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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 λ 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 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 λ Red functions, Exo, Beta and Gam. The λ 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 λ 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^R, chloramphenicol resistant; Ap^R, ampicillin resistant; Tc^R, tetracycline resistant; *cat*, chloramphenicol drug cassette; *amp*, ampicillin drug cassette; BAC, bacterial artificial chromosome.

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Fig. 1. Standard defective λ 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 λ 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 {λ *cI857*Δ(*cro-bioA*)} (Yu et al., 2000). DY406 was constructed by replacing *kil* to *sieB* of λ 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 λ DNA sequence using oligo recombination to generate a deletion from *N* to *kil* and fusing nt 33169 to 35446 of λ. 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 (Cm^R) SIMD3 or ampicillin resistant (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 λ nt 35828–37114 (Daniels et al., 1983). These drug cassettes are in single copy on the chromosome, thus lowered levels of antibiotic are used for selection (30 μg/ml ampicillin and 10 μg/ml chloramphenicol). These prophage-carrying strains are deleted for *bioA* and require biotin for growth on minimal media.

HME6 is W3110 Δ(*argF-lac*)*U169* $galK_{TYR145UAG}$ {λ*cI857*Δ(*cro-bioA*)} (Ellis et al., 2001) while HME57 is Δ(*argF-lac*)*U169* $galK_{TYR145UAG}$. P1 transduction was used to create HME57 from HME6 by bringing in the wild-type λ attachment site *attB* and the *bioA* region from W3110, selecting for Bio^+ recombinants. HME57 thus lacks the λ 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 ($polA_{am}$ 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 λ (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 λ (Daniels et al., 1983).

Likewise, the *ori* of plasmids pSC101 $repA^{ts}$, 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 *ori* flanked by λ homology
SD3–SD4	pSC101 *ori* flanked by λ homology
SD5–SD6	pBBR1 *ori* flanked by λ homology
SD7–SD8	RK2 *ori* flanked by λ homology
SD9–SD10	*amp* cassette targeting *galK* of *E. coli*
SD11–SD12	*amp* cassette targeting *galK* of *S. enterica*

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