

## Cloning of thermostable DNA polymerases from hyperthermophilic marine Archaea with emphasis on *Thermococcus* sp. 9°N-7 and mutations affecting 3'–5' exonuclease activity.

(mutation/purification/intein/protein splicing)

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**ABSTRACT** Five extremely thermophilic Archaea from hydrothermal vents were isolated, and their DNA polymerases were cloned and expressed in *Escherichia coli*. Protein splicing elements (inteins) are present in many archaeal DNA polymerases, but only the DNA polymerase from strain GB-C contained an intein. Of the five cloned DNA polymerases, the *Thermococcus* sp. 9°N-7 DNA polymerase was chosen for biochemical characterization. *Thermococcus* sp. 9°N-7 DNA polymerase exhibited temperature-sensitive strand displacement activity and apparent  $K_m$  values for DNA and dNTP similar to those of *Thermococcus litoralis* DNA polymerase. Six substitutions in the 3'–5' exonuclease motif I were constructed in an attempt to reduce the 3'–5' exonuclease activity of *Thermococcus* sp. 9°N-7 DNA polymerase. Five mutants resulted in no detectable 3'–5' exonuclease activity, while one mutant (Glu143Asp) had <1% of wild-type activity.

Thermostable enzymes have been characterized from eubacteria and Archaea isolated from geothermal habitats. DNA polymerase (pol) genes from three extremely thermophilic marine Archaea, *Thermococcus litoralis* (1), *Pyrococcus* sp. GB-D (accession no. U00707), and *Pyrococcus furiosus* (2), have been cloned. DNA pols isolated from Archaea are family B, pol  $\alpha$ -like, whereas DNA pols isolated from thermophilic eubacteria are family A, pol I-like (3, 4). Two of these archaeal DNA pols contain protein splicing elements, termed inteins. Inteins are inframe insertions that are expressed and eventually spliced as proteins instead of as RNA (reviewed in refs. 5–7). This paper examines the DNA pols from five marine vent isolates.

DNA pols may contain 3'–5' and/or 5'–3' exonuclease (exo) activities. Previously cloned archaeal DNA pols have 3'–5' exo activity but not 5'–3' exo activity (4). The 3'–5' exo activity enables the DNA pol to proofread—i.e., to correct mistakes at the 3'-end of a growing DNA chain during replication or repair. Three 3'–5' exo motifs have been identified by sequence comparisons (8, 9) and mutagenesis (reviewed in ref. 10). Substitution of any of the conserved Asp or Glu residues in these motifs by Ala abolishes exo activity (10). Conservative substitution of these same residues results in variable reductions in exo activity (10). This paper examines whether conservative substitutions of essential amino acids in exo motif I (Asp-Xaa-Glu, where Xaa = variable residue) might result in reduced 3'–5' exo activity in *Thermococcus* sp. 9°N-7 DNA pol.

### MATERIALS AND METHODS

**Origin and Isolation of Strains.** All strains were isolated from sampling material collected with the aid of the research submersible ALVIN from oceanic hydrothermal vent sites.

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Strains GI-J, GB-C, and GB-H were isolated from samples obtained at Guaymas Basin vent. Situated in the Gulf of California, this site is located at the northern end of the East Pacific Rise tectonic spreading zone (11) at depths from 1990 to 2010 m. The samples represented scrapings from a hot black smoker surface (strain GB-C) and a worm tube (strain GI-J). Strain GI-H was isolated from sediment covered by a dense mat of *Beggiatoa* (12, 13). Strain 9°N-7 originated from scrapings of a smoker chimney collected at the 9°N East Pacific Rise vent site,  $\approx$ 500 miles south of Acapulco, at a depth of 2500 m. This vent site erupted  $\approx$ 3 years before the sampling, yet the typical bacterial populations associated with hydrothermal vents appeared to be similar to long-established vent sites (14). Isolate 9°N-7 was identified as a *Thermococcus* species by 16S rRNA sequence analysis (H.W.J. and F.B.P., unpublished data). Strain MAR-13, similar to strain MAR-7C (15), was isolated from polymetal sulfide deposits collected by ALVIN at a Mid-Atlantic Ridge hydrothermal vent site at a depth of 3600 m.

The isolation media and procedures were identical to those described earlier (15). Strictly anaerobic procedures were used during enrichment and growth studies in 10-ml Hungate tubes. Pure cultures were obtained by dilution to extinction in liquid media. When growth at temperatures  $>100^\circ\text{C}$  was tested, cultures were kept in syringes with no gas phase at a pressure of 5 bar (1 bar = 100 kPa). Growth characteristics of the strains, whenever tested, were similar to those of comparable isolates (15).

**Primers and DNA Sequencing.** All primers (Table 1), except NEB1224 (New England Biolabs), were custom synthesized by New England Biolabs. The DNA pol gene from *Thermococcus* sp. 9°N-7 was sequenced from deletion clones generated with the Exo-Size deletion kit (New England Biolabs). Both strands of the *Thermococcus* sp. 9°N-7 DNA pol gene (accession no. U47108; pol gene starts at nucleotide 40), a fragment of the DNA pol gene from strain GI-J (accession no. U00429) generated from genomic DNA by PCR, and all other PCR fragments were sequenced using standard *Taq* DyeDeoxy Terminator Cycle Sequencing Kit protocols, an Applied Biosystems Division 373A Automated DNA Sequencer (Perkin-Elmer), and Applied Biosystems Division Mac-Assembler Programs (FRACTURA and AUTOASSEMBLER).

**Cloning DNA pol Genes.** Genomic DNA from each isolate was prepared, digested with either *Eco*RI or *Bam*HI, and hybridized with *T. litoralis* DNA pol probes (1) representing the 5'-end of the gene (base pairs 1–1274), the 3'-end of the gene (base pairs 4718–5437), and portions of *Tli* pol intein-1 (base pairs 2448–2882) and *Tli* pol intein-2 (base pairs 3666–4242) as described (16). Genomic DNA libraries were con-

**Abbreviations:** pol, polymerase; exo, exonuclease.

**Data deposition:** The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U47108 and U00429).

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Table 1. Oligonucleotides used in this paper

Name of primer	Sequence	exo I	Cut site
<b>General</b>			
NEB1224	5'-CGCCAGGGTTTCCCAGTCACGAC-3'		
Gap primer	5'-CGTAATCATGGTCATAGCTGTTTCCTG-3'		
9°N-7FOR	5'-TGGTGAAGCTTCATATGATTCTCGATACCG-3'		
9°N-7REV	5'-GCCGGGGGATCCCTGCAGAACTTGTGTCC-3'		
32-mer	5'-CGAGTGATGATGATGATGATGACTAGTGCCCA-3'		
<b>exoI mutagenic</b>			
9°N-7WT	5'-CTCGCCTTC <b>GACATCGAA</b> ACGCTCTATCAC-3'	<b>DIE</b>	
9°N-7MUT1	5'-CTCGCCTTC <b>GCGATCGCA</b> ACGCTCTATCAC-3'	<b>AIA</b>	<i>PvuI</i>
9°N-7MUT2	5'-CTCGCCTTC <b>GCGATCGAA</b> ACGCTCTATCAC-3'	<b>AIE</b>	<i>PvuI</i>
9°N-7MUT3	5'-CTCGCCTTC <b>GAAATCGAA</b> ACGCTCTATCAC-3'	<b>EIE</b>	<i>BstBI</i>
9°N-7MUT4	5'-CTCGCCTTC <b>GAAATCGAC</b> ACGCTCTATCAC-3'	<b>EID</b>	<i>BstBI</i>
9°N-7MUT5	5'-TTC <b>GACATCGAC</b> ACGTTGTATCACGAGGGC-3'	<b>DID</b>	<i>AflIII</i>
9°N-7MUT6	5'-TTC <b>GACATCGCA</b> ACGTTGTATCACGAGGGC-3'	<b>DIA</b>	<i>Psp1406I</i>

exo I indicates mutations made in the exo motif I of *Thermococcus* sp. 9°N-7 DNA pol (9°N-7WT), and cut site refers to the restriction enzyme site (underlined) included in each primer. The conserved exo motif I amino acids and coding sequences are in boldface. D, Asp; I, Ile; E, Glu; A, Ala.

structured (17) by ligating DNA partially digested with either *Bam*HI or *Eco*RI into  $\lambda$ DASH (Stratagene). Plaque lifts were screened with the four *T. litoralis* DNA pol probes (16, 17). Phage positive for both the 5' and 3' DNA pol probes were plaque-purified, and the inserts were subcloned into pUC19. Clones were assayed for thermostable DNA pol activity as described (1). Briefly, lysates were heat treated for 20 min at 72°C to kill endogenous DNA pols, and 1–5  $\mu$ l of cleared lysates was then assayed at 72°C for incorporation of a radiolabeled dNTP into trichloroacetic acid-precipitable DNA. pol assays were performed in a solution containing 10 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM Tris (pH 8.8), 2 mM MgSO<sub>4</sub>, 0.1% Triton X-100, 10–20  $\times$  10<sup>6</sup> cpm of [<sup>32</sup>P]-dCTP per ml, 33  $\mu$ M dNTP, and activated DNA (0.2 mg/ml). One DNA pol unit was defined as the amount of enzyme that will incorporate 10 nmol of dNTP in acid-insoluble material in 30 min at 72°C.

**Overexpression of the *Thermococcus* sp. 9°N-7 DNA pol.** *Thermococcus* sp. 9°N-7 DNA pol was overexpressed in *Escherichia coli* under the control of a phage T7 promoter in a modified pET11c vector, pAII17 (1, 18). A three-part ligation was performed with a *Nde*I/*Stu*I fragment from a pLITMUS38 subclone (see below) containing the 5'-end of the DNA pol (nucleotides 1–892), a *Stu*I/*Bam*HI fragment containing the 3'-end of the DNA pol (nucleotides 893–2328) and *Nde*I/*Bam*HI-digested pAII17. The 5'-fragment was generated by PCR using the 9°N-7FOR primer (to include *Hind*III and *Nde*I sites and the start codon of the pol gene) and the reverse primer, 9°N-7REV (adding a *Bam*HI site to nucleotide 1328 of the pol gene). Two units of Vent pol (New England Biolabs) were added to a 100- $\mu$ l reaction containing Vent buffer, 200 mM each dNTP, 100 ng of pUC19/9°N-7 DNA, and 100 ng each of primers 9°N-7FOR and 9°N-7REV. Reaction conditions were 94°C for 30 s, 48°C for 30 s, and 72°C for 2 min for 15 cycles in a Perkin-Elmer Thermal Cycler 480. The resultant 1.35-kb fragment was cloned into pLITMUS38 (New England Biolabs) after *Hind*III and *Bam*HI digestion to create pLITMUS38/9°N-7. The 3'-end of the gene was obtained by digesting the deletion clone pE1 $\Delta$  (which deletes unrelated DNA sequences 3' to the pol gene) with *Stu*I and *Bam*HI. After ligation (17), the mixture was transformed into ER2169plysS (New England Biolabs), and the resultant clone, pAII17/9°N-7 (NEB915), was found to express heat-stable *Thermococcus* sp. 9°N-7 DNA pol activity.

**Construction of 3'–5' exo Motif I Mutants in *Thermococcus* sp. 9°N-7 DNA pol.** Site-directed mutagenesis of single-stranded pLITMUS38/9°N-7 was performed (19) with the 9°N-7MUT1–6 primers (Table 1). Each primer mutated the wild-type exo motif I (Asp-Ile-Glu) and introduced a new

restriction enzyme site for easy identification (Table 1). The *Nde*I/*Stu*I fragment from each pLITMUS38/9°N-7MUT clone was subcloned into pAII17 using the same three-part ligation strategy described above.

**exo Assays.** In assay 1, crude lysates were analyzed for exo activity by measuring the release of <sup>3</sup>H from uniformly *in vivo*-labeled *E. coli* DNA (specific activity, 200,000 cpm/ $\mu$ g), as described (20). 3'–5' exo assays contained Vent buffer (New England Biolabs), 25  $\mu$ g of [<sup>3</sup>H]DNA per ml, and 1–5  $\mu$ l of crude extract. Samples were incubated at 72°C for up to 1 h. exo activity was detected by the release of acid-soluble radioactivity. 5'–3' exo activity was determined using assay 1 in the presence of 200 mM dNTP, which stimulates 5'–3' exo activity and inhibits 3'–5' exo activity.

Assay 2 uses a heterogeneous mixture of *Sau*3AI restriction fragments labeled at the 3'-terminal or penultimate phosphodiester bond by partially filling in the restriction site with [ $\alpha$ -<sup>32</sup>P]-dATP or [ $\alpha$ -<sup>32</sup>P]-dGTP (10). The substrate was treated with inorganic pyrophosphatase (Boehringer Mannheim) before removal of unincorporated dNTPs. exo assays contained Vent buffer, <sup>32</sup>P-labeled substrate (3–30 nM), and various units of DNA pol at 72°C. The percent trichloroacetic acid-soluble cpm or percent nmol labeled dNTP released was determined over time.

Assay 3 uses a single-stranded 32-mer (Table 1) labeled at the 5'-end with [ $\gamma$ -<sup>32</sup>P]-dATP by T4 polynucleotide kinase (New England Biolabs). Labeled DNA substrate (80 nM) and various amounts of DNA pol were incubated in Vent buffer at 72°C for up to 30 min and then mixed with DNA sequencing stop dye (New England Biolabs). Samples were heated at 90°C, electrophoresed through a 20% urea denaturing gel, and the dried gel was autoradiographed.

**Purification and Characterization of *Thermococcus* sp. 9°N-7 DNA pol.** NEB915 (wild-type *Thermococcus* sp. 9°N-7 DNA pol) was induced in mid-logarithmic phase with 0.3 mM isopropyl  $\beta$ -D-thiogalactoside. The cell pellet (432 g) was resuspended in 1700 ml of buffer A [75 mM NaCl, 10 mM KPO<sub>4</sub> (pH 6.8), 0.1 mM EDTA, 1 mM DTT, and 10% glycerol] and sonicated. The cleared lysate was heat-treated at 75°C for 5 min, and precipitated protein was removed by centrifugation. The supernatant containing 4230 mg of protein was applied to a DEAE-sepharose column (8.5  $\times$  5 cm) linked in series to a phosphocellulose column (10.5  $\times$  5 cm). After the linked columns were washed with buffer A, the columns were separated, and the phosphocellulose column was washed further with buffer A before elution of the bound proteins with a 2-liter linear gradient from 75 mM to 800 mM NaCl in buffer A. The fractions containing maximal DNA pol activity were centered at 550 mM NaCl and contained 1350 mg of protein.

After dialysis against buffer B [100 mM NaCl, 20 mM Tris (pH 7.5), 0.1 mM EDTA, 1 mM DTT, and 10% glycerol], the pool was loaded onto a second DEAE-sepharose column (8 × 2.5 cm) at 25°C. After washing the column with buffer B, the flow-through containing 1220 mg of protein was loaded onto a heparin-sepharose column (11 × 2.5 cm), washed with buffer B, and eluted with a 540-ml linear gradient from 100 mM to 800 mM NaCl. The fractions containing maximal DNA pol activity were centered at 600 mM NaCl and contained 410 mg of protein. Fractions containing the DNA pol were pooled and dialyzed into storage buffer containing 100 mM KCl, 10 mM Tris HCl (pH 7.4), 1 mM DTT, 0.1 mM EDTA, 0.1% Triton-X 100, and 50% glycerol.

Kinetic parameters, specific activity, and strand displacement assays were determined as described for *T. litoralis* DNA pol (20). In strand displacement assays, 5'-end-labeled NEB1224 and unlabeled Gap primer (Table 1) were annealed to M13 mp18 single-stranded DNA creating a 79-nt gap between primers. pol reactions were then performed at either 55°C or 72°C. Samples were electrophoresed in 6% acrylamide/6 M urea gels and examined after autoradiography.

## RESULTS

**Cloning of the DNA pol Genes from Five Hydrothermal Vent Isolates.** On the assumption that a probe prepared from the DNA pol gene of *T. litoralis* would cross-hybridize with the DNA pol genes of the five new marine vent isolates, DNA probes were prepared from the 5'- or 3'-end of the pol gene and from each intein. Genomic DNA from all five isolates hybridized to both *T. litoralis* DNA pol probes, but only DNA from strain GB-C hybridized to the *Tli pol intein-1* probe. No new isolate hybridized to the *Tli pol intein-2* probe. *EcoRI* digests of the five genomic DNAs were cloned into  $\lambda$ Dash (Table 2). Each library was screened with the 5'- and 3'-pol probes, yielding 0.6–3.2% phage positive for both probes. After plaque purification, DNA inserts were subcloned into pUC19 and tested for thermostable DNA pol activity. Induced cells were lysed and heat-treated to precipitate heat-labile proteins, especially the endogenous *E. coli* DNA pols. Samples were assayed for thermostable DNA pol activity at 72°C for 40 min (Table 2). DNA pol activity ranged from  $\approx$ 120-fold higher than the pUC19 control in strains 9°N-7 and GI-H (318–344 pmol in 40 min) to only 2-fold higher in MAR-13 samples. The MAR-13 DNA pol gene mapped  $\approx$ 9 kb from the pUC19 *lac* promoter. An 8-kb *AflIII* deletion that placed the DNA pol gene closer to the *lac* promoter resulted in a 7-kb subclone with  $\approx$ 7-fold higher heat-stable DNA pol activity than the original clone. After preliminary biochemical characterization of the five DNA pols, *Thermococcus* sp. 9°N-7 DNA pol was chosen for further study.

**DNA Sequencing and Overexpression of *Thermococcus* sp. 9°N-7 DNA pol.** The position of the DNA pol gene in the 6.5-kb

*EcoRI* genomic DNA fragment was determined by making random terminal deletions. Five clones with representative deletions of 0.6, 1.7, 2.2, 2.7, and 3.0 kb from the downstream end of the 6.5-kb fragment (clones E1 $\Delta$ , A1 $\Delta$ , N14 $\Delta$ , C2 $\Delta$ , and N20 $\Delta$ , respectively) were then used to sequence the DNA pol gene. The sequence indicated that the *Thermococcus* sp. 9°N-7 DNA pol gene did not contain any inteins.

The *Thermococcus* sp. 9°N-7 DNA pol gene was subcloned for overexpression in a three-part ligation. The first 1.35 kb of the DNA pol gene was amplified by PCR introducing a *NdeI* site at the start codon and subcloned into pLITMUS38. The 5'-end of the DNA pol gene (nucleotides 1–892) was isolated after digesting pLITMUS38/9°N-7 with *NdeI* and *StuI*. The 3'-end of the DNA pol gene was generated by digesting the deletion clone pE1 $\Delta$  with *StuI* and *BamHI*. The two halves of the *Thermococcus* sp. 9°N-7 DNA pol gene were then ligated concurrently into *NdeI/BamHI*-digested pAII17, resulting in clone NEB915.

Recombinant *Thermococcus* sp. 9°N-7 DNA pol was purified by standard column chromatography. The purified DNA pol had an apparent molecular mass of  $90 \pm 5$  kDa and was >95% pure, as estimated by observation of a Coomassie blue-stained SDS/polyacrylamide gel. DNA pol ( $\approx$ 400 mg) was purified from 432 g of cells. The recombinant DNA pol had a specific activity on activated DNA (1) of 3.5 units/ $\mu$ g.

**Reduction of 3'-5' exo Activity by Site-Directed Mutagenesis of exo Motif I.** Previous studies have indicated that mutation of the 3'-5' exo motif I (Asp-Xaa-Glu) can reduce or eliminate exo activity (10), but the effect of all possible conservative (Glu  $\leftrightarrow$  Asp) mutations has not been examined. exo motif I in *T. litoralis* DNA pol (Asp-Ile-Glu) was mutated to Ala-Ile-Ala to generate an exo<sup>-</sup> enzyme (20). Site-directed mutagenesis (19) of *Thermococcus* sp. 9°N-7 exo motif I with primers 9°N-7-MUT1–6 (Table 1) yielded clones that express heat-stable DNA pol activity. Five of these six mutants showed no detectable 3'-5' exo activity. However, the Asp-Ile-Asp mutant exhibited <5% of the wild-type 3'-5' exo activity using tritium-labeled *E. coli* DNA as substrate.

**Characterization of the Asp-Ile-Asp exo Motif I Mutant.** More accurate 3'-5' exo assays were used to quantify the reduction in activity in the Asp-Ile-Asp mutant. The four-base 5'-extension produced by *Sau3AI* digestion was partially filled in with two nucleotides such that either the terminal or penultimate 3'-nucleotide was radiolabeled. The substrate was then treated with varying amounts of DNA pol in the absence of added dNTPs (to inhibit pol activity). Similar pmol of dNTPs were released by  $4 \times 10^{-5}$  units of wild-type enzyme and 0.01 units of the Asp-Ile-Asp mutant, indicating that the Asp-Ile-Asp mutant has  $\approx$ 0.4% of wild-type exo activity in this assay (Fig. 1).

The Asp-Ile-Asp mutant was also assayed with a 5'-end-labeled 32-nt single-stranded DNA as the substrate. The 32-mer was treated with dilutions of each enzyme, the products were electrophoresed, and the gel was autoradiographed. A comparable pattern of degradation from the 3'-end was observed with 0.02 units of wild-type enzyme and 2.5–5 units of the Asp-Ile-Asp mutant, indicating that in this assay the Asp-Ile-Asp mutant enzyme has  $\approx$ 0.4–0.8% of wild-type activity (Fig. 2).

**Biochemical Characterization of Recombinant *Thermococcus* sp. 9°N-7 DNA pol.** Examination of the *Thermococcus* sp. 9°N-7 DNA pol DNA sequence failed to detect any of the conserved 5'-3' exo motifs (21). exo activity assay 1 was performed with wild-type and Ala-Ile-Ala mutant *Thermococcus* sp. 9°N-7 DNA pol using uniformly labeled *E. coli* DNA in the presence or absence of dNTP. In the case of the wild-type enzyme, addition of dNTP to the reaction mixture reduced the pmol of dNTP solubilized after 90 min by 7-fold, which is suggestive of 3'-5' exo activity in the absence of 5'-3' exo activity. The Ala-Ile-Ala mutant had no detectable exo

Table 2. Summary of recombinant archaeal DNA pols

Isolate	Fragment size*	DNA pol act.†	
		pmol	cpm
GI-J	4	36	13,450
MAR-13	7	36	13,600
9°N-7	6.5	344	129,700
GB-C	5.5	64	24,000
GI-H	8.5	318	119,000
<i>E. coli</i>		0	100

\*Size (in kb) of the *EcoRI* genomic DNA fragment present in the pUC19 construct expressing thermostable DNA pol activity.

†Thermostable DNA pol activity in heat-treated cleared lysates measured by pmol or acid-insoluble counts (cpm) incorporated after 40 min at 72°C (1).

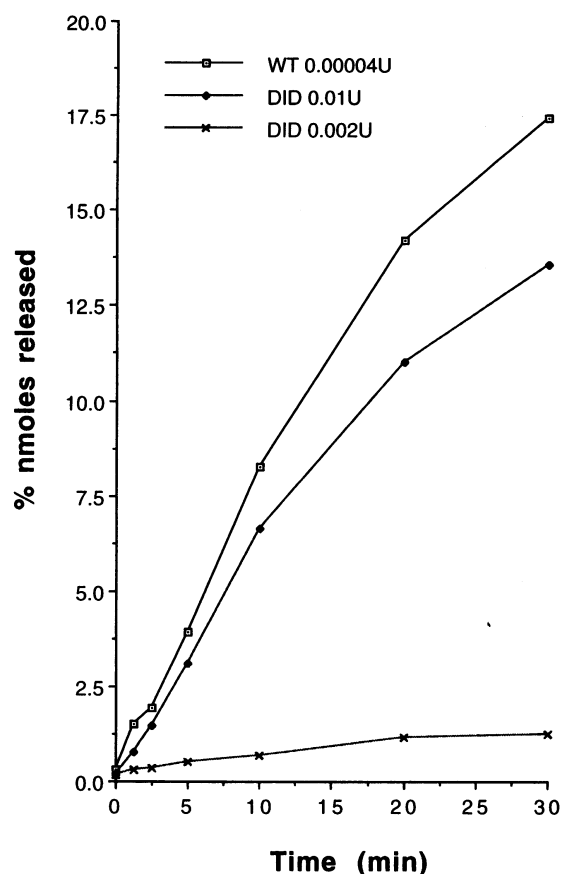


FIG. 1. Mutation of *exo* motif I from Asp-Ile-Glu (DIE) to Asp-Ile-Asp (DID) resulted in reduced 3'-5' *exo* activity using *exo* assay 2. In this assay, the 3'-ends of DNA fragments are labeled by partially filling in the 5'-extension produced by *Sau*3AI. The percent pmol of labeled dNTP released after incubation at 72°C was determined for  $4 \times 10^{-5}$  DNA pol units of wild-type recombinant DNA pol and 0.01 or 0.002 units of Asp-Ile-Asp (DID) mutant DNA pol.

activity in the presence or absence of dNTP, confirming the absence of 5'-3' *exo* activity in 9°N-7 DNA pol.

Biochemical parameters of recombinant wild-type (*exo*<sup>+</sup>), Ala-Ile-Ala mutant (*exo*<sup>-</sup>), and low *exo* activity Asp-Ile-Asp mutant (*exo*<sup>DID</sup>) versions of *Thermococcus* sp. 9°N-7 DNA pol were compared with *T. litoralis* DNA pol. All had similar low apparent *K<sub>m</sub>* values for DNA and high apparent *K<sub>m</sub>* values for nucleotides (Table 3). The ratio of pol-specific activity on primed single-stranded M13 DNA versus specific activity on activated DNA was 10-fold higher with *Thermococcus* sp. 9°N-7 DNA pol than with *T. litoralis* DNA pol (Table 4).

Strand displacement activity was studied in a gap-filling assay. DNA pols that lack a 5'-3' *exo* must either displace the 5'-end of a double-stranded DNA template to continue synthesis or stop polymerization at the end of a gap. Strand displacement activity of *Thermococcus* sp. 9°N-7 DNA pol was temperature-sensitive (Fig. 3). At 55°C, DNA synthesis halted upon reaching the downstream primer. At 72°C, polymerization products extended beyond the downstream primer but still stopped after extensions of about 150 nt. *exo*<sup>-</sup> and *exo*<sup>DID</sup> 9°N-7 DNA pol showed greater strand displacement activity than the wild-type enzyme.

## DISCUSSION

Five DNA pols from extremely thermophilic Archaea were expressed in *E. coli* without prior linkage to *E. coli* promoters.

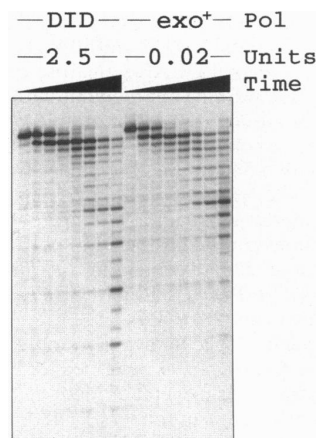


FIG. 2. The Asp-Ile-Asp (DID) mutant *Thermococcus* sp. 9°N-7 DNA pol has reduced 3'-5' *exo* activity on single-stranded DNA substrates. Wild-type enzyme (0.02 DNA pol units) degraded the 5'-end labeled 32-mer from the 3'-end with kinetics similar to that of 2.5 DNA pol units of the mutant enzyme (DID). In this assay, single-stranded DNA (Table 1) was incubated at 72°C in Vent pol buffer without added dNTP for 0, 1, 2, 5, 10, 20, 30, or 60 min. After incubation with either the DID mutant or wild-type recombinant (*exo*<sup>+</sup>) enzyme, the oligomer was electrophoresed in a 20% urea denaturing gel and autoradiographed.

However, the fact that expression of MAR-13 DNA pol was improved  $\approx 7$ -fold by bringing the start of the gene closer to the vector promoter suggests that these genes may be relying on vector promoters for transcription. To date, nine other archaeal DNA pol sequences have been reported (*T. litoralis*, accession no. M74108; *Pyrococcus* sp. GB-D, accession no. U00707; *Pyrococcus* sp. KOD, accession no. D29671; *Pyrococcus furiosus*, accession no. D12983; two from *Sulfolobus solfataricus*, accession nos. X64466 and X71597; two from *Pyrodicticum occultum*, accession nos. D38573 and D38574; and *Methanococcus voltae*, accession no. L33366). Inteins have been found in three of these DNA pols (*T. litoralis*, *Pyrococcus* sp. GB-D, and *Pyrococcus* sp. KOD). In this study, only isolate GB-C hybridized to the *Tli* pol intein-1 probe, while the other four isolates failed to hybridize to either *T. litoralis* intein probe. It is possible that some of these isolates contain inteins that do not cross-hybridize with *T. litoralis* inteins. The presence of inteins in only a subset of species has been documented in *Mycobacterium* (22) and may be attributed to lateral transmission of intein genes (23, 24).

Of the five enzymes cloned, *Thermococcus* sp. 9°N-7 DNA pol was chosen for further study. Although the 16S ribosomal RNA sequence indicated that it is a *Thermococcus* species, the *Thermococcus* sp. 9°N-7 DNA pol was most similar to the *Pyrococcus* sp. KOD DNA pol (90% identical). Despite the similarity in the deduced amino acid sequences of the *Thermococcus* and *Pyrococcus* DNA pols (4), they have very different biochemical properties. For example, there is a 10-fold difference in the ratio of activity on primed single-

Table 3. Comparison of kinetic parameters

pol	<i>K<sub>m</sub></i> DNA*, nM	<i>K<sub>m</sub></i> dNTP†, $\mu$ M
9°N-7 <i>exo</i> <sup>+</sup>	$0.05 \pm 0.03$	$75 \pm 36$
9°N-7 <i>exo</i> <sup>DID</sup>	$0.05 \pm 0.009$	$87 \pm 20$
9°N-7 <i>exo</i> <sup>-</sup>	$0.04 \pm 0.013$	$71 \pm 23$
Vent <i>exo</i> <sup>+</sup>	$0.06 \pm 0.005$	$65 \pm 19$

\*Apparent *K<sub>m</sub>*, moles of template in the presence of an excess of annealed primer.

†Apparent *K<sub>m</sub>*, moles of each nucleotide in an equimolar mixture of the four nucleotides.

Table 4. Specific activity on different DNA substrates

	9°N-7 exo <sup>+</sup>	9°N-7 exo <sup>DID</sup>	9°N-7 exo <sup>-</sup>	Vent exo <sup>+</sup>	Vent exo <sup>-</sup>
M13 DNA	25,000	33,000	17,000	17,000	6,300
Act. DNA	3,600	7,900	6,100	24,000	37,000
Ratio*	6.9	4.2	2.8	0.71	0.17

Specific activity (unit/mg) of polymerization on primed single-stranded M13 DNA or activated calf thymus DNA (Act. DNA).

\*Specific activity with the M13 DNA substrate divided by specific activity with the Act. DNA substrate.

stranded DNA versus activated DNA between *T. litoralis* and *Thermococcus* sp. 9°N-7 DNA pols (77% identical, 89% similar amino acid sequences). *Thermococcus* sp. 9°N-7 DNA pol has a 5-fold greater 3'-5' exo activity per unit pol activity than *T. litoralis* DNA pol (M.W.S. and H.K., unpublished data).

The apparent  $K_m$  values for DNA and dNTP of *Thermococcus* sp. 9°N-7 DNA pol are very similar to those of *T. litoralis* DNA pol (Table 3); the temperature-sensitive strand displacement profiles are also very similar. It is remarkable that polymerization and exonucleolytic degradation occur readily at 55°C, but that strand displacement is blocked at this temperature. The observed increase in strand displacement in the exo<sup>-</sup> *Thermococcus* sp. 9°N-7 DNA pol mutant has also been observed with *T. litoralis* DNA pol (20) and may be due to the substrate spending less time in the exo active site or to the absence of pol stuttering (repeated cycles of polymerization and exo activity).

Mutations in exo motif I generally have a large negative effect on 3'-5' exo activity because both conserved residues

(Asp and Glu) are intimately involved at the exo active site, interacting with the two catalytic metal ions, among other contacts (10). Of the six mutations in exo motif I, all but Asp-Ile-Asp knocked out exo activity. Even the very conservative substitution of Asp by Glu in the Glu-Ile-Glu mutant decreased exo activity by at least three orders of magnitude. In the Asp-Ile-Asp mutant, exo activity was reduced to ≈0.4–0.8% of the wild-type activity, while pol activity was not affected (Table 4). These experiments suggest that it should be possible to change the level of exo activity in any DNA pol by mutating conserved exo motif residues.

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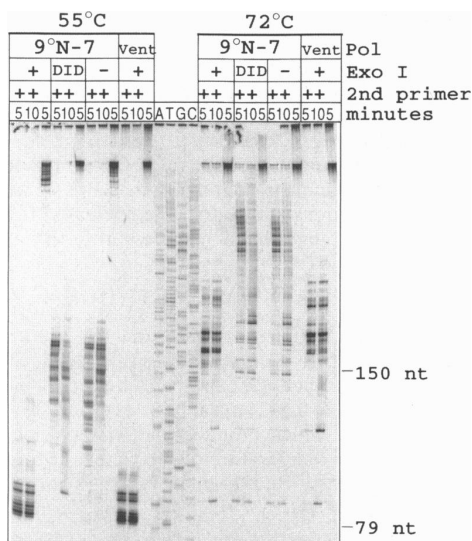


FIG. 3. *Thermococcus* sp. 9°N-7 DNA pol has temperature-sensitive strand displacement activity. M13 mp18 single-stranded DNA was annealed in Vent pol buffer with 5'-end labeled NEB1224 primer in the presence or absence of a second primer 79 nt downstream of the first primer (Table 1). Reactions contained Vent pol buffer, 0.2 mM dNTPs, and 58 units of DNA pol per ml. Reactions were incubated at 55°C or 72°C, as indicated, and aliquots were withdrawn at 5 or 10 min for analysis on denaturing polyacrylamide gels. The pol row indicates the DNA pol used. The exo I row indicates whether the enzyme was exo<sup>+</sup> (+), exo<sup>-</sup> (-), or contained the Asp-Ile-Asp mutation in exo motif I (DID). In the row labeled 2nd primer, a plus (+) indicates the presence of the blocking primer, and a blank space indicates the absence of the blocking primer (3rd sample in each group).

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