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Thermostable DNA Ligase-Mediated PCR Production of Circular Plasmid (PPCP) and Its Application in Directed Evolution via *In situ* Error-Prone PCR

Yilin Le[1], Huayou Chen[1], Robert Zagursky[2], J.H. David Wu[2,*], and Weilan Shao[1,*]

Biofuels Institute, School of Environment, Jiangsu University, Zhenjiang, Jiangsu 212013, PR China[1] and Department of Chemical Engineering, University of Rochester, Rochester, NY 14627-0166, USA[2]

*To whom correspondence should be addressed. Tel. +86 25-85891836, +585-275-8499.
E-mail: weilanshao@gmail.com (W.S.), davidwu@che.rochester.edu (J.H.D.W.)

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Abstract

Polymerase chain reaction (PCR) is a powerful method to produce linear DNA fragments. Here we describe the *Tma* thermostable DNA ligase-mediated PCR production of circular plasmid (PPCP) and its application in directed evolution via *in situ* error-prone PCR. In this thermostable DNA ligase-mediated whole-plasmid amplification method, the resultant DNA nick between the 5′ end of the PCR primer and the extended newly synthesized DNA 3′ end of each PCR cycle is ligated by *Tma* DNA ligase, resulting in circular plasmid DNA product that can be directly transformed. The template plasmid DNA is eliminated by 'selection marker swapping' upon transformation. When performed under an error-prone condition with *Taq* DNA polymerase, PPCP allows one-step construction of mutagenesis libraries based on *in situ* error-prone PCR so that random mutations are introduced into the target gene without altering the expression vector plasmid. A significant difference between PPCP and previously published methods is that PPCP allows exponential amplification of circular DNA. We used this method to create random mutagenesis libraries of a xylanase gene and two cellulase genes. Screening of these libraries resulted in mutant proteins with desired properties, demonstrating the usefulness of *in situ* error-prone PPCP for creating random mutagenesis libraries for directed evolution.

Key words: thermostable DNA ligase; directed evolution; amplification of circular plasmids; random mutagenesis libraries; error-prone PCR

1. Introduction

Directed evolution methods are increasingly employed to improve properties of an enzyme, including substrate specificity, activity, thermostability, high-temperature activity.[1–4] Success of directed protein evolution hinges on the efficiency in creating mutagenesis libraries and screening the libraries for the desired protein properties.[5] The library diversity is a key parameter and various protocols have been developed to achieve the goal of making high-quality mutagenesis libraries.[5] Random mutagenesis coupled to high-throughput screening[6] has become a powerful tool for both basic study on the structure–function relationship of proteins and development of novel proteins with desired properties.

Many methods used for directed evolution of enzymes were reviewed by Lutz and Patrick.[7] *In vitro* mutagenesis approaches have been described in both polymerase chain reaction (PCR)-based and non-PCR-based categories.[8] One of the most commonly used methods is error-prone mutagenic PCR,[9] which introduces random mutations by reducing the fidelity of the DNA polymerase. Early techniques of error-prone PCR generally involved the following steps: (i) amplifying the target gene under error-prone conditions to

generate randomly mutated sequences; (ii) digesting the ends of the amplified DNA fragment with restriction endonucleases; (iii) ligating the digested DNA fragment into a suitable expression vector with DNA ligase and (iv) transforming the ligated vector into a suitable host cell to obtain a mutant library. This process is similar to standard PCR cloning of a target sequence except that, for constructing a diverse mutagenesis library, a much larger pool of transformants are needed to increase the diversity of the library, making the process tedious and inefficient. As a result, various methods have been developed to streamline the process.

Previously reported procedures for whole-plasmid, mutagenic PCR using the entire plasmid as template and back-to-back primer set resulted in linear DNA products, necessitating a separate ligation step to generate circular plasmids before transforming the host cell.[10,11] MegaWHOP uses mutated target sequence as the PCR primer (Megaprimers), expression vector harbouring the wild-type gene as the template, and a high-fidelity DNA polymerase to produce whole length of plasmid.[12,13] However, the process mainly yields linear single-strand DNA (ssDNA), which is released from its circular template at the denaturation step during PCR. Only some of the ssDNA strands can anneal to form nicked circular double-strand DNA (dsDNA) to be directly transformed without subsequent ligation step (Step iii mentioned above). In addition, the newly extended, linear ssDNA cannot serve as a template to achieve exponential amplification. Finally, the procedure depends on methylation of template DNA and later on digestion of methylated DNA by *Dpn*I, which degrades the methylated plasmid template, before the mutated plasmid products are transformed.

To obtain nick-free, circular plasmid production by PCR and improve the efficiency of random mutagenesis or directed evolution, we report a novel method designated as 'thermostable DNA ligase-mediated PCR production of circular plasmid' (PPCP) for applications in directed evolution via *in situ* error-prone PCR. The PPCP method employs a long dsDNA primer pair to amplify the entire template plasmid. The dsDNA primer pair is designed to carry a selection marker different from the original selection marker of the template plasmid. Transformants are plated under the pressure of the new selection marker, resulting in the elimination of the original template plasmid without the need to pretreat the DNA with *Dpn*I. Furthermore, thermostable DNA ligase from *Thermotoga maritima* (*Tma* DNA ligase), which we previously reported,[14] is employed to ligate the nick of the newly extended DNA strand, resulting in the formation of circular plasmid DNA as the amplification product that could directly be transformed. When *Taq* DNA polymerase is used under error-prone conditions, the method generates random mutagenesis libraries in a fast and efficient manner. By using this method, we performed directed evolution on XynA (*xylanase A*) from *Thermomyces lanuginosus* and CelA (*cellulase A*), CelB (*cellulase B*) from *T. maritima*. The results demonstrated that *in situ* error-prone PPCP is an efficient random mutagenesis method for directed evolution of genes and proteins.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth media

Thermotoga maritima (ATCC43589) was grown anaerobically at 80°C in a medium described previously.[15] *Escherichia coli* BL21(DE3) was used as the host for gene cloning. *Escherichia coli* was cultured in Luria-Bertani (LB) medium which contained (per litre): 10 g Tryptone, 5 g yeast extract, 5 g NaCl, pH 7, and supplemented with 100 μg/ml ampicillin or 50 μg/ml kanamycin for selection. The Hsh (heat shock) system of expression vectors was employed for PPCP tests, which contains an Hsh promoter recognizable by an alternative sigma factor (σ^{32}) of *E. coli*, and the expression of foreign genes was induced by a temperature up-shift.[16]

2.2. Generating long complementary primer pair for swapping plasmid selection maker by PPCP

The complementary PPCP primer pair containing the kanamycin resistance gene (884 bp) used in the experiment shown in Fig. 2 was generated by regular PCR using the plasmid pET-28a(+) as template. The regular PCR primers were KanFwd, 5′-*CCCTGATAAAT GCTTCAATAATATTGAAAAAGGAAGAGT*ATGAGCCATATTCAACGGG-3′, and KanRev, 5′-*ATCAATCTAAAGTATATATG AGTAAACTTGGTCTGAC AG*TTAGAAAAACTCATCGAGC-3′ (italicized DNA sequences complementary to the flanking sequences of the ampicillin gene (863 bp) in the PPCP template pHsh described below; underlined DNA sequences complementary to the kanamycin resistance gene from pET-28a(+)). PCR was performed in 50 μl reaction solution containing 1.25 U *Pyrobest* DNA polymerase (TaKaRa) for 30 cycles, each cycle consisting of incubation at 94°C for 40 s, 52°C for 40 s and 72°C for 1 min 10 s. The 894 bp dsDNA fragment generated was subsequently used as PPCP primer pair for amplifying whole pHsh plasmid DNA as described below.

2.3. Amplification of whole pHsh plasmid DNA by PPCP with simultaneous selection maker swapping

The PPCP experiment shown in Fig. 2 was performed in 20 μl reaction solution containing 50 ng pHsh-*Amp* (2.44 kb) as template, 0.5 U *Taq* DNA polymerase (TaKaRa), 0.1 μg/μl *Tma* DNA ligase,[14] 80 ng PPCP primer pair prepared by PCR as described above,

0.2 mM each deoxynucleotide triphosphate, 2.0 mM $MgCl_2$ and 0.5 mM NAD^+. PCR was performed for 25 cycles with each cycle consisting of incubation at 94°C for 30 s (denaturation), 62°C for 30 s (annealing), 72°C for 1.5 min (extension) and 60°C for 2 min (ligation).

2.4. *Preparation of long primer pair from pHsh for* in situ *error-prone PPCP mutagenesis*

To amplify the entire pHsh expression vector by regular PCR for preparing the long primer pair used for the PPCP experiment shown in Fig. 4, the following regular PCR primers were used: pHshRev 5′- pCCTCC ATGGGTATATCTCCTT-3′ and pHshFwd 5′-pAAGCTTGA AGGCCGCTTCCGA-3′. The plasmid pHsh-*kan* (GenBank accession no. FJ571621) or pHsh-*amp* (GenBank accession no. FJ571619) was used as template. PCR of 30 cycles was performed in 50 μl of reaction solution containing 1.25 U *Pyrobest* DNA polymerase (TaKaRa), each cycle consisting of incubation at 94°C for 30 s, 54°C for 30 s and 72°C for 2 min.

2.5. In situ *error-prone PPCP mutagenesis*

Error-prone PPCP was performed in 20 μl reaction solution containing 30 ng plasmid pHsh-*xynA*1, pHsh-*celA* or pHsh-*celB* (see below for their preparations) as template as described in the above, 0.5 U *Taq* DNA polymerase (TaKaRa), 0.1 μg/μl *Tma* DNA ligase,[14] 200 ng PPCP primer pair from pHsh described above, 0.2 mM each deoxynucleotide triphosphate, 2.0 mM $MgCl_2$, 0.5 mM $MnCl_2$ and 0.5 mM NAD^+. Each cycle consisted of heating at 94°C for 1 min, annealing and extension at 72°C for 1.5 min and ligation at 60°C for 2 min.

2.6. *Construction of plasmids pHsh-celA, pHsh-celB and pHsh-xynA1*

Endoglucanase gene *celA* was amplified by PCR using the genomic DNA of *T. maritima* as template and the following primers: 5′-CTGTGGTACTGATGACAAAACCGG GAACATC-3′ and 5′-GGGAAGCTTTCATTCTCTCACCTC CAGATC-3′. The amplicon was inserted into expression vector pHsh-*amp* at *Stu*I/*Hin*dIII sites to generate plasmid pHsh-*celA*. Endoglucanase gene *celB* of *T. maritima* was similarly amplified using primers 5′-CTA GCGTTGGTGCAACGGAC-3′ and 5′-GGGCTCGAGTTA TTTTACAACTTCGACAG-3′, and cloned into pHsh-*amp* at *Stu*I/*Xho*I sites, resulting in pHsh-*celB*. pHsh-*xynA*1 was generated by cloning the *xynA*1 from *Thermomyces lanuginosus* as previously described.[17]

2.7. *Screening xylanase and cellulase random mutant libraries*

Mutant libraries of *celA*, *celB* and *xynA*1, respectively, were constructed by *in situ* error-prone mutagenic PPCP as described above. The resulting mutant libraries were transformed into *E. coli* by electroporation and screened for xylanase or cellulase activity by overlay assays. For detecting xylanase activity, *E. coli* colonies were overlaid with LB top agar containing 2% xylan and kanamycin (50 μg/ml), incubated at 30°C overnight and then the temperature shifted to 42°C for 5–6 h to induce gene expression. Positive clones were identified by a clear zone surrounding xylanase-expressing colonies. Cellulase activity was similarly detected as follows. *Escherichia coli* colonies were overlaid with LB top agar containing 2% carboxymethylcellulose (CMC, Sigma) and kanamycin (50 μg/ml). After incubation at 30°C overnight, the temperature was shifted to 42°C for 5–6 h to induce gene expression. Positive clones were identified by a depressed zone surrounding cellulase-expressing colonies.

2.8. *Cellulase activity assay*

Cell lysates from cells harbouring a cellulase gene were prepared by sonication and used as the source of cellulase. The mutant and wild-type enzyme preparations were prepared from the same amount of cells. For cellulase assay, reducing sugars released from CMC by the cellulase activity in the cell lysate were quantified by using the 4-hydroxybenzoic acid hydrazide method.[18] The reaction mixture comprises 100 μl of 0.5% (w/v) CMC in water, 90 μl of phosphate buffer (50 mM, pH 6.0) and 10 μl of properly diluted enzyme. After incubation at 90°C for 10 min, the reaction was stopped by adding 600 μl of 4-hydroxybenzoic acid hydrazide solution to the reaction mixture. The test tubes were incubated for 10 min in boiling water and chilled on ice. Reducing sugars were determined by measuring optical density at 410 nm using a spectrophotometer. One unit of cellulase activity is defined as the amount of enzyme releasing 1 μmol/ml reducing sugars using glucose as the standard.

3. Results

3.1. *Swapping plasmid selection marker by thermostable DNA ligase-mediated PPCP*

PCR is a powerful method to amplify linear DNA fragments, which consists of repeated cycles of denaturation–annealing–elongation. Recently, we reported that adding a ligation step mediated by thermostable *Thermotoga maritima* (*Tma*) DNA ligase to form the repeated cycles of 'denaturation–annealing–elongation–ligation' enhances the amplification of long DNA fragment.[14] In both cases, the resulting PCR products are linear DNA. Here we describe that *Tma* thermostable DNA ligase can be employed in a new design for PCR amplification of circular plasmids. The procedure for thermostable DNA ligase-mediated PPCP is schematically illustrated in Fig. 1. In this

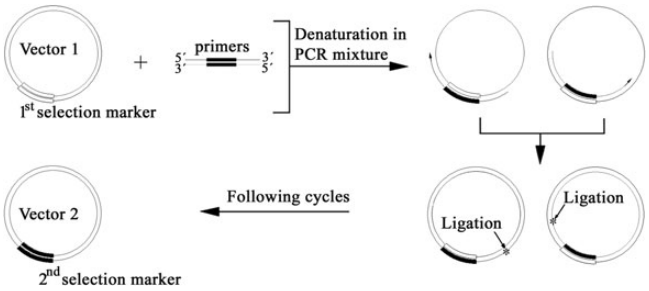

Figure 1. Schematic of swapping selection marker of a plasmid by thermostable DNA ligase-mediated PPCP. The white boxed-region of the template plasmid (Vector 1) represents the original selection marker, which after the whole-plasmid PCR is replaced by a second selection marker built in the double-stranded PPCP primers (the black boxed-region in the primers and Vector 2). The procedure features the incorporation of a thermostable ligation step into the standard PCR procedure to ligate the nick remaining in the extended DNA strand, resulting in nick-free, circular plasmid DNA products. At the end of the PCR cycles, the template plasmid is eliminated by plating the plasmid-transformed host cells under the second selection pressure.

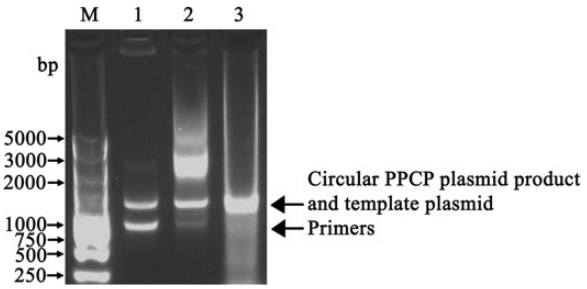

Figure 2. Agarose gel analysis of the thermostable DNA ligase-mediated PPCP using pHsh-*amp* as template. Lane M, DL5000 markers; Lane 1, control reaction containing template plasmid and primer but no *Taq* DNA polymerase or ligase; Lane 2, control reaction containing *Taq* DNA polymerase but no ligase; Lane 3, PPCP reaction containing both *Taq* DNA polymerase and *Tma* DNA ligase. The upper band in lane 2 is presumed to be linear PCR product. Incorporating the nick ligation step resulted in a nick-free, circular PPCP product (lane 3). The size of the PPCP primer pair was 962 bp.

method, circular PCR products are generated by using a long complementary primer pair, which can be generated by regular PCR or synthesized, to extend over a circular template, followed by DNA ligation of the resultant nick between the 3′ end of the newly extended strand and the 5′ end of the primer. Furthermore, if the complementary primer set is designed to contain a second selection marker, the resulting plasmid product will harbour this new marker (Fig. 1). The template plasmid carrying the original marker is eliminated when transformed host cells are cultured under the second selection pressure, eliminating the need of digesting PCR products with a methylated DNA-specific restriction enzyme mentioned above. The ability to swap the selection marker facilitates multiple rounds of mutagenesis and selection as will be discussed below.

To demonstrate the PPCP and marker swapping concepts, we used a 2.44 kb plasmid, pHsh-*amp*, harbouring the ampicillin-resistant gene (*amp*^{r}) as the PPCP template. The PPCP primer pair was a linear 894 bp dsDNA fragment generated by standard PCR using pET-28a(+) as template as described in Materials and methods. The PPCP primer pair thus prepared harboured the kanamycin-resistant gene (*kan*^{r}) flanked by regions (39 bp each) complementary to the flanking sequences of *amp*^{r} in pHsh-*amp*. The PPCP primer pair was thus able to amplify the entire pHsh while simultaneously swapping the selection marker from Amp^r to Kan^r as illustrated in Fig. 1.

PPCP was carried out with 25 cycles each consisting of denaturation of double-stranded template and DNA primer pair, annealing of primers to the respective complementary regions of the template extension of each primer over the template sequence, and ligation of the nick between the 3′ end of the newly extended strand to the 5′ end of the primer. The ligation step took advantage of the thermostable nature of *Tma* DNA ligase,[14] allowing the enzyme to maintain its activity during multiple heating steps of PCR at 94°C and resulting in a nick-free, circular plasmid product ready to serve as a template in the subsequent PPCP cycles.

To verify that nick-free, circular DNA plasmids were obtained, the PPCP products were analysed by electrophoresis on 1.0% agarose gel. As shown in Fig. 2, the double-stranded PPCP primer pair ran faster than the circular template pHsh DNA (lane 1). When *Tma* DNA ligase was not included in the amplification reaction, PCR DNA product ran slower than the circular plasmid DNA, which is presumed to be linear DNA product (lane 2). In contrast, with the inclusion of *Tma* DNA ligase in the amplification mixture (PPCP), the DNA product appears to be circular as it migrated to the same position as the circular template pHsh-*amp* DNA (lane 3), demonstrating that PPCP is capable of producing nick-free, circular DNA products. Furthermore, *E. coli* cells transformed by the PPCP amplification product were able to grow on LB plates containing kanamycin (data not shown), demonstrating successful swapping of the antibiotic selection marker.

To demonstrate the important role of *Tma* DNA ligase in PPCP, we studied the effect of varying the amount of *Tma* DNA ligase on generating circular amplification product. We again observed that, without *Tma* DNA ligase, no circular DNA product was obtained (lane 1, Fig. 3). In contrast, an increasing amount of circular DNA product was obtained with increasing amount of *Tma* DNA ligase added to the reaction mixture (lanes 2–5, Fig. 3), indicating the need to determine the sufficient amount of DNA ligase required for optimal ligation to compensate for enzyme inactivation at high temperature during the denaturation step of the cycle.

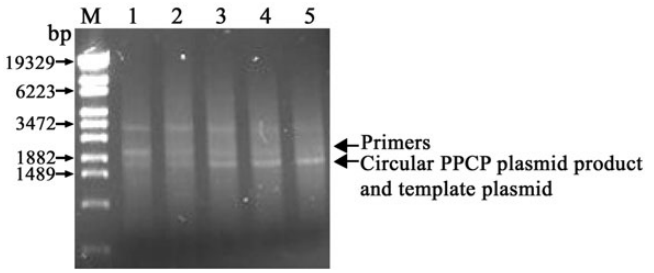

Figure 3. Optimization of thermostable DNA ligase-mediated PPCP by varying the amounts of *Tma* DNA ligase in 20 μl reaction. Lane M: λ EcoT14\xE2\x85markers; Lanes 1–5: 0, 0.06, 0.1, 0.2 and 0.3 μg of *Tma* DNA ligase, respectively. The PPCP long primer pair (2361 bp) was generated using pHshRev and pHshFwd as PCR primers, pHsh-*kan* as template, and Pyrobest DNA polymerase as described in Materials and methods. PPCP was carried out using pHsh-*xynA*1 as template and *Taq* DNA polymerase as described in Materials and methods. The unlabelled, upper band is presumed to be un-ligated, open circle plasmid as its amount diminished with the increasing amount of *Tma* ligase.

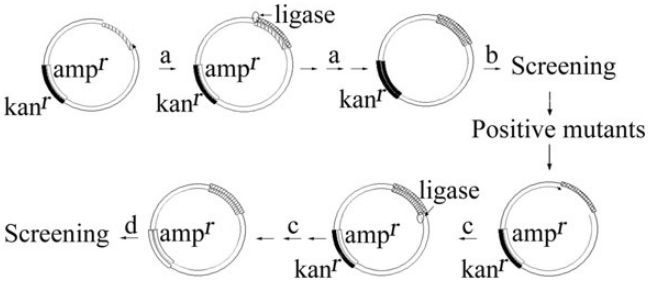

Figure 4. Schematic of directed evolution by *in situ* error-prone PCR using the thermostable DNA ligase-mediated PPCP method to create random mutagenesis libraries. (a) *In situ* error-prone PCR using PPCP. Primers (amplified from the vector with a swapped selection marker, e.g. from *amp^r* to *kan^r* by PPCP as described in the legend to Fig. 1) anneal to and cover the entire template plasmid sequence minus that of the target sequence (slashed boxed region); PPCP is performed as described in the legend to Fig. 1, except that the target DNA sequence is synthesized under error-prone conditions by using mutagenic DNA polymerase. (b) Transforming the circular PCR products into host cells and selecting for the second marker while screening mutants for desired mutation. (c and d) The process of (a) and (b) can be repeated for multiple rounds of PPCP mutagenesis, marker selection and functional screening.

3.2. in situ *error-prone PPCP for creating random mutagenesis libraries*

When *Taq* DNA polymerase is employed under error-prone conditions, e.g. in the presence of Mn^{2+} (mutagenic PCR), PPCP can be used for generating random mutagenesis libraries. Since mutagenesis occurs *in situ* during the PCR reaction, eliminating the need of a separate process for generating or sub-cloning mutant DNA, we designated the procedure as *in situ* error-prone PPCP. In addition, since the extended DNA strand is ligated and serves as the template for the next round of amplification, mutations can accumulate over the subsequent PCR cycles. Furthermore, the target gene or gene segment can precisely be chosen by proper design of the long PPCP primer pair. Finally, the ability to swap a selection marker in PPCP facilitates the removal of the template plasmid during multiple rounds of mutagenesis. The *in situ* error-prone mutagenic PPCP can be coupled to a screening or selection procedure, facilitating directed evolution of the target gene or protein.

Figure 4 depicts the procedure of *in situ* error-prone PPCP. The target gene is cloned into the first vector carrying a selection marker, which serves as template. A second vector, which is the same as the first vector except for the selection marker and missing the DNA segment to be mutagenized, is linearized by regular PCR and serves as PPCP primer pair.

To experimentally demonstrate the *in situ* error-prone PPCP concept, we chose a pair of identical expression vectors comprising different selection markers. Expression plasmid pHsh-*xynA*1 was constructed by cloning the xylanase gene (*xynA*1) from *T. lanuginosus* into vector pHsh-*amp* and used as the PPCP template. To generate the PPCP primer pair, a linear segment of pHsh-*kan* was amplified by regular PCR using pHshFwd and pHshRev PCR primers designed to amplify the entire pHsh-*kan* except for the multiple cloning sites. Error-prone PPCP using *Taq* DNA polymerase in the presence of Mn^{2+}, *Tma* DNA ligase and the PPCP primer pair from pHsh-*kan* was performed over the *xynA*1 region of pHsh-*xynA*1 template (Fig. 4).

The resulting PPCP products were analysed by agarose gel electrophoresis on 1.0% agarose gel (Fig. 5A). Again, circular DNA molecules were observed only when *Tma* DNA ligase was added (lane 3). Transformants of circular PPCP products were obtained by selection on LB plates containing kanamycin. The xylanase activity of the transformants was determined by the xylan-overlay assay. The clear zones around colonies indicate active clones (Fig. 5B). The size of the clear zone reflects the relative activity of the mutant while the lack of it indicates null mutation.

Using the xylan-overlay assay, we were able to determine the frequency of the null mutation as a function of Mn^{2+} concentration and the number of thermal cycles. The results showed that the frequency of null mutations increased with the concentration of Mn^{2+} and the cycle number of error-prone PPCP within the ranges tested (Table 1).

3.3. *Directed evolution of cellulase genes by* in situ *error-prone PPCP mutagenesis*

We used *in situ* error-prone PPCP to improve the activities of two cellulases from *T. maritima*. The *T. maritima celA* and *celB* genes were individually cloned into expression vector pHsh-*amp* to generate pHsh-*celA*

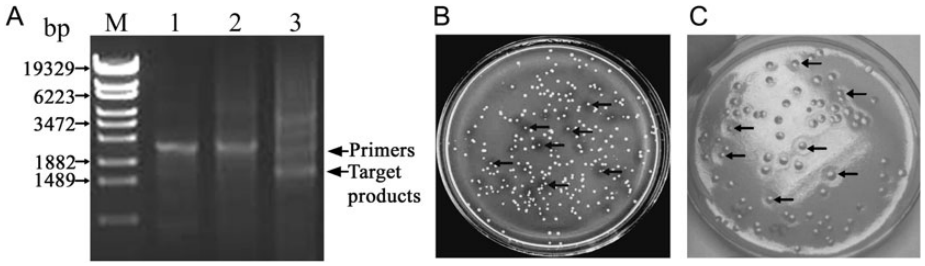

Figure 5. Directed evolution of xylanse and cellulase as examples of *in situ* error-prone mutagenic PCR mutagenesis using thermostable DNA ligase-mediated PPCP. (A) Agarose gel analysis of the PPCP mutagenesis products using pHsh-*xynA*1 as template and the PPCP primer pair from pHsh-*kan*. Lanes: M, λ EcoT14\xE2\x85 markers; 1, control reaction containing no *Taq* DNA polymerase or *Tma* ligase; 2, control reaction containing *Taq* DNA polymerase but no *Tma* ligase; 3, PCR containing both *Taq* DNA polymerase and *Tma* ligase. (B) Functional screening of *E. coli* transformants for xylanase activity using xylan-overlay assay as described in Materials and methods. Positive clones were identified by clear zones surrounding xylanase-expressing colonies (arrows). The mutants were derived from pHsh-*xynA*1. The size of *xynA*1 was 591 bp. (C) Functional screening of *E. coli* transformants for cellulase activity by CMC-overlay assay as described in Materials and methods. Positive clones were identified by depressed area surrounding cellulase-expressing colonies (arrows). The mutants shown were derived from pHsh-*celB*.

Table 1. Effects of Mn^{2+} concentration and PPCP cycle number on null mutation frequency of xylanase

In situ error-prone PPCP	Total number of transformants	Frequency of the null mutant clones (%)
Mn^{2+} concentration (mM)[a]		
0	446	15
0.1	509	58
0.3	461	76
0.5	561	89
Number of cycles[b]		
15	471	59
25	1028	85

[a]15 PCR cycles at various Mn^{2+} concentration.
[b]0.1 mM Mn^{2+} concentration with various number of PCR cycles.

and pHsh-*celB* as described in Materials and methods. With each of these two plasmids serving as template, the same primer pair from pHsh-*kan* described above was used to construct random mutagenesis libraries by *in situ* error-prone PPCP. The amplification was performed over *celA* and *celB* using *Taq* DNA polymerase in the presence of 0.1 mM Mn^{2+} and *Tma* DNA ligase. The amplification products were transformed into competent *E. coli* cells, and the mutant libraries screened on the LB plates containing kanamycin and CMC. Colonies producing larger depressed zones than wild-type transformants were identified as positive mutant clones displaying higher cellulase activities (see Fig. 5C showing plate of *celB* mutants).

Approximately 2000 *celA* and *celB* clones were screened. The positive mutants were further cultured at 30°C and then 42°C. A positive mutant of *celA*, designated as *celA*-m1, exhibited 4.4 U/ml cellulase activity while the wild-type strain exhibited 2.9 U/ml activity when cells were grown under the same culture condition to the same cell density ($OD_{600} = 3.2$). DNA sequence analysis revealed that *celA*-m1 contained five-point mutations, but only one non-synonymous codon change that resulted in the substitution of serine (TCC) for phenylalanine (TTC) at residue 102 (F102S). On the other hand, the synonymous (silent) mutations occurred at amino acid positions L203 (CTT → CTA), L222 (CTT → CTC), G234 (GGA → GGT) and A241 (GCA → GCT). A positive mutant of *celB*, designated as *celB*-m1, produced cellulase activity of 3.88 U/ml while the wild-type strain produced 0.89 U/ml under the same culture conditions. DNA sequence analysis revealed a single-point alteration at codon 61, H61L (CAT → CTT). These results confirm the *in situ* error-prone PPCP procedure in being able to rapidly mutate a target gene.

4. Discussion

Tma DNA ligase from the hyperthermophilic bacterium *T. maritima* has a half-life of over 30 min at 95°C and thus is an ideal DNA ligase for PCRs.[14] *Tma* DNA ligase requires NAD^+, and a divalent cation such as Mg^{2+}, Mn^{2+} or Ca^{2+}. Therefore, the ligase activity is enhanced when Mn^{2+} is added to the reaction mixture, which has an added benefit of increasing the mutation rate of error-prone PCR. Our finding that Mn^{2+} increased the rate of null mutation (Table 1) is consistent with the error rate of *Taq* polymerase being dependent on the concentration of the cation. It is of note that *Tma* DNA ligase exhibits activity on DNA fragments with cohesive termini, but not on blunt-ended DNA molecules. Thus the ligase is unable to ligate the double-stranded PPCP primer pairs used in the reaction.

In conventional mutagenic PCR employing *Taq* DNA polymerase under error-prone conditions, the PCR products have single-base overhangs at the 3′ end of each

strand,[19] which often needs to be removed or filled-in before cloning. In addition, mutant DNA fragments must be digested and ligated to a vector to obtain a mutant library.[13,20–22] Low efficiency of the digestion and ligation steps is a common challenge, and the library is often plagued with plasmids with no or multiple inserts.[23] In the *in situ* error-prone PPCP method described here, thermostable DNA ligase repairs the nick, forming a nick-free, circular plasmid and circumventing many of the issues associated with conventional mutagenic PCR.

The double-stranded PPCP primer pair can be synthesized by conventional PCR. Specifically, a pair of conventional PCR primers can be designed to amply the corresponding region of an expression vector, which comprises the central region harbouring the selection marker, and the flanking regions of the cloning site. This dsDNA PCR product can be used as the primer pair in subsequent *in situ* error-prone mutagenic PPCP. For the experiments reported in this work, the primer pairs were amplified by a high-fidelity polymerase such as *Pyrobest* or *Pfu* DNA. The primer pair should be phosphorylated at 5′ ends for the ligase reaction.

PPCP primer strands are complementary to each other, thus self-annealing of the primer strands may compete with the annealing between the primer strands and the DNA template, decreasing the efficiency of the process. This problem can be alleviated by optimizing the ratio of primer pair to template DNA. Another important parameter is the temperature setting for the ligation step. Although DNA polymerase and DNA ligase have the maximal activities at 72 and 60°C, respectively, both enzymes exhibit sufficient activity within the range of 60–72°C. Even though we found that setting ligation temperature at 60°C works well for the purpose, it is likely that annealing, elongation and ligation all occur at all of the three temperature settings albeit to a different degree of activity.

In contrast to the conventional mutagenic PCR protocols, the PPCP method eliminates background transformants including those without the insert or target sequence that reduce the efficiency in library screening.[24] Thus the need to run vector-only control to gauge the level of background resulting from vector self-ligation is eliminated.

In contrast to the whole-plasmid PCR of the MegaWHOP procedure, the PPCP method reported in this work facilitates exponential amplification. In the PPCP procedure, a newly extended DNA strand is ligated to form a circular DNA, which serves as a new template in the next PCR cycle. Furthermore, the product mixture of MegaWHOP must be digested by a *dam*-methylated DNA-specific restriction enzyme, such as *Dpn*I, to reduce the template plasmid.[13,25] In the *in situ* error-prone mutagenic PPCP method reported in this work, the use of the primer pair harbouring a selection marker, e.g. a drug resistance gene that is different from that of the template plasmid, allows marker swapping that completely eliminates the growth of the transformants of template plasmids without the need of *Dpn*I digestion. Thus no further treatment is needed for the PPCP products, which can be directly transformed into host cells. The differences between MegaWHOP and PPCP are summarized in Table 2.

In PPCP, a generic primer pair can be employed for different target genes cloned into the multi-cloning sites of an expression plasmid vector. Such generic primer pair contains the entire vector sequence but missing the target gene in the cloning site (Fig. 4). A second pair of primers containing a different selection marker can be similarly prepared so that multiple rounds of 'error-prone PPCP – transformation – selection' process can be conveniently performed over a target gene for directed evolution (Fig. 4).

In conclusion, we demonstrated that a thermostable DNA ligase can be employed to mediate the amplification of circular DNA by adding a ligation step to conventional PCR cycles resulting in nick-free, circular plasmid DNA PCR products. We further showed that PPCP can be used to swap the selection marker of the template plasmid negating the need to pretreat the PCR DNA with *Dpn*I. Finally, when an error-prone DNA polymerase is used, PPCP can be used for *in situ* mutagenesis to create random mutagenesis libraries (*in situ* error-prone mutagenic PPCP). The method offers the following advantages: (i) nick-free, circular PCR products can be directly transformed into competent *E. coli* cells and easily selected by using an alternative antibiotic; (ii) a mutant library can be created by one-step error-prone amplification of variable DNA region in a

Table 2. Comparison of thermostable DNA ligase-mediated PPCP with MegaWHOP

Method	Primer pair	Extension region	PCR condition	Products	Template removing	Exponential amplification	Multiple round mutagenesis	Ref.
MegaWHOP	Mutated target gene (0.2–1 kb)	Vector sequence	High fidelity	Linear ssDNA and their hybrids (nicked dsDNA)	*Dpn*I digestion	No	No	12,13
PPCP	Vector sequence	Target gene	High fidelity or error-prone	Nick-free, circular dsDNA	Plate selection	Yes	Yes	This work

plasmid and (iii) accumulation of positive mutations in one sequence can be obtained by multiple rounds of *in situ* error-prone mutagenic PPCP. This method offers a novel, convenient and efficient approach for improving genes and proteins through directed evolution. In this work, we showed how biomass-degrading enzymes can be improved by mutagenic PPCP for the development of green industries.

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