



# Manganese transporters regulate the resumption of replication in hydrogen peroxide-stressed *Escherichia coli*

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**Abstract** Following hydrogen peroxide treatment, ferrous iron ( $Fe^{2+}$ ) is oxidized to its ferric form ( $Fe^{3+}$ ), stripping it from and inactivating iron-containing proteins. Many mononuclear iron enzymes can be remetallated by manganese to restore function, while other enzymes specifically utilize manganese as a cofactor, having redundant activities that compensate for iron-depleted counterparts. DNA replication relies on one or more iron-dependent protein(s) as synthesis abates in the presence of hydrogen peroxide and requires manganese in the medium to resume. Here, we show that manganese transporters regulate the ability to resume replication following oxidative challenge in *Escherichia coli*. The absence of the primary manganese importer, MntH, impairs the ability to resume replication; whereas deleting the manganese exporter, MntP, or transporter regulator, MntR, dramatically increases the rate of recovery. Unregulated manganese import promoted recovery even in the absence of Fur, which maintains iron homeostasis. Similarly, replication was not restored in *oxyR* mutants, which cannot upregulate manganese import following hydrogen peroxide stress. Taken together, the results define a central role for manganese

transport in restoring replication following oxidative stress.

**Keywords** Manganese · DNA replication · Oxidative stress · Metal homeostasis

## Introduction

Ferrous iron is bound to mononuclear, heme and iron-sulfur enzymes and serves as an essential cofactor for a variety of metabolic pathways, including cellular respiration, DNA synthesis, and the tricarboxylic acid cycle (McHugh et al. 2003; Andreini et al. 2008). During oxidative stress, iron rapidly oxidizes to its ferric form, stripping this catalytic metal from iron-dependent proteins, rendering them inactive (for a review, see (Imlay 2014)). High intracellular levels of free iron interact with hydrogen peroxide and superoxide to generate free radicals that can damage DNA, proteins, and lipids (Haber and Weiss 1934; Imlay et al. 1988; Touati et al. 1995; Tamarit et al. 1998; Daly et al. 2007). In contrast, manganese is far less reactive in the presence of oxygen and its byproducts, and can substitute for iron in iron-poor or oxidizing conditions (Kehres and Maguire 2003; Sobota and Imlay 2011; Anjem and Imlay 2012). Yet, manganese can also induce toxicity when cells accumulate too much of this metal (reviewed in (Kehres and Maguire 2003; Waters 2020)). Thus, cells tightly regulate the intracellular amounts of iron and manganese to

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manage both the functionality of metal-cofactored metabolic enzymes and the toxicity associated with excess transition metals.

The manganese-binding transcriptional regulator, MntR, controls intracellular manganese levels under normal growth conditions in *Escherichia coli* (Patzer and Hantke 2001; Kehres et al. 2002; Waters et al. 2011; Martin et al. 2015). Manganese-bound MntR functionally represses expression of the primary manganese importer, MntH, while also activating transcription of the manganese exporter, MntP. MntP expression levels are further modulated by a manganese-binding riboswitch found in the 5'-untranslated region of *mntP* mRNA (Dambach et al. 2015).

Iron homeostasis in *E. coli* is controlled by the global regulator Fur, which regulates expression of over 80 genes, including those involved in iron storage, utilization, and transport, through reversible binding to free ferrous iron (Bagg and Neelands 1987; Angerer and Braun 1998; McHugh et al. 2003; Seo et al. 2014) or a [2Fe–2S] cluster as has been suggested by recent work (Fontenot et al. 2020). In addition, Fur directly binds the promoter and regulatory regions of several genes encoding DNA synthesis and energy metabolism enzymes, coordinating cellular processes in response to iron availability (Seo et al. 2014). Curiously, the  $\text{Fe}^{2+}$ –Fur complex in *E. coli* also regulates intracellular manganese levels by repressing *mntH* expression (Kehres et al. 2000, 2002; Patzer and Hantke 2001).

Hydrogen peroxide rapidly reacts with ferrous iron to form hydroxyl radical and hydroxide anion with the concomitant production of ferric iron. This reaction both inactivates iron-dependent proteins and generates hydroxyl radicals that damage cellular targets, including DNA (Haber and Weiss 1934; Imlay et al. 1988; Touati et al. 1995; Tamarit et al. 1998; Daly et al. 2007; Sobota and Imlay 2011; Anjem and Imlay 2012). The redox sensor, OxyR, regulates the adaptive response of *E. coli* to peroxide stress, inducing expression of DNA repair enzymes, peroxide-scavenging enzymes, *fur*, and *mntH* (Aslund et al. 1999; Zheng et al. 1999, 2001; Kehres et al. 2002; Anjem et al. 2009; Sobota and Imlay 2011; Anjem and Imlay 2012). Hydrogen peroxide-activated OxyR thus modifies metal homeostasis through these latter two gene products to limit free iron in the cell, thus reducing free-radical formation due to Fenton chemistry, and increasing manganese concentrations to

restore enzymatic activities depleted by the loss of iron (Kehres et al. 2000; Anjem et al. 2009; Faulkner and Helmann 2010; Seo et al. 2015).

In *E. coli*, manganese protection from hydrogen peroxide toxicity is mediated through its ability to restore ferro-metallated protein function either through Mn-remetallation of inactivated, mononuclear iron proteins or through induction of functionally redundant manganese-dependent enzymes (Hasan and Fridovich 1977; Beyer and Fridovich 1991; Anjem et al. 2009; Martin and Imlay 2011; Sobota and Imlay 2011). Consistent with this, although the presence of manganese in the growth medium does not affect the amount of oxidative DNA damage or its repair rate, it is required to restore replication following hydrogen peroxide treatment in cells (Hutfilz et al. 2019; Hoff et al. 2021). These results indicate an essential role in re-metallating iron-dependent enzymes or activating manganese-dependent counterparts that are required for DNA replication. Although adding manganese to media was shown to be sufficient to restore replication, whether manganese levels and homeostasis play a role in regulating replication following oxygen stress has not been examined. Here, we characterized the role manganese and iron transporters have on replication following hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) treatment and demonstrate that transporters and intracellular manganese levels play a critical role in regulating the time and rate that DNA replication recovers following oxidative stress.

## Materials and methods

### Bacterial strains

*E. coli* strain BW25113 (wild-type) and isogenic single gene deletion mutants in *oxyR* (JW3933), *soxR* (JW4024), *mntH* (JW2338), *yebN* (*mntP*, JW5830), *mntR* (JW0801) and *fur* (JW0669) belong to the Keio Collection and were obtained from the National BioResource Project at SHIGEN (Japan) (Baba et al. 2006). The kanamycin-resistance cassettes of JW0801, *mntR*:FRT-kan-FRT, and JW2338, *mntH*:FRT-kan-FRT, were excised following transformation with the temperature-sensitive, ampicillin-resistant pCP20 plasmid expressing FLP recombinase as described previously (Datsenko and Wanner 2000). Briefly, JW0801 and JW2338 were transformed with

pCP20 and selected for ampicillin resistance at 30 °C. Several colonies were purified on non-selective plates at 43 °C and tested subsequently for loss of all antibiotic resistance. BW25113 *mntR* kan<sup>S</sup> (CL4444) derived in this manner was then transduced with the *fur*::FRT-kan-FRT allele from JW0669 using standard P1 transduction protocols to generate BW25113 *mntR fur*::FRT-kan-FRT (CL4460). BW25113 *mntH* kan<sup>S</sup> (CL4462) was transduced with the *mntP*::FRT-kan-FRT allele from JW5830 to generate BW25113 *mntH mntP*::FRT-kan-FRT (CL5425).

#### DNA synthesis rate

Overnight cultures were diluted 1:100 and grown at 37 °C in Davis medium supplemented with 0.4% glucose, 0.2% casamino acids (DGC), and 200 μM MnCl<sub>2</sub> as indicated, to an OD<sub>600</sub> of 0.25–0.35. At time zero, half of the cells were mock-treated, while the remaining half was treated with 10 mM H<sub>2</sub>O<sub>2</sub> for 5 min. All cells were then filtered through 0.45 μm nitrocellulose membranes to remove H<sub>2</sub>O<sub>2</sub> from the media, and resuspended in fresh DGC media either supplemented with MnCl<sub>2</sub> or without MnCl<sub>2</sub>, corresponding to the initial growth conditions. Cultures were allowed to recover at 37 °C. At the indicated time points, duplicate 0.5-ml aliquots of culture were pulse-labeled with 0.5 μCi/ml [<sup>3</sup>H]thymidine for 2 min at 37 °C. Cells were then lysed and DNA precipitated with cold 5% trichloroacetic acid and filtered onto Millipore glass fiber filters. The amounts of <sup>3</sup>H on each filter were determined by scintillation counting.

#### H<sub>2</sub>O<sub>2</sub> survival assays

Fresh overnight cultures were diluted 1:100 in DGC medium either with or without manganese supplementation as described above, grown at 37 °C to an OD<sub>600</sub> of 0.3, and then treated with 10 mM H<sub>2</sub>O<sub>2</sub>. At the times indicated, 0.1 ml aliquots of each culture were removed and serially diluted in 10-fold increments into DGC medium with or without manganese according to initial subculture conditions. Triplicate 10 μl-aliquots of each dilution were then spotted on Luria–Bertani (LB) plates. Viable colonies were counted following overnight incubation at 37 °C.

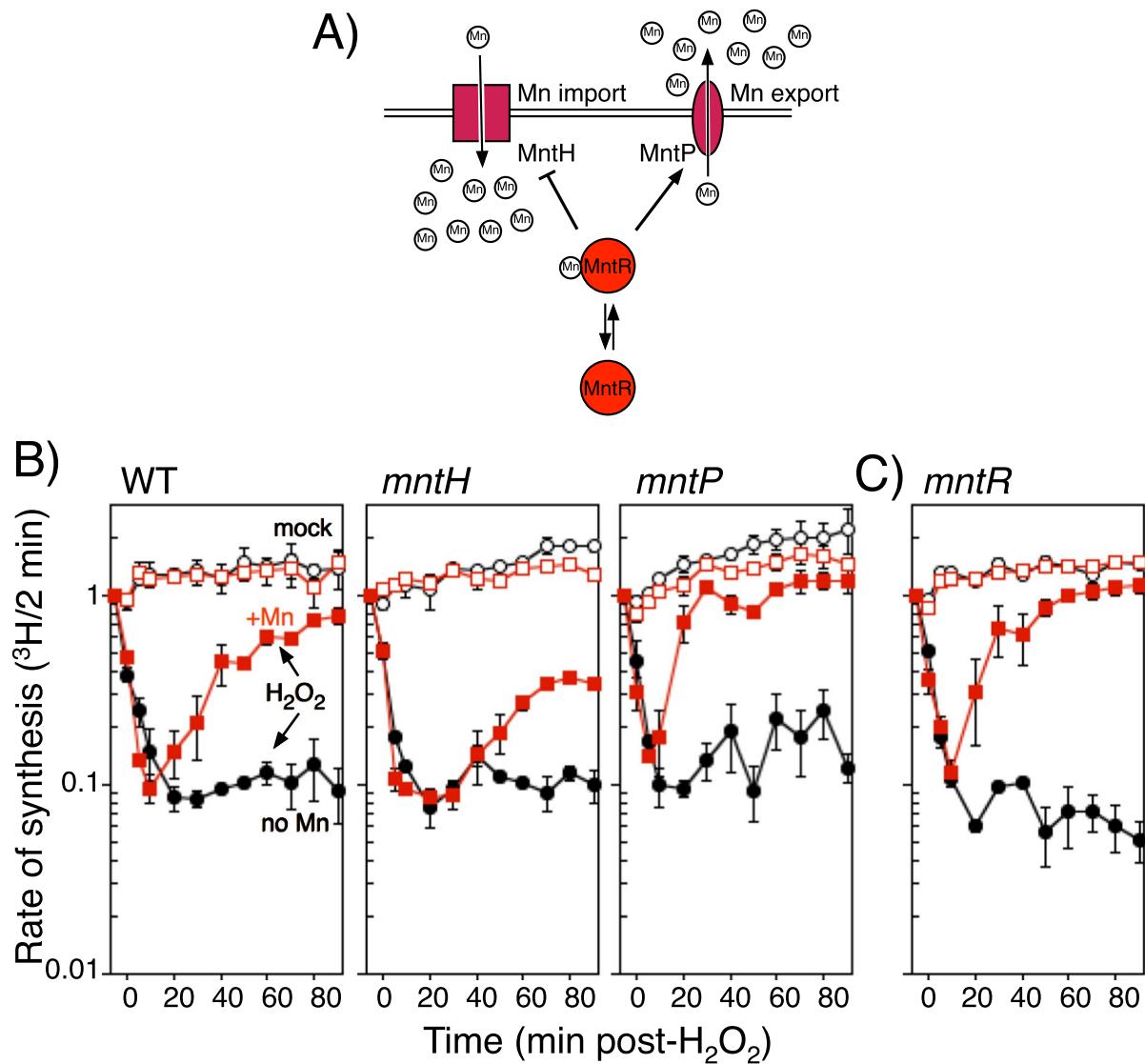
#### Inductively coupled plasma-mass spectrometry (ICP-MS)

Fresh overnight cultures were diluted 1:100 in 10 ml DGC medium either with or without manganese supplementation as described above, grown at 37 °C to an OD<sub>600</sub> of 0.5, and then cells were collected by centrifugation. ICP-MS was adapted from (Martin et al. 2015). Briefly, cells were washed twice with two-volumes of ice-cold 20 mM Tris, 1 mM EDTA (pH 7.4) and once with two-volumes of ice-cold 20 mM Tris (pH 7.4). Cells were resuspended in 1 ml ice-cold 20 mM Tris (pH 7.4), lysed by sonication and cell debris was cleared by centrifugation. The metal content in the cleared lysates was determined by the Trace Element Analytical Laboratory at Portland State University and intracellular concentrations were then calculated. Intracellular metal concentrations were normalized to the intracellular protein concentration value of ~300 mg/ml as derived from (Imlay and Fridovich 1991). Unsupplemented DGC medium contained 26 μM manganese and 268 μM iron as determined by ICP-MS.

## Results

Active manganese import and retention is required for the rapid recovery of DNA synthesis after oxidative stress

*mntH* and *mntP* encode the primary manganese importer and exporter, respectively (Makui et al. 2000; Waters et al. 2011). Their activity and expression is regulated under iron-replete conditions or in the absence of oxidative stress by the regulator, MntR, which binds manganese and limits intracellular manganese concentrations by repressing *mntH* transcription and upregulating *mntP* expression (Fig. 1A and (Patzer and Hantke 2001; Waters et al. 2011; Yamamoto et al. 2011)). To determine how intracellular manganese concentrations regulate replication after oxidative stress, we compared the ability of cells to restore DNA synthesis when challenged with H<sub>2</sub>O<sub>2</sub> in wild-type cells and mutants lacking the manganese importer, exporter, or regulator. Cultures grown in defined medium with or without manganese supplementation (200 μM manganese (II) chloride) were exposed to 10 mM H<sub>2</sub>O<sub>2</sub> for 5 min. To



**Fig. 1** Conditions that favor high intracellular manganese levels facilitate the restoration of DNA synthesis following oxidative stress. **A** MntR bound to Mn<sup>2+</sup> represses MntH expression and upregulates MntP expression to limit intracellular manganese concentrations under manganese-rich conditions. (**B** and **C**) [<sup>3</sup>H]thymidine was added to cultures for 2 min at the indicated times following treatment at time zero. The rate of synthesis (<sup>3</sup>H incorporation/2 min) relative to the amount incorpo-

rated immediately prior to exposure are plotted for **B** wild-type (WT) cells, *mntH*, and *mntP* mutants, and **C** *mntR* mutants exposed to mock treatment (open symbols) or 10 mM H<sub>2</sub>O<sub>2</sub> for 5 min (filled symbols) in manganese-supplemented (red squares) and -unsupplemented (black circles) media. Graphs show an average of at least two independent trials. Error bars represent one standard error of the mean. (Color figure online)

determine the rate of DNA replication (<sup>3</sup>H incorporation in the DNA/2 min) during the period of recovery, aliquots were pulse-labeled for 2 min with [<sup>3</sup>H] thymidine before the DNA was precipitated and the amount of <sup>3</sup>H incorporated was quantified. All experiments included a mock-treated control to ensure that

any observed differences in the rate of replication were due to H<sub>2</sub>O<sub>2</sub> treatment, and not due to changes in the overall growth phase of the cultures over the time course examined.

As shown Fig. 1B, wild-type cultures require the presence of manganese in the medium to restore

replication following  $\text{H}_2\text{O}_2$  treatment. Immediately following treatment, replication was inhibited by greater than 90%. In the presence of manganese, we observed the rate of replication begin to recover at 20 min post- $\text{H}_2\text{O}_2$  exposure and approach pre-exposure levels within 90 min of treatment. However, in the absence of manganese, replication did not resume for the duration of the 90-min time course. Inactivation of either the importer, MntH, or exporter, MntP, affected the recovery of replication, even when manganese was present in the media. Deletion of *mntH* delayed and prevented replication from fully recovering after  $\text{H}_2\text{O}_2$  exposure, with synthesis suppressed by ~70% throughout the time course.

By contrast, recovery occurred much more rapidly in *mntP* mutants, with DNA synthesis fully restored to pre-treatment rates within 30 min after  $\text{H}_2\text{O}_2$  removal (Fig. 1B). The observation that the recovery of replication is affected in the absence of the manganese importer and exporter even when manganese is present in the media demonstrates that this metal must be actively transported and retained by the cell to restore replication, and argues that Mn regulation plays a critical role in restoring replication during oxidative stress.

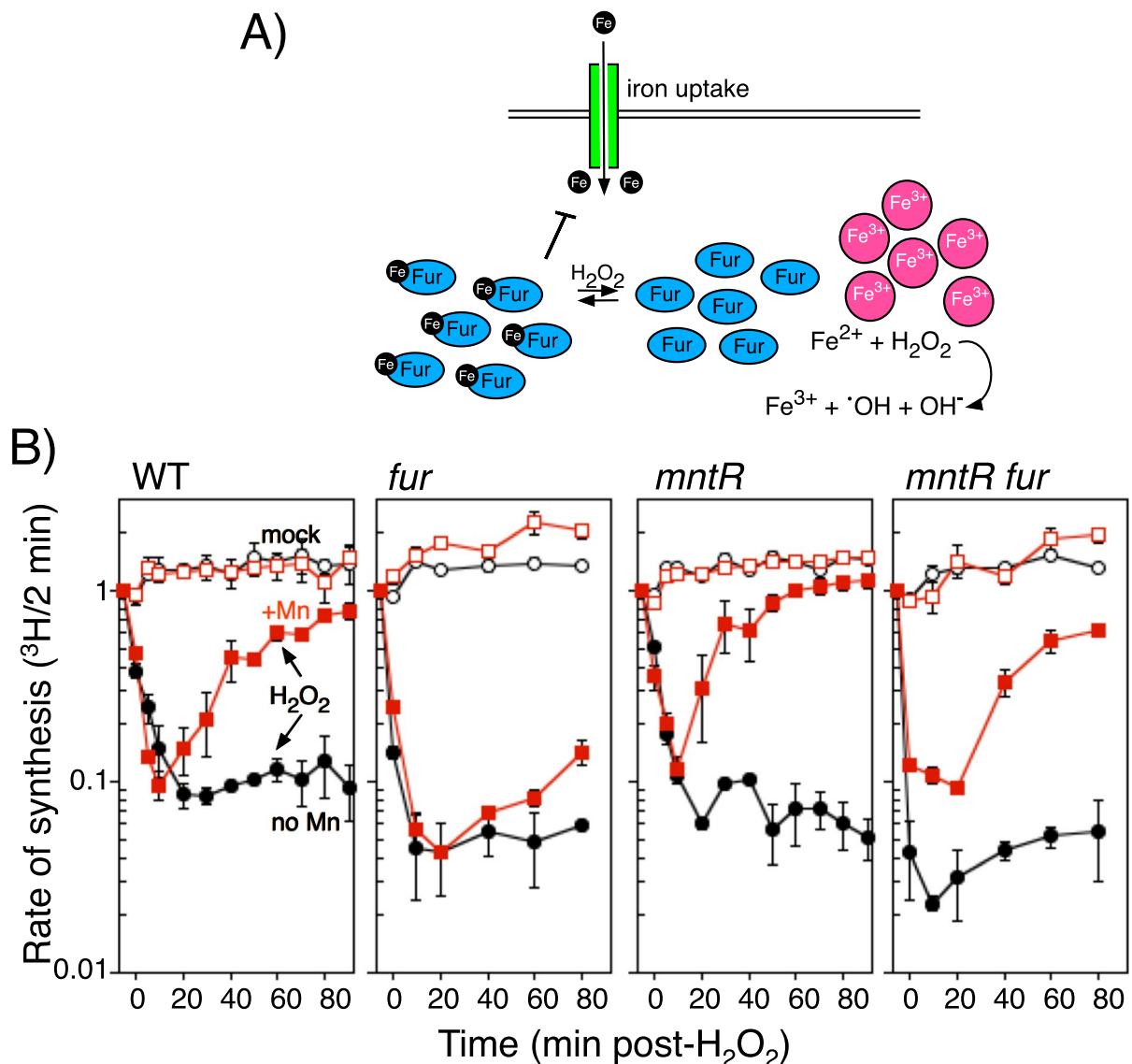
To further characterize how manganese regulation affects replication resumption, we also examined its recovery in the absence of MntR. When sufficient manganese is present in the cell, MntR-bound to  $\text{Mn}^{2+}$  suppresses the expression of the MntH importer and activates expression of the MntP exporter. Thus, in the absence of MntR regulation, manganese accumulates to higher levels in cells (Patzer and Hantke 2001; Kehres et al. 2002; Waters et al. 2011). Consistent with these roles, the rate of replication in *mntR* mutants grown in manganese-supplemented medium recovered more rapidly than in the corresponding parental strain, fully restoring replication to pre-exposure rates by 60 min post- $\text{H}_2\text{O}_2$  challenge (Fig. 1C). Together, these results indicate that active manganese import and exporter suppression facilitates the recovery of DNA synthesis following oxidative stress. We infer that the increased manganese levels allow for the remetallation of oxidized iron-cofactor proteins restoring cellular metabolism and DNA synthesis when strains are cultured in the presence of manganese.

Manganese, but not iron, promotes the recovery of replication after oxidative stress, and upregulated import of this metal can partially compensate for dysregulated iron uptake

Considering that both iron and manganese are used as enzymatic co-factors (Levin et al. 1991; Andreini et al. 2008; Cvetkovic et al. 2010; Cotruvo and Stubbe 2011), we wondered if upregulation of either metal could be utilized to restore replication. To examine this possibility we monitored the recovery of replication occurring in mutants lacking Fur. Fur represses the expression of iron uptake genes through a feedback loop involving reversible  $\text{Fe}^{2+}$  binding (Bagg and Neilands 1987; Angerer and Braun 1998; McHugh et al. 2003; Seo et al. 2014) and Fig. 2A). Thus, in the absence of Fur, intracellular levels of iron become unregulated and increase (Varghese et al. 2007; Martin et al. 2015). Following  $\text{H}_2\text{O}_2$  challenge, the absence of Fur impaired, rather than promoted the resumption of replication (Fig. 2B), consistent with the idea that iron is not as effective a substitute for manganese in promoting replication after oxidative stress. We interpret the result to also suggest that the excess iron present in *fur* mutants leads to increased  $\text{H}_2\text{O}_2$ -induced DNA, protein, and membrane damage. Irrespective, this result suggests that iron, unlike manganese, is unable to promote the recovery of replication during oxidative stress. Consistent with this interpretation, increasing the manganese concentrations by deletion of *mntR*, partially compensated for the absence of Fur, allowing replication to resume in these mutants. Taken together, the results are consistent with the idea that manganese, but not iron, co-factors are required to restore replication following oxidative stress.

OxyR, but not SoxR, is required for replication to recover after  $\text{H}_2\text{O}_2$  challenge

OxyR is a known redox sensor that is activated following  $\text{H}_2\text{O}_2$  treatment and mediates the cellular adaptive response to oxidative stress by regulating genes involved in peroxide scavenging, DNA repair, as well as regulating both iron and manganese import (Aslund et al. 1999; Zheng et al. 1999, 2001; Kehres et al. 2002; Anjem et al. 2009; Sobota and Imlay 2011; Anjem and Imlay 2012) and Fig. 3A). SoxR, is another redox sensor regulator that upregulates



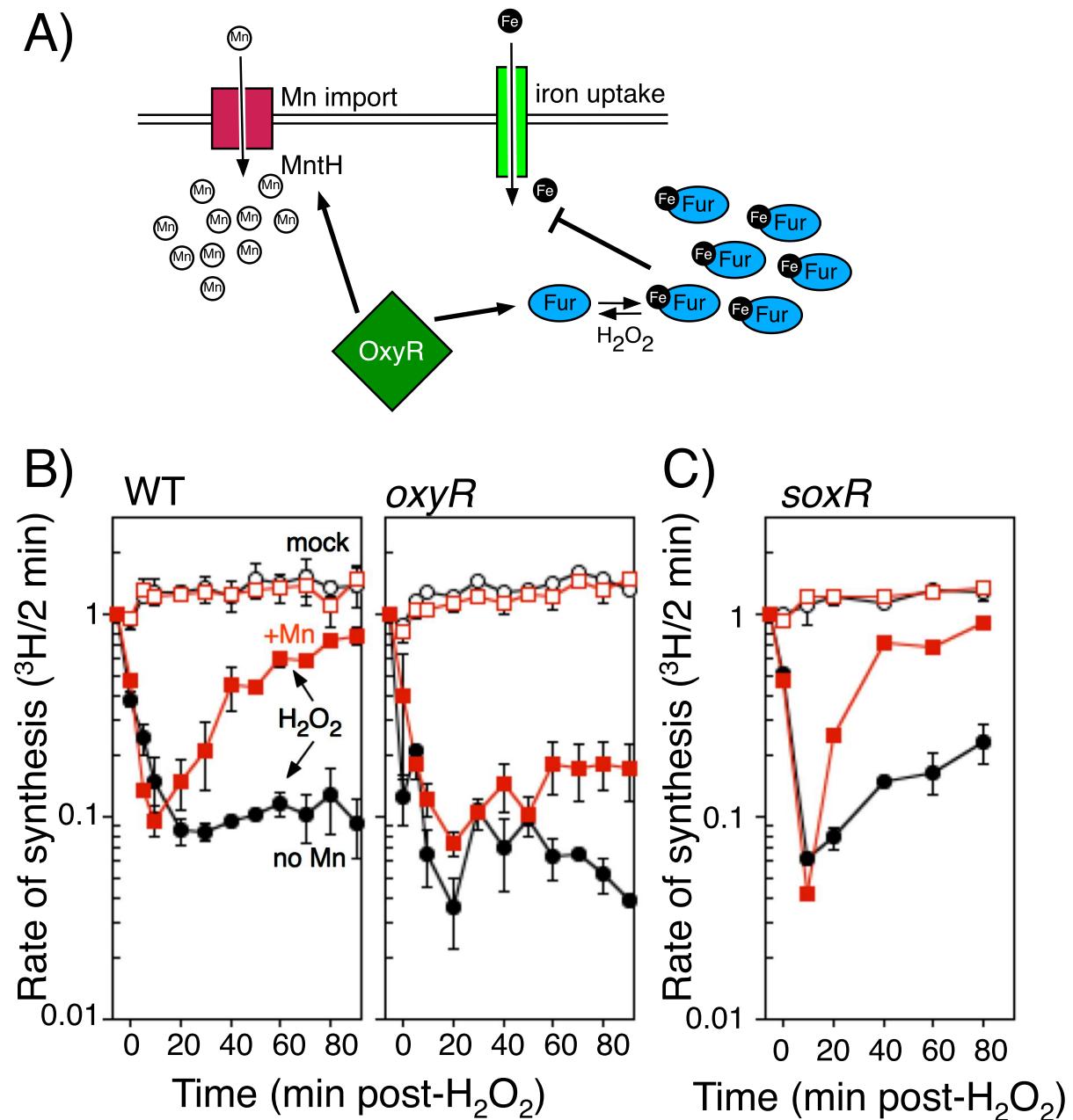
**Fig. 2** Manganese import promotes replication recovery even in the absence of iron uptake regulation in  $H_2O_2$ -treated cells. **A** Fur limits iron uptake following oxidative stress. **B** Data were obtained and plotted as for Fig. 1. The rate of synthesis ( $^3H$  incorporation/2 min) relative to the amount incorporated immediately prior to exposure are plotted for WT, fur, *mntR*, and *mntR fur* cells grown in medium with (red squares)

and without (black circles) manganese supplementation and exposed to mock treatment (open symbols) or 10 mM  $H_2O_2$  for 5 min (filled symbols). Wild-type and *mntR* plots are reproduced from Fig. 1 for comparison. Graphs show an average of at least two independent trials. Error bars represent one standard error of the mean. An abbreviated time course was performed for *fur* and *mntR fur* strains. (Color figure online)

several DNA repair and scavenging genes in response to superoxide, but is not known to regulate manganese (Wu and Weiss 1992; Li and Demple 1994; Zheng et al. 1999, 2001). To examine the contribution of these sensors to the regulation of replication after oxidative challenge, we compared the DNA synthesis

occurring in wild-type cells to that occurring in *oxyR* and *soxR* mutants following oxidative challenge in cells grown with or without manganese supplementation using the replication assay described above.

Cells deficient in *oxyR* were impaired in their ability to restore replication following  $H_2O_2$  exposure,



**Fig. 3** OxyR, but not SoxR, is required for replication to recover after  $\text{H}_2\text{O}_2$  challenge. Data were obtained and plotted as for Fig. 1. A OxyR upregulates MntH and Fur expression after  $\text{H}_2\text{O}_2$  treatment to allow increased manganese import and decreased iron uptake, respectively. **B** The rate of synthesis ( ${}^3\text{H}$  incorporation/2 min) relative to the amount incorporated immediately prior to exposure are plotted for WT cells and

*oxyR* mutants, and **(C)** *soxR* mutants grown in manganese-supplemented (red squares) or -unsupplemented (black circles) media exposed to 0 mM (open symbols) or 10 mM (filled symbols)  $\text{H}_2\text{O}_2$  for 5 min. Wild-type plot is reproduced from Fig. 1 for comparison. Graphs represent an average from at least two independent experiments. Error bars represent one standard error of the mean. (Color figure online)

even in the presence of manganese supplementation (Fig. 3B). In contrast, *soxR* mutants cultured in the presence of manganese resumed replication with kinetics that were as rapid as wild-type cells (Fig. 3C). The impaired recovery of replication in *oxyR* mutants following  $H_2O_2$  treatment indicated the sensor regulates the recovery of replication. Given that replication recovery only occurs in the presence of manganese and OxyR is known to upregulate MntH and Fur, it seems likely that OxyR's ability to restore replication likely occurs through direct regulation of these divalent metals.

#### Accumulation of intracellular manganese increases cell survival to acute oxidative stress

To examine how these manganese and iron transporters/regulators affected  $H_2O_2$  survival, mutants were grown in defined medium either with or without manganese supplementation to early exponential phase, and exposed to 10 mM  $H_2O_2$ . The presence or absence of manganese, by itself, did not have a large effect on the survival of wild-type cells over the time period examined (Fig. 4A). Similarly, the absence of either the manganese importer MntH or exporter MntP did not have large effects on survival, with differences remaining within an order of magnitude over the time course examined irrespective of whether manganese was present in the media. However, in the absence of MntR regulation of these transporters, survival was noticeably reduced by ~100-fold after a 30 min exposure. Interestingly, manganese supplementation compensated for this and restored survival to near wild-type levels in *mntR* mutants. We found an additive effect of *mntH* and *mntP* mutations on  $H_2O_2$  survival in the absence of manganese that generally mirrored that of *mntR* at shorter exposure times, but observed a ~10-fold difference in viability after 30-min of treatment. This result would suggest that additional *mntR*-regulated genes become essential for tolerance during prolonged exposure. Similar to what we observed with *mntR* cells, manganese supplementation allowed *mntH mntP* mutants to survive oxidative stress to near wild-type levels. The observations demonstrate that rapidly restoring or delaying replication does not directly correlate with viability. Consistent with what we've observed previously, cells that fail to restore replication when manganese is absent, remain viable for several hours (Hutfilz

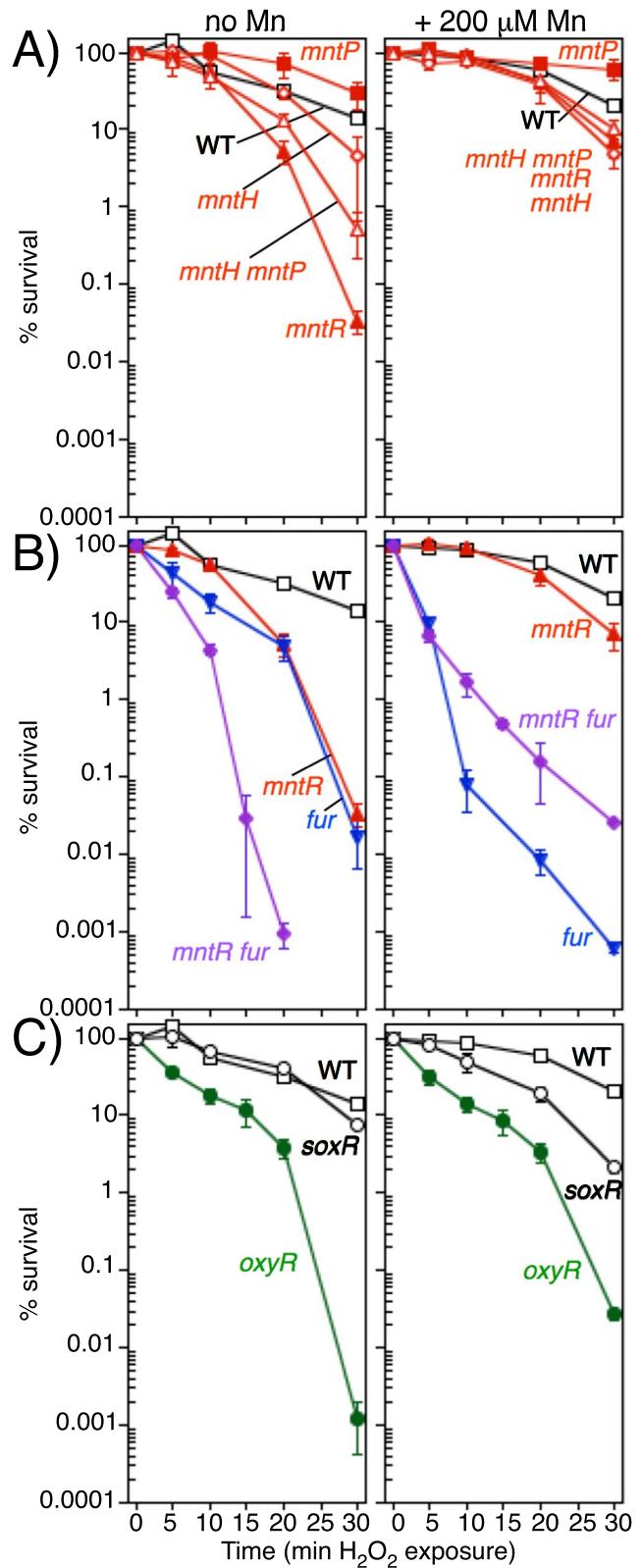
et al. 2019). The observation indicates that at least under some conditions, cells may remain static for growth until iron and manganese homeostasis can be restored, even if this occurs inefficiently.

Regulation of iron by Fur had a much more pronounced effect on survival following  $H_2O_2$ , with the deletion of *fur* reducing survival several orders of magnitude after a 30-min exposure. This effect was further exacerbated in the presence of manganese (Fig. 4B). Additionally, the absence of both iron and manganese regulation (*mntR fur*) severely compromised survival in medium without manganese, reducing viability beyond the limits of detection in our assay after 30 min. However, addition of manganese restored survival in this mutant to levels similar to that of the *fur* single mutant.

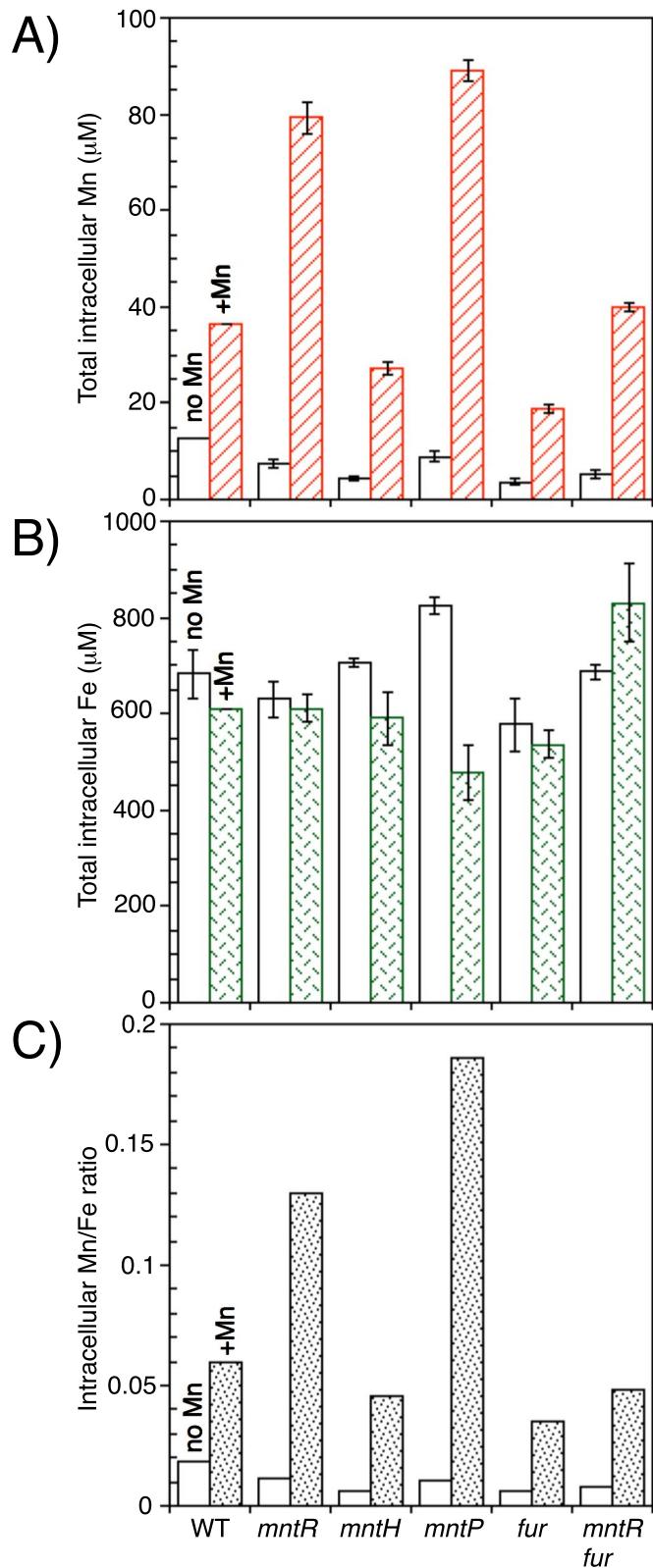
Consistent with the regulation of MntH and Fur by OxyR, survival of the *oxyR* mutant matched that of the *mntR fur* mutants in most respects, with survival being severely compromised in the absence of manganese, but modestly improved when this metal was present in the media (Fig. 4C). A modest reduction in survival of *soxR* was observed when manganese was present in the media. The basis of this observation was not clear, but may relate to expression of adjacent genes in the *soxR* operon or in its regulon.

To determine whether intracellular manganese and iron concentrations correlated with the ability of cells to survive  $H_2O_2$  exposure and restore replication after oxidative challenge, we measured the metal content of strains grown with and without manganese supplementation by ICP-MS. In the absence of manganese supplementation, total intracellular manganese concentrations were ~5–10  $\mu$ M across all strains (Fig. 5A). An increase in intracellular levels of manganese was observed when strains were grown in medium supplemented with this metal. Wild-type, *mntH*, and *fur* cells accumulated approximately 3-fold more intracellular manganese when cultured in the presence of this metal, while *mntR*, *mntP*, and *mntR fur* mutants contained ~10-fold greater manganese concentrations compared to their unsupplemented counterparts. Intracellular iron concentrations in manganese-supplemented wild-type, *mntR*, *mntH*, *fur*, and *mntR fur* cells were within 10–15% of the corresponding cells cultured without manganese (Fig. 5B). Consistent with previous observations (Martin et al. 2015), we found that manganese supplementation reduced intracellular iron levels in *mntP*

**Fig. 4** Accumulation of intracellular manganese increases cell survival to acute oxidative stress. The survival of **A** WT (open squares), *mntP* (filled squares), *mntH* (open diamonds), *mntR* (filled triangles), *mntH mntP* (open triangles), **B** *fur* (filled inverted triangles), *mntR fur* (filled diamonds), and **C** *soxR* (open circles), and *oxyR* (filled circles) cells grown in manganese-supplemented (right panels) and -unsupplemented media (left panels) following exposure to 10 mM H<sub>2</sub>O<sub>2</sub> for the indicated times is shown. Graphs represent an average from at least three independent experiments. Error bars represent one standard error of the mean



**Fig. 5** The absence of MntR or MntP increases Mn/Fe ratio in manganese-supplemented medium. **A** Total intracellular manganese and **B** total intracellular iron measured by ICP-MS in WT, *mntR*, *mntH*, *mntP*, *fur*, and *mntR fur* strains cultured in the absence (unfilled bars) or presence (filled bars) of 200  $\mu$ M manganese is plotted. Graphs represent an average of two independent measurements normalized to the total protein present in cell lysates. Error bars represent one standard error of the mean. **C** The ratio of manganese/iron concentrations for WT, *mntR*, *mntH*, *mntP*, *fur*, and *mntR fur* strains cultured in the absence (unfilled bars) or presence (filled bars) of 200  $\mu$ M manganese is plotted



cells, although we observed a more modest effect of approximately 1.7-fold, a change from  $\sim 825$  to  $\sim 480$   $\mu\text{M}$ . We did not observe any significant changes in iron concentration in *fur* cells compared to wild-type irrespective of manganese supplementation, but were only able to determine total intracellular iron bound to protein by ICP-MS and not free iron levels. Manganese supplementation increased the intracellular manganese/iron ratios for all strains, however this effect was much greater in *mntR* and *mntP* strains, increasing by 11- and 17-fold over unsupplemented cultures, respectively, compared to 3-fold in wild-type cells (Fig. 5C). Taken together, our data support the idea that increased intracellular manganese, but not iron, levels play a protective role in replication recovery and survival when cells face oxidative challenge.

## Discussion

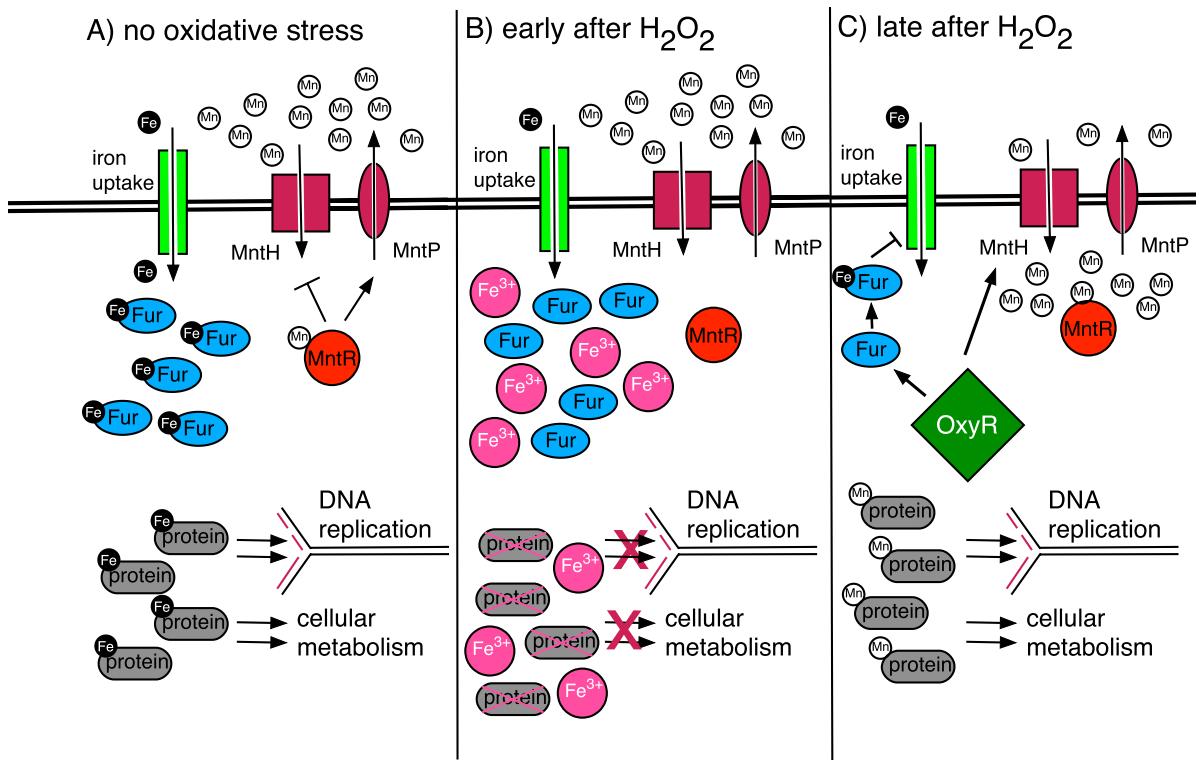
Previous work has shown that *E. coli* cells require manganese in the growth media to restore replication following oxidative stress (Hutflz et al. 2019; Hoff et al. 2021). Here, we demonstrate that active transport of manganese is necessary and regulates the recovery from oxidative challenge. Taken together with earlier work showing that manganese import is needed for proper enzyme metallation following oxidative stress (Anjem et al. 2009), our results define a central role for manganese homeostasis in cellular tolerance to oxygen toxicity.

Manganese has been implicated in protection against oxidative stress in multiple bacterial species and high intracellular manganese/iron ratios are associated with increased resistance to oxidative stress (Kehres et al. 2000; Tseng et al. 2001, 2002; Daly et al. 2004; Seib et al. 2004; Anjem et al. 2009). However, under iron-replete or non-oxidative growth conditions, intracellular manganese concentrations are kept low ( $\sim 5$ – $15$   $\mu\text{M}$ ) in *E. coli* by the activity of manganese-bound MntR to prevent mismetallation of iron- and other metal ion-cofactored enzymes (Fig. 6A and (Patzer and Hantke 2001; Kehres et al. 2002; Waters et al. 2011; Martin et al. 2015)).  $\text{Mn}^{2+}$ -bound MntR represses expression of the primary manganese importer MntH, while activating export through increased *mntP* transcription. Manganese concentrations are further controlled by  $\text{Fe}^{2+}$ -bound Fur acting on MntH expression. When

sufficient iron is present in the growth environment and cells are not undergoing oxidative stress, Fur activity maintains iron homeostasis through transcriptional control of iron transport and utilization to allow the multiple cellular processes that are co-factored by ferrous iron, including DNA synthesis and energy metabolism, while also limiting toxicity that can arise from free radical formation (Fig. 6A and (Bagg and Neilands 1987; Touati et al. 1995; Angerer and Braun 1998; McHugh et al. 2003; Seo et al. 2014)).

At immediate early times following hydrogen peroxide stress, ferrous iron becomes oxidized to its ferric form and disassociates from iron-centered enzymes (Fig. 6B and (Imlay 2014)). The stripping of ferrous iron from mononuclear iron, heme, and iron-sulfur proteins results in rapid inactivation of these proteins and the inhibition/stalling of cellular metabolism, consistent with the rapid inhibition in replication that we observe. The  $\text{Fe}^{2+}$  or  $[\text{2Fe}-\text{2S}]$  bound to Fur itself could also be a target of oxidation (Varghese et al. 2007; Fontenot et al. 2020) and result in inactivation of Fur and de-repression of iron uptake, leading to increased intracellular levels of iron that could then induce further Fenton chemistry and cellular damage. In the absence of Fur, the influx of iron into the cell (Touati et al. 1995; Keyer and Imlay 1996; Varghese et al. 2007) would also be predicted to further exacerbate the effects of oxidative stress and this is what we observe with the increased replication inhibition and  $\text{H}_2\text{O}_2$  sensitivity in *fur* mutants.

The dedicated reactive oxygen species sensor OxyR is activated following hydrogen peroxide stress through the formation of an intramolecular disulfide bond in each subunit of a working OxyR tetramer (Storz et al. 1990; Aslund et al. 1999; Zheng and Storz 2000). The resulting conformational change enhances interactions between OxyR and RNA Polymerase, thereby activating expression of genes in the OxyR regulon including those involved in peroxide scavenging, DNA repair, and iron and manganese import. The effects of this upregulation are seen at late times after peroxide challenge and result in increased intracellular manganese levels ( $\sim 150$   $\mu\text{M}$ ), allowing remetallation of iron-cofactored enzymes by manganese, and the restoration of replication and cellular metabolism (Fig. 6C) and (Anjem et al. 2009)). In addition to controlling manganese levels in the presence of hydrogen peroxide, OxyR upregulation of Fur expression is required to restore levels of both



**Fig. 6** Model for the replication response to H<sub>2</sub>O<sub>2</sub> treatment under manganese-rich growth conditions. **A** In manganese-rich environments where oxidants are absent, both Mn<sup>2+</sup>-bound MntR and Fe<sup>2+</sup>-bound Fur inhibit expression of the primary manganese importer, MntH. At the same time, MntP expression is upregulated by Mn<sup>2+</sup>-bound MntR and binding of free manganese to a riboswitch in the *mntP* promoter to export excess manganese from the cytoplasm. The dual activity of Mn<sup>2+</sup>-bound MntR on manganese import and export prevents toxicity from excess intracellular manganese. **B** Exposure

to H<sub>2</sub>O<sub>2</sub> results in rapid oxidation and inactivation of ferrous iron-cofactor enzymes, inhibiting cellular metabolism and hence DNA replication immediately following oxidative stress. **C** H<sub>2</sub>O<sub>2</sub> also oxidizes a sensory cysteine residue in OxyR, activating this transcription factor. OxyR upregulates expression of MntH and Fur, resulting in increased manganese import and Fur-mediated repression of further iron uptake, respectively. In cells under oxidative stress, rapid remetallation of oxidized mononuclear iron enzymes occurs using available manganese as the active metal cofactor, thus restoring metabolism

iron-bound and -unbound Fur to control the presence of free intracellular iron in peroxide-stressed cells and limit damage (Zheng et al. 1999; Varghese et al. 2007). In the absence of OxyR, both Fur and MntH expression remain at basal levels after hydrogen peroxide stress (Zheng et al. 1999; Varghese et al. 2007; Anjem et al. 2009), resulting in poor recovery of replication and decreased viability in *oxyR* cells.

Our results are consistent with the idea that manganese transport promotes hydrogen peroxide survival overall. Manganese supplementation has been shown to rescue iron overload in cells chronically exposed to endogenous sources of peroxide by replacing an oxidation-susceptible divalent metal with one that is stable (Anjem et al. 2009). Similarly, we find that the

absence of MntR and consequently manganese transport regulation rescues the ability of a *fur* mutant to restore replication and survive oxidative challenge when sufficient manganese is present in the growth medium. Unlike manganese-centric organisms that encode both an active primary ABC (ATP-binding cassette) transporter, e.g. MntABC and SitABC, and a secondary NRAMP (natural resistance-associated macrophage protein) family importer, MntH, iron-centric *E. coli* encodes only *mntH* containing distinct manganese-, iron-, and oxidative species-responsive elements within its promoter (for a review, see (Bosma et al. 2021; Martin and Waters 2022)). Despite this tight regulation of manganese import, *E. coli* cells deficient in MntH have been shown to be

able to use a secondary, low-affinity mechanism of import in the presence of manganese concentrations greater than 50  $\mu\text{M}$  (Anjem et al. 2009). The identity of this transporter remains to be determined, however a few candidates include the broad specificity divalent metal ion transporter, ZupT, and zinc transporter, ZnuABC. A *zupT* *Salmonella* mutant completely abolishes both residual manganese import in a *mntH sitABC* background and all resistance to nitrosative stress (Yousuf et al. 2020). On the other hand, *znuA* and *znuC* mutants suppress the manganese sensitivity of *hfx* *E. coli* cells, which accumulate manganese in an MntR-independent manner, by inhibiting both zinc and manganese import (Kaur et al. 2014). Whatever this secondary pathway, the small amount of manganese that can be non-specifically imported into the intracellular space could account for the partial replication recovery we observe at late times in manganese-supplemented *mntH* cells. In the same way, while *oxyR* mutants only produce basal levels of MntH after oxidative stress, manganese-rich conditions could potentially allow for enough of this metal to accumulate, leading to the slight increase in survival of manganese-supplemented *oxyR* cells that we observe following prolonged exposure to hydrogen peroxide. Consistent with this interpretation, Imlay and others have found that unsupplemented rich medium, which contains more manganese than defined medium, supports growth in the presence of oxidative or nitrosative stress even when primary manganese import is impaired (Anjem et al. 2009; Yousuf et al. 2020).

The disruption of replication by DNA damage is a primary cause of lethality in cells exposed to DNA damaging agents (Rupp and Howard-Flanders 1968; Howard-Flanders et al. 1969; Courcelle and Hanawalt 2001; Courcelle et al. 2001). Pausing replication to allow more time for repair to occur decreases the frequency of these potentially lethal events and has been demonstrated to be beneficial both for reducing mutations and increasing survival (Castellani et al. 1964; Ganesan and Smith 1969; Courcelle et al. 1997). Additionally, cells lacking RecF, a protein specifically required for replication resumption after disruption by DNA damage, are hypersensitive to UV-irradiation and other forms of damage (Courcelle et al. 1997, 1999, 2003; Chow and Courcelle 2004; Ona et al. 2009), yet are modestly resistant to hydrogen peroxide-induced DNA

lesions (Hoff et al. 2021). Thus, one possibility is that the iron sensitivity of replication represents an adaptive response to prevent genomic replication from occurring during periods of oxygen stress when potentially lethal or mutagenic damage may be encountered. In cultures lacking manganese, this inhibition can last for several hours without compromising viability (Imlay and Linn 1986; Hutzil et al. 2019).

The iron-dependent component required for replication in *E. coli* remains unknown. However, the results presented here demonstrate that manganese transport actively regulates the timing and ability to restore DNA synthesis after hydrogen peroxide treatment. Restoration may involve either direct remetallation of this protein or could involve a redundant manganese-specific counterpart. In eukaryotes, the polymerase responsible for leading strand DNA synthesis, Pol  $\epsilon$ , has been shown to contain an iron-sulfur center required for replication (Netz et al. 2011; Lisova et al. 2022). It is not known, if Pol  $\epsilon$ , plays a role in the oxidative response, or if this enzyme can utilize manganese during the recovery period. This and the identity of the proteins responsible for stalling replication after oxidative stress remain important questions to be addressed in future work for both bacteria and humans.

## Conclusions

Reactive oxygen species generated by metabolic pathways or from exogenous sources damage DNA, proteins and lipids in both microbes and human cells. In this study, we show that regulation of manganese transport directly modulates the timing and ability of cells to resume DNA replication following oxidative challenge. In addition, we find that manganese transport promotes survival to hydrogen peroxide exposure particularly when intracellular free iron levels are perturbed. Taken together, our results define a role for manganese homeostasis in the bacterial response and recovery from oxidative stress. Identification of the iron-dependent replication component that is inactivated by acute oxidative challenge will be required to further our understanding of oxidative stress effects outside of DNA damage.

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## Declarations

**Conflict of interest** The authors have no relevant conflicts of interest to disclose.

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