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The role of reactive oxygen species in UVA-mediated killing of *Escherichia coli*

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Solar Water Disinfection (SODIS) is a simple method used for the microbial decontamination of water using ultraviolet-A radiation. Microbial killing in this method is proposed to occur by the light-dependent increase in reactive oxygen species (ROS) production by intracellular photo-sensitizing enzymes. ROS can irreversibly damage DNA leading to cell death. To test the potential role of ROS, strains with different catalase phenotypes were subjected to SODIS. *Escherichia coli* with wild type and mutated catalase genes were exposed to UV-A radiation for 90 minutes, with vigorous bottle agitation every 15 minutes. Increased killing was observed in *kat* mutants in absence of catalase. It was demonstrated that the absence of the *katE* gene (upregulated in aerobic conditions) causes a large increase in UV-A mediated cell killing. Catalase helps prevent bacterial cell death, likely by destroying the oxygen radicals created by UV-A exposure. Adding exogenous catalase to the environment generally caused a dosage-dependent increase in survival rates. However, these results were not reproducible and variation was observed. Varying oxygen concentration did not appear to effect survival rates uniformly; though this might have arisen because the bottles were not agitated to distribute oxygen and bacteria in this part of the experiment.

Pathogenic microbes such as enteropathogenic *Escherichia coli* are a common source of morbidity and mortality in developing countries, as over 4 billion diarrhea cases occur annually, with 6000 children dying per day due to diarrhea-induced dehydration (http://www.sodis.ch/). Simple, cost-effective methods to reduce the pathogenic microbe contamination of water sources are currently being researched. Solar Water Disinfection (SODIS) is an effective process that relies on exposure of the contaminated water in glass or PET (polyethylene tetraphtalate) bottles to sunlight, causing photo-inactivation and killing of microbes (http://www.sodis.ch/Text2002/The Method.htm).

In previous studies (8) aimed at improving the SODIS system it was observed that the killing effect of UV-A radiation on *E. coli* cell suspensions could be increased by vigorously shaking the samples every 15 minutes. This effect was attributed to an increase in the amount of dissolved oxygen in the suspension causing an increase in the light dependent production of reactive oxygen species (ROS) by intracellular photo-sensitizers. UV-A radiation is outside the absorbed wavelength of DNA, which is the main mechanism for cell death cause by UV-B and UV-C radiation (7). ROS are extremely germicidal and provide an indirect mechanism for UV-A mediated killing of bacteria by eliciting DNA damage within the cell (3).

It has been found that catalase, superoxide dismutase and peroxidase enzymes decrease the concentration of steady-state levels of ROS (1). Catalase is found in wild type *E. coli* and other aerobic living organisms. It protects the cell from oxidative damage (6) by catalyzing the conversion of hydrogen peroxide, H_2O_2, to water (H_2O) and oxygen (O_2). Hydrogen peroxide is produced from the addition of two protons (H^+) to the superoxide radical ($O_2^{\bullet-}$) produced by transfers of one electron to oxygen (6). The production of hydrogen peroxide can be catalyzed by superoxide dismutase or occur spontaneously, and along with the superoxide radical can cause lethal damage to DNA (6).

Strains with *katG* and *katE* mutations, the two genes encoding for catalase in *E. coli*, have an increased sensitivity to oxygen and hydrogen peroxide. In the following experiments, the potential role of ROS in SODIS was tested by comparing the killing of four different *E. coli* strains (a *katG* mutant; its isogenic partner; a *katG* and *katE* mutant and B23, a commonly used laboratory strain). In addition, decreasing killing rates in all strains by the addition of exogenous catalase enzyme into the media was attempted. Furthermore, varying the oxygen concentration provided additional insight into the role of ROS. If the oxygen concentration is increased, it was expected that more oxygen will be available for cellular conversion into ROS (8). Thus, we expected that if ROS were involved in killing, an increase in oxygen would result in increased bacterial killing. The catalase and oxygen experiments together determined if the production of reactive oxygen species (ROS) is in fact the mechanism by which bacteria killing occurs when exposed to UV-A light.

MATERIALS AND METHODS

Bacterial culture: Four strains of *E. coli* were obtained: B23 as wild type; UM197 with a *katG* mutation; its isogenic partner Chi760; and UM2, which has mutations in *katE and katG*. Catalase tests were performed to verify the above phenotypes. The four strains (see Table 1) were plated on Luria-Bertania (LB) media with 1.5% agar (5 g yeast extract, 10 g tryptone, 5 g NaCl, 2 g glucose, 15 g agar in one litre of distilled water) and incubated overnight at 37°C. Colonies from these plates were used as inoculum for overnight cultures.

TABLE 1. The four strains of *E. coli* used in the experiments with corresponding genotypes and sources.

Strain	Genotype	Source
B23	None	University of British Columbia Department of Microbiology & Immunology laboratory stock
Chi760	*araC14, leuB6*(Am), *secA206*(aziR), *fhuA23, lacY1, proC83, tsx-67, purE42, gbnV44*(AS), *galK2*(Oc), *LAM-, trpE38, xthA15, his-208, rfbC1, mgl-51, argG77, rpsL109*(strR), *glpR201, xylA5, mtl-1, ilvA681, thi-1, metA160*	*E. coli* Genetic Resource Center
UM197	*araC14, leuB6*(Am), *secA206*(aziR), *fhuA23, lacY1, proC83, tsx-67, purE42, gbnV44*(AS), *galK2*(Oc), *LAM-, trpE38, xthA15, his-208, rfbC1, mgl-51, argG77, rpsL109*(strR), *glpR201, xylA5, mtl-1, ilvA681, thi-1, metA160, katG17::Tn10*	*E. coli* Genetic Resource Center
UM2	*araC14, leuB6*(Am), *secA206*(aziR), *fhuA23, lacY1, proC83, tsx-67, purE42, gbnV44*(AS), *galK2*(Oc), *LAM-, trpE38, xthA15, his-208, rfbC1, mgl-51, argG77, RPSI109*(strR), *glpR201, xylA5, mtl-1, ilvA681, thi-1, metA160, katE2, katG15*	*E. coli* Genetic Resource Center

Preparation of Bottles: Overnight cultures were prepared by inoculating 10 ml of LB and incubating at 37°C overnight. This was done to ensure that cultures would be in stationary phase. The next day, 1 ml of each culture was centrifuged at 7500 g for 10 minutes and the pellet was resuspended in 1 ml of sterile distilled water. This step was repeated and the 1 ml suspension was diluted 1/20 in sterile distilled water.

Five hundred milliliter PET plastic bottles of water from Superstore© were emptied and labels were removed; then 242.5 ml of sterile distilled water was put into the bottles and 7.5 ml of the bacterial suspension was added. The bottles were shaken to distribute the bacteria. All starting cultures throughout the experiment were within the 10^5 range.

Effect of UV-A Irradiation on E. coli Survival: One bottle for each strain was prepared as above and each bottle was laid on its side under the 20 W Sylvania Blacklight-blue emitting UV-A light at a peak wavelength of 366 mm and an intensity of 213 w/m^2 as measured by a UV-A sensor (Vernier, order code UVA-BTA) at the height of the top of the PET bottles. Before exposure, samples were taken from each bottle and plated at final dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} on LB agar. The bottles were exposed to the UV-A light for 90 minutes while being shaken vigorously at 15-minute intervals. After 90 minutes, samples were taken and plated on LB agar to the final plated dilutions as above. All plates were incubated overnight at 37°C.

Effect of Complementation with Exogenous Catalase: One mg of catalase from bovine liver (Sigma-Aldrich, catalogue number C1345-1G) was suspended in 1 ml of sterile distilled water to make a solution of 2950 units/ml. Three bottles for each strain were prepared as above. For each strain, one bottle received 10 units of catalase (3.39 µl), one bottle received 100 units (33.9 µl) and as a control, one bottle did not receive catalase. Six bottles at a time were placed under the UV-A light at an average intensity of 260 w/m^2 for 90 minutes. The bottles were shaken vigorously every 15 minutes and samples were taken at the 0 minute and 90 minute time points. For the 0-time point, final plated dilutions were 10^{-3} and 10^{-4} for all strains. For the 90-minute time point, final plated dilutions were 10^{-2} and 10^{-3} for all strains while the UM2 strain was additionally plated at 10^{-1} dilution. All plates were spread in duplicates and incubated at 37°C overnight.

Effect of Oxygen Concentration of the Water: Three bottles for each strain were prepared as above. For each strain, one bottle was bubbled with an anaerobic gas mixture of CO_2 and N_2 (Praxair) for one hour at a flow rate of approximately 5 bubbles per second. The other two bottles per strain were bubbled with compressed air (Praxair) for 10 minutes, one at a low flow rate of 29 ml/min and the other at a high flow rate of 175 ml/min. The airflow was measured using a gas flow regulator (Cole-Parmer Instrument Company). Six bottles at a time were placed under the UV-A light at an average intensity of 760 w/m^2 for 90 minutes with no shaking. At time point 0, samples were taken from each bottle and plated in duplicate at final dilutions of 10^{-3} and 10^{-4}. After 90 minutes of treatment, samples were taken from each bottle and spread on LB agar plates in duplicate at final plated dilutions of 10^{-2}, 10^{-3} and 10^{-4}. All plates were incubated overnight at 37°C.

RESULTS

The first part of the experiment was carried out to determine differential killing between strains by exposure to UV-A light.

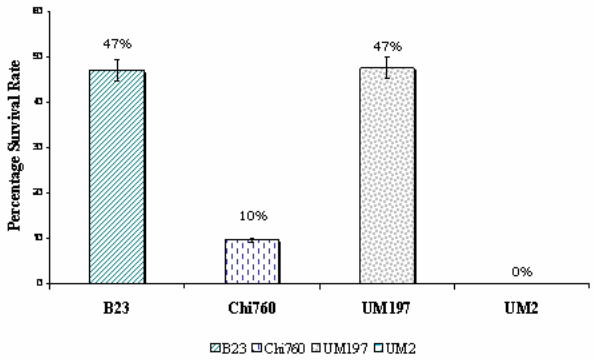

FIG. 1. Observed survival rates for the four *E. coli* strains after 1.5-hour exposure to UV-A light.

The B23 strain with the wild type catalase phenotype showed better survival than the Chi760 strain with the wild type catalase genes and the UM2 strain (FIG 1). The UM2 strain with the double mutation showed zero percent survival. Unexpectedly, the UM197 strain with a mutation in KatG showed the same survival as the wild type B23 strain and had a survival rate 5 times higher than its isogenic wild type strain, Chi760.

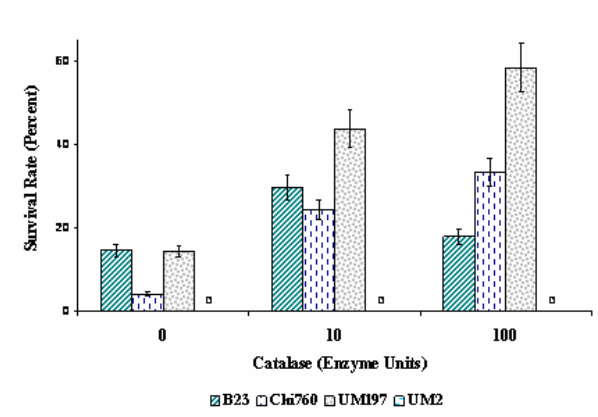

FIG. 2. Effects of exogenous supplied catalase on the observed survival rate for the four *E. coli* strains after 90 minutes of exposure to UV-A light

The results of the addition of exogenous catalase (FIG. 2) showed a general trend of increased survival rate with the addition of exogenous catalase. With the exception of the B23 strain supplemented with 100 units of catalase and all treatments of the UM2 strain, the survival rate increased with the amount of catalase that was added.

In accordance with the previous experiment, the UM2 strain was the most susceptible to UV-A mediated killing. Unlike the other strains, catalase addition was unable to increase the number of surviving cells in this strain. The UM197 strain was the most receptive to the treatments resulting in an increase of the survival rate 1.5 times greater than that of its isogenic partner (Chi760) for both 10 and 100 unit catalase additions.

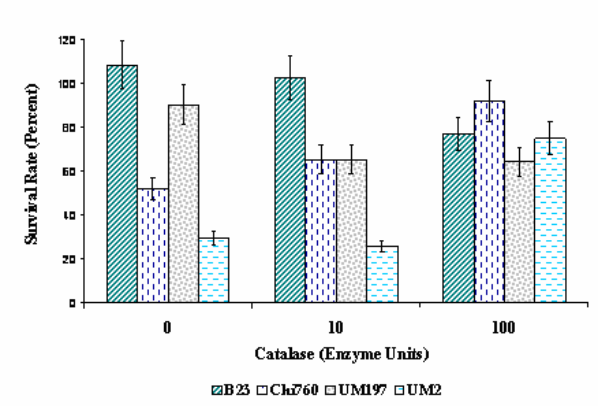

FIG. 3. Effects of exogenous supplied catalase on the observed survival rate for the four *E. coli* strains after 90 minutes of exposure to UV-A

To confirm the results of the initial catalase test, the experiment was repeated (FIG. 3). This test showed very limited killing and limited effects of exogenous catalase. However, the general trend seen before, where the UM2 strain experienced lower survival rates was still evident. In contrast, in the second catalase test it was observed that addition of 100 units of catalase increased the amount of survival of the UM2 strain, while 10 units of catalase was unable to complement the lack of catalase found in the UM2 strain.

The Chi760 strain showed an increase in survival with the addition of catalase. However, this trend was not shared with the B23 and UM197 strains. The UM197 showed an opposite response to catalase in comparison to the first test. In this test, addition of catalase decreased the amount of survival.

Very limited killing was observed in the test where altering the air concentration in the plastic water bottles was altered (FIG. 4). The overall result was an increased survival rate for all of the strains tested when compared to previous experiments. This increased survival was seen regardless of the air concentration present in the water bottle. Increasing the air concentration did not noticeably change the survival rate amongst the four strains. However, as in previous tests, the UM2 strain was among the lowest in survival rate at all air concentrations.

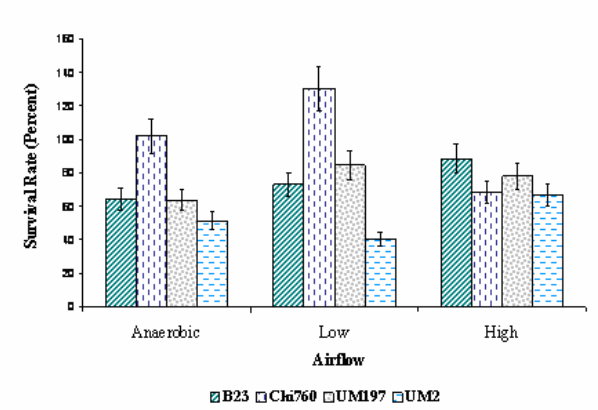

FIG. 4. Effects of oxygen levels on the observed survival rate for the four *E. coli* strains after 90 minutes of exposure to UV-A light

DISCUSSION

E. coli strain UM2 (*katG* mutant and *katE* mutant) was the most susceptible to UV-A mediated killing. This result supports our hypothesis that reactive oxygen species play a major role in cell death caused by UV-A radiation since the catalase genes confer protection against oxidative damage to the cell (5, 6). Because of this effect, we also expected that the UM197 strain (*katG* mutant) would have been more susceptible to UV-A radiation than its isogenic partner, Chi760. However, the UM197 strain was observed to have a survival rate similar to that of the B23 strain. This result can potentially be explained by examining differences in the expression of both catalase genes. In *E. coli*, *katG* is induced by the presence of hydrogen peroxidase and is only produced when the cells are growing anaerobically (8). The *katE* expression on the other hand, is induced when cells growing aerobically enter stationary phase (8). Because all cultures were grown in the presence of oxygen, the *katG* mutant (the UM197 strain) would still be expressing *katE* and therefore, we should not expect to see increased death in that culture as compared to the B23 and Chi760 strains.

Interestingly, the UM197 strain had the second lowest killing efficiency out of all four strains tested. A potential reason for observing this increased killing in the Chi760 strain compared to the UM197 strain may be the larger initial concentration of the UM197 strain. In the starting culture, the UM197 strain had 1.5 times the initial concentration of the Chi760 strain. When investigating the effect of ROS in electrochemical inactivation of *E. coli*, Jeong *et al.* determined that an increase in the initial concentration of bacteria resulted in decreased inactivation of microorganisms (2). This was explained by noting that *E. coli* has the ability to consume the reactive oxygen species itself. Therefore, as the starting population of *E. coli* increases, a reduction in the amount of ROS may occur, resulting in decreased killing. In that case, there would be a lower percentage of ROS for the UM197 population, and hence increased survival rate would be observed in this particular strain.

In addition, the difference may partially be explained by variation in the light intensity across the black light. If each bottle was not exposed to the same intensity, this would effect the amount of ROS produced, and thereby the amount of killing. When the catalase test was performed, it was observed that while both the Chi760 and UM197 strains were positive for catalase, the UM197 strain had a stronger response. This was not quantifiable as it may be due to different amounts of bacteria tested. However, if the UM197 strain produced more catalase than its isogenic partner, this may also have explained the increased survival seen in this strain.

Exogenous photosensitizers may be important in the generation of ROS in natural water (4). Sterile distilled water was used to wash all cells in order to remove such compounds. Also, sterile distilled water was the bacterial environment in which all radiation experiments were conducted. This leaves intracellular photosensitizers as the main generators of ROS in our experiments. Because the enzymes producing the ROS are intracellular, we believe that the majority of ROS are causing intracellular damage (e.g. DNA damage) resulting in subsequent cell death (7).

Previous experiments have shown that the addition of catalase to solid growth media significantly increased colony counts of *E.coli* exposed to UVA radiation (4). These cells were previously treated to remove any traces of exogenous photosensitizers. We expected to see a similar effect when catalase was added prior to exposure to UVA.

E. coli strain UM2, which lacks both catalase genes was the most susceptible to UV-A mediated killing. This result supports our hypothesis that reactive oxygen species play a major role in cell death caused by this type of radiation since the catalase genes confer protection against oxidative damage to the cell. Further support lies with the trend of increasing survival rates for all strains with the addition of catalase. Also, survival rates for strains B23, Chi760 and UM197 increased in a dose-dependent manner, such that addition of 100 units of catalase prior to UV-A exposure resulted in greater survival than 10 units.

As discussed above, it was expected that the UM197 strain, the *katG* knockout, would have been more susceptible to UV-A radiation than its isogenic partner, Chi760. However, this was not the case, possibly due to differences in the expression of both catalase genes in *E. coli*. We were unable to replicate the catalase test results, as there seemed to be definite variation in the results, indicating that there are other

factors involved that differed between the two tests. The major trends, however, conform to those already described.

By increasing the air concentration in the PET bottles, we expected to see an increase in bacterial killing, as it has been previously demonstrated that aerobic conditions in SODIS causes greater bacterial inactivation (8). A potential explanation for the overall increased survival rate of these *E.coli* strains compared to the catalase experiments is that the bottles were not shaken at intervals throughout the duration of UV-A exposure. It has been previously demonstrated that shaking the PET bottles causes a 7% increase in bacterial killing (9). This observation was explained by considering the effect of greater incorporation of oxygen into the water. This causes an increased chance of dissolved oxygen reaching the bacterial cells suspended within the water and, therefore, increased intracellular reactive oxygen species (ROS) formation. Without shaking, oxygen may have only reached the bacteria on the top layer of the water that was exposed to the highest intensity of UV-A light, decreasing the overall killing rates drastically. Even if there was an increase in oxygen within the PET bottle, the non-uniform UV-A light exposure due to not shaking meant the bacterial cells throughout the water were not exposed to the same intensity and had unequal access to dissolved oxygen, with the microorganisms at the surface receiving the greatest amount of UV-A radiation and being exposed to the most oxygen. The PET bottles that were exposed to N_2:CO_2 airflow likely still had dissolved oxygen present in the water, explaining why this treatment did not result in an increased survival rate. This may explain the discrepancies in survival rate between the varying air concentrations.

Although SODIS and photo-inactivation of bacteria are not new ideas, the exact mechanism has never been firmly established. Determination of the effect that ROS have on bacterial killing when exposed to sunlight and UV radiation will provide information that can be used to enhance the SODIS protocol. This would result in better, more efficient and cost-effective ways to provide safe drinking water for individuals in under-developed regions of the world. Our results demonstrated that while there are variations, presence of reactive oxygen species seems to play an important role in UV-A mediated killing of microorganisms.

FUTURE EXPERIMENTS

Potential future experiments would involve further study of *E.coli* UM2, as it appeared to have a drastic reduction in survival rate compared to other strains tested. To confirm that the *katG* and *katE* genes are indeed responsible for this observed phenomenon, a plasmid construct containing these genes could be made and transformed into *E. coli* UM2. Restoration of survival rates, compared to the Chi760 strain, would provide more information on the molecular basis for UV-A killing. Furthermore, repeating the experiment using varying oxygen concentrations would be beneficial. Measuring the oxygen concentration and shaking the PET bottles at 15-minute intervals in order to increase the bacterial exposure to the dissolved oxygen would provide a better indicator of the effect of dissolved oxygen in UV-A mediated killing. In addition, further investigation into the effect of initial bacterial concentrations on the amount of killing would provide valuable information. By varying the initial population and measuring ROS concentrations, the hypothesis that increases in bacterial populations reduce the ROS concentration, thereby reducing the amount of killing, may be investigated.

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