

# A set of recombineering plasmids for gram-negative bacteria

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## Abstract

We have constructed a set of plasmids that can be used to express recombineering functions in some gram-negative bacteria, thereby facilitating in vivo genetic manipulations. These plasmids include an origin of replication and a segment of the bacteriophage  $\lambda$  genome comprising the *red* genes (*exo*, *bet* and *gam*) under their native control. These constructs do not require the anti-termination event normally required for Red expression, making their application more likely in divergent species. Some of the plasmids have temperature-sensitive replicons to simplify curing. In creating these vectors we developed two useful recombineering applications. Any gene linked to a drug marker can be retrieved by gap-repair using only a plasmid origin and target homologies. A plasmid origin of replication can be changed to a different origin by targeted replacement, to potentially alter its copy number and host range. Both these techniques will prove useful for manipulation of plasmids in vivo. Most of the Red plasmid constructs catalyzed efficient recombination in *E. coli* with a low level of uninduced background recombination. These Red plasmids have been successfully tested in *Salmonella*, and we anticipate that they will provide efficient recombination in other related gram-negative bacteria.

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## 1. Introduction

Recombineering is a new means of in vivo genetic engineering that allows DNA modifications to be made easily and efficiently. It is a highly effective method for functional genomic analysis, engineering a wide variety of DNA rearrangements and combining genes with special genetic elements or tags (Copeland et al., 2001; Court et al., 2002). Recombineering allows a researcher to carry out DNA modifications and cloning without restriction enzymes or DNA ligases (Yu et al., 2000; Copeland et al., 2001; Court et al., 2002). The modifying DNA for

recombineering is either a double-stranded (ds) PCR product (Murphy et al., 2000; Yu et al., 2000; Lee et al., 2001; Court et al., 2002) or a single-stranded oligonucleotide (oligo) (Ellis et al., 2001; Swaminathan et al., 2001) carrying short regions of target homology at the ends which can be precisely recombined in vivo with its substrate sequences onto any episome within the cell. Recombination between the short homologies is catalyzed by the  $\lambda$  Red functions, Exo, Beta and Gam. The  $\lambda$  Gam protein prevents degradation of transformed linear dsDNA by the host RecBCD and SbcCD nucleases (Unger and Clark, 1972; Kulkarni and Stahl, 1989) while Exo resects the 5' ends of the dsDNA (Little, 1967) to generate 3' ssDNA overhangs. Beta binds to these ssDNA overhangs, as well as to oligos, ultimately pairing them with a complementary ssDNA target (Karakousis et al., 1998; Li et al., 1998).

Our laboratory uses a defective  $\lambda$  prophage for optimal expression of the Red functions in *E. coli* (Yu et al., 2000; Ellis et al., 2001). This defective prophage contains the phage immunity region and the main leftward operon under control of the  $p_L$  promoter (Fig. 1). The rightward operon encoding the DNA replication genes, lysis cassette, and the structural genes has been

**Abbreviations:** bp, base pair(s); nt, nucleotide(s); PCR, polymerase chain reaction; *ts*, temperature-sensitive; *ori*, origin of replication; ds, double-stranded; ss, single-stranded; Cm<sup>R</sup>, chloramphenicol resistant; Ap<sup>R</sup>, ampicillin resistant; Tc<sup>R</sup>, tetracycline resistant; *cat*, chloramphenicol drug cassette; *amp*, ampicillin drug cassette; BAC, bacterial artificial chromosome.

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Fig. 1. Standard defective  $\lambda$  prophage used for recombineering. The *red* genes *exo*, *bet* and *gam* are expressed from the  $p_L$  promoter under the control of the *ts* CI857 repressor. *N* anti-termination results in the expression of the Red system at 42 °C. Transcription terminators  $t_{L1}$ ,  $t_{L2}$  and  $t_{L3}$  are indicated.

removed by a deletion that extends from *cro* through the right attachment site, *attR* and into the bacterial biotin gene *bioA*. The *exo*, *bet* and *gam* genes are expressed from the  $p_L$  operon under the control of the temperature-sensitive (*ts*) repressor, CI857. At low temperature (30–34 °C) the repressor remains active and blocks the  $p_L$  promoter, shutting off transcription of the *red* genes. A brief temperature shift to 42 °C results in a transient denaturation of the repressor, allowing Red expression. On shifting back to low temperature the repressor renatures, binds to  $p_L$ , and again turns off the Red system. Following heat inactivation of the repressor, the expression of *gam*, *bet* and *exo* are initially prevented by the transcription terminators present between  $p_L$  and the *red* genes unless the *N* anti-termination function modifies RNA polymerase to prevent transcription termination (Gottesman et al., 1980).

Here we report a modification of the prophage strain and derive from it a set of plasmids carrying a minimal Red expression cassette under endogenous  $\lambda$  repressor control. These new vectors can be introduced by transformation to different bacterial backgrounds, and in some of them, a *ts* replication defect provides a means for easy curing from the host. We also report two recombineering applications that are useful for plasmid engineering in general: 1) a technique to clone any gene with a linked drug marker to a multicopy vector by gap-repair and 2) a means to alter the origin of replication (*ori*) of a plasmid, hence changing its copy number and host specificities.

## 2. Materials and methods

### 2.1. Bacterial strains

Unless otherwise specified, strain construction was done using recombineering technology (Yu et al., 2000; Ellis et al., 2001). Strain DY378 is W3110  $\{\lambda$  *cI857* $\Delta$ (*cro-bioA*) $\}$  (Yu et al., 2000). DY406 was constructed by replacing *kil* to *sieB* of  $\lambda$  nucleotide (nt) 33246–35015 (Daniels et al., 1983) in DY378 with a cassette containing both the chloramphenicol (*cat*) and *sacB* genes (Lee et al., 2001). DY432 was constructed from DY406 by replacing the *cat-sacB* cassette plus adjacent  $\lambda$  DNA sequence using oligo recombination to generate a deletion from *N* to *kil* and fusing nt 33169 to 35446 of  $\lambda$ . The loss of the *cat-sacB* cassette makes DY432 resistant to 5% sucrose and sensitive to chloramphenicol.

The *rex* genes downstream of *cI857* in DY432 were replaced by a drug cassette, either *cat* or *amp*, to generate chloramphenicol resistant ( $\text{Cm}^R$ ) SIMD3 or ampicillin resistant ( $\text{Ap}^R$ ) SIMD4. Both the *cat* and *amp* cassettes contain their own promoter region as described by Yu et al. (2000) and Thomason et al. (2005). The cassettes replace the DNA segment from the ATG of *rexA* to the stop codon of *rexB*, deleting  $\lambda$  nt 35828–37114 (Daniels et al., 1983). These drug cassettes are in single copy on the chro-

mosome, thus lowered levels of antibiotic are used for selection (30  $\mu\text{g/ml}$  ampicillin and 10  $\mu\text{g/ml}$  chloramphenicol). These prophage-carrying strains are deleted for *bioA* and require biotin for growth on minimal media.

HME6 is W3110  $\Delta$ (*argF-lac*)*U169 galK*<sub>TYR145UAG</sub>  $\{\lambda$  *cI857* $\Delta$  (*cro-bioA*) $\}$  (Ellis et al., 2001) while HME57 is  $\Delta$ (*argF-lac*)*U169 galK*<sub>TYR145UAG</sub>. P1 transduction was used to create HME57 from HME6 by bringing in the wild-type  $\lambda$  attachment site *attB* and the *bioA* region from W3110, selecting for  $\text{Bio}^+$  recombinants. HME57 thus lacks the  $\lambda$  prophage but retains the *galK* amber mutation.

*Salmonella enterica* serovar Typhimurium LT2 (*S. enterica*) and its derivative TS616 (*his6165 ilv452 metA22 metE551 mal616::Tn10 galE496 xyl404 rpsL120 fla66 hsdL6 hsdSA29*) were obtained from Dr. L. Thomason. The *E. coli* bacterial strain BR825 (*polA<sub>am</sub>* Tn10) having the *resA1* mutation with the Gln codon at position 298 converted to an amber (Kelley and Joyce, 1983) was obtained from the laboratory of Dr. S. Austin.

### 2.2. Amplification of the origin of replication of plasmids

Standard PCR conditions were used to amplify the DNA replication origins of different plasmids using a high fidelity Taq DNA Polymerase. Each primer used for the PCR is a hybrid where the 5' end contains the target homology and the 3' end primes the origin fragment. The *ori* of pBR322, from nt 2348–3296 (GenBank accession no. 208958), was amplified as a 949 bp fragment using primers SD1 and SD2 (Table 1) with pPCR-Script Amp (Stratagene, La Jolla, CA) as template. The 5' end of SD1 has 54 bases of homology to DNA downstream of the stop codon of *exo* including the transcription terminator  $t_{L3}$ , corresponding to nt 31232–31285 of  $\lambda$  (Daniels et al., 1983). SD2 has 41 bases of homology including the  $p_R$  promoter and part of *cro* corresponding to nt 38011–38051 of  $\lambda$  (Daniels et al., 1983).

Likewise, the *ori* of plasmids pSC101 *repA<sup>6s</sup>*, which corresponds to nt 4524–6736 of pSC101 (GenBank accession no. 47833) and pBBR1, nt 800–2517 of pBBR1 (GenBank accession no. X66730) were amplified by PCR using primer-pairs SD3–SD4

Table 1  
Primer pairs used for PCR amplification

Primer pair	PCR product
SD1–SD2	pBR322 <i>ori</i> flanked by $\lambda$ homology
SD3–SD4	pSC101 <i>ori</i> flanked by $\lambda$ homology
SD5–SD6	pBBR1 <i>ori</i> flanked by $\lambda$ homology
SD7–SD8	RK2 <i>ori</i> flanked by $\lambda$ homology
SD9–SD10	<i>amp</i> cassette targeting <i>galK</i> of <i>E. coli</i>
SD11–SD12	<i>amp</i> cassette targeting <i>galK</i> of <i>S. enterica</i>

Sequences are available on request. The primers were obtained from Integrated DNA Technologies as salt-free but otherwise unpurified.

and SD5–SD6, respectively (Table 1). The RK2 replication origin, *oriV*, and a *trfA<sup>ts</sup>* gene were amplified by PCR with primer pair SD7 and SD8 using pRR10-*ts97* as a template, which corresponds to nt 7478–9962 of pRR54, a derivative of pRR10 (GenBank accession no. AY297462, (Roberts et al., 1990). For all PCR amplifications of plasmid origins the 5' ends of each primer contained the same  $\lambda$  sequences. All PCR products were purified with a PCR clean-up kit (Qiagen, Valencia, CA) and introduced into the recombinogenic bacterial cells by electroporation.

### 2.3. Preparation of cells for recombineering

The cultures to be used in recombineering were prepared as described previously (Yu et al., 2003; Thomason et al., 2005). For DNA retrieval by gap-repair, the PCR-amplified linear *ori* fragment of pBR322 was electroporated into SIMD3 or SIMD4 that had been induced 15 min at 42 °C for Red expression and subsequently made electro-competent. Following electroporation the culture was diluted with 10 ml LB and grown non-selectively overnight at 32 °C. Plasmid DNA was then purified and used to transform strain W3110, selecting for Cm<sup>R</sup> or Ap<sup>R</sup> as appropriate. Plasmids (pSIM2, Cm<sup>R</sup> or pSIM4, Ap<sup>R</sup>) from these purified transformants were isolated and checked by restriction analysis to show the presence of the  $\lambda$  genes and pBR322 origin.

To replace the pBR322 *ori* with another origin, W3110 carrying pSIM2 or pSIM4 was induced for Red expression at 42 °C followed by electroporation of PCR product containing the desired plasmid origin and target homologies. After overnight growth of the electroporated culture in 10 ml LB at 32 °C, plasmid DNA was extracted and used to transform the *polA* mutant strain, BR825, selecting for the appropriate drug resistance. Plasmid DNA was again isolated from BR825 and checked by restriction analysis to confirm the loss of the pBR322 origin and the gain of the new origin.

### 2.4. Recombination assays with an oligo and dsDNA

The oligo #144 (Costantino and Court, 2003) used for recombineering corrects the amber TAG stop codon of the *E. coli galk* gene to a TAC tyrosine codon. HME57 was transformed with the different Red plasmids from this study. A saturating level of oligo (5 pmol) was electroporated into these plasmid-containing strains that had been either induced for Red expression by growth at 42 °C for 15 min or that had remained uninduced. After electroporation 1 ml LB was added and cells were incubated at 32 °C for 2 h with shaking. Gal<sup>+</sup> recombinant colonies were selected on M63 minimal galactose plates with biotin, and total viable cells were counted on LB agar (Costantino and Court, 2003).

For recombineering in *S. enterica*, oligo #337 (5'-AAGTGGCGGTGGGCACCGTCTTCCAGCAGCTTTAgtgttCACACCTGCCGCTGGACGGCGCGCAAATTGCGCTCAA) was used to generate a mutation in the *galk* gene. In this oligo the 145th codon of the *Salmonella galk* (nt 19679–20840, GenBank accession no. AE006468) was changed from a tyrosine to a stop codon followed by 4 adjacent base changes, as indicated by nucleotides in lower-case. A correcting oligo #336 (5'-AAGTGGCGGTGGGCACCGTCTTCCAGCAGCTTTAC-

CACCTGCCGCTGGACGGCGCGCAAATTGCGCTCAA) was used to repair this *galk* mutation by restoring the five base changes to the wild-type sequence. *S. enterica* was transformed with a Red expressing plasmid and recombineering with an oligo was done as described above. Gal<sup>-</sup> *Salmonella* recombinants were selected on minimal glycerol plates containing 2-deoxy-galactose (DOG) (Alper and Ames, 1975) while Gal<sup>+</sup> recombinants were selected on M63 minimal galactose plates.

The *amp* cassette used to replace the *galk* gene of *E. coli* or *S. enterica* was amplified from pBluescript SK (+) (Stratagene, La Jolla, CA) with primer-pairs SD9, SD10 and SD11, SD12 respectively (Table 1). Each primer contains two parts: a 5' end homologous to the flanking regions of *galk* of *E. coli* or *Salmonella* and a 3' end that primes the *amp* cassette for PCR amplification. LB agar plates containing 30  $\mu$ g/ml of ampicillin were used to select drug resistant *galk* $\Delta$ *amp* recombinants at 32 °C.

To compare the recombineering efficiencies of Red expression plasmids constructed in this study with other plasmid Red expression systems, the plasmid pKD119 (Datsenko and Wanner, 2000) was obtained from the *E. coli* Genetic Stock Center. It expresses Gam, Beta and Exo from the arabinose-inducible *p<sub>araBAD</sub>* promoter on a pSC101 *repA<sup>ts</sup>* replicon and carries a tetracycline drug marker. 1 mM L-arabinose was used for Red induction as recommended unless otherwise noted (Datsenko and Wanner, 2000).

### 2.5. Plasmid curing

W3110 carrying plasmids with *ts*-replicons (pSIM5 *repA<sup>ts</sup>* or pSIM9 *trfA<sup>ts</sup>*) was grown overnight at 32 °C in LB chloramphenicol (30  $\mu$ g/ml). The cultures were diluted 1000-fold in LB without antibiotic and allowed to grow at 37 °C with aeration. After 2, 4 and 8 h of growth the cultures were diluted and plated on LB-plates for well-isolated colonies. Twenty-five colonies were randomly selected from each time point at different temperatures and tested for chloramphenicol resistance/sensitivity.

## 3. Results

### 3.1. Construction of a minimal Red expression prophage

The minimal prophage was created by removal of the  $\lambda$  N through *kil* genes in the *p<sub>L</sub>* operon and replacement of *rexA* and *rexB* by a drug resistance cassette, either *cat* or *amp* (see Section 2.1). The N through *kil* deletion removes all transcription terminators between *p<sub>L</sub>* and the Red genes and makes Red expression N-independent. In this minimal construct, *p<sub>L</sub>* is regulated by CI857 with operators *O<sub>L</sub>* and *O<sub>R</sub>* ensuring tight repressor control. Raising the temperature to 42 °C inactivates the repressor and directly induces Red expression. We do not expect the removal of these terminators to affect the level of Red expression or recombination in *E. coli*, however, removal of the terminators will eliminate transcription termination and allow expression in other species where the  $\lambda$  N anti-termination system does not function (Baron et al., 1970). The prophage in strain

SIMD3 carries the *cat* cassette while the prophage in SIMD4 contains the *amp* cassette.

### 3.2. Creating Red expression plasmids

Two different in vivo techniques, described below, were used to create a set of Red expression plasmids. These Red expression vectors were constructed by combining the minimal defective  $\lambda$  prophage from either SIMD3 or SIMD4 with various plasmid origins.

#### 3.2.1. Gap-repair method

The replication origin of pBR322 was amplified by PCR to generate a linear *ori* DNA flanked by homology to the prophage. This 949 bp linear fragment contains the minimal plasmid *ori* and lacks both the copy number control region and a drug marker. The fragment was used to retrieve the prophage from SIMD3 and SIMD4 in a gap-repair reaction catalyzed by Red (Fig. 2B). Recombination of the linearized *ori* vector with the prophage target restores a circular plasmid that is able to replicate. Only those plasmid clones that have undergone successful recombineering will also inherit the antibiotic resistance marker from the prophage, allowing selection in the presence of appropriate drug.

Table 2  
Red expression vectors

Plasmid	Origin	Copies/cell	Host range	Drug marker
pSIM2	pBR322	220 <sup>a</sup>	Narrow	Chloramphenicol
pSIM4	pBR322	220 <sup>a</sup>	Narrow	Ampicillin
pSIM5	pSC101	16 <sup>b</sup>	Narrow	Chloramphenicol
pSIM6	pSC101	16 <sup>b</sup>	Narrow	Ampicillin
pSIM7	pBBR1	30–40 <sup>c</sup>	Broad	Chloramphenicol
pSIM8	pBBR1	30–40 <sup>c</sup>	Broad	Ampicillin
pSIM9	RK2	5–8 <sup>d</sup>	Broad	Chloramphenicol

<sup>a</sup> Lin-Chao et al. (1992).

<sup>b</sup> Hashimoto-Gotoh et al. (1981).

<sup>c</sup> Antoine and Locht (1992).

<sup>d</sup> Pogliano et al. (2001).

This method was used to create pSIM2 (Cm<sup>R</sup>) and pSIM4 (Ap<sup>R</sup>) (Table 2).

#### 3.2.2. Exchanging plasmid origins by recombineering

We created a lower copy number Red expression vector with the DNA replication origin of pSC101 by engineering a precise replacement of the pBR322 *ori* segment in pSIM2 and pSIM4 with the *ori* and *repA<sup>ts</sup>* gene of pSC101<sup>ts</sup>. After recombineering and overnight growth at 32 °C in non-selective LB medium,

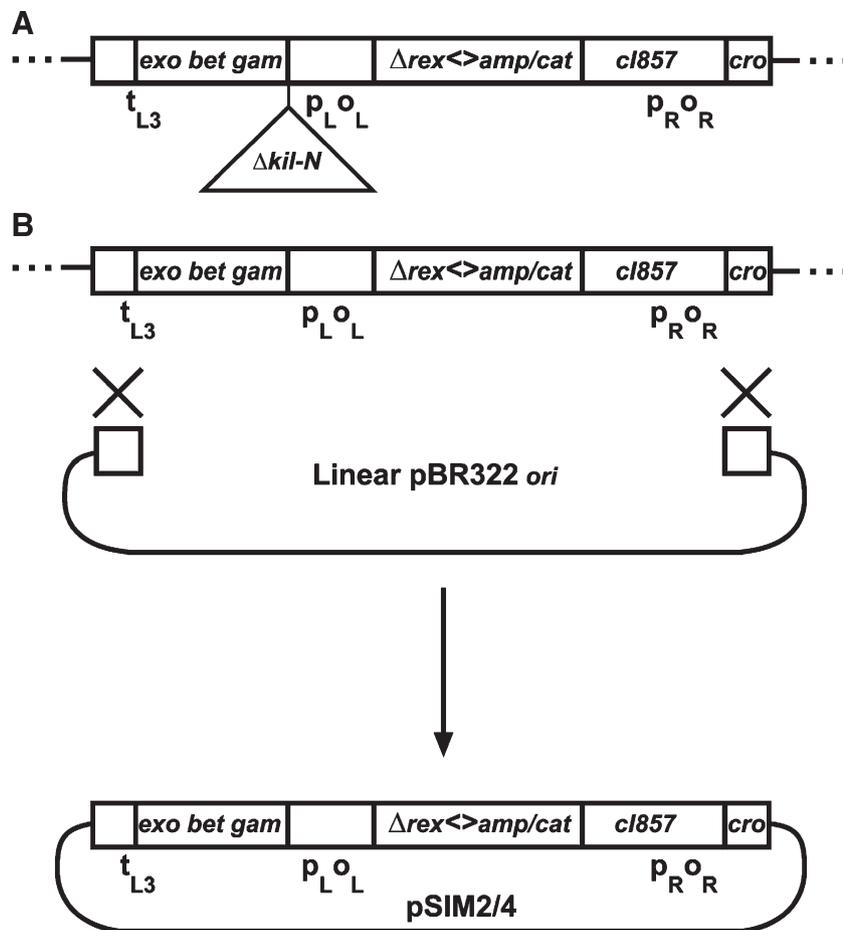


Fig. 2. (A) Minimal defective prophage created by the removal of the *N* through *kil* genes and replacement of *rexA* and *rexB* by a drug cassette (either *amp* or *cat*). Raising the temperature allows the transcription of the  $p_L$  operon directly without *N*-mediated anti-termination. (B) The minimal prophage moved onto a plasmid. The DNA replication origin of pBR322 was PCR amplified and used to clone the minimal prophage by a gap-repair reaction. The linear 949 bp pBR322 fragment contains only the *ori* and lacks any antibiotic resistance gene; only clones undergoing successful recombineering will carry the drug marker inherited from the prophage.

plasmid DNA was isolated and used to transform the *polA* mutant strain, BR825, selecting for the appropriate drug resistance at 32 °C. Since the pBR322 origin does not replicate in a *polA* mutant strain (Kingsbury and Helinski, 1970), only clones in which the pBR322 *ori* was replaced by the pSC101 *ori* replicate, generating pSIM5 and pSIM6 (Table 2). This same technique of selecting for recombinants in a *polA* mutant was also used to replace the pBR322 *ori* segment of pSIM2 and pSIM4 with the broad host range pBBR1 origin, creating pSIM7 and pSIM8, and with the broad host range RK2 *ts* origin, creating pSIM9.

### 3.3. Recombineering with an oligo

We tested the recombination efficiency of the various Red expression plasmids by correcting a *galK* amber mutation in HME57 with oligo #144. Similar experiments were performed with the defective chromosomal prophage strains, HME6 and SIMD3, to compare the recombineering efficiencies of these strains with the Red plasmid systems. All Red plasmids catalyzed recombination efficiently yielding  $\geq 10^7$  Gal<sup>+</sup> recombinants per  $10^8$  viable cells (Table 3). Upon annealing, oligo #144 creates a C:C pairing that is not repaired by the host mismatch repair system and thus gives this very high recombination frequency (Costantino and Court, 2003).

We also used HME57 carrying pKD119 (Datsenko and Wanner, 2000) to carry out similar recombination assays. Using 1 mM arabinose to induce Red expression, Gal<sup>+</sup> recombinants were generated at a frequency of  $2.8 \times 10^5/10^8$  viable cells; this is about 100-fold lower than that obtained using the prophage-expression plasmids (Table 3). HME57 is Ara<sup>+</sup> and will metabolize the arabinose, affecting the induction of *P<sub>paraBAD</sub>*. We found that raising the arabinose concentration to 10 mM increased the frequency of Gal<sup>+</sup> transformants (Table 3) but pKD119 was still less efficient than the prophage regulated systems described here.

### 3.4. Control of Red expression-background recombination

The expression of Gam, Beta and Exo from multicopy plasmids may not be as tightly controlled as in the chromosomal context.

Table 3  
Red-mediated oligo recombineering in *E. coli*

Source of Red	Plasmid origin	Gal <sup>+</sup> recombinants/ $10^8$ viable cells <sup>a</sup>	
		Red induced	Red uninduced
HME6 <sup>b</sup>	NA <sup>c</sup>	$1.5 \times 10^7$	$1.8 \times 10^3$
SIMD3 <sup>b</sup>	NA <sup>c</sup>	$9.0 \times 10^6$	$2.7 \times 10^3$
pSIM2	pBR322	$1.7 \times 10^7$	$1.1 \times 10^6$
pSIM5	pSC101	$1.8 \times 10^7$	$1.5 \times 10^3$
pSIM7	pBBR1	$2.7 \times 10^7$	$1.5 \times 10^4$
pSIM9	RK2	$1.4 \times 10^7$	$1.6 \times 10^3$
pKD119	pSC101	$2.8 \times 10^5$ <sup>d</sup> $4.9 \times 10^6$ <sup>e</sup>	$7.3 \times 10^2$

<sup>a</sup> Values indicated are the average of 3 or more experiments.

<sup>b</sup> See Section 2.1 for complete genotype.

<sup>c</sup> Not applicable.

<sup>d</sup> 1 mM arabinose.

<sup>e</sup> 10 mM arabinose.

Table 4  
Red-mediated dsDNA recombineering using *galK*<→*amp* PCR product

Source of Red	Plasmid origin	Ap <sup>R</sup> recombinants/ $10^8$ viable cells <sup>a</sup>		
		<i>E. coli</i>	<i>S. enterica</i>	
			TS616	LT2
HME6 <sup>b</sup>	NA <sup>c</sup>	$2.5 \times 10^4$	NA <sup>c</sup>	NA <sup>c</sup>
SIMD3 <sup>b</sup>	NA <sup>c</sup>	$1.2 \times 10^4$	NA <sup>c</sup>	NA <sup>c</sup>
pSIM2	pBR322	$2.4 \times 10^4$	ND <sup>d</sup>	ND <sup>d</sup>
pSIM5	pSC101	$2.9 \times 10^4$	$9.0 \times 10^3$	$1.9 \times 10^3$
pSIM7	pBBR1	$2.3 \times 10^4$	$1.0 \times 10^4$	$5.7 \times 10^3$
pSIM9	RK2	$4.3 \times 10^4$	$2.7 \times 10^3$	$1.9 \times 10^3$
pKD119	pSC101	$3.2 \times 10^3$ <sup>e</sup>	$8.5 \times 10^2$ <sup>e</sup>	ND <sup>d</sup>

<sup>a</sup> Values indicated are the average of 3 or more experiments.

<sup>b</sup> See Section 2.1 for complete genotype.

<sup>c</sup> Not applicable.

<sup>d</sup> Not done.

<sup>e</sup> 10 mM arabinose.

Constitutive expression of Gam would inactivate RecBCD, which can affect cell growth and interfere with plasmid replication (Sergueev et al., 2001; Court et al., 2002) while leaky expression of recombination functions leads to unwanted recombination events, especially at repetitive sequences (Murphy and Campellone, 2003). We assayed the oligo recombination in uninduced cells for an indication of background Red expression. In the strains that carry chromosomal prophages, the background recombination was  $2 \times 10^3$  Gal<sup>+</sup> recombinants per  $10^8$  viable cells (Table 3). The pBR322-based plasmid (pSIM2) lacking copy number control gave an unacceptably high number of recombinants ( $1 \times 10^6$ ) in the absence of Red induction. The strain carrying pSIM2 grew abnormally slow even in rich media, which is likely the result of unwanted expression of the Red functions. In strains carrying the lower copy number pSC101-derived pSIM5 and RK2-derived pSIM9, the level of unwanted background recombinations was as low as that observed with the chromosomal prophages, making them ideal candidates for recombineering. pSIM7, having a pBBR1 origin and intermediate copy number, generated an intermediate  $1.5 \times 10^4$  Gal<sup>+</sup> recombinants/ $10^8$  viable cells.

### 3.5. Recombineering with dsDNA

We compared the recombineering efficiency of the various plasmids with linear dsDNA by assaying the frequency of *galK* gene replacement with an *amp* cassette. Ap<sup>R</sup> recombinants were selected at 32 °C and their Gal<sup>-</sup> phenotype was confirmed by streaking on Mac-Gal indicator agar. All Ap<sup>R</sup> colonies tested gave colorless (Gal<sup>-</sup>) colonies as expected. All pSIM expression plasmids yielded more than  $10^4$  Ap<sup>R</sup> recombinants/ $10^8$  viable cells, levels similar to the defective prophages (Table 4). Induction of pKD119 with 10 mM arabinose generated recombinants, but again at a 10-fold lower level than the pSIM plasmids.

### 3.6. Recombineering in *Salmonella*

*S. enterica* was transformed with pSIM5 and a Gal<sup>-</sup> derivative was made by recombineering with oligo #337. This Gal<sup>-</sup> strain was used to test the efficiency of pSIM5 in catalyzing oligo

recombineering in *Salmonella* using oligo #336 to repair the mutation in *galK*, thereby converting the strain back to Gal<sup>+</sup>. On average,  $5 \times 10^6$  Gal<sup>+</sup> recombinants were observed per  $10^8$  viable cells, while the background recombination in uninduced cells was  $4 \times 10^2$  Gal<sup>+</sup> colonies/ $10^8$  cells surviving electroporation.

*S. enterica* and its derivative TS616, transformed with various Red expressing plasmids, were used to study dsDNA recombineering using a PCR-generated *amp* cassette targeted to replace the *S. enterica galK* gene. Ap<sup>R</sup> *Salmonella* recombinants were recovered at a frequency of  $10^3$  per  $10^8$  viable cells (Table 4). *Salmonella* carrying pKD119 again gave about a 10-fold lower frequency of Ap<sup>R</sup> transformants relative to pSIM5.

### 3.7. Curing of plasmids

The *ts*-replicons of pSIM5 and pSIM9 permit curing of plasmids by growing the host strains at 37 °C once the desired recombination is confirmed. W3110 carrying either plasmid was first grown at 32 °C then shifted to 37 °C (see Section 2.5). After 2 h growth at 37 °C pSIM9 was lost from 60% of the colonies screened while pSIM5 was lost from only 4% of the colonies. However, when allowed to grow for 4 h at 37 °C, both pSIM5 and pSIM9 were lost from 92% of the colonies. After 8 h of growth at 37 °C all colonies tested had lost the plasmid. For plasmid curing, it is preferable to use 37 °C as the *p<sub>L</sub>* operon remains mostly repressed by CI857 at this temperature, blocking unwanted Red expression in the host strain.

## 4. Discussion

Here we report the construction of a series of Red expression plasmids, retaining the native  $\lambda$  control, but with a wider host range. Recombineering techniques were used to create these vectors by *in vivo* plasmid cloning. We utilized the  $\lambda$  Red system to both gap-repair a DNA segment of choice onto a vector and exchange the plasmid origin to alter copy number and/or host range.

The plasmids constructed in this study coordinately express *gam*, *bet* and *exo* of  $\lambda$  in their natural prophage context under tight regulation, thereby achieving high levels of recombination with a low uninduced background similar to that of the chromosomal prophage system. Modifications ranging from point mutations to large substitutions can be created using these plasmids. These plasmids can readily transform and catalyze recombination in *recA* mutant strains where the defective  $\lambda$  prophage cannot be easily introduced by P1 transduction. They will be particularly useful in engineering BACs or P1 artificial chromosome (PACs) carrying large DNA clones, which are usually propagated in the *recA* mutant strain DH10B (Lee et al., 2001). The plasmids can be easily introduced where a special *E. coli* background is required; plasmids with the *ts*-replicons can be easily eliminated from such strains allowing the newly created modifications to be retained in a plasmid-free background.

All the plasmid vectors constructed here contain a minimal  $\lambda$  prophage in which most of the unwanted regions of the *p<sub>L</sub>* operon have been removed, including the toxic *kil* gene, transcription terminators, and the anti-termination gene *N*. The *p<sub>L</sub>* promoter on these constructs is regulated by the *ts* CI857

repressor, and the presence of the  $\lambda$  *o<sub>L</sub>* and *o<sub>R</sub>* operators ensure the tightest repression control (Dodd et al., 2001). We have retained the native *t<sub>L3</sub>* transcription terminator downstream of *exo* in all the plasmids. This prevents excessive transcription beyond *exo* into the *ori* region; such transcription interferes with plasmid replication. A plasmid identical to pSIM2 but without the *t<sub>L3</sub>* terminator could not be established in a standard strain lacking a  $\lambda$  repressor.

Tight regulation of gene expression by the  $\lambda$  repressor and the low copy number of the plasmid expression system appear to be critical for effective recombineering. The pSIM2 and pSIM4 plasmids have unacceptably leaky expression of Red and should not be used for recombineering. On the other hand, the most promising of the plasmid systems are pSIM5 and pSIM9; these have a *ts*-replicon and catalyze recombination efficiently with a low level of unwanted constitutive recombination. pSIM7 gave a recombineering efficiency similar to pSIM5 and pSIM9 but generated a 10-fold higher background. Using the native  $\lambda$  control for Red expression in our vectors we observed higher recombination frequencies than we obtained with pKD119, another pSC101-based *ts-ori* plasmid, where Red genes are taken completely out of the context of  $\lambda$  control and are transcribed from the *P<sub>araBAD</sub>* promoter (Datsenko and Wanner, 2000).

The pSIM2 plasmid was generated by recombineering technology using a gap-repair method. This method entailed the retrieval the *red* genes from the defective  $\lambda$  prophage onto a linear 949 bp DNA segment carrying the pBR322 *ori* flanked by appropriate homologies for recombination with the prophage. This modified application of gap-repair makes it convenient to clone almost any region linked to a drug marker. This method can replace standard cloning technology and has the advantage that the cloned segment is not replicated *in vitro*, eliminating the potential for mutations caused by PCR. A second advantage is that the presence of a drug marker at the target DNA rather than on the linear retrieval fragment allows only the desired recombinant plasmids to emerge with the drug marker, minimizing unwanted background of end-joined vector (Zhang et al., 2000).

We changed the plasmid origin of our pBR322-based plasmids by the precise replacement with origins of other plasmids to alter both copy number and confer different host ranges. The ability to “swap out” a pBR322 origin relies on the inability of pBR322 to propagate in a *polA* mutant. This is a very useful trick to get rid of the pBR322 backbone and replace it with another origin of lower copy number, of different host range, or carrying special elements such as expression promoters and gene tags for example. Such an *in vivo* trade of plasmid DNA is not limited to origins but can also be used to exchange drug markers. In fact two other pSIM5 derivatives, pSIM18 and pSIM19 have been made by changing the Cm<sup>R</sup> marker in pSIM5 to hygromycin resistant and spectinomycin resistant respectively (Dr. Pentao Liu and Dr. David Friedman, personal communication).

We expect that our Red expression plasmids will be able to catalyze recombineering in Gram-negative bacteria closely related to *E. coli* and *S. enterica*. It is preferable to first attempt oligo recombineering in different organisms as this requires only  $\lambda$  Beta protein (Ellis et al., 2001) and is more likely to be successful. Intelligent design of oligos helps to increase the

number of recombinants, even in strains competent for mismatch repair. For example, if the recombining oligo forms a C:C mismatch when annealed to the target sequence (Costantino and Court, 2003) or has 4–6 adjacent base changes covering the desired mutational site (N. Costantino, unpublished), it evades the methyl-directed mismatch repair system, maximizing recombination. If oligo recombineering is successful then dsDNA engineering should be attempted. In dsDNA recombineering,  $\lambda$  Gam is able to protect the electroporated linear dsDNA from degradation by inhibiting RecBCD of *E. coli* and *Salmonella*, but in other organisms this may not be the case. For more distantly related Gram-negative bacteria and Gram-positive species, development and use of an alternate phage recombination system may be required. Such systems could be modified and adapted for use on plasmid vectors similar to that shown in this study.

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## References

- Alper, M.D., Ames, B.N., 1975. Positive selection of mutants with deletions of the *gal-chl* region of the *Salmonella* chromosome as a screening procedure for mutagens that cause deletions. *J. Bacteriol.* 121, 259–266.
- Antoine, R., Loch, C., 1992. Isolation and molecular characterization of a novel broad-host-range plasmid from *Bordetia bronchiseptica* with sequence similarities to plasmids from gram-positive organisms. *Mol. Microbiol.* 6, 1785–1799.
- Baron, L.S., Penido, E., Ryman, I.R., Falkow, S., 1970. Behavior of coliphage lambda in hybrids between *Escherichia coli* and *Salmonella*. *J. Bacteriol.* 102, 221–233.
- Copeland, N.G., Jenkins, N.A., Court, D.L., 2001. Recombineering: a powerful new tool for mouse functional genomics. *Nat. Rev. Genet.* 2, 769–779.
- Costantino, N., Court, D.L., 2003. Enhanced levels of  $\lambda$  Red-mediated recombinants in mismatch repair mutants. *Proc. Natl. Acad. Sci. U. S. A.* 100, 15748–15753.
- Court, D.L., Sawitzke, J.A., Thomason, L.C., 2002. Genetic engineering using homologous recombination. *Annu. Rev. Genet.* 36, 361–388.
- Daniels, D.L., et al., 1983. Complete annotated lambda sequence. In: Hendrix, R.W., Roberts, J.W., Stahl, F.W., Weisberg, R.A. (Eds.), *Lambda II*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 519–676.
- Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* 97, 6640–6645.
- Dodd, I.B., Perkins, A.J., Tsemitsidis, D., Egan, J.B., 2001. Octamerization of  $\lambda$  CI repressor is needed for effective repression of  $P_{RM}$  and efficient switching from lysogeny. *Genes Dev.* 15, 3013–3022.
- Ellis, H.M., Yu, D., DiTizio, T., Court, D.L., 2001. High efficiency mutagenesis, repair, and engineering of chromosomal DNA using single-stranded oligonucleotides. *Proc. Natl. Acad. Sci. U. S. A.* 98, 6742–6746.
- Gottesman, M.E., Adhya, S., Das, A., 1980. Transcription antitermination by bacteriophage lambda N gene product. *J. Mol. Biol.* 140, 57–75.
- Hashimoto-Gotoh, T., Franklin, F.C., Nordheim, A., Timmis, K.N., 1981. Specific-purpose plasmid cloning vectors. I. Low copy number, temperature-sensitive, mobilization-defective pSC101-derived containment vectors. *Gene* 16, 227–235.
- Karakousis, G., Ye, N., Li, Z., Chiu, S.K., Reddy, G., Radding, C.M., 1998. The beta protein of phage  $\lambda$  binds preferentially to an intermediate in DNA renaturation. *J. Mol. Biol.* 276, 721–731.
- Kelley, W.S., Joyce, C.M., 1983. Genetic characterization of early amber mutations in the *Escherichia coli* *polA* gene and purification of the amber peptides. *J. Mol. Biol.* 164, 529–560.
- Kingsbury, D.T., Helinski, D.R., 1970. DNA polymerase as a requirement for the maintenance of the bacterial plasmid colicinogenic factor E1. *Biochem. Biophys. Res. Commun.* 41, 1538–1544.
- Kulkarni, S.K., Stahl, F.W., 1989. Interaction between the *sbcC* gene of *Escherichia coli* and the *gam* gene of phage  $\lambda$ . *Genetics* 123, 249–253.
- Lee, E.C., et al., 2001. A highly efficient *Escherichia coli*-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* 73, 56–65.
- Li, Z., Karakousis, G., Chiu, S.K., Reddy, G., Radding, C.M., 1998. The beta protein of phage  $\lambda$  promotes strand exchange. *J. Mol. Biol.* 276, 733–744.
- Lin-Chao, S., Chen, W.T., Wong, T.T., 1992. High copy number of the pUC plasmid results from a Rom/Rop-suppressible point mutation in RNA II. *Mol. Microbiol.* 6, 3385–3393.
- Little, J.W., 1967. An exonuclease induced by bacteriophage  $\lambda$ . II. Nature of the enzymatic reaction. *J. Biol. Chem.* 242, 679–686.
- Murphy, K.C., Campellone, K.G., 2003. Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic *E. coli*. *BMC Mol. Biol.* 4, 11.
- Murphy, K.C., Campellone, K.G., Poteete, A.R., 2000. PCR-mediated gene replacement in *Escherichia coli*. *Gene* 246, 321–330.
- Pogliano, J., Ho, T.Q., Zhong, Z., Helinski, D.R., 2001. Multicopy plasmids are clustered and localized in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* 98, 4486–4491.
- Roberts, R.C., Burioni, R., Helinski, D.R., 1990. Genetic characterization of the stabilizing functions of a region of broad-host-range plasmid RK2. *J. Bacteriol.* 172, 6204–6216.
- Sergueev, K., Yu, D., Austin, S., Court, D., 2001. Cell toxicity caused by products of the pL operon of bacteriophage lambda. *Gene* 272, 227–235.
- Swaminathan, S., et al., 2001. Rapid engineering of bacterial artificial chromosomes using oligonucleotides. *Genesis* 29, 14–21.
- Thomason, L.C., et al., 2005. Recombineering: genetic engineering in bacteria using homologous recombination. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (Eds.), *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc., Hoboken, N.J., pp. 1–21. Chapter 1, Unit 16.
- Unger, R.C., Clark, A.J., 1972. Interaction of the recombination pathways of bacteriophage  $\lambda$  and its host *Escherichia coli* K12: effects on exonuclease V activity. *J. Mol. Biol.* 70, 539–548.
- Yu, D., Ellis, H.M., Lee, E.C., Jenkins, N.A., Copeland, N.G., Court, D.L., 2000. An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* 97, 5978–5983.
- Yu, D., Sawitzke, J.A., Ellis, H., Court, D.L., 2003. Recombineering with overlapping single-stranded DNA oligonucleotides: testing a recombination intermediate. *Proc. Natl. Acad. Sci. U. S. A.* 100, 7207–7212.
- Zhang, Y., Muylers, J.P., Testa, G., Stewart, A.F., 2000. DNA cloning by homologous recombination in *Escherichia coli*. *Nat. Biotechnol.* 18, 1314–1317.