SHORT COMMUNICATION

Integrative Vectors for Gene Deletion and Replacement

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Abstract

An improved method for gene deletion or replacement in *Escherichia coli* was developed. It employs a set of integrative vectors and two helper plasmids, as a temporary source of RecA and Flp activities. The integrative vectors combine several useful features including three different selection markers placed between two parallel oriented Flp recombinase target (*FRT*) sites. Each marker is flanked by two MCSs, for cloning the chosen homologous fragments of DNA to gene targeting. The vectors contain two properly oriented *E. coli* Chi sites for recombination enhancement. When required, selection markers can be excised from the chromosome resulting in unmarked strains.

K e y   w o r d s: gene deletion; gene replacement, integrative rectors

All techniques for quick and precise modification of the bacterial chromosome to make it more useful with indispensable genes are very important for optimizing biotechnological processes. Genetic manipulations like gene replacement or gene knockout can be achieved by a variety of techniques (Sektas, 2000). Since *Escherichia coli* strains serve as the host organisms for many research and commercial applications (Feher *et al.*, 2012), the need for specific host with modified genome still exists. To transfer a gene from the plasmid to the chromosome in homologous recombination-mediated reaction some methods use strains defective in *polA* (Gay, 1984) or temperature-sensitive replicons (Hamilton *et al.*, 1989; Posfai *et al.*, 1997, Martinez-Morales *et al.*, 1999). The procedure may also rely on integrative vector devoid of origin of replication (Posfai *et al.*, 1994, Sektas and Szybalski, 2001) or Rep function depletion (Wild *et al.*, 1998). Another approach is to use linear DNA that contains a target gene flanked by homologous regions of chromosome to replace the specific locus. It requires a certain genetic background, *e.g. recBC* (Jasin and Schimmel, 1984) or *recD* genotypes (Biek and Cohen, 1986; Cherepanov and Wackernagel, 1995), or a means of inhibition of ExoV activity (Datsenko and Wanner, 2000). Alternatively, the presence of properly oriented Chi sequences on the DNA substrates is required (Dabert and Smith, 1997; Karoui *et al.*, 1999). Dabert and Smith (1997) reported strong stimulation of

gene replacement in *E. co*li using relatively short DNA fragments with Chi sequences near each end. RecBCD multifunctional enzyme complex approaches Chi by its diffusion from a double-stranded end in orientation-dependent manner, from right of Chi sequence 5'-GCTGGTGG-3' (Smith, 2012). By continuing unwinding of the DNA, the RecBCD enzyme produces single-stranded DNA substrate for the RecA protein (Roca and Cox, 1997). Recombination between a linear DNA fragment and the circular chromosome requires an even number of exchanges to maintain circularity of the chromosome and hence viability of the cell.

Our integrating system is based on prevention of DNA substrate degradation in RecBCD+ bacteria and stimulation of recombinantion by supplying RecA from helper plasmid pRecAts(Ap^R). The *recA* gene expression is driven by IPTG-dependent P_{lac} promoter. Since this plasmid was constructed based on pSC101 temperature-sensitive replicon it can be easily cured by cultivation under non-permissive conditions (Posfai *et al.*, 1997). To test our method for gene targeting in wild-type *E. coli* MM294 (Meselson and Yuan, 1968), we constructed three basic integrative vectors differing by the presence of resistance genes: Cm^R, Km^R or Tc^R, respectively. The antibiotic resistance genes are flanked by two parallel oriented *FRT* sites allowing for excision *in vivo* under conditions of transient expression of *FLP* gene from a conditional suicidal vector pFT-Km

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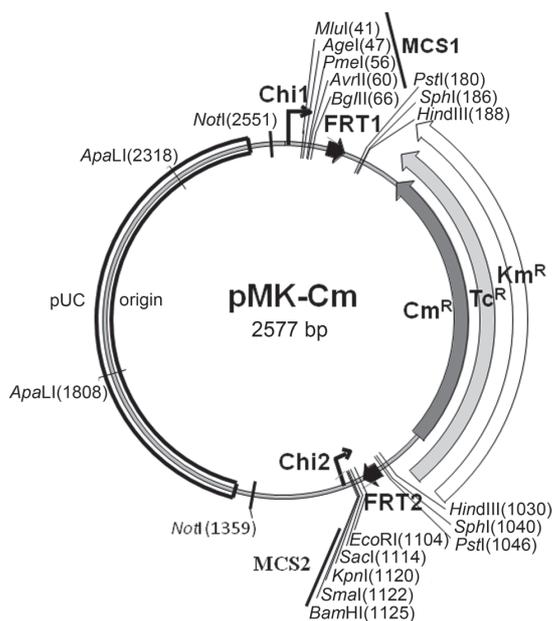
Fig. 1. Structural features of the pMK-family integrative vectors. Only key and unique restriction sites are marked.

Abbreviations: Chi, 5'-GCTGGTGG sequence; Cm^R, chloramphenicol resistance gene; *FRT*, 5'-GAAGTTCCTATTCTCTAGAAAGTATATAG-GAACTTC sequence; Km^R, kanamycin resistance gene; Tc^R, tetracycline resistance gene.

(Posfai *et al.*, 1997). Moreover, there are two polylinkers, MCS1 and MCS2, with five unique cloning sites each and two *E. coli* Chi sequences outside of MCSs. The presence of Chi sites on both ends of integration cassette in productive orientation may also stimulate gene replacement (Dabert *et al.*, 1992). These vectors can be used in broad spectrum of hosts, both as a linear or non-replicating *ori*$^-$ circle. Fig. 1 shows all essential elements of pMK-vectors. The integrative plasmids were constructed in a series of steps starting with the pCANTS as a backbone (Posfai *et al.*, 1994), which is a pBR322 derivative (Bolivar *et al.*, 1977), possessing *ori*$_{pBR}$ as a cassette between two NotI restriction sites. To alter medium copy *ori*$_{pBR322}$ to copy-up of *ori*$_{pUC19}$, PCR method was used. We designed two primers specific to pUC19 plasmid (Yanish-Perron *et al.*, 1985), bearing NotI sites at either end (underlined): PUC3' (5'-AAA GCGGCCGC AGGAAAGA *ACATGT* GAGCA) complementary to the AflIII site (italic) [806 bp of pUC19]) and PUC5' (5'-AAAT GCGGCCGC TAGGT-GAAGATCCTTTTTG) close to the DraI site (1563 bp). 781 bp PCR-product was cut by NotI and inserted between NotI sites of pCANTS to give rise to pMS-Cm. Then, 430 bp fragment of AflIII-NotI of pCANTS was inserted between the analogous sites of pMS-Cm (prepared by incomplete digestion of NotI), to extend the *ori* region to *pas* sequences (Bolivar *et al*, 1977). Thus

obtained plasmid was named pMSS-Cm. To introduce *E. coli* Chi sequence (underlined) into the construct, two oligonucleotides were synthesized (Chi1 5'-TCGA-CA GCTGCTGG TGGA-3' and Chi2 5'-AGCTTC-CA CCAGCAGC TG) to fit between SalI and HindIII of pMSS-Cm. Digestion with HindIII, DNA polymerase Klenow filling in and re-ligation allowed to alter HindIII site to NheI specific sequence.

To insert a *cat* gene (HincII fragment from pKRP10 vector, Reece and Phillips, 1995) between two parallel minimal *FRT* sites, plasmid pminFRTlac1.1 (originally derived from pMSlacZ.1, Sektas and Szybalski, 1998) was used. This plasmid was first prepared by BamHI and XhoI cutting followed by Klenow fragment of DNA I polymerase filling-in. Then, Chi site from pMSS-Cm was inserted between SalI and NheI sequences. Next, 41 bp SalI-XhoI fragment of MCS of pBend2 (Kim *et al.*, 1989) was inserted into the SalI sites. Next, EcoRI-XhoI fragment of DNA (blunted by Klenow polymerase) containing the following set of genetic elements: *FRT-cat-FRT*-MCS1-Chi, was subcloned into pMSS-Cm between DraI and EcoRI sites. To broaden the set of unique restriction sites of MCS2, a polylinker was designed to introduce new AgeI, PmeI, AvrII sites between unique MluI and BglII (BglII primer 5'-GATCTCCTAGGTTTAAACACCGGTA and MluI primer 5'-CGCGTACCGGTGTTTAAAC-CTAGGA) and thus final plasmid pMK-Cm was created. Plasmids pMK-Tc and pMK-Km are identical to pMK-Cm in all details but have genes conferring tetracycline or kanamycin resistance (Reece and Phillips, 1995), respectively (Fig. 1).

The integrative plasmids can recombine by a single or double crossover with homologous targeted sites, either as circular or linear form of DNA. The way of vector utilization is depended on whether one or two homologous fragments of DNA are to be cloned. All vectors contain removable pUC *origin* of replication, located between two NotI sites, giving the possibility of its deletion. A rapid construction of the *ori*-less (Δ*ori*) "suicide" plasmid was carried out by using NotI and ApaLI digestions, followed by agarose-gel purification procedure. Upon introduction of the homologous DNA into *E. coli* MM294 by the standard transformation procedure, such Δ*ori* circle can integrate by a means of homologous recombination to the chromosomal target site. To test the insertion efficiency of linear form of DNA pMK-Cm vector was chosen, where truncated *lacI* gene (450 bp MluI-EcoRV gene fragment) was cloned into MCS1 site, and similarly, internal segment of *lacZ* (1716 Bsu36I-SacI gene fragment) was inserted into MCS2 site (pMK-CmlacIZ, Fig. 2B). Homologous replacement of the chromosomal *lac*$^+$ locus with these sequences should generate a Lac$^-$Cm$^+$ phenotype.
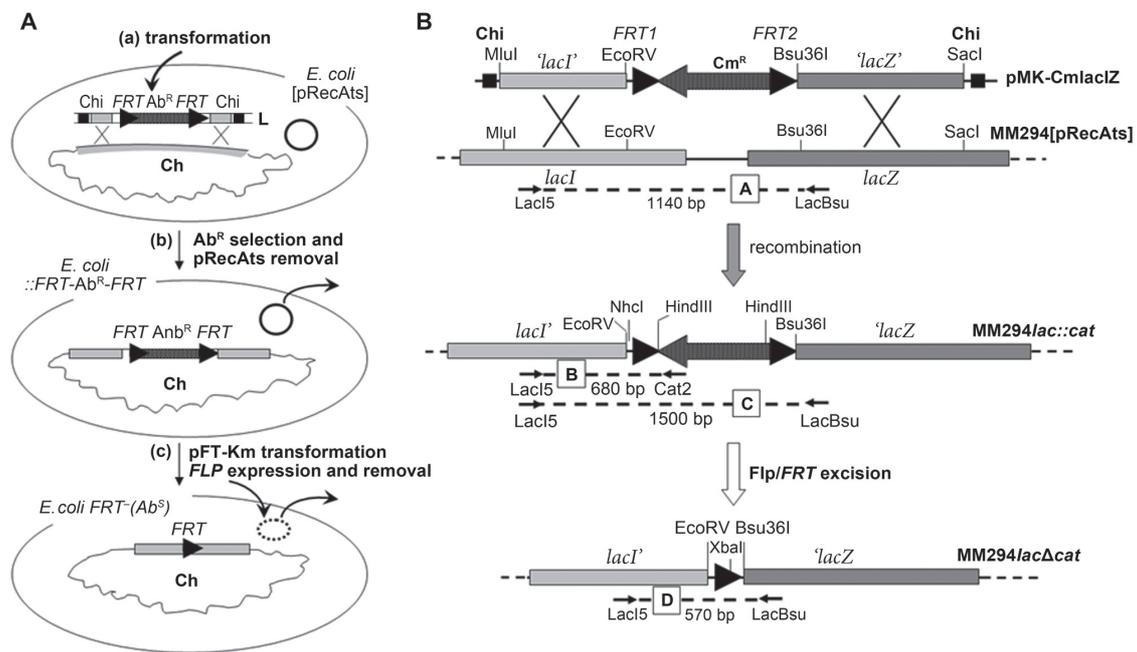
Fig. 2. Strategy and validation
of unmarked gene disruption method.

A. Schematic representation of the three step method: (a) transformation of integrative DNA, (b) selection of recombinants and pRecAts removal, and (c) marker excision and pFT-Km removal. Abbreviations: Ab^R – antibiotic resistance, Ab^S – antibiotic sensitivity, Ch – chromosome, L – linear dsDNA. **B**. Illustration of recombination-mediated chromosomal integration/removing of *cat* gene in *lac* locus of *E. coli* MM294. pMK-CmlacIZ integrative plasmid containing truncated *lacI* and *lacZ* genes was used along with two helper plasmids pRecAts (Ap^R) and pFT-Km (Km^R), to provide the RecA and Flp functions, respectively. **C**. PCR analysis of allelic exchange and subsequent marker removal corresponding to panel B. Total DNA from MM294 and its recombinant derivatives was isolated and subjected to PCR reactions. The PCR products were separated on 0.8% agarose gel. Lane 1 – product A of 1140 bp (MM294), lane 2, product C of 1500 bp (MM294*lac::cat*[Cm^R]), lane 3, DNA fragments from product C after HindIII digestion (840– and 580 bp), lane 4, product D of 570 bp (MM294*lacΔcat*[Cm^S]), lane 5, negative control with Cat2 and LacI5 pair of primers (MM294), lane 6, product B of 680 bp (MM294*lac::cat*[Cm^R]), lane 7, negative control with Cat2 and LacI5 pair of primers (MM294*lacΔcat*[Cm^S]).

Linear DNA fragments were prepared as follows. Typically, plasmid DNA (1 μg) was cut with ApaLI and NotI restriction endonucleases in standard conditions. The mixture was electrophoresed on a 0.7% agarose preparative gel to remove DNA fragments containing origin of replication. Appropriate DNA fragment was recovered by electroelution. The DNA pellet was suspended in TE [Tris-HCl (pH 8), 1 mM EDTA] buffer. Competent MM294 [pRecAts(Ap^R)] cells were prepared by chemical method (Miller, 1972) after IPTG(0.1 mM)-mediated induction of *recA* expression. For transformation, linear DNA of pMK-CmlacIZ (250 ng) and 120 μl of chilled cells were used (10^8 viable cells/ml). An appropriate amount of transformation mixture was
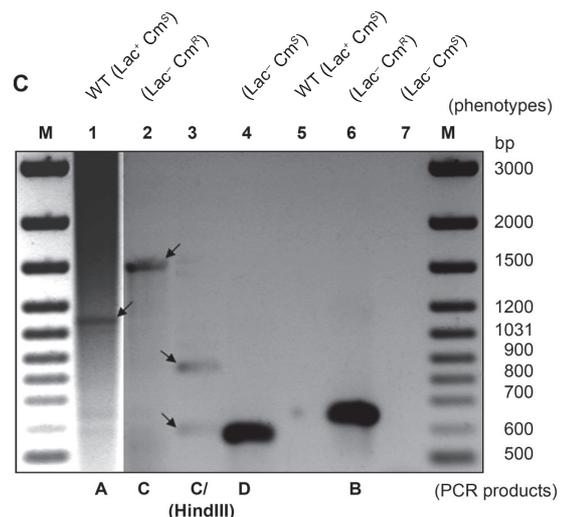
plated onto selective medium (chloramphenicol and ampicillin at concentration of 12.5 μg/ml and 25 μg/ml, respectively) supplemented with X-Gal/IPTG (40 μg/ml and 0.1 mM/ml, respectively) at 30°C. The RecA⁺ helper plasmid was subsequently eliminated from Cm-positive recombinants (MM294*lac::cat*[Cm^R], white colonies) by increasing the temperature to 42°C (Wild *et al.*, 1998, Fig. 2A). To achieve unmarked recombinants we could delete the segment carrying antibiotic resistance gene *in vivo* by transient expression of *FLP* gene from a suicidal helper plasmid pFT-Km (Posfai *et al.*, 1997) simply by addition of autoclaved chlortetracycline (20 μg/ml). Flp promotes recombination at specific 13 bp sites within 65 bp sequence, termed *FRT*. The resulting strain was

named MM294*lac*Δ*cat*[Cm^S]. Gene disruption mutants were verified by *lac* phenotype analysis and tested for chloramphenicol susceptibility. Also, analytical PCR before and after *cat* gene excision was performed with primers specific to *lacI*, *cat* or *lacZ* genes in engineered region of the chromosome, as shown at Fig. 2C. In addition, the mutants were checked for the absence of any plasmids. The frequency of 4–12 gene replacements was obtained, when normalized to 1 μg of DNA. Our results are in the range of recombination efficiency obtained for linear (Chi)-DNA used to target plasmid or chromosome, 2.8 gene replacements using 200–400 ng of DNA (Dabert and Smith, 1997) and 14–64 gene replacements with 1 μg of DNA (Jessen *et al.*, 1998), respectively.

Taken together we report on the construction and use of a set of integration vectors that enable introduction of any DNA fragment into the bacterial genome regardless of the actual RecA phenotype. In conjunction, Flp recombinase-based system offers opportunity for precise *in vivo* excision of an antibiotic resistance gene, served initially as a selection marker. Recombination between *FRT* sites situated on either side of the resistance determinant leads to its deletion, resulting in an unmarked mutant. Remaining *FRT* site can be used as a target for new exchangeable insertions.

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