ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 5, pp. 557-561. © Pleiades Publishing, Ltd., 2009.
Published in Russian in Biokhimiya, 2009, Vol. 74, No. 5, pp. 685-690.

DNA Ligases from Thermophilic Bacteria Enhance PCR Amplification of Long DNA Sequences

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Received April 4, 2008
Revision received June 19, 2008

Abstract—Bacterial NAD-dependent Taq and Tth DNA ligases are capable of significantly increasing the yield of long PCR products when the amplification is carried out using bacterial family A DNA polymerases, e.g. Taq or Tth DNA polymerases, or with enzymatic blends containing these polymerases. We also show that Taq and Tth DNA ligases improve the results of PCR in the absence of NAD and therefore in the absence of DNA ligase activity. These observations suggest that bacterial DNA ligases can interact with these DNA polymerases, presumably as accessory proteins, thereby enhancing the efficiency of DNA polymerization.

DOI: 10.1134/S0006297909050113

Key words: long PCR, Taq DNA polymerase, Taq DNA ligase

Amplification of long DNA fragments (over 5 kb) by polymerase chain reaction (long PCR) is an important and widely used tool in molecular genetics, fundamental and applied molecular biology, molecular medicine, and biotechnology. The most popular and robust enzymes for use in ordinary PCR amplification are bacterial Taq and Tth DNA polymerases from polymerase family A (DNA polymerase I family). Unfortunately, the aforementioned polymerases alone are unable to provide high yields of long PCR products. In 1994 Barnes published a novel method for performing long PCR [1, 2] which helped to resolve the problem in question by adding to those DNA polymerases lacking 3′→5′ exonuclease (proofreading) activity, such as Taq or Tth DNA polymerase, a DNA polymerase which possesses proofreading activity, e.g. Pfu DNA polymerase [2]. The proofreading activity of the added polymerase eliminates mismatched 3′-nucleotides. A mismatched 3′-terminal base can be misincorporated by Taq polymerase from time to time, and as a result the synthesis of the DNA chain is terminated [1, 3]. Thus, elimination of misincorporated nucleotides by the proofreading activity of the secondary polymerase enhances PCR efficiency, especially in long PCR [1, 2].

Since the publications from Barnes' laboratory [1, 2], there have been several methods performed for improving long PCR technology. The majority of them were aimed at the elimination of factors that can be responsible for premature termination of DNA synthesis during PCR. Among the suggested methods were those for the elimination of damaged DNA templates by exonuclease III [4], the removal of inhibitors, e.g. dUTP by dUTPase [5], and the engineering of a polymerase that is tolerant to inhibiting factors such as uracil in DNA templates [6].

Another approach for improving long DNA synthesis *in vitro* is the use of polymerase accessory proteins [7]. These proteins can dramatically increase the processivity of DNA polymerases from eukaryotes [8], archaea [7, 9], bacteria [10], and bacteriophages [11]. Unfortunately, the natural bacterial family A DNA polymerases, including the customarily used Taq and Tth DNA polymerases, do not associate with any known accessory proteins.

We initially set out to improve long PCR by the repair of nicked DNA templates through the nick-closing ligase activity of thermophilic bacterial DNA ligases [12]. However, during our work we noticed that bacterial NAD-dependent Taq and Tth DNA ligases greatly enhance the efficacy of Taq and Tth DNA polymerases in long PCR, even without NAD, whose presence is essential for exhibiting DNA ligase activity. In this report we describe the use of Taq and Tth DNA ligase proteins for enhancing long PCR.

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MATERIALS AND METHODS

DNA ligases and DNA polymerases. In the present work, we used recombinant Taq and Tth DNA ligases purified from *Escherichia coli* strains containing the cloned genes of the enzymes. Tth DNA ligase was obtained as described by Barany and Gelfand [13]. Purification of the thermostable recombinant enzyme included a stage of an irreversible heat denaturation of *E. coli* proteins and stages of DEAE- and phospho-cellulose chromatography [13]. Purity of the obtained Tth DNA ligase was at least 95% as indicated by SDS-PAGE. Recombinant Taq DNA ligase was supplied by New England Biolabs (USA).

AmpliTaq DNA polymerase (a recombinant Taq DNA polymerase) and recombinant Tth DNA polymerase were purchased from Perkin-Elmer (USA); the TaqPlus DNA polymerase mixture was obtained from Stratagene (USA), and the TripleMaster Enzyme Mix DNA polymerase was from Eppendorf (Germany).

Template DNA and nucleotides. An undamaged DNA template and high-quality dNTPs are indispensable reagents for successful long PCR. Bacteriophage λ DNA was from GeneCraft (Germany). A mix of the four dNTPs (pH 7.5) was supplied by Bioline (UK).

Long PCR experiments. Long PCR reactions were performed by amplifying 10, 15, and 20 kb fragments of λ DNA. The 10 kb fragment was amplified with primers Pr1 (5′-ctgatcagttcgtgtccgtacaactggcgtaatc) and Pr2 (5′-atacgctgtattcagcaacaccgtcaggaacacg); the 15 kb fragment was amplified with primers Pr1 and Pr3 (5′-ccagccgcaatatctggcggtgcaatatcggtac), and the 20 kb fragment was amplified with primers Pr1 and Pr4 (5′-gtgcaccatgcaacatgaataacagtgggttatc).

The reaction mixtures contained 2.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 0.05% Triton X-100, and 0.5 mM of each dNTP. The reaction mixture (50 μl) contained 20 pmol of each primer, 7.5-30 ng of phage λ DNA template, and 2.5 U of respective DNA polymerase. The PCR reactions were performed with or without Taq or Tth DNA ligase. The DNA ligase proteins were added to the reaction mixtures in amounts corresponding to 0-120 U per 50 μl. (One ligase unit is defined as the amount of DNA ligase required to obtain 50% ligation of the 12-nucleotide cohesive ends of 1 μg of λ DNA in a total reaction volume of 50 μl in 15 min at 45°C.) In the absence of DNA ligase in the reaction mixture, an equal volume of ligase storage buffer was added.

Amplifications of the 10 kb DNA fragment with Taq DNA polymerase and the 15 kb fragment with Tth DNA polymerase were carried out in 50 μl reactions containing 7.5 ng of phage λ DNA in 32 cycles: 93°C, 45 sec; 58°C, 45 sec; 70°C, 8 min (preheating for 2 min at 92°C). The reaction mixtures contained DNA ligase in amounts corresponding to 0, 30, 60, 90, or 120 U.

Amplifications of the 10 and 15 kb DNA fragments with TaqPlus DNA polymerase were carried out in 25 μl reactions containing 15 ng of phage λ DNA template in 33 cycles: 93°C, 45 sec; 58°C, 45 sec; 70°C, 10 min (preheating for 2 min at 92°C). The reactions were catalyzed with 1.25 U TaqPlus polymerase, either without Taq DNA ligase or in the presence of 60 U of Taq DNA ligase.

Amplification of the 20 kb DNA fragment with the TripleMaster Enzyme Mix was carried out in 50 μl reactions containing 7.5 ng of phage λ DNA in 32 cycles: 93°C, 45 sec; 58°C, 45 sec; 70°C, 10 min (preheating for 2 min at 92°C). The reaction mixtures contained Taq DNA ligase in amounts corresponding to 0, 40, 50, 60, 80, or 120 U.

Resulting PCR products (20 μl) were visualized via electrophoresis on 0.8% agarose gels containing ethidium bromide.

RESULTS

Taq DNA ligase improves efficiency of Taq DNA polymerase in long PCR amplification. For evaluation of the effect of Taq DNA ligase protein on long PCR with Taq DNA polymerase, a 10 kb phage λ DNA fragment was amplified using 2.5 U of recombinant Taq DNA polymerase (AmpliTaq from Perkin-Elmer), either in the absence or presence of Taq DNA ligase. Figure 1 presents the electrophoretic analysis of the amplification products. Remarkably, the addition of NAD-dependent Taq DNA ligase greatly enhances the yield of the target 10 kb PCR product (Fig. 1), even though the reaction mixture did not contain NAD, and therefore the enzyme was unable to exhibit DNA ligase activity. However, adding more than 120 U of Taq DNA ligase per 2.5 U of Taq DNA polymerase did not produce any additional beneficial effects (data not shown).

Tth DNA ligase enhances efficiency of Tth DNA polymerase in long PCR amplification. To study the effect of Tth DNA ligase on the efficiency of long PCR with Tth DNA polymerase, a 15 kb phage λ DNA fragment was amplified with 2.5 U of recombinant Tth DNA polymerase (Perkin-Elmer), either in the presence of Tth DNA ligase or, for comparison, without DNA ligase. The results obtained with Tth DNA polymerase and Tth DNA ligase were similar to those obtained with the Taq enzymes: the target 15 kb DNA fragment was amplified only in the presence of Tth DNA ligase protein (Fig. 2). The best PCR amplification was obtained at the following ratio of the enzymes: 120 U of Tth DNA ligase per 2.5 U of Tth DNA polymerase.

Taq DNA ligase favors long PCR with DNA polymerase blends containing Taq DNA polymerase and a DNA polymerase with proofreading activity. Long PCR is commonly performed using blends of enzymes containing a bacterial family A DNA polymerase, e.g. Taq or Tth DNA polymerase, and an archaeal "proofreading" DNA polymerase from the B polymerase family, such as Pfu DNA polymerase [2]. Here we studied the effect of Taq DNA ligase on the efficiency of long PCR using such blends.

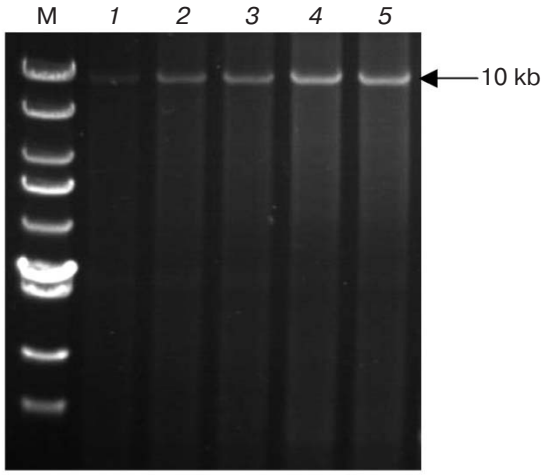

Fig. 1. Taq DNA ligase protein confers a beneficial effect on long PCR performed with Taq DNA polymerase. A 10 kb DNA fragment was amplified from 7.5 ng of phage λ genomic DNA in 32 cycles. Reactions were carried out in 50 μl with 2.5 U of Taq DNA polymerase without extra additives (lane *1*) and in the presence of 30, 60, 90, or 120 U of Taq DNA ligase (lanes *2-5*, respectively). Lane M represents a 1 kb DNA ladder.

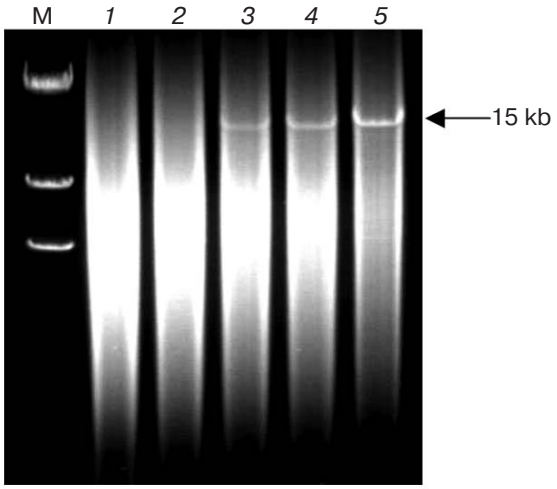

Fig. 2. Tth DNA ligase protein enhances long PCR with Tth DNA polymerase. A 15 kb DNA fragment was amplified from 7.5 ng of phage λ genomic DNA in 32 cycles. Reactions were carried out in 50 μl with 2.5 U of Tth DNA polymerase without extra additives (lane *1*) and in the presence of 30, 60, 90, or 120 U of Tth DNA ligase (lanes *2-5*, respectively). Lane M represents Hind III-digested λ DNA.

For the amplification of a 20 kb DNA fragment, use was made of the TripleMaster Enzyme Mix from Eppendorf (a blend of Taq DNA polymerase, a polymerase-enhancing factor, and a proofreading DNA polymerase). The target DNA fragment was amplified in 50 μl reactions, with 2.5 U of the TripleMaster Enzyme Mix in the presence of 40, 50, 60, 80, or 120 U of Taq DNA ligase and, as a control, without any Taq DNA ligase. Under the PCR conditions that were employed here, successful amplification of the target 20 kb fragment was achieved only in the presence of 80 and 120 U of Taq DNA ligase (Fig. 3a). The best results were obtained at a ratio of

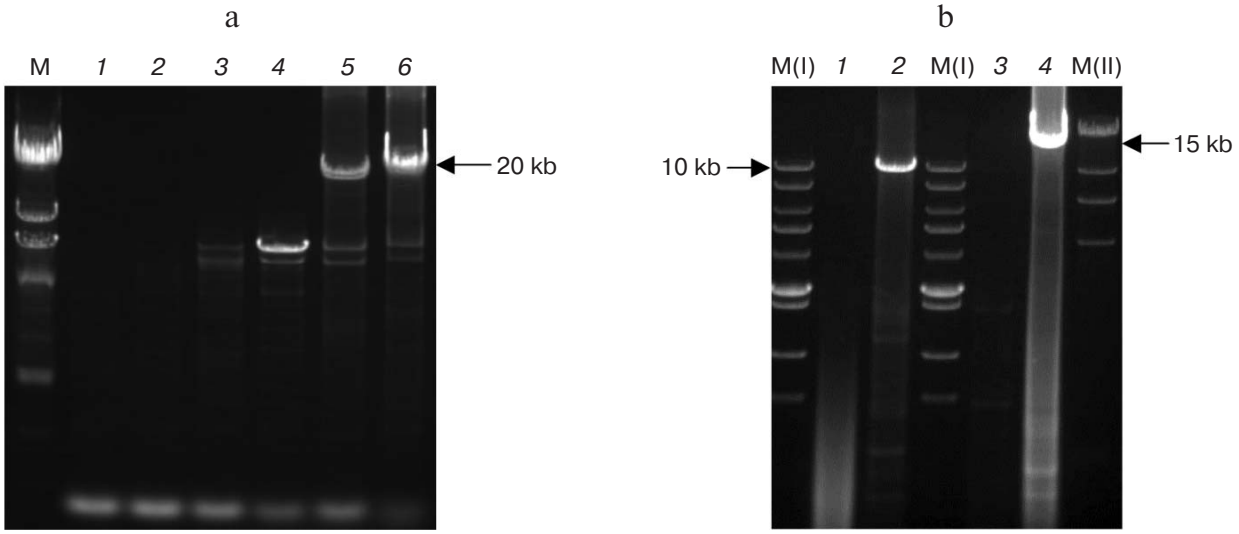

Fig. 3. Taq DNA ligase protein has a beneficial effect on long PCR performed with Taq DNA polymerase in combination with a proofreading DNA polymerase. a) A 20 kb DNA fragment was amplified from 7.5 ng of phage λ genomic DNA in 32 cycles. PCR reactions were carried out in volume of 50 μl with 2.5 U of TripleMaster Enzyme Mix (Eppendorf) in the presence of 40, 50, 60, 80, or 120 U of Taq DNA ligase (lanes *2-6*, respectively) and without Taq DNA ligase (lane *1*). Lane M represents Hind III-digested λ DNA. b) 10 kb (lanes *1* and *2*) and 15 kb (lanes *3* and *4*) DNA fragments were amplified from 15 ng of phage λ genomic DNA in 33 cycles. Reactions were carried out in 25 μl with 1.25 U of the TaqPlus DNA polymerase blend (Stratagene) without Taq DNA ligase (lanes *1* and *3*) and in the presence of 60 U of Taq DNA ligase (lanes *2* and *4*). Lane M(I) represents a 1 kb DNA ladder; lane M(II) represents Hind III-digested λ DNA.

120 U of Taq DNA ligase per 2.5 U of TripleMaster Enzyme Mix (units of TripleMaster Mix refer to the units of Taq DNA polymerase in the blend).

The TaqPlus DNA polymerase mixture from Stratagene (a blend of Taq and Pfu DNA polymerases) was used for PCR amplification of the 10 and 15 kb DNA fragments. Reactions were carried out in volume of 25 μl with 1.25 U of TaqPlus DNA polymerase either in the presence of Taq DNA ligase (60 U) or in its absence. In our experiments, amplification of the target products of this long PCR was observed only in the presence of Taq DNA ligase protein (Fig. 3b).

Our data demonstrate that Taq DNA ligase can appreciably improve the results of long PCR by the use of polymerase blends containing the Taq DNA polymerase and a proofreading DNA polymerase, such as Pfu. At the same time, the addition of Taq or Tth DNA ligase to PCR mixtures containing Pfu DNA polymerase without Taq polymerase did not produce any beneficial effect on the PCR (data not shown). Thus, Taq DNA ligase improves long PCR amplification through enhancing the efficiency of Taq DNA polymerase.

DISCUSSION

Bacterial DNA ligase and bacterial family A DNA polymerase (bacterial DNA polymerase I) are key enzymes in bacterial DNA repair and in the synthesis of the lagging DNA strand during the replication of the bacterial chromosome [14]. Bacterial family A DNA polymerases, such as Taq and Tth polymerases, possess 5′→3′ exonuclease activity in addition to DNA polymerase activity, which provides for the replacement of RNA fragments or damaged DNA with newly synthesized DNA by a process of nick-translation. An NAD-dependent DNA ligase, such as Taq or Tth DNA ligase [12, 13, 15], ensures the joining of 5′ and 3′ ends of the synthesized DNA chains by virtue of nick-closing ligase activity. In a bacterial cell DNA polymerase I and NAD-dependent DNA ligase work in close cooperation [16].

The present work shows that the Taq and Tth DNA ligase proteins considerably enhance the efficiency of long PCR performed with Taq or Tth DNA polymerase, or with polymerase blends containing these polymerases. The improvement is manifested as a many-fold increase in the yield of a long PCR product. This effect is observed without NAD in the reaction mixtures, although this compound is necessary for exhibiting DNA ligase activity; therefore, this activity itself does not play an important role in our enhanced long PCR experiments.

The fact that DNA ligase is required for the synthesis of long DNA in a bacterial cell is well known, but this fact is usually explained only through the joining of short DNA fragments by the nick-closing ligase activity. In contrast, our data indicate that bacterial DNA ligases can stimulate the synthesis of long DNA fragments independently of the ligase activity. These results correlate with the experimental data obtained by Sakakibara [17]. In a study of replication of Col E1 plasmid in an *E. coli* cell extract containing a thermolabile DNA ligase, Sakakibara showed that heat denaturation of the thermolabile DNA ligase, by increasing the reaction temperature from 25 to 32°C, resulted in the production of short DNA fragments [17]. He also reported that the synthesis of long DNA chains during the elongation stage of replication was not affected by the blocking of the bacterial DNA ligase activity by nicotinamide mononucleotide (NMN), which is a specific inhibitor of NAD-dependent DNA ligases. In both cases, the accumulation of newly formed open circular Col E1 DNA molecules indicated the inactivation of the ligase activity [17]. Therefore, the synthesis of long DNA chains during the replication of Col E1 depended on the presence of the intact ligase protein but was independent of the ligase activity.

Thus, based on our results and those obtained by Sakakibara [17], we conclude that bacterial DNA ligases can stimulate the synthesis of long DNA by bacterial DNA polymerases independently of the ligase activity. For explanation of this fact, it can be supposed that a bacterial DNA ligase associates as an accessory protein with a bacterial family A DNA polymerase, thereby improving its processivity. In this case, the NAD-dependent activation of DNA ligase is not important in enhancing the synthesis of long DNA fragments. We can also assume that bacterial DNA ligase associates with bacterial DNA polymerase during the elongation stage of DNA synthesis. The possible existence of this complex in a bacterial cell allows us to speculate that after the binding of a bacterial family A DNA polymerase to primed DNA, bacterial DNA ligase forms a complex with the abovementioned polymerase and increases its processivity. When, in the process of DNA synthesis, the complex reaches the 5′-end of DNA, the ligase joins the 5′- and 3′-ends of the DNA chains and the complex dissociates. Thus, DNA polymerase delivers DNA ligase directly to the DNA-DNA nick. At the same time, DNA ligase functions as a processivity factor for this DNA polymerase and prevents unnecessary nick-translation.

It is also worth mentioning that the possible existence of a complex comprising a bacterial family A DNA polymerase (bacterial DNA polymerase I) and a bacterial DNA ligase was first suggested by Kornberg in 1974 [18].

Our results described here suggest the use of DNA ligase proteins from thermophilic bacteria as protein enhancers when using thermostable bacterial family A DNA polymerases, such as Taq and Tth DNA polymerases, or with blends of these polymerases with proofreading enzymes in long PCR amplifications.

We are grateful to Prof. David P. Hornby from the University of Sheffield (UK) and Prof. Bernard A. Connolly from the University of Newcastle (UK) for comments and discussions.

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