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Escherichia coli RecG and RecA proteins in R-loop formation

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Escherichia coli rnhA **mutants devoid of RNase HI exhibit constitutive stable DNA replication, cSDR, which is thought to be initiated from R-loops stabilized in the absence of RNase HI. We found that a combination of an *rnhA* and a *recG* mutation is lethal to the cell. *recG* mutations that inactivate the helicase activity of RecG protein and inhibit reverse branch migration of Holliday junctions impart phenotypes resembling those of *rnhA* mutants. Thus, *recG* mutants display cSDR activity, and *recG polA* double mutants are inviable as are *rnhA polA* double mutants. These results suggest that the RecG helicase has a role in preventing R-loop formation. A model that R-loops are formed by assimilation of RNA transcripts into the duplex DNA is discussed. The model further postulates that RecA protein catalyzes this assimilation reaction and that RecG protein counteracts RecA in this reaction, resolves R-loops by its helicase activity, or does both.**

Key words: cSDR/RecA/RecG/R-loops/transcription

Introduction

Normally, a very short RNA–DNA hybrid no more than 10 bp is thought to form during transcription in *Escherichia coli* cells. However, the hybrid is only transient and the nascent transcript dissociates from the template (Yager and von Hippel, 1987; Rice *et al.*, 1991). There is accumulating evidence pointing to the existence of large persistent RNA–DNA hybrids between the transcript RNA and template DNA displacing the opposite DNA strand and giving rise to an R-loop. For example, a precursor primer RNA (RNA II) for ColE1 plasmid replication hybridizes to the template strand in the *ori* region forming an R-loop (Itoh and Tomizawa, 1980). RNase H recognizes the hybrid and cleaves it to generate the primer for subsequent DNA synthesis. At certain specific sites on the chromosome of *E.coli rnhA* mutants which are devoid of RNase HI, persistent R-loops are thought to form and become origins of DNA replication (see below). An involvement of R-loops in initiation of chromosome replication at the origin, oriC, has also been suggested (Baker and Kornberg, 1988; Skarstad *et al.*, 1990). Furthermore, evidence has been presented that persistent R-loops are lethal to the cell if they are not removed (Itaya and Crouch, 1991; Kogoma *et al.*, 1993). How R-loops are formed or what factors are involved in the process is not known at present.

Chromosome replication in *E.coli* is triggered by melting of the duplex at the unique site (*oriC*) on the chromosome. The melting is effected by binding of the DnaA protein, encoded by the *dnaA* gene, to the specific sequence at *oriC* (for a review, see Messer and Weigel, 1995). The process of the duplex opening and subsequent replisome assembly culminating in initiation of chromosome replication requires concomitant protein synthesis and transcription. Thus, chloramphenicol (Cam) or rifampicin (Rif) inhibits initiation at *oriC*. *Escherichia coli* cells can achieve duplex opening for initiation of chromosome replication by two other mechanisms under certain specific conditions. In SOS induced cells, a normally repressed form of DNA replication, termed inducible stable DNA replication (iSDR), is activated (for a review, see Asai and Kogoma, 1994a). iSDR can occur in the presence of both Cam and Rif. Initiation of iSDR occurs at D-loops generated by the actions of the RecBC(D) enzyme and RecA protein (Asai *et al.*, 1993). Thus, *recA* and *recB* mutations completely block iSDR (Magee and Kogoma, 1990; Asai *et al.*, 1993). iSDR is considered to be a special type of the newly discovered DNA replication form (homologous recombination-dependent DNA replication) which is triggered by double strand breaks (Asai *et al.*, 1994a). Major origins (*oriM1* and *oriM2*) of iSDR map in the *oriC* and *terC* regions of the chromosome, and one of them (*oriM1*) is located within the minimal *oriC* (Magee *et al.*, 1992; Asai *et al.*, 1994b).

In *rnhA* mutants, another form of DNA replication, termed constitutive stable DNA replication (cSDR), is activated (for a review, see Asai and Kogoma, 1994a). Unlike iSDR, cSDR requires transcription and is therefore inhibited by Rif, though it is resistant to Cam. Like iSDR, the RecA recombinase is essential for cSDR (Kogoma *et al.*, 1994). RecA has been suggested to act at a step in initiation (Kogoma *et al.*, 1985). In contrast to the demonstrated involvement of homologous recombination functions in iSDR, mutations in other *rec* genes including *recB*, *recC*, *recF*, *recJ*, *ruvA*, *ruvB* and *ruvC* do not affect cSDR activity (Kogoma *et al.*, 1994). The dispensability of these *rec* gene products suggests that RecA is uniquely involved in the process of cSDR initiation. It has been proposed that RecA catalyzes annealing between a transcript and the template DNA strand, leading to an R-loop (Cao and Kogoma, 1993; Kogoma *et al.*, 1994). These R-loops become origins (*oriK*s) of chromosome replication, at which DNA synthesis is primed by a ϕX174-type of primosome (Masai *et al.*, 1994).

RecG protein is a helicase that specifically dissociates synthetic X and Y junctions (Lloyd and Sharples, 1993a,b; Whitby *et al.*, 1994) and is thought to catalyze reverse branch migration of Holliday junctions (Whitby *et al.*,

1993). The protein effectively opposes the assimilation reaction catalyzed by RecA (Whitby *et al.*, 1993). The *recG258*::Tn*10*mini-*kan* (*recG258*::*kan*) mutation imparts modest sensitivity to UV light, mitomycin C and ionizing radiation, and reduces the efficiency of conjugational recombination and P1 transduction (Lloyd and Buckman, 1991). In this report we describe evidence showing that the combination of a *recG* and an *rnhA* mutation is lethal to the cell. We discuss the possibility that the RecG helicase prevents R-loop formation by counteracting RecA protein which catalyzes the DNA−RNA hybrid-forming reaction.

Results

A combination of recG and rnhA mutations is lethal

In the course of our previous studies on possible roles of homologous recombination on cSDR (Hong and Kogoma, 1993; Kogoma *et al.*, 1994), we had attempted and failed to introduce, by P1 phage-mediated transduction, the *recG285*::*kan* mutation into *rnhA224* mutants although we had been able to combine any other *rec* mutations with the *rnhA224* mutation. This suggested that simultaneous loss of RecG and RNase HI activities is lethal to the cell. We substantiated this supposition by demonstrating that the *recG258*::*kan* mutation can be introduced into *rnhA339*::*cat* mutants only in the presence of active RNase HI, but removal of the RNase HI activity leads to cell death. Thus, the *recG258*::*kan* mutation was successfully transduced into *rnhA*::*cat* mutant cells which harbored plasmid pRNH carrying P_{lac}-*rnhA*$^+$ (see Materials and methods) and expressed the *rnhA* gene from the *lac* promoter in the presence of IPTG. In the absence of IPTG, no transductants were obtained because the *rnhA* gene expression in the strain was repressed by the presence of another plasmid (pLacI^q) which carried the *lacI*^{q} gene (data not shown). The plating efficiency (PE) of one of the transductants (AQ9899) was then examined in the presence of varying amounts of IPTG (Figure 1). With IPTG below 0.1 mM, PE was $<10^{-4}$. As the IPTG concentration increased, the PE gradually improved. Thus, a 100-fold increase in IPTG concentrations resulted in a 10^3-fold increase in the PE. When the pLacI^q plasmid was absent, cells exhibited full viability independent of the IPTG concentration (Figure 1). Therefore, the survival of the *recG258*::*kan rnhA*::*cat* double mutant depends on the expression of the *rnhA* gene but not on IPTG *per se*. At the maximum concentration of IPTG used (i.e. 9 mM), the PE of the double mutant was below 10%, indicating that the *rnhA* gene was not fully expressed at the highest IPTG concentration used.

The dependence of *recG258*::*kan* mutants on RNase HI for viability was also demonstrated by use of two other strains. The first strain was AQ8152 (*rnhA224*) harboring a plasmid (pMAK-rnhA$^+$) which is temperature-sensitive for replication and carries an *rnhA*$^+$ gene. Thus, this strain was RnhA$^+$ at 30°C but RnhA$^-$ at 42°C, the restrictive temperature for plasmid replication. The second strain was AQ7539 [*rnhA199*(Am) *supF6*(Ts)] which synthesized an active RNase HI at 30°C owing to the nonsense suppressor, *supF6*, despite the amber mutation in the *rnhA* gene. At 42°C, the amber suppression failed because of the temperature-sensitive nature of *supF6* (Murakami *et al.*, 1987), and the cells became RnhA$^-$. Both strains could accept the *recG258*::*kan* mutation at 30°C. However, the resulting transductants were unable to grow at 42°C (data not shown).

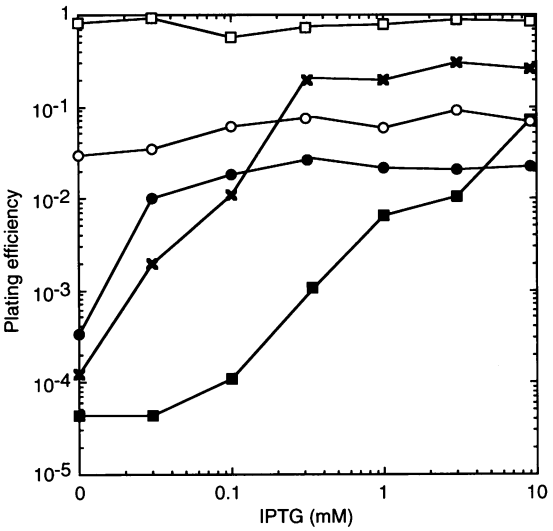

Fig. 1. Plating efficiencies of *rnhA*::*cat recG* and *rnhA*::*cat recB* double mutants in the presence of varying amounts of IPTG. Cultures were grown overnight to stationary phase in the presence of IPTG (9 mM), diluted and plated on CAA plates containing various amounts of IPTG. All plates were incubated for ~42 h before colonies were counted. AQ9899 (■, *recG258*::*kan rnhA*::*cat*/pRNH-Sp/pLacI^q), AQ9897 (□, *recG258*::*kan rnhA*::*cat*/pRNH-Sp), AQ10137 (●, *recG162 rnhA*::*cat*/pRNH-Sp/pLacI^q), AQ10150 (○, *recG162 rnhA*::*cat*/pRNH-Sp/pTKQ27), AQ9768 (×, *recB21 rnhA*::*cat*/pRNH-Km/pLacI^q).

The combination of the *recB21* and *rnhA*::*cat* mutations is also lethal (Itaya and Crouch, 1991; Kogoma *et al.*, 1993). For comparison purposes, a *recB21 rnhA*::*cat* double mutant carrying pRNH was constructed and tested for IPTG dependence (Figure 1). In the absence of IPTG, the strain (AQ9768) had a PE of ~10^{-4}, similar to that of the *recG258*::*kan rnhA*::*cat* strain. However, the PE reached a plateau at 0.3 mM IPTG (Figure 1). The plateau is a maximum PE for the double mutant because *recB* mutants in general have a PE of 20–30% of the wild type (Capaldo-Kimball and Barbour, 1971). This comparison between the *recG258*::*kan rnhA*::*cat* and *recB21 rnhA*::*cat* double mutants indicates that *recG258*::*kan* mutants require more RNase HI activity for survival than do *recB* mutants (see Discussion).

The RecG helicase activity is essential

The *recG162* mutation causes an Ala-to-Val change at the 428th residue of the RecG protein which inactivates the branch-migration activity of the protein *in vitro* (Sharples *et al.*, 1994). We found that the *recG162* mutation also severely reduces the viability of *rnhA*::*cat* mutants. The *recG162* mutation could be introduced into an *rnhA*::*cat* strain only when pRNH carrying P_{lac}-*rnhA*$^+$ and IPTG were present, as was the case with the *recG258*::*kan* mutation (see above). *recG162 rnhA*::*cat* double mutants harboring pRNH and pLacI^q had a PE of ~3×10^{-3} in the absence of IPTG (Figure 1). With a small amount (<0.1 mM) of IPTG added, the PE reached a plateau.

Table I. Chronic SOS induction in *recG* mutants

Strain	Relevant genotype	β-galactosidase (Miller units)
AQ8107	*recG*$^+$	41.17 ± 2.05[a]
AQ8353	*recG258*::*kan*	172.54 ± 4.27
AQ10180	*recG162*	76.55 ± 2.68

[a]An average of two determinations ± standard error.

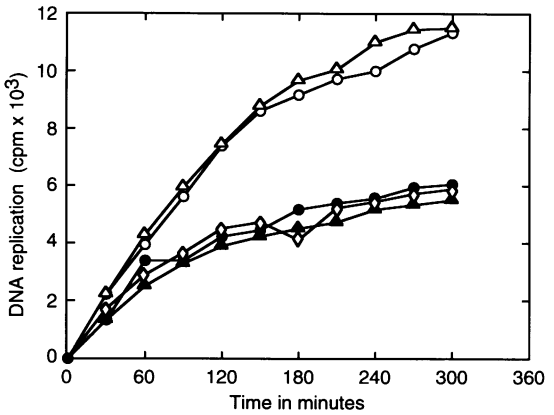

Fig. 2. DNA replication in the presence of Cam. Cultures were grown to ~2×10^8 cells/ml, and a mixture of Cam (150 μg/ml) and [^{3}H]thymine (10 μCi/8 μg/ml) was added. At intervals, samples (100 μl) were taken, and radioactivity in the acid-insoluble fraction was determined as previously described. ○, AQ666 (*rnhA224*); △, AQ8130 (*recG258*::*kan*); ●, AQ10051 (*rnhA224 ΔrecA*); ▲, AQ9964 (*recG258*::*kan ΔrecA*); ◇, AQ634 (wt).

Thus, *recG162* mutants require much less RNase HI activity than do *recG*::*kan* mutants. This is most likely due to the leakiness of the *recG162* mutation; *recG162* mutants are significantly less sensitive to mitomycin C and UV light than *recG258*::*kan* mutants (Sharples *et al.*, 1994; our unpublished results). Because of the failure to resolve occasional homologous recombination intermediates, i.e. Holliday junctions, which block DNA replication fork movement, *recG258*::*kan* mutants are chronically induced for the SOS response (Asai and Kogoma, 1994b; Table I). The *recG162* mutant exhibited a considerably smaller degree of SOS (Table I), again reflecting the leakiness of the mutation. A striking response of the *recG162 rnhA*::*cat* mutant is that PE does not improve with additional IPTG beyond 0.1 mM (Figure 1). Even when RNase HI was fully synthesized (i.e. in the absence of pLacI^q), the PE was $<6\times10^{-2}$.

recG mutants exhibit SDR activities

cSDR has thus far been seen only in *rnhA* mutants. The dependence of *recG258*::*kan* mutants on active RNase HI as demonstrated above suggested that RecG protein might have a function similar to RNase HI, i.e. removal of R-loops. The loss of the RecG activity might therefore activate the normally repressed origins of replication in a manner similar to *rnhA* mutations. This idea was tested by examining *recG258*::*kan* mutants for the ability to replicate DNA in the presence of chloramphenicol (Cam), i.e. SDR. The results shown in Figure 2 indicate that *recG258*::*kan* mutant cells were indeed capable of DNA replication in Cam, and the activity was as high as the

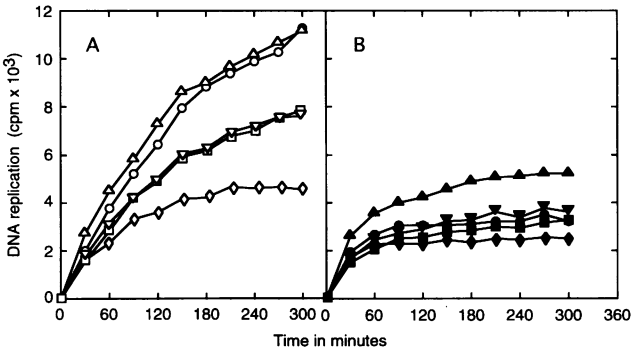

Fig. 3. DNA synthesis in the presence of Cam and/or Rif. Cultures were grown and DNA synthesis was measured as in Figure 2 in the presence of Cam (**A**, open symbols) and in the presence of Cam and Rif (**B**, solid symbols). AQ634, diamonds; AQ666, circles; AQ8130, triangles; AQ10053 and AQ10054 (two clones of *recG258*::*kan recB21*), reverse triangles and squares, respectively.

level exhibited by *rnhA224* mutant cells. The Cam-resistant replication in *recG258*::*kan* cells was as dependent on *recA*$^+$ as the cSDR in *rnhA224* cells (Figure 2).

Figure 3A shows that the Cam-resistant replication in *recG258*::*kan* mutants was significantly reduced by the *recB21* mutation. Since iSDR depends on RecB$^+$, the result suggests a presence of iSDR in the activity. This was confirmed by the detection of a Rif-resistant replication in *recG258*::*kan* mutants (Figure 3B). This Rif-resistant replication was not seen in the *recG recB* mutant, indicating the dependence on RecB$^+$. This is consistent with our previous report that the SOS response is chronically induced in *recG258*::*kan* mutants giving rise to a low level of iSDR which is resistant to Rif and Cam (Asai and Kogoma, 1994b). We estimated that ~50% of the Cam-resistant replication in *recG258*::*kan* mutants could be attributed to iSDR and the remaining 50% to cSDR. The *recG162* mutant also exhibited about one half the SDR activity in the *recG258*::*kan* mutant in the presence of Cam. In contrast to *recG258*::*kan* mutants, most of the SDR activity in the *recG162* mutant was sensitive to Rif (data not shown). This was expected from the much smaller extent of chronic SOS induction in this mutant (Table I).

recG mutants cannot tolerate inactivation of the oriC site or DnaA protein

rnhA mutants can suppress the lethal effects of deletion of the *oriC* site or insertional inactivation of the *dnaA* gene owing to an alternative DNA replication pathway (cSDR) activated by *rnhA* mutations (Kogoma and von Meyenburg, 1983). Our several attempts to introduce Δ*oriC* or *dnaA*::Tn*10* into *recG258*::*kan* strains by P1 transduction failed (data not shown). The *dnaA46*(Ts) mutation was then introduced at 30°C. The resultant *recG258*::*kan dnaA46* mutant (AQ10119) was temperature-sensitive (Figure 4). We conclude that inactivation of normal replication from *oriC* is lethal to *recG258*::*kan* mutants.

A combination of recG258::kan and polA mutations is lethal

The *polA12* mutant allele encodes a DNA polymerase I which is active at 30°C but is severely defective in the

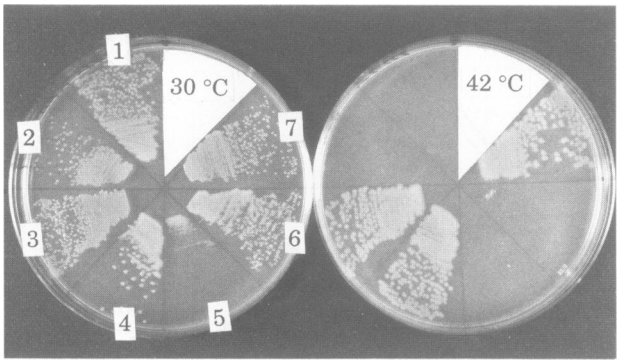

Fig. 4. The presence or absence of growth of *recG258*::*kan dnaA46* and *recG258*::*kan polA12* mutants at 30 or 42°C. Cells were grown at 30°C to saturation, and ~10^5 cells were spotted on CAA plates and spread with toothpicks. One plate was incubated at 30°C and the other at 42°C for ~30 h. 1, AQ705 (*dnaA46*); 2, AQ10119 (*recG258*::*kan dnaA46*); 3, AQ634 (wt); 4, AQ8130 (*recG258*::*kan*); 5, AQ8428 (*rnhA*::*cat polA12*); 6, AQ10075 (*recG258*::*kan polA12*); 7, AQ1524 (*polA12*).

nick translation activity at 42°C (Monk and Kinross, 1972). *rnhA*::*cat polA12* double mutants are temperature-sensitive for growth (Figure 4). This confirms our previous result (Kogoma *et al.*, 1993). The combination of the *recG258*::*kan* and *polA12* mutations also rendered cells temperature-sensitive (Figure 4), indicating that active DNA polymerase I is essential for the viability of *recG258*::*kan* mutants. This conclusion was supported by our failure to combine *polA1*(Am) with *recG258*::*kan* (data not shown).

RNase H activity in recG mutants

The results described above indicate that *recG* and *rnhA* mutants share several common phenotypes. We therefore examined the possibility that *recG* mutations indirectly inactivate the RNase HI activity. We developed an agarose gel-based assay which detects disappearance (dissociation) of ^{32}P-labeled 700-nt RNA hybridized to single-strand circular DNA (see Materials and methods). The procedure could measure not only the degradation of RNA by RNase H activities but also the removal of RNA by RNA−DNA helicase activities. Cell extracts were prepared from saturated cultures. The result is shown in Figure 5. The radioactivity in the bands was quantified, and RNase H activity was estimated from the standard of purified RNase HI (Figure 5). The extract from *rnhA*$^+$ cells gave an activity of 1700 U/mg whereas *rnhA*::*cat* cells exhibited ~50 U/mg. This residual activity in *rnhA*::*cat* cells may reflect the RecG helicase activity as well as RNase HII (Itaya, 1990). Extracts from *recG258*::*kan* and *recG162* cells had ~1250 and 2000 U/mg, respectively. Thus, *recG* mutations did not drastically reduce the RNase H activity.

Discussion

The mechanisms of R-loop formation: models

The initiation of ColE1 plasmid replication requires synthesis of RNA II which hybridizes to the template DNA strand near the *ori* site, forming an R-loop. The hybridized RNA II is cleaved by RNase H to serve as a primer for DNA synthesis by DNA polymerase I (Itoh and Tomizawa, 1980). Thus, formation of a persistent R-loop at *ori* is essential for plasmid replication. Masukata and Tomizawa (1990) proposed that an interaction between a stretch of 6 rG residues located around −265 of RNA II and the 6 dC residues around −20 of the template DNA prevents rewinding of the duplex behind the transcribing RNA polymerase, allowing formation of the persistent hybrid downstream (Figure 6A). This *de novo* formation model accounts for the essentiality of a precise secondary structure of RNA II for hybrid formation (Masukata and Tomizawa, 1986).

Previously we presented a model (Cao and Kogoma, 1993; Kogoma *et al.*, 1994) which is radically different from the *de novo* formation model above. It is hypothesized that a persistent R-loop is formed by invasion of the duplex by a transcript that has just been synthesized and displaced from the template (Figure 6B). Nascent RNA molecules synthesized *in vitro* by *E.coli* RNA polymerase on highly supercoiled circular DNA can hybridize to the template strand if RNA polymerase is removed from the transcription complex (Richardson, 1975). The stabilization of the hybrid involves up to 600 nucleotides of the bound RNA in base-pairing with the DNA. Failure to detect such a long hybrid before the removal of RNA polymerase implicates the polymerase as a factor that dissociates nascent RNA from the DNA as the enzyme moves along the template (Richardson, 1975). The conclusion that R-loops are formed not during but after transcription was also reached by an independent study (Drolet *et al.*, 1994). The involvement of RNA polymerase in hybrid formation was further supported by the isolation of the *rpoB* mutant alleles (encoding altered β subunits of RNA polymerase) that ostensibly enhance or diminish the amount of R-loops in cells (Kogoma, 1994). The model further postulates that the invasion, i.e. assimilation of a transcript into the duplex, is catalyzed by the RecA recombinase (Figure 6B). It has been demonstrated *in vitro* that RecA protein efficiently promotes annealing between RNA and complementary ssDNA (Kirkpatrick *et al.*, 1992; Kirkpatrick and Radding, 1992).

The combination of *recG258*::*kan* and *rnhA*::*cat* is lethal (Figure 1). Like *rnhA* mutants, *recG258*::*kan* mutants exhibit the cSDR phenotype (Figures 2 and 3) and require an active DNA polymerase I for survival (Figure 4). In *recG* mutants, RNase H activity remains as high as in *recG*$^+$ cells (Figure 5). These results strongly suggest that RecG protein has an activity which promotes removal of R-loops and restores the duplex. Since the *recG162* mutation which appears specifically to inactivate the RecG helicase activity (Sharples *et al.*, 1994) also affects the viability of *rnhA*::*cat* strains, this function of RecG involves the helicase activity of the protein. We suggest that RecG protein either counteracts the RecA-catalyzed invasion of a transcript into the duplex or removes RNA from R-loops or both. This would be analogous to the proposed RecG function in the resolution of Holliday junctions (Whitby *et al.*, 1993).

A striking effect of the *recG162* mutation on the plating efficiency of *rnhA*::*cat* cells is that *recG162 rnhA*::*cat* double mutants plate very poorly even in the presence of a full RNase H activity. Although RecG162 protein is defective in dissociation of synthetic junctions, it retains as much DNA binding activity as RecG$^+$ protein (Sharples *et al.*, 1994). It is likely that RecG162 protein, owing to

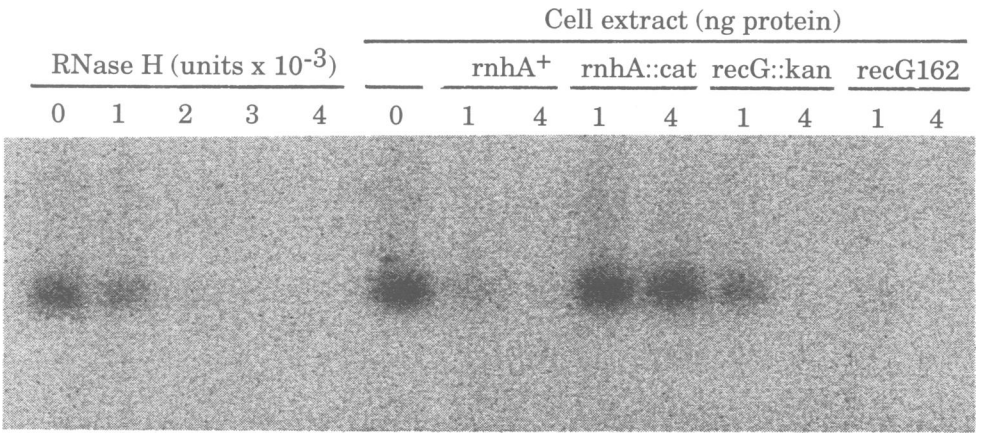

Fig. 5. An autoradiogram of the agarose gel-based RNase H assay. Purified *E.coli* RNase HI ($0–4\times10^{-3}$ U) was incubated with the substrate ([^{32}P]RNA–DNA hybrid) as described in Materials and methods. Similarly, cell extracts (1 and 4 ng protein) of AQ634 (wt), AQ8032 (*rnhA::cat*), AQ8130 (*recG258::kan*) and AQ10092 (*recG162*) were reacted with the substrate. The reaction mixture was then electrophoresed in a 1% agarose gel, and an autoradiogram was made by scanning the gel with a PhosphorImager. A portion of the autoradiogram containing images of substrate bands is shown.

the retained binding activity, interferes with the action of RNase HI at R-loops. This is consistent with the observed dominance of *recG162* over *recG*$^+$, i.e. expression of RecG162 protein rendering *recG*$^+$ cells UV-sensitive (Sharples *et al.*, 1994).

The present results indicating functional interactions between RNase HI and RecG protein, as described above, lend strong support to the invasion model (Figure 6B). Whether or not RecG protein opposes the demonstrated ability of RecA to stimulate annealing between RNA and DNA remains to be tested. R-loop formation detected after transcription of genes on plasmid *in vitro* is very sensitive to the supercoiling status of the plasmid duplex DNA (Drolet *et al.*, 1994). Since topoisomerase I is a crucial factor in adjusting the supercoiling of DNA by its relaxing activity, it has been suggested that this enzyme may play an important role in R-loop formation. Thus, there are at least five factors that may play crucial roles in R-loop formation: RNA polymerase, RecA, RecG, topoisomerase I and RNase HI.

SDR activities in recG mutants

The alternative DNA replication pathway (cSDR) in *rnhA* mutants enables cells to survive the complete loss of normal chromosome replication resulting from *oriC* deletion or *dnaA* inactivation (Kogoma and von Meyenburg, 1983). In contrast, *recG* mutations cannot suppress *dnaA* defects (Figure 4). The reason for the failure is not clear at present. The cSDR activity in *recG258::kan* mutants (about one half that of *rnhA224* mutants) may not be sufficient for survival. Alternatively, the mode of the cSDR (e.g. the origin usage and directions of replication) in *recG258::kan* mutants may not be exactly suitable to replicate the entire chromosome. iSDR is initiated from two major origins (*oriM1* and *oriM2*) located in the *oriC* and *terC* regions (Magee *et al.*, 1992; Asai *et al.*, 1994b). It was also possible, therefore, that iSDR activity in *recG258::kan* mutants interfered with cSDR. This possibility was ruled out because *recG258::kan recB21* double mutants, in which iSDR is not seen (Figure 2B), could not tolerate *dnaA* defects (data not shown).

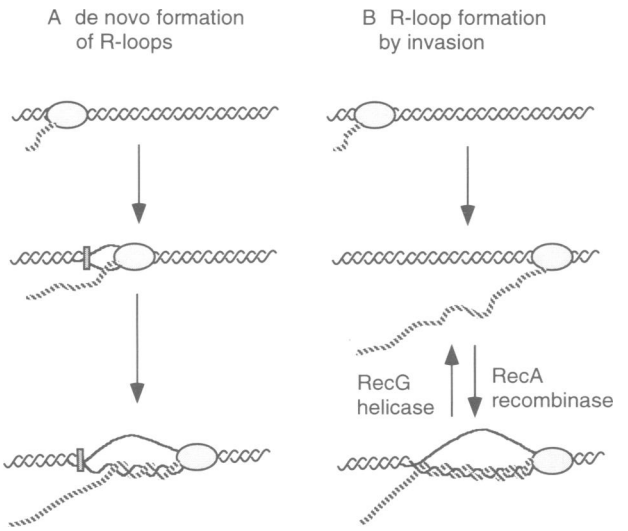

Fig. 6. Models for R-loop formation. Two models for the mechanisms of R-loop formation are schematically shown. Solid wavy lines, DNA duplex; stippled line, RNA transcript; shaded oval, RNA polymerase. See text for details.

The pathways for R-loop removal

RNase HI effectively removes persistent R-loops. Only in the absence of RNase HI activity, does the effect of accumulating R-loops become manifest (for a review, see Kogoma and Foster, 1995). We previously proposed that R-loops can be removed by two other pathways (Kogoma *et al.*, 1993). One pathway involves DNA polymerase I (Pol I) and exonuclease V (RecBCD enzyme) or exonuclease I, and the other is a recombinational repair pathway (Figure 7). Thus, a combination of a *polA* and an *rnhA* mutation is lethal (Kogoma *et al.*, 1993; Figure 4). A *recB* mutation which inactivates both the exonuclease V activity and the recombination function of RecBCD is lethal when combined with the *rnhA::cat* mutation (Kogoma *et al.*, 1993). We have now demonstrated that a combination of *recG258::kan* and *rnhA* or *recG258::kan* and *polA* mutations is lethal. The results indicate that the

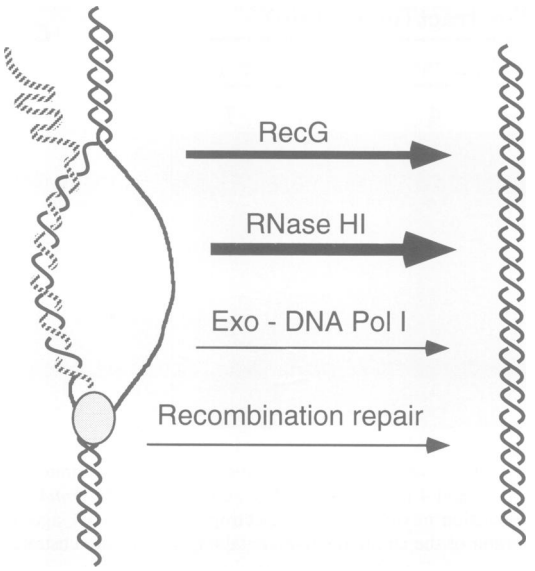

Fig. 7. Pathways for removal of R-loops. Four pathways which contribute to removal of persistent R-loops are shown. The thickness of the arrows represents the approximate relative contribution of each pathway. Symbols are as in Figure 6.

contribution of the postulated RecG action to the overall R-loop removal activity is very significant. In fact, the observation that *recG258*::*kan* mutants require much more RNase HI activity for survival than *recB* mutants (Figure 1) implies that the contribution of the RecG pathway exceeds the combined contributions by the Exo−Pol I and recombinational repair pathways (Figure 7).

The invasion model implies that persistent R-loops are normally formed with a certain frequency. The accumulation of persistent R-loops is detrimental to cellular processes and thus, *E.coli* cells are equipped with several pathways to remove them. Besides the demonstrated role for initiation of DNA replication in *rnhA* mutants, are there any functions of R-loops? What are the genetic elements that dictate where R-loops are formed? Or is it a random process, i.e. accidental? Why is R-loop accumulation lethal? These questions await further investigations.

Materials and methods

Media and growth conditions

Unless otherwise stated, cells were grown at 37°C in M9 salts−glucose medium (Miller, 1992) supplemented with casamino acids (0.2%; Difco Laboratories, Detroit, MI), required amino acids (50 μg/ml), thymine (8 μg/ml).

Table II. *E.coli* strains used

Strain	Relevant genotype	Source, reference or construction[b]
AQ634[a]	*recG*$^+$ *rnhA*$^+$	Ogawa *et al.* (1984)
AQ663	Δ*srlR-recA*	Csonka and Clark (1979)
AQ666[a]	*rnhA224*	Ogawa *et al.* (1984)
AQ705[a]	*dnaA46*(Ts)	this laboratory
AQ1409	*polA1*	as CM5280; Kelley (1980)
AQ1524[a]	*polA12*(Ts) *zig*::Tn*10*	Monk and Kinross (1972)
AQ3519[a]	*rnhA339*::*cat dnaA850*::Tn*10*	Kline *et al.* (1986)
AQ3788	*ori*Cdel-*1071 zif90*::pML31	Kogoma and Kline (1987)
AQ4432	*recB270*(Ts) *zga*::Tn*10*	Kogoma *et al.* (1993)
AQ5486	*dnaA46*(Ts) *tnaA*::Tn*10*	as CM742; Hansen *et al.* (1984) but Tc^r
AQ6460	*zic-4901*::Tn*10*	as CAG18492; Singer *et al.* (1989)
AQ7539	*rnhA199*(Am) *supF6*(Ts)	as YT378; Murakami *et al.* (1987)
AQ8032[a]	*rnhA339*::*cat*	Kogoma *et al.* (1993)
AQ8034	*recG258*::Tn*10*mini-*kan*	as N2731; Lloyd and Buckman (1991)
AQ8058[a]	*rnhA339*::*cat recB270*(Ts) *zga*::Tn*10*	Kogoma *et al.* (1993)
AQ8072[a]	*rnhA*$^+$ *recB21 zga*::Tn*10*	Kogoma *et al.* (1993)
AQ8107	lp[*sfiA*::*lacZ*$^+$ *cI*(Ind$^-$)]	as GC4597; Huisman and D'Ari (1981)
AQ8130[a]	*recG258*::Tn*10*mini-*kan*	AQ634×P1.AQ8034 → Km^r, UV^s
AQ8152	*rnhA-224*(pMAK-Ap-*rnhA*$^+$)	this work
AQ8353	*recG258*::Tn*10*mini-*kan*	AQ8107×P1.AQ8034 → Km^r, UV^s
AQ8428[a]	*rnhA339*::*cat polA12 zig*::Tn*10*	Kogoma *et al.* (1993)
AQ8964	*rnhA339*::*cat ori*Cdel-*1071*	Hong and Kogoma (1993)
AQ9768[a]	*rnhA339*::*cat recB21*(pRNH-Km, placI^q)	this work
AQ9897[a]	*rnhA339*::*cat recG258*::Tn*10*mini-*kan* (pRNH-Sp)	this work
AQ9899[a]	*rnhA339*::*cat recG258*::Tn*10*mini-*kan* (pRNH-Sp, placI^q)	this work
AQ9947	*recG162*	as N2973; Lloyd and Buckman (1991)
AQ9964[a]	*recG258*::Tn*10*mini-*kan* Δ*recA*	AQ8130×P1.AQ663 → Tc^r, UV^s
AQ10051[a]	*rnhA-224* Δ*recA*	AQ666×P1. AQ663 → Tc^r, UV^s
AQ10053/4[a]	*recG258*::Tn*10*mini-*kan recB21*	AQ8130×P1.AQ8072 → Tc^r, UV^s, T4.2^s
AQ10070	*recG162 zic*::Tn*10*	AQ9947×P1.6460 → Tc^r, UV^s
AQ10075[a]	*rceG258*::Tn*10*mini-*kan polA12*	AQ8130×P1.AQ1524 → Tc^r, at 30°C(Ts)
AQ10092[a]	*recG162*	AQ634×P1.AQ10070 → Tc^r, UV^s
AQ10119[a]	*rceG258*::Tn*10*mini-*kan dnaA46*	AQ8130×P1.AQ5486 → Tc^r at 30°C(Ts)
AQ10137[a]	*recG162 rnhA339*::*cat* (pRNH-Km, placI^q)	this work
AQ10150[a]	*recG162 rnhA339*::*cat* (pRNH-Km, pTK27)	this work
AQ10180	*recG162*	AQ8107×P1.AQ10070 → Tc^r, UVs

[a]The remaining genotypes, F$^-$ *ilv metB his-29 trp9605 pro thyA deoB* (or *C*).
[b]Tc^r and Km^r are resistant to tetracycline and kanamycin respectively. Ts, temperature-sensitive growth; UV^s, sensitivity to UV; T4.2^s, sensitivity to T4 gene 2 mutant phage.

Chemicals and radioisotopes

Chemicals were purchased from Sigma Chemical (St Louis, MO). [Methyl-^{3}H]thymine, [α-^{35}S]deoxyadenosine-5′-triphosphate and [α-^{32}P]-uridine-5′-triphosphate were from New England Nuclear Corp. (Boston, MA).

Escherichia coli strains

Escherichia coli strains used in this study are listed in Table II. Strains were constructed by phage P1-mediated transduction (Miller, 1992).

Plasmid construction

To fuse an *rnhA*$^+$ gene to the *lac* promoter (P_{lac}), the 650-bp *AgeI*–*Eco*RI fragment of pSK760 (Kanaya and Crouch, 1983) was cloned into pHSG576 (Takeshita *et al.*, 1987) at the multicloning site between *Sma*I and *Eco*RI after the *Age*I end of the fragment was blunt-ended by treatment with Klenow fragment. The *AgeI*–*Eco*RI fragment contains a 109-bp upstream sequence and the entire *rnhA* coding sequence (Kanaya and Crouch, 1983). The *Pst*I–*Eco*RI fragment containing *rnhA*$^+$ of the plasmid was then recloned into pHK (a Km^r-derivative of pHSG576; Asai *et al.*, 1994a), placing the *rnhA* sequence downstream of P_{lac}. To delete the *rnhA* promoter, the resulting plasmid was linearized by digestion with *Pst*I and *Hin*cII, and treated with lambda exonuclease III for a few minutes at 25°C. The *Pst*I end is resistant to the exonuclease and deletion was expected to occur only in the direction towards *rnhA*$^+$ (Sambrook *et al.*, 1989). The DNA was further treated with mung bean nuclease, Klenow fragment and T4 DNA ligase in succession. The treated plasmid DNA was screened for IPTG-dependent expression of RNase HI activity by transforming an *rnhA*::*cat recB270*(Ts) strain harboring pLacI^q (see below) to Km^r at 42°C in the presence of IPTG (5 mM). *rnhA*::*cat recB*(Ts) mutants are temperature-sensitive for growth due to the lack of RNase HI (Itaya and Crouch, 1991). Thus, the screening selects plasmids that express RNase HI in the presence of IPTG. The plasmid from one of the transformants which grew only in the presence of IPTG at 42°C was designated pRNH-Km (pAQ9691). Nucleotide sequencing revealed that the deletion included the *rnhA* promoter, SD sequence and first four codons. Thus, the plasmid presumably encodes a LacZ′–′RnhA fusion protein which contains the first eleven residues of LacZ at the N terminus. The construct encoding the fusion protein was moved to a spectinomycin-resistant (Spc^r) derivative of pHSG576 in which a part of the *cat* sequence was replaced with the Ω fragment coding for Spc^r (Prentki and Krisch, 1984). This plasmid was designated pRNH-Sp. pLacI^q plasmid was constructed by L.Walkup in this laboratory by inserting a 1.2-kb *Eco*RI fragment containing the *lacI*^{q} gene of pDT1-22 (Touati, 1988) at the *Eco*RI site of pTKQ27, a derivative of pBR322 (Kogoma, 1984).

To construct pMAK-*rnhA*$^+$, the *Pvu*I–*Eco*RI fragment containing *rnhA*$^+$ from pSK760 was blunt-ended and cloned at the *Hin*cII site of pMAK705. pMAK705, a derivative of pSC101, is temperature-sensitive for replication (Hamilton *et al.*, 1989). Subsequently, a part of the *cat* gene of the plasmid was replaced with a *bla* gene cassette. The plasmid was designated pMAK-Ap-*rnhA*$^+$.

RNase H assay

RNase H extracts were prepared from 2 ml of saturated cultures according to Carl *et al.* (1980). RNase H was extracted with 200 μl of solution E [10 mM Tris–HCl (pH 7.9), 2 M NaCl, 10 mM DTT and 5% (wt/vol) polyethylene glycol 6000] (Carl *et al.*, 1980). Purified RNase HI (United States Biochemical Corp., Cleveland, OH) and RNase H extracts were diluted with solution H [20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA and 0.1 mM DTT] to 0.001 U/ml and ~1 ng protein/μg, respectively. 2 μl of the RNA–DNA hybrid substrate (see below) were mixed with either purified RNase HI (0–0.004 U) or RNase H extract (1 and 4 ng). 1/30×solution E was added such that the salt concentration was the same for each reaction. Buffer H was then added to bring the reaction volume to a total of 15 ml. The reaction was incubated for 30 min at 37°C and a solution (3 μl) of 50% glycerol, 10 mM sodium phosphate (pH 7.0), 0.25% bromophenol blue, 0.25% xylene cyanol FF and 0.1 mM EDTA was added to quench the reaction and to serve as a marker in the gel. The reaction (17 μl) was then loaded onto a 1% agarose gel (15×15 cm) and electrophoresed at 100 V for ~2 h. The gel was then rinsed with water and wrapped in a hybridization bag. The gel was then exposed to a phosphorimaging screen for 31 h. Bands were visualized and quantified by a PhosporImager (model 425E-120, Molecular Dynamics, Sunnyvale, CA). RNase H activity was calculated by the disappearance of radioactivity from hybrid bands.

Preparation of RNA–DNA hybrid

Hybrids between an ~700-nt transcript and single-strand (ss) circular DNA were prepared using the GeneScribe-Z^{TM} system (USB Corp.). A 752-bp *Pst*I–*Eco*RI fragment containing a part of the *bla* gene from pBR322 was cloned at the multicloning site of pTZ18R, placing the sequence under the T7 promoter carried by the plasmid. The plasmid was named pAQ8662. ^{32}P-labeled transcript was synthesized *in vitro* with *Pst*I-digested pAQ8662 DNA and T7 RNA polymerase in the presence of [α-^{32}P]deoxyuridine-5′-triphosphate under the condition recommended by the manufacturer. To prepare ssDNA, a strain harboring pAQ8662 and an F episome was infected with a helper phage M13K07. pAQ8662 has the replication origin of phage f1 which allows replication as ssDNA and generation of particles containing circular ssDNA. The ssDNA was purified according to the protocol recommended by the manufacturer. The hybrid between the ^{32}P-labeled transcript and ss circular DNA was prepared by the procedure previously described (Nygaad and Hall, 1964).

DNA sequencing

DNA nucleotide sequences were determined by use of the Sequenase Version 2.0 (USB Corp.) and [α-^{35}S]deoxyadenosine-5′-triphosphate.

DNA synthesis measurement

Incorporation of [^{3}H]thymine into the acid-insoluble fraction in the presence of Cam and Rif was measured as described previously (Torrey and Kogoma, 1987).

Measurement of chronic SOS induction

The *recG258*::*kan* and *recG162* mutations were introduced into AQ8107, a λp[*sfiA*::*lacZ*$^+$ *c*I(Ind$^-$)] lysogen (Huisman and D'Ari, 1981). The cultures were grown to 2–3×10^8 cells/ml, and β-galactosidase activity was determined as previously described (Kogoma *et al.*, 1993).

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References

Asai,T. and Kogoma,T. (1994a) *J. Bacteriol.*, **176**, 1807–1812.
Asai,T., and Kogoma,T. (1994b) *Genetics*, **137**, 895–902.
Asai,T., Sommer,S., Bailone,A. and Kogoma,T. (1993) *EMBO J.*, **8**, 3287–3295.
Asai,T., Bates,D.B. and Kogoma,T. (1994a) *Cell*, **78**, 1051–1061.
Asai,T., Imai,M. and Kogoma,T. (1994b) *J. Mol. Biol.*, **235**, 1459–1469.
Baker,T. and Kornberg,A. (1988) *Cell*, **55**, 113–123.
Cao,Y. and Kogoma,T. (1993) *J. Bacteriol.*, **175**, 7254–7259.
Capaldo-Kimball,F. and Barbour,S.D. (1971) *J. Bacteriol.*, **106**, 204–212.
Carl,P. L., Bloom,L. and Crouch,R.J. (1980) *J. Bacteriol.*, **144**, 28–35.
Csonka,L.N. and Clark,A.J. (1979) *Genetics*, **93**, 321–343.
Drolet,M., Bi,X. and Liu,F. (1994) *J. Biol. Chem.*, **269**, 2068–2074.
Hamilton,C.M., Alder,M., Washburn,B.K., Babitzke,P. and Kushner,S.R. (1989) *J. Bacteriol.*, **171**, 4617–4622.
Hansen,E.B., Atlung,T., Hansen,F.G., Skovgaard,O. and von Meyenburg,K. (1984) *Mol. Gen. Genet.*, **196**, 378–396.
Hong,X. and Kogoma,T. (1993) *J. Bacteriol.*, **175**, 6731–6734.
Huisman,O. and D'Ari,R. (1981) *Nature*, **290**, 797–799.
Itaya,M. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 8587–8591.
Itaya,M. and Crouch,R.L. (1991) *Mol. Gen. Genet.*, **277**, 424–432.
Itoh,T. and Tomizawa,J. (1980) *Proc. Natl Acad. Sci. USA*, **77**, 2450–2454.
Kanaya,S. and Crouch,R.J. (1983) *J. Bacteriol.*, **258**, 1276–1281.
Kelley,W.S. (1980) *Genetics*, **95**, 15–38.
Kirkpatrick,D.P. and Radding,C.M. (1992) *Nucleic Acids Res.*, **20**, 4347–4353.
Kirkpatrick,D.P., Rao,B.J. and Radding,C.M. (1992) *Nucleic Acids Res.*, **20**, 4339–4346.
Kline,B.C., Kogoma,T., Tam,J.E. and Shields,M.S. (1986) *J. Bacteriol.*, **168**, 440–443.
Kogoma,T. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 7845–7849.
Kogoma,T. (1994) *J. Bacteriol.*, **176**, 1521–1523.

Kogoma,T. and Foster,P.L. (1995) In Toulme,J.J. and Crouch,R.J. (eds), *Ribonucleases H.* John Libbey, London, in press.
Kogoma,T. and Kline,B.C. (1987) *Mol. Gen. Genet.*, **210**, 262–269.
Kogoma,T. and von Meyenburg,K. (1983) *EMBO J.*, **2**, 463–468.
Kogoma,T., Skarstad,K., Boye,E., von Meyenburg,K. and Steen,H.B. (1985) *J. Bacteriol.*, **163**, 439–444.
Kogoma,T., Hong,X., Cadwell,G.G., Barnard,K.G. and Asai,T. (1993) *Biochimie*, **75**, 89–99.
Kogoma,T., Barnard,K.G. and Hong,X. (1994) *Mol. Gen. Genet.*, **244**, 557–562.
Lloyd,R.G. and Sharples,G.J. (1993a) *Nucleic Acids Res.*, **21**, 1719–1725.
Lloyd,R.G. and Sharples,G.J. (1993b) *EMBO J.*, **12**, 17–22.
Magee,T.R. and Kogoma,T. (1990) *J. Bacteriol.*, **172**, 1834–1839.
Magee,T.R., Asai,T., Malka,D. and Kogoma,T. (1992) *EMBO J.*, **11**, 4219–4225.
Masai,H., Asai,T., Kobuta,Y., Arai,K. and Kogoma,T. (1994) *EMBO J.*, **13**, 5338–5345.
Masukata,H. and Tomizawa,J. (1986) *Cell*, **44**, 125–136.
Masukata,H. and Tomizawa,J. (1990) *Cell*, **62**, 331–338.
Messer,W. and Weigel,C. (1995) In Ingraham,J.L., Low,K.B., Magasanik,B., Neidhardt,F.C., Schaechter,M. and Umbarger,H.E. (eds), *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology.* 2nd edn. American Society for Microbiology, Washington, DC, in press.
Miller,J.H. (1992) *A Short Course in Bacterial Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Monk,M. and Kinross,J. (1972) *J. Bacteriol.*, **109**, 971–978.
Murakami,Y., Ohmori,H., Yura,T. and Nagata,T. (1987) *J. Bacteriol.*, **169**, 1724–1730.
Nygaard,A.P. and Hall,B.D. (1964) *J. Mol. Biol.*, **9**, 125–142.
Ogawa,T., Pickett,G.G., Kogoma,T. and Kornberg,A. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 1040–1044.
Prentki,P. and Krisch,H.M. (1984) *Gene*, **29**, 303–313.
Rice,G.A., Kane,G.M. and Chamberlin,M.J. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 4245–4249.
Richardson,J.P. (1975) *J. Mol. Biol.*, **98**, 565–579.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual.* 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 5.84–5.85.
Sharples,G.J., Whitby,M.C., Ryder,L. and Lloyd,R.G. (1994) *Nucleic Acids Res.*, **22**, 308–313.
Singer,M. *et al.* (1989) *Microbiol. Rev.*, **53**, 1–24.
Skarstad,K., Baker,T.A. and Kornberg,A. (1990) *EMBO J.*, **9**, 2341–2348.
Takeshita,S., Sato,M., Masahashi,W. and Hashimoto-Gotoh,T. (1987) *Gene*, **61**, 63–74.
Torrey,T.A. and Kogoma,T. (1982) *Mol. Gen. Genet.*, **187**, 225–230.
Torrey,T.A. and Kogoma,T. (1987) *Mol. Gen. Genet.*, **208**, 420–427.
Touati,D. (1988) *J. Bacteriol.*, **170**, 2511–2520.
Whitby,M.C., Ryder,L. and Lloyd,R.G. (1993) *Cell*, **75**, 341–350.
Whitby,M.C., Vincent,S.D. and Lloyd,R.G. (1994) *EMBO J.*, **13**, 5220–5228.
Yager,T.D. and von Hippel,P.H. (1987) In Ingraham,J.L., Low,K.B., Magasanik,B., Neidhardt,F.C., Schaechter,M. and Umbarger,H.E. (eds), *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology.* American Society for Microbiology, Washington, DC, pp. 1241–1275.

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