The SOS and RpoS Regulons Contribute to Bacterial Cell Robustness to Genotoxic Stress by Synergistically Regulating DNA Polymerase Pol II

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ABSTRACT Mitomycin C (MMC) is a genotoxic agent that induces DNA cross-links, DNA alkylation, and the production of reactive oxygen species (ROS). MMC induces the SOS response and RpoS regulons in *Escherichia coli*. SOS-encoded functions are required for DNA repair, whereas the RpoS regulon is typically induced by metabolic stresses that slow growth. Thus, induction of the RpoS regulon by MMC may be coincidental, because DNA damage slows growth; alternatively, the RpoS regulon may be an adaptive response contributing to cell survival. In this study, we show that the RpoS regulon is primarily induced by MMC-induced ROS production. We also show that RpoS regulon induction is required for the survival of MMC-treated growing cells. The major contributor to RpoS-dependent resistance to MMC treatment is DNA polymerase Pol II, which is encoded by the *polB* gene belonging to the SOS regulon. The observation that *polB* gene expression is controlled by the two major stress response regulons that are required to maximize survival and fitness further emphasizes the key role of this DNA polymerase as an important factor in genome stability.

KEYWORDS *Escherichia coli*; SOS; RpoS; DNA polymerase II; mitomycin C

ALL organisms are continuously exposed to multiple environmental stresses throughout their life cycles (Foster 2007). Moreover, many physiological functions generate toxic compounds, which are potentially harmful to cells (Iuchi and Weiner 1996). Consequently, all living organisms possess a plethora of mechanisms that allow them to respond to and counteract the deleterious effects of external and internal stressors. The ubiquitous presence in nature of molecular mechanisms that maintain genome integrity strongly suggests that genome stability is one of the most relevant fitness determinants for all living organisms (Friedberg *et al.* 2005). One of the best-understood systems for the maintenance of genome integrity is the SOS response, which is widespread among bacteria (Michel 2005).

The SOS response in *Escherichia coli* is a coordinated cellular response to DNA damage that involves at least 40 genes, many of which encode proteins with DNA repair functions,

e.g., nucleotide excision repair (NER), translesion synthesis (TLS), and homologous recombination (HR) (Radman 1975; Little and Mount 1982; Walker *et al.* 1982). During normal growth, genes belonging to the SOS regulon are repressed by the LexA protein. The SOS response can be induced by a wide variety of stressors, with one common characteristic: they all result in the appearance of persistent single-stranded DNA (ssDNA). The persistent contact with ssDNA activates the coprotease activity of the RecA protein, which promotes proteolytic auto-cleavage of the repressor, LexA protein, thus activating the SOS response (Little and Mount 1982; Walker *et al.* 1982). When DNA damage is repaired, persistent ssDNA disappears, and the SOS regulon is again repressed.

Interstrand DNA cross-links are potent inducers of the SOS response in bacteria. These DNA lesions are extremely dangerous because even a single unrepaired DNA cross-link per genome can be lethal for the bacterium (Szybalski and Iyer 1964). Several exogenous and endogenous DNA damage agents cause DNA interstrand cross-links (Ali-Osman *et al.* 1995; Rudd *et al.* 1995; Poklar *et al.* 1996; Wu *et al.* 2005; Colis *et al.* 2008). In *E. coli*, two known pathways are capable of repairing interstrand DNA cross-links. The first pathway, which involves NER, HR, and DNA polymerase I (PolA), has

been described to successfully repair psoralen-induced DNA cross-links (Van Houten *et al.* 1986; Sladek *et al.* 1989; Slater and Maurer 1993). The second pathway involves NER, which acts together with the SOS-regulated DNA polymerase II, and repairs nitrogen mustard (HN2)-induced DNA cross-links (Berardini *et al.* 1999).

Mitomycin C (MMC) strongly induces the SOS response in *E. coli*. MMC is a mono- and bifunctional alkylating agent, and a potent cross-linker with a remarkable capacity to generate interstrand DNA cross-links by reacting specifically with guanines of the complementary DNA at CpG sequences (Yu and Pan 1993; Tomasz 1995; Tomasz and Palom 1997). In addition, MMC can also act as an oxidizing agent, because its reduction by $NaBH_4$ or by NADPH, in the presence of O_2, results in the generation of H_2O_2 (Tomasz 1976; Doroshow 1986). MMC can oxidize when it is present in a free state in the cell, or when it is irreversibly bound to DNA (Tomasz 1976). Consequently, MMC causes a myriad of deleterious effects in eukaryotic and prokaryotic cells, including inhibition of DNA synthesis, increased mutagenesis, chromosome breakage, stimulation of genetic recombination, and sister chromatid exchange (Tomasz 1995). Thus, MMC is widely used in cancer therapies as an antitumor drug (Bradner 2001). In *E. coli*, DNA cross-links are repaired by the NER, HR, and TLS systems (Van Houten *et al.* 1986; Sladek *et al.* 1989; Slater and Maurer 1993; Berardini *et al.* 1999), whereas damage caused by monofunctional alkylation and oxidation is repaired by DNA base excision repair (Cunningham *et al.* 1986; Suvarnapunya *et al.* 2003; Hornback and Roop 2006).

However, MMC treatment does activate other pathways beyond the SOS response in *E. coli*. Transcriptomic analysis has shown that > 1000 genes are activated in response to MMC (Khil and Camerini-Otero 2002), many of which have not been previously associated with DNA damage. For example, the list of genes activated by MMC includes many members of the general stress response network, which is regulated by the alternative RNA polymerase (RNAP) σ factor RpoS (σ^S) (Weber *et al.* 2005). This stress response is induced when bacterial growth decreases or when cells are exposed to starvation or to various stresses, such as antibiotics and osmotic or oxidative stress (Bouveret and Battesti 2011; Gutierrez *et al.* 2013). The induction of this regulon results in the downregulation of genes required for rapid growth and upregulation of genes involved in the protection, repair, and maintenance of the cell. Consequently, cells enter a multi-resistant state.

RpoS regulon induction is subjected to complex regulation, which is controlled at all levels of *rpoS* gene expression, *i.e.*, transcription, translation, and protein stability, as well as through the control of RNAP activity (Hengge-Aronis 1996). Approximately 10% of the *E. coli* genome is directly or indirectly controlled by RpoS (Weber *et al.* 2005). Interestingly, only two of the known RpoS-regulated genes are directly involved in DNA repair: the *ada* gene, which encodes an O^6-methylguanine-DNA methyltransferase that removes methyl groups from methylated bases and methyl-phosphates

(Nieminuszczy and Grzesiuk 2007), and the *xthA* gene, which encodes exonuclease III (Demple *et al.* 1986; Centore *et al.* 2008). RpoS also controls the induction of the *katE, dps*, and *gor* genes, which protect cells against reactive oxygen species (ROS) and therefore decrease ROS-induced DNA damage (Hengge-Aronis 1996).

Induction of the RpoS regulon by MMC may merely be a collateral consequence of the MMC treatment, which slows growth, but its activation after MMC treatment may also be a specific adaptive response that contributes to cell survival under genotoxic stress. In this study, we show that the RpoS regulon is primarily induced by MMC-induced ROS production and that RpoS regulon induction is required for the survival of growing cells treated with MMC. Moreover, we show that the expression of the *polB* gene, which encodes SOS-regulated polymerase Pol II, is also coregulated by RpoS. Our data show that Pol II is a major contributor to the repair of MMC-induced DNA lesions. This finding further demonstrates the involvement of the RpoS regulon in the response to genotoxic stress. Therefore, our study shows that the SOS and RpoS regulons synergistically contribute to the robustness of bacterial cells facing genotoxic stressors.

Materials and Methods

Bacterial strains

All strains used in this study were derivatives of the *E. coli* K12 MG1655 strain (Table 1). One of the consequences of SOS induction is SulA-mediated cell filamentation (Gottesman *et al.* 1981). Because filamentation impedes precise quantification of different MMC-induced phenotypes, we used the MG1655 Δ*sulA* strain as a reference strain (RS). Owing to the lack of filamentation, the Δ*sulA* strain had a higher minimum inhibitory concentration (MIC) than does the wild-type strain MG1655, *i.e.*, 1.5 and 0.5 μg ml^{-1}, respectively.

All other strains were derivatives of the RS. The strains were constructed by P1vir transduction (Silhavy *et al.* 1984) of the mutant alleles from either the Keio collection or from our own laboratory collection. Strain TD15 Δ*lexA* [*lexA*(def)] was constructed using the λ Red recombination system (Datsenko and Wanner 2000; Murphy *et al.* 2000; Yu *et al.* 2000) in a Δ*sulA* background. The sequences of the primers used for the *lexA*(def) mutant were as follows: OTD5, 5′-ATGAAAGCGTTAACGGCCAGGCAACAAGAGGTGTTTGTGT AGGCTGGAGCTGCTTC-3′; OTD6, 5′-TTACAGCCAGTCGCC GTTGCGAATAACCCCAACCGCCATATGAATATCCTCCTTAG-3′. The *lexA*(def) construct was subsequently transferred to the RS through P1vir transduction. When needed, the Kan^R cassette was removed by expressing Flp recombinase from plasmid pCP20 as described in (Datsenko and Wanner 2000). All strains were verified by PCR.

The complementation plasmid for Pol II (p*polB*+) was kindly provided by the laboratory of Robert Fuchs. The plasmid was constructed by amplifying the *polB* gene expressed

Table 1 Bacterial strains and plasmids used in this study

	Relevant Genotype	Reference or Source
Strain		
MG1655	Wild-type	Laboratory stock
RpoSmCherry	MG1655 *rpoS-mCherry*::FRT	Maisonneuve *et al.* (2013)
TD6	MG1655 Δ*sulA*::KanR	MG1655 × P1 (KEIO Δ*sulA*::KanR)
TD7, reference strain	TD6 Δ*sulA*::FRT	
TD598	TD7 Δ*cysC*::KanR	TD7 × P1 (KEIO Δ*cysC*::KanR)
TD603[a]	TD598 *rpoS-mCherry*::FRT	TD598 × P1 (RpoSmCherry)
TD318	TD7 Δ*rpoS*::KanR	TD7 × P1 (KEIO Δ*rpoS*::KanR)
TD612	TD318 Δ*rpoS*::FRT	
TD322	TD7 Δ*rssB*::KanR	TD7 × P1 (KEIO Δ*rssB*::KanR)
TD615	TD322 Δ*rssB*::FRT	
TD677	TD7 Δ*katG*::KanR	TD7 × P1 (KEIO Δ*katG*::KanR)
TD678	TD7 Δ*katE*:: TetR	TD7 × P1 (NEC445 Δ*katE*:: TetR)
TD679	TD677 Δ*katE*::TetR	TD677 × P1 (NEC445 Δ*katE*:: TetR)
NEC445	Δ*katG*::CmR Δ*katE*::TetR Δ*ahpCF*::KanR	Hébrard *et al.* (2009)
TD624	TD7 Δ*iraD*::KanR	TD7 × P1 (KEIO Δ*iraD*::KanR)
TD632	TD603 Δ*iraD*::KanR	TD603 × P1 (KEIO Δ*iraD*::KanR)
TD417	TD7 Δ*uvrY*::KanR	TD7 × P1 (KEIO Δ*uvrY*::KanR)
TD669	TD603 Δ*uvrY*::KanR	TD603 × P1 (KEIO Δ*uvrY*::KanR)
TD653	TD7 Δ*barA*::KanR	TD7 × P1 (KEIO Δ*barA*::KanR)
TD654	TD653 Δ*barA*::FRT	
TD668	TD603 Δ*barA*::KanR	TD603 × P1 (KEIO Δ*barA*::KanR)
TD15	TD7 Δ*lexA*::KanR	TD7 × FRT*aph*FRT from pKD4 Datsenko and Wanner (2000)
TD16	TD15 Δ*lexA*::FRT	
TD319	TD16 Δ*rpoS*::KanR	TD16 × P1 (KEIO Δ*rpoS*::KanR)
TD613	TD319 Δ*rpoS*::FRT	
TD292	TD7 Δ*polB*::KanR	TD7 × P1 (KEIO Δ*polB*::KanR)
TD640	TD292 Δ*polB*::FRT	
TD619	TD615 Δ*polB*::KanR	TD615 × P1 (KEIO Δ*polB*::KanR)
TD252	TD7 Δ*dinB*::KanR	TD7 × P1 (KEIO Δ*dinB*::KanR)
TD617	TD615 Δ*dinB*::KanR	TD615 × P1 (KEIO Δ*dinB*::KanR)
TD272	TD7 Δ*umuD*::KanR	TD7 × P1 (KEIO Δ*umuD*::KanR)
TD618	TD615 Δ*umuD*::KanR	TD615 × P1 (KEIO Δ*umuD*::KanR)
Plasmid		
p*polB*+ KanR		Becherel and Fuchs (2001)
p*bolA-gfp* KanR		Plasmids carrying transcriptional fusions of
p*polB-gfp* KanR		the *gfpmut2* gene to promoters of
p*dinB-gfp* KanR		different genes Zaslaver *et al.* (2006).
p*umuD-gfp* KanR		

KanR, kanamycin resistance; TetR, tetracycline resistance.
[a] This strain does not carry the Δ*cysC*::KanR, because *rpoS-mCherry*::FRT was cotransduced with the *cysC* gene.

from its native promoter/operator region and cloning into the low-copy number vector pWKS130 derived from pSC101 (Becherel and Fuchs 2001).

Liquid cultures were grown in Lysogeny Broth medium (LB, Difco, Detroit, MI) at 37° with shaking at 150 rpm. When needed, the medium was supplemented with the appropriate antibiotic at the following concentrations: ampicillin (Amp) 100 μg ml^{-1}, chloramphenicol (Cm) 30 μg ml^{-1}, and kanamycin (Kan) 100 μg ml^{-1}. MMC was purchased from Sigma ([Sigma Chemical], St. Louis, MO). The antioxidant thiourea (Sigma) was added directly to the growing cultures to a final concentration of 50 mM.

Growth curves and fluorescence analysis

Bacterial cultures were inoculated from independent frozen stocks (at $^-80°$). Next, 4.0×10^4 of bacterial cells from the overnight culture were incubated in 96-well black microtiter plates with a flat bottom containing 100 μl of LB broth per well and covered with 50 μl of mineral oil (Sigma). The layer of mineral oil was added to prevent evaporation. It has previously been found that mineral oil does not affect the bacterial growth rate or increase the expression from the gene promoters, which are induced under anaerobic conditions (Zaslaver *et al.* 2006). When the effects of MMC were tested, MMC was added to the culture at a final concentration of 1 μg ml^{-1}. Microtiter plates were incubated at 37° in a microplate reader incubator (SPARK 10 M, Tecan Trading AG, Switzerland) with orbital shaking for a duration of 220 sec per interval, an amplitude of 4 mm, and shaking at 150 rpm. The optical density at 600 nm (OD$_{600}$) and fluorescence were measured every 5 min for 12 hr. The plates were vigorously shaken prior to each measurement. For the fluorescence

analysis, we used a RpoS-mCherry protein fusion to directly estimate the amount of RpoS protein (Maisonneuve *et al.* 2013), and low-copy plasmids carrying *E. coli* promoter regions inserted upstream of a gene encoding a fast-folding *gfpmut2* were used to measure the activity of promoters of genes of interest in this study (Zaslaver *et al.* 2006). Excitation/emission was 590/635 nm for red fluorescence (mCherry) and 485/510 nm for green fluorescence (GFP). Expression levels were normalized and are presented as the ratio of the fluorescence/OD_{600}.

Catalase enzymatic activity assay

To assess the activity of KatE catalase (Iwase *et al.* 2013), bacterial cultures were inoculated from three individual colonies and grown overnight in LB broth at 37° with shaking at 150 rpm. Overnight cultures were diluted 1/1000 (v/v) and inoculated in 5 ml of plain LB broth or LB broth containing 1 μg ml^{-1} of MMC, and incubated at 37° with shaking at 150 rpm until they reached stationary phase (OD_{600} ~5; ~18 hr). Afterward, the OD_{600} of the cultures was recorded, and 1 ml of culture was washed and resuspended in 100 μl of phosphate buffer (50 mM potassium phosphate and 0.1 mM EDTA, pH 7.8). The cell suspension was incubated at 55° for 15 min. Lysates were then transferred to clean glass test tubes (diameter 12 mm; length 100 mm) and mixed with 100 μl of 1% Triton X-100. Finally, 100 μl of 30% hydrogen peroxide was added to the glass tube. The tubes were then vortexed and incubated at room temperature for 5 min to allow the formation of oxygen foam. The height of the maximal foam was measured using a ruler. The height in cm was normalized to the recorded OD_{600} of the culture of interest (HPII activity/OD_{600}) (Iwase *et al.* 2013).

Mutation frequency assay

To perform the fluctuation test (Luria and Delbruck 1943) based on the appearance of rifampicin-resistant mutants (Rif^R) (Jin and Gross 1988), bacterial cultures were inoculated from at least six single colonies in LB broth and incubated overnight at 37° with shaking at 150 rpm. Next, either 5 ml of plain LB broth, or LB broth containing 1 μg ml^{-1} of MMC, was inoculated with 20 μl of a 10^{-6} dilution of the overnight culture (to obtain 20–100 bacterial cells in the final inoculum). Cultures were incubated for 20 hr at 37° with shaking at 150 rpm. Subsequently, 200 μl of each culture was spread on an LB plate containing 100 μg ml^{-1} of rifampicin. The colonies were scored after 2 days of incubation at 37°, and the Rif^R mutant frequency was calculated (CFU on LB Rif/CFU on LB).

Evaluation of survival in response to MMC

The survival assay was performed by inoculating bacterial cultures from three single colonies into LB broth and incubation overnight at 37° with shaking at 150 rpm. The cultures were then diluted 1/1000 (v/v) and inoculated into 5 ml of plain LB broth. The cultures were incubated for 2 hr at 37° with shaking at 150 rpm until the OD_{600} was ~0.2. Next,
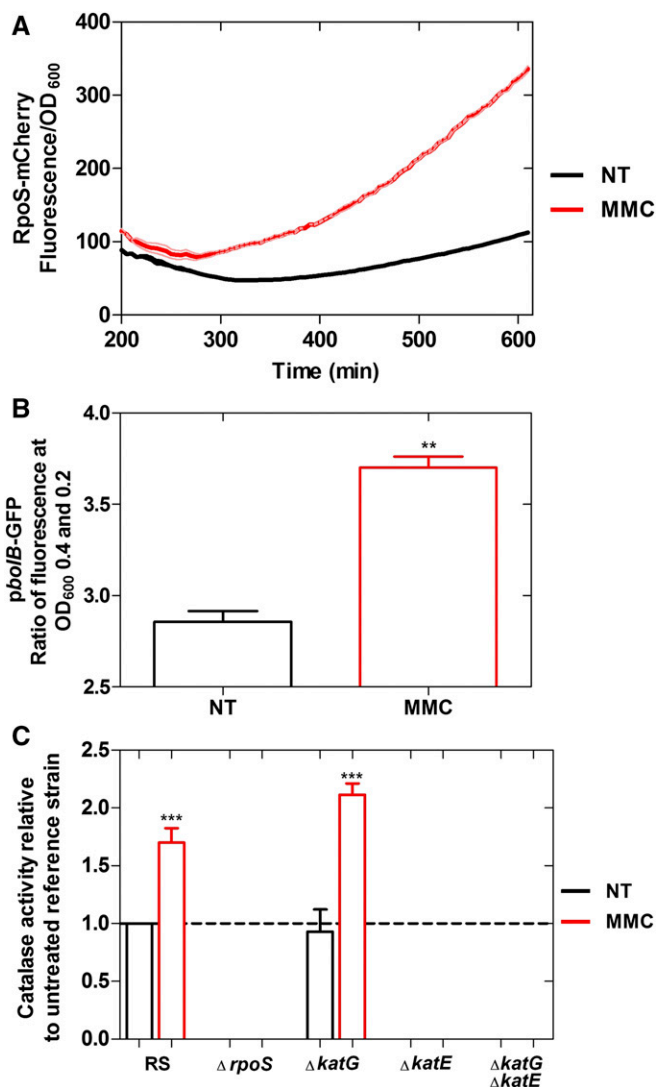
Figure 1 Induction of the RpoS regulon with MMC. The general stress response regulated by RpoS is induced during exponential growth of bacteria in response to the DNA-damaging agent MMC. (A) RS carrying RpoS-mCherry protein fusion was grown in LB (black line) or LB supplemented with MMC (red line). (B) Expression of the RpoS-regulated p*bolA*-GFP reporter in the RS. An increase in fluorescence between OD_{600} 0.2 and 0.4 in MMC-treated and NT cells is shown. (C) Catalase activity of the RpoS-regulated monofunctional catalase HPII encoded by *katE* in different genetic backgrounds. All measured catalase activities were normalized with respect to that of the NT RS. For all measures, each point represents the mean ± SEM of at least three independent experiments. MMC, treated with 1 μg ml^{-1} MMC. ** $P < 0.01$, *** $P < 0.001$, two-tailed Student's *t*-test. MMC, mitomycin C; NT, nontreated; RS, reference strain.

MMC (at a final concentration of 4 and 5 μg ml^{-1}) was added to the tubes, and the cultures were incubated for an additional 2 hr. Serial dilutions of the cultures were generated in MgSO$_4$ 10^{-2} M and plated onto LB agar plates. When the plasmid p*polB*$^+$ was used, cultures were grown in LB broth + Kan and plated onto LB agar + Kan plates. The plates were incubated at 37° for 12 hr; then, the colonies were counted, and survival was calculated (CFU treated/CFU nontreated).
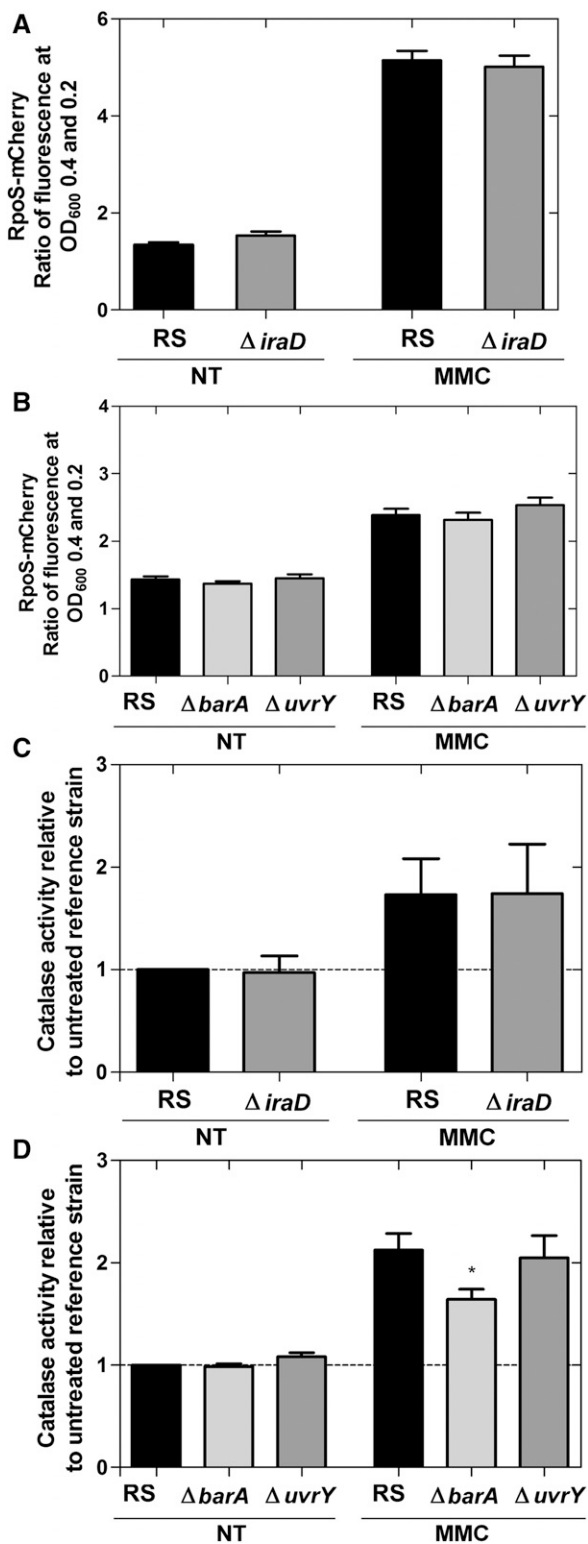
Figure 2 Role of the two-component system BarA-UvrY in RpoS regulon activation. An increase in RpoS-mCherry protein fusion expression between OD$_{600}$ 0.2 and 0.4 in mitomycin C (MMC)-treated and nontreated (A) reference (RS) and $\Delta iraD$ strains, and (B) the $\Delta barA$ and $\Delta uvrY$ strains. Catalase activity of the RpoS-regulated monofunctional catalase HPII encoded by $katE$ in MMC-treated and NT (C) RS and $\Delta iraD$ strains, and (D) in the $\Delta barA$ and $\Delta uvrY$ strains. All measured catalase activities were normalized to that of the NT RS. For all results, each point represents the

(caption continues at bottom right) mean $\pm$ SEM of at least three independent experiments. MMC, treated with 1 μg ml^{-1} MMC. *$P < 0.05$, two-tailed Student's t-test.

Statistical analyses

All analyses were conducted using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). For all set comparisons, unpaired Student's t-tests were used. A nonparametric Mann–Whitney U test was used for the mutation frequency analysis.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. Strains and plasmids are available upon request.

Results

MMC treatment induces the RpoS-mediated general stress response

First, we tested whether MMC induced the synthesis of RpoS protein and the expression of RpoS-regulated genes under our experimental conditions. The MIC of MMC for the RS was determined to be 1.5 μg ml^{-1}. For these experiments, we used a sub-MIC of MMC 1 μg ml^{-1}, which did not significantly affect bacterial growth (data not shown). First, we exposed the strain carrying a RpoS-mCherry protein fusion (Maisonneuve *et al.* 2013) to a sub-MIC of MMC, and we measured RpoS levels over time. We observed that cells growing in the presence of MMC induce RpoS synthesis at an earlier time point and produced higher levels than nontreated cells (Figure 1A).

Second, we used a promoter fusion of the RpoS-regulated gene *bolA* (Lange and Hengge-Aronis 1991) to a fluorescent protein (p*bolA*-GFP) (Zaslaver *et al.* 2006) to quantify induction of the RpoS regulon by MMC. We observed significantly higher activation of the p*bolA*-GFP reporter fusion in the cultures treated with the sub-MIC of MMC than in nontreated cultures (Figure 1B).

Finally, we measured the activity of monofunctional catalase HPII encoded by the *katE* gene, which is positively regulated by RpoS (Loewen *et al.* 1985). Because KatE is the only thermostable catalase, incubation of a suspension of bacterial cells at a high temperature inactivated the other RpoS-independent KatG catalase, thus allowing us to specifically measure RpoS-dependent catalase activity (Iwase *et al.* 2013). Catalase activity was 50% higher in cultures grown in the presence of sub-MIC of MMC compared with the nontreated controls (Figure 1C). As controls, we used $\Delta rpoS$, $\Delta katG$, and $\Delta katE$ mutants, and the double mutant $\Delta katG$ $\Delta katE$. As expected, the $\Delta katG$ mutant exhibited the same level of catalase activity as the RS, whereas no catalase activity was observed for the $\Delta rpoS$, $\Delta katE$, and $\Delta katG$ $\Delta katE$ strains (Figure 1C).

These data demonstrated that *E. coli* cells growing in the presence of a sub-MIC of MMC induced RpoS-mediated stress response sooner and to a greater extent than nontreated cells.

BarA is involved in RpoS activation by MMC

The transcription of RpoS-dependent genes has a crucial role in entry into stationary phase, as well as in the protection of cells during the stationary phase (Hengge-Aronis 1996; Weber *et al.* 2005). Different regulators, which are involved in the induction of the RpoS regulon, modulate responses to different inducers (Battesti *et al.* 2011). The only known regulator of RpoS regulon induction mediated by various DNA-damaging agents is IraD (Merrikh *et al.* 2009). However, we found that IraD was not responsible for the induction of RpoS protein synthesis by MMC (Figure 2A), in agreement with previously published results (Merrikh *et al.* 2009). IraD did not affect the levels of RpoS, as measured with the RpoS-mCherry fusion (Figure 2A), nor did it affect genes under RpoS regulation, *e.g.*, *katE* (Figure 2C), during genotoxic stress (Student's *t*-tests, $P = 0.8352$).

We observed that genotoxic stress, caused by MMC, activated the RpoS regulon in growing cells. Consequently, we tested the involvement of the two-component system BarA-UvrY, which is known to be involved in the regulation of *rpoS* gene transcription during the early exponential phase (Mukhopadhyay *et al.* 2000), by using the RpoS-mCherry protein fusion. Levels of the RpoS protein were not altered in the absence or presence of MMC in Δ*uvrY* or Δ*barA* mutants (Figure 2B). Although our data showed that BarA-UvrY did not control the cellular amount of the RpoS protein in MMC-treated cells, it nonetheless can still participate in the activation of RpoS-regulated genes. For this reason, we tested catalase activity in cultures of the RS, Δ*barA*, and Δ*uvrY* strains treated with MMC or left nontreated. We found that catalase activity was not significantly different among the three strains in the absence of MMC (Figure 2D). However, when the cultures were treated with MMC, catalase activity was significantly lower in the Δ*barA* mutant (40%, Student's *t*-tests, $P = 0.0256$), but not in the Δ*uvrY* mutant (Student's *t*-tests, $P = 0.7730$), relative to the RS. This result further indicated that BarA, a sensory histidine kinase, is necessary for full activation of RpoS during MMC treatment.

MMC-induced ROS production is responsible for RpoS induction

It has been reported that, in addition to causing DNA cross-links and alkylation, MMC also produces ROS (Tomasz 1976; Doroshow 1986). To determine whether, under our experimental conditions, MMC also produced ROS, we examined the effects of MMC on the growth of the Δ*katG* Δ*katE* Δ*ahpCF* strain, which is highly sensitive to oxidative stress (Park *et al.* 2005). We found that growth of this strain was severely affected by MMC (Figure 3A). Next, we examined the effects of the antioxidant thiourea (Kelner *et al.* 1990) on the growth of treated and nontreated strains, and we observed a great attenuation of the effect of MMC on growth (Figure 3A).
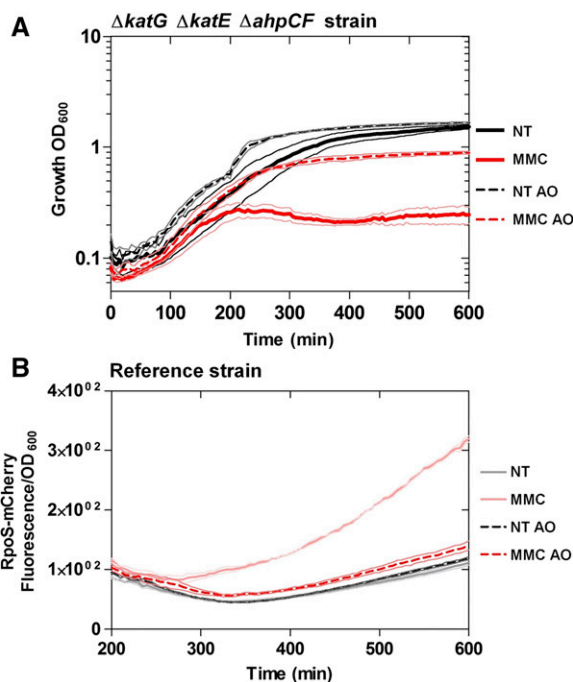
Figure 3 MMC generates oxidative stress in treated cells. (A) Growth of the Δ*katG* Δ*katE* Δ*ahpCF* strain in LB in the presence or absence of MMC, and with or without the addition of the antioxidant AO. (B) Expression of the RpoS-mCherry protein fusion in the reference strain (RS) grown in LB in the presence or absence of MMC, and with or without the addition of the antioxidant thiourea. Each curve represents the mean ± SEM of at least three independent experiments. AO, addition of 50 mM thiourea. MMC, treated with 1 μg ml^{-1} mitomycin C; NT, nontreated.

This observation allowed us to test whether MMC-induced ROS production was responsible for RpoS induction. We incubated cells carrying the RpoS-mCherry fusion and treated with MMC with or without thiourea. Thiourea prevented induction of the RpoS-mCherry reporter fusion (Figure 3B), thus indicating that ROS production was the main factor responsible for activation of the RpoS regulon during MMC treatment.

Activation of the RpoS regulon caused by MMC is an adaptive response to cope with genotoxic stress

As mentioned above, MMC causes a myriad of deleterious effects in treated cells, including slow growth, increased mutagenesis, and cell death (Tomasz 1995). Induction of the RpoS regulon during exposure to MMC (Figure 1) might be a mere consequence of the stress generated in the cell by the genotoxic agent, or in contrast, it might be an adaptive response allowing cells to cope with the effects of MMC. To determine whether the induction of the RpoS-regulated general stress response network was adaptive, we measured the effect of MMC on cell death and mutagenesis in strains with altered regulation of this regulon. Thus, we used the RS, which was wild-type with respect to the regulation of the RpoS regulon, a Δ*rpoS* mutant, and a Δ*rssB* mutant, which had a constitutively induced RpoS regulon, because the RssB protein promotes RpoS degradation (Hengge-Aronis 2002).
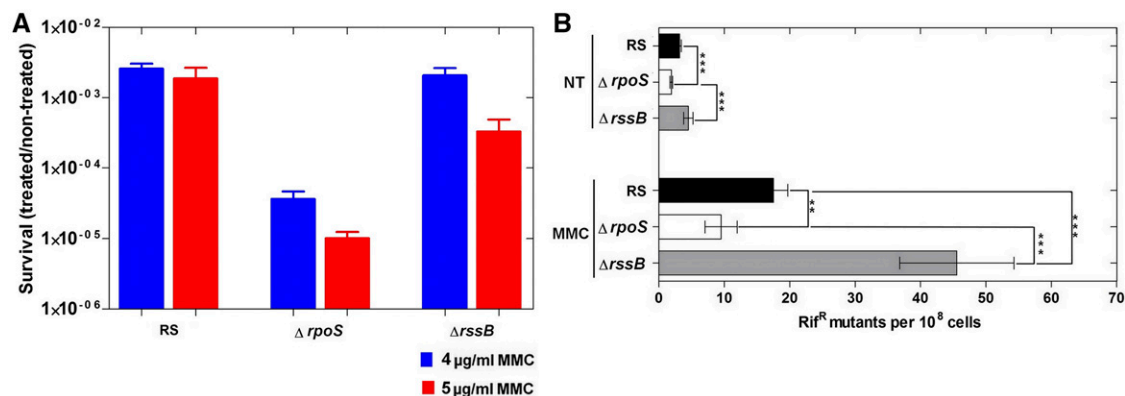
Figure 4 Effect of RpoS-regulon induction on the survival and mutagenesis of MMC-treated cells. (A) Survival of the MMC-treated RS, ΔrpoS, and ΔrssB strains. For a dose of 4 μg ml^{-1} of MMC, the following differences were significant: RS *vs.* ΔrpoS, *** *P*; ΔrssB *vs.* ΔrpoS, ***P*. For a dose of 5 μg ml^{-1} of MMC, the following differences were significant: RS *vs.* ΔrpoS, * *P*; ΔrssB *vs.* ΔrpoS, * *P*. * *P* < 0.05, ** *P* < 0.01, and *** *P* < 0.001, two-tailed Student's *t*-test. (B) The effect of the RpoS regulon on MMC-induced mutagenesis. The frequency of Rif^R mutants arising in MMC-treated cells was measured. Each result represents the mean ± SEM of at least 18 independent cultures in at least three independent experiments. ***P* < 0.01, ****P* < 0.001, nonparametric Mann-Whitney *U* test. MMC, treated with 1 μg ml^{-1} mitomycin C; NT, nontreated; Rif^R, rifampicin-resistant; RS, reference strain.

First, we measured survival of exponentially growing cells to lethal doses of MMC for 2 hr. The survival of the ΔrpoS mutant was > 100-fold lower than that of the RS or the ΔrssB mutant (Figure 4A). Thus, induction of the RpoS regulon was adaptive because it conferred a greater capacity to deal with the cytotoxic effects of MMC.

Second, we measured mutagenesis induced by the sub-MIC of MMC, *i.e.*, 1 μg ml^{-1}. To assess the mutation frequencies, we used the fluctuation test (Luria and Delbruck 1943) based on calculations of the frequency of appearance of Rif^R mutants (Jin and Gross 1988) in MMC-treated cultures and in nontreated controls. The nontreated ΔrpoS mutant had a significantly lower mutation frequency compared with that in the nontreated RS (1.6-fold, Mann–Whitney *U* test, *P* = 0.0003) or the nontreated ΔrssB mutant (2.4-fold, Mann–Whitney *U* test, *P* < 0.0001) (Figure 4B, three top bars). There was no significant difference in spontaneous mutagenesis between the RS and the ΔrssB mutant (1.4-fold, Mann–Whitney *U* test, *P* = 0.0900). When cells were treated with MMC, as expected, mutagenesis increased for all tested strains (Figure 4B). The MMC-treated ΔrssB strain had significantly higher mutagenesis than the MMC-treated RS (2.1-fold, Mann–Whitney *U* test, *P* = 0.0004). The MMC-treated ΔrpoS strain displayed a significantly lower mutation frequency compared with that of the treated RS (2.0-fold, Mann–Whitney *U* test, *P* = 0.0076) and with the ΔrssB strain (4.1-fold, Mann–Whitney *U* test, *P* < 0.0001).

The results presented above indicated that one or more genes regulated by RpoS are crucial for processing the DNA damage caused by MMC in growing cells.

Pol II is under double regulation by SOS and RpoS stress responses

We showed that BarA is involved in RpoS regulon induction by MMC (Figure 2). Among the genes predicted to be regulated by BarA (Sahu *et al.* 2003), only *polB*, which encodes SOS-regulated DNA polymerase II, is also regulated by SOS (Bryan

et al. 1988; Chen *et al.* 1990; Iwasaki *et al.* 1990). Pol II polymerase has been reported to be involved, together with NER, in the repair of HN2-induced DNA cross-links and oxidative damage (Escarceller *et al.* 1994; Berardini *et al.* 1999). Thus, we first investigated whether *polB* gene induction by MMC was regulated by RpoS. To do so, we monitored the expression of the reporter fusion of the *polB* promoter with a gene encoding a green fluorescent protein (Zaslaver *et al.* 2006) in different genetic backgrounds. As expected, *polB* gene expression was induced by MMC in the RS (Figure 5A, RS). However, in cells treated with MMC, *polB* expression was significantly lower in the ΔrpoS mutant compared with the level in the RS and with that in the ΔrssB mutant (Figure 5A).

E. coli possesses two other SOS-controlled TLS DNA polymerases, Pol V and Pol IV, which are encoded by the *dinB* and *umuDC* genes, respectively (Friedberg *et al.* 2005). We evaluated whether RpoS plays a role in the induction of *dinB* and *umuD* genes by MMC. We found that inactivation of the *rpoS* gene slightly increased *dinB* gene induction by MMC relative to that in the RS, whereas expression of the *umuD* gene was only marginally affected by *rpoS* gene inactivation (Figure 5, B and C, respectively).

Next, we explored the involvement of RpoS in the regulation of *polB* expression. We monitored expression of this gene in the *lexA*(def) background, in which the SOS response is constitutively derepressed. In this background, all genes involved in the SOS response, including *polB*, are fully expressed. However, inactivation of the *rpoS* gene in the *lexA*(def) background significantly decreased the expression of the *polB* gene (Figure 5D), this indicating that RpoS is necessary for full expression of *polB* even when the SOS response is derepressed in the absence of DNA damage. Therefore, we showed that RpoS positively regulates the expression of *polB*, but not of the other two SOS-controlled polymerases (*dinB* and *umuD*), in actively growing bacteria.
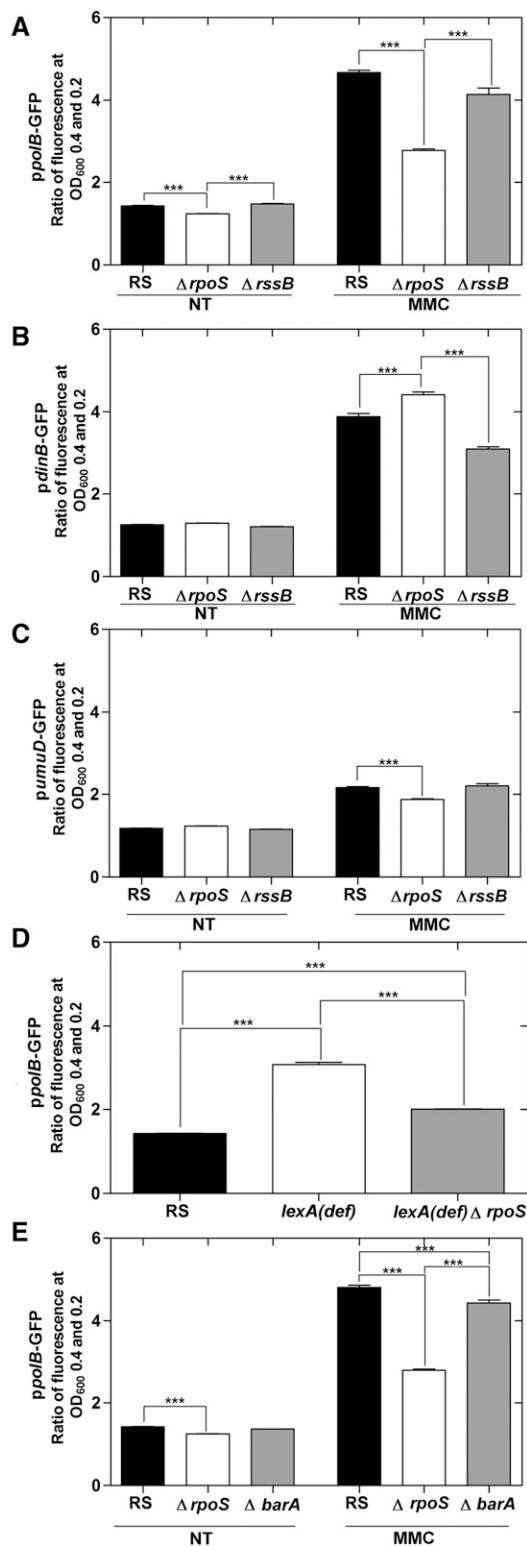
Figure 5 Expression of the *polB* gene encoding SOS regulated Pol II is under RpoS regulation. *polB*, but not genes encoding the other two SOS-dependent polymerases, *dinB* and *umuD*, are under RpoS regulation in the exponentially growing phase during MMC treatment. An increase in green fluorescence levels during growth of the RS, Δ*rpoS*, and Δ*rssB* strains carrying reporter fusions. The bars represent the ratio of the fluorescence at OD_{600} 0.2 and 0.4. (A) Expression of the *polB* promoter fusion to fluorescent protein. (B) Expression of the *dinB* promoter fusion to fluorescent protein. (C) Expression of the *umuD* promoter fusion to fluorescent protein. (D) Expression of the *polB* promoter fusion to fluorescent protein in a constitutively induced SOS background, *lexA*(def). (E) Expression of the *polB* promoter fusion to fluorescent protein in Δ*barA* and Δ*rpoS* mutants. For all experiments, each point represents the mean ± SEM of at least three independent experiments. *** $P < 0.001$, two-tailed Student's *t*-test. MMC, treated with 1 μg ml^{-1} mitomycin C; NT, nontreated; RS, reference strain.

Because we showed that BarA is involved in RpoS regulon induction by MMC (Figure 2) and that RpoS is involved in the expression of the *polB* gene (Figure 5, A and D), we tested whether BarA regulates *polB* expression during genotoxic stress. We found that *polB* gene expression was lower in the Δ*barA* mutant than in the RS (Figure 5E). However, *polB* expression in the Δ*barA* mutant was still higher than that in the Δ*rpoS* mutant (Figure 5E). These data indicated that BarA is only partially responsible for the induction of *polB* in response to MMC.

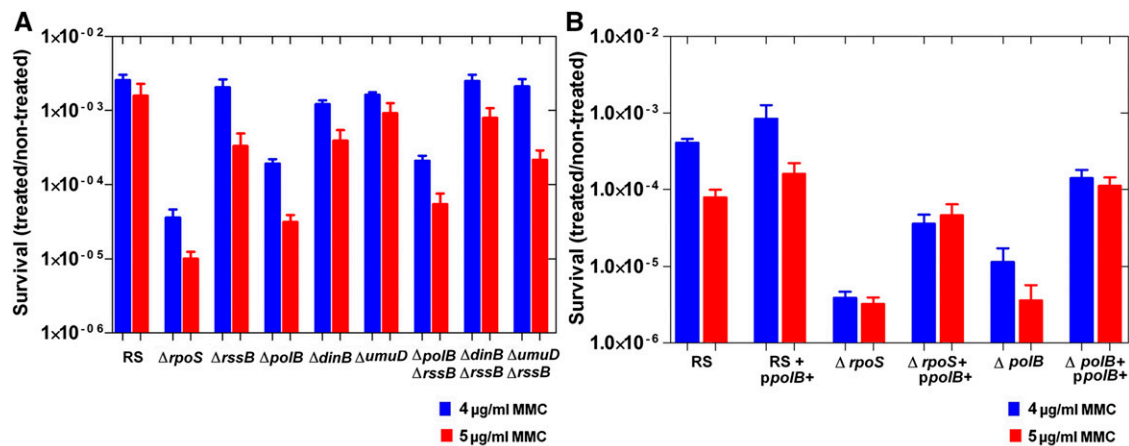### RpoS-mediated induction of polB is required for the adaptive response to MMC treatment

To determine whether Pol II plays a role in the RpoS-regulated adaptive response during MMC-induced genotoxic stress, we measured the survival of the Δ*polB* mutant after MMC treatment in different genetic backgrounds. We also included mutants of the other two SOS-regulated alternative DNA polymerases, Δ*dinB* and Δ*umuD*. We found that the Δ*polB* mutant showed higher sensitivity to MMC than did the RS, and a sensitivity similar to that of the Δ*rpoS* mutant (Figure 6A). Inactivation of *dinB* and *umuD* genes had a much milder impact on survival than *polB* inactivation. The finding that inactivation of the *polB* gene in the Δ*rssB* genetic background decreased the survival of MMC similarly to the increased sensitivity of the Δ*polB* mutant relative to the RS suggested that no other RpoS-regulated functions significantly contributed to the resistance to MMC treatment. This hypothesis was supported by the observation that the introduction of a plasmid carrying a functional *polB* gene under the control of its native promoter (p*polB*+) restored the survival of the MMC-treated Δ*rpoS* mutant nearly to the level of the RS (Figure 6B). The small difference in survival between the MMC-treated Δ*rpoS* p*polB*$^+$ strain and the RS was probably due to the decreased expression of the *polB* gene in the Δ*rpoS* background, as shown in Figure 5A.

Discussion

Genotoxic agents, such as the MMC used in this study, cause a decrease in growth rate and increased mortality as a result of DNA damage and DNA replication blockage. To eliminate DNA damage and restart DNA replication, all organisms have evolved numerous DNA repair pathways. Constitutively expressed DNA repair mechanisms allow organisms to cope with low levels of DNA damage, whereas a large amount of DNA damage requires induction of complex DNA damage response regulons, such as the SOS response regulon in the

Figure 6 Pol II is responsible for the RpoS-adaptive response during the genotoxic stress caused by MMC. Survival of different MMC-treated strains. (A) For a dose of 4 μg ml^{-1} of MMC, the following differences were significant: RS *vs.* $\Delta polB$, ** P; RS *vs.* $\Delta rssB$ $\Delta polB$, *** P; $\Delta rssB$ *vs.* $\Delta polB$, * P; $\Delta rssB$ *vs.* $\Delta rssB$ $\Delta polB$, ** P. For a dose of 5 μg ml^{-1} of MMC, the following differences were significant: RS *vs.* $\Delta polB$, * P; RS *vs.* $\Delta rssB$ $\Delta polB$, * P. (B) Susceptibility of the Pol II-complemented strains to MMC. For a dose of 4 μg ml^{-1} of MMC, the following differences were significant: $\Delta rpoS$ *vs.* $\Delta rpoS$+p$polB$+, * P; $\Delta polB$ *vs.* $\Delta polB$+p$polB$+, * P. For a dose of 5 μg ml^{-1} of MMC, the following differences were significant: $\Delta rpoS$ *vs.* $\Delta rpoS$+p$polB$+, * P. Relevant genotypes are indicated. Each value represents the mean $\pm$ SEM of at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, and ***$P < 0.001$, two-tailed Student's *t*-test. MMC, treated with 1 μg ml^{-1} mitomycin C; NT, nontreated; RS, reference strain.

bacterium *E. coli*. Interstrand cross-links, which are one type of DNA damage caused by MMC, are in *E. coli* repaired by proteins involved in the HR, NER, and TLS pathways (Van Houten *et al.* 1986; Sladek *et al.* 1989; Slater and Maurer 1993; Berardini *et al.* 1999), all of which are upregulated as part of the SOS regulon (Radman 1975; Little and Mount 1982; Walker *et al.* 1982; Friedberg *et al.* 2005). Indeed, we observed that the impossibility of inducing the SOS response in the *lexA*(Ind$^-$) genetic background resulted in a very high sensitivity of the cells to MMC (data not shown), as previously reported (Shanmughapriya *et al.* 2014).

In addition to the SOS response, MMC treatment also induces other stress response pathways, such the *E. coli* RpoS-mediated general stress response [Khil and Camerini-Otero (2002) and Figure 1]. RpoS-regulated genes are involved in morphological and metabolic modifications, as well as in the protection of cellular macromolecules, *e.g.*, DNA, that endow cells with a multi-stress-resistant phenotype (Bouveret and Battesti 2011). Although the RpoS regulon controls up to one-tenth of *E. coli* genes, only two genes are known to be directly involved in DNA repair, *i.e.*, *ada* and *xthA*. Thus, the question addressed herein was whether RpoS regulon induction is a by-product of MMC-induced cellular perturbations without affecting the repair of DNA lesions, or in contrast, whether a specific mechanism is involved in protection against genotoxic stress. We found that induction of the RpoS regulon by MMC treatment is an important part of the cellular adaptive response (Figure 4). We identified the SOS-regulated gene *polB*, which encodes Pol II DNA polymerase, to also be regulated by RpoS. Moreover, we showed that Pol II is the most important RpoS-regulated contributor to the repair of MMC-induced cytotoxic lesions in growing cells (Figure 5 and Figure 6).

The finding that the RpoS regulon controls the expression of *polB* in growing cells further emphasizes the importance of this regulon during growth. Although the RpoS regulon is generally associated with the morphological and metabolic modifications of stationary phase cells, there is growing evidence of its induction and importance in growing cells. For example, it has been shown that subinhibitory concentrations of different antibiotics induce the RpoS regulon (Gutierrez *et al.* 2013) and that this induction protects cells against subsequent lethal stresses (Mathieu *et al.* 2016).

E. coli possesses five DNA polymerases, three of which are regulated by the SOS system: alternative DNA polymerases Pol II, Pol IV, and Pol V, encoded by the *polB*, *dinB*, and *umuDC* genes, respectively (Friedberg *et al.* 2006). When a DNA lesion blocks the replicative DNA polymerase Pol III, these alternative DNA polymerases instead copy the DNA template across the lesion. After the DNA lesion is bypassed, Pol III can resume replication of the chromosome. Each alternative DNA polymerase has a specific function and participates in the processing of a particular type of DNA damage (Knippers 1970; Kornberg and Gefter 1971; Wang and Smith 1985; Rangarajan *et al.* 1999; Wagner *et al.* 1999; Ohmori *et al.* 2001). Specifically, one of the functions of the alternative DNA polymerases, Pol II, includes participation, together with NER, in the processing of HN2-induced cross-links (Berardini *et al.* 1999). This same phenomenon may have occurred during the processing of MMC-induced cross-links (Figure 6). However, it is likely that Pol II also contributes to the processing of MMC-induced oxidative DNA damage, because the $\Delta polB$ mutant has been shown to be sensitive to oxidative stress (Escarceller *et al.* 1994).

Alternative DNA polymerases also contribute to chromosomal replication, even when Pol III is blocked in the absence of DNA lesions. For example, utilization of the *dnaE*^{ts} allele indicates that alternative DNA polymerases continue to carry out chromosomal replication when Pol III is not functional at the nonpermissive temperature (Delmas and Matic 2006).

Interestingly, it has been shown that when this alternative usage occurs, Pol II is the first alternative polymerase that has access to the blocked DNA replication forks. This phenomenon may explain why Pol II is required for the rapid replication restart after UV irradiation, although *polB*-deficient cells are not sensitive to UV radiation (Rangarajan *et al.* 1999). Thus, when UV radiation-induced replication-blocking lesions are bypassed by Pol V, Pol II gains access to replication forks and restarts chromosomal replication.

Pol II also has an important role in evolutionary fitness, as supported by the observation that the *polB* gene is more evolutionarily constrained than the *dinB* and *umuC* genes (Delmas and Matic 2006). It has also been observed that Pol II, unlike the other two alternative polymerases, confers a fitness advantage during rapid growth in the absence of SOS induction by external DNA-damaging agents (Corzett *et al.* 2013). Furthermore, it is important to underscore that, unlike the other two alternative polymerases, Pol II possesses $3' \rightarrow 5'$ exonuclease activity (Gefter *et al.* 1972; Wickner *et al.* 1972; Ishino *et al.* 1992). Consequently, Pol II has greater fidelity than Pol IV and Pol V.

In conclusion, in the present study, we showed that two different transcriptional regulators, the LexA repressor and the RpoS alternative σ factor, coregulate the expression of *polB*. The LexA and RpoS regulons, *i.e.*, the SOS and general stress responses, have generally been studied separately. Thus, prior to our study, little information was available concerning the coregulated genes. For example, it has been shown that in adaptive mutagenesis, both Pol II and Pol IV are regulated by both the SOS response and the RpoS regulon (Frisch *et al.* 2010; Storvik and Foster 2010; Shee *et al.* 2011). Because the regulatory strategies are selected for during evolution, the finding that the *polB* gene is connected to the two stress response regulons that are required to maximize survival and fitness further emphasizes its role as a key factor in the maintenance of genome stability.

Acknowledgments

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