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

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Abstract

Background

Co-trimoxazole (SXT) is increasingly used to treat skin and soft-tissue infections caused by methicillin-resistant *Staphylococcus aureus*. However, the spontaneous emergence of resistance to this antibiotic is a frequent complication. Previous work has implicated DNA damage as an important contributor to the bactericidal activity of SXT, although the mechanism responsible was unclear.

Objectives

The aim of this work was to identify the mechanisms by which SXT damages *S. aureus* DNA, the repair systems used to withstand the damage and the consequences of repair for the emergence of resistance.

Methods

The susceptibility of *S. aureus* wild-type and mutants defective for DNA repair to SXT were determined through MIC and bactericidal activity assays. A *gfp-recA* reporter system enabled the detection of induction of the SOS response when exposed to SXT under various environmental conditions.

Results

Exposure of *S. aureus* to SXT caused DNA damage in *S. aureus* via both thymidine limitation and the generation of reactive oxygen species. 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 spontaneously-resistant strains during SXT therapy.

Conclusions

This work identified two distinct mechanisms by which SXT causes DNA damage in *S. aureus*, which is processed by the RexAB complex, leading to mutagenesis. Small molecule inhibitors of RexAB could have therapeutic value by increasing the efficacy of SXT and decreasing the emergence of drug-resistance.

52 ## Introduction

53 *Staphylococcus aureus* is responsible for a wide spectrum of diseases ranging from superficial skin
54 infections to life-threatening bacteraemia, endocarditis and toxic shock syndrome (1). Whilst β-lactam
55 antibiotics are first choice therapeutics for *S. aureus* infections, the prevalence of skin and soft tissue
56 infections (SSTIs) caused by methicillin-resistant *S. aureus* (MRSA) strains has necessitated the use of
57 second line therapeutics including co-trimoxazole (SXT), which is comprised of trimethoprim (TMP)
58 and sulfamethoxazole (SMX) (2). However, SXT therapy is ineffective against deep-seated infections
59 and long-term use often leads to the emergence of resistance.

60 SXT has been proposed to cause DNA damage by limiting the production of thymine,
61 leading to stalled replication forks (3, 4). Whilst the bacterium attempts to repair this damage by
62 inducing the mutagenic SOS response, it is not clear whether this contributes to staphylococcal survival
63 during antibiotic exposure. This is important because a greater understanding of the mechanisms by
64 which *S. aureus* survives SXT exposure may identify targets for new therapeutics that enhance
65 treatment efficacy. Therefore, the aims of this work were to identify the mechanisms by which SXT
66 damages *S. aureus* DNA, the repair systems that this pathogen uses to withstand this damage and the
67 consequences of repair for the emergence of resistance.

68

69 ## Materials and Methods

70 **Bacterial strains and culture conditions**

71 The bacterial strains used in this study are listed in Table 1. *S. aureus* was cultured in Tryptic Soy Broth
72 (TSB) or Mueller Hinton Broth (MHB) supplemented with calcium (25 mg L^{-1}) and magnesium (12.5 mg
73 L^{-1}), to stationary phase (18 h) at 37 °C, with shaking (180 RPM). Media were supplemented with
74 antibiotics as required.

75 Mutants lacking functional *rexB* were complemented using p*itet* (5) containing the wild-type
76 *rexBA* operon. Primers (Table 2) were used to amplify the *rexBA* operon from USA300 JE2 genomic

77 DNA. The PCR product and p*itet* vector were digested and ligated using T4 ligase and transformed into
78 $CaCl_2$-competent *E. coli* DH5α cells. Plasmid DNA was passaged through *E. coli* DC10B before
79 electroporation into S*. aureus rexB* mutants. Empty vector that did not contain the *rexBA* operon was
80 used as a control.
81
82 **Minimum Inhibitory Concentration (MIC) Assay**
83 The MIC was determined using the broth microdilution protocol described previously (6). The
84 concentration of SXT was calculated based on the concentration of TMP. MICs were determined by
85 incubation in air or in anaerobic jars containing Anaerogen gas packs at 37 °C for 18 h.
86
87 **SXT killing kinetics**
88 Washed stationary-phase *S. aureus* cells were inoculated into fresh MHB or TSB (3 ml) to a density of
89 2 x 10^8 CFU ml^{-1} containing 4 µg ml^{-1} SXT and incubated at 37°C with shaking (180 RPM). Bacterial
90 survival was determined over time by quantification of colony-forming units using the Miles and Misra
91 method (7). Percentage survival was calculated by comparing CFU counts at specific time points with
92 the starting inoculum. Similar studies were performed anaerobically in pre-reduced broth in bijoux
93 tubes, supplemented with 4 µg ml^{-1} SXT, and incubated statically at 37 °C with the vessel caps loosened
94 in an anaerobic jar containing an Anaerogen gas pack.
95
96 **Disc-diffusion Assay**
97 Stationary phase *S. aureus* cultures were adjusted to an OD_{600} of 0.063 (0.5 McFarland) in TSB and used
98 to inoculate TSA plates using a cotton swab. Antibiotic discs containing 2.5 µg of either SXT or TMP
99 (Oxoid) were placed onto the inoculated agar and incubated at 37 °C under aerobic or anaerobic
100 conditions for 17 h. The zone of inhibition was measured as the total diameter of the cleared bacterial
101 lawn, minus the diameter of the antibiotic disc.
102

103 **Mutation rate analyses**

104 Mutation rates were determined as described previously (8, 9). Briefly, thirty parallel cultures of *S.*
105 *aureus* adjusted to ~5 x 10^5 CFU ml^{-1} for each condition or strain were inoculated into TSB with or
106 without 0.05 µg ml^{-1} or 0.1 µg ml^{-1} SXT and incubated at 37 °C, with shaking, for 24 h. After incubation,
107 10 cultures were selected at random, and total CFU counts determined. Subsequently, 100 µl of each
108 of the 30 cultures were spread on TSA plates containing rifampicin (100 µg ml^{-1}) before incubation at
109 37 °C for 24 h. The number of rifampicin-resistant colonies on each plate were counted, and the
110 mutation rate with 95% confidence intervals calculated using the maximum-likelihood setting of the
111 FALCOR mutation rate calculator (8).

112

113 ***recA*-reporter assay**

114 Induction of the SOS response in *S. aureus* was determined using strains containing a *recA-gfp* reporter
115 construct. This was constructed by transforming *S. aureus* with pCN34 containing *gfp* under the control
116 of the *recA* promoter. Primers (Table 2) were used to amplify the *recA* promoter region (3) from JE2
117 wild-type genomic DNA, and the *gfp* gene from the pCL55 P3 GFP plasmid (10). These products were
118 combined by overlapping extension PCR, digested and ligated into phosphatase-treated and digested
119 pCN34. Empty vector without the *recA-gfp* construct served as a control. Vectors were constructed in
120 *E. coli* DC10B and transformed directly with into *S. aureus* WT JE2 or *rexB*::Tn mutant.

121 Stationary phase cultures of *recA-gfp* reporter strains washed twice and resuspended in TSB
122 supplemented with kanamycin (90 µg ml^{-1}) to maintain the plasmid. Cultures at ~3.33 x 10^8 CFU ml^{-1}
123 were exposed to a range of SXT concentrations (0.01 µg ml^{-1} to 8 µg ml^{-1}) in black-walled microtiter
124 plates with shaking at 37 °C for 17 h in a TECAN Infinite® 200 PRO microplate reader, where OD_{600} and
125 GFP relative fluorescent units (RFU) (Excitation: 475 nm; Emission: 525 nm) was measured every 1000
126 seconds. Measurements were blanked against uninoculated wells and GFP fluorescence normalised to
127 OD_{600} readings to determine *recA* expression levels.

128

129 **Endogenous Reactive oxygen species (ROS) production**

130 Production of ROS was detected using the cell permanent 2',7'-dichlorodihydrofluorescein diacetate
131 dye (H_2DCFDA), which is converted to fluorescent DCF by oxidative cleavage of acetate groups (11). A
132 96 well microtitre plate assay was prepared in a similar format to the *recA-gfp* reporter assay, with the
133 addition of 25 µM H_2DCFDA and various concentrations of SXT. Bacterial strains were washed and
134 inoculated to ~3.33 x 10^8 CFU ml^{-1}. Plates were incubated with shaking at 37 °C as described previously,
135 and OD_{600} and RFU (Excitation: 495 nm; Emission: 525nm) was measured every 1000 s. OD_{600} values
136 were blanked against uninoculated wells and RFU values blanked to untreated wells. Fluorescence
137 values were normalised to OD_{600} readings to determine production of ROS relative to cell number.

138

139 **Statistical Analysis**

140 Means or medians were calculated from at least three biologically independent replicates and
141 analysed by Student's t test (two tailed, unpaired, assuming equal variances), One-way ANOVA or Two-
142 way ANOVA corrected for multiple comparison using GraphPad Prism (V7.0) as described in the figure
143 legends.

144

145 Results

146 **Inactivation of *rexB* sensitises *S. aureus* to SXT**

147 To investigate the role of bacterial DNA repair in the susceptibility of *S. aureus* to SXT, a panel of *S.*
148 *aureus* transposon mutants previously used to study mutagenic DNA repair during exposure to H_2O_2
149 (*rexA, rexB*, *recF*, *umuC* and *dinB*) (9), were subjected to MIC and disc diffusion susceptibility assays.
150 Strains from two distinct genetic backgrounds were used: SH1000, a methicillin-sensitive *S. aureus*
151 (MSSA) strain, and JE2, a community-associated methicillin-resistant *S. aureus* (CA-MRSA) strain of the
152 USA300 lineage, responsible for severe skin infections that are often treated with SXT (12, 13).

153 The MIC values for both wild-type (WT) strains indicated susceptibility to SXT and TMP (< 2 µg
154 ml^{-1}), but resistance to SMX (>512 µg ml^{-1}) (14) (Fig. 1A). Disruption of *rexB* in both JE2 and SH1000

155 strains lowered the MIC of SXT and TMP by 2-4-fold and 16-fold for SMX, whilst complementation of
156 the *rexB* mutant either fully or partially restored WT levels of susceptibility (Fig. 1A). The JE2 *recF*
157 mutant was also more susceptible than the WT to all three antibiotics, however, *dinB* or *umuC* mutants
158 only resulted in a slightly increased susceptibility to SXT.

159 Consistent with the MIC data, disc diffusion assays showed that inactivation of *rexB*
160 significantly increased the susceptibility of *S. aureus* to SXT and TMP, relative to the WT (Fig. 1B,C).
161 Complementation of the *rexB* mutant (p*rexBA*) reduced susceptibility close to WT levels, confirming
162 that changes in susceptibility were attributed to the function of RexAB complex (Fig. 1B,C). By contrast,
163 inactivation of *dinB or umuC* did not significantly increase zone sizes for either antibiotic (Fig. 1B,C).
164 However, the *recF*-deficient mutant showed an increase in susceptibility to TMP in the JE2 strain (Fig.
165 1C), indicating that both *rexB* and *recF* contribute to the repair of DNA damage caused by TMP.

166 Next, we investigated the role of DNA repair in the survival of *S. aureus* exposed to SXT at the
167 clinical breakpoint concentration (4 µg ml^{-1}) (14). There was a ~20-fold reduction in survival over 24 h
168 for the WT, *umuC* or *dinB* mutants in the JE2 background (Fig. 1D), indicative of relatively high tolerance
169 to SXT. By contrast, survival of the *recF* mutant was reduced by >20-fold, whilst the *rexB* mutant
170 showed >5000-fold reduction in CFU counts relative to the WT after 24 h. Complementation of the
171 *rexB* mutant increased survival 10-fold relative to a plasmid only control (Fig. 1E). Studies with SH1000
172 confirmed a role for *rexBA* in modulating susceptibility of *S. aureus* to SXT, with a 12-fold greater loss
173 of CFU counts of the *rexB* mutant relative to WT (Fig. 1F), whilst complementation of the *rexB* mutant
174 restored survival levels close to those of the WT (Fig. 1F). Combined, these findings indicate that SXT
175 causes DNA double-strand breaks in *S. aureus*, the repair of which by RexAB significantly promotes
176 staphylococcal survival during exposure to the antibiotic.

177

178 **Thymidine limitation contributes to SXT-mediated DNA damage**

179 Inhibition of folate biosynthesis by SXT results in abrogation of endogenous thymidine biosynthesis,
180 which could provide an explanation for the DNA damage phenotype described above (Fig. 1).

181 Experiments detailed thus far were performed in TSB, which contains a low concentration of thymidine
182 (0.25 µM, (15)). To investigate the influence of exogenous thymidine on SXT activity, the growth of WT
183 and *rexB* mutants were determined in TSB supplemented with doubling dilutions of SXT and thymidine
184 via chequerboard assays (Fig. 2A-D). As expected, the presence of high concentrations of thymidine
185 enabled growth of WT strains of both JE2 and SH1000 at normally growth inhibitory concentrations of
186 SXT (15) (Fig. 2A,C).

187 The thymidine-dependent activity of SXT means that culture media compositions greatly affect
188 antibiotic susceptibility (15, 16). For example, Mueller-Hinton Broth (MHB) contains >16-fold more
189 thymidine than TSB (15). Consistent with the chequerboard data, the efficacy of SXT in MHB was
190 reduced relative to TSB, with detectable bacterial growth of both WT strains observed even at high
191 concentrations of SXT, likely attributable to the elevated thymidine concentration (Fig. 2E,F).

192 To explore the effect of exogenous thymidine on the bactericidal activity of SXT, killing assays
193 were undertaken in MHB. In contrast to TSB (Fig. 1D) there was almost no killing of the *S. aureus* JE2
194 WT and *dinB*, *umuC* and *recF* mutants by SXT (4 µg ml^{-1}) in MHB over 24 h (Fig. 2G). However, despite
195 the presence of exogenous thymidine, survival of the *rexB* mutant was >2000-fold lower than that of
196 the WT, (Fig. 2G), whilst complementation of the mutant (p*rexBA*) significantly promoted survival
197 relative to a plasmid only control (Fig. 2H). Similar results were seen for the SH1000 strain, with the
198 WT surviving, whilst viability of the *rexB* mutant was reduced >6000-fold relative to the inoculum (Fig.
199 2I), with complementation significantly promoting survival (> 10-fold) (Fig. 2I).

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

203

204 **Inactivation of *rexBA* sensitises *S. aureus* to SXT-mediated growth inhibition but not killing under**
205 **anaerobic conditions**

206 To determine whether the production of reactive oxygen species (ROS) contributes to SXT activity
207 against *S. aureus*, the susceptibility of WT and DNA repair mutants to the antibiotic was determined
208 under both aerobic and anaerobic conditions in low-thymidine TSB. In the absence of oxygen, the MIC
209 of WT *S. aureus* was increased 4-fold for both strains (Fig. 3A). By contrast, the SXT MIC of the JE2 *rexB*
210 mutant only doubled under anaerobic conditions, whilst the MIC of the SH1000 *rexB* mutant was
211 unaffected by the presence of oxygen (Fig. 3A). However, zones of inhibition from SXT antibiotic discs
212 were significantly reduced in the absence of oxygen for all strains and mutants, implying reduced
213 susceptibility (Fig. 3B). Despite the reduced antibiotic activity of SXT under anaerobic conditions, the
214 *rexB* mutants were still more susceptible than the corresponding WT, with >10 and 2.5-fold greater
215 zone sizes for JE2 and SH1000 strains respectively (Fig. 3B).

216 Next, we assessed the contribution of ROS to the bactericidal activity of SXT. In contrast to
217 assays performed in air, the WT strains were able to grow slightly under these conditions, however,
218 SXT prevented growth of the *rexB* mutants >2-fold by 24 h post inoculation (Fig. 3C,D).
219 Complementation of the *rexB* mutant restored the WT phenotype (Fig. 3C,D). Therefore, SXT exposure
220 causes bacteriostasis in the *rexBA* mutant but not the WT under anaerobic conditions.

221 The reduced activity of SXT under anaerobic conditions suggested that ROS contribute to the
222 bactericidal activity of SXT. Therefore, H_2DCFDA dye was added to growth inhibition assays (Fig. 3E,F)
223 to quantify and study the kinetics of ROS production during incubation under aerobic conditions. SXT
224 induced a dose-dependent fluorescent peak 4 h post inoculation for both WT and *rexB* mutant strains
225 in the JE2 background (Fig. 3G,H), indicative of the generation of ROS in response to the antibiotic. A
226 second peak in ROS production after 13 h is apparent at lower concentrations of SXT as cells reach
227 stationary phase, which is likely a result of the bacterial starvation response associated with increased
228 endogenous oxidative stress (17).

229 Combined, these data provide strong evidence that SXT induces ROS that contribute to the
230 growth inhibitory activity and are essential for the bactericidal activity of the drug. Furthermore, these
231 findings demonstrate that SXT triggers similar levels of oxidative stress in both WT and *rexB* mutant,

232 indicating that the increased susceptibility of the mutant to the antibiotic is due to an inability to repair
233 damage, rather than increased generation of ROS.
234
235 **RexAB is required for SXT-triggered induction of the SOS response**
236 Given the importance of RexAB for the repair of DNA damage caused by SXT, we wanted to determine
237 how it affected the induction of the mutagenic SOS response associated with spontaneous antibiotic
238 resistance emergence.
239 Using a range of concentrations of SXT that induced a dose-dependent inhibition of growth for
240 both the JE2 WT and *rexB* mutant (Fig. 4A-D), the induction of *recA* expression was determined using
241 a *recA-gfp* reporter construct (Fig. 4E-H). In low-thymidine TSB, SXT exposure resulted in a dose-
242 dependent increase in GFP signal (Fig. 4E) similar to that observed for ROS production (Fig. 3G), which
243 peaked at ~4 h, and then subsided before a second peak as the bacteria entered stationary phase (17,
244 18). However, whilst SXT triggered ROS production in the *rexB* mutant (Fig. 3H), the antibiotic did not
245 induce *recA* expression in this strain (Fig. 4F), revealing that RexAB is required for activation of the SOS
246 response.
247 In contrast to experiments done in TSB, *recA* was only weakly expressed during exposure to SXT in
248 high-thymidine MHB (Fig. 4D), which is in keeping with the reduced activity of the antibiotic in this
249 medium (Fig. 2E,F). Consistent with the data from studies in TSB, there was a lack of *recA* expression
250 in the JE2 *rexB* mutant in response to SXT in MHB (Fig. 4E).
251
252 **RexAB is required for SXT-induced mutagenic DNA repair**
253 De-repression of the SOS regulon leads to expression of the error-prone translesion DNA polymerase
254 UmuC, which catalyses mutagenic DNA repair that contributes to the acquisition of antibiotic
255 resistance (9, 19).
256 To assess whether SXT promoted SOS-dependent mutagenesis in *S. aureus*, JE2 WT and DNA-
257 damage repair mutants were exposed to sub-inhibitory concentrations of SXT in low-thymidine TSB.

258 Since the *rexB* mutant was much more susceptible to SXT treatment than the WT, only a low
259 concentration of the antibiotic (0.05 µg ml^{-1}, 0.2 x MIC of the mutant) could be used in this analyses.
260 SXT treatment resulted in an increase in the mutation rate of WT *S. aureus* (Fig. 5A). By contrast to the
261 WT, SXT exposure did not increase the mutation rate of the *rexB* mutant, consistent with the inability
262 of the antibiotic to trigger induction of the SOS response in this strain (Fig. 4B, 5A).

263 To determine whether the SXT-mediated increase in the mutation rate was due to induction
264 of the SOS response, we exposed the WT and mutants lacking *umuC* or *dinB* (an error prone
265 polymerase that is not part of the SOS response in *S. aureus* (19)) to SXT at 0.2 x MIC of the mutants
266 (0.1 µg ml^{-1}). Consistent with the *recA* reporter data, the higher concentration of SXT used in these
267 experiments promoted the mutation rate of the WT strain to a greater extent than the lower dose (Fig.
268 4A, 5A,B). SXT caused a similar increase in the mutation rate of the *dinB* mutant but had no effect on
269 the mutation rate of the *umuC* mutant (Fig. 5B).

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

273

274 Discussion

275 SXT is effective in the treatment of skin and soft tissue infections caused by MRSA (20). However, its
276 efficacy is limited by the pathogens' ability to by-pass SXT-mediated inhibition of folate biosynthesis,
277 either via the uptake of extracellular metabolites or via resistance-conferring mutations (21).

278 The bactericidal effect of SXT has been ascribed to DNA damage, which leads to the induction
279 of the SOS response (3, 4). Our data support DNA damage as a key contributor to SXT bactericidal
280 activity against *S. aureus* and highlight the key role of RexAB in combatting this genotoxicity. Since
281 RexAB is proposed to be a member of the AddAB family of DNA repair enzymes (9, 22, 23), this finding
282 indicates that SXT causes DNA DSBs in *S. aureus*, which are lethal if not repaired. The protective role

283 of RexAB for *S. aureus* contrasts with recent findings for *E. coli*, where DNA repair contributes to the
284 lethality of trimethoprim (24).

285 The discovery that *S. aureus* is more susceptible to SXT in thymidine-poor medium is in keeping
286 with previous reports, which suggest that thymidine depletion contributes to the lethality of SXT via
287 the collapse of replication forks during DNA synthesis, which lead to DNA DSBs (4, 15, 25). However,
288 there was still a very large reduction in CFU counts of the *rexB* mutant exposed to SXT in the presence
289 of thymidine, indicating that thymidine limitation is not the only element responsible for DNA damage
290 in *S. aureus*.

291 In agreement with several reports that bactericidal antibiotics, including TMP, exert part of
292 their antibacterial effect via the generation of ROS (24, 26), we determined that the bactericidal effect
293 of SXT against *S. aureus* is dependent upon the availability of oxygen. Indeed, our data indicate that
294 ROS-mediated DNA damage makes a greater contribution to the bactericidal activity of SXT than the
295 inhibition of DNA-synthesis caused by thymidine depletion in aerobic conditions. However, whilst ROS
296 clearly make an important contribution to the bactericidal activity of SXT, the increased susceptibility
297 of the *rexB* mutant to this antibiotic under anaerobic conditions confirms that DNA damage can occur
298 independently of ROS. This may explain why there are phenotypic differences in the effects of SXT
299 exposure and thymineless death observed in *thyA* mutants deprived of thymine (24).

300 One drawback of SXT is the frequent emergence of resistance during long-term therapy (27).
301 In keeping with the induction of the SOS response, exposure of *S. aureus* to sub-inhibitory
302 concentrations of SXT was found to increase the mutation rate via UmuC, a low-fidelity DNA
303 polymerase, previously shown to mediate mutagenic DNA repair of bacteria exposed to ciprofloxacin
304 or H_2O_2 (9). Similar to what has been reported for ciprofloxacin and H_2O_2, SXT-induced increases in the
305 mutation rate was dependent upon RexAB (9).

306 In summary, the identification of RexAB as central to both SXT susceptibility and SOS
307 induction suggests that this protein complex is a promising target for novel therapeutic approaches

308 that could improve the efficacy of SXT and reduce the emergence of resistance. Such an approach may
309 also increase the range of MRSA infections that could be treated with SXT.
310

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407 **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	(28)
USA300 LAC JE2 *rexB*::Tn	USA300 LAC JE2 with a *bursa aurealis* transposon insertion in *rexB*, Ery^r	
USA300 LAC JE2 *umuC*::Tn	USA300 LAC JE2 with a *bursa aurealis* transposon insertion in *umuC*, Ery^r	
USA300 LAC JE2 *recF*::Tn	USA300 LAC JE2 with a *bursa aurealis* transposon insertion in *recF*, Ery^r	
USA300 LAC JE2 *dinB*::Tn	USA300 LAC JE2 with a *bursa aurealis* transposon insertion in *dinB*, Ery^r	
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	(29)
SH1000 *rexB*::Tn	SH1000 with a *bursa aurealis* transposon insertion in *rexB*, Ery^r	(9)
SH1000 *rexB*::Tn p*itet* empty	SH1000 with a *bursa aurealis* transposon insertion in *rexB* with integrated p*itet*	This study

	empty plasmid, Ery^r	
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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428 **Table 2. Primers used for construction of complemented *rexB* mutants and *recA-gfp* reporter**

Oligonucleotide	**Sequence 5'-3'**	429
Primers used for construction *rexB*::Tn p*rexBA* complemented mutant (restriction sites underlined)		430
rexB-F AvrII	GGCCCTAGGATGACATTACATGCTTATTTAG	431
rexA-R PmeI	GCCGTTTAAACCTATAGTTGCAATGTACCAAATTTG	432
pCL55 Fwd seq	GGATCCCCTCGAGTTCATG	433
pCL55 Rev seq	CTCGTAGTATCTATACTTCG	434
Lipase *geh* F	GTTGTTTTTGTACATGGATTTTTAG	435
Lipase *geh* R	CTTGCTTTCAATTGTGTTCC	436
pCL55 R	GCGCATAGGTGAGTTATTAGC	437
Primers used for construction of the *recA-gfp* reporter (restriction sites underlined)		438
P*recA*-F BamHI	GAGGATCCTATGGTTCAGATGACACAT	439
P*recA*-R 7xT-GFP	CATTTTTTTTCCTCCTAATTGAAATTGC	440
GFP-F 7xA-P*recA*	AGGAAAAAAAATGAGTAAAGGAGAAGAACT	441
GFP-R KpnI	GCGGGTACCTTATTTGTATAGTTCATCCATG	442
pCN34 seq F	GTTATCCCCTGATTCTGTGGATAAC	443
pCN34 seq R	CCAGAATTATATTCAGAACAGGAAC	444

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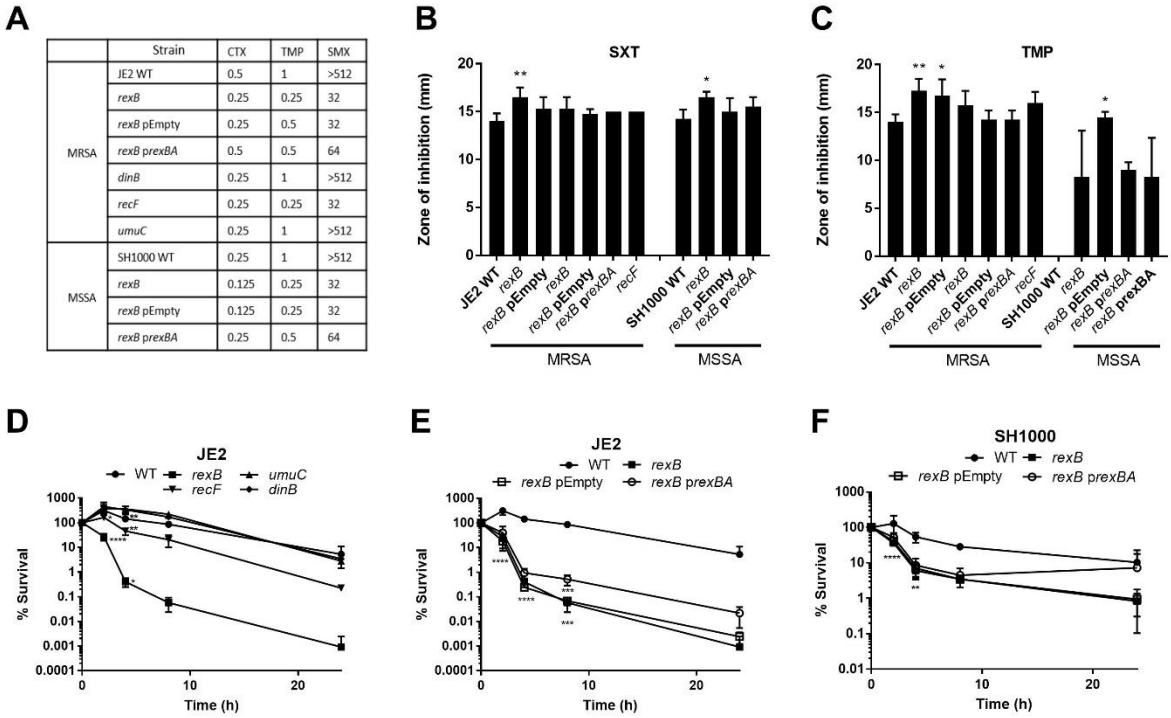
454 **Figures**



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456 **Figure 1. Mutants defective for DNA repair have increased susceptibility to SXT and its constituent**
457 **antibiotics TMP and SMX.** (A) MIC values for SXT, TMP and SMX for WT *S. aureus* JE2 (MRSA) and
458 SH1000 (MSSA) and mutants defective for DNA damage repair in TSB. Data represent median values
459 from n=3. (B,C) Zones of growth inhibition of WT MRSA and MSSA WT *S. aureus* strains or damage-
460 repair mutants from paper discs impregnated with 2.5 µg SXT (B) or TMP (C) after 16 h of incubation
461 on TSA plates. Graphs represents mean±SD from n=4. Values that are significantly different from the
462 WT within each antibiotic-exposure were identified by one-way ANOVA). (D,E,F) Time-course survival
463 assays of *S. aureus* JE2 MRSA (D,E) and SH1000 MSSA (F) and their derived DNA damage-repair mutants
464 during incubation in TSB supplemented with 4 µg ml^{-1} SXT. Data are split between D and E for clarity.
465 Percentage survival at each time point was calculated relative to the starting inoculum. Graphs
466 represent mean±SD from n=3. Values that are significantly different from the WT were identified by
467 two-way ANOVA corrected for multiple comparison using the Dunnett method. $P \leq 0.05$(*), $P \leq$
468 0.01(**), $P \leq 0.001$(***), $P \leq 0.0001$(****).

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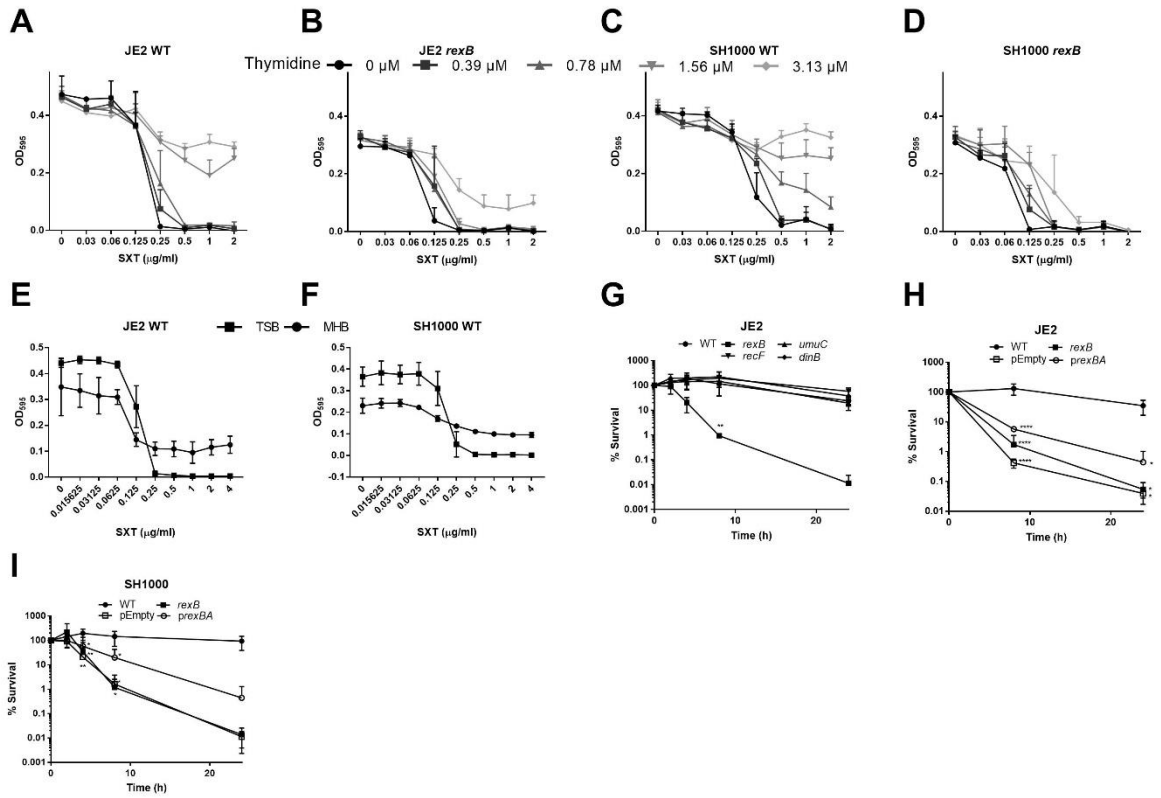

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471 **Figure 2. Mutants defective for *rexB* have increased susceptibility to SXT under thymidine-rich**
472 **conditions.** (A-D) Growth (OD_{600}) of *S. aureus* JE2 WT (A) a JE2-derived *rexB* mutant (B), WT SH1000 (C)
473 and SH1000-derived *rexB* mutant (D) in the presence of doubling concentrations of SXT in TSB
474 supplemented with 0 - 3.13 µM thymidine as indicated. Graphs represent mean±SD from n=3. (E-F)
475 Growth (OD_{595}) of S. *aureus* JE2 WT (E) and WT SH1000 (F) in TSB and MHB supplemented with doubling
476 dilution concentrations of SXT. Graphs represent mean±SD from n=3. (G-I) Time-course survival assays
477 of *S. aureus* JE2 WT or mutants defective for DNA damage repair (G), *S. aureus* JE2 WT, *rexB* mutant
478 only, or transformed with empty vector (pEmpty) or complemented mutant (p*rexBA*) (H) and SH1000
479 WT, *rexB* mutant only and *rexB* mutant transformed with pEmpty or p*rexBA* (I). For (G-I) all strains
480 were incubated in MHB containing 4 µg ml^{-1} SXT. Graphs represent mean±SD from n=3. Values that
481 are significantly different from the WT were determined by two-way ANOVA corrected for multiple
482 comparison using the Dunnett method. $P \leq 0.05$(*), $P \leq 0.01$(**), $P \leq 0.001$(***), $P \leq 0.0001$(****).

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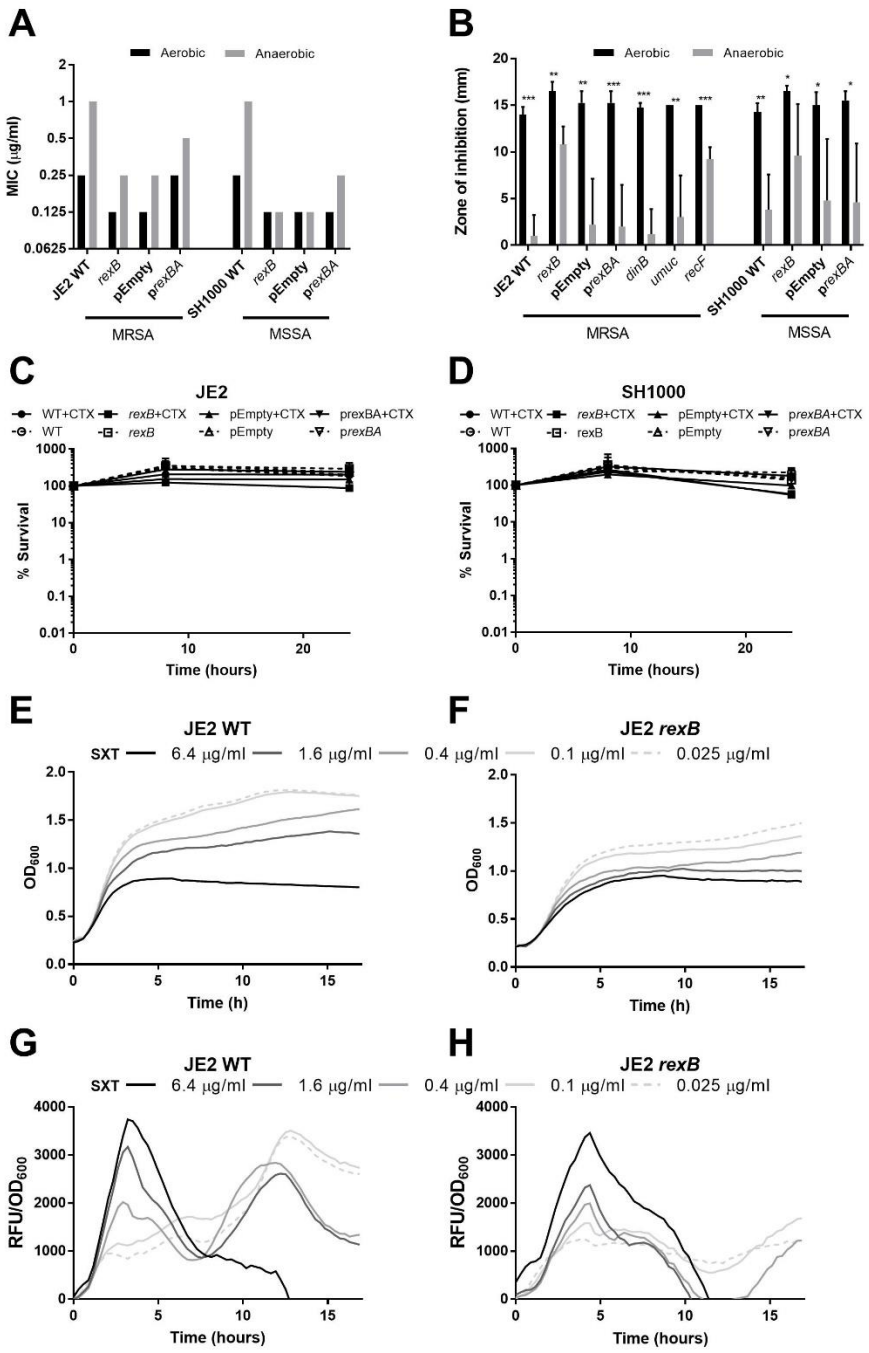

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485 **Figure 3. Reactive oxygen species are essential for SXT-mediated killing of *S. aureus*.** (A) MIC of SXT
486 for WT *S. aureus* JE2 and SH1000 and their derived mutants defective for DNA repair determined in
487 TSB under aerobic or anaerobic conditions. Graphs represent median values from n=3. (B) Zones of
488 inhibition around paper discs containing SXT (2.5 µg) on agar plates inoculated with *S. aureus* JE2 WT
489 (MRSA) and SH1000 WT (MSSA) and associated DNA repair mutants on TSA after 16 h of incubation
490 under aerobic (black) or anaerobic (grey) conditions. The graph represents mean±SD from n=4. Zones

491 which are significantly different in size between aerobic and anaerobic conditions were determined by
492 a two-tailed Student's t-test. (C,D) Time-course survival assays of *S. aureus* JE2 (MRSA) (C) and SH1000
493 (MSSA) (D), *rexB* mutants without or with empty vector (pEmpty) or complemented (p*rexBA*) in TSB
494 with (+SXT) or without 4 µg ml^{-1} SXT, under anaerobic conditions. Graphs represent mean±SD from
495 n=3. Values that are statistically different from the WT were determined by two-way ANOVA corrected
496 for multiple comparison using the Sidak method. $P \leq 0.05$(*), $P \leq 0.01$(**), $P \leq 0.001$(***), $P \leq$
497 0.0001(****). (E-F) Detection of growth inhibition (OD_{600}) of WT JE2 (E) and *rexB* mutant (F) in TSB in
498 the presence of SXT. (G-H) Detection of ROS production by bacteria using the DCF fluorophore for *S.*
499 *aureus* JE2 WT (G) or *rexB* mutant (H) in the presence of SXT. RFU generated by ROS were normalised
500 to OD_{600} data (E,F) to determine ROS production relative to cell density. (E-H) Error bars were omitted
501 for clarity.

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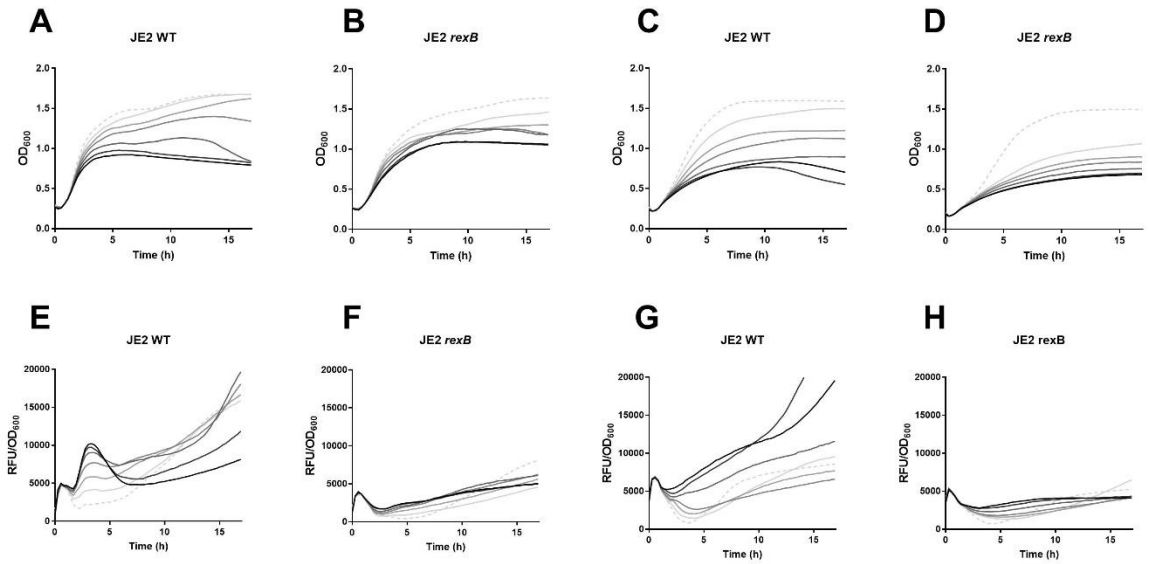

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515 **Figure 4. RexAB is essential for the initiation of the SOS response in response to SXT.** (A-D) Growth
516 (OD_{600}) of WT JE2 (A,C) and *rexB* mutant (B, D) in low thymidine TSB (A,B) and high thymidine MHB
517 (C,D) in the presence of a range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

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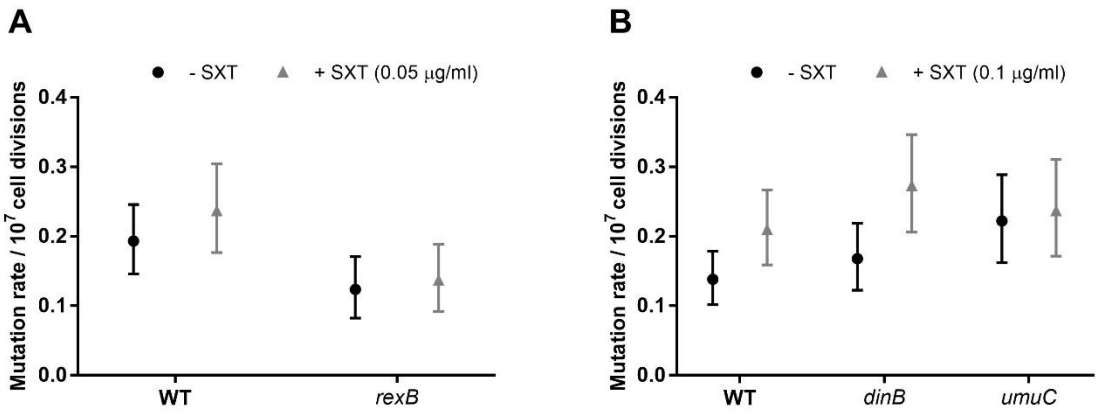

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529 **Figure 5. RexAB and UmuC are required for the increased mutation rate mediated by SXT.** (A,B) The
530 mutation rate of *S. aureus* JE2 or mutants lacking components of DNA repair in TSB in the absence or
531 presence of 0.25 µg ml^{-1} (A) or 0.5 µg ml^{-1} (B) SXT. Error bars represent 95% confidence intervals.