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ORIGINAL PAPER

An Alternative Hot Start PCR Method Using a Nuclease-Deficient ExoIII from *Escherichia coli*

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Abstract

The Hot Start polymerase chain reaction (Hot Start PCR) is designed to reduce off-target amplification by blocking DNA polymerase extension at room temperature until the desired temperature is reached. In this study, we investigated a new method of Hot Start PCR that uses a modified *Escherichia coli* Exonuclease III (EcoExoIIIM) by substituting residues in the DNA-binding pocket and catalytic center. The results showed that PCR amplification yield and specificity were significantly promoted by the addition of EcoExoIIIM. We hypothesize that non-specific binding of primers at room temperature is prevented by binding of the primed template by EcoExoIIIM, which is then released from the DNA by heat denaturation before the first PCR cycle. Through this mechanism, PCR would be enhanced by reducing off-target extension at room temperature.

Keywords Polymerase chain reaction · Hot start · Exonuclease III

Introduction

The polymerase chain reaction (PCR) is one of the most ubiquitous and well-developed molecular biology tools and is used in a wide range of fields such as forensics, biodefence, and molecular diagnostics [1–8]. In a specific PCR assay, primer sequences are selected that hybridize to both ends of the intended target sequence. However, because of sequence similarities between the target and non-targeted regions, primers may anneal to undesired sequences, especially while setting up the PCR reactions on the bench at room temperature [9, 10]. Although polymerase activity at room temperature is relatively low, even a single base being incorporated into off-target annealed primers can produce extension products that compete with amplification of the desired target sequence, thereby suppressing the efficiency and specificity of PCR, especially for low copy number targets [9, 11].

"Hot Start" strategies have been developed to reduce off-target amplification in PCR and optimize the yield of the desired amplified products. This approach is usually achieved by preventing polymerase extension until the Hot Start activation conditions are reached [9]. Generally, there are three existing techniques for performing Hot Start PCR. The first is to withhold one of the key components such as the dNTPs, primers, Mg^{2+}, or the DNA polymerase until the reaction mixture reaches the desired elevated temperature [12, 13]. The second is to temporarily block DNA polymerase activity through chemical modifications [14, 15] or by employing anti-DNA polymerase antibodies or oligonucleotide aptamers that bind to the DNA polymerase [16–18], or through the addition of accessory proteins such as single-strand DNA-binding proteins (SSB) that sequester the primers at lower temperatures [19]. The third approach is the use of chemically modified oligonucleotide primers [11] and dNTPs [20]. Every approach has its own pros and cons and is discussed later.

In *Escherichia coli* (*E. coli*), the wild-type ExoIII has 3′→5′ exonuclease, 3′-phosphomonoesterase, 3′-esterase, and RNase H activity that targets at least some of the following polynucleotide structures: 1, the Apurinic/apyrimidinic

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site; 2, double-stranded DNA with 3′ extension (less than 4 nt); 3, blunt-end double-stranded DNA; and 4, double-stranded DNA with 3′ recession [21–24]. The last DNA substrate is structurally similar to primed DNA in a PCR mixture, which can be extended by the limited activity of the DNA polymerase at room temperature. It was previously reported that wild-type ExoIII enhances the PCR of damaged DNA templates, and even heat-denatured ExoIII has this effect. This observation implies the possibility of the enhancement of PCR by ExoIII through a mechanism independent of nuclease activity. Herein, we describe the design of a new Hot Start strategy that uses a modified *E. coli* ExoIII protein without nuclease activity, which we termed EcoExoIIIM. We propose that EcoExoIIIM enhances PCR through the inhibition of DNA extension by binding to off-target primed DNA.

Materials and Methods

Plasmid Construction

The gene encoding EcoExoIIIM (ECBD_1895) was amplified by PCR using *E. coli* BL21 genomic DNA (ACT28942.1) as the template. Using four pairs of primers (P1148 & P1465; P1466 & P1467; P1468 & P1469; and P1470 & P1149; see Supplementary Table 1), four amplified fragments (208, 172, 134, and 365 bp) were obtained and used as the template for overlapping PCR using P1148 and P1149 as the primers. The four mutated residues were shown in Fig. 1. The full-length DNA fragment encoding EcoExoIIIM was thereby acquired and cloned into a modified pET-15b vector [25] to construct recombinant His-tagged EcoExoIIIM. The nucleotide sequence of the inserted EcoExoIIIM

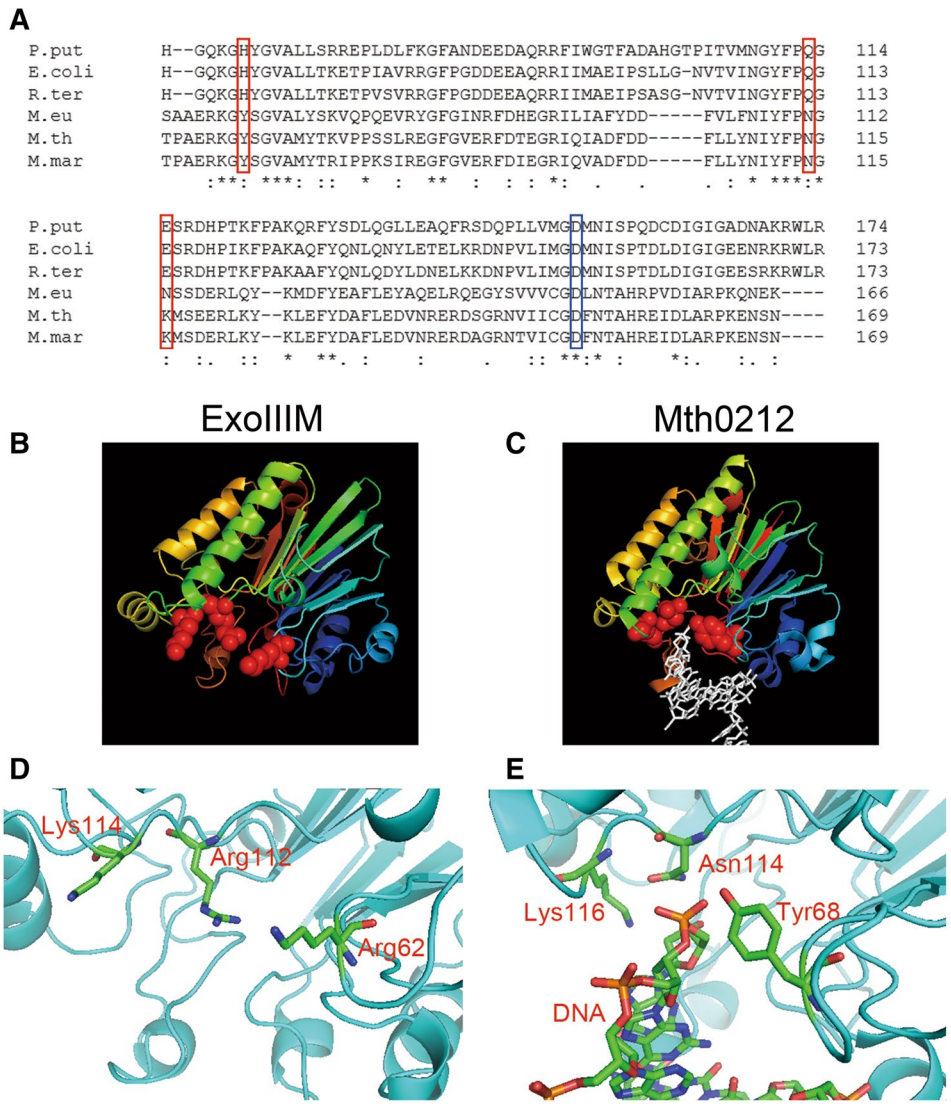

Fig. 1 Multiple sequence alignment and structural models of Exonuclease III. **a**, Multiple sequence alignment of six partial Exonuclease III amino acid sequences from *Escherichia coli* (*E. coli*), *Raoultella terrigena* (*R. te*), *Pseudomonas putida* (*P. pu*), *Methanothermobacter thermautotrophicus* (*M. th*), *Methanothermobacter marburgensis* (*M. ma*), and *Methanohalophilus euhalobius* (*M. eu*) by Clustal Omega (v. 1.2.4). Full-length alignments are shown in Supplementary Fig. 1. **b** and **c**, tertiary structures of *E. coli* ExoIII and *M. th* ExoIII, respectively. The red-sphered amino acids indicate the location of residues R62-R112-K114 in *E. coli* ExoIII and Y68-N114-K114 in *M. th*. D and E, zoomed view of the DNA-binding region corresponding to (**b**) and (**c**) (Color figure online)

gene was confirmed by Sanger sequencing (Meiji, Shanghai, China).

EcoExoIIIM Protein Expression and Purification

Recombinant His-tagged EcoExoIIIM protein was produced in the *E. coli* strain BL21-CodonPlus (DE3)-RIL (Invitrogen, Shanghai, China) grown in 2.5 L Luria–Bertani (LB) medium containing ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL). The cells were grown until they reached an OD_{600} of 0.8 at 37 °C. Protein expression was then induced with 0.5 mM IPTG for 6 h at 37 °C. The cells were harvested and disrupted by sonication in Buffer A (50 mM Tris–HCl pH 8.0, 200 mM NaCl). The soluble fractions were precipitated with 80% saturated ammonium sulfate. The precipitated proteins were re-suspended in Buffer A and dialyzed against Buffer A to remove the ammonium sulfate. After dialysis, the samples were loaded onto a 2-mL Ni–NTA column. The column was washed with 10 column volumes of Buffer A containing 40 mM imidazole and eluted with three column volumes of Elution Buffer (Buffer A containing 250 mM imidazole). The fractions containing the proteins were pooled and dialyzed against Buffer A and loaded onto a 5-mL HiTrap Q column, which was pre-equilibrated with Buffer A. The eluted fractions (between 100 and 200 mM NaCl) were pooled, concentrated, and further purified on a gel filtration column (Sephacryl S-200 HR) pre-equilibrated with Buffer A. The collected peak fractions were dialyzed against Storage Buffer (25 mM Tris–HCl pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 0.1 mM DTT, 50% (v/v) glycerol). EcoExoIIIM proteins were highly purified based (Fig. 2a) on SDS-PAGE analysis, and the final concentration was 12 mg/mL based on the Bradford method.

Nuclease and Binding Assays

Two DNA substrates (sequences shown in Fig. 2b) were annealed to test structure-specific cleavage and binding of EcoExoIIIM in a standard PCR reaction. The two oligonucleotides were synthesized by BGI Bioscience (Shenzhen, China). The shorter oligonucleotide was labeled by Cy5 at the 5′ end. The final concentration of the Cy5-labeled shorter strand was 10 pmol/μL and that of the non-labeled longer strand was 20 pmol/μL to ensure that all the fluorescently labeled strands were paired with non-labeled strands. Annealing was performed in 0.5 mM EDTA pH 8.0. The

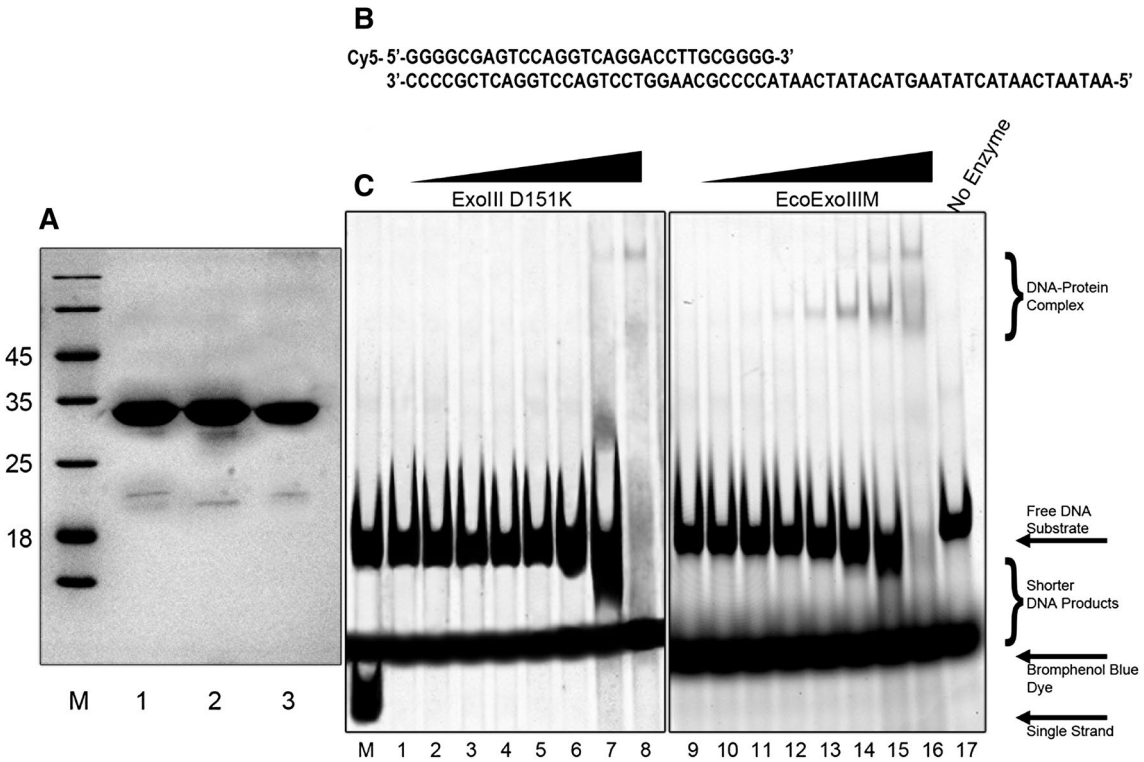

Fig. 2 Assays of the nuclease and DNA-binding activity of the wild-type ExoIII and EcoExoIIIM. **a** The expressed recombinant,wild-type ExoIII, EcoExoIIIM, and ExoIII-D151 K proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. **b** DNA substrates used to test the binding and exonuclease activity of EcoExoIIIM. **c** Native gel analysis showing the nuclease and DNA-binding activity of EcoExoIIIM and wild-type ExoIII toward the DNA substrate indicated in (**b**). In these reactions, the final concentrations of ExoIII-D151 K and EcoExoIIIM were 0.3, 0.5, 1, 2, 4, 8, 16, and 32 pmol/μL (lanes 1–8 and lanes 9–16, respectively)

mixtures were heated at 95 °C for 5 min, gradually cooled to room temperature in the dark, and stored at 4 °C until further use.

The DNA-binding reactions comprised a 20-μL mixture containing 25 mM Tris–HCl pH 8.5, 0.2 mM dNTPs, 2 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 20 mM KCl, 2 pmol of the DNA substrates, and various concentrations of ExoIII (ExoIII-D151 K or EcoExoIIIM) as indicated. Reactions were incubated at 37 °C for 30 min and stopped by the addition of an equal volume of stop buffer containing 10 mM EDTA, 1 × TBE, and 0.1% (w/v) bromophenol blue. Half the final volume of each reaction (20 μL) was then loaded onto a 10% polyacrylamide gel in 1 × TBE buffer and electrophoresed at 150 V for 90 min. Gels were imaged with a Typhoon 9410 scanner using Cy5 channel (GE Healthcare, US).

Standard PCR Assay

PCR components were mixed in 50 μL reaction systems containing 25 mM Tris–HCl pH 8.5, 0.2 mM dNTPs, 2 mM $MgCl_2$, 10 mM $(NH4)_2SO_4$, 20 mM KCl, 200 pmol of each primers, and 10 ng template DNA as indicated in Supplementary Tables 2 and 3. The indicated amounts of EcoExoIIIM or wild-type ExoIII protein were added into the PCR mixtures before adding 5 U of Taq DNA polymerase, in Fig. 3. The amplification parameters were as follows: 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for different lengths of time according to the target product length, followed by a final 2-min extension at 72 °C. The temperature was then reduced to 4 °C and 5.5 μL of 10 × Loading Buffer (Takara, Dalian, China) were added to the reactions. The PCR products (5 μL each) were resolved on a 1.5% agarose gel in 1 × TAE containing 40 μg/mL EB and run at 5 v/cm for 25 min.

Results

Preparation of the EcoExoIIIM Mutant

Wild-type ExoIII targets and digests 3′-recessed double-stranded DNA, which simulates the structure of primer–primer and primer-template duplexes (Fig. 4, panel A). We propose that by binding these duplexes (Fig. 4, panel C), this enzyme prevents DNA polymerase extension of primed DNA at lower temperatures, whether it be on- or off-target, until the first PCR cycle is initiated at an elevated temperature (Fig. 4, panels B and D). However, besides binding to the primed DNA template, wild-type ExoIII also digests the oligonucleotide primers and the DNA template. To produce an *E. coli* ExoIII with DNA-binding activity but without exonuclease activity,

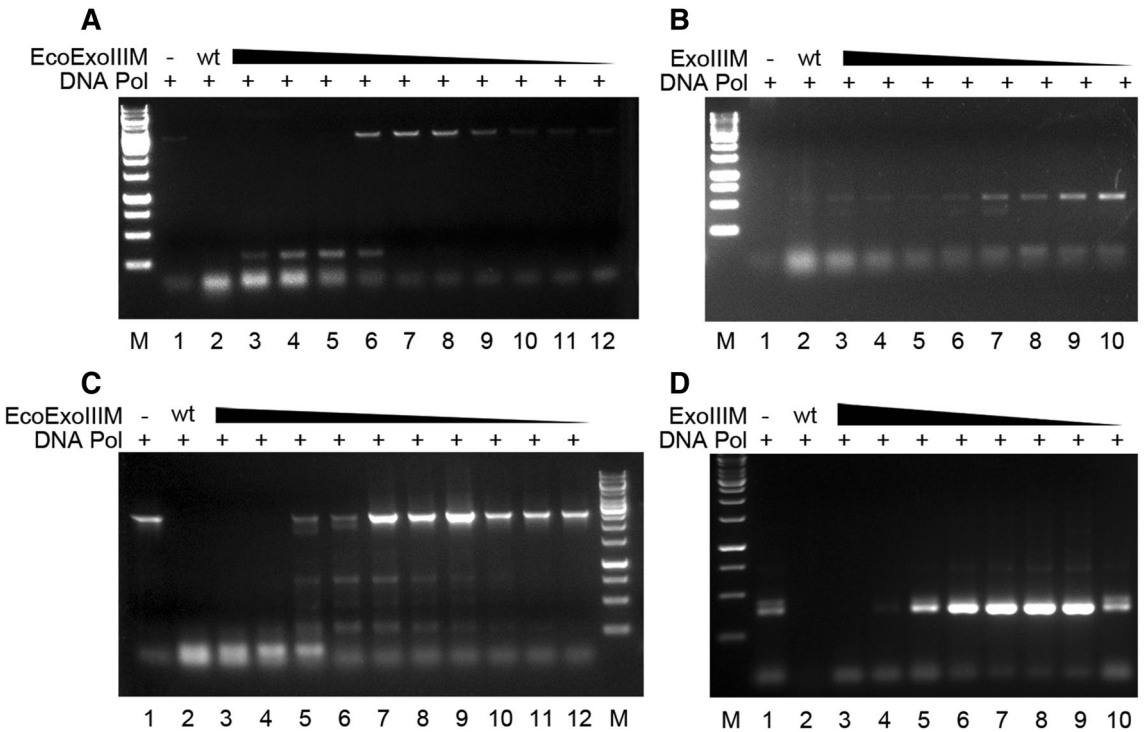

Fig. 3 PCR enhancement by EcoExoIIIM. The PCR reactions contained 125 ng wild-type ExoIII (lane 2) and 4 μg, 2 μg, 1 μg, 500 ng, 250 ng, 125 ng, 60 ng, 30 ng, 15 ng, and 8 ng of EcoExoIIIM (lanes 3–12, respectively). Primer and template sequences are available in Supplementary Tables 2 and 3, respectively. After PCR, 5 μL of each sample were separated by agarose gel electrophoresis

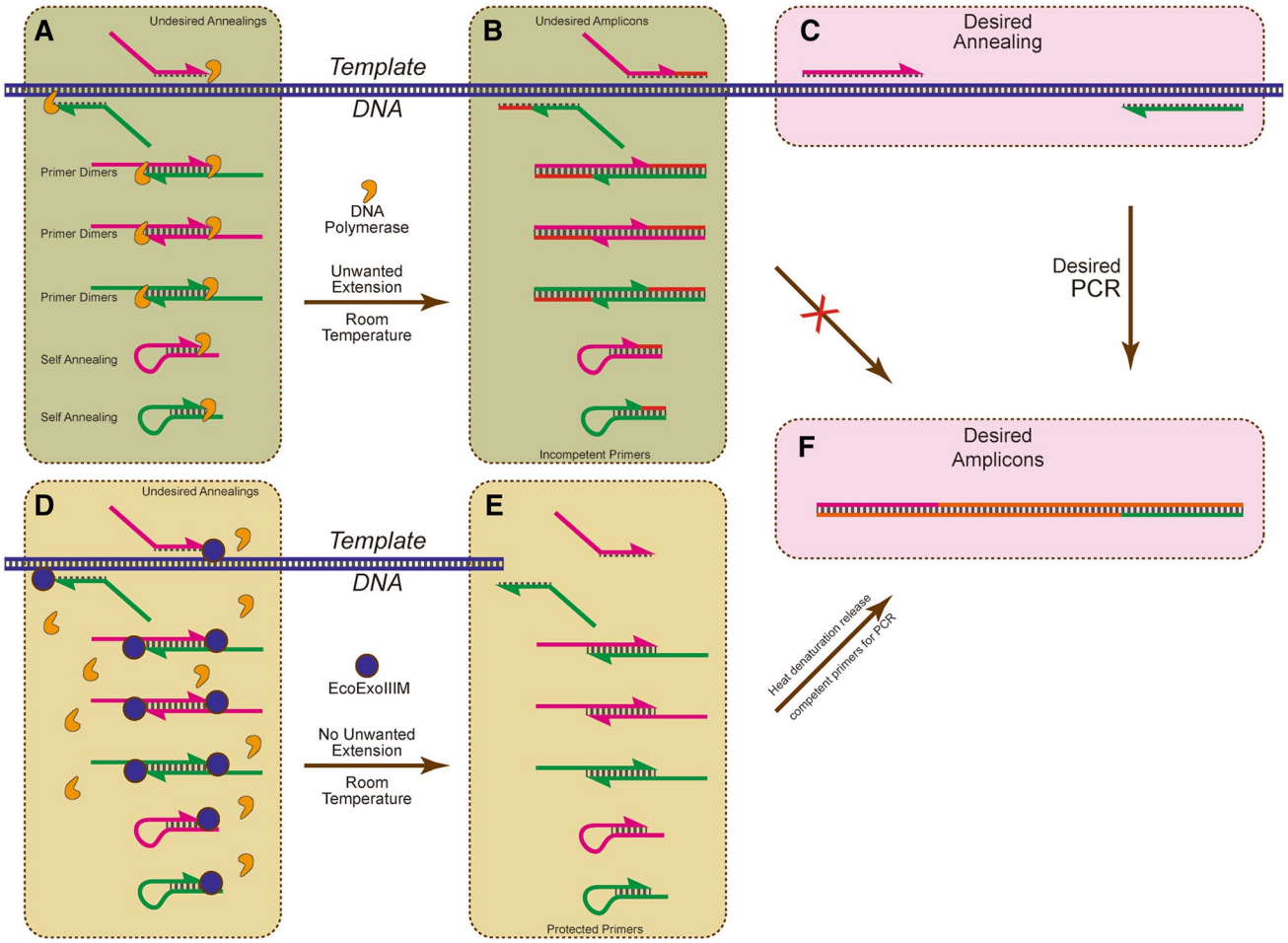

Fig. 4 Proposed mechanism of EcoExoIII enhancement of PCR. At room temperature, primers have the potential to undergo non-specific annealing (**a**), which can be extended by DNA polymerase to generate short oligonucleotides that are off-target (**b**). In the presence of EcoExoIIIM (**c**), non-specifically annealed DNA is blocked and protected from unwanted extension by DNA polymerase. When the reaction temperature is elevated to initiate PCR, denaturation of ExoIII releases the primed template, which itself deanneals and is available for the subsequent PCR reaction

we analyzed the published ExoIII structure (1AKO) and sequences of ExoIII homologues from bacteria and archaea from Protein Data Bank. As shown in Fig. 1a, residue D151 (blue box in Fig. 1a) was reported to be the metal cofactor-binding residue required for exonuclease activity [26]. For this reason, we substituted D151 with lysine to inactivate the nuclease activity of ExoIII. Furthermore, since tight binding to primed DNA was also important to achieve our goal of preventing off-target extension at room temperature, we introduced additional mutations into *E. coli* ExoIII to increase its binding affinity to primed DNA. Based on the available structure of MthExoIII complexed with DNA (ExoIII from *Methanothermobacter thermautotrophicus*, PDB: 3G2D), we located the three residues within MthExoIII that were closest in proximity to the DNA ligand: Tyr68, Asn114, and Lys116 (Fig. 1a, red rectangle; C, red spheres; and D, the three residues). Although no *E. coli* ExoIII-DNA complexed structure has yet been published, other published data have shown that MthExoIII and EcoExoIII are highly similar both in terms of their three-dimensional structure and amino acid sequence (Fig. 1a–c). H62, Q112, and E114 (Fig. 1a, red boxes) of EcoExoIII are located at equivalent positions to the three residues identified in MthExoIII (Y68, N114, and K116). For this reason, besides the D151 K mutation, we further incorporated the mutations H62R, Q112R, and E114 K (Fig. 1d) to increase the DNA-binding affinity of *E. coli* ExoIII, and we designated the mutant H62R-Q112R-E114 K-D151 K as EcoExoIIIM. The plasmids for expressing wild-type *E. coli* ExoIII and EcoExoIIIM were verified by sequencing and the corresponding proteins were overexpressed in *E. coli* and purified. Proteins were analyzed by SDS-PAGE (Fig. 2a).

EcoExoIIIM is Deficient in Nuclease Activity but Maintains DNA-Binding Activity

To verify the predicted biochemical activities of EcoExoIIIM, a DNA cleavage assay was performed. Duplex DNA with 3′ recession was used to mimic a primer duplex (Fig. 2b). EcoExoIIIM was mixed with the above substrate in the same reaction mixture used for standard PCR. DNA-binding analysis was performed after the substrates were incubated with ExoIII-D151 K or EcoExoIIIM in the presence of 2 mM Mg^{2+} (Fig. 2c). The wild-type ExoIII exhibited strong nuclease activity and was able to completely digest the primer duplex substrate (Supplementary Fig. 2, lanes 1–8), which agrees with expectation. In contrast, EcoExoIIIM showed no obvious nuclease activity by 30 min at a concentration of 0.3–8 pmol/μL (Fig. 2c, lanes 9–14). Interestingly, retarded DNA bands were observed, indicating that EcoExoIIIM bound stably to the substrate (Fig. 2c, lanes 13–16). These data showed that EcoExoIIIM lost its nuclease activity but retained its DNA-binding activity.

PCR Efficiency Enhanced by EcoExoIIIM

During previous routine PCR experiments, we experienced that certain PCR reactions never generated satisfactory products (data not shown), which is an issue commonly encountered by most researchers. Eight of these previously failed/poorly performing PCR experiments were selected to test the effect of ExoIIM. Four of the PCR assays did not exhibit any significant improvement (data not shown). Notably, the remaining four PCRs showed an obvious improvement following titration with EcoExoIIIM, even when the reactions were placed at room temperature for 30 min before PCR was initiated (Fig. 3a–d). The predicted product sizes ranged from 600–3000 bp. In contrast, the presence of wild-type ExoIII did not improve the same PCR reactions, although in some cases, we did observe a decrease in primer dimers (Fig. 3d), implying the digestion of primer dimers by wild-type ExoIII. It was interesting to note that in the presence of 60–250 ng EcoExoIIIM, even though the primer dimers were not reduced, the product yield was significantly increased in these PCR reactions (Fig. 3d, lanes 7–9). For the PCR assays shown in Fig. 3a–c, the yield of the targeted PCR products increased significantly in the presence of EcoExoIIIM compared with lane 1 where no EcoExoIIIM was added, although no obvious decrease in primer dimers was observed. These results clearly indicate that EcoExoIIIM increases PCR yields.

Discussion

As described in this work, by depleting the exonuclease activity while preserving the DNA-binding ability of ExoIII (Fig. 2b), we generated a mutant ExoIII from *E. coli* designated as EcoExoIIIM, which significantly promotes the efficiency of PCR. The use of EcoExoIIIM provides a new strategy for performing Hot Start PCR. We hypothesize that EcoExoIIIM exerts its affect through the following mechanism. At lower temperatures, EcoExoIIIM binds to the 3′ ends of DNA structures, including primer self-dimers, primer heterodimers, and oligonucleotides formed by primers annealing to the template DNA at both desired and undesired locations (see Fig. 4). Theoretically, the binding of EcoExoIIIM to the 3′ end of oligonucleotides blocks polymerase extension at room temperature while the PCR mixtures are being prepared. Once the PCR mixture reaches the desired elevated temperature, EcoExoIIIM is denatured and releases the 3′ end of DNA oligonucleotides. The primers also deanneal at this elevated temperature and are, therefore, free to participate in the specific annealing and extension steps of the PCR. According to the proposed mechanism, off-target amplification would thereby be inhibited, and the specificity and efficiency of PCR would be accordingly promoted. This putative mechanism is consistent with the observed enhancement of PCR following the addition of EcoExoIIIM.

A previous report by Fromenty et al. described the application of wild-type ExoIII in long-range PCR [27]. Interestingly, in Fig. 1 of Fromenty's paper, the heat-denatured ExoIII, as well as the wild-type ExoIII, enhanced PCR efficiency. This agrees with our observation that the nuclease-deficient EcoExoIIIM enhanced PCR, which implies that the two different ExoIIIs employed by these two studies may share the same mechanism. Further investigation would be necessary to reveal the mechanisms of this enhancement and to further extend the applications of exonuclease III in PCR.

To improve the efficiency and specificity of PCR, many approaches have been developed to eliminate the extension of non-specific/undesired annealed primers at room temperature [28]. Hot Start PCR is one of the methods devised to suppress this undesired polymerase activity. However, various drawbacks are associated with current approaches to Hot Start PCR. Introducing Anti-polymerase antibodies to inhibit polymerase at the same time introduce risk related to the use of animal-sourced materials. A Cold Start strategy, whereby all the reagents are mixed on ice and the tube is transferred to a PCR machine preheated to the melting temperature stage, which would eliminate polymerase activity during PCR preparation. However, the requirement for ice cold manipulation makes Cold Start PCR unfeasible. Single-strand DNA-binding (SSB) proteins can be used to block all single-stranded DNA at room temperature, only to release the DNA for PCR following denaturation of SSB during the melting stage of PCR [19]. However, SSB proteins bind not only to the primed region, but also non-specifically to any region of single-stranded DNA. For this reason, a large amount of SSB is needed

to completely protect the DNA template. In comparison, EcoExoIIIM binds specifically to the primed region of the DNA template and should, therefore, offer more efficient protection from unwanted extension.

In this study, we reported the use of bioinformatic and structural information on ExoIII from *E. coli* and *M. thermautotrophicus* to engineer a protein with no nuclease activity and remains affinity for DNA, designated as EcoExoIIIM. EcoExoIIIM increased the yields of four poorly performing PCR experiments. This protein could, therefore, act as a potential enhancer for PCR. A similar mechanism utilizing other proteins that block the 3′ end of DNA could also be explored in future work to further enhance PCR.

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