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Development of new versatile plasmid-based systems for λRed-mediated *Escherichia coli* genome engineering

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ABSTRACT

Plasmid-based systems are the most appropriate for multistep lambda Red (λRed)-mediated recombineering, such as the assembly of strains for biotechnological applications. Currently, the widely used λRed-expressing plasmids use a temperature-sensitive origin of replication or temperature shift control of λRed expression. In this work, we have constructed a new, conditionally replicating vector that can be efficiently eliminated from the host strain through passaging in medium containing isopropyl-β-D-thiogalactopyranoside. Using the new vector, we have developed two improved helper plasmids (viz., pDL17 and pDL14) for dsDNA and oligonucleotide-mediated recombineering, respectively. The plasmid pDL14 contains a dominant negative *mutS*^{K622A} allele that suppresses methyl-directed mismatch repair (MMR). The coexpression of λRed and *mutS*^{K622A} provides efficient oligonucleotide-mediated recombineering in the presence of active host MMR. The expression of λRed was placed under the control of the tightly regulated P_{rhaB} promoter. Because of their low expression level under uninduced conditions, both plasmids could be maintained without elimination for multiple recombineering steps. The temperature-independent replication of the plasmids and control of λRed expression by L-rhamnose allow for all procedures to be performed at 37 °C. Thus, the new plasmids are robust, convenient, and versatile tools for *Escherichia coli* genome editing.

1. Introduction

Presently, the homologous recombination system of lambda (λ) bacteriophage (λRed) is the most widely used, convenient, and powerful tool for DNA molecular engineering in *Escherichia coli* cells. The λRed system allows the efficient introduction of extended modifications (Datsenko and Wanner, 2000; Yu et al., 2000) and point mutations (Ellis et al., 2001; Costantino and Court, 2003) into the chromosome of the cell as well as into exogenous autonomously replicating plasmids (Thomason et al., 2007) and bacterial artificial chromosomes (Court et al., 2003) for application in other organisms. Therefore, the λRed-based technique is the method of choice among many scientists conducting research on the biology of various organisms and their biotechnological applications.

The λRed system catalyzes the recombination of linear double-stranded DNA (dsDNA) fragments, using regions of shorter than 50-bp sequence homology (Yu et al., 2000) that can be added in the polymerase chain reaction (PCR) using synthetic primers, thus skipping the cloning procedure. The generation of an integrative cassette in a single PCR step greatly simplifies the construction of recombinant DNA molecules. Thus, the λRed system allows for the easy manipulation of DNA molecules in *E. coli* cells by successive steps of insertion and excision of the selective markers without the application of restriction enzymes and ligase. Moreover, any DNA fragment, such as that of heterologous genes, can be fused to a selective marker and directly cloned into the chromosome (Juhas and Ajioka, 2016). The isolation of chromosomal genes via gap-repair cloning has a great advantage over conventional PCR-based cloning methods (Datta et al., 2006). All λRed-utilizing applications form a group of methods that are known as recombineering (from "recombination" and "engineering").

Besides its use in linear dsDNA recombineering, the λRed system is able to promote recombination between chromosomal DNA and short

Abbreviations: bp, base pair; PCR, polymerase chain reaction; ori, origin of replication; ds, double-stranded; Ap^R, ampicillin resistant; Cm^R, chloramphenicol resistant; *cat*, chloramphenicol acetyltransferase; *bla*, beta-lactamase; MMR, methyl-directed mismatch repair

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synthetic single-stranded oligonucleotides (oligos) (Ellis et al., 2001). Oligo-mediated recombination allows for the introduction of point mutations as well as long deletions and short (up to 30 bp) insertions (Ellis et al., 2001; Wang et al., 2009). Only λRed Beta function is absolutely required for oligo recombination (Ellis et al., 2001). It is surmised that Beta binds to the oligo and preferably anneals it to the lagging strand of the replication fork (Ellis et al., 2001).

Host methyl-directed mismatch repair (MMR) (Schofield and Hsieh, 2003) is the main factor limiting oligo recombination efficiency (Costantino and Court, 2003). The DNA mismatch repair proteins MutS, MutH, and MutL participate in the recognition and correction of mismatches that are generated when the oligo is annealed to the complementary strand of chromosomal DNA. MMR restores the wild-type sequence, because the DNA of an oligo is unmethylated. Whereas some mismatches can be efficiently corrected, others are refractory to the MMR machinery (Dohet et al., 1985). Therefore, the influence of MMR on recombination efficiency depends on the oligo sequence. The highest and lowest recombination efficiencies were observed using oligos generating A:G and G:G mismatches, respectively (Wang et al., 2011; Nyerges et al., 2014). In MMR-deficient strains, the frequency of point mutation introduction reached 3×10^7 recombinants per 10^8 cells (Costantino and Court, 2003). However, MMR deficiency causes an increase of the mutation rate by two orders of magnitude (Lee et al., 2012), leading to the accumulation of undesirable mutations in the bacterial genome. Several approaches have been proposed to minimize the effect of MMR on oligo recombination efficiency. These include (i) oligo sequence design (Sawitzke et al., 2011), (ii) oligo DNA modifications (Wang et al., 2011; van Ravesteyn et al., 2016), and (iii) the use of temperature-sensitive MMR mutants (Nyerges et al., 2014). A more versatile way is the temporary inhibition of MMR *in trans*. It has been surmised that MMR activity can be transiently inhibited by expression of the dominant negative allele of the *mutS* gene (Costantino and Court, 2003). Recently, a work was published that described a similar approach of using the dominant negative allele of the *mutL* gene (Nyerges et al., 2016).

Although a number of λRed-expressing systems are currently available (Murphy, 2016), all of them have some drawbacks or limitations. First, a convenient system should be able to be easily transferred into and also eliminated from a strain. In this regard, a prophage-based system (Yu et al., 2000) is inappropriate for routine use. Efficient elimination could be provided through the application of conditionally replicating vectors. Among these, the temperature-sensitive derivative of the pSC101 replicon is the most widely used (Phillips, 1999; Datsenko and Wanner, 2000; Datta et al., 2006; Yang et al., 2014). However, it requires all cultivation procedures to be performed at 30 °C, which imposes a substantial constraint on its application for poorly growing strains and those with reduced viability. Second, owing to the multicopy state of plasmids, the tight control of λRed expression is required. Otherwise, constitutive expression can lead to growth inhibition (Sergueev et al., 2001; Datta et al., 2006) and increased mutation rates (Murphy and Campellone, 2003), which complicate the manipulation with plasmid-carrying strains. One of the most tightly regulated expression systems used to control λRed expression is the P_L promoter with the CI^{857} repressor of λ bacteriophage (Lanzer and Bujard, 1988; Yu et al., 2000). However, as the system uses temperature-shift control of expression, it possesses the same drawback as described above for plasmids with a temperature-sensitive origin of replication.

In this article, we describe the development of plasmids for λRed-mediated recombineering. To provide independent control of the replication and λRed expression, we have developed a novel multicopy conditionally replicating vector. Replication of the vector is temperature-independent and sensitive to isopropyl-β-D-thiogalactopyranoside (IPTG). This allows its simple elimination from the host strain. On the basis of this vector, we have constructed plasmids that provide λRed expression under the control of the L-rhamnose-inducible P_{rhaB} promoter, one of the most tightly regulated promoters known (Egan and Schleif, 1993; Haldimann et al., 1998). The plasmids allow dsDNA integration and MMR-independent oligo-mediated recombineering due to the simultaneous expression of λRed and the dominant negative allele of the *mutS* gene (Haber and Walker, 1991). The recombination frequency with dsDNA and oligos was not inferior to the best results of well-known prophage-carrying strains (Yu et al., 2000; Costantino and Court, 2003; Sawitzke et al., 2011). We observed that in the absence of induction, the expression of λRed was negligible despite the high copy number of the plasmids. Therefore, the developed plasmids can be introduced into bacterial cells and safely maintained for many recombineering steps.

2. Materials and methods

2.1. Media and reagents

For general manipulation, the *E. coli* cells were grown in Lysogeny Broth (LB) (10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride per 1 L of water). To prepare LB plates, 20 g of agar was added per 1 L of the medium. Low-salt LB medium (10 g of tryptone, 5 g of yeast extract, and 5 g of sodium chloride per 1 L of water) was used for the preparation of electrocompetent cells. For counterselection against cells carrying the levansucrase (*sacB*) gene, LB agar without sodium chloride was supplemented with 6% sucrose. When required, the medium was supplemented with antibiotics: 8 mg/L chloramphenicol, 40 mg/L nalidixic acid, 30 mg/L ampicillin, and 100 mg/L ampicillin for strain HME63 and the other strains, respectively. IPTG was added up to 1.0 mM for plasmid elimination. Tetrazolium agar (10 g of tryptone, 5 g of yeast extract, 10 g of sodium chloride, 25 mg of 2,3,5-triphenyltetrazolium chloride, and 20 g of agar per 1 L) and Endo agar (10 g of tryptone, 2.5 g of dipotassium phosphate, 3.3 g of sodium sulfite, 0.3 g of basic fuchsin, and 20 g of agar per 1 L, pH 7,4) were used for the screening of recombinants. The tetrazolium agar and Endo agar were supplemented with 10 g/L of appropriate carbohydrates. M9 minimal medium (Sambrook et al., 1989) with 1 mg/L of biotin and 2 g/L of D-galactose was used for the direct selection of Gal^+ recombinants. Stock solutions of carbohydrates and 2,3,5-triphenyltetrazolium chloride were sterilized by filtration through 0.22-μm pore-sized membranes. Sterile 0.9% sodium chloride solution was used for dilution of the cells before plating.

Restriction endonucleases, T4 ligase, and T4 polymerase were purchased from Thermo Fisher Scientific (Vilnius, Lithuania). Amplification of the DNA fragments for cloning purposes was performed using KAPA HiFi DNA Polymerase (Kapa Biosystems, Wilmington, MA, USA). Cassettes for dsDNA recombineering were amplified with DreamTaq DNA polymerase (Thermo Fisher Scientific, Vilnius, Lithuania). NEBuilder HiFi DNA Assembly Master Mix was purchased from New England Biolabs (Ipswich, MA, USA).

2.2. Bacterial strains and plasmids

All *E. coli* strains used in this study are derived from strain MG1655, except for strains HME6 and HME63 (11), kindly provided by Dr. Donald L. Court (National Cancer Institute, Frederick, MD, USA). The genotype of HME6 is W3110 $galK^{Tyr145UAG}$ Δ*(argF-lac)U169* λcI^{857} Δ(*cro-bioA*), and that of HME63 is HME6 Δ*mutS::bla*. For λRed-mediated recombineering, pKD46 (Datsenko and Wanner, 2000) was used as a helper plasmid. Strain B928 ($galK^{Tyr145UAG}$) was constructed by transforming strain MG1655 with oligo 2250 and then further screening for clones unable to utilize D-galactose on tetrazolium-galactose plates. The presence of the correct mutation was confirmed by sequencing the modified region. Strain B954 ($galK^{Tyr145UAG}$ Δ*mutS::cat-sacB*) was constructed by replacing the *mutS* gene in B928 with the *cat-sacB* cassette amplified from pICA using primers 2246 and 2247. Strain B1310 (λcI^{857} Δ*cro-attR*) was constructed in several steps. First, strain MG1655