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Biochimica et Biophysica Acta 1307 (1996) 178–186

BBA Biochimica et Biophysica Acta

Construction of single amino acid substitution mutants of cloned *Bacillus stearothermophilus* DNA polymerase I which lack 5′ → 3′ exonuclease activity

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Received 20 November 1995; accepted 15 February 1996

Abstract

Two individual amino acid substitutions were engineered at a selected site in the 5′ → 3′ exonuclease domain of the cloned *Bacillus stearothermophilus* DNA polymerase I gene. These mutations resulted in the expression of enzymes lacking the 5′ → 3′ exonuclease activity while maintaining normal polymerizing activity. The mutated and non-mutated enzymes were each constitutively expressed in an *Escherichia coli* host without the use of an exogenous or inducible promoter, and the mutated enzymes were demonstrated to be equivalent to the subtilisin large fragment of the native holoenzyme in sequencing reactions.

Keywords: DNA polymerase I gene; Exonuclease activity; Substitution mutant; (*B. stearothermophilus*)

1. Introduction

The DNA polymerase I enzyme is used in a variety of biochemical applications including sequencing and amplification reactions. DNA polymerases obtained from thermophiles are particularly useful since higher reaction temperatures not only increase the kinetics of the reaction, but may be helpful in eliminating artifacts due to secondary structure of the template or to mispriming. The large fragment of DNA polymerase I from *Bacillus stearothermophilus* (Bst) has been shown to be useful in performing sequencing reactions due to the decreased secondary structure of the template at the 65°C reaction temperature, the low concentration of template required and the high processivity of the enzyme [1,2]. This fragment is normally prepared by purification of the holoenzyme from *B. stearothermophilus* cells, followed by digestion with subtilisin to remove the 5′ → 3′ exonuclease domain. We were interested in constructing a clone of this enzyme in which the 5′ → 3′ exonuclease activity was absent. In this report, we describe the cloning and sequencing of the Bst DNA polymerase I gene as well as the construction of mutant enzymes in which the 5′ → 3′ exonuclease activity was eliminated by single critical amino acid substitutions, while leaving the polymerizing activity intact. In contrast to reports describing the expression of similar cloned polymerases, all of these clones constitutively expressed enzyme at high levels without any apparent effect on cell growth. The mutant enzymes were also shown to be equivalent to the subtilisin large fragment of the holoenzyme in sequencing reactions, but did not require subtilisin digestion and repurification.

2. Materials and methods

2.1. Bacterial strains, media and plasmids

Escherichia coli strain XL1-Blue MRF cells were obtained from Stratagene Cloning Systems (San Diego, CA) and strain 1200 was from the Yale University *E. coli* Genetic Stock Center (# 4449, genotype F⁻ *rna-1 endA1 thi-1* I⁻ *supE44*). *E. coli* cultures were grown at 37°C in LB medium (10 g tryptone, 5 g yeast extract and 5 g NaCl per liter) with 0.5 mM IPTG (isopropylthio-β-galactoside), 50 μg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 100 μg/ml ampicillin or 12 μg/ml tetracycline as required. *B. stearothermophilus* cells (ATCC type strain 12980) were obtained from American

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PII S0167-4781(96)00051-6

Type Culture Collection (Rockville, MD). *B. stearothermophilus* cultures were grown in LB medium at 55°C. Plasmid vector pGem-3Z was obtained from Promega Corp. (Madison, WI) and pUC 18 was obtained from Life Technologies (Gaithersburg, MD).

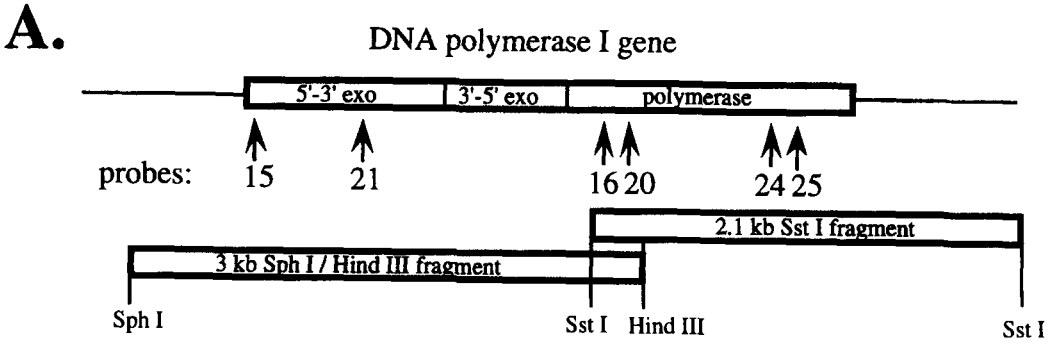
2.2. DNA methods

Plasmid and genomic DNA preparations were made using a miniprep boiling procedure [3] or commercial kit (Qiagen Inc, Chatsworth, CA). DNA oligomer probes 16, 24 and 25 were labeled by [^{32}P]5′-phosphorylation [3]. Probes 15, 21 and 20 were labeled using the LIGHTSMITH™ I high stringency chemiluminescent system (Promega Corp.).

2.3. Identification and cloning of Bst DNA polymerase I gene fragments

Genomic DNA prepared from cultures of *B. stearothermophilus* was digested with restriction endonucleases and Southern blotted. Hybridization probes were synthesized with sequences based on the *Bacillus caldotenax* (Bca) DNA polymerase I sequence [4] in regions known to have strong homology among polymerase enzymes. The probes and their locations are indicated in Fig. 1. Some mismatches between the Bca sequence and the oligomers were introduced either to match a codon preferred by *B. stearothermophilus* or to better match a consensus of the nucleotides at that position in other DNA polymerases. Probes 16, 20, 24 and 25 hybridized with a 2.1 kb *Sst*I fragment. Probes 15 and 21 hybridized to lower molecular weight fragments. Since the full length of the gene was assumed to be approx. 2.6 kb (the length of similar polymerase genes), this indicated that the 3′ end of the gene was located in the 2.1 kb *Sst*I fragment, and that the 5′ end of the gene was present on one or more smaller *Sst*I fragments.

To clone this 3′ gene fragment, Bst genomic DNA was digested with *Sst*I and the 2.1 kb size fraction was gel isolated and ligated with the pGem-3Z vector. *E. coli* XL1-Blue MRF cells were transformed and screened with probe 20. A clone was identified (pGem Bst 3′ end) which was confirmed to contain the 3′ end of the polymerase gene by hybridization with probe 16. Plasmid DNA was purified and sequenced using the dideoxy chain termination method [5].



B.

Probe 15	5'-	TTAATCGACGGCAGCAGCGTGGCGTACCGCGCCTTTTTCGCCTTG-3'
Bca (19-63)	5'-	TTAATCGACGGCAGCAGCGTGGCGTACCGCGCCTTTTTCGCCTTG-3'
Probe 21	5'-	TTGATGGGTGATAAGTCGGATAACATTCCTGGGGT-3'
Bca (544-578)	5'-	TTGATGGGCGACAAATCGGACAACATTCCCGGAGT-3'
Probe 16	5'-	GAGCAGCGCATTTATGAGCTCGCCGGCCAAGAATTCAA-3'
Bca (1543-1580)	5'-	GAGCAGCGCATTTATGAGCTCGCCGGCCAAGAATTCAA-3'
Probe 20	3'-	TTTTGGCCGATGAGGTGTAGTCGCCTACACGACCTT-5'
Bca (1651-1686)	5'-	AAAACCGGCTACTCCACTTCGGCGGATGTGCTTGAA-3'
Probe 24	3'-	GCCGCGGCGATAAACGGCCTATAATGCTCGGCGTTGAAGTT-5'
Bca (2308-2348)	5'-	CGGCGCCGCTATTTGCCGGATATTACGAGCCGCAACTTCAA-3'
Probe 25	3'-	GGCTAAGTTCCCTCGCGGCGACTGTAATAATTTTTCCGCTAC-5'
Bca (2308-2348)	5'-	CCGATTCAAGGGAGCGCCGCTGACATTATTAAAAAGGCGATG-3'

Fig. 1. Probes used for identifying genomic fragments encompassing the Bst DNA polymerase I gene. (A) The locations of the probes and cloned fragments are shown schematically in relation to a full-length DNA polymerase I gene, and to the boundaries of the three structural domains of the protein. (B) The probe sequences are shown aligned with the Bca gene counterparts and coordinates.

A *Hin*dIII restriction site was located near the 5′ end of the cloned insert on the 3′ side of probe 20. This site provided a means for cloning the 5′ end of the gene. Bst genomic DNA was digested with *Hin*dIII plus *Sph*I, Southern blotted and hybridized with probes 21 and 20. A single hybridized fragment approximately 2.8–3 kb long was identified which was long enough to contain the remaining 5′ end of the gene. Bst genomic DNA was digested with *Hin*dIII plus *Sph*I and the 3 kb size fraction was gel-isolated and ligated with the pGem-3Z vector. A clone was identified (pGem Bst 5′ end) which was hybridization positive with probes 20 and 21 and which contained the remaining 5′ portion of the gene as well as some 5′ flanking sequence.

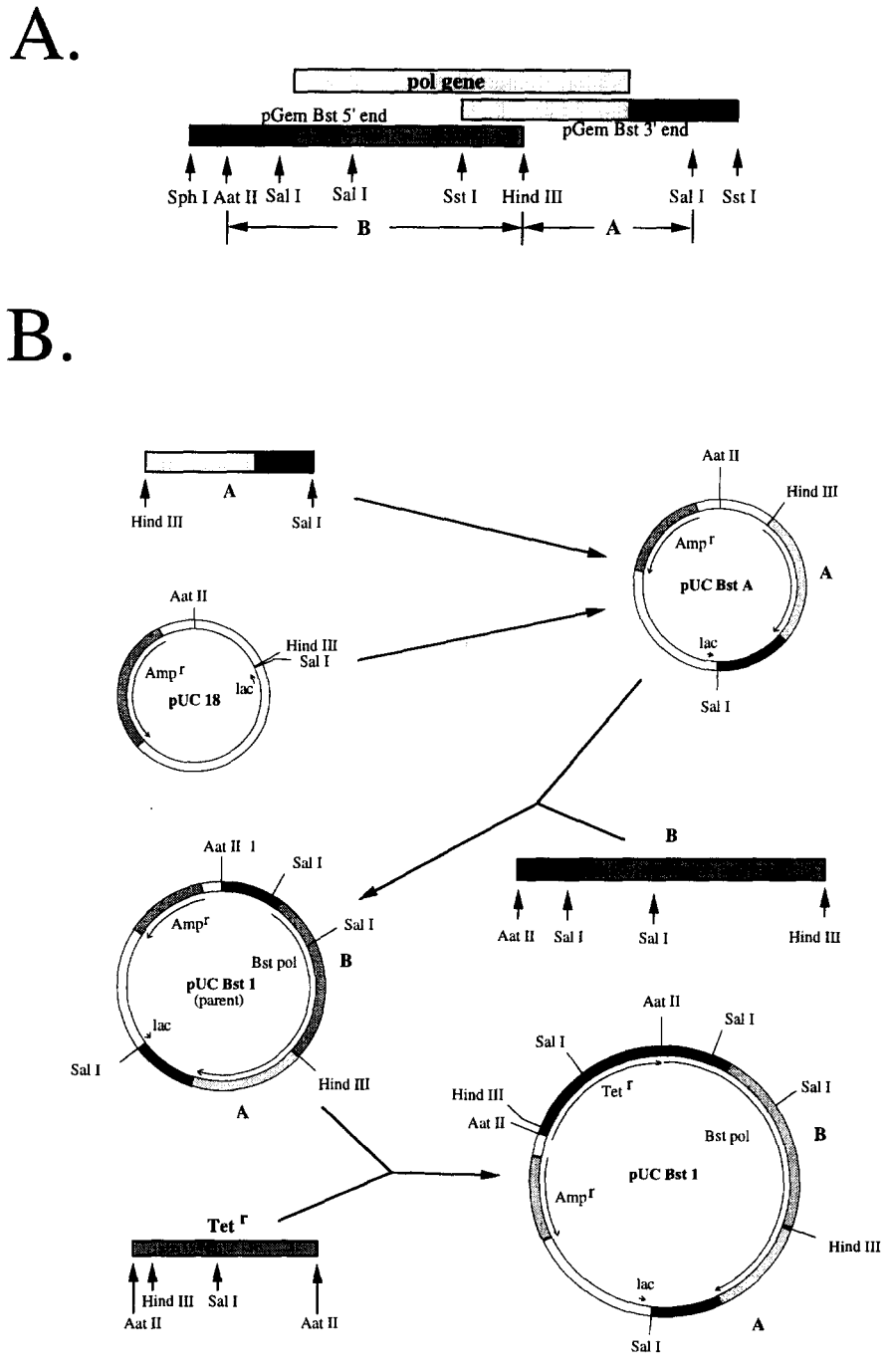


Fig. 2. Strategy for the construction of pUC Bst 1. (A) Physical map showing the cloned genomic fragments and relevant restriction endonuclease sites. Flanking regions are shown in black. (B) Steps in the construction. Fragment A was isolated from pGem Bst 3′ end and inserted into pUC 18 to create pUC Bst A. Fragment B was isolated from pGem Bst 5′ end and ligated with pUC Bst A to reconstruct the intact gene in the parent clone of pUC Bst 1. The *Aat*II linkered tetracycline resistance gene from pBR322 was then inserted to complete clone pUC Bst 1.

2.4. Construction of Bst DNA polymerase clones

A precursor clone (designated pUC Bst A) was constructed which contained the 3′ end of the gene shown as fragment A in Fig. 2. pGem Bst 3′ end DNA was digested with *Hin*dIII plus *Sal*I and the approx. 1.6 kb long fragment A was gel isolated and ligated with pUC 18.

Clone pGem Bst 5′ end was then used for isolating the 5′ end of the gene shown as fragment B in Fig. 2. Purified pGem Bst 5′ end DNA was digested with *Hin*d III plus *Aat*II and the 2.3 kb fragment B was inserted in pUC Bst A. A clone containing the reassembled full-length Bst DNA polymerase gene plus additional 5′ and 3′ flanking sequences (designated pUC Bst 1 parent) was identified by *Sal*I restriction mapping.

Two clones containing amino acid substitutions (designated pUC Bst 5 and 6) were constructed using a common strategy. DNA from the parent clone was partially digested with *Acc*I plus *Xmn*I to remove the 153 base pair region from gene coordinate 103 to 256. Clones containing the desired nucleotide substitutions were constructed by the insertion of annealed and extended oligomers as shown in Fig. 3.

In order to prevent overgrowth of plasmid negative cells due to ampicillin degradation by β-lactamase, the *Eco*RI to *Ava*I tetracycline resistance gene fragment from pBR322 was cloned into pUC 18 following the addition of *Aat*II linkers. The Bst clones were each converted to tetracycline resistance by insertion of this fragment at the unique vector *Aat*II site. Clones containing the tetracycline resistance gene in the same orientation as the polymerase gene were identified by restriction mapping. *E. coli* strain 1200 was transformed and used as the host cell line for all enzyme purifications.

2.5. Purification of the enzymes

Cultures to be used for purification of the enzymes were grown for 16 h at 37°C in N-Z amine A medium (Sheffield Products, Quest International, Norwich, NY) containing 12 μg/ml tetracycline. The pelleted cells were washed once with 0.1 mM EDTA, 20 mM Tris-HCl (pH 7.5) (buffer A)

A.

oligo # 1

5′-AACGCAGTCTACGGGTTTACGATGATGTTAAACAAAATTTTGGCGGAAGAGCAGC
CGACCCACATTCTCGTGGCGTTTGACGCCGGGAAAACGACGTTC -3′

oligo # 2

5′-GCAG CGG AAA CTG TTC CGA CAG TTC CGG CGG CGT CTG CTG CCG CCC GCC
TTT (AaA) GTC TTG GAA CGT TTC ATG GCG GAA CGT CGT TTT CCC GGC GTC -3′
new Dra I site

oligo # 3

new Eag I site
5′-GCAG CGG AAA CTG TTC CGA CAG TTC CGG CGG CGT CTG CTG CCG gCC GCC
TTT (cgc) GTC TTG GAA CGT TTC ATG GCG GAA CGT CGT TTT CCC GGC GTC -3′

B.

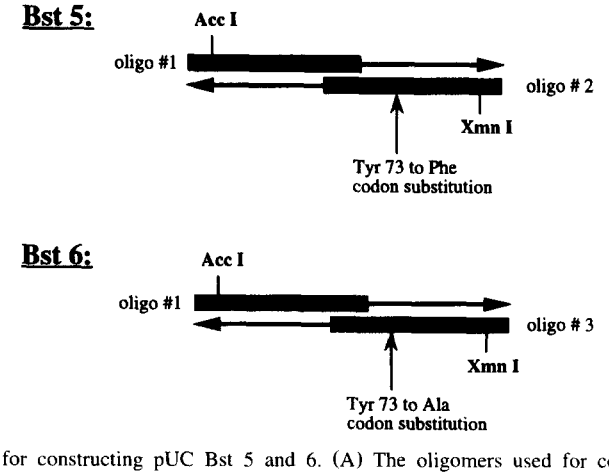


Fig. 3. Replacement fragments for constructing pUC Bst 5 and 6. (A) The oligomers used for constructing the replacement fragments are shown. Nucleotides mutated from the native sequence are shown in lower case. New diagnostic restriction enzyme sites are underlined and the mutated codons are enclosed in parentheses. Note that oligomers 2 and 3 are (-) sense. (B) Schematic diagram showing the location of the mutations and the orientation of the oligomers used in the construction of the replacement fragments.

and resuspended in lysis buffer consisting of 10 mM EDTA, 1 mM dithiothreitol (DTT), 1% Triton X-100, 10 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 mM Tris-HCl (pH 7.5). The cells were lysed by passing the suspension through an APV Gaulin Inc. (Wilmington, MA) model 15MR laboratory homogenizer two times at 8000 psi. The lysate was centrifuged at $12000 \times g$ for 15 min and the supernatant was collected. The supernatant was applied to a Poros-HQ anion exchange column (PerSeptive Biosystems, Cambridge, MA) and washed with 20 mM Tris-HCl (pH 7.5). The bound proteins were eluted with a linear gradient of 0 to 0.5 M NaCl in buffer A and the polymerases were found to elute between 0.1 and 0.2 M NaCl. These fractions were collected, diluted with three volumes of buffer A and applied to a P11 phosphocellulose (Whatman International Ltd., Maidstone, UK) cationic exchange column equilibrated in buffer A plus 50 mM NaCl. The column was washed with the same buffer and the bound proteins were eluted with a linear gradient of 0.1–0.7 M NaCl in buffer A. Fractions between 0.25 and 0.3 M NaCl were pooled, dialyzed against buffer A and applied to a SynChropak AX-300 anion exchange HPLC column (Rainin Corp., Emeryville, CA) equilibrated in buffer A. The bound proteins were eluted with a linear gradient of 0.1 to 0.7 M NaCl in buffer A. The polymerases were found to elute between 0.2 and 0.4 M NaCl. All steps were performed at room temperature.

2.6. Subtilisin digestion

Bst DNA polymerase I holoenzyme purified as above was further digested with subtilisin to produce the large fragment of the enzyme. The holoenzyme was incubated with subtilisin (200:1 w/w) in buffer A at 25°C for 40 min and the reaction was terminated by the addition of 1 mM

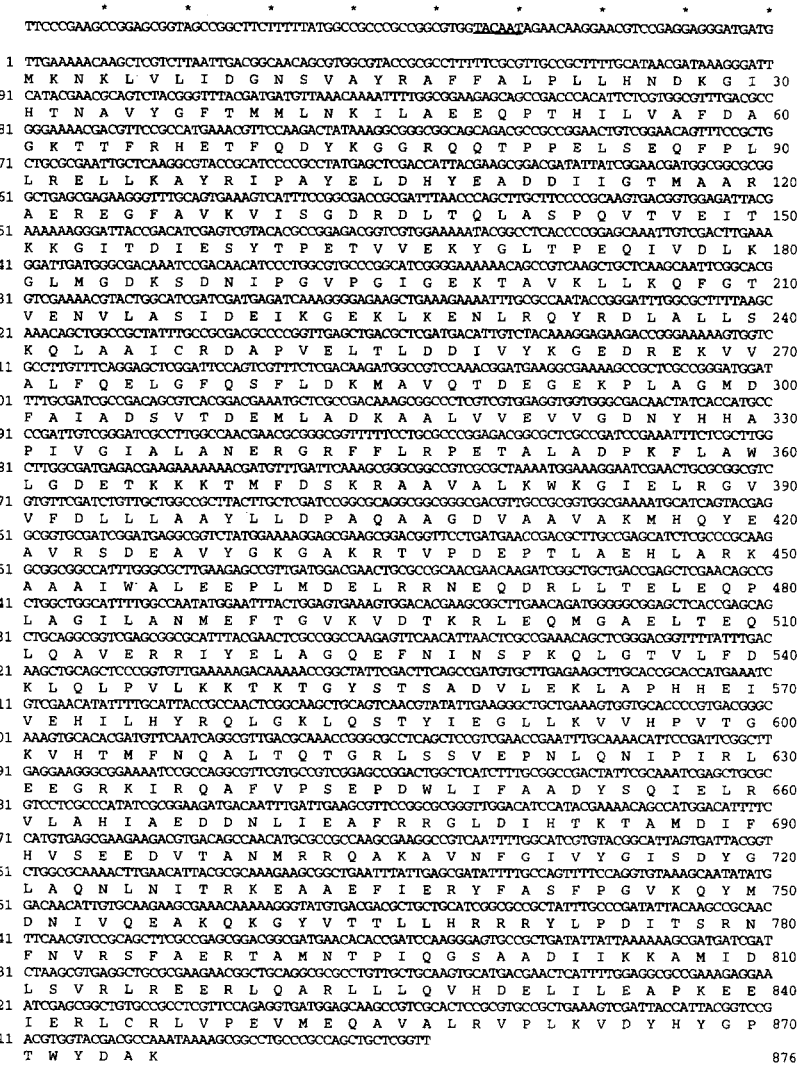


Fig. 4. Sequence of the Bst DNA polymerase I gene and flanking regions. The deduced amino acid translation is also indicated. The -10 promoter region utilized by *E. coli* in expressing the Bca gene [4] is underlined. This sequence has been deposited in the GenBank database under accession number L42111.

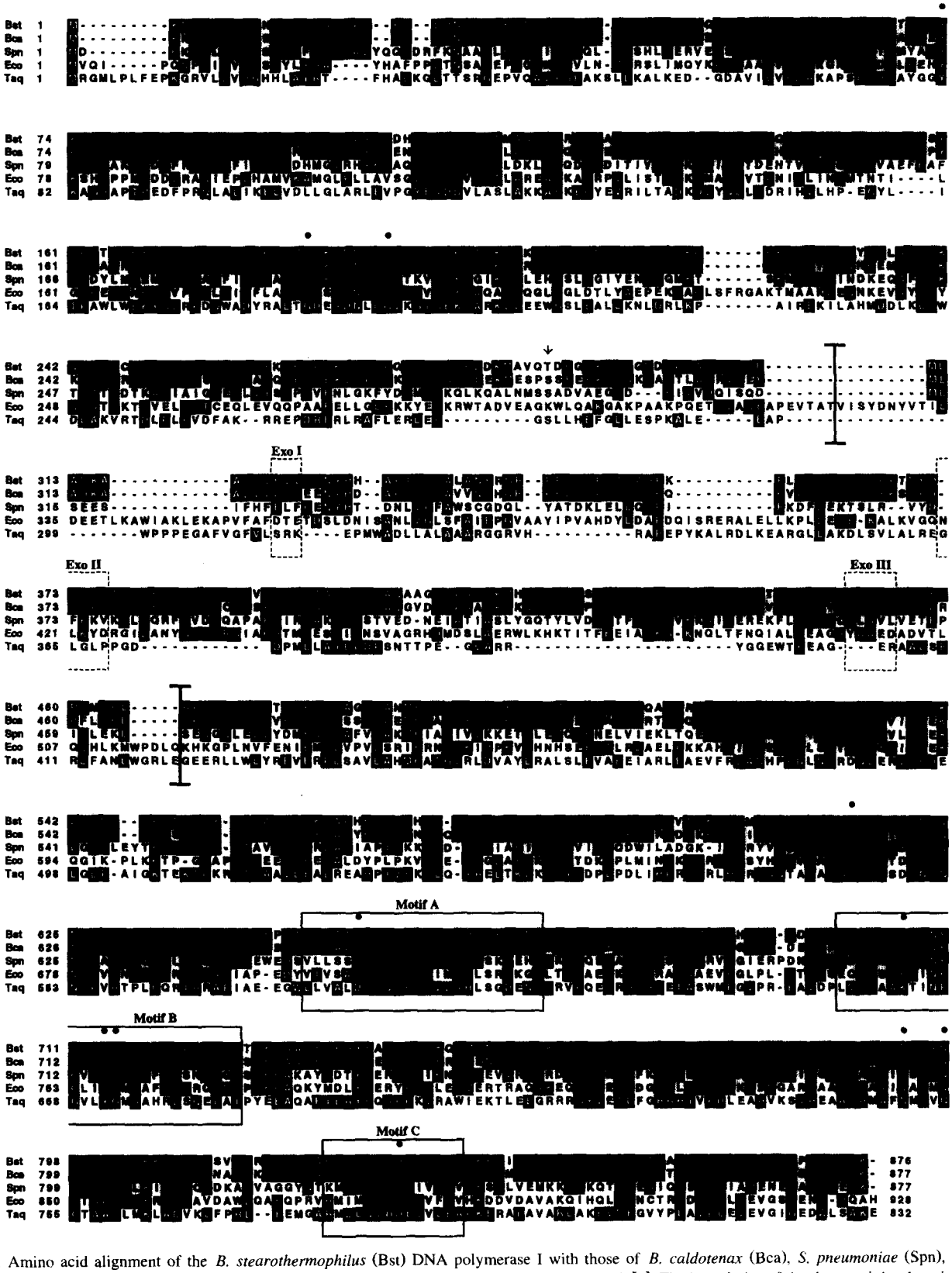


Fig. 5. Amino acid alignment of the *B. stearothermophilus* (Bst) DNA polymerase I with those of *B. caldotenax* (Bca), *S. pneumoniae* (Spn), *E. coli* (Eco) and *T. aquaticus* (Taq). The Bst sequence alignment is superimposed on that of Uemori et al. [4]. The boundaries of the three activity domains in *E. coli* (10) are indicated by heavy bars, and the start of the Bst subtilisin large fragment is indicated by an arrow. Residues identical to the Bst enzyme are shaded, and the highly conserved residues in the 5′ → 3′ exonuclease and polymerase domains mentioned in the text are indicated with closed circles. The aligned regions corresponding to the *E. coli* 3′ → 5′ exonuclease Exo I, Exo II and Exo III motifs [9] are boxed with dotted lines. The conserved polymerase A, B and C motifs [8] are boxed with solid lines.

PMSF. The large fragment was subsequently purified by hydroxyapatite chromatography [6].

2.7. DNA polymerase activity assays

Purified enzymes were incubated at 60°C for 30 min in a 50-μl reaction containing 50 mM Hepes (pH 7.6), 5 mM $MgCl_2$, 100 mM KCl, 0.2 mM each dNTP, 0.8 μCi [^{32}P]dATP, 1 mM 2-mercaptoethanol and 10 μg activated salmon sperm DNA prepared as described [7]. The reactions were chilled and 20 μl aliquots were spotted on Whatman GF/C filter discs and dried. The filters were sequentially washed in 5% trichloroacetic acid (TCA) plus 0.2% sodium pyrophosphate, 5% TCA alone and three times in ethanol. The [^{32}P] incorporation was measured and used to calculate units of polymerase activity. One unit was defined as the amount of enzyme which incorporates 10 nmol of dATP into acid insoluble material in 30 min at 60°C.

2.8. 5′ → 3′ exonuclease assays

Linearized and dephosphorylated plasmid DNA was 5′ end-labeled with [^{32}P] using T4 polynucleotide kinase, and approx. 0.015 pmol of product (containing approx. 130 000 cpm) was used as substrate in each reaction. Purified Bst DNA polymerases were added to the substrate in a 50 μl total reaction containing 0.5 mM each dNTP, 1.5 mM $MgCl_2$, 90 mM KCl and 10 mM Tris-HCl (pH 8.3). The mixture was incubated at 60°C for 3 h and chilled on ice. Bovine serum albumin was added as a carrier and the mixture was precipitated by the addition of 20 μl of cold 50% TCA. Following a 20-min incubation on ice, the mixture was spun 5 min in a microfuge and the supernatant and pellet were separated and counted in a scintillation counter. The percentage of total cpm released in the supernatant was calculated as a measure of 5′ → 3′ exonuclease activity. Recombinant *Thermus thermophilus* DNA polymerase from Perkin Elmer Corp. (Norwalk, CT) was used as a positive control and assayed similarly except that the buffer condition and reaction temperature were altered to conform with the manufacturer's specifications for optimal activity.

3. Results and discussion

3.1. Construction of a Bst DNA polymerase I expression clone

Genomic fragments encompassing the 5′ and 3′ halves of the Bst DNA polymerase I gene were identified by hybridization of Southern blots of Bst genomic DNA with oligonucleotide probes homologous to the *B. caldotenax* (Bca) DNA polymerase I gene sequence [4]. A 3 kb *Sph*I to *Hin*dIII fragment was identified which contained the 5′ portion of the gene and a 2.1 kb *Sst*I fragment was identified which contained the 3′ portion of the gene, as outlined in Fig. 1. Clones of these two genomic fragments (designated pGem Bst 5′ end and pGem Bst 3′ end) were constructed and sequenced. A new clone containing the intact DNA polymerase I gene, as well as 5′ and 3′ flanking regions (designated pUC Bst 1) was then constructed by recombining the relevant portions of the two overlapping genomic fragment clones followed by insertion of a tetracycline resistance gene, as outlined in Fig. 2.

3.2. Sequence of the Bst DNA polymerase I gene

The sequence of the Bst DNA polymerase I gene was determined from the two overlapping genomic fragment clones by the dideoxy chain termination method. The sequence of the gene plus additional 5′ and 3′ flanking regions is shown in Fig. 4. The gene has a GC content of 55.4%. It is similar to other eubacterial DNA polymerase I genes and shows 84% nucleotide and 88% amino acid similarity with the gene from the closely related *B. caldotenax*, and shows 51%, 41% and 38% amino acid similarity with the genes from *Streptococcus pneumoniae*, *Thermus aquaticus* and *E. coli* respectively.

The deduced amino acid translation aligned with other DNA polymerase I genes is shown in Fig. 5. Amino acids known to be highly conserved in the 5′ → 3′ exonuclease and polymerase domains of Pol I genes [4,8] are present, including: Tyr-73, Gly-184 and Gly-192 in the 5′ → 3′ exonuclease domain and Arg-615, Asp-653, Lys-706, Tyr-714, Gly-715, Asn-793, Gln-797 and Asp-830 in the polymerase domain. All of the strictly and highly conserved residues of polymerase motifs A, B and C [8] are present. The Exo I (DXE), Exo II ($NX_{2-3}F/YD$) and Exo III (YX_3D) conserved 3′ → 5′ exonuclease active site residues [9] are not present in the Bst enzyme, however.

The Bst DNA polymerase I gene is 3 nucleotides shorter than the Bca gene and lacks an amino acid residue at the Bca Gln-576 position in the polymerase domain of the enzyme. This residue is not generally conserved. The corresponding *E. coli* residue (Glu-629) is known to be near the N-terminus of α helix I of the Klenow fragment [10]. If the Bst residue is in a similar structure, the tip of the 'thumb' region which is thought to fold over the template DNA would be shortened slightly.

3.3. N-terminal amino acid sequence of the holoenzyme and subtilisin large fragment

N-terminal amino acid sequencing of the purified holoenzyme yielded the sequence Met Lys Asn Lys Leu, indicating that the TTG codon at coordinate 1 in Fig. 4 is translated as Met as is the case in the corresponding Bca gene [4]. The holoenzyme was then digested with subtilisin and the N-terminal amino acid sequence of the purified large fragment was determined to be Thr Asp Glu Gly Glu,

indicating that this fragment starts at amino acid residue 289.

3.4. Construction of amino acid substitution clones

Initial attempts at constructing clones of the subtilisin large fragment by truncating the full-length clone were unsuccessful, as we were not able to obtain stable expression of this portion of the gene. An alternative approach was to specifically eliminate the 5′ → 3′ exonuclease activity of the enzyme using appropriate amino acid substitutions. Four amino acid mutations in the 5′ → 3′ exonuclease domain of *E. coli* have been shown to result in enzymes defective in that activity [11]. Three of these mutations (*polA*4113, *polA*214 and *polA*480ex) also affect the DNA polymerase activity of the enzyme. The fourth mutation (*polA107*) does not affect the DNA polymerase activity of the enzyme, presumably because there is less distortion of the molecule than occurs in the other three mutations. The *polA107* mutation consists of a single nucleotide substitution (A 230 to G) which results in the amino acid substitution tyrosine 77 to cysteine. This tyrosine is conserved among DNA polymerases and is also present in the Bst DNA polymerase I gene at amino acid coordinate 73.

We constructed two clones of Bst DNA polymerase with individual amino acid substitutions at this location using synthetic oligonucleotide replacement fragments as described in Section 2. The first clone (pUC Bst 5) contained a tyrosine 73 to phenylalanine substitution. Phenylalanine was selected since the hydroxyl group of the tyrosine would be missing and therefore unavailable for substrate binding reactions, while the space filling phenyl ring would still be present to help maintain the overall conformation of the enzyme. The second clone (pUC Bst 6) contained a tyrosine 73 to alanine substitution. Alanine was selected as a substitution more likely to affect enzyme conformation than would phenylalanine, since both the hydroxyl group and the phenyl ring would be absent. In both cases, the mutations were also selected so as to avoid introduction of a reactive or charged amino acid side chain which might cause unwanted conformational distortions.

3.5. Expression of the cloned enzymes

To evaluate the expression of the cloned enzymes, whole cell lysates were analyzed by SDS PAGE. As can be seen in Fig. 6, there was a strong band of equal intensity in each of the Bst clone samples running at approximately the calculated molecular weight of the enzyme (98 kDa). This band was absent in the lysate of the negative control clone containing vector alone (lane 2). These results indicated that all three clones constitutively expressed the Bst DNA polymerase I gene at reasonably high levels.

Interestingly, we found that stable constitutive expression of the full length Bst DNA polymerase I holoenzyme was not only possible, but that it appeared to be quite robust judging from the band intensities on SDS gels. A repressor was not required, and growth of the cultures appeared to be normal. This finding was surprising in two regards. First, it has been generally reported that stable expression of full-length Pol I on multicopy plasmids in *E. coli* is not possible unless the gene is under the control of an inducible promoter, whereas the corresponding Klenow fragment can be stably expressed [4,12–15]. The toxicity effect in the host cell is apparently due to the 5′ → 3′ exonuclease activity of the enzyme. The most likely explanation of our success would seem to be that at 37°C, the cloned enzyme has only about 10% of the maximum activity [16] and is not sufficiently active to interfere with growth of the host cell. This may not be the sole explanation, however, since Uemori et al. [4] state that they were not successful in directly cloning the entire Bca DNA polymerase gene in their multicopy vector, even though that enzyme has a similar temperature optimum. Second, the expression of the Bst DNA polymerase I enzyme was under the control of either the native Bst promoter sequence or other sequences recognized by *E. coli* RNA polymerase, since an exogenous promoter was not present. The pUC 18 vector does contain a *lac* promoter, but it is present in the opposite orientation and at the 3′ end of the gene in our clone. We have not identified the promoter sequence utilized by the *E. coli* RNA polymerase to transcribe the gene in this clone. The same −10 region

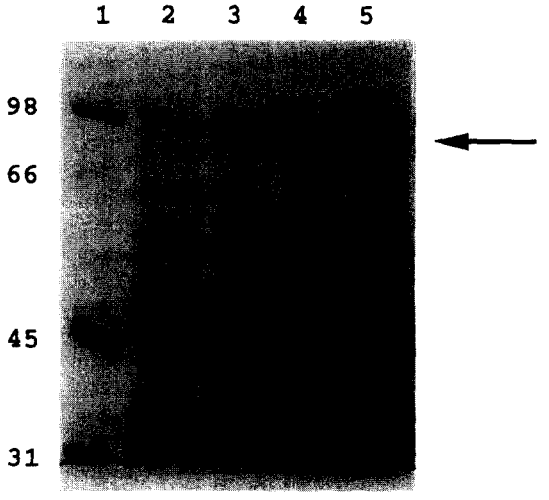


Fig. 6. SDS polyacrylamide gel patterns of whole cell lysates of the Bst DNA polymerase I clones. Cultures of tetracycline-resistant clones pUC Bst 1, pUC Bst 5, pUC Bst 6 and plasmid vector alone in *E. coli* cell line 1200 were grown at 37°C for about 16 h. Cell pellets from 75 μl of culture were lysed with SDS gel loading buffer, run on an 8% SDS polyacrylamide gel and stained with Coomassie blue for visualization of total cell proteins. The arrow indicates the position of the expressed Bst DNA polymerase. Lane 1: molecular weight markers with sizes in kilodaltons indicated on the left; lane 2: negative control plasmid clone without insert, lane 3: pUC Bst 1, lane 4: pUC Bst 5, and lane 5: pUC Bst 6.

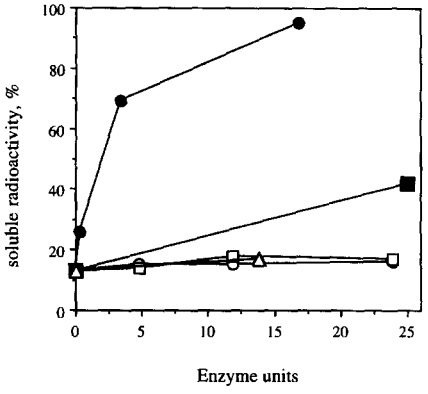


Fig. 7. 5′ → 3′ exonuclease activity of the purified enzymes. Bst enzymes were purified and assayed for 5′ → 3′ exonuclease activity as described in Section 2. A commercial preparation of *T. thermophilus* (Tth) was included as a positive control. Bst 1 (●), Bst 5 (○), Bst 6 (□), Tth (■), and the subtilisin large fragment of Bst 1 (△).

reportedly utilized by *E. coli* in transcribing the Bca gene [4] is present in this clone, although the −35 region they identified is absent (Fig. 4).

3.6. Enzyme characterization

The 98 kDa holoenzymes from pUC Bst 1, 5 and 6 cultures were purified and judged to be greater than 95% pure by SDS PAGE. The 5′ → 3′ exonuclease activities of the purified holoenzymes were measured to assess the effect of the amino acid substitutions. The results, shown in Fig. 7, demonstrate that the non-mutated holoenzyme Bst 1 had a high level of 5′ → 3′ exonuclease activity when compared to *Thermus thermophilus*. As expected, the subtilisin large fragment of the Bst DNA polymerase was devoid of this activity since the relevant domain had been enzymatically removed. The single amino acid substitution clones also showed no detectable activity, indicating that these mutations have completely eliminated the 5′ → 3′ exonuclease activity while leaving the polymerase activity and expression level intact. We were not able to detect 3′ → 5′ exonuclease activity in any of the Bst DNA polymerases, indicating that this activity is absent (data not shown). This is consistent with the observation that the conserved Exo I, Exo II and Exo III motifs forming the 3′ → 5′ exonuclease active site [9] are absent in the Bst enzyme.

Finally, in order to assess the utility of the mutated enzymes, the purified Bst 1, 5 and 6 holoenzymes as well as the subtilisin large fragments of the cloned Bst 1 and native Bst polymerases were used in side by side sequencing reactions (data not shown). Plasmid DNA was sequenced using each enzyme as specified in the Bio-Rad (Hercules, CA) Bst sequencing kit. As expected, the Bst 1 holoenzyme did not produce a sequencing ladder due to the high level of 5′ → 3′ exonuclease activity present. In contrast, the other enzymes all produced clear and identical sequencing ladders, confirming that the amino acid substitutions in Bst clones 5 and 6 have eliminated the 5′ → 3′ exonuclease activity to the extent that these enzymes are comparable to the subtilisin large fragment in these reactions. We expect that the methods described here should be generally applicable to the cloning and modification of other DNA polymerases.

Acknowledgements

We thank Cindy Hasselkus-Light, Denise Riggan and Jaime Blanco for help with enzyme purification and analysis; George Schneider, Kiyotada Nunomura and Dawn Lavka for providing reagents and assistance with enzyme assays; Mehrdad Majlessi, Irene Andruszkiewicz, Lisa Fukunaga and Anna Lio for oligonucleotide synthesis; Linda Korb for help with sequencing and Larry Mimms for critical reading of the manuscript.

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