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Characterization of DNA Polymerase from *Pyrococcus* sp. Strain KOD1 and Its Application to PCR

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The DNA polymerase gene from the archaeon *Pyrococcus* sp. strain KOD1 (KOD DNA polymerase) contains a long open reading frame of 5,013 bases that encodes 1,671 amino acid residues (GenBank accession no. D29671). Similarity analysis revealed that the DNA polymerase contained a putative 3′-5′ exonuclease activity and two in-frame intervening sequences of 1,080 bp (360 amino acids; KOD *pol* intein-1) and 1,611 bp (537 amino acids; KOD *pol* intein-2), which are located in the middle of regions conserved among eukaryotic and archaeal α-like DNA polymerases. The mature form of the DNA polymerase gene was expressed in *Escherichia coli*, and the recombinant enzyme was purified and characterized. 3′-5′ exonuclease activity was confirmed, and although KOD DNA polymerase's optimum temperature (75°C) and mutation frequency (3.5×10^{-3}) were similar to those of a DNA polymerase from *Pyrococcus furiosus* (*Pfu* DNA polymerase), the KOD DNA polymerase exhibited an extension rate (100 to 130 nucleotides/s) 5 times higher and a processivity (persistence of sequential nucleotide polymerization) 10 to 15 times higher than those of *Pfu* DNA polymerase. These characteristics enabled the KOD DNA polymerase to perform a more accurate PCR in a shorter reaction time.

Recently, it was shown that archaea constitute a third domain of living organisms that is distinguishable from the domains *Bacteria* and *Eucarya* (46). On the phylogenetic tree, the hyperthermophiles are the deepest and shortest branches, and the last common ancestor on this tree most likely thrived in extreme thermal environments (37, 47).

We are currently screening hyperthermophilic archaea to find new thermostable enzymes useful for research, diagnosis, and industrial applications. One strain, *Pyrococcus* sp. strain KOD1, isolated from a solfataric hot spring at Kodakara island in Japan, was identified, and characterization of many genes and their products was performed (10, 20, 28, 32–36, 40, 44, 48). A physical map of its whole genome was also constructed (11). Most of the cloned gene sequences were similar to eukaryotic genes, indicating that the gene structure of this archaeon is more closely related to that of eukarya than that of bacteria.

Enzymes from hyperthermophiles are extremely thermostable and of industrial importance. PCR, which uses the thermostable DNA polymerase, is one of the most important contributions to protein and genetic research (38). Thus, isolation and identification of valuable DNA polymerases from thermophilic species will have both academic and industrial rewards.

More than 50 DNA polymerase genes have been cloned from various organisms, including thermophiles and archaea, and sequenced. Amino acid sequences deduced from their nucleotide sequences can be classified into four major groups: *Escherichia coli* DNA polymerase I (family A), DNA polymerase II (family B), DNA polymerase III (family C), and others (family X) (19). DNA polymerases from the largest family, family B, are called α-like DNA polymerases because they have the amino acid sequences of conserved eukaryotic DNA polymerase α (2). In this paper, we report the cloning of a new α-like DNA polymerase gene from *Pyrococcus* sp. strain KOD1 and characterization of the recombinant enzyme. The DNA polymerase exhibited characteristics superior to those of previously reported DNA polymerases. Application of this DNA polymerase to an accurate and short-time PCR was also investigated.

MATERIALS AND METHODS

Strains, plasmids, and media. The archaeon *Pyrococcus* sp. strain KOD1 (28) was used as a donor for molecular cloning of the DNA polymerase gene. *E. coli* JM109 {*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ* M15]} was used for plasmid propagation and nucleotide sequencing. *E. coli* BL21(DE3), which carries the T7 RNA polymerase gene under the control of a chromosomal *lacUV5* gene (42), and the expression vector pET-8c were used for overexpression of the cloned genes.

pUC18 and pUC19 were used as vectors for cloning of the DNA polymerase gene. *E. coli* JM109 was grown in L broth (1% Bacto Tryptone, 0.5% yeast extract, 0.5% NaCl; adjusted to pH 7.3 with NaOH) at 37°C. *E. coli* BL21(DE3) was grown in NZCYM medium (1% NZ amine, 0.5% NaCl, 0.5% yeast extract, 0.1% Casamino Acids, 0.2% $MgSO_4 \cdot 7H_2O$; adjusted to pH 7.0 with NaOH) at 37°C.

PCR. For PCR, we designed two primers based on the conserved region II of α-like DNA polymerases (50) (forward primer, 5′-GGATCCTGGGAGAA[C/T]ATAGTTTA-3′; reverse primer, 5′-CTGCAGTGC[T/C/G]GG[G/A]GA[G/A]ACGTTGTG-3′). Genomic DNA (1 μg) was predenatured by heating at 94°C for 3 min in 100 μl of reaction buffer containing 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton X-100, 200 μM deoxyribonucleoside triphosphates (dNTPs), 200 pmol of each primer, and 2 U of Vent DNA polymerase (New England Biolabs Inc., Beverly, Mass.). Subsequently, 30 cycles

Sce VMA	210	YLLGLWIGDGLS	318	FLAGLIDSDGYV
Mtu recA	113	RLLGYLIGDGRD	212	LLFGLFESDGWV
Tli pol 2	147	ELVGLIVGDGNW	245	FLRGLFSADGTV
Tli pol 1	281	KLLGYYVSEGYA	376	FLEAYFTGDGDI
KOD *pol* intein−2	280	KLLGYYVSEGYA	375	FLEGYFIGDGDV
KOD *pol* intein−1	117	ELAGILLAEGTL	217	VLRGFFEGDGSV

FIG. 1. Conserved dodecapeptide motifs in inteins. Sequences from *Sce VMA* intein (*Sce VMA*), *M. tuberculosis recA* intein (*Mtu recA*), *Tli DNA* polymerase inteins (*Tli pol* 1 and *Tli pol* 2), and *Pyrococcus* sp. strain KOD1 DNA polymerase inteins (KOD *pol* intein-1 and KOD *pol* intein-2) are aligned. Numbers indicate distance from the start of the intein.

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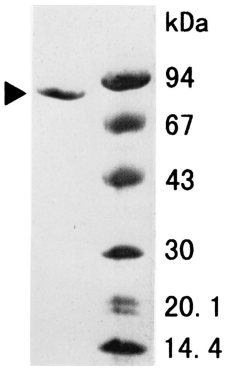

FIG. 2. Sodium dodecyl sulfate-PAGE of purified KOD DNA polymerase. The gel was stained with Coomassie brilliant blue R-250.

consisting of denaturing at 94°C (2 min), primer annealing at 55°C (2 min), and primer extension at 72°C (3 min) were carried out with a fully automated DNA thermal cycler (GeneAmp PCR System 2400; Perkin-Elmer, Foster City, Calif.). After the last cycle, all samples were incubated for an additional 10 min at 70°C, to ensure completion of the extension step.

DNA manipulation and transformation. Genomic DNA from *Pyrococcus* sp. strain KOD1 was isolated by the procedure of Imanaka et al. (16). Plasmid DNA from *E. coli* was prepared by the rapid alkaline method (3). Restriction endonucleases and T4 DNA ligase were purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan) and were used as recommended by the manufacturer. Competent cells of *E. coli* JM109 and BL21(DE3) were prepared as described before (17). Ampicillin (final concentration, 100 μg/ml) was added for plasmid harboring *E. coli* cells. Recombinant and native *Pfu* DNA polymerases and recombinant *Taq* DNA polymerase were purchased from Toyobo Co. Lmt. (Osaka, Japan). Deep Vent DNA polymerase was purchased from New England Biolabs.

Nucleotide sequence determination and analysis. DNA fragments of interest were subcloned into M13mp18 or M13mp19. Single-stranded DNA (ssDNA) was prepared as described in reference 49. The nucleotide sequence was determined by the dideoxy chain termination method (39). Nucleotide and amino acid sequence analyses, including open reading frame searches, molecular weight calculations, and homology searches, were performed by using the DNASIS software (Hitachi Software Co., Ltd., Yokohama, Japan).

Protein assay. The protein concentration was measured by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.). Purity of the enzyme was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) (24). Samples and molecular weight marker proteins were mixed with an equal volume of sample buffer and then boiled for 5 min. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. The molecular weight standards (Pharmacia Biotech, Uppsala, Sweden) (and their molecular masses in kilodaltons) were α-lactalbumin (14.0), trypsin inhibitor (20.1), bovine carbonic anhydrase (30.0), ovalbumin (43.0), albumin (67.0), and phosphorylase *b* (94.0).

Overexpression of the mature KOD DNA polymerase gene in *E. coli* and purification of the enzyme. A DNA fragment coding for the mature form of KOD DNA polymerase that did not contain intervening sequences was constructed by combining three DNA fragments. DNA regions encoding N-terminal and middle portions of mature KOD DNA polymerase were separately amplified with primers containing the overlapping sequences. Likewise, fusion of the DNA region encoding the C-terminal portion of KOD DNA polymerase was also performed, and the DNA fragment encoding the entire mature KOD DNA polymerase was obtained. The fragment was cloned between the *Nco*I site, whose cohesive end was filled in prior to ligation, and the *Bam*HI site of pET-8c vector. The resultant recombinant plasmid, pET-*pol*(mature), was used to transform *E. coli* BL21(DE3). Overexpression was induced by addition of isopropylthiogalactopyranoside (final concentration, 1 mM), and cells were harvested after 3 h of incubation. The cells were disrupted by sonication, and after centrifugation, a crude enzyme sample was prepared by heat treatment of the cytoplasmic fraction at 85°C for 10 min. A heparin column (1.6 by 2.5 cm; HiTrap; Pharmacia) was equilibrated with the buffer (10 mM sodium phosphate [pH 7.0], 0.1 M NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol), and the crude enzyme sample was applied to the column. Protein fractions were eluted by a linear gradient of 0.1 to 1.5 M NaCl. KOD DNA polymerase was recovered at approximately 0.6 to 0.7 M.

Assay for DNA polymerase activity. DNA polymerase activity was determined by a trichloroacetic acid precipitation assay (22). The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 50 μg of bovine serum albumin (BSA) per ml, 0.15 mM each dNTP, [*methyl*-^{3}H]TTP (0.13 mCi/nmol, final concentration), 150 mg of activated calf thymus DNA per ml, and 7.5 mM dithiothreitol. One unit of enzyme activity is defined as the amount of enzyme required to incorporate 10 nmol of dNTP into an acid-insoluble form at 75°C in 30 min.

Exonuclease activity assays of the DNA polymerase. 3′-5′ and 5′-3′ exonuclease activity assays of DNA polymerase quantified the release of ^{3}H and ^{32}P from the 3′ and 5′ ends, respectively, of labeled *Hin*dIII-digested λ DNA fragments. Ten micrograms of λ DNA completely digested with *Hin*dIII was labeled at its 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase by the standard method. After ethanol precipitation, 5′-labeled DNA fragments were labeled at the 3′ end with [^{3}H]dTTP and DNA polymerase Klenow fragment. The exonuclease reaction mixture (50 μl) contained 0.2 μg of double-labeled DNA fragments and PCR buffer for each enzyme. The buffer components were 120 mM Tris-HCl (pH 8.0), 1.2 mM $MgCl_2$, 6 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1% Triton X-100, and 0.01% BSA for KOD DNA polymerase; 20 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 6 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1% Triton X-100, and 0.01% BSA for *Pfu* DNA polymerase; and 10 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 50 mM KCl, and 0.1% Triton X-100 for *Taq* DNA polymerase. These buffers were used for all experiments except the processivity measurements. The mixture was preincubated at 75°C for 5 min, and then DNA polymerase (KOD, *Pfu* [1 U], or *Taq* [5 U]) was added. All reactions were carried out at 75°C, and the reaction was terminated by addition of 50 μl of 1% BSA and 100 μl of 10% trichloroacetic acid. The reaction mixture was kept in ice for 10 min and then centrifuged for 10 min at 12,000 rpm. The supernatant was obtained, and the radioactivity was measured in a liquid scintillation counter.

Terminal transferase activity. Existence of a terminal transferase activity in KOD DNA polymerase was examined by using the 36-mer oligonucleotide (5′-GGAATTCGCGGCCGCGCATGCGCGGCCGGGAATTCC-3′), which forms a palindromic structure, labeled with ^{32}P at the 5′ end, and self-annealed. The annealed oligonucleotides were incubated with various DNA polymerases (KOD, *Pfu*, and *Taq*) in the PCR buffers described above in the presence of 2 mM dNTPs. Reaction mixtures were subjected to denaturing PAGE, and addition of ^{32}P-labeled base at the 3′ end by terminal transferase activity was detected by autoradiography.

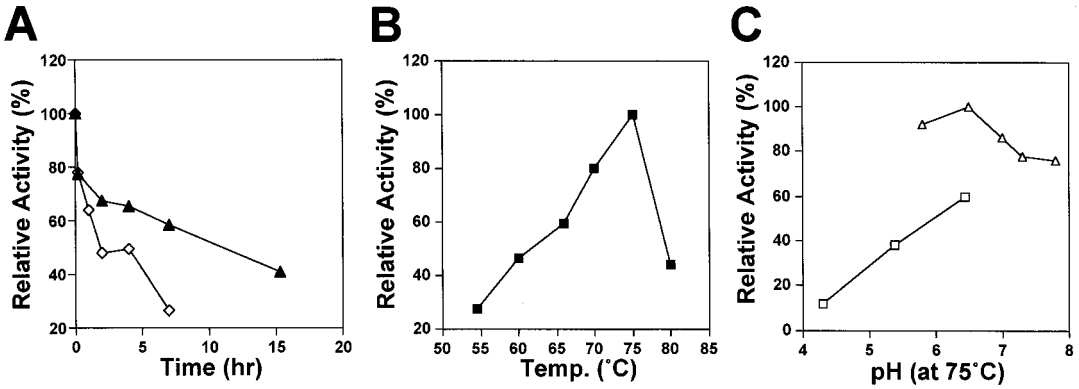

FIG. 3. Thermostability and effects of temperature and pH on activity of KOD DNA polymerase. (A) Thermostability of KOD DNA polymerase at 95°C (▲) and 100°C (◇). After incubation at 95 or 100°C, remaining activities were assayed at 75°C. (B) Effect of temperature on DNA polymerase activity. (C) Effect of pH on DNA polymerase activity. The DNA polymerase activity was assayed at 75°C with morphalineethanesulfonic acid (□) and Tris-HCl (△) buffer.

TABLE 1. Comparison of fidelities of thermostable DNA polymerases by the method of Kunkel et al. (23)

Enzyme	No. of plaques scored[a]		Mutation frequency[b] (%)
	Mutant	Total	
KOD	23	6,619	0.35
Pfu	30	7,691	0.39
Taq	54	4,141	1.3
Tth	115	7,375	1.6
Taq-Pfu (20:1)	30	4,238	0.71
Taq-Pfu (50:1)	23	4,489	0.51

[a] Mutant, colorless plaques plus lighter blue plaques; total, blue plaques plus mutant plaques.
[b] Mutant plaques/total plaques.

Fidelity of DNA polymerase activity. Fidelity of the DNA polymerase reaction was analyzed by the method of Kunkel et al. (23). Fifty micrograms of M13mp18 double-stranded DNA was digested with *Pvu*I and *Pvu*II, and the 6.5-kb band was purified. The digested double-stranded DNA was heated with 100 μg of M13 ssDNA at 95°C for 10 min, cooled in an ice bath, and then incubated at 65°C for 30 min. The gapped M13mp18 molecule was then purified by agarose gel electrophoresis (1% low-melting-point agarose gel), phenol extraction, and ethanol precipitation. One hundred nanograms of the purified gapped M13mp18 molecule was added into a DNA polymerase reaction mixture containing 200 μM dNTPs and 5 U of DNA polymerase and kept at 75°C for 30 min to fill in the gap. Two microliters (approximately 4 ng) of the remaining product was used for transfection. In calculation of the mutation frequencies, light blue and white plaques were counted as mutants.

Extension rate measurements. The extension rate of the DNA polymerase was calculated from the length of DNA synthesized in a fixed time. M13 ssDNA primed with the P7 primer (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) was used as the substrate. The reaction mixture (80 μl), containing 1.6 μg of M13 ssDNA, 16 pmol of P7 primer, 0.2 mM each dATP, dGTP, and dTTP, 0.1 mM [α-^{32}P]dCTP (1.11 MBq), 5 U of DNA polymerase, and the suitable PCR buffer for each enzyme, was incubated at 75°C. After a fixed time, 12.5 μl of the reaction mixture was taken and an equal volume of stop solution containing 60 mM EDTA and 60 mM NaOH was added. A 10-μl aliquot of each sample was analyzed by agarose gel electrophoresis. The buffer for reaction of Deep Vent DNA polymerase contained 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$ and 0.1% Triton X-100.

Processivity measurements. Processivity was measured as described by Tabor et al. (43). Processivity is one of the most important parameters for DNA polymerases and is defined as the number of nucleotides that can be extended in one catalytic reaction by one DNA polymerase molecule. The reaction was carried out with 200 fmol of 5′-biotin-labeled M13 primer (5′-CGCCAGGGTT TTCCCAGTCACGAC-3′), annealed to 100 fmol of the M13 ssDNA template, and two different ratios of DNA polymerase (a 10:1 ratio and a 100:1 ratio of primer-template to DNA polymerase) at 75°C. Aliquots were taken at 20 s, 1 min, and 3 min and placed into stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). The products were analyzed by denaturing PAGE on a 6% polyacrylamide gel and detected by the chemical luminescence method. The buffer used for the processivity measurement contained 20 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 6 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1% Triton X-100, and 0.01% BSA.

Measurements of fidelity in PCR. Fidelity in PCR was compared as a mutation frequency in PCR products, using the whole length (5.2 kbp) of plasmid pUR288, an expression vector containing the *lacZ* gene, as a template. Two adjacent primers were annealed in the opposite direction at the replication origin region, and PCR was performed with KOD, *Pfu*, and *Taq* DNA polymerases, each at optimized conditions, with a Perkin-Elmer GeneAmp PCR System 2400. Twenty-five cycles were carried out as follows: 20 s at 99°C, 2 s at 65°C, and 90 s at 74°C for KOD DNA polymerase; 20 s at 99°C, 2 s at 65°C, and 5 min at 74°C for *Pfu* DNA polymerase; and 20 s at 99°C and 3 min at 68°C for *Taq* DNA polymerase. The PCR products were blunted with T4 DNA polymerase, treated with *Dpn*I, which cleaves sequences including methylated adenine to eliminate template plasmids, and then self-ligated and used to transform *E. coli* cells. Colorless and light blue colonies were counted as mutated plasmids because of the mutations in the *lacZ* gene during PCR and blue colonies were considered to be intact plasmids. The mutation frequency was calculated as the ratio of number of colonies with mutated plasmids to the number of total colonies.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data banks with accession no. D29671.

RESULTS AND DISCUSSION

Cloning and nucleotide sequencing of the DNA polymerase gene from *Pyrococcus* sp. strain KOD1. Based on the conserved amino acid sequence found in α-like DNA polymerases of different sources, two oligonucleotide primers were designed.

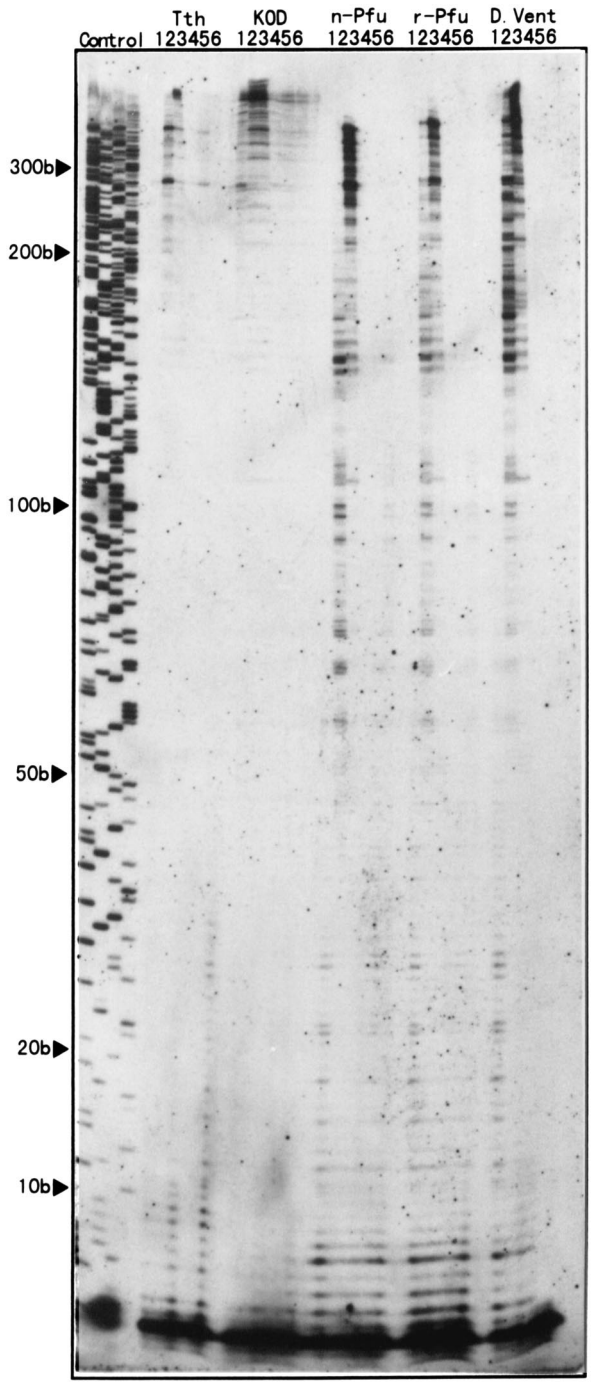

FIG. 4. Comparison of processivity of KOD DNA polymerase and other thermostable DNA polymerases. Tth, *Tth* DNA polymerase; KOD, KOD DNA polymerase; n-Pfu, native *Pfu* DNA polymerase purified from *P. furiosus*; r-Pfu, recombinant *Pfu* DNA polymerase expressed in *E. coli*; D. Vent, Deep Vent DNA polymerase. For each enzyme, ratios of substrate to enzyme and reaction times are as follows: lane 1, 10:1, 20 s; lane 2, 10:1, 60 s; lane 3, 10:1, 180 s; lane 4, 100:1, 20 s; lane 5, 100:1, 60 s; lane 6, 100:1, 120 s. b, bases.

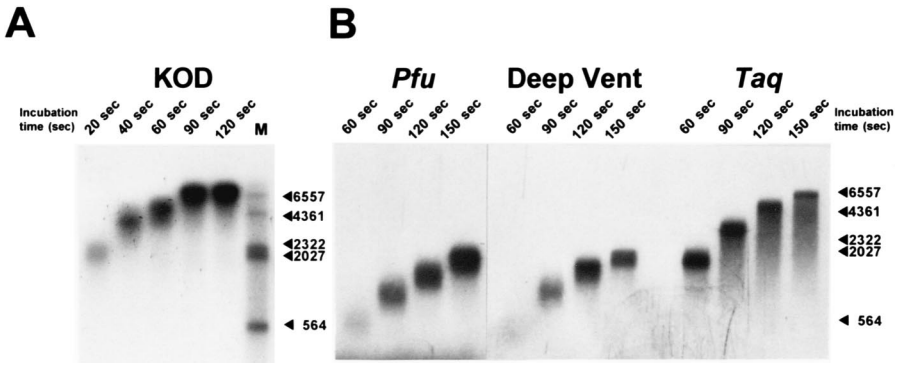

FIG. 5. Comparison of elongation rates of KOD DNA polymerase, *Pfu* DNA polymerase, Deep Vent DNA polymerase, and *Taq* DNA polymerase. The elongation rate was measured according to the length of synthesized DNA, using M13 ssDNA as the template at 75°C. Results of agarose gel electrophoresis are shown. Enzymes and elongation reaction times used are indicated at the top. Lane M, size marker (λ DNA digested with *Hin*dIII). Sizes are indicated in base pairs.

PCR was performed, and a product around the predicted DNA size (94 bp) was obtained. The PCR product was cloned into pUC18, and the nucleotide sequence (88 bp) was determined. The amino acid sequence (WENIVYLDYKALYPSIIITH NVSPD) derived from the DNA sequence was similar to the amino acid sequence of the corresponding region (region II). The PCR fragment was used as a probe to obtain the whole DNA polymerase gene from KOD1.

The nucleotide sequence of the entire open reading frame for the DNA polymerase was determined. The open reading frame consists of 5,013 bases coding for a protein with 1,671 amino acid residues. The molecular mass of the protein derived from this amino acid sequence was 193.2 kDa, which is much larger than the size predicted for the average molecular mass of thermostable DNA polymerases (see below).

Similarity analysis of the amino acid sequence of the DNA polymerase. An amino acid sequence derived from the DNA sequence of the KOD DNA polymerase was compared with those of other DNA polymerases from different sources. The amino acid sequence from the open reading frame had two conserved regions. One was the 3′-5′ exonuclease motif consisting of Exo I, II, and III, which is responsible for proofreading ability (4). The other region was the DNA polymerase domain consisting of five regions (region I to V) that are conserved among α-like DNA polymerases (50). It was shown that this amino acid sequence included two very long intervening sequences of 360 (KOD *pol* intein-1) and 537 (KOD *pol* intein-2) amino acid residues at conserved regions II and III and that the reading frame was maintained throughout the coding region. The size of the mature form of the DNA polymerase gene would be 2,322 bases encoding 774 amino acid residues. The amino acid sequence of the mature form of KOD DNA polymerase was similar to those from *Thermococcus litoralis* (*Tli* DNA polymerase; 78% identity) (22, 29), *Pyrococcus furiosus* (*Pfu* DNA polymerase; 79% identity) (45), *Pyrococcus* sp. strain GB-D (Deep Vent DNA polymerase; 81% identity) (GenBank accession no. U00707), and *Thermococcus* sp. strain 9°N-7 (90% identity) (41).

Sequence and similarity analysis of two inteins in KOD DNA polymerase. One of the most interesting features of the KOD DNA polymerase gene is the existence of two intervening sequences. Sequences similar to those of KOD1 were found in the *Tli* DNA polymerase (29), and they are designated inteins. However, the *Tli* inteins are in conserved regions III (*Tli pol* intein-1, 538 amino acid residues) and I (*Tli pol* intein-2, 390 amino acid residues). The amino acid sequence of intein-2 from KOD DNA polymerase exhibited lower similarity, 62% identity, to intein-1 from *Tli* DNA polymerase than to that of the mature DNA polymerase portion (78% identity). KOD *pol* intein-1 did not show significant similarity to either *Tli pol* intein-1 or *Tli* intein-2 (<32% identity). The splice sites of these inteins could be predicted by amino acid sequence, since they are found in conserved regions. Cys or Ser at the N-terminal and His-Asn-Cys/Ser/Thr at the C-terminal splice junctions are very well conserved (15). These residues might be important for the inteins to be spliced out from the KOD DNA polymerase precursor at the protein level. Inteins, intervening sequences spliced at the protein level, are found in bacteria,

TABLE 2. Properties of thermostable DNA polymerases

Property	Value for indicated DNA polymerase			
	KOD	*Pfu*	Deep Vent	*Taq*
Deduced molecular mass (kDa)	90.0	90.1	90.6	93.9
Optimum temp[a] (°C)	75	75	75	75
Optimum pH at 75°C[a]	6.5	6.5	7.5	8.0–8.5
Thermostability (half-life)[a]	95°C, 12 h; 100°C, 3.0 h	95°C, 6 h; 100°C, 2.9 h	95°C, 13.5 h; 100°C, 3.4 h	95°C, 1.6 h
3′-5′ exonuclease activity	+	+	+	−
Fidelity[b]	3.5×10^{-3}	3.9×10^{-3}	ND[c]	1.3×10^{-2}
Terminal transferase activity	−	−	−	+
Processivity (bases)[b]	>300	<20	<20	ND
Elongation rate (bases/s)[b]	106–138	25	23	61

[a] Our experimental results, using KOD DNA polymerase and commercially available DNA polymerases.
[b] Data from this study.
[c] ND, not determined.

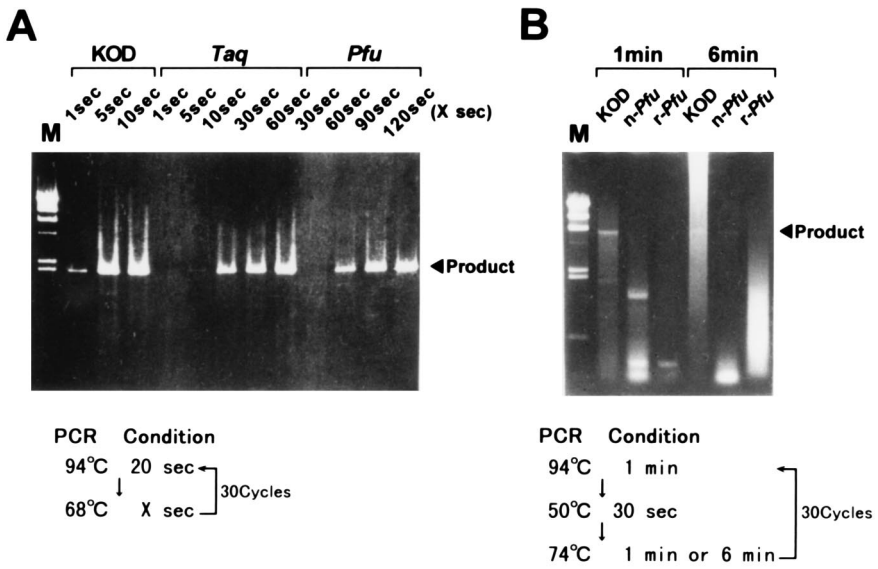

FIG. 6. (A) PCR using KOD DNA polymerase. By changing the elongation reaction time, shuttle PCR (two-step PCR) was carried out in a GeneAmp PCR System 2400 (Perkin-Elmer). Denaturation temperature and time were 94°C and 20 s, and the elongation reaction temperature was 68°C. λ DNA (0.2 ng/μl) as the template and 0.4 μM forward (5′-GGG-CGG-CGA-CCT-CGC-GGG-TTT-TCG-CTA-TTT-ATG-AAA-3′) and reverse (5′-GCC-CAT-AAT-AAT-CTG-CCG-GTC-AAT-3′) primers were used. Enzymes and elongation reaction times used are indicated at the top. Lane M is λ DNA digested with *Hin*dIII. The sizes from the top are 23,130, 9,416, 6,557, 4,361, 2,322, 2,027, and 564 bp. (B) Comparison of PCR between KOD and *Pfu* (native [n-*Pfu*] and recombinant [r-*Pfu*]) DNA polymerases on amplification of 6 kb in λ DNA. Forward (5′-GAT-GAG-TTC-GTG-TCC-GTA-CAA-CT-3′) and reverse (5′-CCA-CAT-CCA-TAC-CGG-GTT-TCA-C-3′) primers were used. Thirty cycles were carried out with conditions of 1 min at 94°C, 30 s at 50°C, and 1 or 6 min at 74°C per cycle. Enzymes and elongation reaction times used are indicated at the top. Lane M, size marker (λ DNA digested with *Hin*dIII).

eukarya, and archaea (6–9, 14, 15, 21, 31). Among the previously identified or reported inteins, some of them exhibit endonuclease activities at specific DNA regions (13, 29, 30). These endonucleases are designated homing endonucleases and are also found in group I introns (reviewed in reference 25). They contain conserved dodecapeptide motifs that are involved in the active centers of these endonucleases (12). One of the homing endonucleases, the *Sce VMA* intein, in the H^+-ATPase gene of the yeast *Saccharomyces cerevisiae*, mediates a gene conversion event initiated by cleaving its insertion site (homing site) in inteinless genes, resulting in *Sce VMA* intein mobility. The inteins of KOD DNA polymerase also contain the dodecapeptide motifs (Fig. 1) and might participate in intein mobility and rearrangement of chromosomal DNA.

Characteristics of KOD DNA polymerase. Recombinant KOD DNA polymerase was purified (Fig. 2), and its characteristics were studied and compared with those of DNA polymerases from different sources (26, 27). The optimum temperature and pH are 75°C and 6.5, respectively. Thermostability of KOD DNA polymerase was assayed at 95 and 100°C, and half-lives at these temperatures were 12 and 3 h, respectively (Fig. 3). Amino acid sequence homology suggested the presence of a 3′-5′ exonuclease domain. Indeed, KOD DNA polymerase could release 47% ^3H from the 3′ end of a standard substrate in 5 min, whereas *Pfu* DNA polymerase took 2 h to release 41% ^3H. The amount of ^{32}P released from 5′ end-labeled DNA by KOD DNA polymerase was very low, suggesting that KOD DNA polymerase had no or very weak 5′-3′ exonuclease activity. Terminal transferase activity could not be detected for KOD DNA polymerase (data not shown).

Frequency of misincorporation of deoxyribonucleotide was 0.35%, indicating that KOD DNA polymerase has high fidelity, equivalent to that of *Pfu* DNA polymerase (Table 1). Processivity (persistence of sequential nucleotide polymerization) of the KOD DNA polymerase appears to be 10- to 15-fold greater than those for *Pfu* DNA polymerase and Deep Vent DNA polymerase. The results shown in Fig. 4 clearly indicated that KOD DNA polymerase is processive, because smaller DNA fragments were detected when DNA polymerases other than KOD DNA polymerase were used. Moreover, even when the amount of enzyme was decreased, large products of the same size could be obtained. The extension rate of KOD DNA polymerase was 106 to 138 bases/s (Fig. 5), which was much higher than those of *Pfu* (25 bases/s), Deep Vent (23 bases/s), and *Taq* (61 bases/s) DNA polymerases. The characteristics of KOD DNA polymerase are summarized and compared with those of representative DNA polymerases (*Pfu*, Deep Vent, and *Taq* DNA polymerases) in Table 2.

Accurate and time-saving PCR by KOD DNA polymerase. The major uses of thermostable DNA polymerases are for in vitro amplification of DNA fragments and for determination of DNA sequence. *Taq* DNA polymerase is used in many cases, but its fidelity is not high. To improve low fidelity in PCR, new thermostable DNA polymerases isolated from several hyperthermophiles have been characterized and used for PCR. These DNA polymerases exhibit proofreading ability based on 3′-5′ exonuclease activity that edits out mismatched nucleotides. In general, these DNA polymerases can amplify DNA more accurately than *Taq* DNA polymerase. However, *Taq* DNA polymerase was not replaced with these DNA polymerases because of their low extension rates among other factors. Our experimental results clearly show that KOD DNA polymerase has excellent characteristics in fidelity (low mutation frequency), processivity, and elongation speed, suggesting that the KOD DNA polymerase is suitable for long, accurate, and time-saving PCR.

Since the high processivity and high extension rate of KOD DNA polymerase may contribute to a decrease in the total reaction time, we performed PCR using various reaction times (1 to 120 s) with λ DNA as a template (Fig. 6A). When the

KOD DNA polymerase was used, an amplified DNA product could be observed even with a very short reaction time (1 s). In the case of *Taq* and *Pfu* DNA polymerases, the DNA band could be observed at the minimum tested reaction times of 5 and 60 s, respectively.

DNA polymerases with higher fidelity are not necessarily suitable for amplification of long DNA fragments because of their potentially strong exonuclease activity (1). Amplification of a longer target DNA sequence (6 kb) by using the KOD DNA polymerase was attempted; the desired DNA fragment could be observed after a 1-min reaction, whereas 6 min was needed for the *Pfu* DNA polymerase (Fig. 6B).

We also compared the relative fidelity in PCR among KOD, *Pfu*, and *Taq* DNA polymerases. Ratios of the number of mutant colonies to total colonies were 380/13,531 for KOD DNA polymerase, 269/7,485 for *Pfu* DNA polymerase, 57/120 for *Taq* DNA polymerase, and 12/10,320 for spontaneous mutation (background). The mutation frequencies calculated from the first three of these ratios were 2.8, 3.6, and 48%, respectively. KOD DNA polymerase showed the lowest mutation frequency in PCR.

One of the technical topics concerning PCR is the long and accurate PCR (LA PCR) (1, 5). In the past, the robust amplification of targets longer than 5 kb was difficult, limiting some applications of PCR. Recently, longer DNA target fragments were effectively amplified by using a two-DNA polymerase system to provide optimal levels of both DNA polymerase activity and proofreading or 3′-5′ exonuclease activity. KOD DNA polymerase could not be used for LA PCR because of the difficulty in optimization of the two activities. In the case of 9°N-7 DNA polymerase, which exhibited the highest similarity to KOD DNA polymerase (90% identical and 95% similar amino acid sequences), Southworth et al. have performed some mutation studies of 3′-5′ exonuclease motif I (41). They constructed six mutated DNA polymerases and showed that five of them resulted in no detectable 3′-5′ exonuclease activity, while one mutant had <1% of wild-type activity. Their experimental results suggested the possibility of changing the level of 3′-5′ exonuclease activity, but it would be difficult to obtain mutant DNA polymerases with the modest level of 3′-5′ exonuclease activity by amino acid substitutions at conserved exonuclease motif residues. We are now constructing several mutated KOD DNA polymerases that show exonuclease activities lower than that of the intact KOD DNA polymerase by mutating residues at and near the exonuclease motif. Application of the mutated KOD DNA polymerases for LA PCR is also under way.

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