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

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Edited by Dr Minoru Yoshida
(Received 4 January 2013; accepted 8 April 2013)

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 screening⁶ 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 (Megaprimer), 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 DNA strand, resulting in the formation of circular plasmid.\(^{12,13}\) However, the process mainly yields DNA strand, resulting in the formation of circular plasmid.\(^{12,13}\) Furthermore, the newly extended, linear ssDNA cannot serve as the 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 DpnI. 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′-CTTCAGATAATGCTTCAATATTTGAAAGAGCATATGACCATATTCAACGGG-3′, and KanRev, 5′-ATCAATCTAAGATATAATGAGTTAAACTTGGCTGACAGTTAGAAAAAATCTCAGACC-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 MgCl₂ 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 Taq DNA polymerase (TaKaRa), 0.2 mM each deoxynucleotide triphosphate, 2.0 mM MgCl₂, 0.5 mM MnCl₂ 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.

2.5. In situ error-prone PPCP mutagenesis

Error-prone PPCP was performed in 20 μl reaction solution containing 30 ng plasmid pHsh-xynA1, 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, 200 ng PPCP primer pair from pHsh described above, 0.2 mM each deoxynucleotide triphosphate, 2.0 mM MgCl₂, 0.5 mM MnCl₂ 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 SruI/HindIII sites to generate plasmid pHsh-celA. Endoglucanase gene celB of T. maritima was similarly amplified using primers 5'-CTA GCCTTGGTGCAACCGCA-3' and 5'-GGGCTCGAGTTA TTTACAACCTGAGCG-3', and cloned into pHsh-amp at SruI/XhoI sites, resulting in pHsh-celB. pHsh-xynA1 was generated by cloning the xynA1 from Thermomyces lanuginosus as previously described.¹⁷

2.7. Screening xylanase and cellulase random mutant libraries

Mutant libraries of celA, celB and xynA1, 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 hydrate 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 hydrate 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...
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 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.

To demonstrate the PPCP and marker swapping concepts, we used a 2.44 kb plasmid, pHsh-amp, harbouring the ampicillin-resistant gene (amp') 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') flanked by regions (39 bp each) complementary to the flanking sequences of amp' in pHsh-amp. The PPCP primer pair was thus able to amplify the entire pHsh while simultaneously swapping the selection marker from Amp' to Kan' 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, 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 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.
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 Mn²⁺ (mutagenic PCR), PPCP can be used for generating random mutagenesis libraries. Since mutagenesis occurs in situ during the PCR reaction, eliminating the need for 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-xynA1 was constructed by cloning the xylanase gene (xynA1) 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 Mn²⁺ and Tma DNA ligase and the PPCP primer pair from pHsh-kan was performed over the xynA1 region of pHsh-xynA1 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 Mn²⁺ concentration and the number of thermal cycles. The results showed that the frequency of null mutations increased with the concentration of Mn²⁺ 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 3. Optimization of thermostable DNA ligase-mediated PPCP by varying the amounts of Tma DNA ligase in 20 µl reaction. Lane M: λ EcoT14×E2×B5 markers; 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-xynA1 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' to kan') 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.
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 → CTA). 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. 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 primer pair from pHsh-xynA1 as template and the PPCP primer pair described above. 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.

![Figure 5](image-url)
strand,\textsuperscript{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.\textsuperscript{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.\textsuperscript{23} In the \textit{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 amplify 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 \textit{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 \textit{Pyrobest} or \textit{Pfu} DNA. The primer pair should be phosphorylated at 5’ ends for the ligation reaction.

PPCP primer pairs 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.\textsuperscript{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 \textit{dam}-methylated DNA-specific restriction enzyme, such as \textit{DpnI}, to reduce the template plasmid.\textsuperscript{13,25} In the \textit{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 \textit{DpnI} 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 \textit{DpnI}. Finally, when an error-prone DNA polymerase is used, PPCP can be used for \textit{in situ} mutagenesis to create random mutagenesis libraries (\textit{in situ} error-prone mutagenic PPCP). The method offers the following advantages: (i) nick-free, circular PCR products can be directly transformed into competent \textit{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

\textbf{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) | \textit{DpnI} 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 |

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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.

**Funding**

This work was supported by grants from the National Natural Science Foundation of China (31170027), PAPD of Jiangsu Higher Education Institutions, Nanjing Program of Science and Technology (201101094), the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (12KBJ180002) and Zhenjiang Agricultural Science and Technology support program (NY2012032). J.H.D.W acknowledges the supports of a grant (DEFG02-94ER64709) from the Office of Science (BER), a grant (DEFG02-08ER64692) from the Office of Science (BES) and a grant (DE-FG02-08ER64692) from the Office of Science (BER), the US Department of Energy.

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