The L409 amino acid residue is important for the DNA replication fidelity of thermostable Pfu DNA polymerase

Chris Hergott, 2009
Adviser: Baek Kim, Ph.D.
Department of Microbiology and Immunology
University of Rochester Medical Center

Effective cell division requires an accurate and efficient mechanism by which genetic information can be replicated and passed on to progeny cells. Excessive errors in genome replication may mutate essential genes and threaten the ability of these daughter cells to survive and proliferate. Organisms that utilize double-stranded deoxyribonucleic acids (dsDNA) as carriers of their genetic code have developed a class of enzymes called DNA polymerases to catalyze this DNA replication process. DNA polymerases incorporate free deoxyribonucleotide triphosphates (dNTPs) into newly synthesized DNA strands in a sequence primarily determined by that of the template DNA strand through Watson-Crick base-pairing rules. However, the wide range of accuracy levels among the known DNA polymerases in faithfully replicating the template strand suggests that simple base-pairing forces are an insufficient explanation for these differences in fidelity. Indeed, the polymerase is actively involved in the dNTP selection, and thus at least partially responsible for replication fidelity.

The DNA polymerase from the thermophilic archaean Pyrococcus furiosus (Pfu Pol) remains one of the most widely known thermostable high fidelity DNA polymerases. It is commonly used as the replicating enzyme for Polymerase Chain Reactions (PCR) in many laboratory protocols because of its high degree of replication accuracy and inherent thermal stability. While the presence of a 3’→5’ exonuclease proofreading domain on Pfu significantly contributes to its superior fidelity among often-used enzymes in PCR, exonuclease-deficient Pfu still confers an error rate within an order of magnitude of Taq polymerase, the other most commonly used PCR enzyme lacking this proofreading nuclease activity. This inherent fidelity makes Pfu polymerase a good model for studying the interaction between active site molecular architecture and replication fidelity.

Pfu polymerase belongs to the α-like family of DNA polymerases, a group of closely related enzymes sharing a structure similar to the eukaryotic DNA polymerase α. The crystal structure of another α family member, the RB69 phage polymerase, has been co-crystallized with template-primer and an incoming dNTP (known as the ternary complex) at a resolution of 2.8 Å. Crystallography confirms that the RB69 and Pfu polymerases are extremely similar in structure, with highly conserved topological features. Several functional domains, separated by clefts, radiate from a central cavity. They have been named the “Thumb,” “Fingers,” “Exo,” “Palm,” and “N-terminal” domains, with the nomenclature noting its shape’s similarity to that of a right hand. The palm domain, thought to be the most highly conserved of the group, has been implicated in the dNTP-binding and nucleotidyl transferase activities central to the accurate polymerization needed for DNA replication. Within this region, the leucine residing at the 409th residue of Pfu Pol has been shown to be highly conserved among α family polymerases and is represented by Leucine-415 in the RB69 polymerase. Conservation of a similarly located amino acid across members of a family of enzymes suggests mechanistic importance and provides a potential target for investigation.

Here, we have created a mutant form of exonuclease-deficient Pfu polymerase in which Leucine-409 was replaced with a methionine residue. This new enzyme (L409M) was assayed for its enzyme fidelity in comparison with the wild-type Pfu Pol. Ongoing work in our laboratory has focused on replacing L409 with bulkier residues, including phenylalanine. Preliminary data has shown that the L409F mutation renders the enzyme unable to complete PCR-based fidelity assays. However, the leucine to methionine substitution presents only approximately four cubic Angstroms in residue volume change and little change in side-chain polarity and this mutant is able to complete a PCR reaction. Therefore, the L409M Pfu polymerase mutant is instructive in determining the role of the 409th residue in maintaining faithful DNA replication in vitro.

Materials and Methods

Site-directed mutagenesis was completed via a two-step overlapping Polymerase Chain Reaction (PCR) method. The first PCR step used a forward primer containing a leucine → methionine substitution (5’-TACCTAGATTATTAGGCCATGTATCCCTGATTATAATTACCACAC-3’) and reverse primer that annealed near the C-Terminal domain (5’-GGCTCTAAAATCTAGGTA-3’) of the pfu gene. A second PCR reaction used the amplicons created in the first step as templates, annealed to a T7 forward primer (5’-TAA TAC GAC TCA CTA TAG GG-3’) and the original Pfu reverse primer. This second PCR extended the insert fragment to a size of approximately 2.4 kb, allowing for it to be more easily
used in cloning procedures. This 2.4 kb amplicon and a pET28a dsDNA plasmid (Novagen, WI) were double-digested with NheI and NcoI restriction endonucleases. The vector and the insert (PCR product) were covalently joined by T4 DNA ligase (Invitrogen, CA) and transformed via electroporation into XLI-Blue E. coli cells (Stratagene, CA). Surviving colonies were harvested and the pfu clones were isolated from the other cellular components by a Miniprep (Promega, WI) purification procedure. Correct insertion of the PCR amplicon insert into the pET28a backbone in the clones was verified by analytical Apal digest, with successful clones yielding a single linear 7.0 kb band upon agarose electrophoresis. These clones were sequenced, and the results were analyzed for similarity to the exon(-) wild type pfu gene with the exception of the L→M mutation at amino acid residue 409.

A clone confirmed by sequencing (ACGT Inc., IL) to contain L409M pfu was transformed into chemically competent BL-21 (DE3) pLysS E. coli cells for over-expression and incubated in 2X-YT rich media under kanamycin/chloramphenicol selection pressure. Kanamycin selects for cells containing the L409M pfu-pET28a clone and chloramphenicol selects for BL-21 cells containing the pLysS plasmid needed for strict transcriptional control over the lacUV5 promoter, which controls expression of the L409M pfu gene. Ten milliliters of 100 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a liter of culture upon an OD reading of 0.1 to induce expression of the mutant protein and the culture was incubated for approximately three more hours. The cells were then pelleted by centrifugation and lysed. Supernatant was isolated from the precipitated impurities in the cell lysate and centrifuged for twenty minutes at 3600 rpm at 4°C.

To isolate and purify the mutant Pfu polymerase protein from the rest of the supernatant, a nickel ion-NTA affinity chromatography method was utilized. This technique involves chelation of hexahistidine tags genetically attached to the L409M Pfu mutant polymerase by embedded nickel ions in a semisolid resin, immobilizing the desired protein while letting all other flows through the column. Subsequent treatment with imidazole, which has a higher affinity for the nickel ions, allows for elution and collection of the purified protein, followed by overnight 4°C dialysis in a Slide-A-Lyzer cassette against 1X-Dialysis Buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 50% glycerol). SDS-PAGE was used to confirm sufficient concentration and purity of Pfu in the eluted fractions.

In order to accurately compare Pfu polymerization activity between L409M and (previously analyzed) wild type, the purified L409M polymerase preparation was normalized for concentration and activity. Differences in activity among mutants must be based solely on the effects of the introduced substitutions, rather than artifactual differences in the concentration and efficiency levels of protein preparations. To this end, single nucleotide extension assays were performed with varying protein concentrations to ascertain the concentrations at which L409M polymerase extended approximately fifty percent of an oligonucleotide primer.

The L409M Pfu polymerase was used in a primer extension reaction with 40 nM 40mer DNA template (5′-AAGCTTGCGCTGAGCACGAGATATTTGCTAGCGGGAATTCCGGCGG-3′) annealed to 80 nM 23mer Extend A primer (5′-CCGCGGGAATTCCGGCGG-TAGCAAT-3′) 5′-radiolabeled with Phosphor-32. These reactions also included 200 µM oligo-dT, 2.5 mM dATP, and 10X Pfu Buffer (200 mM Tris HCl pH 8.8, 20 mM Mg2SO4, 100 mM KCl, 100 mM (NH4)2SO4, 1% Triton X-100, and 1 mg/mL BSA) with the approximately 40 nM template:primer. The template:primer existed in 250-fold excess over Pfu enzyme in the extension reactions. Other dNTPs were excluded from the reaction mixture so that only an adenine residue could be added onto the primer.

At the commencement of each reaction, the diluted protein was added to the reaction mixture and incubated for five minutes at 55°C. After this incubation, 2x-TC Stop Dye (containing 40 mM EDTA) was added and the reaction was kept at 95°C for five minutes. The EDTA chelates the magnesium ion cofactor from the polymerase enzyme and halts catalysis. For each reaction, a 4 µL sample was electrophoresed through a 16% urea denaturing polyacrylamide gel and visualized by phosphorimaging analysis.

Once the appropriate L409M protein amount was found for comparison to wild type Pfus at fifty percent primer extension, this concentration was used in dATP titration reactions in order to establish the mutant’s Michaelis Constant (K_M), or the enzyme concentration at which L409M polymerized at half-maximal velocity. These reactions were performed under identical conditions as those for the Activity Normalization Assays with the exception that polymerase concentration was now fixed at approximately 800 pM and dATP concentration differed between reactions, ranging from final concentrations of 1 µM to 250 µM (See Figure 2). The results were once again electrophoresed in 16% polyacrylamide. Products were then observed via phosphorimaging and quantified by densitometry. The extent of extended primer formation was plotted against dATP concentration and fit to the Michaelis-Menten equation (V_MAX = V = [S] / K_M + [S]) to determine the K_M value for L409M Pfu. This process was completed in duplicate to ensure precision. The wild type polymerase K_M had been previously elucidated by similar means.

To measure the extent of mutation conferred by L409M upon a newly synthesized DNA strand, and thus its fidelity, we performed a PCR-based Forward Mutation Assay. This experiment involved the L409M (without the 3′-exo domain) and WT (+/- 3′-exo) Pfu polymerase as the replicating enzymes in PCR reactions upon a template fragment (pUC18 plasmid linearized by restriction endonuclease AfIII) containing the lacZα reporter gene. The lacZ gene encodes beta-galactosidase, a hydrolase that can cleave the chromogenic reporter X-GAL to yield a blue product. Primers flanking the lacZ region on the pUC18 fragment (5′-AAAAAAGATCTCTTCTTTCTGCTGGTTATCCC-3′ and 5′-AAAAAAGATCTTAGCGCCAGC-3′) were used with 50 µM dNTPs and 10X-Pfu Buffer to complete PCR in a BioRad Thermal Cycler. Q-PCR had been previously utilized to normalize for template duplications among the mutants in the PCR reactions.

After this PCR was completed and verified by agarose electrophoresis, the amplicon DNA fragment was purified with a Qiagen PCR Purification Kit and double-digested by DpnI and BglII. DpnI specifically degrades dam-methylated adenine bases within a (5′-GA−3′) palindrome, thereby destroying any residual plasmid template remaining from the reaction while BglII made two cut sites within the PCR products, leaving complementary single-stranded overhangs at each end. This digestion fragment was purified by phenol–ethanol extraction and then subjected to an intramolecular ligation by T4 DNA Ligase (Invitrogen, CA).
This newly ligated pUC18 construct was then electroporated into XL1-Blue E. coli cells (Stratagene, CA). These were spread onto plates under carbenicillin selection pressure with IPTG as an expression inducer and X-GAL as the chromogenic substrate of beta-galactosidase action. When cells expressing this lacZ protein dilution factors to normalize for any concentration and extension reactions were performed with varying L409M Pfu polymerase at a concentration of 800 pM (a dilution of 1:28,000). The fraction of extended primer over the total primer added per lane was quantified by densitometry and the observed product concentrations were fit to Michaelis-Menten kinetics.

Results and Discussion

Normalization of L409M and Wild Type DNA polymerase Activities

As described in the Methods section, the single nucleotide extension reactions were performed with varying L409M Pfu protein dilution factors to normalize for any concentration and efficiency differences between the L409M and wild type proteins brought about by preparation procedures. The goal of this assay was to find the concentration of polymerase displaying 50% extension of 32P-labeled 23mer at the maximal dATP concentration used in our single nucleotide extension reactions (250 uM final). This protein concentration will be used for the following enzyme characterizations in comparison to wild type Pfu. After electrophoresis, through 16% urea denaturing polyacrylamide, the phosphor-image of 50% extension should show two similarly radioactive bands representing the small unextended primer (P) and extended primer (E). A scan of these reactions with increasing L409M dilutions from 1:10 to 1:100,000 is depicted in Figure 1.

While the wild type exo(-) enzyme used for comparison was found to reach 50% extension at the 1:180,000 dilution factor mark, Figure 1 demonstrates that the fifty percent extension point for L409M Pfu lied between 1:25,000 and 1:30,000. This point was later found to reside at 1:28,000 by a more refined set of protein dilution reactions, yielding a concentration of 70 pg/µL. This would be the dilution factor used for this mutant in subsequent kinetics assays. The multi-nucleotide extension seen in Figure 1 is unexpected as the template:primer anneals such that the template thymidine residues to further discourage multi-nucleotide extension. The correct concentration of L409M needed for 50/50 enzyme control assays were counted and the ratios of white:blue colonies for the L409M (exo-), WT (exo-), WT (exo+), and a no-enzyme negative control reaction, expectedly showing no primer extension. The eight subsequent treatment groups were treated with a gradually decreasing concentration of dATP. The final dATP concentrations represented in these lanes are: 250 µM, 100 µM, 50 µM, 25 µM, 10 µM, 5 µM, 2.5 µM, and 1.0 µM. The fraction of extended primer over the total primer added per lane was quantified by densitometry and the observed product concentrations were fit to Michaelis-Menten kinetics.

**Figure 1.** This image is a phosphorimager visualization of a 16% urea denaturing polyacrylamide gel for a L409M Pfu polymerase dilution series. Single nucleotide extension reactions were performed upon 40 nM 32P-labeled 23mer DNA primer annealed to 80 nM 40mer template combined with 2.5 mM dATP, 200 uM oligodT, and 10X Pfu Buffer. Unextended primers (23mers) are represented in the row labeled with a P while single-nucleotide extended primers (24mers) are labeled with an E. The reactions were performed for 5 minutes at 55° C, then quenched with 2X-Stop Dye containing 40 mM EDTA. On the left, the N.E. lane contains a no-enzyme negative control reaction, predictably showing no primer extension. The thirteen subsequent treatment groups were treated with a gradually decreasing concentration of L409M Pfu polymerase. The dilution factors (from 2ug/µL protein) for these lanes were: 1:10, 1:256, 1:4000, 1:15000, 1:20000, 1:25000, 1:30000, 1:35000, 1:40000, 1:45000, 1:50000, and 1:100000. The goal of this assay was to determine the protein concentration at which 50% of the primers were singly extended to form 24mers. This would allow normalization of enzyme activity between L409M and wild type Pfu proteins. The correct concentration of L409M needed for 50/50 was extension was 70 pg/µL or 800 pM, from a 1:28,000 dilution of 2 µg/µL stock protein. Multinucleotide extension beyond the E row illustrates a tendency for L409M Pfu to misincorporate nucleotides onto the primer without respect to template.

![Figure 1](image1.png)

**Figure 2.** This image is a phosphorimager visualization of a 16% urea denaturing polyacrylamide gel for a L409M Pfu polymerase dATP Titration Assay. Reactions were completed similarly to those in the Protein Dilution Series (Figure 1) except that protein concentration was fixed at 71pg/µL and dATP concentration was gradually decreased. Single nucleotide extension reactions were performed upon 40 nM 23mer DNA primer annealed to 80 nM 40mer template combined with varying dATP concentrations, 200 uM oligodT, and 10X Pfu Buffer. Unextended primers (23mers) are represented in the row labeled with a P while single-nucleotide extended primers (24mers) are labeled with an E. The reactions were performed for 5 minutes at 55° C, then quenched with 2X-Stop Dye containing 40 mM EDTA. On the left, the N.E. lane contains a no-enzyme negative control reaction, expectedly showing no primer extension. The eight subsequent treatment groups were treated with a gradually decreasing concentration of dATP. The fraction of extended primer over the total primer added per lane was quantified by densitometry and the observed product concentrations were fit to Michaelis-Menten kinetics.
Extension ability among the enzyme samples added to the reactions, pre-steady state kinetic analysis would be required to arrive at a quantitative percent activity figure for each polymerase. This will be a topic of future research on the L409M mutant, as fractional activity data is needed to accurately assess L409M turnover number (kcat). However, the present kinetics data shows that any observed fidelity differences between L409M and wild type Pfu are not coupled with drastic difference in Km value.

**Fidelity Assay Results**

A Forward Mutation Assay was performed to measure the mutation rate of L409M, and thus its fidelity, within a ~600 bp lacZα gene fragment. The first step of this procedure was to use the mutant L409M polymerase in a PCR reaction on a pUC18 fragment containing the lacZα gene. The subsequent steps are detailed in the Methods section. Successful completion of the PCR and corresponding Q-PCR experiments (not shown) show that L409M is functional enough to extend primers and allows for enough processivity to extend them many thousands of bases. However, this result speaks only to the effectiveness of the enzyme at completing the reaction, not the extent to which its products are mutated.

As described in the Methods section, the PCR product was digested and ligated to itself to form a circular pUC18 fragment (containing the lacZα gene region) that was transformed via electroporation into XL1-Blue E.coli. These cells were spread on plates containing the chromogenic beta-galactosidase substrate X-GAL, allowing a blue-white assay to be completed in which blue colonies represented functional lacZα expression while white colonies represented functional lacZα expression while white colonies represented functional lacZα expression.

**Figure 3.** (A) After quantification of the extended and unextended bands from the dATP Titration Experiment for L409M Pfu polymerase (duplicated), the concentration of extended product (over five minute reactions) was graphed as a function of dATP concentration and fit to the Michaelis-Menten equation. The nonlinear fit provides the Michaelis Constant (Km) by calculation of the dATP concentration needed for half-maximal velocity, or that needed to reach half of the maximal product formed in the fixed five minute reactions. (B) This chart compiles the mean Km value for L409M Pfu calculated from Figure 4A with the previously determined Km for wild type (exo-) Pfu polymerase. At 1.6 µM for L409M and 2.5 for wild type (exo-), these two values differ by less than 1 µM. This provides initial data suggesting similar dATP incorporation kinetics in the steady state (to be confirmed by pre-steady state analysis). All Km values shown have been confirmed by Lineweaver-Burk linear regression analysis.
colonies represented mutation. This conferred to the extent that beta-galactosidase was functionally impaired. These procedures were completed in triplicate for the WT (exo+), WT (exo-), and L409M Pfu proteins, along with a no-enzyme negative control to assess background white colony frequency, in order to elucidate the extent of these blue-white assays are depicted in chart and graph form in Figures 4A and 4B, respectively.

One would expect a wild type Pfu polymerase with proofreading ability to effectively replicate the genome of its host organism with a limited amount of error. This is supported by Figure 4, in which wild type exo(+) plates showed only slightly higher mutation rate than plates with no enzyme at all. When the proofreading exonuclease function is disabled, mutation rate should increase. Figure 4 shows that indeed it does, with wild type exo(-) presenting a 3.6-fold increase in white colonies per blue colony over wild type exo(+). For L409M, the white:blue colony ratio averaged at 1.1. Its white:blue ratio lies 1.8-fold above wild type exo(-), 6.6-fold above exo(+), and 9.6-fold above the no-enzyme control. Therefore, the leucine → methionine substitution at residue 409 decreases the fidelity of the polymerase approximately two-fold, as its 1.1 white:blue ratio is 1.9 times higher than 0.56, the ratio for wild type (exo-).

The L409M mutation reduces the ability of Pfu polymerase to faithfully replicate the lacZ reporter gene in PCR without damaging the function of the enzyme expressed from it, beta-galactosidase. Unpublished work from this laboratory suggests that replacing L409 with isoleucine produces similar fidelity aberrations. These observations suggest that L409 is an important component in maintaining the replication fidelity of Pfu polymerase, as even slight modification to this residue yields significant loss of fidelity.

Conclusion

Faithful replication of DNA requires polymerase enzymes with sufficient fidelity to minimize mutations that may hinder the fitness of progeny cells. Pfu DNA polymerase ranks among the most accurate enzymes commonly used in PCR reactions and therefore represents an appropriate target for investigation into the role of the active site architecture in determining this fidelity. We introduced a leucine-to-methionine substitution at residue 409, within the highly conserved dNTP-binding motif, to investigate whether subtly changing the geometry of this region would affect the Pfu polymerase's ability to correctly replicate a lacZ reporter gene in a PCR-based Forward Mutation Assay. The results of these experiments show that the L409M (exo-) mutant presents replication fidelity nearly two-fold below exonuclease-deficient wild type Pfu polymerase, as E. coli colonies containing the damaged beta-galactosidase are 1.83 times more common in L409M than WT (exo-). Furthermore, steady state kinetic analysis of L409M yielded a Michaelis Constant (K_M) of 1.6 uM versus 2.5 uM for wild type (exo -). This means L409M Pfu functions at half-maximal catalytic velocity at a comparable, if not lower, dATP concentration than the exonuclease-deficient wild type enzyme while still severely hindering the fidelity with which the polymerase pairs the incoming nucleotide with the correct template base. Future work will focus on a mechanistic understanding of this altered state through pre-steady state kinetic analysis and elucidation of L409M Pfu dNTP-binding parameters. In any case, the correlation between this subtle change in the Pfu polymerase active site and its drastic
effects on the mutant's ability to faithfully replicate DNA speaks to the refined nature of this enzyme as a product of evolution.

References


