Improvements of rolling circle amplification (RCA) efficiency and accuracy using *Thermus thermophilus* SSB mutant protein

Jin Inoue3, Yasushi Shigemori and Tsutomu Mikawa1,2,3,*

Aisin Cosmos R&D Co. Ltd. 2-1-5 Kazusa-kamatarı, Kisarazu, Chiba 292-0818 Japan, 1RIKEN Harima Institute/SPRing-8, Mikazuki cho, Hyogo 679-5148, Japan, 2RIKEN Discovery Research Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan and 3International Graduate School of Arts and Science, Yokohama City University, 1-7-29, Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan

Received December 13, 2005; Revised February 6, 2006; Accepted April 19, 2006

**ABSTRACT**

Rolling circle amplification (RCA) of plasmid or genomic DNA using random hexamers and bacteriophage phi29 DNA polymerase has become increasingly popular in the amplification of template DNA in DNA sequencing. We have found that the mutant protein of single-stranded DNA binding protein (SSB) from *Thermus thermophilus* (*Tth*) HB8 enhances the efficiency of amplification of DNA templates. In addition, the *Tth*SSB mutant protein increased the specificity of phi29 DNA polymerase. We have over-expressed the native and mutant forms of *Tth*SSB protein in *Escherichia coli* and purified them to homo-geneity. In vitro, these proteins were found to bind specifically to single-stranded DNA. Addition of *Tth*SSB mutant protein to RCA halved the elongation time required for phi29 DNA polymerase to synthesize DNA fragments in RCA. Furthermore, the presence of the *Tth*SSB mutant protein essentially eliminates nonspecific DNA products in RCA reactions.

**INTRODUCTION**

Rolling circle amplification (RCA) is an isothermal method that amplifies circular DNA by a rolling circle mechanism (1–4). The reaction products are high molecular weight, linear, double-stranded, tandem repeat copies of the input template that can subsequently be used for DNA sequencing, restriction endonuclease digestion and other methods used in cloning, labeling and detection. Kits that use this method for preparation of circular DNA templates for direct use in cycle sequencing are commercially available. RCA variants have been employed for the genotyping of single nucleotide polymorphisms through ligation of circularizable DNA probes and for whole bacterial or human genome amplification via multiple-displacement amplification (5–8).

Although the utility of RCA is beginning to be accepted widely, several technical problems still remain to be solved. Perhaps the most significant is the appearance of nonspecifically amplified products. In practice, even with the most optimized reaction conditions (e.g. using modified random oligonucleotides) (9), nonspecific RCA products cannot be completely eliminated, particularly in instances where complex and/or very low amounts of the template DNA are used. Nonspecific RCA products are most likely to be derived from false priming at sites where primer dimers are formed.

Recently, a heat stable SSB protein from a thermophilic bac-teria, *Thermus thermophilus* (*Tth*), has been isolated and fully characterized (10–12). Bacterial SSB proteins are required for DNA replication and repair (13,14). Most SSB proteins bind nonspecifically to single-stranded DNA (ssDNA) and prevent the formation of secondary structures. Wild-type and mutant SSB-encoding genes from *T.thermophilus* HB8 were cloned and the proteins they encode have undergone preliminary character-ization (J.I. Inoue, manuscripts in preparation). We attempted to eliminate nonspecific RCA products by employing these heat-stable SSB proteins. We speculated that the presence of SSB protein could greatly stimulate polymerization (isothermal strand-displacement), and therefore reduce the formation of primer dimer and lead to the elimination of nonspecific RCA products. Mutated SSB proteins would be ideal candidates for such an application owing to their predicted ssDNA binding efficiency.

In this report, we demonstrate that a mutant SSB protein from *T.thermophilus* strain HB8 (*Tth*SSB) increases the effi-
ciency of DNA synthesis and the accuracy of RCA using the bacteriophage phi29 DNA polymerase.

MATERIALS AND METHODS

Cloning of the ssb wild-type gene

*T.thermophilus* genomic DNA was purchased from Takara-Bio. Amplification of the ssb gene was performed by PCR with the genomic DNA. *Nde*-ssb (5'-GGCGGATCCCAT ATGGCTCGAG GCTGGAACCG-3'), *Bam*-ssb (5'-TAAG- GATCCT TATTTAACC GCAACTCCTC CTCCG- GGCGGA AA-3') and *Kod* DNA polymerase (Toyobo). The primers included *Nde*I and *Bam*HI restriction sequences (underlined) for cloning purposes and were based on the sequence of the gene encoding the *T.thermophilus* HB8 ssb gene (GenBank accession number NC006461). A single DNA fragment of the expected size (792 bp) was obtained. For construction of the expression plasmid, the PCR product was digested by *Nde*I and *Bam*HI, purified and inserted into plasmid pET17b. The resulting plasmid was named pET17b-ssb.

Cloning of the ssb mutant gene

For construction of the expression plasmid of the mutant protein carrying one amino acid replacement (F255P), the ssb mutant gene (nt 763–764, TT[GTCTTCCA AGCCTTCGTC-3]) was prepared -mercaptoethanol and 1.0 M (NH₄)₂SO₄. The SSB protein was purified by a kit (DNA purification kit, Amersham Biosciences). Primers used for PCR were purchased from Sigma-Aldrich Japan. PCR was performed using DNA polymerase from *Thermus aquaticus* (*Taq*) according to the manufacturer’s instructions (QIAGEN HotSartTaq DNA polymerase kit and QIAGEN Multiplex PCR kit, QIAGEN). Primers were used at concentrations of 0.4 μM. PCR thermal cycles used were 15 min at 95°C for DNA denaturation, followed by 35 cycles of 30 s at 94°C, 90 s at 60°C and 90 s at 72°C. PCR products were purified by a kit (DNA purification kit, Amersham Biosciences). Multiplex PCR was performed in the presence of *T.thermophilus* RecA as described previously (16).

Gel electrophoresis of DNA and protein

DNA was electrophoresed on 1.2% agarose gels and visualized by staining with ethidium bromide. Loading buffer contained 60 mM Tris–HCl pH 6.8, 10% (v/v) glycerol, 5 mM EDTA and 0.01% bromophenol blue (BPB). The samples and the DNA size marker (*Bst*BI digested Lambda DNA fragments) were run for 1 h on an agarose gel.

Protein samples were heated for 3 min at 85°C before being loaded onto the gel. Proteins were electrophoresed on 12.5% polyacrylamide gels containing 0.1% SDS under reducing conditions and visualized by staining with Coomassie brilliant blue R-250 (CBB). Loading buffer for gels contained 30 mM Tris–HCl pH 6.8, 5% (v/v) glycerol, 1% SDS, 2.5% β-mercaptoethanol and 0.01% BPB. The samples and the protein markers were run for 1 h on SDS–PAGE to ensure that all proteins had achieved mobility according to their respective sizes.
Standard RCAs

RCAs were performed with DNA polymerases from phi29 in accordance with the manufacturer’s instructions (Amersham Biosciences). We used 1 ng pUC19 DNA (included in the kit as the control DNA template) as template. The RCA conditions used were 3 min at 95°C for denaturation, followed by 24 h at 30°C. An enzyme inactivation step was performed for 10 min at 65°C.

RCA whole genome amplification

We used 10 ng of human genomic DNA (Promega) as templates in the genomic DNA amplifications. Template DNA was mixed with 0.5 μl hexamers (400 ng/μl, Sigma Genosys) and 0.5 μl binding buffer (400 mM Tris–HCl at pH 8.0 and 160 mM KCl) and denatured at 95°C for 4 min. The denatured DNA was amplified using 0.3 μl Phi29 DNA polymerase (10 U/μl, New England Biolabs) complemented with 2 μl of 10× Phi29 DNA polymerase buffer, 0.2 μl of 100× BSA, 3.2 μl of 2.5 mM dNTP and 1 μl of 20% DMSO (Sigma Aldrich) in a volume of 20 μl at 30°C for ~24 h. The phi29 DNA polymerase was inactivated at 65°C for 10 min and the amplification product was purified using a spin-column (Sephadex G-50, Amersham Biosciences) to eliminate the un-reacted primers.

DNA spot hybridization

To perform DNA spot hybridizations, DNA samples were mixed with 30 μl of DNA denaturation solution. The mixtures were then incubated for 5 min at 65°C and cooled on ice. Equal volumes of 20× SSPE (0.2 M sodium phosphate, pH 7.4, 3.0 M NaCl and 0.02 M EDTA) was added to each sample, which were spotted onto a nylon membrane. The DNA was then fixed to the membrane by ultraviolet irradiation. The membrane was incubated for 2 h at 68°C in 20 ml of prehybridization solution in a hybridization chamber. The radiolabeled probes were prepared with [γ-32P]dCTP (6000 Ci/mmol, Amersham Pharmacia Biotech, Buckinghamshire, UK), a kit (BcaBEST™ Labeling kit, Takara Bio) and pUC19 or a human gene site (3121 bp). Following addition of the radiolabeled probes, the membrane was incubated at 68°C for a further 12 h. After hybridization, the membrane was washed with 200 ml 0.5× SSC (7.5 mM sodium citrate pH 7.0 and 75 mM NaCl), 0.1% SDS at 68°C. Finally, the membrane was dried and exposed to an imaging plate (BAS2000 Image analyzer, Fuji Photo Film) for 24 h.

RESULTS

Purification of wild-type and mutant TthSSB protein

The wild-type T.thermophilus SSB and its mutant were prepared as described in Materials and Methods, which were...
named TthSSB and TthSSB-255 protein, respectively. Each step of the purification of TthSSB was analyzed by 12.5% SDS–PAGE with CBB staining (Figure 1). Almost all proteins from E.coli could be removed by heat treatment. The TthSSB-255 mutant protein was also purified using the same strategy (data not shown). The final products were shown to be 99% pure.

Properties of TthSSB-255 protein

The TthSSB-255 protein has a single amino acid replacement (F255P) and the same pI (5.06) as the wild-type protein. The strategies of purification for both proteins were identical. Therefore, the mutant would not be expected to have significantly different properties compared with the wild-type. To assess the effect of the F255P mutation on the DNA binding properties of SSB, we determined the dissociation constants for the binding of TthSSB-255 to ssDNA. As shown in Figure 2a, the TthSSB-255 protein showed weak binding to ssDNA in comparison with the wild-type TthSSB and EcSSB proteins. Figure 2b summarizes the dissociation constants for the binding of each SSB protein to ssDNA.

Effect of SSBs on RCA

To examine whether the presence of either wild-type or TthSSB-255 or EcSSB was able to reduce the formation of nonspecific RCA products and thus affect the RCA reaction, we compared the patterns of RCA products obtained in the presence (or absence) of these proteins. As shown in Figure 3a, high molecular weight DNA is generated as a result of isothermal strand-displacement amplification. However, RCA produces nonspecific amplification artifacts (Figure 3c, lane 1 for no protein, lane 2 for EcSSB and lane 3 for TthSSB) in the absence of input template DNA. In contrast, RCA products in the presence of the TthSSB-255 protein were free of such nonspecific amplification products (Figure 3c, lane 4) and the presence of high molecular weight DNA is a clear indication of template-specific amplification (Figure 3a and b, each of lane 4).

Effects of the DNA synthesis in the presence of TthSSB-255 protein

Since the above effects on DNA synthesis were observed with short elongation times, we examined whether the TthSSB-255 protein was able to affect the mean rate of DNA synthesis of DNA polymerase. Conventional RCA and RCA in the presence of the TthSSB protein produce nonspecific amplification artifacts in the absence of input template DNA (Figure 4b and d). Therefore, the RCA electropherogram is not indicative of successful amplification. In contrast, RCA in the presence of the TthSSB-255 protein was free of such nonspecific amplification products (Figure 4f) and the presence of high molecular weight DNA is a clear indication of template-specific amplification (Figure 4e). From the results of spot hybridization analysis, with 30 min elongation times, the DNA fragment was not amplified in the absence (Figure 4a) or presence of the TthSSB protein (Figure 4c). In contrast, the DNA fragment was amplified in the presence of TthSSB-255 protein (Figure 4e). These results support the idea that the DNA polymerase at least doubles the mean rate of DNA synthesis in the presence of TthSSB-255 protein.

Whole genome amplification

Figure 5 demonstrates the amplification products obtained following whole genome amplification via RCA, using intact human genomic DNA. High molecular weight DNA is generated as a result of isothermal strand-displacement amplification. However, conventional RCA and RCA in the presence of TthSSB protein produced nonspecific amplification artifacts (Figure 5a and b, each of lanes 3 and 4) in the absence of template DNA. RCA in the presence of the TthSSB-255 protein was free of such nonspecific amplification products (Figure 5c, lane 4).
of input genomic DNA. In contrast, RCA in the presence of
*Tth*SSB-255 protein was free of such nonspecific amplification
products (Figure 5c, lanes 3 and 4) and the presence of
high molecular weight DNA is a clear indication of template-
specific amplification (Figure 5c, lanes 1 and 2).

**The effect of template concentration**

We expected that RCA in the presence of *Tth*SSB-255 protein
would proceed with a considerably lower concentration of
template DNA than that required for conventional RCA. As
shown in Figure 6, high molecular weight DNA is generated as
a result of isothermal strand-displacement amplification.

However, RCA produces nonspecific amplification artifacts
(Figure 6a, lane 5) in the absence of input genomic DNA. In
contrast, RCA in the presence of *Tth*SSB-255 protein
was free of such nonspecific amplification products
(Figure 6b, lane 5), and the presence of high molecular
weight DNA is a clear indication of template-specific ampli-
fication (Figure 6b, lanes 1–4).

The results were further validated by Southern hybridiza-
tion. Figure 6a shows that the amount of RCA products
decreased drastically as template concentration was reduced
to 1/10 in the control experiment (without *Tth*SSB-255,
Figure 6a, bottom). In contrast, in the presence of *Tth*SSB-
255, substantial amounts of RCA products were still obtained.
even when the template concentration was reduced to 1/1000 of the original concentration (Figure 6b, bottom). It is clear that *Tth*SSB-255 greatly stimulates polymerization (isothermal strand-displacement), particularly at lower template concentrations, indicating that in the presence of *Tth*SSB-255, the pairing of primers to template sequences occurs with high efficiency. Consequently, considerably lower template concentrations are enough to perform the RCA in the presence of *Tth*SSB-255.

**PCR examinations of RCA-amplified whole genome**

To estimate the quality of DNA obtained following RCA of human genomic DNA, a comparison was made between conventional RCA and RCA in the presence of *Tth*SSB-255 protein. To validate the effect of the *Tth*SSB-255 protein, a region in human genomic DNA was equally subdivided into six 1346 bp sites (designated a-1–a-6, see Figure 7a). Using primers (20 bp) complementary to the terminal sequence of the subdivided site, PCR was performed. When we employed the DNA obtained by conventional RCA as a template, no amplification or nonspecific PCR products were observed (Figure 7b). As seen in Figure 7d, the employment of the amplified DNA in the presence of *Tth*SSB-255 eliminated nonspecific PCR products in most of the six PCR sites examined. These results suggest that the products by RCA in the presence of *Tth*SSB-255 protein are suitable for following PCR.

**Multiplex PCR examinations of RCA-amplified whole genome**

Encouraged by the elimination of nonspecific PCR products and the particularly low concentrations of template DNA
required for RCA in the presence of TthSSB-255 protein, we
further examined the effect of the protein in RCA amplification
from very small DNA amounts. To estimate the extent of
amplification obtained following RCA of human genomic
DNA, multiplex PCR examinations were performed using
serially diluted template DNA concentrations.

We show the results of multiplex PCR for the randomly
selected 12 human genes (derived from different chromo-
some) ranging from 57 to 360 bp in which 12 parallel PCR
corresponding to each gene were conducted in the same reaction
mixture (Figure 8a). The multiplex PCR were carried out
on 0.5 \( \mu l \) samples of RCA products using human genomic
dNA as the template in the absence or presence of TthSSB-255
mutant protein (Figure 8b and c). While the amount of PCR products
amplified from RCA products decreased drastically as tem-
plate concentration in the RCA was reduced to 1/1000 of
that in lane 1 (Figure 8b), in the presence of TthSSB-255
substantial amounts of PCR products were still obtained even
when the template concentration was reduced to 1/100 000
of the original concentration (Figure 8c).

It is clear that TthSSB-255 protein greatly stimulates
polymerization particularly at lower template concentrations.
This indicates that in the presence of TthSSB-255, elimination
of nonspecific DNA products in RCA reactions occurs
with high efficiency, resulting in a considerably lower tem-
plate concentration requirement and homogeneous amplifica-
tion of template than conventional RCA.

**DISCUSSION**

A thermophilic SSB protein and its mutant form from *T.ther-
omophilus* HB8 were purified and its effects on the activity of
phi29 DNA polymerases were assayed. The presence of
TthSSB-255 mutant protein shortened the elongation time
required to synthesize a DNA fragment by phi29 DNA
polymerase. Such stimulation could be the result of direct
interactions between TthSSB and DNA polymerase. However, as an increase in DNA synthesis was also observed when a Bst DNA polymerase was used (Supplementary Figure S1), it follows that the effect of TthSSB-255 was not due to specific interactions with a DNA polymerase, but was most probably due to an indirect consequence of its binding to ssDNA. There are several putative explanations for such an indirect effect on DNA synthesis stimulation since the TthSSB-255 protein showed weak binding to ssDNA. For example, the TthSSB-255/ssDNA complexes could avoid the formation of folded ssDNA structures, which are generally considered to block the progression of DNA polymerase. In addition, the primers could hybridize not only free ssDNA region but also ssDNA on the TthSSB-255/ssDNA complexes. Then, TthSSB-255 could prevent non-productive binding of DNA polymerase to ssDNA, and direct it to specific dsDNA regions where the primers hybridize, thus allowing more effective strand-displacement in the RCA reaction.

**Figure 8.** Multiplex PCR examinations of RCA amplified whole genome. (a) Control, multiplex PCR amplifications for the 12 randomly selected human genes using human genomic DNA as the template. Amount of the template DNA for the PCR is indicated. Multiplex PCR amplifications of the 12 human genes using RCA products as the template in the absence (b) or presence (c) of TthSSB-255 protein. Amount of the template DNA for RCA is serially diluted as indicated. Throughout (a–c), all samples were amplified with primers at the same primer pair concentrations (0.1 μM per pair). Aliquots of 5 μl volume were electrophoresed through 12.5% acrylamide gel in Tris–borate/EDTA buffer, and stained with SYBR Green (SYBR Green I, Novagen). The signals were detected using a Fluoro Imager (Fluoro Imager 595, Molecular Dynamics). Product sizes (from 57 to 360 bp) are indicated on the right or left side of each panel. The oligonucleotide sequences used for the primers are as follows. Primer sets 1 (chromosome 11), 5'-GGGCA GAGCC ATCTA TTGCT TACA-3', 5'-GGTTG CTAGT GAACA CAGTT GTGTC A-3'; Primer sets 2 (chromosome 16), 5'-GCACT CTCTT GTGCC CCACA GA, 5'-TTGGT CTAGT GAACA CAGTT GTGTC A-3'; Primer sets 3 (chromosome 8), 5'-GTCTCC CGCTG GAAAC-3', 5'-GGAGC AGAGG TCATC GCGCC-3'; Primer sets 4 (chromosome 7), 5'-CACAG ATTTC CAAGG ATGCG CTG, 5'-CCGTGC TCTTG TCCAG ACTTG-3'; Primer sets 5 (chromosome 10), 5'-CGCTCC GTTGCC GAAAC-3', 5'-GGGCA GTTGT GATCC ATGAG AA-3'; Primer sets 6 (chromosome 17), 5'-GCGTC TGATT CCTCA AGTCA AG, 5'-GGAGG GGTGG GGGTT AATGG TTA-3'; Primer sets 7 (chromosome 20), 5'-TTGGT GGGG TGGTG GTCC AATTG TTA-3'; Primer sets 8 (chromosome 13), 5'-GAAGT AAGAC ACGGC TGGGT T-3', 5'-AGCAA GGCAG GGCAG GCAAG T-3'; Primer sets 9 (chromosome 1), 5'-GCCGA CCATT CTCAG GGAAT CT-3', 5'-GCCGA CCATT CTCAG GGAAT CT-3'; Primer sets 10 (chromosome 1), 5'-CCACC ATGTT GGTCC CTA-3', 5'-CCACC ATGTT GGTCC CTA-3'; Primer sets 11 (chromosome 6), 5'-GTCTA GCATG GTGGT GGGTG GGGTG GGGGT T-3', 5'-CACAG CCACC AGATC CAATC-3'.

| (a) Control | (b) Amount of template DNA for RCA (ng) | (c) Amount of template DNA for RCA (ng) |
|------------|----------------------------------------|----------------------------------------|
| M 2 3      | 1 2 3 4 5 6 7                         | 1 2 3 4 5 6 7                         |
| PCR        | 100 10 1 0.1 0.01 0.001               | 100 10 1 0.1 0.01 0.001               |
| RCA->PCR   | 360 350 300 250 200 150 100           | 360 350 300 250 200 150 100           |
| RCA->PCR   | 360 350 300 250 200 150 100           | 360 350 300 250 200 150 100           |

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Although several groups have reported that DNA binding proteins, such as T4gene 32, enhance RCA reactions, there are no reports on the application of SSB protein for RCA where the protein reduces nonspecific RCA products (17,18). Under the conditions we employed here, the inclusion of wild-type SSB protein either from E.coli or T.thermophilus in the reaction mixture for RCA neither reduced nonspecific products nor template concentrations required for RCA. TthSSB-255 could only enhance RCA reactions drastically, which showed weak ssDNA binding affinity. This characteristic of the protein would be critical for reduction of nonspecific products. As shown in Supplementary Figure S2, TthSSB has a significant cluster consisting of acidic residues in its C-terminus although the role has not been cleared. Since F255P substitution is in the cluster, it may be related to a function of ssDNA binding in spite of acidic environment.

The utility of RCA-based whole genome amplification has been demonstrated for a variety of uses including quantitative PCR, SNP genotyping, Southern blot analysis of restriction fragments, chromosome painting (FISH), subcloning and DNA sequencing. The usefulness of whole genome amplification depends on its ability to give complete coverage of the genome with little regional bias, which is critical when the product is to be used for SNP genotyping (19,20). At extremely low template concentrations, regions of the genome are rarely lost, resulting in loss of one allele despite amplification of the locus (21). At a considerably lower template concentration, RCA in the presence of TthSSB-255 protein could amplify template homogeneously than conventional RCA. Therefore, this method may be applicable to a genotyping application.

Finally, the homology between TthSSB and TaqSSB proteins is very high especially in the C terminal domain (see Supplementary Figure S2) (22). Therefore, we strongly suspect that the same effects will be achieved by the TaqSSB mutant protein, which has the mutated amino acid at the same site.

ACKNOWLEDGEMENTS

The authors thank Drs T. Shibata and M. Oishi for valuable suggestions and Drs K. Okumura and K. Kondo for their help and encouragement. The authors are particularly indebted to Dr S. Kuramitsu for providing T.thermophilus genes. Funding to pay the Open Access publication charges for this article was provided by RIKEN.

Conflict of interest statement. None declared.

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