The EMBO Journal (2006) 25, 868–879 | © 2006 European Molecular Biology Organization | All Rights Reserved 0261-4189/06
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THE EMBO JOURNAL

Y-family DNA polymerases respond to DNA damage-independent inhibition of replication fork progression

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In *Escherichia coli*, the Y-family DNA polymerases Pol IV (DinB) and Pol V ($UmuD_2'C$) enhance cell survival upon DNA damage by bypassing replication-blocking DNA lesions. We report a unique function for these polymerases when DNA replication fork progression is arrested not by exogenous DNA damage, but with hydroxyurea (HU), thereby inhibiting ribonucleotide reductase, and bringing about damage-independent DNA replication stalling. Remarkably, the *umuC122*::Tn*5* allele of *umuC*, *dinB*, and certain forms of *umuD* gene products endow *E. coli* with the ability to withstand HU treatment (HU^R). The catalytic activities of the UmuC122 and DinB proteins are both required for HU^R. Moreover, the lethality brought about by such stalled replication forks in the wild-type derivatives appears to proceed through the toxin/antitoxin pairs *mazEF* and *relBE*. This novel function reveals a role for Y-family polymerases in enhancing cell survival under conditions of nucleotide starvation, in addition to their established functions in response to DNA damage.

The EMBO Journal (2006) **25,** 868–879. doi:10.1038/sj.emboj.7600986; Published online 16 February 2006
Subject Categories: genome stability & dynamics; microbiology & pathogens
Keywords: DinB; hydroxyurea; *mazEF*; UmuC; UmuD

Introduction

In both eukaryotes and prokaryotes (Boye *et al*, 1996; Bell and Dutta, 2002), initiation of DNA replication is exquisitely regulated, and sophisticated systems have evolved to contend with the potentially lethal consequences of inhibition of replication fork progression (Elledge, 1996). Depletion of deoxyribonucleotide triphosphate (dNTP) pools leads to arrest of cell division in eukaryotes (Tercero *et al*, 2003) and prokaryotes (Foti *et al*, 2005) until DNA replication is properly restored. Mutations in components of such checkpoints result in genomic instability and elevated mutation frequencies that may lead to cancer in higher organisms (Hartwell and Kastan, 1994). Responses to arrest of fork progression include induction of DNA damage tolerance pathways. Although the rationale for such a response is clear when stalling is brought about by exogenous DNA damage, it is more enigmatic (Kai and Wang, 2003a, b) when replication fork progression is inhibited in a DNA damage-independent manner.

Y-family polymerases possess properties that are advantageous for the resolution of replication forks stalled by DNA damage as they have the ability to insert nucleotides opposite DNA lesions that block replicative DNA polymerases, a process termed translesion synthesis (TLS) (Friedberg *et al*, 2002). TLS often ensues with comparatively low fidelity, meaning that bypass of DNA damage takes place at a potentially mutagenic cost (Goodman, 2002). Notable exceptions exist, however, such as eukaryotic Pol η bypassing cyclobutane pyrimidine dimers (Washington *et al*, 2001).

The Y-family DNA polymerases are encoded in *Escherichia coli* by the *dinB* and *umuDC* genes, which are both regulated by the LexA transcriptional repressor as part of the SOS response to DNA damage (Sutton *et al*, 2000). Initially, full-length UmuD is expressed from the *umuDC* operon. The UmuD homodimer interacts with UmuC to effect a DNA damage checkpoint function (Opperman *et al*, 1999), and cold sensitivity due to overproduction of *umuDC* (Marsh and Walker, 1985) appears to result from an exaggeration of this function (Opperman *et al*, 1999; Sutton and Walker, 2001b). UmuD thereafter undergoes removal of its first 24 amino acids, dependent on the RecA nucleoprotein filament (Burckhardt *et al*, 1988; Shinagawa *et al*, 1988), to form UmuD′. The UmuD′ homodimer ($UmuD_2'$) is a positive effector of UmuC, the catalytic subunit of Pol V (Nohmi *et al*, 1988). Transcription of the *dinB* gene is weakly repressed by LexA, so that basal levels of DinB are high compared to those of UmuC (Woodgate and Ennis, 1991; Kim *et al*, 2001). Indeed, upon SOS induction Pol IV is the most abundant DNA polymerase in the cell (Kim *et al*, 2001). Among Y-family polymerases, the DinB subfamily is strikingly conserved, and it is the only branch present in all domains of life (Ohmori *et al*, 2001).

Hydroxyurea (HU) has been widely used to investigate responses to DNA damage-independent replication arrest (Lopes *et al*, 2001; Sogo *et al*, 2002). HU inhibits class I ribonucleotide reductases (RNR), such as that of aerobically grown *E. coli* (Stubbe, 2003), by scavenging a stable di-iron tyrosyl radical that is essential for catalysis. RNRs catalyze the conversion of ribonucleotides into deoxyribonucleotides—the rate-limiting step in DNA biosynthesis in most organisms (Stubbe, 2003). Levels of intracellular dNTPs are thought to decline upon HU treatment such that DNA replica-

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Received: 8 September 2005; accepted: 10 January 2006; published online: 16 February 2006

tion is arrested through substrate starvation (Sneeden and Loeb, 2004).

We report that the *E. coli* Y-family polymerases Pol IV and Pol V play a role upon DNA damage-independent replication stalling. Strains bearing novel *umuC* alleles are unexpectedly HU^R, challenging the notion that replication inhibition by HU arises solely from dNTP starvation. Genetic analyses demonstrate that the *dinB* and *umuD* gene products also participate in the DNA damage-independent response to inhibition of replication fork progression. Together, these data suggest combined action of the UmuC derivatives together with the *dinB* and *umuD* gene products at these stalled replication forks. Moreover, we also find that the lethality of such replication fork arrest in wild-type derivatives is alleviated independently by mutation of the *mazEF* and *relBE* toxin/antitoxin pairs, suggesting that the action of these Y-family polymerases may prevent *mazEF*- or *relBE*-mediated lethality under conditions of nucleotide starvation.

Results

E. coli carrying the umuC122::Tn5 allele are unexpectedly resistant to HU

We were interested in whether the $umuC^+$ gene product might be part of the cellular response when replication fork progression is inhibited in a DNA damage-independent manner by dNTP depletion. We therefore examined a set of strains carrying null alleles of *umuC* for their sensitivity to killing by HU (Figure 1A). A strain in which the *umuDC* operon has been deleted is as sensitive to killing by HU as its $umuD^+C^+$ parent. Intriguingly, a strain carrying a precise Δ*umuC* deletion that leaves the $umuD^+$ gene intact displays a modest level of resistance to killing (Figure 1A). Perhaps, either or both of the $umuD^+$ gene products might contribute to HU^R in the absence of UmuC (see below).

We also tested *umuC122*::Tn*5* (*umuC122*), which is known to behave as a *umuC* null allele with respect to induced mutagenesis caused by UV radiation and many chemicals (Elledge and Walker, 1983; Sargentini and Smith, 1984; Christensen *et al*, 1988; Bates *et al*, 1991). We found that strains carrying *umuC122* are at least 100-fold more resistant to killing by HU than their $umuC^+$ parents and can in fact multiply during HU treatment (Figure 1A). We observed this HU^R phenotype in all strain backgrounds tested, including AB1157 (Figure 1B; Bachmann, 1987). These observations indicate that *umuC122* is a gain-of-function *umuC* allele with regard to cell survival after HU treatment. This is plausible as the Tn*5* insertion results in a missense mutation followed immediately by a termination codon giving rise to a predicted 32 kDa UmuC protein lacking its last 102 residues (Koch *et al*, 1992). The truncation occurs downstream of the conserved polymerase domain common to Y-family DNA polymerases (Boudsocq *et al*, 2002). Immunoblotting confirmed that the *umuC122* allele indeed encodes a UmuC derivative of this molecular weight (Figure 1C). We observed that the truncated UmuC122 protein appears to be expressed at higher levels than wild-type UmuC (data not shown), although this may be because one of the synthetic peptides used to raise antibodies against UmuC

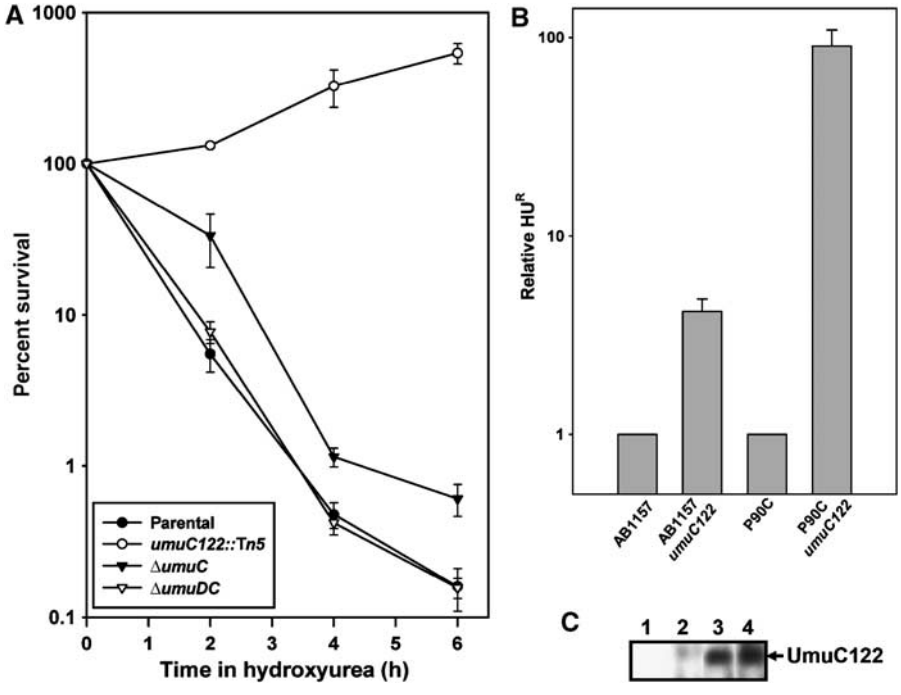

Figure 1 Bacterial cells bearing the *umuC122* allele are HU^R. (**A**) Survival time course in hydroxyurea reveals HU^R of a strain bearing the *umuC122* allele (open circles). In comparison, a strain bearing a Δ*umuC* allele (closed triangles) is slightly HU^R, whereas both parental (closed circles) and Δ*umuDC* strains (open triangles) are sensitive to the reagent. CFUs were determined by serial dilution. Error bars represent the standard deviation determined from at least five samples. (**B**) Comparison of survival in HU of both AB1157 and P90C backgrounds. Error bars represent the standard deviation determined from at least five samples. (**C**) The truncated UmuC122 protein is expressed *in vivo* as determined by immunoblotting. Lane 1 shows a cell-free extract from a Δ*umuDC* strain with vector only (pGB2), lane 2 shows the same strain but instead bearing the plasmid p*DC*, and lanes 3 and 4 show two independent isolates of the same strain bearing p*DC122*. Plasmid-borne copies were used to facilitate detection of UmuC in the absence of SOS induction.

lies immediately at the C-terminus of the UmuC122 protein, perhaps resulting in a more accessible epitope relative to full-length UmuC. The UmuC122 protein may also lack one or more C-terminal motifs that would normally target the protein for Lon-mediated proteolytic degradation (Frank *et al*, 1996). Overexpression of UmuC did not confer statistically significant HU^R (data not shown).

The umuC122 allele alleviates the lethal effects of class I RNR inhibition by HU

The observation of an HU^R phenotype as a consequence of a *umuC* mutation was unanticipated as most previously reported HU^R mutants affect RNR (Sneeden and Loeb, 2004). By immunoblotting, we showed that the levels of the small and large subunits of RNR are not affected during HU treatment in strains bearing the *umuC122* allele (Figure 2A). Also, we found that the protective effect of *umuC122* is observed with other RNR inhibitors such as guanazole (Figure 2B). We sought evidence that the *umuC122* mutation helps cells recover from the lethal consequences of HU-mediated RNR inhibition instead of acting by some other mechanism. Therefore, we took advantage of the fact that anaerobically grown *E. coli* utilize an HU-insensitive class III RNR rather than the HU-sensitive class I RNR used during aerobic growth (Fontecave *et al*, 1989). As shown in Figure 2C, we found that the anaerobically grown HU-treated *umuC*$^+$ and *umuC122* strains were both insensitive to HU. These observations indicate that the *umuC122* mutation alleviates the lethality caused by HU inhibition of the class I RNR in *E. coli* through a mechanism that does not involve alteration of RNR protein levels.

Resistance to HU requires the catalytic activity of the truncated UmuC122 protein

To facilitate further analysis of the genetic requirements for *umuC122*-mediated HU^R, we tested whether a plasmid-borne *umuDumuC122* (p*DC122*), expressed in a Δ*umuDC* derivative, conferred HU^R. This was indeed the case (Figure 3A). To determine whether this HU^R requires the catalytic activity of UmuC122, we used the *umuC104* allele (D101N) (Figure 3D) (Koch *et al*, 1992), which alters a conserved catalytic residue common to all Y-family polymerases (Boudsocq *et al*, 2002). The addition of p*DC104* had little effect on resistance to killing by HU (Figure 3A). However, introduction of the D101N mutation into p*DC122* eliminated HU^R (Figure 3A), indicating that the UmuC122 protein must be catalytically active to observe this phenotype.

A unique umuC missense allele also confers resistance to HU

We also tested the response to HU of *umuC125*, a *umuC* allele bearing an A39V mutation, which does not affect the ability of UmuC to function in UV mutagenesis, but eliminates the cold sensitivity observed when it is overexpressed together with UmuD (Marsh *et al*, 1991; Sutton and Walker, 2001b). We found that Δ*umuDC* cells containing p*DC125*, the plasmid-borne version of *umuC125*, are also resistant to HU, although not to as high a level as observed with p*DC122* (Figure 3A). This observation indicates that HU^R is not a unique property of the *umuC122* allele, but can be mimicked, at least in part, by a simple missense mutation affecting the N-terminus of UmuC.

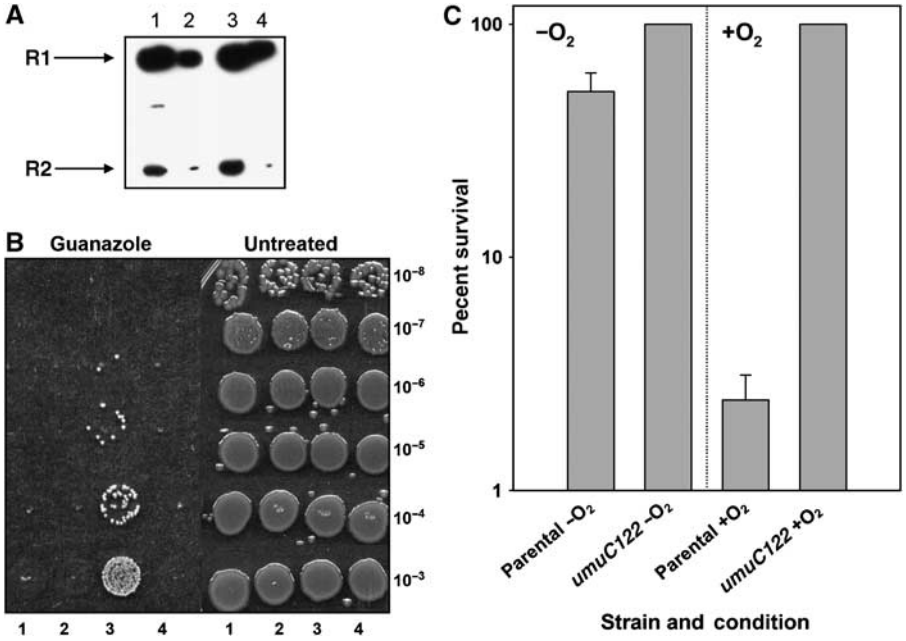

Figure 2 HU^R proceeds through RNR inhibition. (**A**) Immunoblot of large and small subunits of RNR shows no difference in levels between wild-type (lanes 1 and 2) and *umuC122* (lanes 3 and 4) strains during HU treatment. Lanes 1 and 3 contain twice as much total protein as lanes 2 and 4 (3.25 μg of total protein). (**B**) *umuC122* also alleviates cell death during challenge with other RNR inhibitors. The left panel shows results of treatment with 100 mM guanazole, whereas untreated results are shown on the right. Lane 1 shows the parental P90C strain, lane 2 shows the Δ*umuDC* strain, lane 3 shows *umuC122* and lane 4 shows the Δ*umuC* strain. (**C**) Class I RNR is sensitive to HU, whereas class III RNR, used exclusively in anaerobic growth, is indifferent to the reagent. The parental (P90C) and *umuC122* strains were treated with HU for 6 h with ($+O_2$) and without ($-O_2$) oxygen. CFUs reported are the average of four samples and error bars represent the standard deviation as determined from these samples.

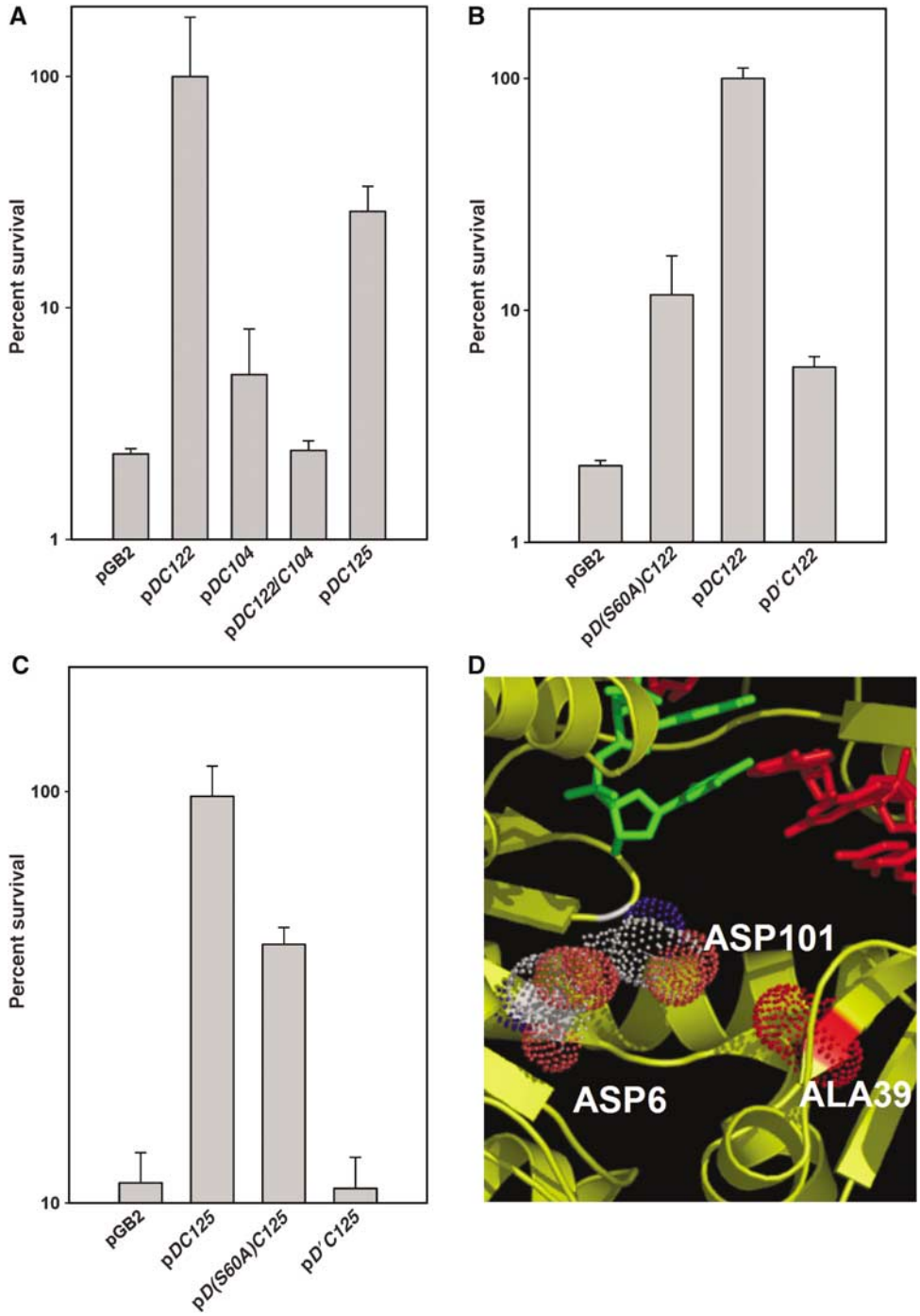

Figure 3 *umuC* requirements for observation of HU^R. (**A**) In a Δ*umuDC* strain, addition of the plasmid-borne *umuC* alleles p*DC122* and p*DC125* confer HU^R. p*DC122* carries the $umuD^+$ gene, but *umuC* has a stop codon at residue 322, thus reconstructing the truncated allele present on the chromosome by virtue of the Tn*5* insertion. p*DC104* encodes UmuC(D101N), rendering UmuC catalytically inactive, whereas p*DC122C104* encodes UmuC122(D101N). p*DC125* encodes UmuC(A39V), an allele that separates the UV-induced mutagenesis and cold-sensitivity phenotypes of *umuC*. CFUs were determined by serial dilution, and treatment was carried out with Sp (for plasmid maintenance) and 50 mM HU. Values reported are the average of three experiments and error bars represent the standard deviation obtained from those values. (**B**) The *umuD* gene products are also required for HU^R. The resistance conferred by a plasmid-borne *umuC122* allele depends upon the *umuD* gene products. p*D(S60A)C122* is as p*DC122* but encodes a UmuD protein with a mutation (S60A) rendering the protein unable to undergo autoproteolysis to become UmuD′. The plasmid p*D′C122* is as p*DC122* but encodes only UmuD′ instead of the full-length protein. Reported values are the average of three experiments and error bars represent the standard deviation as determined from those experiments. (**C**) The *umuD* gene products are also required for the HU^R conferred by *umuC125*. Plasmids and data analysis are as in (B). (**D**) A structural representation of the UmuC active site reveals the proximity of A39 to residues essential for catalysis (D6, D101). The template is shown in red, and the primer in green. Model is courtesy of Dr D Barksy (LLNL, Livermore, CA).

The umuD$^+$ gene is required for HU resistance

The data presented in Figure 1A suggest that the $umuD^+$ gene product(s) might contribute to HU^R in the absence of UmuC. Furthermore, the $umuD^+$ gene products influence the biological function of UmuC (Nohmi *et al*, 1988; Woodgate *et al*, 1989). We therefore assessed whether either form of the

umuD$^+$ gene product is required for the high level of HU^R we observed in *umuC122* bearing strains. The *umuD*(S60A) mutation (Koch *et al*, 1992; McLenigan *et al*, 1998) eliminates the serine that serves as the nucleophile in RecA-mediated UmuD autocleavage, so that only full-length UmuD is produced [p*D(S60A)C*]. Alternatively, the DNA encoding the first 24 amino acids in the N-terminus of UmuD can be deleted so that UmuD′ is synthesized directly (p*D′C*) (Nohmi *et al*, 1988).

As shown in Figure 3B, Δ*umuDC* cells with a plasmid carrying *umuD(S60A)umuC122* [p*D(S60A)C122*] exhibited a lower level of HU^R than the corresponding cells bearing the *umuC122* plasmid (p*DC122*), but nevertheless were substantially HU^R. Similarly, Δ*umuDC* cells bearing p*D′C122* exhibited a lower level of HU^R than the corresponding p*DC122* bearing strain, but were still HU^R. These results suggest that the full degree of HU^R displayed by a *umuD*$^+$*umuC122* strain requires both forms of the *umuD*$^+$ gene product. The two forms might act sequentially, first the $UmuD_2$ homodimer and then the $UmuD_2'$ homodimer. If so, it would appear that the component of HU^R requiring $UmuD_2$ is more substantial than the component requiring $UmuD_2'$. Another possibility is that a component of the HU^R requires the action of the UmuD·UmuD′ heterodimer, which is known to be the most stable form *in vitro* (Battista *et al*, 1990).

We performed similar experiments with the *umuC125* plasmid-borne allele (Figure 3C, note *y*-axis scale) in which we examined HU^R when *umuD(S60A)* and *umuD′* were combined with *umuC125*. Interestingly, in contrast to the situation with *umuC122*, the strain bearing p*D(S60A)C125* displayed comparable HU^R relative to the strain bearing p*DC125*, whereas the strain bearing p*D′C125* showed substantially less HU^R. These data, combined with the fact that the level of HU^R of a *umuC125* strain is less than that of a *umuC122* strain (Figure 3A), suggest that the UmuC125 protein is less proficient at the UmuD′-dependent component of HU^R than the UmuC122 protein.

The dinB$^+$ *gene is required for HU*^{R}

The results presented to this point indicate that the high-level resistance of certain *umuC* mutants to killing by HU also requires certain forms of the UmuD protein. Involvement of DinB in HU^R would be consistent with reports that DinB cooperates with UmuC in TLS past certain lesions (Napolitano *et al*, 2000; Sommer *et al*, 2003). Furthermore, under both induced and uninduced conditions, the intracellular levels of the *umuD* gene products are much higher than the estimated intracellular concentrations of UmuC, but are approximately equal to those of DinB (Woodgate and Ennis, 1991; Kim *et al*, 2001). Therefore, we constructed a strain with a precise deletion of the *dinB*$^+$ gene in *umuC*$^+$ and *umuC122* backgrounds. In a *umuC*$^+$ strain, loss of *dinB*$^+$ results in a slight sensitivity to HU (Figure 4A). However, introduction of the Δ*dinB* mutation into the strain carrying the *umuC122* allele eliminates the high level of HU^R observed in this strain (Figure 4A). Thus, the *dinB*$^+$ gene product is essential for the HU^R exhibited in *umuC122* strains.

We asked whether HU^R could be restored in a *umuC122*Δ*dinB* mutant by introducing plasmids carrying the *dinB*$^+$ gene. We were unable to complement HU^R *in trans* with low- or high-copy number plasmids bearing *dinB*$^+$. However, by transducing the wild-type copy of the

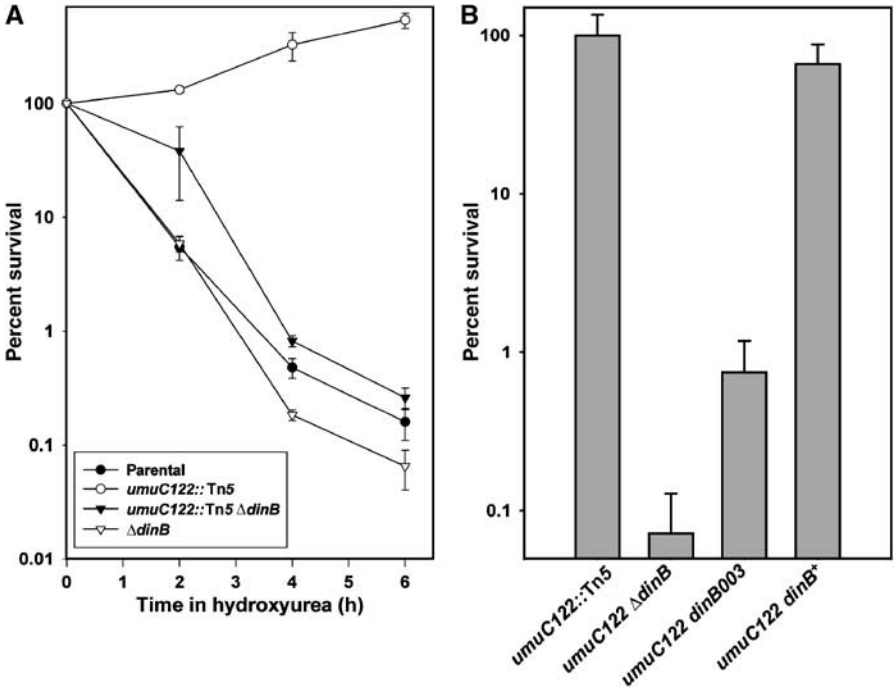

Figure 4 The *dinB* gene and its catalytic activity are necessary to avert HU lethality. (**A**) The HU^R of a *umuC122 dinB*$^+$ strain (open circles) is eliminated by deletion of the *dinB* gene (closed triangles). In contrast, deletion of the *dinB* gene has only a mild effect on the parental strain (open triangles and closed circles). (**B**) Reconstruction of the *dinB*$^+$ locus on the chromosome restores HU^R to the *umuC122* Δ*dinB* strain. However, transduction of the *dinB003* allele, which encodes a catalytically inactive DinB(D103N), does not restore HU^R, indicating that the catalytic activity of DinB is required. Treatment was for 6 h with 100 mM HU in rich medium. *umuC122 dinB*$^+$ refers to the reconstructed wild-type gene with a linked *cat* gene upstream the *dinB* promoter. Reported values are the average of three experiments and error bars represent one standard deviation.

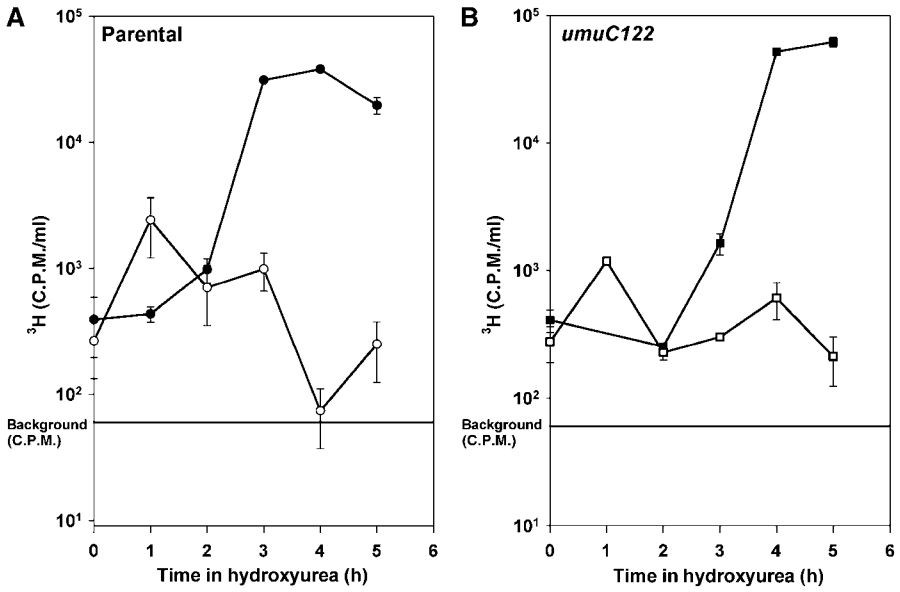

Figure 5 DNA synthesis is slowed in both wild-type and *umuC122* strains. Thymidine-requiring derivatives of both strains were used for the experiments shown. ^{3}H-Thy was added at 1 μCi/ml for 10 min at each time point shown, after which cells were immediately precipitated with 10% TCA. For both the wild-type shown in (**A**) (circles) and *umuC122* shown in (**B**) (squares) strains, bulk DNA replication is slowed during hydroxyurea treatment. The straight line represents the background c.p.m. Error bars represent the standard deviation of three samples.

dinB$^+$ gene into the *umuC122*Δ*dinB* mutant, the HU^R phenotype was restored (Figure 4B). The possibility that the restoration is due to a closely linked locus rather than to *dinB*$^+$ is inconsistent with the data presented in the following section. These observations suggest that the level of DinB expression or a *cis*-regulatory element is critical for the ability of *dinB*$^+$ to contribute to the HU^R of a *umuC122* strain. Perhaps, DinB cannot contribute to HU^R if its levels do not correlate with those of the products of the *umuD*$^+$ gene.

The catalytic activity of DinB is required for HU resistance

To test whether DinB must be catalytically active to contribute to HU^R, we introduced the *dinB003* mutation into the chromosome of a *umuC122* strain. This mutation (D103N) alters a conserved aspartic acid residue required for phosphodiester bond formation (Wagner and Nohmi, 2000). The large loss of HU^R we observed (Figure 4B) suggests that DinB is indeed acting as a DNA polymerase as it contributes to HU^R. Thus, it appears that HU^R results from the combined action of two DNA polymerases, DinB and a mutant form of UmuC, acting together with UmuD and UmuD′.

DNA synthesis is slowed in both parental and umuC122 strains during HU challenge

To explain the observation that both Y-family polymerases are required for HU^R, we asked whether HU^R was simply due to an extensive alteration in the rate of DNA replication. We measured DNA synthesis by examining the ability of thymidilate synthase-negative (*thyA*$^-$) derivatives of wild-type and *umuC122* strains to incorporate thymidine (^{3}H-Thy) in 10 min pulses during HU treatment. We found that the amount of DNA synthesis is reduced during HU treatment in both wild-type and *umuC122* strains compared to untreated controls (Figures 5A and B). Any minor changes that we observe in the ability to incorporate ^{3}H-Thy into the DNA do not appear to account for the striking difference in viability, that is, competence to develop colonies, between the HU-treated wild-type and *umuC122* strains. This remarkable and unexpected result led us to examine the cells microscopically during HU treatment (see below).

A strain bearing a mazEF or relBE mutation is also resistant to HU

Although wild-type and *umuC122* strains display comparable levels of bulk DNA synthesis during HU treatment, only in the *umuC122* mutant is this activity beneficial for survival. It seemed possible that the wild-type strain loses viability not directly due to stalled replication forks that arise during HU treatment, but instead due to events that occur downstream of such stalled forks. Examination by microscopy of an HU-treated parental culture revealed drastically fewer cells (>90% reduction at 5 h) than in the *umuC122* strain, most likely due to cell lysis. Hence, we considered the phenomenon of thymineless death, which is also thought to be the product of stalled replication forks formed by substrate starvation (Ahmad *et al*, 1998). In *E. coli* strain MC4100, thymineless death is mediated at least in part by the *mazEF* genes (Sat *et al*, 2003), which encode a toxin–antitoxin pair. We speculated that HU^R and thymineless death may proceed through similar mechanisms.

Therefore, we examined the sensitivity to HU of an MC4100 derivative harboring a deletion of the *mazEF* genes (Aizenman *et al*, 1996). Not only does deletion of these genes protect cells from the lethal consequences of HU challenge (Figure 6A), but the mechanism of HU^R is also likely to be related to that of the *umuC122* strain. Microscopical examination during HU treatment indicates that *umuC122* and *mazEF* strains appear quite similar at the single-cell level (Figure 6B). No morphological difference is visible among the strains 1 h into HU treatment (panels A–D), but each HU^S parental strain had to be concentrated an additional five-fold to analyze comparable numbers of cells relative to its HU^R derivative. Finally, at 5 h (panels E–H), we observed similar

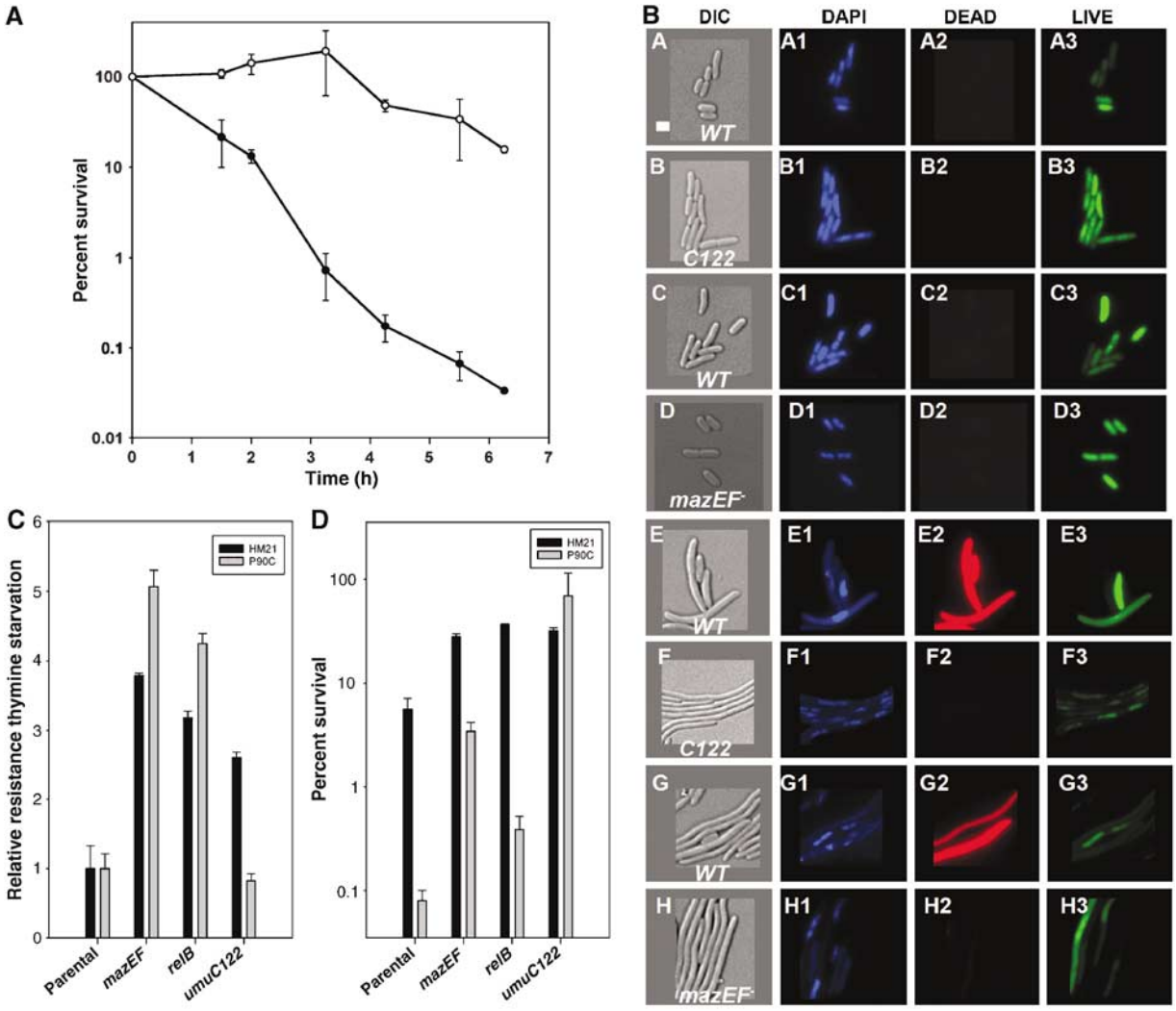

Figure 6 Survival phenotypes under dNTP starvation. (**A**) Survival time course in 100 mM HU of the parental MC4100 derivative (closed circles) and the *mazEF* mutant strain (open circles) in LB. Error bars shown represent the standard deviation of two samples. (**B**) Strains bearing the indicated alleles and wild-type control backgrounds were treated with 100 mM HU to determine cell morphology under HU treatment. Micrographs are presented for treated cells only because untreated samples of each strain showed indistinguishable morphologies over 5 h without HU. Panels A–D show representative images of cells after 1 h of HU treatment. (A, B) P90C wild-type and *umuC122* control DIC image, (C, D) MC4100 wild-type and *mazEF*⁻ control DIC image. Images labeled 1 show DAPI staining, images labeled 2 show DEAD staining and images labeled 3 show LIVE staining. Panels E–H are corresponding representative images of cells following treatment with HU for 5 h. Images were colorized using OpenLab software (Improvision) and were sized in Canvas (Deneba Systems). The white bar in (A) represents 2 μm. Exposure times for the images were as follows: DIC, 0.03 s; DAPI, 0.13 s; LIVE, 0.01 s; and DEAD, 0.13 s. The LIVE/DEAD stain was used according to the manufacturer's recommendations (Molecular Probes). (**C**) Mutation in the *relBE* gene products protects (denotes relB in this figure) cells from thymine starvation. Error bars represent the standard deviation of three samples. (**D**) Deletion of the *relBE* genes also promotes HU^R. Treatment with Tp in HM21 (4 μg/ml) and P90C (7 μg/ml) strains was performed in appropriately supplemented M9 minimal medium. CFUs were determined after 16 h incubation. HU challenge (100 mM) was carried out in LB appropriately supplemented medium. Error bars shown represent the standard deviation of three samples.

responses in both HU^S parental strains (concentrated 15-fold relative to their HU^R derivatives). In comparison, the *umuC122* and *mazEF* strains show extreme elongation and no dead cells, suggesting that HU^R may arise through a similar mechanism in both strains. Therefore, it is plausible that the HU^R phenotype of the *umuC122* mutant may be due to a failure to transduce a signal in a *mazEF*-dependent pathway leading to cell death and lysis (Aizenman *et al*, 1996).

We then tested whether a different TA pair may protect cells from the lethality caused by thymine starvation or HU challenge. Thus, we constructed P90C derivatives that harbored deletions of either the *mazEF* or *relBE* genes (Gerdes *et al*, 2005). We also transduced the *umuC122* allele into the *E. coli* strain HM21, the donor of the *mazEF* and *relBE* deletion alleles. We tested the *mazEF* and *relBE* strains in both backgrounds for HU^R and response to thymine starvation using trimethoprim (Tp) to inhibit *thyA*. We found that the *relBE* deletion protects cells from inhibition of fork progression upon thymine starvation similarly to *mazEF* (Figure 6C), and that both strains showed comparable responses upon HU challenge (Figure 6D). Moreover, we found the *umuC122* allele confers resistance to both Tp (Figure 6C) and HU (Figure 6D) in the HM21 strain background, although this HU^R is of a lower magnitude than that observed in the P90C strain. In contrast, the *umuC122* allele does not confer resistance to Tp in the P90C background. This results suggest

that there may be communication between pathways that couple HU- and Tp-induced stalled replication forks to cell death, and that a factor(s) involved in such communication is/are absent in the P90C strain, which bears a ~105 kb deletion on its chromosome. Moreover, both pathways appear to utilize the *relBE* and *mazEF* TA pairs as their ultimate executioners.

HU-treated strains bearing the truncated UmuC protein have a high mutation frequency

Our findings raise the possibility that the four proteins we have identified as being critical for HU^R—DinB, a UmuC derivative, UmuD, and UmuD′—enhance cell survival under conditions of low dNTP concentrations. They may even take over much of DNA replication, thereby helping cells to replicate even in the presence of HU (Figure 1A). If DNA replication upon HU challenge is DinB- and UmuC dependent, one would expect such DNA synthesis on undamaged DNA to be less accurate than that carried out by the DNA Pol III holoenzyme. Therefore, we tested whether the mutation frequency to rifampicin resistance is changed before or after HU treatment in a *umuC122* strain. We determined that untreated strains encoding UmuC122 protein have a spontaneous mutation frequency of $4 \pm 2 \times 10^{-7}$, identical to the mutation frequency of the untreated *umuC*$^+$ parental strain ($4 \pm 3 \times 10^{-7}$). However, after HU treatment, the mutation frequency of the *umuC122* strain increases ca. 100-fold to $7 \pm 3 \times 10^{-5}$, whereas the mutation frequency of the *umuC*$^+$ parental strain remains at ca. 10^{-7}. These data suggest that it may be possible to explain the HU^R phenotype of strains bearing the *umuC122* allele by a model in which one or both of the Y-family polymerases are responsible for a significantly greater proportion of DNA replication during HU treatment than under normal conditions.

Discussion

We examined the effect of inhibiting replication fork progression in a DNA damage-independent manner with HU in strains bearing different alleles of the *umuC* gene and found that cells bearing a carboxy-terminal truncation allele *umuC122*::Tn*5* (Elledge and Walker, 1983) are strikingly resistant to HU treatment (Figure 1A). Moreover, an unusual point mutation in UmuC (*umuC125* allele, A39V) (Marsh *et al*, 1991) displays a similar phenotype (Figures 3A and D). We have shown that *umuC122* is a gain-of-function allele that mediates HU^R and encodes a gene product that could, in principle, perform DNA polymerization as its polymerase domain is intact (Boudsocq *et al*, 2002). DNA polymerase activity in such a mutant protein is not unprecedented as truncations of the carboxy-terminal domain of human Y-family polymerase η are TLS proficient *in vitro* (Broughton *et al*, 2002). XP-V patients (Masutani *et al*, 1999) bearing these C-terminal truncations tend to have more tumors than those carrying other Pol η alleles (Broughton *et al*, 2002). Indeed, we show that cells expressing a catalytically inactive UmuC122 protein are sensitive to HU (Figure 3A). We have also shown that the DinB protein (Figure 4A), and its catalytic activity (Figure 4B), is needed to observe the phenotype. In addition, we have learned that certain *umuD* gene products are required for the HU^R phenotype (Figures 3B and C).

Analysis of HU-treated cultures by microscopy (Figure 6B) revealed not only that the HU-treated wild-type cells die, but that many also disappear over the course of treatment, presumably through cell lysis. These data challenged our expectation that stalled replication forks would simply arrest cell division and prevent colony formation. We had not anticipated that they would bring about cell lysis in and of themselves.

We have shown that cells treated with HU are affected in a process downstream of RNR inhibition (Figures 2B and C). The current model for replication stalling elicited by dNTP depletion is that substrate starvation brings about fork arrest and concomitant cell death (Roy *et al*, 2004). However, HU-treated *Saccharomyces cerevisiae* cells have been shown to exhibit both normal replication forks that can still sustain very slow DNA synthesis, as well as stalled replication forks (Sogo *et al*, 2002; Lopes *et al*, 2003). Moreover, HU-treated *S. cerevisiae* show a reduction in levels, but not an absence, of dNTPs (Koc *et al*, 2004). Hence, the dNTP starvation model may be too simplistic to account for all these observations.

Therefore, we considered whether the HU^R mediated by these gain-of-function alleles of *umuC* is due to an abrogation in a pathway that would normally lead to cell death under conditions of dNTP starvation. We found that *E. coli* strains bearing a deletion of such a function (*mazEF*::Kan) (Aizenman *et al*, 1996) are also HU^R (Figures 6A and B). We also found that deletion of *relBE* protects cells from both thymine starvation and HU challenge (Figures 6C and D). It is likely that the function of the *mazEF* and *relBE* gene products is to slow metabolism, thereby enabling stasis and resumption of balanced growth (Pedersen *et al*, 2002; Gerdes *et al*, 2005). However, when challenged with dNTP starvation, cells are unable to recover from this stasis and eventually perish. Based on these data, HU-induced death of *E. coli* may be brought about not by stalled replication forks directly, but rather through a series of downstream processes involving the TA pairs *mazEF* and *relBE*. The UmuC variants, acting in combination with the *dinB* and *umuD* gene products, may mitigate such *mazEF*- or *relBE*-induced death, either directly or indirectly. Further studies will be needed to establish whether and to what extent replication fork collapse is required to signal such lethal pathways, as well as other factors that might be involved. It will be interesting to look for a function that would bestow Tp^R in the P90C *umuC122* derivative (Figure 6D). This strain harbors a large deletion (Δ(*lac-pro)*, ca. 105 kb) compared to the HM21 background, where the *umuC122* derivative is Tp^R (Figure 6C).

In *E. coli*, intracellular dNTP pools are at least 10-fold lower (10 μM) in the presence of HU than in untreated cells (100 μM) (Sinha and Snustad, 1972; Mathews and Sinha, 1982). One explanation for HU-induced stalled replication forks is that the replicative DNA polymerase cannot catalyze efficient DNA synthesis as its K_m for dNTPs (3–40 μM for DNA Pol III) (Kornberg and Baker, 1991) is higher than the concentrations of dNTPs present in the HU-treated cells. In comparison, the K_m for dNTPs of Pol IV (0.12 μM for His-DinB with the β processivity clamp) and Pol V (0.08 μM with RecA versus 1200 μM without) are much lower (Tang *et al*, 2000; Wagner *et al*, 2000). Therefore, it appears the *E. coli* Y-family DNA polymerases have the potential to operate efficiently at low dNTP concentrations, conditions at which

DNA Pol III would operate poorly. Furthermore, such capabilities seem to be dramatically regulated through protein–protein interactions.

All these data are consistent with the notion that DinB, UmuC, and the *umuD* gene products are recruited to stalled replication forks upon HU treatment. We propose that the UmuC derivatives alter the highly dynamic process of polymerase switching, so that Y-family polymerases are defective in the switch back to the replicative polymerase. Ordinarily, UmuC, UmuD, and DinB would be part of a transient complex relieving arrested replication forks, regardless of how they arise. Both Y-family polymerases would work together to enhance cell survival, perhaps with DinB extending primers that are misaligned on their templates (Wagner *et al*, 1999) and UmuC continuing replication before hand off of the primer terminus to the replicative DNA polymerase. Such polymerase switching is regulated by numerous factors in *E. coli* including the *umuD* gene products (Sutton and Walker, 2001a). In contrast, the UmuC variants would be recruited to HU-induced stalled forks and would be proficient to catalyze DNA synthesis, but would be unable to sense the signal to hand off the primer terminus to the replicative DNA polymerase. Hence, these UmuC derivatives would retain access to the replication fork unlike the wild-type protein. The unexpected finding (Figure 5A) that wild-type cells still carry out DNA replication upon HU challenge may be explained by a futile cycling of Y-family polymerase recruitment and subsequent handoff to the replicative DNA polymerase which cannot function effectively at the low dNTP levels of the cell. Furthermore, although *umuC122* is nonmutable *in vivo* with respect to UV, its gene product may be able to catalyze DNA polymerization on undamaged templates. Under normal circumstances, such prolonged access to the fork would be detrimental, but during the unique stress of HU treatment (low dNTPs), it is advantageous for survival, albeit at a mutagenic penalty.

Why does this apparent failure to hand off to the replicative polymerase in the *umuC* mutants prevent HU-induced death? Although it is possible that UmuC communicates directly with either or both of the *mazEF* and *relBE* gene products, thereby signaling cell death in response to stalled replication forks, it is perhaps more likely that the prolonged action of the UmuC derivatives at the replication fork prevents the generation of an intermediate that would lead to the *mazEF*- and *relBE*-dependent process of cell death and lysis. We suggest a factor that responds to one of these intermediates that is specific to thymineless death is missing in the P90C strain, explaining why the *umuC122* derivative behaves as the wild type upon Tp challenge. The carboxy-terminus of UmuC harbors interaction sites for both $UmuD_2$ and $UmuD_2'$ (Jonczyk and Nowicka, 1996; Sutton and Walker, 2001b), which are absent in the UmuC122 protein. Perhaps, the lack of this domain alters the ability of the UmuC122 protein to return the primer terminus to the replicative DNA polymerase. Moreover, the data in Figures 3B and C highlight the role of UmuD cleavage in HU^R. Alternatively, the truncated UmuC122 protein may remain at the replication fork due to altered interaction with the β-subunit of Pol III as deletion of its C-terminus may modify the accessibility of its β-binding motif (residues 357–361) (Becherel *et al*, 2002). It is clear that *umuC122* and Δ*umuC* are both loss of function alleles for UV- and chemical-induced mutagenesis in exponentially growing cells. However, phenomena tested using *umuC122* should be reevaluated. In comparison, the A39V mutation in the UmuC125 protein is in close proximity to the active site (ca. 6 Å; Figure 3D). The phenotype conferred by the *umuC125* allele may be due to either disruption of regulatory protein–protein interactions with similar consequences to the *umuC122* mutation or to alteration of the biochemical properties of the protein, such as a reduction in k_{off} for the primer/template, K_m for dNTP substrates, or both. In either case, the consequence is prolonged access to the replication fork under conditions of nucleotide starvation, resulting in survival during HU challenge.

If these polymerases replicate DNA in the presence of HU, mutability should be markedly higher in the mutant strains relative to the wild type. Indeed, the *umuC122* bearing strain displays a 100-fold higher mutation frequency upon HU treatment than its untreated counterpart or the wild-type strain. Intriguingly, before the discovery of Y-family polymerases, it has been reported that imbalances in dNTP pools increase mutagenesis, perhaps by decreasing the fidelity of DNA synthesis (Sargent and Mathews, 1987; Ji and Mathews, 1991; Mun and Mathews, 1991; Zhang *et al*, 1996). This reduction in fidelity could perhaps now be attributed to the recruitment of such Y-family polymerases to the replication forks under conditions of nucleotide imbalance.

Materials and methods

Strains and plasmids

We used different *E. coli* K12 strains and their isogenic derivatives (Table I): P90C (Cairns and Foster, 1991), AB1157 (Bachmann, 1987), and HM21 (Moyed and Bertrand, 1983). A precise deletion of *dinB* was constructed using the method described by Wanner *et al* (Datsenko and Wanner, 2000) with primers FW2 (5′acgcgttaaatgctg aatctttacgcatttctcaaacc3′) and RW2 (5′gtgatattgaccgatttttcagcgagaatt cgatgcat3′). The deletion was transduced by P1 (Miller, 1974) into the appropriate strains from BW25113 (Datsenko and Wanner, 2000). P1 transduction was also used to transfer the *umuC122* allele (Elledge and Walker, 1983), a deletion of the *umuDC* operon (Woodgate, 1992), and a precise deletion of *umuC*. Wild-type and *umuC122 thyA*$^-$ derivatives were constructed by P1 transduction from the strain EGSC#6827. The *dinB003* allele (Wagner *et al*, 1999) was constructed on the chromosome of BW25113 using the plasmid-borne allele as a template. The *umuDC*-containing plasmids are derivatives of pGB2 (Sutton and Walker, 2001b). The noncleavable *UmuD(S60A)* allele (Nohmi *et al*, 1988) was introduced by site-directed mutagenesis using a Quickchange kit (Stratagene, La Jolla, CA) with the following oligonucleotide (5′gcaagtggtgatgctatgattga tggtgg3′) and its reverse complement. The *umuC122* allele was reconstructed in the same plasmid system using the primer (5′ccactcaggacagcagggattgaatagatagttaaacgcgatctctggatgc3′) and its reverse complement.

Strains were grown routinely in liquid or solid media (LB) or in minimal M9 medium with the addition of HU (30–100 mM), ampicillin (Amp; 100 μg/ml), spectinomycin (Sp; 60 μg/ml), chloramphenicol (Cm; 10–20 μg/ml), kanamycin (Kan; 50 μg/ml), rifampicin (Rif; 100 μg/ml), trimethoprim (Tp; 3–7 μg/ml), diaminopimelic acid (DAP; 30 μg/ml) and thymine (Thy; 50 μg/ml) whenever required. The *dinB*$^+$ locus was reconstructed on the chromosome using the same approach as the *dinB003* construction in the Cm^S derivative of *umuC122*Δ*dinB* mutant. The locus was transduced with P1 phage, and the presence of the full-length *dinB*$^+$ gene was verified by PCR with the primers dinBF, 5′atgcgtaaaatcattcatgtgga3′ and dinBR, 5′tcataatcccagcaccagttgt3′.

HU treatment

Cultures were routinely treated in LB broth containing HU (Calbiochem) by diluting saturated cultures 1:1000. Treatment of ca. 10^6 bacteria/ml was for 6 h or as noted in the text or figure legends. Viability was checked throughout treatment. For anaerobic

Table I Strains and plasmids used in this study

	Description	Reference
Bacterial strains		
P90C	Δ*(lac-pro)$_{XIII}$ thi ara*	Cairns and Foster (1991)
P90C *umuC122*::Tn*5*	As P90C, but with Tn*5* insertion in the *umuC* gene	This work
P90C Δ*dinB*	As P90C, but bearing a precise deletion of the *dinB* gene and replacement by *cat*	This work
P90C Δ*umuC*	As P90C, but bearing a deletion of the *umuC* gene	S Lovett
P90C Δ*umuDC*	As P90C, but bearing a deletion of the *umuDC* genes and replacement by *cat*	This work
P90C *umuC122*Δ*dinB*	As P90C *umuC122*, but bearing a precise deletion of *dinB* gene and replacement by *cat*	This work
P90C *umuC122dinB003*	As P90C *umuC122*, but with *dinB003* encoding DinB D103N	This work
P90C *umuC122dinB+*	As P90C *umuC122*, but with *cat* upstream of *dinB*	This work
P90C Δ*umuDC*Δ*dinB*	As P90CΔ*dinB*, but bearing a deletion in the *umuDC* genes	This work
P90C *thyA*	As P90C, but with a deletion in the *thyA* gene linked to Tn*10*	This work
P90C *umuC122*::Tn*5 thyA*	As P90C *umuC122*::Tn*5*, but with a deletion in the *thyA* gene linked to Tn*10*	This work
P90C Δ*relBE*	As P90C, but with a deletion of the *relBE* genes and replacement by a Kan^R marker	This work
P90C Δ*mazEF*	As P90C, but with a deletion of the *mazEF* genes and replacement by a Kan^R marker	This work
AB1157	F$^-$ *thr-1 leuB6 proA2 his4 thi1 argE3 lacY1 galK2 rpsL supE44 ara-14 xyl-15 mtl-1, txs-33*	Walker Lab Stock
AB1157 *umuC122*::Tn*5*	As AB1157, but bearing a Tn*5* insertion in the *umuC* gene	This work, Walker Lab Stock
HM21	F$^+$ *dapA zde-264*::Tn*10*	K Lewis
HM21 Δ*relBE*	As HM21, but bearing a deletion of the *relBE* genes and replacement by a Kan^R marker	K Lewis
HM21 Δ*mazEF*	As HM21, but bearing a deletion of the *mazEF* genes and replacement by a Kan^R marker	K Lewis
HM21 *umuC122*::Tn*5*	As HM21, but carrying a Tn*5* insertion in *umuC* gene	This work
MC4100 *relA*$^+$	*araD139* Δ*(argF-lac)205 flb-5301 pstF25 rpsL150 deoC1*	H Engelberg-Kulka
MC4100 *relA*$^+$ Δ*mazEF*	As MC4100, but bearing a deletion of the *mazEF* genes and replacement by a Kan^R marker	H Engelberg-Kulka
Plasmids		
p*GB2*	pSC101 derivative, bearing an Sp^R marker	Walker Lab Stock
p*DC*	As p*GB2* bearing the *umuDC* genes	Walker Lab Stock
p*DC125*	As p*DC*, but the *umuC* gene carries a A39V mutation	Walker Lab Stock
p*D′C125*	As p*DC125*, but the *umuD* gene encodes only the 24 aa shorter protein UmuD′	This work
p*DC122*	As p*DC*, but carrying a truncation in the *umuC* gene	This work
p*D′C122*	As p*DC122*, but the *umuD* gene encodes only for the 24 aa shorter protein UmuD′	This work
p*DC104*	As p*DC*, but carrying a D104N mutation in the *umuC* gene	Walker Lab Stock
p*DC122/C104*	As p*DC*, but carrying a truncation in the *umuC* gene and a D104N mutation	This work
p*D(S60A)C122*	As p*DC*, but carrying an S60A mutation in the *umuD* gene	This work
p*D(S60A)C125*	As p*DC125*, but carrying a S60A mutation in the *umuD* gene	This work

treatment with HU, cultures were treated as above for 6 h with 55 mM HU in an anaerobic chamber (Coy Laboratory Products) with a mixture of 5% carbon dioxide, 10% hydrogen, and 85% nitrogen. Samples for Western blotting were either TCA precipitated (20%) or concentrated 100-fold. The αUmuC antibody was used at a dilution of 1:20 000. The secondary antibody dilution and further detection were performed following the manufacturer's instructions (Pierce Biotechnology).

For the thymidine incorporation during HU treatment (100 mM), we used a 1:1 mixture of M9 medium (Miller, 1974) with 0.3% casein to LB with 10 μg/ml of thymidine. The ^{3}H-Thy (Perkin-Elmer) incorporation was carried out in 10 min pulses, after which the sample was immediately TCA precipitated (10% final).

Acknowledgements

We would like to thank Sue Lovett (Brandeis) for providing us with the Δ*umuC* mutant strain, Hannah Engelberg-Kulka (Hebrew University) for the *mazEF* strains, Kim Lewis (Northeastern University) for the HM21 *mazEF* and *relBE* strains, and the *E. coli* genetic stock center for the #6827 strain. We also thank JoAnne Stubbe (MIT) for the antibodies to RNR subunits, Alan Grossman (MIT) for use of the microscope, and Michael Malamy (Tufts Medical School) for use of the anaerobic chamber. LAS was supported in part by a postdoctoral fellowship from NCI. This work was supported with the NIH Grant No. CA21615-27. GCW is an American Cancer Society Research Professor.

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