried to ensure a strong counter-selection. A sucrose-sensitive isolate is chosen for the second round of recombineering, from which sucrose-resistant colonies are selected and screened to confirm that they are chloramphenicol sensitive and true recombinants. Those that are still resistant to chloramphenicol may have a spontaneous mutation in the *sacB* gene (normally found at a frequency of 1 in 10⁴), and thus are “false positives.” If recombination conditions have been optimized, the number of chloramphenicol-sensitive recombinants should be greater than these chloramphenicol-resistant false positives.

Recently, *galK* and *thyA* have been developed for the same purpose as *cat-sacB* (Warming *et al.*, 2005; Wong *et al.*, 2005); however, in these cases, either *galK* or *thyA* is used for both selections. To use *galK* as a dual selection cassette, the recombineering takes place in cells that are deleted for the *galK* gene, and thus are unable to utilize galactose as a sole carbon

source. In the first step, recombineering inserts the *galK* gene, allowing growth on minimal galactose agar. The *galK* gene product, galactokinase, also effectively catalyzes the phosphorylation of the galactose analog, 2-deoxy-galactose (DOG), leading to a toxic buildup of 2-deoxy-galactose-1-phosphate (Alper and Ames, 1975). Thus, the second round of recombineering with the *galK* system is selection against *galK* on agar containing DOG (see “Media” section).

When *thyA* is used as the dual selection cassette, the cells must be deleted for *thyA* (Wong *et al.*, 2005). Cells containing a *thyA* deletion are unable to grow on minimal medium in the absence of thymine. Thus, in the first recombineering step, *thyA* is inserted in the target sequence of cells that contain a *thyA* deletion, selecting for growth on minimal medium. Cells containing a functional *thyA* gene, however, are sensitive to trimethoprim in the presence of thymine, which is the basis for the counter-selection in the second recombineering event.

There is one minor change to the “basic protocol” for the second recombineering event when using a selection/counter-selection. The electroporated cells should be suspended in a final volume of 10 ml of LB and incubated with aeration at 34 °C for at least 3 to 4 h, and preferably overnight. The longer outgrowth allows for complete segregation of recombinant chromosomes that no longer contain the toxic counter-selectable marker. The presence of a sister chromosome with an intact counter-selectable marker will prevent growth of the cell even though one chromosome is recombinant. We note, however, the standard recombineering protocol that includes outgrowth for 3 hr in 1 ml of broth does produce some recombinants.

All of the dual selection systems have strengths and weaknesses. The *cat-sacB* product is large (3 kb), and thus the PCR product can be more difficult to make than the single gene (*galK* and *thyA*) systems. The *cat-sacB* dual cassette system will work in any strain and has the added advantage that loss of the *cat* cassette can easily be screened. In contrast, the *galK* or *thyA* systems work only in strains lacking these genes, and PCR must be used to distinguish true recombinants from spontaneous mutations. Note that the *cat* gene can be replaced by another drug-resistance marker in the *cat-sacB* dual selection cassette.

Duplications

Recombineering can be used to identify duplications, which are tandem diploid regions often being multiple kilobases in size. Duplications naturally occur and exist for any region at frequencies from 10^{-4} to 10^{-2} in a culture (Haack and Roth, 1995). Cells with such duplications can be identified by engineering a gene replacement with a selectable drug cassette in which the gene being replaced is either essential or is conditionally

essential (Yu *et al.*, 2000). The duplication is stabilized by maintaining simultaneous selection for the essential gene and the drug cassette. If one targets genes in the chromosome, two classes of recombinants are found based on frequencies alone. Replacement of a nonessential gene is straightforward and occurs at high efficiency, whereas replacement of an essential gene occurs but is found at much reduced frequency ($<100/10^8$ viable). Such rare recombinants contain large duplications with a second WT copy of the essential gene present. PCR analysis using primers that flank the targeted essential gene is useful for identifying the duplication, as two products will be seen corresponding to the essential gene and the modified copy (M. Bubunencko, unpublished results).

Recombineering can also be used to engineer duplication of a defined region by designing the linear substrate with the appropriate homologies (Sawitzke, unpublished results). This technique is described in Slechta *et al.* (2003) for generating duplications in *S. enterica*.

Inversions

Making a defined inversion using recombineering is most easily achieved with a two-step process. In the first step, the region to be inverted is deleted, perhaps while inserting a selectable/counter-selectable cassette. In the second step, a PCR product of the region, containing the appropriate flanking homologies, is recombineered and replaces this counter-selectable cassette (or deleted region). The final product must be sequenced as PCR can create mutations. A similar approach was used for inverting the *gal* operon (Ellis *et al.*, 2001).

Annealing Oligos In Vivo

Two or more overlapping oligos can be simultaneously electroporated into Red-expressing cells. These oligos have two parts, an end with homology to the target sequence and an end complementary to the other oligo (Yu *et al.*, 2003). The oligos anneal *in vivo*, perhaps with the help of Beta, which would also protect them from degradation. The oligos must overlap by six or more bases to anneal and longer overlaps increase efficiency. If the annealed oligos have 5' single-stranded overhangs (the target homology), they recombine efficiently. Using this technique, multiple overlapping oligos can be used to construct longer DNA substrates (Yu *et al.*, 2003). This reaction is very similar to *in vitro* PCR assembly (Stemmer *et al.*, 1995) but occurs *in vivo*.

Gene-Specific Random Mutagenesis Using Recombineering

Recently, a useful protocol that includes recombineering to generate random, site-directed (a specific gene, for example) mutations has been

published (De Lay and Cronan, 2006). Briefly, a mutagenized PCR product of your gene of interest is made (product 1). A PCR product of a nearby gene containing a selectable marker is also made (product 2). The two PCR products overlap by 20 bases, are gel purified, mixed together, and overlapping extension PCR is performed (Ho *et al.*, 1989). Finally, the overlapping extension PCR product is used as a substrate for recombineering, inserting both the mutagenized fragment and the selectable marker into the chromosome. Mutations in your gene are then screened for. Such a targeted mutagenesis should be useful for many genes. De Lay and Cronan (2006) developed this technique to isolate temperature-sensitive mutations in an essential gene.

We imagine that gene-specific random mutagenesis can be done without a selectable marker. The gene can again be amplified by mutagenic PCR and used directly for recombineering in a mismatch-repair mutant host, thereby ensuring very high levels of recombination and relatively easy screening for mutant phenotypes.

Targeting Recombineering to Plasmids: Modifications to the Standard Protocol

Although we have emphasized modifying genes on the chromosome, the techniques discussed thus far can be used to modify plasmids as well. In addition, direct *in vivo* cloning can be accomplished with recombineering.

Recombineering on Plasmids

Recombineering targeted to a pBR322-type plasmid has been characterized, and frequencies similar to those obtained when targeting the *E. coli* chromosome are observed for both ds- and ss-DNA recombination. These results will be detailed in Thomason *et al.* (submitted), but the key findings are summarized here. It is critical to start with a pure monomer species plasmid for recombineering. Optimally, the plasmid should be introduced into *recA* mutant cells expressing the Red system by co-electroporation rather than targeting a resident plasmid. A low-plasmid DNA concentration should be used; 10 ng is usually sufficient for maximal transformation efficiency. After recombinant colonies are identified, they should be purified, under selective conditions if possible, before they are used to inoculate cultures from which to isolate candidate modified plasmid DNA. This DNA should be introduced into a *recA* mutant standard cloning strain at a low DNA concentration, once again selecting or screening for the desired modification.

Circular plasmid multimers arise when targeting plasmids. One source of these circular multimers is recombination catalyzed by the host RecA

protein; these can be eliminated through the use of a *recA* mutant host for the recombineering. Another source of circular multimers is Red recombination acting on both double- and single-stranded linear substrate DNA; these multimers cannot be eliminated. In a *recA* mutant recombineering host, circular multimers are rarely, if ever, found among non-recombinants. Co-electroporation of the plasmid with the modifying DNA minimizes, but does not eliminate, the formation of these plasmid multimers. It is important to screen recombinant plasmids by gel electrophoresis to determine their multimeric state. Multimeric recombinant plasmid products that have been converted on only one copy of the region to be altered have been observed. If a recombinant plasmid has multimerized, the DNA can be digested, re-ligated under dilute conditions, and then introduced into a *recA* mutant host lacking the Red system in order to obtain a recombinant monomer clone. It has been reported (Cohen and Clark, 1986) that extended expression of the Gam protein can give rise to linear plasmid multimers, but the circular multimers we have observed depend only on Beta expression and the presence of linear substrate DNA during recombination.

Gap Repair of Plasmids: In Vivo Cloning

Recombineering using a gapped plasmid with homology to the target can be used to clone genes or regions from the chromosome or other replicons (e.g., BACs). A gapped plasmid is a linear DNA fragment containing a plasmid origin. Gap repair of this linear plasmid is useful to retrieve a mutated gene for sequencing, allow expression of a gene under the control of a chosen promoter, or to create a gene fusion to a tag or reporter (Fig. 3). A gapped linear plasmid can also recombine with a co-electroporated linear fragment (Court *et al.*, 2002).

Oligos for PCR amplification of a gapped plasmid are designed as outlined in “preparing linear dsDNA.” In this case, however, the 3⁰ ends prime synthesis of a plasmid origin, and the 5⁰ ends have homology flanking the target sequence to be cloned. Two methods can be used that differ in the location of the drug-resistance cassette, which can be either on the gapped plasmid itself or linked to the target sequence. If the target contains a linked drug-resistance cassette, the gapped plasmid need only contain a plasmid origin and homologies to the target (Datta *et al.*, 2006). The region to be cloned can be either co-transformed with the linear origin fragment or be already present in the cell. After recombineering, the electroporation mix is diluted in 10 ml of LB and incubated overnight nonselectively. Plasmid DNA is isolated and transformed into a standard *recA* mutant cloning strain using a low concentration of DNA to ensure that only one plasmid enters the cell. Select for the marker retrieved onto the origin

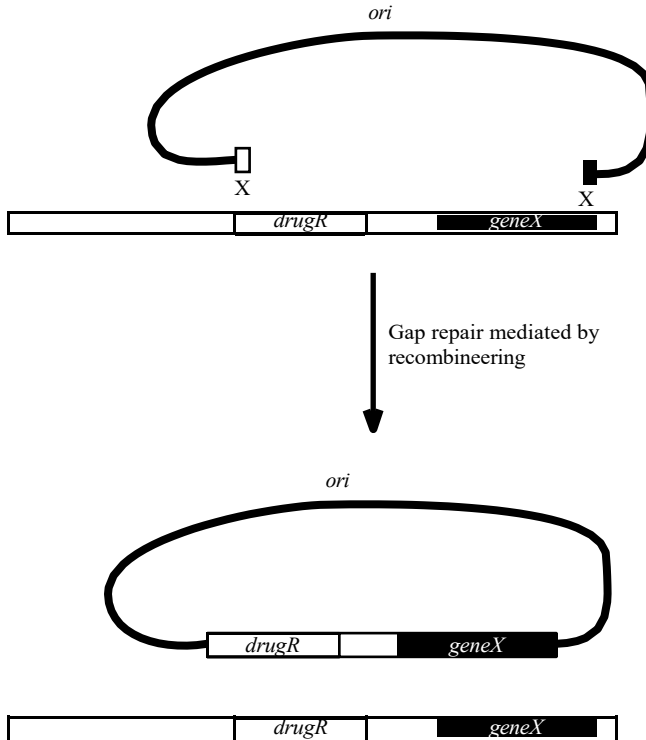


FIG. 3. Cloning by retrieval onto a gapped plasmid with recombineering. A linear DNA fragment containing a plasmid origin and homologies (50 bp on each end) to a region of interest can be used to clone sequences from the chromosome, other plasmids, BACs, or even a co-electroporated linear DNA fragment. In this illustration, a drug resistance is linked to the gene of interest, and thus the gapped linear plasmid need not contain a selectable marker. As the chromosome will still contain this drug resistance, plasmid DNA must be isolated and screened to find the desired recombinants as described in the text.

vector and confirm candidate recombinants by PCR (Thomason *et al.*, 2005a).

If there is no drug resistance linked to the target sequence, then the drug cassette must be on the gapped plasmid. The linear DNA “vector” containing a plasmid origin, a drug-resistance cassette, and ending in homologies to the target sequence is transformed into a cell that has been induced for the Red system. The target can be either co-transformed or already resident in the Red-producing strain and selection is for the drug resistance on the gapped plasmid. After purifying drug-resistant candidates, the recombinant plasmids must be checked since false positives can

be caused by nonhomologous end joining of the linear vector. Repeats longer than 5 bp near the ends enhance nonhomologous end joining (Zhang *et al.*, 2000), which can be minimized by careful primer design.

Gap repair is less efficient than ssDNA or gene replacement recombineering; typically we see a few hundred recombinants per 10^8 viable cells. Because of this low frequency, it is important to eliminate false positives (see “Preparing Linear dsDNA” section). The small effort involved makes gap-repair cloning techniques very appealing as compared to traditional cloning methods. An important advantage is that DNA retrieved by gap repair from the chromosome is not subject to PCR-generated mutations.

Replacing Plasmid Origins

During genetic studies, one often encounters the problem that a plasmid is incompatible for use with another plasmid (or the chromosome) because it has the same drug resistance or origin of replication. Recombineering can be used to change the drug resistance of one of the plasmids. It can also be used to exchange one plasmid origin for another, thereby making one plasmid compatible with the other. Changing the origin can also be used to alter the copy number and/or extend the host range (Datta *et al.*, 2006).

Many clones are found in pBR322-based plasmids. Since the pBR322 origin does not replicate in a *polA* mutant strain (Kingsbury and Helinski, 1970), the origin of these plasmids can be selected against and replaced with other origins. A linear DNA fragment containing a new origin and any necessary replication functions (e.g., pSC101 or the RK2 origin) with homologies flanking the pBR322 origin can be electroporated into a strain containing the Red functions and the pBR322-based plasmid. After recombineering, the culture is grown nonselectively overnight in 10 ml of LB, plasmid DNA is prepared, and then used to transform a *polA* mutant strain with selection for the plasmid drug marker. Only plasmids that have acquired the new origin will be able to replicate in the *polA* mutant strain (Datta *et al.*, 2006). We note that origin replacement is mechanistically the same as retrieval by gap repair.

Targeting Recombineering to Phage: Modifications to the Standard Protocol

Like other replicons, the phage λ chromosome can also be modified by recombineering. The Red proteins can be supplied by a prophage on the chromosome (Court *et al.*, 2003; Oppenheim *et al.*, 2004), by a defective prophage on a plasmid (Datta *et al.*, 2006) or by the infecting phage itself (Oppenheim and Costantino, unpublished results). The “standard recombineering protocol” has been modified (Oppenheim *et al.*, 2004). Cells containing a defective prophage are grown to mid-log at 32 and then

harvested by centrifugation at 4600 *g* for 7 min at 4 before suspending them in 1 ml of TMG buffer (see “Media” section). The λ phage to be modified are added at a multiplicity of infection of one to three phage per cell and are adsorbed at room temperature for 15 min. The infected cells are added to 5 ml of LB prewarmed to 42 °C, which will induce production of the Red proteins. Cultures are shaken in baffed fasks at 200 rpm for 15 min. After 15 min, the cultures are chilled on ice and processed as described in the standard recombineering protocol. The cells are electroporated with either a PCR product or oligo, diluted into 5 ml of 39 °C LB, and allowed to incubate at 39 °C with shaking to finish the lambda lytic cycle (60 to 90 min). As a negative control, include an electroporation without PCR or oligo. The lysates are diluted and titered on appropriate bacteria to obtain single plaques. The desired mutation can be selected or screened for (Oppenheim *et al.*, 2004).

If recombineering is done with an intact (cI857) prophage, then induction at 42 °C should only be for 4 to 5 min to prevent cell killing. The shorter time minimizes expression of the prophage DNA replication genes, which are toxic to the host when expressed for longer periods (Court *et al.*, 2003). The rest of the protocol is as outlined.

Prophage-Containing Recombineering Plasmids

Recombineering has already proven very useful for bacterial genetics in *E. coli*, pathogenic *E. coli* (Murphy and Campellone, 2003), and *S. enterica* (Bunny *et al.*, 2002; Uzzau *et al.*, 2001). This technology has also been used to modify plasmids or BACs in *E. coli* before moving the altered constructs to other organisms such as mice (Lee *et al.*, 2001; Warming *et al.*, 2005), and *Aspergillus nidulans* (Chaveroche *et al.*, 2000). Pioneering studies have been done in *Yersinia pseudotuberculosis* (Derbise *et al.*, 2003), and will undoubtedly be tried in other prokaryotes and perhaps eukaryotes soon.

Recently, we have made a series of plasmids that should aid recombineering in *E. coli* and certain other gram-negative bacteria (Datta *et al.*, 2006). These plasmids contain a defective prophage in which the *pL* promoter has been directly fused to the Red genes, thereby removing some of the normal regulatory elements (Fig. 1B). The *pL* promoter and Red expression on these plasmid vectors are still tightly regulated by the temperature-sensitive repressor, CI857. These vectors are available with different plasmid origins of replication and drug-resistant markers as described in Table II. Another vector, mini-1, was developed to move the defective prophage system between *E. coli* strains (Court *et al.*, 2003). However, the plasmid vectors just described are more efficient for this purpose and still maintain tight control of Red gene expression.

Strains and Plasmids

Many bacterial strains and plasmid vectors that are useful for using λ Red recombineering have been constructed. [Table IV](#) lists several recombineering strains and their genotypes. [Table II](#) describes the key attributes of several recombineering plasmids that are currently available.

Media

The growth media for the various protocols, in quantities per liter, follow. As indicated in [Table IV](#), many recombineering strains are biotin auxotrophs, and biotin must be added to a final concentration of 0.0001% (w/v) to all minimal media.

Luria Broth (LB)

- 10 g Bacto-typtone (Difco)
- 5 g yeast extract (Difco)
- 5 g NaCl (not 10 g, as used by many)

Note: Add 15 g Bacto-agar (Difco) for plates.

L ρ Sucrose (No NaCl) Plates

L plates are supplemented with 6% (w/v) sucrose for selecting against *sacB*. NaCl should be omitted from this medium ([Blomfeld et al., 1991](#)).

M63 Minimal Glycerol ρ Sucrose Plates

- 3 g KH_2PO_4
- 7 g K_2HPO_4
- 2 g $(\text{NH}_4)_2\text{SO}_4$
- 0.5 ml FeSO_4 (1 mg/ml solution)
- 1 ml 1M MgSO_4
- 10 ml 20% glycerol
- 5% (w/v) sucrose
- 5 ml 0.2 mg/ml (0.02%) D-biotin (Sigma)
- 1 ml 1% thiamine (vitamin B₁)
- 15 g Bacto-Agar

M63-DOG (for Selecting GalK Mutants)

- 3 g KH_2PO_4
- 7 g K_2HPO_4
- 2 g $(\text{NH}_4)_2\text{SO}_4$

TABLE IV
USEFUL RECOMBINEERING STRAINS

Strain	Genotype	Special purpose	References
DY329	W3110 <i>lacU169 nadA::Tn10 gal490 pgI8</i> [1 <i>cl857 (cro bioA)</i>]	Useful for moving prophage by P1 transduction using linked <i>Tn10</i>	(Yu <i>et al.</i> , 2000)
DY330	W3110 <i>lacU169 gal490 pgI8</i> [1 <i>cl857 (cro-bioA)</i>]		(Yu <i>et al.</i> , 2000)
DY331	<i>lacU169 (srIA-recA)301::Tn10 gal490 pgI8 cl857 (cro-bioA)</i>		(Yu <i>et al.</i> , 2000)
DY378	W3110 [1 <i>cl857 (cro-bioA)</i>]		(Yu <i>et al.</i> , 2000)
DY380	DH10B <i>mcrA (mrr-hsdRMS-mcrBC) ø80dlacZM15 lacX74 deoR recA1 endA1 araD139 (ara, leu)7697 galU gal490 pgI8 rpsL nupG</i> [1 <i>cl857ind1 (cro-bioA)<> tet</i>]	Useful for BAC transformation and manipulations	(Lee <i>et al.</i> , 2001)
HME5	W3110 <i>lacU169</i> [1 <i>cl857 (cro-bioA)</i>]		(Ellis <i>et al.</i> , 2001)
HME6	W3110 <i>galK_{tyr145UAG} lacU169</i> [1 <i>cl857 (cro-bioA)</i>]	Assay system for oligo recombineering.	(Ellis <i>et al.</i> , 2001)
HME43	W3110 <i>galK_{tyr145UAG} lacU169</i> [1 <i>cl857 (exo-int)<> cat <> (gam-N)</i>]	Strain makes only Red Beta	(Ellis <i>et al.</i> , 2001)
HME51	W3110 <i>galK_{tyr145UAG} lacU169</i> [1 <i>cl857 (exo-int)<> cat <> (gam-N)</i>] (<i>srIA-recA</i>)301::Tn10		N. Costantino, personal communication
HME63	W3110 <i>galK_{tyr145UAG} lacU169 mutS<> amp</i> [1 <i>cl857 (cro-bioA)</i>]	Defective for mismatch repair; therefore, high-level oligo recombineering	(Costantino and Court, 2003)
HME68	W3110 <i>galK_{tyr145UAG} lacU169</i> [1 <i>cl857 (cro-bioA)</i>] <i>mutS<> cat</i>	Defective for mismatch repair	N. Costantino, personal communication
HME70	W3110 <i>galK_{tyr145UAG} lacU169</i> [1 <i>cl857 (cro-bioA)</i>] <i>mutS<> cat (srIA-recA)301::Tn10</i>	Oligo recombineering with plasmids	(Thomason <i>et al.</i> , submitted)
HME71	W3110 <i>galK_{tyr145UAG} lacU169</i> [1 <i>cl857 (cro-bioA)</i>] (<i>srIA-recA</i>)301::Tn10	Oligo recombineering with plasmids	N. Costantino, personal communication
SIMD3	W3110 [1 <i>cl857 rex<> cat (N-kil) (cro-bioA)</i>]	Contains N-independent minimal prophage (Fig. 1B)	(Datta <i>et al.</i> , 2006)
SIMD4	W3110 [1 <i>cl857 rex<> amp (N-kil) (cro-bioA)</i>] DH10B	Contains N-independent minimal prophage (Fig. B)	(Datta <i>et al.</i> , 2006)
SW102	<i>mcrA (mrr-hsdRMS-mcrBC) ø80dlacZM15 lacX74 deoR recA1 endA1 araD139 (ara, leu)7697 galK pgI8 rpsL nupG</i> [1 <i>cl857ind1 (cro-bioA)<> tet</i>]	Use for <i>galK</i> selection/counter-selection	(Warming <i>et al.</i> , 2005)

0.5 ml FeSO₄ (1 mg/ml solution)
1 ml 1 M MgSO₄
10 ml 20% glycerol
5 ml 0.2 mg/ml (0.02%) D-biotin (Sigma)
1 ml 1% thiamine (vitamin B₁)
5 ml 40% 2-deoxy-galactose (DOG) (Ferro Pfanstiehl)
15 g Bacto-agar

TMG Buffer

10 mM Tris base
10 mM MgSO₄
0.01% gelatin

Antibiotics

When antibiotics are added to select for single copy markers (i.e., on the chromosome), they are used at lower concentrations than for plasmid selection. Using a too-high drug concentration will reduce the number or even prevent detection of recombinants. The following is for single copy use: ampicillin, 30 g/ml; chloramphenicol, 10 g/ml; kanamycin, 30 g/ml; tetracycline, 12.5 g/ml; and spectinomycin, 30 to 50 g/ml. These concentrations have been used in *E. coli* and *S. enterica*, but the proper concentrations in other bacteria must be determined.

Concluding Remarks

Recombineering has made complex genetic manipulations possible. Large DNA molecules such as BACs and the chromosome can be directly modified. In contrast to site-specific recombination systems that leave a *loxP* or *frt* site at the modified region, recombineering does not necessarily leave “scars” behind. Although recombineering has been primarily developed in *E. coli*, it is starting to be used in other bacteria and soon perhaps even in eukaryotes. New advances in the understanding of the mechanisms as well as new ways to use recombineering are rapidly being developed. See <http://RedRecombineering.ncifcrf.gov/> and <http://recombineering.ncifcrf.gov/> to download protocols as well as to check for updates of techniques, and to request strains or plasmids.

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[16] 1-Red Genetic Engineering in *Salmonella enterica* serovar Typhimurium

By JOYCE E. KARLINSEY

Abstract

The use of the recombination system from bacteriophage lambda, 1-Red, allows for PCR-generated fragments to be targeted to specific chromosomal locations in sequenced genomes. A minimal region of homology of 30 to 50 bases flanking the fragment to be inserted is all that is required for targeted mutagenesis. Procedures for creating specific insertions, deletions, and site-directed changes are described.