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Engineering of DNA polymerase I from *Thermus thermophilus* using compartmentalized self-replication

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Although compartmentalized self-replication (CSR) and compartmentalized partnered replication (CPR) are powerful tools for directed evolution of proteins and gene circuits, limitations remain in the emulsion PCR process with the wild-type *Taq* DNA polymerase used so far, including long run times, low amounts of product, and false negative results due to inhibitors. In this study, we developed a high-efficiency mutant of DNA polymerase I from *Thermus thermophilus* HB27 (*Tth* pol) suited for CSR and CPR. We modified the wild-type *Tth* pol by (i) deletion of the N-terminal 5′ to 3′ exonuclease domain, (ii) fusion with the DNA-binding protein Sso7d, (iii) introduction of four known effective point mutations from other DNA polymerase mutants, and (iv) codon optimization to reduce the GC content. Consequently, we obtained a mutant that provides higher product yields than the conventional *Taq* pol without decreased fidelity. Next, we performed four rounds of CSR selection with a randomly mutated library of this modified *Tth* pol and obtained mutants that provide higher product yields in fewer cycles of emulsion PCR than the parent *Tth* pol as well as the conventional *Taq* pol.

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1. Introduction

Polymerase chain reaction (PCR), a powerful technique for the amplification of DNA, has been elaborated upon in many ways since its introduction in 1985 [1]. The applications of PCR range broadly across many research fields, including medicine and forensics [2]. Since the first description and presentation of PCR at Cold Spring Harbor [3,4], scientists have been attempting to improve PCR techniques. A major breakthrough was the replacement of DNA polymerase (pol) with an enzyme from a thermostable species to eliminate the requirement of new enzyme addition to the reaction after each cycle [5]. Several thermostable DNA polymerases have been isolated from a handful of thermophilic bacteria, including *Thermus aquaticus* (*Taq*) [6], *Thermus thermophilus* (*Tth*) [7–10] and *Pyrococcus furiosus* (*Pfu*) [11]. These enzymes are extremely thermostable at a high temperature of 95 °C. Among the commercially available enzymes, *Tth* DNA polymerase (*Tth* pol) possesses reverse transcriptase activity in the presence of Mn^{2+} ions and can thus be used in RT-PCR. *Tth* pol shows optimal activity at temperatures between 70 °C and 74 °C, with an extension rate of 1.5 kb/min [7]. Although its half-life is comparatively shorter (20 min at 95 °C), *Tth* pol tolerates higher concentrations of inhibitory components than *Taq* pol [12].

Despite the promising performance of PCR, the remaining limitations to PCR are its product yield, the length of DNA that can be amplified, the speed of the polymerase, and the fidelity of the process. Ultimately, PCR run time is solely influenced by the kinetic properties of the PCR enzyme. Currently, faster PCR throughput can be achieved by reducing the time of the denaturation, annealing and extension steps or by using 2-step cycling that combines the annealing and extension steps. However, shorter cycle times yield lower detection signals and higher failure rates. Alternatively, increasing the enzyme concentration, adjusting buffer conditions and reducing the reaction volume can all improve the resultant yields. Another approach to enhance the processivity of polymerase is its fusion to a sequence non-specific dsDNA-binding protein, such as Sso7d from *Sulfolobus solfataricus*, whose fusion does not affect the catalytic activity and thermal stability of the *Taq* and *Pfu* polymerases but enhances their processivity [13].

In this study, we attempted to improve the performance of DNA polymerase I from *Tth* HB27 [14] for compartmentalized self-replication (CSR) [15] (Fig. 1). CSR is a powerful method for directed evolution of polymerase with desired functions such as

Abbreviations: CPR, compartmentalized partnered replication; CSR, compartmentalized self-replication.

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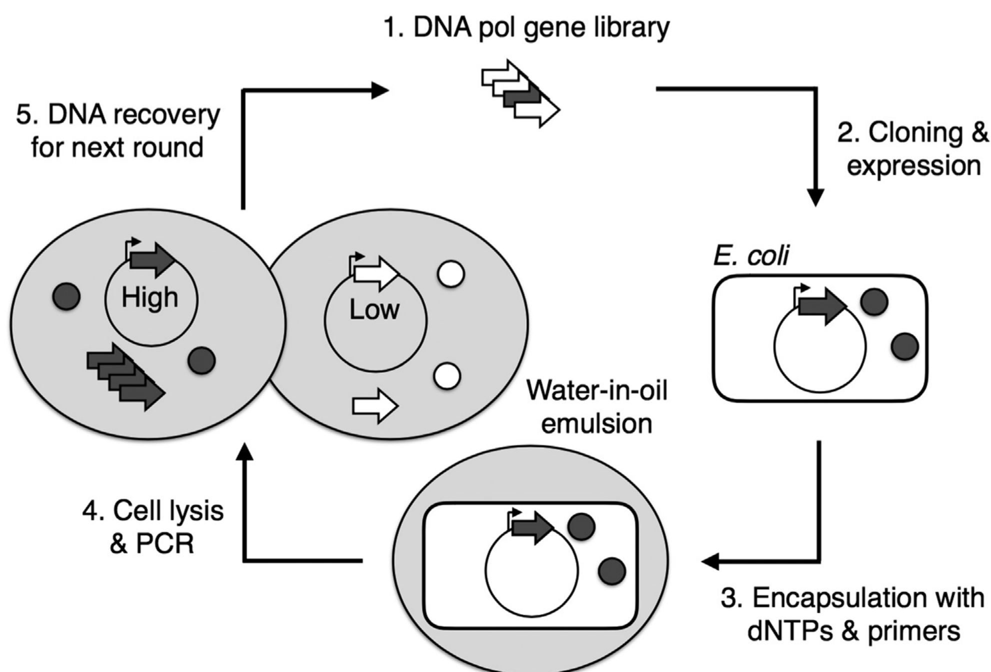

Fig. 1. Schematic representation of the directed evolution of DNA polymerase (pol) using CSR [15]. (1) A randomly-mutated DNA pol gene library was prepared by error-prone PCR. (2) The library was cloned and transformed into *E. coli* cells, and the expression of DNA pol was induced. (3) After harvesting, the cells were resuspended in PCR buffer with dNTPs and primers, and then encapsulated in a water-in-oil emulsion. (4) After the heat lysis of each cell in each micelle, genes encoding highly active DNA pol mutants were amplified by their own enzyme. (5) After the emulsion-PCR, the amplified genes were recovered and cloned for further successive selection rounds.

enhanced thermostability, increased resistance to inhibitors and increased DNA-binding activity [16–18]. CSR is performed by compartmentalizing self-replication reactions into individual non-interactive aqueous compartments of a water-in-oil emulsion to ensure a linkage between genotype and phenotype. Recently, the CSR concept was expanded to compartmentalized partnered replication (CPR) for the directed evolution of genetic circuits that can be linked to the production of *Taq* pol [19]. We expect that the improved *Tth* pol mutants constructed in this study will also be useful for the emulsion PCR step of CPR.

2. Materials and methods

2.1. Bacterial strains and culture condition

Escherichia coli strain XL10-Gold (Agilent Technologies, California, USA) was used for DNA cloning, and *E. coli* strain BL21(DE3) codon-plus RIPL (Stratagene, California, USA) was used for protein expression. Bacterial cultures were grown at 37 °C in 2 × YT or LB media containing kanamycin (25 μg/ml) (Wako, Japan), ampicillin (100 μg/ml), or both according to the required protocol. IPTG (Sigma Aldrich Japan, Tokyo, Japan) was added to the culture at the required concentration when necessary.

2.2. Plasmids, DNA manipulation and transformation

DNA polymerase I (*Tth* pol) gene was amplified from the genome of *Thermus thermophilus* HB27 strain. A modified *Tth* gene (Supplementary Fig. S1a) and the wild-type *Taq* DNA polymerase gene (*Taq* pol) were synthesized by Thermo Fisher Scientific (MA, USA). The details of the construction of all plasmids encoding *Tth* pol mutants were described in Supplementary Methods.

For cloning purposes, we applied the Gibson assembly protocol [20] using primers with overlapped regions. In detail, for each ligation reaction, 50 ng of each PCR amplified insert and vector DNA (2 μl) were mixed with an equal volume of 2 × Gibson Assembly (GA) mixture. The ligation reaction was kept at 50 °C for 15 min and maintained on ice before transformation. Transformation into XL10-Gold cells was performed using a heat shock protocol at 42 °C for 30–45 s. After shaking for 1 h at 37 °C in SOC, cells were plated on agar plates containing the appropriate antibiotic(s) and were incubated overnight at 37 °C.

Sequencing of the plasmids was confirmed by using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Framingham, MA) and the primers T7promoter-F, OptFwdforSeq, OptRevforSeq and T7terminator-R (Supplementary Table S1) with ABI PRISM 3130xl genetic analyzer (Applied Biosystems).

2.3. Selection by CSR

CSR was performed essentially as described [15]. Between CSR rounds, collected genes were re-diversified by staggered extension process (StEP) PCR [21]. The detail of the CSR procedure was described in Supplementary Methods.

2.4. Enzyme activity assay

For confirmation of DNA polymerase extension efficiency, a set of primers (Supplementary Table S1) was used to amplify DNA fragments 0.5 kb (T7promoter-F and T7terminator-R), 2 kb (OptFwd and OptRev), 4.65 kb (lambda-F and lambda-4.65k-R) and 7.5 kb (lambda-F and lambda-7.5k-R) in size. PCR was performed at 94 °C for 2–5 min, followed by 10–30 cycles of 94 °C for 15 s, 60 °C for 15 s and 72 °C for 10–60 s/kb. The PCR reactions were performed in a total volume of 200 μl, and the mixture contained 1 × *ExTaq* PCR buffer, 2 mM dNTPs, 0.2 μM of primers, 50 ng of template DNA (lambda-DNA HindIII digest; New England Biolabs) and 0.2×10^8 expressed cells induced with 0.1 mM IPTG for 16 h at 30 °C. The

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