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RESEARCH ARTICLE

Manganese Is Required for the Rapid Recovery of DNA Synthesis following Oxidative Challenge in *Escherichia coli*

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ABSTRACT Divalent metals such as iron and manganese play an important role in the cellular response to oxidative challenges and are required as cofactors by many enzymes. However, how these metals affect replication after oxidative challenge is not known. Here, we show that replication in *Escherichia coli* is inhibited following a challenge with hydrogen peroxide and requires manganese for the rapid recovery of DNA synthesis. We show that the manganese-dependent recovery of DNA synthesis occurs independent of lesion repair, modestly improves cell survival, and is associated with elevated rates of mutagenesis. The Mn-dependent mutagenesis involves both replicative and translesion polymerases and requires prior disruption by H_2O_2 to occur. Taking these findings together, we propose that replication in *E. coli* is likely to utilize an iron-dependent enzyme(s) that becomes oxidized and inactivated during oxidative challenges. The data suggest that manganese remetallates these or alternative enzymes to allow genomic DNA replication to resume, although with reduced fidelity.

IMPORTANCE Iron and manganese play important roles in how cell's cope with oxygen stress. However, how these metals affect the ability of cells to replicate after oxidative challenges is not known. Here, we show that replication in *Escherichia coli* is inhibited following a challenge with hydrogen peroxide and requires manganese for the rapid recovery of DNA synthesis. The manganese-dependent recovery of DNA synthesis occurs independently of lesion repair and modestly improves survival, but it also increases the mutation rate in cells. The results imply that replication in *E. coli* is likely to utilize an iron-dependent enzyme(s) that becomes oxidized and inactivated during oxidative challenges. We propose that manganese remetallates these or alternative enzymes to allow genomic DNA replication to resume, although with reduced fidelity.

KEYWORDS DNA repair, DNA replication, manganese, oxygen toxicity

Iron serves as a cofactor for enzymes involved in a broad variety of metabolic pathways but is easily oxidized to its inactive form (1). At the same time, excess or unbound intracellular iron is a prooxidant that is deleterious to cell viability when peroxides are present, generating hydroxyl radicals via Fenton chemistry that damage DNA, proteins, and lipids (2–7). In contrast, manganese is relatively stable in aerobic environments and can often, but not always, remetallate iron-dependent enzymes or homologous Mn-dependent enzymes to carry out the required catalytic function with a similar or modestly compromised efficiency (reviewed in references 8, 9, and 10). Thus, cells tightly regulate the intracellular concentrations of iron and manganese when growing in aerobic environments to optimize the metal-dependent enzymes required for metabolism and minimize the toxicity associated with metabolically activated oxygen species.

Escherichia coli contains several interdependent regulatory genes to ensure that

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metal concentrations allow for proper protein function and limit oxygen free radical damage (11–14). The ferric uptake regulator (Fur) controls the expression of more than 60 genes encoding proteins performing functions required for iron transport and storage (*fecABCDE*) and manganese import (*mntH*), proteins performing essential metabolic functions such as ribonucleotide reductase (*nrdEF*) and cytochrome bo_3 ubiquinol oxidase (*cyoABCDE*), and proteins performing detoxification of metabolically activated oxygen species such as hydroperoxidase II (*katE*), iron-dependent superoxide dismutase (*sodB*), and others (11, 12, 14–18). Other regulators include the redox sensors OxyR and SoxRS, which respond to oxidative stress and regulate expression of genes that enhance survival under these conditions, including *fur* itself, peroxide-scavenging enzymes (*katG* products), DNA repair enzymes, and a proton-dependent manganese(II) import channel (MntH) (9, 10, 12, 19–25). Increased intracellular manganese pools also provide the optimal conditions for incorporation of the correct metal into several enzymes, including SodA, the manganese-dependent superoxide dismutase, while activating several other enzymes that specifically require manganese for activity, such as the manganese-dependent ribonucleotide reductase (NrdEF) (18, 26, 27). The *in vivo* metallation state of many enzymes, as well as how their metallation status and activity are altered during oxidative stress, remains an active area of investigation.

In addition to metabolically activated oxygen species, other environmental agents can interact with, alter, and damage DNA, including ionizing radiation, UV light, and a variety of chemical agents (28–35). Replication in the presence of DNA damage is thought to produce most of the mutagenesis, genomic rearrangements, and lethality that occur in all cells. Of the agents described above, how replication processes and recovers from UV-induced damage has been the most extensively characterized. Irradiation with short-wave UV light forms cyclobutane pyrimidine dimers and 6-4 photoproducts in DNA that block the progression of the replication fork (31, 36–38). After arrest, RecA and several RecF pathway proteins are required to process the replication fork such that the blocking lesion can be removed and replication can resume (38–44). Cells lacking RecA or any of several RecF pathway proteins are hypersensitive to UV-induced damage and fail to recover replication following disruption (38, 39, 42, 45). The time and ability to resume DNA synthesis following UV-induced damage also correlates with the time at which the UV-induced lesions are removed from the DNA (38, 41, 43, 44). In cells deficient in nucleotide excision repair, the UV-induced lesions are not removed, and the recovery of replication is severely impaired. These cultures exhibit elevated levels of mutagenesis, strand exchanges, and cell lethality (46–50).

The *E. coli* genome also encodes three translesion DNA polymerases, polymerase II (Pol II), Pol IV, and Pol V. Although mutations in these proteins do not affect the ability of DNA replication to recover after DNA damage or the time required, they do contribute to the overall mutagenesis that occurs in the presence of lesions (43, 44, 51–55). In the case of UV irradiation, DNA Pol V is responsible for nearly all the mutagenesis that occurs and contributes modestly to survival at high UV doses that exceed the repair capacity of the cell (43, 44, 56–59).

Less is known about how replication recovers following DNA damage induced by oxygen free radical species. Further, although both iron and manganese are known to regulate the cellular response after oxidative challenges, how these divalent metals affect replication under these conditions has not been examined. Here, we investigated the survival, recovery, and mutagenic phenotypes exhibited by *E. coli* exposed to 10 mM H_2O_2 when supplemented with 200 μM iron, manganese, or no metals. We found that the rapid recovery of DNA synthesis was independent of lesion repair but depends on the presence of manganese. The manganese-dependent recovery modestly improves survival and is associated with an increased mutation frequency that likely involves both replicative and translesion DNA polymerases.

RESULTS

Replication recovery is not dependent on lesion repair following oxidative challenge in minimal growth medium. To begin to characterize how replication responds to and recovers from oxidative stress, we monitored the rate of DNA synthesis in *E. coli* treated with H_2O_2. Wild-type cultures, grown in a defined phosphate-buffered medium, were exposed to 10 mM H_2O_2 for 5 min before the cells were filtered to remove excess H_2O_2, resuspended in fresh medium, and allowed to recover. To monitor replication, duplicate aliquots of culture were pulsed for 2 min with [^{3}H]thymidine at various times before and after H_2O_2 treatment, and the rate of replication (^{3}H incorporation into DNA/2 min) was then determined for each time point. For the purposes of comparison and as a control, we also monitored the recovery of cells following UV irradiation, which has been well characterized in previous work (41, 43, 44). Consistent with previous observations, the rate of DNA synthesis was initially inhibited by >90% following UV irradiation with 50 J/m^2 in wild-type cells. However, within 20 min the DNA synthesis began to resume, and that rate continued to increase over the remaining time course (Fig. 1A). In contrast, in H_2O_2-treated cultures, although replication was inhibited to a similar degree, DNA synthesis did not resume, and no recovery was observed during the 60-min time course (Fig. 1A).

The failure to recover DNA synthesis following H_2O_2 treatment was not due to elevated levels of lethality. Consistent with our previous work, under the conditions used in these assays, more than 90% of the cells survived both the H_2O_2 and the UV treatments that were used (Fig. 1B) (39, 41, 60).

The lack of recovery following H_2O_2 treatment was also not due to persisting oxidative lesions that prevented the resumption of DNA synthesis. To monitor the repair of both UV- and H_2O_2-induced lesions in cultures over time, cells were either UV irradiated or treated with H_2O_2 and allowed to recover as before. At various times during the recovery period, total genomic DNA was purified and treated with either T4 endonuclease V (T4 Endo V) or Fpg glycosylase/AP-lyase. These enzymes recognize and incise DNA at sites where the predominant lesions produced by UV irradiation and H_2O_2, cyclobutane pyrimidine dimers, and 8-oxoguanines occur, respectively (61–64). Genomic DNA samples from each time point were then electrophoresed in a denaturing alkali agarose gel, where the presence of UV- or H_2O_2-induced lesions was observed as the loss of high-molecular-weight DNA in the enzyme-treated samples. In the case of UV, a loss of high-molecular-weight DNA was observed immediately after UV irradiation in T4 Endo V-treated samples (Fig. 1C), demonstrating the presence of UV-induced DNA lesions. As the UV-induced lesions were repaired during the recovery period, the number of T4 Endo V-sensitive sites decreased, and high-molecular-weight DNA was restored to >60% by 40 min post-UV irradiation (Fig. 1D). The rate at which DNA synthesis resumed (Fig. 1A) correlated with the repair of the DNA damage (Fig. 1C and D), consistent with previous work (38, 41, 43, 44).

In H_2O_2-treated cultures, a similar induction of oxidative DNA lesions was observed at times immediately after treatment with H_2O_2. These lesions were removed, and high-molecular-weight fragments were restored at a rate that was similar to that observed for the UV-treated samples (Fig. 1C and D). However, despite the removal of the oxidative lesions, no resumption in DNA synthesis occurred (Fig. 1A).

To determine whether the timing of the recovery was simply delayed beyond 60 min in H_2O_2-treated cultures, we repeated the assay and extended the time course. We observed that the rate of synthesis remained suppressed for over 3 h, with a modest increase in DNA synthesis possibly beginning 4 h posttreatment (Fig. 1E). Taken together, the results demonstrate that replication is inhibited by H_2O_2 and fails to recover in defined minimal medium. The inability to recover DNA synthesis is not due to cell lethality or the presence of replication-blocking lesions in the genomic template.

Manganese promotes the recovery of DNA synthesis after treatment with H_2O_2. The results presented above were unexpected, as we have previously observed that replication recovers when cultures are similarly treated with H_2O_2 in rich Luria-Bertani (LB) medium (60, 65). Therefore, we hypothesized that the lack of recovery after

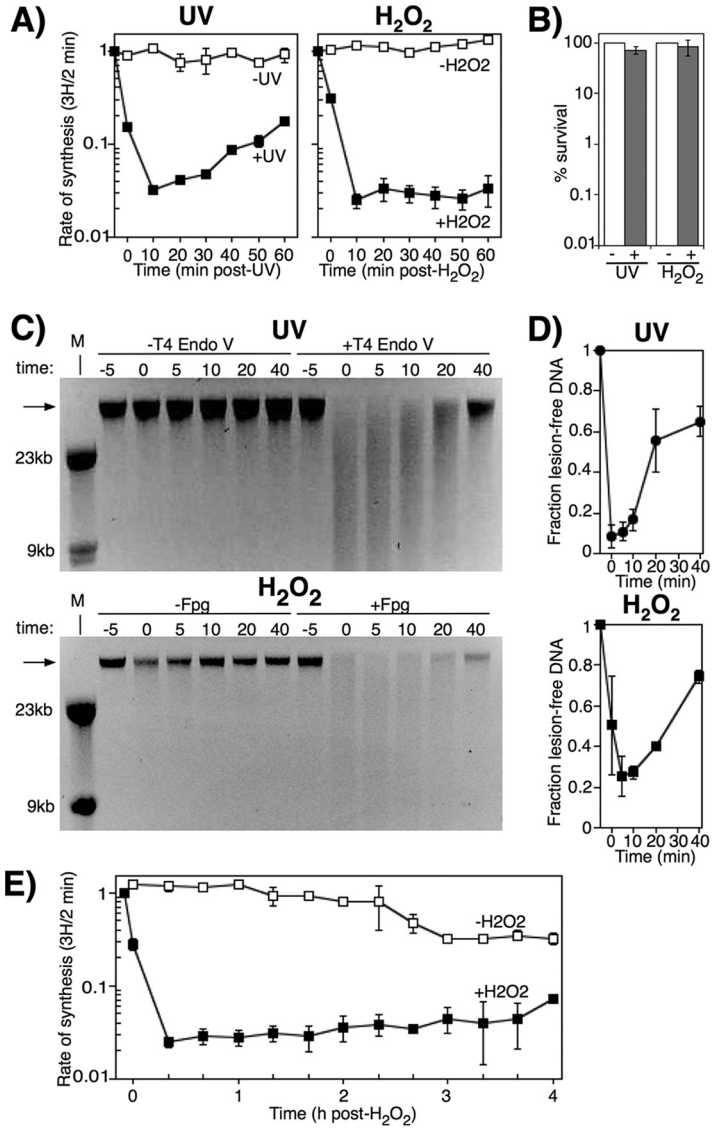

FIG 1 Lesion removal does not necessarily correlate with the onset of replication recovery following oxidative stress. (A) [^{3}H]thymidine was added to cultures for 2 min at the indicated times following either UV irradiation or H_2O_2 treatment at time zero. The rate of DNA synthesis (^{3}H/2 min) relative to the amount incorporated immediately prior to exposure are plotted for wild-type cells exposed to mock treatment (open symbols), 50 J/m^2 UV (filled symbols), or 10 mM H_2O_2 (filled symbols). Graphs represent an average from at least two independent experiments. Error bars represent one standard error of the mean. (B) Wild-type cultures were either UV irradiated at 50 J/m^2 or treated with 10 mM H_2O_2 for 5 min. The survival of wild-type cultures after UV irradiation or oxidative challenge is plotted. Bar graphs represent the averages from three independent experiments. Error bars represent one standard error of the mean. (C) Wild-type cultures were either UV irradiated at 50 J/m^2 (top panel) or treated with 10 mM H_2O_2 for 5 min (bottom panel) and allowed to recover, and then genomic DNA was purified at the indicated times. For UV-irradiated samples, DNA was either treated with T4 endonuclease V (T4 Endo V) or no T4 Endo V for 1 h at 37°C and then analyzed on alkali agarose gels. For H_2O_2-treated samples, DNA was either treated with Fpg or no glycosylase for 1 h at 37°C and then analyzed on alkali agarose gels. A representative gel is shown for each treatment. Arrows indicate lesion-free DNA. (D) The fraction of lesion-free, high-molecular-weight (HMW) DNA in T4 Endo V-treated (top graph) or Fpg-treated (bottom graph) samples is plotted for each time point relative to mock-treated samples. Graphs represent the averages from at least two independent experiments. Error bars represent one standard error of the mean. (E) Cells were either exposed to 10 mM H_2O_2 for 5 min (filled symbols) or mock treated (open symbols) at time zero and then allowed to recover. At the indicated times, [^{3}H]thymidine was added to cultures for 2 min. The amount of DNA synthesis/2 min (^{3}H) is plotted. The graph represents an average from at least two independent experiments. Error bars represent one standard error of the mean.

TABLE 1 Metal concentrations in minimal and rich media as measured by inductively coupled plasma mass spectrometry

	Metal concn							
Medium	Mg (mM)	Cr (μM)	Mn (μM)	Fe (μM)	Ni (μM)	Cu (μM)	Zn (mM)	Pb (μM)
Minimal	392.1	66.92	117.04	489.75	39.87	67.67	1.17	0.92
Rich	81.87	307.9	184.21	4,326.98	64.91	231.8	8.26	1.35

H_2O_2 treatment was due to a difference between using defined minimal medium and rich LB medium.

The cellular toxicity of H_2O_2 arises through the oxidation and reduction of iron in a disproportionation reaction that produces a hydroxyl radical species, which then reacts with and damages DNA, proteins, and lipids in the cell (2–6). The oxidation of iron also inactivates a range of Fe-dependent enzymes which play essential roles in a number of metabolic processes that are still being characterized (9, 10, 23, 66, 67). Manganese plays a large role in ameliorating the toxic effects of iron, since it does not react with H_2O_2 and can replace iron in many Fe-proteins to restore activity (9, 10, 23, 68–71). In addition, cells contain several genes devoted to limiting intracellular iron concentrations and upregulating manganese import during oxidative stress (23, 72–74). We noted that our rich medium was significantly enriched for both iron and manganese relative to our defined media (23, 68) (Table 1) and speculated that these metals may affect the ability to restore replication following H_2O_2 treatment. To examine this, cultures were grown in defined [^{14}C]thymine medium, supplemented with 200 μM ferrous sulfate ($FeSO_4{\cdot}7H_2O$) or 200 μM manganese (II) chloride ($MnCl_2$) or without additional metals, and treated with 10 mM H_2O_2 for 5 min as before. At various times during the recovery period, aliquots from each culture were pulse-labeled for 2 min with [^{3}H]thymidine before the DNA was precipitated and the amount of ^{14}C and ^{3}H incorporated was quantified. In this way, both replication fork speed (^{3}H incorporation per 2 min) and overall DNA accumulation (^{14}C incorporation) could be monitored over time. All experiments included a mock-treated control to ensure that any differences observed were due to H_2O_2 treatment and not an effect of thymine addition or differences in growth phase. As shown in Fig. 2, cultures in nonsupplemented medium or iron-containing medium did not resume DNA synthesis for the duration of the experiment. However, cultures supplemented with manganese began to recover DNA synthesis between 20 and 30 min after H_2O_2 was removed (Fig. 2). The total DNA accumulation correlated with the rate of DNA synthesis in each case, indicating that the newly made DNA was stable and not associated with degradation or DNA turnover. From these results, we infer that manganese promotes the recovery of DNA synthesis after acute exposure to H_2O_2.

Manganese-dependent recovery increases survival and mutagenesis. To determine how the ability to resume DNA synthesis affects survival in the presence of H_2O_2, cultures were grown in medium containing no metal supplements, 200 μM iron, or 200 μM manganese as before and then treated with 10 mM H_2O_2 and sampled at various times after exposure to determine the fraction of cells surviving to form colonies. When manganese was present in the growth medium, a modest increase in cell viability was observed compared to either no metal or iron supplementation, particularly with longer H_2O_2 exposure times (Fig. 3). Thus, the manganese-dependent recovery of DNA synthesis correlates with improved survival in H_2O_2-treated cultures. However, since manganese also affects several other enzymes and metabolic pathways, we do not rule out the possibility that other metabolic processes are contributing to the enhanced survival.

We also examined whether the manganese-promoted recovery of replication affected mutagenesis after oxidative challenge. To do this, we measured the frequency at which mutations conferring resistance to rifampin arose after H_2O_2 treatment. There are at least 69 base substitutions within the *rpoB* gene that confer rifampin resistance to cells, making this phenotype a robust system to monitor mutagenesis at multiple sites

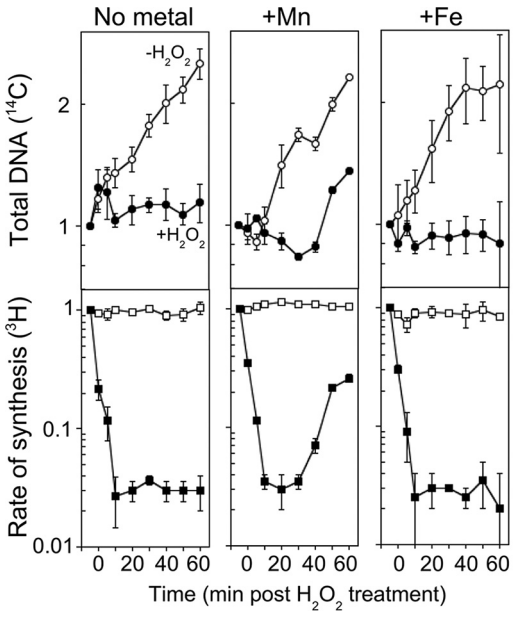

FIG 2 Manganese promotes replication recovery after H_2O_2 treatment. [^{3}H]thymidine was added to [^{14}C]thymine-prelabeled cultures for 2 min at the indicated times following treatment at time zero. The total DNA accumulation (^{14}C) and rate of DNA synthesis (^{3}H) relative to the amount incorporated immediately prior to exposure are plotted for wild-type cells grown in medium supplemented with manganese, iron, or no metal and exposed to 0 mM (open symbols) or 10 mM (filled symbols) H_2O_2. Graphs represent an average from at least three independent experiments. Error bars represent one standard error of the mean.

and in various sequence contexts (75). Replicating cultures containing no metal supplements, 200 μM iron, or 200 μM manganese were either mock treated or exposed to 10 mM H_2O_2 for 5 min and then allowed to recover overnight before the fraction of rifampin-resistant mutations per viable cells in each culture was determined. We found that a higher rate of mutations occurred after H_2O_2 treatment when manganese was present in the medium, relative to media containing either iron or no additional metals (Fig. 4A). In medium containing no metal supplements or in iron-supplemented medium, H_2O_2 treatment increased the frequency of rifampin-resistant mutations ~3-fold. In comparison, in the presence of manganese, the frequency of rifampin-resistant

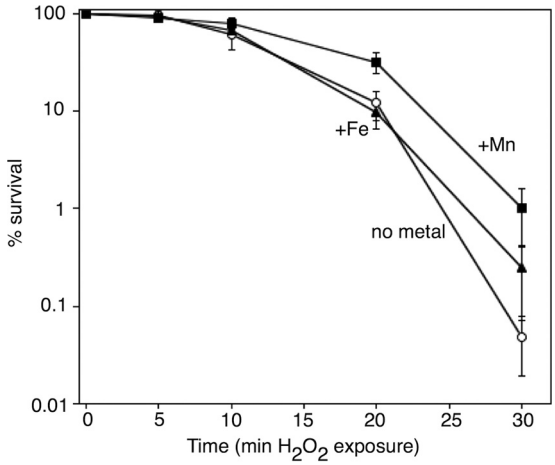

FIG 3 The presence of manganese increases survival of cells chronically exposed to H_2O_2. The survival of wild-type cells grown in medium containing manganese, iron, or no metal additive after exposure to 10 mM H_2O_2 is plotted after treatment for the indicated times. Graphs represent an average from five independent experiments. Error bars represent one standard error of the mean.

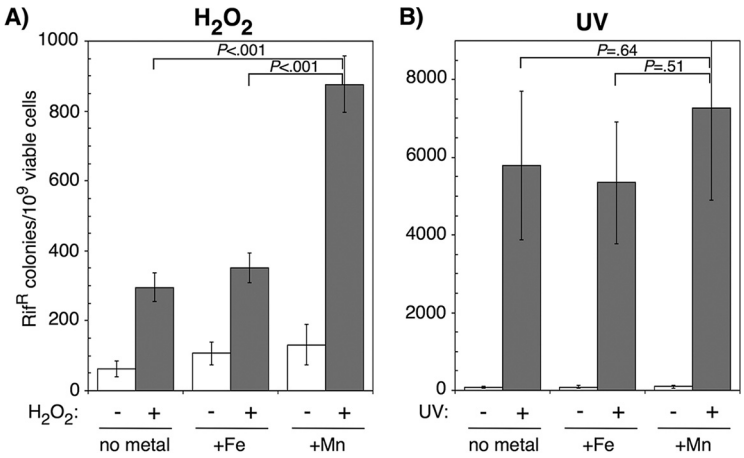

FIG 4 Manganese-dependent replication recovery after oxidative challenge is mutagenic. (A) Wild-type cultures grown in medium supplemented with manganese, iron, or no metals were treated with 10 mM H_2O_2 for 5 min and examined for the number of rifampin (Rif)-resistant colonies that appeared after overnight incubation. (B) Wild-type cultures grown in medium supplemented with manganese, iron or no metals were irradiated with 27 J/m^2 UV and examined for the number of rifampin-resistant (Rif^r) colonies that appeared after overnight incubation. The number of rifampin-resistant colonies per 10^9 surviving cells is plotted for each treatment. Graphs represent averages from six independent experiments. Error bars represent one standard error of the mean. *P* values represent the likelihood the observed difference could arise by chance, as determined by two-way analysis of variance (ANOVA).

mutations increased ~9-fold. No significant increase in mutation frequency was caused by the addition of manganese alone, indicating that replication must first be inhibited by H_2O_2 before mutagenesis can occur. To determine whether manganese increased mutagenesis after other forms of DNA damage, we repeated the experiment treating cells with 27 J/m^2 UV irradiation instead of H_2O_2. After UV treatment, the frequency of mutants increased ~50-fold in unsupplemented medium and increased by a similar amount in the presence of either iron or manganese (Fig. 4B). Thus, although UV irradiation was a more potent mutagen overall, the presence of manganese did not significantly increase the frequency of mutants observed compared to cultures containing no additional metals or supplemented with iron, respectively.

Mutagenesis following many forms of DNA damage depends upon specialized polymerases that are capable of replicating across DNA lesions with lower fidelity (52, 55, 76). *E. coli* contains three such translesion DNA polymerases: Pol II (a *polB* gene product), Pol IV (a *dinB* gene product), and Pol V (a *umuDC* gene product) (52). We hypothesized that the mutagenic replication occurring after H_2O_2 may be due to a manganese-dependent translesion DNA polymerase. To address this, we examined how the absence of these polymerases affected mutagenesis in the presence or absence of manganese supplementation as before. We observed that the frequency of mutagenesis after H_2O_2 treatment was only partially reduced by the absence of *polB*, *dinB*, and *umuDC* compared to wild-type cells in the manganese medium (Fig. 5A). Further, no single translesion polymerase appeared to be responsible for partial reduction in manganese-dependent mutagenesis. Single *polB*, *dinB*, and *umuDC* mutants each displayed partially reduced mutation frequency when manganese-supplemented cultures were exposed to H_2O_2 (Fig. 5C). In comparison, when the translesion DNA polymerase mutants were UV irradiated with 27 J/m^2, we observed a nearly complete lack of UV-induced mutagenesis irrespective of metal supplementation in the growth medium (Fig. 5B). Thus, the translesion DNA polymerases contribute to a portion of the manganese-dependent mutagenesis that occurs in the presence of oxidative damage. However, a significant portion of the mutagenesis seems likely to be due to the replicative and/or repair polymerases, and the overall magnitude of the mutations induced is relatively modest compared to UV-induced damage.

We also tried to assess the contribution of the replicative polymerase to manganese-dependent mutagenesis by examining a *mutD5* mutant, which inactivates the proof-

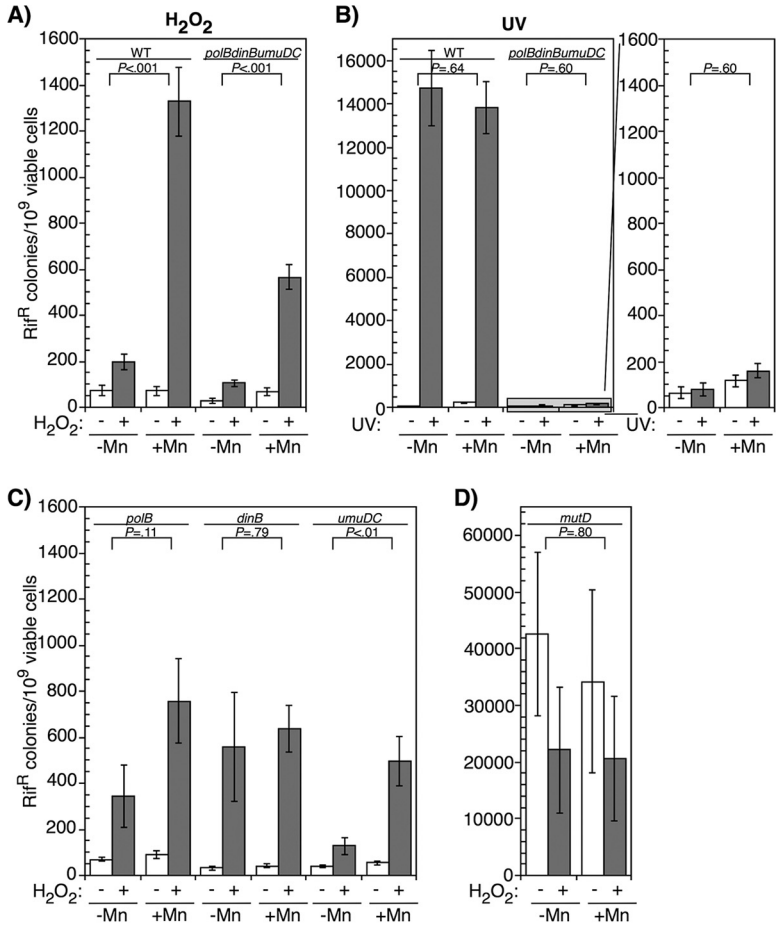

FIG 5 The absence of translesion DNA polymerases reduces, but does not completely inhibit, H_2O_2-induced mutagenesis in the presence of manganese. (A) Wild-type cultures and *polB*, *dinB*, and *umuDC* mutant cultures grown in medium supplemented with manganese or no metals were treated with 10 mM H_2O_2 for 5 min and examined for the number of rifampin-resistant (Rif^r) colonies that appeared after overnight incubation. (B) Wild-type cultures and *polB*, *dinB*, and *umuDC* mutant cultures grown in medium supplemented with manganese or no metals were treated with 27 J/m^2 UV and examined for the number of rifampin-resistant colonies that appeared after overnight incubation. (C) Cultures of *polB*, *dinB*, and *umuDC* mutants grown in medium supplemented with manganese or no metals were treated with 10 mM H_2O_2 for 5 min and examined for the number of rifampin-resistant colonies that appeared after overnight incubation. (D) *mutD* cultures were grown in the presence or absence of manganese and treated with 10 mM H_2O_2 for 5 min, then examined for the number of rifampin-resistant colonies after overnight incubation. The number of rifampin-resistant colonies per 10^9 surviving cells is plotted for each treatment. Graphs represent averages from six independent experiments. Error bars represent one standard error of the mean. *P* values represent the likelihood the observed difference could arise by chance, as determined by two-way ANOVA.

reading exonuclease of Pol III and results in a high rate of spontaneous mutations. If the Mn-dependent mutations result from Pol III, we hypothesized that the mutation rate may increase after H_2O_2 treatment in the presence of manganese. However, contrary to this expectation, we observed the mutation rate decreased ~2-fold in *mutD5* after oxidative challenge irrespective of the presence of manganese (Fig. 5D). Although no manganese-dependent effect was observed, the basal mutation rate of *mutD5* was 2 orders of magnitude higher than that seen after H_2O_2 exposure in any other strain we examined, making it unlikely that we would be able to detect any oxidative-stress-dependent mutagenesis occurring in this background. Nevertheless, given the manganese-dependent induction of mutations in wild-type cells and the partial reduction of mutations in the absence of translesion polymerases, we suggest that the reduced fidelity in the presence of manganese may generally apply to both replicative and translesion polymerases during recovery from H_2O_2 treatment.

Taken together, these results indicate that replication is sensitive to oxidative challenge and likely depends upon one or more oxygen-sensitive, iron-dependent enzymes. Manganese is required for the rapid resumption of DNA replication and presumably remetallates this or an alternative enzyme of similar function that allows replication to resume. The manganese-dependent recovery of replication modestly improves survival, but it is also associated with elevated levels of mutagenesis involving both translesion and replicative polymerases.

DISCUSSION

Ferrous iron (Fe^{2+}) serves as a cofactor in a broad variety of mononuclear, heme, and iron-sulfur proteins. However, in the presence of H_2O_2, it can oxidize to its ferric (Fe^{3+}) form and is stripped from these proteins (for a review, see reference 1). This has the dually detrimental effect of inactivating enzymes involved in multiple metabolic pathways and increasing the free intracellular iron concentration, which through Fenton chemistry produces free radicals that damage DNA, proteins, and lipids (2, 3, 5–7). The presence of intracellular manganese has been shown to correlate with increased resistance to oxidative DNA damage in a wide range of bacteria (68–71, 77, 78). Similarly, we observed a modest increase in the survival of *E. coli* when manganese is present in the media. In *E. coli*, manganese import is upregulated ~10-fold in response to oxidative challenge (23, 79). The typical intracellular manganese concentration of cells grown in minimal medium is ~15 μM, whereas manganese supplementation increases this concentration to ~35 μM, and oxidative stress can further increase the concentration to ~150 μM (23, 79). Whereas a manganese importer (*mntH*) mutant grows as well as wild-type cells under nonoxidizing growth conditions, manganese import is essential for viability under oxidizing or low-iron growth conditions (23). Manganese protection from H_2O_2 toxicity is thought to be conferred by remetallating inactivated, mononuclear iron enzymes, as well as through the activation of functionally redundant enzymes that specifically utilize manganese. In both cases, manganese acts to restore required enzymatic activities to essential metabolic pathways (9, 10, 23). We did not find any influence of iron supplementation on either survival or mutagenesis following oxidative challenge despite the potential for elevated free iron pools to induce DNA damage via Fenton chemistry. We speculate that the tight regulation of Fe^{2+} import by iron homeostasis genes is sufficient to repress iron uptake and limit additional iron-induced DNA damage.

A wide range of mononuclear iron enzymes involved in diverse cellular pathways have been characterized that can be remetallated by manganese to restore function, including those required for pyrimidine metabolism, amino acid catabolism, and carbohydrate metabolism (9, 10). In addition, several enzymes that specifically utilize manganese as a cofactor and are important in the response to oxidative stress or iron starvation have been identified. Many appear to have redundant activities with iron enzymes that are oxygen sensitive. These include the alternative manganese-dependent class Ib ribonucleotide reductase, NrdEF, the Mn-dependent superoxide dismutase, MnSOD, and the apurinic/apyrimidinic (AP) endonuclease IV (26, 27, 80–82). In this study, we show that the metabolic process of DNA replication likely requires oxygen-sensitive iron-enzymes that are inactivated by H_2O_2 but can be restored by the presence of manganese. Although we were unable to identify the specific enzyme(s) that allows replication to resume, it seems likely that they will fall into either the class of metabolic mononuclear iron enzymes or manganese-cofactored enzymes. At the time of writing, the ecocyc.org database listed approximately 90 proteins for which manganese serves as a cofactor (83). Several of these proteins function in nucleotide metabolism. These enzymes represent potential candidates which can be examined and include the alternative class Ib ribonucleotide reductase, NrdEF, that is required for cell replication under low-iron conditions (18).

Although the manganese-promoted recovery of replication increased survival after H_2O_2 treatment, it also compromised genomic stability as a higher frequency of mutations occurred in these cultures. We believe the increased mutagenesis most likely

results from manganese reducing the overall fidelity of the DNA polymerases. A large number of studies have shown that the fidelity of multiple DNA polymerases is altered by the presence of manganese *in vitro*. The fidelity of *E. coli* DNA polymerase I, as well as human polymerases ι, Dpo4, and Primpol are all altered by manganese supplementation in a concentration-dependent manner (84–87). Further, the reduced fidelity of DNA polymerases in the presence of manganese has been frequently utilized for *in vitro* PCR-mediated mutational screens (88, 89). The observations we report here suggest that manganese also affects the fidelity of both translesion and replicative polymerases of *E. coli in vivo*.

The increased mutagenesis could also partially result from more frequent encounters of the replication machinery with oxidized base damage. Since replication resumes more rapidly when manganese is present, it is likely that replication would more frequently encounter lesions. Several polymerases have been shown to incorporate the wrong base on templates containing several common oxidized bases induced by H_2O_2, including 8-oxoguanine, thymine glycol, and 5-hydroxycytosine (90–94).

Following oxidative challenges, manganese has been shown to restore function to a range of metabolic pathways that utilize monofunctional iron enzymes (9, 10, 23). The results presented here extend these observations to the process of DNA replication. Following many forms of DNA damage, replication inhibition and recovery correlate with the presence and removal of blocking DNA lesions, respectively (36, 38, 41). Our results suggest that in the case of H_2O_2, the inhibition of replication may occur independent of blockage by DNA damage, and result from an inactivation of oxygen-sensitive iron enzymes. Eukaryotic DNA polymerases also contain iron-sulfur clusters that are required for function, raising the possibility that similar mechanisms operate in humans (95). These observations demonstrate that the mechanisms by which replication responds to and recovers from oxidative challenges are distinct from other agents that primarily target DNA. It will be important to consider these potential differences and broaden our understanding of the metallation state of proteins in order to understand how genomic stability is maintained during oxidative stress or acute oxidative challenges.

MATERIALS AND METHODS

Bacterial strains and plasmids. SR108, a *thyA36 deoC2* derivative of W3110, and CL646 (SR108 *polB*::Ω Sm-Sp *dinB*::Kan^r *umuDC595*::*cat*) have been previously described (43, 96). CL716 (SR108 *mutD5*) was constructed by P1 transduction of *mutD5 zaf-13*::Tn*10* from NR9458 (97) into SR108. pBR322 is a medium-copy-number, ColE1-based, 4.4-kb plasmid (Promega).

DNA synthesis and accumulation. UV irradiation used a 15-W germicidal lamp (254 nm) at an incident dose of 0.9 J/m^2/s. For experiments using UV irradiation, overnight cultures were diluted 1:100 and grown at 37°C in Davis medium supplemented with 0.4% glucose, 0.2% Casamino Acids, and 10 μg/ml thymine (DGCthy) to an optical density at 600 nm (OD_{600}) of 0.25 to 0.35. At this time, half of the cells were mock irradiated, while the other half of the culture was irradiated with 50 J/m^2.

For experiments using hydrogen peroxide as a DNA-damaging agent, overnight cultures were diluted 1:100 and grown at 37°C in DGCthy to an OD_{600} of precisely of 0.25 to 0.35. Where DNA accumulation was also monitored, cultures were grown in DGCthy supplemented with 0.1 μCi/ml [^{14}C]thymine and, where indicated, either 200 μM ferrous sulfate ($FeSO_4{\cdot}7H_2O$) or 200 μM manganese(II) chloride ($MnCl_2{\cdot}4H_2O$) was added to the medium as well. At this time, half of the cells grown in each type of medium were mock treated, while the remaining culture was exposed to 10 mM H_2O_2 for 5 min at 37°C. After either mock or H_2O_2 treatment, cells were filtered on 0.45-μm membranes (Fisher) to remove excess H_2O_2 from the medium and resuspended in fresh DGCthy medium. As before, DGCthy medium was supplemented with 0.1 μCi/ml [^{14}C]thymine in the case of DNA accumulation assays and, where indicated, the corresponding metal additive in which cells were cultured.

For both UV irradiation and H_2O_2 experiments, cultures were returned immediately to 37°C after treatment to allow recovery and continued growth. At the times indicated, duplicate 0.5-ml aliquots of culture were pulse-labeled with 0.5 μCi/ml [^{3}H]thymidine for 2 min at 37°C. The cells were then lysed, and the DNA was precipitated in cold 5% trichloroacetic acid and filtered onto Millipore glass fiber filters. The amounts of ^{3}H and ^{14}C on each filter were determined by scintillation counting.

Metal concentration analysis. Metal concentrations in minimal and rich media were determined by inductively coupled plasma mass spectrometry through Oregon Health Sciences University's Elemental Analysis Core services.

H_2O_2 survival assays. Fresh overnight cultures were diluted 1:100 in DGCthy medium supplemented with either iron or manganese or without metals as described above, grown at 37°C to an OD_{600} of 0.3, and then treated with 10 mM H_2O_2. At the times indicated, 0.1-ml aliquots of each culture were removed

and serially diluted in 10-fold increments into DGCthy medium supplemented with the corresponding metal. Triplicate 10-μl aliquots of each dilution were then spotted onto LB plates supplemented with 10 μg/ml thymine (LBthy). Viable colonies were counted after overnight incubation at 37°C.

UV survival assays. Fresh overnight cultures were diluted 1:100 in DGCthy medium, grown at 37°C to an OD_{600} of 0.3, and then treated with 0 or 50 J/m^2 UV irradiation. Immediately after irradiation, 0.1-ml aliquots of each culture were removed and serially diluted in 10-fold increments into DGCthy medium. Triplicate 10-μl aliquots of each dilution were then spotted onto LBthy plates. Viable colonies were counted after overnight incubation at 37°C.

Lesion frequency. For UV irradiation, fresh overnight cultures were diluted 1:100 and grown at 37°C in DGCthy medium to an OD_{600} of 0.3. At this time, cultures were irradiated with an incident dose of 50 J/m^2 and then returned to 37°C to allow recovery. For H_2O_2 challenge, fresh overnight cultures were diluted 1:100, grown at 37°C in DGCthy medium to an OD_{600} of 0.3, and then treated with 10 mM H_2O_2 for 5 min at 37°C. Cells were filtered on 0.45-μm membranes to remove excess H_2O_2 from the medium, resuspended in fresh DGCthy medium, and returned to 37°C for the duration of the time course. At the times indicated, a 0.75-ml aliquot was transferred to an equal volume of 2× NET (200 mM NaCl, 20 mM Tris [pH 8.0], 40 mM EDTA [pH 8.0]). Cells were pelleted, resuspended in 0.14 ml of lysis buffer (1 mg/ml lysozyme and 0.5 mg/ml RNase A in 10 mM Tris [pH 8.0]–1 mM EDTA [pH 8.0]), and incubated for 30 min at 37°C. Then, 0.01-ml portions of 10-mg/ml proteinase K and 0.01-ml portions of 20% Sarkosyl were added to the samples, and incubation was continued for 30 min at 37°C. Samples were then extracted once with 4 volumes phenol-chloroform, followed by 2 volumes of chloroform, and dialyzed against 200 ml of 1 mM Tris (pH 8.0)–1 mM EDTA (pH 8.0) for 30 min using 47-mm Millipore (0.025-μm-pore) disks.

For UV-irradiated samples, 15 μl of each DNA sample was treated in reaction buffer (12.5 mM sodium phosphate [pH 6.8], 5 mM EDTA [pH 8.0], 50 mM NaCl, 0.5 mM dithiothreitol, 0.005% Triton X-100, 0.1 mg/ml bovine serum albumin) supplemented with either no enzyme or 2 U of T4 endonuclease V (T4 Endo V; Trevigen) for 1 h at 37°C. For H_2O_2-treated samples, 15 μl of each DNA sample was treated in reaction buffer (30 mM EDTA [pH 8.0], 22.5 mM NaCl, 5 mM Tris [pH 8.0]) supplemented with either no enzyme or 0.53 μM Fpg glycosylase for 1 h at 37°C. Enzyme preparations were titrated using purified undamaged genomic DNA as a template. The highest enzyme concentration that did not exhibit nonspecific activity on undamaged DNA was used. For the preparations in our lab, this corresponded to 2 U of T4 Endo V and 0.53 μM Fpg glycosylase. Treated samples were then electrophoresed on a 0.5% alkaline agarose gel in 30 mM NaOH–1 mM EDTA at 30 V for 16 h, stained, and visualized with ethidium bromide.

H_2O_2- and UV-induced mutagenesis. Mutagenesis induced by either H_2O_2 or UV was measured by the appearance of rifampin-resistant colonies as a result of exposure to these agents. For experiments using H_2O_2 treatment, fresh overnight cultures were diluted 1:100 and grown at 37°C in DGCthy medium supplemented with iron, with manganese, or without metals to an OD_{600} of 0.3. At this time, cultures were divided equally and mock treated or exposed to 10 mM H_2O_2 for 5 min. After either mock or H_2O_2 treatment, the cells were filtered on 0.45-μm membranes to remove excess H_2O_2 from the medium and resuspended in fresh DGCthy medium supplemented with the corresponding metal additive in which they were cultured.

For experiments using UV irradiation, fresh overnight cultures were diluted 1:100 and grown at 37°C in DGCthy medium supplemented with iron, with manganese, or without metals to an OD_{600} of 0.3. At this time, cultures were divided equally and irradiated with an incident dose of 0 or 27 J/m^2.

For both H_2O_2 treatment and UV irradiation, cultures were returned to 37°C after exposure to allow growth and recovery overnight. After overnight incubation, cultures were plated on LBthy plates containing 100 μg/ml rifampin to determine the mutagenic effect, as well as on LBthy plates to determine the number of viable colonies. Rifampin-resistant colonies and viable cells were counted after overnight incubation at 37°C.

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