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Cloning, Sequence Analysis and Three-dimensional Structure Prediction of DNA Pol I from Thermophilic *Geobacillus* sp. *MKK* Isolated from an Iranian Hot Spring

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Abstract Molecular phylogenetic analysis of a novel thermophilic eubacterium isolated from an Iranian hot spring using 16S rDNA sequence showed that the new isolate belongs to genera *Geobacillus*. DNA pol I gene from this isolate was amplified, cloned, sequenced, and the three-dimensional (3D) structure of deduced amino acid sequence was predicted. Sequence analysis revealed the gene is 2,631 bp long, encodes a protein of 876 amino acids with a calculated molecular mass of 99 kDa, and belongs to family A DNA polymerases. Comparison of 3′–5′exonuclease domain of Klenow fragment (KF) with corresponding region of newly identified DNA pol I (MF), the large fragment of *Bacillus stearothermophilus* DNA pol I (BF) and Klentaq1, revealed not only deletions in three regions compared to KF, but that three of the four critical metal-binding residues in KF (Asp355, Glu357, Asp424, and Asp501) are altered in MF as well. Predicted 3D structure and sequence alignments between MF and BF showed that all critical residues in the polymerase active site are conserved.

Keywords Molecular phylogenetic · Thermostable DNA pol I · Cloning · 3D structure prediction · *Gsm pol I*

Abbreviations

KF	Klenow fragment of *E. coli* DNA pol I
BF	large fragment of *Bacillus stearothermophilus* DNA pol I
Klentaq1	Klenow-like fragment of *Taq* DNA polymerase
MF	Klenow-like region of *Geobacillus* sp. *MKK* DNA pol I
Gsm pol I	DNA pol I of *Geobacillus* sp. *MKK*

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Introduction

During the past decade, a large number of DNA polymerase sequences from all three domains of life (Archaea, Bacteria, and Eukarya) have been deposited in the databases. There is considerable diversity among DNA polymerases and this gene sequences can be arranged into six major groups of DNA polymerases A, B, C, D, X, and Y [1]. The first thermostable DNA polymerase (*Taq* DNA polymerase) was isolated from thermophilic bacterium *Thermus aquaticus YT-1* [2, 3, 4]. This polymerase made the polymerase chain reaction (PCR) feasible and introduced a powerful technology that complemented recombinant DNA studies and aided in the diagnosis of inherited and infectious diseases [5, 6]. Since the identification of *Taq* DNA polymerase, a number of thermophilic DNA polymerases have been isolated from both thermophilic eubacteria and archaea sources and characterized [7]. These enzymes have several different notable properties in common [2].

Three-dimensional (3D) structure prediction and homology modeling using resolved crystal structures as templates has been a common approach to structural studies. Homology modeling can be a successful tool for structure prediction and various theoretical models turned out to be very similar to the crystal structures resolved later [8]. Much of the biology of DNA polymerases have been elucidated through the study of the first isolated polymerase, DNA polymerase I (pol I), from *Escherichia coli* [9, 10]. Pol I has three enzymatic activities: 5′–3′ exonuclease, 3′–5′ proofreading exonuclease, and polymerase activity. Each activity is localized to a separate structural domain of the protein [9]. There is little identity between DNA polymerase sequences [1] and it appears that only three catalytically essential carboxylate residues in their polymerase active sites are strictly conserved [9, 11]. In spite of the diversity among polymerase sequences, the three-dimensional structures of enzymes as distantly related as KF [9], HIV [12, 13], and Moloney murine leukemia virus (MMLV) [14] reverse transcriptase have similar topology and organization of their polymerase active sites.

In this study, the identification, cloning, sequence analysis, and structural modeling of deduced amino acid sequence of a new thermostable DNA pol I derived from a thermophilic eubacterium isolated from an Iranian hot spring are presented. Molecular phylogenetic analysis method based on 16S rDNA sequence was used for the preliminary identification of new isolate and degenerate oligonucleotide-based PCR [5, 15] was subsequently utilized to obtain the full-length sequence of thermophilic pol I gene. Moreover, the vestigial 3′–5′exonuclease domain was examined and compared with that of the KF, BF, and Klentaq1. Finally, homology modeling and sequence comparison were used to predict presence or absence of potential enzymatic activities of new isolate.

Materials and Methods

Bacterial strains, vectors, and culture conditions *Geobacillus* sp. *MKK* was isolated from a hot spring north of Iran by R. H. Sajedi et al. [16] and was kindly supplied for this study. *E coli DH5-α* and pTZ57R/T plasmid vector (Fermentas, Lithuania) was obtained from CinnaGen Inc., Iran. *Geobacillus* sp. *MKK* was cultured in nutrient agar plates containing 1% (*w*/*v*) agar and *E. coli* cells were grown in Luria–Bertani medium at 37°C. The plates were solidified with 1.5% (*w*/*v*) agar; ampicillin (100 μg/ml) was added when needed.

Genomic DNA extraction Fresh bacterial colonies were used to extract genomic DNA. The colonies were suspended in a sterile 1.5-ml tube containing 500 μl ddH_2O under laminar

hood and mixed thoroughly. Subsequently, three cycles of freezing in liquid nitrogen and thawing at room temperature were conducted to release DNA from the microbial cells. To precipitate the proteins and cell debris, tubes were heated to 100°C for 10 min and centrifuged at 13,000×*g* for 5 min. The supernatant was transferred to a new sterile tube and the ratio of absorbance was measured at 260/280 nm by UV spectrophotometer (Windaus, GmbH & Co. KG). The quality and integrity of the genomic DNA was evaluated by electrophoresis on 1% agarose gel.

16S rDNA analysis A 850-bp 16S rDNA fragment was amplified using primers Fu16: 5′- CCT ACG GGA GGC AGC AG -3′ as forward and Ru16: 5′-GAC GTC RTC CNC DCC TTC CTC -3′ as reverse primers where R = (A/G), D = (A/G/T), N = (A/C/G/T). These primers are specific for two universally conserved regions of bacterial 16S rDNA sequences. PCR mixture was prepared, containing 0.15 µl of *Taq* DNA polymerase 5 U/µl, 0.5 µl of 20 pmol of each primer, 0.8 µl of 50 mM $MgCl_2$ solution, 2.5 µl of a 2 mM dNTPs, 2.5 µl of 10× PCR buffer, and 5 µl of purified genomic DNA as template, and was made up to 25 µl total volume with ddH_2O. PCR was performed at 96°C for 4 min as the denaturation step, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 30 s, and primer extension at 72°C for 50 s. The final extension step was 4 min at 72°C. Amplified products were analyzed on 1% agarose gel, and the desired 850 bp fragment was purified using DNA extraction kit (Fermentas, Lithuania). The purified DNA fragment was then sequenced (3700 ABI Capillary system Microgene Inc., Korea).

Molecular phylogenetic analysis and primer designing The obtained 16S rDNA partial sequence was subjected to Blast software (http://www. ncbi. nlm. nih.gov/) and, subsequently, all homologous 16S rDNA sequences were retrieved but only a few were selected along with other thermophilic members of *Bacillaceae* family for the in-group sampling. Corresponding regions of retrieved 16S rDNA sequences for the obtained 16S rDNA partial sequence of the unknown isolate were aligned using ClustalW software (http://www.ebi.ac.uk) and the remaining nucleotides were excluded. The phylogenetic tree was constructed using AliBee-Multiple Alignment software (http://www2.genebee.msu.su/). The DNA pol I gene sequences of the closest bacterial species to *Geobacillus* sp. *MKK*, (GenBank accession nos. BACPOL, BSU33536, BSU93028, AY247636, and BACPOLYTG) were aligned using ClustalW software; subsequently, a degenerate primer set was designed, FDPOL1: 5′-ATG AAA AAM AAG CTT GTY TTA ATY GAC A-3′ as forward primer and RDPOL1: 5′-TTA TTT CGC ATC ATA CCA TGT TGG GCC GTA A-3′ as reverse primer, where M = (A/C) and Y = (T/C), and used to amplify pol I gene. PCR reaction mix was prepared using GC-rich PCR system (Roche, Germany) containing a *Taq* and *Tgo* polymerase blend for high fidelity amplification of GC-rich and long templates, as recommended by the manufacturer. PCR reaction was performed using MyGene96 thermal cycler (Bioneer Inc., South Korea) at 96°C for 4 min as denaturation step followed by 35s cycle of denaturation at 94°C for 1 min, primer annealing at 48.5°C for 30 s, and primer extension at 72°C for 2.5 min. The final extension step was carried out for 4 min at 72°C. PCR products were analyzed on 1% agarose gel, and the desired 2.6-kb band was excised from the gel and purified using DNA extraction kit (Fermentas, Lithuania).

Cloning and sequencing of DNA polymerase I gene All steps of ligation and transformation were carried out using InsT/A cloning kit (Fermentas). Purified PCR products were ligated into pTZ57R/T plasmid vector and the ligation product was used to transform *E. coli* strain DH5-α, followed by the selection of recombinant cells. The integrity of the insert was

tested by PCR, double digestion, and subsequently by the sequencing of purified recombinant plasmid DNA (3700 ABI capillary system Microgene Inc., Korea).

Bioinformatics and sequence analysis All the handling and the analysis of nucleotide sequences were performed with tools available at the ExPASy Molecular Biology Server (http://www.expasy.ch). Pair-wise and multiple sequence alignments were carried out using the Needleman–Wunch algorithm and ClustalW softwares, respectively. 3D structure model of DNA polymerase I from *Geobacillus* sp. *MKK* and 3′–5′exonuclease domains were predicted using Geno3D server [17] (www.expasy.ch) and the crystal structure of large fragment of DNA polymerase I from *Geobacillus stearothermophilus* within the Protein Data Bank (pdb *1XWL*) as template. Analysis and comparison of the structures were carried out using the Swiss-PdbViewer software (http://www.expasy.org/spdbv).

Results and Discussion

Phylogenetic Analysis

The use of 16S rDNA sequence for identification and phylogenetic classification purposes of eubacteria has long been established [18, 19, 20]. A 779-bp-long 16S rDNA partial fragment was amplified, sequenced, and submitted to GenBank under accession no. DQ309334. Sequence analysis showed G + C contents of 55.3% for this fragment. Figure 1 depicts the phylogenetic tree, which represents deduced position of X, the new thermophilic isolate, among some members of *Bacillaceae* family. Based on Fig. 1, the new thermophilic isolate, X, is in the same sister taxon of *Geobacillus proteolyticus* and *G. stearothermophilus*.

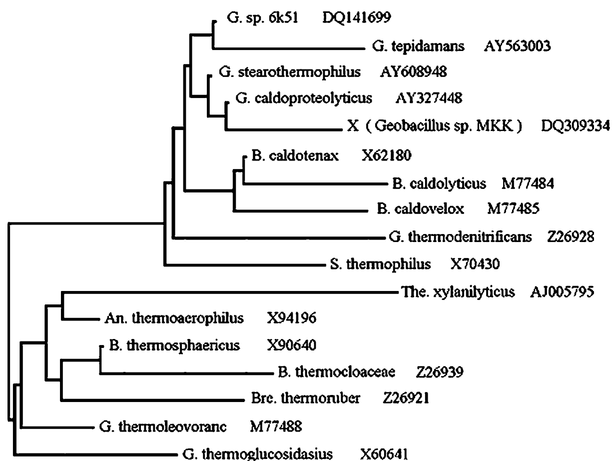

Fig. 1 Phylogenetic position of X, new isolate, (*Geobacillus* sp. *MKK*) among some other thermophilic members of family *Bacillaceae* based on the 16S rDNA sequence of *Geobacillus* sp. *MKK*, determined by our group. Other sequences were retrieved from public nucleotide databases. Corresponding regions of retrieved 16S rDNA sequences for the obtained 779 nucleotides 16S rDNA of the new isolate were subjected to phylogenetic tree construction, and the remaining nucleotides were excluded. Codes at the *right* of organism names are sequence accession numbers. The phylogenetic tree was constructed using AliBee-Multiple Alignment software (http://www2.genebee.msu.su/). *G.*, *Geobacillus*; *B.*, *Bacillus*; *An.*, *Aneurinibacillus*; *Bre.*, *Brevibacillus*; *The.*, *Thermobacillus*; *S.*, *Streptococc*

Moreover, it is allied with a clade of another species of *Bacillaceae* family such as *Bacillus caldotenax*, *Bacillus caldolyticus*, and *Bacillus caldovelox*. Phylogenetic and distance matrix analysis (data not shown) revealed that the new isolate is related to genus *Geobacillus* so it was named *Geobacillus* sp. *MKK*.

Cloning and Sequence Analysis of DNA Polymerase I from *Geobacillus* sp. *MKK*

The polymerase chain reaction resulted in amplification of a fragment 2.6 kb long as depicted on agarose gel (Fig. 2). This fragment was cloned and sequenced, and the new pol I gene sequence was submitted to GenBank (GenBank accession no. DQ244056). Sequence analysis revealed a 2,631 nucleotide open reading frame encoding a 876-aa polypeptide with an estimated mass of 99 kDa. Amino acid sequence analysis of *Geobacillus* sp. *MKK* DNA pol I, using the Needleman–Wunch algorithm, indicated 94.3 and 92.5% identity to DNA pol I of *G. stearothermophilus* (GenBank accession no. AAB52611) and *G. stearothermophilus* (GenBank accession no. AAB62092), respectively. MotifScan analysis showed crucial motif and domains including DNA polymerase A signature, 3′–5′ exonuclease domain, leucine zipper pattern, helix–hairpin–helix motif, and other motifs, but not the 3′–5′exonuclease domain. Presence of DNA polymerase A signature was verified using ScanProSite that resulted in (702-RrqAKavnFGivYgiSdygL-721) as DNA polymerase A signature. It can therefore be proposed that *Gsm pol I* (*Geobacillus* sp. *MKK* DNA pol I) belongs to the family A DNA polymerases. DNA polymerase A signature of some family A polymerases were retrieved and compared in Table 1; among 20 residues of the DNA polymerase A signature, three amino acids R, K, and Y (Arg702, Lys706, and Tyr714 in *Gsm pol I*) are strictly conserved. Figure 3 illustrates a diagram which depicts conserved and alternatively variable sequences of the DNA polymerase A signature.

Bioinformatics and Structural Modeling

1-Geobacillus sp. MKK DNA polymerase I 3D Structure

The ribbon model of 3D structure for *Gsm pol I* has been predicted using the deduced amino acid sequence and *Bacillus stearothermophilus* DNA polymerase I large fragment (BF) (pdb *1XWL*) as template. The schematic representation of the homology model of *Gsm*

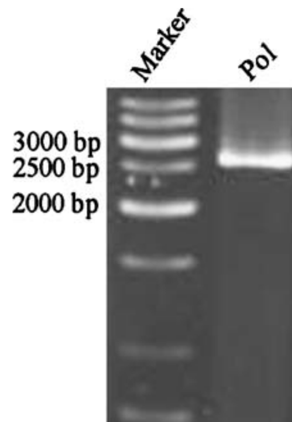

Fig. 2 PCR product of amplified *Geobacillus* sp. *MKK* DNA polymerase I gene on 1% agarose gel. *Lane 1*, DNA size marker; *lane 2*, Pol I PCR product, 2,631 bp in length

Table 1 Multiple alignment of DNA polymerase A signature from 12 well-known members of the DNA polymerase A family together with *Geobacillus* sp. *MKK* DNA polymerase I.

DNA polymerase A signature	Organism
703-RrqAKavnFGivYgiSdygL-722	*B. caldotenax*
704-RrqAKavnFGivYgiSdygL-723	*G. caldolyticus*
704-RrqAKavnFGivYgiSdygL-723	*G. kaustophilus*
406-RrqAKavnFGivYgiSdygL-425	*G. stearothermophilus*
655-RraAKtinFGvlYgmSahrL-677	*T. thermophilus*
706-RrqAKavnFGivYgiSdygL-725	*B. subtilis*
702-RrqAKavnFGivYgiSdygL-721	*B. Rhodothermus*
749-RrrAKmvnYGipYgiSawgL-768	*T. thermophilus* HB2
659-RraAKtvnFGvlYgmSahrL-678	*T. filiformis*
658-RraAKtinFGvlYgmSahrL-677	*T. aquaticus*
658-RraAKtinFGvlYgmSahrL-677	*B. halodurans* C-125
773-RrsAKainFGliYgmSafgL-792	*E. coli* K12
702-RrqAKavnFGivYgiSdygL-721	*G.* sp. *MKK*

B. Bacillus, *G. Geobacillus*, *T. Thermus*, *E. Escherichia*

pol I (residues 297–876) showing the arrangements of secondary structural elements is depicted in Fig. 4. The crystallography and 3D structure of BF, the large fragment of DNA polymerase I derived from *G. stearothermophilus*, were previously reported [9]. Pair-wise sequence alignment between BF and the corresponding region of *Geobacillus* sp. *MKK* DNA polymerase I (MF) revealed 89% sequence identity and, as expected, the folding pattern strongly resembles to the template. The root mean square deviation of Cα positions between all residues of MF and BF is 0.8 A°, reflecting comparatively local and total structural similarities. As previously reported, the theoretical model of a protein molecule turns out to be very similar to its defined crystal structure, indicating that homology modeling can be used as one of the reliable tools for structure prediction [8]. Therefore, it has been useful to utilize BF as a favorite paradigm to compare and elucidate the putative features of *Gsm pol I*. The C-terminal polymerase domain of MF (residues 297–876) is topologically similar to the corresponding domain of BF. Like other polymerases [12, 13, 21, 22], the MF polymerase domain resembles a right hand with "fingers", "palm", and "thumb" subdomains (Fig. 4). These three subdomains define a cleft that is predicted to bind duplex DNA [9, 21]. The fingers and thumb regions of the structure are α-helical, and the palm region is formed

Fig. 3 Schematic MotifScan representation of the DNA polymerase A signature demonstrating maintained and variable residues. Three residues R, K, and Y are strictly conserved

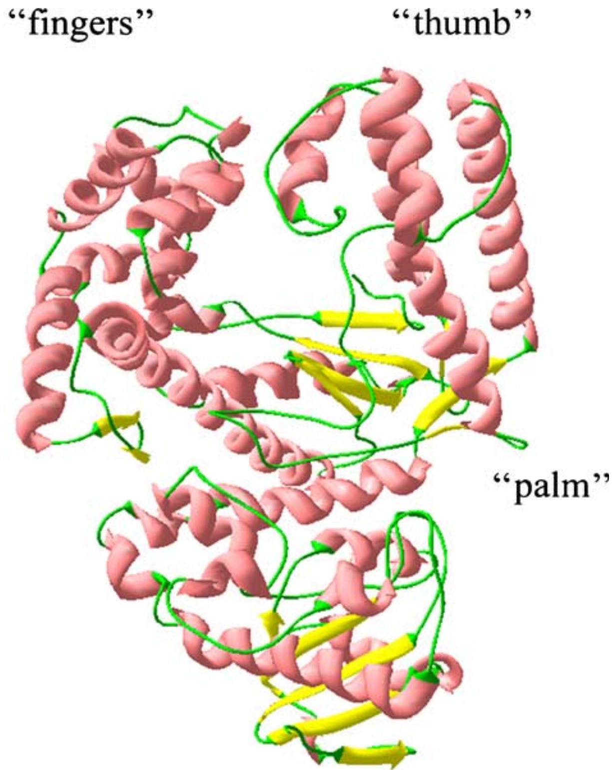

Fig. 4 Three-dimensional structure of *Geobacillus* sp. *MKK DNA* polymerase I (*Gsm pol I*). 3D structure was predicted using Geno3D server (http://www.expasy.ch) and the crystal structure of BF, the large fragment of *G. stearothermophilus* DNA polymerase I (pdb *1XWL*) as a template. The Swiss-PdbViewer was used to make ribbon model, demonstrating secondary structure elements. The helixes are in *pink*, strands are in *yellow*, and turns are in *light green*. Essential residues for polymerase activity are R319, Q328, D357, S359, Q360, E362, Q501, Q531, H533, and H534. The overall fold of *Gsm pol I* is strongly similar to that of BF

primarily from five antiparallel sheets. The active site of polymerase domain is positioned on the palm region width of the cleft (Fig. 4). All conserved residues in BF are maintained in MF as well and located in the same positions. Most DNA polymerases have three conserved carboxylate residues which are essential for catalysis [9, 11, 24, 25]. In MF, these residues correspond to Asp653, Asp830, and Glu831 which have the same positions in BF and these three residues are interconnected through hydrogen bonds to water molecules and other highly conserved residues in the active site as reported in BF [9], KF [23, 26], Klentaq1 [9, 27], HIV-RT [13, 28], and MMLV [14].

3′–5′ Exonuclease Domain

As mentioned above, MotifScan analysis of *Gsm pol I* did not reveal any 3′–5′exonuclease domain. To further substantiate this fact, the 3′–5′exonuclease domain of Klenow fragment, the corresponding region of BF, Klentaq1, and *Gsm pol I* were compared with each other (Fig. 5). The root mean square deviation of Cα positions between vestigial 3′–5′exonuclease domain residues of MF and BF, KF, and Klentaq1 are 0.69, 3.6, and 2.56 A°, respectively. It has been shown that the 3′–5′exonuclease of KF requires two bound divalent cations for catalysis. In KF, these metal ions are bound by residues Asp355, Glu357, Asp424, and Asp501 [9, 26, 29, 30] which correspond to Val319, Glu321, Ala376, and Lys450 from MF and BF, respectively, showing a change in three of the four critical metal-binding residues. All four of these critical residues are also different in *Taq* [27, 31]. Mutation of any one of the metal-binding residues in KF can disrupt metal binding and catalysis [29, 30]. A multiple sequence alignment of the N-terminal domain of KF with the analogous 3′–5′

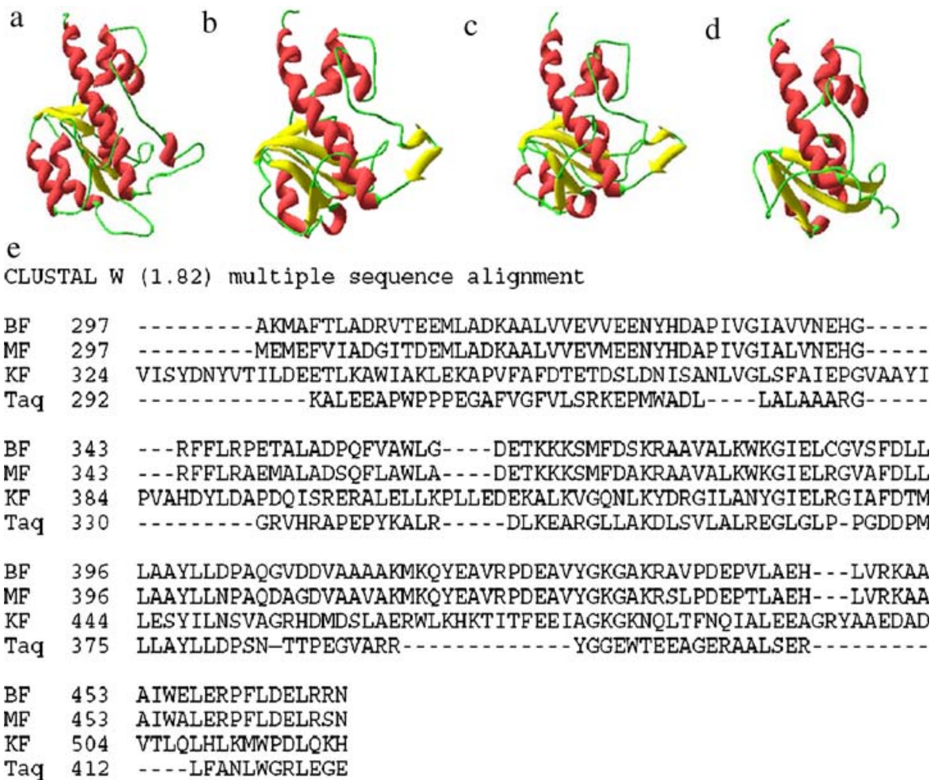

Fig. 5 Comparison of 3′–5′ exonuclease domains. Ribbon schematics of (**a**) the 3′–5′exonuclease domain of KF and the homologous regions of (**b**) *Geobacillus* sp. *MKK* DNA pol I (**c**) BF and (**d**) Klentaq1 polymerases. (**e**) Sequence alignments of the 3′–5′exonuclase domains representing sequence deletion regions in MF, BF, and Klentaq1 polymerases as compared to KF

exonuclease region of MF, BF, and Klentaq1 revealed that sequences were deleted from KF in three regions to generate the topology observed in MF and BF (Fig. 5). As a result of these sequence deletions in BF, klentaq1 [9], and MF, the fold of protein at the 3′–5′ exonuclease active site cleft was altered and, consequently, the size of cleft was reduced resulting in the disruption of any potential DNA and nucleotide-binding sites. It is therefore reasonable to conclude that the disruption of metal binding and any potential DNA and nucleotide binding sites can result in the loss of 3′–5′exonuclease activity. Based on this data, we propose that the newly identified DNA polymerase I, *Gsm pol I*, as BF possibly lacking proofreading activity. Choice of a DNA polymerase for PCR is usually dependent on its application. For example, DNA polymerases with proofreading activity such as *Pfu* polymerase [32] and *Vent* polymerase facilitate PCR in cloning, but those lacking proofreading activity are more valuable in PCR for detection purposes or in cases where fidelity and accuracy of the amplified fragments are less important [33]. Therefore, the newly identified DNA polymerase, *Gsm pol I*, has potential application not only in PCR but in sequencing as well.

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