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

Rebecca S. Clarke¹, Maya S. Bruderer¹,², Kam Pou Ha¹ and Andrew M. Edwards¹*

¹ MRC Centre for Molecular Bacteriology and Infection, Imperial College London, Armstrong Rd, London, SW7 2AZ, UK.

² Current address: Department of Infection Biology, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland.

* These authors contributed equally.

* For correspondence:

Tel: 0044 (0)207 594 2072

Fax: 0044 (0)207 594 3096

a.edwards@imperial.ac.uk

Keywords: Reactive oxygen species / DNA damage / SOS response / *Staphylococcus aureus*.

Running title: Co-trimoxazole damages staphylococcal DNA
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. We found that mutants lacking the DNA repair complex RexAB had a modest 2-fold lower SXT MIC than wild-type strains but were killed 50-5000-fold more efficiently by the combination antibiotic at the breakpoint concentration. SXT-mediated DNA damage occurred via both thymidine limitation and the generation of reactive oxygen species, and triggered induction of the SOS response in a RexAB-dependent manner. SOS induction was associated with a 50% increase in the mutation rate, which may contribute to emergence of resistant strains during SXT therapy. In summary, this work determined that SXT caused DNA damage in *S. aureus* via both thymidine limitation and oxidative stress, which was 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*.
Introduction

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

SXT is a combination of two antibiotics; trimethoprim (TMP) and sulfamethoxazole (SMX) at a 1:5 ratio, which target sequential steps in the tetrahydrofolate biosynthetic pathway (5). SMX inhibits dihydropteroate synthetase (DHPS), which prevents the production of dihydropteroic acid, while TMP binds and inhibits dihydrofolate reductase (DHFR), blocking the conversion of dihydrofolic acid to tetrahydrofolate (6). Since the production of tetrahydrofolate is essential for the biogenesis of thymidine, purines and some amino acids bacteria exposed to SXT will experience disrupted metabolic activity and stalled DNA replication (7).

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

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

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

The SOS-regulon of *S. aureus* consists of 16 LexA-regulated genes (17), including *recA*, *lexA* and *umuC*, which encodes a low-fidelity DNA polymerase. UmuC catalyses translesion DNA synthesis but lacks proofreading ability and permits DNA replication across unresolved lesions which often introduces mutations. Such mutagenic DNA repair may be advantageous to the pathogen by conferring resistance to antibiotics or adaptation to host stresses (17, 20). For example, mutations in the gene encoding DHFR resulted in reduced enzyme activity
and were associated with TMP resistance, with MICs of 8->512 µg ml⁻¹ (21). Similarly, mutations in the gene encoding DHPS resulted in SMX resistance with MICs of ≥256 µg ml⁻¹ (22). Therefore, exposure of S. aureus to SXT at concentrations that do not kill the pathogen may promote the emergence of resistance via induction of the mutagenic SOS response.

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

Materials and Methods

Bacterial strains and culture conditions

A full list of the bacterial strains used in this study is provided in Table 1. S. aureus was cultured in either Tryptic Soy Broth (TSB) or Mueller Hinton Broth (MHB) supplemented with calcium (25 mg/L) and magnesium (12.5 mg/L), to stationary phase for 18 h at 37 °C, with shaking (180 RPM). Culture media were supplemented with erythromycin (10 µg ml⁻¹) for growth of transposon mutants, kanamycin (90 µg ml⁻¹) for growth of the recA reporter strains, or anhydrotetracycline hydrochloride (AHT) (10 or 100 ng ml⁻¹) to induce expression of rexBA on plasmids, as required (Table 1).
S. aureus SH1000 and JE2 mutants deficient for rexB were complemented using pitet (25) containing the wildtype rexBA operon. Since insertion of the transposon into rexB is expected to block the expression of the downstream rexA gene, the rexB mutants were complemented with the entire rexBA operon. The pitet vector is a single-copy plasmid that integrates into the geh locus of the S. aureus genome. It contains a tetracycline-inducible promoter that was used to control rexBA gene expression. Primers containing the AvrII or Pmel restriction sites (Table 2) were used to amplify the rexBA operon from USA300 JE2 genomic DNA. The PCR product and pitet vector were digested using AvrII and Pmel restriction enzymes, ligated using T4 ligase and transformed into CaCl₂-competent E. coli DH5α cells. Successful ligation of rexBA into pitet was confirmed with DNA sequencing. Plasmid DNA was subsequently transformed into the DC10B E. coli strain which lacks cytosine methylation to allow bypassing of the staphylococcal restriction-modification barrier (26) and allowed successful electroporation into electrocompetent S. aureus rexB mutants in the JE2 and SH1000 genetic backgrounds. Empty vector that did not contain the rexBA operon was used as a control. Successful integration into the S. aureus genome was confirmed by PCR amplification of the geh gene followed by DNA sequencing.

Minimum Inhibitory Concentration (MIC) Assay

The minimum inhibitory concentration was determined according to the broth microdilution protocol described previously (27). Briefly, 95 µl aliquots of either MHB or TSB were supplemented with doubling concentrations of Co-trimoxazole (SXT), Trimethoprim (TMP) or Sulfamethoxazole (SMX) ranging from 0.008 µg ml⁻¹ to 512 µg ml⁻¹ in 96 well plates. In some assays, the culture medium was also supplemented with thymidine. In this study, the concentration of SXT was calculated based on the concentration of TMP present within the
combination therapeutic. Stationary phase cultures of *S. aureus* were diluted and inoculated into the antibiotic-containing media to a final cell density of $1 \times 10^5$ CFU ml$^{-1}$. For complemented mutants containing *rexBA* under the control of the *pitE* promoter, the medium was supplemented with AHT (100 ng ml$^{-1}$) to induce gene expression. Plates were incubated for 17 h at 37 °C before OD$_{600}$ readings were taken. The MIC was determined as the lowest concentration of antibiotic required to cause growth inhibition. To determine MICs under anaerobic conditions, 96 well plates were incubated in anaerobic jars containing Anaerogen gas packs at 37 °C for 18 h.

**SXT Killing Assay**

Stationary phase *S. aureus* cultures were washed twice by alternate rounds of centrifugation and resuspension in fresh MHB or TSB (3 ml) to a density of $2 \times 10^8$ CFU ml$^{-1}$ containing 4 μg ml$^{-1}$ SXT, the antibiotic clinical breakpoint concentration (28). Some experiments investigating the inoculum effect used lower starting bacterial cell densities, such as $\sim 10^7$, $10^6$ and $10^5$ CFU ml$^{-1}$. Bacteria were incubated with SXT at 37°C with shaking (180 RPM) and survival determined over time by quantification of CFUs using the Miles and Misra method, which consists of serial dilution followed by plating onto agar plates to enable the enumeration of colonies (29). Data were converted to % survival by comparing CFU counts at specific time points with the starting inoculum. To investigate SXT-mediated killing under anaerobic conditions, washed stationary phase cultures were inoculated into pre-reduced broth in bijoux tubes supplemented with 4 μg ml$^{-1}$ SXT, and incubated statically at 37 °C with the vessel caps loosened in an anaerobic jar containing an Anaerogen gas pack. The growth of *S. aureus* strains in TSB, under aerobic or anaerobic conditions was examined using a similar protocol using TSB or MHB without antibiotics.
Disc-diffusion Assay

Stationary phase *S. aureus* cultures were adjusted to an OD$_{600}$ of 0.063 (0.5 McFarland) in TSB and used to inoculate TSA plates using a cotton wool swab. Antibiotic discs containing 2.5 μg of either SXT or TMP (Oxoid) were placed onto the inoculated agar and incubated at 37 °C under aerobic or anaerobic conditions and incubated at 37 °C for 17 h. The zone of inhibition was measured as the total diameter of the cleared bacterial lawn, minus the diameter of the antibiotic disc.

Mutation rate analyses

Mutation rates were determined as described previously (20, 30). Briefly, 30 parallel cultures of *S. aureus* were used for each condition or strain in bijoux tubes containing 1 ml TSB inoculated with *S. aureus* at ~ 5 x 10$^5$ CFU ml$^{-1}$ and incubated with or without 0.05 μg ml$^{-1}$ (0.1 X wild-type MIC) or 0.1 μg ml$^{-1}$ (0.2 X wild-type MIC) SXT with shaking at 37 °C for 24 h. After incubation, 10 cultures were selected at random, and total CFU counts determined through serial dilution in PBS followed by plating onto TSA plates. Subsequently, 100 μl of each of the 30 undiluted cultures were spread on TSA plates containing rifampicin (100 μg ml$^{-1}$) before incubation at 37 °C for 24 h. The number of rifampicin-resistant colonies on each plate were counted, and the mutation rate with 95% confidence intervals calculated using the maximum-likelihood setting of the FALCOR mutation rate calculator (30). Since these assays were done in the presence of SXT it was important to use rifampicin resistance (rather than SXT resistance) to measure the mutation rate to avoid selecting for resistance emergence. Rifampicin resistance is a well-established marker for mutation-rate analysis and is not selected for or against under the conditions used (20, 30).
In vitro SXT resistance emergence assay

This assay was based on that described previously for the emergence of daptomycin resistance (31). *S. aureus* was inoculated to \( \sim 10^8 \text{CFU ml}^{-1} \) in 3 ml TSB containing co-trimoxazole (4 µg ml\(^{-1}\)) for 8 h per exposure. After this time, bacterial survival was determined by CFU counts, which were compared to the inoculum. For repeated antibiotic exposure, 1 ml of each culture was centrifuged (3 min, 17,000 x \(g\)) and the resulting pellet washed once in PBS to remove the antibiotic. The pellet was resuspended in 100 µl TSB and \( \sim 10^5 \text{CFU} \) (based on survival data) used to inoculate 3 ml TSB before incubation for 16 h at 37°C with shaking (180 RPM) in the absence of antibiotics to enable bacterial recovery. Co-trimoxazole MIC assays were undertaken on these cultures to detect changes in susceptibility. Recovered bacterial cultures were then re-exposed to co-trimoxazole for a total of three repeated exposures.

RecA-reporter assay

Induction of the SOS response in *S. aureus* in response to TMP, SMX and SXT combination exposure was determined through the use of strains containing a *recA*-gfp reporter construct. These were generated by transforming *S. aureus* with pCN34 containing the *recA* promoter upstream of *gfp*. Primers, detailed in Table 2, were used to amplify the *recA* promoter region (sequence detailed in (14) from JE2 wildtype genomic DNA, and the *gfp* gene from the pCL55 P3 GFP plasmid (32). These products were combined by overlapping extension PCR using primers ‘PrecA-F BamHI’ and ‘GFP-R KpnI’, digested with BamHI and KpnI (relevant restriction sites incorporated into amplicon primers), and ligated into phosphatase-treated, BamHI and KpnI digested low copy shuttle vector pCN34. Empty vector
without the recA-gfp construct served as a control. The recA-gfp pCN34 vectors were constructed in E. coli DC10B and confirmed by sequencing. Cultures of DC10B containing the recA-gfp pCN34 vector and empty vector were cultured in LB broth supplemented with ampicillin (100 μg/ml) to select for plasmid maintenance. Using methods described in (26), recA-gfp and empty vectors were transformed directly with into S. aureus WT JE2 and the rexB::Tn mutant. Reporter strains were grown in the presence of 90 μg/ml of kanamycin to maintain the plasmid.

Stationary phase cultures of recA-gfp reporter strains were pelleted by centrifugation, washed twice, and resuspended in TSB supplemented with kanamycin (90 μg/ml). Cultures at ~ 3.33 x 10^8 CFU ml^-1 were exposed to a range of TMP, SMX and SXT concentrations (0.01 μg ml^-1 to 512 μg ml^-1) in black-walled microtiter plates. These were incubated, with shaking, at 37 °C for 17 h in a TECAN Infinite® 200 PRO microplate reader, where OD_{600} and GFP relative fluorescent units (RFU) (Excitation: 475 nm; Emission: 525 nm) was measured every 1000 seconds (~17 minutes). The values were blanked against values generated from uninoculated wells, and GFP fluorescence was normalised to OD_{600} readings to determine recA expression levels.

Endogenous ROS production

Production of ROS was detected using the cell permanent 2',7'-dichlorodihydrofluorescein diacetate dye (H_{2}DCFDA), which is converted to fluorescent DCF by oxidative cleavage of acetate groups (33). A 96 well microtitre plate assay was prepared in a similar format to the recA-gfp reporter assay, with the addition of 25 μM H_{2}DCFDA and various concentrations of SXT. Wildtype and rexB::Tn mutant strains were washed and inoculated at ~ 3.33 x 10^8 CFU ml^-1. Plates were incubated with shaking at 37 °C as described previously, and OD_{600} and
fluorescence (Excitation: 495 nm; Emission: 525 nm) was measured every 1000 s (~17 minutes). OD$_{600}$ values were blanked against uninoculated wells, and fluorescence data was blanked to untreated wells. Fluorescent values were normalised to OD$_{600}$ readings to determine production of ROS relative to cell number. Bacterial growth rates ($\mu$, expressed as $\Delta$OD$_{600}$ min$^{-1}$) were calculated for logarithmic phase growth using the formula $\mu = \frac{(\ln \text{OD}_2 - \ln \text{OD}_1)}{(t_2 - t_1)}$.

**Statistical Analysis**

Means or medians were calculated from at least three biologically independent replicates and analysed by Student’s t test (two tailed, unpaired, assuming equal variances), One-way ANOVA or Two-way ANOVA corrected for multiple comparison using GraphPad Prism (V7.0) as described in the figure legends. MIC-bar graphs show the median value. All remaining graphs were plotted to show mean ± SD. Error bars were omitted on recA-reporter data for clarity.

Data were not log transformed, with the exception of growth rate analyses.

**Results**

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

To investigate the role of bacterial DNA repair in the susceptibility of *S. aureus* to SXT, a panel of *S. aureus* transposon mutants were subjected to MIC and disc diffusion susceptibility tests, including SXT and its constituent antibiotics, TMP and SMX. These mutants lacked components of DNA repair (*rexA, rexB, recF, umuC or dinB*) previously shown to contribute to the repair of damage caused by the quinolone antibiotic ciprofloxacin, or acting as controls (20). Mutants in two distinct genetic backgrounds were used in this study;
SH1000, a well-established laboratory strain, and JE2, a community-associated methicillin-resistant S. aureus (CA-MRSA) strain of the USA300 lineage, responsible for severe skin infections that are often treated with SXT (3, 34).

The MIC values for both the SH1000 and JE2 wild-type (WT) strains indicated susceptibility to SXT and TMP (< 2 µg ml⁻¹), but resistance to SMX (>512 µg ml⁻¹) based on EUCAST breakpoints for S. aureus [EUCAST, 2019] (Fig. 1A). Despite the resistance to SMX, the sulphonamide appeared to synergise with TMP since the MIC for SXT was 2-fold lower than that of TMP for JE2 and 4-fold lower for SH1000, which is in keeping with previous work (35). Disruption of rexB in both JE2 and SH1000 strains lowered the MIC of SXT and TMP by 2-4-fold and at least 16-fold for SMX, whilst complementation of the rexB mutant led to a doubling in the MICs for SXT for both JE2 and SH1000 relative to the mutant strain containing the empty plasmid control (Fig. 1A). The mutant lacking recF in the JE2 strain was also more susceptible than the WT to all three antibiotics, but the disruption of dinB or umuC only resulted in a slightly increased susceptibility to SXT but not TMP or SMX.

Consistent with the MIC data, disc diffusion assays with both JE2 and SH1000 strains showed that inactivation of rexB caused a small but significant increase in the susceptibility of S. aureus to SXT and TMP, relative to the WT (Fig. 1B,C). Complementation of the rexB mutant with (prexBA) reduced susceptibility close to WT levels, confirming the role of the RexAB complex (Fig. 1B,C). By contrast, inactivation of dinB or umuC did not significantly increase zone sizes for either antibiotic (Fig. 1B,C). However, the recF-deficient mutant showed an increase in susceptibility to TMP in the JE2 strain (Fig. 1C), indicating that both rexBA and recF contribute to the repair of DNA damage caused by TMP.

The finding that RexAB and RecF reduced the susceptibility of S. aureus to SXT confirmed that DNA repair modulated susceptibility to SXT and prompted us to undertake a
screen of mutants with disrupted copies of recJ, uvrA, uvrB, nth, mutS, mutL, sbcC or sbcD, to identify any additional repair processes that might be involved. However, none of these mutants had a SXT MIC that was different from that of the wild-type. In summary, mutants lacking rexA, rexB or recF had small but statistically significantly increased levels of susceptibility to the growth inhibitory activity of SXT and each of its two components, TMP and SMX.

Despite the increased susceptibility of mutants lacking rexA, rexB or recF to SXT in MIC and zone of inhibition assays, the differences observed were minimal and of questionable clinical relevance. Therefore, we next investigated the contribution of DNA repair to the survival of *S. aureus* (~10⁸ CFU ml⁻¹) exposed to the breakpoint concentration of the antibiotic (4 µg ml⁻¹) (EUCAST, 2019). Despite the reported bactericidal activity of SXT, there was only a 20-fold reduction in survival over 24 h for the JE2 strain (Fig. 1D), indicating a relatively high level of tolerance to SXT at this concentration (35, 36). Similar levels of survival were seen for mutants lacking umuC or dinB in the JE2 background (Fig. 1D). By contrast, survival of the recF mutant was reduced by >20-fold, relative to the WT, indicating increased susceptibility. Most notably however, the rexB mutant showed >5000-fold reduction in CFU counts relative to the WT at 24 h. Complementation of the rexB mutant increased survival 10-fold relative to a plasmid only control (Fig. 1E). Subsequent experiments with the SH1000 confirmed a role for rexBA in modulating susceptibility of *S. aureus* to SXT. There was a 12-fold greater loss of CFU counts of the rexB mutant relative to WT (Fig. 1F). However, similar to what was seen for the MIC assays, complementation of the rexB mutant with prexB restored survival levels close to those of the WT (Fig. 1F).

For both JE2 and SH1000 strains, complementation of the rexAB::Tn mutants employed an AHT-inducible promoter system that enabled control of rexAB expression,
enabling dose-response studies to be undertaken. In the absence of AHT, bacterial survival was similar to that seen for the rexAB::Tn mutant after 24 h. However, the addition of 10 or 100 μg ml⁻¹ AHT resulted in a dose-dependent increase in survival, providing further evidence that RexAB promotes the survival of S. aureus strains exposed to co-trimoxazole at 4 μg ml⁻¹ (Fig. 1G,H). It is unclear why the complementation system fully restored the WT phenotype in the SH1000 strain but only partially in the JE2 strain (Fig. 1G,H). However, it is not uncommon for complementation of S. aureus to confer a stronger or weaker phenotype than the WT, including when AHT inducible systems such as pitet are used (37).

In Summary, despite small differences in the susceptibility of wild-type and rexB mutant to the growth inhibitory activity of SXT (as defined by MIC values), the DNA repair mutant was significantly more susceptible to the combination antibiotic when used at the breakpoint (8X wild-type MIC). These findings indicate that SXT causes DNA double-strand breaks in S. aureus when used at the breakpoint concentration, the repair of which by RexAB significantly promotes staphylococcal survival during exposure to the antibiotic.

Thymidine limitation contributes to SXT-mediated DNA damage

Inhibition of folate biosynthesis by SXT results in abrogation of endogenous thymidine biosynthesis (38), which could provide an explanation for the DNA damage phenotype described above (Fig. 1). In support of this, previous studies have reported that the in vitro inhibitory activity of TMP and SXT is inversely correlated with the availability of thymidine in the culture medium (10, 39). The presence of exogenous thymidine allows S. aureus to continue with canonical DNA synthesis and allow effective DNA repair to occur, despite SXT-mediated inhibition of tetrahydrofolate production (13).
Whilst the precise composition of media can vary between manufacturers and from batch to batch, TSB contains a low concentration of thymidine (reported previously to be 0.04 µg ml$^{-1}$, 0.25 µM, (39)). To investigate the influence of exogenous thymidine on SXT activity in our assays, the growth of WT and rexB mutant strains was determined in TSB supplemented with doubling dilutions of SXT and thymidine via checkerboard assays (Fig. 2A-D). As expected, the presence of high concentrations of thymidine enabled growth of WT strains of both JE2 and SH1000 at higher concentrations of SXT (39) (Fig. 2A,C).

Given the thymidine-dependent activity of SXT, different culture media compositions may greatly affect antibiotic susceptibility (10, 39). For example, Mueller-Hinton Broth (MHB), the gold standard medium for antibiotic susceptibility testing, contains 0.46 µg ml$^{-1}$ thymidine compared to 0.04 µg ml$^{-1}$ thymidine in TSB, which can greatly affect susceptibility testing (39). Therefore, susceptibility tests were undertaken using MHB to compare with those described above using TSB. Consistent with the checkerboard data and the literature (39), the efficacy of SXT in MHB was reduced relative to TSB, with detectable bacterial growth of both WT strains observed even at high concentrations of SXT, most likely due to the increased levels of thymidine (Fig. 2E,F).

To explore the effect of exogenous thymidine on the bactericidal activity of SXT, time-course killing assays were undertaken using MHB. As expected from the MIC assays, and by contrast to assays done in TSB (Fig. 1D) there was almost no killing of the S. aureus JE2 WT strain by SXT (4 µg ml$^{-1}$) in MHB over 24 h (Fig. 2G). Similarly high levels of survival were observed for the dinB, umuC and recF mutants (Fig. 2G). By contrast, survival of the rexB mutant in MHB containing SXT was >2000-fold lower than that of the WT, despite the presence of exogenous thymidine (Fig. 2G). As for TSB, complementation of the rexB mutant (prexBA) significantly promoted survival in MHB containing SXT, relative to a plasmid only
control (Fig. 2H). Similar results were seen for the SH1000 strain, with the WT surviving in MHB containing SXT (4 μg ml⁻¹), whilst viability of the rexB mutant was reduced >6000-fold relative to the inoculum (Fig. 2I). As for the JE2 strain, complementation of the SH1000 rexB mutant significantly promoted survival (> 10-fold) (Fig. 2I).

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

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

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

To examine whether the production of ROS contributes to SXT activity against S. aureus, the susceptibility of WT and DNA repair mutants to the antibiotic was determined under both aerobic and anaerobic conditions in low-thymidine TSB. In the absence of oxygen, the MIC of WT S. aureus was increased 4-fold for both JE2 and SH1000 strains (Fig. 3A). By contrast, the SXT MIC of the JE2 rexB mutant only doubled under anaerobic conditions, whilst the MIC of the SH1000 rexB mutant was unaffected by the presence of oxygen (Fig. 3A). To explore this further, zone of inhibition assays were done under aerobic and anaerobic conditions. Zones of inhibition were significantly reduced in the absence of
oxygen for all strains and mutants, implying reduced susceptibility (Fig. 3B). However, despite the reduced antibiotic activity of SXT under anaerobic conditions, the rexBA mutants were still more susceptible than the corresponding WT, with >10 and 2.5-fold greater zone sizes for JE2 and SH1000 strains respectively (Fig. 3B).

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

Next, we assessed the contribution of ROS to the bactericidal activity of SXT. In contrast to assays done in air, none of the strains examined were killed by SXT (4 μg ml\(^{-1}\)) under anaerobic conditions (Fig. 3C,D). However, whilst the WT strains were able to grow slightly under these conditions, SXT prevented growth of the rexB mutants >2-fold by 24 h post inoculation (Fig. 3C,D). Complementation of the rexB mutant, but not the empty vector, rescued SXT mediated growth inhibition for both strains at 24 h (Fig. 3C,D). Therefore, SXT exposure caused bacteriostasis in the rexBA mutant but not the WT under anaerobic conditions.

We hypothesised that the ability of the WT strains to grow in the presence of an SXT concentration above the MIC (Fig. 3C,D) likely reflected the 1000-fold larger inoculum used in time kill experiments (~\(10^8\) CFU ml\(^{-1}\)) compared to MIC assays (~\(10^5\) CFU ml\(^{-1}\)) (40-42). Since this phenomenon, known as the inoculum effect, has been reported to affect co-trimoxazole susceptibility of Haemophilus influenzae (43) we investigated whether it applied to S. aureus.

TSB was inoculated with various concentrations of S. aureus WT or rexB::Tn mutant and growth assessed in the absence or presence of co-trimoxazole (4 μg ml\(^{-1}\)). As described
above, the growth of *S. aureus* WT at high bacterial densities was almost completely unaffected by the presence of co-trimoxazole relative to growth medium alone (Fig. 3E,F). However, co-trimoxazole caused a larger degree of growth restriction of *S. aureus* when lower inoculums were used (Fig. 3E,F). For both JE2 and SH1000 strains, the inoculum effect was more pronounced for the *rexB*:Tn mutant than the WT (Fig. 3E,F).

The reduced activity of SXT under anaerobic conditions suggested that ROS make an important contribution to the bactericidal activity of the antibiotic. Therefore, H$_2$DCFDA dye, which is converted to the fluorescent ‘DCF’ in the presence of ROS, was added to growth inhibition assays to quantify and study the kinetics of ROS production during incubation under aerobic conditions (Fig. G,I). SXT induced clear concentration dependent inhibition growth for both the JE2 WT and *rexB* mutant (Fig. 3H,J). When these data were used to normalise to the relative cell density, SXT also induced a dose-dependent fluorescent peak at 4 h post inoculation for both WT and *rexB* mutant strains in the JE2 background (Fig. 3G,I), indicative of the generation of ROS in response to the antibiotic. A second peak in ROS production after 13 h is apparent at lower concentrations of SXT as cells reach stationary phase, which is likely a result of the bacterial starvation response, which is associated with increased endogenous oxidative stress (44).

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

**RexAB is required for SXT-triggered induction of the SOS response**
The SOS response is a DNA damage-inducible regulon that includes several genes that encode DNA repair components and is regulated by RecA and LexA (17). Consistent with our findings that SXT causes DNA damage in \textit{S. aureus}, previous work has shown that TMP triggers \textit{recA} expression, which is indicative of induction of the SOS response (14). This is important because the SOS response increases the mutation rate, promoting the likelihood of drug resistance emerging, which is a frequent limitation of SXT therapy (17, 45-47).

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

Irrespective of thymidine concentration, SXT induced a dose dependent inhibition of growth for both the JE2 WT and \textit{rexB} mutant (Fig. 4A-D). In low-thymidine TSB, SXT exposure resulted in a dose-dependent increase in GFP signal from the \textit{precA-gfp} reporter construct in the \textit{S. aureus} WT strain (Fig. 4E), which peaked at about 4 h and then subsided before the signal slowly increased once more as the bacteria entered stationary phase, presumably due to SOS induction in response to internal DNA-damaging events as a consequence of nutrient limitation and other metabolic stresses (44, 48).

The temporal profile of \textit{recA} expression is similar to that observed for ROS production by WT \textit{S. aureus} in response to SXT (Fig. 3G), providing additional evidence that antibiotic-induced oxidative stress contributes to DNA damage. However, although SXT also triggered ROS production in the \textit{rexB} mutant (Fig. 3H), the antibiotic did not induce \textit{recA} expression in this strain (Fig. 4F), revealing that RexAB is required for activation of the SOS response under these conditions.
In contrast to experiments done in TSB, recA was only weakly expressed during exposure to SXT in high-thymidine MHB (Fig. 4D), which is in keeping with the reduced activity of the antibiotic in this medium (Fig. 2E,F). Consistent with the data from studies in TSB, there was a lack of recA expression in the JE2 rexB mutant in response to SXT in MHB (Fig. 4E).

Next, we investigated whether SOS induction was due to a single component of co-trimoxazole or the combination of the two antibiotics. Therefore, S. aureus JE2 strains were exposed to a range of concentrations of trimethoprim or sulphamethoxazole sufficient to cause dose-dependent reductions in growth (Fig. 4I, J,K,L). Analysis of recA expression revealed that trimethoprim caused a dose-dependent induction of the SOS response in WT S. aureus JE2, but not the rexB::Tn mutant (Fig. 4M,N). By contrast, sulphamethoxazole did not trigger the SOS response at any of the concentrations examined in either the WT or rexB::Tn mutant (Fig. 4O,P). Therefore, it appears that trimethoprim is both necessary and sufficient to triggers the SOS response induced by co-trimoxazole.

RexAB is required for SXT-induced mutagenic DNA repair

De-repression of the SOS regulon leads to expression of the error-prone translesion DNA polymerase UmuC, which catalyses mutagenic DNA repair that contributes to the acquisition of antibiotic resistance (17, 20).

To assess whether SXT promoted SOS-dependent mutagenesis in S. aureus, JE2 WT and DNA-damage repair mutants were exposed to sub-inhibitory concentrations of SXT in low-thymidine TSB. The resulting mutation rate was calculated by fluctuation analysis using the emergence of rifampicin resistance, which occurs via point mutations in the rpoB gene encoding the RNA polymerase β-subunit (30, 49). Since the rexBA mutant was more
susceptible to SXT treatment than the wild-type, only a very low concentration of the antibiotic (0.05 µg ml$^{-1}$, 0.2 x MIC of the mutant) could be used in mutation rate analyses with this strain. However, even at this concentration, SXT treatment resulted in an increase in the mutation rate of WT *S. aureus* (Fig. 5A). By contrast to the WT, SXT exposure did not increase the mutation rate of the *rexB* mutant, consistent with the inability of the antibiotic to trigger induction of the SOS response in this strain (Fig. 4B, 5A).

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

To explore whether RexAB might contribute to the emergence of co-trimoxazole resistance, we exposed 6 independent cultures of JE2 WT or *rexB::Tn* mutant to 3 rounds of exposure to the combination antibiotic, followed by a recovery period in TSB alone. After two rounds of exposure to co-trimoxazole, the MIC of 5 of the WT cultures had increased (2-16 fold above WT) (Fig. 5C). This was associated with an increased level of WT survival during the third exposure to co-trimoxazole (Fig. D). By contrast, there was no change in the co-trimoxazole MIC after 3 rounds of antibiotic exposure, nor was there any increase in bacterial survival after 8 h exposure to the combination antibiotic (Fig. E,F).
Taken together, these data demonstrate that SXT exposure results in RexAB-dependent induction of the SOS response in S. aureus, which triggers UmuC-dependent mutagenic DNA repair that promotes the rate at which antibiotic resistance arises. In keeping with these findings, RexAB appears to be important for the emergence of SXT resistance in vitro.

**Discussion**

Multi-drug resistance in S. aureus is a major global health concern associated with high treatment failure rates (50). Many second or third-line treatments such as vancomycin or daptomycin can lack efficacy, require intravenous administration and are associated with toxicity (12). By contrast, SXT is a safe and orally administered antibiotic that is effective in the treatment of skin and soft tissue infections caused by MRSA (4). However, the efficacy of this antibiotic is limited by the pathogens’ ability to by-pass SXT-mediated inhibition of folate biosynthesis, either via the uptake of metabolites from the environment or by the frequent acquisition of resistance-conferring mutations (9). Therefore, it is hoped that an improved understanding of SXT and its mechanism of action will enable the development of novel approaches aimed at expanding the therapeutic use of this antibiotic and reducing the frequency at which resistance emerges.

The bactericidal effect of SXT has been ascribed to DNA damage, which leads to the induction of the SOS response in bacterial cells (14, 15). The data we present here support DNA damage as a central component of the mechanism of SXT bactericidal activity in S. aureus and highlight the key role of RexAB in combatting this genotoxicity. In contrast to the WT, mutants defective for rexB were significantly more susceptible to SXT in both growth inhibition and killing assays.
It should be noted however, that the increased susceptibility of the *rexB* mutants to co-trimoxazole, relative to the wild type, was much more apparent in killing assays than those that measured growth inhibition (MIC and zone of inhibition). This may indicate that the genotoxicity of co-trimoxazole is dose-dependent, which is supported by data showing that both ROS generation and *recA* expression increase with the dose of the combination antibiotic. Fortunately, the low toxicity of co-trimoxazole enables high doses to be used therapeutically, with mean serum concentrations above the breakpoint (51).

RexAB is proposed to be a member of the AddAB family of DNA repair enzymes required for the processing of DNA double strand breaks on the basis of protein sequence identity (19, 20, 52). Therefore, the increased susceptibility of *rexB* mutants to SXT indicate that DNA DSBs are caused by the antibiotic and are lethal if not repaired. Additional evidence that SXT causes DNA damage in *S. aureus* came from analysis of the *recF* mutant, which was more susceptible to the antibiotic than the WT, albeit to a lesser extent than the *rexB* mutant. The function of RecF has been ascribed to the repair of DNA lesions that arise from DNA single-strand gaps. However, whilst this function is well established in canonical model organisms for bacterial DNA damage repair, including *E. coli* and *Bacillus subtilis*, the evidence that supports RecF performing a similar function in *S. aureus* is less strong (18). Therefore, a greater understanding of how RecF repairs damage in staphylococci will provide insight into the type of DNA damage caused by SXT in *S. aureus*.

Apart from *rexA* and *rexB* mutants, none of the other strains lacking DNA repair components had increased SXT susceptibility. Whilst it is possible that SXT causes DNA damage beyond DSBs, these other types of damage do not appear to contribute to growth inhibition or bacterial killing. This finding also implies that DNA repair does not contribute to the lethality of SXT, as has been reported for trimethoprim-mediated killing of *E. coli* (13).
The discovery that *S. aureus* is more susceptible to SXT when incubated in thymidine-poor medium is in keeping with previous reports, which suggest that thymidine depletion makes a crucial contribution to the lethality of SXT (15, 39). Specifically, nucleotide imbalance, caused by thymidine depletion, can result in the collapse of replication forks during DNA synthesis, which can lead to DNA DSBs (8). In support of this, we observed SOS induction under thymidine-limited but not thymidine-replete conditions. This is thought to have important implications for the efficacy of SXT-treatment. Whilst the concentration of free thymidine in plasma is relatively low (~0.2 µM/~48 ng ml\(^{-1}\)) (53), elevated thymidine concentrations are likely to occur in human tissues containing necrotic cells or neutrophil extracellular traps (9, 54). To acquire this thymidine, *S. aureus* expresses DNase to break down DNA and NupC, a nucleoside transporter that has been shown to support the growth of *S. aureus* during SXT exposure by enabling the uptake of extracellular thymidine (55, 56).

The presence of thymidine and other folate-dependent metabolites including serine, methionine and glycine in host tissues modulate the susceptibility of SXT-treated cells and restrict the types of infection that can be treated with this antibiotic (9). However, whilst the presence of thymidine reduced the susceptibility of the WT to SXT, there was still a >1000-fold reduction in CFU counts of the rexB mutant exposed to the antibiotic, suggesting that thymidine limitation is not solely responsible for DNA damage in *S. aureus* (Fig. 2G,I).

In agreement with several reports that bactericidal antibiotics, including TMP, exert part of their antibacterial effect via the generation of ROS (13, 57), we determined that the bactericidal effect of SXT against *S. aureus* is dependent upon the availability of oxygen. This was evident by the complete lack of bacterial killing under anaerobic conditions and suggests that ROS-mediated DNA damage makes a greater contribution to the bactericidal activity of SXT than the inhibition of DNA-synthesis caused by thymidine depletion in aerobic conditions.
conditions. However, it should also be considered that bacterial replication and physiology are significantly different under anaerobic conditions relative to aerobic growth. Therefore, future work will attempt to further define the role of ROS in SXT-mediated killing, for example by testing whether SXT exposure under aerobic conditions leads to oxidative DNA damage. Antibiotic-induced oxidative stress has been shown to increase the intracellular level of 8-oxodeoxyguanosine (8-oxo-dG), which is incorporated into DNA, and paired with deoxyctydine or deoxyadenosine (58). Incomplete repair of DNA-incorporated 8-oxo-dG can lead to DSB in *E. coli*, which contributes to the lethality of TMP (13). Additional experiments using mutants defective for the repair of 8-oxo-dG will determine whether a similar mechanism mediates the genotoxicity of SXT in *S. aureus* under aerobic conditions.

However, whilst ROS appear to make an important contribution to the bactericidal activity of SXT, the increased susceptibility of the rexB mutant to this antibiotic under anaerobic conditions confirms that DNA damage can occur independently of ROS. This may explain why there are phenotypic differences in the effects of SXT exposure and thymineless death observed in thyA mutants deprived of thymine (13).

One drawback of SXT is the frequent emergence of resistance during long-term therapy (59). In keeping with the induction of the SOS response, exposure of *S. aureus* to sub-inhibitory concentrations of SXT was found to increase the mutation rate via UmuC, a low-fidelity DNA polymerase, previously shown to mediate mutagenic DNA repair of bacteria exposed to ciprofloxacin or H$_2$O$_2$ (20). Similar to what has been reported for ciprofloxacin and H$_2$O$_2$, SXT-induced increases in the mutation rate were dependent upon RexAB (20). Therefore, the processing of DNA DSBs by RexAB is central to the survival of *S. aureus* exposed to SXT as well as SOS-mediated increases in the mutation rate associated with drug resistance.
In summary, our data demonstrate that SXT causes DNA damage in *S. aureus* via thymidine depletion and ROS generation, the repair of which via RexAB enables bacterial survival and induction of the SOS response, and appears to contribute to the emergence of drug-resistance *in vitro*. The identification of RexAB as central to both SXT susceptibility and SOS induction suggests that this protein complex may provide a target for novel therapeutic approaches that could improve the efficacy of SXT and reduce the emergence of resistance. Such an approach may also increase the range of MRSA infections that could be treated with SXT.

**Acknowledgements**

A.M.E. and R.S.C. acknowledge funding from Shionogi & Co., Ltd. A.M.E. also acknowledges support from the National Institute for Health Research (NIHR) Imperial Biomedical Research Centre (BRC).

K.P.H. is supported by a PhD scholarship funded by a Medical Research Council award to the Centre for Molecular Bacteriology and Infection (MR/J006874/1). All authors acknowledge the provision of strains by the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) Program: under NIAID/ NIH Contract No. HHSN272200700055C. The funders had no role in the study design, interpretation of the findings or the writing of the manuscript.

**References**
1. Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG. 2015. Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management. Clinical microbiology reviews 28:603-661.

2. Cenizal MJ, Skiest D, Luber S, Bedimo R, Davis P, Fox P, Delaney K, Hardy RD. 2007. Prospective randomized trial of empiric therapy with trimethoprim-sulfamethoxazole or doxycycline for outpatient skin and soft tissue infections in an area of high prevalence of methicillin-resistant Staphylococcus aureus. Antimicrobial agents and chemotherapy 51:2628-2630.

3. Liu C, Bayer A, Cosgrove SE, Daum RS, Fridkin SK, Gorwitz RJ, Kaplan SL, Karchmer AW, Levine DP, Murray BE. 2011. Clinical practice guidelines by the Infectious Diseases Society of America for the treatment of methicillin-resistant Staphylococcus aureus infections in adults and children. Clinical infectious diseases 52:e18-e55.

4. Bowen AC, Carapetis JR, Currie BJ, Fowler Jr V, Chambers HF, Tong SY. Sulfamethoxazole-Trimethoprim (Cotrimoxazole) for Skin and Soft Tissue Infections Including Impetigo, Cellulitis, and Abscess, p ofx232. In (ed), Oxford University Press US.

5. Minato Y, Dawadi S, Kordus SL, Sivanandam A, Aldrich CC, Baughn AD. 2018. Mutual potentiation drives synergy between trimethoprim and sulfamethoxazole. Nature communications 9:1003.

6. Hitchings GH. 1973. Mechanism of action of trimethoprim-sulfamethoxazole—I. Journal of Infectious Diseases 128:S433-S436.

7. Harvey R, Dev I. 1975. Regulation in the folate pathway of Escherichia coli. Advances in enzyme regulation 13:97-124.

8. Khodursky A, Guzmán EC, Hanawalt PC. 2015. Thymineless death lives on: new insights into a classic phenomenon. Annual review of microbiology 69:247-263.

9. Goldstein EJ, Proctor RA. 2008. Role of folate antagonists in the treatment of methicillin-resistant Staphylococcus aureus infection. Clinical Infectious Diseases 46:584-593.
10. Jones C, Stevens D, Ojo O. 1987. Effect of minimal amounts of thymidine on activity of trimethoprim-sulfamethoxazole against *Staphylococcus epidermidis*. Antimicrobial agents and chemotherapy 31:144-147.

11. Indiveri MC, Hirsh DC. 1992. Tissues and exudates contain sufficient thymidine for growth of anaerobic bacteria in the presence of inhibitory levels of trimethoprim-sulfamethoxazole. Veterinary microbiology 31:235-242.

12. Lee Y-J, Dai N, Walsh SE, Müller S, Fraser ME, Kauffman KM, Guan C, Corrêa IR, Weigele PR. 2018. Identification and biosynthesis of thymidine hypermodifications in the genomic DNA of widespread bacterial viruses. Proceedings of the National Academy of Sciences 201714812.

13. Giroux X, Su W-L, Bredeche M-F, Matic I. 2017. Maladaptive DNA repair is the ultimate contributor to the death of trimethoprim-treated cells under aerobic and anaerobic conditions. Proceedings of the National Academy of Sciences 114:11512-11517.

14. Mesak LR, Miao V, Davies J. 2008. Effects of subinhibitory concentrations of antibiotics on SOS and DNA repair gene expression in *Staphylococcus aureus*. Antimicrobial agents and chemotherapy 52:3394-3397.

15. Sangurdekar DP, Zhang Z, Khodursky AB. 2011. The association of DNA damage response and nucleotide level modulation with the antibacterial mechanism of the anti-folate drug trimethoprim. BMC genomics 12:583.

16. Michel B. 2005. After 30 years of study, the bacterial SOS response still surprises us. PLoS biology 3:e255.

17. Cirz RT, Jones MB, Gingles NA, Minogue TD, Jarrahi B, Peterson SN, Romesberg FE. 2007. Complete and SOS-mediated response of *Staphylococcus aureus* to the antibiotic ciprofloxacin. Journal of bacteriology 189:531-539.

18. Alonso J, Fisher L. 1995. Nucleotide sequence of the recF gene cluster from *Staphylococcus aureus* and complementation analysis in Bacillus subtilis recF mutants. Molecular and General Genetics MGG 246:680-686.
19. Wigley DB. 2013. Bacterial DNA repair: recent insights into the mechanism of RecBCD, AddAB and AdnAB. Nature Reviews Microbiology 11:9.

20. Painter KL, Strange E, Parkhill J, Bamford KB, Armstrong-James D, Edwards AM. 2015. Staphylococcus aureus adapts to oxidative stress by producing H2O2-resistant small colony variants via the SOS response. Infection and immunity:IAI. 03016-14.

21. Dale GE, Broger C, D’Arcy A, Hartman PG, DeHoogt R, Jolidon S, Kompis I, Labhardt AM, Langen H, Locher H. 1997. A single amino acid substitution in Staphylococcus aureus dihydrofolate reductase determines trimethoprim resistance 1. Journal of molecular biology 266:23-30.

22. Hampele IC, D’Arcy A, Dale GE, Kostrewa D, Nielsen J, Oefner C, Page MG, Schönfeld H-J, Stüber D, Then RL. 1997. Structure and function of the dihydropteroate synthase from Staphylococcus aureus 1. Elsevier.

23. Gill EE, Franco OL, Hancock RE. 2015. Antibiotic adjuvants: diverse strategies for controlling drug-resistant pathogens. Chemical biology & drug design 85:56-78.

24. Wright GD. 2016. Antibiotic adjuvants: rescuing antibiotics from resistance. Trends in Microbiology 24:862-871.

25. Grundling A, Schneewind O. 2007. Genes required for glycolipid synthesis and lipoteichoic acid anchoring in Staphylococcus aureus. J Bacteriol 189:2521-30.

26. Monk IR, Shah IM, Xu M, Tan M-W, Foster TJ. 2012. Transforming the untransformable: application of direct transformation to manipulate genetically Staphylococcus aureus and Staphylococcus epidermidis. MBio 3:e00277-11.

27. Wiegand I, Hilpert K, Hancock RE. 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature protocols 3:163.

28. EUCAST. 2019. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 9.0, 2019. http://www.eucast.org.
29. Miles AA, Misra S, Irwin J. 1938. The estimation of the bactericidal power of the blood. Epidemiology & Infection 38:732-749.

30. Hall BM, Ma C-X, Liang P, Singh KK. 2009. Fluctuation Analysis CalculatOR: a web tool for the determination of mutation rate using Luria–Delbrück fluctuation analysis. Bioinformatics 25:1564-1565.

31. Pee CJE, Pader V, Ledger EVK, Edwards AM. 2019. A FASII Inhibitor Prevents Staphylococcal Evasion of Daptomycin by Inhibiting Phospholipid Decoy Production. Antimicrobial Agents and Chemotherapy 63:e02105-18.

32. James EH, Edwards AM, Wigneshweraraj S. 2013. Transcriptional downregulation of agr expression in Staphylococcus aureus during growth in human serum can be overcome by constitutively active mutant forms of the sensor kinase AgrC. FEMS microbiology letters 349:153-162.

33. Wang H, Joseph JA. 1999. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic Biol Med 27:612-6.

34. Pate AJ, Terribilini RG, Ghobadi F, Azhir A, Barber A, Pearson JM, Kalantari H, Hassen GW. 2014. Antibiotics for methicillin-resistant Staphylococcus aureus skin and soft tissue infections: the challenge of outpatient therapy. Am J Emerg Med 32:135-8.

35. Elwell LP, Wilson HR, Knick VB, Keith BR. 1986. In vitro and in vivo efficacy of the combination trimethoprim-sulfamethoxazole against clinical isolates of methicillin-resistant Staphylococcus aureus. Antimicrobial Agents and Chemotherapy 29:1092-1094.

36. Kaka AS, Rueda AM, Shelburne SA, 3rd, Hulten K, Hamill RJ, Musher DM. 2006. Bactericidal activity of orally available agents against methicillin-resistant Staphylococcus aureus. J Antimicrob Chemother 58:680-3.

37. Bowman L, Zeden MS, Schuster CF, Kaever V, Grundling A. 2016. New Insights into the Cyclic Di-adenosine Monophosphate (c-di-AMP) Degradation Pathway and the Requirement of the
Cyclic Dinucleotide for Acid Stress Resistance in *Staphylococcus aureus*. J Biol Chem 291:26970-26986.

38. Wormser GP, Keusch GT, Heel RC. 1982. Co-trimoxazole (trimethoprim-sulfamethoxazole): an updated review of its antibacterial activity and clinical efficacy. Drugs 24:459-518.

39. Koch AE, Burchall JJ. 1971. Reversal of the antimicrobial activity of trimethoprim by thymidine in commercially prepared media. Applied microbiology 22:812-817.

40. Marks MI, Weinmaster G. 1975. Influences of media and inocula on the in vitro susceptibility of Haemophilus influenzae to co-trimoxazole, ampicillin, penicillin, and chloramphenicol. Antimicrobial agents and chemotherapy 8:657-663.

41. Najjar A, Murray BE. 1987. Failure to demonstrate a consistent in vitro bactericidal effect of trimethoprim-sulfamethoxazole against enterococci. Antimicrobial agents and chemotherapy 31:808-810.

42. Widmer A, Wiestner A, Frei R, Zimmerli W. 1991. Killing of nongrowing and adherent Escherichia coli determines drug efficacy in device-related infections. Antimicrobial agents and chemotherapy 35:741-746.

43. Sinai R, Hammerberg S, Marks MI, Pai CH. 1978. In vitro susceptibility of Haemophilus influenzae to sulfamethoxazole-trimethoprim and cefaclor, cephalaxin, and cephradine. Antimicrob Agents Chemother 13:861-4.

44. Horsburgh MJ, Ingham E, Foster SJ. 2001. In *Staphylococcus aureus*, fur is an interactive regulator with PerR, contributes to virulence, and is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. Journal of bacteriology 183:468-475.

45. Eliopoulos GM, Huovinen P. 2001. Resistance to trimethoprim-sulfamethoxazole. Clinical infectious diseases 32:1608-1614.

46. Galhardo RS, Hastings PJ, Rosenberg SM. 2007. Mutation as a stress response and the regulation of evolvability. Critical reviews in biochemistry and molecular biology 42:399-435.
47. Painter KL, Hall A, Ha KP, Edwards AM. 2017. The electron transport chain sensitises Staphylococcus aureus and Enterococcus faecalis to the oxidative burst. Infection and immunity:IAI. 00659-17.

48. Nakamura MM, Liew SY, Cummings CA, Brinig MM, Dieterich C, Relman DA. 2006. Growth phase- and nutrient limitation-associated transcript abundance regulation in Bordetella pertussis. Infect Immun 74:5537-48.

49. Goldstein BP. 2014. Resistance to rifampicin: a review. The Journal of antibiotics 67:625.

50. Dombrowski JC, Winston LG. 2008. Clinical failures of appropriately-treated methicillin-resistant Staphylococcus aureus infections. Journal of Infection 57:110-115.

51. Muhammed Ameen S, Rolain JM, Le Poullain MN, Roux V, Raoult D, Drancourt M. 2014. Serum concentration of co-trimoxazole during a high-dosage regimen. J Antimicrob Chemother 69:757-60.

52. Halpern D, Gruss A, Claverys J-P, El Karoui M. 2004. rexAB mutants in Streptococcus pneumoniae. Microbiology 150:2409-2414.

53. Tavazzi B, Lazzarino G, Leone P, Amorini AM, Bellia F, Janson CG, Di Pietro V, Ceccarelli L, Donzelli S, Francis JS, Giardina B. 2005. Simultaneous high performance liquid chromatographic separation of purines, pyrimidines, N-acetylated amino acids, and dicarboxylic acids for the chemical diagnosis of inborn errors of metabolism. Clin Biochem 38:997-1008.

54. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. 2004. Neutrophil extracellular traps kill bacteria. science 303:1532-1535.

55. Berends ET, Horswill AR, Haste NM, Monestier M, Nizet V, von Köckritz-Blickwede M. 2010. Nuclease expression by Staphylococcus aureus facilitates escape from neutrophil extracellular traps. Journal of innate immunity 2:576-586.

56. Kriegeskorte A, Block D, Drescher M, Windmüller N, Mellmann A, Baum C, Neumann C, Lorè NI, Bragonzi A, Liebau E. 2014. Inactivation of thyA in Staphylococcus aureus attenuates
virulence and has a strong impact on metabolism and virulence gene expression. MBio 773:e01447-14.

57. Dwyer DJ, Collins JJ, Walker GC. 2015. Unraveling the physiological complexities of antibiotic lethality. Annual review of pharmacology and toxicology 55:313-332.

58. Foti JJ, Devadoss B, Winkler JA, Collins JJ, Walker GC. 2012. Oxidation of the guanine nucleotide pool underlies cell death by bactericidal antibiotics. Science 336:315-319.

59. Kahl BC, Belling G, Becker P, Chatterjee I, Wardecki K, Hilgert K, Cheung AL, Peters G, Herrmann M. 2005. Thymidine-dependent Staphylococcus aureus small-colony variants are associated with extensive alterations in regulator and virulence gene expression profiles. Infection and immunity 73:4119-4126.

60. Fey PD, Endres JL, Yajjala VK, Widhelm TJ, Boissy RJ, Bose JL, Bayles KW. 2013. A genetic resource for rapid and comprehensive phenotype screening of nonessential Staphylococcus aureus genes. MBio 4:e00537-12.

61. Horsburgh MJ, Aish JL, White IJ, Shaw L, Lithgow JK, Foster SJ. 2002. sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from Staphylococcus aureus 8325-4. J Bacteriol 184:5457-67.
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.                                | (60)              |
| USA300 LAC JE2 rexB::Tn   | USA300 LAC JE2 with a *bursa aurealis* transposon insertion in rexB, Ery’                  | (60)              |
| USA300 LAC JE2 umuC::Tn   | USA300 LAC JE2 with a *bursa aurealis* transposon insertion in umuC, Ery’                  | (60)              |
| USA300 LAC JE2 recF::Tn   | USA300 LAC JE2 with a *bursa aurealis* transposon insertion in recF, Ery’                  | (60)              |
| USA300 LAC JE2 dinB::Tn   | USA300 LAC JE2 with a *bursa aurealis* transposon insertion in dinB, Ery’                  | (60)              |
| USA300 LAC JE2 rexB::Tn pitet empty | USA300 LAC JE2 with a *bursa aurealis* transposon insertion in rexB with integrated pitet empty plasmid, Ery’ | This study |
| USA300 LAC JE2 rexB::Tn pitet rexB | USA300 LAC JE2 with a *bursa aurealis* transposon insertion in rexB with integrated pitet with AHT-inducible rexB, Ery’ | This study |
| USA300 LAC JE2 +pCN34 recA-gfp | USA300 LAC JE2 containing pCN34 with gfp under the control of the recA promoter, Kan’ | This study |
| USA300 LAC JE2 +pCN34 recA-gfp | USA300 LAC JE2 with a *bursa aurealis* transposon insertion in rexB containing pCN34 with gfp under the control of the recA promoter, Kan’ | This study |
| USA300 LAC JE2 +pCN34 Empty | USA300 LAC JE2 containing pCN34 empty vector, Kan’                                         | This study |
| USA300 LAC JE2 +pCN34 Empty | USA300 LAC JE2 with a *bursa aurealis* transposon insertion in rexB containing pCN34 empty vector, Kan’ | This study |
| **Methicillin-sensitive S. aureus** |                                                                                         |                   |
| SH1000                    | Methicillin sensitive *S. aureus* strain                                                   | (61)              |
| SH1000 rexB::Tn           | SH1000 with a *bursa aurealis* transposon insertion in rexB, Ery’                          | (20)              |
| SH1000 rexB::Tn pitet empty | SH1000 with a *bursa aurealis* transposon insertion in rexB with integrated pitet empty plasmid, Ery’ | This study |
**Table 2. Primers used for construction of complemented rexB mutants and recA-gfp reporter**

| Oligonucleotide          | Sequence 5'-3'                                                                 |
|--------------------------|-----------------------------------------------------------------------------|
| SH1000 rexB::Tn pitet rexB | SH1000 with a *bursa aureolis* transposon insertion in rexB with *This study* integrated pitet with AHT-inducible rexB, Ery' |
| SH1000 containing pCN34 with gfp under the control of the recA | *This study* promoter, Kan' |

Primers used for construction rexB::Tn prexB complemented mutant (restriction sites underlined)

- **rexB-F AvrII**
  - **GGCCCTAGGATGACATTACATGCTTATTTAG**

- **rexB-R PmeI**
  - **GCCGGTTTAAACCTATAGTTGCAATGTACCAAATTG**

- **pCL55 Fwd seq**
  - **GGATCCCTCGAGTTCATG**

- **pCL55 Rev seq**
  - **CTCGTAGTATCTATACCTCG**

- **Lipase geh F**
  - **GTGTTTTTTGTACATGGATTITTTAG**

- **Lipase geh R**
  - **CTTGCTTTCAATTGTGTTC**

- **pCL55 R**
  - **GCGCATAGGTGAGTTATTAGC**

Primers used for construction of the recA-gfp reporter (restriction sites underlined)

- **PrecA-F BamHI**
  - **GAGGATCCATTGATGATGACACAT**

- **PrecA-R 7xT-GFP**
  - **CATTTTTTTCCTCTGTTGGAAATTGC**

- **GFP-F 7xA-PrecA**
  - **AGGAAAAATAATGAGTAAGAGAAGAAAC**

- **GFP-R KpnI**
  - **GCGGTTACCTATTGATGATTGCATCCAT**

- **pCN34 seq F**
  - **GTTATCCCCTGATTCTGTGGATAAC**

- **pCN34 seq R**
  - **CCAGAATTATATTCAGAACAGGAAC**
Figures

| Strain   | SXT   | TMP   | SMX   |
|----------|-------|-------|-------|
| JE2      |       |       |       |
| WT       | 0.5   | 1     | >512  |
| recB     | 0.25  | 0.25  | 32    |
| recB pEMpy | 0.25 | 0.5   | 32    |
| recB prxA | 0.5   | 0.5   | 64    |
| dinA     | 0.25  | 1     | >512  |
| recC     | 0.25  | 0.25  | 32    |
| omuc     | 0.25  | 1     | >512  |
| SH1000   |       |       |       |
| WT       | 0.25  | 1     | >512  |
| recB     | 0.125 | 0.25  | 32    |
| recB pEMpy | 0.125 | 0.25  | 32    |
| recB prxA | 0.25  | 0.5   | 64    |

A) Strain comparison table

B) Zone of inhibition for SXT

C) Zone of inhibition for TMP

D) Survival of JE2 over time

E) Survival of JE2 over time with different mutants

F) Survival of SH1000 over time

H) JE2 survival after 24 hours

I) SH1000 survival after 24 hours
Figure 1. Mutants defective for DNA repair have increased susceptibility to SXT and its constituent antibiotics TMP and SMX. (A) MIC values for SXT, TMP and SMX for wild-type S. aureus JE2 and SH1000 and mutants defective for DNA damage repair in TSB. Data represent median values from n=3. (B,C) Zones of growth inhibition of WT JE2 and SH1000 S. aureus strains or damage-repair mutants from paper discs impregnated with 2.5 μg SXT (B) or TMP (C) after 16 h of incubation on TSA plates. Graphs represent mean±SD from n=4. Values that are significantly different from the WT within each antibiotic-exposure were identified by one-way ANOVA). (D,E,F) Time-course survival assays of S. aureus JE2 (D,E) and SH1000 (F) and their derived DNA damage-repair mutants during incubation in TSB supplemented with 4 μg ml⁻¹ SXT. Data are split between D and E for clarity. Percentage survival at each time point was calculated relative to the starting inoculum of ~1x10⁸ CFU ml⁻¹. Graphs represent mean±SD from n=3. (H,I) Survival of S. aureus JE2 (H) and SH1000 (I) at 24 h in TSB supplemented with 4 μg ml⁻¹ SXT. Survival data include WT, rexB mutants and rexB mutants transformed with pEmpty or prexB, which were grown to stationary phase in the presence of either 0, 10 or 100 ng/ml AHT to regulate rexBA expression. Graphs represent mean±SD from n=4. Values that are significantly different from the WT were identified by two-way ANOVA corrected for multiple comparison using the Dunnett method. P ≤ 0.05(*), P ≤ 0.01(**), P ≤ 0.001(***), P ≤ 0.0001(****).
Figure 2. Mutants defective for *rexB* have increased susceptibility to SXT under thymidine-rich conditions. (A-D) Growth (OD$_{600}$) of *S. aureus* JE2 WT (A) a JE2-derived *rexB* mutant (B), WT SH1000 (C) and SH1000-derived *rexB* mutant (D) after 16 h static incubation at 37°C in the presence of doubling concentrations of SXT in TSB supplemented with 0 - 3.13 μM thymidine as indicated. Graphs represent mean±SD from n=3. (E-F) Growth (OD$_{595}$) of *S. aureus* JE2 WT (E) and WT SH1000 (F) in TSB and MHB supplemented with doubling dilution
concentrations of SXT. Graphs represent mean±SD from n=3. (G-I) Time-course survival assays of *S. aureus* JE2 WT or mutants defective for DNA damage repair (G), *S. aureus* JE2 WT, *rexB* mutant only, or transformed with empty vector (pEmpty) or complemented mutant (*prexB*A) (H) and SH1000 WT, *rexB* mutant only and *rexB* mutant transformed with pEmpty or *prexB*A (I). For (G-I) all strains were incubated in MHB containing 4 μg ml⁻¹ SXT. Percentage survival at each time point was calculated relative to the starting inoculum of ~1x10⁸ CFU ml⁻¹. Graphs represent mean±SD from 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 ≤ 0.05(*), P ≤ 0.01(**), P ≤ 0.001(***), P ≤ 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 after 16 h of incubation under aerobic (black) or anaerobic (grey) conditions. The graph represents mean±SD from n=4. Zones which are significantly different in size between aerobic and anaerobic conditions were determined by a two-tailed Student’s t-test. (C,D) Time-course survival assays of *S. aureus* JE2 (C) and SH1000 (D), *rexB* mutants without or with empty vector (pEmpty) or complemented (p*rexBA*) in TSB with (+SXT) or without 4 μg ml⁻¹ SXT, under anaerobic conditions. Percentage survival at each time point was calculated relative to the starting inoculum of ~1x10⁸ CFU ml⁻¹. Graphs represent mean±SD from n=3. Values that are statistically different from the WT were determined by two-way ANOVA corrected for multiple comparison using the Sidak method. (E-F) Survival of *S. aureus* JE2 (E) and SH1000 (F) WT and *rexB* mutants at 24 h (T24) in TSB with (+SXT) and without (-SXT) 4 μg ml⁻¹ SXT under anaerobic conditions. Starting inoculums (T0) varied from 1x10⁸ to 1x10⁵ CFU ml⁻¹. Graph represents mean±SD from n=4. Statistical significance between T24 growth +/- SXT was determined by multiple two-tailed t-tests. P ≤ 0.05(*), P ≤ 0.01(**), P ≤ 0.001(***) P ≤ 0.0001(****). (G-H) Detection of ROS production by bacteria using the DCF fluorophore for *S. aureus* JE2 WT (G) or *rexB* mutant (H) in the presence of SXT. RFU generated by ROS were normalised to OD₆₀₀ data (I,J) to determine ROS production relative to cell density. (G-J) Error bars were omitted for clarity. For panels H and J, logarithmic growth rates (ΔOD₆₀₀ min⁻¹) for each SXT concentration are shown adjacent to the line graphs.
Figure 4. RexAB is essential for the initiation of the SOS response in response to SXT. (A-D, I-L) Growth (OD$_{600}$) of WT JE2 (A,C, I, K) and rexB mutant (B, D, J, L) in low thymidine TSB (A,B, I-L) and high thymidine MHB (C,D) in the presence of a range of SXT (A-D), TMP (I-J) and SMX (K,L) 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). (M-P) Expression of recA-gfp in response to TMP (M,N) and SMX (O,P) concentrations in JE2 WT (M,O), rexB mutant (N,P) in TSB. Fluorescence generated by GFP was measured and normalized to OD$_{600}$ readings to relate recA expression relative to cell number. (A-P) Graphs represent mean values from n=3. Error bars were omitted for clarity.
Figure 5. RexAB and UmuC are required for the increased mutation rate mediated by SXT.

(A,B) The mutation rate of *S. aureus* JE2 or mutants lacking components of DNA repair in TSB in the absence or presence of 0.05 μg ml⁻¹ (A) or 0.1 μg ml⁻¹ (B) SXT. Error bars represent 95% confidence intervals. (C-F) Survival and MIC values of 6 independent cultures of JE2 (C, D) and rexB mutant (E, F) upon 3 rounds of SXT exposure at 4 μg ml⁻¹ for 8 h followed by ~16 hour recovery in TSB. Percentage survival at 8 h was calculated relative to the starting inoculum of ~1x10⁸ CFU ml⁻¹. Statistical significance was determined by one-way ANOVA.
corrected for multiple comparison using the Dunnett method. P ≤ 0.05(*), P ≤ 0.01(**), P ≤ 0.001(***), P ≤ 0.0001(****).
