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.

Key words: gene deletion; gene replacement, integrative vectors

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. coli 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 recombination by supplying RecA from helper plasmid pRecAts(ApR). The recA gene expression is driven by IPTG-dependent P_ 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: CmR, KmR or TcR, 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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resistance gene; each and two elements of pMK-vectors. 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 (Δori) “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 Δori 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.
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(ApR)] 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 [CmR]], 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 chlorotetracycline (20 µg/ml). Flp promotes recombination at specific 13 bp sites within 65 bp sequence, termed FRT. The resulting strain was

![Fig. 2. Strategy and validation of unmarked gene disruption method.](image-url)

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 (ApR) and pFT-Km (KmR), 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 [CmR]]), lane 3, DNA fragments from product C after HindIII digestion (840 and 580 bp), lane 4, product D of 570 bp (MM294lac::cat [CmS]), lane 5, negative control with Cat2 and LacI5 pair of primers (MM294), lane 6, product B of 680 bp (MM294lac::cat [CmS]), lane 7, negative control with Cat2 and LacI5 pair of primers (MM294lac::cat [CmS]).
named MM294lacCatt[CM^2]. 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.

Literature


