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Bst polymerase — a humble relative of Taq polymerase

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ABSTRACT

DNA polymerases are a superfamily of enzymes synthesizing DNA using DNA as a template. They are essential for nucleic acid metabolism and for DNA replication and repair. Modern biotechnology and molecular diagnostics rely heavily on DNA polymerases in analyzing nucleic acids. Among a variety of discovered DNA polymerases, Bst polymerase, a large fragment of DNA polymerase I from Geobacillus stearothermophilus, is one of the most commonly used but is not as well studied as Taq polymerase. The ability of Bst polymerase to displace an upstream DNA strand during synthesis, coupled with its moderate thermal stability, has provided the basis for several isothermal DNA amplification methods, including LAMP, WGA, RCA, and many others. Bst polymerase is one of the key components defining the robustness and analytical characteristics of diagnostic test systems based on isothermal amplification. Here, we present an overview of the biochemical and structural features of Bst polymerase and provide information on its mutated analogs.

1. Introduction

DNA polymerases play a crucial role in DNA replication and repair, ensuring the transmission of genetic information to progeny and maintaining genome integrity. They form a superfamily of enzymes consisting of seven separate families: A, B, C, D, X, Y, and RT. Each family includes numerous enzymes with unique characteristics, with every particular DNA polymerase playing a specific role in the cell, requiring an exclusive set of enzymatic properties. For example, DNA polymerases from the B, C, and D families specialize in replication, with enzymes from other families participating in DNA repair. DNA polymerases of the A family are considered unique due to their chimeric properties and 5′–3′ exonuclease activity in a separate N-terminus domain. They are single-subunit enzymes involved in DNA replication and repair by filling gaps after the removal of RNA primers or DNA lesions. Given their relative simplicity, DNA polymerases of the A family became a model for studying DNA synthesis and are widely used in practice.

Since their discovery by Arthur Kornberg in 1956, DNA polymerases have become one of the most commonly used enzymes in modern science, biotechnology, and diagnostics. While DNA polymerase I from a thermophilic bacterium *Thermus aquaticus* is the most known enzyme, other DNA polymerases of the A family are also regarded as convenient tools for practical applications. Thus, specific niches have been found for DNA polymerase I from *E. coli*, the oldest known DNA polymerase, T7 DNA polymerase, and a large fragment of DNA polymerase I from *Geobacillus stearothermophilus*. The latter, the Klenow fragment of *E. coli* DNA polymerase I and the Stoffel fragment of Taq polymerase are homological enzymes comprising a polymerase domain and a 3–5′ exonuclease domain. They retain DNA polymerase activity but lack an N-terminal 5–3′ exonuclease domain with the corresponding enzymatic activity.

Several methods of isothermal nucleic acid amplification have become popular since they allow DNA and RNA to be analyzed outside clinical laboratories without requiring expensive equipment. In the early 1990 s, NASBA, TMA, 3SR, and SDA methods were developed as alternatives to PCR due to its limitations, including the need for a special PCR machine. Rapid testing is particularly advantageous during the pandemic since a timely reaction is crucial in preventing the transmission of pathogens. Isothermal amplification approaches allow a rapid search for infected individuals in crowded areas, which is a gigantic task for conventional clinical laboratories. The cost-effectiveness of bedside testing is of great importance in developing countries with a high prevalence of infectious diseases. In this regard, a large fragment of DNA polymerase I from *Geobacillus stearothermophilus* (BF) has attracted increasing attention. The strand displacement activity of BF is robust and efficient enough to separate DNA strands at a constant temperature without requiring thermal cycling, which is essential for isothermal amplification.

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Several methods of isothermal DNA amplification are based on the strand displacement activity of BF: strand displacement amplification (SDA) [1], rolling circle amplification (RCA) [2], linear target isothermal multimerization and amplification (LIMA) [3], loop-mediated isothermal amplification (LAMP) [4], nicking enzyme amplification reaction (NEAR) [5], and recombinase-assisted amplification (RAA) [6]. The schemes of several well-known methods are given in Fig. 1. The LAMP method is based on 2 or 3 primer pairs forming a dumbbell-shaped structure during synthesis under the action of Bst polymerase, with the structure obtained to serve as a template for further amplification. The resulting LAMP products are concatemers containing multiple copies of that stem structure. The NEAR method uses pairs of primers with nicking endonuclease recognition sites, the corresponding nicking endonuclease, and DNA polymerase with a strand-displacement activity. After annealing, the primers are elongated by the action of the polymerase. At the same time, the nicking endonuclease produces free 3′-OH ends that are recognized by the polymerase, further continuing amplification. Thus, multiple short DNA fragments are produced. It should be emphasized that all these methods do require the strand displacement activity of DNA polymerases.

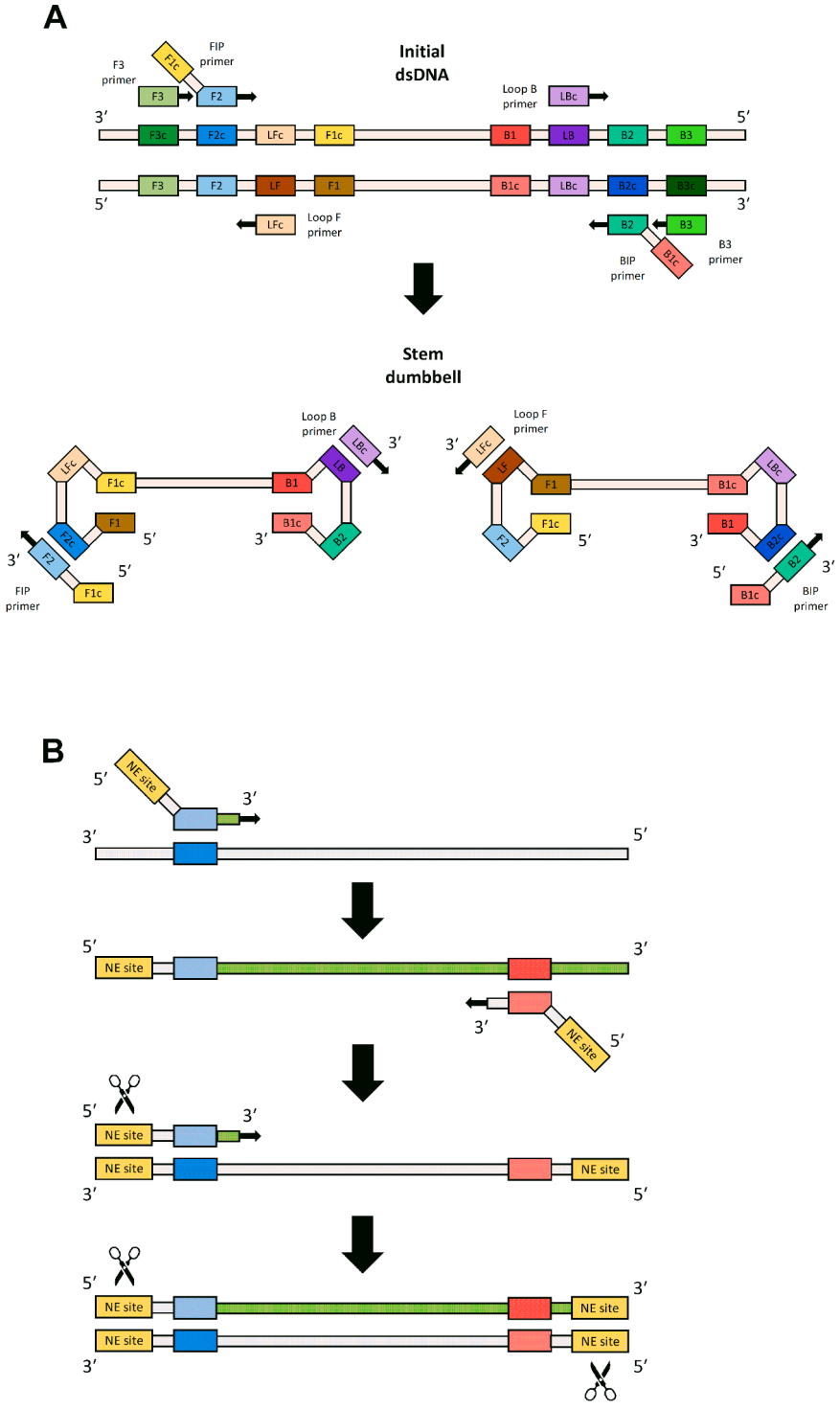

Fig. 1. Schematic representation of LAMP amplification (A), NEAR (B), and RCA.

Without strand displacement, the amount of primed template in the reaction mixture will be insufficient for the polymerase to continue the synthesis, causing the reaction to stop.

Despite being increasingly applied in practice, BF still remains relatively poorly studied in contrast to cognate enzymes, such as the Klenow fragment of *E. coli* DNA polymerase I (KF) and Taq-polymerase. Information on the biochemical properties of BF, such as processivity, optimal buffer conditions, and others, is limited. The difference becomes even more striking when mutants of BF are considered. While BF is an indispensable component of isothermal amplification methods, only a few articles have reported various mutated BF variants with improved qualities. This lack of knowledge remains a challenge for future studies aimed at providing a more comprehensive characterization of BF and ways to improve it.

Here, we review the structural and biochemical features of DNA polymerase I from *Geobacillus stearothermophilus*. A separate paragraph is dedicated to the attempts to improve BF for practical usage. Thus, our aim is to provide a basis for future endeavors in designing mutant BF enzymes that will be superior to the native enzyme that is currently in use.

2. Discovery

The Bst polymerase discovery history is complicated. Several articles reported DNA polymerases from *G. stearothermophilus* with contradicting properties. The main issue was the proofreading exonuclease activity described as weak yet detectable in some papers, with other studies failing to detect any. The first DNA polymerase from *G. stearothermophilus* (former *Bacillus stearothermophilus*) was reported by J. Stenesh and G.R. McGowan in 1972 [7]. It happened six years after the discovery of M-MuLV reverse transcriptase [8] and four years before the discovery of Taq-polymerase [9]. The enzyme was purified directly from *G. stearothermophilus* and had a temperature optimum of 65 °C, which was 10 degrees below the growth optimum of the host bacterium. Later, the same authors demonstrated their enzyme to be prone to misincorporate dATP and dTTP [10]. In 1981, Kaboev et al. described the biochemical properties of a DNA polymerase from *G. stearothermophilus* with the following properties: a molecular weight of 76 kDa, a maximum activity at 60 °C, a pH of 8–9, and a 90 min half-life at 60 °C [11]. Other divalent ions, such as Mn^{2+} and Co^{2+}, but not Ca^{2+} ions, could also be used as cofactors. The exonuclease activity of the purified enzyme was also low, indicating the absence of exonuclease activity centers. Those authors also assumed their protein to differ from the one previously described by J. Stenesh and G.R. McGowan. In the same year, A.F. Trofimenko and A.I. Gaziev reported two DNA polymerases from *G. stearothermophilus* [12] with different molecular weights of 135 kDa or 95 kDa, the pH optimum of 7.3 or 9.0, the temperature optimum of 40 °C or 56–60 °C, and with or without the ATP-dependent exonuclease activity.

In 1992, Sellmann et al. purified and characterized DNA polymerases I from several bacteria: *Bacillus stearothermophilus*, *Bacillus caldotenax*, *Bacillus caldovelox*, and *Thermus thermophilus* [13]. The molecular weight of DNA polymerase from *Bacillus stearothermophilus* was 95 kDa, and the optimal conditions were 65 °C, 30 mM $MgCl_2$ or 0.4 mM $MnCl_2$, and pH 8.8. The enzyme was inactivated by heating at 80 °C, showed no exo- and endonuclease activity, and was inhibited by 49% ddNTP. In the same year, Uemori et al. reported DNA polymerase I from *Bacillus caldotenax*, a close relative to *G. stearothermophilus* [14]. The enzyme with a molecular weight of 99 kDa possessed polymerase activity with maximal efficacy at 65 °C and pH 7.5, 5–3′ exonuclease activity, and a weaker 3–5′ exonuclease activity. Bca-polymerase was inactivated at temperatures higher than 70 °C. The large fragment (65 kDa) could synthesize DNA and retained only a low 3–5′ exonuclease activity. Thus, several groups reported DNA polymerases from *G. stearothermophilus* with different features. The discrepancy mentioned above may have been the result of either the traces of other enzymes in purified DNA polymerases or of different DNA polymerases. *Bacillus* (and *Geobacillus*) cells are known to possess several such enzymes. Another reason that is worth mentioning is that the researchers could have used different bacterial strains.

The controversy was solved in 1995 when two independent research groups cloned the complete gene of DNA polymerase I from *G. stearothermophilus*. Thus, Phang et al. simultaneously cloned and sequenced the polA gene from *G. stearothermophilus* along with its Klenow-like fragment [15]. The authors claimed that both proteins were similar in DNA sequencing, but no data were shown. Aliotta et al. cloned and biochemically characterized Bst polymerase and its large fragment produced by subtilisin digestion [16]. They reported a 99 kDa protein, with the molecular weight of the large fragment being 67 kDa. The authors revealed the absence of aspartate residues in the 3–5′ exonuclease domain necessary for the 3–5′ exonuclease activity and proved it through a direct biochemical analysis. The cloned Bst polymerase was determined to possess a 5–3′ exonuclease activity. The large fragment without a 5–3′ exonuclease domain could synthesize DNA but could not digest it. These observations provided an answer to the question concerning the activities of Bst polymerase and its molecular weight.

The DNA polymerase from *G. stearothermophilus* was first referred to as a “large fragment” in 1987 when S.Y. Ye and G.F. Hong digested the purified polymerase using subtilisin [17]. The resulting protein was most active at 65 °C and was used for sequencing a single-stranded DNA template derived from the M13 vector. Later, the thermostable DNA polymerase from *G. stearothermophilus,* named Bst polymerase, was applied for highly sensitive sequencing of single-stranded and double-stranded DNA [18]. Ye et al. demonstrated that, in the dried state, the large fragment was stable at ambient temperatures [19], which also was convenient for DNA sequencing [20–22]. Hereafter, this truncated polymerase will be designated as BF or Bst polymerase, with the latter often used in related articles referring not to the native enzyme but to its shortened form. Without an N-terminal domain, BF does not possess a 5–3′-exonuclease activity, which degrades an upstream DNA strand during the synthesis. Instead, BF displaces the upstream DNA strand, allowing BF to be used in various isothermal DNA and RNA amplification methods.

3. Structural features of Bst polymerase

Despite their distinct origins and relatively low amino acid sequence homologies, all known DNA polymerases share a common 3D structure resembling a right human hand. A unique feature of A family DNA polymerases is an N-terminal 5′–3′ exonuclease originating from a separate enzyme. The schematic representation of the Bst polymerase structure with functionally important amino acid residues is given in Fig. 2.

The crystal structure of BF was solved by Kiefer et al. in 1998 [23]. They cloned and crystallized N-terminal 592 amino acids fragment of Bst polymerase (67.7 kDa) homological to the Klenow fragment of *E. coli* DNA polymerase (Fig. 3). The biochemical characteristics of the two enzymes and Taq-polymerase were also compared (Table 1). The specific activity, k_{cat}, and processivity of Bst polymerase were significantly higher than those of Taq-polymerase and KF. At the same time, The K_M values for DNA and dNTP were closer across the different enzymes. Surprisingly, BF was found to be catalytically active when co-crystallized with a suitable DNA substrate [24]. In a crystallized form, Bst polymerase also retained the ability to discriminate correct Watson-Crick pairs from mismatches. This discovery contributed to the investigation of the interactions between polymerase and DNA, including various DNA modifications to be discussed below.

The N-terminal 3′–5′ exonuclease domain of BF resembles a similar domain of KF (Fig. 4). Compared to KF, three deletions in the exonuclease domain of Bst polymerase span the last 7 amino acids of helix A, 11 amino acids between sheet 4 and helix B, and 3 amino acids preceding helix F. Among the 4 anionic residues crucial for catalysis and

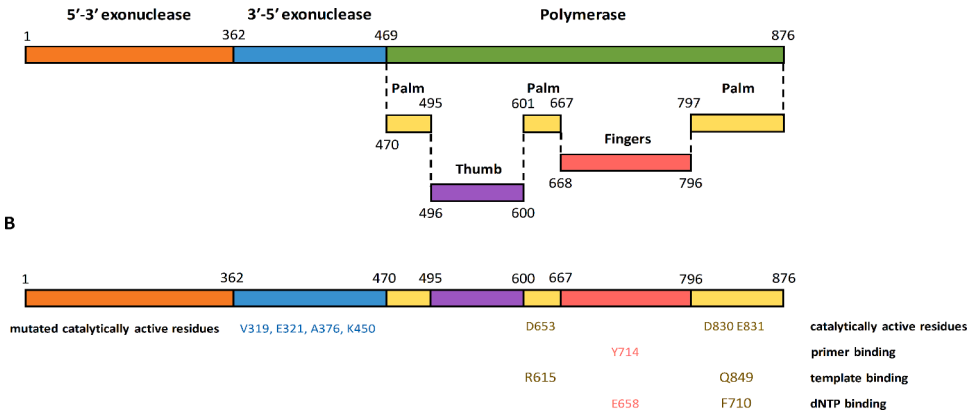

Fig. 2. Schematic representation of Bst polymerase structure (A) and functionally important amino acid residues (B).

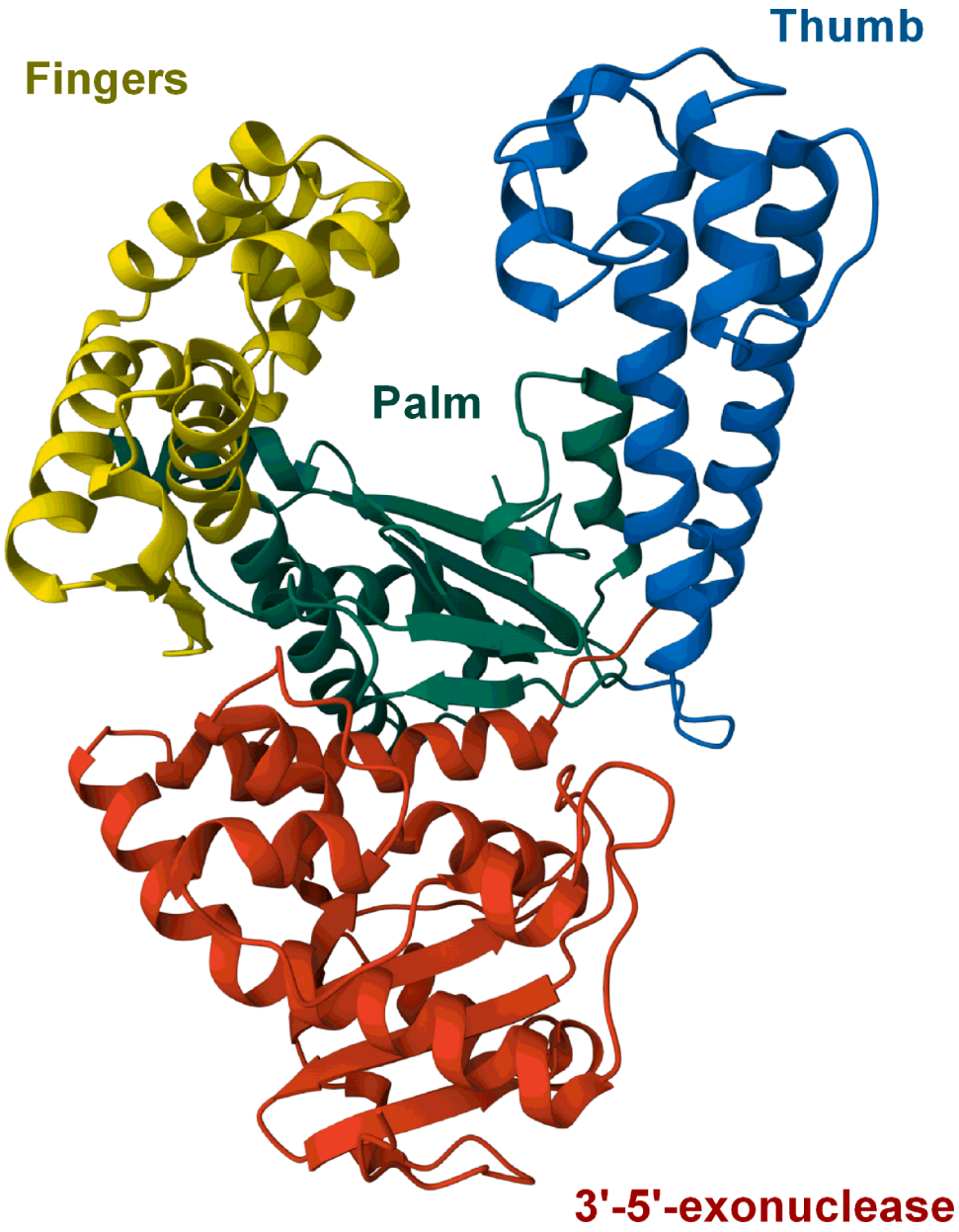

Fig. 3. 3D-structure of Bst polymerase without N-terminal 5′–3′ exonuclease domain. The separate domains are marked in color: 3′–5′-exonuclease is red, palm is green, thumb is blue, and fingers are yellow. The image was taken from the RCSB PDB (RCSB.org) of PDB ID 1XWL [23].

Table 1
Comparison of biochemical features for the large fragment of Bst polymerase (BF), Taq-polymerase, Stoffel fragment of Taq-polymerase (SF), and Klenow fragment (KF) based on Kiefer et al. [23] and other works.

Enzyme	Bst	BF	Taq	SF	PolI *E. coli*	KF
Molecular mass, kDa	97	67	94	63	103	68
Specific activity, U/mg	1.5×10^5 [16]	1.5×10^5 [23] 0.87×10^5 [112] 4.9×10^5[23]	2.2×10^4 (22) 4.9×10^4 [23]	-	9.4×10^4 [113]	9.7×10^3 [23] 9.8×10^3 [114] 5.5×10^3 [23] 2.8×10^4 [114]
Optimal reaction temperature, °C	65 [13]	65 [17]	75 [115]	75 [116]	37[117]	
k_{cat}, s^{-1}	-	191.2 (39.3) [23]	46.6 [23] 21 ± 1 [99]	18 ± 2 [99]	5.7 ± 0.7[118]	5.2 [23] 2.8 [119]
K_m DNA, nM	-	3.4 (1.6) [23]	3.5 [23] 9.6 ± 0.6 [99]	32 ± 10 [99]	35[120]	1.8 [23]
K_m dNTP, μM	-	13 (5.5) [23]	24 (2.3) [23]	-	1.2–14.7[121]	2.3 [119]
Processivity, nt	-	111 [23] 98 [112]	10 [23] 22 ± 3 [99]	2.9 ± 0.7 [99]	15–40[122]	7.7 [119]
Fidelity	-	-	$1.1*10^{-4}$ [123]	-	$1.1*10^{-7}$[124]	$1.1*10^{-6}$ [125]

-*- no information is available

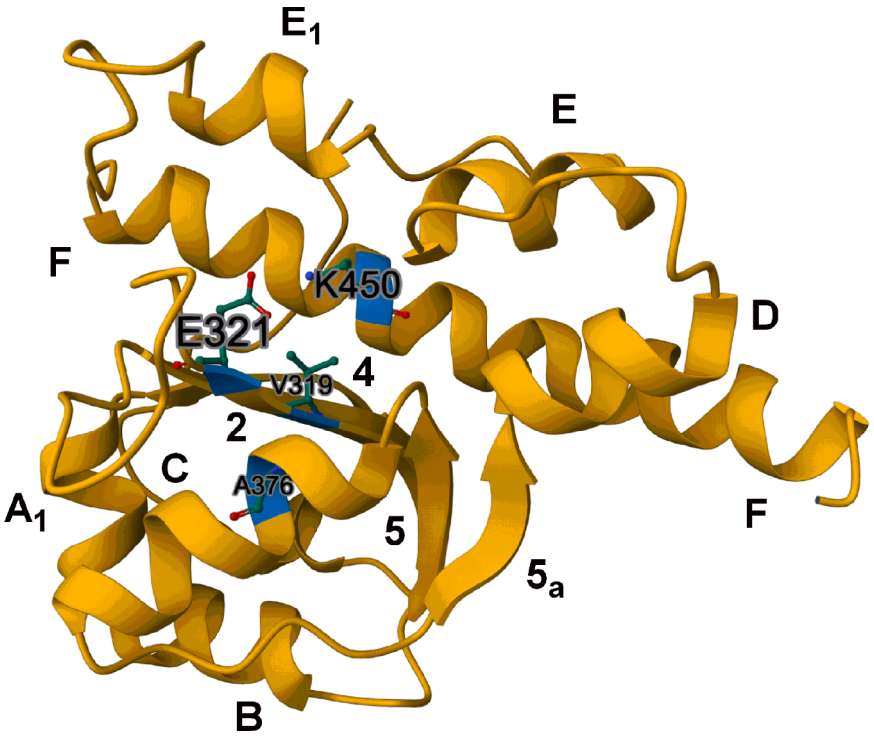

Fig. 4. 3D-structure of the 3′–5′ exonuclease domain. The residues corresponding to those catalytically active in the Klenow fragment are labeled and marked in blue, with their side chains also shown. The secondary structures are also marked by the corresponding numbers and letters. The image was taken from the RCSB PDB (RCSB.org) of PDB ID 1XWL [23].

coordinating divalent ions in KF, 3 residues are replaced by amino acids from other classes, such as V319, E321, A376, and K450. Thus, no binding of metal ions to the active site of the exonuclease was detected in KF crystals grown with MgSO4 or MnSO4. The key amino acids involved in DNA and nucleotide binding, such as L361, D424, F473, and Y497 in KF, were different in Bst polymerase. The helices E and E1 (Fig. 4) reduced the size of the active site cleft, which could involve P403-P424-P438-P441 unique to BF, and the enzyme failed to bind dCMP at the exonuclease site.

The polymerase domain of BF is also similar to that of KF and Taq-polymerase (Fig. 5A). In the thumb, the H and I helices form an anti-parallel coiled-coil structure stabilized by hydrophobic interactions between predominantly leucine residues (Fig. 5B). Most of the conserved residues are located on the surface of the polymerase cleft (Fig. 5C, D). These are 9 amino acids coordinating with the DNA phosphate backbone (N675, N678, K635, R631, E611, T609, R835, D827, S582, N579 of KF) and residues, participating in substrate binding or catalysis (D705, E710, Y766, R841, N845, Q849, R668, D882, E883 of KF). Many conserved amino acids are located in the region spanning the residues 611–666, including helices J–L and sheets 8 and 9, accounting for the bulk of the highly conserved polymerase motif A.

The palm subdomain harbors a polymerase active site with catalytically active residues D653, D830, and E831, corresponding to D705, D882, and E883 in KF. Several hydrogen bonds between these residues, other residues in the active site, and water molecules stabilize a strained conformation of residue H829. Other conservative residues in the active site, Q797, R615, E658, and D830, are close to each other and participate in an electrostatic interaction. Mutations of their counterparts Q849, R683, E710, and D883 disable substrate binding or catalysis of KF.

In the thumb subdomain, a single residue acts like a breaking point between helices I and I_1 and is thought to limit the flexibility of the

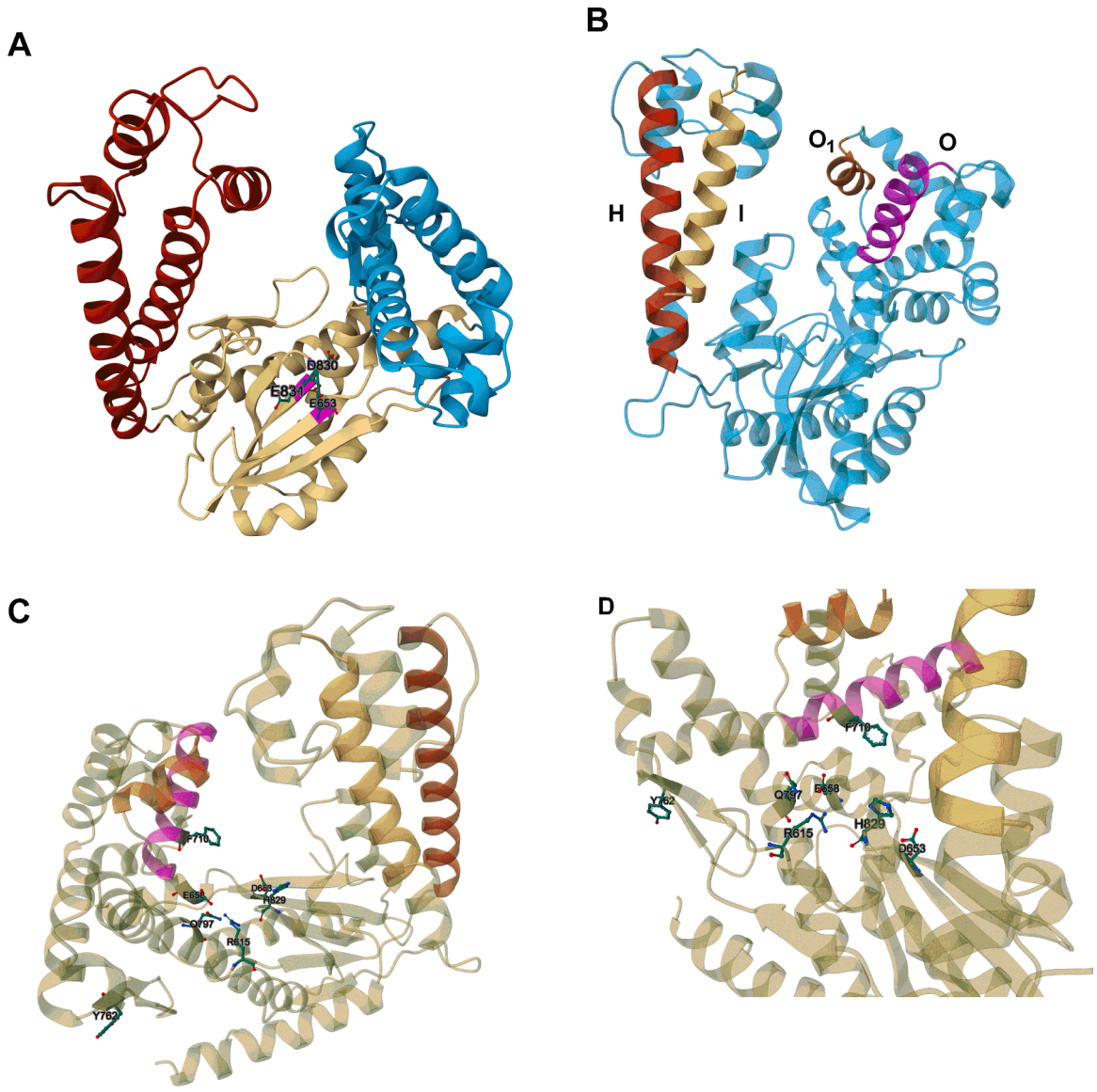

Fig. 5. 3D structure of the polymerase domain. A represents the polymerase subdomains. The separate subdomains are marked in color: the palm is beige, the thumb is red, and the fingers are blue. The catalytically active residues are labeled and marked by magenta, with their side chains also shown. B represents the important α-helices. The helices are marked in color: H in red, I in yellow, O in violet, and O1 in orange. C and D show several amino acid residues participating in the polymerase functioning. The residues are labeled and marked by yellow, with their side chains also shown. The image was taken from the RCSB PDB (RCSB.org) of PDB ID 1XWL [23].

thumb domain of Bst polymerase. A more rigid structure could restrict the transition of DNA between polymerase and exonuclease active sites and be responsible for the higher processivity of BF. Another reason for the increased processivity could be an altered position of a C-terminal turn in the I_1 helix, which is not maintained by hydrogen bonds and is disrupted by K600 instead of G558 in KF. This altered turn could affect the thumb subdomain, stabilizing DNA in the polymerase active site.

In the fingers subdomain, the C-terminal end of the O-helices harbors two highly conservative residues: F710 and Y714. This region is involved in template and/or dNTP binding. In KF, a mutation of the homological F762 residue decreases discrimination between dNTP and ddNTP [25]. Additionally, BF is flexible in the region that connects helices O and O_1, the residue I716. The exact conformation here can be stabilized by DNA binding. The finger subdomain also closely resembles the Mrf-2 domain, found in various DNA-binding proteins, and consists of 6 α-helices and several loops [26].

In terms of thermostability, no significant advantages of structural features were found. Thus, the same percentage of 3′–5′ exonuclease surface area is buried, as in KF. The hydrophobic cores of the enzymes and the ratios of residues E/D, L/I, and R/K are similar. BF also contains fewer ion pairs than KF, although the latter has more unfavorable interactions. The secondary structure of BF is tighter than KF, but their surface/volume ratios are similar, with the C-ends of both enzymes exposed to solvents. Most KF, Bst polymerase, and Taq-polymerase atoms are also in favorable conformations. Conservative deletions in the 3′–5′ exonuclease are the only consistent structural alterations between mesophilic KF on one side and thermophilic polymerases, Bst polymerase, and Taq-polymerase on the other.

The minor groove of the template contacts in a sequence-independent manner with the enzyme by the first 4 nucleotides from the 3′-end of a primer. N3 of purines and O2 of pyrimidines form hydrogen bonds either directly with highly conserved residues or indirectly through water molecules. The major groove is accessible to the solvent and is free from contact with the protein. The terminal base of a primer binds with Y714 by stacking interactions. Removing this interaction in KF by Y766A or Y766S mutations decreases fidelity. The

template base is bound by van der Waals interactions with amino acids of the palm subdomain in the polymerase active site. These interactions and hydrogen bonds with highly conserved R615 and Q849 residues provide high specificity when a mismatched primer has a misaligned 3′-OH-end. DNA in this interaction seems to be in a loosened transition from A to B form spanning up to −5 nucleotide pair and facilitates base-specific interactions of DNA and BF. A divalent ion coordinates with invariant residues D653 and D830, stabilizing the α-, β-, and γ-phosphates of the incoming dNTP. The 3′-OH-end of the primer forms a hydrogen bond with D830 that is invariant in all DNA polymerases. This hydrogen bond with the 3′-OH is subsequently replaced by interactions with the newly introduced Mg^{2+} ion. The 3′-OH-end of the dNTP is positioned above E658 and adjacent to F710 residues. In KF, the counterpart of the latter residue, F762, is involved in the discrimination of sugars.

In 2003, Jonson et al. demonstrated prolonged DNA synthesis by Bst polymerase in crystals [27]. The contacts of D329 and D598A stabilized an open conformation in crystals, and a closed conformation was obtained in the D329A mutants. The authors described four sites interacting with DNA and a new fifth site.

1. A nucleotide “insertion site” is located close to the 3′-end of the primer, where the incoming nucleotide participates in a Watson–-Crick interaction with the acceptor base (n) on the template strand.
2. A “catalytic site,” close to the insertion site, where the new phosphodiester bond is formed. Together, insertion and catalytic sites form a “replication site.”
3. A “postinsertion site” that borders the insertion site and contains the previously synthesized base pair and base pair n-1 in the extending DNA duplex.
4. A “DNA duplex binding region” where the newly synthesized base pairs still interact with the enzyme (base pairs from n-2 to n-11 in BF).
5. A “template preinsertion site,” where the acceptor template base (n) is located before moving into the replication site.

When BF is in an open conformation, the acceptor template base is not present in the insertion site. A conserved Y714 residue stacks against the n-1 template base, blocking access of the acceptor template base to the insertion site. Also, in that conformation, the DNA template turns away, and the acceptor base is located in an additional pocket made by the loop connecting the O and O_1-helices of the finger subdomain. When shifting between opened and closed conformations, the finger subdomain moves, causing the O-helix to rotate. In the closed state, the insertion site is freed from Y714, with the preinsertion site being blocked by the connecting loop of O and O_1-helices. Thus, the transition of the O-helix, the controlling Y714 residue, and the preinsertion site shape define the enzyme conformation.

Two divalent ions are necessary for a new phosphodiester bond to be formed. However, only one Mg^{2+} was observed at the active site of the polymerase. The incoming nucleotide and the acceptor template base are necessary for the metal center to participate in the two-ion catalysis mechanism. The incoming nucleotide is involved in the catalytic Mg^{2+} coordination, displacing the hydrogen bond between the 3′-OH and D830. The displacement leads to an unfavorable geometry of the coordinating center. Thus, this process is involved in nucleotide discrimination and serves as the first checkpoint. The second checkpoint occurs in the postinsertion site where hydrogen bonds between the enzyme and DNA are formed correctly, only given a proper Watson-Crick pairing. When a misincorporation occurs, amplification is probably halted due to a decreased binding in the postinsertion site or a change in the geometry of the 3′-OH primer. The preinsertion site is conserved among DNA polymerases from the A family. The end of the O-helix encompassing Y714 and flanking glycine residues is a part of the highly conserved motif B. The more erroneous pol β (X-family polymerase), an HIV reverse transcriptase, and error-prone Y-family DNA polymerases were found to lack a preinsertion site, emphasizing the role of the latter in the overall replication fidelity.

To sum up, the structural features of Bst polymerase are relatively well-studied, providing a solid basis for biochemical studies and polymerase mutagenesis.

4. Biochemical properties of Bst polymerase

4.1. Overview

DNA polymerase I from *G. stearothermophilus* is a typical A-family DNA polymerase with a molecular mass of 99 kDa, length of 876 amino acids, possessing two enzymatic activities, namely DNA-dependent DNA polymerase (DDDP) activity, and 5–3′ exonuclease activity. The enzyme also possesses limited RNA-dependent DNA polymerase activity (RDDP) [28]. Additionally, Bst polymerase can use xeno nucleic acids as a template for DNA synthesis [29,30]. The 5–3′ exonuclease activity can be eliminated by mutations or by truncating the respective N-terminal domain, leaving the C-terminal polymerase domain active, such as the large fragment (BF) [31]. BF displaces the upstream DNA strand during polymerization and harbors a 3–5′ exonuclease active site inactivated by the exchanges of several catalytic residues [23]. BF also acts as a terminal transferase, adding nucleotides to a 3′-terminus of a nascent DNA strand, preferably dATP [32]. The enzyme can switch templates during synthesis, shifting from one strand to another [33]. Bst polymerase is more active in the presence of Mg^{2+} cations rather than those of Mn2 + , with a pH optimum of 8.8 and a temperature optimum of 65 °C, and is inactivated at temperatures higher than 70 °C [13]. The reaction buffers for Bst polymerase from popular vendors (including New England Biolabs and Lucigene) contain KCl and $(NH_4)_2SO_4$ at low concentration (10 mM), detergent (Triton X-100), no reduction agents such as DTT, and Tris-HCl as a buffer reagent with a pH of 8.5–8.8. The optimal temperature for commercial enzymes is also in the range of 60–65 °C. These specifications agree well with original articles describing the optimal conditions for Bst polymerase. However, some reaction buffers are proprietary, and their characteristics are not disclosed. The salt concentration or temperature range can be higher for mutated analogs of BF, such as Bst 2.0.

While reviewing the biochemical features, it should also be mentioned that the Bst polymerase available on the market is a recombinant protein purified from *E. coli*. Thus, BF is subjected to multiple purification steps to ensure that no host DNA or any other enzymatic activity is present, especially that of nuclease. One of the most common approaches to facilitating the purification of recombinant proteins is attaching the histidine tract (His-Taq) to the protein of interest. However, multiple other tags have been suggested, including maltose-binding protein (MBP), GST, and silica-binding protein (SBP). The latter can be used not only for purifying the enzyme but also for performing enzymatic reactions without the elution of the enzyme. In 2022, Seevaratnam et al. reported on the fusion of Bst polymerase with a doubled R5 peptide at the N-terminus, followed by mCherry, a tract of 10 histidine residues or a flexible linker, and BF at the C-terminus [34]. The mCherry part served for the real-time monitoring of the recombinant protein accumulation, while the R5 peptide allowed the fusion proteins to be purified using silica particles. The fusions were performed by LAMP without BF desorption by directly adding silica with Bst polymerase. However, due to strong adhesion, a high volume of silica was required to be added with the enzyme absorbed to catalyze the LAMP reaction. His-Tag or flexible linker did not affect the absorption, and Ni-resin affinity chromatography could be omitted to reduce the analysis cost. Using PBS during absorption increased the protein yield. However, adding Mg^{2+} and Mn^{2+} increased the activity of silica-bound BF in PBS. The absorbed BF outperformed a commercial enzyme with 4 out of 8 primer sets in detecting malaria plasmodium with LoD up to 10 copies per reaction. In a clinical trial with 500 samples tested for *Plasmodium falciparum* by both PCR and LAMP, a chimeric Bst polymerase with the

flexible linker demonstrated 69% or 50% sensitivity and 85% or 95% specificity depending on the primer set for LAMP. Thus, the silica-absorbed BF was suitable for LAMP, with that purification approach likely to reduce the cost of enzyme production and the overall cost of the assay. The approach developed seems promising for creating cheap and robust tests for developing countries, as it can facilitate the purification and transportation of the most unstable component, such as DNA polymerase. Thus, reagents for testing can be delivered at room temperature without requiring lyophilization of the enzyme.

4.2. Divalent cations

Divalent cations are essential for polymerase catalysis, with polymerase active sites requiring two such metal ions. The first resides in the "A site" and facilitates nucleophilic attack of the 3′-OH group of the primer on the α-phosphorous atom of the incoming dNTP. The second is located in the "B site" and helps to neutralize the developing negative charge as the ternary complex and facilitates the PP_i departure [35]. The polymerase efficacy depends on the type of the divalent cations, with the latter also affecting the DNA synthesis fidelity.

Vashishtha and Konigsberg studied the effect of various divalent ions on Bst polymerase [36]. They found Mg^{2+}, Co^{2+}, Mn^{2+}, and Cd^{2+} to support the polymerase activity of BF, while the enzyme was inactive with Fe^{2+}, Ca^{2+}, Zn^{2+}, Ni^{2+}, Sr^{2+}, Ba^{2+}, Cu^{2+}, and Cr^{2+}. The authors assumed that the ability of Mg^{2+}, Co^{2+}, Mn^{2+}, and Cd^{2+} to be the BF cofactors depends on 1) their ability to effectively reduce the water molecule pK_a, 2) the similar ionic radii of these metal ions, and 3) the ability of these metal ions to form octahedral complexes.

The incorporation efficiency was much higher with Co^{2+} and Mn^{2+} compared to Mg^{2+}. Thus, the $K_{d,app}$ values for correct nucleotide incorporation were very similar, with the k_{pol} values decreasing in the $Mn^{2+} > Co^{2+} > Mg^{2+} > Cd^{2+}$ order. The base selectivity decreased in the $Mg^{2+} > Co^{2+} > Cd^{2+} >$ and Mn^{2+} order, resulting from an increased k_{pol} with incorrect dNTPs for non-Mg^{2+} cations. The authors assumed that this result was due to the formation of a tautomeric cognate base pair between non-matching nucleotides, which is virtually the same as a proper Watson-Crick pair with triphosphates properly positioned for catalysis. The higher mutation rate observed with Mn^{2+} can be explained by the more polarizable nature of Mn^{2+}, the lower energy penalty for coordination number change, and the tighter binding to dNTP triphosphates and carboxylate groups. Template-independent DNA synthesis occurred in the presence of Mn^{2+} and Cd^{2+}, in contrast to Mg^{2+} and Co^{2+}. The processivity decreased in the $Cd^{2+} > Mg^{2+} > Co^{2+}$ and Mn^{2+} order.

To summarize, the incorporation efficiency was higher with Co^{2+} and Mn^{2+}, while the error rate was the lowest with Mg^{2+} ions. These findings resemble the results of similar studies with Taq-polymerase and KF, where non-Mg^{2+} ions decreased the fidelity of DNA synthesis. However, the exact set of cations suitable for catalysis is enzyme-specific. Thus, KF and DNA polymerases from *Pyrococcus abyssi* were demonstrated to work with Ca^{2+} ions [37], which is not possible for BF.

4.3. Strand displacement

For DNA polymerases, strand displacement is the ability to separate the DNA duplex and displace the upstream strand during DNA synthesis. This activity allows elongation to continue without stalling caused by the 5′-end of DNA. Strand displacement may be necessary for *in vivo* functions of DNA polymerases, as in the case of mitochondrial Polγ or phage Φ29 polymerase. In vitro, truncated enzymes lacking a proofreading domain can also exhibit strong strand displacement activity, as can, for example, the large fragment of Bst-polymerase. Strand displacement is a key activity defining the suitability of BF for practical applications. To our knowledge, there have been no studies conducted on the structural basis of the BF's ability to separate DNA strands without other proteins. Therefore, the following paragraph will provide a short glimpse into strand displacement using other DNA polymerases as examples.

In eukaryotes, replicative DNA polymerases tend to have only a weak ability to divide DNA strands, as reported for ε and δ DNA polymerases [38,39]. In the case of phage replicative T4 and T7 DNA polymerases, a regression pressure generated by an upstream DNA fork causes the enzyme to be stalled. A distorted template strand in the active site of the polymerase switches the enzyme to exonuclease mode [40]. This mechanism is assumed to prevent the excessive movement of replicative polymerases and allow replication forks to be synchronized. In mitochondria of higher eukaryotes, Polγ strand displacement is stimulated by inactivation of proofreading activity and the presence of SSB protein [41,42]. A cognate enzyme from Saccharomyces cerevisiae possesses a long C-terminal extension (CTE), and the partial deletion of this extension decreases the strand displacement and increases the preference for exonuclease activity [43].

In prokaryotes, strand displacement seems to be more common among DNA polymerases. It may be due to specific subdomains, as in Φ29 polymerase, or caused by amino acid motives, as in KF. In the highly processive Φ29 polymerase, a terminal protein region 2 (TPR2, long β-hairpin) located between the fingers and the palm and opposite to the thumb is responsible for the unique, strong strand displacement activity [44]. TPR2 is involved in tunnel formation around downstream DNA, promoting the DNA strand separation and generating a ring-like structure with a palm and a thumb conferring processivity. The TPR2 subdomain removal decreases the processivity and strand displacement activity of Φ29 polymerase. The covalent linkage of TPR2 with the thumb inhibits the polymerization on circular templates and the transition from the replication initiation to the elongation phase [45]. In KF, the conservative amino acid residues S769, F771, and R841, being located in the fingers, provide strand displacement activity [46]. The mutations of these amino acid residues impaired the ability of KF to separate DNA strands in an upstream duplex. However, the exchanges of S769 and F771 only slightly affected the polymerase activity [47]. S769 and F771 residues were suggested to participate in strand separation, while R841 was assumed to facilitate that process and interact with the template strand. Also, S769, F771, and R841 were found to form functionally important, relatively weak contacts of the fingers with a single-stranded template [48]. The F771 residue of KF corresponds to H678 Taq-polymerase and to Y719 in BF. The other two amino acids, S769 and R841, are conservative among these three enzymes. Given that KF and BF possess strand displacement activity and Taq-polymerase does not, it could be speculated that histidine and alanine cannot separate DNA strands like phenylanine and tyrosine. However, a single D732N mutation enables the Taq-polymerase strand displacement activity, suggesting the existence of other mechanisms of strand separation by DNA polymerases [50]. The strand displacement activity of KF could be reduced by the β-clamp, which interferes with the interaction between the fingers and the downstream template-primer junction [49]. This observation should be taken into account when trying to stimulate the polymerase activity of A-family DNA polymerases using processivity factors.

In reverse transcriptases, another family of DNA polymerases, the finger subdomain also participates in strand displacement. Thus, in HIV-1 RT, the F61 mutations caused the changes in the ability to separate DNA strands in the following order: F61Y≥F61L>wild-type-=F61A>F61W. However, the effect of these mutations on HIV-1 RT processivity was the opposite. Plausibly, F61 stabilizes the first pair in an upstream DNA duplex in a melted state [51]. In M-MuLV RT, a Y64A substitution demonstrated the same processivity and RNAseH activity as the wild-type enzyme, while the strand displacement activity was impaired [52]. It is worth noting that despite the L99 amino acid residue of M-MuLV RT corresponding to F61 of HIV-1 RT (involved in the strand separation), the strand displacement remained unaffected by the Y64F and L99 mutations.

The exact mechanism of strand separation by BF remains unknown.

However, structural studies of KF allow one to confidently assume Y719 to be the key amino acid residue in this process. This residue, together with S717 and R789, is located in the finger subdomain responsible for strand displacement in reverse transcriptases. However, Φ29 polymerase and Polγ of Saccharomyces cerevisiae indicate the existence of other structural features enabling strand displacement, for example, additional subdomains. Of interest is the case of the D732N mutation in Taq-polymerase, also enabling the enzyme to divide DNA strands, demonstrating, in turn, another unstudied way of strand displacement.

4.4. Fidelity

In the characterization of DNA polymerases, the term "fidelity" refers to the rate of errors occurring during DNA synthesis caused by the enzyme, whether on a DNA or RNA template. These *de novo* errors are mismatches, deletions, insertions, and undesirable template switching. A-family DNA polymerases lack any proofreading activity, with the fidelity of these enzymes being relatively moderate. Thus, a large fragment of DNA polymerase I from *Geobacillus anatolicus* was cloned and characterized by Çağlayan and Bilgin [53]. They found a 3-times higher sulfur elemental effect for misincorporation and suggested that it was a consequence of the phosphoryl transfer serving as a rate-limiting step for the incorporation. LacZ assay demonstrated a significantly higher error rate directly depending on the reaction temperature. The same effect of reaction temperature on fidelity was demonstrated earlier for Taq-polymerase [54]. According to the results of Sanger sequencing, the error rate of Bst-polymerase was reported to be similar to that of other cognate DNA polymerases, specifically 15×10^{-6} [55]. NGS provided other estimations for commercially available variants of Bst-polymerase. Bst 2.0 and Bst 3.0 produced $62 \pm 5 \times 10$–6 and $70 \pm 23 \times 10^{-6}$ errors per base, respectively, with 92–89% of substitutions, 7–8% of deletions, and 1–3% insertions in DNA-directed synthesis. In RNA-directed synthesis, the fidelity of these enzymes was significantly lower, $179 \pm 105 \times 10^{-6}$ and $181 \pm 102 \times 10^{-6}$ errors per base, respectively, with 78–82% of substitutions, 16–15% of deletions, and 6–4% insertions [56].

In contrast to the biochemical studies, no misincorporation was detected in BF in the crystallized state. Moreover, no blunt-end template-independent primer extension occurred [27]. The latter contradicts the terminal transferase activity of the enzyme mentioned in several works. Such a discrepancy highlights a specific limitation of crystallographic studies when existing enzymatic activities are not observed during crystal catalysis. The ability to synthesize DNA in crystals made BF a valuable model for studying the lesion bypass by non-translesion DNA polymerases. Many papers were published describing the details of conformational changes that BF undergoes when encountering various DNA modifications. Below, we present a brief overview of these findings.

Crystallographic studies with BF provided a comprehensive explanation of the polymerase interactions with various DNA lesions and mismatches, such as bulky carcinogenic adducts, N-2-(2′-deoxyguanosin-8-yl)-acetylaminofluorene (G-AAF) and N-(2′-deoxyguanosin-8-yl)-aminofluorene (G-AF) [57], oxidative lesion 8-oxoguanine (8oxoG) [58], benzo[*a*]pyrene ([BP]dG) [59], O^6-methyl-guanine (O6MeG) [60], and a 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPydG) lesion [61]. In solution, G-AAF completely stopped BF, while G-AF reduced the synthesis efficiency, with only dCTP incorporated into the nascent strand. Against 8oxoG, a misincorporation of dATP was 9 times more efficient than dCTP incorporation, with the opposite result for an unmodified template. Despite a high enzyme concentration and prolonged incubation, the [BP]dG adduct almost completely blocked the primer elongation. In crystals, O^6-methyl--guanine (O6MeG) formed stable pairs with either C or T bases, mimicking canonical Watson-Crick interactions and favoring the misincorporation of dTTP rather than dCTP. The fact that BF preferred dCTP over dATP in the presence of β-cFaPydG indicates a low mutagenic potential of the latter. In the presence of α-cFaPydG, only with a significant excess of the enzyme, a longer elongation time, and a higher dNTP concentration could BF continue the synthesis, with dNTPs having the incorporation efficacy range as dCTP > dATP > dGTP > dTTP.

In crystals, G-AF, 8oxoG, and [BP]dG changed the conformation from *syn* in a preinsertion site to *anti* in a postinsertion site with incoming dCTP. However, the consequences of this transition were different. With G-AF, BF did overcome the modification despite the distortion of the enzyme conformation. With 8oxoG, the proper Watson–Crick base pair blocked the synthesis, while the Hoogsteen A·8oxoG base pair did not cause any distortion, and BF continued the elongation of the nascent strand. [BP]dG extensively distorted both the template and the enzyme, stalling the polymerase. G-AAF was sterically unable to transform into an *anti*-conformation, leaving the enzyme in the open conformation and resulting in the absence of dCTP incorporation. Unlike other lesions, O6MeG is a promiscuous base that can form stable pairs with either T or C, accommodated by binding with the polymerase.

Another issue is determining the structural features of dNTPs that BF uses to distinguish between nucleotides. In 2009, Trostler et al. demonstrated that four nitrogen atoms (N-1, N^2, N-3, or N^6) in purines are required for discriminating between correct and incorrect dNTPs [62]. The nitrogen atoms involved in the formation of correct Watson-Crick pairs, namely N-1, N-3, N^2, and N^6, were necessary for efficient polymerization. Their deletion led to less effective DNA synthesis and increased misincorporations. In a similar work, Patro et al. described the interactions of human DNA polymerase α and BF with hypoxanthine, 8-oxoguanine, and a set of adenine analogs [63]. For BF, N7 modifications in dNTPs decreased the polymerization efficiency to a greater extent than the similar changes in the A:T base pair formation template. Additionally, N7 modifications did not prevent the generation of A:G mispairs. N^6 and N7 atoms were necessary for the A:hypoxanthine base pair formation. The incorporation of dATP against 8-oxoG was much less effective than the opposite process, requiring N-1, N^6, and N7 atoms. These observations suggest that BF "scans" the entire base to prevent misincorporation.

A further issue is the ability of BF to discriminate between dNTPs, ddNTPs, and NTPs. It is known that a concentration of NTPs in cells is 1000-fold higher than that of dNTPs, and DNA polymerases can incorporate NTP into a nascent DNA, making a replication error. Being unnatural substrates, ddNTPs often require specific mutations in DNA polymerases for better incorporation. It should be noted that BF and several other thermostable DNA polymerases can use dNDP as substrates for DNA synthesis. However, pausing was observed before dADP and dCDP incorporation [64]. In 2012, Wang et al. reported on the structural factors for discriminating between dNTPs, ddNTPs, and NTPs by Bst polymerase [65]. They demonstrated that native Bst polymerase incorporated dCTP four times and three times better than rCTP and ddCTP, respectively. The "steric gate" E658A mutation disturbed the active site, decreasing the selectivity and catalytic rate for dCTP. The F710Y mutation, located in the O-helix, led to the efficient incorporation of ddCTP, boosted the catalytic rate for dCTP, and resulted in almost zero usage of rCTP. The same observation was made earlier for other A-family DNA polymerases: *E. coli* PolI [25] and Taq-polymerase [66]. The importance of the F710 residue was also confirmed by Sandali et al. when using a close homolog of BF, namely DNA polymerase I from *Geobacillus caldoxylosilyticus* TK4 [67]. This enzyme has a cognate mutation F712Y that disables the discrimination between ddNTP and dNTP. Thus, both the "steric gate" residue and O-helix prove to be involved in the discrimination of incoming nucleotides in BF.

Finally, several studies have reported BF residues that are involved in error prevention or are potentially important for fidelity maintenance. In 2012, Graham et al. described residues comprising a putative post-insertion site in Bst polymerase and KF using energy decomposition analysis (EDA), electrostatic free energy response analysis (EFER), and noncovalent interaction analysis (NCI). EDA allowed them to identify ten residues interacting electrostatically with mispairs and being totally

conserved in all A-family DNA polymerases. These residues were R615, R629, D653, E658, R702, K706, Y714, R770, K805, and H829. NCI revealed eight other conserved residues featuring noncovalent interactions with mismatches. Among them, only P627 was found to interact with all mismatches when they were at the site of validation. Two residues, R629 and H829, were found by EDA and NCI. Using EFER, the authors determined a set of conserved residues with variable interaction energy for all mispairs, including 615, 629, 653, 658, 702, 706, 770, and 805. In KF, the R668 and N845 residues (R615 and N793 in BF) recognized matched base pairs [68]. Mutations of R668, R682, E710, and N845 residues in KF (R615, R629, R658, and N793 in BF) were found to decrease the fidelity [69]. Thus, the mutation E710A was favorable for the A:C mispair, R668A increased the probability for dGMP insertion errors, R682A and N845A increased the frequency of T:C mismatch, and N845A resulted in a higher frequency of A:A error. Additionally, Q849A, R754A, and H881A (Q797, R702, H829 in BF) were less prone to the T:G misinsertion. These findings may help determine a BF "core" consisting of residues directly involved in catalyzing and preventing misincorporation. Hence, these residues can be excluded from mutagenesis to avoid an undesirable fidelity loss.

All the studies referred to above have provided detailed insights into how BF can distinguish correct/incorrect nucleotides to prevent misincorporation, how the enzyme acts when confronted with various DNA lesions, and what structural features of the polymerase, templates, and dNTPs are essential to ensure fidelity. This knowledge is expected to facilitate the development of novel, improved analogs of Bst polymerase without affecting its fidelity, which is important for practical applications.

4.5. Replication slippage

Replication slippage is one of the reasons for DNA rearrangements *in vivo* and *in vitro*. In cells, the template and primer mismatch can lead to rearrangements and replication arrest. *In vitro*, polymerase slippage results in various mutations, including frameshift mutations in homopolymeric stretches and deletions between repeated sequences. These events can hinder NGS data analysis due to the accumulation of artificial deletions and fusions. The slippage mechanism is closely related to DNA polymerase arrest by secondary structures or DNA lesions promoting enzyme dissociation, formation of heteroduplex, and their elongation. High processivity, in turn, results in fewer amplification errors caused by multiple binding of a polymerase to a template.

The first report on the replication slippage of BF was published in 2001 by Viguera et al. [70]. They tested several DNA polymerases (Taq, Pfu, Pab exo$^-$, Vent, Vent exo$^-$, Tfu, and BF) from thermophilic organisms. As expected, BF with a strong strand displacement activity did not form heteroduplex products in a model system, whereas enzymes with low or absent strand displacement slipped significantly. However, it is still unclear whether BF is more prone to slippage errors compared to a highly processive Φ29 DNA polymerase with a strand displacement activity. Both enzymes are applied in protocols of isothermal DNA amplification, including whole genome amplification (WGA), which is especially vulnerable to various amplification errors. While Φ29 DNA polymerase is preferably used in WGA due to its speed, fidelity, and processivity, Bst polymerase is likely to become a valuable option when damaged DNA is used as a template [71].

4.6. Terminal transferase activity

DNA polymerases that lack proofreading activity can incorporate nucleotides into a nascent DNA strand in a template-independent manner. This synthesis was named a "terminal transferase activity," and some A-family DNA polymerases can also elongate blunt-end DNA molecules. Following the so-called "A-rule," dATP is considered to be the preferential substrate for terminal transferase synthesis. Despite being convenient for TA-cloning by Taq-polymerase or template switching by M-MuLV RT, terminal transferase may be disadvantageous in other reactions. The elongation of blunt ends produces overhangs capable of becoming primers for undesirable amplification byproducts. Therefore, along with misincorporation and replication slippage, terminal transferase activity is the third source of errors arising during amplification. Overhangs can be especially dangerous during isothermal amplification, performed at a relatively moderate temperature when new sticky ends can anneal on potential templates. In this regard, the terminal transferase activity of Bst polymerase is ambivalent in nature and requires careful consideration to prevent the formation of undesirable products.

One possible strategy to reduce terminal transferase activity is to introduce the chemical modifications of DNA to inhibit template-independent synthesis. A single report on such modifications was published in 2016 by Güixens-Gallardo et al. [32]. They studied the influence of ortho-twisted intercalating nucleic acid (ortho-TINA) and 4, 4′-dimethoxytrityl (DMT) modifications at the 5′-end of a template on the terminal transferase activity of several DNA polymerases, including Bst 2.0. The ortho-TINA modification was observed to completely inhibit template-independent incorporation with almost all enzymes, with a single exception of Therminator polymerase. The DMT modification resulted in polymerases retaining various levels of terminal transferase activity. With DMT and ortho-TINA being sizeable modifications, the size of a modification at the 5′-end of a template does not *per se* prevent the formation of a 5′-overhang. It is plausible for the ortho-TINA modification to interact with DNA *via* π–π stacking between pyrene in ortho-TINA and nucleobases in DNA. As a result, a chemical-based approach to control the terminal transferase activity of Bst polymerase was substantiated. Other modifications with a similar effect, particularly at the 3′-end of the primer, capable of preventing the formation of unwanted amplification products, may be discovered in the future.

4.7. Ab initio DNA synthesis

Prokaryotic thermophilic DNA polymerases can synthesize DNA *ab initio*. For example, they can synthesize highly polymeric double-stranded DNA from free dNTPs. This synthesis was registered for A-family DNA polymerases (Bst polymerase) and B-family archaeal enzymes [72,73]. Terminal transferase activity is thought to be one of the main reasons for false-positive results in LAMP and possibly in other amplification methods [74]. However, *ab initio* synthesized DNA molecules can also be formed as byproducts in various methods of isothermal DNA amplification, leading to false-positive results. Therefore, studying this process would allow diagnostic errors to be reduced.

The first to report on *ab initio* synthesis by BF were O.K. Kaboev and L.A. Luchkina in 2004. They discovered the ability of several DNA polymerases, including BF, to synthesize DNA in a template- and primer-independent manner in the presence of *E. coli* helicase DnaB, 4 dNTPs, and ATP. However, the ATPase and helicase activities of DnaB were not involved in the template-independent synthesis by BF. The reaction products were AT-rich DNAs with randomly incorporated dCTP and dGTP. Later, in 2007–2018, a number of studies described the effect of various proteins on *ab initio* synthesis by BF: N.BspD6I nickase [75,76], SSB from *E. coli* and T4 phage [77], and engineered nickases Nt.*Alw*I, Nb.BbvCI, Nb.*Bsm*I [78]. All nickases were found to stimulate *ab initio* DNA synthesis by BF, with no DNA products accumulated in the presence of the heat-inactivated nickase. A*b initio* synthesis was more efficient at 37 °C. When the concentration of nickase was increased, the synthesis products were transformed from net-like molecules of various lengths to shorter and more linear DNA molecules. Depending on the nickase, the *ab initio* products were non-palindromic repeats or short repeated palindromes with recognition sites of the corresponding nickase. The effect of SSBs from *E. coli* and T4 phage on *ab initio* DNA synthesis was contradicting. In rolling circle amplification, T4 SSB increased the reaction yield compared to *E. coli* SSB. T4 SSB was also found to increase the efficacy of template-dependent amplification with

N.BspD6I nickase and decrease the amount of non-specific products. Although *E. coli* SSB did not affect *ab initio* synthesis, regardless of the presence of N.BspD6I, T4 SSB completely inhibited the synthesis. The discrepancy in the results is probably due to the different nature of DNA binding by SSB: *E. coli* SSB binds DNA as a tetramer, while T4 SSB acts as a monomer.

Taken together, the findings described above demonstrate that BF can perform *ab initio* DNA synthesis, with this process stimulated or inhibited by various heterologous proteins. Interestingly, the enzymatic activity of DnaB helicase is not essential for BF stimulation, with only active nickases being able to enhance *ab initio* DNA synthesis. This finding may indicate the existence of various mechanisms for stimulating the template-free DNA synthesis by BF. Whether traces of host DNA in enzymes affect this process remains to be determined. It is well known that DNA-binding proteins naturally e copurify with DNA from host cells, and contaminant DNA can be disadvantageous for practical applications, particularly for microbiome studies. Therefore, the relationship between residual DNA and *ab initio* DNA synthesis needs to be studied, and more detailed information can help prevent the accumulation of unwanted products during isothermal amplification by BF.

4.8. Reverse transcriptase activity

A-family DNA polymerases, being DNA-dependent enzymes, have the ability to use RNA as a template. This characteristic was observed for KF, Taq-polymerase [79], and Tth-polymerase [80]. This activity can be stimulated by Mn^{2+} ions. However, the latter can reduce the fidelity and efficacy of amplification. Direct synthesis on an RNA template can be advantageous for practical applications, as it allows one to dispense with reverse transcription in a separate tube with a specific buffer and an enzyme.

In 2015, Shi et al. reported BF to exhibit weak reverse transcriptase activity, similar to cognate enzymes [28]. The authors confirmed the reverse transcriptase ability of BF, Bst 2.0, and Warm Start Bst 2.0. The enzymes synthesized 65 b cDNA with the same efficacy as AMV RT but were less effective for reverse transcription of longer templates. Several years before, in 2004, Shandilya et al. cloned thirteen and biochemically characterized nine A-family DNA polymerases, including a close homolog of Bst polymerase, DNA polymerase I from *Bacillus caldolyticus* EA1 [81]. The enzyme was stable up to 65 °C and demonstrated an RDRP activity, albeit 20 times lower than that observed in DNA-directed synthesis. Bca polymerase also synthesized a 679 b cDNA fragment despite the presence of 1.5 M betaine, which improved the reverse transcription activity of Tth- and Tne-polymerases. The descriptions of commercial enzymes closely related to BF, such as Bst 2.0 and Bst 3.0, mention their ability to use RNA as a template. Several mutations were mentioned in patents as increasing reverse transcriptase activity of BF. Thus, the ability of BF to recognize RNA as a template could be enhanced by introducing specific mutations, as it was done for Taq-polymerase [50].

4.9. Xeno nucleic acid synthesis

Xeno nucleic acids (XNAs) are synthetic nucleic acid analogs with a sugar backbone different from natural deoxyribose and ribose. They can broaden the genetic alphabet and are superior in stability to DNA and RNA, acting as aptamers and therapeutic agents. Efficient XNA transcription and replication require specific enzymes to recognize them as a template and incorporate artificial nucleotides. The application of BF for XNA synthesis will be described below.

Chaput et al. were the first to report on XNA transcription by Bst polymerase in 2003 [29]. They found BF to be able to use threose nucleic acid as a template for DNA synthesis. Later, in 2016, Dunn and Chaput thoroughly investigated the TNA-dependent polymerase activity of Bst polymerase [30]. The enzyme proved to outperform Superscript II reverse transcriptase and was further enhanced by the addition of $MgCl_2$. In a full DNA–TNA–DNA replication cycle, BF was found to be more erroneous when $MnCl_2$ was added. Meanwhile, BF was more accurate than Superscript II without manganese ions. In 2007, Tsai et al. demonstrated that BF synthesized DNA when glycerol nucleic acids (GNAs) were used as a template and due to template switching [33]. The fidelity of the synthesis with a GNA template was 4 times lower than with a DNA template, with the presence of $MnCl_2$ leading to a two-fold decrease in fidelity. In the presence of 2′-deoxydiaminopurine-5′-triphosphate (dDTP), the addition of $MnCl_2$ increased the efficacy of Bst polymerase and reduced template switching. In 2009, Chen et al. demonstrated that Bst polymerase incorporated glycerol-nucleoside triphosphates (gNTPs) onto a DNA template with a relatively moderate efficiency [82]. Wang et al. showed that wild-type Bst polymerase, Bst 2.0, and Bst 3.0 exhibited strong FANA (2′-fluoroarabino nucleic acid) reverse transcriptase activity [83]. Yang et al. successfully applied Bst polymerase in a α-L-threofuranosyl nucleic acid data retrieval method [84]. The method developed allowed the researchers to store up to 23 kilobytes of information resistant to nucleases. Medina et al. found that BF was able to synthesize a small amount of DNA product using arabino nucleic acid (ANA) and 1′,5′-anhydrohexitol nucleic acid (HNA) as a template. In a complete replication DNA–ANA–DNA cycle, BF demonstrated a fidelity of 99.7% for more than 1000 incorporated nucleotides [85]. Vengut-Climent et al. demonstrated that Bst 2.0 elongated a primer despite the presence of glucose in the template [86]. If the next base after glucose was dA, the enzyme primarily incorporated dTTP. However, if the next base was dT, it incorporated dGTP.

Bst polymerase can incorporate unnatural dNTPs into DNA and is considered one of the best polymerases for this task. Sun et al. reported that BF could use miRNA as a primer and dATPαS (2′-deoxyadenosine-5′-O-(1-thiotriphosphate)) instead of dATP [87]. Later, Matyašovský et al. demonstrated that Bst polymerase could incorporate a set of dATP derivatives with Cl, NH2, CH3, vinyl, and ethynyl modifications in position 2 of adenine; however, phenyl modifications were not incorporated [88]. Cahová et al. reported that Bst polymerase sometimes incorporated modified dNTPs bearing methyl and π-electron-containing substituents (vinyl, ethynyl, phenyl) more effectively than cognate natural dNTPs [89]. Among the studied modifications, phenyl-purine nucleotides proved to be better substrates than natural dNTPs, as opposed to phenyl-pyrimidines.

Bst polymerase is regarded as a promising tool for XNA studies. The enzyme can use several XNAs (TNA, GNA, FNA, ANA, and HNA) as templates and various modified dNTPs as substrates. Thus, BF can become a scaffold for engineering more specific and efficient XNA polymerases.

5. Mutated variants of Bst polymerase

The innate strand-displacement activity of Bst polymerase has made it a popular tool for isothermal DNA amplification. The classical biochemical features of BF are relatively moderate and may limit the application of the enzyme. Specifically, unlike Taq polymerase, BF is inactivated when heated at 70 °C and is less processive than Φ29-polymerase. Another feature is the ability of polymerases to work in the presence of various amplification inhibitors. Bst polymerase is commonly used in diagnostics, especially in point-of-care testing, when DNA samples can be contaminated by various substances. Therefore, more stable and robust enzymes are necessary as key components for future diagnostic tests without advanced DNA purification. The subsequent subsections provide a concise overview of various attempts to improve BF based on publications from peer-reviewed scientific journals. A summary of these works is presented in Table 2.

5.1. Exonuclease activity

Full-length Bst polymerase, being an A-family DNA polymerase, harbors two separate exonuclease active sites. One of them, 3–5′-

Table 2
Summary of improved mutants of Bst polymerase.

Authors	Object	Method	Mutation	Functional consequences
Riggs et al.	Bst polymerase	Site-directed mutagenesis	Y73F and Y73A	Loss of 5–3′ exonuclease activity
Rastgoo et al.	PolI from *Geobacillus* sp. MKK	Site-directed mutagenesis	V319D, E325L, A367D, D425F, insY455, K450D	Gain of 5–3′ exonuclease activity
		Protein chimerization	3′–5′ exonuclease domain from KF	Gain of 5–3′ exonuclease activity
Pavlov et al.	Bst polymerase	Protein chimerization	C2 domain of topoisomerase V from *Methanopyrus kandleri*	Increased thermostability
Milligan et al.	Bst polymerase, KF	Directed evolution	14 amino acids insertion from Bst polymerase in KF	Increased thermostability
Paik et al.	Bst polymerase	Site-directed mutagenesis	S371D/T493N/A552G	Increased thermostability, efficiency in LAMP
		Protein chimerization	Villin from *Gallus gallus*	
Paik et al.	Bst polymerase	Site-directed mutagenesis	S371D/T493N/A552G	Increased thermostability, efficiency in LAMP, tolerance to urea, loss of reverse transcriptase activity (restored by K9D)
		Protein chimerization	Villin from *Gallus gallus* with A20K/ N31R/E43K/N39K or N31R/E43K/N39K	
Ma et al.	Bst polymerase	Site-directed mutagenesis	G310A and G310L	Increased polymerase activity
Piotrowski et al.	Bst polymerase	Site-directed mutagenesis	D422A	Increased strand displacement activity
Sandalli et al.	PolI from *Geobacillus caldoxylosilyticus* TK4	Site-directed mutagenesis	Y721F	Increased strand displacement activity
Seevaratnam et al.	Bst polymerase	Protein chimerization	R5 silica-binding peptide	Purification and performance of LAMP using silica particles

exonuclease, is inactive due to the exchange of catalytically important three amino acid residues. The other, 5–3′-exonuclease, is active. This activity can be disabled by truncation of the whole N-terminal exonuclease domain. However, the 5–3′ exonuclease activity could also be turned off by substituting two amino acids, Y73F and Y73A, as reported by Riggs et al. [31]. They introduced Y73F and Y73A mutation in Bst polymerase based on the *E. coli* DNA polymerase I Y77C exchange. The mutants did not exhibit any 5–3′ exonuclease activity. Hence, a question arises about the strand displacement activity of such point-mutated enzymes and their biochemical properties. In this respect, truncated enzymes may differ from native enzymes, similar to Taq-polymerase and its Stoffel fragment [90]. To date, no comparisons of full-length Bst polymerase and BF have been found in the open literature, suggesting an opportunity for future studies.

The lack of 3–5′ exonuclease activity in Bst polymerase is beneficial for whole genome amplification. The site-directed mutagenesis of residues in the 3–5′ exonuclease active site of BF may restore this activity. No studies were reported for BF. However, Rastgoo et al. managed to restore the proofreading activity of a homologous DNA polymerase I from *Geobacillus* sp. MKK [91]. The enzyme is a close 3–5′ exonuclease-deficient homolog of BF (94% homology). The lack of proofreading activity is due to the exchange of catalytically important negatively charged residues, namely V319, A376, and K450, in the conservative Exo I, Exo II, and Exo III motifs. Other conservative amino acids were also replaced with E325 and D425 or were deleted (Y446). The authors introduced several mutations in various combinations, V319D, E325L, A367D, D425F, insY455, and K450D, and designed a chimeric enzyme containing a 3–5′ exonuclease domain from KF. Most mutants were found to exhibit similar levels of polymerase activity. The chimeric enzyme was less active, which may be due to less thermostable exonuclease domain from KF. In contrast to other mutants, the enzyme with all mutations mentioned above and the fusion protein exhibited 3–5′ exonuclease activity. This finding proves that the proofreading activity of Bst polymerase can also be restored in the same manner.

5.2. Thermal stability

Thermostability affects the overall performance of DNA polymerases by defining their ability to synthesize DNA at elevated temperatures and bypass complex secondary structures of DNA templates. Therefore, most studies aimed at improving Bst polymerase are focused on its thermal stability.

The first to report on Bst polymerase with enhanced thermostability were Pavlov et al. [92]. Specifically, they designed chimeric Bst polymerase with a C2 domain of topoisomerase V (Topo V) from *Methanopyrus kandleri* at the C-terminus. The fusion protein was 8-fold more thermostable than the native enzyme. Later, Milligan et al. used high-temperature isothermal compartmentalized self-replication (HTI-CSR) to develop a novel thermostable enzyme with a strand displacement activity from Bst polymerase and KlenTaq polymerase [93]. The authors prepared a shuffling library using coding sequences of BF and codon-optimized KlenTaq polymerase. Two mutants, 5.9 and 7.16, were capable of performing LAMP and RCA. The 5.9 enzyme comprised a KlenTaq backbone with a 14 amino acids region from BF at the base of the thumb domain, possibly stabilizing a specific conformation favorable for strand displacement activity. The 7.16 enzyme was BF with 4 substitutions, with Q249R, N416S, and E453G located immediately at or near known Taq mutations related to decreased fidelity. The analog of the Q249R led to unnatural nucleobase incorporation. In LAMP, the 5.9 variant was slower but more thermostable than the wild-type Bst polymerase, while the 7.16 mutant was faster but was inactivated by preheating at 85 °C. However, the 5.9 mutant partially lost the thermostability of KlenTaq. In hyperbranched RCA with a relaxed template, the 5.9 variant and BF operated almost equally and were 2–3 times faster than KlenTaq. When a supercoiled template was used, only the 5.9 enzyme demonstrated efficient amplification.

Rational protein design methods are considered a promising alternative for random mutagenesis and directed evolution. For Bst polymerase, the rational design was first used by Paik et al. in 2021 [94] as a machine-learning approach. The authors engineered a fusion enzyme consisting of 3 parts: a thermostable 47 amino acids from villin, a flexible glycine-serine linker, and BF. Villin is an actin-binding protein from *Gallus gallus* with a transition midpoint (T_m) of 70 °C and clusters of positively charged amino acids. The chimeric enzyme (Br512) performed LAMP faster than the parental BF and was more thermostable. Adding the villin component resulted in a 3.5 times increase in the fusion protein yield after purification. With the original MutCompute convolutional neural network, several mutations were found,

withN528E and Y303H inactivating the chimeric enzyme, T493N and A552G increasing thermostability, and S371D not found to increase thermostability. The activity of a triple mutant S371D/T493N/A552G was close to commercial Bst 2.0 and Bst 3.0 and exceeded that of the chimeric polymerase. The triple mutant also proved to be more thermostable than Bst 2.0.

One year later, the same group reported the variants of Bst polymerase with a supercharged additional domain [95]. The authors supercharged the villin part from the early constructed chimeric BF, adding positive and negative amino acids to existing surfaces with the respective charges. All four individual positive substitutions additively increased the thermostability of the enzyme, while negative mutations had no effect on activity. The A20K/N31R/E43K/N39K and N31R/E43K/N39K mutants performed LAMP faster than the parental enzyme after preheating. The authors also constructed combined mutants, namely S371D/T493N/A552G/A20K/N31R/E43K/N39K (Br512 g3.1) and S371D/T493N/A552G/N31R/E43K/N39K (Br512 g3.2). Without preheating, these enzymes were 5 min faster than Br512 in LAMP. The mutants also retained more polymerase activity compared to Br512, its mutated variant, and parental enzymes. Br512 and its various mutants were more active at temperatures higher than 65 °C. However, the mutants produced spurious products that were previously reported for commercial enzymes like Bst 3.0. Considering the inhibitors, Br512 g3.1 and Br512 g3.2 were active in the presence of up to 2 M urea, while Br512 proved to be inactive. The presence of 1 M urea also facilitated LAMP with Br512 g3.1 and Br512 g3.2. EMSA demonstrated the higher DNA binding ability of the mutants compared to Br512. However, Br512 g3.1 and Br512 g3.2 lost their reverse transcriptase activity, while Br512 and the S371D/T493N/A552G mutant retained it. A balancing negative K9D substitution restored the reverse transcriptase activity without affecting the improved thermostability and inhibitor resistance of the mutants.

Over decades, some progress has been achieved in increasing the thermostability of Bst polymerase by several methods, including rational design, fusion with other proteins, and direct evolution. However, the failure of most stable mutants of BF to work at temperatures higher than 85 °C is disadvantageous for the Bst polymerase application.

5.3. Polymerase activity

The polymerization rate is closely related to the structural features of the polymerase active site with a well-balanced geometry, with most mutations here causing the loss of polymerase activity. However, in 2016, Ma et al. found mutations that increase the polymerase activity of BF [96]. The first target was a D540 residue involved in the formation of a new phosphodiester bond, and the second target was a G310 residue located in the preinsertion site and involved in fidelity maintenance. The mutation D540E exhibited a specific activity similar to the wild-type enzyme, while mutants G310A and G310L were more effective. Other mutations, such as D540A, R412A, R412E, K416A, K416D, G310A/D540E, and G310L/D540E, were found to almost completely disable the polymerase activity. In 2020, Hamm et al. found the I716M mutation in the presinsertion site to increase polymerization efficacy 40-fold. Plausibly, the mutated residue does not impede the entrance of the incoming nucleotide in the insertion site. The I716A substitution led to a 30-fold decrease in activity, reduced fidelity, and denaturation after incubation at 65 °C [97]. Thus, mutations of catalytically active residues were deleterious, while the changes in the preinsertion site were not fatal. Broad mutagenesis could identify other mutations advantageous for the specific activity of Bst polymerase. In that light, residues located in the polymerase active site but involved in the catalysis would be a preferential target.

5.4. Strand displacement

Strand displacement activity is one of the key features of Bst polymerase, defining its popularity for isothermal DNA amplification. Piotrowski et al. found a single amino-acid substitution capable of increasing the strand-displacement activity of 3 family A DNA polymerases, including BF [98]. The D422A substitution in a conserved position located in the O_1-helix 2.1-fold was found to increase the strand displacement activity of Bst polymerase. The authors assumed the mutation to be likely to disrupt a hypothetical salt bridge between closely located D422 and R433, affecting the enzyme flexibility. For a cognate DNA polymerase I from *Geobacillus caldoxylosilyticus* TK4, Sandalli et al. described a Y721F mutation that enhanced strand displacement [67]. However, this activity was lower than for *E. coli* PolI, prompting future attempts to improve the strand displacement ability of BF.

5.5. Processivity

Polymerase processivity defines the length of amplification products and their quality. For replicative polymerases, high processivity allows long DNA stretches to be produced in a short period of time. *In vivo*, Bst polymerase mostly participates in DNA repair and replication, filling relatively short gaps. Thus, this enzyme has a relatively low intrinsic processivity. The central sphere of application for Bst polymerase is molecular diagnostics, which involves the synthesis of predominantly short DNA products and does not require highly processive enzymes. However, processivity itself can be synonymous with a high turnover rate, and the high processivity of Bst polymerase could provide an opportunity to use the enzyme in other applications, for example, for whole genome amplification of chemically modified DNA from FFPE specimens [71].

In 2004, Wang et al. successfully increased the processivity of B-family Pfu-polymerase by fusing it with a small protein Sso7d [99]. In 2017, Oscorbin et al. applied the same strategy and obtained a chimeric Bst polymerase with increased processivity and tolerance to inhibitors. The authors fused the Sso7d-like protein Sto7d from *Sulfolobus tokodaii* with either N- or C-termini of BF. One of the chimeric polymerases constructed, namely BF with Sto7d at a C-terminus, demonstrated a 3-fold increase in processivity and 4 times higher efficacy in whole genome amplification. The fused polymerase was also more tolerant to inhibitors, including urea, whole human blood, heparin, and NaCl. However, in contrast to the work of Paik et al. [78], no effect on thermostability and temperature optimum was observed. Such a discrepancy could be explained by the different positions of the N-terminal villin domain and C-terminal Sto7d. It is possible that a broad screening of other DNA-binding domains fused with BF would give even more thermostable and effective polymerases.

5.6. Patented variants of Bst polymerase

Bst polymerase and its analogs are valuable and highly demanded commercial products, with multiple companies engaged in manufacturing. Several patents have been published by vendors related to Bst polymerase, its purification, and its improvement. It is not within the scope of this review to provide a detailed account of the patents. However, a few examples summarizing the history and current state-of-the-art of BF mutagenesis are provided below.

The first variant of Bst polymerase lacking 5′–3′ exonuclease activity was patented in 1994. The resulting enzyme exhibited activity similar to that of a subtilisin-digested fragment of full-length Bst polymerase [100]. The authors also tested two mutants, Y73F and Y73A, demonstrating their polymerase activity and the absence of 5′–3′ exonuclease activity. In the next few years, a number of patents for various recombinant variants of BF were published [101–105]. Surprisingly, several of them repeatedly reported BF to possess 3′–5′ exonuclease activity [101–103]. It may have been the residual exonuclease activity, as reported in the peer-reviewed articles. Although mentioned in the literature only in 2015, the reverse transcriptase activity of BF was described in patents much earlier, starting from 1997 [101,106–108].

Later, some biochemical features of BF were improved, with patents obtained for corresponding alterations. Thus, Hong et al. reported T342L, P343E, L344E, and Y422F mutations in BF that enhanced its ability to incorporate fluorescently labeled ddATP and ddCTP [103]. The mutated enzyme was also more thermostable than the native polymerase. Śpibida et al. patented fusion variants of Bst polymerase with N-terminal SSB protein from *Nanoarchaeum equitans,* namely full-length polymerase, the large fragment, and a short fragment lacking both exonuclease domains [109]. Then, chimeric enzymes were obtained with increased thermostability, tolerance to inhibitors, affinity to a DNA template, and 3-fold higher processivity. In 2012, NEB patented Bst 2.0, which proved to be superior to BF in various aspects, such as polymerization speed, temperature stability, salt tolerance, fidelity, storage stability, dUTP tolerance, reverse transcriptase activity, and modified nucleotide incorporation [106,107]. The patent claimed several dozens of mutations in all structural domains of Bst polymerase and attachment of various N- and C-terminal domains: DNA binding domains (including Sso7d), native or disabled exonuclease domain from full-length Bst polymerase, His-Tag, chitin-binding domain, intein, and maltose binding domain. It is unclear whether Bst 2.0 has all the listed mutations and other changes. Currently, the most popular Bst-like enzymes are Bst 2.0 and Bst 3.0, with the latest version of Bst polymerase produced by NEB. Bst 3.0, the latest version, is Bst 2.0 fused with an additional DNA binding domain that further increases the enzyme's affinity to a template nucleic acid. Roche claimed two mutations increasing 3′-mismatch discrimination by thermophilic DNA polymerases. For Bst polymerase, these mutations were F(Y)466X and E(D)547X [110]. Later, Roche reported the E(D)547 L/G/T/Q/A/S/N/R/K and M678K/R/S/G/A mutations in BF and cognate mutations in other DNA polymerase and stated them to enhance the reverse transcription activity and tolerance to inhibitors [108].

Particular progress has been achieved in the improvement of BF. Commercial enzymes, being more thermostable and more tolerant to inhibitors, are more advantageous for practical applications than native BF. However, as shown in several works mentioned above, these commercial polymerases can be improved even further, especially in terms of thermostability and processivity. It should be noted that patents provide much information about Bst polymerase and its mutants. A good example is the reverse transcriptase ability known already in the middle of the 1990 s. In the future, any efforts to alter BF should take into account patented mutations and other known modifications to avoid rediscovering and duplicating previously published findings.

6. Future perspectives

Bst polymerase has become a crucial element in practical applications and modern science. The combination of mesophilic temperature optimum and strand displacement activity became a basis for many methods of isothermal DNA amplification. Advancements in molecular diagnostics have changed the way we diagnose diseases. The convenience of conducting tests at a patient's bedside instead of specialized laboratories is now a reality. However, Bst polymerase remains relatively poorly studied. This lack of information becomes striking when Bst polymerase is compared to two other enzymes widely used in practice, Taq-polymerase and M-MuLV RT. There are only a few research papers that focus on the biochemical properties of Bst polymerase, with a strong bias toward fidelity-driven crystallography studies. Further exploration and development of Bst-polymerase is needed to fully understand its capabilities, particularly regarding terminal transferase activity, reverse transcriptase activity, and optimal buffer composition. Also, the differences between the biochemical properties of native Bst polymerase and its large fragment have not been fully identified. The analog of BF, the Stoffel fragment of Taq-polymerase, is more thermostable than a full-length Taq-polymerase [90]. M-MuLV RT with a disabled RNAse H domain is more thermally stable than the native enzyme or H^- M-MuLV RT [111]. However, to the best of our knowledge, there is no information about the thermostability of BF and native Bst polymerase. Enzymatic characteristics directly affect the performance of Bst polymerase in various applications and a deeper understanding of how BF may facilitate the development of new tests.

Another issue is a relatively small number of reports on alterations capable of improving the properties of Bst polymerase. Only a few mutations are known to increase the thermostability of BF. However, even a few mutations can enhance the other polymerase characteristics, such as exonuclease and polymerase activities and strand displacement. One possible reason for this gap could be a relatively late development of protocols for isothermal DNA amplification compared to PCR and reverse transcription. Isothermal amplification has been rapidly developing since the beginning of the 2000 s, when PCR was already a routine method for over a decade. The increasing demand for point-of-care testing has led to a heightened emphasis on Bst-polymerase. As a result, its superior mutations are expected to be developed with growing speed. Another reason could be that advantageous alterations have a high commercial value and are protected by patents. Thus, these mutations escape the attention of scientists who are searching for information mostly in scientific articles.

In this review, we have provided a description of Bst polymerase discovery and its biochemical properties. In addition, we have presented details on attempts to improve the Bst polymerase characteristics. We believe this review will provide valuable insights for designing more advantageous Bst-polymerase variants in the future.

Author contributions

Igor Oscorbin conducted the literature search, created figures, and wrote the manuscript. Maxim Filipenko devised the topic, supervised the manuscript structure, conducted the literature search, created figures, and wrote the manuscript.

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Author statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript.

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The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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