View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by KnowledgeBank at OSU

The Protective Effects of the Violacein Pigment Against UV-C Irradiation in *Chromobacterium violaceum*

Andrew N. Abboud[1], Tippecanoe High School, Tipp City, OH and Anthony Arment, Deptartment of Natural Sciences, Central State University, Wilberforce, OH, USA

Abstract. Chromobacterium violaceum is a gram-negative bacteria found in tropical regions. *C. violaceum* has the distinct phenotypic characteristic of a deep violet pigment called violacein. Violacein has a high molar extinction in methanol, suggesting that it is protective against visible light. The purpose of this study was to establish the protective effects of violacein against UV-induced cellular damage. It was hypothesized that violacein protects DNA and proteins (e.g. catalase) from UV-C induced damage. Wild-type (*WT*) *C. violaceum* was mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine to produce mutants with varying amounts of violacein. Mutants CV9, CV13, and CV14 (non-pigmented) produced less pigmentation than *WT* and retained colony morphology, while mutants H19, H20, and H21 (hyper-producers) over-expressed violacein but had an altered petite morphology. UV-induced DNA damage was assayed through sub-culture post-irradiation at 6,000μW*s^{-1}*cm^{-2} at λ=253.7nm. Sub-cultures of *WT* and hyper-producers showed reduced viability after 48 hours; nonpigmented mutants showed no growth, suggesting violacein is protective against UV-induced DNA damage. UV-induced catalase damage was assayed pre- and post-irradiation. Catalase activity in *WT* and hyper-producers significantly decreased post-irradiation; catalase activities of non-pigmented mutants significantly increased post-irradiation. Increased catalase activity in non-pigmented mutants can potentially be explained by the increased induction of catalase genes in response to elevated reactive oxidative species, presumably from lack of pigmentation. Taken together, these results support the hypothesis that violacein is protective against UV-induced cellular damage.

Date of publication: January 2013

OHIO J SCI 111 (2-5): 28-32

INTRODUCTION

Chromobacterium violaceum is a gram negative, facultative anaerobic, non-spore forming bacteria found in tropical and sub-tropical regions. It is an opportunistic pathogen that may cause diseases in individuals with compromised immune systems (Lee 1999). *C. violaceum* has the distinct phenotypic characteristic of a violet, metallic pigment called violacein. Violacein is one of several antibiotics produced by *C. violaceum*; violacein has been proven effective against amoeba and trypanosome infection.

Violacein has a high molar extinction in methanol (ε =1.7 x 10^4 L*mol^{-1}*cm^{-1},λ=577nm), suggesting that it could be protective against visible radiation (Antonio 2004). Additionally, violacein has a comparable peak absorbance in the UV-visible range (λ=260.3nm), suggesting that it could be protective against ultraviolet radiation (Salas 2006). The protective effects of violacein against ultraviolet irradiation have not been widely studied.

Conclusions about bacterial activity can be drawn through studying changes in cellular enzymatic activity (e.g. catalase). Catalase is an aerobic enzyme that is nearly ubiquitous among organisms (Li 2007). Its main function is to prevent the accumulation of toxic levels of hydrogen peroxide formed as by-products of metabolic processes. Catalase employs a two-electron transfer in the dismutation of hydrogen peroxide (H_2O_2) to oxygen gas and water, thereby protecting the cell from the harmful effects of H_2O_2. There have been at least two catalases identified from *Escherichia coli*, hyperperoxidase I (HPI) and hyperperoxidase II (HPII). HPI (*katG*) is expressed under both aerobic and anaerobic conditions and its synthesis is induced under oxidative stress or exposure to sub-lethal levels of hydrogen peroxide. HPII hyperperoxidase (*katE*) is expressed under aerobic conditions, beginning in the stationary phase (Meir and Ezra 1985). Catalase is generally quantified by measuring the decrease in absorbance of hydrogen peroxide or by measuring oxygen release with Clark-type electrodes (Li 2007). In this study, the protective effects of the violacein pigment were examined by observing DNA damage (viability) and changes in catalase activity pre- and post- UV-C (λ=253.7nm) irradiation. It was hypothesized that the violacein pigment protects DNA and enzymes (e.g. catalase) from UV-C induced damage in *C. violaceum*.

[1]Address correspondence to Andrew N. Abboud, 748 Oak Lea Drive, Tipp City, OH 45371. Email: andrew.abboud9@gmail.com

MATERIALS AND METHODS

Bacterial Strains

All bacterial mutants were derivatives of the wild type strain *Chromobacterium violaceum* (*WT*) provided by Central State University (Wilberforce, OH). *C. violaceum* mutants *H19, H20* and *H21* expressed more violaceum than *WT,* and were considered hyper producers. These hyper producers displayed a change in colony morphology. *C. violaceum* mutants *CV9, CV13,* and *CV14* had little to no pigmentation, compared to *WT,* and were considered partially or non-pigmented mutants. Non-pigmented mutants displayed the parental colony morphology but demonstrated reduced growth at *WT* incubation temperature.

Media and Chemicals

Trypticase Soy Agar and Broth were used as the standard media. Na_2PO_4, NaH_2PO_4, NaCl, H_2O_2 30 percent, sodium dodecyl sulfate 10 percent, glycerol (for freeze-away media), 1-butanol, and all lab equipment were generously provided by Central State University Department of Natural Sciences. N-methyl-N'-nitro-N-nitrosoguanidine (NG) was purchased from Sigma-Aldrich (St. Louis, MO). A Vernier LabQuest™ and Dissolved Oxygen Sensor™ (Beaverton, OR) were kindly provided by Tippecanoe High School.

Growth and Culture of Bacteria

Bacterial strains *WT* and hyperproducers were grown at 37°C and non-pigmented mutants were grown at 25°C. All broth cultures were grown aerobically with shaking. Cell densities were determined by absorbance at 600 nm in a ThermoFisher Scientific SPECTRONIC™ 20D+ Digital Spectrophotometer using a standard curve correlating absorbance and colony forming units (cfu).

Mutagenesis and Isolation

Mutagenesis procedures were adapted from Oeschger and Berlyn (1974). Log phase cultures of *WT* growing aerobically in trypticase soy broth (TSB) were treated with NG. NG was added to a final concentration of 0.1 mg*mL^{-1} using a freshly prepared solution of NG (10 mg*mL^{-1} in acetone) and the cells were incubated at 37°C for 10 minutes without aeration. The mutagenized cells were collected by centrifugation, washed, and resuspended in one ml freeze-away TSB medium. The mutagenized cells were plated on TSA at a serial dilution of 10^{-10} cfu and grown at 25°C overnight. The plates were visually inspected for mutants that lacked pigmentation and were streaked and plated for isolation.

Violacein Extraction and Quantification

The pigment extraction and photometric quantification of violacein were adapted from a method described by Matz (2004). One milliliter samples of *C. violaceum* mutants were grown to A_{600}=1.0 and were harvested by centrifugation. The pellets were resuspended in one mL aliquots of TSB. One milliliter of the bacterial suspensions was mixed in equal volume with one mL of 10 percent sodium dodecyl sulfate (SDS) and then incubated for five minutes at room temperature. Violacein was separated from the cell debris by vortexing with two mL of H_2O-saturated butanol. The upper phase, containing violacein, was separated from the aqueous phase by centrifugation in a Hamilton Bell VanGuard V6000 clinical centrifuge for two minutes. The violacein content of the butanol phase was quantitatively measured as absorbance at 585nm.

Initial DNA/Catalase Assays

Assays were run in a system of a 0.0625M solution of one percent phosphate buffered saline (1xPBS) and three percent hydrogen peroxide. One milliliter cultures of *WT, CV9, CV13, CV14, H19, H20, and H21* were grown aerobically overnight and diluted to A_{600}=1.000. Bacteria were harvested by centrifugation (8000 rpm x five minutes), and the pellets were brought up in one mL PBS pH 7.0, to be used as the catalase source. One hundred microliters of bacteria was added to the system, and oxygen gas generated was recorded in mg*mL^{-1} on a Vernier LabQuest™ with a Dissolved Oxygen Sensor™ over a time course of 180 seconds. The rates of change of oxygen gas dissolution were used to determine catalase activity. Ten microliters of the bacteria was plated on TSA and grown overnight at 25°C to determine initial viability.

UV-C Irradiation and Post-Irradiation Assays

Bacterial samples were prepared as previously described. One hundred microliters of bacteria was added to PBS and irradiated in a SpectroLinker™ XL-1000 Microprocessor-Controlled UV Crosslinker (Spectronics Corporation, Westbury, NY) at 6,000μWs^{-1}cm^{-2} at λ=253.7nm. Post-irradiation assays were run as previously described and the rates of change of oxygen gas dissolution were used in comparing catalase activity.

Statistical Analysis

Assays were compared statistically with the Paired T Test at the five percent significance level. Paired t-test statistic for H_0: $\mu_1=\mu_2$ (paired sample, and normal differences or large sample).

Table 1

Violacein Quantification in Absorbance 585

Strain	A_{585}
WT	0.620
H19*	0.888
H20*	0.956
H21*	1.304
CV9**	0.436
CV13**	0.380
CV14**	0.390

* - Mutants *H19, H20, H21* hyperproducers produced more violacein than *WT* as seen by higher absorbance at A_{585}.

** - Mutants *CV9, CV13,* and *CV14* (non -pigmented) produced less violacein than *WT* as seen by lower absorbance at A_{585}.

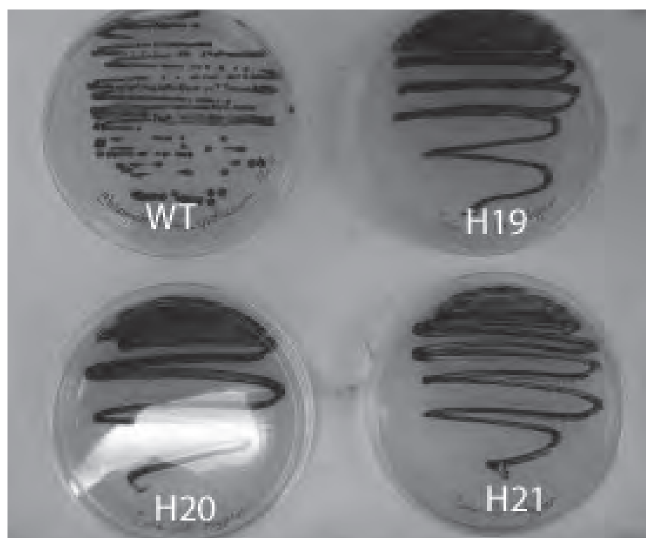

Figure 1. Isolated hyper-producer strains *H19, H20* and *H21*. These strains contain more violacein than the wild type strain.

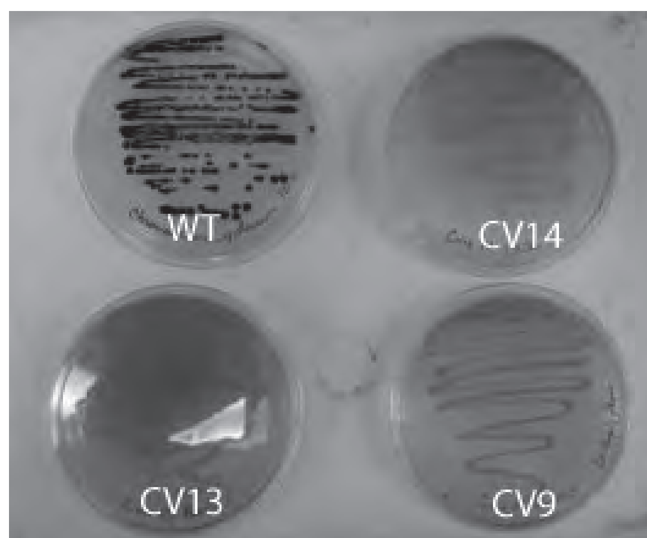

Figure 2. Isolated non-pigmented strains *CV9, CV13* and *CV14*. These strains contain less violacein than the wild type strain.

RESULTS

Violacein Extraction and Quantification

The amounts of violacein in *WT* and mutants are detailed in Table 1. Mutants *H19, H20,* and *H21* hyper producers produced more violacein than *WT* as seen by higher absorbance at 585 nm. Mutants *CV9, CV13,* and *CV14* (non-pigmented) produced less violacein than *WT* as seen by lower absorbance at 585 nm. (Fig. 1 and 2)

DNA Assays

UV-induced DNA damage was assayed through sub-culture post-irradiation. Sub-cultures of *WT* and hyper-producers showed growth with the same average of 53 cfu after 48 hours; nonpigmented mutants showed no growth, suggesting violacein protected against UV-induced DNA damage (Fig. 3 and 4).

Catalase Assays

Catalase activities of *WT* and hyperproducers significantly decreased post-irradiation. *WT*, *H19*, *H20 and H21* are all pigmented and assume the average negative t values of 20.4058, -15.9284, -12.7082 and -11.1229, respectively. These t values are all significantly less than the five percent significance value of ±1.960. Therefore, this data suggests that catalase activities of the pigmented are significantly less post-irradiation (Fig. 5). Catalase activities of

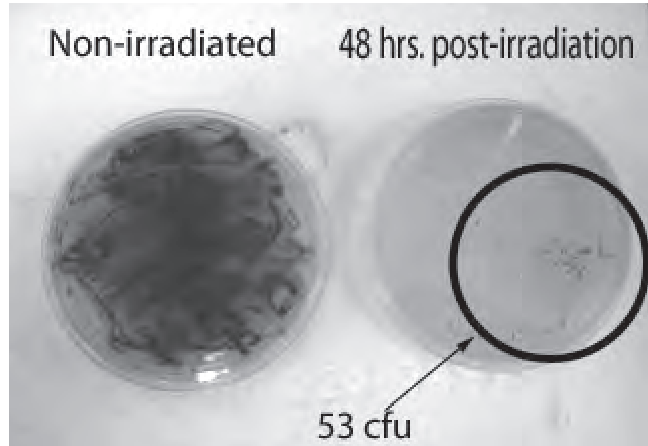

Figure 3. A pigmented strain of *Chromobacterium violaceum* shows growth 48 hours after UV-C irradiation, suggesting violacein is protective.

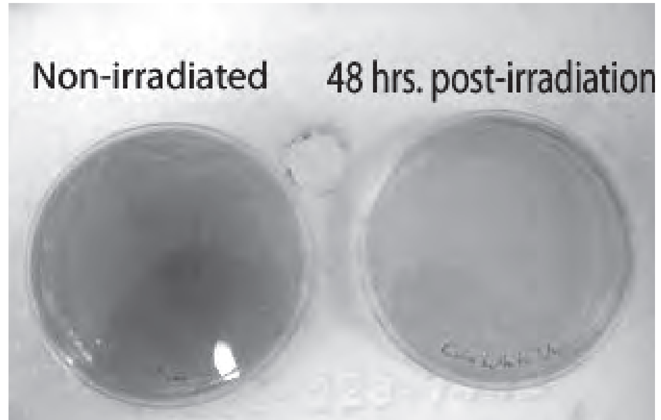

Figure 4. A non-pigmented strain of *Chromobacterium violaceum* shows no growth over 48 hours after UV-C irradiation, suggesting the lack of violacein made bacterial DNA more susceptible to UV-induced damage.

Catalase Activities of Pigmented Strains Before and After Irradiation

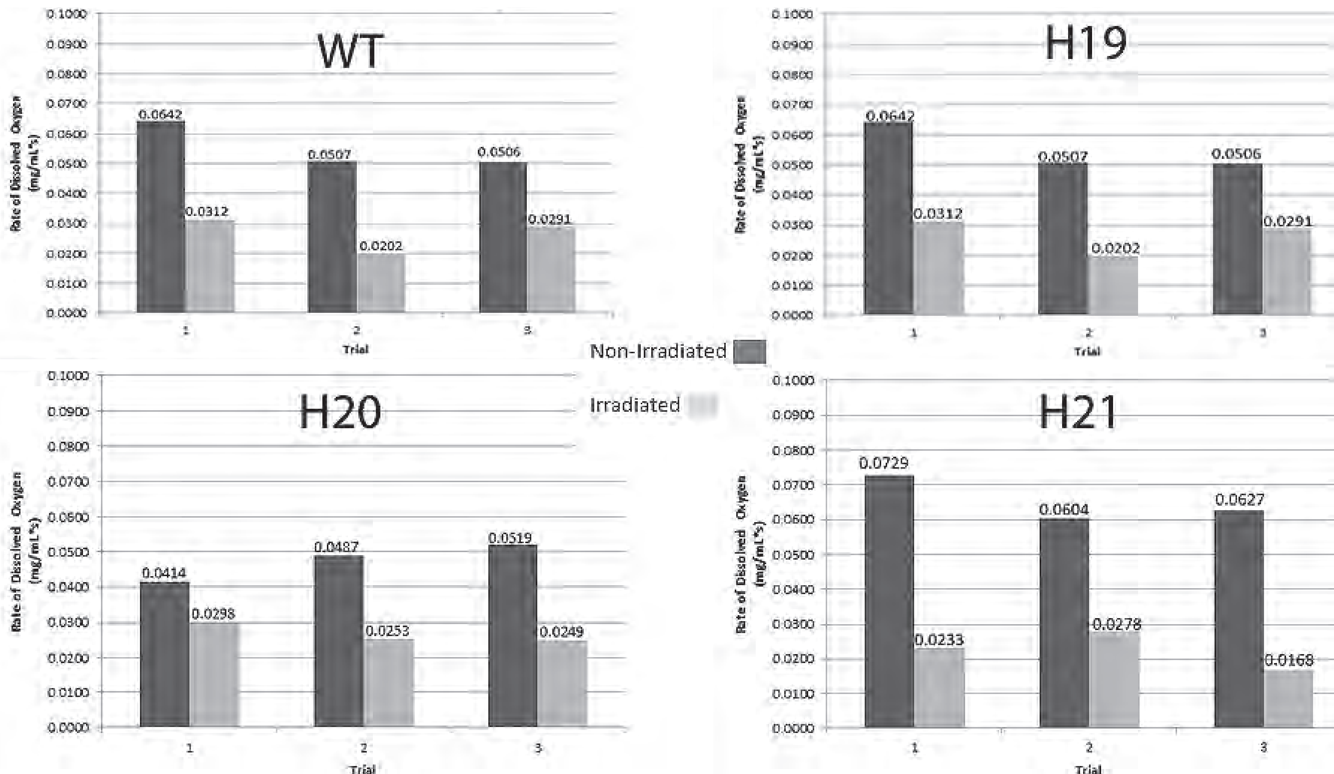

Figure 5. Catalase activities of pigmented strains before and after irradiation statistical analysis: H_0: $\mu_1=\mu_2$; H_A: $\mu_1\neq\mu_2$; *WT*: $t_0=\pm1.960$ $t_1=-22.8719$ $t_2=-17.5127$ $t_3=-20.8327$; H19: $t_0=\pm1.960$ $t_1=-11.0643$ $t_2=-20.9391$ $t_3=-15.7817$; H20: $t_0=\pm1.960$ $t_1=-11.0643$ $\mathbf{t}_2=-13.4002$ $t_3=-13.6600$; H21: $t_0=\pm1.960$ $t_1=-12.1711$ $\mathbf{t}_2=-8.1113$ $t_3=-13.0863$

non-pigmented mutants significantly increased post-irradiation. *CV9*, *CV13 and CV14* are all non-pigmented and assume the average positive t values of 16.2441, 27.0759 and 26.2194, respectively. These t values are all significantly greater than the five percent significance value of ±1.960. Therefore, this data suggests that catalase activities of the pigmented are significantly greater post-irradiation (Fig. 6). These data are summarized in Figure 7.

DISCUSSION

DNA Assays

DNA damage was assayed through sub-culture post-irradiation. Twenty-four hours after sub-culture, none of the bacterial strains showed growth. Bacterial strains continued growing, and after 48 hours, only sub-cultures of *WT* and hyperproducers showed growth. The UV dosage was not germicidal to the pigmented strains. However, the UV-C irradiation did cause significant UV-induced DNA damage, as evidenced by the loss of cell viability. After sub-culture for more than 48 hours, the non-pigmented strains showed no growth, post-irradiation. This data suggests that the lack of bacterial pigmentation made the bacterial DNA more susceptible to UV-induced DNA damage (Fig. 3 and 4). Although both pigmented and non-pigmented strains of *C. violaceum* were exposed to the same dose of UV-C radiation, the pigmented strains showed less DNA damage and higher cell viability. It is hypothesized that the lack of violacein in the non-pigmented strains allowed more DNA damage as evident by the loss of all cell viability. It is reasonable to question whether mutagenesis with NG affected cell viability; however, multiple assays produced congruous results respective to each strain, suggesting that the mutagenesis with NG did not significantly affect genes that are important to cell function or cell growth. Because the only variable changed was the amount of violacein, it is assumed that the violacein pigment is protective against UV-induced DNA damage.

Catalase Assays

UV-induced damage was further studied through an enzymatic catalase assay post-irradiation. Prior to experimentation, it was expected that the pigmented mutants would be protected from UV-C irradiation, showing insignificant protein inhibition. It was also expected that non-pigmented mutants would be more susceptible to UV-induced damage and, therefore, catalase activities would be significantly reduced or inactivated. All pigmented strains (*WT* and hyperproducers) showed significant decreases in catalase activity post-irradiation, according to the paired t-test at the five percent significance level. This data suggests that violacein was not completely protective of catalase (Fig. 5). All non-pigmented strains (non-pigmented mutants) showed significant increases in catalase activity post-irradiation according to the paired t-test at the five percent significance level. Increased catalase activity in non-pigmented bacteria can potentially be explained by the lack of violacein (Fig. 6). The lack of the UV-protective pigmentation may have rendered the cells more susceptible to ultraviolet damage through the production of free radicals. Free radicals created reactive oxidative species (ROS), such as hydrogen peroxide, which exposed the cells to sub-lethal levels of hydrogen peroxide and to oxidative stress. Consequently, oxidative stress induced one or more catalase

Catalase Activities of Non-Pigmented Strains Before and After Irradiation

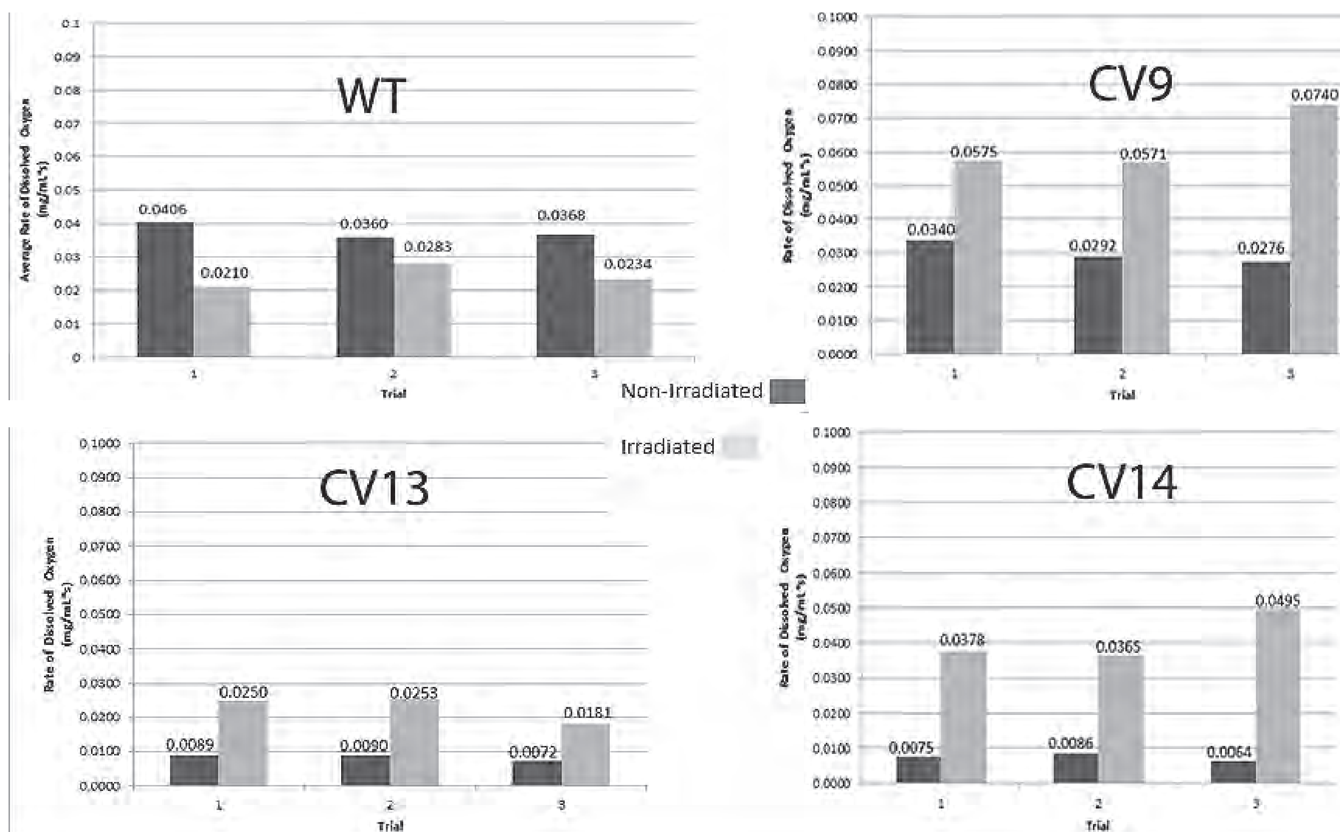

FIGURE 6. Catalase Activities of Non-Pigmented Strains Before and After Irradiation Statistical Analysis: H_0: $\mu_1=\mu_2$; H_A: $\mu_1\neq\mu_2$; *WT*: $t_0= \pm1.960$ $t_1 = -22.8719$ $t_2 = -17.5127$ $t_3 = -20.8327$; CV9: $t_0= \pm1.960$ $t_1 = 14.8614$ $t_2 = 16.1382$ $t_3 = 17.7326$; CV13: $t_0= \pm1.960$ $t_1 = 21.1191$ $t_2 = 33.6032$ $t_3 = 26.5055$; CV14: $t_0= \pm1.960$ $t_1 = 29.9897$ $t_2 = 24.0647$ $t_3 = 24.6037$

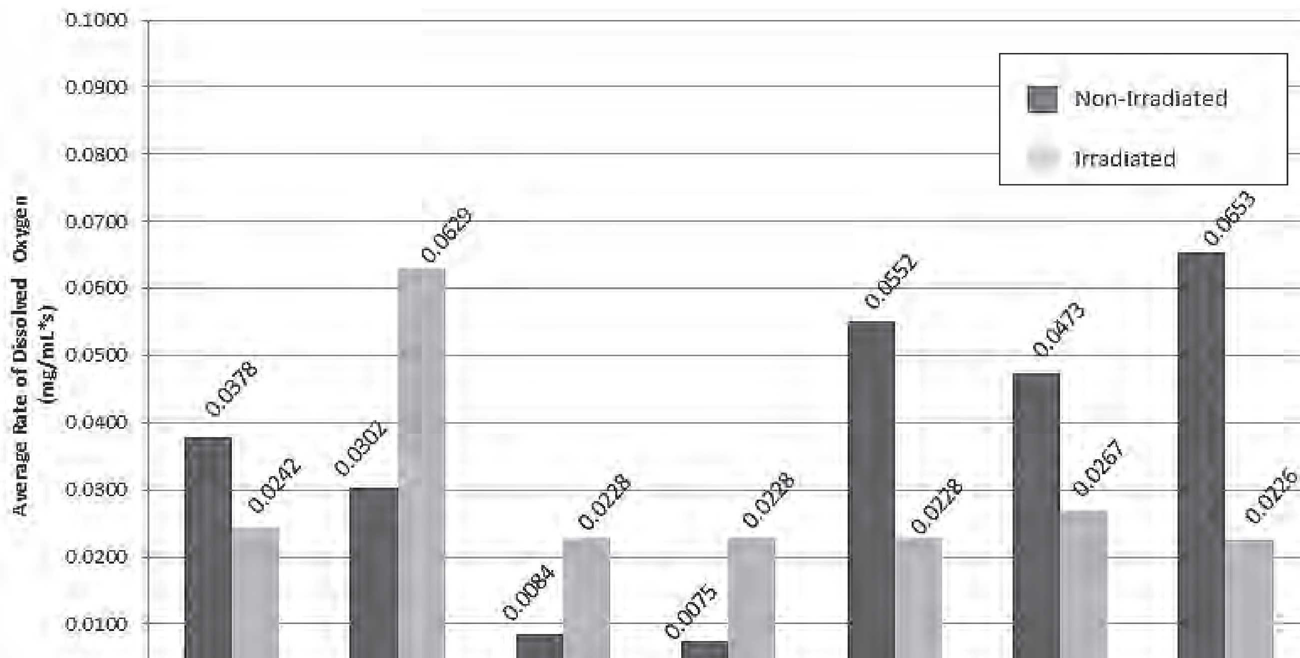

Figure 7. Average Catalase Activity in *Chromobacterium violaceum* mutants

genes, potentially HPI hyperperoxidase *(katG)* - a stress induced enzyme also found in *E. coli* - which was activated in order to handle the newly introduced toxicity. The non-pigmented mutants potentially expressed more catalases (*katE*, *katG*, etc.) because of the lack of violacein, higher susceptibility to UV-induced damage and, therefore, exposure to ROS and oxidative stress. *WT* and hyperproducers expressed primarily the constitutively expressed hyperperoxidase, potentially HPII (*katE*), and catalases present under normal conditions, and therefore showed less catalase activity than the highly catalase-active non-pigmented mutants. Although hyperproducers showed significant decrease in enzymatic activity, it is assumed that violacein is UV-protective because it renders the hyperproducers less susceptible to UV-induced damage by free radicals. The lack of violacein and its protective effects against UV-C irradiation allowed the ultraviolet free radicals to induce more catalase activity in the non-pigmented mutants. These results support the hypothesis that the violacein pigment is protective against UV-induced DNA and catalase damage (Fig. 7). The effects of UV-induced catalase damage are being further studied in varying catalase deficient mutants. Violacein and other bacterial pigments are also being studied in microorganisms for their applications in UV-protection.

Acknowledgements. I thank Central State University Department of Natural Sciences for allowing me to use their microbiology and genetics laboratories. I also thank Tippecanoe High School for allowing me to use Vernier LabQuests and Dissolved Oxygen Sensors.

LITERATURE CITED

Antonio RV and others. 2004. Genetic Analysis of Violacein Biosynthesis by Chromobacterium Violaceum. Genet Mol Res 3(1):85-91.

August PR and others. 2000. Sequence Analysis and Functional Characterization of the Violacein Biosynthetic Pathway from Chromobacterium Violaceum. J Mol Microbiol Biotechnol 2(4):513-19.

Cheng M and others. 2009. *KatE* Complementation Fails to Protect Against UV-A-Mediated Killing in Catalase-Deficient *Escherichia Coli*. Journal of Experimental Microbiology and Immunology 13:63-66.

Goldblith SA and BE. Proctor. 1950. Photometric Determination of Catalase Activity. J Biol Chem 187:705-09.

Lee J and others. 1999. Two Cases of Chromobacterium Violaceum Infection after Injury in a Subtropical Region. J Clin Microbiol 37(6):2068-070. Abstract.

Li Y, Schellhorn HE. 2007. Rapid Kinetic Microassay for Catalase Activity. Journal of Biomolecular Techniques 18(4):185-87.

Liu GY, Nizet V. 2009. Color Me Bad: Microbial Pigments as Virulence Factors. Trends Microbiol 17(9):406-13.

Loewen PC. 1983. Isolation of Catalase-Deficient *Escherichia Coli* Mutants and Genetic Mapping of *KatE*, a Locus That Affects Catalase Activity. J Bacteriol 157(2):622-26.

Matz C and others. 2004. Impact of Violacein-Producing Bacteria on Survival and Feeding of Bacterivorous Nanoflagellates. Appl Environ Microbiol 70(3):1593-599.

Main ER, Shinn LE. 1939. The Determination of Hydrogen Peroxide in Bacterial Cultures. Journal of Biological Chemistry 128:417-23.

Meir E, Yagil E. 1985. Further Characterization of the Two Catalases in *Escherichia Coli*. Curr Microbiol 12(6):315-19.

Oeschger MP, Berlyn MKB. 1974. A Simple Procedure for Localized Mutagenesis Using Nitrosoguanidine. Molecular & General Genetics 134(1):77-83.

Pick E, Keisari Y. 1980. A Simple Colorimetric Method for the Measurement of Hydrogen Peroxide Produced by Cells in Culture. J Immunol Methods 38(1-2):161-70.

Salas JA and others. 2006. Reevaluation of the Violacein Biosynthetic Pathway and its Relationship to Indolocarbazole Biosynthesis. Chembiochem 7(8):1231-40.

Schellhorn HE. 1995. Regulation of Hydroperoxidase (catalase) Expression in *Escherichia Coli*. FEMS Microbiol Lett 131(2):113-19.

Zigman S and others.1996.Structural and Functional Changes in Catalase Induced by Near-UV Radiation. American Society for Photochemistry and Photobiology 63(6):818-24.