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Effect of Ultraviolet-A Radiation on Water Disinfection and the Activity of Catalase and Superoxide Dismutase in *Escherichia coli* B23

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The lack of safe potable water in many developing countries puts their citizens at high risk of contracting water-borne diseases. Solar water disinfection (SODIS) offers a simple, inexpensive method of sanitizing water by utilizing the ultraviolet-A radiation (UV-A) from sunlight to generate highly reactive, bacteriocidal forms of oxygen known as reactive oxygen species (ROS). However, previous studies have shown that some *Escherichia coli* can survive extended UV-A exposure. It is known that *E. coli* possess the enzymes catalase and superoxide dismutase (SOD), which convert specific ROS into less lethal forms for the bacteria. As such, we hypothesized that some *E. coli* cells can survive prolonged UV-A exposure by up-regulating catalase and/or SOD activity. To test this hypothesis, *E. coli*-inoculated water samples were exposed to UV-A for 1.5 hours. The catalase activity in the exposed cells was assessed by a potassium dichromate colorimetric assay performed on their lysates, while SOD activity was assessed by a nitro blue tetrazolium colorimetric assay. The assays for catalase, SOD and protein yields between similarly-treated samples showed high variability. A two-tailed t-test at 95% confidence revealed no significant difference in catalase activity between control and treatment groups; however, the low number of replicates limits the significance of this finding. Accumulated oxidative stress may explain the lack of up-regulation in treated cells, as excessive accumulated oxidative stress can lead to the repression/inactivation of regulons responsible for catalase expression. The activity of SOD was also assayed, but due to the lack of an optimized protocol and a suitable positive control, the results were inconclusive.

The World Health Organization estimates that 17% of the global population lack access to safely consumable water sources with at least one third of the population in developing countries having no access to safe drinking water. Data shows that about 1.8 million people worldwide die every year from diarrheal diseases. Improvements in safe drinking water can lead to reduction of diarrhea episodes by 35-39% (http://www.who.int/water_sanitation_health/publications/facts2004/en/index.html)

Solar water disinfection (SODIS), also known as solar water pasteurization, is a simple, inexpensive method of purifying water using only sunlight. Ultraviolet-A (UV-A) from sunlight (320-400nm) reacts with dissolved oxygen in water, producing highly reactive forms of oxygen known as reactive oxygen species (ROS), which can damage and kill microorganisms. UV-A is also known to inhibit bacterial growth (6). The solar heat, associated with SODIS usually significantly accelerates the disinfection process (up to three times faster) (12).

Although the solar water disinfection process reduces the number of pathogens in drinking water, some bacteria can nonetheless survive the prolonged exposure to UV-A (12, 19). It has been shown that prior exposure to certain ROS could encourage the development of pathogenic strains resistant to UV-A killing (6).

ROS production causes oxidative stress in microorganisms. *Escherichia coli* has independent multigene responses to two kinds of oxidative stress (3): hydrogen peroxide (H_2O_2) triggers the oxyR regulon, and excess O_2^- or nitric oxide (NO') radicals trigger the soxRS regulon, which coordinates the transcriptional induction of at least 12 promoters (4).

Catalase is a microbial enzyme that functions in the decomposition of H_2O_2 to water and oxygen. During the process of solar water disinfection, hydrogen peroxide is among the several ROS produced by UV-A-water interaction (10).

In *E. coli*, the detoxification of hydrogen peroxide is achieved by two types of catalase: hydroperoxidase I and II (HPI and HPII). HPI is encoded by the *katG* gene, and is induced by

hydrogen peroxide in an OxyR-dependent manner (13). OxyR functions as a peroxide-sensing positive regulator. HPII is encoded by the *katE* gene, whose expression is regulated by the sigma factor RpoS (7). RpoS is described as a general stress response regulator that controls expression of genes that increase resistance to various stresses, such as high concentrations of hydrogen peroxide.

In addition to catalase, superoxide dismutase (SOD) is another enzyme that is known to help cells defend against ROS (1). SOD can convert free radical O_2^- ions into H_2O_2 and O_2, followed by the breakdown of H_2O_2 by catalase, and minimizing the damaging effects by ROS.

We hypothesize that microorganisms survive in UV-A treated water by upregulating catalase and SOD genes. The catalase and SOD activity were measured for an *Escherichia coli* B23 sample that successfully survived UV-A irradiation. Colorimetric assays for measuring catalase and SOD activity were tested, optimized and applied. The effect of UV-A exposure on catalase and SOD regulation was assessed by comparing the levels of SOD and catalase between UV-A treated and UV-A untreated organisms.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* B23 obtained from the MICB 421 culture collection in the Department of Microbiology and Immunology at the University of British Columbia was used for all tests and was cultured in Luria Bertani Broth (for 1L: Tryptone 10 g; Yeast Extract 5 g; NaCl 10 g; adjusted to pH 7.0 with NaOH). LB agar contained 7.5 g agar in 0.5 L. E. coli were grown on an LB agar streak plate overnight and a single colony was used to inoculate either 100 mL or 200 mL of LB medium (100 mL in the first trial , 200 mL in subsequent trials) for an overnight culture grown at 37 ˚C with shaking.

Preparation of cells for UV-A exposure. Polyethylene terephthalate (PET) bottles (Dasani) were rinsed successively in 70% ethanol and in sterilized dH_2O. Overnight cultures were centrifuged in a Beckman J2-21 high-speed centrifuge at 7,500 x g for 10 minutes at room temperature. Half the supernatant was removed and replaced with sterilized dH_2O and the cells resuspended. Cells were then centrifuged at 13,500 x g for 5 minutes at room temperature and all supernatant discarded. The pellet was resuspended in 4 mL sterile dH_2O and separated equally into four sterile PET bottles filled with 200 mL sterile dH_2O. Two bottles were exposed to a 20W Sylvania Black light (UV-A light source) placed 6 cm from the top surface of the bottles for 1.5 hours at an intensity of 807.7 W/m^2. The two control bottles were placed into the dark for the same time period.

Protein isolation. All bottles were centrifuged in a Beckman J2-21 high-speed centrifuge at 7,500 xg for 10 minutes and pellets were washed twice with PBS (for 1L: 0.2 g KCl, 8 g NaCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4, and pH adjusted to 7.4 with NaOH). Pellets were resuspended each in 1 mL of 50 mM phosphate buffer, pH 7. Phosphate buffer contained 1.25 g NaH_2PO_4 and 2.05 g Na_2HPO_4 in 500 mL. Approximately 100 µL of glass beads were added to each tube and cells were disrupted by FastPrep at level 6 for three 30 s periods with 1 min of chilling on ice between each period. Cell debris was removed by centrifugation at 10,000 ×g for 20 min at room temperature. Supernatant was filtered through particle filters with 0.2 µm pores using syringes. All lysates were kept on ice thereafter. Protein concentrations were determined by Bradford protein assay as described (9).

Superoxide dismutase assay. The superoxide dismutase assay was performed as described (2). At a final volume of 3 mL the following was mixed in each test tube: 50 mM phosphate buffer, pH 7.8, 13 mM methionine, 75 µM nitrobluetetrazolium (NBT), 2 µM riboflavin, 0.1 mM EDTA and 100 µl of protein extract. Tubes were placed 30 cm below a fluorescent lamp at 15W for 10 minutes to initiate reaction and were placed in the dark to end the enzyme reaction. Absorbance was read at 560 nm in a Spectronic 20 spectrophotometer.

Catalase assay. Small beakers containing the reaction mixture were prepared which contained: 2 mL of 0.2 M H_2O_2, 2.5 mL of 0.01 M phosphate buffer, pH 7.8, and 0.5 mL of enzyme solution. The mixture was gently swirled to mix and successive 1 mL of reaction was removed to a test tube containing 2 mL of the dichromate/acetic acid solution, at one minute intervals to stop the reaction. The dichromate/acetic acid was prepared as a 50 mL aqueous solution of 5% potassium dichromate in distilled water with 150 mL of glacial acetic acid. The blue precipitate formed was decomposed to a green solution by heating the tubes for 10 min in a boiling water bath. Samples were measured at 570 nm in a Spectronic 20 spectrophotometer. A standard curve containing 0 to 160 µmol of H_2O_2 was prepared to determine the amount of H_2O_2 present in each tested sample.

RESULTS

The catalase activity present in the cell lysates can be viewed below in Figure 1.

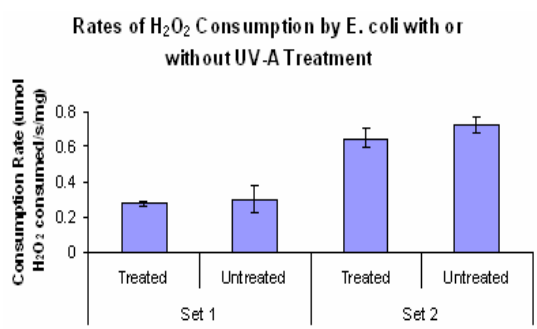

FIG. 1. Comparison of the rate of H_2O_2 consumption between UV-A treated and UV-A untreated enzyme samples extracted and measured in two trials. N=2 for each trial. Error bars represent standard deviation.

Set 2 had a significantly higher overall consumption rate than Set 1, and thus data could only be compared within each set. Initial data suggested that untreated cell lysates have a higher H_2O_2 consumption rate than treated lysates. Hence a t-test was performed to determine if the difference between the treatments are significant. The value of t_{act} for a two-tailed test at 95% confidence for 1 degree of freedom is 12. The calculated t_{exp}'s for Sets 1 and 2 (0.7 and 1.5) are

much lower than the t_{act}=12. These results suggest that there is no significant difference between the catalase activity per mg of cell extract between the treated and untreated samples.

Due to the lack of adequate number of replicates for the catalase assay, it is not possible to detect a significant difference of catalase activity between the untreated and treated samples.

Insignificant difference in superoxide dismutase activity between UV-irradiated and non-irradiated *E. coli* B23. Due to insufficient samples from Day 1, the SOD assay was only performed on samples from Days 2 and 3. The late arrival of the required nitroblue tetrazolium reagent and the absence of a positive control (in the form of industrially purified SOD) meant that the assay remains to be fully optimized. Nonetheless, while the results need to be validated by a positive control, some observations should be noted.

Table 1 lists the absorbance readings obtained from the assays; the data is again grouped by days and compared within each group.

Table 1. Comparison of SOD assay readings for enzyme extracts from UV-A treated and UV-A untreated *E.coli* B23

UV-A Treated and Date		Average Concentration of Chromagen (A_{560})
Set I	Untreated	0.60 ± 0.01
	Treated	0.59 ± 0.05
	UV-treated blank	0.73
	Untreated blank	0.00
Set II	Untreated	0.13 ± 0.03
	Treated	0.08 ± 0.02
	UV-treated blank	0.74
	Untreated blank	0.00

The SOD assay performed in this experiment is based on the principle that SOD from the enzyme extract will compete with nitro blue tetrazolium (NBT) to oxidize the superoxide molecules that are photochemically generated by riboflavin. NBT, upon oxidizing superoxide, becomes a blue formazan that absorbs at 560nm. Thus, given that the same quantity of superoxide molecules were generated in each assayed sample, a lower A_{560} reading would represent decreased NBT oxidation, which can be translated here as higher SOD activity in the assayed sample. The enzyme extracts were replaced with phosphate buffer in the blank samples, which served as our controls.

The different sets of data showed different results. While the results from Set 2 showed that UV-A treated samples has a higher SOD activity than untreated samples, results from Set 1 suggested that there is little to no difference (Figure 1). However, for both sets, the differences between the untreated and treated samples were deemed statistically insignificant (based on two-tailed t-tests with 95% confidence).

The SOD results cannot be fully validated due to the lack of a positive control in the form of purified SOD. However, the controls used in this experiment supports that SOD could affect the assay readings. The UV-treated controls was expected to show a high A_{560} reading (i.e. high NBT oxidation) due to lack of competition from SOD, while the untreated control with low level of superoxide molecules was expected to have a lower A_{560} reading. The readings of the controls matched these expectations (Table 1), suggesting that SOD does exert an effect on the readings.

DISCUSSION

The results suggest that UV-A does not upregulate catalase. Ideally, the trials were intended to be combined into a single statistical analysis; however, unforeseen differences in the samples depending on the day of analysis made it impossible. The variation in the assays is perplexing as all assays were performed the same, with the only change being in a fresh reagent made each day. New standard curves were made for each new reagent to prevent this from affecting the assays. More likely, the differences observed stemmed from the preparation of the cultures for exposure and preparation of cell lysates.

All organisms contain proteases and the lysis of cells releases them into the environment along with the desired proteins. It has been known that high levels of ROS, especially oxygen radicals and hydrogen peroxide, induce cellular apoptosis (5). During apoptosis, cells undergo an organized degradation of cellular products by activated proteolytic caspases. Furthermore, it is common for cells to subsequently break apart into several vesicles called “apoptotic bodies”. If the bacterial cells were to break apart in a similar response, it is possible that some of the catalase and SOD were not recovered after the purification process. This could very well be the cause of the variation in protein recovery in our experiment (data not shown).

In this study, high levels of ROS were introduced to the cells due to the UV-A radiation. It is possible that this scenario stimulated the cells to break apart. In turn, proteolytic enzymes would be released, leading to degradation of cellular products. Proteases can be inactivated by adding chelators, DIPF or PMSF to inhibit metalloproteases and serine dependant proteases. These agents would also inhibit enzymes; alternatively, the method of chilling the samples was chosen to limit proteolysis. It is important to note that inactivation by proteases would be consistent with our findings of a low rate of catalase and SOD activity in the treated samples. This activity could also explain the increase in activity in catalase and SOD in the second set of results (FIG. 1, Table 1), as it is possible that the procedure was performed faster each day, resulting in less time spent by the lysates at room temperature.

H_2O_2 is known to oxidize sulfhydryl and iron-sulfur moieties (11) and could be inactivating the very enzyme that is trying to decrease the levels of H_2O_2. It seems counterintuitive that an enzyme could be susceptible to the molecular species it is meant to destroy, but the levels produced by the UV-A could be higher than the levels that bacteria would usually encounter in the environment. However, since there is variation in the UV-A untreated samples and all treated samples were exposed to the same amount or radiation, the inactivation of catalase by UV-A is unlikely to be causing the variation seen between days.

In recent studies, time course studies were performed on stress regulons, OxyR and SoxRS respectively (8). Results displayed an induction maximum of these regulons after a defined period of applied oxidative stress. The maximum induction was followed by repression after a threshold of oxidative stress was obtained. Furthermore, the study revealed different response patterns and expression rates of the genes regulated by the OxyR and SoxRS regulons, respectively (8).

Our results show a low rate of production of catalase and SOD in the treated samples. This can be explained if a threshold of oxidative stress was obtained due to UV-A radiation. This threshold might have led to the repression/inactivation of regulons responsible for catalase and SOD expression, respectively.

The high variability in catalase activity was likely due to preparation of cultures for exposure and preparation of cell lysates and not reagent differences. Inactivation by released proteases during treatment likely caused the variation between days, as the procedure was performed faster each day and the lysates spent less time at room temperature and less inactivation occurred each day. It appears that there was a higher activity of catalase and SOD in the untreated samples, opposite of our hypothesis; however, a two-tailed t-test revealed no significant difference in the catalase activity per mg of cell extract between the treated and untreated samples. This analysis is made with low confidence due to the low number of replicates. The low activity in treated samples might have been due to a threshold of oxidative stress being obtained due to UV-A radiation.

Activity of SOD was also assayed, but as the assay was not optimized and positive controls were absent, the results cannot be fully validated and are thus inconclusive. As such, the data obtained is not fit for further interpretation and analysis. Though the negative controls suggest that the SOD assay readings do reflect the presence of SOD, a positive control will be needed to completely verify these results.

FUTURE EXPERIMENTS

Further optimization of methods are needed to determine the significant differences in catalase and SOD protein activity in UVA-treated and untreated samples. More replicates for the assays could be done to significantly detect differences between the samples if they exist.

As mentioned before, the SOD assay needs validation by a positive control. A standard curve using commercially purified SOD may be needed to ascertain that the assay is indeed quantitative.

Comparison of two different catalase assays using the same samples could determine whether the variation seen between days was due to sample differences or assay differences. Alternatively, catalase could be detected by running an SDS-PAGE gel with a catalase standard to observe the amount of catalase protein if a band was visible.

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