# Creation of genetic information by DNA polymerase of the archaeon *Thermococcus litoralis*: influences of temperature and ionic strength

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Received June 25, 1998; Revised and Accepted September 7, 1998

DDBJ/EMBL/GenBank accession nos Y17520-Y17551

#### ABSTRACT

DNA polymerase of the archaeon Thermococcus litoralis can synthesize a long stretch of linear doublestranded DNA in the complete absence of added primer and template DNAs. This finding suggests that genetic information can potentially be created by protein. We report here the effects of temperature, ionic strength and pH on this ab initio DNA synthesis by the protein in vitro. When the temperature of the reaction was changed, the sequence of the product DNA changed markedly. For instance, the reaction products were (TAAT)<sub>n</sub> at 69°C, (TATCCGGA)<sub>n</sub> at 84°C and (TATCGCGATAGCGATCGC)<sub>n</sub> at 89°C. The ionic strength of the reaction condition also affected the sequence: it was  $(TATCTAGA)_n$  with 0 mM KCl,  $(TATATACG)_n$  with 50 mM KCl and  $(TATAGTTATAAC)_n$ with 100 mM KCl at 74°C. When the pH of the reaction condition was changed from 6.8 to 10.8, the size of the product DNA decreased, but its sequence did not. These results demonstrate that DNA synthesized ab initio by DNA polymerase of T.litoralis is markedly influenced by the reaction conditions. The results also suggest that genetic information that might have been created by protein on the early earth is strongly influenced by environmental factors.

### INTRODUCTION

Genetic information, whether small or large, is encoded in an RNA or a DNA strand as a defined sequence of mostly four kinds of nucleotide bases (1). One region of this sequence is translated *en bloc* into mRNA and further into a protein. Other regions are believed to regulate transcription or other functions, such as control of chromosomal conformation, control of meiosis, etc. in a cell (2–4). The genetic information on the DNA strand is copied in a complementary way to another new DNA strand by a protein enzyme DNA polymerase (5,6). DNA polymerases in highly developed organisms are extremely accurate in their copying ability. This means that novel genetic information is made on rare occasions, such as enzyme error during replication of a genome

(5), recombination of chromosomes or DNA damage by various environmental factors (7,8).

Ogata and Miura (9) reported that DNA polymerase of Thermococcus litoralis (Tli), a hyperthermophilic archaeon (archaebacterium) discovered in a submarine thermal vent in the Bay of Naples (10), can synthesize long double-stranded linear DNA of complex sequences at 74°C in the complete absence of added primer and template DNAs in vitro, demonstrating that genetic information can potentially be created by protein. They substantiated the independence of this DNA synthesis of the pre-existing primer and template DNAs by vigorous exclusion of the possibility of contamination of DNA or RNA in the reaction mixture (9). The DNAs thus created by the protein had various tandem repetitive sequences, such as  $(CTAGATAT)_n$  and  $(TAGATATCTATC)_n$  (9). The crucial importance of this finding is that it suggests that some or a major part of primordial genetic information was created on the early earth by DNA polymerase. If this is really the case, one might well ask what environmental factors may have influenced such ab initio DNA synthesis by this protein. To clarify the environmental factors that may affect this primer-template-independent DNA synthesis by Tli DNA polymerase, we carried out the reaction without added primer and template DNAs as before (9) but changed temperature, ionic strength and pH, and analyzed the reaction products. This paper reports the results of the influence of such environmental factors on this DNA synthesis and on the sequence of the reaction products. The implication of the results in terms of evolution of genetic information is also discussed. In the accompanying paper (11), similar ab initio DNA synthesis by the thermophilic bacterium Thermus thermophilus is also reported.

### MATERIALS AND METHODS

#### **DNA** accession numbers

The EMBL DNA accession numbers of the sequences of the 32 clones in this paper are Y17520–Y17551.

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Figure 1. DNA synthesis without added primer and template DNAs by *Tli* DNA polymerase at various reaction temperatures. (A) A standard reaction mixture was incubated in a buffer of pH 8.8 and with 10 mM KCl at indicated temperatures for 3 h as described in the text. Reaction products were electrophoresed on an agarose gel and stained with ethidium bromide. DNA size markers are shown on the left in kb. (B) The gel in (A) is scanned by a fluorescence densitometer. The size of the DNA at maximal fluorescence intensity is plotted against the reaction temperature (inset).

# *Tli* DNA polymerase reaction without primer and template DNAs

A standard reaction mixture contained 0.4 U of T.litoralis DNA polymerase (12,13) (recombinant and expressed in Escherichia coli, New England Biolabs, >98% pure as determined by SDS-PAGE) and 200 µM each of dATP, dTTP, dGTP and dCTP (Pharmacia, >99% pure as determined by Mono Q ion-exchange chromatography) in 20 µl of polymerase buffer containing 10 mM (unless otherwise specified) KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 6 mM MgSO<sub>4</sub>, 20 mM Tris-HCl (pH 8.8 at 25°C and pH 7.3 at 74°C) (or another buffer where specified) and 0.1% Triton X-100. The mixture was incubated for 3 h at 74°C or at another temperature where specified. In some experiments, which were designed to examine the effect of pH on this reaction, the Tris-HCl buffer (pH 8.8) in the above reaction mixture was replaced by 20 mM MOPS-NaOH (pH 6.8), Tris-HCl (pH 7.8), CAPS-NaOH (pH 9.8) or CAPS-NaOH (pH 10.8). The products of the above reactions were recovered by phenol treatment, ethanol precipitation (14) and dissolved in 2 µl of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. It was next electrophoresed on a 0.8% agarose gel, and then stained with 0.5 µg/ml ethidium bromide solution (14). The stained gel was photographed and scanned by a fluorescence densitometer under ultraviolet illumination (14).

# Molecular cloning of *Tli* DNA polymerase reaction products

The reaction products of Tli DNA polymerase were cloned as described (9). Clones containing an insert DNA were sequenced by a dideoxy chain-termination method (15) for both strands. Homology searches of the insert DNA sequences were carried out by FASTA (16) and BLAST (17).

## RESULTS

When *Tli* DNA polymerase was incubated without added primer and template DNAs at various temperatures ranging from 64 to



**Figure 2.** DNA synthesis without added primer and template DNAs by *Tli* DNA polymerase at various pHs. (**A**) A standard reaction mixture with 10 mM KCl and in a 20 mM buffer of various pHs indicated was incubated at  $74^{\circ}$ C for 3 h. The reaction products were electrophoresed on an agarose gel and stained with ethidium bromide. DNA size markers are shown on the left in kb. (**B**) The gel in (A) is scanned by a fluorescence densitometer. The size of the DNA at maximal fluorescence intensity is plotted against the reaction pH (inset).



Figure 3. DNA synthesis without added primer and template DNAs by *Tli* DNA polymerase under various ionic strengths. (A) DNA was synthesized in the standard reaction mixture at  $74^{\circ}$ C for 3 h in a buffer of pH 8.8 and with various concentrations of KCl as indicated. The reaction products were electrophoresed on an agarose gel and stained with ethidium bromide. DNA size markers are shown on the left in kb. (B) The gel in (A) was scanned by a fluorescence densitometer. The size of the DNA at maximal fluorescence intensity is plotted against the KCl concentration in the reaction mixture (inset).

94°C in a standard reaction mixture (pH 8.8, 10 mM KCl), DNAs of various sizes (0.5–100 kb) were produced (Fig. 1A), partially confirming previous results (9). The identity of the reaction product as double-stranded linear DNA was extensively confirmed as described by Ogata and Miura (9). The amounts as well as the sizes of the DNA product (hereafter called 'pol product') varied greatly depending upon the reaction temperature. The pol product was maximally synthesized at 74–84°C (Fig. 1B).

When the pol product was synthesized as above with fixed KCl concentration (10 mM) and at fixed temperature (74°C) but at various pHs by changing the reaction buffer from pH 6.8 to pH 10.8, it was maximally synthesized at pH 8.8, and the sizes of DNA became smaller upon the increase of the reaction pH (Fig. 2A).

69°C     pTL692     AAT TAAT TAAT TAAT TAAT TAAT TAAT T(24)       pTL694     AAT TAAT TAAT TAAT TAAT TAAT (19)       74°C     pTL45     A TATCTAGA TATCTAGA TATCTAGA TATCTAGA TATCTAGA TA(43)       pTL88     A TATCTAGA	(TAAT)n (TAAT)n (TATCTAGA)n (TATCTAGA)n (TATAGC)n (TATAGC)n (TATCCGGA)n		
pTL694     AAT TAAT TAAT TAAT TAAT (19)       74°C     pTL45     A TATCTAGA TATCTAGA TATCTAGA TATCTAGA TATCTAGA TA(43)       pTL88     A TATCTAGA TATCTAGA TATCTAGA TATCTAGA TATCTAGA TA(43)	(TAAT)n (TATCTAGA)n (TATCTAGA)n (TATCTAGA)n (TATAGC)n (TATAGC)n (TATCCGGA)n		
74°C pTL45 A TATCTAGA TATCTAGA TATCTAGA TATCTAGA TATCTAGA TA(43) pTL88 A TATCTAGA TATCTAGA TATCTAGA TATCTAGA TATCTAGA TA(43)	(TATCTAGA)n (TATCTAGA)n (TATAGC)n (TATAGC)n (TATAGC)n (TATCCGGA)n		
PTL88 A TATCTAGA TATCTAGA TATCTAGA TATCTAGA TATCTAGA TA(43)	(TATCTAGA)n (TATAGC)n (TATAGC)n (TATAGC)n		
	(TATAGC)n (TATAGC)n (TATCCGGA)n		
79°C pTL792 GC TATAGC T	(TATAGC)n (TATCCGGA)n		
pTL793 TATAGC TATAGC TATAGC TATAGC TATAGC TATAGC TATA(40)	(TATCCGGA) n		
84°C pTL842 TCCGGA TATCC(11)	(		
pTL845 TATCCGGA TATCCGGA TATCCGGA TATCCGGA TATCCGGA TATCCGGA(48)	(TATCCGGA) n		
89°C pTL892 GATCGC TATCGCGATAGCGATCGC TATCGCGATAGCGATCGC TATCGCGATAGCGATCG(59)	(TATCGCGATAGCGATCGC)		
pTL893 GC TATCGCGATAGC(14)	(TATCGCGATAGCGATCGC)		
94°C pTL942 C GATCGC GATCGC GATCGC GATCGC GATCGC GATC(35)	(GATCGC) n		
pTL943 ATCGC GATCGC GATCGC GATCGC GATCGC GATCGC(35)	(GATCGC) n		
[KC1]			
0 mM pTLK2 AGA TATCTAGA TATCTAGA TATCTAGA(27)	(TATCTAGA) n		
pTLK3 TATCTAGA TATCTAGA TATCTAGA TATCTAGA TATCTA(38)	(TATCTAGA) n		
10 mM pTL53 A TATCTAGA TATCTAGA TATCTAGA TATCTAGA TATCTAGA(41)	(TATCTAGA) n		
pTL93 TATCTAGA TATCTAGA TATCTAGA TATCTAGA TAT(35)	(TATCTAGA) n		
50 mM pTLK52 TCTAGA TATCTAGA TATCTAGA TATCTAGA TA(32)	(TATCTAGA) n		
pTLK53 TATCTAGA TATCTAGA TATCTAGA TATCTAGA(32)	(TATCTAGA) n		
pTLK56 TATACG TATATACG TATATACG(22)	(TATATACG) n		
pTLK57 TATCTAGA TATCTAGA TATCTAGA(24)	(TATCTAGA)n		
75 mM pTLK752 TATCTAGA TATCTAGA TATCTAGA TATCTAGA TA(34)	(TATCTAGA) n		
pTLK753 TATCTAGA TATCTAGA TATCTAGA TATCTAGA T(33)	(TATCTAGA)n		
pTLK757 AGA TATCTAGA TATCTAGA TATCTAGA TATCTAGA TA(37)	(TATCTAGA) <i>n</i>		
100 mM pTLK83 AC TATAGTTATAAC TATAGT(20)	(TATAGTTATAAC) <i>n</i>		
Н			
7.8 DTLP72 TATCTAGA TATCTAGA TATCTAGA(24)	(TATCTAGA)n		
pTLP74 TATCTAGA TATCTAGA TATCTAGA(24)	(TATCTAGA) n		
8.8 pTL37 A TATCTAGA TATCTAGA TATCTAGA TATCTAGA TATCTAGA TATCTAGA TAT(44)	(TATCTAGA) n		
DTL43 A TATCTAGA TATCTAGA TATCTAGA TATCTAGA TATCTAGA(41)	(TATCTAGA) n		
9.8 pTLP82 TCTAGA TATCTAGA TATCTAGA TATCTAGA TATCTAGA(38)	(TATCTAGA) n		
pTLP83 TCTAGA TATCTAGA TATCTAGA TATCTAGA TATCTAGA TATCTAGA(46)	(TATCTAGA) n		
10.8 pTLP92 TCTAGA TATCTAGA TATCTAGA TATCTAGA TATCTAGA(38)	(TATCTAGA)n		
pTLP93 AGA TATCTAGA TATCTAGA TATCTAGA TATCTAGA(35)	(TATCTAGA)n		

Figure 4. Sequences of the DNA synthesized by *Tli* DNA polymerase without added primer and template DNAs. The reaction products of *Tli* DNA polymerase as shown in Figures 1–3 under various conditions were cloned in a plasmid and the sequence of each insert DNA is shown. The salient points of the reaction conditions are shown in the first column. The names of the clones are shown in the second column. The length of each cloned DNA in bp is shown just after the sequence in parentheses. The last column shows the sequence of each cloned DNA as a general form of a repetitive sequence.

We next examined the effect of ionic strength on this reaction. When the reaction was carried out at fixed pH (8.8) and at fixed temperature (74°C) but in different KCl concentrations, the amount of pol product synthesized decreased markedly above 100 mM KCl (Fig. 3A), but the size of DNA did not change significantly (Fig. 3B). The reaction was optimal in 0–10 mM KCl.

To characterize the pol product, we next cloned the pol product DNA in a SmaI (a blunt-end cutter) site of a plasmid pUC19 by partially digesting the pol product with deoxyribonuclease I in the presence of manganese ion, under which most of the cut ends are rendered blunt-ended. Cloning efficiency was very low and the size of the insert DNA was very small as we had experienced before (9), although one could expect their lengths to be 0.5-2 kb from the cloning procedure employed. All the insert DNAs, which were derived from the pol product, had tandem repetitive sequences of a unit length of 4-18 bp (Fig. 4). There was no difference in the sequences when pH of the reaction for pol product synthesis was changed. When the ionic strength was changed, there was a moderate change in the sequence of the cloned DNA between 0 and 75 mM KCl, namely (TATCTAGA)<sub>n</sub> or  $(TATATACG)_n$ , but the sequence changed markedly at 100 mM KCl, namely (TATAGTTATAAC)<sub>n</sub> (Fig. 4). The sequence of the pol product varied greatly when the temperature

of the reaction was changed. For instance, it was  $(TAAT)_n$  at 69°C, (TATCCGGA)<sub>n</sub> at 84°C and (GATCGC)<sub>n</sub> at 94°C (Fig. 4). All of the repeat unit sequence had a palindromic structure. For instance, the sequence  $(TATCTAGA)_n$  (clone pTL37, Fig. 4), which can also be written as  $(ATCTAGAT)_n$ , has the center of the palindrome between italicized T and A. The remarkable feature of this temperature dependence of the pol product DNA sequence is that a GC content (mole percent of the sum of G and C) of the pol product increases almost linearly upon the increase of the reaction temperature; the GC content was 25% at 74°C, 50% at 84°C and 66.7% at 94°C. Furthermore, this temperature dependence of the GC content parallels very well with a double strand-to-single strand dissociation temperature (also called melting temperature,  $T_{\rm m}$ ) of linear DNA calculated from the equation  $T_{\rm m}$  (°C) = 16.6logM + 0.41C + 81.5, where M is salt concentration in mol/l and C is GC content in mole percent (18). Nearest neighbour frequency of the pol product calculated from all the cloned sequences (Fig. 4), as double-stranded DNA, showed that sequences TG and CA never appeared, while sequences AT and TA appeared frequently (Table 1). Homology searches of the pol product sequences revealed that similar sequences were present in natural genes of a variety of organisms (data not shown).

Temperature (°C)	69	74	74	74	79	84	89	94
KCl (mM)	10	10	50	100	10	10	10	10
Nearest neighbour	Frequency (%)							
AA	25.6	0	0	5.3	0	0	0	0
AT	26.8	25.2	23.8	15.8	17.4	22.8	16.9	17.6
AG	0	12.1	0	10.5	15.8	0	5.6	0
AC	0	0	11.9	10.5	0	0	0	0
ТА	22.0	25.2	38.1	31.6	34.8	12.3	11.3	0
TT	25.6	0	0	5.3	0	0	0	0
TG	0	0	0	0	0	0	0	0
TC	0	13.1	0	0	0	13.2	11.3	16.9
GA	0	12.1	0	0	0	13.2	11.3	16.9
GT	0	0	11.9	10.5	0	0	0	0
GG	0	0	0	0	0	13.2	0	0
GC	0	0	0	0	16.3	0	16.9	16.2
CA	0	0	0	0	0	0	0	0
CT	0	12.4	0	10.5	15.8	0	5.6	0
CG	0	0	14.3	0	0	12.3	21.1	32.4
СС	0	0	0	0	0	13.2	0	0

Table 1. Nearest neighbour frequency of DNA synthesized by Tli DNA polymerase without added primer and template DNAs

The nearest neighbour frequencies were calculated from the sequences of insert DNAs of all the pol product clones shown in Figure 4 as double-stranded.

### DISCUSSION

The DNA inserts obtained by cloning of the pol product in this study were very short just as we had found previously (9); we reasoned this was because of repetitive structures of the DNA (9). It is reported that only short DNA inserts are obtained due to frequent deletion during cloning of repetitive DNAs (19,20). We think that similar deletion occurs during the cloning of the pol product as it has repetitive structures (Fig. 4), and that this is the reason why only short DNA inserts were obtained. The fact that most of the pol products have repetitive sequences was further supported by the result that they were hardly digested with restriction enzymes HaeIII, AluI, Sau3AI or HhaI, which require four bases as a recognition sequence; chromosomal DNA of E.coli used as a control was digested into small fragments by these enzymes under the same reaction conditions (data not shown). If the pol product were not repetitive with a short repeat unit sequence, such a huge DNA as pol product (Figs 1-3) would have been digested to small fragments. One possible mechanism by which the pol product is synthesized is proposed for the sequence  $(TATCCGGA)_n$  in Figure 5 [clone pTL845 which can also be written as  $(ATCCGGAT)_n$ ; Fig. 4] as an example. According to this model, a trace amount of oligonucleotide, a 'seed oligomer', is first synthesized (ATCC in this example, Fig. 5A) enzymatically or non-enzymatically; non-enzymatic reaction is energetically possible, although its rate may be very low without an enzyme catalyst. A complementary sequence is next synthesized enzymatically using the seed oligomer as a non-added (synthesized ab initio) template, and eventually a hairpin structure is made (Fig. 5B). The hairpin is next melted into a single strand, because the reaction temperature is near  $T_{\rm m}$ , and then a second round of



**Figure 5.** A hypothetical model of DNA synthesis by *Tli* DNA polymerase without added primer and template DNAs. A model for the synthesis of  $(TATCCGGA)_n$  [Fig. 4, clone pTL845, which can also be written as  $(ATCCGGAT)_n$ ] is shown as an example. (A) An initial oligonucleotide ('seed oligomer') is synthesized enzymatically or non-enzymatically. A 5'-triphosphate residue is shown as 'ppp'. (B) A hairpin structure is next made enzymatically using the seed oligomer as a non-added template. (C) The hairpin is melted, because the reaction temperature is near its  $T_{\rm m}$ . (D) The second round of a hairpin synthesis occurs. (E) The second hairpin is melted. (F) The third hairpin is made. Such hairpin-melting rounds are repeated and the seed oligomer is elongated.

complementary sequence is synthesized (Fig. 5D) using the melted single-stranded DNA (Fig. 5C) as the next template. We speculate that such a hairpin-melting round is repeated and the oligonucleotide is elongated.

The hairpin-melting rounds will occur only if the  $T_{\rm m}$  expected from the GC content of a seed oligomer is close to its reaction temperature. If the actual reaction temperature is much higher than the  $T_{\rm m}$ , the hairpin structure will never be formed, because A:T and G:C base pairs are not stably formed. On the other hand, if the reaction temperature is much lower than  $T_{\rm m}$ , once made, the hairpin cannot be melted and cannot serve as a template in the next round of synthesis. In other words, the reaction will occur only in a small zone, a 'melting zone', of temperature around the  $T_{\rm m}$  expected of the GC content of each seed oligomer. This means that there may be many kinds of, but in a trace amount, seed oligomers having various GC contents soon after the initiation of the reaction, and that only those seed oligomers having an optimal GC content at the reaction temperature are 'selected' for elongation. We propose a term 'melting zone synthesis' for this kind of *ab initio* DNA synthesis. Such a chemical selection of DNAs can also explain the tandem repetitive sequences found in pol product created by DNA polymerase (Fig. 4).

Tandem repetitive sequences consisting of a short repeat unit are found in genes of various organisms, for instance in a silk fibroin gene (21), antifreeze protein of antarctic cod (22) and telomeres (6). Some are in the coding regions (21,22) and some are outside the coding regions (6). It may be possible that such tandem repetitive sequences were created by DNA polymerase at a certain stage of evolution of genome of organisms on the early earth. In the accompanying paper (11), we demonstrate that DNA polymerase of thermophilic bacterium (eubacterium) *T.thermophilus* can also synthesize a variety of tandem repetitive DNAs in the complete absence of added primer and template DNAs, demonstrating that protein of a thermophilic bacterium as well as that of a thermophilic archaeon can potentially create genes in a hightemperature environment in which these organisms are living.

#### ACKNOWLEDGEMENT

We thank Ms. M. Shimokawa for contribution to this work.

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