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Reactive oxygen species are major contributors to SOS-mediated mutagenesis induced by fluoroquinolones

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Certain antibiotics, particularly fluoroquinolones, induce the mutagenic SOS response and increase the levels of intracellular reactive oxygen species (ROS), which have been correlated with antibiotic lethality. Both SOS and ROS increase mutagenesis in treated bacteria, likely resulting in the appearance of resistant mutants during antibiotic treatments. However, the relative contribution of ROS and SOS on this antibiotic-mediated mutagenesis is currently unknown. We used the antioxidant molecule N-acetylcysteine (NAC) to study the contribution of ROS on the SOS response and mutagenesis mediated by the fluoroquinolone antibiotic ciprofloxacin (CIP). We show that NAC is able to reduce intracellular ROS levels, mitigating as well the SOS response caused by treatment with subinhibitory concentrations of CIP. This effect leads to the reduction of antibiotic-induced mutagenesis to levels comparable to a translesion DNA-polymerases (TLS) deficient strain, suggesting that ROS play a major role in SOS-induced mutagenesis. Collectively, our results shed light on the mechanisms underlying antibiotic-induced mutagenesis and pave the way for the use of NAC as a safe adjuvant in antibiotic therapy to inhibit antibiotic-induced mutagenesis, hence augmenting our capacity to fight against threatening bacterial infections.

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Introduction

In recent years, there has been accumulating evidence that antibiotics, besides their antimicrobial action, can promote genetic variability in bacteria as an undesirable side effect (Blázquez, Rodríguez-Beltrán, and Matic 2018). In turn, genetic variability increases the chances of bacteria of acquiring resistance and jeopardize the success of antimicrobial therapies. Understanding the bacterial physiological responses that promote genetic variability is thus crucial to hinder resistance spread (Cirz and Romesberg 2007; Cirz et al. 2005).

In some cases, the physiological response of the cells is directly related with the mechanism of action of the antibiotic. This is the case of the SOS response, which is a coordinated genetic response to DNA damage. Several antibiotics produce DNA damage. Quinolones such as ciprofloxacin (CIP) block DNA gyrase on DNA, causing the stalling of replication forks and leading to cell death (K. Drlica and Zhao 1997). This process produces double strand breaks (DSB) that are processed into single strand DNA, which forms a complex with RecA protein to trigger the SOS response. SOS induction up-regulates the expression of more than 40 genes, including its own co-regulator *recA* (Fernández De Henestrosa et al. 2000). The SOS genes encode proteins whose functions include mechanisms for cell-cycle checkpoint, DNA-damage tolerance and non-mutagenic DNA repair (Janion 2008; Courcelle, Crowley, and Hanawalt 1999; Friedberg et al. 2006). However, when DNA damage is persistent, the error-prone DNA translesion synthesis (TLS) takes place. TLS is a process by which heavily damaged DNA can be replicated at the cost of a reduced fidelity. In *Escherichia coli*, TLS is accomplished by the specialized DNA polymerases Pol II, Pol IV and Pol V, encoded respectively by the *polB*, *dinB* and *umuDC* genes (Vaisman et al. 2012). Notably, RecA-mediated recombination is also induced by fluoroquinolone antibiotics (López et al. 2007). Hence, some antibiotics can promote mutagenesis and recombination (i.e. genetic instability) by directly inducing DNA damage and, in turn, the SOS response.

However, the responses of bacterial physiology to antibiotics are not always directly related with the mechanism of action. For instance, several bactericidal antibiotics have been shown to produce a perturbation of the intracellular redox homeostasis. This perturbation is caused by an increased intracellular respiration rate accompanied by destabilization of the Iron-Sulfur clusters,

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which leads to production of reactive oxygen species (ROS) via Fenton chemistry (Dwyer, Collins, and Walker 2014; Dwyer et al. 2007; Kohanski et al. 2007). ROS are highly reactive chemical species capable of rapidly oxidizing key cellular components, including proteins, lipids and DNA. ROS have been argued as a common cause of bacterial cell death for several antibiotic families (Kohanski et al. 2007; Foti et al. 2012; Dwyer et al. 2007), although this notion has been further challenged (Liu and Imlay 2013; Keren et al. 2013). Oxidation of DNA by ROS produces a wide variety of lesions that, if not repaired, are mutagenic and can even cause cell death (Keyer and Imlay 1996; Linn and Imlay 1987; Imlay 2008).

In summary, previous studies have shown that there are at least two routes to antibiotic-triggered bacterial mutagenesis; SOS-mediated TLS and ROS-induced mutagenesis. Interestingly, these two routes are probably not independent but highly intertwined. For instance, oxidation of the nucleotide pool after antibiotic treatment leads to Pol IV mediated incorporation of 8-oxo-dGTP into DNA creating a mutagenic lesion (Kohanski, DePristo, and Collins 2010; Foti et al. 2012). Furthermore, ROS are good SOS inducers because they directly damage DNA (Goerlich, Quillardet, and Hofnung 1989; Konola, Sargent, and Gow 2000; Carlsson and Carpenter 1980). Hence, SOS-mediated TLS mutagenesis might be fueled by the presence of oxidative damage in both DNA and the nucleotide pool (McBride, Preston, and Loeb 1991; Kottur and Nair 2016)

N-acetylcysteine (NAC) is a clinically safe antioxidant drug, which is currently used to treat numerous disorders (Samuni et al. 2013). The well-known antioxidant activity of NAC has been attributed to its ability to act as a scavenger of oxidant species and as a precursor of glutathione synthesis (Samuni et al. 2013; Elbini Dhouib et al. 2016). Here, we hypothesized that NAC could reduce the ROS produced by CIP treatment and consequently, both SOS induction and subsequent mutagenesis.

Results

CIP induces mutagenesis at subinhibitory levels

The mutagenic activity of antimicrobials is expected to occur within a window of concentrations around their minimal inhibitory concentration (MIC), because higher levels would kill cells or stop their growth and lower concentrations would not have a stimulatory effect (Blázquez et

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al. 2012). To determine the concentration of CIP that induces the highest increase in mutagenesis, we treated exponentially growing cultures of *E. coli* strain IBDS1 (Bjedov et al. 2007) with different concentrations of CIP ranging from 0.25 to 4 times the MIC for 8 hours, and determined mutation rates using two independent selective markers. Cells treated with 8 ng/ml of CIP (which correspond to ½ of the MIC) showed the highest increase on the rate of mutations conferring rifampicin (Rif-R; 24-fold increase) and tetracycline resistance (Tet-R; 2.5-fold increase) **(Supplementary figure S1)**. Higher concentrations of CIP barely produced any increase in mutagenesis, probably because these concentrations hampered growth of most treated cells (**Supplementary figure S2)** drastically reducing effective population size and hence limiting evolvability (Frenoy and Bonhoeffer 2018). We decided to use 8 ng/ml of CIP hereafter to maximize antibiotic-induced mutagenesis.

NAC reduces CIP-mediated induction of the SOS response

We then analyzed how this CIP concentration induces the SOS response. To this end, we used the strain IBDS1 pRecA::*gfp* that harbours a transcriptional fusion P*recA*::*gfp* contained in a low copy number plasmid (Ronen et al. 2002; Thi et al. 2011). As expected, CIP strongly induced the SOS response approximately 14-fold compared to untreated controls (Tukey multiple comparisons after significant ANOVA, P>0.001; **Fig 1a**). Following the hypothesis that antioxidant compounds can be effective inhibitors of SOS induction (Peng et al. 2011), we tested the effect of different NAC concentrations on CIP-mediated SOS induction (**Fig 1a**). NAC inhibited SOS induction caused by CIP at all concentrations, with an IC50 of 0.5% (Tukey multiple comparisons after significant ANOVA, P>0.001; **Fig 1c**). This concentration is in the range of attainable physiological values after inhaled administration (Szkudlarek et al. 2004), strongly suggesting that inhibition of the SOS response by NAC could be a feasible therapeutic approach. Importantly, NAC did not reduce CIP bactericidal activity, as stated by MIC results **(Supplementary table 1),** growth curves **(Fig1b)** and a checkerboard assay (**Fig1d)**. On the contrary, NAC alone inhibited bacterial growth without inducing the SOS response (Tukey multiple comparisons after significant ANOVA, P=0.0003). This result is in line with previous studies that reported NAC antibacterial properties against a range of bacterial pathogens (Jang, Bak, and Cha 2017; Landini et al. 2016; Zhao and

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Liu 2010). Additionally, we verified that the differences in the final optical density observed upon different treatments do not directly bias SOS induction measured as GFP fluorescence **(Supplementary figure S3).**

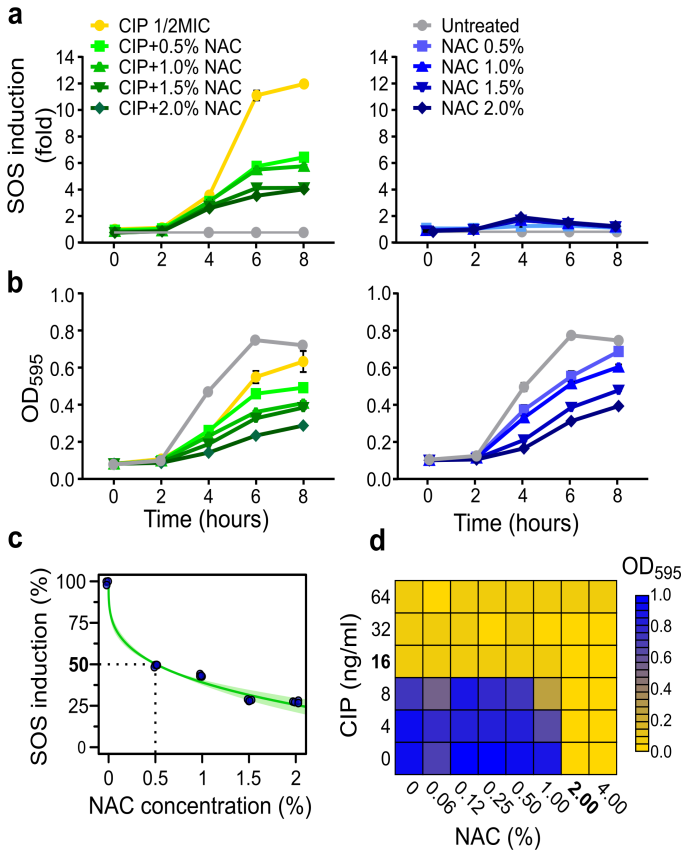

Figure 1. NAC reduces ciprofloxacin-induced SOS response. *Bacterial growth and SOS induction were monitored during treatment with varying concentrations of CIP alone or in combination with NAC (a), or NAC alone (b). Samples were taken at indicated time points and SOS induction (upper panels) and absorbance (lower panels) were quantified. Error bars represent standard deviation and are not shown when smaller than data points. (c) Potential interactions of CIP with NAC were determined by the checkerboard method, following EUCAST guidelines. MIC concentrations for each compound alone are shown in bold typeface. No synergistic or antagonistic effect was found. (d) CIP-mediated SOS induction at 8h was assessed in combinations with a range of NAC concentrations giving rise to a dose-response curve. Experimental data was fitted to the following formula* $SOS=a*NAC^b$ *by a non-linear model (nls function in R,* $R^2=0.988$*). The concentration of NAC that inhibits 50% of SOS response (*IC_{50}*) was found to be 0.5%. Green shaded area represent 95% confidence interval of the fit.*

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SOS induction leads to the overexpression of *sulA* (*sfiA*), whose product inhibits FtsZ ring formation and hence cell division (Huisman, D'Ari, and Gottesman 1984). The phenotypic consequence of cell division inhibition is filamentation, offering an additional SOS-dependent measurable phenotype. We assessed whether 0.5% NAC was able to inhibit CIP-mediated cell filamentation by both flow cytometry and direct observation of Gram-stained cultures. **Figure 2** shows that, as expected, CIP treatment produces a vast increase in the fraction of the population with filamented cells compared with untreated cultures (33% versus 0.3% of filamented cells). Crucially, administration of 0.5% NAC together with CIP, prevented filamentation in a large fraction of cells (14% versus 33%, for CIP+NAC versus CIP alone). We qualitatively confirmed these results by microscopy observation of Gram-stained cells **(Fig 2b)**.

in summary, our results demonstrated that NAC is able to significantly reduce a 50% CIP-mediated induction of *recA* transcription and the production of cell filamentation, both hallmarks of SOS induction, without decreasing bacterial susceptibility to CIP.

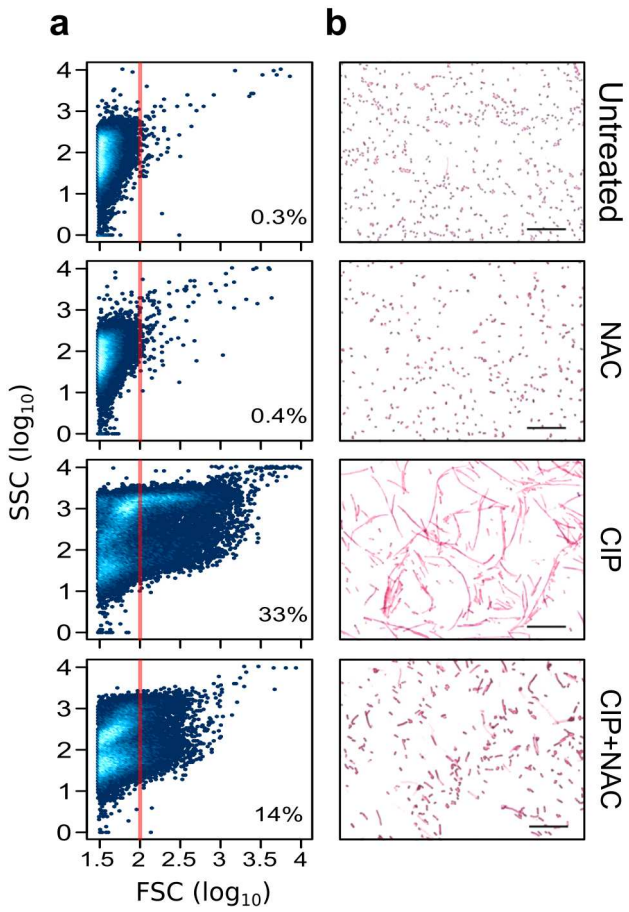

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Figure 2. NAC reduces CIP-induced filamentation*. a) The fraction of filamented cells after the stated treatments is shown by means of flow cytometric analysis of 30,000 cells (SSC; side scatter FSC; forward scatter). The percentage on every graph represents the filamented fraction of the population (log10(FSC)>2; red vertical line). b) Representative microscopy fields of Gram-stained cells. Scale bars is 20µm.*

NAC reduces ciprofloxacin-induced intracellular ROS

To ascertain whether NAC inhibition of SOS induction was mediated by its antioxidant activity we used 2′,7′-Dichlorofluorescein diacetate (H_2DCFDA), a ROS sensitive dye that emits fluorescence when it is oxidized intracellularly (Dwyer et al. 2014). H_2DCFDA has been previously shown to be an extremely sensitive probe for the detection of ROS caused by fluoroquinolones, detecting with great sensitivity H_2O_2, ROO· and $ONOO^-$ (Dwyer et al. 2014). **Figure 3** shows the fluorescence of H_2DCFDA in 30,000 cells using a flow cytometer with either 8 ng/ml CIP, 0.5% NAC and both agents in combination. As expected, CIP produced a massive increase of ROS levels compared to untreated cells. Crucially, the induction of ROS was reverted to nearly basal levels when NAC was added. This result suggests that NAC, by reducing the levels of ROS upon CIP exposure, concomitantly decreases DNA damage, leading to a lower SOS induction.

Finally, because it has been described previously that quinolone treatment produce autofluorescence in *E. coli* cells (Renggli et al. 2013), we assessed whether it can influence our ROS measurements. Our results with CIP confirm such autofluorescence in all the strains used in this work. However, cells treated with CIP consistently showed increased fluorescence levels over those produced by the antibiotic-mediated autofluorescence validating our experimental approach **(Supplementary figure S4**).

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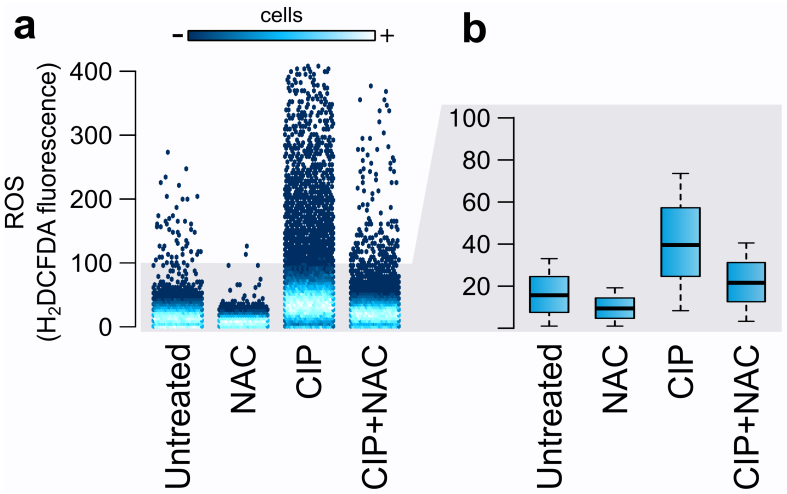

Figure 3. NAC reduces ciprofloxacin-induced intracellular ROS. *ROS were assessed by individually capturing the fluorescence of H_2DCFDA in 30,000 cells using a flow cytometer after 8 hours of treatment with either 8 ng/ml of CIP, 0.5% NAC or both agents in combination. An untreated control is shown as a reference. a) Dot plot showing the distribution of fluorescence signal in the treated populations. At least 99% of the events recorded are shown. The color scale allows visualization of the density of events at every fluorescence level. b) To allow better comparison, the data is depicted as boxplots, in which the horizontal line represents the median value, the depth of the box represents the interquartile range (50% of the population), and the whiskers extend to 0.5 times the interquartile range. Note that shaded areas in both panels represent the same data.*

NAC inhibits SOS response in a ROS-dependent manner

Although the above results compellingly suggest that the reduction of CIP-induced ROS underlie SOS inhibition by NAC, we can not rule out other possibilities. NAC could potentially perturb the activity of the SOS regulatory machinery, for example inhibiting RecA-ssDNA nucleation or LexA self-cleavage. If that were the case, we expect that NAC would reduce SOS induction also when DNA damage is independent of ROS. To test this possibility, we used the *E. coli* strain SMR14354 (Shee et al. 2013), whose chromosome carries a unique cutting site for the restriction enzyme I-SceI. In the presence of 0.1% L-arabinose (Ara), I-SceI is produced, generating DSB and consequently inducing SOS response (**Figure 4a**). Using flow cytometry and H_2DCFDA we first verified that generation of DSB by I-SceI does not increase ROS levels as a side effect (**Figure 4c and Supplementary figure S4).** We then measured SOS induction at different time-points after DSB induction. Our results demonstrate that the addition of NAC causes no

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measurable inhibition of the SOS response (Two tailed Student's *t* test, t=0.64, df=4, P=0.56 for Ara vs Ara+NAC after eight hours of treatment), indicating that NAC inhibition of the SOS response is ROS-dependent (**Figure 4b**). Although the experimental conditions used here have been shown to cause at least a single DSB in 90% of the cells (and more than one in 50% of cells) (Shee et al. 2013), induction of the SOS response is lower than that caused by CIP 8 ng/ml. (~6 versus ~14 fold). An alternative explanation for our results could be that at lower SOS inductions, NAC is unable to decrease SOS induction. To discard this possibility and to match the level of induction caused by I-SceI mediated-DSB, we tested the effect of NAC in cultures treated with lower CIP concentrations**.** Our results show that NAC is able to reduce CIP-induced SOS response in all cases **(Supplementary figure S5).**

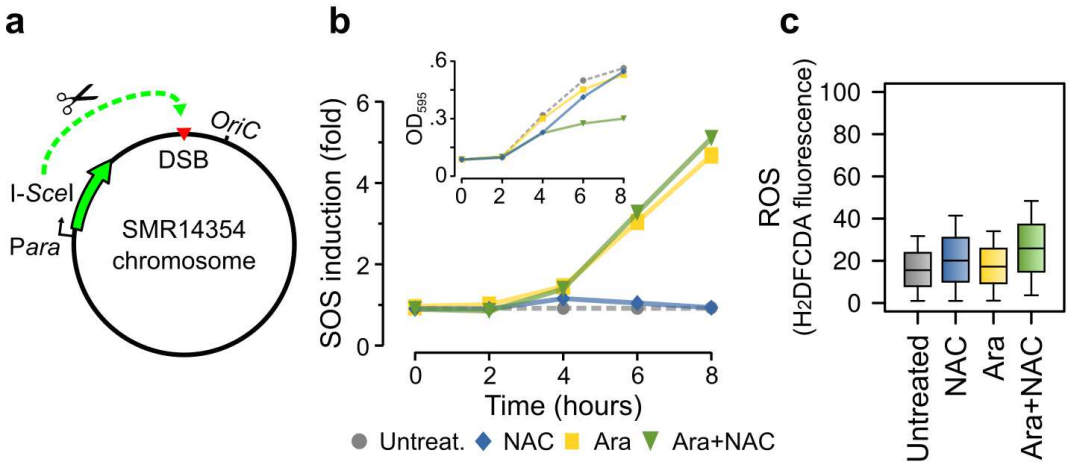

Figure 4. Artificially generated double-strand breaks (DSB) induce the SOS response but do not generate ROS. *a) Schematic diagram of the experimental setting. To generate in vivo DSB, the strain SMR14354 carries a unique cutting site (red triangle) close to OriC of the restriction enzyme I-SceI (scissor), whose expression is induced by L-arabinose (Ara). b) The addition of 0.1% L-arabinose generates DSB that concomitantly induce the activation of the SOS response (yellow curves), measured here by means of an PrecA::gfp transcriptional fusion. The addition of NAC alone (blue) or in combination with Ara (yellow) does not alter SOS induction. Error bars (sd) smaller than data points are not shown for the sake of clarity. Inset graph represents optical density (OD_{595}) under the same conditions. c) ROS levels detected by the use of the fluorescent H_2DCFDA probe and flow cytometry show no significant elevation after the induction of DSB for eight hours.*

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NAC reduces the SOS-mediated mutagenesis promoted by CIP

The quinolone-mediated increase in mutagenesis has been attributed to the activity of TLS DNA-polymerases, whose transcription is induced as part of the SOS response (Vaisman et al. 2012; Goodman 2002). However, high levels of ROS are also mutagenic (Keyer and Imlay 1996; Linn and Imlay 1987; Imlay 2008). To gain knowledge on the contribution of each of these two mechanisms, we used the strain IBDS1 and its TLS⁻ derivative devoid of the three TLS error-prone DNA-polymerases to quantify CIP-mediated mutagenesis (Bjedov et al. 2007). We verified that the TLS⁻ strain showed similar SOS induction and ROS production levels to the wild-type strain when treated by CIP and NAC alone or in combination (**Supplementary figure S4 and S6)**. Mutation rates of treated cultures showed that treatment with CIP induced mutagenesis in both WT and TLS⁻ strain, although at lower levels in the TLS⁻ strain **(Figure 5).** This result indicates that a fraction of CIP-mediated mutagenesis is not dependent on SOS TLS-polymerases. The concomitant treatment with CIP and NAC decreased up to 40% CIP-mediated mutagenesis in the wild-type strain for both Rif-R and Tet-R selective markers (**Fig. 5a**), highlighting the importance of ROS as a major contributor to CIP-induced mutagenesis. On the contrary, NAC was unable to alter CIP-induced mutagenesis in the TLS⁻ strain, indicating that TLS-indendent mutagenesis is also ROS-independent **(Fig. 5b)**.

Together, these results suggest that treatment in wild-type *E. coli* TLS polymerases act synergistically with ROS in a highly intertwined mutagenesis pathway. Accordingly, abolition of ROS causes a dramatic reduction in mutagenesis, reaching similar levels to those found in the TLS⁻ strain.

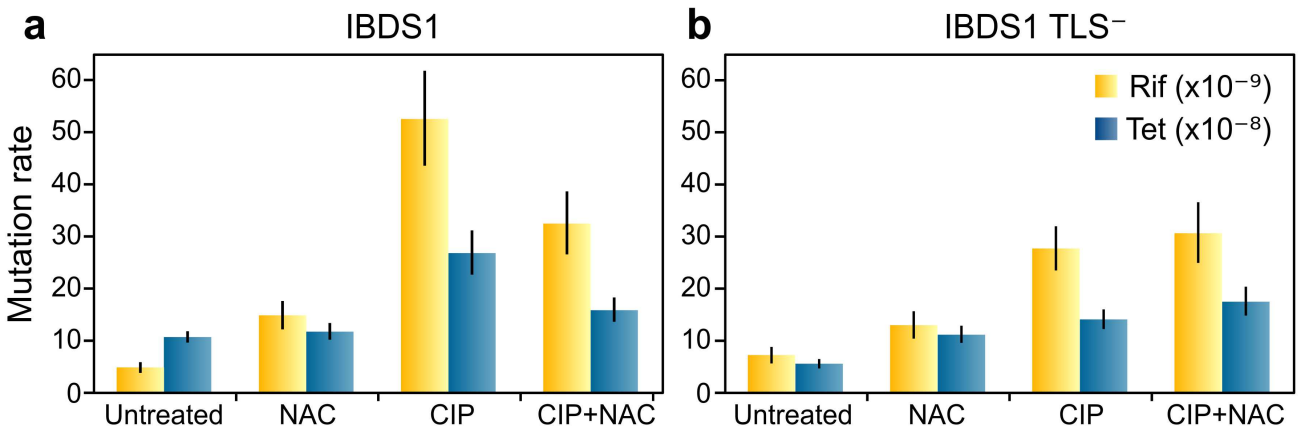

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Figure 5. NAC reduces CIP-induced mutagenesis in the wild-type but not in its TLS⁻ derivative. *Wild-type (a) and TLS⁻ cells (b) were treated with 8 ng/ml of CIP and 0.5% of NAC alone or in combination. After 20 hours of recovery in antibiotic-free medium, mutation rates (mutations per site per generation) were calculated using rifampicin (yellow bars) or tetracycline (blue bars) as selective markers. Differences are considered statistically significant when error bars (95% CI) do not overlap.*

Discussion

Fluoroquinolones such as CIP provoke the blockage of DNA gyrase on DNA causing the stalling of replication forks, producing DSB and leading to cell death (Karl Drlica et al. 2008). DSB are processed to single strand DNA which activates the SOS response causing the upregulation of the SOS genes, including the error-prone TLS DNA polymerases (Vaisman et al. 2012; Fernández De Henestrosa et al. 2000). Because TLS DNA replication is mutagenic (Vaisman et al. 2012; Goodman 2002), fluoroquinolones promote mutagenesis by directly inducing DNA damage and, as a result, the SOS-controlled TLS. Additionally, bactericidal antibiotics such as fluoroquinolones increase the intracellular levels of ROS, causing cell death (Dwyer et al. 2007; Kohanski et al. 2007). Moreover, high levels of ROS, as DNA-damaging agents, can additionally induce the SOS response and cause mutagenesis (Foti et al. 2012; Linn and Imlay 1987). However, the relative contribution of both pathways to antibiotic-induced mutagenesis remains unclear.

Our results show that the decrease of ROS caused by the treatment with the antioxidant NAC attenuates the induction of the SOS response. This effect was to some extent anticipated since it is known that ROS are inducers of SOS response (Goerlich, Quillardet, and Hofnung 1989; Konola, Sargent, and Gow 2000; Carlsson and Carpenter 1980). However, the magnitude of the effect observed in this study (i.e. a reduction of up to 75% of SOS induction by NAC), suggests that ROS are major contributors to DNA-damage and the subsequent activation of SOS in fluoroquinolone-treated bacteria.

This idea is further supported by our mutagenesis results in which a significant reduction, but not abolition, of CIP-induced mutagenesis was observed upon NAC treatment in the wild-type strain. Crucially, NAC treatment reduced wild-type mutagenesis to similar levels to those seen in the TLS⁻ strain, suggesting that the residual CIP-induced mutagenesis is independent of TLS

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repair. Consistent with this view, we observed an increase in mutagenesis in the TLS⁻ strain when submitted to CIP treatment. This result agrees with recent work in which an increased frequency of indels was found upon CIP treatment in a TLS⁻ strain (Song et al. 2016). Together, these results support the existence of a TLS-independent mutagenic pathway. Pomerantz et al. suggested that Pol I can be highly error-prone at RecA-mediated D-loops produced by DSB repair (Pomerantz, Goodman, and O'Donnell 2013). Hence, it is likely that CIP, by generating DSB, can fuel the creation of these RecA-mediated D-loops and the subsequent Pol I mutagenesis. This mechanism is expected to be ROS-independent and could explain the TLS-independent mutagenesis observed in our study. Further experimentation will be needed in order to contrast this hypothesis.

As a clinical application of our results, we propose that NAC could be used as a promising adjuvant in CIP treatment, and possibly extended to other quinolones. NAC is a clinically safe, FDA-approved drug widely used for the treatment of numerous disorders (Elbini Dhouib et al. 2016). We have shown that physiologically attainable concentrations of NAC inhibited SOS induction without compromising CIP bactericidal activity. On the contrary, NAC itself has been shown to present antibacterial properties against *Helicobacter pylori* (Jang, Bak, and Cha 2017), *Haemophilus influenzae* (Landini et al. 2016) and *Pseudomonas aeruginosa* biofilms (Zhao and Liu 2010). From the clinical point of view, inhibition of SOS induction could provide several important benefits besides reducing mutagenesis. Bacterial filamentation is an SOS controlled process crucial for the development of some bacterial infections, such as urinary tract infections (Li et al. 2010). Therefore, inhibition of filamentation has been proposed as a desirable therapeutic target to improve the prognosis of bacterial infections. In this regard, our results also show that NAC significantly reduced the fraction of filamented cells after antibiotic treatment. Therefore, NAC could be used as an SOS inhibitor whose beneficial effects go beyond reducing antibiotic-induced mutagenesis.

In a broader context, our study opens new avenues for the investigation of antioxidant molecules such as NAC as potential inhibitors of the SOS response, reducing the chances of the development of bacterial resistance and decreasing pathogenesis without compromising antibiotic activity.

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Methods

Bacterial strains, plasmids and media

Mutation rate experiments, as well as growth curves and flow cytometry assays, were performed with the *Escherichia coli* MG1655 *att*λ::cI (Ind -) λpR *tet* Δ*ara*::FRT Δ*met*RE::FRT strain (IBDS1) and its derivative deficient in error prone polymerases (TLS-). Mutations that inactivate λ cI (Ind -) represor gene allow the expression of λpR*tetA* gene, which confers resistance to tetracycline (TET) (Bjedov et al. 2007). The strain SMR14354 (*E. coli* MG1655 Δ*araBAD*567 Δ*att*λ::PBADI-SceI *zfd*2509.2::PN25*tetR* FRT ΔattTn7::FRT *cat*FRT PN25*tetOgam-gfp* I-site was used to measure the SOS induction induced by DSB. This strain carries a unique I-SceI restriction site close to the chromosomal OriC. I-SceI expression is regulated by the Ara inducible P*ara* promoter (Shee et al. 2013). The plasmid pSC101-Pr*ecA*::*gfp* (Ronen et al. 2002) was used to monitor SOS induction by fluorescence experiments. Bacterial strains were grown in LB Broth media, supplemented when needed with ciprofloxacin (CIP; at various concentrations), kanamycin (KAN; 30 µg/ml) or 0.5 % V/V of N-Acetylcysteine (NAC).

Minimal Inhibitory Concentration (MIC), as well as checkerboard assay to determine the interaction between ciprofloxacin and NAC, were performed according to standard susceptibility testing, but using LB broth instead of Müller-Hinton. Absorbance at 595 nm was determined using a TECAN Infinite® F200 spectrophotometer after 20h of incubation at 37ºC.

Induction of SOS Response

Three independent overnight cultures of the strain containing the plasmid pSC101-P*recA*::*gfp* were diluted 1:100 in 5 ml of LB supplemented with KAN and grown to exponential phase (OD_{600} 0.5-0.6) at 37ºC and 200 r.p.m. Then, the cultures were diluted 1:50 in LB+KAN. 2ml aliquots were treated with various concentrations of CIP (with or without NAC 0.5%) during 8 hours at 37ºC and 250 r.p.m. The procedure was repeated with the strain SMR14354, but adding 0.1% V/V of Ara instead of CIP as SOS inducer. We verified that higher Ara concentrations do not increase SOS induction, probably because at 0.1% the concentration of I-SceI is enough to cause DSB in most cells (Shee et al. 2013) **(Figure S7)**. Controls without treatment were also included in

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every experiment. Absorbance at 595 nm and green fluorescence (485/520 nm) were monitored using a TECAN Infinite F200 plate reader. SOS induction was obtained by normalizing GFP-fluorescence by the absorbance of each sample. To determine fold change, the average SOS induction of three replicas per condition was divided by the average value of untreated samples.

Flow cytometry

Intracellular ROS levels were determined by the use of the oxidation sensitive probe 2′,7′-Dichlorofluorescein diacetate (H_2DCFDA, Sigma-Aldrich). Overnight cultures of the strain IBDS1 or its TLS- derivative were diluted 1:100 in 5 ml of LB media containing H_2DCFDA, 100 µg/ml, and grown to exponential phase (OD_{600} 0.5-0.6) at 37ºC and 250 r.p.m. Controls without probe were included to monitor autofluorescence. 2 ml aliquots from 1:50 dilutions from both cultures (with and without H_2DCFDA) were treated with CIP 8 ng/ml or Ara 0.1%, with or without NAC 0.5%, during 8 hours at 37ºC and 250 r.p.m. Three replicas of each condition were included in the assay. Green fluorescence emitted by the intracellular oxidation of the dye was determined using a guava easyCyte cytometer (Millipore). Three replicas of 10,000 events each one, with a concentration of 200-400 cells/µl, were analyzed for each one of the conditions. For estimation of filamentation, forward scatter was analyzed in samples without H_2DCFDA. Data analysis was performed using custom scripts in R (www.R-project.org/).

Microscopy

Cultures were treated with CIP, NAC or both agents exactly as in the mutation rate experiments. After 8 hours of treatment, a frotis of every sample was prepared as follows: 10µl of culture was spread with a loop on a microscope slide. Samples were fixated by heat, stained with safranin for 1minute and then washed with distilled water. Slides were observed under a Olympus BX61 microscope using the 100x objective.

Mutation Rate Assays

Three biological replicates of 1:100 dilutions from overnight cultures were grown in LB media to exponential phase (OD_{600} 0.5-0.6) at 37ºC and 200 r.p.m. Then, 2ml aliquots from an 1:50 dilution were treated with CIP 8 ng/ml, with or without NAC 0.5% during 8 hours at 37ºC and 250

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r.p.m. After treatment 1 ml of culture was centrifuged for 6 min at 8,000 r.p.m. Cells were resuspended in fresh LB media and incubated 20 hours at 200 r.p.m to allow resolution of filaments. Appropriate dilutions were plated onto LB-dishes containing tetracycline (TET; 15 µg/ml) or rifampicin (RIF; 100 µg/ml) as selective markers, and LB agar for viable counting. Plates were incubated at 37ºC for 24 hours. The expected number of mutations per culture (m) and 95% confidence intervals were calculated using the maximum likelihood estimator applying the *newton.LD.plating* and *confint.LD.plating* functions that account for differences in plating efficiency implemented in the package rSalvador (Zheng 2017) for R (www.R-project.org/). Mutation rates (mutations per cell per generation) were then calculated by dividing m by the total number of generations, assumed to be roughly equal to the average final number of cells. Differences are considered statistically significant when 95% confidence intervals do not overlap.

Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding authors on reasonable request.

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