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 NaBH₄ or by NADPH, in the presence of O₂, results in the generation of H₂O₂ (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*. Transcriptional 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 (σS) (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 O₆-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 (Dempel 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 ΔsulA strain as a reference strain (RS). Owing to the lack of filamentation, the ΔsulA 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 ΔlexA [lexA(def)] was constructed using the λ Red recombinase system (Datsenko and Wanner 2000; Murphy et al. 2000; Yu et al. 2000) in a ΔsulA background. The sequences of the primers used for the lexA(def) mutant were as follows: OTD5, 5′-ATGAAAGCGTTAAGCCGACCAAGAGGTGTTTGTG TGGCTGGACTGCTTC-3′; OTD6, 5′-TTACAGCCAGTCGCC GTTGCGAATACCCCAACGCGCATATGAATTCCTTTAG-3'. The lexA(def) construct was subsequently transferred to the RS through P1vir transduction. When needed, the Kan⁶ 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 (ppolB+) 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

| Strain          | Relevant Genotype                  | Reference or Source                                      |
|-----------------|-----------------------------------|----------------------------------------------------------|
| MG1655          | Wild-type                         | Laboratory stock                                         |
| RpoSmCherry     | MG1655 rpoS-mCherry::FRT          | Maisonneuve et al. (2013)                                 |
| TD6             | MG1655 ΔsuA::KanR                 | MG1655 × P1 (KEIO ΔsuA::KanR)                            |
| TD7, reference strain | TD6 ΔsuA::FRT                      |                                                          |
| TD598           | TD7 ΔcysC::KanR                   | TD7 × P1 (KEIO ΔcysC::KanR)                               |
| TD603           | TD598 rpoS-mCherry::FRT           | TD598 × P1 (RpoSmCherry)                                 |
| TD318           | TD7 ΔrpoS::KanR                   | TD7 × P1 (KEIO ΔrpoS::KanR)                               |
| TD612           | TD318 ΔrpoS::FRT                  |                                                          |
| TD322           | TD7 ΔrpsB::KanR                   | TD7 × P1 (KEIO ΔrpsB::KanR)                               |
| TD632           | TD7 ΔrpsB::FRT                    |                                                          |
| TD677           | TD7 ΔkatE::KanR                   | TD7 × P1 (KEIO ΔkatE::KanR)                               |
| TD678           | TD7 ΔkatE::TetR                   | TD7 × P1 (NEC445 ΔkatE::TetR)                            |
| NEC445          | ΔkatG::CmR, ΔkatE::TetR, ΔahpCF::KanR | Hébrard et al. (2009)                                      |
| TD624           | TD7 ΔrpoA::KanR                   | TD7 × P1 (KEIO ΔrpoA::KanR)                               |
| TD632           | TD603 ΔrpoA::KanR                 | TD603 × P1 (KEIO ΔrpoA::KanR)                             |
| TD417           | TD7 ΔuvrY::KanR                   | TD7 × P1 (KEIO ΔuvrY::KanR)                               |
| TD669           | TD603 ΔuvrY::KanR                 | TD603 × P1 (KEIO ΔuvrY::KanR)                             |
| TD653           | TD7 Δbara::KanR                   | TD7 × P1 (KEIO Δbara::KanR)                               |
| TD654           | TD653 Δbara::FRT                  |                                                          |
| TD668           | TD603 Δbara::KanR                 | TD603 × P1 (KEIO Δbara::KanR)                             |
| TD15            | TD7 ΔlexA::KanR                   | TD7 × FRTapFRT from pKD4                                   |
|                 |                                   | Datsenko and Wanner (2000)                                |
| TD16            | TD15 ΔlexA::FRT                   |                                                          |
| TD319           | TD16 ΔrpoS::KanR                  | TD16 × P1 (KEIO ΔrpoS::KanR)                              |
| TD613           | TD319 ΔrpoS::FRT                  |                                                          |
| TD292           | TD7 ΔpolB::KanR                   | TD7 × P1 (KEIO ΔpolB::KanR)                               |
| TD640           | TD292 ΔpolB::FRT                  |                                                          |
| TD619           | TD615 ΔpolB::KanR                 | TD615 × P1 (KEIO ΔpolB::KanR)                             |
| TD252           | TD7 ΔdinB::KanR                   | TD7 × P1 (KEIO ΔdinB::KanR)                               |
| TD617           | TD615 ΔdinB::KanR                 | TD615 × P1 (KEIO ΔdinB::KanR)                             |
| TD272           | TD7 ΔumuD::KanR                   | TD7 × P1 (KEIO ΔumuD::KanR)                               |
| TD618           | TD615 ΔumuD::KanR                 | TD615 × P1 (KEIO ΔumuD::KanR)                             |
| Plasmid         |                                   |                                                          |
| ppolB+ KanR     |                                   |                                                          |
| pbolA-gfp KanR  |                                   |                                                          |
| ppolB-gfp KanR  |                                   |                                                          |
| pdinB-gfp KanR  |                                   |                                                          |
| pumuD-gfp KanR  |                                   |                                                          |

KanR, kanamycin resistance; TetR, tetracycline resistance.

* This strain does not carry the ΔcysC::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°C 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°C). Next, 4.0 × 10⁴ 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°C 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 (OD₆₀₀) 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/OD600.

**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°C 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°C with shaking at 150 rpm until they reached stationary phase (OD600 ~5; ~18 hr). Afterward, the OD600 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°C 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 OD600 of the culture of interest (HPII activity/OD600) (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⁺) (Jin and Gross 1988), bacterial cultures were inoculated from at least six single colonies in LB broth and incubated overnight at 37°C 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°C 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°C, and the Rif⁺ 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°C 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°C with shaking at 150 rpm until the OD600 was ~0.2. Next, 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 MgSO₄ 10⁻² M and plated onto LB agar plates. When the plasmid ppoB⁺ was used, cultures were grown in LB broth + Kan and plated onto LB agar + Kan plates. The plates were incubated at 37°C for 12 hr; then, the colonies were counted, and survival was calculated (CFU treated/CFU nontreated).
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 $1.5 \mu g \cdot ml^{-1}$. For these experiments, we used a sub-MIC of MMC $1 \mu g \cdot ml^{-1}$, 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 non-treated cells (Figure 1A).

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

As expected, the ΔkatG mutant exhibited the same level of catalase activity as the RS, whereas no catalase activity was observed for the ΔrpoS, ΔkatE, and ΔkatGΔkatE strains (Figure 1C).

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 ΔiraD strains, and (B) the ΔbarA and ΔuvrY strains. Catalase activity of the RpoS-regulated monofunctional catalase HPII encoded by katE in MMC-treated and NT (C) RS and ΔiraD strains, and (D) in the ΔbarA and ΔuvrY strains. All measured catalase activities were normalized to that of the NT RS. For all results, each point represents the mean ± SEM of at least three independent experiments. MMC, treated with $1 \mu g \cdot ml^{-1}$ MMC. *P < 0.05, two-tailed Student’s t-test.
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-mCherry protein fusion in the absence or presence of MMC in ΔuvrY or ΔbarA 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, ΔbarA, and ΔuvrY 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 ΔbarA mutant (40%, Student’s *t*-tests, *P* = 0.0256), but not in the ΔuvrY 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 ΔkatG ΔkatE ΔahpCF 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).

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 ΔrpoS mutant, and a ΔrssB mutant, which had a constitutively induced RpoS regulon, because the RssB protein promotes RpoS degradation (Hengge-Aronis 2002).
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 RifR 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.
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 ΔbarA mutant than in the RS (Figure 5E). However, polB expression in the ΔbarA mutant was still higher than that in the ΔrpoS 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 ΔpolB mutant after MMC treatment in different genetic backgrounds. We also included mutants of the other two SOS-regulated alternative DNA polymerases, ΔdinB and ΔumuD. We found that the ΔpolB mutant showed higher sensitivity to MMC than did the RS, and a sensitivity similar to that of the ΔrpoS 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 ΔrpoS genetic background decreased the survival of MMC similarly to the increased sensitivity of the ΔpolB 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 ΔrpoS mutant nearly to the level of the RS (Figure 6B). The small difference in survival between the MMC-treated ΔrpoS p-polB+ strain and the RS was probably due to the decreased expression of the polB gene in the ΔrpoS 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

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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, ΔrpoS, and ΔrpoS ΔrSSB strains carrying reporter fusions. The bars represent the ratio of the fluorescence at OD600 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 ΔbarA and ΔrpoS 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.
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 (Shannmugapriya et al. 2014).

In addition to the SOS response, MMC treatment also induces other stress response pathways, such as the E. coli RpoS-mediated general stress response [Khil and Camerin-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 Gelder 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 ΔpolB 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<sup>ts</sup> allele indicates that alternative DNA polymerases continue to carry out chromosomal replication when Pol III is not functional at the non-permissive 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′→5′ exonuclease activity (Gefer 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 (Frish 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

We thank Roberto Balbontín (Instituto Gulbenkian de Ciência, Portugal) and Xavier Giroux (Institut de Biologie Intégrative de la Cellule, Université Paris-Saclay, France) for critical reading of the manuscript. This work was supported by grants from the International Development Exchange (IDEX) ANR-11-IDEX-0005-01/ANR-11-LABX-0071, IDEX-Sorbonne Paris Cité, AXA Research Fund, Mériteux Research, and DIM Malin (Domaine d’Intérêt Majeur Maladies Infectieuses)-Ile de France.

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Communicating editor: S. J. Sandler