The Role of Catalase HPII Levels in Protection against UV-A Damage in a Catalase Knockout Escherichia coli Strain

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E. coli strains show low sensitivity to ultraviolet-A (UV-A) irradiation, a common method of decontamination. The two known catalases in E. coli, hydroperoxidase I (HPI) and hydroperoxidase II (HPII) are suspected to protect against UV-A inactivation by removing destructive reactive oxygen species (ROS). Catalase HPI knockout UM197 cells, subjected to UV-A, previously showed wildtype survival suggesting that only HPII, encoded by katE, protects against UV-A. However, katE overexpression in catalase double knockout strain UM2 did not protect against UV-A possibly due to the photosensitizing properties of HPII at high concentrations. In this study, a UM197 strain containing antisense katE (a katE) on the pBAD24 construct, CKLW09W2 cells were differentially induced at mid-log phase to create decreasing HPII levels. The araC promoter on pBAD24 was induced using zero, low, medium, and high L-arabinose concentrations followed by UV-A irradiation of respective bacterial solutions in water. Survival was determined as a percentage of colony counts of control treatments incubated in the dark. Compared to the wildtype plasmid control, uninduced CKLW09W2 cells were twice as susceptible to UV-A killing suggesting negative effects of the a katE insert and/or genetic manipulations conducted to create the strain; addition of L-arabinose did not affect growth rates. At all induction levels, CKLW09W2 survival was similar and HPII expression did not correlate with UV-A survival. Some variations in the data were observed and can be attributed to nutrient deprivation in water, loss of inducibility and/or degradation of a katE mRNA during mid-log phase growth. The overall results of this study indicate that HPII does not provide E. coli protection against UV-A and other gene(s) may be involved.

Solar Water Disinfection (SODIS) is a World Health Organization recommended method that renders clear water safe for drinking after merely six hours under direct sunlight (http://www.sodis.ch). Although SODIS is an ancient method, research on its mechanism only began in the 1970s. A comprehensive study by Arca et al. conducted in the early 1980s revealed that heat and ultraviolet-A radiation (UV-A; λ 320- 400nm) inactivate bacteria (1). Further research in the late-1980s by Eisenstark described generation of damaging reactive oxygen species (ROS), like peroxide, through excitation of photosensitizers such as amino acids after exposure to visible light (8). UV-A damage may be caused by ROS-generation but proving this has been a difficult task with contradicting results.

The ROS hypothesis for UV-A damage has been studied using catalases, which destroy peroxide and other ROS (8). Because wildtype Escherichia coli strains are able to resist complete inactivation by SODIS, the two known catalases in E. coli are suspected to provide protection (8). The 84 kDa catalase, hydroperoxidase-I (HPI), is membrane-bound, encoded by katG and has catalase and peroxidase activity (13). HPI is expressed only during anaerobic growth is predicted to repair after UV-A damage by increasing cell permeability to anti-oxidants (9). The 93 kDa catalase, hydroperoxidase-II (HPII), is cytoplasmic, encoded by katE, and has only catalase activity (14). HPI is expressed during aerobic exponential growth (11) but its role in UV-A damage remains unclear due to contradicting evidence. The katG, katE double knockout E. coli strain, UM2, has previously shown increased sensitivity to UV-A consistent with a role in UV-A resistance (2). However, the katG knockout strain, UM197, did not suffer after UV-A exposure compared to its isogenic parent, suggesting that only katE is required for protection (2). However, the UM2 strain complemented with katE also failed to protect against UV-A (5) speculatively because of over-expression of HPII by the pBAD24 construct (3). Lack of protection was thought to be because of the photosensitizing properties of HPII at high concentrations (5). Another study successfully created an alternative catalase double knockout of the UM197 strain, CKLW09W2, using antisense katE DNA (a katE) in the pBAD24 vector (4); however, the authors did not test the strain’s ability to resist UV-A damage.
To test for HPII-mediated UV-A protection at different HPII concentrations, this study controlled expression of the akatE message in CKLW09W2 using the inducible arac promoter on the pBAD24 construct. The aim of this study was to control expression levels of HPII in HPI knockout cells to elucidate whether HPII alone has protective properties against UV-A damage at lower concentrations and determine the expression level above which photosensitizing properties of HPII take over.

**MATERIALS AND METHODS**

**Strains and growth conditions.** The following *E. coli* K12 strains were obtained from the MICB 421 Culture Collection (Department of Microbiology and Immunology, University of British Columbia): χ<sup>W3</sup>, UM2, CKLW09W2, and CKLW09W3 (described in Table 1). Strains with the pBAD24 plasmid were grown on Luria-Bertani (LB) broth or agar (1% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl, pH 7) containing 10 µg/mL ampicillin. Untransformed χ<sup>W3</sup> and UM2 strains were grown in LB media without ampicillin. All strains were grown at 37°C, with shaking for broth cultures. For induction, media was supplemented with L-arabinose at stated concentrations (% w/v).

Transformation of χ<sup>W3</sup> and UM2 strains with pBAD24. The Purelink<sup>™</sup> Quick Miniprep kit (Invitrogen K2100-10) was used as per manufacturer instructions to extract the pBAD24 plasmid from 3 ml of CKLW09W3 overnight culture. The concentration and quality of DNA in the final eluent was determined by absorbance at 260 nm and 280 nm. Aliquots of pBAD24 were stored at -20°C until needed. Competent χ<sup>W3</sup> and UM2 cells were prepared using the CaCl<sub>2</sub> method. Both strains were inoculated at 1/20 in LB media with ampicillin: 2 ml of overnight culture in 38 ml of media. At O.D.<sub>600</sub> 0.6, cultures were pelleted using Du Pont Instruments Sorval<sup>™</sup> SS-34 rotor at 4°C and 7000 rpm in a pre-chilled tube for 10 minutes. Cells were resuspended in 10 ml of cold 10 mM NaCl, pelleted as above and resuspended in 10 ml of cold 75 mM CaCl<sub>2</sub>; (pH 7). Following a 30-minute incubation on ice, cells were pelleted and resuspended in 1 ml of CaCl<sub>2</sub> solution. Cells (34 µl) were mixed with 166 µl ice-cold 100% glycerol and stored at -80°C till needed. Competent χ<sup>W3</sup> and UM2 cells and 50 ng of pBAD24 DNA were incubated together for 10 minutes on ice. Mixtures were subjected to heat shock at 42°C for 1 minute followed by 2 minutes on ice. LB broth (1 ml) was added to each tube and allowed to recover for 1 hour at 37°C. Transformation mixtures (100 µl) were plated on LB agar with ampicillin.

Screening Transformants. Putative positive colonies were screened using the following toothpick method. Two colonies per transformation were transferred to 50 µl of 10 mM EDTA. Fifty µl of NSS Solution (0.2 N NaOH, 0.5% w/v SDS, 20% w/v sucrose) were added, tubes were vortexed and incubated in a 70°C water bath for 5 minutes. After cooling, 150 µl of 4 M KCl were added. Vortexed tubes were incubated on ice for 5 minutes and pelleted for 3 minutes at 4°C at 4000 x g. For restriction digestion, salt was removed from the DNA extract by ethanol precipitation. Sodium acetate (3 M) was added at 1/10 of total volume and inverted to mix. Two volumes of 100% ethanol were added and tubes were spun at 4000 x g for 15 minutes at 4°C. Ethanol was aspirated and DNA was allowed to dry. Wash was repeated with 70% ethanol and DNA was resuspended in 4.6 µl H2O. 0.5 µl 8fg (New England Biolabs R0143L) and 0.7 µl 10X NEB Buffer 3. After 1.5 hours digestion at 37°C reactions with loading dye were inactivated for 5 minutes at 70°C and electrophoresed on a 1% (w/v) agarose gel at 75 V for 1 hour in 1X TAE Buffer (40 mM tris base, 0.01% glacial acetic acid, 1.3 mM EDTA). 1X DNA Ladder GeneRuler™ 1 Kb (Fermentas SM0314) was used (10 µl) to estimate size. Gels were stained in 0.2 µg/ml ethidium bromide for 2 hours and visualized using the Cell Biosciences AlphaImager.

**Table 1. Summary of E. coli K12 strains used in this study.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>χ&lt;sup&gt;W3&lt;/sup&gt;</td>
<td>Wildtype for katE and katG</td>
</tr>
<tr>
<td>F&lt;sup&gt;−&lt;/sup&gt;, araC14, leuB6(Am), secA206(azlR), fhuA23, lacY1, proC38, tex-67, purE42, glnV44(AS), galK20(Oc), LAM-, trpE38, xylA15, his-208, mlg-t1, rpsL109 (strR), glnB201, xylA15, mlt-1, dfrA681, thi-1, metA160.</td>
<td></td>
</tr>
<tr>
<td>PW10-1</td>
<td>χ&lt;sup&gt;W3&lt;/sup&gt; with pBAD24</td>
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<tr>
<td>F&lt;sup&gt;−&lt;/sup&gt;, araC14, leuB6(Am), secA206(azlR), fhuA23, lacY1, proC38, tex-67, purE42, glnV44(AS), galK20(Oc), LAM-, trpE38, xylA15, his-208, rfbC1, mlg-t1, argG77, rpsL109(strR), glnB201, xylA15, mlt-1, dfrA681, thi-1, metA160, kanG15, katE2</td>
<td></td>
</tr>
<tr>
<td>PW10-2</td>
<td>UM2 with pBAD24</td>
</tr>
<tr>
<td>F&lt;sup&gt;−&lt;/sup&gt;, araC14, leuB6(Am), secA206(azlR), fhuA23, lacY1, proC38, tex-67, purE42, glnV44(AS), galK20(Oc), LAM-, trpE38, xylA15, his-208, rfbC1, mlg-t1, argG77, rpsL109(strR), glnB201, xylA15, mlt-1, dfrA681, thi-1, metA160, kanG15::Tn10</td>
<td></td>
</tr>
<tr>
<td>CKLW09W3</td>
<td>UM197 with pBAD24</td>
</tr>
<tr>
<td>CKLW09W2</td>
<td>UM197 with pBAD24 containing antisense katE (akatE) insert</td>
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</table>

Flasks measured at O.D.<sub>600</sub> 2.2 – 2.5 were inoculated 1/3 of mid-log phase culture, LB broth, ampicillin when needed, and L-arabinose in 35 ml final volume. Four ml of induced culture was pelleted at 4800 x g for 10 minutes and subjected to 50 µl of 30% H<sub>2</sub>O<sub>2</sub> while gently pipetting the pellet. Resulting bubbling was timed and qualified using the following scale: - (no bubbles), +/- (2-5 bubbles), + (quick small bubbles), ++ (large bubbles), +++ (continuous large bubbles). The floating disk catalase assay was attempted by coating 5 mm disks of 3 mm filter (Whatman 1030021) with induced culture pellet and placing in 3 cm liquid height of 1% H<sub>2</sub>O<sub>2</sub>. Enzyme activity was correlated to time taken in seconds for the disk to reach the top of the liquid. A range of inducer concentrations were used for this assay to determine low, medium and high akatE expression level before UV-A exposure.

**UV-A Exposure.** This method was adapted from (2) to simulate the SODIS method. Overnight starter cultures O.D.<sub>600</sub> 0.3 – 0.4 were used. For the induction procedure see the “UV-A Exposure” section above. Four ml of induced culture was pelleted at 4800 x g for 10 minutes and subjected to 50 µl of 30% H<sub>2</sub>O<sub>2</sub> while gently pipetting the pellet. Resulting bubbling was timed and qualified using the following scale: - (no bubbles), +/- (2-5 bubbles), + (quick small bubbles), ++ (large bubbles), +++ (continuous large bubbles). The floating disk catalase assay was attempted by coating 5 mm disks of 3 mm filter (Whatman 1030021) with induced culture pellet and placing in 3 cm liquid height of 1% H<sub>2</sub>O<sub>2</sub>. Enzyme activity was correlated to time taken in seconds for the disk to reach the top of the liquid. A range of inducer concentrations were used for this assay to determine low, medium and high akatE expression level before UV-A exposure.
and to normalize results. Each induction culture was then diluted 1/300: 40 µl in 12 ml of sterile dH₂O and vortexed resulting in a solution containing approximately 1 × 10⁶ cells/ml. Each of five (2 dark room controls, 3 replicates) 60 × 15 mm Polystyrene Tissue Culture plates (BD Falcon™) were filled with 2 ml of the microbe solution; this was repeated for each induction and strain combination. Three dishes per condition were exposed to UV-A radiation with lids off, 10 cm under a 20 W Sylvania Black light (UV-A source with an intensity of 200 W/m²) for 90 minutes at room temperature. Two dishes per condition were placed in the dark for 90 minutes with lids off to determine background survival rates. All dishes were swirled gently every 20 minutes during treatment and plated at 10⁻² and 10⁻³ on LB agar with ampicillin if needed. Survival rates in each replicate were calculated as a percentage of the average dark room counts per induction condition.

RESULTS

BgII digestion of pBAD24. To measure potential decreases in survival due to introduction of pBAD24 and to create appropriate control strains, χ₇₆₀ and UM2 strains were transformed with pBAD24. The generated strains were named PW10-1 and PW10-2 respectively. A transformation efficiency of approximately 500 transformants/µg of DNA was achieved for both strains (data not shown). Fig. 1 shows that pBAD24 DNA isolated from positive transformants and linearized by BgII digestion travels between 4.0 kb and 5.0 kb as expected (actual size: 4.54 kb). The lighter undigested plasmid band was seen much above the ladder representing open circular pBAD24 DNA as observed by (6). Out of six putative positive colonies screened, 3 per strain, all six DNA isolates showed bright linearized bands at approximately 4.5 kb (data not shown).

Inducing pBAD24 with L-arabinose. The χ₇₆₀ strain showed high catalase activity at all levels of L-arabinose induction as expected since this strain is wildtype for both catalases (Table 1). A similar trend was seen in the χ₇₆₀ + pBAD24 strain suggesting that introduction of pBAD24 did not interfere with normal catalase production levels or the catalase assay. The χ₇₆₀ descendant and knockout strain for both catalases, UM2 transformed with pBAD24, only showed the expected no catalase phenotype at all induction levels also suggesting that addition of pBAD24 did not interfere with catalase production or the catalase assay used. The experimental strain UM197 + pBAD24-akatE (aka CKLW09W2) also from the χ₇₆₀ lineage showed predicted catalase levels. Increased induction of the araC promoter correlated with decreased catalase production; 0.02% L-arabinose and lower showed wildtype catalase activity; 0.01% showed “medium” levels of catalase; and 0.2% and 0.3% induction showed low catalase activity as expected. A complete catalase knockout phenotype could not be achieved even using 0.4% L-arabinose (data not shown). To better quantify this trend the floating disk assay was conducted, however CKLW09W2 showed wildtype catalase levels at all L-arabinose levels (data not shown) suggesting inconsistency.

Simulating the SODIS method. SODIS simulation was achieved by exposing mid-log phase cells at 1 × 10⁶ cells/ml in dH₂O to UV-A exposure for 90 minutes. The χ₇₆₀ + pBAD24 and CKLW09W2 strains were subjected to UV-A exposure as in the SODIS method. Observed survival after 90 minutes of UV-A exposure in χ₇₆₀ + pBAD24 cells showed no significant difference at all induction levels (Fig. 2). Survival of χ₇₆₀ + pBAD24 cells was approximately twice as high compared to the CKLW09W2 strain without induction suggesting lower survival ability in CKLW09W2 cells also observed by (4). Comparing survival of uninduced cells to survival at low, medium and high L-arabinose concentrations, E. coli CKLW09W2 survival rates were not significantly different. Despite the different catalase activities achieved by differential induction (Table 1), all conditions were equally susceptible to UV-A damage (Fig. 2). The large error bars seen in Fig. 2 were due to the small

<table>
<thead>
<tr>
<th>Induction [L-arabinose] (%)</th>
<th>Catalase Activity</th>
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<tr>
<td></td>
<td>χ₇₆₀</td>
</tr>
<tr>
<td>0</td>
<td>+++</td>
</tr>
<tr>
<td>0.0002</td>
<td>+++</td>
</tr>
<tr>
<td>0.002</td>
<td>+++</td>
</tr>
<tr>
<td>0.01</td>
<td>+++</td>
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<tr>
<td>0.02</td>
<td>+++</td>
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<tr>
<td>0.2</td>
<td>+++</td>
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<tr>
<td>0.3</td>
<td>+++</td>
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</tbody>
</table>

FIG. 1. BgII digestion of plasmid DNA isolated from positive χ₇₆₀ and UM2 colonies transformed with pBAD24. Arrows show linearized pBAD24, larger bands represent relaxed form of the plasmid. Lane 1 contains GeneRuler™ 1 kb ladder.

TABLE 1. Direct catalase activity assay on 4 ml of mid-log phase culture pellets after 20 minute induction described as amount of bubbling observed after addition of 30% H₂O₂ to cell pellets.
magnitude of colonies per plate (<30) causing uncertainty. Growth rates during induction were similar at each induction level as measured by O.D. 600 (data not shown) suggesting that addition of L-arabinose does not affect observed survival rates. Overall Fig. 2 survival was independent of catalase activity indicating that katE at all expression levels did not protect against UV-A.

DISCUSSION

In this study, the inducible araC promoter on pBAD24-akatE was used to create changing HPII levels and to measure the effect on UV-A protection. The UM197 katG knockout E. coli strain was previously transformed with pBAD24-katE to create a double knockout phenotype in the CKLW09W2 strain and confirmed using the catalase assay (4). Wildtype $\chi^{760}$ and $\gamma^{760}$ + pBAD24 strains showed expected phenotypes with high catalase activity at all four L-arabinose levels (Table 2). Since katG is only expressed during anaerobic growth (8) it is expected that wildtype strains would not produce HPI in the aerobic mid-log phase cultures tested. This is supported by similar catalase activity in wildtype cultures and at low induction levels in CKLW09W2 cells, expected to only produce HPII. The control UM2 + pBAD24 strain showed no catalase activity in all conditions supporting lack of catalases (Table 2).

The highest induction level used, 0.3% L-arabinose, showed presence of catalase activity in CKLW09W2 mid-log phase cells (Table 2) not seen previously by (4). This may be caused by insufficient induction because although pBAD24 is quickly induced it is also quickly repressed when L-arabinose is removed (7). Leaving cells in induction media during the catalase assay or overnight induction may combat this effect. Compounding this may be the degradation of akatE message during mid-log phase growth when the cell normally expresses katE (13) and is growing rapidly. The subsequent floating disk catalase assay showed that the CKLW09W2 strain had high catalase activity at all induction levels (data not shown). Contradictory evidence could be explained by loss of pBAD24 inducibility or loss of pBAD24-akatE combined with the gain of ampicillin resistance. Alternatively, assay incompatibility could also lead to observed inconsistencies.

Results of SODIS simulation (Fig. 2) show that survival of CKLW09W2 cultures without induction is significantly lower than in the $\chi^{760}$ + pBAD24 strain. This suggests that either the akatE insert DNA or the katG knockout caused detrimental effects on survival. Given that UM197 and UM2 strains are descendants of the $\gamma^{760}$ strain (Table 1), and were prepared using transposon mutagenesis (10), it is possible that E. coli UM197 and UM2 contain additional altered genes compared to E. coli $\chi^{760}$ that cause the observed differences in survival. This warrants the need for E. coli strains prepared using more controlled mutagenesis methods in further study. For this study, however, calculating survival rates as a percentage of darkroom colony counts provides a mode to avoid and compare strain-to-strain inconsistencies.

Fig. 2 also shows that survival of CKLW09W2 cells after UV-A treatment was similar at all induction levels. These results support the conclusion that HPI does not play a role in protection against UV-A. This suggests that either ROS removal using catalase is not the mechanism for UV-A protection in E. coli or that repair or dormancy genes may be involved. Alternatively, another gene or biochemical pathway capable of removing ROS might be involved. Although HPI is not expected in aerobic culture it may still be involved here. The UV-A survival evidence is convoluted by weak catalase activity results, which may be caused if akatE was not induced. In addition, large error bars seen in Fig. 2 due to low, uncertain colony counts may have been caused because 90 minutes in dH2O can deprive cells of nutrients and set off unpredictable cell death, dormancy or rescue pathways further convoluting the results. A previously conducted study on $\Delta$katE and UV-A survival (5), also shows inconsistency between replicates possibly due to the unpredictability of such pathways. For the small volumes used in this study (2 ml) it may be possible to use shorter UV-A exposure times to combat this effect provided some UV-A killing still occurs.

The results of this study indicate that HPII does not protect against UV-A damage in the E. coli strains used and that (an)other gene(s) may provide protection against UV-A exposure. However, the trends are variable and need to be further
FUTURE DIRECTIONS

Results of this study can lead the researcher in two directions. The first path involves confirming the survival rates and catalase levels observed in this study because of the qualitative and inconsistent nature of the results. This can be achieved by repeating this study using strains with predictable genetics and more robust catalase assays. Because of unexpected genome changes between \( \chi^{506} \), UM2 and UM197 cells, an *E. coli* \( \Delta katG \) strain like JW1721 from the Keio collection, prepared using systematic site-directed mutagenesis (http://ecoli.naist.jp) with fewer unexpected genome mutations is recommended. Chosen strains can be transformed with a compatible inducible vector containing the \( katE \) insert and can be tested as described in this study. In addition, use of shorter UV-A exposure time, a lower UV-A intensity or increased starting *E. coli* concentrations are recommended to avoid low colony counts caused by nutrient deprivation and the resulting onset of rescue/dormancy pathways. Also, comparing UV-A survival in mid-log phase versus overnight culture due to predicted \( katE \) mRNA degradation at mid-log phase should also be considered as a procedural modification. A more quantitative catalase assay is recommended to combat inconsistencies seen using the direct and floating disk methods. Previously recommended is a spectrophotometric assay, which converts methanol to a chromogen in the presence of catalase (5). This method allows the inducer to remain in culture avoiding the issue of a repressed vector. Since some vector constructs are repressed once the activator molecule is removed, pelleting the cells from the induction culture is not recommended before testing for catalase levels.

The second recommended future direction is the search for another gene or pathway capable of protecting against UV-A. This can be conducted by searching the *E. coli* genome against an annotated database of all known bacterial genes like Gene Ontology. Genes involved in UV-A or light damage present in *E. coli* can be screened for the most likely candidate. The candidate should be chosen keeping in mind that ROS resistance may not be the only mechanism for UV-A protection; ROS resistance or an efficient repair mechanism may be at play. The chosen gene(s) can then be knocked out from the genome and tested for survival against UV-A damage using well-characterized strains. By identifying and characterizing the gene or pathway involved, we may be able to modify the SODIS method or create another simple way to inactivate *E. coli* strains.

ACKNOWLEDGEMENTS

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