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DNA Polymerase I in Constitutive Stable DNA Replication in *Escherichia coli*

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We examined the effects of mutations in the *polA* (encoding DNA polymerase I) and *polB* (DNA polymerase II) genes on inducible and constitutive stable DNA replication (iSDR and cSDR, respectively), the two alternative DNA replication systems of *Escherichia coli*. The *polA25*::miniTn*10spc* mutation severely inactivated cSDR, whereas *polA1* mutants exhibited a significant extent of cSDR. cSDR required both the polymerase and 5′→3′ exonuclease activities of DNA polymerase I. A similar requirement for both activities was found in replication of the pBR322 plasmid in vivo. DNA polymerase II was required neither for cSDR nor for iSDR. In addition, we found that the lethal combination of an *rnhA* (RNase HI) and a *polA* mutation could be suppressed by the *lexA*(Def) mutation.

Escherichia coli is known to possess two alternative DNA replication systems which are normally repressed but can be activated under certain specific conditions (see reference 2 for a review). One is inducible stable DNA replication (iSDR), which is activated upon the SOS response to DNA damage and can occur in the presence of chloramphenicol and rifampin. Initiation of iSDR requires $recA^+$ and $recBC^+$ (33, 37). It is thought that upon induction of the SOS response, a double-strand break is introduced at *oriM*, the origin of iSDR. The resulting ends are processed by RecBCD enzyme to generate single-stranded DNA with a 3′ end, which is then assimilated into an intact *oriM* site on a homology by the action of RecA recombinase, yielding a D-loop (3). DNA replication can be initiated from D-loops in a manner which depends on the DNA replication priming protein, PriA (37). It has been demonstrated that artificially generated double-strand breaks can trigger recombination-dependent replication in normal cells (not induced for the SOS response) and that *priA* null mutations significantly reduce P1 transduction frequency and sensitize cells to agents that cause double-strand breaks. These observations have led to the proposal that this $recA^+$,$recBC^+$-dependent replication is involved in homologous recombination and double-strand break repair (1, 25, 26, 45).

The second alternative replication system is constitutive stable DNA replication (cSDR), which occurs in *rnhA* mutants lacking RNase HI. RecA protein is likely to catalyze invasion of the duplex by an RNA transcript, leading to formation of an R-loop (14). In *rnhA* mutants, the R-loop is stabilized and becomes an initiation site for cSDR. Initiation of cSDR can occur in the presence of chloramphenicol, which blocks initiation of normal replication at *oriC*. Activation of cSDR in *rnhA* mutants can suppress initiation defects of normal DNA replication. Thus, for example, *dnaA5*(Ts) *rnhA* double mutants are viable at 42°C despite the inactivation of the initiator DnaA protein at the restrictive temperature (30). The RecG helicase which specifically recognizes Holliday intermediates in homologous recombination is proposed to be involved in removal of R-loops (14). Evidence supporting this proposal has been reported (11, 48a). As expected from these observations, mutations in the *recG* gene have also been shown to activate cSDR (14). Furthermore, it is recently demonstrated that a cSDR-like activity can be transiently activated in rapidly growing $rnhA^+$ $recG^+$ cells at the time of cell's entry to stationary phase (15).

E. coli possesses three DNA polymerases, DNA Pol I, Pol II, and Pol III. DNA Pol III, the replicase of *E. coli*, is essential for iSDR (28) and cSDR (22a). Involvement of DNA Pol I and Pol II in iSDR and cSDR has not been extensively examined except for a report which suggested a modulating role of DNA Pol I in iSDR (44). DNA Pol I is involved in excision repair of damaged DNA and in replication of ColE1-type plasmids (for a review, see reference 32). DNA Pol I is a single polypeptide (the *polA* gene product) which possesses three enzymatic activities: a 5′→3′ exonuclease (exo), a 3′→5′ exo, and a 5′→3′ polymerase (pol) located in three distinct domains (32). Limited proteolysis of the protein yields two fragments of unequal sizes: the larger of the two (the Klenow fragment) contains the 3′→5′ exo and pol activities, and the smaller one contains the 5′→3′ exo activity (22). *polA1* is an amber mutation, and this allele encodes a truncated protein which has little pol activity but possesses a nearly normal level of the 5′→3′ exo activity (40). The *polA25*::miniTn*10spc* allele has a miniTn*10* insertion at the end of the coding region and encodes a truncated protein missing the C-terminal 57 amino acid residues (6). This portion of the enzyme includes the last α helix and three β sheets which contain at least two residues crucial for the pol activity (42). It was concluded from a genetic analysis that the PolA25 polymerase lacks the pol activity but retains the 5′→3′ exo activity (4). In this study, we have compared the effects of the *polA1*, *polA25*::miniTn*10spc*, and Δ*polA*::*kan* mutations on cSDR and iSDR. DNA Pol II, the *polB* gene product, has been implicated in repair of oxidative damage and in adaptive mutation (9). Since possible roles of R-loops (46) and iSDR (10, 43) in adaptive mutation have been suggested, we have also examined a *polB* null mutation (*polBΔ1*) in cSDR and iSDR.

MATERIALS AND METHODS

Media and growth conditions. Unless otherwise stated, cells were grown at 37°C with aeration by shaking. Growth was monitored by measuring cell numbers with a particle counter (Particle Data Inc., Elmhurst, Ill.). M9G is M9 salts-glucose medium (39) supplemented with required amino acids (50 μg/ml), thiamine (2 μg/ml), and thymine (8 μg/ml). CAA is M9G supplemented with

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TABLE 1. *E. coli* strains used

Strain	Relevant genotype						Reference, source, or construction[d]
	dnaA	*rnhA*	*polA*	*polB*	*lexA*	Other	
AQ634[a]	+	+	+	+	+		41
AQ666[a]	+	224	+	+	+		41
AQ2547[a]	5	+	+	+	+		Laboratory collection
AQ9013[a]	+	+	Δ::*kan*	+	+		AQ634 × P1.AQ8866, select Km^r, screen UV^s
AQ9016	+	+	Δ::*kan*	+	+	F′ D355A E357A(Cm^r) *zih-35*::Tn*10*	5
AQ10810[a]	+	+	1	+	+	*zih*::Tn*10*	AQ634 × P1.AQ1409, select Tc^r, screen UV^s
AQ10811[a]	+	224	1	+	+	*zih*::Tn*10*	AQ666 × P1.AQ1409, select Tc^r, screen UV^s
AQ10812[a]	+	+	25::*spc*	+	+	*zih-35*::Tn*10*	AQ634 × P1.AQ8534, select Tc^r, screen UV^s
AQ10814[a]	+	+	+	Δ1	+	*zac-3051*::Tn*10*	AQ666 × P1.AQ9239, select Tc^r, screen $H_2O_2^s$
AQ10836[a]	+	224	25::*spc*	+	+	*zih-35*::Tn*10*	AQ666 × P1.AQ8534, select Tc^r, screen UV^s
AQ10838[a]	+	224	+	Δ1	+	*zac-3051*::Tn*10*	AQ666 × P1.AQ9239, select Tc^r, screen $H_2O_2^s$
AQ10850[a]	+	+	25::*spc*	+	+	*zih-35*::Tn*10*	AQ634 × P1.AQ8534, select Tc^r, screen UV^s
AQ10884[a]	5	224	+	+	+	*pro*$^+$	AQ2547 × P1.AQ677, select Pro$^+$, screen Tr L^s
AQ10903[a]	+	+	Δ::*kan*	+	+	F′*polA*$^+$(Cm^r)	AQ9013 × AQ8862, select Tc^r Cm^r
AQ10904[a]	+	+	Δ::*kan*	+	+	F′exo(Cm^r)	AQ9013 × AQ8863, select Tc^r Cm^r
AQ10905[a]	+	+	Δ::*kan*	+	+	F′Klenow(Cm^r)	AQ9013 × AQ8864, select Tc^r Cm^r
AQ10907[a]	5	224	1	+	+	*zih*::Tn*10*	AQ10884 × P1.AQ1409, select Tc^r, screen UV^s
AQ10915[a]	5	224	25::*spc*	+	+	*zih-35*::Tn*10*	AQ10884 × P1.AQ8534, select Tc^r, screen UV^s
AQ10924[a]	+	224	+	+	+	F′vector(Cm^r)	AQ666 × AQ8865, select Tc^r Cm^r
AQ10925[a]	+	224	+	+	+	F′*polA*$^+$(Cm^r)	AQ666 × AQ8862, select Tc^r Cm^r
AQ10926[a]	+	224	+	+	+	F′exo(Cm^r)	AQ666 × AQ8863, select Tc^r Cm^r
AQ10927[a]	+	224	+	+	+	F′Klenow(Cm^r)	AQ666 × AQ8864, select Tc^r Cm^r
AQ10955[a]	5	224	25::*spc*	+	+	F′Klenow(Cm^r) *zih-35*::Tn*10*	AQ10884 × AQ8864, select Tc^r Cm^r
AQ10957[a]	5	224	25::*spc*	+	+	F′exo(Cm^r) *zih-35*::Tn*10*	AQ10884 × AQ8863, select Tc^r Cm^r
AQ10991[b]	5	102	+	+	71::Tn*5*	*recA*$^+$ *sfiA11*	AQ8937 × SL521, select Trp$^+$ Sm^r, screen UV^r
AQ11007[b]	+	102	+	+	71::Tn*5*	*recA*$^+$ *sfiA11*	AQ3476 × SL521, select Trp$^+$ Sm^r, screen UV^r
AQ11047[c]	+	+	Δ::*kan*	+	+	*zih-35*::Tn*10*	AQ4335 × P1AQ9016, select Tc^r, screen UV^s
AQ11048[b]	+	102	Δ::*kan*	+	71::Tn*5*	*zih-35*::Tn*10 sfiA11*	AQ11007 × P1.AQ9016, select Tc^r, screen UV^s
AQ11058[c]	+	+	Δ::*kan*	+	+	F′*polA*$^+$(Cm^r) *zih-35*::Tn*10*	AQ11047 × AQ8862, select Tc^{r}Cm^r
AQ11059[c]	+	+	Δ::*kan*	+	+	F′Klenow(Cm^r) *zih-35*::Tn*10*	AQ11047 × AQ8864, select Tc^{r}Cm^r
AQ11060[c]	+	+	Δ::*kan*	+	+	F′exo(Cm^r) *zih-35*::Tn*10*	AQ11047 × AQ8863, select Tc^{r}Cm^r
AQ11062[b]	5	102	Δ::*kan*	+	71::Tn*5*	*zih-35*::Tn*10 sfiA11*	AQ10991 × P1.AQ9016, select Tc^r, screen UV^s
Other strains used for construction							
AQ677	+	224	+	+	+	*pro*$^+$	Laboratory collection
AQ1409	+	+	1	+	+	*zih*::Tn*10*	20
AQ3476	+	102	+	+	71::Tn*5*	*recA200 sfiA11*	6
AQ4335	+	+	+	+	+		As MC1000 (7)
AQ8534	+	+	25::spc	+	+	*zih-35*::Tn*10*	Laboratory collection
AQ8862	+	+	+	+	+	F′*polA*$^+$(Cm^r) (=JC207)	As RM2425
AQ8863	+	+	+	+	+	F′exo(Cm^r) (=JC219)	As RM2426
AQ8864	+	+	+	+	+	F′Klenow(Cm^r) (=JC222)	As RM2427
AQ8865	+	+	+	+	+	F′vector(Cm^r) (=JC251)	As RM2428
AQ8866	+	+	Δ::kan	+	+	F′ D355A E357(Cm^r) A (=JC386)	As RM2429
AQ8937	5	102	+	+	71::Tn*5*	*recA200 sfiA11*	6
AQ9239	+	+	+	Δ1	+	*zac-3051*::Tn*10*	6
SL521	+	+	+	+	+	HfrKL16 *trp*$^+$	Laboratory collection

[a] Remaining genotype: F$^-$ *thyA his-29 trpA9605 pro ilv metB deoB* (or *-C*).
[b] Remaining genotype: F$^-$ *metE90 trpA9605 thyA708 deo29 lacZ118 lacI22 rnhA102 rpsL.*
[c] Remaining genotype: F$^-$ *araD139* Δ(*araABIOC leu*)*7697* Δ(*lacIPOXY*)*X74 galU galK rpsL.*
[d] Ts, temperature-sensitive growth; Tc^r, Cm^r, Km^r, and Sm^r, resistance to tetracycline, chloramphenicol, kanamycin, and streptomycin, respectively; UV^s and UV^r, sensitive and resistant to UV, respectively; L^s, slow growth on L plates.

Casamino Acids (0.2%; Difco Laboratories, Detroit, Mich.), required amino acids, thiamine, and thymine. LB is L broth (39) supplemented with 0.1% glucose.

Chemicals and isotopes. The chemicals used were from Sigma Chemical (St. Louis, Mo.). [*methyl*-^{3}H]thymidine was from Du Pont Company (Boston, Mass.).

***E. coli* strains and plasmids.** The *E. coli* strains used in this study are listed in Table 1. Strains were constructed by phage P1*vir*-mediated transduction (34). For strains which were sensitive to broth, CAA medium was used for transduction. The Δ*polA*::*kan* mutation was previously described (18). Strains RM2425 (AQ8862) to RM2429 (AQ8866) harboring F′ episomes carrying various alleles of the *polA* gene (18) were a gift of Russell Maurer (35). F′ episomes were introduced to several strains by mating in CAA medium at 30°C for 60 min. The exconjugants were then selected for chloramphenicol resistance (Cm^r) and tetracycline resistance (Tc^r) on CAA plates. AQ10991 was constructed by mating AQ8937 (*dnaA5 rnhA102 recA200 trpA9605* Sm^r) with SL521 (HfrKL16 *recA*$^+$ *trp*$^+$), selecting streptomycin-resistant (Sm^r) Trp$^+$ cells and screening for UV^r. The *polB*Δ*1* mutation was previously described (9).

Measurement of SDR. cSDR and iSDR activities were measured by determining incorporation of [^{3}H]thymidine into the acid-insoluble fraction as described previously (37, 48). Unless otherwise stated, SDR was measured at 37°C.

Determination of transformation frequencies. Cultures were grown in CAA medium, and transformation was carried out by electroporation in an electroporator (Invitrogen Corp., San Diego, Calif.). pBR322 DNA (1.7 μg) or pOC23 DNA (2.5 μg) was mixed with 40 μl of washed and concentrated cells and electroporated. Cell suspensions were plated on CAA plates containing ampicillin (20 μg/ml).

RESULTS

Effects of *polA* and *polB* mutations on cSDR. The *polA25*::miniTn*10spc* mutation was found to severely, if not completely, inhibit cSDR, whereas *polA1* had a moderate in-

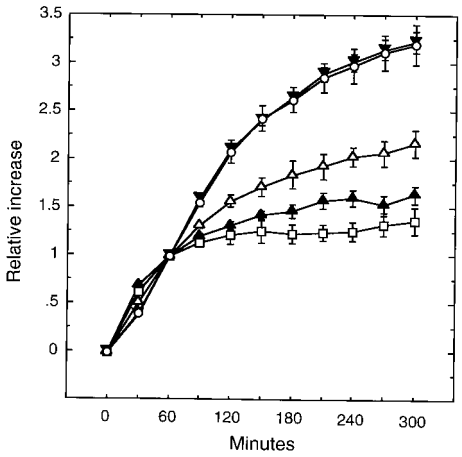

FIG. 1. Effects of *polA* and *polB* mutations on cSDR. AQ634 (*rnhA*$^+$ *polA*$^+$; □), AQ666 (*rnhA224 polA*$^+$; ○), AQ10811 (*rnhA224 polA1*; △), AQ10836 (*rnhA224 polA25*::miniTn*10spc*; ▲), and AQ10838 (*rnhA224 polBΔ1*; ▼) were grown in CAA medium to 1×10^8 to 2×10^8 cells/ml. At time zero, chloramphenicol (final concentration, 150 μg/ml) and [^{3}H]thymidine (5 μCi/10 μg/ml) were added; 100-μl samples were taken at the times indicated, and radioactivity in the acid-insoluble fraction was measured as previously described (48). The relative increase in radioactivity was determined by dividing each cpm value by that of the 60-min sample, which was between 5,000 and 15,000 cpm. The data for AQ634, AQ666, AQ10811, AQ10836, and AQ10838 are averages (± standard errors of means) of two, five, three, three, and five independent determinations, respectively.

hibitory effect and allowed cSDR to occur to a significant extent (Fig. 1). *polA25*::miniTn*10spc* has a miniTn*10* insertion at the end of the *polA* coding region (6), and it is inferred from genetic data that the mutant DNA Pol I lacks the pol activity but retains the 5′→3′ exo activity (4). Thus, this result suggests that cSDR requires the pol activity of DNA Pol I. The result shown in Fig. 2 indicates that the PolA25 activity for cSDR is somewhat temperature dependent: cSDR in this mutant was completely inhibited at 42°C, whereas at 30°C it occurred to a small but significant degree.

cSDR activated in *rnhA* mutants can suppress initiation de-

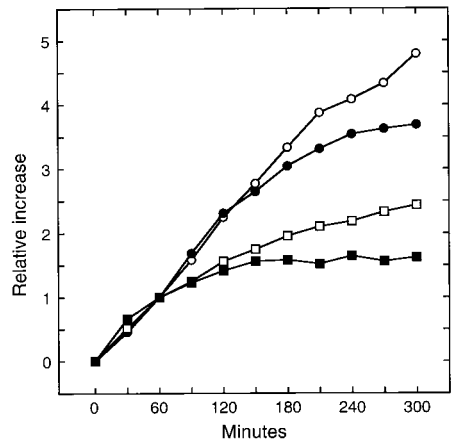

FIG. 2. Temperature-dependent effects of *polA25*::miniTn*10spc* on cSDR. AQ666 (*rnhA224*; ● and ○) and AQ10836 (*rnhA224 polA25*::miniTn*10spc*; ■ and □) were grown in CAA medium to 10^8 cells/ml at 30°C. After addition of chloramphenicol and [^{3}H]thymidine, the cultures were split into two halves; one was incubated at 30°C (open symbols), and the other was incubated at 42°C (closed symbols). Samples were withdrawn at intervals, and radioactivity in the acid-insoluble fraction was determined as for Fig. 1.

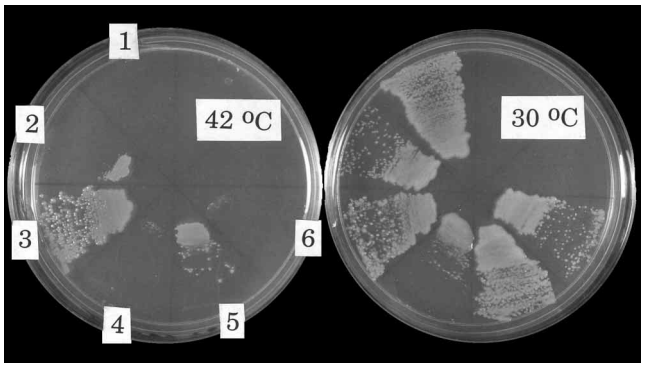

FIG. 3. Temperature-sensitive growth of *dnaA rnhA polA* mutants. Overnight cultures grown in CAA medium (containing 50 μg of chloramphenicol per ml for strains carrying F′ episomes but no IPTG) at 30°C were diluted to ~5×10^8 cells/ml. Aliquots (2 μl) of the diluted cultures were spotted on CAA plates and spread with toothpicks. The plates were incubated at 42°C for 25 h or at 30°C for 39 h. Sectors: 1, AQ2547 (*dnaA5*); 2, AQ10907 (*dnaA5 rnhA224 polA1*); 3, AQ10884 (*dnaA5 rnhA224*); 4, AQ10915 (*dnaA5 rnhA224 polA25*::miniTn*10spc*); 5, AQ10955 (*dnaA5 rnhA224 polA25*::miniTn*10spc*/F′Klenow); 6, AQ10957 (*dnaA5 rnhA224 polA25*::miniTn*10spc*/F′exo).

fects at *oriC* (30). Thus, a *dnaA5*(Ts) *rnhA224* double mutant could grow well at 42°C (Fig. 3). Introduction of the *polA25*::miniTn*10spc* mutation into the *dnaA5*(Ts) *rnhA224* mutant rendered the growth temperature sensitive (Fig. 3). Since AQ10836, an *rnhA224 polA25*::miniTn*10spc* (*dnaA*$^+$) strain, grew at 42°C (data not shown), the temperature sensitivity of the *dnaA5 rnhA224 polA25*::miniTn*10spc* triple mutant must have stemmed from the temperature-sensitive *oriC* initiation, indicating the absence of *rnhA*-mediated suppression due to the *polA25*::miniTn*10spc* mutation. The result corroborates the previous result that *polA25*::miniTn*10spc* severely inactivates cSDR. An F′ factor carrying the Klenow fragment (F′Klenow) complemented the defect and reverted the temperature-sensitive growth to the wild type (Fig. 3). An F′ factor carrying fragment encoding the 5′→3′ exo activity (F′exo) did not complement. These results indicate that cSDR requires the pol activity of DNA Pol I and that the exo activity alone is insufficient. In contrast to the severe *polA25*::miniTn*10spc* effect, *polA1* allowed some growth of the *dnaA5*(Ts) *rnhA224* mutant at 42°C (Fig. 3), reflecting the significant degree of cSDR detected with this mutant (Fig. 1).

Figure 1 includes the results of experiments which show that the *polBΔ1* mutation had no effect on cSDR.

Either 5′→3′ exo or pol activity is sufficient for *rnhA* mutant viability. It was previously demonstrated that *rnhA339*::*cat polA12*(Ts) double mutants were temperature sensitive for growth (27), although *rnhA224 polA12*(Ts) could grow at 42°C (24). Our repeated attempts to construct *rnhA224* Δ*polA*::*kan* double mutants were unsuccessful. Since *rnhA339*::*cat* confers no detectable RNase H activity whereas *rnhA224*, a UGA nonsense mutation, may give rise to a trace amount of activity due to occasional readthrough (16), these observations suggest that complete inactivation of either of the *rnhA* or *polA* gene with simultaneous partial inactivation of the other is lethal. To investigate which activity of DNA Pol I becomes essential for viability in the absence of RNase HI, F′*polA*$^+$, F′exo, and F′Klenow episomes were brought into an *rnhA224* mutant, and introduction of Δ*polA*::*kan* by P1 transduction into these constructs was attempted (Table 2). Transductants were first selected for a nearby Tn*10* insertion marker (*zih-35*::Tn*10*) and then screened for kanamycin resistance (Km^r) (Δ*polA*::*kan*). When the *rnhA224*/F′*polA*$^+$ strain was used, about 80% of the

TABLE 2. Transduction of Δ*polA*::*kan* into *rnhA224* strains harboring various F′ episomes[a]

Strain	F′ episome	Km^r/Tc^r (%)	UV^s/Tc^r Km^r
AQ10924	F′vector	3/47 (6)	0/3
AQ10925	F′*polA*$^+$	365/450 (81)	8/8
AQ10926	F′exo	20/142 (14)	5/7
AQ10927	F′Klenow − IPTG	21/230 (9)	1/11
AQ10927	F′Klenow + IPTG	441/1,440 (31)	24/24

[a] Derivatives of AQ666 (*rnhA224*) harboring various F′ episomes were transduced with P1 lysate grown on AQ9016 (Δ*polA*::*kan zih-35*::Tn*10*). Tc^r transductants were selected and screened for Km^r.

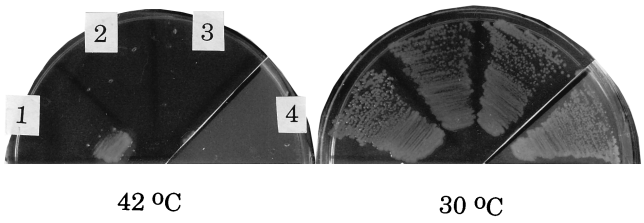

FIG. 4. Temperature sensitivity of *dnaA5*(Ts) *rnhA102* Δ*polA* mutants harboring F′ episomes. The plates were prepared as described for Fig. 3 except that the medium was M9G containing chloramphenicol (50 μg/ml). Sectors: 1, AQ11062 plus *F′polA*$^+$; 2, AQ11062 plus F′vector; 3, AQ11062 plus F′Klenow; 4, AQ11062 plus F′exo.

Tc^r transductants were Km^r, as expected from the linkage between the Tn*10* marker and *polA*, and all Tc^r Km^r transductants tested were UV^s (Table 2). With the *rnhA224*/F′exo strain, 14% of Tc^r transductants were Km^r and most Km^r transductants tested were UV^s, indicating that Δ*polA* could be successfully introduced into a strain containing F′exo. In contrast, with *rnhA224*/F′Klenow, less than 10% were Km^r, and only 1 of 11 Tc^r Km^r transductants tested was UV^s. The expression of the Klenow fragment on the F′ factor is driven by a *lac* promoter (18). To see if an increase in Klenow fragment expression might ease the introduction of Δ*polA*, the experiment was repeated with use of medium containing isopropylthiogalactopyranoside (IPTG; 5×10^{-3} M). In the presence of IPTG, 31% were Km^r and all of the Tc^r Km^r transductants tested were UV^s. With a control (*rnhA224* F′vector) strain, a few of the Tc^r transductants were Km^r, none of which were UV^s (Table 2). These results indicate that in the absence of RNase HI activity, either the exo or pol activity of DNA Pol I is sufficient for viability. This is consistent with the fact that *rnhA224 polA25*::miniTn*10spc* could be constructed as described above (Fig. 1).

***lexA*(Def) suppresses the *rnhA polA* lethality.** The derepression of the LexA regulon by a *lexA*(Def) mutation was previously demonstrated to suppress the lethal effect of a *recA polA* combination (5). In this study, we found that the presence of *lexA*(Def) in *rnhA102* mutants permits introduction of Δ*polA* (e.g., AQ11048 in Table 1). Therefore, derepression of the LexA regulon also suppresses the *rnhA polA* lethality.

Both the exo and pol activities of DNA Pol I are required for cSDR. We constructed a *dnaA5*(Ts) *rnhA102 lexA*(Def) mutant and introduced Δ*polA*. The resultant strain (AQ11062) was found to be temperature sensitive. Presumably, the temperature sensitivity arose from the defect in the *oriC* system, which was rendered temperature sensitive due to *dnaA5* and from the defective *oriK* system (cSDR) which was inoperative due to Δ*polA*. The temperature sensitivity of the strain indicates that *lexA*(Def) does not suppress the requirement of DNA Pol I for cSDR although *lexA*(Def) can alleviate the lethal effect of the *rnhA polA* combination. This mutant strain permitted us to test whether the F′Klenow or F′exo episome complements the Δ*polA* defect in cSDR. Figure 4 shows that neither episome complemented the defect although F′*polA*$^+$ rendered the strain temperature resistant as expected. The addition of IPTG to the medium did not change the outcome for the F′Klenow strain (data not shown). These results indicate that neither exo nor pol activity alone is sufficient to rectify the Δ*polA* defect in cSDR. We conclude that cSDR requires both the exo and pol activities of DNA Pol I.

Pol I function requirements in UV damage repair and pBR322 replication. *polA* mutants are sensitive to DNA-damaging agents such as UV radiation and methyl methanesulfonate and fail to support replication of ColE1-type plasmids such as pBR322. To gain some insights into the requirement of DNA Pol I activities for cSDR, we examined, for comparison, DNA Pol I function requirements for UV damage repair and pBR322 replication. The results shown in Fig. 5A indicate that *polA1* and *polA25*::miniTn*10spc* mutants were as sensitive to UV light as the Δ*polA* mutant despite their different effects on cSDR (Fig. 1 and 3). The presence of F′Klenow made the Δ*polA* mutant more resistant to UV than its absence, indicating that the pol activity alone partially restores excision repair (Fig. 5B). Increased synthesis of the Klenow fragment further improved the repair capacity. On the other hand, F′exo did not alleviate the UV sensitivity of Δ*polA* (Fig. 5B), indicating that the exo activity alone is not sufficient for excision repair.

The ability of *polA* strains to support pBR322 replication was also tested by transformation, with pOC23 (a minichromosome [36]), which does not require DNA Pol I activities for replication, as a control (Table 3). Clearly, pBR322 replication required both the pol and exo activities of DNA Pol I. Increased synthesis of the Klenow fragment made no difference in this case.

Effects of *polA* and *polB* mutations on iSDR. The various *polA* mutations were examined for the effects on iSDR induced by 60-min thymidine starvation (Fig. 6). iSDR was strongly induced in the *polA1* mutant. The iSDR activity in *polA25*::miniTn*10spc* was somewhat reduced but was not inhibited. When thymidine starvation of the *polA25*::miniTn*10* mutant was extended to 90 min, the activity was significantly increased (Fig. 6). Similar results were obtained when iSDR was induced by UV irradiation (data not shown).

No effect was detected for the *polBΔ1* mutation when iSDR

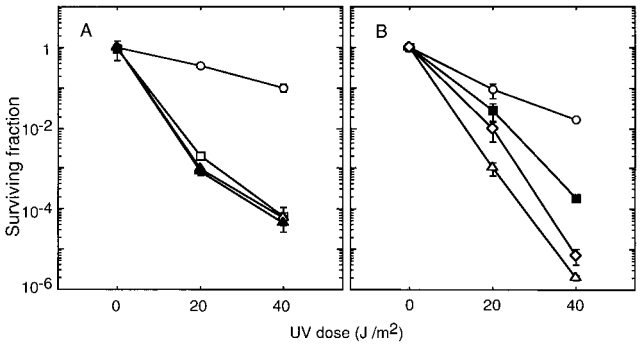

FIG. 5. UV sensitivity of *polA* mutants. (A) AQ634 (*polA*$^+$; ○), AQ10810 (*polA1*; □), AQ10812 (*polA25*::miniTn*10spc*; △), and AQ9013 (Δ*polA*; ▲) were grown in CAA medium to 2×10^8 cells/ml and irradiated with UV light. (B) AQ10903 (Δ*polA* F′*polA*$^+$; ○), AQ10905 (Δ*polA* F′Klenow with IPTG; ■), AQ10905 (Δ*polA* F′Klenow without IPTG; ◇), and AQ10904 (Δ*polA* F′exo; △).

TABLE 3. Transformation frequencies with pBR322 and pOC23

Strain	F′ episome	No. of transformants/μg of DNA		pBR322/pOC23
		pBR322	pOC23	
AQ11058	F′*polA*$^+$	9.24×10^3	1.07×10^2	8.64×10^1
AQ11059	F′Klenow	$<5.88 \times 10^{-1}$	0.708×10^2	$<8.31 \times 10^{-3}$
AQ11059	F′Klenow + IPTG	$<5.88 \times 10^{-1}$	0.566×10^2	$<1.04 \times 10^{-2}$
AQ11060	F′exo	$<5.88 \times 10^{-1}$	4.88×10^2	$<1.20 \times 10^{-3}$

was induced by thymidine starvation (Fig. 6) or by UV irradiation (data not shown).

DISCUSSION

cSDR can occur in the presence of chloramphenicol but is inhibited by addition of rifampin, indicating a requirement for transcription (23, 49). It was proposed that an R-loop, a structure deriving from a transcript hybridizing to the template DNA strand and thereby displacing the opposite strand, becomes a site of initiation of chromosome replication (2, 49). RecA protein is known to act in a step in initiation of cSDR (29) and is likely to be involved in generation of such an R-loop (14). Recently, we have obtained in vitro evidence that RecA protein can indeed catalyze assimilation of an RNA transcript into the duplex (19). The R-loop can be primed for semiconservative DNA replication by a PriA-catalyzed priming system, which loads the DnaB replicative helicase and DnaG primase on the R-loop (2, 38). Marker frequency analysis revealed several sites (termed *oriK*s) on the chromosome from which cSDR can be initiated in *rnhA* mutants (8). Since the marker frequencies of the sequences flanking a peak (*oriK*) taper off symmetrically in both sides (8), the replication is likely to proceed bidirectionally.

In this study, we have demonstrated that DNA Pol I is essential for cSDR. The results strongly suggest that both the pol and 5′→3′ exo activities of the polymerase are required.

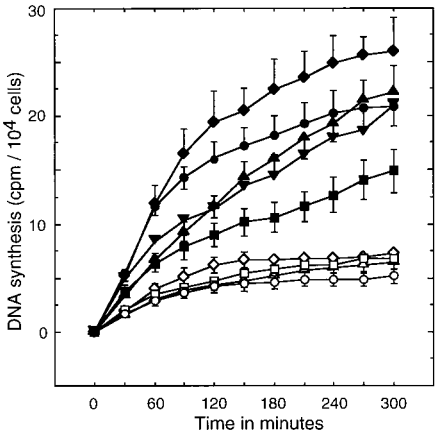

FIG. 6. Induction of iSDR in *polA* and *polB* mutants by thymidine starvation. Cultures of AQ634 (*polA*$^+$; ○ and ●), AQ10810 (*polA1*; △ and ▲), AQ10850 (*polA25*::miniTn*10spc*; □ and ■), and AQ10814 (*polB*Δ*1*; ◇ and ◆) were grown in CAA medium to 10^8 cells/ml. A portion (solid symbols) was subjected to thymidine starvation for 60 min (except for the second portion of AQ10850 [▼] which was starved for 90 min), and another portion (open symbols) was not starved. iSDR was measured in the presence of [^{3}H]thymidine (5 μCi/10 μg/ml) and chloramphenicol (150 μg/ml) as described previously (34). The data for AQ634, AQ10810, AQ10850, and AQ10814 are averages (± standard errors of means) of four, two, three, and two independent determinations, respectively.

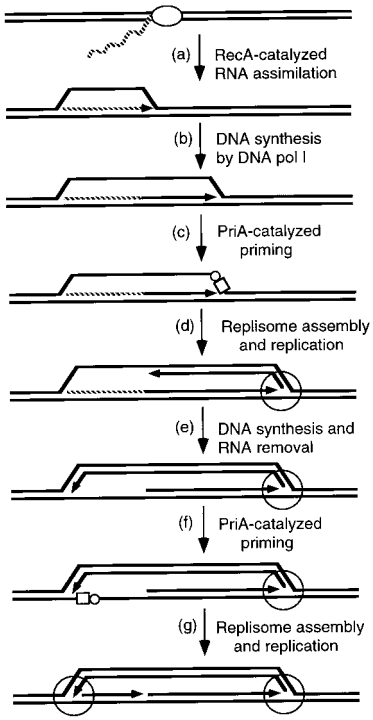

FIG. 7. A model for initiation of bidirectional replication at *oriK*. Solid and stippled lines represent DNA and RNA strands, respectively, with small arrows indicating 3′ hydroxyl ends. Ovals, small squares, and small circles designate RNA polymerase, DnaB helicase, and DnaG primase, respectively. Large circles, replication forks.

Based on the present as well as previous findings, we wish to propose the following mechanism for initiation of bidirectional replication at an *oriK* site (Fig. 7). A transcript is assimilated into the duplex by the strand assimilation activity of RecA protein to form an R-loop (step a). This structure is normally recognized by RNase HI and efficiently removed, but in *rnhA* mutants it is stabilized and persists for a period of time. DNA Pol I then synthesizes DNA from the 3′ end of the hybridizing RNA, enlarging the loop (step b). The PriA-catalyzed priming reaction loads the DnaB helicase, with which DnaG primase interacts (step c). A replication fork can then be established by replisome assembly at the site and semiconservative replication begins in one direction (step d). Meanwhile, a DNA Pol I molecule extends the 3′ end of the newly synthesized lagging strand while another molecule removes the hybridizing RNA with its 5′→3′ exo activity (step e). These two reactions are not necessary to be simultaneous or sequential; either one of the two can precede the other. PriA-catalyzed priming followed by replisome assembly then establishes a second replication fork which replicates the chromosome in the opposite direction (steps f and g). According to this model, DNA Pol I plays a crucial role in initiation of bidirectional replication at *oriK*.

The requirement for RecA in cSDR can be bypassed by derepression of the LexA regulon by introduction of a *lexA*(Def) mutation, leading to activation of the Rip (RecA-independent process) bypass pathway (6, 48). Thus, *dnaA*::Tn*10 rnhA102 recA*(Ts) *lexA*(Def) quadruple mutants are viable at 42°C despite the nonfunctional *oriC* system due to *dnaA*::Tn*10* and despite the inactivation of the *oriK* system by *recA*(Ts) at the restrictive temperature. To gain some insights into the Rip pathway, a mutation which rendered the quadruple mutant temperature sensitive was isolated and designated *polA25*::

miniTn*10spc* (6). A genetic manipulation to replace *recA*(Ts) with *recA*$^+$ in this multiple mutant by Hfr conjugation yielded a strain (AQ8876) which was temperature resistant. Based on these results, it was concluded that *polA25*::miniTn*10spc* inactivated the Rip pathway (6). In this study, we found that the *polA25*::miniTn*10spc* mutation inactivates cSDR in *recA*$^+$ strains in a temperature-dependent manner (Fig. 1 and 2) and renders a *dnaA*(Ts) *rnhA224* (*recA*$^+$ *lexA*$^+$) strain temperature sensitive (Fig. 3), indicating that the *polA* mutation directly inactivates cSDR. In light of this finding, it is most likely that the particular *recA*$^+$ derivative (AQ8876) which showed temperature-resistant growth acquired an additional mutation(s) which permitted growth at the restrictive temperature. Consistent with this interpretation, P1 transduction of a strain [*dnaA5 rnhA102 recA*(Ts) *lexA*(Def) *polA25*::miniTn*10spc*] with lysate grown on a strain of *srlC300*::Tn*10 recA*$^+$ (>90% linked) did not yield temperature-resistant Tc^r transductants (3a). The temperature-dependent effect of *polA25*::miniTn*10spc* on cSDR (Fig. 2) explains why cSDR in the *dnaA*::Tn*10 rnhA102 recA* (Ts) *lexA*(Def) quadruple mutant was temperature sensitive (6).

The function of DNA Pol I required for cSDR involves both the pol and 5′→3′ exo activities (Fig. 4). Similar requirements for both activities are seen in excision repair after UV irradiation (31) (Fig. 5B) and in pBR322 plasmid replication in vivo (Table 3). The requirement for the exo as well as pol activities in excision repair was previously demonstrated with *polA480ex* (formally *polAex1*) mutants defective in the exo activity, and the requirement was interpreted to mean that the lack of the exo activity yields an unligatable product during gap filling by DNA Pol I after an excision event by UvrABC (50). The partial restoration of UV resistance to Δ*polA* mutants by an enhanced synthesis of the Klenow fragment (Fig. 5B) suggests presence of a factor which functionally substitutes for the lack of the exo activity of DNA Pol I.

In contrast to the present result (Table 3), the exo activity of DNA Pol I was previously suggested to be dispensable for ColE1-type plasmid replication in vivo (12, 47). It is likely, however, that the residual exo activity in the *polA480ex* mutants used in these studies may have been sufficient for the requirement. In fact, the Klenow fragment was demonstrated to be inactive in an in vitro ColE1 DNA synthesis (17), suggesting a requirement for the exo activity. The reason for the exo requirement in plasmid replication is not known. As the defect of a *polA1* cell extract could not be complemented by addition of the Klenow fragment in the in vitro ColE1 DNA synthesis, the possibility that an intact DNA Pol I molecule is required, perhaps, for a specific concerted action of both activities was suggested (17). In the case of cSDR, the lack of the pol activity in the *polA25*::miniTn*10spc* mutant can be complemented by F′Klenow (Fig. 3). Therefore, the structural intactness of the polymerase is not essential. The exo activity of DNA Pol I is perhaps necessary for the removal of the hybridizing RNA in an R-loop as proposed above.

It is striking that the *polA1* mutant is as sensitive to UV radiation as *polA25*::miniTn*10spc* and Δ*polA* mutants (Fig. 5A) yet displays a significant degree of cSDR (Fig. 1 and 3). As the *polA1* mutation is known to be leaky (21), the observations suggest that the amount of the DNA Pol I function required is much less in cSDR than in excision repair.

A combination of a *recA* and *polA* mutation is lethal (13). Both the pol and exo activities of DNA Pol I are essential for the viability of *recA* mutants (5). We previously proposed that the *recA polA* lethality culminates from the failure in repair of double-strand breaks, in the absence of RecA function, which the mutant suffers as a result of the defective Okazaki fragment processing due to the DNA Pol I defect (5). The lethality can be suppressed by a *lexA*(Def) mutation which activates a RecA-independent repair pathway capable of compensating for the RecA defect. A combination of an *rnhA* and a *polA* mutation is also lethal (27). In this study, we have found that either the exo or the pol activity of DNA Pol I alone is sufficient for the viability of *rnhA* mutants (Table 2). It was proposed that lesions (e.g., double-strand breaks) deriving from the accumulating R-loops in the absence of RNase HI fail to be repaired because a repair pathway requiring DNA Pol I is defective in the double mutant (27). It is also possible that the lesions which derive both from the accumulating R-loops and from the defective Okazaki fragment processing overwhelm the RecA-dependent recombination repair system. Since the *rnhA polA* lethality, like the *recA polA* lethality, can be suppressed by the *lexA*(Def) mutation (this study), it is likely that the RecA-independent repair pathway augments the RecA-dependent recombination repair, thus enhancing the chance of survival for the double mutant cells.

It is clear that DNA Pol II is required neither for cSDR nor for iSDR (Fig. 1 and 6). The *polB* null mutation stimulates adaptive mutation, suggesting that in the absence of DNA Pol II, another replication system with less fidelity might be involved in the process (9). It is possible that either iSDR or cSDR is such a replication system (10, 43).

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REFERENCES

1. **Asai, T., D. B. Bates, and T. Kogoma.** 1994. DNA replication triggered by double-stranded breaks in E. coli: dependence on homologous recombination functions. Cell **78:**1051–1061.
2. **Asai, T., and T. Kogoma.** 1994. D-loops and R-loops: alternative mechanisms for the initiation of chromosome replication in *Escherichia coli*. J. Bacteriol. **176:**1807–1812.
3. **Asai, T., S. Sommer, A. Bailone, and T. Kogoma.** 1993. Homologous recombination-dependent initiation of DNA replication from DNA damage-inducible origins in *Escherichia coli*. EMBO J. **12:**3287–3295.

3a. **Cao, Y., and T. Kogoma.** Unpublished data.

4. **Cao, Y., and T. Kogoma.** 1993. Requirement for the polymerization and 5′→3′ exonuclease activities of DNA polymerase I in initiation of DNA replication at *oriK* sites in the absence of RecA in *Escherichia coli rnhA* mutants. J. Bacteriol. **175:**7254–7259.
5. **Cao, Y., and T. Kogoma.** 1995. The mechanism of *recA polA* lethality: suppression by RecA-independent recombination repair activated by the *lexA*(Def) mutation in *Escherichia coli*. Genetics **139:**1483–1494.
6. **Cao, Y., R. R. Rowland, and T. Kogoma.** 1993. DNA polymerase I and the bypassing of RecA dependence of constitutive stable DNA replication in *Escherichia coli rnhA* mutants. J. Bacteriol. **175:**7247–7253.
7. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138:**179–207.
8. **de Massy, B., O. Fayet, and T. Kogoma.** 1984. Multiple origin usage for DNA replication in *sdrA* (*rnh*) mutants of *Escherichia coli* K12: initiation in the absence of *oriC*. J. Mol. Biol. **178:**227–236.
9. **Escaceller, M., J. Hicks, G. Gudmundsson, G. Trump, D. Touati, S. Lovett, P. L. Foster, K. McEntee, and M. Goodman.** 1994. Involvement of *Escherichia coli* DNA polymerase II in response to oxidative damage and adaptive mutation. J. Bacteriol. **176:**6221–6228.
10. **Foster, P. L.** 1993. Adaptive mutation: the uses of adversity. Annu. Rev. Microbiol. **47:**467–504.
11. **Fukuoh, A., H. Iwasaki, and H. Shinagawa.** 1997. ATP-dependent resolution of the ColE1 replication origin by *Escherichia coli* RecG protein, a Holliday junction-specific helicase. EMBO J. **16:**203–209.
12. **Grindley, N. D. F., and W. S. Kelly.** 1976. Effects of different alleles of the *E. coli polA* gene on the replication of non-transferring plasmids. Mol. Gen. Genet. **143:**311–318.
13. **Gross, J. D., J. Grunstein, and E. M. Witkin.** 1971. Inviability of *recA*-derivatives of the DNA polymerase mutant of De Lucia and Cairns. J. Mol. Biol. **58:**631–634.

14. **Hong, X., G. W. Cadwell, and T. Kogoma.** 1995. *Escherichia coli* RecG and RecA proteins in R-loop formation. EMBO J. **14:**2385–2392.
15. **Hong, X., G. W. Cadwell, and T. Kogoma.** 1996. Activation of stable DNA replication in rapidly growing *Escherichia coli* at the time of entry to stationary phase. Mol. Microbiol. **21:**953–961.
16. **Itaya, M., D. McKelvin, S. Chatterjie, and R. J. Crouch.** 1991. Selective cloning of genes encoding RNase H from *Salmonella typhimurium*, *Saccharomyces cerevisiae* and *Escherichia coli rnh* mutants. Mol. Gen. Genet. **227:** 438–445.
17. **Itoh, T., and J. Tomizawa.** 1978. Initiation of replication of plasmid ColE1 DNA by RNA polymerase, ribonuclease H, and DNA polymerase I. Cold Spring Harbor Symp. Quant. Biol. **48:**409–417.
18. **Joyce, C. M., and N. D. Grindley.** 1984. Method for determining whether a gene of *Escherichia coli* is essential: application to the *polA* gene. J. Bacteriol. **158:**636–643.
19. **Kasahara, M., D. B. Bates, and T. Kogoma.** Unpublished data.
20. **Kelly, W. S.** 1980. Mapping of the *polA* locus of *Escherichia coli* K-12: genetic fine structure of the cistron. Genetics **95:**15–38.
21. **Kelly, W. S., and C. M. Joyce.** 1983. Genetic characterization of early amber mutations in the *E. coli polA* gene and purification of the amber peptides. J. Mol. Biol. **164:**529–560.
22. **Klenow, H., and I. Henningsen.** 1970. Selective elimination of the exonuclease activity of the deoxyribonucleic acid polymerase from *Escherichia coli* B by limited proteolysis. Proc. Natl. Acad. Sci. USA **65:**168–175.

22a. **Kogoma, T.** Unpublished data.

23. **Kogoma, T.** 1978. A novel *Escherichia coli* mutant capable of DNA replication in the absence of protein synthesis. J. Mol. Biol. **121:**55–69.
24. **Kogoma, T.** 1984. Absence of RNase H allows replication of pBR322 in *Escherichia coli* mutants lacking DNA polymerase I. Proc. Natl. Acad. Sci. USA **81:**7845–7849.
25. **Kogoma, T.** 1996. Recombination by replication. Cell **85:**625–627.
26. **Kogoma, T., G. W. Cadwell, K. G. Barnard, and T. Asai.** 1996. The DNA replication priming protein, PriA, is required for homologous recombination and double-strand break repair. J. Bacteriol. **178:**1258–1264.
27. **Kogoma, T., X. Hong, G. W. Cadwell, K. G. Barnard, and T. Asai.** 1993. Requirement of homologous functions for viability of the *Escherichia coli* cell that lacks RNase HI and exonuclease V activities. Biochimie **75:**89–99.
28. **Kogoma, T., and K. G. Lark.** 1975. Characterization of the replication of *Escherichia coli* DNA in the absence of protein synthesis: stable DNA replication. J. Mol. Biol. **94:**243–256.
29. **Kogoma, T., K. Skarstad, E. Boye, K. von Meyenburg, and H. B. Steen.** 1985. RecA protein acts at the initiation of stable DNA replication in *rnh* mutants of *Escherichia coli* K12. J. Bacteriol. **163:**439–444.
30. **Kogoma, T., and K. von Meyenburg.** 1983. The origin of replication, *oriC*, and the *dnaA* protein are dispensable in stable DNA replication (*sdrA*) mutants of *Escherichia coli* K12. EMBO J. **2:**463–468.
31. **Konrad, E. B., and I. R. Lehman.** 1974. A conditional mutant of *Escherichia coli* K12 defective in the 5′→3′ exonuclease associated with DNA polymerase I. Proc. Natl. Acad. Sci. USA **71:**2048–2051.
32. **Kornberg, A., and T. A. Baker.** 1992. DNA replication. W. H. Freeman and Company, New York, N.Y.
33. **Lark, K. G., and C. A. Lark.** 1978. *recA*-dependent DNA replication in the absence of protein synthesis: characteristics of a dominant lethal mutation, *dnaT*, and requirement for *recA*$^+$ function. Cold Spring Harbor Symp. Quant. Biol. **48:**537–549.
34. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1:**190–206.
35. **Lifsics, M. R., E. D. Lancy, Jr., and R. Maurer.** 1992. DNA replication defect in *Salmonella typhimurium* mutants lacking the editing (ε) subunit of DNA polymerase III. J. Bacteriol. **174:**6965–6973.
36. **Magee, T. R., T. Asai, D. Malka, and T. Kogoma.** 1992. DNA damage-inducible origins of DNA replication in *Escherichia coli*. EMBO J. **11:**4219–4225.
37. **Magee, T. R., and T. Kogoma.** 1990. Requirement of RecBC enzyme and an elevated level of activated RecA for induced stable DNA replication in *Escherichia coli*. J. Bacteriol. **172:**1834–1839.
38. **Masai, H., T. Asai, Y. Kubota, K. Arai, and T. Kogoma.** 1994. *Escherichia coli* PriA protein is essential for inducible and constitutive stable DNA replication. EMBO J. **13:**5338–5346.
39. **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
40. **Monk, M., and J. Kinross.** 1972. Conditional lethality of *recA* and *recB* derivatives of a strain of *Escherichia coli* K-12 with a temperature-sensitive deoxyribonucleic acid polymerase I. J. Bacteriol. **109:**971–978.
41. **Ogawa, T., G. G. Pickett, T. Kogoma, and A. Kornberg.** 1984. RNase H confers specificity in the *dnaA*-dependent initiation of replication at the unique origin of the *Escherichia coli* chromosome *in vivo* and *in vitro*. Proc. Natl. Acad. Sci. USA **81:**1040–1044.
42. **Polesky, A. H., T. A. Steitz, N. D. F. Grindley, and C. M. Joyce.** 1990. Identification of residues critical for the polymerase activity of the Klenow fragment of DNA polymerase I from *Escherichia coli*. J. Biol. Chem. **265:** 14579–14589.
43. **Rosenberg, S. M., R. S. Harris, and J. Torkelson.** 1995. Molecular handles on adaptive mutation. Mol. Microbiol. **18:**185–189.
44. **Ruscitti, T., and S. Linn.** 1992. DNA polymerase I modulates inducible stable DNA replication in *Escherichia coli*. J. Bacteriol. **174:**6311–6313.
45. **Sandler, S. J., H. S. Samra, and A. J. Clark.** 1996. Differential suppression of *priA2*::*kan* phenotypes in *Escherichia coli* K-12 by mutations in *priA*, *lexA*, and *dnaC*. Genetics **143:**5–13.
46. **Stahl, F. W.** 1992. Unicorn revised. Genetics **132:**865–867.
47. **Tacon, W., and D. Sharratt.** 1976. ColE1 plasmid replication in DNA polymerase I-deficient strains of *Escherichia coli*. Mol. Gen. Genet. **147:**331–335.
48. **Torrey, T. A., and T. Kogoma.** 1987. Genetic analysis of constitutive stable DNA replication in *rnh* mutants of *Escherichia coli* K12. Mol. Gen. Genet. **208:**420–427.

48a. **Vincent, S. D., A. A. Mahdi, and R. G. Lloyd.** 1996. The RecG branch migration protein of *Escherichia coli* dissociates R-loops. J. Mol. Biol. **264:** 713–721.

49. **von Meyenburg, K., E. Boye, K. Skarstad, L. Koppes, and T. Kogoma.** 1987. Mode of initiation of constitutive stable DNA replication in RNase H-defective mutants of *Escherichia coli* K-12. J. Bacteriol. **169:**2650–2658.
50. **Wahl, A. F., J. W. Hockensmith, S. Kowalski, and R. A. Bambara.** 1983. Alternative explanation for excision repair deficiency caused by the *polAex1* mutation. J. Bacteriol. **155:**922–925.