Staphylococcal DNA repair is required for infection

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Abstract

The repair of DNA damage is essential for bacterial viability and contributes to adaptation via increased rates of mutation and recombination. However, the mechanisms by which DNA is damaged and repaired during infection are poorly understood. Using a panel of transposon mutants, we identified the *rexBA* operon as important for the survival of *Staphylococcus aureus* in whole human blood. Mutants lacking *rexB* were also attenuated for virulence in murine models of both systemic and skin infections. We then demonstrated that RexAB is a member of the AddAB family of helicase/nuclease complexes responsible for initiating the repair of DNA double strand breaks. Using a fluorescent reporter system, we were able to show that neutrophils cause staphylococcal DNA double strand breaks via the oxidative burst, which are repaired by RexAB, leading to induction of the mutagenic SOS response. We found that RexAB homologues in *Enterococcus faecalis* and *Streptococcus gordonii* also promoted survival of these pathogens in human blood, suggesting that DNA double strand break repair is required for Gram-positive bacteria to survive in host tissues. Together, these data demonstrate that DNA is a target of host immune cells, 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.

52 **Introduction**

53

54 The integrity of bacterial DNA is essential for viability. However, the causes of DNA damage
55 during infection and the mechanisms employed by bacteria to repair this damage are poorly
56 understood, particularly in the case of Gram-positive pathogens such as *Staphylococcus*
57 *aureus*. This pathogen is responsible for a raft of serious invasive infections including
58 bacteraemia, infective endocarditis and osteomyelitis[1]. Despite a potent immune response,
59 many infections become chronic or recurrent[2], implying that either *S. aureus* does not
60 experience DNA damage during infection or that it has efficient mechanisms for damage
61 repair.

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

75 Despite the importance of the oxidative burst in combatting staphylococcal infection,
76 relatively little is known about how it kills the pathogen. Studies with single oxidants such as
77 H_2O_2 indicate that the molecular targets of the oxidative burst are broad and include proteins,
78 lipids and DNA[18]. To survive this damage, *S. aureus* employs several stress response regulators
79 and repair systems. For example, previous work has shown that DNA damage caused by *S.*
80 *aureus* exposure to H_2O_2 leads to the initiation of the DNA repair SOS response[19], which
81 facilitates the excision of damaged bases or repair of double-strand breaks (DSBs) by
82 homologous recombination[20]. However, H_2O_2 is a sub-optimal model for the oxidative burst
83 because it is typically used at concentrations that exceed those generated by the oxidative

84 burst[21]. Therefore, it is unclear whether staphylococcal DNA is damaged by neutrophil-
85 generated ROS, what the nature of this damage is, how it is repaired by *S. aureus*, and the
86 impact of this damage on infection.
87 To address this gap in our knowledge, we examined mutants defective for DNA repair and
88 found that a member of the AddAB helicase/nuclease family of enzymes was required for
89 staphylococcal survival in blood and murine models of systemic and skin infections. We also
90 demonstrated that this complex is required for the repair of DNA double strand breaks caused
91 by the oxidative burst of neutrophils, which leads to the induction of the mutagenic SOS
92 response. Similar complexes were required for the survival of infective endocarditis
93 pathogens *Streptococcus gordonii* and *Enterococcus faecalis* in human blood, demonstrating
94 that DNA damage repair is an important mechanism by which Gram-positive pathogens
95 withstand host defences.
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98 **Results**
99 **RexAB is required for staphylococcal survival in host tissues**
100 To determine whether DNA damage occurs under infection conditions, we assembled a panel
101 of *S. aureus* mutants from the ordered NARSA transposon library[22]. Each mutant was
102 defective for a different protein associated with DNA repair, which would enable the nature
103 of any damage to be identified. We then assessed the contribution of each repair protein to
104 bacterial survival in the host by measuring CFU counts of mutants in an *ex vivo* whole human
105 blood model of infection. Previous characterisation of this model system by our group and
106 others has shown that *S. aureus* is rapidly phagocytosed by neutrophils and exposed to the
107 oxidative burst and other killing mechanisms[23–25].
108 Wild type *S. aureus* JE2 survived relatively well in human blood, with >60% of the inoculum
109 viable after 2 h (Supplementary Fig. S1). By contrast, survival of mutants defective for *rexA* or
110 *rexB* was <5% of the inoculum (Supplementary Fig. S1). Since the transposon insertion in *rexB*
111 contains a terminator sequence that prevents transcription of *rexA*, the second gene in the
112 operon, *rexB* mutants are defective for both *rexB* and *rexA*, and are effectively *rexBA*
113 mutants[22]. Therefore, we confirmed the importance of RexAB for the survival of two distinct
114 strains (JE2 and SH1000) in blood by complementation of the *rexB* mutants with a plasmid
115 containing the *rexBA* operon (p*rexBA*), which restored bacterial survival to wild type levels

116 (Fig. 1A,B). By contrast, the survival of *rexB* mutants transformed with the vector alone
117 (pEmpty) was not changed from that of the mutant (Fig. 1A,B).
118 Having shown that RexAB contributed to staphylococcal tolerance of neutrophil-mediated
119 killing in human blood, we then tested its role in staphylococcal survival *in vivo* using a murine
120 model of systemic infection. Mice were infected via the peritoneal cavity, which results in the
121 recruitment of neutrophils within 2 h, with wild type or *rexB* mutants of *S. aureus* SH1000 or
122 JE2[26]. After 6 h, the mice were sacrificed and the peritoneal cavity washed with PBS to recover
123 bacteria, which were quantified by CFU counts. This revealed that *rexB* mutants in both
124 genetic backgrounds were significantly attenuated for survival *in vivo*, with ~5-fold lower CFU
125 counts than the respective wild type bacteria ($p \leq 0.05$), confirming that RexAB contributes to
126 staphylococcal resistance to host immune defences and the progression of systemic infection
127 (Fig. 1C,D).
128 Because *S. aureus* causes many different types of infection and the associated immune
129 responses might vary, we next assessed survival of wild type strain JE2 and *rexB* mutant in a
130 murine skin infection model. Mice were infected via sub-cutaneous injection and infection
131 allowed to progress for 5 days before CFU counts at inoculation sites were determined. This
132 revealed that wild type bacteria were present at 2-3-fold higher levels than the *rexB* mutant
133 ($p \leq 0.05$) (Fig. 1E). We also measured the size of the skin lesions generated by injected *S.*
134 *aureus*. Wild type JE2 caused a lesion that progressively increased in size over time (Fig. 1F).
135 By contrast, the lesion caused by the *rexB* mutant did not increase after day 2 and was
136 significantly smaller than that caused by the wild type for days 2, 3, 4 and 5 (Fig. 1F;
137 Supplementary Fig. S2). This indicated that RexAB was also required for skin infection
138 progression.
139 To understand whether the reduced CFU counts of the *rexB* mutants relative to wild type in
140 animal models were due to differences in growth rate or reduced virulence factor production,
141 we measured bacterial growth, haemolysin production, staphyloxanthin levels and catalase
142 across JE2 and SH1000 wild type and mutant strains. For both JE2 and SH1000, the *rexB*
143 mutant did replicate at a slightly slower rate than wild type, but there were no significant
144 differences in production of haemolysin, staphyloxanthin or catalase (Supplementary Fig. 3).
145 Taken together, these findings demonstrate that RexAB significantly promotes staphylococcal
146 survival in host tissues and is required for infection progression.
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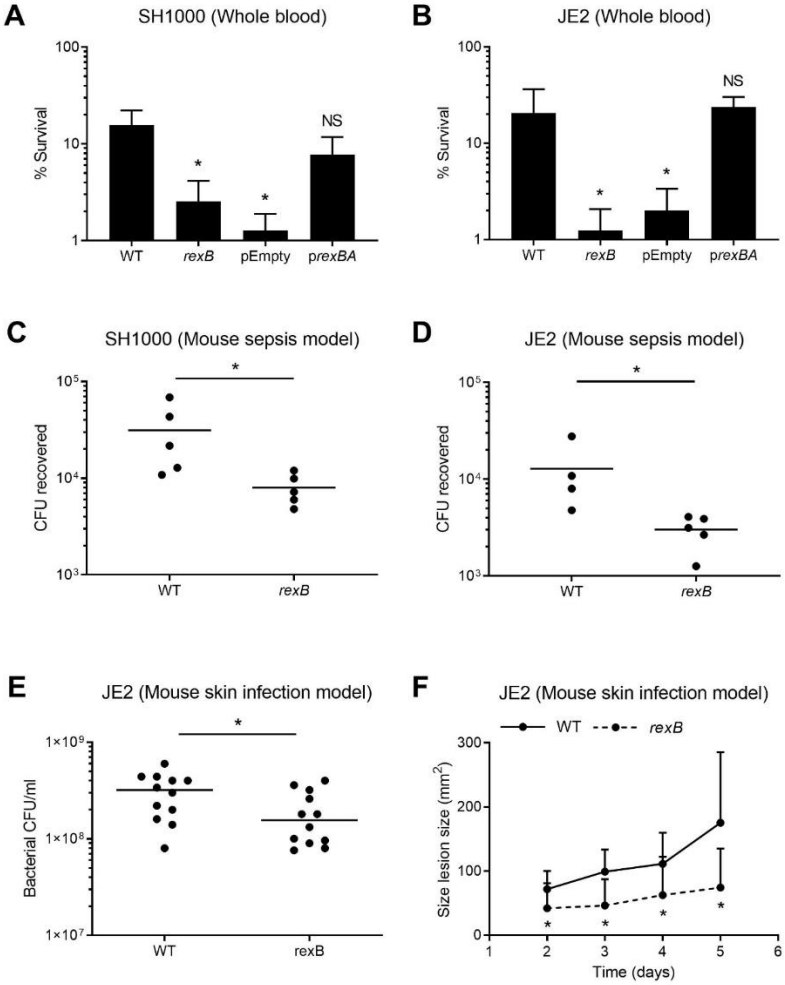

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149 **Figure 1. Survival of the *rexB* mutant is significantly reduced in human blood and *in vivo***
150 **murine infection models. (A-B)** Survival of *S. aureus* wild type (WT), *rexB* mutant, empty
151 vector (pEmpty) and complemented mutant (p*rexBA*) in SH1000 (A) and JE2 (B) backgrounds
152 after 6 h incubation in whole human blood. Empty vectors and complemented mutants were
153 supplemented with 100 ng/µl AHT to induce *rexBA* expression (n ≥ 3). **(C-D)** Number of CFU
154 recovered from the peritoneal cavities of mice at 6 h post infection (each circle represents a
155 single mouse; n = 4-5). **(E)** CFU/ml recovered from mouse skin biopsies at 5 days post infection
156 (each circle represents a single mouse; n = 12). **(F)** Size of skin lesions observed on mice up to
157 5 days post infection (n = 12). Data in **A** and **B** were analysed by one-way ANOVA with Tukey's
158 post-hoc test relative to WT (*, $P < 0.05$). Data in **C-F** were analysed by Mann-Whitney test (*,
159 $P < 0.05$).

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

167 The *rexA* and *rexB* genes form a two gene operon (*rexBA*) on the staphylococcal chromosome,
168 and are proposed to encode an AddAB helicase/nuclease enzyme on the basis of sequence
169 homology[27–30]. However, this had not been demonstrated experimentally.

170 Our initial *in silico* structural analysis of the predicted *rexBA* gene products supported
171 predictions that this operon encodes an AddAB-type ATP-dependent helicase/nuclease that
172 contributes to the processing and repair of DNA double strand breaks (Supplementary Fig.
173 S4). AddAB enzymes process DSBs to generate a 3' single-stranded DNA overhang that is
174 necessary for RecA-mediated homologous recombination[31]. In support of the structural
175 predictions, phenotypic testing of *rexBA* mutants showed that they were >8-fold more
176 susceptible than wild type strains to the DNA-damaging antibiotics ciprofloxacin and
177 mitomycin C, both of which cause DNA DSBs[32,33] (Supplementary Fig. S5).

178 To confirm the ATP-dependent helicase/nuclease activity of the *S. aureus* RexAB complex,
179 recombinant RexAB protein was generated and the helicase and nuclease activities were
180 measured over 1 h. Nuclease activity assays were performed under conditions of high free
181 Mg^{2+}, which has been previously shown to activate nuclease activity in AddAB enzymes[34]. We
182 found that DNA was degraded over time by the recombinant complex in the presence of ATP,
183 whereas this degradation was minimal in its absence, demonstrating that RexAB has ATP-
184 dependent nuclease activity (Fig. 2A).

185 We measured helicase activity at conditions of low free Mg^{2+} and observed loss of dsDNA,
186 concomitant with increased ssDNA formation over time (Fig. 2B,C). These experiments were
187 repeated in the absence of single-stranded DNA binding protein (SSB), which is required to
188 prevent reannealing of DNA. In the absence of SSB, no DNA unwinding or ssDNA formation
189 were observed, demonstrating that RexAB has ATP-dependent helicase activity (Fig. 2D,E).

190 Combined, these results confirm that RexAB is a functional member of the AddAB family of
191 DNA repair complexes. In turn, this demonstrates that DNA DSBs occur during staphylococcal
192 infection and must be repaired for bacterial survival.

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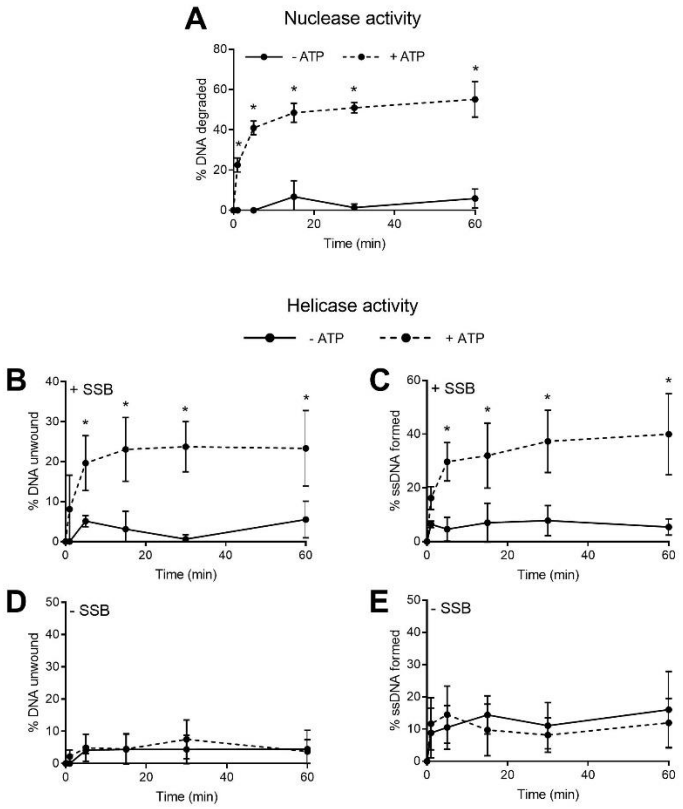

Figure 2. RexAB possesses ATP-dependent nuclease and helicase activity. (A) Nuclease activity of recombinant *S. aureus* RexAB protein in the presence (dotted line) or absence (solid line) of ATP (n = 4). **(B-E)** Helicase activity of recombinant *S. aureus* RexAB protein in the presence (dotted line) or absence (solid line) 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 comparing without versus with ATP (*, $P < 0.05$). Error bars represent standard deviation of the mean.

214 **RexAB enables staphylococcal tolerance of the oxidative burst**

215 Having confirmed that DNA DSBs occur in *S. aureus* during infection, we wanted to determine
216 whether this was due to the oxidative burst of neutrophils. Therefore, we incubated wild type
217 and *rexB* mutants in whole human blood in the presence of diphenyleneiodonium chloride
218 (DPI) to block the NADPH oxidase generated oxidative burst, or DMSO alone as a solvent
219 control.

220 As already described in Fig. 1A,B, the survival of *rexB* mutants in whole human blood was
221 significantly reduced relative to wild-type bacteria (Fig. 3A,B). However, the presence of DPI
222 promoted the survival of *rexB* mutants to wild type levels, indicating that the survival deficit
223 observed for bacteria lacking RexAB was due to increased sensitivity to the oxidative burst
224 (Fig. 3A,B). To ensure that the killing of *rexB* mutants in blood was due to neutrophils, *S.*
225 *aureus* strains were incubated with purified neutrophils and survival measured via CFU
226 counts. Similar to whole blood, *rexB* mutants were more susceptible to neutrophil-mediated
227 killing than wild type, but the presence of DPI restored survival of the *rexB* mutants to wild
228 type levels (Fig. 3C,D). These data strongly indicated that RexAB contributed to staphylococcal
229 survival of the oxidative burst by repairing DNA damaged by the oxidative burst.

230 To confirm that *rexB* mutants were more susceptible to oxidative damage, we measured
231 survival of *S. aureus* strains in H_2O_2, which is one of the ROS produced in the oxidative burst[4].
232 As observed for whole human blood and purified neutrophils, *rexB* mutants were more
233 susceptible to H_2O_2 than wild type or complemented strains.

234 Together, these data demonstrate that neutrophils cause DNA DSBs in *S. aureus* via the
235 oxidative burst. This damage must be repaired by RexAB to enable staphylococcal survival in
236 the host.

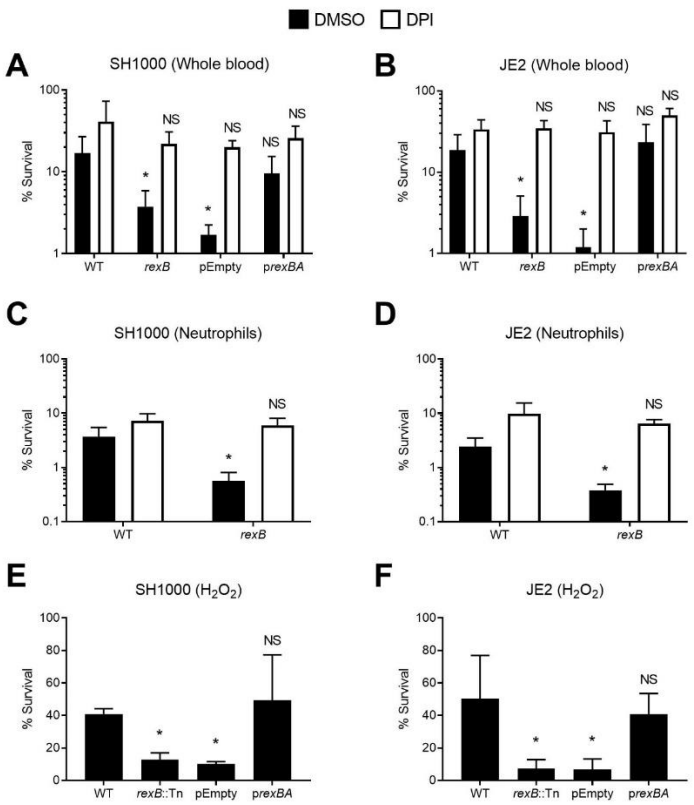

Figure 3. RexAB protects *S. aureus* from the oxidative burst of neutrophils and when exposed to H_2O_2. (A-B) Survival of *S. aureus* WT, *rexB* mutant, empty vector (pEmpty) and complemented mutant (p*rexBA*) in SH1000 (A) and JE2 (B) backgrounds in whole human blood after 6 h of incubation, in the 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 AHT to control *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). **(E-F)** Survival of *S. aureus* WT, *rexB* mutant, empty vector (pEmpty) and complemented mutant (p*rexBA*) after 1 h incubation with 10 mM of H_2O_2. Empty vectors and complemented mutants were supplemented with 100 ng/µl AHT to induce *rexBA* expression (n = 3). Data in **A** and **B** were analysed by one-way ANOVA with Tukey's post-hoc test relative to WT (*, $P < 0.05$). Data in **C** and **D** were analysed by Student's t-test relative to WT (*, $P < 0.05$). Error bars represent standard deviation of the mean. Data in **E** and **F** were analysed by one-way ANOVA with Dunnett's post-hoc test relative to WT (*, $P < 0.05$).

260 **RexAB is required for induction of the SOS response during exposure to the oxidative burst**
261 Processing of DNA DSBs by AddAB proteins leads to the generation of a 3' overhang. This
262 results in the formation of a RecA filament, which triggers the SOS response, a multi-
263 component DNA repair mechanism that mediates repair of the DNA DSB[20,35]. However,
264 induction of the SOS response also leads to a transient increase in the mutation rate, which
265 promotes the emergence of mutants with resistance to antibiotics or host-adapted
266 phenotypes such as small colony variants[36,37].
267 Therefore, we tested whether the neutrophil oxidative burst triggers the SOS response and
268 whether this was dependent upon RexAB. To do this we used a P*recA-gfp* reporter construct
269 and validated it by showing dose-dependent activity with mitomycin C, a well-established
270 trigger of the SOS response[38] (Supplementary Fig. S6). We then incubated TRITC-labelled *S.*
271 *aureus* JE2 wild type and *rexB* mutants containing the reporter strain with neutrophils for 30
272 min and used flow cytometry to measure phagocytosis and reporter activity (GFP
273 fluorescence) (Supplementary Fig. S7). As reported previously, >95% of *S. aureus* cells were
274 phagocytosed by neutrophils within 30 min (Fig. 4A)[24]. Also, by 30 min, there was an increase
275 in the GFP signal from wild type *S. aureus* relative to the start of the assay (Fig. 4B). By
276 contrast, there was no increase in GFP signal from the *rexB* mutant (Fig. 4B), indicating that
277 neutrophils trigger the SOS response in *S. aureus* via DNA processing by RexAB.
278 To further explore the requirement for RexAB for the induction of the SOS response during
279 exposure to oxidative stress, wild type and *rexB* mutant bacteria were incubated with various
280 sub-inhibitory doses of paraquat, which results in the generation of superoxide. For wild type
281 bacteria, there was a clear dose-dependent increase in GFP-mediated fluorescence, indicative
282 of SOS induction (Fig. 4C). For the *rexB* mutant, whilst there also appeared to be a dose-
283 dependent induction of the SOS response, it was at considerably lower levels than that seen
284 for the wild type (Fig. 4C). Therefore, the induction of SOS in response to oxidative stress is
285 almost entirely dependent upon RexAB-mediated processing of DNA DSBs.
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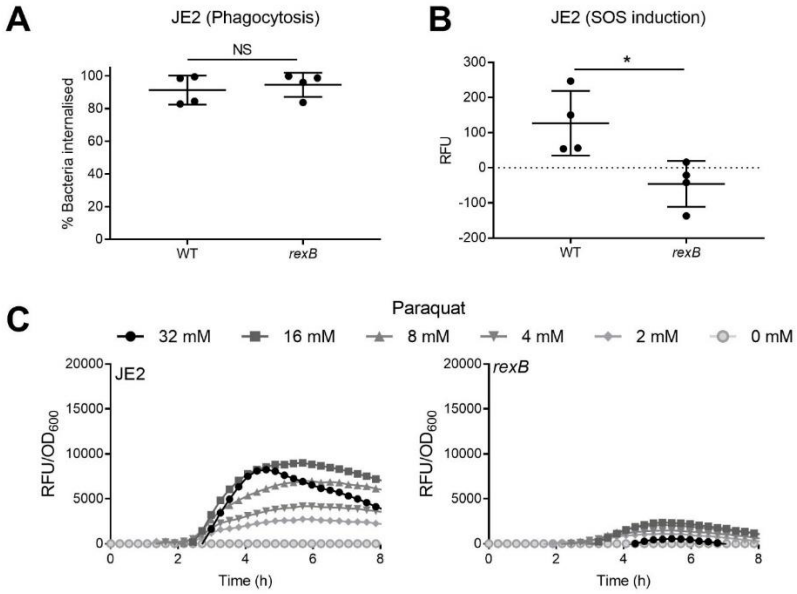

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288 **Figure 4. RexAB is required for induction of the SOS response during exposure to the**
289 **oxidative burst of neutrophils and when exposed to the ROS generator paraquat. (A)**
290 Phagocytosis of JE2 wild type and *rexB* mutant *recA-gfp* reporter strains by neutrophils and
291 **(B)** resulting induction of the SOS response measured by GFP expression (RFU) after 30 min
292 of exposure (n = 4). **(C)** Induction of SOS response of JE2 wild type and *rexB* mutant as
293 measured by GFP expression upon exposure to sub-lethal concentrations of paraquat. GFP
294 fluorescence was normalised by OD_{600} to determine induction of SOS relative to cell density
295 (n = 4). OD_{600} measurements alone are shown in Fig. S5. Data in **A** and **B** were analysed by
296 Mann-Whitney test (*, $P < 0.05$). Where shown, error bars represent standard deviation of
297 the mean. Error bars were omitted in **C** for clarity.

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300 **RexAB is required for survival of streptococci and enterococci in human blood**

301 Since homologues of RexAB are present in most Gram-positive bacteria[29–31], we next tested
302 whether this repair complex contributes to the survival of other bacteria exposed to
303 neutrophils. Like *S. aureus*, *Enterococcus faecalis* and *Streptococcus gordonii* are frequent
304 causes of infective endocarditis, which brings these species into close contact with
305 neutrophils[39,40]. Therefore, *rexBA* was deleted in representative strains of each species and
306 their sensitivity to the microbicidal activity of neutrophils determined using the *ex vivo* whole
307 human blood model.

308 Wild type *E. faecalis* survived at high levels in human blood with ~100% of the inoculum
309 remaining viable during the full 6 h duration of the assay, but the loss of *rexBA* reduced
310 enterococcal survival by ~50% (Fig. 5A). This indicated that *E. faecalis* suffers DNA damage

311 whilst in blood, but it can be tolerated via DNA repair (Fig. 5A). However, in contrast to *S.*
312 *aureus*, DNA damage in *E. faecalis* was not due to the oxidative burst since survival of both
313 wild type and *rexB* mutant in blood was unaffected by the presence of DPI (Fig. 5A).
314 *S. gordonii* was much more susceptible to host defences in blood than *E. faecalis*, with ~5%
315 wild type bacteria remaining viable after 6 h (Fig. 5A). However, survival of the *rexBA* mutant
316 was still reduced relative to the wild type, with ~1% streptococci surviving after 6 h, indicating
317 that DNA repair also contributes to the survival of *S. gordonii* in blood (Fig. 5A). As for *E.*
318 *faecalis*, the presence of DPI did not increase the survival of *S. gordonii* in blood, indicating
319 that DNA damage was not due to the oxidative burst in either bacteria. In support of these
320 findings, the *rexBA* mutants of both *S. gordonii* and *E. faecalis* were no more susceptible to
321 H_2O_2 than wild type bacteria (Fig. 5B).
322 Therefore, as for *S. aureus*, neutrophils damage DNA of both *S. gordonii* and *E. faecalis*, the
323 repair of which by RexAB promotes bacterial survival. However, in contrast to *S. aureus*, the
324 neutrophil oxidative burst does not appear to contribute to DNA damage in these bacteria.
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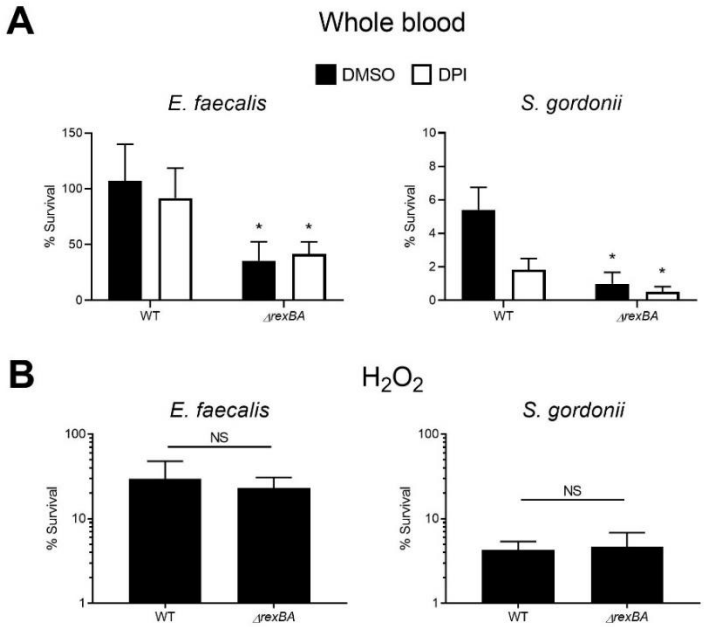

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327 **Figure 5. RexAB protects *Enterococcus faecalis* and *Streptococcus gordonii* from host**
328 **defences in blood. (A)** Survival of *E. faecalis* and *S. gordonii* wild type and *ΔrexBA* mutant in
329 whole human blood after 6 h in the presence of the oxidative burst inhibitor DPI (white) or an
330 identical volume of the DMSO solvent alone (black) (n = 3). **(B)** Survival of *E. faecalis* and *S.*
331 *gordonii* wild type and Δ*rexBA* mutant exposed to H_2O_2 for 1 h (n = 3). Data in **A** and **B** were
332 analysed by were analysed by Student's t-test comparing *ΔrexBA* mutant versus WT (*, $P <$
333 0.05). Error bars represent standard deviation of the mean.

334 **Discussion**

335 Neutrophils are an essential host defence against *S. aureus* and many other bacterial
336 pathogens. However, our understanding of the mechanisms by which these immune cells kill
337 staphylococci is limited. The data presented here demonstrate that neutrophils cause DNA
338 damage in *S. aureus* via the oxidative burst, the repair of which requires the RexAB complex
339 and leads to induction of the SOS response. RexAB is a member of the AddAB
340 helicase/nuclease family of enzymes involved in the processing of DNA DSBs for repair via
341 homologous recombination. Thus, the oxidative burst of neutrophils causes DNA DSBs in *S.*
342 *aureus*, which are lethal if not repaired. This provides new information on both the target of
343 the oxidative burst, and the mechanisms by which *S. aureus* repairs and survives this damage.
344 The importance of RexAB for staphylococcal survival during infection was demonstrated in
345 murine models of both systemic and skin infection, as well as an *ex vivo* whole human blood
346 model of bacteraemia. Since *S. aureus* is a frequent cause of both superficial and systemic
347 infections[1], these findings confirm the importance of DNA repair for staphylococcal survival
348 in relevant host tissues.

349 Previous work has demonstrated the requirement of DNA DSB repair for the survival of Gram-
350 negative pathogens *in vivo*. For example, AddAB was shown to be required for the infection
351 of chickens and mice by *Campylobacter jejuni* and *Helicobacter pylori* respectively[41,42].
352 Furthermore, the virulence of *Salmonella enterica* in a murine model of bacteraemia was
353 dependent upon the RecBCD DNA DSB repair complex[43]. However, the importance of such
354 systems for Gram-positive bacteria was unclear.

355 DNA damage occurred within 30 min of phagocytosis of *S. aureus* by neutrophils, which
356 corresponds to the time at which ROS are maximally generated in these immune cells[44,45].
357 The finding that the oxidative burst leads to DNA DSBs in *S. aureus* is in keeping with previous
358 reports that ROS damage the DNA of Gram-negative pathogens such as *Escherichia coli,*
359 *Salmonella enterica* and *Coxiella burnetii*, with the survival of bacteria phagocytosed by
360 macrophages dependent upon RecBCD[43,46,47]. Therefore, DNA is a common target of the
361 oxidative burst of phagocytic immune cells for several different human pathogens.

362 The RexAB system was also demonstrated to be important for survival of the infective
363 endocarditis pathogens *E. faecalis* and *S. gordonii*. However, whilst neutrophils in blood
364 caused DNA DSBs in these pathogens, this did not appear to be due to the oxidative burst,
365 because the inhibition of NADPH oxidase with DPI had no effect on the survival of the wild

366 type or *rexBA* mutants. Neutrophils employ several different antibacterial elements to kill
367 invading pathogens, including reactive oxygen and nitrogen species, proteases and
368 antimicrobial peptides. Several studies have indicated that bacteria vary in their susceptibility
369 to each of these microbicides. In agreement with our findings, Standish *et al.* showed that *S.*
370 *aureus* but not *Streptococcus pneumoniae* was killed by the oxidative burst[48]. However, whilst
371 *S. aureus* can grow in the presence of nitric oxide, the replication of several other pathogens
372 including *Pseudomonas aeruginosa* and *Streptococcus pyogenes* is inhibited[49]. Since reactive
373 nitrogen species can cause DNA damage, this may provide an explanation for the finding that
374 mutants of *E. faecalis* and *S. gordonii* lacking RexAB are more susceptible to killing by
375 neutrophils in which the oxidative burst is blocked.
376 In addition to providing protection from oxidative damage caused by neutrophils, we have
377 previously shown that RexAB provides tolerance to the combination antibiotic co-
378 trimoxazole[38]. That work showed that DNA damage was partly due to endogenous oxidative
379 stress that occurred during exposure to the combination antibiotic, in addition to direct
380 damage to DNA caused by thymidine limitation[38]. The data presented here show that loss of
381 RexAB also sensitises *S. aureus* to ciprofloxacin, even though the JE2 strain is resistant. In
382 addition, there is growing evidence that multiple classes of antibiotics cause endogenous ROS
383 production in *S. aureus*, suggesting that RexAB may provide an important defence against
384 damage caused by both host defences and multiple classes of antibiotics, the two key threats
385 to staphylococcal survival in the host. Further studies are needed to test this, but the
386 identification of RexAB as important for staphylococcal survival during exposure to both
387 neutrophils and at least two antibiotics makes this complex a potential target for novel
388 therapeutics, particularly as the lack of RexAB homologues in eukaryotes reduces the
389 likelihood of host toxicity[50].
390 Inhibitors of AddAB and RecBCD have been previously reported in the literature, but problems
391 include limited *in vivo* stability, poor oral bioavailability and a sub-optimal mechanism of
392 action[51–54]. However, Amundsen *et al.* (2012) identified several small-molecule inhibitors of
393 *Helicobacter pylori* AddAB and *E. coli* RecBCD, in particular ML328[55], indicating that the
394 development of stable, potent inhibitors is possible. More recently, a derivative of ML328
395 (IMP-1700) was found to be capable of potentiating antibiotic activity such that a resistant *S.*
396 *aureus* strain was sensitised to ciprofloxacin[56]. However, the ability of IMP-1700 to inhibit
397 AddAB/RecBCD activity under *in vivo* conditions remains to be determined. Further work in

398 this area may lead to broad-spectrum therapeutics that promote bacterial susceptibility to
399 both host defences and antibiotics as well as inhibiting induction of the mutagenic SOS
400 response associated with the acquisition of drug resistance and host adaptation[37].
401 In summary, staphylococcal, streptococcal and enterococcal DNA is damaged by the host
402 immune system, leading to DNA DSBs that are lethal if not repaired by RexAB. These findings
403 suggest that the RexAB complex is a potentially viable target for novel therapeutics, capable
404 of sensitising Gram-positive pathogens to neutrophil-mediated killing and blocking the SOS
405 response associated with the emergence of drug resistance.
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410 **Materials and Methods**
411 **Bacterial strains and culture conditions**
412 The bacterial strains used in this study are listed in Table 1. *S. aureus* was cultured in Tryptic
413 Soy Broth (TSB) to stationary phase (18 h) at 37 °C, with shaking (180 rpm). *S. gordonii* and *E.*
414 *faecalis* were grown in Todd Hewitt Broth (THB) supplemented with 1% (w/v) yeast extract
415 (THB-Y) at 37 °C, statically in 5% CO_2. *E. coli* was grown in Lysogeny Broth (LB) or in Terrific
416 Broth (TB) (1.2% (w/v) tryptone, 2.4% (w/v) yeast extract, 0.5% glycerol, 0.17 M KH_2PO_4, 0.72
417 M K_2HPO_4) for protein expression at 37 °C with shaking (180 rpm). Media were supplemented
418 with antibiotics as required. When appropriate, bacteria were grown on Columbia Blood Agar
419 (CBA) made with 5% defibrinated sheep blood.
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430 **Table 1. Bacterial strains used in this study.**

Strain	Description	Source
Staphylococcus aureus		
SH1000	*rsbU+* derivative of the laboratory strain 8325-4	[57]
SH1000 *rexB*::Tn	SH1000 with a *bursa aurealis* transposon insertion in *rexB,* Ery^r	[38]
SH1000 *rexB*::Tn p*itet* empty	SH1000 with a *bursa aurealis* transposon insertion in *rexB* with integrated p*itet* empty plasmid, Ery^r	[38]
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	[38]
JE2	A derivative of CA-MRSA USA300 LAC, cured of plasmids	[22]
JE2 *rexB*::Tn	JE2 with a *bursa aurealis* transposon insertion in *rexB,* Ery^r	[22]
JE2 *rexB*::Tn p*itet* empty	JE2 with a *bursa aurealis* transposon insertion in *rexB* with integrated p*itet* empty plasmid, Ery^r	[38]
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	[38]
JE2 pCN34 *PrecA-gfp*	JE2 containing pCN34 with *gfp* under the control of the *recA* promoter, Kan^r	[38]
JE2 *rexB*::Tn pCN34 *PrecA-gfp*	JE2 *rexB*::Tn containing pCN34 with *gfp* under the control of the *recA* promoter, Kan^r	[38]
Streptococcus gordonii		
DL1 (Challis)	Wild type	[58]
DL1 *ΔrexBA*	DL1 with the *rexA* and *rexB* genes deleted	This study
Enterococcus faecalis		
CK111 (pCF10-101)	Conjugative donor strain with pCF10-101	[59]
OG1X	Gelatinase deficient	[60]
OG1X *ΔrexBA*	OG1X with the *rexA* and *rexB* genes deleted	This study
Escherichia coli		
EC1000	Cloning host that provides RepA in trans	[61]
SoluBL21 (DE3)	Derivative of BL21 (DE3) for the expression of challenging proteins	Genlantis

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433

434

435 **P*recA-gfp* fluorescent reporter assay**

436 As detailed previously[38], promoter-reporter gene constructs in the JE2 background was used
437 to directly assess expression of *recA*. Antibiotic two-fold dilutions were made in flat-bottomed
438 black-walled 96-well plates containing TSB and kanamycin (90 µg ml^{-1}) and inoculated with
439 1/10 dilution of a stationary phase culture of the reporter strains. Plates were placed into an
440 Infinite M200-PRO microplate reader (Tecan) where cultures were grown for 17 h at 37 °C
441 (700 rpm), and both absorbance at 600 nm (OD_{600}) and GFP relative fluorescence units (RFU)
442 were measured every 30 min.

443 OD_{600} data and RFU data were normalised to no antibiotic controls. To account for differences
444 in cell-density, RFU values were normalised by OD_{600} data at each time point.

445

446 **Neutrophil phagocytosis and measurement of DNA damage**

447 Whole human blood (15 ml) was collected from individual healthy donors in EDTA-treated
448 tubes (BD Biosciences) and layered over 20 ml of room-temperature Polymorph prep (Alere
449 Limited) before centrifugation at 500 x g for 45-60 min (brake off, 30 °C) until a clear
450 separation of red blood cells (RBCs), peripheral blood mononuclear cells (PBMCs) and
451 polymophonuclear leukocytes (PMNs, or neutrophils) was seen. The PBMCs were discarded
452 and the PMNs were transferred to a fresh centrifuge tube. Hanks' Balanced Salt Solution
453 (HBSS) was added to the PMNs to a total volume of 50 ml and cells were pelleted at 500 x g
454 for 10 min (brake off, 30 °C). The cells were resuspended in 3 ml of HBSS, counted using a
455 haemocytometer and adjusted to 5 x 10^6 cells ml^{-1} in HBSS containing 10% human serum, 0.1
456 mM calcium and 0.1 mM magnesium. To the neutrophil suspension, 5 x 10^6 cells ml^{-1} of
457 bacteria (stationary or exponential-TRITC-stained) were added. The bacterial and neutrophil
458 suspension was then incubated at 37 °C with tumbling. At each time point (0.5, 1, 2, 3 h) 100-
459 150 µl was taken and resuspended in 4% PFA in PBS for a minimum of 1 h. Before analysis
460 using flow cytometry, samples were washed and resuspended in PBS. Samples were analysed
461 on the FACS Aria or LSRFortessa flow cytometer (BD Biosciences), and at least 100,000 events
462 were captured, except for bacteria-only samples where at least 50,000 events were captured.
463 Green (GFP bacteria) fluorescence was detected at 530 (30) nm, and TRITC labelling at 586
464 (16) nm. Full gating strategies are detailed in Figures S6 and S7.

465

466

467 **Determination of minimum inhibitory concentration (MIC)**

468 Minimum inhibitory concentrations (MICs) were determined using a serial broth dilution
469 protocol as described previously[62]. Bacteria were diluted to 1 x 10^5 CFU ml^{-1} and incubated in
470 flat-bottomed 96-well plates with a range of antibiotic concentrations for 17 h at 37 °C under
471 static conditions (aerobic, anaerobic or 5% CO_2). Media containing daptomycin was
472 supplemented with 1.25 mM $CaCl_2$. The MIC was defined as the lowest concentration at which
473 no growth was observed.

474

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

476 Bacteria were washed twice with PBS, adjusted to 10^6 CFU ml^{-1} in HBSS and 10^4 CFU (10 µl)
477 used to inoculate 90 µl of freshly donated human blood (collected in EDTA-treated tubes; BD
478 Biosciences) or freshly diluted H_2O_2 (10 mM in PBS) in 96 well plates. Ethical approval for
479 drawing and using human blood was obtained from the Regional Ethics Committee and
480 Imperial NHS Trust Tissue Bank (REC Wales approval no. 12/WA/0196, ICHTB HTA license no.
481 12275). In some assays blood was pre-treated for 10 min with diphenyleneiodonium (DPI; 50
482 µM) or an equivalent volume of DMSO as a solvent control. After 6 h incubation, bacterial
483 survival was determined by CFU counts of blood-bacteria mixtures on CBA plates. For H_2O_2
484 assays, survival was measured after 1 h at 37 °C (static) in the dark Survival for both assays
485 was calculated as a percentage of the number of bacteria in the starting inoculum.

486

487 **Neutrophil survival assay**

488 Neutrophils were adjusted to 5 x 10^6 cells ml^{-1} in HBSS containing 10% human serum, 0.1 mM
489 calcium and 0.1 mM magnesium. Stationary phase bacterial cultures were washed in PBS and
490 1 x 10^6 CFU was added to the neutrophil suspension (MOI 1:5) to a total volume of 1 ml.
491 Neutrophils were treated for 10 min prior to the addition of bacteria with either
492 diphenyleneiodonium (DPI; 50 µM) or an equivalent volume of DMSO (solvent control) as
493 needed. The bacterial and neutrophil suspension was subsequently incubated at 37 °C with
494 tumbling. At relevant time points (0.5, 1, 2, 3 h), 50 µl of the suspension was transferred to a
495 96-well plate and serially diluted ten-fold in PBS up to 10^{-3} dilution. All dilutions (including
496 neat) were then plated onto CBA and incubated for 24 h at 37 °C before counting. Survival
497 was calculated as a percentage of the number of bacteria in the starting inoculum.

498

499 **Murine systemic infection model**

500 Animal work was conducted in accordance with the Animals (Scientific Procedures) Act 1986
501 outlined by the UK Home Office regulations. Work was approved by the UK Home Office after
502 ethical approval by the Imperial College Animal Welfare and Ethical Review Body (AWERB).
503 Six- to eight-week-old female C57BL/6 mice (Charles River) were infected via the
504 intraperitoneal route with wild type or *rexB*::Tn mutant in JE2 and SH1000 backgrounds.
505 Stationary phase bacterial cultures were washed twice with PBS and adjusted to 10^7 CFU ml^{-}
506 ^{1}. Subsequently, 400 µl (4 x 10^6 CFU) of the washed bacterial suspensions were injected into
507 the peritoneal cavity of each mouse (5 mice for each strain, 20 in total). After 6 h, the mice
508 were humanely sacrificed by cervical dislocation and death confirmed by severing the femoral
509 artery. The peritoneal cavity was washed with PBS to release the bacteria and CFU counts
510 were determined by plating onto TSA. Sample size was determined prior to the experiment
511 using power analysis based on *in vitro* data[63]. Tubes containing the bacterial suspensions were
512 blinded before starting the experiment. Mice were randomly allocated to group cages and
513 each group was randomly allocated to a treatment. Following Home Office regulations, any
514 animals that displayed two or more of the following symptoms were humanely killed using a
515 Schedule 1 method and excluded from the study: shivering, hunched posture, reduced
516 movement, cyanosis, circling or difficulty breathing.

517

518 **Murine skin infection model**

519 Six- to -eight-week-old mice were subcutaneously infected with 2 x 10^6 CFU of exponential
520 phase cultures of *S. aureus* as previously described[64]. Skin infection animal work was
521 performed according to the Guidelines for the Care and Use of Laboratory Animals of the
522 National Institutes of Health, the Animal Welfare Act, and U.S. Federal law. Protocol was
523 approved by the Animal Care and Use Committee of Rutgers New Jersey Medical School.

524

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

526 A Δ*rexBA* mutant was generated in *S. gordonii* by in-frame allelic replacement with the
527 erythromycin resistance determinant *ermAM*. Flanking regions directly upstream and
528 downstream of the *rexBA* operon were amplified by PCR from *S. gordonii* DL1 genomic DNA
529 with primer pairs Sg.rexAB.F1/Sg.rexAB.R1 and Sg.rexAB.F2/Sg.rexAB.R2 (Table 2),
530 respectively, while the *ermAM* cassette was amplified from plasmid pVA838[65] using primers

531 ermAM.SgF/ermAM.SgR (Table 2). The resulting amplimers were then joined together via 20-
532 bp overlapping regions by stitch PCR using primers Sg.rexAB.F1/Sg.rexAB.R2, and transformed
533 into *S. gordonii*. Erythromycin-resistant transformants were confirmed by sequencing and the
534 strain designated UB3018.
535 A *rexBA* mutant was generated in *E. faecalis* by markerless exchange using a 2-step
536 homologous recombination approach, as previously described[59]. In brief, flanking regions
537 directly upstream and downstream of the *rexBA* operon were amplified by PCR from *E.*
538 *faecalis* OG1X genomic DNA with primer pairs Ef.rexAB.pheF1/Ef.rexAB.pheR1 and
539 Ef.rexAB.pheF2/Ef.rexAB.pheR2 (Table 2), respectively, and then joined together via 20-bp
540 overlapping regions by stitch PCR using primers Ef.rexAB.pheF1/Ef.rexAB.pheR2. The
541 resultant amplimer was cloned into donor plasmid pCJK47[59] via unique restriction sites
542 *Pst*I/*Nco*I to generate pCJK47-*rexAB*, propagated in *E. coli* EC1000 and then introduced into
543 conjugative donor strain *E. faecalis* CK111 (pCF10-101) by electroporation. Plasmid pCJK47-
544 *rexAB* was transferred to *E. faecalis* OG1X by conjugation. Transconjugants carrying the
545 integrated plasmid were confirmed by colony PCR, before counterselection based on the P-
546 *pheS** marker was used to identify secondary recombinants in which the integrated plasmid
547 had been excised and lost, leaving the desired Δ*rexBA* allele. This was confirmed by
548 sequencing and the strain designated UB2948.
549
550 **Construction of *S. aureus* RexAB expression vector**
551 Cloning of the *rexA* and *rexB* genes from *S. aureus* was achieved by PCR from wild-type
552 genomic JE2 DNA using the "rexB-F BamHI" and "rexA-R SalI" primers listed in Table 2, which
553 allowed the amplification of the *rexBA* operon immediately flanked by suitable restriction
554 endonuclease recognition sequences (BamHI and SalI). The *rexA* and *rexB* genes were inserted
555 into the pET28b$^+$ vector (Novagen) using standard cloning techniques and site-directed
556 mutagenesis (SDM) was performed to insert a StrepII-tag and a thrombin site in front of the
557 *rexA* gene. This enabled RexA and RexB proteins to be detected individually, via an N-terminal
558 His_6-tag for RexB (His_6 from the pET28b$^+$ vector) and an N-terminal StrepII-tag for RexA. PCR
559 primers for SDM are listed in Table 2. DNA sequencing was used on the "pET28b$^+$ *rexBA*"
560 expression plasmid to confirm that the sequences of the entire *rexA* and *rexB* genes, tags and
561 promoter regions were as expected.
562

563 **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	CCCGCAGTTCGAAAAAAGCGCTGGCCTGGTGCCGCGCGGCAGCGGCACAATTCCAGAGAAACCACAAGG
Thr rexAB SDM-R	CCTTGTGGTTTCTCTGGAATTGTGCCGCTGCCGCGCGGCACCAGGCCAGCGCTTTTTCGAACTGCGGG
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

564 * Underlined regions indicate bp overlaps for stitch PCR; italicised regions indicate restriction
565 endonuclease sites for ligation into pCJK47
566
567 **Expression and purification of recombinant *S. aureus* RexAB**
568 Cells from single colonies of *E. coli* SoluBL21 (DE3), freshly transformed with pET28b$^+$ *rexAB*
569 expression plasmid coding for N-terminally StrepII-tagged RexA and N-terminally His$_6$-tagged
570 RexB, were used to inoculate an overnight starter culture in LB supplemented with 50 µg ml$^-$
571 ^{1} of kanamycin. The starter culture was diluted to an OD_{600} of 0.05 into 4 L of TB containing
572 50 µg ml^{-1} of kanamycin. Cells were grown at 37 °C with shaking at 180 rpm until an OD_{600} of
573 0.5 was reached prior to induction with 1 mM of isopropyl β-D-thiogalactoside (IPTG).
574 Following induction, the temperature was reduced to 20 °C and cultures were further
575 incubated for 20 h. Cells were harvested by centrifugation at >10,000 x *g* and 4 °C for 30 min
576 and pellets were resuspended in 100 ml of 50 mM Tris, 150 mM NaCl (pH 7.5).
577 For protein purification, cells were disrupted by sonication and cell debris was cleared by
578 centrifugation at 32,000 x g 4 °C and for 30 min. The resulting supernatant was added to 5 ml
579 of Chelating Sepharose Fast Flow resin (GE Healthcare), which had been loaded with 0.1 M

580 $NiCl_2$ and equilibrated with 100 ml of 50 mM Tris, 150 mM NaCl, 20 mM imidazole (pH 7.5).
581 The supernatant-resin mixture was left at 4 °C overnight with gentle stirring to optimize the
582 binding of the His-tagged protein to the nickel-charged resin. Then, the mixture was washed
583 9x with 40 ml of 50 mM Tris, 150 mM NaCl, 20mM imidazole (pH 7.5) and 1x with 20 ml of 50
584 mM Tris, 150 mM NaCl, 70 mM imidazole (pH 7.5). His-tagged protein was eluted with 50 mM
585 Tris, 150 mM NaCl, 150 mM imidazole (pH 7.5); each 10 ml fraction was tested with Bradford
586 reagent (Bio-Rad) for protein content until no more protein could be detected. Fractions
587 containing protein were pooled, buffer exchanged and concentrated using an Amicon 100
588 kDa cut-off concentrator (Merck Millipore); RexAB is >250 kDa in size. The concentrating
589 device was centrifuged at 2000 x g (4 °C), and the protein solution was exchanged into 50 mM
590 Tris, 150 mM NaCl (pH 7.5) by four serial concentration and re-dilution steps. The total protein
591 concentration was quantified using the Pierce BCA protein assay kit (Thermo Fisher Scientific)
592 following the manufacturer's instructions, and presence of intact recombinant RexAB was
593 confirmed via SDS-PAGE and Western blot analysis.
594
595 **Nuclease and helicase activity assays**
596 The nuclease and helicase activity of RexAB was measured to confirm the AddAB-like activity
597 in our recombinant RexAB protein. Staphylococcal DNA was amplified from the JE2 whole
598 genome by colony PCR using the primer pairs "Chi control F" (5'- TCAGTGAATTAGATGATTCGC
599 -3') and "Chi control R" (5'- TTCATACGTATGAATGTTATTTGC-3') where the amplicon lacked a
600 Chi site region to be used as the DNA substrate in these assays.
601 Reactions were set up in either nuclease assay buffer (25 mM Tris-acetate pH 7.5, 2 mM Mg
602 acetate, 1 mM DTT) or helicase assay buffer (25 mM Tris-acetate pH 7.5, 0.25 mM Mg acetate,
603 1 mM DTT), along with 5 ng μl^{-1} of DNA, 1 mM ATP or equivalent volume of nuclease-free
604 water and either 20 nM or 50 nM of recombinant RexAB for the nuclease and helicase assay
605 respectively. Additionally, for the helicase assay, 2 µM of SSB protein was added to each
606 sample. The DMSO content was maintained at 1% (v/v) when compounds were being tested.
607 Samples were incubated statically at 37 °C and at 0, 5, 15, 30, 60 and 120 min, 5 µl was
608 removed and pipetted into 20 µl of STEB buffer (40 % (w/v) sucrose, 100 mM Tris-HCl, 10 mM
609 EDTA, 0.5 mg/ml bromophenol blue, pH 8) to stop the reaction. 20 µl of chloroform/isoamyl
610 alcohol (24:1) was added to each tube, vortexed for 10 sec and centrifuged for 2 min at 17,000
611 x g to remove the protein and any compounds used for inhibition. The aqueous (upper blue)

612 phase was loaded onto a 1% (w/v) agarose gel prepared in TBE buffer and electrophoresis
613 was carried out at 85 V for 1 h. The gels were subsequently stained with SYBR Safe DNA gel
614 stain (Invitrogen) at 1/10,000 dilution in TBE buffer for 2 h with rocking and visualised using a
615 Gel Doc EZ Imager (Bio-Rad). Band intensity was quantified using ImageJ software. For
616 nuclease activity, values were normalised to no ATP controls at 0 h. For helicase activity,
617 values were normalised to a ssDNA control that lacked the RexAB protein, which had been
618 heated at 95 °C for 2 min to denature the dsDNA and allow the SSB protein to bind and
619 stabilise the two ssDNA strands.
620
621 **Measurement of bacterial growth**
622 To measure growth of *S. aureus*, bacterial cultures were first grown to stationary phase in TSB
623 at 37 °C (180 rpm), then inoculated 1/50 for growth curves, or 1/10 for growth inhibition
624 assays (supplementary) into a flat-bottomed 96-well plate (200 µl total volume) and placed
625 into a POLARstar Omega plate reader (BMG Labtech). Bacteria were grown for 17 h at 37 °C
626 (700 rpm), and absorbance at 600 nm was measured every 30 min.
627
628 **Haemolytic activity**
629 The haemolytic activity of culture supernatants was determined as described previously[66].
630 Briefly, stationary phase *S. aureus* cultures were pelleted for 5 min at 17,000 x g, and 400 µl
631 of the supernatant pipetted into microcentrifuge tubes. An equal volume of 2% defibrinated
632 sheep blood in PBS was added and the mixture incubated statically for 1 h at 37 °C. Fresh TSB
633 containing 2% defibrinated sheep blood was used as a negative control. After incubation, the
634 samples were centrifuged for 5 min at 500 x g and 200 µl of supernatant was transferred into
635 a flat-bottomed 96-well plate. The absorbance of released haemoglobin was measured at 540
636 nm using a POLARstar Omega plate reader (BMG Labtech). Percentage haemolysis was
637 calculated relative to the wild type control.
638
639 **Staphyloxanthin extraction and quantification**
640 To extract and quantify staphyloxanthin, *S. aureus* stationary phase cultures grown in TSB
641 were harvested by centrifugation at 17,000 x g for 2 min. Staphyloxanthin was extracted by
642 incubating the culture in methanol at 42 °C for 30 min. Cells were pelleted by centrifugation
643 at 17,000 x g for 2 min, and 100 µl of supernatant was transferred into a flat-bottomed 96-

644 well plate. The released staphyloxanthin was quantified by measuring the absorbance at 462
645 nm using a POLARstar Omega plate reader (BMG Labtech).
646
647 **Catalase activity**
648 To determine the level of catalase activity, overnight cultures were washed three times in PBS
649 and 10^7 CFU were inoculated into 1 ml of 100 µM H_2O_2 (diluted in PBS). Samples were
650 incubated at 37 °C protected from light for 15 min. 200 µl of the sample was centrifuged at
651 17,000 x g for 3 min, and 20 µl of the supernatant was added to a 96-well microtitre plate.
652 PBS containing no bacteria was used as a negative control.
653 Catalase activity was determined indirectly by measuring the concentration of H_2O_2 over time,
654 using the Pierce Quantitative Peroxide Assay kit (aqueous-compatible formulation) (Thermo
655 Fisher Scientific). Following the manufacturer's instructions, reagents A and B were mixed at
656 a ratio of 1:100 and 200 µl was added to each sample in the 96-well plate. The plate was then
657 incubated for 30 min at room temperature and the absorbance was measured at 595 nm
658 using an iMark microplate reader (Bio-Rad). The H_2O_2 concentration was determined using a
659 standard curve of known concentrations (up to 1 mM).
660
661 **Computational analyses**
662 Multiple sequence alignments were generated using Clustal Omega via the EMBL-EBI web
663 server[67]. Parameters were left in their default settings with the exception of alignment
664 "order", which was set to consider the input order. Protein structures were predicted using
665 the Phyre2 protein fold recognition server[68]. Phyre2 structural models were viewed and
666 manipulated using the PyMOL Molecular Graphics System Version 2.3 (Schrödinger).
667
668 **Statistical analyses**
669 Data are represented as the mean or median from three or more independent experiments
670 and analysed by Student's t test (two tailed, unpaired, assuming equal variances), one-way
671 ANOVA or two-way ANOVA corrected for multiple comparison, as described in the figure
672 legends. For each experiment, "n" refers to the number of independent biological replicates.
673 CFU counts from murine experiments are presented as the value obtained from each animal
674 and significance was assessed using the Mann-Whitney test. $P < 0.05$ was considered
675 significant between data points (GraphPad Prism 7 for Windows).

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