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The *Escherichia coli* Histone-like Protein HU Has a Role in Stationary Phase Adaptive Mutation

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ABSTRACT

Stationary phase adaptive mutation in *Escherichia coli* is thought to be a mechanism by which mutation rates are increased during stressful conditions, increasing the possibility that fitness-enhancing mutations arise. Here we present data showing that the histone-like protein, HU, has a role in the molecular pathway by which adaptive Lac$^+$ mutants arise in *E. coli* strain FC40. Adaptive Lac$^+$ mutations are largely but not entirely due to error-prone DNA polymerase IV (Pol IV). Mutations in either of the HU subunits, HUα or HUβ, decrease adaptive mutation to Lac$^+$ by both Pol IV-dependent and Pol IV-independent pathways. Additionally, HU mutations inhibit growth-dependent mutations without a reduction in the level of Pol IV. These effects of HU mutations on adaptive mutation and on growth-dependent mutations reveal novel functions for HU in mutagenesis.

BACTERIA exist in rapidly changing environmental microcosms and are constantly subjected to stresses such as DNA damage and nutrient depletion. As a consequence, bacteria have evolved powerful and efficient mechanisms to respond to these stresses. While these mechanisms may appear to function independently, evidence is accumulating that stress response pathways are more intertwined than previously thought. For example, the RpoS-dependent response to nutrient depletion (Hengge-Aronis 2002) and mutagenesis by error-prone DNA polymerase IV (Pol IV), encoded by the *dinB* gene (Wagner *et al.* 1999), are interconnected (Layton and Foster 2003). While most DNA repair functions serve to maintain the integrity of the genetic information, Pol IV activity can introduce mutations into the genome. The resulting genetic fluidity may transiently increase the mutation rate, making the random appearance of beneficial mutations that relieve selective pressures more likely. Adaptive mutation is one mechanism that affords *Escherichia coli* such a survival advantage.

It was long thought genome replication was required to generate mutations. However, adaptive mutations occur in nutritionally starved cells when the chromosome is not being actively replicated (Foster 1994). Adaptive mutation is studied most frequently in *E. coli* strain FC40 (Cairns and Foster 1991). FC40 is deleted for the *lac* operon on the chromosome but carries a constitutively expressed fusion of *lacI* to *lacZ*, Φ(*lacI33-lacZ*) (Müller-Hill *et al.* 1964; Brake *et al.* 1978), on an F′ episome (F'_{128}). FC40 is unable to utilize lactose as a carbon or energy source (Lac$^-$) because of a +1 frameshift in the *lacI* region of the fusion that inactivates the β-galactosidase activity encoded by *lacZ* (Calos and Miller 1981). The Lac$^-$ allele is leaky, however, and cells exhibit a low level of lactose metabolism. When FC40 is grown nonselectively, the mutation rate to lactose utilization (Lac$^+$) on the episome is $\sim 1/10^9$ cells/generation. The types of growth-dependent mutations leading to the Lac$^+$ phenotype vary and >50% are rearrangements, duplications, or deletions. When FC40 is plated on minimal medium with lactose as the sole carbon source, Lac$^+$ mutations arise at the rate of 10–$50/10^8$ cells/day, and this rate remains constant for ~5 days. The mutations to Lac$^+$ that occur during lactose selection are almost entirely −1-bp deletions in runs of repeated bases (Foster and Trimarchi 1994; Rosenberg *et al.* 1994). Adaptive Lac$^+$ mutations require recombination functions and expression of F plasmid conjugal genes, but not conjugation (Foster and Trimarchi 1995a,b; Foster 2004). Between 50 and 80% of these frameshifts are due to the error-prone Y-family DNA Pol IV (Foster 2000; McKenzie *et al.* 2001).

Although the mechanism by which adaptive Lac$^+$ reversion occurs is controversial, our current model is as follows (Foster 2004). We hypothesize that the energy generated by the low level of lactose metabolism in FC40, while not sufficient for chromosomal replication, allows episomal replication to occasionally be initiated from one of the episomal vegetative origins (*oriS* or *oriV*). Because of the persistent nicking at the episomal origin of transfer (*oriT*) by the episome-encoded *Tra*I nickase, the progressing replication fork can encounter a nick, resulting in a double-strand break. The blunt end of the double-strand break is

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processed by the RecBCD complex. RecA then binds to the resulting single-stranded DNA and mediates strand invasion of an intact homologous duplex. Next, new DNA synthesis is primed by the PriA primosome and carried out by a combination of Pol II, Pol IV, and Pol III using the homologous sequence as a template. Eventually, a Holliday junction is formed, translocated by RecG or RuvAB, and resolved by the RuvABC complex, ultimately restoring the replication fork. If during this process error-prone DNA synthesis involving Pol IV traverses the Φ(*lacI33-lacZ*) allele, frameshifts that revert the *lac* allele may arise.

In *E. coli*, even in the absence of DNA damage, the basal levels of Pol IV are high compared to the replicative Pol III and other repair polymerases (Nohmi 2006). Pol IV is further upregulated in late stationary phase cells under control of the general stress response sigma factor RpoS (Layton and Foster 2003). This growth-phase regulation suggests that Pol IV serves some function during long-term survival in nutrient-limited conditions. This hypothesis is supported by a study showing that after prolonged incubation in stationary phase, strains lacking Pol IV exhibit no growth-defective phenotype in pure culture but compete poorly for survival with wild-type *E. coli* when grown in mixed cultures (Yeiser *et al.* 2002; Finkel 2006). Thus, Pol IV may be required to introduce mutations that confer a fitness advantage under adverse conditions (Foster 2000). To further explore the mechanism by which adaptive mutation occurs, we searched the literature for proteins that are growth-phase regulated and are also involved in DNA related processes. One such protein is the histone-like protein, HU.

HU is a small positively charged DNA-binding protein that is highly conserved among bacteria. It is associated with the nucleoid (Wery *et al.* 2001) and is involved in regulating DNA supercoiling (Brunetti *et al.* 2001; Kar *et al.* 2005). In most bacteria HU is a homodimer; however, in *E. coli* and certain other enteric bacteria, HU is encoded by two paralogous genes: *hupA* and *hupB*, encoding HUα and HUβ, respectively (Kano *et al.* 1986, 1988). Thus, in *E. coli*, there are three forms of HU: HUα2, HUαβ, and HUβ2 (Rouviere-Yaniv and Kjeldgaard 1979). The reason for this unique genetic arrangement is unknown; however, the relative abundances of the three forms of HU vary with cell growth phase (Claret and Rouviere-Yaniv 1997). During exponential growth, *hupA* is expressed at a higher level than *hupB*, resulting in a predominance of HUα2. As nutrients become depleted and cells approach stationary phase, expression of *hupB* is upregulated, and with the increased levels of HUβ, the heterodimer HUαβ becomes most abundant. HUβ2 represents only a small component of the HU pool even in stationary phase, leading to the hypothesis that the actual role of HUβ is to titrate the relative amounts of the HUα2 homodimer and the HUαβ heterodimer.

In addition to this growth-phase regulation, the composition of HU is regulated in response to certain stresses; for example, HUαβ is required for long-term survival of *E. coli* in nutrient-depleted conditions (Claret and Rouviere-Yaniv 1997), and HUβ2 becomes prevalent when cells are cold shocked (Giangrossi *et al.* 2002). These results suggest that different forms of HU may serve different functions under different environmental conditions. This possibility is further supported by data showing that the three forms of HU differ in their biochemical properties and activities. The affinities of the three forms of HU for various DNA structures (linear, nicked, and gapped, for example) differ (Pinson *et al.* 1999). Also, while HUα2 and HUαβ can introduce negative supercoiling into DNA (in the presence of topoisomerase I), HUβ2 cannot (Claret and Rouviere-Yaniv 1997). The common trait shared by all three forms of HU appears to be the ability to bind to DNA and induce bends (van Noort *et al.* 2004).

HU has diverse physiological roles. Perhaps best characterized is stabilization of specific protein-DNA architectures during Mu phage transposition (Kano *et al.* 1989; Lavoie and Chaconas 1994) and during repression of the *gal* operon (Aki *et al.* 1996; Aki and Adhya 1997; Lewis and Adhya 2002; Roy *et al.* 2005; Semsey *et al.* 2006). Mutants defective for HU are more sensitive to UV light and γ-ray irradiation than are wild-type cells (Boubrik and Rouviere-Yaniv 1995; Li and Waters 1998), suggesting that HU is involved in DNA protection and/or repair. HU also has regulatory roles in *E. coli*. HU has been shown to have a robust ability to bind RNA (Balandina *et al.* 2002). In this capacity, HU promotes efficient translation of RpoS by destabilizing secondary structure in the *rpoS* mRNA that blocks the ribosome-binding site (Balandina *et al.* 2001). HU regulates the abundance of SeqA, a protein involved in preventing reinitiation of chromosome replication (Lee *et al.* 2001). Interestingly, while *hupA* inactivation results in a twofold increase in SeqA abundance, *hupB* inactivation has little effect. The mechanism of this regulation is not known.

Here we report that proper HU composition is required for normal levels of adaptive mutation. HU exerts this effect by an unknown mechanism with Pol IV-independent and Pol IV-dependent components. The HU effect is also independent of RpoS. Our results suggest that HU acts in a pathway with the RecG helicase, providing new evidence for a RecG function in adaptive mutation. In addition to its role in adaptive mutation, HU is required for the increase in mutation rate during growth caused by Pol IV overexpression. In contrast, *hup* mutations do not cause a substantial decrease in the growth-dependent mutation rate in the absence of Pol IV. These parallel effects of *hup* mutations on adaptive mutation and growth-dependent mutations suggest that there is a functional relationship

TABLE 1

***E. coli* strains and plasmids used in this study**

Strain/plasmid	Relevant phenotype or genotype	Reference
E. coli strains		
FC29	*ara* Δ(*gpt-lac*)5 *thi*/F′ Δ(*lacIZ*) Pro^+	Cairns and Foster (1991)
FC36	F^- *ara* Δ(*gpt-lac*)5 *thi* Rif^R	Cairns and Foster (1991)
FC40	FC36/F′ Φ(*lacI33-lacZ*) Pro^+	Cairns and Foster (1991)
FC722	FC40 carrying a Tc^S *Tn*10 on the episome	Foster (1997)
FC1373	Φ(*lacI33-lacZ*) Pro^+ Tc^S *Tn*10 on the chromosome	Stumpf *et al.* (2007)
FC1408	FC40 *recG265*::Cm	This study
FCG150	FC40 *rpoS*::Kn	This study
GC7620	*hupA*::Cm *hupB*::Kn	Huisman *et al.* (1989)
BW25113[a]	Host strain for PCR-mediated gene inactivation.	Datsenko and Wanner (2001)
PFV247	BW25113/pKD46	This study
PFB236	C36 Δ*dinB*::Zeo	This study
PFB243	FC40 Δ*dinB*::Zeo on chromosome and episome	This study
PFB570	FC40 *hupB*::Kn	This study
PFB574	FC40 *hupA*::Cm	This study
PFB610	FC40 Δ*hupA*::Kn	This study
PFB611	FC40 Δ*hupB*::Kn	This study
PFB612	FC40 Δ*hupA* (Kn^S)	This study
PFB613	FC40 Δ*hupB* (Kn^S)	This study
PFB644	FC40 Δ*hupA*::Kn *recG265*::Cm	This study
PFB645	FC40 Δ*hupB*::Kn *recG265*::Cm	This study
PFB638	PFB243 Δ*hupA*::Kn	This study
PFB639	PFB243 Δ*hupB*::Kn	This study
PFG265	FC722/pBAD24	Stumpf and Foster (2005)
PFG266	FC722/pPFG96	Stumpf and Foster (2005)
PFB622	FC722 Δ*hupA*::Kn	This study
PFB623	FC722 Δ*hupB*::Kn	This study
PFB628	PFB622/pPFG96	This study
PFB629	PFB623/pPFG96	This study
PFB665	PFB236/F′ Δ*dinB*::Zeo with Tc^S *Tn*10 on the episome	This study
PFB673	PFB236 Δ*hupA*::Kn/F′ Δ*dinB*::Zeo with Tc^S *Tn*10 on the episome	This study
PFB674	PFB236 Δ*hupB*::Kn/F′ Δ*dinB*::Zeo with Tc^S *Tn*10 on the episome	This study
PFB656	FC1373/pPFG96	This study
PFB657	FC1373 Δ*hupA*::Kn/pPFG96	This study
PFB658	FC1373 Δ*hupB*::Kn/pPFG96	This study
PFG338	TE8197[b] Arg^+ Tc^S	Layton and Foster (2003)
PFG339	TE8222[c] Arg^+ Tc^S	Layton and Foster (2003)
PFB563	PFG338 *hupA*::Cm	This study
PFB564	PFG339 *hupA*::Cm	This study
Plasmids		
pBAD24	Vector for arabinose-inducible gene expression	Guzman *et al.* (1995)
pPFG96	*dinB*$^+$ on pBAD24	Stumpf and Foster (2005)
pKD46	λ Red recombinase helper plasmid	Datsenko and Wanner (2001)
pKD4	Kn^R template plasmid	Datsenko and Wanner (2001)
pCP20	FLP helper plasmid	Datsenko and Wanner (2001)

[a] BW25113 is (*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$).
[b] TE8197 is MG1655 Δ(*lacIZ*) *argA*::*Tn*10 *trpDC700*::*putPA1303*::Kn^R-*rpoS-lac* (protein fusion) (Hirsch and Elliott 2002).
[c] TE8222 is MG1655 Δ(*lacIZ*) *argA*::*Tn*10 *trpDC700*::*putPA1303*::Kn^R-*rpoS-lac* (operon fusion) (Hirsch and Elliott 2002).

between Pol IV function and HU that is important for both pathways.

MATERIALS AND METHODS

Bacterial strains and plasmids: All bacterial strains are *E. coli* K-12 derivatives and are described in Table 1. Genetic manipulations were performed as described previously (Miller 1992). The *hupA*::Cm and *hupB*::Kn mutations were obtained from R. D'Ari (Huisman *et al.* 1989). These alleles are insertions of chloramphenicol resistance (Cm^R) and kanamycin resistance (Kn^R) cassettes. The Δ*hupA*::Kn and Δ*hupB*::Kn alleles were made using the λ Red recombinase system described by Datsenko and Wanner (2001). Linear PCR products containing sequences upstream and downstream of

hupA and *hupB* flanking a Kn^R cassette were generated by amplification from the template plasmid pKD4. The PCR oligos used were: 5′-GCTTAGCAAGCGATAAACACATTGTAAGGA TAACTTTGTGTAGGCTGGAGCTGCTT-3′ and 5′-CACCCC TTCGTTAAAACTGTTCACTGCCACGCAATCCATATGAATA TCCTCCTTAG-3′ for *hupA*; 5′-TCAAGTGCGATATAAATTA TAAAGAGGAAGAGAAGATGTGTAGGCTGGAGCTGCTT-3′ and 5′-CCTTGAACTTCGTCACATCCCCACTGGGGACAAC GCCATATGAATATCCTCCTTAG-3′ for *hupB*. Linear PCR products were transformed by electroporation into PFV247 selecting for Kn^R. Deletion of the *hup* ORFs was confirmed by PCR. To construct PFB610 and PFB611, the Δ*hupA*::Kn and Δ*hupB*::Kn alleles were transduced into FC36 using P1$_{vir}$ bacteriophage. The F′ episome carrying the Φ(*lacI33-lacZ*) allele was then mated into FC36 from a methionine auxotroph (Met$^-$) donor carrying F′ Φ(*lacI33-lacZ*), which encodes proline prototrophy (Pro$^+$). Selection was for resistance to rifampicin (Rif) to select against the donor and Pro$^+$ to select for the presence of the F′ episome.

To construct PFB612 and PFB613, F$^-$ derivatives of FC40 carrying the Δ*hupA*::Kn and Δ*hupB*::Kn alleles were transformed with the plasmid pCP20 and screened for loss of Kn^R, as described in Datsenko and Wanner (2001). The F′ episome carrying the Φ(*lacI33-lacZ*) allele was then mated into these new strains as described above.

PFG150 was constructed by P1$_{vir}$ transduction of the *rpoS*::Kn allele (originally identified as *katF*::Kn) from strain ZK1000 (Bohannon *et al.* 1991) into FC40 selecting for Kn^R. PFB236 and PFB243 were constructed by the method described in Layton and Foster (2003) except that the *dinB* allele was Δ*dinB*::Zeo, designated first as Δ*dinB61*::ble in Borden *et al.* (2002). Selection was for zeomycin resistance (Zeo^R). PFB673 and PFB674 were constructed by first transducing either the Δ*hupA*::Kn or Δ*hupB*::Kn alleles into PFB236 using P1$_{vir}$ bacteriophage selecting for Kn^R. The episome carrying the Δ*dinB*::Zeo and the tetracycline sensitive (Tc^S) *Tn*10 was mated by conjugation with PFB373 selecting for methionine and proline prototrophy into these new strains as well as FCB236.

PFG338 and PFG339 were constructed as described in Layton and Foster (2003). The construction of pPFG96 is described in Stumpf and Foster (2005).

Mutation and viability assays Protocols and media were performed as described previously (Cairns and Foster 1991; Miller 1992; Foster 1994; Foster *et al.* 1996). Rich media were supplemented with antibiotics in the following concentrations: carbenicillin, 100 μg/ml; Kn, 30 μg/ml; Cm, 10 μg/ml; Tc, 20 μg/ml; Rif, 100 μg/ml. The concentrations were reduced by one-half in minimal media, with the exception of Cm, which was unchanged. For the adaptive mutation experiments in Figures 1–3, ~10^8 cells for *recG*$^+$ strains or 10^6 cells for *recG*$^-$ strains from four independent cultures of each strain plus 10^9 FC29 scavenger cells were spread on M9 minimal lactose plates. New Lac$^+$ colonies appearing on days 2–5 were counted and the results averaged. To monitor the viability of Lac$^-$ cells while incubating on lactose minimal plates (Figures 1B and 3B), plugs were removed from between Lac$^+$ colonies and the number of Lac$^-$ cells determined by plating dilutions on LB plus Rif (Rif selects against the scavenger cells). Because it takes 2 days for a Lac$^+$ colony to grow, the numbers of Lac$^+$ colonies were divided by the mean numbers of Lac$^-$ cells present on the plate 2 days earlier. For Figure 2, the numbers of Lac$^+$ colonies were divided by the mean number of cells initially plated.

Growth-dependent mutation rates (Figure 6) were determined as follows: overnight cultures of each strain were diluted 10^{-5} in LB broth plus the appropriate antibiotics. Then, 0.1-ml aliquots were distributed in 96-well microtiter plates and incubated overnight at 37°. The 10-μl samples of 40 independent cultures (microtiter wells) were spread on LB plates supplemented with tetracycline (Tc) and incubated at 37°. Tc-resistant (Tc^R) colonies arising after 48 hr were counted. Mutation rates were calculated using the Jones median estimator to determine the number of mutations per culture and then dividing this by twice the total number of cells in the culture, as described in Foster (2006). To confirm that the microaerobic growth conditions in the microtiter wells did not influence the experimental results, the experiment in Figure 6A was repeated with 20 1-ml cultures incubated with shaking. The results were similar; however, the larger number of cultures used for the generation of Figure 6A increases the statistical reliability of the data. The 95% confidence limits were calculated as described in Foster (2006).

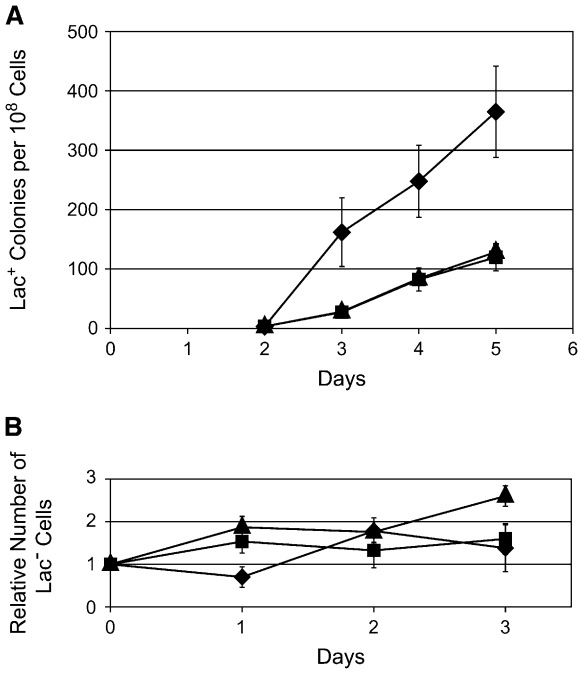


Figure 1.—Inactivation of *hupA* or *hupB* decreases adaptive mutation. (A) The cumulative mean number of Lac$^+$ revertants per 10^8 Lac$^-$ cells. About 10^8 cells from four independent cultures of FC40 and its *hupA*::Cm and *hupB*::Kn derivatives were plated on lactose minimal plates and newly arising colonies were counted on days 2–5. Data points are the cumulative mean number of Lac$^+$ colonies appearing on each day divided by the number of Lac$^-$ cells on the plates 2 days earlier. Error bars are ± SEM. (♦) Wild type = FC40; (■) FC40 *hupA*::Cm = PFB574; (▲) FC40 *hupB*::Kn = PFB570. (B) Mean number of the Lac$^-$ cells during incubation on lactose plates. Three plates per strain were used to determine the number of viable Lac$^-$ cells (see materials and methods). Data points are the mean number of Lac$^-$ cells on each day divided by the number of Lac$^-$ cells plated on day 0. Error bars are ± SEM. Symbols are the same as in A.

Immunoblots Standard molecular biology techniques were as described (Ausubel *et al.* 1988). For the immunoblots in Figures 2, 3, and 6, overnight cultures were grown in LB broth plus appropriate antibiotics and diluted 10^{-5} into M9 minimal medium with glycerol and grown to saturation at 37° for ~48 hr. For the immunoblots in Figure 6, overnight cultures were grown in LB broth plus appropriate antibiotics and then diluted 1:1000 in the same medium but containing only carbenicillin to maintain selection for the plasmid. Cells were

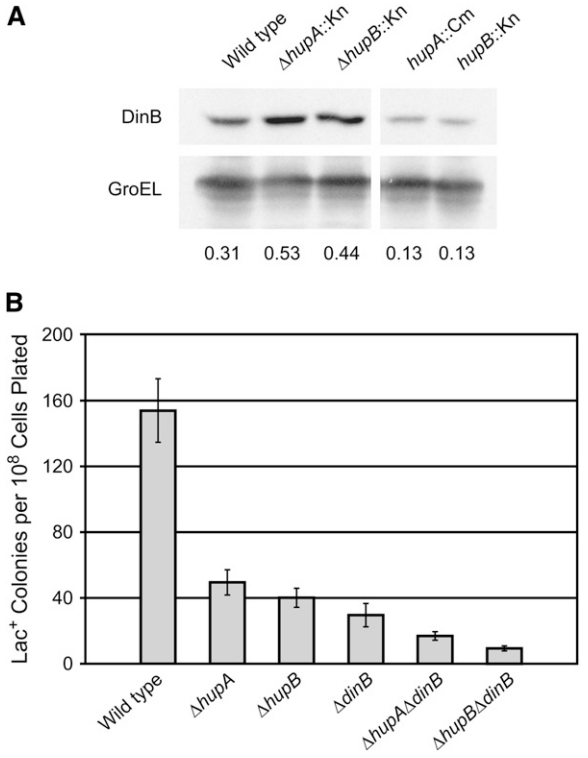


FIGURE 2.—The decrease in adaptive mutation in *hup* mutants is not due solely to an effect on Pol IV activity. (A) Immunoblot showing that derivatives of FC40 carrying different *hup* null mutations have different amounts of Pol IV. Forty micrograms of total protein from each strain was loaded in each lane. The blot was probed with anti-Pol IV antibody, stripped, and then reprobed with anti-GroEL antibody. Band intensities were quantified and the numbers below the lanes give the ratios of the densities of the Pol IV bands to the GroEL bands (see MATERIALS AND METHODS). (B) Approximately 10^8 cells from four independent cultures were plated on lactose minimal plates. The data are the means of the total number of Lac^+ colonies appearing on days 3–5 divided by the number of cells plated on day 0. Error bars are ± SEM. Wild type = FC40; Δ*hupA*::Kn = PFB610; Δ*hupB*::Kn = PFB611; Δ*dinB* = PFB243; Δ*hupA*::Kn Δ*dinB* = PFB638; Δ*hupB*::Kn Δ*dinB* = PFB639.

grown to mid-log phase (OD_{600} ~0.6) without aeration to mimic the microaerobic conditions in the microtiter wells used in assays for growth-dependent mutation rates. In all cases, cells were harvested by centrifugation, resuspended in 1× SDS–PAGE sample loading buffer without dye, and boiled for 15 min. Protein concentrations were determined by Bradford assays (Bio-Rad Laboratories, Hercules, CA) and the volume of each sample required to give 40 μg of total protein for Pol IV immunoblots or 25 μg for the RpoS immunoblot was diluted into 1× sample loading buffer with dye and loaded onto SDS-12% polyacrylamide gels. Proteins were separated by electrophoresis and then electrotransferred to Immobulin-P membranes (pore size, 0.45 μm; Millipore, Bedford, MA). For Pol IV immunoblots, membranes were probed with rabbit anti-Pol IV polyclonal antiserum (obtained from H. Ohmori). The antiserum was first clarified using acetone powder made from a Δ(*dinB*) strain by the procedure described in HARLOW and LANE (1988). For RpoS immunoblots, monoclonal mouse anti-RpoS antibody (NeoClone) was used. Blots were stripped with BlotFresh Western blot stripping

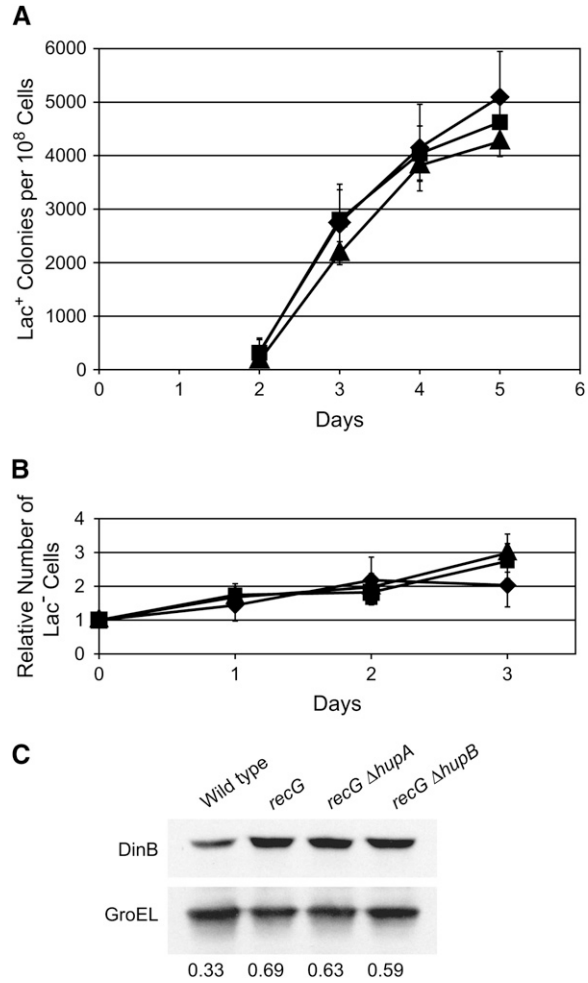


FIGURE 3.—Inactivation of *hupA* or *hupB* does not decrease adaptive mutation in *recG*⁻ cells. (A) The cumulative mean number of Lac^+ revertants per 10^8 Lac^- cells. Approximately 10^6 cells from each of four independent cultures of a *recG*⁻ derivative of FC40 and the isogenic strain carrying either the Δ*hupA*::Kn or Δ*hupB*::Kn allele were plated on lactose minimal plates; newly arising colonies were counted on days 2–5. Data points are the cumulative mean number of Lac^+ colonies appearing on each day divided by the number of Lac^- cells on the plates 2 days earlier. Error bars are ± SEM. (♦) FC40 *recG* = FC1408; (■) FC40 *recG* Δ*hupA*::Kn = PFB644; (▲) FC40 *recG* Δ*hupB*::Kn = PFB645. (B) Mean number of Lac^- cells during incubation on lactose plates. Three plates per strain were used to determine the number of viable Lac^- cells (see MATERIALS AND METHODS). Data points are the mean number of Lac^- cells on each day divided by the number of Lac^- cells on day 0. Error bars are ± SEM. Symbols are the same as in A. (C) Immunoblot showing that *hup* inactivation does not substantially affect the amount of Pol IV in the *recG*⁻ strains. Forty micrograms of total protein from FC1408, PFB644, and PFB645 was loaded in each lane. The blot was probed with anti-Pol IV antibody, stripped, and then reprobed with anti-GroEL antibody. Band intensities were quantified and the numbers below the lanes give the ratios of the densities of the Pol IV bands to the GroEL bands (see MATERIALS AND METHODS).

reagent (SignaGen Laboratories) according to the manufacturer's suggested protocol and reprobed with mouse monoclonal anti-GroEL antibody (Stressgen Bioreagents). Antibody binding was visualized using alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (both from Promega, Madison, WI) and developed using the Western-light chemiluminescence reagent (Applied Biosystems, Foster City, CA). Densitometric analysis was performed using ImageJ software (version 1.36b) (Abramoff *et al.* 2004). Because chemiluminescent intensity can be affected by a number of factors, such as the age of the reagents and the length of exposure, the apparent amount of a protein in the same strain can vary between blots. For this reason, band intensities can only directly be compared within a single blot. However, we normalized the band intensities of Pol IV to those of the GroE protein, which was not affected by our manipulations, to allow the amount of Pol IV to be compared among blots.

β-Galactosidase assays: For each experiment, four independent cultures of each strain were grown to saturation at 37° in M9 glycerol minimal medium supplemented with antibiotics when appropriate. β-Galactosidase assays were performed as in Miller (1992).

Southern blot analysis: Total DNA was extracted by the method described in Pushnova *et al.* (2000) and digested with the restriction endonuclease *Eco*RV (New England Biolabs, Beverly, MA). DNA fragments were separated by electrophoresis in 1% agarose gels. The DNA was then transferred to Zeta-Probe membrane (Bio-Rad Laboratories) by upward alkaline transfer. The membrane-bound DNA was then hybridized to [^{32}P] end-labeled *lac*, *traT*, and *dnaQ* probes by the method described in the Zeta-Probe instruction manual. The blot was then exposed to a storage phosphor screen (Kodak, Rochester, NY) and visualized using a Typhoon phosphor imaging system (Molecular Dynamics). Densitometric analysis was performed using ImageQuant TL software (GE Healthcare).

All probes were generated by PCR: the 661-bp *lac* probe was generated using the oligos 5′-TGAATTACATTCCCAACCG CGT-3′ and 5′-ACCAACGCGCAGCCCGGAC-3′; the 1050-bp *traT* probe was generated using the oligos 5′-ATATCAATT TGTTGGTG-3′ and 5′-GTCGGACTGATTATTAATG-3′; and the 639-bp *dnaQ* probe was generated using the oligos 5′-GCTTAAGCGCGATATTCC-3′ and 5′-CTTGCGGCTCTCTG AAC-3′. The PCR products were dephosphorylated with shrimp alkaline phosphatase (New England Biolabs) and 5′-end labeled using [γ-^{32}P]dATP catalyzed by T4 polynucleotide kinase (New England Biolabs). The labeled PCR products were purified using the QIAquick nucleotide removal kit (QIAGEN, Valencia, CA) prior to hybridization.

In-gel catalase assays: Assays for catalase activity were performed as described in Clare *et al.* (1984). Cultures were grown to saturation in M9 minimal medium with glycerol and the appropriate antibiotics. The cells in 1 ml of culture from each strain were pelleted and resuspended in Tris–EDTA buffer (pH 8). Protease inhibitor cocktail (Sigma, St. Louis, P8465) was added to the suspension per the manufacturer's recommendations. Lysozyme was added to a concentration of 1 mg/ml and incubated at 37° for 30 min. The lysate was cleared by centrifugation for 20 min at 4°, and then 40 μg of total protein for each strain was loaded onto a 12% nondenaturating polyacrylamide gel and separated by electrophoresis. The gel was then soaked in a 50 μg/ml solution of HRP (Sigma-Aldrich, P8375) in a 50 mm potassium phosphate buffer (pH 7.0) for 45 min without agitation. Hydrogen peroxide was then added to a final concentration of 5.0 mm and the gel was allowed to soak without agitation for an additional 10 min. The gel was then briefly rinsed twice in water and transferred to a solution of 0.5 mg/ml diaminobenzidine (Sigma-Aldrich, D8001) in the same phosphate buffer. Staining was carried out until catalase bands were clearly visible and the gel was then rinsed in water.

RESULTS

Inactivation of *hupA* and *hupB* decreases adaptive Lac$^+$ reversion in FC40: To test if HU protein has a role in adaptive mutation, we introduced into FC40 mutant alleles of *hupA* and *hupB* interrupted by insertions of drug-resistance cassettes conferring resistance to Cm or Kn (Huisman *et al.* 1989). As shown in Figure 1A, cells with the *hupA*::Cm or *hupB*::Kn alleles had an ~70% reduction in adaptive mutation. During this experiment the numbers of viable Lac$^-$ cells on the minimal lactose plates were monitored each day. Figure 1B shows that the *hup* mutations did not have an adverse effect on the survival of the Lac$^-$ cells, eliminating the possibility that the decrease in adaptive mutation was due to poor survival of the *hup* mutant strains. In fact, the *hupB* mutant appeared to have a slight growth advantage on the minimal lactose plates; however, this does not affect the interpretation of the adaptive mutation data.

To determine if this effect on adaptive mutation was unique to these particular *hup* alleles, we made null alleles of *hupA* and *hupB* by replacing the *hup* ORFs with Kn^R cassettes using the λ Red recombinase system (see materials and methods) (Datsenko and Wanner 2001). The new alleles, designated Δ*hupA*::Kn and Δ*hupB*::Kn, resulted in similar reductions in adaptive mutation of FC40 (Figure 2B).

Double mutations of *hupA* and *hupB* have pleiotropic effects (Huisman *et al.* 1989), and double mutant strains do not grow on minimal media (Huisman *et al.* 1989). Thus, the effect on adaptive mutation of total loss of HU could not be evaluated.

The decrease in Lac$^+$ adaptive mutation of *hup* mutant strains is not due to lower levels of Pol IV: Because up to 80% of adaptive Lac$^+$ mutations are due to Pol IV (Foster 2000; McKenzie *et al.* 2001), the decrease in adaptive mutation of *hup* mutant strains could be caused by a decrease in the amount of Pol IV. We used immunoblots with anti-Pol IV antibody to compare the amount of Pol IV in various strains that were grown to saturation in M9-glycerol minimal medium. As shown in Figure 2A, the amount of Pol IV was slightly decreased in *hupA*::Cm and *hupB*::Kn mutant cells compared to wild-type cells, but both the Δ*hupA*::Kn and Δ*hupB*::Kn null alleles resulted in slight increases in Pol IV abundance. The reason for these different effects on Pol IV levels is currently under investigation. Nevertheless, the amount of Pol IV and the rate of adaptive mutation are not correlated in the different *hup* mutants, suggesting that the effects of the hup mutants on adaptive mutation are independent of the levels of Pol IV.

The decrease in Lac$^+$ adaptive mutation of *hup* mutant strains is not solely due to effects on Pol IV: The results described above suggested that at least some

of the effect of *hup* mutations on adaptive mutation is independent of Pol IV. To test this hypothesis, we examined the epistatic relationships between *dinB* and the *hup* mutant phenotypes. As shown in Figure 2B, ~80% of the Lac$^+$ adaptive mutations that appeared during a 3-day experiment were eliminated in Δ*dinB* mutant cells. The Δ*hupA and* Δ*hupB* mutants reduced both the *dinB*-dependent and the *dinB*-independent Lac$^+$ mutations. Although the relative effects of the *hup* mutants were approximately the same in *dinB*+ and Δ*dinB* mutant cells (~70 and 60%, respectively), because of the higher mutation rate in *dinB*$^+$ cells the absolute magnitude of the *hup* effect on the *dinB*-dependent mutations was 5-fold greater. Nonetheless, that the negative effects of the Δ*hup* alleles are diminished but not eliminated in cells lacking Pol IV suggests that HU is involved in both Pol IV-independent and Pol IV-dependent pathways leading to Lac$^+$ reversion.

The HU role in the pathway leading to Lac$^+$ adaptive mutation may require RecG helicase function: Adaptive mutation to Lac$^+$ is increased 10- to 100-fold in *recG*$^-$ derivatives of FC40 (Foster *et al.* 1996; Harris *et al.* 1996). This increase is mostly or entirely due to the increased levels of Pol IV that result from the low-level SOS activation in *recG* mutant cells (Lloyd and Buckman 1991; Layton and Foster 2003). As shown in Figure 3A, Δ*hupA*::Kn and Δ*hupB*::Kn alleles had little effect on adaptive mutation in a *recG* mutant strain. Although small decreases in adaptive mutation were detected in the *recG* Δ*hup* double mutant strains, these differences were not statistically significant. As the immunoblot in Figure 3C shows, the Δ*hup* alleles did not cause detectable changes in the amount of Pol IV in the *recG* mutant cells. During the adaptive mutation experiment the *recG hup* double mutants had no survival defect; indeed, some increase in the number of Lac$^-$ cells was detected (Figure 3B). These data support two alternative hypotheses: first, that *hup* mutations affect adaptive mutation via a mechanism that requires the presence of RecG; or second, that the effects of *hup* mutations are masked in the *recG* strain by the high mutation rate due to elevated Pol IV levels.

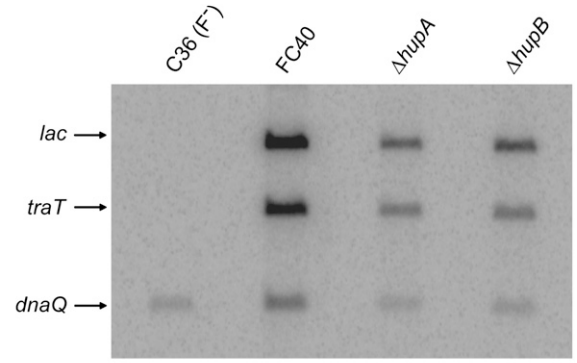
To discriminate between these possibilities, we attempted to measure adaptive mutation in *recG dinB hup* triple mutants. Unfortunately, these experiments were not successful. Cells with all 3 mutations had severe growth defects in minimal liquid culture, reaching titers of only 10^7–10^8 CFU/ml (*vs.* 10^9/ml for wild-type cells). Because single mutants, or any combination of double mutants (*dinB hupA*, *dinB hupB*, *recG hupA*, *recG hupB*, or *recG dinB*), exhibit no pronounced growth defect, it appears that the presence of all three mutations has a severe synergistic effect on fitness. The reason for this synthetic lethality is not clear at this time, but it suggests that these genes act together in a pathway required for optimal growth, at least in minimal medium. The triple mutants appeared to accumulate suppressors at a high rate, resulting in nonreproducible adaptive mutation and varied colony morphology.

Although analysis of a triple mutant was not possible, the individual epistatic relationships between the *hup* mutants and *dinB* and *recG* mutants strongly suggest that HU affects both Pol IV-dependent and Pol IV-independent adaptive mutation and that RecG is required for this effect, at least when Pol IV is present.

The copy number of F′$_{128}$ is stable in *hup* mutants: Our current model for adaptive mutation requires some amount of episomal replication. Thus, *hup* mutations could cause a decrease in adaptive mutation by perturbing episome replication, resulting in a reduced copy number of the *lac* target or reduced levels of some episomal function required for maximal adaptive mutation. This possibility is supported by data showing that F plasmids in *Salmonella typhimurium* are 25-fold less stable in *hupB* mutant cells than in wild-type cells or *hupA* mutant cells when selection for the plasmid is not maintained (Hillyard *et al.* 1990). Whether this result applies to *E. coli* has not been tested, but reconstitution of F plasmid replication from *oriV in vitro* with purified *E. coli* proteins required HU (Zzaman *et al.* 2004). Purified HUαβ was used in these experiments, but it is possible that HUα2 or HUβ2 could have functioned as well as HUαβ.

We used Southern blot analysis to determine if the F′$_{128}$ episome carried by FC40 is stable in *hup* mutants. Two episome-specific probes were used: one that hybridizes to a 1592-bp *Eco*RV fragment of the *traT* gene and one that hybridizes to a 2510-bp *Eco*RV fragment of the *lac* region. A probe that hybridizes to a 794-bp *Eco*RV fragment of the chromosomal *dnaQ* gene was used to normalize the episomal DNA to the chromosomal DNA. As shown in Figure 4, the ratios of the intensities of the *traT* and *lac* bands to the intensity of the *dnaQ* band are similar in FC40 and the Δ*hup* mutants. This result suggests that the copy number of F′$_{128}$ is stable in cells lacking one of the two *hup* genes and eliminates the possibility that a decrease in the copy number of the episome or the *lac* mutational target underlies the decrease in adaptive mutation in *hup* mutants.

Inactivation of *hup* genes does not affect *rpoS* expression or RpoS function: In *E. coli* HU is required for efficient translation of RpoS, the stationary phase sigma factor (Balandina *et al.* 2001). Cells lacking *rpoS* have a low rate of adaptive mutation mostly because RpoS is required to maintain normal levels of Pol IV during stationary phase (Layton and Foster 2003; Lombardo *et al.* 2004). Therefore, we tested if *hup* mutations decrease adaptive mutation via an effect on *rpoS* expression or, possibly, RpoS activity. As shown in Table 2, expression of β-galactosidase from an *rpoS-lacZ* operon fusion (*rpoS′-lacZ*$^+$) was not significantly affected but expression of β-galactosidase from an *rpoS-lacZ* protein fusion (*rpoS′-lacZ′*) was slightly decreased in the *hupA*::Cm mutant strain. It is unlikely that this 11% drop



lac / *dnaQ*	–	5.5	5.2	5.9
traT / *dnaQ*	–	3.6	3.3	3.2

FIGURE 4.—Inactivation of *hupA* or *hupB* does not decrease the copy number of the F'_{128} episome. C36 (the F^- parent of FC40), FC40, and the FC40 Δ*hup* derivatives PFB610 and PFB611 were grown to saturation (~48 hr) in minimal M9-glycerol liquid medium. The DNA was purified and digested with *Eco*RV restriction endonuclease and separated by agarose gel electrophoresis. A Southern blot was performed using radiolabeled probes to the chromosomal *dnaQ* gene and two episomal makers, the *lac* region and the *traT* gene (see MATERIALS AND METHODS). The respective bands are indicated by labeled arrows. The ratios of the intensities of the *lac* and *traT* bands to the *dnaQ* band are given below the blot. About four times as much DNA was loaded for FC40 compared to the other strains. The difference in the ratios of *lac* to *dnaQ* and *traT* to *dnaQ* is likely an artifact resulting from differences in the labeling efficiency of the two probes.

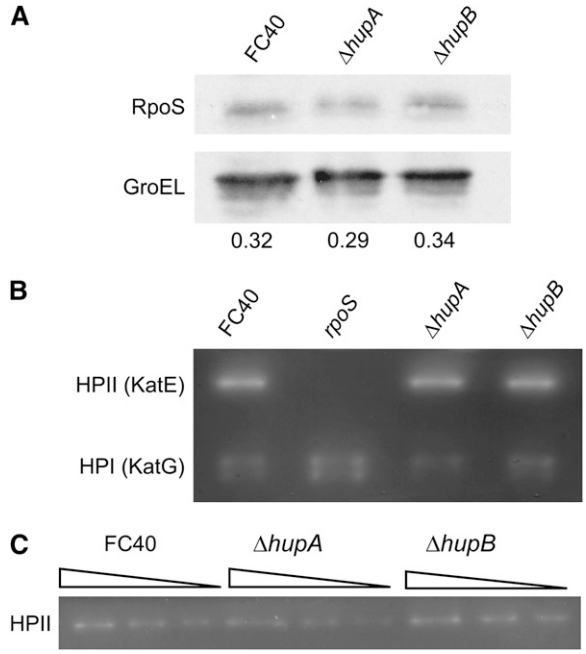


FIGURE 5.—The negative effects of inactivating *hupA* or *hupB* are not due to decreased RpoS levels or RpoS function. (A) Immunoblot showing that the cellular levels of RpoS are not affected by inactivation of *hupA* and *hupB*. Twenty-five micrograms of total protein from FC40, PFB610 (Δ*hupA*::Kn), and PFB611 (Δ*hupB*::Kn) was loaded in each lane. The blot was probed with anti-RpoS antibody, stripped, and then reprobed with anti-GroEL antibody. Band intensities were quantified and the numbers below the lanes give the ratios of the densities of the Pol IV bands to the GroEL bands (see MATERIALS AND METHODS). (B) In-gel negative stain for *katE*-encoded HPII catalase activity (top band) in wild-type and *hup*⁻ strains. Forty micrograms of total protein from FC40, PFG150, PFB610, and PFB611 was separated using nondenaturing polyacrylamide gel electrophoresis and stained for catalase activity (see MATERIALS AND METHODS). The bottom running band is HPI catalase, encoded by *katG*. (C) Serial twofold dilutions of the protein samples shown in B showing that consistent intensities of the bands in B are not the product of saturation beyond the linear range of detection. Only the relevant KatE bands are shown.

in expression of the protein fusion could explain the large decrease in adaptive mutation in the *hupA*::Cm background.

Because of duplicate antibiotic resistance markers, we could not test these *lacZ* fusions in the *hupB*::Kn mutant strain. In addition, the fusions lack the site that controls RpoS protein stability (HENGGE-ARONIS 2002). Therefore, we used additional methods to further explore the possibility of an RpoS-dependent effect. As shown in Figure 5A, immunoblotting demonstrated that the cellular levels of RpoS were not detectably changed in *hupA*::Cm or *hupB*::Kn mutant cells. These results suggest that the decrease in adaptive mutation in *hup* mutants is not due to an effect on the amount or expression of *rpoS*.

The *hup* mutations could affect the activity of RpoS instead of its amount. The *E. coli* chromosome encodes two catalase enzymes, HPI and HPII, encoded by *katG* and *katE*, respectively; the *katE* gene is tightly regulated by RpoS (SCHELLHORN 1995; SCHELLHORN and HASSAN 1988; TANAKA *et al.* 1997). A defect in RpoS activity would reduce or eliminate *katE* expression, resulting in a decrease in the *katE*-encoded HPII catalase activity in cell-free extracts. KatE activity can be measured by using a semiquantitative in-gel negative stain assay (GREGORY and FRIDOVICH 1974; CLARE *et al.* 1984; TOUATI *et al.*

TABLE 2

β-Galactosidase activity of wild-type and *hupA* mutant strains

Strain	Fusion	Relevant genotype	β-Galactosidase activity (Miller units)[a]	SEM
PFG339	*rpoS′-lac*$^{+}$[b]	*hup*$^{+}$	7656	134
PFB564	*rpoS′-lacZ*$^{+}$	*hupA*::Cm	7885	215
PFG338	*rpoS′-lacZ′*[c]	*hup*$^{+}$	1928	44
PFB563	*rpoS′-lacZ′*	*hupA*::Cm	1716	24

[a] Four independent cultures were assayed for each strain. The data are means and SEMs.

[b] *rpoS′-lacZ*$^{+}$ is an operon fusion placing *lacZ* under the control of the *rpoS* promoter (HIRSCH and ELLIOTT 2002).

[c] *rpoS′-lacZ′* encodes a protein fusion of LacZ to RpoS under the control of the *rpoS* promoter (HIRSCH and ELLIOTT 2002).

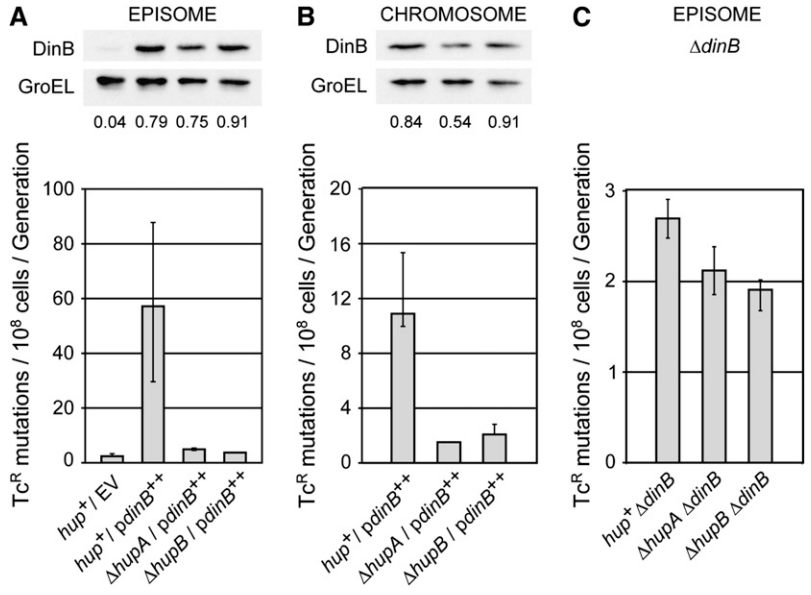


FIGURE 6.—Deletion of *hupA* or *hupB* reduces the rate of growth-dependent mutations when *dinB* is expressed from a multicopy plasmid. (A) Mutation rates when the *tetA* allele is carried on the F'_{128} episome. Data are mutation rates to Tc^R; error bars are 95% confidence levels. (B) Mutation rates when the *tetA* allele is carried on the chromosome. Data are mutation rates to Tc^R; error bars are 95% confidence levels. Shown above the graphs are immunoblots probed with anti-Pol IV antibody, stripped, and then reprobed with anti-GroEL antibody. Band intensities were quantified and the numbers below the lanes give the ratios of the densities of the Pol IV bands to the GroEL bands (see MATERIALS AND METHODS). Bands are in the same order as the strains in the graphs. For episomal *tetA* alleles: *hup*$^+$/EV = PFG265; *hup*$^+$/p*dinB*$^{++}$ = PFG266; Δ*hup*A/p*dinB*$^{++}$ = PFB628; Δ*hup*B/p*dinB*$^{++}$ = PFB629. For chromosomal *tetA* alleles: *hup*$^+$/p*dinB*$^{++}$ = PFB656; Δ*hup*A/p*dinB*$^{++}$ = PFB657; Δ*hupB*/p*dinB*$^{++}$ = PFB658. (C) Inactivation of *hup* genes causes only small decreases in the growth-dependent mutation rate in the absence of Pol IV. Data are mutation rates to Tc^R with the *tetA* allele on the F'_{128} episome; error bars are 95% confidence levels. *hup*$^+$ Δ*dinB* = PFB665; Δ*hupA*::Kn Δ*dinB* = PFB673; Δ*hupB::Kn* Δ*dinB* = PFB674.

1991). As shown in Figure 5B, KatE activity (top band) was absent in an *rpoS* mutant strain, but KatE activity was normal in the Δ*hupA*::Kn and Δ*hupB*::Kn mutant strains. To ensure that the KatE bands in Figure 5B were not saturated, obscuring smaller differences in KatE activity, serial twofold dilutions of the cell-free extract were tested in the same assay. As shown in Figure 5C, the amount of catalase activity was not decreased to any significant extent in either *hup* mutant. Taken together, these results suggest that *hup* mutations do not affect adaptive mutation via a negative effect on *rpoS* expression or RpoS activity.

Inactivation of *hup* genes decreases spontaneous mutations due to Pol IV overexpression: When Pol IV is overexpressed in exponentially growing cells, the rate of spontaneous mutation can increase as much as 1000-fold (KIM *et al.* 1997). This growth-dependent Pol IV-dependent mutation rate can be easily measured in strain FC722 (FOSTER 1997; STUMPF and FOSTER 2005), which carries on the F'_{128} episome a *tetA* allele with a +1 frameshift mutation that renders the cells Tc^S. Like the frameshift that renders the Φ(*lacI-lacZ33*) allele Lac$^-$, the +1 frameshift in *tetA* is in a run of repeated G:C base pairs (FOSTER 1997). Because of this similarity, the mechanism by which the two alleles are reverted by Pol IV is likely to be essentially the same at the DNA base level. However, unlike adaptive mutation, Tc^R mutations occur during exponential growth when the episome and chromosome are being actively replicated.

Overexpression of Pol IV under control of the arabinose promoter on a multicopy plasmid (pBAD24-*dinB)* increased the reversion rate to Tc^R in FC722 by ~20-fold relative to the strain carrying the empty vector (Figure 6A). In otherwise isogenic Δ*hupA*::Kn and Δ*hupB*::Kn derivatives of FC722, the spontaneous mutation rate in the presence of pBAD24-*dinB* was increased only about twofold. Immunoblots (Figure 6A, top) revealed that the decreases in growth-dependent mutation are likely not due to decreases in the amount of Pol IV; in fact, the Δ*hupB* mutant actually shows a slight elevation in the level of Pol IV. When the mutant *tetA* allele was on the chromosome rather than on the episome, the *hup* mutant alleles had a similar effect on the spontaneous mutation rate (Figure 6B). An immunoblot for Pol IV levels revealed a similar trend to that in Figure 6A.

These results suggest that the effects of the *hup* mutations on growth-dependent mutation rates are due to an effect on the mutagenic activity of Pol IV. To further test this possibility, growth-dependent mutation rates were measured in FC722 derivatives lacking *dinB*. As shown in Figure 6C, when Pol IV is absent, inactivation of the *hup* genes resulted in decreases that were much smaller than observed for Pol IV-independent adaptive mutation (Figure 2B). This result further supports the hypothesis that when Pol IV is overproduced, most of the drop in growth-dependent mutation rate in *hup* mutants is due to a negative effect on Pol IV mutagenesis. However, as in the adaptive mutation experiments, the effects of the *hup* mutations on growth-dependent mutation are reduced, but not eliminated, in the absence of *dinB*, suggesting that *hup* mutations also affect growth-dependent mutation by a Pol IV-independent mechanism.

DISCUSSION

The precise mechanism by which adaptive Lac$^+$ reversions occur is still controversial (FOSTER 2004;

Rosenberg and Hastings 2004; Roth and Andersson 2004); however, in our model adaptive mutation in *E. coli* involves upregulation of mutation rates during stressful conditions, thus increasing the possibility that fitness-enhancing mutations may arise. The data presented here provide a new insight into the molecular pathway by which Lac$^+$ mutations arise during nutrient limitation. Specifically, we have demonstrated that the histone-like protein, HU, is involved in adaptive mutation in *E. coli* strain FC40.

Strains carrying null alleles of one of the two genes encoding HU protein, *hupA* or *hupB*, have decreased levels of adaptive mutation (Figure 1). The *hup* mutations do not affect the viability of the Lac$^-$ cells, and thus they must affect the mechanism by which adaptive Lac$^+$ revertants arise. HU is also involved in growth-dependent mutations when Pol IV is expressed from a multicopy plasmid (Figure 6). In addition, we ruled out the possibilities that the effects of *hup* mutations on adaptive mutation are due to a negative effect on the copy number of the episome or the *lac* mutational target (Figure 4) or to a decrease in *rpoS* expression (Table 2) or RpoS function (Figure 5).

Most adaptive Lac$^+$ mutations are Pol IV dependent. However, immunoblot analysis with anti-Pol IV antibody revealed that while various *hupA* and *hupB* alleles have similar effects on adaptive mutation, they have small but reproducible differences in their effects on the cellular amounts of Pol IV (Figure 2). The *hupA*::Cm and *hupB*::Kn alleles decrease Pol IV levels slightly, while the Δ*hupA*::Kn or the Δ*hupB*::Kn alleles increase Pol IV levels slightly. The reason for these differences is not clear; however, the nature of the mutant alleles may play a role. Both the *hupA*::Cm and *hupB*::Kn alleles have insertions of drug resistance cassettes. Thus, these alleles may express truncated forms of the subunits that retain residual function and negatively affect Pol IV levels by an unknown mechanism. In contrast, the strains carrying the Δ*hupA*::Kn and Δ*hupB*::Kn deletions are null alleles and thus should reveal any effect that complete loss of an HU subunit has on the amount of Pol IV. One possible explanation for why the deletion mutants increase in the amount of Pol IV is that the SOS response is induced to some extent in the mutant cells. However, induction of the SOS response causes cell filamentation (Huisman *et al.* 1984) and microscopic examination of the *hup* mutant strains used in this study showed no filamentation in stationary phase cells (data not shown). Thus, the SOS response is not induced to a level sufficient to induce filamentation and probably does not explain the increase in Pol IV in the Δ*hupA*::Kn and Δ*hupB*::Kn strains. Possibly small differences in HU composition affect a yet-unknown regulator of *dinB*. Nevertheless, the uncoupling of the amount of Pol IV from the level of adaptive mutation indicates that *hup* mutations affect adaptive mutation independently of Pol IV.

Epistasis analyses reinforced the conclusion that the *hup* mutations have both Pol IV- dependent and Pol IV-independent inhibitory effects on adaptive mutation (Figure 2). Interestingly, Δ*hupA*::Kn and Δ*hupB*::Kn had essentially the same inhibitory effect, suggesting that the heterodimer HUαβ is more functional in promoting adaptive mutation to Lac$^+$ than either of the homodimers. These results have interesting implications for the growth-phase regulation of the different forms of HU. During early exponential growth, HUα2 is most prevalent, but as cells progress toward stationary phase, expression of *hupB* is upregulated and HUαβ becomes the dominant species (Claret and Rouviere-Yaniv 1997). As described in the Introduction, the various dimeric forms of HU have different biochemical properties and appear to have different biological functions. The requirement for the proper balance of the three species of HU for maximal adaptive mutation may reflect a mechanism to selectively enhance different DNA-related functions during different growth phases of the cell. Although this hypothesis is speculative, it is interesting to consider that cells may have an ability to regulate the activities of various pathways for recombination, repair, and mutagenesis during different growth phases by regulating the relative composition of the three forms of a small and abundant DNA-binding protein.

In contrast to their large effects in *recG*$^+$ cells, the Δ*hupA*::Kn and Δ*hupB*::Kn cause only small, possibly insignificant, decreases in Lac$^+$ adaptive mutation in *recG* mutant cells (Figure 3). This result suggests that *hup* mutations normally reduce adaptive mutation by a RecG-dependent pathway. Such an interaction would be interesting because the high levels of Pol IV and the resulting high mutation rates caused by *recG* mutations have prevented genetic analysis of the true role of the RecG helicase in adaptive mutation. RecG can process recombination intermediates independently of the major Holliday junction resolution complex, RuvABC (Briggs *et al.* 2004). But cells mutant in *ruvA*, *B*, or *C* are severely defective for adaptive mutation even with functional RecG (Foster *et al.* 1996; Harris *et al.* 1996). Thus, RuvABC is required to process the majority of the recombination intermediates that lead to Lac$^+$ adaptive mutations. Since the *hup* mutations do not significantly affect the normal elevated level of adaptive mutation in *recG* mutant cells, RuvABC function is apparently not affected by HUαβ in the absence of RecG. However, our data are consistent with the hypothesis that RecG normally inhibits RuvABC function, but this inhibition is relieved by HUαβ. Since to our knowledge no direct involvement of RecG in the RuvABC pathway has been shown, RecG must inhibit RuvABC indirectly. One possibility is that in the absence of HUαβ, RecG binds to recombination intermediates, blocking further processing by RuvABC. RecG could simply sequester the intermediate, or RecG could process the intermediate

by a pathway that does not produce Lac$^+$ mutations (*e.g.*, see Foster *et al.* 1996). At biologically relevant salt concentrations *in vitro*, HUαβ binds model Holliday junctions with a higher affinity than either of the HU homodimers; thus, HUαβ might out-compete RecG for binding to recombination intermediates, preventing RecG from blocking RuvABC access. HUαβ could then be displaced by RuvABC, allowing further processing. This model makes HUαβ a central regulator of protein trafficking during recombination.

In our current model of the mechanism of adaptive Lac$^+$ reversion (Foster 2004), Pol IV competes with other polymerases for access to a 3′ DNA terminus during the replication primed by the PriA primosome following replication fork failure. In the event that Pol IV gains access to the terminus and replicates a sufficient length of DNA to traverse the Φ(*lacI33-lacZ*) allele, potentially reverting frameshifts occur due to template slippage at runs of identical bases. The error is immortalized in the DNA sequence by the subsequent formation, migration, and resolution of a Holliday junction, which leads to replication restart, retention of the Lac$^+$ mutation, and expression of the Lac$^+$ phenotype. Other polymerases could be involved in this process and could also produce frameshifts, which would explain why the *hup* mutations decrease Lac$^+$ adaptive mutation in the absence of Pol IV. For example, our results are consistent with HU acting upstream in the mutagenic pathway, possibly by providing the substrate for polymerase activity. However, all pathways are likely to converge at a DNA intermediate requiring further processing by RuvAB and/or RecG. In the absence of Pol IV, loss of *recG* has a small negative effect on adaptive mutation (Layton and Foster 2003), consistent with the hypotheses that RecG has a positive role in adaptive mutation and that other polymerases can participate in the process. The partial dependencies of adaptive mutation on Pol IV and RecG are similar to the roles that these proteins have been hypothesized to play in recombination stimulated by replication arrest (Lovett 2006).

An alternative model for the generation of adaptive Lac$^+$ mutations proposes that the initiating event is a spontaneous duplication event followed by amplification of the leaky *lac* allele on the minimal lactose plates. This amplification pathway potentially leads to two outcomes: true Lac$^+$ revertants that result from a mutation among the amplified array and pseudo-revertant Lac$^+$ clones that, when plated on a nonselective medium, will revert to Lac$^-$ by deamplification of the *lac* region (Roth and Andersson 2004). While it was possible that the *hup* mutants inhibited the amplification pathway, we found that formation of unstable Lac$^+$ revertants was not impaired in *hup* mutants (data not shown). In addition, the amplification pathway is irrelevant to the *tetA* experiments (Figure 6) because under the conditions of this assay there is no selection for amplification.

In addition to their effects on adaptive mutation, the *hup* mutant alleles decreased growth-dependent Tc^R mutations due to Pol IV overproduction from a multicopy plasmid (Figure 6). The *hup* mutations did not substantially affect the amount of Pol IV (Figure 6) nor did they affect the plasmid copy number (data not shown). As described above, the frameshift mutation in the mutant *tetA* allele has a similar local sequence context as that in Φ(*lacI33-lacZ*); thus, Pol IV activity at the sequence level is likely to be identical at the two mutational targets. The most significant difference in the two assays is that Tc^R reversions arise when the cells are dividing and actively replicating their DNA. During DNA replication, the sliding β-clamp is proposed to be responsible for polymerase switching under various conditions (Naktinis *et al.* 1996; Bunting *et al.* 2003; Lopez De Saro *et al.* 2003). Overexpressing Pol IV will increase the probability that it gains access to the clamp at a stalled replication complex and continues DNA synthesis from the misaligned primer, an activity that it has been shown to possess (Kobayashi *et al.* 2002). Pol III could then resume normal replication downstream. As Pol IV levels are unchanged in the *hup* mutants, one possible explanation for the decrease in the rate of Pol IV-generated frameshifts leading to Tc^R reversion is that the absence of the HUαβ heterodimer restricts Pol IV access to these stalled replication complexes. A second possibility is that HUαβ is required in a downstream processing step during restart of replication after Pol IV activity. In either case, our results show an additional context in which the HUαβ heterodimer appears to have a unique function that is absent in the homodimers.

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LITERATURE CITED

Abramoff, M. D., P. J. Magelhaes and S. J. Ram, 2004 Image processing with ImageJ. Biophotonics Intl. **11:** 36–42.

Aki, T., and S. Adhya, 1997 Repressor induced site-specific binding of HU for transcriptional regulation. EMBO J. **16:** 3666–3674.

Aki, T., H. E. Choy and S. Adhya, 1996 Histone-like protein HU as a specific transcriptional regulator: co-factor role in repression of *gal* transcription by GAL repressor. Genes Cells **1:** 179–188.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman *et al.*, 1988 *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.

Balandina, A., L. Claret, R. Hengge-Aronis and J. Rouviere-Yaniv, 2001 The *Escherichia coli* histone-like protein HU regulates *rpoS* translation. Mol. Microbiol. **39:** 1069–1079.

Balandina, A., D. Kamashev and J. Rouviere-Yaniv, 2002 The bacterial histone-like protein HU specifically recognizes similar structures in all nucleic acids. DNA, RNA, and their hybrids. J. Biol. Chem. **277:** 27622–27628.

Bohannon, D. E., N. Connell, J. Keener, A. Tormo, M. Espinosa-Urgel *et al.*, 1991 Stationary-phase-inducible "gearbox"

promoters: differential effects of *katF* mutations and role of sigma 70. J. Bacteriol. **173:** 4482–4492.

Borden, A., P. I. O'Grady, D. Vandewiele, A. R. Fernandez de Henestrosa, C. W. Lawrence *et al.*, 2002 *Escherichia coli* DNA polymerase III can replicate efficiently past a T-T *cis-syn* cyclobutane dimer if DNA polymerase V and the 3′ to 5′ exonuclease proofreading function encoded by *dnaQ* are inactivated. J. Bacteriol. **184:** 2674–2681.

Boubrik, F., and J. Rouviere-Yaniv, 1995 Increased sensitivity to gamma irradiation in bacteria lacking protein HU. Proc. Natl. Acad. Sci. USA **92:** 3958–3962.

Brake, A. J., A. V. Fowler, I. Zabin, J. Kania and B. Muller-Hill, 1978 β-galactosidase chimeras: primary structure of a *lac* repressor-β-galactosidase protein. Proc. Natl. Acad. Sci. USA **75:** 4824–4827.

Briggs, G. S., A. A. Mahdi, G. R. Weller, Q. Wen and R. G. Lloyd, 2004 Interplay between DNA replication, recombination and repair based on the structure of RecG helicase. Philos. Trans. R. Soc. Lond. B. Biol. Sci. **359:** 49–59.

Brunetti, R., G. Prosseda, E. Beghetto, B. Colonna and G. Micheli, 2001 The looped domain organization of the nucleoid in histone-like protein defective *Escherichia coli* strains. Biochimie **83:** 873–882.

Bunting, K. A., S. M. Roe and L. H. Pearl, 2003 Structural basis for recruitment of translesion DNA polymerase Pol IV/DinB to the beta-clamp. EMBO J. **22:** 5883–5892.

Cairns, J., and P. L. Foster, 1991 Adaptive reversion of a frameshift mutation in *Escherichia coli*. Genetics **128:** 695–701.

Calos, M. P., and J. H. Miller, 1981 Genetic and sequence analysis of frameshift mutations induced by ICR-191. J. Mol. Biol. **153:** 39–66.

Clare, D. A., M. N. Duong, D. Darr, F. Archibald and I. Fridovich, 1984 Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. Anal. Biochem. **140:** 532–537.

Claret, L., and J. Rouviere-Yaniv, 1997 Variation in HU composition during growth of *Escherichia coli*: the heterodimer is required for long-term survival. J. Mol. Biol. **273:** 93–104.

Datsenko, K. A., and B. L. Wanner, 2001 One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:** 6640–6645.

Finkel, S. E., 2006 Long-term survival during stationary phase: evolution and the GASP phenotype. Nat. Rev. Microbiol. **4:** 113–120.

Foster, P. L., 1994 Population dynamics of a Lac$^-$ strain of *Escherichia coli* during selection for lactose utilization. Genetics **138:** 253–261.

Foster, P. L., 1997 Nonadaptive mutations occur on the F′ episome during adaptive mutation conditions in *Escherichia coli*. J. Bacteriol. **179:** 1550–1554.

Foster, P. L., 2000 Adaptive mutation in *Escherichia coli*. Cold Spring Harb. Symp. Quant. Biol. **65:** 21–29.

Foster, P. L., 2004 Adaptive Mutation in *Escherichia coli*. J. Bacteriol. **186:** 4846–4852.

Foster, P. L., 2006 Methods for determining spontaneous mutation rates. Methods Enzymol. **409:** 195–213.

Foster, P. L., and J. M. Trimarchi, 1994 Adaptive reversion of a frameshift mutation in *Escherichia coli* by simple base deletions in homopolymeric runs. Science **265:** 407–409.

Foster, P. L., and J. M. Trimarchi, 1995a Adaptive reversion of an episomal frameshift mutation in *Escherichia coli* requires conjugal functions but not actual conjugation. Proc. Natl. Acad. Sci. USA **92:** 5487–5490.

Foster, P. L., and J. M. Trimarchi, 1995b Conjugation is not required for adaptive reversion of an episomal frameshift mutation in *Escherichia coli*. J. Bacteriol. **177:** 6670–6671.

Foster, P. L., J. M. Trimarchi and R. A. Maurer, 1996 Two enzymes, both of which process recombination intermediates, have opposite effects on adaptive mutation in *Escherichia coli*. Genetics **142:** 25–37.

Giangrossi, M., A. M. Giuliodori, C. O. Gualerzi and C. L. Pon, 2002 Selective expression of the beta-subunit of nucleoid-associated protein HU during cold shock in *Escherichia coli*. Mol. Microbiol. **44:** 205–216.

Gregory, E. M., and I. Fridovich, 1974 Visualization of catalase on acrylamide gels. Anal. Biochem. **58:** 57–62.

Guzman, L. M., D. Belin, M. J. Carson and J. R. Beckwith, 1995 Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. **177:** 4121–4130.

Harlow, E., and D. Lane, 1988 *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Harris, R. S., K. J. Ross and S. M. Rosenberg, 1996 Opposing roles of the Holliday junction processing systems of *Escherichia coli* in recombination-dependent adaptive mutation. Genetics **142:** 681–691.

Hengge-Aronis, R., 2002 Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. Microbiol. Mol. Biol. Rev. **66:** 373–395.

Hillyard, D. R., M. Edlund, K. T. Hughes, M. Marsh and N. P. Higgins, 1990 Subunit-specific phenotypes of *Salmonella typhimurium* HU mutants. J. Bacteriol. **172:** 5402–5407.

Hirsch, M., and T. Elliott, 2002 Role of ppGpp in *rpoS* stationary-phase regulation in *Escherichia coli*. J. Bacteriol. **184:** 5077–5087.

Huisman, O., R. D'Ari and S. Gottesman, 1984 Cell-division control in *Escherichia coli*: specific induction of the SOS function SfiA protein is sufficient to block septation. Proc. Natl. Acad. Sci. USA **81:** 4490–4494.

Huisman, O., M. Faelen, D. Girard, A. Jaffe, A. Toussaint *et al.*, 1989 Multiple defects in *Escherichia coli* mutants lacking HU protein. J. Bacteriol. **171:** 3704–3712.

Kano, Y., M. Wada, T. Nagase and F. Imamoto, 1986 Genetic characterization of the gene *hupB* encoding the HU-1 protein of *Escherichia coli*. Gene **45:** 37–44.

Kano, Y., M. Wada and F. Imamoto, 1988 Genetic characterization of the gene *hupA* encoding the HU-2 protein of *Escherichia coli*. Gene **69:** 331–335.

Kano, Y., N. Goshima, M. Wada and F. Imamoto, 1989 Participation of *hup* gene product in replicative transposition of Mu phage in *Escherichia coli*. Gene **76:** 353–358.

Kar, S., R. Edgar and S. Adhya, 2005 Nucleoid remodeling by an altered HU protein: reorganization of the transcription program. Proc. Natl. Acad. Sci. USA **102:** 16397–16402.

Kim, S. R., G. Maenhaut-Michel, M. Yamada, Y. Yamamoto, K. Matsui *et al.*, 1997 Multiple pathways for SOS-induced mutagenesis in *Escherichia coli*: an overexpression of *dinB/dinP* results in strongly enhancing mutagenesis in the absence of any exogenous treatment to damage DNA. Proc. Natl. Acad. Sci. USA **94:** 13792–13797.

Kobayashi, S., M. R. Valentine, P. Pham, M. O'Donnell and M. F. Goodman, 2002 Fidelity of *Escherichia coli* DNA polymerase IV. Preferential generation of small deletion mutations by dNTP-stabilized misalignment. J. Biol. Chem. **277:** 34198–34207.

Lavoie, B. D., and G. Chaconas, 1994 A second high affinity HU binding site in the phage Mu transpososome. J. Biol. Chem. **269:** 15571–15576.

Layton, J. C., and P. L. Foster, 2003 Error-prone DNA polymerase IV is controlled by the stress-response sigma factor, RpoS, in *Escherichia coli*. Mol. Microbiol. **50:** 549–561.

Lee, H., H. K. Kim, S. Kang, C. B. Hong, J. Yim *et al.*, 2001 Expression of the *seqA* gene is negatively modulated by the HU protein in *Escherichia coli*. Mol. Gen. Genet. **264:** 931–935.

Lewis, D. E., and S. Adhya, 2002 *In vitro* repression of the *gal* promoters by GalR and HU depends on the proper helical phasing of the two operators. J. Biol. Chem. **277:** 2498–2504.

Li, S., and R. Waters, 1998 *Escherichia coli* strains lacking protein HU are UV sensitive due to a role for HU in homologous recombination. J. Bacteriol. **180:** 3750–3756.

Lloyd, R. G., and C. Buckman, 1991 Genetic analysis of the *recG* locus of *Escherichia coli* K-12 and of its role in recombination and DNA repair. J. Bacteriol. **173:** 1004–1011.

Lombardo, M. J., I. Aponyi and S. M. Rosenberg, 2004 General stress response regulator RpoS in adaptive mutation and amplification in *Escherichia coli*. Genetics **166:** 669–680.

Lopez de Saro, F. J., R. E. Georgescu, M. F. Goodman and M. O'Donnell, 2003 Competitive processivity-clamp usage by DNA polymerases during DNA replication and repair. EMBO J. **22:** 6408–6418.

Lovett, S. T., 2006 Replication arrest-stimulated recombination: dependence on the RecA paralog, RadA/Sms and translesion polymerase, DinB. DNA Repair **5:** 1421–1427.

McKenzie, G. J., P. L. Lee, M. J. Lombardo, P. J. Hastings and S. M. Rosenberg, 2001 SOS mutator DNA polymerase IV functions in adaptive mutation and not adaptive amplification. Mol. Cell **7:** 571–579.

Miller, J. H., 1992 *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Müller-Hill, B., H. V. Rickenberg and K. Wallenfels, 1964 Specificity of the induction of the enzymes of the *lac* operon in *Escherichia coli*. J. Mol. Biol. **10:** 303–318.

Naktinis, V., J. Turner and M. O'Donnell, 1996 A molecular switch in a replication machine defined by an internal competition for protein rings. Cell **84:** 137–145.

Nohmi, T., 2006 Environmental stress and lesion-bypass DNA polymerases. Annu. Rev. Microbiol. **60:** 231–253.

Pinson, V., M. Takahashi and J. Rouviere-Yaniv, 1999 Differential binding of the *Escherichia coli* HU, homodimeric forms and heterodimeric form to linear, gapped and cruciform DNA. J. Mol. Biol. **287:** 485–497.

Pushnova, E. A., M. Geier and Y. S. Zhu, 2000 An easy and accurate agarose gel assay for quantitation of bacterial plasmid copy numbers. Anal. Biochem. **284:** 70–76.

Rosenberg, S. M., and P. J. Hastings, 2004 Adaptive point mutation and adaptive amplification pathways in the *Escherichia coli* Lac system: stress responses producing genetic change. J. Bacteriol. **186:** 4838–4843.

Rosenberg, S. M., S. Longerich, P. Gee and R. S. Harris, 1994 Adaptive mutation by deletions in small mononucleotide repeats. Science **265:** 405–407.

Roth, J. R., and D. I. Andersson, 2004 Adaptive mutation: how growth under selection stimulates Lac$^+$ reversion by increasing target copy number. J. Bacteriol. **186:** 4855–4860.

Rouviere-Yaniv, J., and N. O. Kjeldgaard, 1979 Native *Escherichia coli* HU protein is a heterotypic dimer. FEBS Lett. **106:** 297–300.

Roy, S., E. K. Dimitriadis, S. Kar, M. Geanacopoulos, M. S. Lewis *et al.*, 2005 Gal repressor-operator-HU ternary complex: pathway of repressosome formation. Biochemistry **44:** 5373–5380.

Schellhorn, H. E., 1995 Regulation of hydroperoxidase (catalase) expression in *Escherichia coli*. FEMS Microbiol. Lett. **131:** 113–119.

Schellhorn, H. E., and H. M. Hassan, 1988 Transcriptional regulation of *katE* in *Escherichia coli* K-12. J. Bacteriol. **170:** 4286–4292.

Semsey, S., K. Virnik and S. Adhya, 2006 Three-stage regulation of the amphibolic *gal* operon: from repressosome to GalR-free DNA. J. Mol. Biol. **358:** 355–363.

Stumpf, J. D., and P. L. Foster, 2005 Polyphosphate kinase regulates error-prone replication by DNA polymerase IV in *Escherichia coli*. Mol. Microbiol. **57:** 751–761.

Stumpf, J. D., A. R. Poteete and P. L. Foster, 2007 Amplification of *lac* cannot account for adaptive mutation to Lac$^+$ in *Escherichia coli*. J. Bacteriol. **189:** 2291–2299.

Tanaka, K., K. Handel, P. C. Loewen and H. Takahashi, 1997 Identification and analysis of the *rpoS*-dependent promoter of *katE*, encoding catalase HPII in *Escherichia coli*. Biochim. Biophys. Acta **1352:** 161–166.

Touati, E., E. Dassa, J. Dassa, P. L. Boquet and D. Touati, 1991 Are *appR* and *katF* the same *Escherichia coli* gene encoding a new sigma transcription initiation factor? Res. Microbiol. **142:** 29–36.

van Noort, J., S. Verbrugge, N. Goosen, C. Dekker and R. T. Dame, 2004 Dual architectural roles of HU: formation of flexible hinges and rigid filaments. Proc. Natl. Acad. Sci. USA **101:** 6969–6974.

Wagner, J., P. Gruz, S. R. Kim, M. Yamada, K. Matsui *et al.*, 1999 The *dinB* gene encodes a novel *Escherichia coli* DNA polymerase, DNA Pol IV, involved in mutagenesis. Mol. Cell **4:** 281–286.

Wery, M., C. L. Woldringh and J. Rouviere-Yaniv, 2001 HU-GFP and DAPI co-localize on the *Escherichia coli* nucleoid. Biochimie **83:** 193–200.

Yeiser, B., E. D. Pepper, M. F. Goodman and S. E. Finkel, 2002 SOS-induced DNA polymerases enhance long-term survival and evolutionary fitness. Proc. Natl. Acad. Sci. USA **99:** 8737–8741.

Zzaman, S., M. M. Abhyankar and D. Bastia, 2004 Reconstitution of F factor DNA replication *in vitro* with purified proteins. J. Biol. Chem. **279:** 17404–17410.

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