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1 # The antioxidant drug N-acetylcysteine abolishes SOS-mediated mutagenesis produced
2 by fluoroquinolones in bacteria

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21 **Abstract**

22 Certain antibiotics, particularly fluoroquinolones, induce the mutagenic SOS response and
23 increase the levels of intracellular reactive oxygen species (ROS), which have been
24 associated with antibiotic lethality. Both SOS and ROS promote bacterial mutagenesis,
25 fueling the emergence of resistant mutants during antibiotic treatments. However, the
26 relative contribution of ROS and SOS on this antibiotic-mediated mutagenesis is currently
27 unknown. We used the antioxidant molecule N-acetylcysteine (NAC) to study the
28 contribution of ROS on the SOS response and the mutagenesis mediated by the
29 fluoroquinolone antibiotic ciprofloxacin (CIP). We show that NAC is able to reduce
30 intracellular ROS levels, as well as the SOS response caused by treatment with
31 subinhibitory concentrations of CIP, without affecting its anti-bacterial activity. This effect
32 reduces antibiotic-induced mutagenesis to levels comparable to a translesion synthesis
33 DNA-polymerases deficient strain, suggesting that ROS play a major role in SOS-induced
34 mutagenesis. Collectively, our results shed light on the mechanisms underlying antibiotic-
35 induced mutagenesis and open the possibility for the use of NAC as adjuvant in antibiotic
36 therapy to hinder the development of antibiotic resistance.

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37 **Importance**

38 The development of antimicrobial resistance, together with the existing paucity in the
39 antibiotic pipeline, renders every antibiotic into a non-renewable resource that should be
40 carefully rationed. This worrisome scenario is exacerbated by the fact that treatment with
41 certain antibiotics, besides killing bacteria, increase the chances of surviving bacteria to
42 acquire resistance as a side-effect. The mechanisms underlying this phenomenon involve
43 complex bacterial physiological responses to antibiotics such as induction of the SOS
44 response and the generation of reactive oxygen species. In this work, we demonstrate that
45 the antioxidant drug N-acetylcysteine inhibits antibiotic-induced mutagenesis by reducing
46 the levels of reactive oxygen species and SOS induction in bacterial cells upon antibiotic
47 treatment. Our results strongly suggest that reactive oxygen species are a key factor in
48 antibiotic-induced SOS mutagenesis and open the possibility of using NAC combined with
49 antibiotic therapy to counteract the development of antibiotic resistance.

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50 **Introduction**

51 Antibiotics, besides their antimicrobial action, can promote genetic variability in
52 bacteria as an undesirable side effect (1). In turn, genetic variability increases the chances
53 for bacteria to acquire resistance and jeopardize the success of antimicrobial therapies.
54 Blocking the bacterial physiological responses that promote genetic variability is thus
55 crucial to hinder resistance spread (2, 3).

56 Most of the genetic variability produced by antibiotics has been attributed to the
57 induction of the SOS response. The SOS response is a coordinated genetic network that
58 responds to DNA damage. Several antibiotics produce DNA damage and consequently
59 induce the SOS response. For instance, fluoroquinolones such as ciprofloxacin (CIP) block
60 DNA gyrase on DNA, which cause the stalling of replication forks and leads to cell death
61 (4). This produces double strand breaks (DSB) that are processed into single strand DNA,
62 that together with RecA trigger the SOS response. SOS induction up-regulates the
63 expression of more than 40 genes whose functions include DNA-damage tolerance and non-
64 mutagenic DNA repair (5–8). However, when DNA damage is persistent, the error-prone
65 DNA translesion synthesis (TLS) takes place. In *Escherichia coli*, TLS is accomplished by the
66 specialized DNA polymerases Pol II, Pol IV and Pol V, encoded respectively by the *polB*,
67 *dinB* and *umuDC* genes (9). TLS polymerases are able to replicate heavily damaged DNA
68 but do so at the cost of a reduced fidelity, therefore increasing mutagenesis (5).
69 Additionally, RecA-mediated recombination is also induced by fluoroquinolone antibiotics
70 (10). Hence, some antibiotics can promote mutagenesis and recombination (i.e. genetic
71 instability) by directly inducing DNA damage and, in turn, the SOS response.

72 Bactericidal antibiotics have been shown to produce a perturbation of the
73 intracellular redox homeostasis. This perturbation is caused by an increased intracellular
74 respiration rate accompanied by destabilization of the Iron-Sulfur clusters, which leads to

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75 production of reactive oxygen species (ROS) via Fenton chemistry (11–13). ROS are highly
76 reactive chemical species capable of rapidly oxidizing key cellular components, including
77 proteins, lipids and DNA. ROS have been argued as a common cause of bacterial cell death
78 for several antibiotic families (12–14), although this notion has been further challenged
79 (15, 16). Oxidation of DNA by ROS produces a wide variety of lesions that, if not repaired,
80 are mutagenic and can even cause cell death (17–19).

81 In summary, previous studies have shown that there are at least two routes to
82 antibiotic-triggered bacterial mutagenesis; SOS-mediated TLS and ROS-induced
83 mutagenesis. Interestingly, these two routes are probably not independent but highly
84 intertwined. For instance, oxidation of the nucleotide pool after antibiotic treatment leads
85 to Pol IV-mediated incorporation of 8-oxo-dGTP into DNA, which creates a mutagenic
86 lesion (14, 20). Furthermore, ROS are good SOS inducers because they directly damage
87 DNA (21–23). Hence, SOS-mediated TLS mutagenesis might be fueled by the presence of
88 oxidative damage in both DNA and the nucleotide pool (24, 25).

89 Recently, there has been growing interest in the development of novel therapies
90 designed not to kill bacteria, but to inhibit the aforementioned routes to antibiotic
91 resistance (2, 26, 27). In particular, most studies have focused on developing SOS
92 inhibitors that target RecA (28–30). RecA offers an appealing target because its inhibition
93 not only reduces bacterial evolvability, but also renders bacteria more sensitive to several
94 antibiotics (31); in some cases leading to complete reversion of antibiotic resistance (32).
95 However, the presence of RecA homologs in mammals in humans suggests LexA as a
96 potentially safer target (33).

97 In this study, we hypothesized that antioxidant molecules could reduce ROS
98 produced by antibiotic treatment and consequently inhibit SOS induction. This combined
99 inhibition might, in turn, reduce antibiotic-induced mutagenesis. To test this idea, we

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100 focused on N-acetylcysteine (NAC), a well-known antioxidant that acts as a scavenger of
101 oxidant species and as a precursor of glutathione synthesis (34, 35). NAC is clinically safe
102 and is currently used in humans therapy to treat numerous disorders (34). Additionally,
103 NAC does not negatively affect the activity of major antibiotic classes, with the exception of
104 carbapenems (36, 37). On the contrary, NAC has shown antimicrobial properties against a
105 range of clinically-relevant pathogens (38–41). Our results show that, due to its
106 antioxidant properties, NAC offers an unique opportunity to disentangle the effects of ROS
107 and SOS in antibiotic-mediated mutagenesis. Additionally, it promises to become a
108 therapeutic alternative to outsmart the evolution of bacterial resistance.

109 **Results**

110 *CIP induces mutagenesis at subinhibitory levels*

111 The mutagenic activity of antimicrobials is expected to occur within a window of
112 concentrations around their minimal inhibitory concentration (MIC), because higher levels
113 would kill cells or stop their growth while lower concentrations would not have a
114 stimulatory effect (42). To determine the concentration of CIP that induces the highest
115 increase in mutagenesis, we treated exponentially growing cultures of *E. coli* strain IBDS1
116 (43) with different concentrations of CIP ranging from 0.25 to 4 times the MIC for 8 hours.
117 We then determined mutation rates using two independent selective markers. Cells treated
118 with 8 ng/ml of CIP (which correspond to ½ of the MIC) showed the highest increase on
119 the rate of mutations conferring resistance to rifampicin (Rif-R; 24-fold increase) and
120 tetracycline (Tet-R; 2.5-fold increase) **(Supplementary figure 1)**. Higher concentrations of
121 CIP barely produced any increase in mutagenesis. This probably occurred because these
122 concentrations hampered growth of most treated cells (**Supplementary figure 2),**

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123 drastically reducing effective population size and hence limiting evolvability (44). We
124 decided to use 8 ng/ml of CIP hereafter to maximize antibiotic-induced mutagenesis.

125 *NAC reduces ciprofloxacin-induced intracellular ROS*

126 We then measured the levels of ROS caused by treatment with CIP and tested if NAC
127 was able to reduce CIP-generated ROS. To this end we used 2′,7′-Dichlorofluorescein
128 diacetate (H_2DCFDA), a ROS sensitive dye that emits fluorescence when it is oxidized
129 intracellularly (45). H_2DCFDA has been previously shown to be an extremely sensitive
130 probe for the detection of ROS caused by fluoroquinolones, detecting with great sensitivity
131 H_2O_2, ROO· and $ONOO^-$ (45). As expected, CIP consistently showed increased fluorescence
132 levels over those produced by antibiotic-mediated autofluorescence (46), indicating a
133 massive increase of ROS levels compared to untreated cells (**Figure 1** and **Supplementary**
134 **figure 3a**). Most important, the induction of ROS was reverted to nearly basal levels when
135 CIP treatment was combined with NAC at 0.5%. This result suggests that NAC, at a
136 physiologically attainable concentration (47), might be able to reduce DNA damage by
137 reducing the levels of ROS upon CIP exposure.

138 *NAC reduces CIP-mediated induction of the SOS response*

139 We then analyzed the effect of CIP and NAC in SOS induction. To this end, we used
140 the strain IBDS1 pRecA::*gfp* that harbours the transcriptional fusion P*recA*::*gfp* contained in
141 a low copy number plasmid (48, 49). As expected, CIP strongly induced the SOS response
142 approximately 14-fold compared to untreated controls (Tukey multiple comparisons after
143 significant ANOVA, P>0.001; **Figure 2a**).

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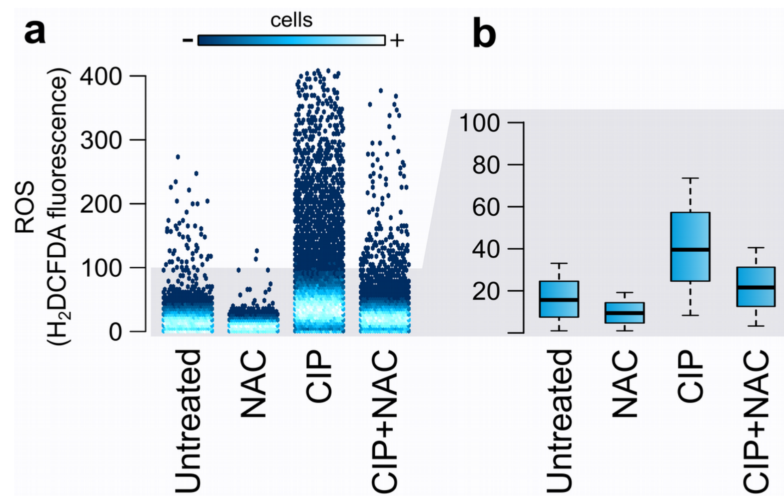

144 ***Figure 1. NAC reduces ciprofloxacin-induced intracellular ROS.*** *ROS were assessed by*
145 *individually capturing the fluorescence of H_2DCFDA in 30,000 cells by flow cytometry after 8*
146 *hours of treatment with either 8 ng/ml of CIP, 0.5% NAC or both agents in combination. An*
147 *untreated control is shown as a reference. a) The dot plot shows the distribution of fluorescence*
148 *signal in the treated populations. At least 99% of the events recorded are shown. The color*
149 *scale displays the density of events at every fluorescence level. b) To allow better comparison,*
150 *the data is depicted as boxplots, in which the horizontal line represents the median value, the*
151 *depth of the box represents the interquartile range (50% of the population), and the whiskers*
152 *extend to 0.5 times the interquartile range. Note that shaded areas in both panels represent the*
153 *same data.*

154 Following the hypothesis that antioxidant compounds can be effective inhibitors of SOS
155 induction (50), we tested the effect of different NAC concentrations on CIP-mediated SOS
156 induction. NAC inhibited SOS induction caused by CIP at all concentrations (Tukey
157 multiple comparisons after significant ANOVA, P>0.001; **Figure 2a**), with an IC_{50} of 0.5%
158 (**Figure 2c**). Again, this concentration is within the range of attainable physiological values
159 after inhaled administration (47), strongly suggesting that inhibition of the SOS response
160 by NAC could be a feasible therapeutic approach. Importantly, NAC did not reduce CIP

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161 bactericidal activity, as stated by MIC results **(Supplementary Table 1),** growth curves
162 **(Figure 2b)** and a checkerboard assay (**Figure 2d)**. On the contrary, NAC alone inhibited
163 bacterial growth without inducing the SOS response (Tukey multiple comparisons after
164 significant ANOVA, P=0.0003). This result is in line with previous studies that reported
165 NAC antibacterial properties against a range of bacterial pathogens (37, 40, 41).
166 Additionally, we verified that the differences in the final optical density observed upon
167 different treatments do not directly influence the measurement of SOS induction using GFP
168 fluorescence **(Supplementary figure 4).**

169 SOS induction leads to the overexpression of *sulA* (*sfiA*), whose product inhibits FtsZ
170 ring formation and hence cell division (51). The phenotypic consequence of cell division
171 inhibition is filamentation, which offers an additional SOS-dependent measurable
172 phenotype. We assessed whether 0.5% NAC was able to inhibit CIP-mediated cell
173 filamentation by both flow cytometry and direct observation of Gram-stained cultures.
174 **Figure 3** shows that, as expected, CIP treatment produces a vast increase in the fraction of
175 the population with filamented cells compared with untreated cultures (33% versus 0.3%
176 of filamented cells). Remarkably, administration of 0.5% NAC together with CIP, prevented
177 filamentation in a large fraction of cells (14% versus 33%, for CIP+NAC versus CIP alone).
178 We qualitatively confirmed these results by microscopy observation of Gram-stained cells
179 **(Figure 3b)**.

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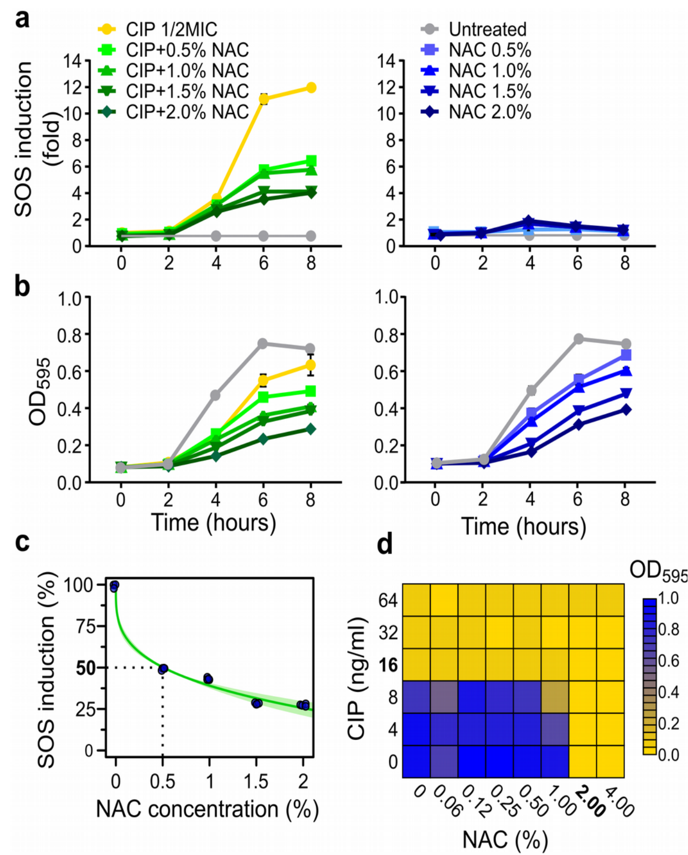

180 ***Figure 2. NAC reduces ciprofloxacin-induced SOS response.*** *Bacterial growth and SOS*
181 *induction were monitored during treatment with varying concentrations of CIP alone or in*
182 *combination with NAC (left panels), or NAC alone (right panels). Samples were taken at*
183 *indicated time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

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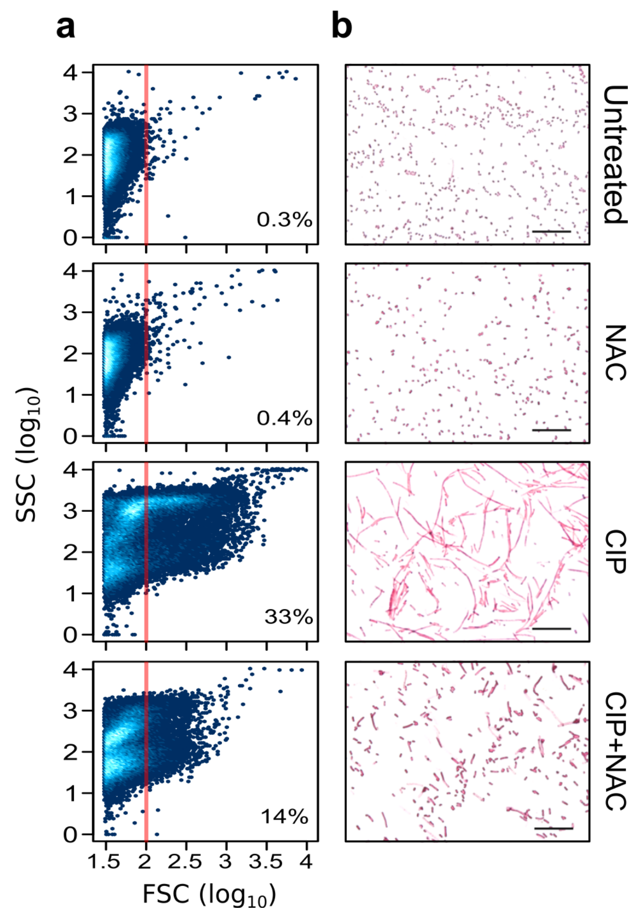
192 In summary, these results demonstrate that NAC does not decrease bacterial
193 susceptibility to CIP. It does, however, significantly reduce up to a 75% CIP-mediated
194 induction of *recA* transcription and cell filamentation, hallmarks of SOS induction.



195 ***Figure 3. NAC reduces CIP-induced filamentation.*** ***(a)*** *The fraction of filamented cells after*
196 *the stated treatments is shown by means of flow cytometric analysis of 30,000 cells (SSC; side*
197 *scatter FSC; forward scatter; proportional to cell size). The percentage on every graph*
198 *represents the filamented fraction of the population (log10(FSC)>2; red vertical line).* ***(b)***
199 *Representative microscopy fields of Gram-stained cells. Scale bars is 20µm.*

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200 *NAC inhibits SOS response in a ROS-dependent manner*

201 Although the above results compellingly suggest that the reduction of CIP-induced
202 ROS underlie SOS inhibition by NAC, we cannot rule out other possibilities. NAC could
203 potentially perturb the activity of the SOS regulatory machinery, for example inhibiting
204 RecA-ssDNA nucleation or LexA self-cleavage. If that were the case, we expect that NAC
205 would reduce SOS induction also when DNA damage is independent of ROS. To test this
206 possibility, we used the *E. coli* strain SMR14354 (52), whose chromosome carries a unique
207 cutting site for the restriction enzyme I-SceI. In the presence of 0.1% L-arabinose (Ara), I-
208 SceI is produced, generating DSB and consequently inducing SOS response (**Figure 4a**).
209 Using flow cytometry and H_2DCFDA we first verified that generation of DSB by I-SceI does
210 not increase ROS levels as a side effect (**Figure 4b** and **Supplementary figure 3c).** We
211 then measured SOS induction at different time-points after DSB induction. Our results
212 demonstrate that the addition of NAC causes no measurable inhibition of the SOS response
213 (Two tailed Student's *t* test, t=0.64, df=4, P=0.56 for Ara vs Ara+NAC after eight hours of
214 treatment), indicating that NAC inhibition of the SOS response is ROS-dependent (**Figure**
215 **4c**). Although the experimental conditions used here have been shown to cause at least a
216 single DSB in 90% of the cells (and more than one in 50% of cells) (52), induction of the
217 SOS response is lower than that caused by 8 ng/ml of CIP (~6 versus ~14 fold). An
218 alternative explanation for our results could be that at lower SOS inductions, NAC is unable
219 to decrease SOS induction. To discard this possibility, and to match the level of induction
220 caused by I-SceI mediated-DSB, we tested the effect of NAC in cultures treated with lower
221 CIP concentrations. Our results show that NAC is able to reduce CIP-induced SOS response
222 in all cases **(Supplementary figure 5).**

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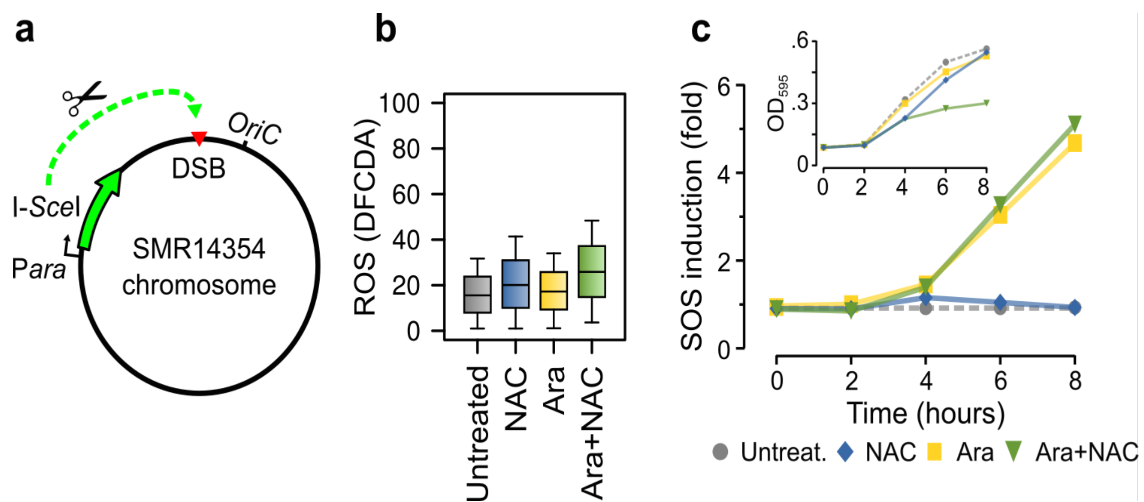

223 ***Figure 4. Artificially generated double-strand breaks induce the SOS response but do*** 224 ***not generate ROS.*** *a) Schematic diagram of the experimental setting. To generate in vivo* 225 *DSB, the strain SMR14354 carries a unique cutting site (red triangle) close to OriC of the* 226 *restriction enzyme I-SceI (scissor), whose expression is induced by L-arabinose (Ara). b) The* 227 *addition of 0.1% L-arabinose generates DSB that concomitantly induce the activation of the* 228 *SOS response (yellow curves), measured here by means of an PrecA::gfp transcriptional fusion.* 229 *The addition of NAC alone (blue) or in combination with Ara (yellow) does not alter SOS* 230 *induction. Error bars (sd) smaller than data points are not shown for the sake of clarity. Inset* 231 *graph represents optical density (OD_{595}) under the same conditions. c) ROS levels detected by* 232 *the use of the fluorescent H_2DCFDA probe and flow cytometry show no significant increase after* 233 *the induction of DSB for eight hours.*

234 *NAC reduces the SOS-mediated mutagenesis promoted by CIP*

235 The quinolone-mediated increase in mutagenesis has been attributed to the activity 236 of TLS DNA-polymerases, whose transcription is induced as part of the SOS response (9, 237 53). However, our results and previous studies strongly suggest that high levels of ROS are 238 also mutagenic (17–19). To gain knowledge on the contribution of each of these two 239 mechanisms we used the strain IBDS1 and its TLS⁻ derivative which lacks the three TLS 240 error-prone DNA-polymerases (43). We verified that the TLS⁻ strain showed similar SOS 241 induction and ROS production levels to the wild-type strain when treated by CIP and NAC 242 alone or in combination (**Supplementary figures 3 and 6**). Mutation rates of treated

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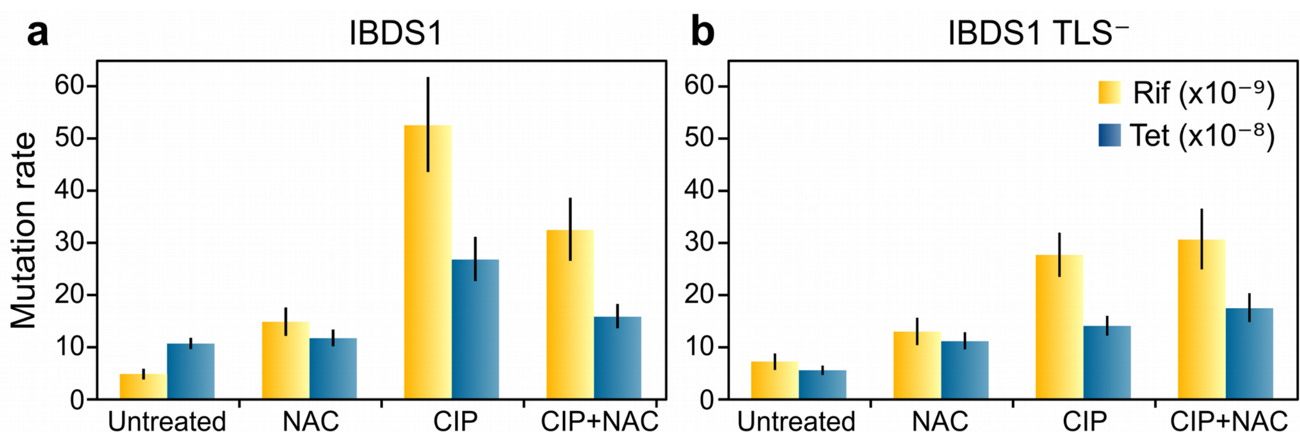
243 cultures showed that treatment with CIP induced mutagenesis in both WT and TLS⁻ strain,
244 although at lower levels in the TLS⁻ strain **(Figure 5).** This result indicates that a fraction
245 of CIP-mediated mutagenesis is not dependent on SOS TLS-polymerases. The combined
246 treatment with CIP and NAC decreased up to 40% CIP-mediated mutagenesis in the wild-
247 type strain for both Rif-R and Tet-R selective markers (**Fig. 5a**). This highlights the
248 importance of ROS as a major contributor to CIP-induced mutagenesis. On the contrary,
249 NAC was unable to alter CIP-induced mutagenesis in the TLS⁻ strain, indicating that TLS-
250 independent mutagenesis is also ROS-independent **(Fig. 5b)**.

251 Together, these results suggest that treatment in wild-type *E. coli* TLS polymerases
252 act synergistically with ROS in a highly intertwined mutagenesis pathway. Accordingly,
253 reduction of ROS by NAC completely abolishes SOS-mediated mutagenesis in CIP-treated
254 bacteria.



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

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261 **Discussion**

262 Fluoroquinolones such as CIP provoke the blockage of DNA gyrase on DNA causing
263 the stalling of replication forks, which produces DSB and leads to cell death (54). DSB are
264 processed to single strand DNA which activates the SOS response causing the upregulation
265 of the SOS genes, including the error-prone TLS DNA polymerases (8, 9). Because TLS DNA
266 replication is mutagenic (9, 53), fluoroquinolones promote mutagenesis by directly
267 inducing DNA damage and, as a result, the SOS-controlled TLS. Additionally, bactericidal
268 antibiotics such as fluoroquinolones increase the intracellular levels of ROS, causing cell
269 death (12, 13). Moreover, high levels of ROS, as DNA-damaging agents, can additionally
270 induce the SOS response and cause mutagenesis (14, 18, 22).

271 Here, we showed that the decrease of ROS caused by the treatment with the
272 antioxidant NAC attenuates the induction of the SOS response. However, the magnitude of
273 the effect observed in this study (i.e. a reduction of up to 75% of SOS induction by NAC),
274 suggests that ROS are major contributors to DNA-damage and the subsequent activation of
275 SOS in fluoroquinolone-treated bacteria.

276 This idea is further supported by our mutagenesis results in which a significant
277 reduction, but not abolition, of CIP-induced mutagenesis was observed upon NAC
278 treatment in the wild-type strain. Most important, NAC treatment reduced wild-type
279 mutagenesis to similar levels to those seen in the TLS^{-} strain, suggesting that the residual
280 CIP-induced mutagenesis is independent of TLS repair. Consistent with this view, we
281 observed an increase in mutagenesis in the TLS^{-} strain when submitted to CIP treatment.
282 This result agrees with recent work in which an increased frequency of indels was found
283 upon CIP treatment in a TLS^{-} strain (55). Together, these results support the existence of a
284 TLS-independent mutagenic pathway. Pomerantz et al. suggested that Pol I can be highly
285 error-prone at RecA-mediated D-loops produced by DSB repair (56). Hence, it is likely that

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286 CIP, by generating DSB, can fuel the creation of these RecA-mediated D-loops and the
287 subsequent Pol I mutagenesis. This mechanism is expected to be ROS-independent and
288 could explain the TLS-independent mutagenesis observed in our study. Further
289 experimentation will be needed in order to contrast this hypothesis.

290 As a clinical application of our results, we propose that NAC could be used as a
291 promising adjuvant in CIP treatment, and possibly with other quinolones. NAC is a
292 clinically safe, FDA-approved drug widely used for the treatment of numerous disorders
293 (35). We have shown that physiologically attainable concentrations of NAC inhibited SOS
294 induction without compromising CIP bactericidal activity. On the contrary, NAC itself has
295 been shown to present antibacterial properties against *Helicobacter pylori* (40),
296 *Haemophilus influenzae* (37), *Stenotrophomonas maltophilia*, *Burkholderia cepacia* (39) and
297 *Pseudomonas aeruginosa* biofilms (41). From the clinical point of view, inhibition of SOS
298 induction could provide several important benefits besides reducing mutagenesis. For
299 instance, SOS-regulated genes control pathogenic processes such as persistence, tolerance,
300 infection, and expression of toxins or virulence factors (57–59). Additionally, it is well
301 known that bacterial filamentation is a SOS controlled process crucial for the development
302 of some bacterial infections, such as urinary tract infections (60). Therefore, inhibition of
303 filamentation could be a desirable therapeutic target to improve the prognosis of bacterial
304 infections. In this regard, our results also show that NAC significantly reduced the fraction
305 of filamented cells after antibiotic treatment.

306 Taken together, our results suggest that NAC could be used as adjuvant in antibiotic
307 treatment to inhibit SOS-mutagenesis, reducing the chances for the development of
308 bacterial resistance and decreasing pathogenesis without compromising antibiotic activity.

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309 **Methods**

310 *Bacterial strains, plasmids and media*

311 Mutation rate experiments, as well as growth curves and flow cytometry assays,
312 were performed with the *Escherichia coli* MG1655 *att*λ::cI (Ind -) λpR *tet* Δ*ara*::FRT
313 Δ*met*RE::FRT strain (IBDS1) and its derivative deficient in error prone polymerases (TLS-).
314 Mutations that inactivate λ cI (Ind-) represor gene allow the expression of λpR*tetA* gene,
315 which confers resistance to tetracycline (TET) (43). The strain SMR14354 (*E. coli* MG1655
316 Δ*araBAD*567 Δ*att*λ::PBADI-SceI *zfd*2509.2::PN25*tetR* FRT Δatt*Tn*7::FRT *cat*FRT
317 PN25*tetOgam-gfp* I-site was used to measure the SOS induction triggered by DSB. This
318 strain carries a unique I-SceI restriction site close to the chromosomal OriC. I-SceI
319 expression is regulated by the Ara inducible P*ara* promoter (52). The plasmid pSC101-
320 P*recA*::*gfp* (48) was used to monitor SOS induction by fluorescence experiments. Bacterial
321 strains were grown in LB Broth media, supplemented with ciprofloxacin (CIP; at various
322 concentrations), kanamycin (KAN; 30 μg/ml) or 0.5 % V/V of N-Acetylcysteine (NAC)
323 when needed.

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

329 *Induction of SOS Response*

330 Three independent overnight cultures of the strain containing the plasmid pSC101-
331 P*recA*::*gfp* were diluted 1:100 in 5 ml of LB supplemented with KAN and grown to
332 exponential phase (OD_{600} 0.5-0.6) at 37ºC and 200 r.p.m. Subsequently, the cultures were

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333 diluted 1:50 in LB+KAN. 2ml aliquots were treated with various concentrations of CIP
334 (with or without NAC 0.5%) during 8 hours at 37ºC and 250 r.p.m. The procedure was
335 repeated with the strain SMR14354, but adding 0.1% V/V of Ara instead of CIP as SOS
336 inducer. We verified that higher Ara concentrations do not increase SOS induction,
337 probably because at 0.1% the concentration of I-SceI is enough to cause DSB in most cells
338 (52) **(Supplementary figure 7)**. Controls without treatment were also included in every
339 experiment. Absorbance at 595 nm and green fluorescence (485/520 nm) were monitored
340 using a TECAN Infinite F200 plate reader. SOS induction was obtained by normalizing GFP-
341 fluorescence by the absorbance of each sample. To determine fold change, the average SOS
342 induction of three replicas per condition was divided by the average value of untreated
343 samples.

344 *Flow cytometry*

345 Intracellular ROS levels were determined by the use of the oxidation sensitive probe
346 2′,7′-Dichlorofluorescein diacetate (H_2DCFDA, Sigma-Aldrich). Overnight cultures of the
347 strain IBDS1 or its TLS- derivative were diluted 1:100 in 5 ml of LB media containing
348 H_2DCFDA 100 μg/ml, and grown to exponential phase (OD_{600} 0.5-0.6) at 37ºC and 250
349 r.p.m. Controls without probe were included to monitor autofluorescence. 2 ml aliquots
350 from 1:50 dilutions from both cultures (with and without H_2DCFDA) were treated with CIP
351 8 ng/ml or Ara 0.1%, with or without NAC 0.5%, during 8 hours at 37ºC and 250 r.p.m.
352 Three replicas of each condition were included in the assay. Green fluorescence emitted by
353 the intracellular oxidation of the dye was determined using a guava easyCyte cytometer
354 (Millipore). Three replicas of 10,000 events each one, with a concentration of 200-400
355 cells/μl, were analyzed for each one of the conditions. For estimation of filamentation,
356 forward scatter (FSC) was analyzed in samples without H_2DCFDA. Data analysis was
357 performed using custom scripts in R (www.R-project.org/).

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358 *Microscopy*

359 Cultures were treated with CIP, NAC or both agents exactly as in the mutation rate
360 experiments. After 8 hours of treatment, a frotis of every sample was prepared as follows:
361 10μl of culture was spread with a loop on a microscope slide. Samples were fixated by heat,
362 stained with safranin for 1minute and then washed with distilled water. Slides were
363 observed under a Olympus BX61 microscope using the 100x objective.

364 *Mutation Rate Assays*

365 Three biological replicates of 1:100 dilutions from overnight cultures were grown in
366 LB media to exponential phase (OD_{600} 0.5-0.6) at 37ºC and 200 r.p.m. Subsequently, 2ml
367 aliquots from 1:50 dilutions were treated with CIP 8 ng/ml, with or without NAC 0.5%
368 during 8 hours at 37ºC and 250 r.p.m. After treatment 1 ml of culture was centrifuged for 6
369 min at 8,000 r.p.m. Cells were resuspended in fresh LB media and incubated 20 hours at
370 200 r.p.m to allow resolution of filaments. Appropriate dilutions were plated onto LB-dishes
371 containing tetracycline (TET; 15 μg/ml) or rifampicin (RIF; 100 μg/ml) as selective
372 markers, and LB agar for viable counting. Plates were incubated at 37ºC for 24 hours. The
373 expected number of mutations per culture (m) and 95% confidence intervals were
374 calculated using the maximum likelihood estimator, applying the *newton.LD.plating* and
375 *confint.LD.plating* functions that account for differences in plating efficiency implemented
376 in the package rSalvador (61) for R (www.R-project.org/). Mutation rates (mutations per
377 cell per generation) were then calculated by dividing m by the total number of generations,
378 assumed to be roughly equal to the average final number of cells. Differences are
379 considered statistically significant when 95% confidence intervals do not overlap.

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380 *Data Availability*

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

383 **Author contributions**

384 J.B and J.R-B conceived the study. A.I.R, E.Y.V, R.S.G, J.B, A.R-R and J.R-B designed the
385 experiments. A.I.R, E.Y.V and C.C performed the experiments. A.I.R and J.R-B analysed the
386 data. J.B and R.S.G provided reagents and materials. A.I.R, J.B. and J.R-B wrote the
387 manuscript. All authors discussed the results and implications and commented on the
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404 **Competing Interests**

405 The authors declare that they have no competing interests.

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