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Use of Pol I-Deficient *E. coli* for Functional Complementation of DNA Polymerase

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1. Introduction

The *E. coli* JS200 strain carries a temperature-sensitive allele of DNA polymerase I that renders this strain conditional lethal. Growth under restrictive conditions is restored by small amounts of DNA polymerase activity. Even mutants with greatly reduced (1–10% of wild-type) catalytic activity or distantly-related polymerases of bacterial, eukaryotic, or viral origin effectively complement JS200 cells. The versatility of this complementation system makes it advantageous for selection of active polymerase mutants, for screening of polymerase inhibitors, or for screening of mutants with altered properties. Here we describe complementation of JS200 cells with the wild-type *E. coli* DNA polymerase I to illustrate such functional polymerase complementation.

Polymerases catalyze the template-directed incorporation of nucleotides or deoxynucleotides into a growing primer terminus. DNA polymerases and reverse transcriptases share a common structure and mechanism of catalysis in spite of low sequence conservation ***(1)***. As central players in replication, repair, and recombination, DNA polymerases have been intensely studied since the early days of molecular biology. Errors in nucleotide incorporation have been recognized as significant sources of mutations, contributing to the generation of genetic diversity, of which HIV reverse transcriptase is a dramatic example. Polymerase errors may also contribute to the genetic instability that characterizes certain disorders, such as cancer and trinucleotide expansion diseases. Finally, polymerases are finding an ever-growing number of applications in sequencing, amplification, mutagenesis, and cDNA library construction.

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E. coli DNA polymerase I is encoded by the *polA* gene. It has two relatively independent functional units: a polymerase (with a 3'→5' exonuclease proofreading domain), and a separate 5'→3' exonuclease subunit. In vitro, the coordinated action of these two subunits results in efficient nick translation. In vivo, pol I is involved in lagging-strand synthesis during chromosomal replication and in DNA excision repair. Pol I mediates the processing of Okazaki fragments by extending from the 3' end of the RNA primer and by excising the RNA primer from the 5' end of the downstream fragment. Removal of all residues of the RNA primer is essential for joining of Okazaki fragments ***(2)***. Similarly, the coordinated action of polymerase and 5'→3' exonuclease activities on an RNA primer initiates ColE1 plasmid replication ***(3)***. On the DNA repair front, pol I catalyzes fill-in reactions in base and nucleotide excision repair. In the latter, pol I also contributes to releasing the oligonucleotide fragment and UvrC protein from the postincision complex ***(4,5)***. Pol I expression is constitutive, with an estimated 400 molecules/cell. It seems, however, that only a fraction of these molecules are engaged in lagging strand synthesis catalysis under normal circumstances, which would leave a substantial cellular complement available for DNA excision repair.

Pol I is not essential for growth in minimal medium, although pol I-deleted strains show slower growth rates. In rich medium, pol I is essential, presumably because cells are unable to complete lagging-strand synthesis before the next round of replication ***(6)***. Expression of either of the polymerase I subunits restores growth in rich media ***(6)***, implying that other enzymes are able to substitute for pol I in lagging-strand synthesis. In agreement with pol I's partial redundancy in vivo, *pol A* shows epistasis with a number of genes involved in DNA repair and recombination, including *rnhA* ***(7)***, *polC* ***(8,9)***, *uvrD* ***(10)***, *recA* ***(11–13)***, and *recB* ***(11)***.

PolA12 encodes a misfolding form of pol I that is a defective in the coordination between the polymerase and 5'-exonuclease activities ***(14)***. *PolA12* also exhibits reduced temperature stability, and in vivo, its polymerase and 5'-exonuclease activities decrease 4-fold at 42°C ***(14)***. In combination with *recA*- and *recB*-inactivating mutations, *polA12* is lethal in rich medium ***(11)***. Surprisingly, RecA-mediated constitutive expression of the SOS response also renders *polA12* cell growth sensitive to high temperature ***(13)***. The *polA12 recA718* temperature-sensitive strain (JS200 strain) probably falls into this category ***(9)***. *RecA718* is a sensitized allele of *recA* ***(15)*** that is likely activated as a result of slow Okazaki fragment joining under conditions that are restrictive for *polA12*. The combination of a 5'→3' exonuclease- inactivating mutation and constitutive SOS expression is viable under restrictive conditions ***(13)***, however, and expression of polymerase activity alone (without 5'→3' exonuclease) relieves *polA12 recA718* conditional lethality ***(9)***. These two observations point to polymerase as the rate-limiting activity in pol I-deficient, SOS-induced cell conditional lethality. Complementing polymerase activity can be provided even by distantly-related polymerases of bacterial, eukaryotic, or viral origin, although polymerase overexpression may be required for complementation in some cases ***(9,17)***. Examples of complementing polymerases include *E. coli* pol III α subunit ***(9)***, *Thermus aquaticus* (*Taq*) polymerase ***(16)***, rat pol β ***(17)***, and HIV and MLV reverse transcriptases ***(18)***. JS200 complementation by some of these polymerases occurs even after partial inactivation by mutagenesis ***(19–22)*** (the threshold being 10% of wild-type activity for *Taq* and pol I, based on colony formation). With its great versatility, the *polA12 recA718* complementation system in *E. coli has* been used for selection of active mutants of *Thermus aquaticus* (*Taq*), and *E. coli* pol I ***(19,21,22)***, pol β ***(20)***, and HIV reverse transcriptase ***(23)***. These mutants were further screened for altered properties. A *TrpE65* ochre mutation was used as a secondary screen for pol β mutators ***(24)***. Finally, expression of low-fidelity pol I mutants in this system achieved in vivo mutagenesis with some specificity for a ColE1 plasmid ***(25)***.

In the following chapter we present a protocol for functional complementation of polA12 recA718 cells by E. coli DNA polymerase I. This protocol can be easily adapted for complementation by other DNA polymerases, for mutator screening and for in vivo mutagenesis.

2. Materials

1. JS200 (*recA718 polA12 (ts) uvrA155 trpE65 lon-11 sulA*) competent cells *see* **Notes 1–3**).
2. pHSG576 empty vector control (*see* **Note 4**) and pECpol I construct containing the *E. coli* pol I gene (or another polymerase) under the tac promoter (*see* **Note 5**) in water solution (from mini, midi, or maxiprep).
3. LB (Luria-Bertani) medium.
4. Tetracycline solution: 12.5 mg/mL stock in 50% ethanol, light-sensitive, keep at –20°C.
5. Chloramphenicol solution: 30 mg/mL stock in 100% ethanol, keep at –20°C.
6. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) solution: 100 m*M* stock in water, sterile-filtered, keep at –20°C.
7. 15-mL plastic, 1.5-mL eppendorf tubes, and racks to hold them.
8. Biorad Gene pulser ™ electroporator and 0.2-cm electroporation cuvets.
9. Sterile toothpicks.
10. LB tetracycline (12 μg/mL) and LB tetracycline (12 μg/mL) chloramphenicol (30 μg/mL) plates.
11. Petri dish turntable, 10 μL inoculation loop, and ethanol for flaming.
12. Bunsen burner.
13. 30 and 37°C incubators.
14. 30°C shakers.

3. Methods

1. Combine 40 µL (5×10^9 cells) competent cells with 1 µL of pHSG576 or pECpolI construct in electroporation cuvets.
2. Electroporate the cells (at 400 Ω, 2.20 V, and 2.5 µFD).
3. Resuspend in 1 mL LB (*see* **Note 6**) immediately after electroporation and transfer to a 15-mL plastic tube.
4. Place in a shaker at 30°C for 1 h (*see* **Note 7**).
5. Plate a 1:0 and a $1{:}10^3$ dilution of cells (to ensure single colony formation) on LB tetracycline chloramphenicol plates (*see* **Note 6**).
6. Incubate at 30°C for 24 h (*see* **Note 7**).
7. Pick at least two single colonies from each electroporation into 5 mL LB with tetracycline (12 µg/mL) and chloramphenicol (30 µg/mL) (*see* **Note 8**).
8. Grow overnight in a 30°C incubator (without shaking). The next morning vortex briefly and shake at 30°C until the culture reaches mid-exponential phase (1– 2 h) (*see* **Note 7**).
9. Test for temperature sensitivity in rich medium: Inoculate a spiral of increasing dilution in two LB agar plates with tetracycline and chloramphenicol (*see* **Note 8**). One of the plates needs to be pre-warmed at 37°C and the other plate pre-warmed at 30°C (*see* **Note 9**). This is done placing the loop of the inoculation rod (~ 2×10^6 cells) in the center of a plate and moving the loop toward the periphery as the plate spins. Incubate 1 plate at 37°C (*see* **Note 10**) and the duplicate plate at 30°C for 24–30 h (*see* **Notes 11** and **12**). Some growth in the center of the plate (where there is a high cell density) is expected, but there should be no growth in low cell density areas (*see* **Fig. 1**, **Note 13**).

4. Notes

1. JS200 cells were originally designated SC18-12 ***(9)*** and are tetracycline-resistant.
2. The *uvrA155* genotype means JS200 cells are deficient in nucleotide excision repair. This might contribute to the relative deficiency in polymerase (compared to 5'→3' exonuclease) activity in these cells, as 5'→3' exonuclease activity has a prominent role in nucleotide excision repair ***(26)***.
3. Competent cells can be prepared as follows: single JS200 colonies growing on LB plates with appropriate antibiotic selection (in this case, 12.5 µg/mL tetracycline) are picked into a flask containing 50 mL of LB plus antibiotic and grown at 30°C overnight without shaking (*see* **Notes 6** and **7**). The next morning, cells are shaken for 1 h at 30°C. All 50 mL of bacterial culture are transferred to a flask containing 450 mL LB with antibiotic, and left in the 30°C shaker for 3–4 h (to an OD_{600} of 0.5–1). Cells are chilled on ice for 20 min, pelleted in a Sorval® RC 5B plus centrifuge (10 min at 6000 rpm 4°C), and washed twice in 10% glycerol. The last spin is performed in bottles with conical bottom for easy removal of the supernatant in a Sorval® RC 3B centrifuge (10 min at 4000 rpm 4°C). The pellet is resuspended in ~2 mL 10% glycerol, stored in 120 µL aliquots, and quick-frozen in dry ice.

4. pHSG576 is a low-copy plasmid encoding chloramphenicol resistance ***(27)***. This plasmid carries the pol I-independent pSC100 origin of replication ***(28)***. Providing the test polymerase in a pol I-independent vector is of relevance, as maintenance of a ColE1 plasmid in JS200 cells under restrictive conditions would compete for residual or redundant pol I activity and effectively increase the threshold for functional complementation. On the other hand, increasing the threshold for complementation might be desirable in some cases (for example to minimize the likelihood of reversion [*see* **Note 6**]).
5. pECpol I construction: the entire open reading frame of the pol I gene (*polA*) of *E. coli* DH5α was amplified with primers 5'-ATATATAT*AAGCTT***ATG**GTT CAGATCCCCCAAAATCCACTTATC-3' (initiating methionine in bold) and 5'-ATATATAATG*AATTC***TTA**GTGCGCCTGATCCCAGTTTTCGCCACT (stop codon in bold) and cloned into the *HindIII EcoRI* sites of the pHSG576 polylinker using *HindIII EcoRI* adapters (italics). This places the pol I gene under transcriptional control of the *tac* promoter.
6. Nutrient Broth has been used instead in the work reported in the literature ***(17–19,21)***. In our hands, growth in LB appears to be similar in the rates of loss of temperature-sensitivity or in the strength of the conditional lethal phenotype.
7. Pol I-deficient strains in combination with alterations in RecA, RecB or UvrD are easily overgrown by suppressors or revertants under non-permissive conditions ***(10)***. This problem is less severe for *polA12 recA718* double mutants ***(9)***, but revertants/suppressors still occur at a detectable frequency (about 1 in 500 after overnight culture). To avoid overgrowth by these revertants, we maintain conditions as permissible as possible, growing the cultures at 30°C, and keeping the cell density to less than $OD_{600} = 1$. The temperature sensitivity of these cells should be checked periodically (*see* **step 9** in **Subheading 3.**). Most of the cells that lose temperature sensitivity appear to be suppressors rather than simple revertants and often exhibit a milder but not wild-type phenotype (Tsai, C.-H., personal communication and our own observations). In the *polA12 uvrE502* background one apparent revertant was found to be an intragenic suppressor ***(10)***.
8. Overexpression of the polymerase can be induced at this point by adding 1 m*M* IPTG to the medium. IPTG induction of transcription was required for complementation in the case of pol III α subunit and pol β ***(9,17)***.
9. Pre-warming of the plates is critical. The temperature-sensitive phenotype of JS200 cells (*see* **Fig. 1**) and that of other *polA12 recA*, *polA12 recB*, or *polA12 uvrD* derivatives is only apparent in isolated cells. These cells lose viability quickly (2–4 h) after switching to the restrictive temperature, at least in liquid culture ***(11,13)***. In consequence, for tests or selections that depend on conditional lethality it is essential that the plates achieve the restrictive temperature before the JS200 cells plated on them reach the local cell density that allows survival.
10. Initially 42°C was chosen as the restrictive temperature for functional complementation in JS200 cells ***(9,17,20,29)***. We have since switched to 37°C ***(16,19,22,23,25)***, as we still see strong conditional lethality at this temperature (*see* **ref.** ***18*** for a comparison).

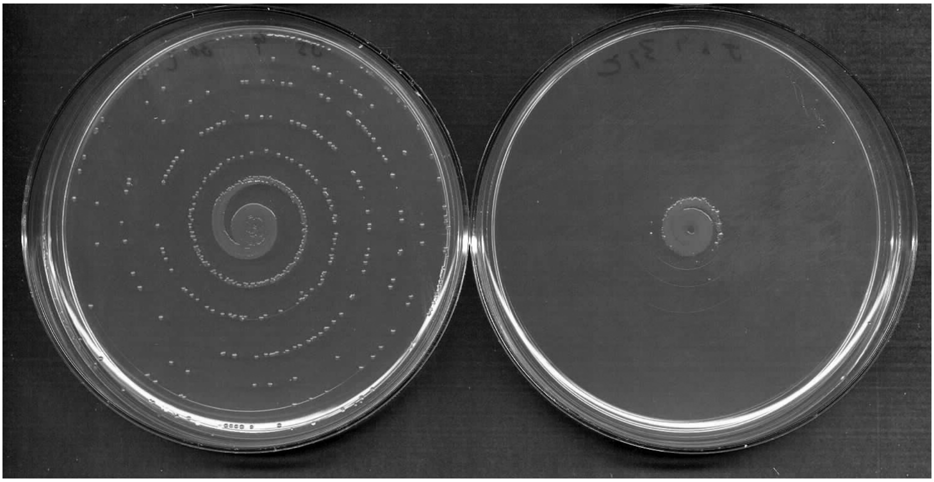

Fig. 1. Spiral assay for temperature sensitivity. *PolA12 rec718* cells were plated and grown as described in **Subheading 3., step 9**. On the left, growth at 30°C, on the right growth at 37°C (Modified from Ern Loh, unpublished).

11. In cases of partial functional complementation plates can be incubated for longer periods of time, up to 48 h, to detect growth at 37°C ***(17–19)***.
12. The plates should be placed upside-down in the incubator to prevent excessive evaporation from the agar.
13. Alternatively, the temperature-sensitivity assay can de done in a quantitative manner by plating approx 10^3 cells (in duplicate or triplicate) instead of inoculating them. Briefly, add 100 µL of a dilution containing 10^4 cells/mL to 4 LB agar plates with tetracycline and chloramphenicol, 2 of them pre-warmed to 30°C, and the other 2 pre-warmed to 37°C. Spin the plate on the turntable while evenly spreading the bacterial dilution with a glass rod (previously flamed in ethanol). Place the duplicate plates in the 30°C and 37°C incubators, and incubate for 24–30 h. No more than 2 or 3 cells should grow at 37°C for every 1000 cells that grow at 30°C.

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