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TiO_2 Photocatalysis Causes DNA Damage via Fenton Reaction-Generated Hydroxyl Radicals during the Recovery Period▽

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Here, we show that resistance of *Escherichia coli* to TiO_2 photocatalysis involves defenses against reactive oxygen species. Results support the idea that TiO_2 photocatalysis generates damage which later becomes deleterious during recovery. We found this to be partly due to DNA attack via hydroxyl radicals generated by the Fenton reaction during recovery.

Studies in the past few years have revealed that classical disinfection by chlorine or ozonation can generate carcinogenic and mutagenic by-products, thereby boosting research into alternative methods, such as photocatalysis (2, 26, 28). This process is based on the ability of a semiconductive catalyst (TiO_2) to kill bacteria upon illumination in aqueous solution (1, 12, 15, 16, 30). However, the basis for the bactericidal effect of photocatalysis is not well established.

Active TiO_2 in anatase crystalline form behaves as a classical semiconductor. The bactericidal effect of photocatalysis with TiO_2 could be due to the presence of reactive oxygen species (ROS), such as superoxide ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO·), generated either by illuminated TiO_2 or by the illumination (mainly UV) of the cells. Most studies have concluded that HO·, directly generated by this process, is the main cause of the bactericidal effect of photocatalysis (5, 19).

To prevent the harmful effects of ROS generated during the normal course of aerobic metabolism, especially that of the extremely reactive HO· able to damage DNA (18), bacteria like *Escherichia coli* are equipped with defenses, including catalases (KatG and KatE) and superoxide dismutases (SodA, SodB, and SodC) (4, 17). These defenses decrease H_2O_2 and $O_2\cdot^-$ steady states and consequently limit the formation of HO·, for which no defense exists (22). Previous reports have shown that HO· is generated via a Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow HO\cdot + HO^- + Fe^{3+}$) and that regulating iron uptake by the transcriptional repressor Fur (3, 7) permits maintenance of low-level HO· production.

Here, we aimed to investigate the resistance of *E. coli* to TiO_2 photocatalysis. Cells were grown in Luria-Bertani broth at 37°C on a rotary shaker (160 rpm) to an absorbance at 600 nm of 0.5. We then washed the cells twice with sodium phosphate (0.05 mol/liter, pH 7, 4°C) and resuspended them in sodium phosphate (0.05 mol/liter, pH 7) solution to a concentration of 2×10^7 CFU/ml. As previously described (14), culture plates were illuminated from 310 nm to 800 nm with a xenon lamp in a Hanau Suntest system (AM1) at 550 W/m^2 light intensity with a filter cutting off wavelengths below 310 nm. Stopped bacterial growth in the exponential phase followed by incubation in phosphate buffer causes starvation and induction of the RpoS regulon, involved in resistance towards many environmental stresses (7, 13). With this in mind, we used a mutant strain of *E. coli* to test whether the induction of the RpoS regulon protects cells against photocatalysis (Degussa P25, 20% rutile 80% anatase crystalline form; Degussa AG, Switzerland). As depicted in Fig. 1, we observed a drastic increase in sensitivity to photocatalysis in the *rpoS*::Tn*10* mutant strain following just 20 min of illumination. This sensitivity was not observed with inactive TiO_2 (Huntsman TR 92, 100% rutile crystalline form; Tioxide Europe Ltd., England), suggesting that light (UV) alone has no effect on bacterium culturability between strains.

The ability of TiO_2 photocatalysis to generate ROS led us to investigate whether RpoS-controlled functions protecting

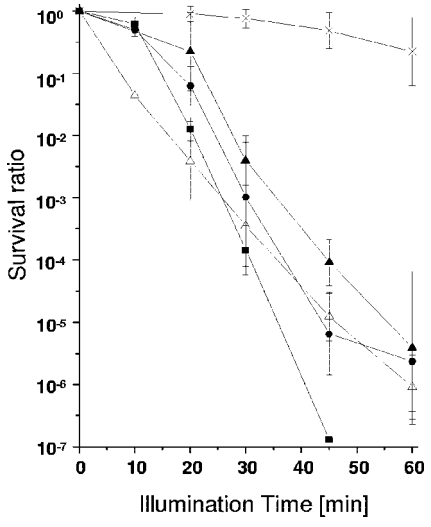

FIG. 1. Survival ratios of wild-type *E. coli* MG1655 (×) and *rpoS*::Tn*10* (■), *dps*::*kan* (●), *katE*::Tn*10* *katG*::Tn*10* (▲), and *katE*::Tn*10* *katG*::Tn*10* *dps*::*kan* (Δ) mutants with 60 min of illumination with active TiO_2 (Degussa P25, 1 g/liter). Data points indicate the mean values and standard deviations of three or more independent experiments.

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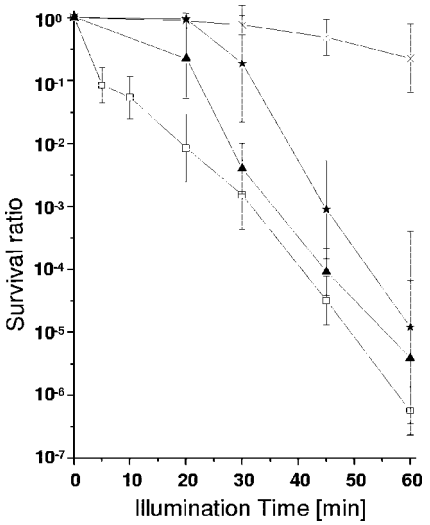

FIG. 2. Sensitivities of wild-type *E. coli* MG1655 (×) and Δ*fur*::*kan* (□), *katE*::Tn*10* *katG*::Tn*10* (▲), and Δ*sodA* *sodB*::MudIIPR3 (★) mutants to TiO_2 photocatalysis (Degussa P25, 1 g/liter). Data points indicate the mean values and standard deviations of three or more independent experiments.

against ROS also participate in the RpoS-dependent protection against TiO_2 photocatalysis. The catalases (HphI and HphII) and Dps protein, all involved in resistance to H_2O_2, are induced during starvation under RpoS control (13). While we observed no differences in terms of sensitivity with inactive TiO_2 (data not shown), *dps*::*kan*, *katE*::Tn*10* *katG*::Tn*10*, and *dps*::*kan* *katE*::Tn*10* *katG*::Tn*10* mutant strains showed high sensitivity to TiO_2 photocatalysis (Fig. 1), indicating the involvement of Dps and catalases in resistance to the toxic effects of TiO_2-mediated production of ROS.

Since RpoS-dependent resistance observed in TiO_2 photocatalysis depends in turn on genes involved in the resistance to H_2O_2, we wished to test the involvement of other defenses against ROS on resistance to TiO_2 photocatalysis. To this end, we tested the sensitivity of the Δ*sodA* *sodB*::MudIIPR3 mutant strain, deficient in cytosolic superoxide dismutases, and also that of the Δ*fur* mutant. As indicated in Fig. 2, we found dramatic sensitivity of both of these mutants to photocatalysis (active TiO_2). Moreover, we detected no difference in culturability after illumination with inactive TiO_2 (data not shown). The concomitant sensitivity of all of these mutants suggests the occurrence of a Fenton reaction, enhanced by iron overload in the presence of active TiO_2. With this in mind, we wanted to test whether DNA damage could be detected after TiO_2 photocatalysis. As depicted in Fig. 3A, we found a sensitivity in the Δ*recA* *srl*::Tn*10* mutant similar to that of the wild type, incapable of SOS induction and homologous recombination, thus rendering it unable to repair DNA strand breaks (20). The *dps*::*kan* Δ*recA* *srl*::Tn*10* mutant, however, showed a high sensitivity to TiO_2 photocatalysis, suggesting a synergistic effect between *dps* and *recA* mutations on DNA, although part of this sensitivity was due to a light-only effect (UV) (Fig. 3B). Finally, we verified that the *srl*::Tn*10* mutation had no effect on Δ*recA* *srl*::Tn*10* or *dps*::*kan* Δ*recA* *srl*::Tn*10* mutant sensitivity (data not shown). Taken together, these results provide evidence in favor of a Fenton reaction occurring, leading to the formation of a hydroxyl radical and consequently DNA damage.

We have shown that resistance of *E. coli* to TiO_2 photocatalysis is mediated largely by genes involved in ROS resistance. Interestingly, either an increase in ROS concentration when plating cells onto a solid growth medium or an exogenous supply of ROS may provoke stress via an imbalance between ROS concentration and the defense mechanisms (6). Since the 1950s, reports have shown that apparently dead cells could be reactivated on addition of ROS scavengers such as catalase or pyruvate to agar plates (8–11, 23–25, 27, 29). As a result of cumulative cellular damage, these so-called "injured cells" are in a transient state, reversible under appropriate conditions to enable resumed growth. We therefore decided to incubate in parallel the illuminated cells with active or inactive TiO_2, with

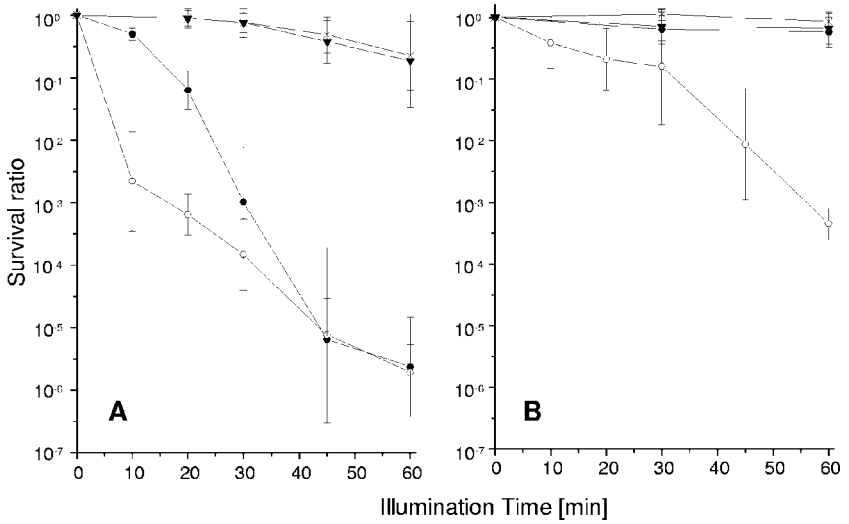

FIG. 3. Sensitivities of wild-type *E. coli* MG1655 (×) and *dps*::*kan* (●), Δ*recA* *srl*::Tn*10* (▼), and *dps*::*kan* Δ*recA* *srl*::Tn*10* (○) mutants to (A) TiO_2 photocatalysis (Degussa P25, 1 g/liter) and (B) light plus inactive TiO_2 (Huntsman, 1 g/liter). Data points indicate the mean values and standard deviations of three or more independent experiments.

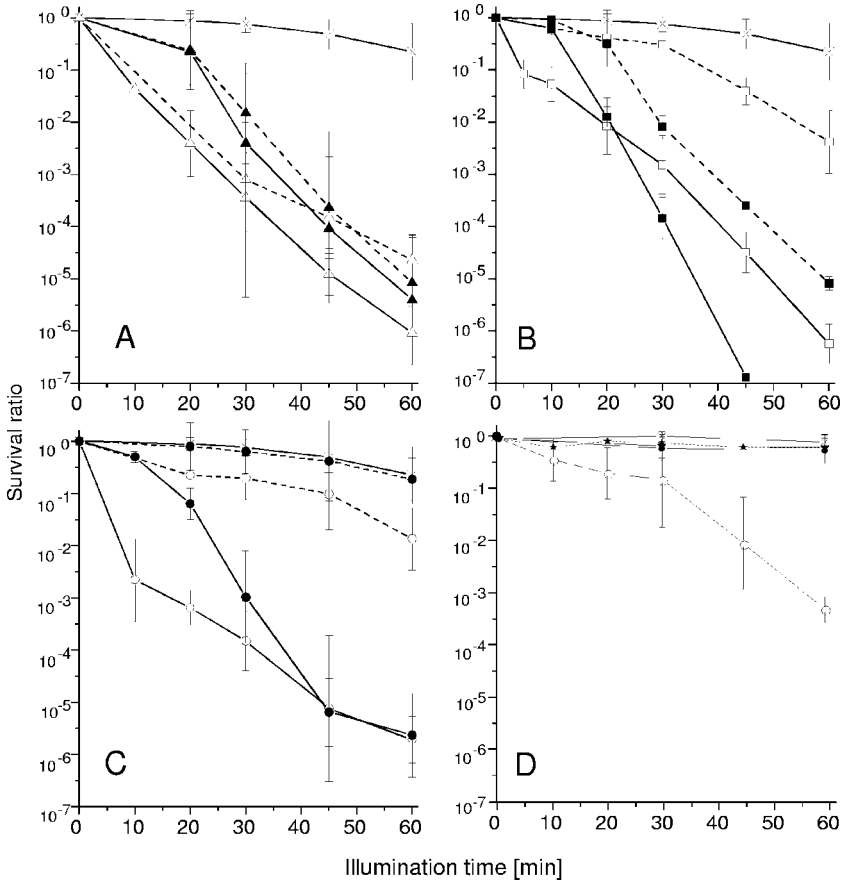

FIG. 4. Sensitivities after incubation either with (dashed lines) or without (plain lines) 20,000 U catalase. Sensitivities are shown for (A) *E. coli* MG1655 (×) and *katE*::Tn*10 katG*::Tn*10* (▲) and *katE*::Tn*10 katG*::Tn*10 dps*::*kan* (Δ) mutants, (B) Δ*fur*::*kan* (□) and *rpoS*::Tn*10* (■) mutants, and (C) *dps*::*kan* (●) and *dps*::*kan* Δ*recA srl*::Tn*10* (○) mutants to TiO_2 photocatalysis (Degussa P25, 1 g/liter) or, (D) with inactive TiO_2 (Huntsman, 1 g/liter), the *dps*::*kan* Δ*recA srl*::Tn*10* mutant (○), the *dps*::*kan* mutant with catalase (●), and the *dps*::*kan* Δ*recA srl*::Tn*10* mutant with catalase (⋆). Data points indicate the mean values and standard deviations of three or more independent experiments.

or without adding 20,000 U of catalase to the petri dish. As depicted in Fig. 4A, we observed no increase in the survival of the wild type or the *katE*::Tn*10 katG*::Tn*10* and *katE*::Tn*10 katG*::Tn*10 dps*::*kan* mutants when plated with catalase. Interestingly, however, the survival rates of the Δ*fur* and *rpoS*::Tn*10* mutants increased more than 1,000-fold when plated with catalase, and these survival rates were identical to that of the wild-type strain during the first 20 or 30 min of illumination (Fig. 4B). Finally, the survival rates of *dps*::*kan* and *dps*::*kan* Δ*recA srl*::Tn*10* mutants increased up to 10,000 times when plated with catalase, and these were the same (or similar) survival rates as that of the wild-type strain (Fig. 4C). Interestingly, adding catalase to the plate restored the light-alone effect on the *dps*::*kan* Δ*recA srl*::Tn*10* mutant (Fig. 4D). These results demonstrate that TiO_2 photocatalysis generates damage that becomes deleterious during recovery from TiO_2 photocatalytic stress, especially for mutants sensitive to ROS species.

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