Red/ET Recombination

λ vs. Mu: base-precise modification of the *E. coli* genome

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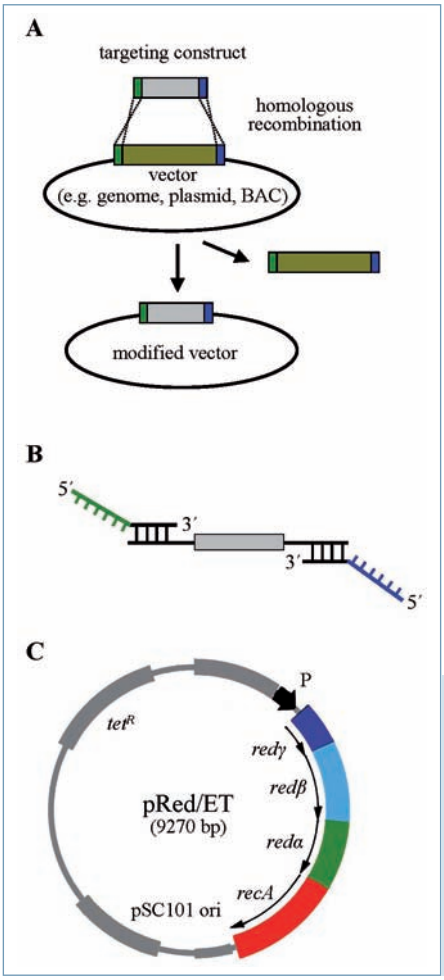
Red/ET recombination is an *in vivo* method for precise modifications of the *E. coli* genome. Markerless DNA-modifications of 1 bp to 80 kb can be introduced in combination with subsequent Flp/Cre recombination steps. The efficiency of this technology is demonstrated here by the removal of a Mu prophage from the *E. coli* genome.

Recombineering: site-directed DNA modification by recombination

■ Homologous recombination results in the conjunction of similar or identical DNA strands within the cell. This mechanism contributes to the repair of the DNA helix and increases the genetic diversity, e.g. by exchange of homologous chromatids during meiosis. Molecular biologists have exploited the endogenous cellular recombination potential for years in order to achieve directed DNA modifications. This method, termed recombineering, requires a DNA molecule which i) encodes for the desired modification(s) and ii) is flanked by appropriate targeting sequences. Once the construct is introduced into the cell, recombination of the flanking homology arms (ha) with the targeting regions on a vector results in a base-precise exchange of the ha-flanked DNA stretch **(Fig. 1A)**.

Recombineering in the „work-horse" *E. coli* is accompanied by certain difficulties: linear DNA is an unsuitable recombination partner since it is rapidly degraded - mainly by exonuclease ExoV[1]. Therefore circular targeting constructs based on suicide plasmids are used to overcome this problem. However, efficient recombination between circular molecules requires long (≧ 500 bp) stretches of sequence homology[2], which certainly complicates cloning of an appropriate vector.



◀ **Fig. 1:** Recombineering. **A,** Double homologous recombination between DNA molecules results in an exchange of the genetic information which is flanked by similar or identical sequence stretches (green, blue). **B,** Preparation of linear targeting constructs for Red/ET cloning. The 50 bp homology arms of any given sequence (green, blue) are attached as primers by PCR. **C.** Plasmid map of pRed/ET. P, promoter.

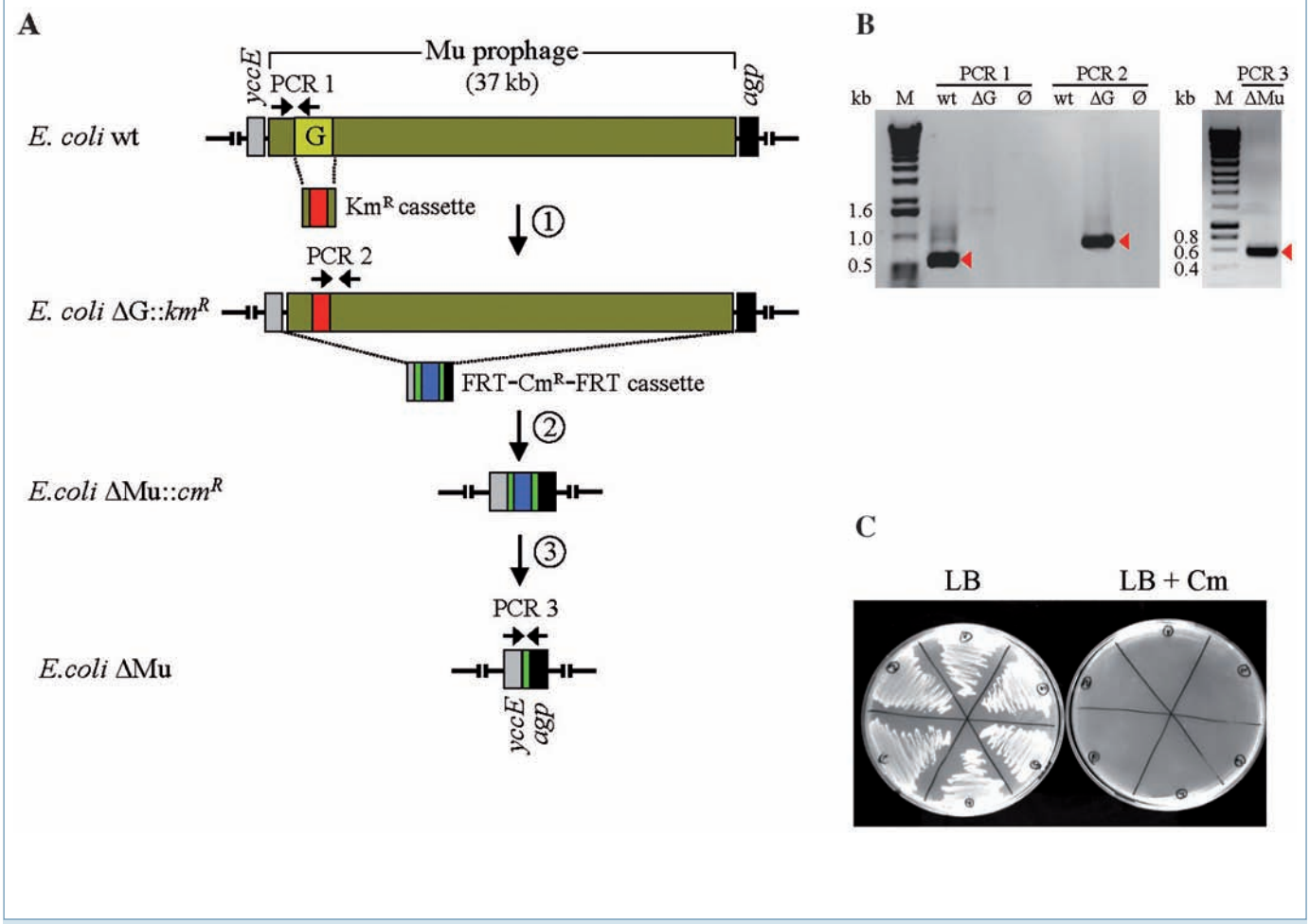
Red/ET Recombination

Red/ET recombination, invented in Francis Stewart's laboratory at EMBL (Heidelberg), provides access to a much more efficient recombineering in *E. coli*, since this technology does not depend on a circular targeting construct[3-5]. Furthermore homology arms of only 50 bp are required, which can easily be synthesized as primers and attached to a linear DNA fragment by PCR. (**Fig. 1B**). Thus a targeting construct for any genomic locus can rapidly be constructed without using the „classical" molecular tools i.e. restrictionendonuclease and ligase.

Plasmid pRed/ET (**Fig. 1C**), which basically can be introduced in any *E. coli* strain represents the core of the Red/ET technology. pRed/ET encodes for the phage λ recombination machinery. Redγ stabilizes the linear recombination partner so that exonuclease Redα and the DNA-binding protein Redβ can mediate recombination between target molecules. Additional copies of the *E. coli* RecA protein increase the number of obtained transformands after electroporation[2]. Expression of the corresponding genes is under control of an inducible promoter. The addition of the inducer, followed by a temperature rise (30°C to 37°C) results in a transient recombineering activity, since pRed/ET is no longer replicated due to its thermosensitive ori pSC101 and finally gets lost. Therefore unspecific recombination events in the cell are kept to a minimum.

λ vs. Mu – elimination of a Mu prophage by Red/ET recombination

Mu is a bacterial virus (phage) which can either multiply via the lytic cycle or enter a quiescent state in the cell. Mu integrates into the genome of certain enterobacteria in an almost sequence-independent manner[6]. In lysogenic cells the prophage is replicated along with the host chromosome and passed on to the daughter cells.



▲ **Fig. 2:** Deletion of prophage Mu from the *E. coli yccE-agp* intergenic region. **A,** In Red/ET step 1 the viral G fragment (3 kb) is replaced by a Km^R cassette (red) resulting in strain *E. coli* ΔG::km^R. The inactivated prophage (37 kb) is thereupon removed by a FRT-flanked Cm^R targeting construct (green-blue-green; Red/ET step 2). Finally the Cm^R marker (blue) is deleted from the *E. coli* ΔMu::cm^R chromosome by Flp recombinase (3). A FRT site (34 bp, green) remains in the *yccE-agp* locus of *E. coli* ΔMu. **B,** PCR verification of recombination steps 1-3. The orientation and position of the PCR primers is indicated by horizontal arrows in A. Gel-separated PCR fragments of the expected size are marked by red triangles. ∅, control PCR without template; ΔG, *E. coli* ΔG::km^R, ΔMu, *E. coli* ΔMu; M, size marker. **C,** Cm-sensitive growth of *E. coli* ΔMu colonies obtained after Flp recombination. LB, Luria-Bertani medium.

Here we show that a lysogenic *E. coli* strain displaying a Mu titer of 10^3 to 10^4 pfu/ml could be cured by deleting the prophage genome (37 kb) in three successive recombination steps (**Fig. 2**). Usually a 37 kb DNA stretch can be replaced by a selection marker in a single Red/ET step. However, we first replaced the G fragment of prophage Mu with a kanamycin resistant (Km^R) marker (**Fig. 2A**, step 1). Since the G fragment encoded tail fibre proteins are essential for cell wall contact and subsequent infection[7] such a procedure prevents re-infection by newly formed virulent phages elsewhere in the genome.

The sequence-specific exchange of the G fragment by a Km^R cassette could be demonstrated by PCR (**Fig. 2B**; PCR 1, 2). A subsequent plaque assay confirmed that the obtained *E.coli* ΔG::km^R culture is free of any infectious Mu particles (not shown).

In a second Red/ET step the inactivated prophage was removed by a chloramphenicol selection (Cm^R) marker flanked with appropriate homology arms and Flp Recognition Target sites (**Fig. 2A**, step 2). FRT sites enable for a subsequent removal of the selection marker by Flp recombinase[8]. Therefore plasmid 706-FLP, whose promoter (cI578) and origin of replication (pSC101) allow for a transient *flp* expression, was electroporated into *E. coli* ΔMu::cm^R. Flp activity was induced by a temperature rise (30 °C to 37 °C). Deletion of the FRT-flanked Cm^R marker (**Fig. 2A**, step 3) could be demonstrated by PCR (**Fig. 3B**; PCR 3) and was confirmed by the Cm-sensitive phenotype of *E. coli* ΔMu (**Fig. 3C**).

Application of the Red/ET technology

Red/ET recombination is a patented platform technology which has been successfully commercialised by Gene Bridges GmbH for more than 7 years. Gene Bridges GmbH offers licences, customer service and kits (www.genebridges.com). The technology allows for an efficient genetic engineering (e.g. insertions, deletions or pointmutations), especially of large DNA molecules, since Red/ET cloning does not depend on restrictionendonucleases or *in vitro* clean up steps. The main fields of application are the construction of *E. coli* production strains (metabolic engineering) as well as the preparation of BAC-based targeting constructs for eukaryotic cells.

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Correspondent footnote


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