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Thermophilic bacterial DNA polymerases with reverse-transcriptase activity

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Abstract Conserved motifs found in known bacterial polI DNA polymerase sequences were identified, and degenerate PCR primers were designed for PCR amplification of an internal portion of *polI* genes from all bacterial divisions. We describe here a method that has allowed the rapid identification and isolation of 13 *polI* genes from a diverse selection of thermophilic bacteria and report on the biochemical characteristics of nine of the purified recombinant enzymes. Several enzymes showed significant reverse-transcriptase activity in the presence of Mg^{2+}, particularly the polymerases from *Bacillus caldolyticus EA1*, *Caldibacillus cellovorans* CompA.2, and *Clostridium stercorarium*.

Keywords DNA polymerase · Reverse transcriptase · Thermophilic bacteria

Introduction

Thermostable DNA polymerase from *Thermus aquaticus* (*Taq*) 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 (Innis et al. 1990). *Taq* DNA polymerase has measurable reverse-transcriptase (RT) activity (Jones and Foulkes 1989). The RT activity of a recombinant DNA polymerase from *T. thermophilus* (r*Tth*) has been reported to be 100-fold greater than that of *Taq* DNA polymerase (Myers and Gelfand 1991). The *Taq* and r*Tth* enzymes have significant amino acid sequence similarity, and it is not clear why their abilities to utilize RNA templates are so different. Reverse transcription by thermophilic DNA polymerases has advantages over mesophilic retroviral RTs such as Moloney murine leukemia virus (MoMLV) and *Avian myeloblastosis virus* (AMV) RTs, which are commonly used for cDNA synthesis because the higher reaction temperatures with thermophilic polymerases help destabilize RNA secondary structures which pose problems for mesophilic RTs (DeStefano et al. 1991; Harrison et al. 1998; Wu et al. 1996). The uses and advantages of employing thermophilic DNA polymerases for reverse transcription and reverse transcription-coupled PCR amplifications (RT-PCR) have been described (Myers and Gelfand 1991). However, one of the disadvantages of using r *Tth* DNA polymerase for copying RNA is the requirement for the use of Mn^{2+} rather than Mg^{2+} as the divalent metal ion. The presence of Mn^{2+} results in higher error rates during cDNA synthesis (Cadwell and Joyce 1992) and in reduced yields of DNA product during PCR amplification (Leung et al. 1989). Special measures must be taken during the PCR step of RT-PCR to remove the influence of Mn^{2+} introduced during the reverse-transcription step (Myers and Gelfand 1991).

Accordingly, we have carried out a survey of a number of thermophilic bacteria to identify DNA polymerases that could be used to copy RNA efficiently at elevated temperatures exclusively in the presence of Mg^{2+}. We have used degenerate oligonucleotide-based PCR (Rose et al. 1998) combined with genomic-walking (GW)-PCR (Morris et al. 1995) to obtain the full-length gene sequences of 13 thermophilic *polI* genes. The degenerate primers were designed to hybridize to DNA

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coding for two conserved regions identified in an alignment of 24 bacterial polI sequences. Three forward and three reverse primers were designed to amplify a PCR product of approximately 570 bp. The cloning of the genes and the purification and preliminary characterization of the gene products are described here. We have identified several thermophilic DNA polymerases that copy RNA efficiently in the presence of Mg^{2+}.

Materials and methods

Microorganisms

Clostridium stercorarium (Cst), *Clostridium thermosulfurogenes* (Cth), *Caldibacillus cellulovorans* CompA.2 (CA2), *Caldicellulosiruptor saccharolyticus*, *Caldicellulosiruptor* sp. strain Tok13B.1 (Tok13B), *Caldicellulosiruptor saccharolyticus* strain Tok7B.1 (Tok7B), *Caldicellulosiruptor* sp. strain Rt69B.1 (Rt69B), *Bacillus caldolyticus* EA1.3 (B.EA1), *Thermus* sp. Rt41A (Rt41A), *Dictyoglomus thermophilum* strain Rt46B.1 (Dth), thermophilic *Spirochaete*, *Thermoanaerobacter* strain AZ3B.1, and *Tepidomonas* sp. were kindly supplied by Professor Hugh Morgan, Thermophile Research Unit, Waikato University, Hamilton, New Zealand.

Enzymes

Taq DNA polymerase was from Invitrogen. r*Tth* was purchased from Applied Biosystems (Foster City, Calif.). *Thermotoga neapolitana* (*Tne*) DNA polymerase mutated to eliminate 3′ to 5′ and 5′ to 3′ exonuclease activity was cloned, engineered, and purified as described elsewhere (Yang et al. 2002). SuperScript II RT (SS II RT) was from Invitrogen.

RNA and DNA

Chloramphenicol acetyl transferase (CAT) cRNA [~900 nucleonucleotides (nt)] with an $(rA)_{40}$ 3′ tail was synthesized by T7 RNA polymerase run-off transcription from linearized plasmid DNA (D'Alessio and Gerard 1988). cDNA synthesis from CAT cRNA was primed with a DNA 24mer complementary to CAT cRNA that annealed between nt 679 and 692 with its 5′ end 146 nt distant from the first base at the 5′ end of the CAT cRNA $(rA)_{40}$ tail. $(rA)_{250}$ and $(dA)_{270}$ were from Amersham Biosciences (Piscataway, N.J.).

SDS-PAGE

Purified DNA polymerases were analyzed by SDS-PAGE. Approximately 1 μg purified protein was loaded onto a 4–20% Tris-glycine gel (Novex, Invitrogen). The gel was run according to the manufacturer's recommendation and was stained using Gelcode Blue (Pierce, Rockford, Ill.). The Benchmark Protein Ladder was used as a standard (Invitrogen).

Removal of DNA from commercial polymerase preparations

Commercial preparations of recombinant *Taq* polymerase were found to contain trace amounts of DNA encoding the *Taq* polymerase gene (Carroll et al. 1999). To digest and remove the contaminating DNA, 2.5 U of the restriction enzyme *Sau*3AI was added to each 50-μl PCR reaction, and the reaction was incubated at 37°C for 30 min. The mixture was then heated to 95°C for 2 min to denature the *Sau*3AI before adding approximately 1 ng genomic template DNA.

PCR and gene cloning

PCRs were performed using Platinum *Taq* (Invitrogen) or Platinum *Pfx* DNA polymerases (Invitrogen) according to the manufacturer's recommendations. All PCRs were performed using a GeneAmp 2400 (Applied Biosystems), using 30 to 35 cycles and 50 to 70°C annealing temperature unless stated otherwise. GW-PCR to obtain full-length gene sequences was carried out as previously described (Morris et al. 1995, 1998; Reeves et al. 2000). When required, PCR products were purified using a Concert gel extraction kit (Invitrogen). When using degenerate primers in the PCR, a step-down method was used where the annealing temperature was lowered from 60 to 45°C by 1°C per cycle, followed by 35 cycles with a 55°C annealing temperature.

Once the complete DNA sequence of each *polI* gene had been obtained, specific oligonucleotide primers were designed to amplify each full-length gene using high-fidelity *Pfx* DNA polymerase. Restriction sites were incorporated into each primer to allow directional in-frame ligation of PCR product into the expression vector pET26B (Novagen). Each pET26B construct was transformed into *Escherichia coli* DH5α cells with selection on LB agar plates containing 30 μg ml^{-1} kanamycin. Individual plasmids confirmed intact by DNA sequencing were subsequently transformed into the expression host BL21-SI (Invitrogen).

DNA sequencing, computer analysis, and GenBank accession numbers

Plasmids and PCR products were sequenced using Perkin Elmer Big Dye Terminator chemistry and run on a Perkin Elmer ABI Prism 377 DNA sequencer. Computer analysis of sequence data was carried out using the Genetics Computer Group Wisconsin (software) Package (Devereux et al. 1984). The sequence accession numbers for all polymerase genes presented here are listed in Table 1.

Subcloning of genes for Cst and Cth DNA polymerase

In order to improve expression and simplify purification of Cst and Cth DNA polymerase, the genes were subcloned downstream of a T7 promoter, and an amino-terminal His_6-tag sequence was introduced using Gateway cloning technology (Invitrogen). The sequence of the DNA oligonucleotide used at the 5′ end of the *Cst* gene was: 5′-GGGGACAACTTTG TACAA AAAAGTTGTCGATCC-AAAAATAATCCTTATAGAC 3′. The sequence of the DNA oligonucleotide used at the 5′ end of the *Cth* gene was: 5′GGGGA-CAACTTTGTACAAAAAAGTTGTCGCGAAATTTTTGATC-ATAGATGGT 3′. The sequence of the DNA oligonucleotide used at the 3′ end of each gene was the same: 5′-GGGGA-CAACTTTGTACAAGAAAGTTGCT CAGGAGGCTTCATA-CCAGTTTTT 3′. Purified pET26B plasmid DNA (Novagen) bearing the gene for Cst or Cth DNA polymerase was amplified by PCR utilizing the primers listed above and Platinum *Taq* HiFi DNA polymerase (Invitrogen). PCR products purified by agarose-gel electrophoresis were cloned into Gateway vector pDON21 and transferred by recombination into vector pDEST17. This resulted in the introduction of a His_6 tag at the amino terminus of the Cst and Cth DNA polymerases and the positioning of a T7 promoter upstream of the genes. Each final recombinant plasmid was transformed into the *E. coli* expression host BL21-AI (Invitrogen).

Subcloning of genes for Tok13B, Tok7B, and Rt69B DNA polymerase

Subcloning of the genes for Tok13B, Tok7B, and Rt69B DNA polymerases was carried out to remove the *pelB* leader sequence derived from pET26B. Each DNA polymerase gene was removed from pET26B by restriction digestion of the plasmid DNA with *Nco*I, which cut at the 5′ end of the gene, and *Bam*HI, which cut

Table 1 Microorganisms used in this study

Source organism	PolA length (amino acids)	%G + C content	CODEHOP[a] primers used to amplify internal sequence	Restriction sites used for cloning	DNA sequence accession no.
Thermoanaerobacter strain AZ3B.1	834[b]	31	PolATF-polATR	*Nco*I-*Bam*HI	AY247643
Dictyoglomus thermophilum strain Rt46B.1	856	33	PolATF-polATR	*Bam*HI-*Sal*I	AY247646
Caldicellulosiruptor saccharolyticus	849	33.3	PolATF-polATR	*Nco*I-*Bam*HI	AY247641
Caldicellulosiruptor saccharolyticus strain Tok7B.1	849	34.2	PolATF-polATR	*Nco*I-*Bam*HI	AY247639
Caldicellulosiruptor sp. strain Rt69B.1	849	34.4	PolATF-polATR	*Nco*I-*Bam*HI	AY247640
Caldicellulosiruptor sp. strain Tok13B.1	849	34.5	PolATF-polATR	*Nco*I-*Bam*HI	AY247638
Clostridium thermosulfurogenes (*Thermoanaerobacterium thermosulfurigenes*)	867	35	PolATF-polATR	*Nco*I-*Bam*HI	AY247642
Clostridium stercorarium	898	44	PolATF-polATR	*Nco*I-*Bam*HI	AY247644
Bacillus caldolyticus EA.1	878	46.5	PolGCF1-polGCR	*Nco*I-*Bam*HI	AY247636
Caldibacillus cellulovorans CompA.2	904	64	PolGCF1- polGCR	*Nco*I-*Bam*HI	AY247637
Thermophilic *Spirochaete*	898	65	PolGCF1- polGCR	*Eco*RI-*Sal*I	AY247648
Tepidomonas sp.	928	68	PolGCF1- polGCR	*Eco*RI-*Sal*I	AY247647
Thermus sp. Rt41A	833	68.3	PolGCF1- polGCR	*Eco*RI-*Sal*I	AY247645

[a]Consensus-degenerate hybrid oligonucleotide primers
[b]The complete sequence of this polymerase gene has not been obtained; approximately 150–200 bp of the 5′ end of the gene is missing

downstream of the translation stop codon at the 3′ end of the gene. The *Nco*I-*Bam*HI fragment was ligated into the *Nco*I and *Bam*HI sites of expression vector pET14B (Novagen). The recombinant plasmids were transformed into the *E. coli* expression host BL21-AI (Invitrogen).

Purification of CA2, B.EA1, Rt41A, Dth, Tok13B, Tok7B, and Rt69B DNA polymerase

E. coli cells (BL21SI, Invitrogen) bearing the plasmid pET26B with the gene for CA2, B.EA1, Rt41A, or Dth DNA polymerase were grown in 2.8-l Fernbach flasks in LB broth containing no salt and 50 μg ml^{-1} kanamycin at 37°C. After the culture reached an A_{590} of 1.2, expression of DNA polymerase was induced with 0.3 M NaCl for 3 h. Cells were harvested by centrifugation and stored at −70°C. *E. coli* cells (BL21AI) bearing the plasmid pET14B with the gene for Tok13B, Tok7B, or Rt69B DNA polymerase were grown in 2.8-l Fernbach flasks in LB broth containing 50 μg ml^{-1} ampicillin at 37°C. After the culture reached an A_{590} of 1.0, expression of DNA polymerase was induced by the addition of 0.2% arabinose for 3 h. Cells were harvested by centrifugation and stored at −70°C.

All purification steps were carried out at 4°C or on ice unless stated otherwise. Frozen cells (7 g) were thawed and suspended in sonication buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 8% (v/v) glycerol, 5 mM β-mercaptoethanol, and 50 μg ml^{-1} PMSF] at a 1:3 ratio (w/v) of buffer. The cell suspension was sonicated until greater than 70% of the total cells were lysed. A 10% (v/v) solution of NP-40 and Tween 20 was added to the sonicated sample to a final concentration of 0.05% of each. The sonicated sample was heated at 55 (CA2 and B.EA1 DNA polymerase), 60 (Tok13B, Tok7B, and Rt69B), or 75°C (Dth and Rt41A DNA polymerase) for 15 min, then cooled on ice for 30 min. NaCl (5 M) was added to a final concentration of 0.25 M, and polymin P was added to a final concentration of 0.2%. The sample was centrifuged at 20,000 *g* for 20 min to remove the precipitate. Solid ammonium sulfate was dissolved in the supernatant (0.326 gm ml^{-1}), and the suspension was stirred overnight. The insoluble protein was collected by centrifugation and resuspended in 5 ml low-salt buffer [25 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5 mM EDTA, 5% (v/v) glycerol, 2 mM β-mercaptoethanol, and 0.05% (v/v) each of NP-40 and Tween 20]. The sample was dialyzed against 200 ml low-salt buffer and centrifuged to remove insoluble material. The protein was fractionated by column chromatography on a 5-ml EMD sulfate column in low-salt buffer eluted with a linear gradient of 50 to 500 mM NaCl. The fractions containing DNA polymerase were determined by SDS-PAGE analysis and assay for DNA-directed DNA polymerase activity. These were pooled and dialyzed overnight against the low-salt buffer. The dialyzed protein was fractionated by column chromatography on a Mono-Q HR 5/5 column (Amersham Biosciences) run in low-salt buffer and eluted using a linear gradient of 50 to 250 mM NaCl. Fractions containing the thermostable DNA polymerase were pooled and dialyzed overnight against storage buffer [20 mM Tris-HCl (pH 8.0), 40 mM KCl, 0.1 mM EDTA, 50% (v/v) glycerol, 1 mM DTT, and 0.04% (v/v) each of NP-40 and Tween 20]. Purified DNA polymerase was stored at −20°C.

Purification of Cst-His and Cth-His DNA polymerase

E. coli cells (BL21AI) bearing the plasmid pDEST17 with the gene for Cst-His or Cth-His DNA polymerase were grown at 37°C in 2.8-l Fernbach flasks in LB broth containing 50 μg ml^{-1} ampicillin. After the culture reached an A_{590} of 1.0, expression of DNA polymerase was induced by the addition of 0.2% arabinose for 3 h. Cells were harvested by centrifugation and stored at −70°C.

All operations were at 4°C unless otherwise specified. Frozen cells (7 g) were thawed and suspended at a 1:2 ratio (w/v) in 50 mM Tris-HCl (pH 7.8), 10% (v/v) glycerol, and 2 mM $MgCl_2$. Cells were disrupted by sonication, and Benzonase (Merck, Darmstadt, Germany) was added at a ratio of 25 U per ml of slurry. After 30 min, NaCl was added to a final concentration of 1 M. The suspension was centrifuged at 13,000 *g* for 30 min. The crude extract was fractionated by column chromatography on a 5-ml HiTrap chelating column charged with Ni^{2+} and washed in Buffer N [25 mM Tris-HCl (pH 7.8), 1 M NaCl, 5 mM imidazole, and 10% (v/v) glycerol]. After loading the sample, the column was washed in Buffer N containing 20 mM imidazole and eluted with a linear gradient from 20 to 450 mM imidazole. Fractions were assayed for DNA-directed DNA polymerase activity, and the peak fractions were pooled. EDTA was added to the pooled fractions to a final

concentration of 1 mM, and the pool was dialyzed against Buffer H [25 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5 mM EDTA, 5% (v/v) glycerol, and 1 mM β-mercaptoethanol]. The dialyzed pool was fractionated on a 1- or 5-ml HiTrap Heparin column (Amersham Biosciences) equilibrated in Buffer H. After loading the sample, the column was washed with Buffer H and eluted with a linear gradient of 50 to 800 mM NaCl. The fractions were assayed for DNA polymerase activity, and the peak fractions were pooled. The pooled fractions were dialyzed against 20 mM Tris-HCl (pH 8.0), 40 mM KCl, 0.1 mM EDTA, 50% (v/v) glycerol, and 1 mM DTT. The final sample was stored at −20°C.

DNA polymerase activity assays

DNA-directed DNA polymerase unit activity

Reaction mixtures (50 µl) contained 25 mM N-Tris[hydroxymethyl]methyl-4-aminobutanesulfonic acid (TAPS, pH 9.3); 2.0 mM $MgCl_2$; 50 mM KCl; 1.0 mM DTT; 0.2 mM each of dATP, dTTP, dGTP, and [α-^{32}P]dCTP (250 cpm pmole^{-1}); 500 µg ml^{-1} activated salmon testes DNA; and 2 to 20 pg (0.02 to 0.2 fmol) DNA polymerase. After incubation at 55 or 72°C for 10 min, the reaction was terminated by addition of 10 µl 0.5 M EDTA. Incorporation of radioactivity into acid-insoluble DNA product was determined. One unit of DNA-directed DNA polymerase activity is the amount of enzyme required to incorporate 10 nmol dNTPs into acid insoluble product in 30 min.

RNA-directed DNA polymerase unit activity

Reaction mixtures (25 µl) contained 10 mM Tris-HCl (pH 8.3); 25 mM KCl; 5 mM $MgCl_2$; 0.5 mM each of dATP, dTTP, dGTP, and [α-^{32}P]dCTP (200 cpm pmol^{-1}); 1 µg (3.2 pmol) chloramphenicol acetyl transferase (CAT) cRNA; and 0.6 µg (80 pmol) DNA 24mer primer. The range of the amount of DNA polymerase used in the assay varied. For CA2, Cst-His and B.EA1 DNA polymerases, 0.25 to 4 DNA-directed DNA polymerase units were used and the reaction was incubated at 55°C for 5 min. For Cth-His DNA polymerase, 5 to 40 DNA-directed DNA polymerase units were incubated at 55°C for 5 min. In the case of Tok13B, Tok7B, Rt69B, Dth, and R41A DNA polymerases, the range was 5 to 40 DNA-directed DNA polymerase units incubated at 72°C for 5 min. The reaction was terminated by addition of 5 µl 0.5 M EDTA. Incorporation of radioactivity into acid-insoluble DNA products was determined. One unit of RNA-directed DNA polymerase activity is the amount of enzyme required to incorporate 10 nmol dNTPs into acid insoluble product in 30 min.

RT functional activity

Reaction mixtures (20 µl) contained 10 mM Tris-HCl (pH 8.3); 25 mM KCl; 5 mM $MgCl_2$; 0.5 mM each of dATP, dTTP, dGTP, and [α-^{32}P]dCTP (200 cpmpmol^{-1}); 1 µg CAT cRNA; and 0.6 µg DNA 24mer primer. The reaction was set up in the presence and absence of 1.5 M betaine. The amount of DNA polymerase activity (DNA-directed DNA polymerase units) added to the reaction was: 1 U (0.05 µg) CA2; 5 U (0.23 µg) Cst–His; and 20 U (1.25 µg) Cth–His or 10 U B.EA1 (0.17 µg), Tok13B (0.2 µg), Tok7B (0.15 µg), Rt69B (0.4 µg), Dth (0,2 µg), Rt41A (0.1 µg), *Tne* (0.15 µg), r *Tth* (0.15 µg), or *Taq* (0.15 µg) DNA polymerase. SS II RT (200 U) was incubated as a control at 42°C, and the other enzymes were incubated at 60°C for 30 min. A portion of the reaction mixture was precipitated with TCA to determine total yield of cDNA synthesized, and the remaining cDNA product was size fractionated on an alkaline 2% agarose gel (McDonell et al. 1977). The gel was dried and exposed to X-ray film.

Thermal inactivation profiles of DNA polymerases

Purified DNA polymerases were analyzed for thermostability at temperatures between 55 and 95°C. An incubation mixture (50 µl) containing 10 mM Tris-HCl (pH 8.3), 25 mM KCl, 5 mM $MgCl_2$, and 2.5 U DNA-directed DNA polymerase activity was incubated at various temperatures for 10 min. The tubes were placed on ice and 5 µl of the sample was tested for residual DNA polymerase activity, using the DNA-directed DNA polymerase unit activity assay. After incubation at 55°C (DNA polymerases CA2, Cst-His, B.EA1, and Cth-His) or 72°C (DNA polymerases Tok13B, Tok7B, Rt69B, Dth, and Rt41A) for 10 min, the reaction was terminated by addition of 5 µl 0.5 M EDTA. Incorporation of radioactivity into acid-insoluble DNA products was determined.

Steady-state kinetic measurements

The steady-state kinetic parameters $K_{M(dTTP)}$ (dissociation constant for dTTP concentration) and k_{cat} (first-order rate constant) were determined as described (Polesky et al. 1990) using $(rA)_{250}$·$(dT)_{30}$ or $(rA)_{250}$·$(dT)_{40}$ and $(dA)_{270}$·$(dT)_{40}$. A range of four to five [^{32}P]dTTP concentrations, which bracketed the $K_{M(dTTP)}$ value, was used for $K_{M(dTTP)}$ determinations. Reaction mixtures (50 µl) contained 10 mM Tris-HCl (pH 8.3), 25 mM KCl, 5 mM $MgCl_2$, 100 to 1,000 µM [α-^{32}P]dTTP, 1 µM $(rA)_{250}$ or $(dA)_{270}$, 3 µM $(dT)_{30}$ or $(dT)_{40}$, and 5 to 50 nM DNA polymerase. In some cases, k_{cat} was determined with $(dC)_n$·$(dG)_{35}$ (Astatke et al. 1995) in reaction mixtures (50 µl) containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 100 to 200 µM [α-^{32}P]dGTP, and 5 nM DNA polymerase.

Results and discussion

Cloning of DNA polymerase genes

All gene constructions were based on PCR amplification rather than the cloning of wild-type genes that may or may not have been revealed in expression libraries. Each gene sequence was compiled by direct sequencing of GW-PCR products to ensure that PCR-derived mutations were not included in the gene sequence. Final expression plasmid constructions were sequenced and compared to the GW-PCR-derived sequence to ensure each gene was free of PCR errors. Careful design of degenerate primers for the initial PCR of the consensus *polI* sequence allowed the amplification and sequencing of an internal gene fragment, which allowed the design of gene-specific primers suitable for genomic walking in the 5′ and 3′ directions so that the entire *polI* gene could be isolated from a variety of bacteria with widely differing %G + C contents, but it was necessary to design a suite of primers to achieve successful amplifications. The high conservation of the motifs against which the degenerate primers were designed means that theoretically, these primers should amplify the *polI* genes from the majority of bacteria across all bacterial divisions. The degenerate PCR method was so sensitive that initial difficulties were encountered due to the presence of trace amounts of the *Taq* polymerase gene in commercial enzyme preparations. We found it was necessary to pretreat the *Taq* enzyme with a temperature-sensitive restriction enzyme to remove the contaminating *Taq polI* DNA. A significant advantage of this method over

isolation of *polI* genes from genomic expression libraries is that no demands are made for expression in the host *Escherichia coli*, which may cause weakly-expressed polA enzymes to be overlooked. Accordingly, the genes can be translated in appropriate expression vectors under optimal conditions for the production of the particular enzyme.

Degenerate oligonucleotide design

The amino acid sequences from 24 bacterial polI DNA polymerases were aligned, and two highly conserved regions were identified within the 5′–3′ DNA polymerase domain of all enzymes (Fig. 1) Consensus-degenerate hybrid oligonucleotide primers (CODEHOP, Rose et al. 1998) were designed to hybridize to DNA coding for the conserved regions. Three forward and three reverse primers were designed to amplify a PCR product of approximately 570 bp (see Fig. 1.) The polGCF1/F2 and polGCR primers were found to work best with organisms with a high %G + C content. The polGCF1 and polGCF2 primers are identical apart from the sequence encoding the serine codon positioned within the motif. The primers polATF and R were based upon the sequences of the polGCF1/F2 and polGCR primers but with a lower %G + C within the 5′ nondegenerate end of each primer. Decreasing the %G + C content of the nondegenerate ends was found to improve the correct amplification of *polI* genes from organisms with a low %G + C content.

The degenerate primers described in Fig. 1 were used to amplify internal portions of *polI* genes from the following bacteria: *Caldicellulosiruptor saccharolyticus*; *Caldicellulosiruptor saccharolyticus* strains Tok7B.1, Rt69B.3, and Tok13B.1; *Thermus filiformis* strain Rt41A1; *Dictyoglomus thermophilum* strain Rt46B.1; *Clostridium stercorarium*; *Clostridium* (*Thermoanaerobacterium*) *thermosulfurogenes*; *Thermoanaerobacter* sp. AZ3B.1; *Bacillus caldolyticus* strain EA1; and *Caldibacillus cellulovorans*. The degenerate primer combination that amplified the internal portion of each polymerase gene is shown in Table 1. In terms of correct amplification of the internal polymerase gene region, there was a direct correlation between the %G + C content of template genomic DNA and the %G + C content of the nondegenerate 5′ portion of the CODEHOP primers. The polATF/R primer combinations were required for correct amplification of *polI* from low %G + C genomic DNA, while the polGCF1/F2/R primers worked most efficiently with high %G + C genomic DNA.

Purification of cloned DNA polymerases

Subcloning of the genes for Cst and Cth DNA polymerases and purification of the enzymes

The level of expression of the cloned DNA polymerase in each of nine *E. coli* strains bearing a thermostable DNA polymerase gene was determined. The expression

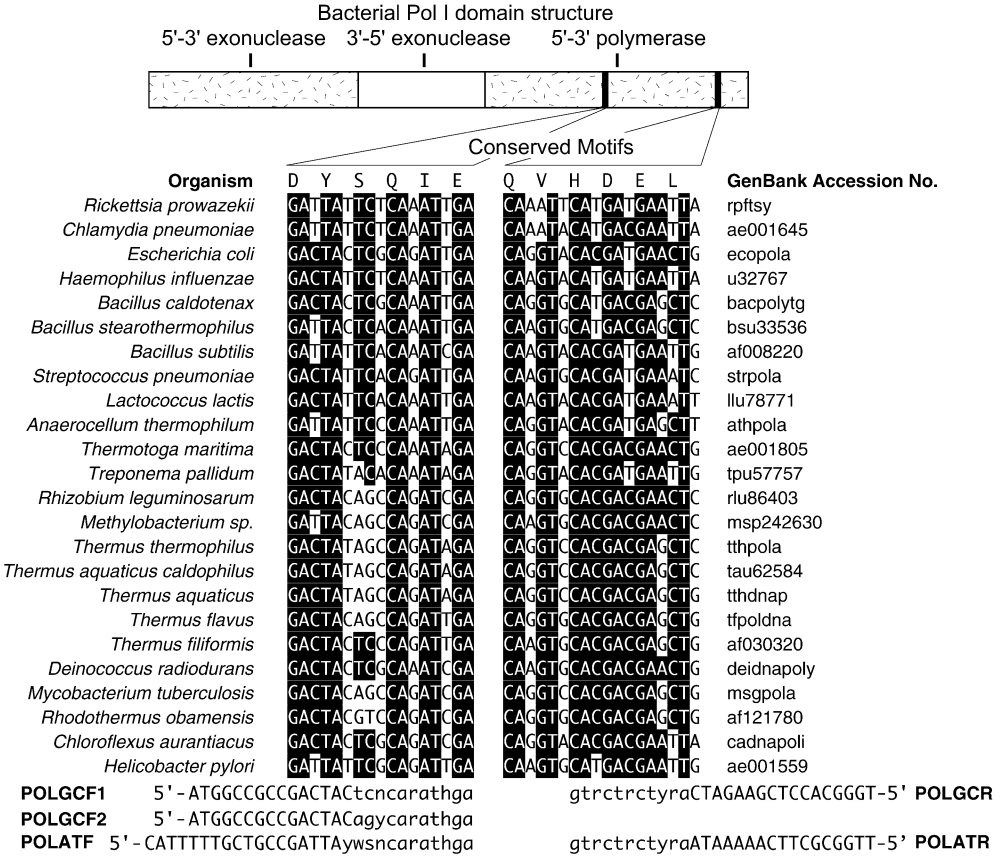

Fig. 1 An alignment of known bacterial DNA *polI* gene sequences at the position of the two highly conserved amino acid motifs. Degenerate oligonucleotides designed to amplify the equivalent region from other bacterial polymerases are shown *beneath the alignment*

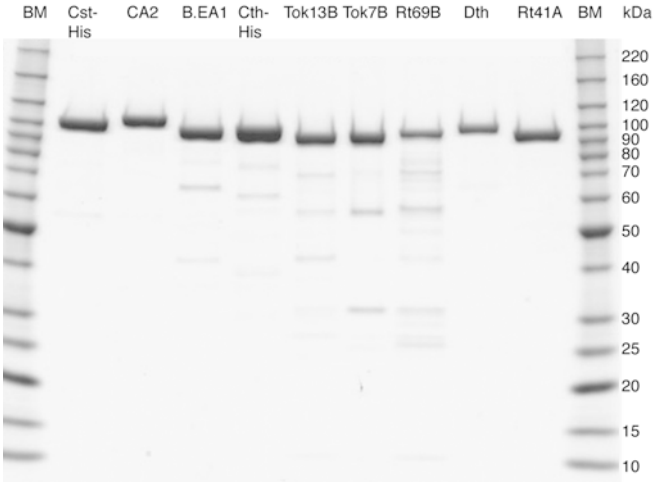

Fig. 2 SDS-PAGE analysis of the purified DNA polymerases. Approximately 1 μg of each purified DNA polymerase was subjected to electrophoresis on a 4–20% Tris-glycine gel and stained using Gel-code Blue (see Materials and methods). The Benchmark Protein Ladder (*BM*) was run as a standard on the left and the right sides of the samples and the molecular weight (kDa) of each band is labeled on the *right side* of the figure

level in each strain was compared before and after induction by SDS-PAGE analysis of cell extracts. In some cases a distinct new protein species migrating at the position expected for a cloned thermostable DNA polymerase (95 to 105 kDa) was observed in induced cells. In strains bearing the Cst or Cth DNA polymerase gene, little apparent induced gene product was observed, and subsequent efforts to purify the thermostable DNA polymerase in quantities adequate for biochemical characterization were not successful. To aid in purification of Cst and Cth DNA polymerases, a His_6-tag fusion was added to the amino end of each gene product. Addition of the His_6 affinity tag expedited purification. A combination of Benzonase nuclease treatment of *E. coli* crude extracts to reduce viscosity, Ni^{2+}-affinity chromatography, and heparin-sepharose chromatography produced cloned Cst–His and Cth–His DNA polymerases that were ~90 and ~80% homogeneous, respectively (Fig. 2).

Subcloning of the genes for Tok13B, Tok7B, and Rt69B DNA polymerases and purification of the all remaining polymerases

Attempts to purify cloned Tok13B, Tok7B, and Rt69B DNA polymerases by classical nonaffinity methods produced only truncated polypeptides of small size, rather than the ~97-kDa-sized polypeptide expected. We speculated this was caused by the presence of the *pelB* leader at the amino end of the gene product introduced during cloning of the gene. The genes for these polymerases were subcloned, removing the leader. This step resulted in reduced levels of breakdown of these polymerase gene products. Tok13B, Tok7B, and Rt69B DNA polymerase, as well as CA2, B.EA1, Dth, and Rt41A DNA polymerase, were purified by a similar procedure. After heating an *E. coli* cell extract to the highest temperature at which a particular cloned DNA polymerase remained stable (Materials and methods; Table 2), polymin P was added to precipitate nucleic acids, and particulate material was removed by centrifugation. The DNA polymerase was precipitated with ammonium sulfate (55% of saturation). Sequential ion-exchange chromatography on Fractogel EMD sulfate (Merck) followed by Mono-Q was then carried out on each enzyme. This procedure was used to produce CA2, Dth, and Rt41A DNA polymerases that were ~90% homogeneous, and B.EA1 polymerase that was ~80% homogeneous (Fig. 2). The purified preparations of cloned Tok13B, Tok7B, and Rt69B DNA polymerases were all ~60 to 70% homogeneous, containing, in addition to the expected band at ~97 kDa, a series of smaller bands (Fig. 2). We speculate that at least some of these smaller protein species were derived from a proteolytically clipped ~97-kDa polymerase that copurified with intact polymerase.

Thermal stability

The intrinsic thermal stability of each cloned, purified DNA polymerase was determined by assaying the amount of DNA-directed DNA polymerase activity remaining after heating the enzyme for 10 min at

Table 2 Thermal inactivation profiles of purified DNA polymerases. (The results of a single experiment are shown; similar results were obtained in at least two other experiments)

[a]DNA polymerases were heated, and activity was determined using the DNA-directed DNA polymerase unit activity assay as described in Materials and methods. A reference sample of each DNA polymerase was kept on wet ice and assayed to establish 100% activity

[b]Optimum growth temperature of the parent organism

Enzyme	Percent activity remaining after heating for 10 min at the temperatures (°C) below[a]									
	Growth temperature[b] (C°)	55	60	65	70	75	80	85	90	95
Cth-His	60	90	60	1	0	-	-	-	-	-
CA2	65	109	101	47	0	-	-	-	-	-
Cst-His	60	95	94	81	0	-	-	-	-	-
B.EA1	70	94	94	65	7	0	-	-	-	-
Tok13B	65	-	-	100	65	33	8	0	-	-
Tok7B	65	-	-	-	105	87	12	0	-	-
Rt69B	70	-	-	100	84	69	37	0	-	-
Dth	73	-	-	-	-	100	93	88	21	1
Rt41A	75	-	-	-	-	100	87	92	87	12

temperatures ranging from 55 to 95°C (Table 2). The DNA polymerases varied widely in resistance to heat inactivation and could be grouped into six categories: active up to 60°C (Cth-His), active up to 65°C (CA2, Cst-His, and B.EA1), active up to 75°C (Tok13B and Tok7B), active up to 80°C (Rt69B), active up to 85°C (Dth), and active up to 90°C (Rt41A).

RNA-directed DNA polymerase activity

To date, conditions have not been described under which known thermophile-derived polI-type DNA polymerases copy RNA efficiently in the presence of Mg^{2+} (Jones and Foulkes 1989; Myers and Gelfand 1991; Yang et al. 2002). Three criteria were used to judge the efficiency with which the nine thermophilic DNA polymerases reported here utilize RNA as a template in the presence of Mg^{2+}. First, the specific activity of each enzyme with a heteropolymeric RNA template was determined and compared with the specific activity determined with a DNA–DNA template primer. Second, the steady-state kinetic parameters, K_M and k_{cat}, of RNA-directed DNA synthesis from an RNA homopolymer template were established and used to calculate the efficiency, k_{cat}/K_M (second-order rate constant), of synthesis. The k_{cat} of DNA-directed DNA synthesis was determined with a homopolymer DNA template for each enzyme as reference. Third, the ability of each enzyme to synthesize a full-length copy of CAT cRNA (~680 nt) was established. Several other DNA polymerases were characterized as references. *Taq* DNA polymerase has minimal activity with an RNA template (Myers and Gelfand 1991); r *Tth* DNA polymerase (Myers and Gelfand 1991) and *Tne* DNA polymerase (Yang et al. 2002) are known to copy RNA in the presence of Mn^{2+}; retroviral SS II RT copies RNA efficiently in the presence of Mg^{2+}.

Table 3 shows the specific enzymatic activity of each of the nine thermophile-derived DNA polymerases as well as *Taq*, r*Tth*, and *Tne* DNA polymerase, with CAT cRNA or activated DNA as template in the presence of Mg^{2+}. Based upon the ratio of specific activity with RNA versus DNA as template, the enzymes can be placed in three categories. Rt41A and Dth DNA polymerases had little or no detectable activity with an RNA template, even though the specific activity of each enzyme was relatively high with a DNA template primer. As expected, *Taq* DNA polymerase fell in this category. Tok7B, Cth-His, Rt69B, and Tok13B DNA polymerases had detectable activity with an RNA template, but the specific activity with RNA was <1% of the specific activity with a DNA template. Again as expected, *Tne* and r*Tth* DNA polymerases had low, but detectable, activity with an RNA template. B.EA1, Cst–His, and CA2 DNA polymerases had substantially more activity with an RNA template; the specific activity with RNA was 10 to 25% of that with a DNA template.

Table 3 DNA polymerase specific activities of purified DNA polymerases with DNA–DNA and RNA–DNA template primers. (The results of a single experiment are shown; similar results were obtained in at least one other experiment)

Enzyme	Temperature[a] (°C)	Specific activity (U mg^{-1})		
		DNA–DNA[b]	RNA–DNA[c]	Ratio (RNA/DNA)
Taq	72	80,000	<1	-
Rt41A	72	84,500	<1	-
Dth	72	37,800	<1	-
r *Tth*	72	150,000[d]	130	0.001
Tok7B	72	33,800	60	0.002
Cth-His	55	12,700	30	0.002
Rt69B	72	16,800	50	0.003
Tok13B	72	34,800	160	0.005
Tne	72	31,300	325	0.01
B.EA1	55	45,800	4,400	0.10
Cst-His	55	19,500	2,100	0.11
CA2	55	20,000	4,900	0.25

[a]Assays were carried out at optimal temperatures
[b]Activity with DNA-DNA was determined with activated salmon testes DNA (Materials and methods)
[c]Activity with RNA-DNA was determined with CAT cRNA·DNA 20-mer (Materials and methods)
[d]Taken from Reeves et al. (2000)

Table 4 Catalytic constants of purified DNA polymerases with DNA–DNA and RNA–DNA template-primer (mean ± standard deviation of two to four determinations is shown). k_{cat} First-order rate, K_M dissociation constant, k_{cat}/K_M second-order rate

Enzyme	Temperature[a] (°C)	k_{cat} (s^{-1})		K_M (μ M)	k_{cat}/K_M
		DNA–DNA[b]	RNA–DNA[c]	RNA–DNA[c]	RNA–DNA[c]
Rt41A	72	187 ± 7	<1	-	-
Dth	72	39 ± 15	<1	-	-
Tne	72	130 ± 32	0.2 ± 0.04	-	-
Rt69B	72	20 ± 5	0.6 ± 0.36	-	-
Tok13B	72	43 ± 9	1.2 ± 0.5	-	-
Cth-His	55	28 ± 1	16 ± 1	1,200 ± 260	0.01
B.EA1	55	73 ± 16	43 ± 7	350 ± 110	0.12
CA2	55	82 ± 9	48 ± 9	270 ± 50	0.18
Cst-His	55	40 ± 13	88 ± 5	67 ± 5	1.3
SS II RT	37	16 ± 2	45 ± 18	17	2.6

[a]Assays were carried out at optimal temperatures
[b]Catalytic constants were determined with $(dA)_{270}\cdot(dT)_{40}$ with the exception that for *Tne* DNA polymerase and SS II RT $(dC)_n\cdot(dG)_{35}$ was used (see Materials and methods)
[c]Catalytic constants were determined with $(rA)_{250}\cdot(dT)_{30}$ at 37 and 55°C or $(rA)\cdot(dT)_{40}$ at 72°C (see Materials and methods)

The k_{cat} values of DNA-directed DNA synthesis for eight of the nine DNA polymerases (Tok7B was not assayed) were determined (Table 4). They varied between 20 and 187 s^{-1}. The k_{cat} values of RNA-directed DNA synthesis varied over a much wider range from not measurable to values comparable with the DNA-directed DNA synthesis k_{cat} values (Table 4). There was no apparent correlation between k_{cat} for DNA-directed and RNA-directed DNA synthesis. With the exception of the Cth-His DNA polymerase (see

below), there was a good correlation between the k_{cat} values. In contrast, there was a good correlation between the k_{cat} of RNA-directed DNA synthesis and the hierarchy of three categories of RNA-directed DNA synthesis specific activities observed in Table 3. For Rt41A and Dth DNA polymerase, the k_{cat} with homopolymeric $(rA)_{250}$ template in the presence of Mg^{2+} could not be measured. Rt69B, Tok13B, and *Tne* DNA polymerases each had a measurable k_{cat} with $(rA)_{250}$, but the values were relatively small. The k_{cat} values determined for the remaining four DNA polymerases—Cth-His, B.EA1, CA2, and Cst-His—were comparable to that of retroviral RT. However when the efficiency (k_{cat}/K_M) of RNA-directed DNA synthesis for these enzymes was compared, they could be further differentiated. The $K_{M(dTTP)}$ of Cth-His DNA polymerase was extremely low, making the efficient synthesis of DNA from RNA at the concentration of dNTPs employed (500 μM) unlikely. The efficiencies of B.EA1 and CA2 were an order of magnitude higher. In the case of Cst-His DNA polymerase, the efficiency with RNA as a template was another log higher and was similar to that of retroviral RT.

The initial rate and steady-state kinetic measurements in Tables 3 and 4 indicate that B.EA1, CA2, and Cst–His DNA polymerases could be used to synthesize long cDNA from mRNA at elevated temperatures in the presence of Mg^{2+}. They also clearly indicate that Rt41A, Dth, and *Taq* DNA polymerases cannot be used. For the remaining DNA polymerases, predictive interpretation of the data is more difficult. In addition, other DNA polymerase properties such as binding affinity for RNA-DNA and processivity play a significant role in determining the ability of a DNA polymerase to copy long stretches of RNA. Therefore, we examined the ability of the DNA polymerases to synthesize a full-length copy of CAT cRNA (679 nt) in the presence of Mg^{2+} at 60°C. This temperature was selected to minimize chemical breakdown of RNA that occurs in the presence of excess Mg^{2+} at a rapid rate above 60°C (Gerard et al. 2002). Synthesis was carried out in the presence and absence of 1.5 M betaine. Betaine has been shown to reduce pausing by DNA polymerases during DNA-directed DNA synthesis caused by particular types of DNA sequences (Mytelka and Chamberlin 1996) or by secondary structure in DNA (McDowell et al. 1998). Figure 3 shows that in the absence of betaine Cst-His, CA2, and B.EA1 DNA polymerases did indeed synthesize full-length CAT cDNA, while Cth-His DNA polymerase did not. Surprisingly, Tok13B DNA polymerase (and to lesser extent Rt69B) also synthesized full-length CAT cDNA. As predicted Dth, Rt41A, and *Taq* DNA polymerase synthesized little detectable product under any conditions tested. With the exception of Cth–His DNA polymerase, betaine enhanced the ability of a particular DNA polymerase to synthesize longer cDNA product. This was especially apparent for Tok7B, Rt69B, r *Tth*, and *Tne* DNA polymerases.

Comparison of the thermal stabilities in Table 2 and the RNA-directed DNA synthesis efficiencies derived from the data in Tables 3 and 4 and Fig. 3 indicate there is an inverse relationship between thermal stability and reverse-transcription efficiency for thermal stable polI-related DNA polymerases. We tested the ability of the thermostable DNA polymerases newly cloned in this study to carry out PCR amplification (data not shown). Only Rt41A DNA polymerase, the most thermal stable of the nine enzymes studied, was stable enough to catalyze PCR. Cst–His and CA2 DNA polymerases in combination with *Taq* DNA polymerase were used to carry out RT-PCR amplification of a 600-bp amplicon of CAT cRNA (data not shown).

Conclusions

Sampling of a cross-section of thermophile-derived polI-like DNA polymerases resulted in the identification of four new enzymes with good Mg^{2+}-dependent RT activity: Cst-His, B.EA1, CA2 DNA, and Tok13B DNA polymerases. Based upon measurement of synthesis of full-length 600-nt cDNA, the addition of betaine to reaction mixtures dramatically improved the ability of

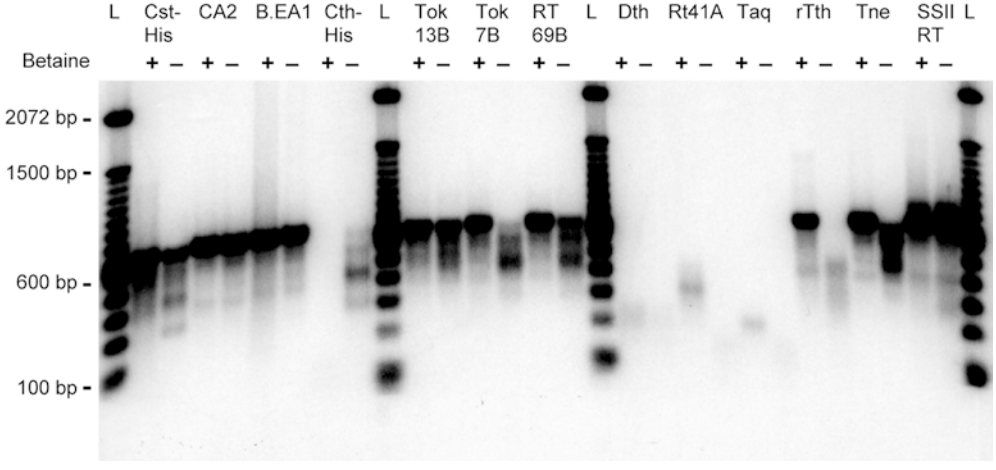

Fig. 3 Alkaline-agarose gel analysis of first-strand cDNA synthesized from CAT cRNA by purified thermostable DNA polymerases. CAT cRNA was reverse transcribed using a 24-bp gene-specific DNA primer in the presence (+) and absence (–) of betaine. The cDNA products were subjected to electrophoresis on an alkaline 2% agarose gel. A 100-bp DNA ladder (*L*) was used as a standard

almost all enzymes tested to synthesize longer cDNA products, including the well-characterized enzymes, r *Tth* DNA polymerase (Meyers and Gelfand 1991) and *Tne* DNA polymerase (Yang et al. 2002). This result suggests that structural features of an RNA–DNA template primer altered by betaine interfere with the ability of thermophile-derived DNA polymerases to remain bound to the template-primer complex during reverse transcription. The inverse relationship between thermal stability and RT activity of thermophile-derived DNA polymerases suggests that those enzymes that are required to function at higher temperatures in nature are less able to accommodate an RNA–DNA hybrid structure at their active site. The least thermal stable of the DNA polymerases studied, Cth-His DNA polymerase, was inhibited rather than stimulated by betaine. However Cth-His DNA polymerase had an extremely high $K_{M(dTTP)}$, and the dNTP concentrations used in cDNA synthesis probably severely limited the enzyme's reverse-transcription efficiency and perhaps also masking the effect of betaine.

Using an alignment of the DNA polymerases described in this work, we have looked for any correlations between conserved residues in the three polymerases showing outstanding RT activity (as a ratio of DNA polymerase/RT-specific activity; *Bacillus* EA1, *C. stercorarium* and *Caldibacillus* CompA2) and excluded any residues also conserved in their closest relative *C. thermocellum* that has minimal RT activity. There are six residues unique to the BEA1, Cst, and CA2 polymerases (data not shown). We have used the known structure of *Taq* polymerase (Li et al. 1999) as a template for the modeling of the differences in primary structure between DNA polymerases showing RT activity and those that do not. None of the six unique residues appear to be in contact with the template strand, and it is unclear if these residues contribute to RT ability. We are currently continuing investigations into which residues might be involved in conferring RT activity to bacterial DNA polymerases.

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