RexAB is essential for the mutagenic repair of *Staphylococcus aureus* DNA damage caused by co-trimoxazole

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Abstract

Co-trimoxazole (SXT) is a combination therapeutic that consists of sulfamethoxazole and trimethoprim that is increasingly used to treat skin and soft-tissue infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA). However, the use of SXT is limited to the treatment of low-burden, superficial *S. aureus* infections and its therapeutic value is compromised by the frequent emergence of resistance. As a first step towards the identification of approaches to enhance the efficacy of SXT, we examined the role of bacterial DNA repair in antibiotic susceptibility and mutagenesis. This revealed that SXT caused DNA damage in *S. aureus* via both thymidine limitation and the generation of reactive oxygen species. Then, using mutants defective for DNA repair, it was found that repair of this damage required the RexAB nuclease/helicase complex, indicating that SXT causes DNA double-strand breaks. Furthermore, RexAB-mediated DNA repair led to induction of the SOS response, which resulted in an increased mutation rate and may explain the frequent emergence of resistant strains during SXT therapy. In summary, this work determined that SXT causes DNA damage in *S. aureus* via both thymidine limitation and oxidative stress, which is repaired by the RexAB complex, leading to induction of the mutagenic SOS response. Small molecule inhibitors of RexAB could therefore have therapeutic value by increasing the efficacy of SXT and decreasing the emergence of drug-resistance during treatment of infections caused by *S. aureus*.

48 # Introduction

49 *Staphylococcus aureus* is responsible for a wide spectrum of diseases ranging from superficial
50 skin infections to life-threatening bacteraemia, endocartidis and toxic shock syndrome (1).
51 Whilst β-lactam antibiotics such as oxacillin are first choice therapeutics for *S. aureus*
52 infections, the prevalence of skin and soft tissue infections (SSTIs) caused by methicillin-
53 resistant *S. aureus* (MRSA) strains has necessitated the use of second line therapeutics such
54 as co-trimoxazole (SXT) (2, 3). SXT has several desirable properties including low cost, the
55 availability of both oral and intravenous formulations and low host toxicity, making it an
56 appealing treatment option (4).

57 SXT is a combination of two antibiotics; trimethoprim (TMP) and sulfamethoxazole
58 (SMX) which target sequential steps in the tetrahydrofolate biosynthetic pathway (5). SMX
59 inhibits dihydropteroate synthetase (DHPS), which prevents the production of dihydropteroic
60 acid, while TMP binds and inhibits dihydrofolate reductase (DHFR), blocking the conversion of
61 dihydrofolic acid to tetrahydrofolate (6). Since the production of tetrahydrofolate is essential
62 for the biogenesis of thymidine, purines and some amino acids bacteria exposed to SXT will
63 experience disrupted bacterial metabolism and stalled DNA replication (7).

64 Previous studies have reported that TMP-induced thymidine depletion contributes to
65 bacterial cell death through stalled DNA replication forks, which together with the continued
66 initiation of replication will result in DNA damage (8). However, one of the limitations of SXT
67 as an antibiotic is that *S. aureus* can bypass SXT-mediated metabolic blockage by utilising
68 exogenous thymidine released from damaged tissues (9). It is therefore hypothesised that the
69 presence of thymidine at infection sites reduces the efficacy of SXT treatment in patients (10-
70 12). As a consequence, SXT is only used for low burden superficial staphylococcal infections.

71 In addition to SXT-mediated DNA damage occurring via stalled replication forks, recent
72 work with *E. coli* has implicated the production of reactive oxygen species (ROS) and
73 maladaptive DNA repair in the bactericidal activity of this antibiotic (13). However, it is not
74 clear if this is specific to *E. coli* or represents a general mechanism of bactericidal activity for
75 many different bacteria.

76 SXT-mediated DNA damage triggers the SOS response, an inducible repair system that
77 enables bacteria to survive genotoxic stress (14, 15). Regulation of the SOS response is highly
78 conserved and occurs primarily via LexA, a transcriptional repressor of the SOS regulon (16).
79 When DNA damage occurs, RecA binds to single-stranded DNA (ssDNA) at the lesion site to
80 form a nucleoprotein filament which, i) facilitates DNA strand invasion that initiates repair by
81 homologous recombination, and ii) induces the autocleavage of LexA, resulting in
82 derepression of the SOS regulon (17). In *S. aureus*, it is believed that RecF assists RecA-binding
83 to ssDNA-gaps from single-nucleotide lesions (18). However, double-strand breaks (DSB) are
84 recognised by the AddAB complex (known as RexAB in *S. aureus*), which it binds and generates
85 a ssDNA overhang through its helicase/exonuclease activity. This ssDNA strand then serves as
86 the substrate for RecA-binding and activation of the SOS response (19).

87 The SOS-regulon of *S. aureus* consists of 16 LexA-regulated genes (17), including *recA*,
88 *lexA* and *umuC*, which encodes a low-fidelity DNA polymerase. UmuC catalyses translesion
89 DNA synthesis but lacks proofreading ability and permits DNA replication across unresolved
90 lesions which often introduces mutations. Such mutagenic DNA repair may be advantageous
91 to the pathogen by conferring resistance to antibiotics or adaptation to host stresses (17, 20).
92 For example, mutations in the genes encoding DHFR and DHPS confer resistance to TMP and
93 SMX respectively (21, 22). Therefore, exposure of *S. aureus* to SXT at concentrations that do

94 not kill the pathogen may promote the emergence of resistance via induction of the mutagenic
95 SOS response.

96 The prevalence of infections caused by drug-resistant pathogens necessitates new
97 therapeutic options. However, given the lack of investment into the development of new
98 antibiotics there is increasing interest in the development of therapeutics that enhance the
99 efficacy of existing antibacterial drugs or by-pass resistance (23, 24). However, such an
100 approach requires a thorough understanding of how antibiotics function and the mechanisms
101 used by bacteria to survive exposure to antibacterial drugs. Therefore, the aims of this work
102 were to identify the mechanisms by which SXT damages *S. aureus* DNA, the repair systems
103 that this pathogen uses to withstand this damage and the consequences of repair for the
104 emergence of resistance.

105

106 # Materials and Methods

107 **Bacterial strains and culture conditions**

108 A full list of the bacterial strains used in this study is provided in Table 1. *S. aureus* was cultured
109 in either Tryptic Soy Broth (TSB) or Mueller Hinton Broth (MHB) supplemented with calcium
110 (25 mg/L) and magnesium (12.5 mg/L), to stationary phase for 18 h at 37 °C, with shaking (180
111 RPM). Culture media were supplemented with erythromycin (10 µg ml^{-1}) for growth of
112 transposon mutants, kanamycin (90 µg ml^{-1}) for growth of the *recA* reporter strains, or
113 anhydrotetracycline hydrochloride (AHT) (100 ng ml^{-1}) to induce expression of *rexBA* on
114 plasmids, as required (Table 1).

115 *S. aureus* SH1000 and JE2 mutants deficient for *rexB* were complemented using p*itet*
116 (25) containing the wildtype *rexBA* operon. Since insertion of the transposon into *rexB* is

117 expected to block the expression of the downstream *rexA* gene, the *rexB* mutants were
118 complemented with the entire *rexBA* operon. The p*itet* vector is a single-copy plasmid that
119 integrates into the *geh* locus of the *S. aureus* genome. It contains a tetracycline-inducible
120 promoter that was used to control *rexBA* gene expression. Primers containing the AvrII or
121 PmeI restriction sites (Table 2) were used to amplify the *rexBA* operon from USA300 JE2
122 genomic DNA. The PCR product and p*itet* vector were digested using AvrII and PmeI restriction
123 enzymes, ligated using T4 ligase and transformed into $CaCl_2$-competent *E. coli* DH5α cells.
124 Successful ligation of *rexBA* into p*itet* was confirmed with DNA sequencing. Plasmid DNA was
125 subsequently transformed into the DC10B *E. coli* strain which lacks cytosine methylation to
126 allow bypassing of the staphylococcal restriction-modification barrier (26) and allowed
127 successful electroporation into electrocompetent S*. aureus rexB* mutants in the JE2 and
128 SH1000 genetic backgrounds. Empty vector that did not contain the *rexBA* operon was used
129 as a control. Successful integration into the *S. aureus* genome was confirmed by PCR
130 amplification of the *geh* gene followed by DNA sequencing.
131
132 **Minimum Inhibitory Concentration (MIC) Assay**
133 The minimum inhibitory concentration was determined according to the broth microdilution
134 protocol described previously (27). Briefly, 95 µl aliquots of either MHB or TSB supplemented
135 with doubling concentrations of Co-trimoxazole (SXT), Trimethoprim (TMP) or
136 Sulfamethoxazole (SMX) ranging from 0.008 µg ml^{-1} to 512 µg ml^{-1} were prepared in wells of
137 96 well plates. In this study, the concentration of SXT was calculated based on the
138 concentration of TMP present within the combination therapeutic. Stationary phase cultures
139 of *S. aureus* were diluted and inoculated into the antibiotic-containing media to a final cell
140 density of 1 x 10^5 CFU ml^{-1}. For complemented mutants containing *rexBA* under the control of

141 the p*itet* promoter, the medium was supplemented with AHT (100 ng ml^{-1}) to induce gene
142 expression. Plates were incubated for 17 h at 37 °C before OD_{600} readings were taken. The
143 MIC was determined as the lowest concentration of antibiotic required to cause growth
144 inhibition. To determine MICs under anaerobic conditions, 96 well plates were incubated in
145 anaerobic jars containing Anaerogen gas packs at 37 °C for 18 h.
146
147 **SXT Killing Assay**
148 Stationary phase *S. aureus* cultures were washed twice by alternate rounds of centrifugation
149 and resuspension in fresh MHB or TSB (3 ml) to a density of 2 x 10^8 CFU ml^{-1} containing 4 µg
150 ml^{-1} SXT, the antibiotic clinical breakpoint concentration (28). Bacteria were incubated with
151 SXT at 37°C with shaking (180 RPM) and survival determined over time by quantification of
152 CFUs using the Miles and Misra method, which consists of serial dilution followed by plating
153 onto agar plates to enable the enumeration of colonies (29). Data were converted to %
154 survival by comparing CFU counts at specific time points with the starting inoculum. To
155 investigate SXT-mediated killing under anaerobic conditions, washed stationary phase
156 cultures were inoculated into pre-reduced broth in bijoux tubes supplemented with 4 µg ml^{-1}
157 SXT, and incubated statically at 37 °C with the vessel caps loosened in an anaerobic jar
158 containing an Anaerogen gas pack. The growth of *S. aureus* strains in TSB, under aerobic or
159 anaerobic conditions was examined using a similar protocol using TSB or MHB without
160 antibiotics.
161
162 **Disc-diffusion Assay**
163 Stationary phase *S. aureus* cultures were adjusted to an OD_{600} of 0.063 (0.5 McFarland) in TSB
164 and used to inoculate TSA plates using a cotton wool swab. Antibiotic discs containing 2.5 µg

165 of either SXT or TMP (Oxoid) were placed onto the inoculated agar and incubated at 37 °C
166 under aerobic or anaerobic conditions and incubated at 37 °C for 17 h. The zone of inhibition
167 was measured as the total diameter of the cleared bacterial lawn, minus the diameter of the
168 antibiotic disc.
169
170 **Mutation rate analyses**
171 Mutation rates were determined as described previously (20, 30). Briefly, 30 parallel cultures
172 of *S. aureus* were used for each condition or strain in bijoux tubes containing 1 ml TSB
173 inoculated with *S. aureus* at ~ 5 x 10^5 CFU ml^{-1} and incubated with or without 0.05 µg ml^{-1} or
174 0.1 µg ml^{-1} SXT with shaking at 37 °C for 24 h. After incubation, 10 cultures were selected at
175 random, and total CFU counts determined through serial dilution in PBS followed by plating
176 onto TSA plates. Subsequently, 100 µl of each of the 30 undiluted cultures were spread on TSA
177 plates containing rifampicin (100 µg ml^{-1}) before incubation at 37 °C for 24 h. The number of
178 rifampicin-resistant colonies on each plate were counted, and the mutation rate with 95%
179 confidence intervals calculated using the maximum-likelihood setting of the FALCOR mutation
180 rate calculator (30). Since these assays were done in the presence of SXT it was important to
181 use rifampicin resistance (rather than SXT resistance) to measure the mutation rate to avoid
182 selecting for resistance emergence. Rifampicin resistance is a well-established marker for
183 mutation-rate analysis and is not selected for or against under the conditions used.
184
185 ***recA*-reporter assay**
186 Induction of the SOS response in *S. aureus* in response to SXT exposure was determined
187 through the use of strains containing a *recA-gfp* reporter construct. These were generated by
188 transforming *S. aureus* with pCN34 containing the *recA* promoter upstream of *gfp*. Primers,

189 detailed in Table 2, were used to amplify the *recA* promoter region (sequence detailed in (14)
190 from JE2 wildtype genomic DNA, and the *gfp* gene from the pCL55 P3 GFP plasmid (31). These
191 products were combined by overlapping extension PCR using primers 'PrecA-F BamHI' and
192 'GFP-R KpnI', digested with BamHI and KpnI (relevant restriction sites incorporated into
193 amplicon primers), and ligated into phosphatase-treated, BamHI and KpnI digested low copy
194 shuttle vector pCN34. Empty vector without the *recA-gfp* construct served as a control. The
195 *recA-gfp* pCN34 vectors were constructed in *E. coli* DC10B and confirmed by sequencing.
196 Cultures of DC10B containing the *recA-gfp* pCN34 vector and empty vector were cultured in
197 LB broth supplemented with ampicillin (100 µg/ml) to select for plasmid maintenance. Using
198 methods described in (26), *recA-gfp* and empty vectors were transformed directly with into *S.*
199 *aureus* WT JE2 and the *rexB*::Tn mutant. Reporter strains were grown in the presence of 90
200 µg/ml of kanamycin to maintain the plasmid.

201 Stationary phase cultures of *recA-gfp* reporter strains were pelleted by centrifugation,
202 washed twice, and resuspended in TSB supplemented with kanamycin (90 µg ml^{-1}). Cultures at
203 ~ 3.33 x 10^8 CFU ml^{-1} were exposed to a range of SXT concentrations (0.01 µg m^{l-1} to 8 µg ml^{-}
204 ^{1}) in black-walled microtiter plates. These were incubated, with shaking, at 37 °C for 17 h in a
205 TECAN Infinite® 200 PRO microplate reader, where OD_{600} and GFP relative fluorescent units
206 (RFU) (Excitation: 475 nm; Emission: 525 nm) was measured every 1000 seconds. The values
207 were blanked against values generated from uninoculated wells, and GFP fluorescence was
208 normalised to OD_{600} readings to determine *recA* expression levels.

209

210 **Endogenous ROS production**

211 Production of ROS was detected using the cell permanent 2',7'-dichlorodihydrofluorescein
212 diacetate dye (H_2DCFDA), which is converted to fluorescent DCF by oxidative cleavage of

213 acetate groups (32). A 96 well microtitre plate assay was prepared in a similar format to the
214 *recA-gfp* reporter assay, with the addition of 25 µM H_2DCFDA and various concentrations of
215 SXT. Wildtype and *rexB*::Tn mutant strains were washed and inoculated at ~ 3.33 x 10^8 CFU
216 ml^{-1}. Plates were incubated with shaking at 37 °C as described previously, and OD_{600} and
217 fluorescence (Excitation: 495 nm; Emission: 525nm) was measured every 1000 s. OD_{600} values
218 were blanked against uninoculated wells, and fluorescence data was blanked to untreated
219 wells. Fluorescent values were normalised to OD_{600} readings to determine production of ROS
220 relative to cell number.
221
222 **Statistical Analysis**
223 Means or medians were calculated from at least three biologically independent replicates and
224 analysed by Student's t test (two tailed, unpaired, assuming equal variances), One-way ANOVA
225 or Two-way ANOVA corrected for multiple comparison using GraphPad Prism (V7.0) as
226 described in the figure legends. MIC-bar graphs show the median value. All remaining graphs
227 were plotted to show mean ± S.E.M. Error bars were omitted on *recA*-reporter data for clarity.
228

229 # Results

230 **Inactivation of *rexB* sensitises *S. aureus* to SXT**
231 To investigate the role of bacterial DNA repair in the susceptibility of *S. aureus* to SXT, a panel
232 of *S. aureus* transposon mutants were subjected to MIC and disc diffusion susceptibility tests,
233 including SXT and its constituent antibiotics, TMP and SMX. These mutants lacked components
234 of DNA repair (*rexA, rexB*, *recF*, *umuC* and *dinB*) previously shown to contribute to the repair
235 of damage caused by the quinolone antibiotic ciprofloxacin, or acting as controls (20). Mutants
236 in two distinct genetic backgrounds were used in this study; SH1000, a well-established

237 laboratory strain, and JE2, a community-associated methicillin-resistant *S. aureus* (CA-MRSA)
238 strain of the USA300 lineage, responsible for severe skin infections that are often treated with
239 SXT (3, 33).

240 The MIC values for both the SH1000 and JE2 wild-type (WT) strains indicated
241 susceptibility to SXT and TMP (< 2 µg ml^{-1}), but resistance to SMX (>512 µg ml^{-1}) based on
242 EUCAST breakpoints for *S. aureus* [EUCAST, 2019] (Fig. 1A). Disruption of *rexB* in both JE2 and
243 SH1000 strains lowered the MIC of SXT and TMP by 2-4-fold and 16-fold for SMX, whilst
244 complementation of the *rexB* mutant either fully or partially restored wild-type levels of
245 susceptibility to each of the antibiotics (Fig. 1A). The mutant lacking *recF* in the JE2 strain was
246 also more susceptible than the WT to all three antibiotics, but the disruption of *dinB* or *umuC*
247 only resulted in a slightly increased susceptibility to SXT but not TMP or SMX.

248 Consistent with the MIC data, disc diffusion assays with both JE2 and SH100 strains
249 showed that inactivation of *rexB* significantly increased the susceptibility of *S. aureus* to SXT
250 and TMP, relative to the WT (Fig. 1B,C). Complementation of the *rexB* mutant with (p*rexBA*)
251 reduced susceptibility close to WT levels, confirming the role of the RexAB complex (Fig. 1B,C).
252 By contrast, inactivation of *dinB or umuC* did not significantly increase zone sizes for either
253 antibiotic (Fig. 1B,C). However, the *recF*-deficient mutant showed an increase in susceptibility
254 to TMP in the JE2 strain (Fig. 1C), indicating that both *rexBA* and *recF* contribute to the repair
255 of DNA damage caused by TMP.

256 The finding that RexAB and RecF reduced the susceptibility of *S. aureus* to SXT
257 confirmed that DNA repair modulated susceptibility to SXT and prompted us to undertake a
258 screen of mutants with disrupted copies of *recJ*, *uvrA*, *uvrB*, *nth*, *mutS*, *mutL*, *sbcC* or *sbcD,* to
259 identify any additional repair processes that might be involved. However, none of these
260 mutants had a SXT MIC that was different from that of the wild-type.

261 Having established that staphylococcal DNA repair reduced the growth-inhibitory
262 activity of SXT, we next investigated its role in the survival of *S. aureus* exposed to the
263 breakpoint concentration of the antibiotic (4 μg ml^{-1}) (EUCAST, 2019). There was a 20-fold
264 reduction in survival over 24 h for the JE2 strain (Fig. 1D), indicating a relatively high level of
265 tolerance to SXT at this concentration. Similar levels of survival were seen for mutants lacking
266 *umuC* or *dinB* in the JE2 background (Fig. 1D). By contrast, survival of the *recF* mutant was
267 reduced by >20-fold, relative to the WT, indicating increased susceptibility. Most notably
268 however, the *rexB* mutant showed >5000-fold reduction in CFU counts relative to the WT at
269 24 h. Complementation of the *rexB* mutant increased survival 10-fold relative to a plasmid
270 only control (Fig. 1E). Subsequent experiments with the SH1000 confirmed a role for *rexBA* in
271 modulating susceptibility of *S. aureus* to SXT. There was a 12-fold greater loss of CFU counts
272 of the *rexB* mutant relative to WT (Fig. 1F). However, similar to what was seen for the MIC
273 assays, complementation of the *rexB* mutant with p*rexBA* restored survival levels close to
274 those of the WT (Fig. 1F). Combined, these findings indicate that SXT causes DNA double-
275 strand breaks in *S. aureus*, the repair of which by RexAB significantly promotes staphylococcal
276 survival during exposure to the antibiotic.
277
278 **Thymidine limitation contributes to SXT-mediated DNA damage**
279 Inhibition of folate biosynthesis by SXT results in abrogation of endogenous thymidine
280 biosynthesis (34), which could provide an explanation for the DNA damage phenotype
281 described above (Fig. 1). In support of this, previous studies have reported that the *in vitro*
282 inhibitory activity of TMP and SXT is inversely proportional to the availability of thymidine in
283 the culture medium (10, 35). The presence of exogenous thymidine allows *S. aureus* to

284 continue with canonical DNA synthesis and allow effective DNA repair to occur, despite SXT-
285 mediated inhibition of tetrahydrofolate production (13).

286 The experiments detailed thus far were performed in Tryptic Soy Broth (TSB), which
287 contains a low concentration of thymidine (0.25 µM, (35)). To investigate the influence of
288 exogenous thymidine on SXT activity in our assays, the growth of WT and *rexB* mutant strains
289 was determined in TSB supplemented with doubling dilutions of SXT and thymidine via
290 chequerboard assays (Fig. 2A-D). As expected, the presence of high concentrations of
291 thymidine enabled growth of WT strains of both JE2 and SH1000 at higher concentrations of
292 SXT (35) (Fig. 2A,C).

293 Given the thymidine-dependent activity of SXT, different culture media compositions
294 may greatly affect antibiotic susceptibility (10, 35). For example, Mueller-Hinton Broth (MHB),
295 the gold standard medium for antibiotic susceptibility testing, contains >16-fold more
296 thymidine than TSB (35). Therefore, similar susceptibility tests were undertaken using this
297 medium. Consistent with the checkerboard data, the efficacy of SXT in MHB was reduced
298 relative to TSB, with detectable bacterial growth of both WT strains observed even at high
299 concentrations of SXT, most likely due to the increased levels of thymidine (Fig. 2E,F).

300 To explore the effect of exogenous thymidine on the bactericidal activity of SXT, time-
301 course killing assays were undertaken using MHB. As expected from the MIC assays, and by
302 contrast to assays done in TSB (Fig. 1D) there was almost no killing of the *S. aureus* JE2 WT
303 strain by SXT (4 µg ml^{-1}) in MHB over 24 h (Fig. 2G). Similarly high levels of survival were
304 observed for the *dinB*, *umuC* and *recF* mutants (Fig. 2G). By contrast, survival of the *rexB*
305 mutant in MHB containing SXT was >2000-fold lower than that of the WT, despite the
306 presence of exogenous thymidine (Fig. 2G). As for TSB, complementation of the *rexB* mutant
307 (p*rexBA*) significantly promoted survival in MHB containing SXT, relative to a plasmid only

308 control (Fig. 2H). Similar results were seen for the SH1000 strain, with the WT surviving in MHB
309 containing SXT (4 µg ml^{-1}), whilst viability of the *rexB* mutant was reduced >6000-fold relative
310 to the inoculum (Fig. 2I). As for the JE2 strain, complementation of the SH1000 *rexB* mutant
311 significantly promoted survival (> 10-fold) (Fig. 2I).

312 Combined, these data demonstrate that the RexAB DNA repair complex is required for
313 the survival of *S. aureus* exposed to SXT, even when thymidine is available, suggesting that
314 thymidine limitation is not the only mechanism by which this antibiotic damages bacterial
315 DNA.

316

317 **Inactivation of *rexB* sensitises *S. aureus* to SXT-mediated growth inhibition but not killing**
318 **under anaerobic conditions**

319 It was recently reported that TMP exposure increases the production of reactive oxygen
320 species (ROS) by *E. coli*, which together with incomplete DNA repair contributes to bacterial
321 cell death (13). Therefore, we hypothesised that SXT-mediated DNA damage could occur via
322 stalled DNA synthesis caused by inhibition of thymidine production and via the generation of
323 ROS.

324 To examine whether the production of ROS contributes to SXT activity against *S.*
325 *aureus*, the susceptibility of WT and DNA repair mutants to the antibiotic was determined
326 under both aerobic and anaerobic conditions in low-thymidine TSB. In the absence of oxygen,
327 the MIC of WT *S. aureus* was increased 4-fold for both JE2 and SH1000 strains (Fig. 3A). By
328 contrast, the SXT MIC of the JE2 *rexB* mutant only doubled under anaerobic conditions, whilst
329 the MIC of the SH1000 *rexB* mutant was unaffected by the presence of oxygen (Fig. 3A). To
330 explore this further, zone of inhibition assays were done under aerobic and anaerobic
331 conditions. Zones of inhibition were significantly reduced in the absence of oxygen for all

332 strains and mutants, implying reduced susceptibility (Fig. 3B). However, despite the reduced
333 antibiotic activity of SXT under anaerobic conditions, the *rexBA* mutants were still more
334 susceptible than the corresponding WT, with >10 and 2.5-fold greater zone sizes for JE2 and
335 SH1000 strains respectively (Fig. 3B).

336 The elevated susceptibility of the *rexB* mutant to SXT under both aerobic and anaerobic
337 conditions indicated that thymidine limitation causes double-strand breaks via a process that
338 is promoted by, but not dependent upon, the presence of oxygen.

339 Next, we assessed the contribution of ROS to the bactericidal activity of SXT. In
340 contrast to assays done in air, none of the strains examined were killed by SXT (4 µg ml^{-1})
341 under anaerobic conditions (Fig. 3C,D). However, whilst the WT strains was able to grow
342 slightly under these conditions, SXT prevented growth of the *rexB* mutants >2- fold by 24 h
343 post inoculation (Fig. 3C,D). The ability of the WT strains to grow in the presence of an SXT
344 concentration above the MIC likely reflects the 1000-fold larger inoculum used in time kill
345 experiments compared to MIC assays (36-38). Complementation of the *rexB* mutant, but not
346 the empty vector, rescued SXT mediated growth inhibition for both strains at 24 h (Fig. 3C,D).
347 Therefore, SXT exposure causes bacteriostasis in the *rexBA* mutant but not the WT under
348 anaerobic conditions.

349 The reduced activity of SXT under anaerobic conditions suggested that ROS make an
350 important contribution to the bactericidal activity of the antibiotic. Therefore, H_2DCFDA dye,
351 which is converted to the fluorescent 'DCF' in the presence of ROS, was added to growth
352 inhibition assays (Fig. 3E,F) to quantify and study the kinetics of ROS production during
353 incubation under aerobic conditions. SXT induced clear concentration dependent inhibition
354 growth for both the JE2 WT and *rexB* mutant (Fig. 3E,F). When these data were used to
355 normalise to the relative cell density, SXT also induced a dose-dependent fluorescent peak at

356 4 h post inoculation for both WT and *rexBA* mutant strains in the JE2 background (Figures 3G
357 and 3H), indicative of the generation of ROS in response to the antibiotic. A second peak in
358 ROS production after 13 h is apparent at lower concentrations of SXT as cells reach stationary
359 phase, which is likely a result of the bacterial starvation response, which is associated with
360 increased endogenous oxidative stress (39).

361 Combined, these data provide strong evidence that SXT-induces ROS that contribute
362 to the growth inhibitory activity of the antibiotic and are essential for the bactericidal activity
363 of the drug. Furthermore, these findings provide evidence that SXT triggers similar levels of
364 oxidative stress in both WT and *rexB* mutant, indicating that the increased susceptibility of the
365 mutant to the antibiotic is due to an inability to repair damage, rather than increased
366 generation of ROS.

367

368 **RexAB is required for SXT-triggered induction of the SOS response**

369 The SOS response is a DNA damage-inducible regulon that includes several genes that
370 encode DNA repair components and is regulated by RecA and LexA (17). Consistent with our
371 findings that SXT causes DNA damage in *S. aureus*, previous work has shown that TMP triggers
372 *recA* expression, which is indicative of induction of the SOS response (14). This is important
373 because the SOS response increases the mutation rate, promoting the likelihood of drug
374 resistance emerging, which is a frequent limitation of SXT therapy (17, 40-42).

375 Since RexAB was important for the repair of DNA damage caused by SXT, we wanted
376 to determine how the complex affected the induction of the SOS response. To investigate this,
377 we used *S. aureus* JE2 WT and *rexB* mutant strains containing a *recA-gfp* reporter construct
378 (p*recA*-gfp) to measure induction of the SOS response (Fig. 4).

379 Irrespective of thymidine concentration, SXT induced a dose dependent inhibition of
380 growth for both the JE2 WT and *rexB* mutant (Fig. 4A-D). In low-thymidine TSB, SXT exposure
381 resulted in a dose-dependent increase in GFP signal from the p*recA*-gfp reporter construct in
382 the *S. aureus* WT strain (Fig. 4E), which peaked at about 4 h and then subsided before a the
383 signal slowly increased once more as the bacteria entered stationary phase, presumably due
384 to SOS induction in response to internal DNA-damaging events as a consequence of nutrient
385 limitation and other metabolic stresses (39, 43).
386 The temporal profile of *recA* expression is similar to that observed for ROS production
387 by WT *S. aureus* in response to SXT (Fig. 3G), providing additional evidence that antibiotic-
388 induced oxidative stress contributes to DNA damage. However, although SXT also triggered
389 ROS production in the *rexB* mutant (Fig. 3H), the antibiotic did not induce *recA* expression in
390 this strain (Fig. 4F), revealing that RexAB is required for activation of the SOS response under
391 these conditions.
392 In contrast to experiments done in TSB, *recA* was only weakly expressed during
393 exposure to SXT in high-thymidine MHB (Fig. 4D), which is in keeping with the reduced activity
394 of the antibiotic in this medium (Fig. 2E,F). Consistent with the data from studies in TSB, there
395 was a lack of *recA* expression in the JE2 *rexB* mutant in response to SXT in MHB (Fig. 4E).
396
397 **RexAB is required for SXT-induced mutagenic DNA repair**
398 De-repression of the SOS regulon leads to expression of the error-prone translesion DNA
399 polymerase UmuC, which catalyses mutagenic DNA repair that contributes to the acquisition
400 of antibiotic resistance (17, 20).
401 To assess whether SXT promoted SOS-dependent mutagenesis in *S. aureus*, JE2 WT
402 and DNA-damage repair mutants were exposed to sub-inhibitory concentrations of SXT in low-

403 thymidine TSB. The resulting mutation rate was calculated by fluctuation analysis using the
404 emergence of rifampicin resistance, which occurs via point mutations in the *rpoB* gene
405 encoding the RNA polymerase β-subunit (30, 44). Since the *rexBA* mutant was more
406 susceptible to SXT treatment than the wild-type, only a very low concentration of the
407 antibiotic (0.05 µg ml^{-1}, 0.2 x MIC of the mutant) could be used in mutation rate analyses with
408 this strain. However, even at this concentration, SXT treatment resulted in an increase in the
409 mutation rate of WT *S. aureus* (Fig. 5A). By contrast to the WT, SXT exposure did not increase
410 the mutation rate of the *rexB* mutant, consistent with the inability of the antibiotic to trigger
411 induction of the SOS response in this strain (Fig. 4B, 5A).

412 To determine whether the SXT-mediated increase in the mutation rate was due to
413 induction of the SOS response, we exposed the WT and mutants lacking *umuC* or *dinB* (an
414 error prone polymerase that is not part of the SOS response in *S. aureus*; (17) to SXT at 0.2 x
415 MIC of the mutants (0.1 µg ml^{-1}). Consistent with the *recA* reporter data, the higher
416 concentration of SXT used in these experiments promoted the mutation rate of the WT strain
417 to a greater extent than the lower dose (Fig. 4A, 5A,B). SXT caused a similar increase in the
418 mutation rate of the *dinB* mutant but had no effect on the mutation rate of the *umuC* mutant
419 (Fig. 5B).

420 Taken together, these data demonstrate that SXT exposure results in RexAB-
421 dependent induction of the SOS response in *S. aureus*, which triggers UmuC-dependent
422 mutagenic DNA repair that promotes the rate at which antibiotic resistance arises.

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426 ## Discussion

427 Multi-drug resistance in *S. aureus* is a major global health concern associated with high
428 treatment failure rates (45). Many second or third-line treatments such as vancomycin or
429 daptomycin can lack efficacy, require intravenous administration and are toxic (12). By
430 contrast, SXT is a safe and orally administered antibiotic that is effective in the treatment of
431 skin and soft tissue infections caused by MRSA (4). However, the efficacy of this antibiotic is
432 limited by the pathogens' ability to by-pass SXT-mediated inhibition of folate biosynthesis,
433 either via the uptake of metabolites from the environment or by the frequent acquisition of
434 resistance-conferring mutations (9). Therefore, it is hoped that an improved understanding of
435 SXT and its mechanism of action will to enable the development of novel approaches aimed
436 at expanding the therapeutic use of this antibiotic and reducing the frequency at which
437 resistance emerges.

438 Although poorly characterised, the bactericidal effect of SXT has been ascribed to DNA
439 damage, which leads to the induction of the SOS response in bacterial cells (14, 15). The data
440 we present here support DNA damage as a central component of the mechanism of SXT
441 bactericidal activity in *S. aureus* and highlight the key role of RexAB in combatting this
442 genotoxicity. In contrast to the WT, mutants defective for *rexB* were significantly more
443 susceptible to SXT in both growth inhibition and killing assays.

444 RexAB is proposed to be a member of the AddAB family of DNA repair enzymes
445 required for the processing of DNA double strand breaks on the basis of protein sequence
446 identity (19, 20, 46). Therefore, the increased susceptibility of *rexB* mutants to SXT indicate
447 that DNA DSBs are caused by the antibiotic and are lethal if not repaired. Additional evidence
448 that SXT causes DNA damage in *S. aureus* came from analysis of the *recF* mutant, which was
449 more susceptible to the antibiotic than the WT, albeit to a lesser extent than the *rexB* mutant.

450 The function of RecF has been ascribed to the repair of DNA lesions that arise from DNA single-
451 strand gaps. However, whilst this function is well established in canonical model organisms
452 for bacterial DNA damage repair, including *E. coli* and *Bacillus subtilis*, the evidence that
453 supports RecF performing a similar function in *S. aureus* is less strong (18). Therefore, a greater
454 understanding of how RecF repairs damage in staphylococci will provide insight into the type
455 of DNA damage caused by SXT in *S. aureus*. Whilst we didn't see increased SXT susceptibility
456 for any of the other mutants examined in MIC assays, this doesn't imply that other types of
457 DNA damage don't occur, simply that they don't contribute to bacterial growth inhibition. This
458 finding does imply however, that DNA repair does not contribute to the lethality of SXT, as has
459 been reported for trimethoprim-mediated killing of *E. coli* (13).

460 The discovery that *S. aureus* is more susceptible to SXT when incubated in thymidine-
461 poor medium is in keeping with previous reports, which suggest that thymidine depletion
462 makes a crucial contribution to the lethality of SXT (15, 35). Specifically, nucleotide imbalance,
463 caused by thymidine depletion, can result in the collapse of replication forks during DNA
464 synthesis, which can lead to DNA DSBs (8). In support of this, we observed SOS induction under
465 thymidine-limited but not thymidine-replete conditions. This has important implications for
466 the efficacy of SXT-treatment since elevated thymidine concentrations are often observed in
467 human tissues containing necrotic cells or neutrophil extracellular traps (9, 47). To acquire this
468 thymidine, *S. aureus* expresses DNase to break down DNA and NupC, a nucleoside transporter
469 that has been shown to support the growth of *S. aureus* during SXT exposure by enabling the
470 uptake of extracellular thymidine (48, 49). The presence of thymidine and other folate-
471 dependent metabolites including serine, methionine and glycine in host tissues modulate the
472 susceptibility of SXT-treated cells and restrict the types of infection that can be treated with
473 this antibiotic (9). However, whilst the presence of thymidine reduced the susceptibility of the

474 WT to SXT, there was still a very large reduction in CFU counts of the *rexB* mutant exposed to
475 the antibiotic, suggesting that thymidine limitation is not the only element responsible for
476 DNA damage in *S. aureus*.

477 In agreement with several reports that bactericidal antibiotics, including TMP, exert
478 part of their antibacterial effect via the generation of ROS (13, 50), we determined that the
479 bactericidal effect of SXT against *S. aureus* is dependent upon the availability of oxygen. This
480 was evident by the complete lack of bacterial killing under anaerobic conditions and suggests
481 that ROS-mediated DNA damage makes a greater contribution to the bactericidal activity of
482 SXT than the inhibition of DNA-synthesis caused by thymidine depletion in aerobic conditions.

483 Antibiotic-induced oxidative stress increases the intracellular level of 8-
484 oxodeoxyguanosine (8 -oxo-dG), which is incorporated into DNA, and paired with
485 deoxycytidine or deoxyadenosine (51). Incomplete repair of DNA-incorporated 8-oxo-dG can
486 lead to DSB in *E. coli*, which contributes to the lethality of TMP (13). Additional experiments
487 using mutants defective for the repair of 8-oxo-dG are needed to determine whether a similar
488 mechanism mediates the genotoxicity of SXT in *S. aureus* under aerobic conditions. However,
489 whilst ROS clearly make an important contribution to the bactericidal activity of SXT, the
490 increased susceptibility of the *rexB* mutant to this antibiotic under anaerobic conditions
491 confirms that DNA damage can occur independently of ROS. This may explain why there are
492 phenotypic differences in the effects of SXT exposure and thymineless death observed in *thyA*
493 mutants deprived of thymine (13).

494 One drawback of SXT is the frequent emergence of resistance during long-term
495 therapy (52). In keeping with the induction of the SOS response, exposure of *S. aureus* to sub-
496 inhibitory concentrations of SXT was found to increase the mutation rate via UmuC, a low-
497 fidelity DNA polymerase, previously shown to mediate mutagenic DNA repair of bacteria

498 exposed to ciprofloxacin or H_2O_2 (20). Similar to what has been reported for ciprofloxacin and
499 H_2O_2, SXT-induced increases in the mutation rate was dependent upon RexAB (20). Therefore,
500 the processing of DNA DSBs by RexAB is central to the survival of *S. aureus* exposed to SXT as
501 well as SOS-mediated increases in the mutation rate associated with drug resistance.

502 In summary, our data demonstrate that SXT causes DNA damage in *S. aureus*
503 via thymidine depletion and ROS generation, the repair of which enables bacterial survival and
504 leads to induction of the SOS-mediated increase in the mutation rate and the emergence of
505 drug-resistance. The identification of RexAB as central to both SXT susceptibility and SOS
506 induction suggests that this protein complex is a promising target for novel therapeutic
507 approaches that could improve the efficacy of SXT and reduce the emergence of resistance.
508 Such an approach may also increase the range of MRSA infections that could be treated with
509 SXT.

510

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666 strain derived from *Staphylococcus aureus* 8325-4. J Bacteriol 184:5457-67.

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670 **Table 1. Bacterial strains used in this study.**

Bacterial strain	Relevant characteristics	Source/ reference
Methicillin-resistant *S. aureus*		
USA300 LAC JE2	LAC strain of the USA300 CA-MRSA lineage cured of plasmids	(53)
USA300 LAC JE2 *rexB*::Tn	USA300 LAC JE2 with a *bursa aurealis* transposon insertion in *rexB*, Ery^r	(53)
USA300 LAC JE2 *umuC*::Tn	USA300 LAC JE2 with a *bursa aurealis* transposon insertion in *umuC*, Ery^r	(53)
USA300 LAC JE2 *recF*::Tn	USA300 LAC JE2 with a *bursa aurealis* transposon insertion in *recF*, Ery^r	(53)
USA300 LAC JE2 *dinB*::Tn	USA300 LAC JE2 with a *bursa aurealis* transposon insertion in *dinB*, Ery^r	(53)
USA300 LAC JE2 *rexB*::Tn p*itet* empty	USA300 LAC JE2 with a *bursa aurealis* transposon insertion in *rexB* with integrated p*itet* empty plasmid, Ery^r	This study
USA300 LAC JE2 *rexB*::Tn p*itet rexB*	USA300 LAC JE2 with a *bursa aurealis* transposon insertion in *rexB* with integrated p*itet* with AHT-inducible *rexB*, Ery^r	This study
USA300 LAC JE2 +pCN34 recA-gfp	USA300 LAC JE2 containing pCN34 with gfp under the control of the *recA* promotor, Kan^r	This study
USA300 LAC JE2 *rexB*::Tn +pCN34 recA-gfp	USA300 LAC JE2 with a *bursa aurealis* transposon insertion in *rexB* containing pCN34 with gfp under the control of the *recA* promotor, Kan^r	This study
USA300 LAC JE2 +pCN34 Empty	USA300 LAC JE2 containing pCN34 empty vector, Kan^r	This study
USA300 LAC JE2 *rexB*::Tn +pCN34 Empty	USA300 LAC JE2 with a *bursa aurealis* transposon insertion in *rexB* containing pCN34 empty vector, Kan^r	This study
Methicillin-sensitive *S. aureus*		
SH1000	Methicillin sensitive *S. aureus* strain	(54)
SH1000 *rexB*::Tn	SH1000 with a *bursa aurealis* transposon insertion in *rexB*, Ery^r	(20)
SH1000 *rexB*::Tn p*itet* empty	SH1000 with a *bursa aurealis* transposon insertion in *rexB* with integrated p*itet* empty plasmid, Ery^r	This study
SH1000 *rexB*::Tn p*itet rexB*	SH1000 with a *bursa aurealis* transposon insertion in *rexB* with integrated p*itet* with AHT-inducible *rexB*, Ery^r	This study
SH1000 +pCN34 recA-gfp	SH1000 containing pCN34 with gfp under the control of the *recA* promotor, Kan^r	This study
SH1000 +pCN34 Empty	SH1000 containing pCN34 empty vector, Kan^r	This study

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675 **Table 2. Primers used for construction of complemented *rexB* mutants and *recA-gfp* reporter**

Oligonucleotide	**Sequence 5'-3'**	676
Primers used for construction *rexB*::Tn p*rexBA* complemented mutant (restriction sites underlined)		677
rexB-F AvrII	GGCCCTAGGATGACATTACATGCTTATTTAG	678
rexA-R PmeI	GCCGTTTAAACCTATAGTTGCAATGTACCAAATTTG	679
pCL55 Fwd seq	GGATCCCCTCGAGTTCATG	
pCL55 Rev seq	CTCGTAGTATCTATACTTCG	680
Lipase *geh* F	GTTGTTTTTGTACATGGATTTTTAG	681
Lipase *geh* R	CTTGCTTTCAATTGTGTTCC	682
pCL55 R	GCGCATAGGTGAGTTATTAGC	
Primers used for construction of the *recA-gfp* reporter (restriction sites underlined)		683
P*recA*-F BamHI	GAGGATCCTATGGTTCAGATGACACAT	684
P*recA*-R 7xT-GFP	CATTTTTTTTCCTCCTAATTGAAATTGC	685
GFP-F 7xA-P*recA*	AGGAAAAAAAATGAGTAAAGGAGAAGAACT	
GFP-R KpnI	GCGGGTACCTTATTTGTATAGTTCATCCATG	686
pCN34 seq F	GTTATCCCCTGATTCTGTGGATAAC	687
pCN34 seq R	CCAGAATTATATTCAGAACAGGAAC	688

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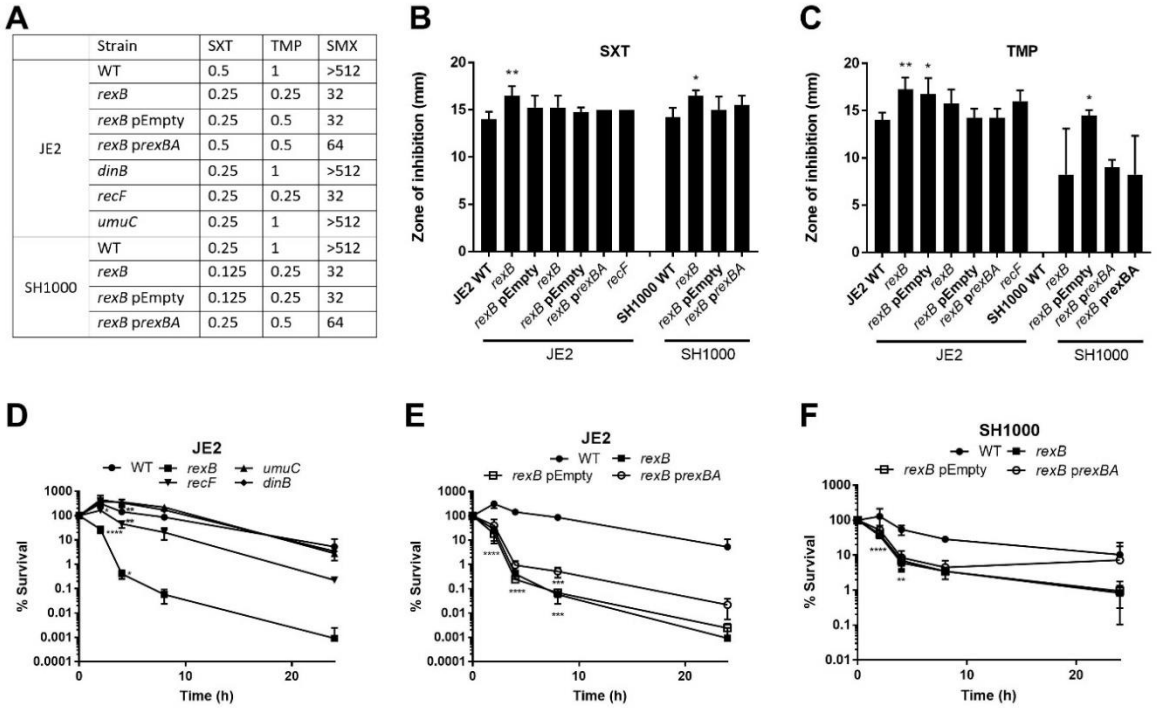
699 **Figures**



	Strain	SXT	TMP	SMX
JE2	WT	0.5	1	>512
	rexB	0.25	0.25	32
	rexB pEmpty	0.25	0.5	32
	rexB prexBA	0.5	0.5	64
	dinB	0.25	1	>512
	recF	0.25	0.25	32
	umuC	0.25	1	>512
SH1000	WT	0.25	1	>512
	rexB	0.125	0.25	32
	rexB pEmpty	0.125	0.25	32
	rexB prexBA	0.25	0.5	64

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701 **Figure 1. Mutants defective for DNA repair have increased susceptibility to SXT and its**
702 **constituent antibiotics TMP and SMX.** (A) MIC values for SXT, TMP and SMX for wild-type *S.*
703 *aureus* JE2 and SH1000 and mutants defective for DNA damage repair in TSB. Data represent
704 median values from n=3. (B,C) Zones of growth inhibition of WT JE2 and SH1000 *S. aureus*
705 strains or damage-repair mutants from paper discs impregnated with 2.5 µg SXT (B) or TMP
706 (C) after 16 h of incubation on TSA plates. Graphs represents mean±s.e.m from n=4. Values
707 that are significantly different from the WT within each antibiotic-exposure were identified by
708 one-way ANOVA). (D,E,F) Time-course survival assays of *S. aureus* JE2 (D,E) and SH1000 (F)
709 and their derived DNA damage-repair mutants during incubation in TSB supplemented with 4
710 µg ml^{-1} SXT. Data are split between D and E for clarity. Percentage survival at each time point
711 was calculated relative to the starting inoculum. Graphs represent mean±s.e.m from n=3.
712 Values that are significantly different from the WT were identified by two-way ANOVA

713 corrected for multiple comparison using the Dunnett method. $P \leq 0.05$(*), $P \leq 0.01$(**), $P \leq$

714 0.001(***), $P \leq 0.0001$(****).

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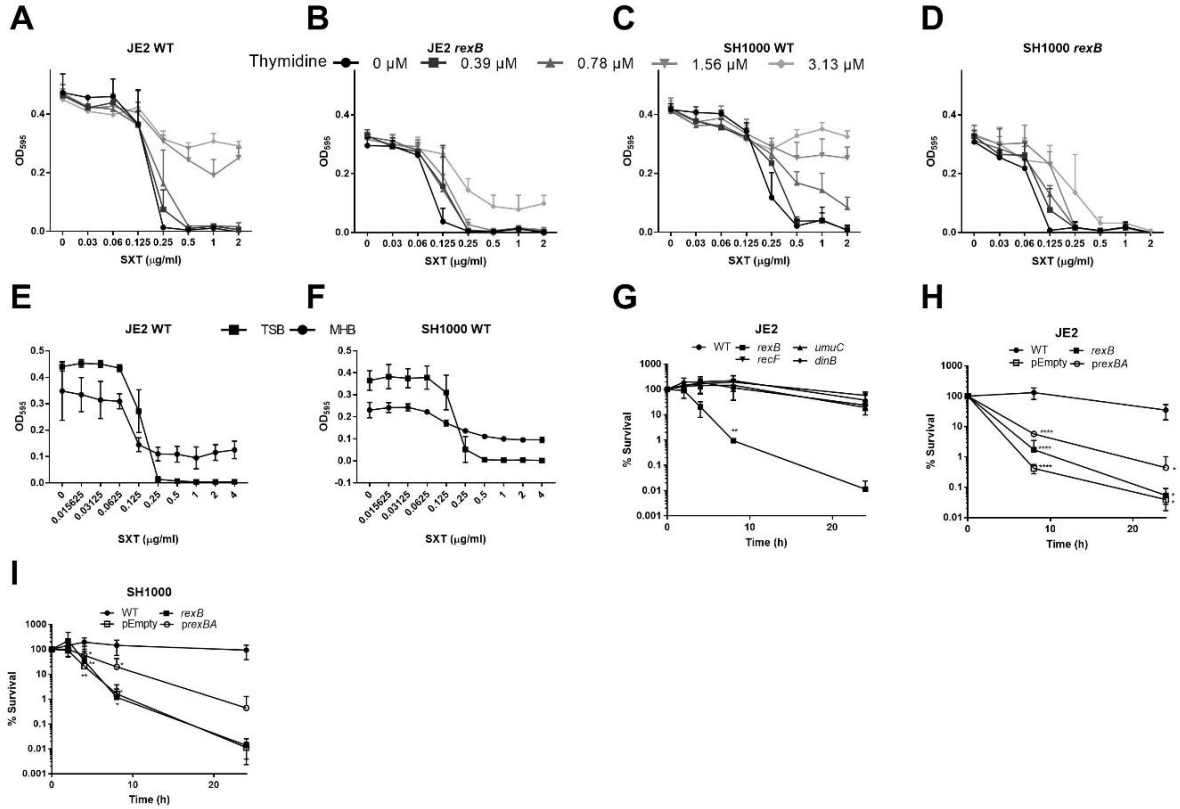

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732 **Figure 2. Mutants defective for *rexB* have increased susceptibility to SXT under thymidine-**
733 **rich conditions.** (A-D) Growth (OD_{600}) of *S. aureus* JE2 WT (A) a JE2-derived *rexB* mutant (B),
734 WT SH1000 (C) and SH1000-derived *rexB* mutant (D) in the presence of doubling
735 concentrations of SXT in TSB supplemented with 0 - 3.13 µM thymidine as indicated. Graphs
736 represent mean±s.e.m from n=3. (E-F) Growth (OD_{595}) of S*. aureus* JE2 WT (E) and WT SH1000
737 (F) in TSB and MHB supplemented with doubling dilution concentrations of SXT. Graphs
738 represent mean±s.e.m from n=3. (G-I) Time-course survival assays of *S. aureus* JE2 WT or
739 mutants defective for DNA damage repair (G), *S. aureus* JE2 WT, *rexB* mutant only, or
740 transformed with empty vector (pEmpty) or complemented mutant (p*rexBA*) (H) and SH1000
741 WT, *rexB* mutant only and *rexB* mutant transformed with pEmpty or p*rexBA* (I). For (G-I) all
742 strains were incubated in MHB containing 4 µg ml^{-1} SXT. Graphs represent mean±s.e.m from
743 n=3. Values that are significantly different from the WT were determined by two-way ANOVA

corrected for multiple comparison using the Dunnett method. $P \leq 0.05$(*), $P \leq 0.01$(**), $P \leq 0.001$(***), $P \leq 0.0001$(****).

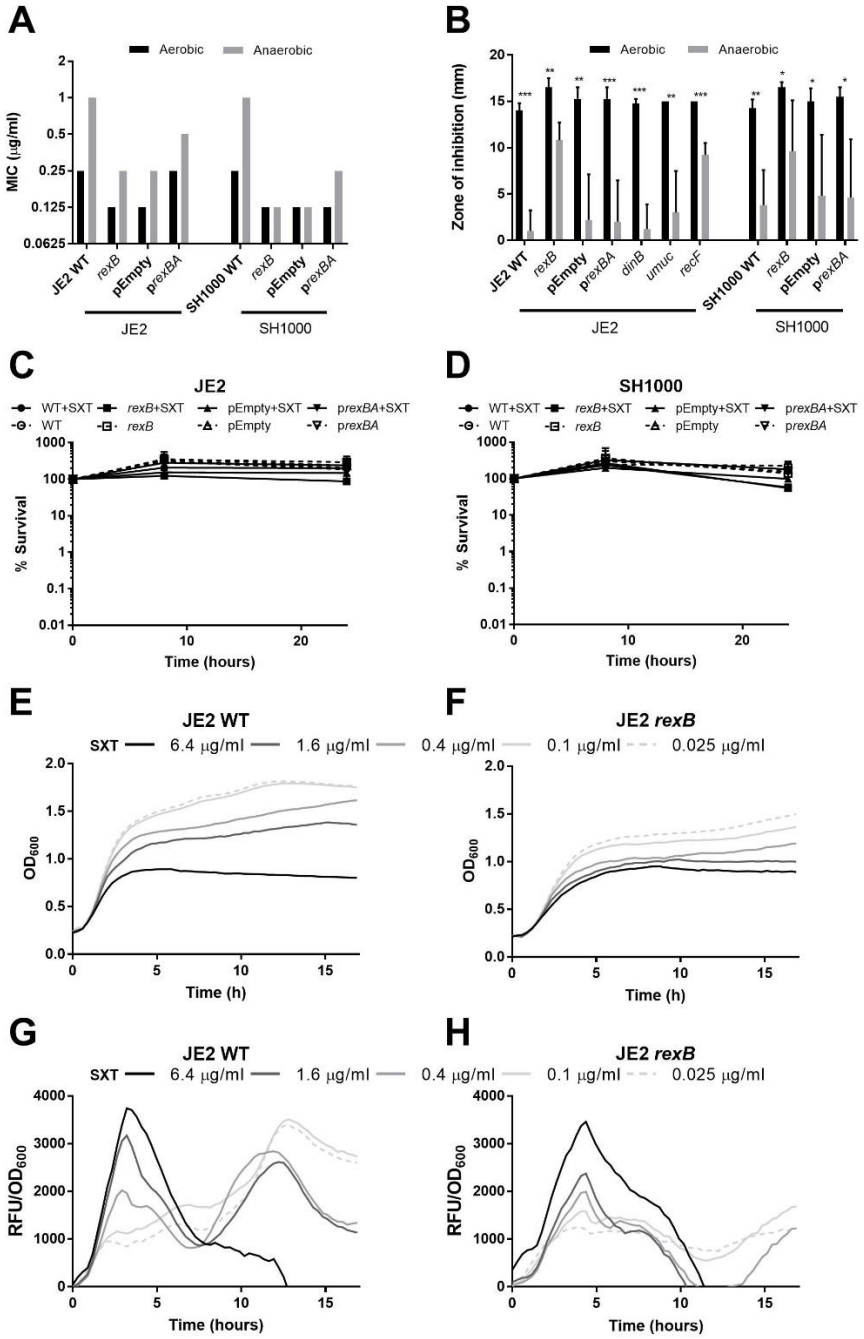

Figure 3. Reactive oxygen species are essential for SXT-mediated killing of *S. aureus*. (A) MIC of SXT for WT *S. aureus* JE2 and SH1000 and their derived mutants defective for DNA repair determined in TSB under aerobic or anaerobic conditions. Graphs represent median values from n=3. (B) Zones of inhibition around paper discs containing SXT (2.5 µg) on agar plates inoculated with *S. aureus* JE2 WT or SH1000 WT and associated DNA repair mutants on TSA

769 after 16 h of incubation under aerobic (black) or anaerobic (grey) conditions. The graph
770 represents mean±s.e.m from n=4. Zones which are significantly different in size between
771 aerobic and anaerobic conditions were determined by a two-tailed Student's t-test. (C,D)
772 Time-course survival assays of *S. aureus* JE2 (C) and SH1000 (D), *rexB* mutants without or with
773 empty vector (pEmpty) or complemented (p*rexBA*) in TSB with (+SXT) or without 4 µg ml^{-1} SXT,
774 under anaerobic conditions. Graphs represent mean±s.e.m from n=3. Values that are
775 statistically different from the WT were determined by two-way ANOVA corrected for multiple
776 comparison using the Sidak method. $P \leq 0.05$(*), $P \leq 0.01$(**), $P \leq 0.001$(***), $P \leq 0.0001$(****).
777 (E-F) Detection of growth inhibition (OD_{600}) of WT JE2 (E) and *rexB* mutant (F) in TSB in the
778 presence of SXT. (G-H) Detection of ROS production by bacteria using the DCF fluorophore for
779 *S. aureus* JE2 WT (G) or *rexB* mutant (H) in the presence of SXT. RFU generated by ROS were
780 normalised to OD_{600} data (E,F) to determine ROS production relative to cell density. (E-H) Error
781 bars were omitted for clarity.

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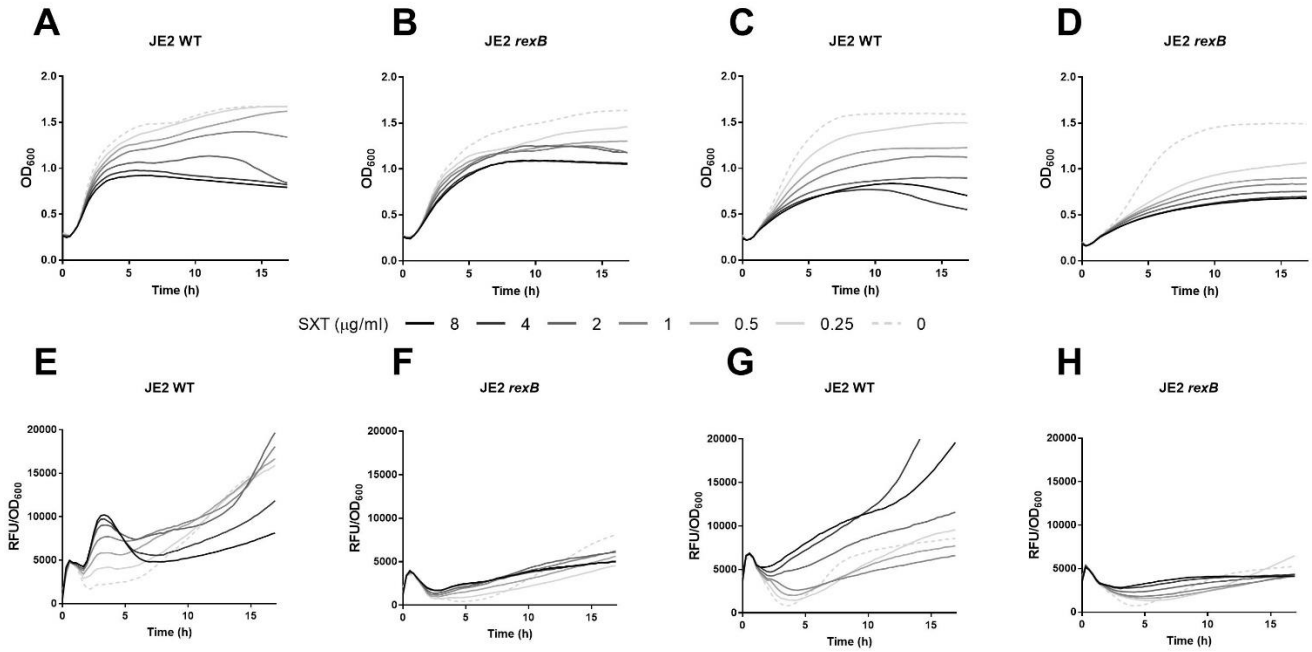
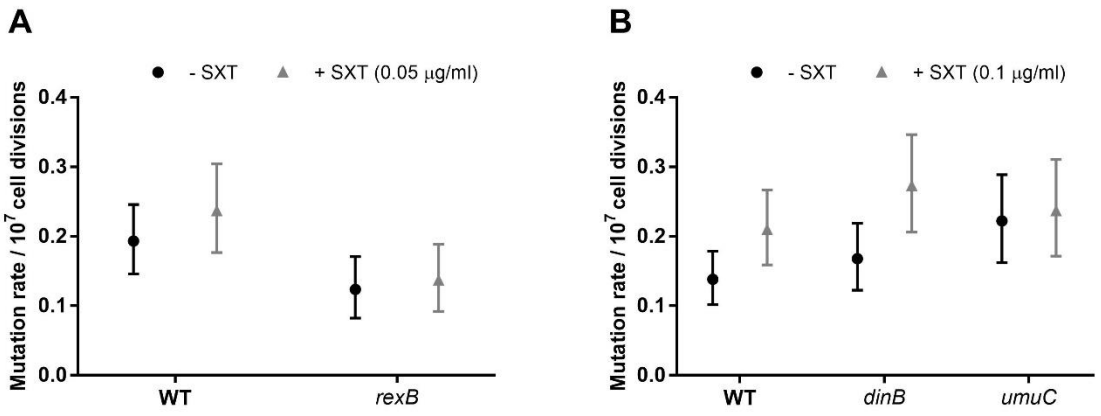


Figure 4. RexAB is essential for the initiation of the SOS response in response to SXT. (A-D) Growth (OD_{600}) of WT JE2 (A,C) and *rexB* mutant (B, D) in low thymidine TSB (A,B) and high thymidine MHB (C,D) in the presence of a range of SXT concentrations. (E-H) Expression of *recA-gfp* in response to SXT concentrations in *S. aureus* JE2 WT (E,G), *rexB* mutant (F,H) in TSB (E, F), or MHB (G-H). Fluorescence generated by GFP was measured and normalized to OD_{600} readings to relate *recA* expression relative to cell number. (A-H) Graphs represent mean values from n=3. Error bars were omitted for clarity.



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804 **Figure 5. RexAB and UmuC are required for the increased mutation rate mediated by SXT.**

805 (A,B) The mutation rate of *S. aureus* JE2 or mutants lacking components of DNA repair in TSB

806 in the absence or presence of 0.25 µg ml^{-1} (A) or 0.5 µg ml^{-1} (B) SXT. Error bars represent 95%

807 confidence intervals.