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ABSTRACT

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

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

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

Introduction

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

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

TiO_2 exists in three crystallographic phases: anatase, brookite and rutile. Their band gaps, mechanisms of light absorption and photocatalytic activities differ (Zhang et al., 2014). All the band gaps are in the violet–ultraviolet region; in actual samples surface and impurity states may shift the absorption to longer wavelengths (Ramsden, 2015). However, most experimental studies use near-ultraviolet light (typically UVA, 320–380 nm) to investigate the photocatalytic antimicrobial action. It is now known that such light itself has some antimicrobial action (Merwald et al., 2005). Shorter-wavelength ultraviolet light (UVC) is already well-established as an antimicrobial agent in healthcare facilities (Rastogi, 2007). However, UVC is harmful to human beings, whereas mild UVA can be used in their presence, hence is more amenable for use in hospitals and in hospitality and catering industries such as food preparation areas to promote continuous disinfection and environmental hygiene. Band-gap irradiation of TiO_2 produces highly reactive oxygen species (ROS), especially superoxide, hydroxyl and perhydroxyl radicals (Hirakawa and Nosaka, 2002; Kikuchi et al., 1997; Ramsden, 2015). There is realization that bacteria may 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

Materials and methods

Chemical reagents

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

Photocatalysis reactor experimental set-up

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

Fabrication of TiO_2 films

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

Characterization of TiO_2 coatings

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

Bacterial strains, media and growth conditions

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

Quantification of hydroxyl radicals and hydrogen peroxide

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

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

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

Photocatalytic activity test

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

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

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

Quantification of total intracellular ROS concentration

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

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

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

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

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

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

Statistical analysis

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

Results

Physical characterization of the coated glass slides

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

Photocatalytic activity of the coatings immersed in solution

TPA was always present in excess (hence zero-order concentration dependence) in comparison with the hydroxyl radicals produced during the photocatalytic process. Hence, the production rate of hydroxyl radicals in solution can be calculated from the gradient of the measured concentration of fluorescent hTPA produced during photocatalysis. Hydroxyl radical production at both LI and HI was found to be linear (Fig. 3a). At LI the average rate of hydroxyl radical production was 0.32 μM h^{-1} (95% 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

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

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

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

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

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

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

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

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

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

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

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

Discussion

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

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

radicals measured here (Liochev and Fridovich, 2010). Bulk •OH radical generation rate was directly related to the level of light irradiance (Fig. 3) and was likely formed by the well-known Haber–Weiss reaction in which H_2O_2 reacts with $•O_2^-$ to give bulk •OH directly in solution (Hirakawa and Nosaka, 2002).

Decoupling the effect of intracellular ROS-induced stress at HI UVA due to TiO_2 photocatalysis compared with that caused by HI UVA alone was not possible (Figs. 4 and 5). Comparison of HI inactivation kinetics for the *ahpC/katA* and *sodA/sodM* mutants suggested that intracellular superoxide $•O_2^-$ formation caused rapid killing of the SOD mutant while the catalase mutant initially showed resistance to HI UVA damage (characteristic shoulder seen on the inactivation curve) but irradiation continuing after about 90 min of initial exposure to HI UVA resulted in cells beginning to rapidly die (the decay rate was faster during this interval in comparison with that of the SOD). The WT strain exposed to HI UVA started showing some viability loss after 4h of exposure (Fig. 4a) increasing to over 2 log reduction after 6 h (Fig. 6a). In the WT strain, intracellular enzymes presumably afford initial protection to UVA-induced ROS damage; however, accumulating levels of ROS have been shown to damage intracellular enzymes making the cells susceptible to oxidative damage if exposure continues (Imlay, 2015).

Bacteria are known to be resistant to short exposures of the near-UV (NUV) component of the solar spectrum (λ = 300–400 nm) at irradiances mimicking natural sunlight (3.5–5 mW/cm^2 corresponding to HI) but begin to die rapidly after 3 to 4 h of exposure (Kramer and Ames, 1987). Exposure to HI UVA may involve photosensitization by endogenous NUV-absorbing chromophores resulting in their excitation followed by reaction with 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

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

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

Conclusions

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

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Conflict of Interest

No conflict of interest is declared.

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