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Enhanced error-prone RCA mutagenesis by concatemer resolution

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ABSTRACT

Error-prone rolling circle amplification (RCA) is a promising alternative to error-prone PCR for random mutagenesis. The main disadvantage of error-prone RCA is the low transformation efficiency of the DNA concatemer produced in the amplification reaction. We improved the method by introducing *loxP* recombination site of bacteriophage P1 Cre recombinase into the target plasmid and reducing the concatemer by Cre recombinase to plasmid-sized units, increasing the number of transformants 50-fold in non-error-prone and 13-fold in error-prone conditions. The efficiency improvement was verified by obtaining 115 ± 57 ceftazidime resistant colonies per recombined RCA reaction from randomly mutated TEM-1 β-lactamase gene library whereas only 9 ± 11 colonies were gained without recombination. Supplementation of the error-prone RCA with Cre/*loxP* recombination is a simple and useful tool to increase the transformable library size.

1. Introduction

The advantages of error-prone RCA in random mutagenesis are that random hexamers can be used for priming, there is no need for a thermal cycler and the base misincorporation preference differs from error-prone PCR in $MnCl_2$-doped reactions (Fujii et al., 2004; Shafikhani et al., 1997). Error-prone RCA is especially suited for whole-plasmid based mutagenesis strategies rivaling the commonly used *Escherichia coli* mutator strains (Greener et al., 1997; Horiuchi et al., 1978) by its speed and consistency. In randomly primed RCA a double-stranded hyper-branched DNA product is formed consisting of tandemly repeated copies of the target plasmid (Dean et al., 2001). In the earlier reports on error-prone RCA the concatemer is directly transformed to host and segregation to single plasmid units is speculated to occur by homologous recombination in the host cells (Fujii et al., 2004, 2006).

We performed error-prone RCA on a 5.2 kb DNA plasmid encouraged by the simplicity of the method (Fujii et al., 2004). With a similar protocol described by Fujii et al. (2004, 2006) we were able to generate none or only few ceftazidime resistant (ctzR) TEM-1 β-lactamases due to very low transformation efficiency. The transformability of the DNA concatemer formed in the multiply-primed RCA reaction can be improved by modifying the DNA into plasmid-sized circular units.

Earlier this has been experimented by cutting the concatemer to plasmid-sized fragments with a unique restriction enzyme and circularizing the fragments in appropriately diluted reaction to favor self-ligation (Christ et al., 2006). The reported method requires two enzymatic reactions and a DNA purification step. We replaced this time-consuming multistep procedure with Cre/*loxP* recombination containing a single recombinase addition and incubation step to yield highly transformable circular DNA. When *loxP* site is present in the target plasmid a concatemer of directly repeated *loxP* sites is produced in the RCA reaction. The DNA concatemer is an ideal substrate

Abbreviations: CTZ, ceftazidime; ctzR, ceftazidime resistant colony; ampR, ampicillin resistant colony.

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for the Cre/*loxP* recombination, because Cre recombinase catalyzes the excision of the intervening DNA of directly repeated *loxP* sites in circular form (Metzger and Feil, 1999).

2. Materials and methods

2.1. Vectors and reagents

Vector pEB91 is a phagemid carrying chloramphenicol-resistance selectable marker and designed for phage display of recombinant proteins on the filamentous phage p9 coat protein (Brockmann, 2010). The vector pAK400ampR was constructed from pAK400 (Deyev et al., 2003) by replacing the chloramphenicol acetyl-transferase gene with *TEM-1* β-lactamase gene from pET-11a (Merck, Darmstadt, Germany). All modifying enzymes, phi29 DNA polymerase and restriction enzymes used in this work were purchased from Fermentas (St. Leon-Rot, Germany), except Cre recombinase (NEB, Ipswitch, UK). Sequencing in this study was performed at the Sequencing service unit of the Center of Biotechnology (Turku, Finland).

2.2. Cloning loxP cassette to target vectors

The target vectors pEB91 and pAK400ampR were digested with *HindIII* and dephosphorylated with Calf Intestine Alkaline Phosphatase. The vector pEB91 was gel extracted (Qiagen, Hamburg, Germany) and pAK400ampR purified with PCR purification kit (Qiagen, Hamburg, Germany).

Two HPLC purified oligonucleotides *LoxP1*-for and *LoxP1*-rev (Biomers, Ulm, Germany) containing the Cre recombinase recognition sequence (sequence given in Fig. 1) were phosphorylated with 10 U T4 Polynucleotide Kinase for 30 min at 37 °C, heat inactivated for 10 min at 70 °C and hybridized at 1:1 M ratio by decreasing temperature 1 °C/ min from 95 °C to 16 °C. The vectors were ligated to the *loxP* cassette at 1:3 M ratio with 2.5 U T4 DNA ligase at RT for 1 h and transformed. Minipreps (Qiagen, Hamburg, Germany) were prepared and the presence of *loxP* among the transformants was verified by *KpnI* restriction analysis and sequencing.

2.3. RCA and recombination of pEB91loxP

10 ng pEB91loxP was amplified in phi29 DNA polymerase buffer with 500 μM dNTPs, 50 μM exo-resistant random primers (Fermentas, St. Leon-Rot, Germany) and 5 U phi29 DNA polymerase in 10 μl reaction volume at 30 °C overnight (o/n). The RCA reaction was heat inactivated at 70 °C for 10 min, diluted in 50 μl Cre recombinase buffer supplemented with 1 U Cre recombinase and incubated at 37 °C for 2 h.

Alternatively, the 10 μl RCA reaction was diluted in 50 μl Red buffer (Fermentas) supplemented with 10 U *HindIII*, incubated at 37 °C for 2 h and purified by PCR purification kit (Qiagen). DNA fragments were self-ligated with 1 U of T4 DNA ligase in 50 μl reaction volume at 16 °C o/n. All reactions were heat inactivated at 70 °C for 10 min and EtOH precipitated with Pellet Paint Co-precipitant (Merck, Darmstadt, Germany) to 10 μl volume. The templates were removed by digesting 100 ng of the precipitated products with 2 U *DpnI* in 10 μl volume at 37 °C for 2 h and transformed to *E. coli* SS320 cells (Sidhu et al., 2000). Dilutions of the recovery were plated on LB agar (25 μg/ml chloramphenicol, 10 μg/ml tetracycline and 0.5% glucose) for colony counting.

2.4. Error-prone RCA and recombination of pAK400ampRloxP

pAK400ampRloxP was heat denatured at 95 °C for 3 min. 25 pg plasmid was amplified in 1x phi29 DNA polymerase buffer with 1.5 mM $MnCl_2$, 1 mM dNTPs, 50 μM random hexamers or exo-resistant random primers (Fermentas, St. Leon-Rot, Germany), 0.05 U Inorganic Pyrophosphatase (Fermentas, St. Leon-Rot, Germany) and 20 U phi29 DNA polymerase in 20 μl reaction volume at 30 °C o/n. The RCA reaction was heat inactivated at 70 °C for 10 min and 10 μl of the reaction was recombined and concentrated as earlier. Samples were transformed to XL-1 Blue cells (Stratagene, La Jolla, CA) for plating on LB agar containing 200 μg/ml ampicillin or 1 μg/ml ceftazidime and colonies counted after o/n incubation at 37 °C.

The MIC values of the TEM-1 variants were determined as described in Baldwin et al. (2008), except that the cultures were grown o/n at 37 °C supplemented with 1 μg/ml ceftazidime and 1% glucose.

3. Results and discussion

3.1. The efficiency of concatemer resolution by recombination

The *loxP* site (Fig. 1) was cloned to two target vectors: 6.1 kb pEB91 for testing recombination efficiency (named pEB91loxP) and 5.2 kb pAK400ampR for applying the method to select for ceftazidime resistant TEM-1 β-lactamase variants by error-prone RCA (named pAK400ampRloxP). To construct the insertion cassette, two complementary oligos were hybridized, creating cohesive *HindIII* sites for direct destination vector insertion. The recognition sequence of the right hand *HindIII* was destroyed by the oligo design (AAGCTT → GAGCTT) leaving only one *HindIII* site in the vector post-integration. The unique *KpnI* site was used for restriction enzyme digestion analysis to screen for positive clones. The described *loxP* insertion method can be modified to suit any vector by changing the boxed *HindIII* sequences in Fig. 1 with the unique restriction enzyme site present in the target vector.

Ligation of both the gel extracted and affinity purified vectors to the *loxP* cassette occurred with equal success estimated by the difference in the number of transformants on the insert and vector ligation plate and the vector ligation control plate. Therefore, gel extraction of the vector is unnecessary for cloning.

In theory, the *loxP* cassette is integrated in both orientations in the palindromic HindIII site with equal probability. In fact, it was also found in both orientations (Fig. 2: *LoxP* and *LoxP* reversed) as well as a tandem repeat (Fig. 2: tandem *LoxP*) among the sequenced pEB91loxP transformants. The three different constructs were amplified by RCA and the