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1 **_Staphylococcus aureus_ resists UVA at low irradiance but succumbs in the**
2 **presence of TiO_2 photocatalytic coatings**

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13 **ABSTRACT**

14 The aim of this study was to evaluate the bactericidal effect of reactive oxygen
15 species (ROS) generated upon irradiation of photocatalytic TiO_2 surface coatings
16 using low levels of UVA and the consequent killing of *Staphylococcus aureus.* The
17 role of intracellular enzymes catalase and superoxide dismutase in protecting the
18 bacteria was investigated using mutant strains. Differences were observed in the
19 intracellular oxidative stress response and viability of *S. aureus* upon exposure to
20 UVA; these were found to be dependent on the level of irradiance and not the total
21 UVA dose. The wild type bacteria were able to survive almost indefinitely in the
22 absence of the coatings at low UVA irradiance (LI, 1 mW/cm^2), whereas in the

23 presence of TiO_2 coatings, no viable bacteria were measurable after 24 hours of
24 exposure. At LI, the lethality of the photocatalytic effect due to the TiO_2 surface
25 coatings was correlated with high intracellular oxidative stress levels. The wild type
26 strain was found to be more resistant to UVA at HI compared with an identical dose
27 at LI in the presence of the TiO_2 coatings. The UVA-irradiated titania operates by a
28 "stealth" mechanism at low UVA irradiance, generating low levels of extracellular
29 lethal ROS against which the bacteria are defenceless because the low light level
30 fails to induce the oxidative stress defence mechanism of the bacteria. These results
31 are encouraging for the deployment of antibacterial titania surface coatings wherever
32 it is desirable to reduce the environmental bacterial burden under typical indoor
33 lighting conditions.

34 **Keywords:** UVA, photocatalysis, reactive oxygen species, *Staphylococcus aureus*,
35 titanium dioxide

36 **Introduction**

37 Surfaces in many industries, including healthcare, hospitality and leisure services,
38 require regular cleaning and disinfection to maintain environmental hygiene and
39 prevention of cross-transmission of pathogenic bacteria (Dancer, 2008).
40 Conventional methods of cleaning and disinfection with wiping are not particularly
41 effective, whilst also being time- and resource-intensive (White et al., 2008). Surface
42 recontamination rates following cleaning are rapid (Hardy et al., 2007). Other
43 methods of environmental surface decontamination include use of steam, hydrogen
44 peroxide vapour, ozone and UV light (Khan et al., 2012). However, the effectiveness
45 of these methods is limited because uniform dispersal of the active agent in a 3-
46 dimensional space is rarely achieved.

47 A recent study evaluated the use of photocatalytic surface coatings to reduce the
48 bioburden of frequently touched surfaces in a healthcare environment and reported a
49 lower microbial burden on surfaces treated with a commercial TiO_2-based
50 photocatalytic coating (Reid et al., 2018). The efficacy of irradiated titania (TiO_2) as
51 an antibacterial agent has long been known (Matsunaga et al., 1985). There have
52 been many laboratory experiments corroborating this photocatalytic effect against
53 both Gram-negative bacteria (e.g., *Escherichia coli* and *Pseudomonas aeruginosa*)
54 and Gram-positive bacteria like *Staphylococcus aureus* (Kühn et al., 2003; Nakano
55 et al., 2013; Sunada et al., 2003).

56 TiO_2 exists in three crystallographic phases: anatase, brookite and rutile. Their band
57 gaps, mechanisms of light absorption and photocatalytic activities differ (Zhang et
58 al., 2014). All the band gaps are in the violet–ultraviolet region; in actual samples
59 surface and impurity states may shift the absorption to longer wavelengths
60 (Ramsden, 2015). However, most experimental studies use near-ultraviolet light
61 (typically UVA, 320–380 nm) to investigate the photocatalytic antimicrobial action. It
62 is now known that such light itself has some antimicrobial action (Merwald et al.,
63 2005). Shorter-wavelength ultraviolet light (UVC) is already well-established as an
64 antimicrobial agent in healthcare facilities (Rastogi, 2007). However, UVC is harmful
65 to human beings, whereas mild UVA can be used in their presence, hence is more
66 amenable for use in hospitals and in hospitality and catering industries such as food
67 preparation areas to promote continuous disinfection and environmental hygiene.
68 Band-gap irradiation of TiO_2 produces highly reactive oxygen species (ROS),
69 especially superoxide, hydroxyl and perhydroxyl radicals (Hirakawa and Nosaka,
70 2002; Kikuchi et al., 1997; Ramsden, 2015). There is realization that bacteria may
71 not be able to develop resistance to all of the different ROS species

72 photocatalytically generated (Ramsden, 2017). This has raised interest in
73 photocatalytic antimicrobial materials, especially because of the global health threat
74 posed by the increasingly prevalent antimicrobial resistance (O'Neill, 2016).

75 *S. aureus* was chosen in the present study as an example of a typical problematic
76 pathogen. It is a Gram-positive bacterium of interest to hospital hygienists, because
77 of the widespread prevalence of methicillin-resistant *S. aureus* strains (MRSA),
78 which are associated with healthcare-associated infections, increased lengths of stay
79 in hospitals, increased healthcare costs and increased mortality (Goodman et al.,
80 2008). Surfaces in rooms occupied by MRSA-positive patients can contaminate the
81 hands of healthcare workers and result in cross-transmission. Studies have
82 demonstrated that these organisms can survive and persist in the environment for
83 prolonged periods despite routine cleaning (Kramer et al., 2006).

84 In the present study, viability of wild type *S. aureus* SH1000 and isogenic mutants
85 defective in either peroxide or superoxide detoxification on P25 titanium dioxide
86 (TiO_2) films at low and high UVA irradiances was investigated to elucidate the
87 mechanisms of bactericidal activity. At low natural irradiance (representative of
88 indoor lighting conditions) UVA has very low, if any, bactericidal action; however, at
89 high irradiance, bactericidal action has been noted (Kramer and Ames, 1987). The
90 effect of photocatalytically induced reactive oxygen species on intracellular oxidative
91 stress in bacteria was investigated and their bactericidal effect was quantified.

92

93 **Materials and methods**

94 **Chemical reagents**

95 P25 TiO_2 was purchased from Evonik Industries AG, Germany. Terephthalic acid
96 (TPA), hydroxyterephthalic acid (hTPA), indigo trisulfonate (ITS), 2,7-
97 dichlorofluorescein diacetate (DCFH-DA) and 2,7-dichlorofluorescein (DCF), ethanol
98 (99.8+% analytical grade), phosphoric acid (99.9+% analytical grade), sodium
99 phosphate monobasic (reagent grade) were purchased from Sigma Aldrich (UK).

100 **Photocatalysis reactor experimental set-up**

101 The photocatalytic experiments were carried out in a specially designed and built
102 photoreactor (Fig. 1). It consists of two identical rectangular boxes equipped with a
103 lid that can be unfastened to allow ease of access to Petri dishes (4 per box). The
104 photoreactor was equipped with a black-light UV-A fluorescent lamp (tubular ~50 cm
105 length, 26 mm diameter, Philips 8W/BLB, wavelength (λ) 360 nm) positioned ~4 cm
106 above the Petri dish in the centre of each box. Inside the irradiation compartment,
107 local measurements of the irradiance were made using a radiometer (ILT 1700,
108 International Light Technologies) equipped with a SED 033 sensor calibrated with
109 appropriate filters. The spatial distribution of light intensity across the four Petri
110 dishes was found to be uniform within measurement error (± 2% of irradiance). The
111 boxes were placed on a platform rocker (Stuart Scientific, UK, 3D Rocking platform,
112 Model STR9) with a frequency of 5 rev min^{-1}. The Petri dishes containing the glass
113 slides (with and without TiO_2 coating) contained 15 ml of sterile deionized water. The
114 liquid depth in the Petri dishes was ~ 2 mm. Samples were exposed at a controlled
115 irradiance of 1.00 ± 0.05 mW cm^{-2} (low irradiance, LI) and 4.00 ± 0.05 mW cm^{-2} (high
116 irradiance, HI).

117 **Fabrication of TiO_2 films**

118 TiO_2 nanoparticles were suspended in ethanol at a concentration of 25 g l^{-1}.
119 Borosilicate glass microscope slides (Sigma Aldrich, UK, 38 mm x 75 mm) were
120 washed with ethanol under sonication and subsequently air-dried in a laminar flow
121 hood. The slides were then coated with TiO_2 using a standard dip-coating procedure
122 (Fig. 1): they were rigidly clamped to a motorised rod that allowed a dipping and
123 withdrawal rate of 3 cm min^{-1}. Coating was carried out at room temperature (25 °C).
124 The slides were dipped in the TiO_2 suspension (100 ml beaker equipped with a
125 magnetic stirrer to ensure uniform dispersion of TiO_2 nanoparticles). The weight gain
126 of the slide after each coating cycle (i.e., dipping and withdrawing) was measured
127 using a six-digit balance (Sartorius, UK). The process was repeated several times
128 until the mass of TiO_2 deposited on each slide reached 0.5 ± 0.05 mg. The ethanol
129 was allowed to evaporate at room temperature between each coating cycle (the
130 drying process took ~45 sec).

131 **Characterization of TiO_2 coatings**

132 The morphology of the TiO_2 particles was examined using a field emission gun
133 scanning electron microscope (FEG-SEM), also used for characterization of surface
134 morphology and coating thickness (Leo Elektronenmikroskopie GmbH model 1530
135 VP equipped with an EDAX Pegasus (EBSD/EDXA) unit). Sputter coating (for 60 s)
136 of the samples prior to SEM imaging was carried out using gold/palladium (Au/Pd)
137 alloy.

138

139 **Bacterial strains, media and growth conditions**

140 The antibacterial photocatalytic coatings were tested against *S. aureus* wild type
141 SH1000 and isogenic mutants defective in peroxide (SH1000 *ahpC/katA*) and
142 superoxide (SH1000 *sodA/sodM)* detoxification (Cosgrove et al., 2007; Karavolos et
143 al., 2003).*S. aureus* strains were grown in a brain-heart infusion (BHI, Oxoid) culture
144 medium at 37 °C overnight. An aliquot of the overnight culture was transferred in
145 fresh BHI broth to reach an optical density (OD) of 0.05 at 600 nm. According to the
146 growth curve of each strain (data not shown), the concentration of bacterial cells was
147 adjusted to a target concentration of 2.5 x 10^6 CFU ml^{-1}. The bacteria were
148 centrifuged at 2500 g for 5 min at 4 ° C and the pellets of bacterial cells were
149 resuspended in 1 ml of deionized sterile water after removing any growth medium
150 traces. The bacterial suspension was then added to 14 ml deionized sterile water in
151 the Petri dishes before the start of each experiment. Suspension samples were
152 taken at intervals during irradiation and plated after serial dilution on BHI + 5% blood
153 agar plates (TCS Biosciences) and incubated at 37 °C for 24 h, to measure cell
154 viability as colony-forming units (CFU ml^{-1}).

155 **Quantification of hydroxyl radicals and hydrogen peroxide**

156 The hydroxyl radical production rate of the coatings was obtained by monitoring the
157 rate of reaction of hydroxyl radicals produced during the photocatalytic process and
158 terephthalic acid (TPA) reagent added to the solution. In alkaline aqueous solution,
159 TPA produces terephthalate anions, these react with hydroxyl radicals to produce
160 highly fluorescent hydroxyl-terephthalate ions (hTPA) (Mason et al., 1994). A
161 solution of 2 mM TPA in phosphate buffer (pH 7) was made and 15 ml were poured
162 into each Petri dish. The fluorescence of each sample was measured using a Perkin-

163 Elmer LS-50 luminescence spectrometer with an excitation wavelength of 315 nm
164 and analysing the emission at 425 nm. A stock solution of 2 mM in phosphate buffer
165 of 2-hydroxyterephthalic acid was prepared for calibration purposes. This is the final
166 product of the chemical reaction between terephthalic acid and the hydroxyl radicals
167 produced during the photocatalytic process. The fluorescent signals of serial
168 dilutions from the stock solution was monitored and used to construct the calibration
169 curve.

170 The aqueous H_2O_2 concentration was measured by the standard titanium sulphate
171 colorimetric method (Machala et al., 2013). The reaction results in a yellow-coloured
172 complex according to the following scheme: $Ti^{4+} + H_2O_2 + 2\ H_2O \rightarrow H_2TiO_4$
173 (pertitanic acid) + $4H^+$. The complex is stable for at least 6 h. Absorbance was read
174 at 407 nm using a UV-Vis spectrophotometer (Shimadzu, UV Mini 1240).

175 **Photocatalytic activity test**

176 The degradation of indigo trisulfonate (ITS) in aqueous solution was monitored to
177 evaluate the photocatalytic activity of the prepared coatings. ITS is a well known
178 redox indicator of oxidative stress. The indigo molecule has only one C=C double
179 bond, which is highly reactive with the ROS produced during the photocatalytic
180 process. Oxidative cleavage of the C=C bond eliminates the absorbance at 600 nm
181 (Dorta-Schaeppi and Treadwell, 1949).

182 An ITS stock solution (0.1 mM) was made in deionized water. A fresh test solution
183 was prepared by mixing 5 g sodium phosphate monobasic, 3.5 ml concentrated
184 phosphoric acid, 20 ml ITS stock solution and pure water up to final volume of 500
185 ml. The pH of the resulting test solution was 3.0. During irradiation 0.5 ml of indigo

186 solution was taken every hour and the indigo concentration was determined
187 spectrophotometrically at 605 nm.

188 **Quantification of total intracellular ROS concentration**

189 Quantification of intracellular ROS generated by the UVA-irradiated TiO_2 coatings
190 and due to UVA irradiation only was estimated with 2,7-dichlorofluorescein diacetate
191 (DCFH-DA). Intracellular ROS convert the nonfluorescent DCFH-DA to fluorescent
192 2,7-dichlorofluorescein, which is monitored. A stock solution of DCFH-DA (10 mM in
193 methanol) was prepared and kept at -80 °C in the dark. Before illumination, bacteria
194 (2.5 x 10^6 CFU ml^{-1}) were centrifuged (2500 g for 5 min at 4 °C) and the pellets
195 resuspended in 2 ml PBS. An aliquot of DCFH-DA stock solution was added to the
196 bacterial suspension and incubated at 37 °C for 1 h under agitation. The solution
197 was then centrifuged (2500 *g* for 5 min at 4 °C), the supernatant was discarded, and
198 the bacteria resuspended in 1 ml ultrapure sterile water. After exposure to UVA or
199 photocatalysis, the bacterial suspension was collected and centrifuged, the
200 supernatant discarded, and the cells resuspended in 500 µl of alkaline solution (0.2
201 M NaOH containing 1 % SDS) and 1 ml Tris-HCl, 40 mM, pH 7.4. Fluorescence
202 intensity was monitored at excitation 488 nm/emission 525 nm after 15 minutes. A
203 calibration curve was constructed using fluorescent 2,7-dichlorofluorescein (DCF) to
204 measure the unknown fluorescence signal and relate this to the final concentration of
205 oxidized probe.

206 **Estimation of parameters of inactivation kinetics using a series-event model**

207 In this model an ‘event’ is a ‘quantum of damage’ inflicted on a bacterial cell. The
208 inactivation of a bacterial cell can be viewed as undergoing a series of damaging
209 reactions or events. Damage is considered to occur in integer steps. A certain

210 number of such events, occurring in series and with kinetics modelled as first order
211 with respect to the cell state, needs to be accumulated by the cell for death to ensue.
212 A series-event model with the following form of model equation (Severin et al., 1983)
213 was used to fit the photocatalytic and UVA inactivation data:

214

215 $$\frac{C}{C_o} = \exp(-kt) \sum_{i=0}^{n-1} \frac{(kt)^i}{i!} \quad \text{[eq. 1]}$$

216 where the magnitude of the inactivation rate constant k (h^{-1}) is dependent on the
217 UVA irradiance, and C_o and C are the concentrations of viable bacteria (CFU/ml) at
218 time zero prior to exposure to UVA and after time t following the start of exposure to
219 UVA or photocatalytically-induced stress. The series-event model has two fitting
220 parameters (rate constant k, and the number of damaging events n), which were
221 numerically varied to achieve a nonlinear least-squares regression fit (using the
222 Levenberg-Marquardt method) to a given set of experimental data (using Datafit
223 software version 9.1.32, Oakdale Engineering, USA).

224 **Statistical analysis**

225 Statistical analysis was carried out using Minitab version 18 (USA). Two-sample t-tests
226 were performed (n=3) with reporting of $p < 0.05$ as statistically significant. Error bars
227 represent a single standard deviation, number of replicates indicated in the Figure
228 captions.

229

230 **Results**

231 **Physical characterization of the coated glass slides**

232 The surface morphology of the coatings was visualized using SEM (Fig. 2). The
233 TiO_2-coated glass slides showed no significant changes in morphology between the
234 starting P25 material suspended in ethanol and the deposited TiO_2 (data not shown).
235 The size of agglomerates on the slide surface is ~ 200 nm (Fig. 2). The thickness of
236 the coatings was typically ~ 3 μm (Fig 2). The TiO_2 surface coverage indicated a
237 relatively even distribution of the nanoparticles, although there were bare patches on
238 the glass surface (Fig. 2). Typically, the number of dipping cycles needed to achieve
239 0.5 mg of TiO_2 deposited per slide was between 8 and 10, giving a coating surface
240 density of 0.02 mg cm^{-2}. Increasing this number did not greatly change the amount of
241 the catalyst deposited on the surface. Complete surface coverage of the catalyst on
242 the glass slide was difficult to achieve without dramatically increasing the number of
243 coating cycles, which was considered unnecessary given that the length scale of the
244 randomly distributed uncoated glass areas was smaller than the size of a typical
245 bacterium. Hence, any bacteria adherent to the glass surface would nevertheless be
246 in at least partial contact with TiO_2 nanoparticles.

247 **Photocatalytic activity of the coatings immersed in solution**

248 TPA was always present in excess (hence zero-order concentration dependence) in
249 comparison with the hydroxyl radicals produced during the photocatalytic process.
250 Hence, the production rate of hydroxyl radicals in solution can be calculated from the
251 gradient of the measured concentration of fluorescent hTPA produced during
252 photocatalysis. Hydroxyl radical production at both LI and HI was found to be linear
253 (Fig. 3a). At LI the average rate of hydroxyl radical production was 0.32 μM h^{-1} (95%
254 CI range 0.31–0.33 μM h^{-1}) and at HI it was 1.09 μM h^{-1} (95% CI range 0.77–1.41

255 μM h^{-1}). Hence the rate of hydroxyl radical production is, within experimental
256 uncertainty, proportional to the UVA irradiance at the surface of the coatings.
257 Controls (UVA irradiation in the absence of a TiO_2 coating) yielded no production of
258 hydroxyl radicals (data not shown).

259 Assessment of the effect of irradiance on the photocatalytic degradation of ITS in the
260 presence of the coated substrates was carried out as an indicator of the overall rate
261 of ROS production (Fig. 3b). 95% of ITS was degraded in 7 h at LI and in 4 h at HI.
262 ITS degradation was found to follow first order kinetics and an exponential
263 regression model (of the form ae^{-bt}) was therefore appropriate. Fitted parameters: for
264 LI, a = 58.6 μM, 95% CI (54–62.6); b = 0.35 h^{-1}, 95% CI (0.38–0.30) and for HI, a =
265 59.9 μM, 95% CI (48.3–71.4); b = 0.61 h^{-1}, 95% CI (0.83–0.40). Initial degradation
266 rates (at t = 0) were 20.5 μM h^{-1} for LI and 36.5 μM h^{-1} for HI. The controls (UVA
267 irradiation in the absence of a TiO_2 coating) showed a modest decrease in ITS
268 concentration (Fig. 3), which was fitted with a linear regression model yielding rates
269 of 1.5 μM h^{-1} for LI and 5.8 μM h^{-1} for HI. Unlike hydroxyl radical production, there
270 was no evidence that *ex vivo* ROS production is proportional to irradiance; it was
271 markedly subproportional.

272 Photocatalytic inactivation of *S. aureus* (wild type and mutants) and 273 intracellular oxidative stress

274 *Inactivation kinetics and intracellular oxidative stress for wild type*

275 At LI over 8 h the viable cell concentration for the WT strain was stable for both UVA-
276 only exposed controls (without coatings) and samples exposed to UVA in the
277 presence of TiO_2 coatings, producing ROS (Fig. 4a). 4 h of HI UVA exposure was
278 needed for a ~1 log reduction in viable cell concentration; it was not possible to

279 discriminate between the level of killing achieved using HI UVA alone and samples
280 exposed to HI UVA in the presence of TiO_2 coatings (Fig. 4a). LI UVA exposure for
281 8h resulted in low intracellular DCF concentrations (< 0.2 mM) in the WT strain (Fig.
282 5a). There was a statistically significant difference ($P<0.05$) in intracellular ROS
283 levels in bacteria exposed to UVA only and those exposed to UVA in the presence of
284 the TiO_2 coatings. This suggests a measurable effect of photocatalytically induced
285 ROS on intracellular oxidative stress levels.

286 No viable *S. aureus* wild type cells were detected in solution upon exposure to LI
287 UVA in the presence of coatings after 24 h (Fig. 6a). Exposure for 6 h at HI UVA (i.e.
288 replicating the 24 h LI dose—irradiance multiplied by exposure time) resulted in a
289 considerable decrease (~2 log) in viable cell concentration. No significant difference
290 in viable bacterial counts was observed between the HI UVA-treated and the TiO_2-
291 coated samples (Fig. 6a). A significant increase in intracellular DCF concentration
292 (~2 mM) was measured for the WT strain in the presence of TiO_2 coatings exposed
293 to 24 h LI UVA (Fig. 6b). Intracellular DCF concentration for the control sample (WT
294 strain exposed to LI UVA for 24 h without TiO_2 coatings) was significantly lower ~ 0.5
295 mM (Fig. 6b). These results suggest a significant increase in intracellular oxidative
296 stress following 24 h LI UVA exposure in the presence of TiO_2 coatings, which
297 correlates with the killing of the WT strain.

298 *Inactivation kinetics and intracellular oxidative stress for the ahpC/katA catalase-*
299 *negative mutant*

300 The *ahpC/katA* mutant strain showed 1.5 log greater inactivation for bacterial
301 samples exposed to LI UVA for 8 h in the presence of TiO_2 coatings compared with
302 UVA controls (Fig. 4b). The inactivation kinetic data was fitted with a series-event

303 model. The optimum fitted value of the threshold number of events was *n*=10 and the
304 fitted inactivation rate constant for the coated samples was *k*=2.30 h^{-1} and for the
305 UVA controls 1.75 h^{-1}, indicating faster inactivation in the presence of the coating,
306 presumably due to the production of ROS (Table S1). A 3 log reduction in viable cell
307 concentration took 4 h upon exposure of the *ahpC/katA* mutant strain to HI UVA and
308 it was not possible to discern differences in lethality between UVA controls (no
309 coating) and the TiO_2-coated samples at any time point, suggesting no additional
310 effect of TiO_2-induced ROS in comparison with HI UVA alone (Fig. 4b). The
311 inactivation kinetics data was fitted by a series-event model with *n*=10. The
312 inactivation rate constant for the coated samples was found to be 4.65 h^{-1} and for the
313 UVA controls 4.56 h^{-1}, indicating faster inactivation at HI compared with LI, but the
314 rate constant for HI (4 mW/cm^2) was not found to be four times that for 1 mW/cm^2
315 (LI). Less than 1 log reduction was observed after 2 h HI exposure compared with a
316 3 log reduction at LI for the same overall dose for samples in the presence of TiO_2
317 coatings (Fig. 4b). A considerably greater degree of lethality was therefore achieved
318 with LI UVA compared with HI for the same radiation exposure dose in the presence
319 of the photocatalytic coatings. This indicates bacteria were more susceptible to LI
320 UVA killing compared with HI for the same total radiation dose and suggests that the
321 bacteria activate a defence mechanism in response to HI UVA , a mechanism that is
322 not activated during LI UVA exposure.

323 Intracellular DCF concentration (~0.3 mM) for the *ahpC/katA* strain exposed to TiO_2
324 was significantly higher compared with the UVA-only controls at LI (Fig. 5a). This
325 suggests photocatalytically induced intracellular oxidative stress due to ROS
326 production by TiO_2. Irradiance of the bacteria at HI for 2 h resulted in a significant
327 increase in intracellular DCF concentrations (~1 mM) in the *ahpC/katA* mutant strain

328 for both UVA controls and TiO_2-coated samples and no significant difference
329 between them (Fig. 5b).

330 *Inactivation kinetics and intracellular oxidative stress for the sodA/sodM mutant*

331 The *sodA/sodM* mutant strain was highly sensitive to LI ROS production by TiO_2
332 showing a ~5 log decrease in viability in the presence of the TiO_2 coating. In the
333 absence of the photocatalytic coating there was no bactericidal effect (Fig. 4c). The
334 inactivation kinetics fitted with a series-event model (n=10) yielded an inactivation
335 rate constant of 2.57 h^{-1} for the coated samples, indicating faster inactivation
336 compared with the catalase mutant strain. 4 h HI exposure resulted in a ~3 log
337 decrease in viable cells and no discernible differences between the viable cell
338 concentrations for UVA controls and TiO_2-coated samples at any time (Fig. 4c). This
339 suggests no additional effect of photocatalytic ROS in comparison with HI UVA
340 alone. The inactivation kinetic data did not fit the series-event model (typical of
341 concave inactivation curves with a shoulder) when n = 10 was used for fitting the
342 data, but did fit with n = 1. The inactivation rate constant for the coated samples was
343 1.31 h^{-1} and for the UVA controls 1.28 h^{-1}. Less than 2 log decrease in viable cells
344 was observed after 2 h exposure to HI UVA in the presence of TiO_2 coatings
345 compared with 5 log reduction at LI for the same dose.

346 Intracellular DCF concentration for 8h LI UVA exposure in the presence of TiO_2
347 coatings for the *sodA/sodM* strain had the highest value (~0.5 mM, Fig. 5a)
348 compared with the WT and *ahpC/katA* strains. This suggests significant
349 photocatalytically induced intracellular oxidative stress due to ROS production by
350 TiO_2. Irradiance of the *sodA/sodM* mutants at HI for 2 h resulted in a significant

351 increase in intracellular DCF concentrations (~1 mM) for both UVA controls and
352 TiO_2-coated samples with no significant difference between them (Fig. 5b).

353 **Discussion**

354 The main product of oxygen reduction by TiO_2 photocatalysis is superoxide $\bullet O_2^-$, which
355 can pick up a proton to form the perhydroxyl radical •OOH (Ramsden, 2015). Meanwhile
356 hydroxyl ions are oxidized to hydroxyl radicals •OH (Ramsden, 2015). Elevation in the
357 intracellular levels of these oxidants, notably superoxide $\bullet O_2^-$, results in enzyme damage
358 and may accelerate mutagenesis (Imlay, 2015). In contrast to some other common
359 bacteria like *E. coli*, *S. aureus* synthesizes only one catalase protein but also uses AhpC
360 alkylhydroperoxide reductase to degrade peroxide (Antelmann et al., 1996; Horsburgh et
361 al., 2001; Loewen, 1984). Catalase is well known for its ability to detoxify intracellular
362 hydrogen peroxide (Mandell, 1975; Pezzoni et al., 2016). However, the most important
363 role of catalase is to avoid formation of hydroxyl radicals through the Fenton reaction
364 between H_2O_2 and iron in the cell (Cosgrove et al., 2007). *S. aureus* has two SOD-
365 encoding genes, *sod*A and *sod*M. The products of translation of mRNA are two
366 homodimers and a heterodimer that combine to give rise to three activity centres for SOD
367 (Clements et al., 1999; Valderas and Hart, 2001). SOD is a metalloprotein that converts
368 O_2^- to H_2O_2 and O_2, preventing not only direct damage caused by O_2^- but also the toxicity
369 of the Fe^{3+}-dependent catalytic reactions leading to OH via the Haber-Weiss reaction
370 (Haber and Weiss, 1934). In the WT strain intracellular ROS concentrations are held in
371 check by the superoxide dismutases that degrade $\bullet O_2^-$ and the peroxidases and catalases
372 that degrade H_2O_2. Mutants that lack either set of enzymes suffer damage to specific
373 enzymes and are unable to grow under conditions requiring their activity (Gu and Imlay,
374 2013).

375

376 TiO_2-coated glass substrates immersed in water and exposed to LI UVA-generated ROS
377 in the water (Fig. 3). It was possible to discriminate between the bactericidal effect of LI
378 UVA alone and that due to ROS production by the TiO_2 coatings. The *sodA/sodM* and to a
379 lesser extent the *ahpC/katA* mutant strains were found to be resistant to LI UVA damage
380 but were highly susceptible to TiO_2-induced ROS over the same exposure period. The WT
381 strain was considerably more resistant; nevertheless, after 24 h LI exposure no viable
382 cells were culturable. Measurement of intracellular DCF formation showed differential
383 levels of intracellular oxidative stress at LI, with the highest measured levels in the
384 *sodA/sodM* mutant strain followed by the *ahpC/katA* mutant strain and considerably lower
385 levels in the WT strain (Fig. 5a). Intracellular levels of DCF for LI UVA-only exposed
386 samples were significantly less in comparison with the TiO_2-exposed samples (Fig. 5a).
387 Intracellular levels of DCF increased in the WT strain after LI exposure for 24 h and were
388 much higher in comparison with WT exposed to LI UVA only (Fig. 6b). Inactivation kinetics
389 and the intracellular oxidative stress data suggest that superoxide dismutases that
390 degrade $\bullet O_2^-$ play a significant role in affording protection against ROS under LI UVA.
391 Hydrogen peroxide levels in solution were below the limit of detection (< 0.1 mM and
392 below the minimum inhibitory concentration > 10 mM) even after 24 h irradiation with UV
393 in the presence of the TiO_2 coatings. Previous studies with NUV corroborate these results;
394 researchers did not find elevated levels (> 1 μM) of hydrogen peroxide at similar low
395 fluence rates (Kramer and Ames, 1987). This does not rule out the involvement of low
396 levels of hydrogen peroxide in the formation of more toxic oxygen species (Pezzoni et al.,
397 2016). Addition of sublethal amounts of hydrogen peroxide during NUV irradiation was
398 found to increase bacterial cell death rates and thought to result from superoxide anion
399 formation which may react further with hydrogen peroxide to yield reactive hydroxyl

400 radicals measured here (Liochev and Fridovich, 2010). Bulk •OH radical generation rate
401 was directly related to the level of light irradiance (Fig. 3) and was likely formed by the
402 well-known Haber–Weiss reaction in which H_2O_2 reacts with $•O_2^-$ to give bulk •OH directly
403 in solution (Hirakawa and Nosaka, 2002).
404
405 Decoupling the effect of intracellular ROS-induced stress at HI UVA due to TiO_2
406 photocatalysis compared with that caused by HI UVA alone was not possible (Figs. 4 and
407 5). Comparison of HI inactivation kinetics for the *ahpC/katA* and *sodA/sodM* mutants
408 suggested that intracellular superoxide $•O_2^-$ formation caused rapid killing of the SOD
409 mutant while the catalase mutant initially showed resistance to HI UVA damage
410 (characteristic shoulder seen on the inactivation curve) but irradiation continuing after
411 about 90 min of initial exposure to HI UVA resulted in cells beginning to rapidly die (the
412 decay rate was faster during this interval in comparison with that of the SOD). The WT
413 strain exposed to HI UVA started showing some viability loss after 4h of exposure (Fig.
414 4a) increasing to over 2 log reduction after 6 h (Fig. 6a). In the WT strain, intracellular
415 enzymes presumably afford initial protection to UVA-induced ROS damage; however,
416 accumulating levels of ROS have been shown to damage intracellular enzymes making
417 the cells susceptible to oxidative damage if exposure continues (Imlay, 2015).
418
419 Bacteria are known to be resistant to short exposures of the near-UV (NUV) component of
420 the solar spectrum (λ = 300–400 nm) at irradiances mimicking natural sunlight (3.5–5
421 mW/cm^2 corresponding to HI) but begin to die rapidly after 3 to 4 h of exposure (Kramer
422 and Ames, 1987). Exposure to HI UVA may involve photosensitization by endogenous
423 NUV-absorbing chromophores resulting in their excitation followed by reaction with
424 dissolved intracellular O_2 resulting in intracellular ROS production (Fig. 5b) and oxidative

425 damage (Kramer and Ames, 1987). Involvement of the oxidative defense regulon oxyR in
426 affording protection to intracellular oxidative stress has previously been shown to be
427 crucial in protecting bacteria against NUV damage (Eisenstark, 1998; Wei et al., 2012).

428 UVA-induced oxidative damage and, ultimately, cytotoxicity has been shown to be
429 dependent on radiation intensity not just the total energy dose (Eisenstark, 1987).
430 UVA radiation generates active oxygen species, including hydrogen peroxide, inside
431 irradiated bacteria (Cunningham et al., 1985; Czochralska et al., 1984; McCormick et
432 al., 1976; Pezzoni et al., 2016). Intracellular oxidative stress at LI and HI (using the
433 same total energy dose) were measured using the DCFH probe (Fig. 5). In the
434 absence of the titania coatings, very low intracellular concentration of fluorescent
435 DCF was detected at LI, indicating low intracellular ROS production (Fig. 5a).
436 Exposed for the same dose of UVA only but using HI, the intracellular concentration
437 of fluorescent DCF increased dramatically (Fig. 5b). It is unclear whether UVA has
438 contributed to the increase in intracellular ROS directly; e.g., through the tryptophan
439 and/or NADP/NADPH pathway, or indirectly through inactivating the bacterial
440 enzymes for disarming ROS. Regulatory gene products are known to be triggered
441 upon excess NUV oxidation leading to synthesis of entire batteries of anti-oxidant
442 enzymes, DNA repair enzymes etc., which may explain the results reported here
443 (Eisenstark, 1998; Pezzoni et al., 2016; Sassoubre et al., 2014).
444

445 Inactivation results at the same 'inactivation dose' for the WT strain at LI (24 h
446 exposure) and HI (6 h exposure) did not follow the Bunsen–Roscoe reciprocity law
447 applicable to simple photochemical processes. This law states that the effect of
448 radiation depends on the total radiant energy received and is independent of
449 irradiance and duration. In the case of *S. aureus* WT strain the photochemical effect

450 was found not to follow the reciprocity law. At high irradiance the presence of the
451 titania had no additional effect on bacterial viability compared with UVA alone, and
452 the degree of intracellular oxidative stress was the same regardless of the presence
453 or absence of catalase/AhpC or SOD. On the other hand, at low irradiance, survival
454 of the mutants lacking catalase/AhpC or SOD was severely compromised by the
455 presence of titania, and all bacterial forms, even the wild-type, had significantly
456 increased internal oxidative stress compared with UVA alone. For the WT strain,
457 after 24 h of exposure at low irradiance (1 mW/cm^2 — cf. ordinary interior lighting,
458 which is typically around 0.1 mW/cm^2) all the bacteria were killed in the presence of
459 the titania coatings (~ 7 log reduction), whereas when the same exposure was
460 delivered at 4 mW/cm^2, about 1% of the bacteria survived (2 log reduction),
461 regardless of the presence of titania; a similar proportion survived at 1 mW/cm^2 (after
462 24 h exposure) in the absence of titania (Fig. 6a). This implies that when the
463 irradiance exceeds a threshold (corresponding to a level somewhere between LI and
464 HI), certain defence mechanisms are activated, which affords protection to the
465 bacterium from the ROS generated both by UVA *and* by the titania coating. LI, which
466 still greatly exceeds typical interior irradiance, failed to activate these defence
467 mechanisms and in consequence the WT strain accumulated damage and was
468 effectively inactivated in the presence of titania after 24 h exposure. A previous study
469 with *E. coli* irradiated with UVA at 365 nm reported a similar result; *E. coli* cells were
470 found to be more resistant at high irradiance in comparison with low irradiance with
471 reciprocity found only at high values above 75 mW/cm^2; considerably higher than
472 those used in the present study (Peak and Peak, 1982). These observations are
473 supported by another study using *E. coli* cells and UVA which showed that
474 increasing the light intensity from 0.48 mW/cm^2 to 3.85 mW/cm^2 i.e. an 8-fold

475 increase, resulted in only halving of the bacteria killing time from 180 min to 90 min
476 respectively (Benabbou et al., 2007).

477 **Conclusions**

478 LI UVA in the presence of surface-immobilized TiO_2 was shown to result in the production
479 of ROS in solution and increased intracellular levels of oxidative stress, which over 24 h
480 was found to be lethal for the WT *S. aureus* strain. These results are encouraging for the
481 deployment of antibacterial titania surface coatings; e.g., for hospital interiors such as
482 wards and surgical theatres as well as in vehicles, hotels and restaurants—wherever it is
483 desirable to reduce the environmental bacterial burden; the titania may be thought to
484 operate by a "stealth” mechanism, generating lethal ROS against which the wild type
485 bacteria are defenceless because at these low light levels the oxidative stress defence
486 mechanisms are not triggered. The LI irradiance used in the present study was an order of
487 magnitude higher than typical indoor irradiance; future studies should investigate whether
488 there is a low irradiance threshold correlating with a minimum photocatalytic induced ROS
489 dose which is needed for inactivation of *S. aureus.* We have, moreover, shown the level of
490 photocatalytic activity and timescales needed to inactivate *S. aureus.* The methods used
491 to evaluate the coatings may help in evaluating the performance of commercial
492 photocatalytic coatings designed to be used in practical indoor settings.

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498

499 **Conflict of Interest**

500 No conflict of interest is declared.

501 **References**

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