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Multicopy plasmid modification with phage λ Red recombineering

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Abstract

Recombineering, *in vivo* genetic engineering using the bacteriophage λ Red generalized recombination system, was used to create various modifications of a multicopy plasmid derived from pBR322. All genetic modifications possible on the *Escherichia coli* chromosome and on bacterial artificial chromosomes (BACs) are also possible on multicopy plasmids and are obtained with similar frequencies to their chromosomal counterparts, including creation of point mutations (5–10% unselected frequency), deletions and substitutions. Parental and recombinant plasmids are nearly always present as a mixture following recombination, and circular multimeric plasmid molecules are often generated during the recombineering.
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1. Introduction

In vivo genetic engineering by homologous recombination, or recombineering, has been well developed in *Escherichia coli* allowing DNA alteration of almost any replicon. Possible modifications include single base mutations, small and large deletions, and heterologous insertions (Copeland et al., 2001; Court et al., 2002). Recombineering utilizes the bacteriophage λ generalized recombination Red functions, Exo and Beta, along with the Gam protein to catalyze recombination with linear DNA substrates. Red Exo and Beta are, respectively, a 5′ → 3′ double strand (ds)-exonuclease (Little, 1967) and a single strand (ss)-annealing protein (Karakousis et al., 1998) that together catalyze recombination between short homologies of around 50 bases (Yu et al., 2000). Gam inhibits the *E. coli* exonuclease RecBCD (Unger and Clark, 1972) allowing intracellular preservation of linear ds DNA, such as PCR products. Both dsDNA (Yu et al., 2000; Lee et al., 2001; Murphy, 1998) and ssDNA (Swaminathan et al., 2001; Ellis et al., 2001) substrates can be used in recombineering;

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insertion of dsDNA requires all three functions, while creation of point mutations, small insertions, and deletions with ssDNA requires only the Beta protein.

It has been previously demonstrated (Ellis et al., 2001) that, for ssDNA recombination with synthetic oligonucleotides (oligos), one of the two possible complementary oligos gives a higher recombination frequency than the other. The oligo identical in sequence to an Okazaki fragment, which corresponds in sequence to the discontinuously replicated lagging strand, displays a higher recombinant frequency than the oligo corresponding in sequence to that replicated as the leading strand (Ellis et al., 2001). It has also been previously demonstrated (Costantino and Court, 2003) that eliminating the host methyl-directed mismatch repair (MMR) system increases the frequency of point mutations made by ss-oligo recombineering by as much as 100-fold. An oligo that creates a C–C mismatch with the target generates high numbers of recombinants, even in the presence of mismatch repair (Costantino and Court, 2003) because the C–C mismatch is not recognized and repaired by the mismatch repair system (Modrich, 1991).

Plasmids are widely used, and thus, it is important to thoroughly characterize plasmid engineering mediated by recombineering. Recombineering of multicopy plasmids has complexities not associated with single copy replicons such as the bacterial chromosome, BACs, or PACs. Almost all recombineering studies have targeted these single copy replicons, although it was demonstrated very early that multicopy plasmids could also be targeted. Zhang et al. (1998) inserted a linear *neo* cassette onto a circular high copy plasmid by RecET recombination. Yu et al. (2000) modified pBR322 by insertion of a chloramphenicol resistance (*cat*) gene and observed multimeric products. Other investigators have utilized recombineering to create plasmid constructs (Yosef et al., 2004; Vetcher et al., 2004; Bartra et al., 2006) and have commented on either problems of multimer formation or mixtures of recombinant and parental plasmids. The purpose of this paper is to further investigate the issues associated with multicopy plasmids. Hence, we have generated several modifications of a pUC-plasmid derivative, using λ Red-mediated recombination: the modifications include repairing an amber point mutation with lagging and leading strand oligos, inserting a 1.2 kb heterologous drug resistance cassette supplied as a PCR product, and removing that same drug cassette by lagging and leading strand oligo recombination. In most experiments both the plasmid and modifying linear DNA are introduced by co-electroporation rather than targeting a resident plasmid (Zhang et al., 1998; Yu et al., 2000). As anticipated from previous work, frequencies of recombineering are similar to those observed when the *E. coli* chromosome is targeted and are independent of the host RecA protein. Circular multimers of the plasmid often form and depend on the presence of input linear DNA and homologous recombination catalyzed by Red Beta. Multimerization apparently arises during the recombineering reaction, although not all recombinant plasmids are multimers. λ Gam-dependent linear multimeric plasmids seen by others (Cohen and Clark, 1986) are not formed with our standard recombineering conditions. Even after selection for drug resistant recombinants, modified plasmids coexist in the same cells with the parental genotype, from which they must be separated.

2. Materials and methods

2.1. Bacterial strains, oligonucleotides, and plasmids

The *E. coli* strains used in this study are listed in Table 1. Oligonucleotides (Table 2) were from Integrated DNA Technologies and supplied as salt free but otherwise unpurified. Oligos for ssDNA recombineering were approximately 70 nt long with the mismatched base centrally located. The plasmid pJS516 (from J. Strathern) is a pUC-derivative bearing the *bla* gene encoding ampicillin resistance (Amp^R) and a defective kanamycin resistance (Kan^R) gene containing an ochre mutation at codon 34 which converted the original serine TCA codon to a stop codon (TAA). Plasmid pJS516 was modified serially with two oligos by recombineering, first repairing the ochre mutation at codon position 34 in the *kan* gene to the wild type (wt) serine codon with oligo LT220, creating pLT59, and from pLT59 creating an amber (TAG) mutation at codon 39 with oligo LT219 to give pLT60 (Fig. 1). The *cat* cassette was as described in Yu et al. (2000) and primers LT232 and LT233 were used for its amplification. Plasmid DNAs were isolated with Qiagen mini or midi prep kits. The plasmid DNA concentration was determined by reading the absorbance at 260 nm assuming A_{260} of 1 equivalent to 50 lg/ml. Agarose gel electrophoresis of plasmid DNAs was in TAE buffer, routinely using 0.7% agarose (Sambrook and Russell, 2001). Monomeric plasmid species have been used for all experiments. They were isolated and maintained by transformation into the *recA* mutant strain DH5a.

Table 1
Bacterial strains and plasmids

Strain	Genotype	Source
DH5a	F , u80d*lacZ*DM15, D(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk , mk$^+$), *phoA*, *supE*44, k , *thi*-1, *gyrA*96, *relA*1	Invitrogen
HME6	W3110 D(*argF-lac*)*U169 galK*$_{tyr145UAG}$ {kcI*857* D(*cro-bioA*)}	Ellis et al. (2001)
HME43	W3110 D(*argF-lac*)*U169 galK*$_{tyr145UAG}$ {kcI*857* D(*cro-bioA*) D<>(*N-gam*) D(*exo-int*)<>*cat*}	Ellis et al. (2001)
HME51	W3110 D(*argF-lac*)*U169 galK*$_{tyr145UAG}$ D(*srlA-recA*)::*Tn*10 {kcI*857* D(*cro-bioA* D<>(*N-gam*) D(*exo-int*)<>*cat*)}	Costantino and Court (2003)
HME57	W3110 D(*argF-lac*)*U169 galK*$_{tyr145UAG}$	Datta et al. (2006)
HME68	W3110 D(*argF-lac*)*U169 galK*$_{tyr145UAG}$ *mutS*<>*cat* {kcI*857* D(*cro-bioA*)}	This work
HME71	W3110 D(*argF-lac*)*U169 galK*$_{tyr145UAG}$ D(*srlA-recA*)::*Tn*10 {kcI*857* D(*cro-bioA*)}	Costantino and Court (2003)
HME70	W3110 D(*argF-lac*)*U169 galK*$_{tyr145UAG}$ *mutS*<>*cat* D(*srlA-recA*)::*Tn*10 {kcI*857* D(*cro-bioA*)}	This work

Table 2
Primer sequences

Primer	Sequence (5^0 to 3^0)
Designation	
LT22	GAGTATTCAACATTTCCGTGTCG
LT23	CAATGCTTAATCAGTGAGGCAC
LT213	AACAACTCTGGCGCATCGGGCTTCCCATACAATCG**G**TAGATTGTGGCGCCTGATTGCCCGACATTATCACG
LT217	CGTGATAATGTCGGGCAATCAGGCGCCACAATCT**A**CCGATTGTATGGGAAGCCCGATGCGCCAGAGTTGTT
LT219	CGTGATAATGTCGGGCAATCAGGCGCCACAATCTA**G**CGATTGTATGGGAAGCCCGATGCGCCAGAGTTGTT
LT220	GGGTATAAATGGGCTCGTGATAATGTCGGGCAAT**C**AGGCGCCACAATCTACCGATTGTATGGGAAGCCCG
LT232	GGTATAAATGGGCTCGTGATAATGTCGGGCAATCAGGCGCCACAATCTAC**TGTGACGGAAGATCACTTCG**
LT233	TTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACAATC**ACCAGCAATAGACATAAGCG**
LT275	CGTGATAATGTCGGGCAATCAGGCGCCACAATCTA**T**CGATTGTATGGGAAGCCCGATGCGCCAGAGTTGTT

For oligos used in ssDNA recombineering, the modifying base is underlined and in bold type. For oligos used in dsDNA recombineering, the portion of the hybrid primer used to amplify the *cat* drug cassette is similarly indicated.

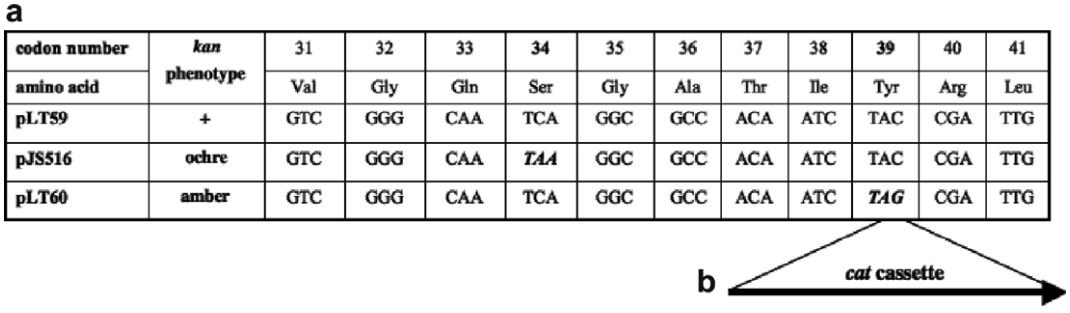

Fig. 1. Sequence modifications introduced to the plasmid-borne kanamycin resistance gene with recombineering. (a) The DNA and amino acid sequences of the *kan* gene in the vicinity of the modified codons are diagrammed. The italicized, bold triplets encode stop codons. To create pLT59, the central A of the ochre stop triplet at codon 34 in pJS516 was changed to a C with the lagging-strand oligo LT220. To create pLT60 from pLT59, the C in the wobble position of codon 39 was changed to a G with oligo LT219, creating an amber mutation that was restored to *wt* with several oligos as described in the text. (b) During recombineering with dsDNA, the *cat* cassette is inserted between the 2nd base of codon 39 and the first base of codon 40, removing the wobble base of codon 39 (not to scale).

2.2. Recombineering methodology

Recombineering was done according to established procedures (Yu et al., 2000; Ellis et al., 2001; Thomason et al., 2005a) with some modifications. (The methods described in this paper will be appended to the latter reference, which is updated quarterly). Briefly, strains containing the defective k prophage with the Red genes expressed from the p_L promoter under control of the *c*I857 repressor, were grown at 32 C in a shaking H_2O bath to mid-log ($A_{600\ nm}$ 0.4–0.6). The cultures were shifted to 42 C for 15 min to induce the Red functions, then quick-chilled in an ice water slurry and the cells washed with sterile ice cold water in preparation for

electroporation. For transformations, monomeric plasmid DNA (ranging between 0.1 and 100 ng) was co-electroporated with 5 pmol ss-oligo or 100 ng purified PCR product. Unless otherwise noted, the plasmid and the modifying linear DNA, either oligo or PCR product, were both introduced by electroporation into bacteria expressing the phage k Red recombination functions, and the cells were outgrown in 1 ml L broth at 30 C for at least 2 h before dilution and plating. The electroporation mixes were plated on L-plates to determine viable cell counts, on L-Amp plates (100 lg/ml) to quantitate plasmid transformants, and on L-Kan (30 lg/ml) or L-Cam (10 or 30 lg/ml) to determine recombinant frequencies. For deletion formation, the same lagging and leading oligos used to repair the amber mutation were used.

2.3. Examining plasmid topology

In order to examine the topology of the products of recombineering, monomeric plasmid and modifying linear substrate DNAs were first introduced by co-electroporation and recombinants selected by drug resistance. Individual colonies were purified on drug plates, overnight cultures were grown, plasmid DNA was isolated and the supercoiled products screened by agarose gel electrophoresis (Sambrook and Russell, 2001). At least 10 isolates of each recombinant class were examined. As controls, the topology of plasmids introduced into cells either induced or repressed for the Red system in the absence of modifying linear DNA was also examined. The partial restriction digestion series were according to Sambrook and Russell (2001).

2.4. Southern analysis

For Southern analysis, total genomic and plasmid DNA was isolated from cells as described by Garcia and Molineux (1995). DNA was resuspended in 10 mM Tris and the DNA concentration determined. Three microgram DNA was digested with restriction enzyme *Kpn*I, which degrades the *E. coli* DNA but does not cut the plasmids used here. Following digestion, 1/10 of the each digest was run on a 0.7% TAE agarose gel to confirm adequate cutting. The remainder of each digest was then run on a 0.7% TAE agarose gel at 5 V/cm, the gel was visualized with ethidium bromide staining, then treated according to conditions described for the digoxigenin (DIG) system of Roche. The *bla* gene was labeled with DIG during PCR amplification with primers LT22 and LT23 and used as a probe.

3. Results

3.1. Description of the system

We have studied recombineering targeted to a multicopy pUC-based plasmid replicating in *E. coli* strains containing a defective k prophage. Our model plasmid, pLT60, has a wild type b-lactamase gene (*amp*), a kanamycin gene (*kan*) carrying an amber mutation, $kan_{tyr39UAG}$, lacks copy number control and is present at 500–700 copies/cell (Sambrook and Russell, 2001). pLT60 was targeted in several different recombineering reactions and the recombinant frequency assessed for each.

The presence or absence of the *E. coli* RecA protein had a minor effect, if any, on recombinant plasmid frequency. Since RecA-dependent plasmid recombination complicates the analysis and isolation of recombinant monomer clones, most of the experiments reported here were performed in a *recA* mutant host unless otherwise indicated.

To examine recombineering in the absence of mismatch repair, either mismatch repair was eliminated by mutation of *mutS*; or the oligo was designed so that, when bound to the target, it created a C–C mismatch that is not subject to mismatch repair even in the presence of an active MMR system (Costantino and Court, 2003). Both methods gave similar results. Plasmid recombinant frequencies are very similar to those observed when the *E. coli* chromosome is targeted (Thomason et al., 2005b), being the highest for lagging-strand oligo recombination occurring in the absence of mismatch repair (Table 3).

Similar frequencies of recombination were obtained whether the plasmid was co-electroporated with the linear substrate DNA or was already established in the cell before linear substrate DNAs were added (data not shown). Since introduction of the plasmid at the time of electroporation has several advantages (see Section 4), the results presented here were obtained by co-electroporation of the plasmid with the linear DNA substrate unless otherwise indicated.

3.2. Recombineering with ssDNA: repair of a point mutation

To repair the $kan_{tyr39UAG}$ point mutation to Kan^+ with recombineering, plasmid pLT60 and one of several ssDNA oligos (LT213, LT217 or LT275) were co-electroporated into competent host cells induced for all three Red functions or into a host strain induced for only Red Beta. The results are presented in Table 3. For recombination in the absence of mismatch repair with a lagging-strand oligo, nearly 60% of the Amp^R colonies were also Kan^R. When mismatch repair was allowed, the

Table 3
Recombinant frequencies for repair of a point mutation on plasmid with oligo recombineering[a]

Strain number	Relevant genotype	Recombineering reaction		
		Repair *kan-am* with lagging-strand oligo LT275 (T:C mismatch)	Repair *kan-am* with leading strand oligo LT213 (G:G mismatch)	Repair *kan-am* with lagging-strand oligo LT217 (C:C mismatch)
HME6	*a*$^+$*b*$^+$*c*$^+$	0.46	0.15	57
HME71	*a*$^+$*b*$^+$*c*$^+$*DrecA*	0.19	0.057	29
HME43	*b*$^+$	0.065	0.009	26
HME51	*b*$^+$ *DrecA*	0.10	0.015	38
HME68	*a*$^+$*b*$^+$*c*$^+$ *mutS<>cat*	63	21	76*
HME70	*a*$^+$*b*$^+$*c*$^+$ *DrecA mutS<>cat*	34	6	49

[a] Expressed as the percentage of Amp^R cells. Recombinants were either selected directly on L-Kan (30 lg/ml) plates or, for experiments in the absence of mismatch repair, screened by testing colonies isolated on L-Amp (100 lg/ml) for growth on L-Kan (30 lg/ml). The number of replicates for each experiment ranged from 3 and 16 with an average s.d. of 42%, with the exception of the value indicated by the asterisk, which was determined only once.

recombinant frequency was about 100-fold lower, with the absolute frequency depending on the efficiency of the MMR system in recognizing the mispaired bases and whether a lagging- or leading-strand oligo was used. Of the Red proteins, only the Red Beta single-strand annealing function is required to catalyze high efficiency oligo recombination (see Table 3, HME43 and HME51).

3.3. Recombineering with dsDNA: insertion of a heterology

The plasmid pLT61 was made from pLT60 with recombineering by insertion of a chloramphenicol resistance gene (*cat*) with its promoter and terminator between the second base of codon 39 and the first base of codon 40, resulting in the removal of the wobble base at codon 39. Plasmid targeting with the linear dsDNA gave similar recombinant frequencies to that observed when targeting the *E. coli* chromosome in a RecA$^+$ host at *galK* with this cassette, $2.5 \cdot 10^4/10^8$ (Yu et al., 2000), as assayed by selection for Cm^R colonies (see Table 4). Since large heterologies are not recognized by the mismatch repair system, strains lacking the MMR system were not assayed.

3.4. Recombineering with ssDNA: creation of a deletion

The chloramphenicol resistance gene, *cat*, a 1.2 kb DNA segment that was inserted by recombineering between two adjacent bases flanking the site of the $kan_{tyr39UAG}$ allele (as described in Section 3.3), was deleted using the lagging- and leading-strand oligos, LT275 and LT213, respectively. Thus,

Table 4
Insertion of a dsDNA drug cassette (*cat* gene) into plasmid with recombineering[a]

Strain Number	Relevant genotype	Recombineering reaction
		Insert dsDNA (*kan<>cat*) PCR product
HME6	*a*$^+$*b*$^+$*c*$^+$	$1.8 \cdot 10^5$
HME71	*a*$^+$*b*$^+$*c*$^+$ *DrecA*	$7.2 \cdot 10^4$

[a] Per 10^8 Amp^R cells. Recombinants selected on L-Cm (10 lg/ml) plates. The number of replicates for each experiment was between 3 and 5 with an average s.d. of 68%.

to obtain Kan^R in this reaction, the *cat* cassette must be removed and the correct base encoded by the oligo must be inserted at the wobble position of codon 39 (see Fig. 1). This reaction was performed in *recA* mutant strains either expressing all three Red genes *exo*, *bet*, and *gam* or expressing only the Red *bet* gene. Deletion of a large DNA segment is not affected by mismatch repair (Costantino and Court, 2003; Parker and Marinus, 1992; and our unpublished data) and the lagging–leading differential is apparent in this reaction, with the lagging-strand oligo frequency 10- to 30-fold higher than that of the leading-strand oligo (Table 5). Both reactions were substantially lower in efficiency than the corresponding frequency observed for repair of a point mutation with these oligos in the absence of mismatch repair (compare Tables 3 and 5).

3.5. Dependence of recombinant formation on plasmid DNA concentration

We assayed the variation in recombinant frequency as a function of input plasmid concentration in the *recA* mutant MMR proficient strain HME71,

Table 5
Recombinant frequencies for creating a large deletion on a plasmid with oligo recombineering[a]

Strain number	Relevant genotype	Recombineering reaction	
		Removal of cat gene with lagging-strand oligo LT275	Removal of cat gene with leading strand oligo LT213
HME6	$a^+b^+c^+$	$5.8 \cdot 10^5$	$2.0 \cdot 10^4$
HME71	$a^+b^+c^+DrecA$	$2.1 \cdot 10^5$	$1.8 \cdot 10^4$
HME43	b^+	$5.0 \cdot 10^5$	$2.0 \cdot 10^4$
HME51	b^+ $DrecA$	$6.2 \cdot 10^5$	$2.7 \cdot 10^4$

[a] Per 10^8 Amp^R cells. Recombinants were selected on L-Kan (30 lg/ml) plates. The number of replicates for each experiment ranged between 3 and 9 with an average s.d. of 60%.

while holding the lagging-strand substrate DNA oligo (LT275) constant at a saturating level of 5 pmol (approximately 3000 molecules/cell). The results are presented in Fig. 2. The recombinant frequency, normalized per 10^8 cells transformed with the plasmid (i.e. Amp^R), was approximately constant over the range tested, from 0.1 to 100 ng. The absolute number of recombinants is not constant, however, since it varies as the number of cells transformed with the plasmid varies. For our conditions,

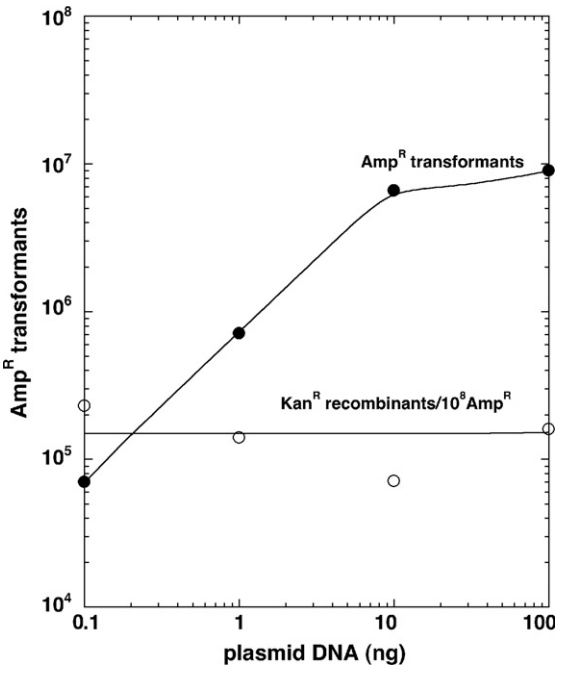

Fig. 2. Transformation efficiency and recombination frequency graphed as a function of amount of plasmid DNA added. Strain HME71 was used as host for recombineering with plasmid pLT60 and oligo LT275. The amount of plasmid DNA introduced was varied from 0.1 to 100 ng while the oligo concentration was held constant at 5 pmol. As can be seen from the graph, the yield of Amp^R transformants is nearly maximal at 10 ng plasmid DNA. The frequency of Kan^R recombinants does not vary appreciably over the range assayed.

10 ng of this plasmid DNA per electroporation (10 molecules/cell) provides maximal recombination.

3.6. Topology of recombinant plasmids

Recombinant plasmids were observed directly by visualization of their differential migration during agarose gel electrophoresis. Repair of the $kan_{tyr39\text{-}UAG}$ allele does not change the size of the plasmid, however, for both insertion of the *cat* cassette and its removal with an oligo, the recombinant products differ in size from the parental plasmid, and thus are distinguishable by size in an agarose gel. Following recombineering and selection on drug plates for plasmid recombinants, colonies were purified on drug plates and used to inoculate cultures for plasmid DNA preparation (see Section 2). When these plasmid DNAs were analyzed by agarose gel electrophoresis, it was observed that for all types of recombination reactions monitored, higher molecular weight plasmid species had been formed. In some individual isolates, a plasmid multimer was the predominant species, but even when the predominant species was a monomer, faint higher molecular weight bands were visible (Fig. 3a). Table 6 presents the relative percentages of monomeric vs. multimeric plasmid species summarized from various experiments. Control experiments demonstrated that circular monomeric plasmids in *recA* mutant cells remained monomeric in the absence of added modifying linear DNA (Fig. 3b). This was true whether such plasmids were already resident or were introduced by electroporation, and whether or not the Red system was induced. The faint higher molecular weight bands were not observed in these control experiments. However, when the Red system was induced and linear modifying DNA, either oligo or PCR product, was added, circular multimers were observed. These species are not

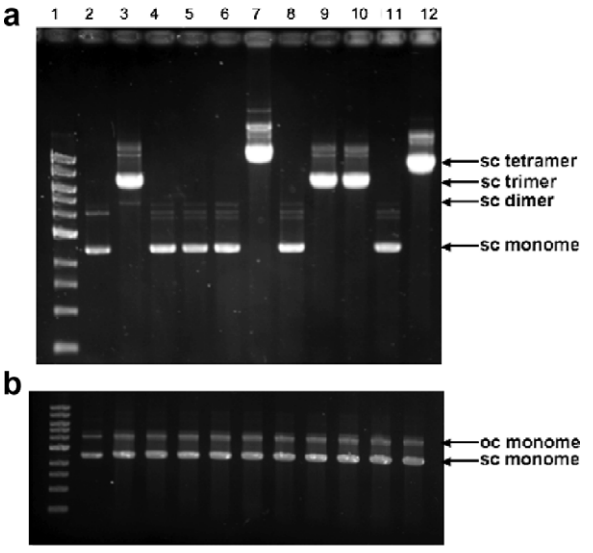

Fig. 3. Representative plasmid species from recombineering reactions, visualized by agarose gel electrophoresis. (a) Lane 1: NEB 1 kb ladder, lane 2: pLT60 plasmid DNA used in the experiment. The supercoiled monomeric species is indicated by the arrow; the other faint band in the lane is open circular monomer. Lanes 3–12: plasmid DNA was isolated from Amp^R Kan^R candidates from a standard recombineering experiment where both pLT60 and oligo LT217 were introduced by co-electroporation into HME71 that had been induced for the Red system for 15 min at 42 C. Lanes 4–6, 8, and 11 contain plasmids that are recombinant and largely monomeric, although each of these lanes contains three faint bands that migrate at the positions expected for linear monomer, open circular monomer, and supercoiled dimer (from bottom to top). The plasmids in lanes 3, 9, and 10 migrate as expected for a supercoiled trimer, that in lane 12 migrates as predicted for a supercoiled tetramer, and the species in lane 7 may be a pentamer. Although none of the plasmid preparations analyzed in this experiment was predominantly dimeric, we did observe such dimers frequently in other experiments. (b) Lane 1: NEB 1kb ladder, lane 2: pLT60 plasmid DNA used in the experiment, lanes 3–12: ten Amp^R plasmids isolated from a control experiment where pLT60 was introduced by electroporation into HME71 that had been induced for the Red system for 15 min at 42 C, in the absence of added linear DNA substrate.

dependent on *gam* expression since they formed whether the bacterial host expressed the complete Red system (Exo, Beta, and Gam) or only Red Beta. That these multimeric species are circular was confirmed by a series of partial restriction digests (data not shown), allowing identification of supercoiled, nicked, and linear species. The circular multimers observed included dimers, trimers, and tetramers; other higher order multimers may be formed as well, although we did not identify them unequivocally.

Table 6
Plasmid topology studies: percentage monomeric plasmids obtained from different recombineering experiments

Reaction relevant genotype	No recombination		Among recombinant clones			
	Amp^R transformants uninduced for Red system	Amp^R transformants induced for Red system	Repair point mutation with leading strand oligo LT 213	Repair point mutation with lagging-strand oligo LT275 or LT217	Create deletion with leading strand oligo LT 213	Create deletion with lagging-strand oligo LT275 or LT217
DrecA	10/10	10/10	56/96	90/126	26/50	39/56
$a^+b^+c^+$	100%	100%	58%	71%	52%	70%
DrecA	10/10	10/10	12/20	22/30	22/30	38/40
b^+	100%	100%	60%	73%	73%	95%
DrecA	*	*	1/10	2/10	2/10	4/10
$a^+b^+c^+$ with resident plasmid	100%	100%	10%	20%	20%	40%

The data are a compilation of a number of individual experiments. Each value represents a single plasmid mini-prep that was analyzed by gel agarose electrophoresis as described in the text. DNA was introduced by co-electroporation unless otherwise indicated.
* These data were determined by Southern analysis of a population.

We routinely observed multimeric recombinant plasmids that had been modified at only one of the potential targets in the multimer. Multimer formation was not obligatory for recombinant formation, however, since we were able to isolate monomeric recombinant plasmids from *recA* mutant strains following recombineering. When the plasmid DNA isolated from a single mini-prep appeared mostly monomeric on an agarose gel, pure recombinant monomeric plasmids were obtained in the following manner: first this DNA was introduced into a *recA* mutant host at a ratio of less than one molecule/cell (0.1 ng DNA/electroporation), then Kan^R colonies were selected and purified. The purified colonies were used to inoculate overnight cultures, and DNA isolated from such cultures was screened on agarose gels to verify a monomer species. To obtain a recombinant monomeric plasmid from the circular multimers that had been modified at only one allele, the DNA was digested with a restriction enzyme that cuts once per unit length plasmid molecule, diluted and ligated under conditions that favor intramolecular ligation. The ligation mix was introduced into a *recA* mutant host, selecting for Kan^R, enabling isolation of pure recombinant monomer species.

3.7. Formation of linear multimeric plasmids

Since k *gam* expression can cause plasmids to enter rolling circle replication by inhibiting the RecBCD exonuclease (Cohen and Clark, 1986; Silberstein and Cohen, 1987) we looked for Gam-dependent linear multimers of the plasmid using the method of Cohen and Clark (1986). With our standard 15-min k induction conditions for recombineering, we saw no evidence of Gam-induced high molecular weight linear multimers by Southern analysis (data not shown). Yet when cultures containing a resident plasmid were incubated at 42 C for extended periods (P1 h), high molecular weight forms that migrated near the wells were observed (data not shown). Thus, if the complete Red system, including *gam*, is expressed for long time periods, large linear forms of the plasmid will arise, complicating isolation of recombinant plasmids.

3.8. Isolation of recombinant plasmids without selection

In the work described here, drug resistance was used to select recombinant plasmid molecules. In many instances, however, a desired genetic modification such as a point mutation will not have a selectable phenotype. Therefore we determined the frequency of isolation of unselected recombinants for the highest efficiency recombination reaction: repair of a point mutation with a lagging-strand oligo in the absence of mismatch repair. (Similar results were obtained whichever means of eliminating mismatch repair was employed). After co-electroporation of either 1 or 10 ng of plasmid DNA with 5 pmol oligo, the 1 ml electroporation mix was allowed a 2-h recovery, then were diluted to 10 ml and grown overnight at 30 C. Plasmid DNA was isolated, diluted to either 1 or 10 ng/ll, and 1 ll of each dilution was introduced into HME71 by electroporation (1 and 10 molecules per cell, respectively). After a 2-h recovery the cultures were assayed for total transformants on L-Amp100 and for KanR recombinants on L-Kan30 plates. The Amp^R colonies were also tested for resistance to kanamycin by patching to L-Kan30 plates. In these experiments the frequency of unselected Kan^R recombinants ranged from 2.7 to 9.0% ($<x> = 5.3\%$) when the cells were plated directly for Kan^R, and from 2–15% ($<x> = 8.5\%$) when established Amp^R colonies were sampled for kanamycin resistance. These values are lower than the 50% recombinants observed when drug resistance was selected (Table 3) because those drug resistant colonies contained a mixture of parental and recombinant plasmids. In a separate experiment a mixture of two oligos was added: one that repairs the mutant *kan* allele to Kan^+, and a second oligo that creates an amber mutation in the *bla* gene. The frequency of Amp among Kan^+ colonies was found to be 8%. Although these experiments allowed scoring of drug resistance, non-selected recombinants could also be found by differential behavior on indicator plates, such as Mac-lac or Mac-gal, by alteration of a restriction site, or by direct sequencing.

4. Discussion

These experiments confirm the early obervations of others (Zhang et al., 1998; Yu et al., 2000): recombineering can be as useful a tool for modifying plasmid replicons as it is for BACs (Lee et al., 2001; Muyrers et al., 1999), the bacterial chromosome (Yu et al., 2000; Ellis et al., 2001; Costantino and Court, 2003; Thomason et al., 2005b), and phage k itself (Oppenheim et al., 2004), but there are complexities associated with multicopy plasmids

that do not arise with the other replicons. ssDNA recombineering in the absence of mismatch repair targeted to plasmids is of such high efficiency that it can be used to isolate point mutations or changes of only a few bases without selection. When a point mutation was created with a lagging-strand oligo in the absence of mismatch repair, we recovered unselected recombinants at a frequency of 5–10%. The recombinant frequencies we obtained were comparable to those observed on the *E. coli* chromosome (Thomason et al., 2005b). Insertion of dsDNA occurs with lower efficiency, and in some cases (i.e. a simple DNA insertion or deletion where convenient restriction sites exist) recombineering may provide no advantage over classical in vitro cloning. Although the experiments described here were performed with a ColE1 replicon in W3110 derivatives, our preliminary results indicate that the technique will be generally applicable to other plasmids and *E. coli* strains expressing Red. We obtained similar recombination frequencies to those shown here for all the reactions using the host strain DY380, which contains the λ prophage in a DH10B background, and when a plasmid with a pBBR1 replication origin (Antoine and Locht, 1992) was targeted (data not shown).

In the experiments described here, the plasmids were usually introduced into the cell at the time of electroporation of the substrate DNA. Although similar recombineering frequencies were obtained with both co-electroporation and modification of a plasmid already resident in the cell, co-electroporation has definite advantages (Zhang et al., 1998; Yu et al., 2000). It provides an internal control for transformation efficiency and allows introduction of a low number of plasmids per cell. It reduces the frequency of multimer formation caused by the recombination (Table 6) and minimizes time of exposure of the plasmid to the Gam-inhibited RecBCD protein, thus minimizing the opportunity for rolling circle replication of the plasmid. If the recombineering is done in a MMR deficient host, co-electroporation also minimizes plasmid exposure to a potentially mutagenic environment.

Unlike the situation for single copy replicons, such as BACs, PACs, and the *E. coli* chromosome, the multicopy nature of plasmids complicates isolation of pure recombinants. This difficulty is illustrated in the context of our model for the recombineering reaction, which proposes that the linear substrate DNA is annealed by the λ Beta protein to a single-stranded region generated at the replication fork during DNA replication. Once the oligo is annealed and ligated into one strand, a heteroduplex plasmid molecule has been formed. A single round of DNA replication will separate this heteroduplex into two populations of parental and recombinant genotypes. Each type will undergo further rounds of replication. Thus, if one plasmid molecule is introduced into a single cell and that molecule is then modified with recombineering, at best the number of recombinants will be only 50% of the population in a single cell. With multicopy plasmids, only a few of the plasmid molecules resident in a cell are likely to be modified during a recombineering event. The frequency of lagging-strand ss-oligo recombineering in the absence of mismatch repair is so high, however, that it is straightforward to identify colonies that contain mixtures of recombinant and parental plasmids and purify the desired recombinant plasmid away from the contaminating parental DNA. If a different genetic alteration is needed, for example insertion of a heterologous DNA segment, but no selection can be applied, other means of identifying the recombinant exist. For example, the colonies containing recombinant plasmids can often be found by colony hybridization, or if the modification removes a unique restriction site the parental DNA can be eliminated by restriction digestion, enriching for the recombinant species.

Another issue specific to plasmid recombineering is the formation of circular multimeric plasmids. One source of such multimers is RecA-dependent recombination (James et al., 1982); this class can be eliminated through the use of a *recA* mutant host. The work presented here demonstrates that circular multimers are a major product of Red recombination acting in the absence of RecA, using either double- or single-stranded linear substrate DNA. Although extended expression of the Gam protein can give rise to linear multimeric plasmids (Cohen and Clark, 1986; and our unpublished results) the circular multimeric forms we have observed during recombineering are formed in the absence of Gam and minimally require only Beta expression and linear substrate ssDNA. It is possible that both circular and linear multimeric forms arise from a similar recombination-dependent mechanism. In practical terms, when targeting plasmids with recombineering, it is important to screen recombinant plasmids by gel electrophoresis to determine their multimeric state. The fact that successfully modified plasmid molecules are often

multimers can actually be helpful in identifying recombinants. Recombinant multimeric plasmids are often modified on only one copy of the targeted region.

In summary, to optimize multicopy plasmid recombineering and recombinant isolation, a monomeric plasmid to be modified should be introduced into *recA* mutant cells expressing the Red system by co-electroporation with the modifying DNA. A low plasmid DNA to cell ratio should be used; this is best determined empirically by titration. Recombinant colonies, once identified, are amplified by growth and plasmid DNA prepared. This plasmid DNA, which will be a mixed population, should be again introduced into a *recA* mutant strain at a low DNA concentration of less than one molecule per cell, once again selecting or screening for the desired modification. At this step the plasmid may be a multimer containing both parental and recombinant alleles. If a recombinant plasmid has multimerized, the DNA can be digested, religated under dilute conditions, and then introduced into a *recA* mutant host in order to obtain a recombinant monomer clone.

This work has addressed problems initially identified by others (Yu et al., 2000; Yosef et al., 2004; Vetcher et al., 2004) that needed to be resolved to adapt the powerful technique of recombineering, *in vivo* genetic engineering, to allow targeting of plasmids. When targeting plasmids, one should be aware of the possible ramifications of both the multicopy nature of plasmids and the circular multimers arising among recombinant products. The steps detailed herein can readily deal with these issues, allowing genetic modification of plasmids by recombineering with the same high efficiency observed on the *E. coli* chromosome.

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