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katE Complementation Fails to Protect Against UV-A-Mediated Killing in Catalase-Deficient *Escherichia coli*

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Solar water disinfection (SODIS) is an inexpensive method whereby bottled water is exposed to sunlight for five hours to inactivate and destroy pathogens, thus rendering it safe for drinking. This is especially practical in developing countries where one third of the population has no access to safe drinking water. The mechanism of SODIS has been proposed to involve two synergetic factors, ultraviolet-A radiation which generates reactive oxygen species (ROS), and increases in water temperature. Previously, the proposed UV-A mechanism was studied by comparing survival rates of *Escherichia coli* strains with mutations of catalase and peroxidase genes. The Δ*katE* Δ*katG* double mutant strain suffered a greater loss of viable cells upon exposure to UV-A radiation than a Δ*katG* mutant, suggesting the importance of the *katE* gene. In this study, we examined whether a *katE*-complemented mutant still lacking *katG* would have a better survival rate compared to the double mutant. Bottles of water containing various *E. coli* mutants were subjected to 90 minutes of UV-A treatment, with vigorous shaking at 15 minute intervals. Survival rates were determined by bacterial enumeration using pour plate technique. Surprisingly, our *katE*-complemented mutant had the lowest survival rate compared to the other strains, suggesting that *katE* alone does not protect cells from UV-A irradiation and that overexpression of catalase may be detrimental.

About one billion people in the world do not have access to safe drinking water, among which the majority live in developing countries. This is a major global health problem as poor water supply is related to diseases such as diarrhea, trachoma, and schistosomiasis (http://www.wssinfo.org/en/141_wshIntro.html). As a result, cost-effective methods to improve the quality of water are very important. Solar water disinfection (SODIS) is an inexpensive treatment method that uses solar radiation to inactivate and destroy pathogens, such as *Escherichia coli*, in contaminated water by exposing transparent plastic bottles of water to full sunlight for five hours (http://www.sodis.ch/).

The mechanism of bactericidal activity occurring during SODIS is not well understood. It is believed that sunlight destroys pathogens via UV-A radiation (wavelength 320-400 nm) and increased water temperature (5). UV-A is harmful to the pathogens because endogenous photosensitizers, including intracellular porphyrins and flavins, and exogenous photosensitizers absorb light and generate reactive oxygen species (ROS) such as superoxide and hydroxyl radicals, as well as toxic derivatives such as hydrogen peroxide (3).

Previously, the role of ROS in UV-A-mediated killing of *E. coli* was investigated using mutant strains of *E. coli* with Δ*katG* and Δ*katE* mutations (1). *katG* and *katE* encode for two catalases, hydroperoxidase I (HPI) and hydroperoxidase II (HPII), respectively, which convert hydrogen peroxide to water and oxygen (4). It was shown that the *E. coli* UM2 strain, a Δ*katE* Δ*katG* double mutant, had a dramatically reduced survival rate after 90 minutes of UV-A irradiation as compared to the UM197 strain, a Δ*katG* mutant, and wild-type (1). However, since there was a lack of proper negative controls, it was ambiguous as to whether ROS played an essential role in UV-A-mediated killing of pathogens or the observed killing was simply due to starvation. The purpose of this experiment is to better assess the importance of the *kat* genes and the role of ROS in UV-A-mediated killing of pathogens by complementing the UM2 strain with a full-length *katE* gene and using non-UV-A-irradiated negative controls. Based on the assumption that ROS do play a critical role in UV-A-mediated killing of pathogens, we hypothesized that the complemented mutant would have increased survival rates after 90 minutes of UV-A irradiation as compared to the parental strain.

MATERIALS AND METHODS

Bacterial strains and plasmid. *E. coli* strains used are listed in Table 1. pAMkatE72 was obtained from Dr. Loewen (University of

Manitoba, Department of Microbiology) and was used as the source of the *katE* gene. The phenotypes of the strains were confirmed by catalase test. Cultures were grown in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0), which was supplemented with 50 µg/mL of ampicillin for the transformed strain, CCHL08W-1.

Isolation of pAMkatE72. *E. coli* UM255 strain carrying the pAMkatE72 plasmid was streaked onto a LB agar plate and incubated overnight at 37°C. Plasmid isolation was carried out using the PureLink Quick Plasmid Miniprep Kit (Invitrogen, #K210010) as per the manufacturer's protocol. DNA quality and concentration were determined by measuring the absorbance at 260 nm and 280 nm. The DNA was stored at -20°C until needed.

Transformation of UM2 strain with pAMkatE72. Preparation of electrocompetent cells and electroporation was carried out as described in section 5 of Bio-Rad's MicroPulser Electroporation Apparatus Operating Instructions and Applications Guide (Bio-Rad, #165-2100), with the exception that the volumes were reduced by ten-fold. Briefly, 50 mL of LB medium was inoculated with 2.5 mL of overnight UM2 culture, and the cells were incubated at 37°C shaking at 300 rpm to an OD_{600} of 0.5-0.7. The cells were then chilled on ice for 20 minutes and harvested by centrifugation at 4,000 × *g* for 15 minutes at 4°C. The cells were washed three times with ice-cold 10% glycerol and resuspended in a final volume of 100-200 µL in ice-cold 10% glycerol, such that the cell concentration was about 1-3×10^{10} cells/mL. Forty µL of cell suspension was mixed with 2 µL of plasmid DNA, incubated on ice for 1 minute, transferred to a cold 0.2 cm electroporation cuvette, and pulsed at 2.5 kV. The cells were then resuspended in 1 mL of SOC medium (20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 2.5 mM KCl, 20 mM $MgCl_2$, 20 mM glucose) and incubated with gentle rotation for 1 hour at 37°C. 50-200 µL of cells were plated onto SOB agar medium (20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 2.5 mM KCl, 20 mM $MgCl_2$, 50 µg/mL ampicillin, 1.5% agar) and incubated overnight at 37°C.

Preparation of polyethylene (PET) bottles. Overnight cultures of the five strains were prepared by inoculating 10 mL of LB medium and incubating overnight at 37°C, with shaking at approximately 180 rpm. One mL of each culture was obtained the next morning, pelleted by centrifuging at 4,000 × *g* for 5 minutes, and resuspended into 20 mL of sterile distilled water. Seven and a half mL of bacterial suspension were then added to 242.5 mL of sterile distilled water in an ethanol-sterilized 500 mL PET bottle. Two PET bottles were prepared for each bacterial strain and the bottles were shaken to evenly distribute the bacteria. Two more PET bottles containing 250 mL of sterile distilled water were prepared as negative controls.

UV-A irradiation of *E. coli*. At t = 0, 1 mL of sample was removed from each of the twelve bottles for dilution plating at final plated dilutions of 10^{-3} to 10^{-5}. One set of bottles was laid on its side and exposed to UV-A irradiation as previously described (1). A second set of bottles, non-UV-A-irradiated negative controls, was laid on its side in the dark. The bottles were shaken vigorously every 15 minutes. After 90 minutes, 1 mL of sample was removed from each bottle for dilution plating at final plated dilutions of 10^{-2} to 10^{-5}. The plates were then incubated overnight at 37°C. The experiment was repeated two more times where final plated dilutions of 10^{-3} to 10^{-4} were used.

RESULTS

***katE*-complemented CCHL08W-1 strain showed greater catalase activity than isogenic wild-type χ^{760} strain.** *E. coli* UM2 strain was transformed with the plasmid pAMkatE72 containing a full-length catalase gene. To test whether the transformation was successful, a catalase test was performed (Table 2). The χ^{760} strain showed similar catalase activity to the UM197 strain and less catalase activity than the CCHL08W-1 strain. This was unexpected, as the χ^{760} strain contains two catalase genes compared to one catalase gene in each of the UM197 and CCHL08W-1 strains. As expected, the UM2 strain did not show any catalase activity, as it is a double mutant that lacks both catalase genes.

UV-A radiation did not affect survival rate in the

TABLE 1. *E. coli* strains used.

Strain	Genotype	Source
B23	Wild-type	University of British Columbia, Department of Microbiology & Immunology, MICB 421 laboratory stock
χ^{760}	F-, *araC14*, *leuB6*(Am), *secA206*(aziR), *fhuA23*, *lacY1*, *proC83*, *tsx-67*, *purE42*, *glnV44*(AS), *galK2*(Oc), *LAM-*, *trpE38*, *xthA15*, *his-208*, *rfbC1*, *mgl-51*, *argG77*, *rpsL109*(strR), *glpR201*, *xylA5*, *mtl-1*, *ilvA681*, *thi-1*, *metA160*	Coli Genetic Stock Center
UM197	F-, *araC14*, *leuB6*(Am), *secA206*(aziR), *fhuA23*, *lacY1*, *proC83*, *tsx-67*, *purE42*, *glnV44*(AS), *galK2*(Oc), *LAM-*, *trpE38*, *xthA15*, *his-208*, *rfbC1*, *mgl-51*, *argG77*, *rpsL109*(strR), *glpR201*, *xylA5*, *mtl-1*, *ilvA681*, *thi-1*, *metA160*, ***katG17::Tn10***	Coli Genetic Stock Center
UM2	F-, *araC14*, *leuB6*(Am), *secA206*(aziR), *fhuA23*, *lacY1*, *proC83*, *tsx-67*, *purE42*, *glnV44*(AS), *galK2*(Oc), *LAM-*, *trpE38*, *xthA15*, *his-208*, *rfbC1*, *mgl-51*, *argG77*, *rpsL109*(strR), *glpR201*, *xylA5*, *mtl-1*, *ilvA681*, *thi-1*, *metA160*, ***katG15***, ***katE2***	Coli Genetic Stock Center
CCHL08W-1	UM2 host with pAMkatE72	This study

χ^{760} strain but drastically reduced survival rate in the CCHL08W-1 strain. After catalase activity was confirmed in the complemented CCHL08W-1 strain, the effect of UV-A-mediated cell killing was tested in the five *E. coli* strains (Fig. 1). The results from the B23, UM197, UM2, and CCHL08W-1 strains showed increased survival in the samples not exposed to UV-A light, suggesting that cell death was caused by exposure to UV-A radiation rather than starvation in water. Surprisingly, the χ^{760} strain did not show a significant difference in survival rate between UV-A-irradiated and non-irradiated samples. It was expected that the χ^{760} strain would be similar to the B23 strain as they both contain the *katE* and *katG* catalase genes.

Comparing the survival rates between the five strains, the CCHL08W-1 strain showed the highest susceptibility to UV-A radiation in all three experimental replicates (Fig. 1). This was unexpected, as this strain had been complemented with a catalase gene. The UM2 strain was expected to have the lowest survival rate, as it did not show any catalase activity. However, it showed a similar survival rate to the UM197 strain. In general, the B23 and χ^{760} strains showed similar survival rates after 90 minutes of UV-A light (Fig. 1A and 1B). These survival rates were better than those observed in the UM197, UM2, and CCHL08W-1 strains, except in one replicate (Fig. 1C).

DISCUSSION

It was hypothesized that the CCHL08W-1 strain would have better survival rates than the parental UM2 strain after 90 minutes of UV-A irradiation; however, this was not observed in this experiment. The CCHL08W-1 strain had the lowest survival rate among the five *E. coli* strains that were tested. This may have been due to many reasons, such as the expression of *katE* in the CCHL08W-1 strain. The plasmid pAMkatE72 contains an inducible *lac* promoter and the full length *katE* gene with its endogenous promoter. Since isopropyl-β-D-thiogalactopyranoside (IPTG) was not used in this experiment, the expression of *katE* was under the control of its endogenous promoter, similar to UM197, rather than being expressed constitutively.

One possible explanation for the observed results is that *katE* may not even be expressed in the CCHL08W-1 strain when the cells were transferred into deionized water. *katE* is supposedly expressed during stationary phase as a result of carbon source depletion; however, it has been shown that just transferring the cells into a nutrient limited environment will not induce *katE* expression. It appears to be the accumulation of metabolites in the stationary phase medium that cause *katE* induction (4), which is absent from deionized water used in this study. However, *katE* appears to be expressed in both UM197 and CCHL08W-1, as both

TABLE 2. Catalase activity in the five strains of *E. coli* used, measured by the amount of bubbles produced after addition of 3% H_2O_2 to colonies on glass slides.

Strain	Catalase Activity*
B23	+++
χ^{760}	+
UM197	+
UM2	-
CCHL08W-1	++

* – indicates no bubbles, + indicates a few bubbles, ++ indicates several bubbles, +++ indicates a lot of bubbles.

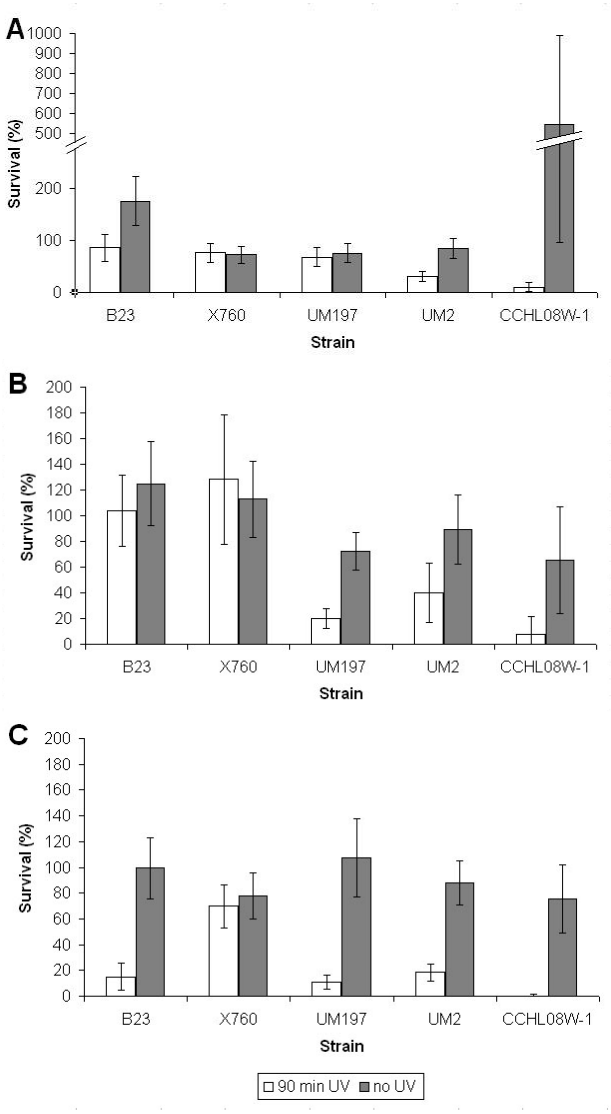

FIG. 1. Observed survival rates for the five *E. coli* strains after 90 min exposure to UV-A light compared to 90 min of no exposure to UV-A light. A, B, and C represent three experimental replicates.

seem unlikely.

A more likely explanation is that rather than lack of expression of *katE*, it may be overexpressed since it is on a high copy number plasmid. This is supported by the results from the catalase test, as the CCHL08W-1 strain showed greater catalase activity than both UM197 and χ^{760}. This overexpression may contribute to the decreased survival during UV-A irradiation as it was suggested by Eisenstark and Perrot (2) that excess endogenous catalase may act as a photosensitizer that absorbs UV-A light and generates reactive oxygen species. They have also found that exogenous bovine catalase added to UV-irradiated cells do not act as sensitizers (2), which explains the protective effect of exogenous catalase seen in the previous study by Amiri *et al.* (1).

The variations in each of our replicates may be due to the slight differences in sampling and plating times. These slight differences may largely affect survival rates of the bacteria based on the assumption that bacterial death is due to accumulated cellular damage caused by UV-A irradiation. It may be possible that there is exponential cell death past a certain threshold of cellular damage. Thus, if our 90 minute time point is near the threshold, then slight variations in the time between samples being taken and being plated would cause large differences in the observed survival rates.

It is interesting to note that we were unable to completely replicate the results by Amiri *et al.* (1). In particular, they observed that the χ^{760} strain had a lower survival rate than the UM197 strain, but we had the opposite result in two of the three replicates. It is reasonable for χ^{760} to have a higher survival rate than UM197 since it has both catalase genes, whereas UM197 has only a functional *katE* gene.

Our results suggest that the complementation of *katE* in the double mutant enhanced sensitivity to UV-A irradiation. Further experiments need to be done to elucidate the reason for this enhanced sensitivity, so it is difficult to assess the importance of the *kat* genes and the role ROS plays in UV-A mediated killing of pathogens.

FUTURE EXPERIMENTS

To assess whether this enhanced sensitivity in CCHL08W-1 is due to the overexpression of *katE*, Western blot analysis could be done to quantify its expression level. However, since a higher protein level does not necessarily correlate with higher levels of active catalase, catalase activity should be measured as well. The amount of catalase activity could be quantified using a spectrophotometric method where catalase is allowed to react with methanol in the presence of hydrogen peroxide and the product, formaldehyde, is measured using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromagen. Furthermore, an extended time course with sampling at 15 minute intervals for two hours should be carried out to determine whether exponential cell death is seen past a certain time point. To better assess the protective functions of *katE* and *katG*, the experiment should be repeated using single mutants. Complementation and overexpression studies could be performed in these mutants once the roles of *katE* and *katG* have been elucidated to assess their interaction (for example, if the presence of *katG* would decrease the sensitivity of a *katE*-complemented mutant to UV-A radiation). Lastly, we could quantify ROS in each of the strains to further support the idea that during UV-A irradiation, the generation of ROS results in cell death and expression of catalase provides protection by reducing the abundance of ROS. To look at the change in concentration of ROS intracellularly, a discontinuous *kat-sod* assay or a luminal-dependent chemiluminescence assay could be done. Alternatively, Invitrogen offers H2DCF-DA staining for ROS which could then be quantified by flow cytometry.

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