Staphylococcal DNA repair confers tolerance of the bactericidal activity of both neutrophils and antibiotics

Kam Pou Ha[1]*, Rebecca S. Clarke[1]*, Jane L. Brittan[2], Jessica E. Rowley[1], Despoina A. I. Mavridou[1,3], Thomas B. Clarke[1], Angela H. Nobbs[2] and Andrew M. Edwards[1#]

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

[2] Bristol Dental School, University of Bristol, Bristol, BS1 2LY, UK.

[3] Department of Molecular Biosciences, University of Texas at Austin, Austin, 78712, Texas, USA

* these authors contributed equally.

[#] For correspondence:
a.edwards@imperial.ac.uk

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Running title: Neutrophils and antibiotics target *S. aureus* DNA

Abstract

Staphylococcus aureus is a leading cause of chronic and recurrent infections of the skin, bones, joints and bloodstream. During infection, *S. aureus* faces the twin threats of the host immune system and therapeutic antibiotics. Since both host immune cells and antibiotics generate reactive oxygen species, we hypothesised that *S. aureus* may employ a common mechanism to repair damage caused by either threat. We found that staphylococcal DNA is damaged during exposure to both human neutrophils and most bactericidal antibiotics. To understand the nature of this damage and how *S. aureus* repairs it, we screened a panel of transposon mutants defective for various DNA repair processes. This revealed that loss of the *rexBA* operon significantly reduced staphylococcal survival in human blood, during incubation with purified neutrophils, in the peritoneal cavity of mice and during exposure to a large panel of bactericidal antibiotics. We then used biochemical assays to demonstrate that RexAB is a member of the AddAB family of ATP-dependent helicase/nucleases that are required for the repair of DNA double strand breaks. Finally, we found that RexAB homologues in *Enterococcus faecalis* and *Streptococcus gordonii* also promoted survival of these pathogens in human blood, suggesting that DNA repair constitutes a broadly conserved defence against neutrophils. Together, these data demonstrate that DNA is a target of host immune cells and several antibiotics, leading to double-strand breaks, and that repair of this damage by an AddAB-family enzyme enables the survival of Gram-positive pathogens during infection.

55 **Introduction**

56 *Staphylococcus aureus* is a major human pathogen, responsible for a raft of serious invasive infections
57 including bacteraemia, infective endocarditis and osteomyelitis[1]. Whilst the combination of a potent
58 immune response and the use of antibiotics significantly reduces mortality, many infections become
59 chronic or recurrent[2]. This implies that *S. aureus* has acquired mechanisms by which it can tolerate
60 the damaging effects of both immune cells and antibiotics, and that disruption of such tolerance
61 mechanisms may therefore provide new therapeutic approaches to combat chronic and recurrent
62 infections.

63 Entry of *S. aureus* into normally sterile tissues triggers a strong immune response, with significant
64 infiltration of neutrophils to control infection[3,4,5]. Neutrophils phagocytose *S. aureus*, exposing them
65 to a bactericidal cocktail of antimicrobial peptides and proteases[3,6], as well as the oxidative burst that
66 consists of reactive oxygen species (ROS) that damages lipids, proteins and DNA [7,8,9,10,11,12]. Whilst the
67 contribution of each ROS to bactericidal activity is the subject of investigation, there is compelling
68 evidence that the oxidative burst is crucial for control of *S. aureus* infection[9,12,13]. For example,
69 individuals with chronic granulomatous disease (CGD) are particularly prone to staphylococcal
70 infections because their neutrophils are defective for the oxidative burst[4,14]. In keeping with this, *S.*
71 *aureus* survives better in mice defective for the oxidative burst, relative to wild-type animals, whilst
72 the treatment of human neutrophils with an inhibitor of the oxidative burst increased staphylococcal
73 survival relative to untreated immune cells[5,9,15]. However, even when the oxidative burst is functional,
74 there is evidence that some *S. aureus* cells can survive in neutrophils, which contributes to the
75 progression and persistence of infection[16,17].

76 Despite the importance of the oxidative burst in combatting staphylococcal infection, relatively little
77 is known about how it kills the pathogen. Studies with single oxidants such as H_2O_2 indicate that the
78 molecular targets of the oxidative burst are broad and include proteins, lipids and DNA[18]. To survive
79 this damage, *S. aureus* employs several stress response regulators and repair systems. For example,
80 previous work has shown that DNA damage caused by *S. aureus* exposure to H_2O_2 leads to the
81 initiation of the DNA repair SOS response[19], which facilitates the excision of damaged bases or repair
82 of double-strand breaks (DSBs) by homologous recombination[20,21]. DSBs are particularly dangerous
83 since they are lethal if not repaired, as evidenced by the rapid bactericidal activity of the antibiotic
84 ciprofloxacin which causes this type of DNA damage[21]. However, H_2O_2 is a sub-optimal model for the
85 oxidative burst as it's typically used at concentrations that exceed those generated by the oxidative
86 burst[22]. Therefore, it is unclear whether DNA is damaged by neutrophil-generated ROS, what the
87 nature of this damage is and how *S. aureus* repairs it.

88 In addition to the threat posed by neutrophil generated ROS during infection, *S. aureus* is also targeted
89 by therapeutic antibiotics such as the β-lactam oxacillin[23]. In addition to the established molecular
90 targets of antibiotics, there is an increasingly large body of evidence that bactericidal antibiotics
91 trigger endogenous ROS production in bacteria, leading to DNA damage [24–31]. However, the degree to
92 which these ROS contribute to bacterial killing is the subject of debate[32-34].
93 Since ROS generated by neutrophils and antibiotics have been linked to bactericidal activity against *S.*
94 *aureus*, we hypothesised that oxidants from both sources target the same bacterial macromolecules
95 and that the mechanisms used by staphylococci to survive exposure to host immune defences may
96 therefore also enable staphylococci to tolerate damage caused by antibiotics. This would explain how
97 *S. aureus* is so successful in surviving the twin threats of neutrophils and antibiotics and may identify
98 targets for novel therapeutics that sensitise *S. aureus* to both host defences and antibiotics, enabling
99 the efficient clearance of the pathogen from infection sites.
100 To test this hypothesis, we determined whether staphylococcal DNA is a target of both antibiotic-
101 induced ROS and the oxidative burst of neutrophils and elucidated the mechanism by which this
102 pathogen repairs damage caused by both threats. This revealed that a single DNA repair pathway
103 conferred tolerance of damage caused by both neutrophils and multiple bactericidal antibiotics.
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106 **Results**
107 **Neutrophils and most antibiotics cause DNA damage in *S. aureus***
108 DNA damage in most bacteria, including *S. aureus,* triggers activation of the SOS response, which leads
109 to the transcription of genes whose products contribute to DNA repair. These include *recA*, a crucial
110 mediator of DNA recombination and a regulator of the SOS response[21]. To determine whether
111 neutrophils and antibiotics cause DNA damage in *S. aureus*, we constructed a P*recA-gfp* reporter
112 system that placed the expression of *gfp* under the control of the *recA* promoter in strains from two
113 distinct genetic backgrounds: SH1000, a methicillin-sensitive *S. aureus* (MSSA) strain, and JE2, a
114 community-associated methicillin-resistant *S. aureus* (CA-MRSA) strain of the USA300 lineage[35,36]. We
115 then demonstrated functionality of this system by showing a dose-dependent fluorescent signal in
116 response to mitomycin C, a well-characterised DNA-damaging antibiotic that triggers the SOS
117 response (Fig. S1A,B).
118 *S. aureus* strains bearing the P*recA-gfp* reporter were incubated with purified human neutrophils to
119 measure both phagocytosis and induction of *recA* expression (Fig. S2). To study the role of the
120 oxidative burst in this process, some of the neutrophils were pre-treated with the NADPH inhibitor
121 diphenyleneiodonium (DPI), whilst others were incubated with the DMSO solvent alone. Incubation

122 of wild-type SH1000 and JE2 strains containing the *recA-gfp* reporter with neutrophils resulted in rapid
123 phagocytosis, with >90% bacteria internalised within 5 min and >99% by 30 min (Fig. 1A). During the
124 first 30 min, there was an increase in GFP-mediated fluorescence for both SH1000 and JE2 strains
125 incubated with neutrophils treated with DMSO (Fig. 1B). There was also a GFP signal from bacteria
126 incubated with DPI-treated neutrophils, but to a lesser degree than that seen for DMSO only (Fig. 1B).
127 Therefore, phagocytosis of *S. aureus* by neutrophils leads to induction of the SOS response, partly but
128 not exclusively due to the oxidative burst.
129 Previous work from our group has shown that co-trimoxazole triggers the SOS response in *S. aureus*
130 in a time and dose-dependent manner[37]. To determine whether other antibiotic classes damaged DNA
131 and thus induced the SOS response, we incubated the *S. aureus* P*recA-gfp* reporter strains with various
132 antibiotics (Fig. S3). We found that ciprofloxacin and nitrofurantoin strongly triggered SOS induction
133 in both the SH1000 and JE2 strains (Fig. 1C,D). DNA damage was also apparent during exposure to
134 oxacillin, daptomycin, chloramphenicol or linezolid, albeit to varying degrees, but not when exposed
135 to gentamicin (Fig. 1E-I).
136 Taken together, these data demonstrated that both neutrophils and almost all clinically relevant
137 antibiotics, including bacteriostatic agents and those that do not directly target bacterial DNA
138 replication triggered the SOS response in *S. aureus*, which is indicative of DNA damage.
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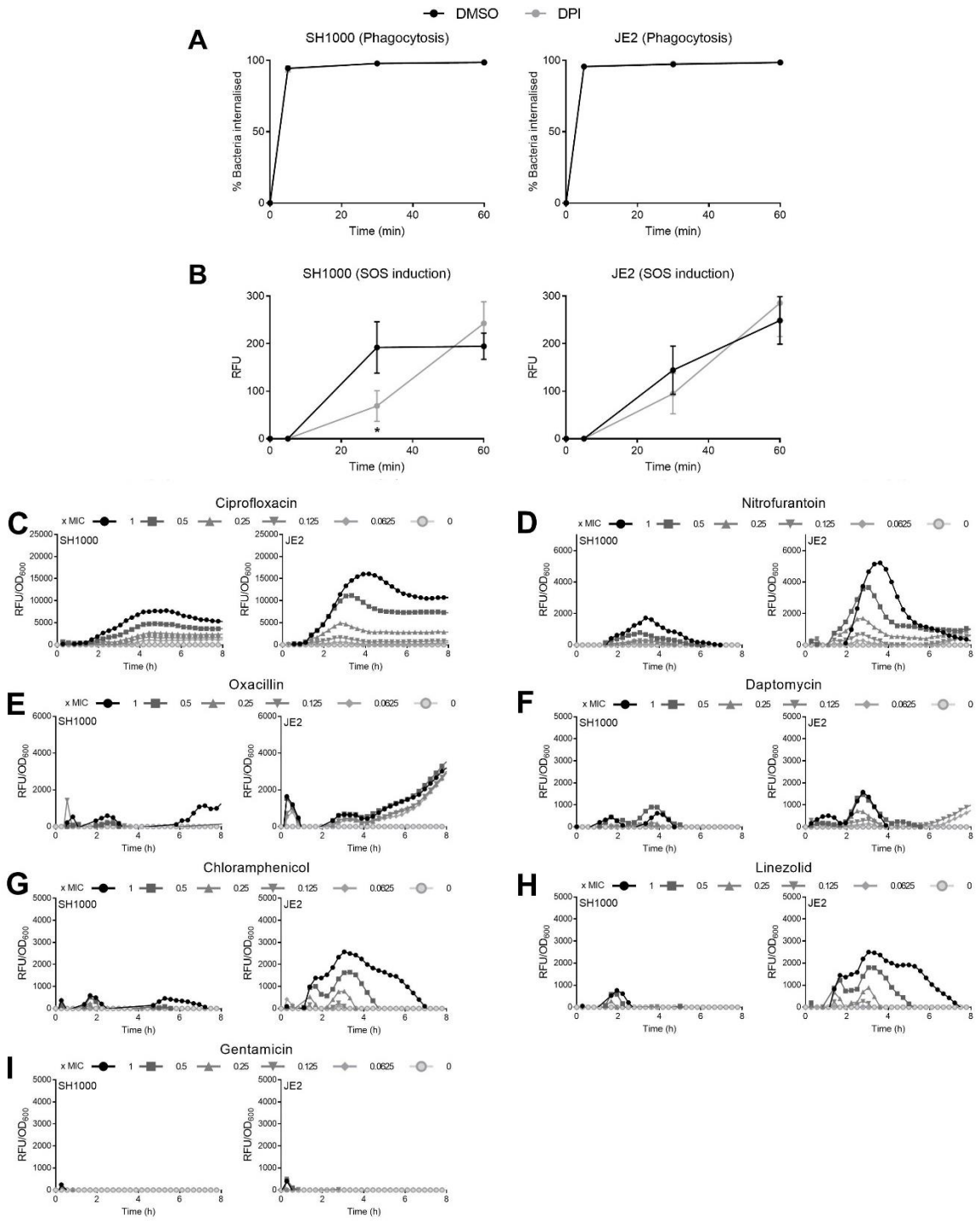

Figure 1. Induction of *S. aureus* SOS response by neutrophils and antibiotics. (A) Phagocytosis of SH1000 and JE2 WT *S. aureus recA-gfp* reporter strains by neutrophils and **(B)** resulting induction of the SOS response measured by GFP expression (RFU) in the presence of the oxidative burst inhibitor DPI (grey) or an identical volume of the DMSO solvent alone (black) (n = 4, with blood from different donors). **(C-I)** Induction of SOS measured by GFP expression upon exposure to a range of concentrations of various antibiotics. Antibiotic concentrations are labelled by multiple of the MIC of the individual strain (x MIC). GFP fluorescence was normalised by OD_{600} to determine induction of SOS relative to cell density (n = 3). OD_{600} measurements alone are shown in Fig. S3. Data in **B** were analysed by two-way ANOVA with Sidak's post-hoc test, for DPI-treated versus DMSO-treated (*, $P < 0.05$). Where shown, error bars represent standard deviation of the mean. Error bars were omitted in **C-I** for clarity.

154 **The RexAB DNA repair complex is required for the survival of *S. aureus* during infection**

155 To understand the nature of the DNA damage caused by neutrophils and how it was repaired by *S.*
156 *aureus*, we assembled a panel of mutants from the ordered NARSA transposon library[35], each of which
157 was deficient for a different protein involved in DNA repair. To understand which of these proteins
158 were important for repairing damage caused by neutrophils, we measured the survival of wild-type
159 and mutant *S. aureus* in an *ex vivo* whole human blood model of infection. Previous characterisation
160 of this model system by our group and others has shown that *S. aureus* is rapidly phagocytosed by
161 neutrophils and exposed to the oxidative burst and other killing mechanisms[38,39,40].

162 Our targeted screen revealed that survival of mutants defective for *rexA* or *rexB* was <5% of the
163 inoculum, compared with >60% for the wild type (Fig. S4). The *rexA* and *rexB* genes form a two gene
164 operon (*rexBA*) on the staphylococcal chromosome, and are proposed to encode an AddAB
165 helicase/nuclease enzyme involved in the processing of DNA DSBs[41–44]. We confirmed a role for RexAB
166 in staphylococcal survival in blood by complementation of mutants with a plasmid containing the
167 *rexBA* operon (p*rexBA*), which restored bacterial survival to wild type levels (Fig. 2A,B). By contrast,
168 the survival of *rexBA* mutants transformed with the vector alone (pEmpty) was not changed (Fig. 2A,B).

169 Having shown that RexAB contributed to staphylococcal tolerance of neutrophil mediated killing in
170 human blood, we then tested its role in staphylococcal survival during exposure to neutrophils *in vivo*.
171 Mice were infected via the peritoneal cavity, which results in the recruitment of neutrophils within 2
172 h, with wild-type or *rexBA* mutants of *S. aureus* SH1000 or JE2[45]. After 6 h, the mice were sacrificed
173 and the peritoneal cavity washed with PBS to recover bacteria, which were quantified by CFU counts.
174 This revealed that *rexBA* mutants in both genetic backgrounds were significantly attenuated for
175 survival *in vivo* relative to wild type bacteria, confirming that RexAB contributes to staphylococcal
176 resistance to host immune defences and the progression of infection (Fig. 2C,D).

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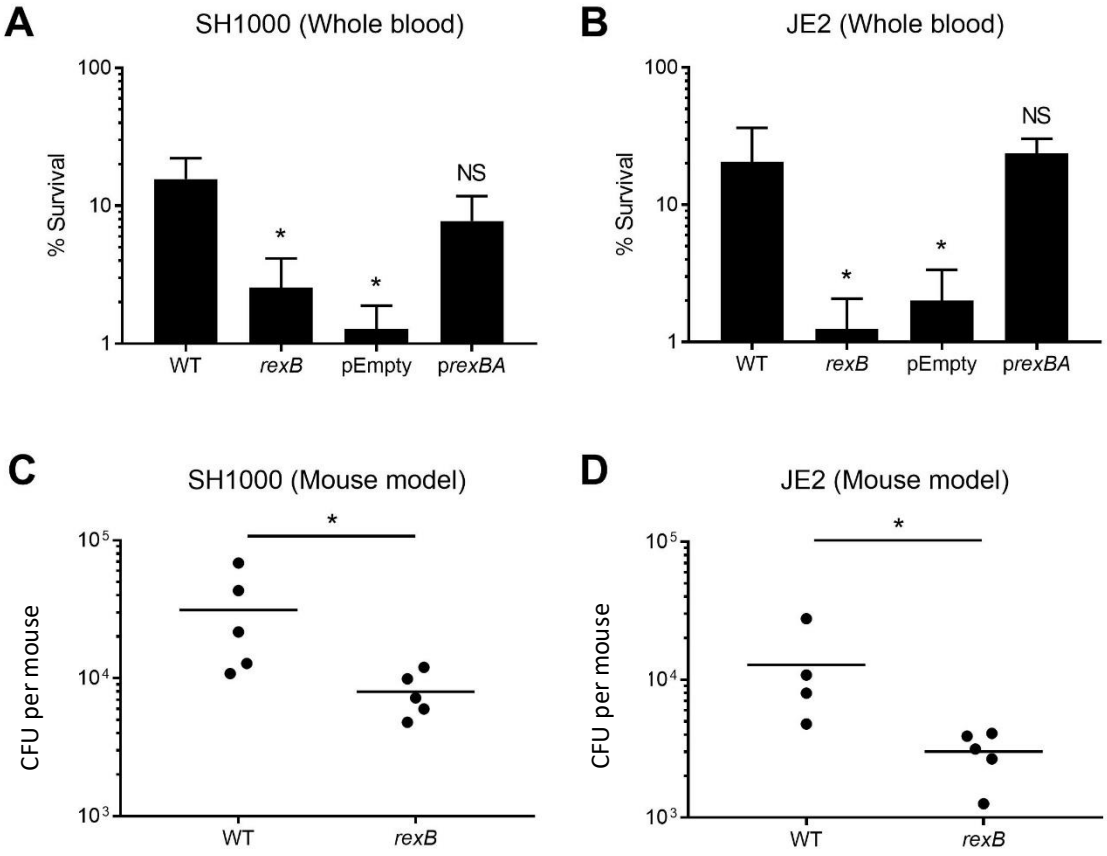

Figure 2. Reduced survival of *rexB* mutants in human blood and in an *in vivo* murine infection model. (A-B) Survival of *S. aureus* WT, *rexB* mutant, empty vector (pEmpty) and complemented mutant (p*rexBA*) in SH1000 (A) and JE2 (B) backgrounds after 6 h incubation in whole human blood. Empty vectors and complemented mutants were supplemented with 100 ng μl^{-1} AHT to control *rexBA* expression ($n \geq 3$). **(C-D)** Number of CFU recovered from the peritoneal cavities of mice 6 h after inoculation (each circle represents a single mouse; n = 4-5). Data in **A** and **B** were analysed by one-way ANOVA with Tukey's post-hoc test, for *rexB,* pEmpty or p*rexBA* versus WT (*, $P < 0.05$). Data in **C** and **D** were analysed by Mann-Whitney test (*, $P < 0.05$). Where shown, error bars represent standard deviation of the mean.

RexAB contributes to staphylococcal tolerance of the oxidative burst

Having confirmed that *rexBA* is important for staphylococcal survival during infection conditions, we wanted to determine whether this was due to repair of DNA damage caused by the oxidative burst of neutrophils. As described above, the survival of *rexBA* mutants in whole human blood was significantly reduced relative to wild-type bacteria (Fig. 2A,B). However, the presence of DPI, which inhibits the NADPH oxidase complex, promoted the survival of *rexBA* mutants to wild-type levels, indicating that the survival deficit observed for bacteria lacking RexAB was due to increased sensitivity to the oxidative burst (Fig. 3A,B). Comparable results were obtained when *S. aureus* was incubated with

224 neutrophils purified from human blood. The *rexBA* mutants were more susceptible to neutrophil
225 mediated killing than wild type, but the presence of DPI restored survival of the *rexBA* mutants to wild
226 type levels (Fig. 3C,D).
227 These data strongly indicated that RexAB contributed to staphylococcal survival of the oxidative burst
228 by repairing DNA. However, several different factors have been shown to affect survival of *S. aureus*
229 incubated in human blood or exposed to oxidative stress such as production of the staphyloxanthin
230 pigment[38]. However, whilst there was a slight growth defect for the *rexBA* mutant relative to the wild
231 type, we did not see significant differences between wild type strains, *rexBA* mutants and
232 complemented strains for toxin production, staphyloxanthin pigment production or catalase activity
233 (Fig. S5). Together, the data shown in Figures 1-3 demonstrate that neutrophils cause DNA damage in
234 *S. aureus*, in part via the oxidative burst, which needs to be repaired by RexAB to enable staphylococcal
235 survival in the host.
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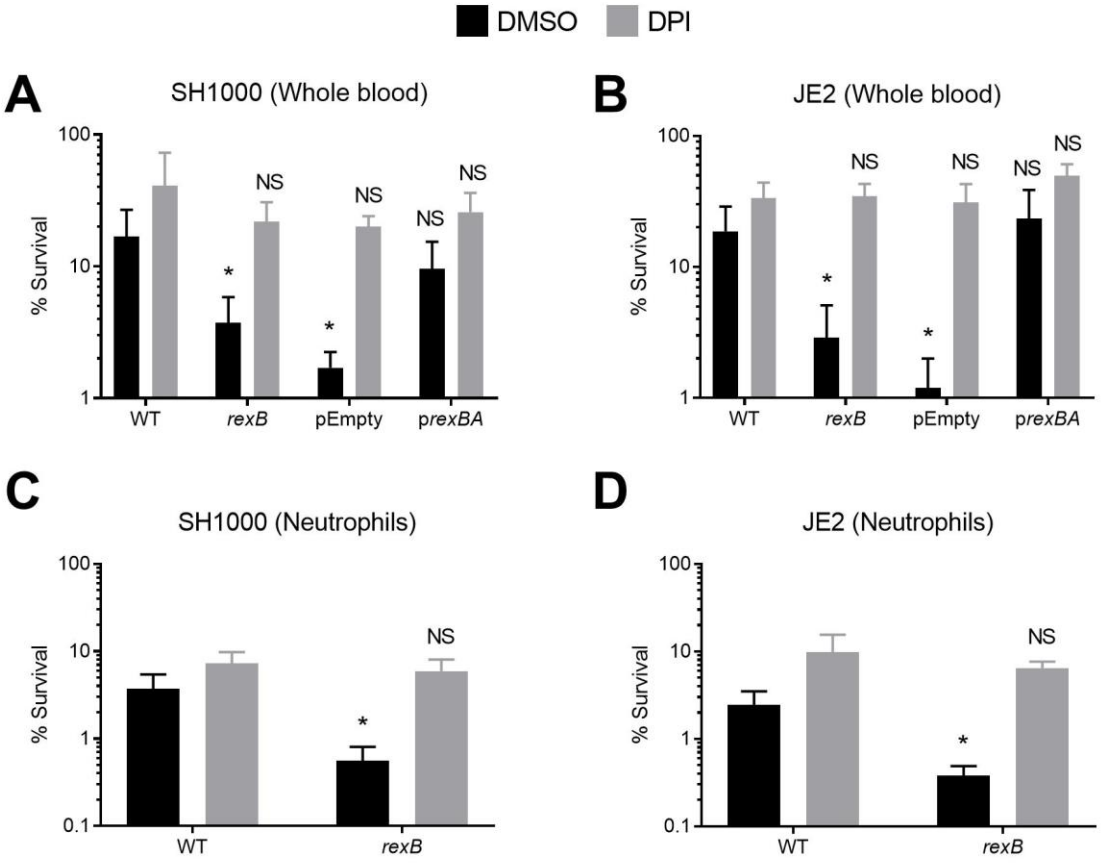

Figure 3. RexAB protects *S. aureus* from the oxidative burst of phagocytes. (A-B) Survival of *S. aureus* WT, *rexB* mutant, empty vector (pEmpty) and complemented mutant (p*rexBA*) in SH1000 (A) and JE2 (B) backgrounds in whole human blood after 6 h of incubation, in the presence of the oxidative burst inhibitor DPI (grey) or an identical volume of the DMSO solvent alone (black). Empty vectors and complemented mutants were supplemented with 100 ng μl^{-1} AHT to induce *rexBA* expression (n = 3). **(C-D)** Survival of *S. aureus* WT and *rexB* mutant when incubated with purified human neutrophils for 3 h (n = 4). Data in **A** and **B** were analysed by one-way ANOVA with Tukey's post-hoc test, for *rexB,* pEmpty or p*rexBA* versus WT (*, $P < 0.05$). Data in **C** and **D** were analysed by Student's t-test, for *rexB* mutant versus WT (*, $P < 0.05$). Error bars represent standard deviation of the mean.

RexAB contributes to the survival of *S. aureus* exposed to bactericidal antibiotics

We have shown previously that RexAB enables staphylococcal tolerance of the combination antibiotic co-trimoxazole[37]. Since most of the DNA damage caused by co-trimoxazole was due to the production of ROS, we hypothesised that RexAB would promote the growth and survival of *S. aureus* when incubated with other bactericidal antibiotics reported to trigger ROS production in bacteria[24–30].

To test this, we exposed wild-type *S. aureus* SH1000 and JE2, along with associated *rexB* mutants, to a panel of bactericidal (ciprofloxacin, nitrofurantoin, oxacillin, daptomycin, gentamicin, vancomycin, fosfomycin) and bacteriostatic (chloramphenicol, linezolid) antibiotics and determined the minimum

263 inhibitory concentration (MIC) of each (Fig. 4A). The *rexB* mutants in both JE2 and SH1000 strains were
264 ≥2-fold more susceptible to 7 of the 9 antibiotics tested under aerobic conditions, the exceptions being
265 vancomycin, and gentamicin for the JE2 strain only (Fig. 4A). When assays were done under anaerobic
266 conditions the lack of oxygen reduced the susceptibility of one or both *S. aureus* wild-type strains to
267 ciprofloxacin, daptomycin, oxacillin and gentamicin, inferring that antibiotic-triggered ROS
268 contributed to growth inhibitory activity. Furthermore, under anaerobic conditions, the only
269 antibiotics that were more active against both *rexB* mutants relative to their parental strains were
270 ciprofloxacin, linezolid and genatmicin, suggesting that ROS are the primary, but not the only,
271 mechanism by which antibiotics cause DNA damage.
272 Next, we exposed wild-type *S. aureus* and *rexB* mutants to the same antibiotics at 10x the MIC and
273 measured survival after 8 h (Fig. 4B-J). Similar to the MIC assays, most antibiotics (6/9) were more
274 active against the *rexB* mutant relative to wild type bacteria under aerobic conditions. This resulted in
275 5-500-fold greater reduction in CFU counts of the *rexB* mutant compared to wild type cells after 8h
276 exposure to ciprofloxacin, nitrofurantoin, oxacillin, daptomycin, gentamicin and fosfomycin (Fig. 4B-
277 I).
278 Next, the role of ROS was investigated by repeating assays under anaerobic conditions. This revealed
279 that the presence of oxygen contributed to the bactericidal activity of gentamicin but none of the
280 other antibiotics examined against wild type bacteria (Fig. 4B-I). However, whilst most antibiotics
281 killed efficiently under anaerobic conditions, in almost all cases they were equally active against both
282 wild type and *rexB* mutants (Fig. 4B-I). The exception to this was ciprofloxacin which directly damages
283 DNA leading to DNA DSBs (Fig. 4B).
284 Taken together, these findings support previous work by showing that several diverse classes of
285 antibiotics cause DNA damage under aerobic conditions, most likely via ROS generation[24-30,37].
286 However, this damage does not contribute to killing of wild type bacteria because damage is repaired
287 via RexAB and the SOS response.
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Minimum inhibitory concentration (µg/ml)								
	Aerobic				Anaerobic			
	SH1000 WT	SH1000 *rexB*	JE2 WT	JE2 *rexB*	SH1000 WT	SH1000 *rexB*	JE2 WT	JE2 *rexB*
Ciprofloxacin	0.125	0.0078	16	4	0.25	0.03	32	2
Nitrofurantoin	8	4	16	4	8	4	8	8
Oxacillin	0.125	0.06	0.125	0.06	0.125	0.125	0.25	0.125
Daptomycin	0.25	0.125	0.25	0.125	1	1	1	0.5
Chloramphenicol	4	1	4	2	4	4	4	4
Linezolid	1	0.25	1	0.25	1	0.25	1	0.5
Gentamicin	0.0625	0.031	0.125	0.125	16	1	4	2
Vancomycin	1	1	1	1	1	1	1	1
Fosfomycin	16	8	32	16	8	4	16	16

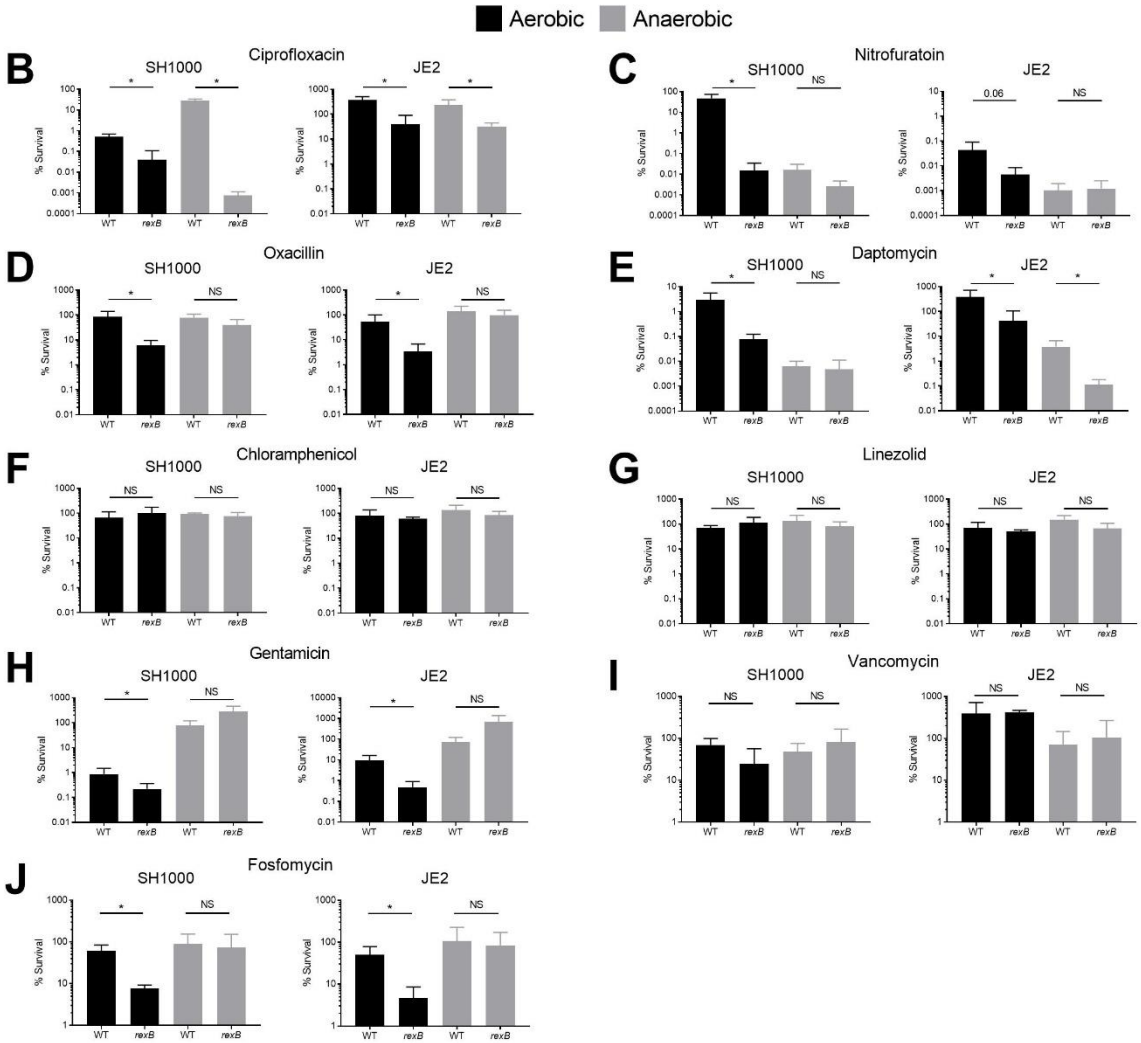

Figure 4. Lack of effective DNA repair increases the sensitivity of *S. aureus* to bactericidal antibiotics in MIC and killing assays. (A) MIC table of *S. aureus* WT and *rexB* mutant in SH1000 and JE2 backgrounds grown under aerobic and anaerobic conditions (n ≥ 3; median MIC is shown). **(B-J)** Survival of *S. aureus* WT and *rexB* mutant in SH1000 and JE2 backgrounds after 8 h incubation at 37°C in TSB supplemented with 10x MIC. Survival was assessed under aerobic (black) or anaerobic (grey) conditions (n = 3). Data in **B-J** were analysed by Student's t-test (*, P < 0.05). Where shown, error bars represent standard deviation of the mean.

300 **RexAB is required for induction of the SOS response during exposure to the oxidative burst and**
301 **bactericidal antibiotics.**
302 The SOS response facilitates homologous recombination and mutagenic DNA repair, promoting the
303 acquisition of adaptive mutations and antibiotic resistance[20,21,37]. To determine the degree to which
304 RexAB-mediated repair of DNA damage caused by neutrophils and antibiotics triggered SOS, we used
305 the P*recA-gfp* reporter system in JE2 wild-type and *rexB* mutant strains (Fig. S6). Both JE2 wild-type
306 and *rexB* mutant were rapidly phagocytosed by the neutrophils, with similar levels of internalised
307 bacteria (Fig. 5A). We detected GFP expression in wild type bacteria, but this was significantly weaker
308 for the *rexB* mutant, indicating that RexAB-mediated repair is required for full induction of the SOS
309 response in phagocytosed *S. aureus* (Fig. 5B).
310 Next, induction of *recA* in the JE2 wild-type and *rexB* mutant was monitored during exposure to a
311 panel of antibiotics. We found that the lack of RexAB reduced *recA* induction relative to the wild-type
312 for the DNA-targeting antibiotic ciprofloxacin (Fig. 5C). Similar trends were observed with
313 nitrofurantoin, oxacillin, daptomycin, chloramphenicol and linezolid, albeit to varying degrees (Fig. 5D-
314 H). As expected from previous data (Fig. 1I), no *recA* induction was observed from either wild-type or
315 mutant when exposed to gentamicin (Fig. 5I).
316 Taken together, these data show that RexAB is required for maximal induction of SOS in response to
317 DNA damage caused by neutrophils or antibiotics, which facilitates repair of DNA damage.
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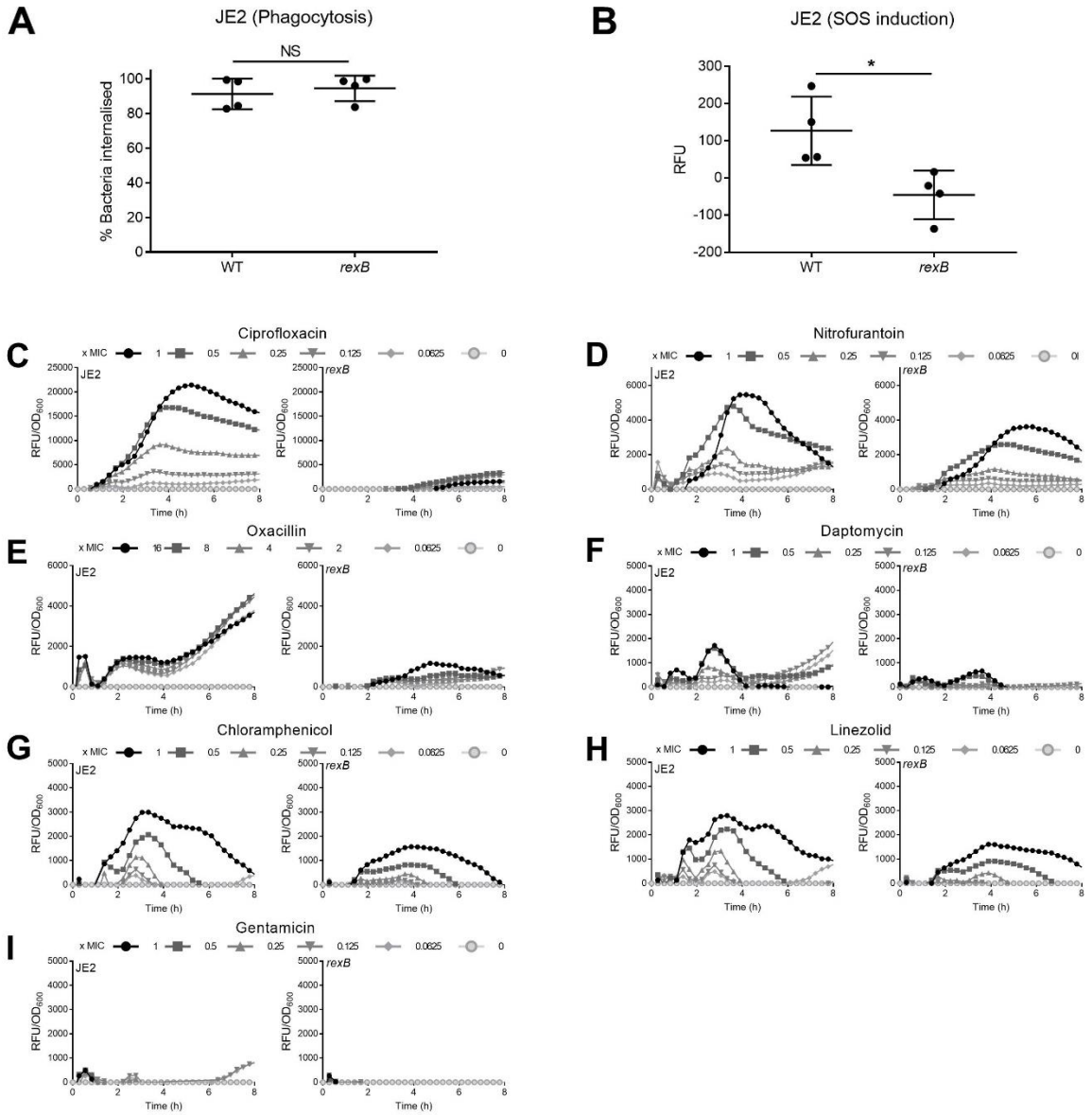

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321 **Figure 5. RexAB is required for induction of the SOS response during exposure to the oxidative burst**
322 **of neutrophils and bactericidal antibiotics. (A)** Phagocytosis of JE2 wild-type and *rexB* mutant *recA-*
323 *gfp* reporter strains by neutrophils and **(B)** resulting induction of the SOS response measured by GFP
324 expression (RFU) after 30 min of exposure (n = 4). **(C-I)** Induction of SOS response of JE2 wild-type and
325 *rexB* mutant measured by GFP expression upon exposure to varying sub-lethal concentrations of
326 bactericidal antibiotic. Concentrations of antibiotic are labelled by multiple of the MIC of the wild-type
327 strain (x MIC). GFP fluorescence was normalised by OD_{600} to determine induction of SOS relative to
328 cell density (n = 3). OD_{600} measurements alone are shown in Fig. S6. Data in **A** and **B** were analysed by
329 Mann-Whitney test (*, $P < 0.05$). Where shown, error bars represent standard deviation of the mean.
330 Error bars were omitted in **C-I** for clarity.

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335 **Staphylococcal RexAB is a member of the AddAB family of ATP-dependent helicase/nucleases**

336 Previous studies have identified staphylococcal RexAB as a member of the AddAB enzyme family via
337 sequence homology[41–44]. In support of this, *in silico* structural analysis of the predicted *rexBA* gene
338 products confirmed that this operon encoded an AddAB-type ATP-dependent helicase/nuclease
339 involved in DSB repair (Fig. S7). However, there is no experimental evidence that RexAB is a functional
340 AddAB homologue.

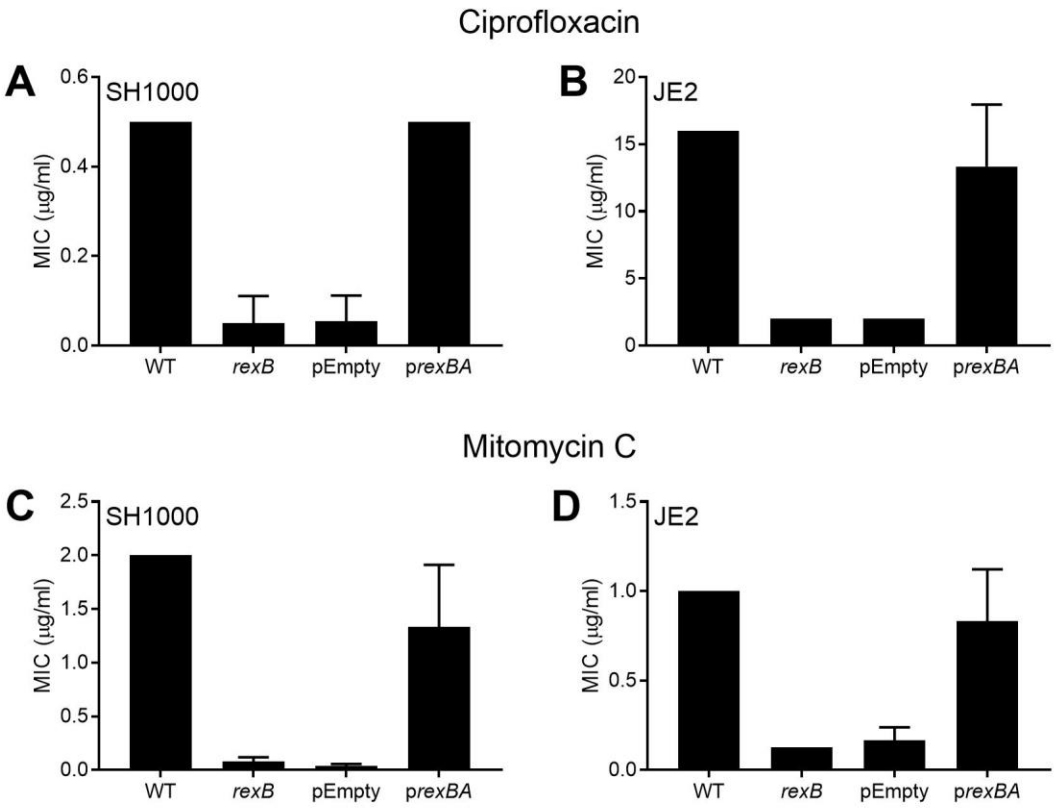
341 AddAB enzymes process DSBs to generate a 3' single-stranded DNA overhang that is necessary for
342 RecA-mediated homologous recombination[46]. In support of the structural predictions, phenotypic
343 testing of *rexBA* mutants showed that they were more sensitive than wild-type strains to the DNA-
344 damaging antibiotics ciprofloxacin and mitomycin C (Fig. 6), both of which cause DSBs[47,48].

345 The generation of the 3' overhang by AddAB proteins results in the formation of a RecA filament,
346 which triggers the SOS response. Therefore, we determined whether RexAB was needed for SOS
347 induction in response to DNA double strand breaks caused by ciprofloxacin or mitomycin C using the
348 *recA-gfp* reporter system described previously. When compared to wild-type bacteria, the JE2 *rexBA*
349 mutant had greatly reduced *recA* expression in response to either antibiotic, providing additional
350 evidence that RexAB is an AddAB-family enzyme (Fig. 5C and S1).

351 To confirm the ATP-dependent helicase/nuclease activity of the *S. aureus* RexAB complex,
352 recombinant RexAB protein was generated and the helicase and nuclease activities were measured
353 over 1 h. Nuclease activity assays were performed under conditions of high free Mg^{2+}, which has been
354 previously shown to activate nuclease activity in AddAB enzymes[49]. We found that DNA was degraded
355 over time by the recombinant complex in the presence of ATP, whereas this degradation was minimal
356 in its absence, demonstrating that RexAB has ATP-dependent nuclease activity (Fig. 7A).

357 At conditions of low free Mg^{2+} to measure helicase activity, we observed a loss of dsDNA concomitant
358 with an increase in ssDNA formation over time (Fig. 7B,C). These experiments were repeated in the
359 absence of single-stranded DNA binding protein (SSB), which is required to prevent reannealing of
360 DNA. In the absence of SSB, no DNA unwinding or ssDNA formation were observed, demonstrating
361 that RexAB has ATP-dependent helicase activity (Fig. 7D,E).

362 Combined, these results show that RexAB is required for the growth of *S. aureus* in the presence of
363 DNA-damaging antibiotics, induction of the SOS response and exhibits ATP-dependent
364 helicase/nuclease activities, demonstrating it is a functional member of the AddAB family of DNA
365 repair complexes.



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367 **Figure 6. RexAB is required for growth of *S. aureus* in the presence of DNA-damaging antibiotics.**
368 MIC of *S. aureus* WT, *rexB* mutant, empty vector (pEmpty) and complemented mutant (p*rexBA*) in
369 SH1000 and JE2 backgrounds, for ciprofloxacin (A-B) and mitomycin C (C-D). Empty vectors and
370 complemented mutants were supplemented with 100 ng μl^{-1} AHT to induce *rexBA* expression (n = 3;
371 median MIC is shown). Error bars represent standard deviation of the mean.

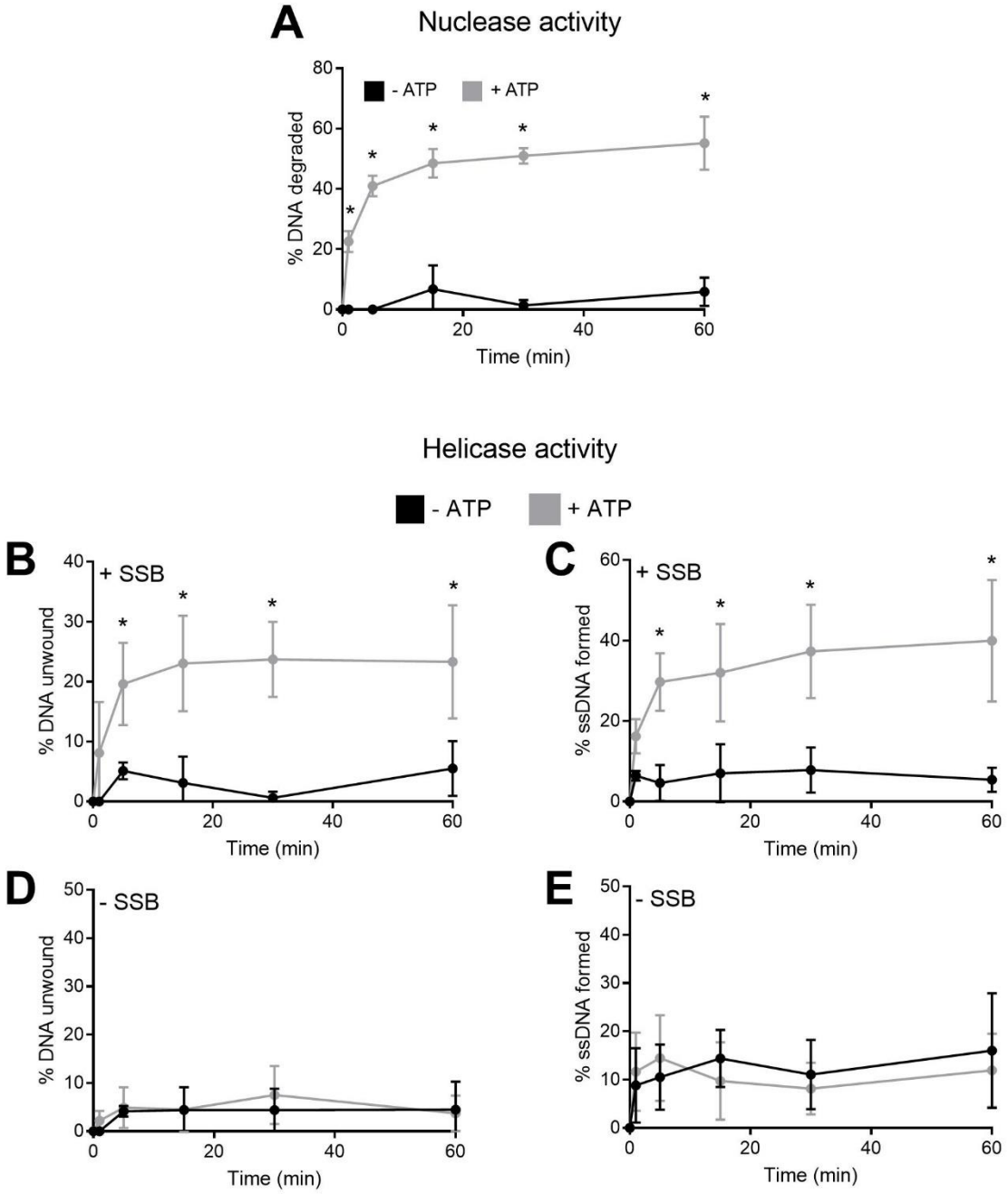

Figure 7. RexAB possesses nuclease and helicase activity. (A) Nuclease activity of recombinant *S. aureus* RexAB protein in the presence or absence of ATP (grey and black, respectively) (n = 4). **(B-E)** Helicase activity of recombinant *S. aureus* RexAB protein in the absence (-) or presence (+) of ATP, by measuring unwinding of dsDNA and formation of ssDNA. Single-stranded DNA binding protein (SSB) was added to prevent reannealing of DNA (B-C) (n = 4). Equivalent assays were performed without SSB to confirm helicase activity (D-E) (n = 4). Data in **A-C** were analysed by two-way ANOVA with Sidak's post-hoc test, for without ATP versus with ATP (*, $P < 0.05$). Error bars represent standard deviation of the mean.

383 **RexAB is required for survival of other Gram-positive pathogens in human blood and during**
384 **exposure to certain antibiotics**
385 Since homologues of RexAB are present in most Gram-positive bacteria[43,44,46], we next tested whether
386 this repair complex contributes to the survival of other bacteria exposed to neutrophils. Like *S. aureus*,
387 *Enterococcus faecalis* and *Streptococcus gordonii* are frequent causes of infective endocarditis, which
388 brings these species into close contact with neutrophils[50,51]. Therefore, *rexBA* was deleted in
389 representative strains of each species and their sensitivity to the microbicidal activity of neutrophils
390 determined using the *ex vivo* whole human blood model.
391 Wild-type *E. faecalis* survived at high levels in human blood with ~100% of the inoculum remaining
392 viable during the full 6 h duration of the assay, but the loss of *rexBA* reduced enterococcal survival by
393 ~50%. This indicated that *E. faecalis* suffers DNA damage whilst in blood, but it can be tolerated via
394 DNA repair (Fig. 8A). By contrast to *S. aureus*, DNA damage in *E. faecalis* did not appear to be due to
395 the oxidative burst, since survival of both wild type and *rexB* mutant in blood was unaffected by the
396 presence of DPI (Fig. 8A).
397 *S. gordonii* was much more susceptible to host defences in blood than *E. faecalis*, with <5% wild-type
398 bacteria remaining viable after 2 h (Fig. 8A). However, survival of the *rexBA* mutant was still reduced
399 relative to the wild type, with <1% streptococci surviving after 2 h, indicating that DNA repair also
400 contributes to the survival of *S. gordonii* in blood (Fig. 8A). As for *E. faecalis*, inhibition of the oxidative
401 burst with DPI did not promote survival of either the wild type or *rexBA* mutant indicating that DNA
402 damage was not due to the oxidative burst. In support of this, the *rexBA* mutants of both *S. gordonii*
403 and *E. faecalis* were no more susceptible to H_2O_2 than wild-type bacteria (Fig. 8B).
404 Next, to determine whether loss of RexAB also increased the susceptibility of *E. faecalis* and *S. gordonii*
405 to bactericidal antibiotics, these strains were exposed to a selection of DNA-damaging (ciprofloxacin,
406 co-trimoxazole) and clinically-relevant (daptomycin, linezolid) antibiotics and survival measured after
407 8 h under aerobic and anaerobic conditions. Consistent with *S. aureus*, *E. faecalis* and *S. gordonii*
408 mutants defective for *rexBA* showed notable reductions in survival during exposure to ciprofloxacin
409 and co-trimoxazole compared with wild type (Fig. 8C,D). However, in keeping with their resistance to
410 ROS, mutants lacking *rexBA* were no more susceptible to daptomycin than wild type (Fig. 8E).
411 Surprisingly, the *E. faecalis rexBA* mutant was killed by the normally bacteriostatic linezolid (Fig. 8F).
412 Similar results were observed under anaerobic conditions (Fig. 8C-F), mimicking the findings in human
413 blood that DNA damage was not caused by the oxidative burst. Therefore, as for *S. aureus*, neutrophils
414 and certain antibiotics cause DNA damage in both *S. gordonii* and *E. faecalis*, the repair of which by
415 RexAB promotes bacterial survival. However, in contrast to *S. aureus*, oxidative stress does not appear
416 to contribute to neutrophil mediated DNA damage in these bacteria.

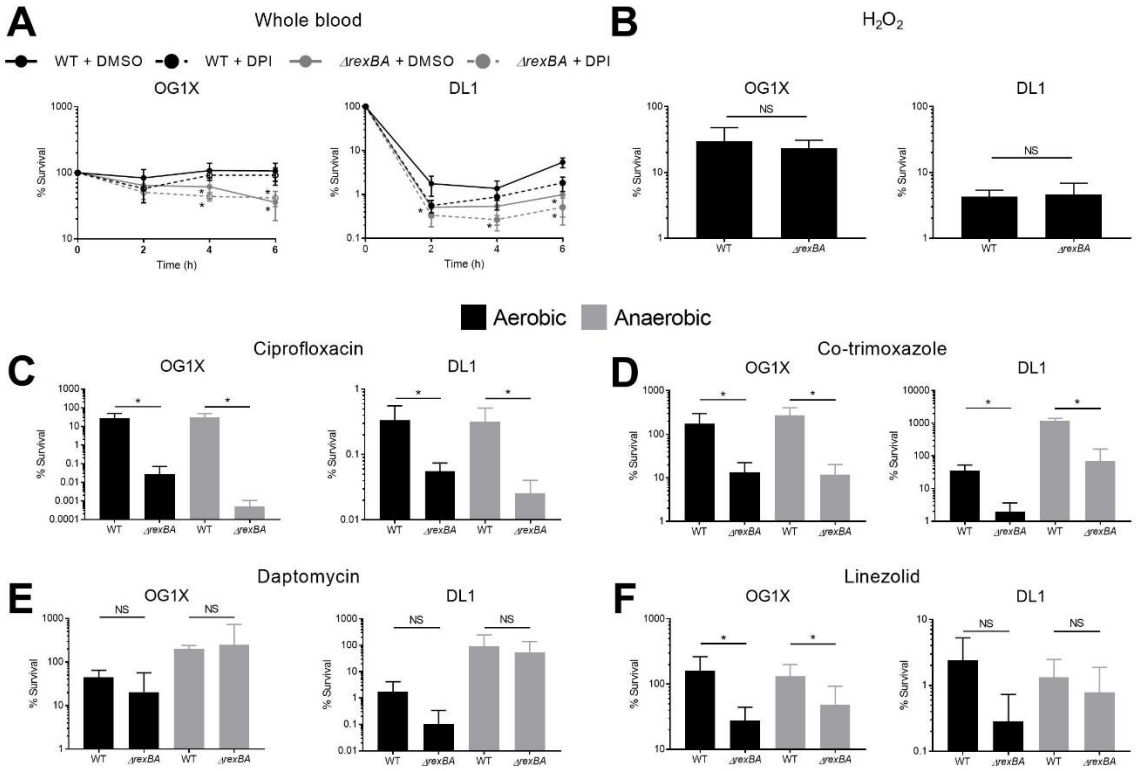

Figure 8. RexAB protects *Enterococcus faecalis* and *Streptococcus gordonii* from host defences in blood and from antibiotics. (A) Survival of *E. faecalis* OG1X and *S. gordonii* DL1 wild-type and *ΔrexBA* mutant in whole human blood over 3 h in the presence of the oxidative burst inhibitor DPI (dashed lines) or an identical volume of the DMSO solvent alone (solid lines) (n = 3). **(B)** Survival of OG1X and DL1 wild-type and Δ*rexBA* mutant exposed to H_2O_2 for 1 h (n = 3). **(C-F)** Survival of OG1X and DL1 wild-type and Δ*rexBA* mutant in THYB supplemented with antibiotic at 10x MIC of the wild-type. Survival was assessed under aerobic (black) or anaerobic (grey) conditions (n ≥ 3). Data in **A** were analysed by two-way ANOVA with Sidak's post-hoc test, for *ΔrexBA* mutant versus WT (*, $P < 0.05$). Data in **C-F** were analysed by Student's t-test (*, $P < 0.05$). Error bars represent standard deviation of the mean.

438 **Discussion**

439 Neutrophils are an essential host defence against *S. aureus*. However, our understanding of the
440 mechanisms by which these immune cells kill staphylococci is limited. Equally, the bactericidal activity
441 of many important anti-staphylococcal antibiotics is only partially understood. The data described
442 above demonstrate that both neutrophils and several different antibiotics cause DNA damage in *S.*
443 *aureus*, leading to induction of the SOS response. Repair of this DNA damage is dependent upon the
444 RexAB complex, which is a member of the AddAB helicase/nuclease family of enzymes involved in the
445 repair of DSBs. This provides new information on both the target of the oxidative burst and individual
446 antibiotics, and the mechanisms by which *S. aureus* repairs and survives this damage.

447 The staphylococcal SOS response was triggered within 30 min of phagocytosis by neutrophils, which
448 corresponds with the time at which ROS are maximally generated in these immune cells[52,53] and
449 establishes bacterial DNA as a target of these cells. This is in keeping with previous reports that ROS
450 damage the DNA of *Escherichia coli* and *Coxiella burnetii* phagocytosed by macrophages[54,55]. The SOS
451 response in *S. aureus* was also triggered by several different classes of antibiotics, which indicated
452 DNA damage. Whilst this was expected for DNA-targeting antibiotics such as the fluoroquinolone
453 ciprofloxacin[21], SOS induction also occurred with antibiotics that do not directly target bacterial DNA.
454 It has been shown that certain β-lactam antibiotics induce the SOS response in *E. coli* via the DpiBA
455 two-component system rather than via DNA damage[56]. Although this mechanism has been
456 hypothesised for *S. aureus*[57,58], our data show that *S. aureus rexBA* mutants were more susceptible to
457 killing by oxacillin, demonstrating that DNA damage does occur during exposure to this beta-lactam
458 antibiotic.

459 Whilst controversial, there is increasing evidence that many classes of antibiotics trigger the
460 production of ROS [24–33]. However, the degree to which these ROS contribute to bactericidal activity is
461 less clear[32,33,34]. Our data provide evidence that many antibiotics cause DNA damage, presumably via
462 ROS. However, this DNA damage appears to be tolerated by wild type bacteria via RexAB-mediated
463 repair.

464 The production of ROS by bactericidal but not bacteriostatic antibiotics has been proposed to explain
465 their differences in lethality. However, we observed SOS induction during exposure of *S. aureus* to the
466 bacteriostatic antibiotics linezolid and chloramphenicol but not the bactericidal antibiotic gentamicin.
467 Interestingly, despite DNA damage, the absence of RexAB did not sensitise *S. aureus* mutants to
468 linezolid or chloramphenicol but did increase susceptibility to gentamicin. This may be explained by
469 differences in the type of DNA damage caused by each of the antibiotics. Whilst several different types
470 of DNA damage trigger SOS, only those leading to DSBs would be expected to promote susceptibility
471 of the *rexBA* mutant. As such, it is possible that bactericidal antibiotics trigger the potentially lethal

472 DNA DSBs, whilst bacteriostatic antibiotics trigger non-lethal types of DNA damage. In keeping with
473 this, the absence of RexAB had only a small effect on SOS induction in *S. aureus* caused by linezolid or
474 chloramphenicol.
475 It is unclear why gentamicin did not trigger SOS during antibiotic exposure, but it may simply be that
476 this antibiotic doesn't trigger endogenous ROS production in *S. aureus*. Combined, our data indicates
477 significant differences between antibiotics in the degree of DNA damage caused, as well as the time
478 required to cause damage. These differences may explain some of the debate around the contribution
479 of ROS to antibiotic-mediated killing.
480 In addition to repair of DNA damage caused by antibiotics, we also found that RexAB contributed to
481 staphylococcal survival during exposure to purified human neutrophils, incubation in whole human
482 blood and in a murine model of infection. Moreover, we also demonstrated that RexAB contributed
483 to the survival of *E. faecalis* and *S. gordonii* in blood. Previous *in vivo* studies have shown that AddAB
484 is required for the infection of chickens and mice by *Campylobacter jejuni* and *Helicobacter pylori*
485 respectively[59,60]. In place of AddAB, many other Gram-negative bacteria employ the closely related
486 RecBCD helicase/nuclease to process DSBs, which also appears to be important for survival in the host.
487 For example, a *Salmonella enterica* mutant lacking RecBCD was found to be less virulent than the wild
488 type in a murine model of bacteraemia and was also defective for replication in macrophages[61].
489 Together, these findings indicate that bacterial DNA is a conserved target of host defences, leading to
490 DSBs that must be repaired for pathogen survival.
491 However, whilst host defences and antibiotics both caused DNA damage, the mechanism seems to
492 vary depending on the species concerned. We found that strains of *E. faecalis* and *S. gordonii* defective
493 for RexAB were more susceptible to host defences in blood, but this was not due to oxidative stress.
494 Neutrophils employ several different antibacterial elements to kill invading pathogens, including
495 reactive oxygen and nitrogen species, proteases and antimicrobial peptides. Several studies have
496 indicated that bacteria vary in their susceptibility to each of these microbicides. In agreement with our
497 findings, Standish *et al.* showed that *S. aureus* but not *Streptococcus pneumoniae* was killed by the
498 oxidative burst[62]. However, whilst *S. aureus* can grow in the presence of nitric oxide, the replication of
499 several other pathogens including *Pseudomonas aeruginosa* and *Streptococcus pyogenes* is
500 inhibited[63]. Since reactive nitrogen species can cause DNA damage, this may provide an explanation
501 for the finding that mutants of *E. faecalis* and *S. gordonii* lacking RexAB are more susceptible to killing
502 by neutrophils in which the oxidative burst is blocked.
503 The identification of RexAB as important for staphylococcal survival during infection and exposure to
504 antibiotics provides insight into the nature of the damage caused by these twin threats and makes this

505 complex a potential target for novel therapeutics, particularly as the lack of RexAB homologues in
506 eukaryotes reduces the likelihood of host toxicity[64].
507 Inhibitors of AddAB and RecBCD have been previously reported in the literature, but problems include
508 limited *in vivo* stability, poor oral bioavailability and a sub-optimal mechanism of action[65–68]. However,
509 Amundsen *et al.* (2012) identified several small-molecule inhibitors of *Helicobacter pylori* AddAB and
510 *E. coli* RecBCD; in particular, ML328[69] indicating that the development of stable, potent inhibitors is
511 possible. More recently, a derivative of ML328 (IMP-1700) was found to be capable of potentiating
512 antibiotic activity such that a resistant *S. aureus* strain was sensitised to ciprofloxacin[70]. However, the
513 ability of IMP-1700 to inhibit AddAB/RecBCD activity under *in vivo* conditions remains to be
514 determined. Further work in this area may lead to broad-spectrum therapeutics that promote
515 bacterial susceptibility to both host defences and antibiotics, and inhibit induction of the mutagenic
516 SOS response.
517 In summary, staphylococcal, streptococcal and enterococcal DNA is damaged by the host immune
518 system and several classes of bactericidal antibiotics leading to DSBs that are lethal if not repaired.
519 This work demonstrates that DNA repair via RexAB is a common mechanism by which these pathogens
520 repair damage caused by both threats and identifies this complex as a potential target for novel
521 therapeutics, capable of sensitising Gram-positive pathogens to both neutrophils and antibiotics.
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539 **Materials and Methods**

540 **Bacterial strains and culture conditions**

541 The bacterial strains used in this study are listed in Table 1. *S. aureus* was cultured in Tryptic Soy Broth
542 (TSB) to stationary phase (18 h) at 37 °C, with shaking (180 rpm). *S. gordonii* and *E. faecalis* were grown
543 in Todd Hewitt Broth (THB) supplemented with 1% (w/v) yeast extract (THB-Y) at 37 °C, statically in
544 5% CO_2. *E. coli* was grown in Lysogeny Broth (LB) or in Terrific Broth (TB) (1.2% (w/v) tryptone, 2.4%
545 (w/v) yeast extract, 0.5% glycerol, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4) for protein expression, at 37 °C with
546 shaking (180 rpm). Media were supplemented with antibiotics as required. When appropriate,
547 bacteria were grown on Columbia Blood Agar (CBA) made with 5% defibrinated sheep blood.

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

Strain	Description	Source
Staphylococcus aureus		
SH1000	*rsbU+* derivative of the laboratory strain 8325-4	36
SH1000 *rexB*::Tn	SH1000 with a *bursa aurealis* transposon insertion in *rexB,* Ery^r	37
SH1000 *rexB*::Tn p*itet* empty	SH1000 with a *bursa aurealis* transposon insertion in *rexB* with integrated p*itet* empty plasmid, Ery^r	37
SH1000 *rexB*::Tn p*itet rexAB*	SH1000 with a *bursa aurealis* transposon insertion in *rexB* with integrated p*itet* with AHT-inducible *rexB*, Ery^r	37
JE2	A derivative of CA-MRSA USA300 LAC, cured of plasmids	35
JE2 *rexB*::Tn	JE2 with a *bursa aurealis* transposon insertion in *rexB,* Ery^r	35
JE2 *rexB*::Tn p*itet* empty	JE2 with a *bursa aurealis* transposon insertion in *rexB* with integrated p*itet* empty plasmid, Ery^r	37
JE2 *rexB*::Tn p*itet rexAB*	JE2 with a *bursa aurealis* transposon insertion in *rexB* with integrated p*itet* with AHT-inducible *rexB*, Ery^r	37
SH1000 pCN34 *PrecA-gfp*	SH1000 containing pCN34 with *gfp* under the control of the *recA* promoter, Kan^r	37
JE2 pCN34 *PrecA-gfp*	JE2 containing pCN34 with *gfp* under the control of the *recA* promoter, Kan^r	37
JE2 *rexB*::Tn pCN34 *PrecA-gfp*	JE2 *rexB*::Tn containing pCN34 with *gfp* under the control of the *recA* promoter, Kan^r	37
Streptococcus gordonii		
DL1 (Challis)	Wild type	71
DL1 *ΔrexBA*	DL1 with the *rexA* and *rexB* genes deleted	This study
Enterococcus faecalis		
CK111 (pCF10-101)	Conjugative donor strain with pCF10-101	72
OG1X	Gelatinase deficient	73
OG1X *ΔrexBA*	OG1X with the *rexA* and *rexB* genes deleted	This study
Escherichia coli		
EC1000	Cloning host that provides RepA in trans	74
SoluBL21 (DE3)	Derivative of BL21 (DE3) for the expression of challenging proteins	Genlantis

574

575 ***recA-gfp* fluorescent reporter assay**

576 As detailed previously[37], promoter-reporter gene constructs in JE2 and SH1000 backgrounds were
577 used to directly assess expression of *recA*. Antibiotic two-fold dilutions were made in flat-bottomed
578 black-walled 96-well plates containing TSB and Kanamycin (90 µg ml^{-1}) and inoculated with 1/10
579 dilution of a stationary phase culture of the reporter strains. Plates were placed into an Infinite M200-

580 PRO microplate reader (Tecan) where cultures were grown for 17 h at 37 °C (700 rpm), and both
581 absorbance at 600 nm (OD_{600}) and GFP relative fluorescence units (RFU) were measured every 30 min.
582 OD_{600} data was normalised to no antibiotic controls and RFU data was normalised to no antibiotic
583 controls. To account for differences in cell-density, RFU values were normalised by OD_{600} data at each
584 time point.
585
586 **Neutrophil phagocytosis and measurement of DNA damage**
587 Whole human blood (15 ml) was collected from independent healthy donors in EDTA-treated tubes
588 (BD Biosciences) and layered over 20 ml of room-temperature Polymorph prep (Alere Limited) before
589 centrifugation at 500 x g for 45-60 min (brake off, 30 °C) until a clear separation of red blood cells
590 (RBCs), peripheral blood mononuclear cells (PBMCs) and polymophonuclear leukocytes (PMNs, or
591 neutrophils) was seen. The PBMCs were discarded and the PMNs were transferred to a fresh
592 centrifuge tube. Hanks' Balanced Salt Solution (HBSS) was added to the PMNs to a total volume of 50
593 ml and cells were pelleted at 500 x g for 10 min (brake off, 30 °C). The cells were resuspended in 3 ml
594 of HBSS, counted using a haemocytometer and adjusted to 5 x 10^6 cells ml^{-1} in HBSS containing 10%
595 human serum, 0.1 mM calcium and 0.1 mM magnesium. To the neutrophil suspension, 5 x 10^6 cells
596 ml^{-1} of bacteria (stationary or exponential-TRITC-stained) were added. The bacterial and neutrophil
597 suspension was then incubated at 37 °C with tumbling. At each time point (0.5, 1, 2, 3 h) 100-150 µl
598 was taken and resuspended in 4% PFA in PBS for a minimum of 1 h. Before analysis using flow
599 cytometry, samples were washed and resuspended in PBS. Samples were analysed on the FACS Aria
600 or LSRFortessa flow cytometer (BD Biosciences), and at least 100,000 events were captured, except
601 for bacteria-only samples where at least 50,000 events were captured. Green (GFP bacteria)
602 fluorescence was detected at 530 (30) nm, and TRITC labelling at 586 (16) nm. Full gating strategies
603 are detailed in Figures S6 and S7.
604
605 **Determination of minimum inhibitory concentration (MIC)**
606 Minimum inhibitory concentrations (MICs) were determined using a serial broth dilution protocol as
607 described previously[75]. Bacteria were diluted to 1 x 10^5 CFU ml^{-1} and incubated in flat-bottomed 96-
608 well plates with a range of antibiotic concentrations for 17 h at 37 °C under static conditions (aerobic,
609 anaerobic or 5% CO_2). Media containing daptomycin was supplemented with 1.25 mM $CaCl_2$. The MIC
610 was defined as the lowest concentration at which no growth was observed.
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614 **Antibiotic survival assay**

615 Bacteria were adjusted to 10^8 CFU ml^{-1} in TSB (*S. aureus*) or THY broth (*S. gordonii* and *E. faecalis*)
616 supplemented antibiotics at 10x MIC, unless otherwise stated. For aerobic incubation, 3 ml of media
617 were inoculated in 30 ml universal tubes and incubated with shaking. For anaerobic conditions 6 ml
618 of pre-reduced media in 7 ml bijou tubes was inoculated and incubated statically in an anaerobic
619 cabinet. Cultures were incubated at 37 °C and bacterial viability determined by CFU counts. Culture
620 media containing daptomycin was supplemented with 1.25 mM $CaCl_2$. Survival was calculated as a
621 percentage of the number of bacteria in the starting inoculum.

622

623 **Whole blood and hydrogen peroxide survival assays**

624 Bacteria were washed twice with PBS, adjusted to 10^6 CFU ml^{-1} in HBSS and 10^4 CFU (10 µl) used to
625 inoculate 90 µl of freshly donated human blood (collected in EDTA-treated tubes; BD Biosciences) or
626 freshly diluted H_2O_2 (10 mM in PBS) in 96 well plates. Ethical approval for drawing and using human
627 blood was obtained from the Regional Ethics Committee and Imperial NHS Trust Tissue Bank (REC
628 Wales approval no. 12/WA/0196, ICHTB HTA license no. 12275). In some assays blood was pre-treated
629 for 10 min with diphenyleneiodonium (DPI; 50 µM) or an equivalent volume of DMSO as a solvent
630 control. After 6 h incubation, bacterial survival was determined by CFU counts of blood-bacteria
631 mixtures on CBA plates. For H_2O_2 assays, survival was measured after 1 h at 37 °C (static) in the dark
632 Survival for both assays was calculated as a percentage of the number of bacteria in the starting
633 inoculum.

634

635 **Neutrophil survival assay**

636 Neutrophils were adjusted to 5 x 10^6 cells ml^{-1} in HBSS containing 10% human serum, 0.1 mM calcium
637 and 0.1 mM magnesium. Stationary phase bacterial cultures were washed in PBS and 1 x 10^6 CFU was
638 added to the neutrophil suspension (MOI 1:5) to a total volume of 1 ml. Neutrophils were treated for
639 10 min prior to the addition of bacteria with either diphenyleneiodonium (DPI; 50 µM) or an equivalent
640 volume of DMSO (solvent control) as needed. The bacterial and neutrophil suspension was
641 subsequently incubated at 37 °C with tumbling. At relevant time points (0.5, 1, 2, 3 h), 50 µl of the
642 suspension was transferred to a 96-well plate and serially diluted ten-fold in PBS up to 10^{-3} dilution.
643 All dilutions (including neat) were then plated onto CBA and incubated for 24 h at 37 °C before
644 counting. Survival was calculated as a percentage of the number of bacteria in the starting inoculum.

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648 **Murine infection model**

649 Animal work was conducted in accordance with the Animals (Scientific Procedures) Act 1986 outlined
650 by the UK Home Office regulations. Work was approved by the UK Home Office after ethical approval
651 by the Imperial College Animal Welfare and Ethical Review Body (AWERB). Six- to eight-week-old
652 female C57BL/6 mice (Charles River) were infected via the intraperitoneal route with wild-type or
653 *rexB*::Tn mutant in JE2 and SH1000 backgrounds. Stationary phase bacterial cultures were washed
654 twice with PBS and adjusted to 10^7 CFU ml^{-1}. Subsequently, 400 µl (4 x 10^6 CFU) of the washed bacterial
655 suspensions were injected into the peritoneal cavity of each mouse (5 mice for each strain, 20 in total).
656 After 6 h, the mice were humanely sacrificed by cervical dislocation and death confirmed by severing
657 the femoral artery. The peritoneal cavity was washed with PBS to release the bacteria and CFU counts
658 were determined by plating onto TSA. Sample size was determined prior to the experiment using
659 power analysis based on *in vitro* data [9]. Tubes containing the bacterial suspensions were blinded
660 before starting the experiment. Mice were randomly allocated to group cages and each group was
661 randomly allocated to a treatment. Following Home Office regulations, any animals that displayed two
662 or more of the following symptoms were humanely killed using a Schedule 1 method and excluded
663 from the study: shivering, hunched posture, reduced movement, cyanosis, circling or difficulty
664 breathing.

665

666 **Construction of Δ*rexBA* mutants of *E. faecalis* and *S. gordonii***

667 A Δ*rexBA* mutant was generated in *S. gordonii* by in-frame allelic replacement with the erythromycin
668 resistance determinant *ermAM*. Flanking regions directly upstream and downstream of the *rexBA*
669 operon were amplified by PCR from *S. gordonii* DL1 genomic DNA with primer pairs
670 Sg.rexAB.F1/Sg.rexAB.R1 and Sg.rexAB.F2/Sg.rexAB.R2 (Table 2), respectively, while the *ermAM*
671 cassette was amplified from plasmid pVA838[76] using primers ermAM.SgF/ermAM.SgR (Table 2). The
672 resulting amplimers were then joined together via 20-bp overlapping regions by stitch PCR using
673 primers Sg.rexAB.F1/Sg.rexAB.R2, and transformed into *S. gordonii*. Erythromycin-resistant
674 transformants were confirmed by sequencing and the strain designated UB3018.

675 A *rexBA* mutant was generated in *E. faecalis* by markerless exchange using a 2-step homologous
676 recombination approach, as previously described[72]. In brief, flanking regions directly upstream and
677 downstream of the *rexBA* operon were amplified by PCR from *E. faecalis* OG1X genomic DNA with
678 primer pairs Ef.rexAB.pheF1/Ef.rexAB.pheR1 and Ef.rexAB.pheF2/Ef.rexAB.pheR2 (Table 2),
679 respectively, and then joined together via 20-bp overlapping regions by stitch PCR using primers
680 Ef.rexAB.pheF1/Ef.rexAB.pheR2. The resultant amplimer was cloned into donor plasmid pCJK47[72] via
681 unique restriction sites *Pst*I/*Nco*I to generate pCJK47-*rexAB*, propagated in *E. coli* EC1000 and then

682 introduced into conjugative donor strain *E. faecalis* CK111 (pCF10-101) by electroporation. Plasmid
683 pCJK47-*rexAB* was transferred to *E. faecalis* OG1X by conjugation. Transconjugants carrying the
684 integrated plasmid were confirmed by colony PCR, before counterselection based on the P-*pheS**
685 marker was used to identify secondary recombinants in which the integrated plasmid had been
686 excised and lost, leaving the desired Δ*rexBA* allele. This was confirmed by sequencing and the strain
687 designated UB2948.
688
689 **Construction of *S. aureus* RexAB expression vector**
690 Cloning of the *rexA* and *rexB* genes from *S. aureus* was achieved by PCR from wild-type genomic JE2
691 DNA using the "rexB-F BamHI" and "rexA-R SalI" primers listed in Table 2, which allowed the
692 amplification of the *rexBA* operon immediately flanked by suitable restriction endonuclease
693 recognition sequences (BamHI and SalI). The *rexA* and *rexB* genes were inserted into the pET28b$^+$
694 vector (Novagen) using standard cloning techniques and site-directed mutagenesis (SDM) was
695 performed to insert a StrepII-tag and a thrombin site in front of the *rexA* gene. This enabled RexA and
696 RexB proteins to be detected individually, via an N-terminal His$_6$-tag for RexB (His$_6$ from the pET28b$^+$
697 vector) and an N-terminal StrepII-tag for RexA. PCR primers for SDM are listed in Table 2. DNA
698 sequencing was used on the "pET28b$^+$ *rexBA*" expression plasmid to confirm that the sequences of the
699 entire *rexA* and *rexB* genes, tags and promoter regions were as expected.
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716 **Table 2. Primers used in this study.**

Oligonucleotide	Sequence 5'-3'
rexB-F BamHI	CCAGGATCCGATGACATTACATGCTTATTTAGG
rexA-R SalI	GCGGTCGACCTATAGTTGCAATGTACC
StrepII rexAB SDM-F	ACAATTCCAGAGAAACCACAAGGCGTGATTTGGACTGACGCGCAATGGC
StrepII rexAB SDM-R	AGCGCTTTTTTCGAACTGCGGGTGGCTCCACATCTATTGCTCACCCCC
Thr rexAB SDM-F	CCCGCAGTTCGAAAAAAGCGCTGGCCTGGTGCCGCGCGGCAGCGGCACAATTCCAGA GAAACCACAAGG
Thr rexAB SDM-R	CCTTGTGGTTTCTCTGGAATTGTGCCGCTGCCGCGCGGCACCAGGCCAGCGCTTTTTTC GAACTGCGGG
Sg.rexAB.F1	CAGCAGGACAAGGGAAGTG
Sg.rexAB.R1	TTTTGTTCATAGCCTGCCTTTTCCTCTAG
Sg.rexAB.F2	AGGTCCCTAGAAGGAAGCATCTGAGTTG
Sg.rexAB.R2	CGTCGAGCACTAGTCTCG
ermAM.SgF	AAGGCAGGCTATGAACAAAAATATAAAATATTCTCA
ermAM.SgR	ATGCTTCCTTCTAGGGACCTCTTTAGCTCC
Ef.rexAB.pheF1	TGACGTCGACGCGT*CTGCAG*GATCGCTAAAACGCTAGAAGC
Ef.rexAB.pheR1	TAAAATCTACCTGCACACTCATGGTTTCAC
Ef.rexAB.pheF2	GAGTGTGCAGGTAGATTTTATAAAGTAGAAAAAATTAGAAG
Ef.rexAB.pheR2	GCTTAGCATG*CCATGG*TCTTAATACTTCGGTGATTGG

717 * Underlined regions indicate bp overlaps for stitch PCR; italicised regions indicate restriction
718 endonuclease sites for ligation into pCJK47
719
720 **Expression and purification of recombinant *S. aureus* RexAB**
721 Cells from single colonies of *E. coli* SoluBL21 (DE3), freshly transformed with pET28b$^+$ *rexAB* expression
722 plasmid coding for N-terminally StrepII-tagged RexA and N-terminally His$_6$-tagged RexB, were used to
723 inoculate an overnight starter culture in LB supplemented with 50 µg ml^{-1} of kanamycin. The starter
724 culture was diluted to an OD_{600} of 0.05 into 4 L of TB containing 50 µg ml^{-1} of kanamycin. Cells were
725 grown at 37 °C with shaking at 180 rpm until an OD_{600} of 0.5 was reached prior to induction with 1 mM
726 of isopropyl β-D-thiogalactoside (IPTG). Following induction, the temperature was reduced to 20 °C
727 and cultures were further incubated for 20 h. Cells were harvested by centrifugation at >10,000 x *g*
728 and 4 °C for 30 min and pellets were resuspended in 100 ml of 50 mM Tris, 150 mM NaCl (pH 7.5).
729 For protein purification, cells were disrupted by sonication and cell debris was cleared by
730 centrifugation at 32,000 x g 4 °C and for 30 min. The resulting supernatant was added to 5 ml of
731 Chelating Sepharose Fast Flow resin (GE Healthcare), which had been loaded with 0.1 M $NiCl_2$ and
732 equilibrated with 100 ml of 50 mM Tris, 150 mM NaCl, 20 mM imidazole (pH 7.5). The supernatant-
733 resin mixture was left at 4 °C overnight with gentle stirring to optimize the binding of the His-tagged
734 protein to the nickel-charged resin. Then, the mixture was washed 9x with 40 ml of 50 mM Tris, 150
735 mM NaCl, 20mM imidazole (pH 7.5) and 1x with 20 ml of 50 mM Tris, 150 mM NaCl, 70 mM imidazole
736 (pH 7.5). His-tagged protein was eluted with 50 mM Tris, 150 mM NaCl, 150 mM imidazole (pH 7.5);

737 each 10 ml fraction was tested with Bradford reagent (Bio-Rad) for protein content until no more
738 protein could be detected. Fractions containing protein were pulled, buffer exchanged and
739 concentrated using an Amicon 100 kDa cut-off concentrator (Merck Millipore); RexAB is >250 kDa in
740 size. The concentrating device was centrifuged at 2000 x g (4 °C), and the protein solution was
741 exchanged into 50 mM Tris, 150 mM NaCl (pH 7.5) by four serial concentration and re-dilution steps.
742 The total protein concentration was quantified using the Pierce BCA protein assay kit (Thermo Fisher
743 Scientific) following the manufacturer's instructions, and presence of intact recombinant RexAB was
744 confirmed via SDS-PAGE and Western blot analysis.
745
746 **Nuclease and helicase activity**
747 The nuclease and helicase activity of RexAB was measured to confirm the AddAB-like activity in our
748 recombinant RexAB protein. Staphylococcal DNA was amplified from the JE2 whole genome by colony
749 PCR using the primer pairs "Chi control F" (5'- TCAGTGAATTAGATGATTCGC -3') and "Chi control R" (5'-
750 TTCATACGTATGAATGTTATTTGC-3') where the amplicon lacked a Chi site region to be used as the DNA
751 substrate in these assays.
752 Reactions were set up in either nuclease assay buffer (25 mM Tris-acetate pH 7.5, 2 mM Mg acetate,
753 1 mM DTT) or helicase assay buffer (25 mM Tris-acetate pH 7.5, 0.25 mM Mg acetate, 1 mM DTT),
754 along with 5 ng μl^{-1} of DNA, 1 mM ATP or equivalent volume of nuclease-free water and either 20 nM
755 or 50 nM of recombinant RexAB for the nuclease and helicase assay respectively. Additionally for the
756 helicase assay, 2 µM of single-stranded binding proteins were added to each sample. The DMSO
757 content was maintained at 1% (v/v) when compounds were being tested.
758 Samples were incubated statically at 37 °C and at 0, 5, 15, 30, 60 and 120 min, 5 µl was removed and
759 pipetted into 20 µl of STEB buffer (40 % (w/v) sucrose, 100 mM Tris-HCl, 10 mM EDTA, 0.5 mg/ml
760 bromophenol blue, pH 8) to stop the reaction. 20 µl of chloroform/isoamyl alcohol (24:1) was added
761 to each tube, vortexed for 10 sec and centrifuged for 2 min at 17,000 x g to remove the protein and
762 any compounds used for inhibition. The aqueous (upper blue) phase was loaded onto a 1% (w/v)
763 agarose gel prepared in TBE buffer and electrophoresis was carried out at 85 V for 1 h. The gels were
764 subsequently stained with SYBR Safe DNA gel stain (Invitrogen) at 1/10,000 dilution in TBE buffer for
765 2 h with rocking and visualised using a Gel Doc EZ Imager (Bio-Rad). Band intensity was quantified
766 using ImageJ software. For nuclease activity, values were normalised to no-ATP controls at 0 h. For
767 helicase activity, values were normalised to a ssDNA control that lacked the RexAB protein, which had
768 been heated at 95 °C for 2 min to denature the dsDNA and allow the SSB protein to bind and stabilise
769 the two ssDNA strands.
770

771 **Statistical analyses**

772 Data are represented as the mean or median from three or more independent experiments and
773 analysed by Student's t test (two tailed, unpaired, assuming equal variances), one-way ANOVA or two-
774 way ANOVA corrected for multiple comparison, as described in the figure legends. For each
775 experiment, "n" refers to the number of independent biological replicates. CFU counts from murine
776 experiments are presented as the value obtained from each animal and significance was assessed
777 using the Mann-Whitney test. $P < 0.05$ was considered significant between data points (GraphPad
778 Prism 7 for Windows).

779

780

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