

UV EFFECTS ON PLANT-ASSOCIATED PSEUDOMONADS

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Abstract

Concerns about dramatic changes in the Earth's atmosphere, such as ozone depletion, make it imperative to study how microbial communities are responding to such changes that will increase UV irradiance. Ultraviolet A (UVA) and UVB irradiation on the plant pathogen, *Pseudomonas syringae* pv *syringae* (*Pss*), and a plant beneficial, saprophytic root-colonizer, *Pseudomonas putida* (*Pp*) impaired their survival. Stationary-phase cells of both *Pss* and *Pp* were more susceptible to fatal UV damage than logarithmic-phase cells. This observation suggests that an active metabolism is involved in responses to protect against UV. Mutants of *Pss* and *Pp*, with insertions in *rpoS*, *cata*, and *sodA* and *B*, were all more severely affected by UV than the wild-types. These findings lead us to speculate that mechanisms to protect against oxidative stress are important in cell survival against UVA/B irradiation.

Introduction

Sunlight results in the radiances of UVA and UVB wavelengths reaching the Earth's surface. The majority of UVC wavelengths are sorbed by the ozone layer. However, with the deterioration of the ozone layer, resulting from activities on Earth, there is a predicted increase in the dose of radiances the Earth's surface will receive, especially in the UVB wavelengths. The biological consequences are not known. This research centers on the possible impacts of enhanced UVA/B radiation on the interactions between plants and microbes. A myriad of microbes have the ability to cause plant disease, which reduces crop yield and quality. The changes in the UV radiances may affect these interactions leading to an increase or decrease in plant disease.

We will focus on the effects of UVA/B on the survival of two pseudomonads, one a plant pathogen and the other a beneficial microbe that helps protect plants from pathogens. These microbes have distinct environmental niches on Earth. Our hypothesis is that the responses of these organisms to UV irradiation will differ due to their specific niches.

These studies also are relevant to prolonged space endeavors where there is no atmosphere to protect organisms from harmful UV radiation. Also, more information in this area could lead to ways to overcome the obstacle of UV-hypersensitive organisms that could otherwise be used beneficially. For example commercial use of the fungal bioinsecticide, *Metarhizium anisopliae* is hampered by the UV sensitivity of the germinating spores (Braga, et al., unpublished). Perhaps such microorganisms would benefit from expression of UV repair and other protective genes.

Plant Associated Bacteria: *Pseudomonas syringae* and *Pseudomonas putida*

Pseudomonas syringae (*Pss*) is a pathogen that infects the leaves and stems of bean plants, causing brown rot symptoms. This pathogen is often directly exposed to UV irradiation under field conditions because it exists partially as an epiphyte on the plant's surface. In contrast, *Pseudomonas putida* (*Pp*) is a saprophytic root colonizer. *Pseudomonas putida* promotes plant growth and renders the plant more resistant to pathogen attack (Kim, et al., 1997). *Pseudomonas putida* is exposed to UV irradiation less often than *Pss* due to its below ground habitat. Thus, our hypothesis is that *Pp* would not have evolved UV repair mechanisms to the same extent as *Pss*, and therefore, should not be as resistant to UV damage. At present little is known about how these types of plant-associated bacteria protect themselves against UV irradiation.

UV-DNA Damage

The major UVA component (320-400 nm) and minor UVB component (290-320 nm) of irradiation that reaches the Earth's surface are thought to induce different types of problems in biological systems. The UVB component represents the most energetic solar radiation reaching our planet, and causes DNA damage by the formation of adducts between adjacent pyrimidines. Photolyase and UvrABC excinuclease are two enzymes that take part in repairing this short wavelength damage.

In contrast, UVA damage is, for the most part, oxygen dependent. The UVA component challenges cells mainly because of the induction of active oxygen species (AOS), which can attack

several cellular components including proteins, lipids, and carbohydrates. Damage can also be caused to DNA by indirect mechanisms involving the production of hydroxyl radicals through non-catalytic interaction of the AOS, hydrogen peroxide (H₂O₂) and superoxide anion (O₂⁻), with redox-active metal ions (Shennan, et al., 1996). Consequently, these AOS are toxic to organisms.

Protective and Repair Mechanisms in Microorganisms

Several enzymes have been detected in microorganisms that can protect or repair the cell from UV damage. In the bacterium *Escherichia coli*, a complex of enzymes, called UvrABC excinuclease and Fpg (Fapy) glycosylase, seek out and repair oxidized nucleotide bases that otherwise might generate mutations (Delagoutte, et al., 1996). Such mutations could have harmful, if not, lethal consequences. It is thought that the glycosylases recognize the oxidative lesions caused by UVA damage.

Deinococcus radiodurans, a gram positive bacterium, is one of the most UV resistant organisms in existence. It has been found to repair DNA damage from ionizing radiation, UV irradiation, and cross-linking. The *uvrA* homologs of *E. coli* and *Micrococcus luteus* are 60% homologous with *D. radiodurans mtcA* and *mtcB* genes (Battista, 1997).

Protection against oxidative stress, induced by UVA, also is correlated with other enzyme systems. Strains of *E. coli* that are deficient in the hydrogen peroxide-inducible Oxy-R response show hypersensitivity to UVA exposure (Shennan, et al., 1996). Oxy-R is a regulator of at least 20 genes in *E. coli*. A gene encoding catalase, an enzyme that degrades H₂O₂, and the gene encoding Mn superoxide dismutase (MnSOD), which will degrade the superoxide anion are regulated by OxyR. Another regulator, an alternative sigma factor for RNA polymerase, is RpoS. RpoS conditions synthesis of a second set of genes encoding proteins that protect against oxidative stress. A second catalase gene and the *uvrA* gene are two of the genes controlled by this system in *E. coli*.

Studies in A.J. Anderson's lab have identified homologous genes in plant-associated pseudomonads. *Pseudomonas putida* produces three catalases: catalase A that is the major catalase, and catalases B and C that are produced in stationary phase growth and in response to oxidative stress (Kim, et al., 1997). The gene for *catA* has been mutated to derive mutants of *Pp* that lack this enzyme. A gene encoding UvrA is present

downstream of the *catA* gene in the gene sequence *catA*, *bfr*, *uvrA*. Studies with *Pss* show that six isozymes of catalase are produced, under different regulatory conditions, and that the *rpoS* gene is present.

It appears that many of these AOS relief proteins work in conjunction with one another. For instance, Miller et al., (unpublished) found RpoS-mutants produce more MnSOD activity than the parent strain, although they lack the expression of the catalase B isozyme (Miura, et al., 1998 and Miller et al., unpublished). The genes encoding both MnSOD and FeSOD have been identified for both *Pp* and *Pss* and mutants prepared that lack either or both of these isozymes.

Proposed project

With this background the proposed project is to:

- 1) Compare the responses of UV exposure between *Pp* and *Pss*. The hypothesis is that *Pss* should be more resistant than *Pp*.
- 2) Use the mutants in *catA*, *rpoS* and *sod* to explore the roles of these gene products in the survival of the microbes against UV challenge.
- 3) Detect whether a *uvrA* gene was present in *Pss* using the *uvrA* gene from *Pp* as a screen. The eventual goal will be to examine the UV survival of *Pss* mutants lacking *uvrA* expression.

Methods

Evaporation Experiments

Studies were performed to minimize the evaporation rate during UV chamber incubations studies. Evaporation was assessed by measuring the weight of the petri dish system with time of chamber incubation.

Three variations in coverings were tested for their ability to reduce water evaporation in the chamber. These variations included a filter lid, the Parafilm-sealed plastic lid, and no lid. The filter lid was a modified plastic petri dish lid with the middle removed and a round piece of Mylar inserted and secured with glue. The Parafilm-sealed plastic lid served as a control for minimal to no evaporation. The absence of a lid was used to measure maximum evaporation.

The petri dishes, each containing three ml of de-ionized water each, were placed in clusters of three (all three lids types included in each cluster) in various regions of the UV-chamber: front right, back right, center, and front left. The purpose of this placement was to see if at any particular location

there was a higher rate of evaporation. The fan was located in the center back just above the rack where the tray of samples was placed. Trays of standing water were placed in the chamber during both studies to try to decrease evaporation by humidifying the air. There were more free water containers used in the second study.

UV Sensitivity Studies

The pseudomonads used in the studies were the wild-type strains of *Pp* and *Pss* and mutants deficient in catalase A, RpoS and both SOD A and B proteins. The mutants were derived as described by Kim et al., (1997) and Miller et al., (1997). All cultures were stored at -70°C and were initiated into growth by transfer into Luria broth to obtain an overnight culture.

UV Chamber Experiments

Pp and *Pss* and their mutants were examined for differences in UV-irradiation sensitivity in logarithmic and stationary growth phases. Laboratory-irradiation experiments were conducted in a chamber at $27 \pm 1^{\circ}\text{C}$. A 0.13 mm-thick cellulose diacetate film was used to allow both UVA and UVB to impinge on the bacterial cultures. This diacetate film eliminated exposure to the bacteriocidal UVC irradiation. Mylar film filter lids were used to create a negative control, to block out the majority of UVA, UVB, and UVC-irradiation. The weighted irradiances of this UV-chamber were 920 mW m^{-2} . According to the Fiscus and Booker model (1998), the chamber conditions simulated sunlight at noon on April 20 at our location (41.5N latitude, 1.5km elevation) with 0% ozone depletion.

The effects of solar irradiation

This study was performed on the roof of the Biology and Natural Resource building at Utah State University at 11:37a.m. - 3:35 p.m. on August 5, 1999. It started out as a sunny day with no clouds and throughout the afternoon clouds gradually became abundant. The cultures that were included in this study were the wild-type *Pss* and *Pp*, and the CatA, SOD AB and RpoS deficient mutants.

The methods were identical to those used in the UV-chamber studies, except the petri dishes were placed on a tray lined with white paper instead of just a white tray and the tray was floated on water after the first sample was taken. The water had been warmed in the sun for the first 33 minutes. The temperature of the water started at 23°C and ended at 38°C . The tray sat low in the container of water to help prevent wind desiccation, but in such a way that

the treatments were always directly exposed to the sun.

UvrA Cloning

DNA manipulations for cloning (library screening, plasmid mini-preps, restriction digests, DNA purification from agarose gel, and ligation of purified DNA into pBluescript vector) were carried out as described in Ausubel, et al., (1989). For the Southern analysis the Genius system (Boehringer Mannheim, Indianapolis, IN) with chemiluminescent detection was used. The blot was probed with a probe of the *uvrA* prepared from *Pp*.

Results and Discussion

Evaporation Experiments

Preliminary studies using the UV-chamber revealed uneven evaporation rates in the front right corner of the UV-chamber, compared with the other locations within the chamber. This spurred us to perform a series of evaporation experiments, so we could document the conditions inside the chamber and develop methods to produce accurate results, while minimizing the inconsistencies in evaporation. Also, during these evaporation experiments we engineered a petri dish lid that permitted the passage of defined UV irradiances. We found (Fig. 1) that rapid evaporation occurred in the petri dishes without the lid but that evaporation was minimized in the covered samples. Our future studies used only the center of the chamber with no more than four dishes in a row.

UV Sensitivity Studies

Once we minimized the effects of evaporation in the UV-chamber, we analyzed for differences in UV damage susceptibility between *Pss* and *Pp* and selected mutants that included the CatA, SOD AB and RpoS deficient cells.

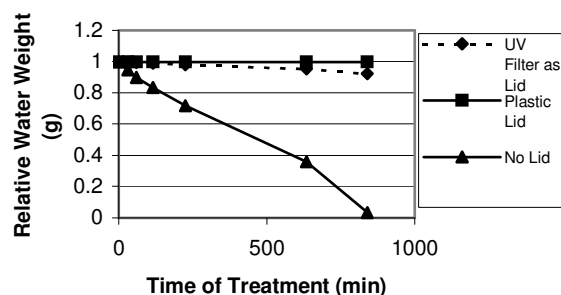


Figure 1. Influences of different lids on sample evaporation in UV chamber.

Survival of all cells decreased with exposure to UV-irradiation in the UV chamber (Fig. 2A and 2B). We found the logarithmic-phase cells were less sensitive than the stationary phase cells for both *Pss* and *Pp*. Also, both *Pss* and *Pp* behaved in a similar manner. This is not what we have would predicted. Generally stationary phase cells are more resistant to stresses than log phase cells. According to Katsuwon and Anderson (1990), pseudomonads tend to have greater defenses against stresses, such as AOS, in stationary phase. These findings suggest to us that active repair mechanisms are required for UV protection and that these were limiting in the stationary-phase cells.

The observation that the two wild-type (Wt) strains of pseudomonads, *Pss* and *Pp*, were relatively similar in their sensitivity to UV does not support our original hypothesis. We suggested that *Pss*, which in its normal habitat as a leaf and stem surface colonizer receives direct UV light, would be less sensitive to UV than the soil inhabiting, *Pp*. However, *Pp* does rely on mechanisms to compete against the oxidative stress imposed by other microorganisms and plant defenses (Kim et al., 1997). If indeed, UV repair mechanisms and oxidative stress mechanisms are correlated, as we suspect, this would be a logical finding.

We observed that the Wt *Pp* cells were more resistant than mutants with the *catA*, *sod AB* or *rpoS* deficiencies (Fig. 3A and 3B). Studies of the populations without UV irradiation showed that both Wt and mutants increased in colony forming units with time of incubation (Fig. 3A). The mutations in *rpoS* appeared to have a more severe effect than that in *catA* and the lack of SOD activities (Fig. 3B). This would support our hypothesis that oxidative stress mechanisms are important in protecting these cultures against UV damage.

Solar irradiation Sensitivity Study

The UV-chamber emits primarily UVB wavelengths. However, the majority of UV reaching the Earth's surface is UVA. Therefore, in order to judge how realistic our results were, we performed natural UV sensitivity studies outdoors. Wildtype *Pp*, and *Pp* mutants lacking *catA* or *rpoS* and *Pss* lacking *sodA* and *B* showed declining viability with exposure to sunlight (Fig. 4). Further studies on the effects of outdoor UV-irradiation should coincide with future UV-chamber studies. Also, acquiring a lamp that emits primarily UVA and less UVB would be beneficial in comparison with natural UV.

Conclusions

- 1) The UV-chamber studies have allowed us to develop reliable materials and methods to be used in laboratory and outdoor solar-irradiation studies. For instance, a modified petri dish with an UV-filter lid was developed to reduce evaporation.
- 2) Unexpectedly a soil and an above ground inhabiting pseudomonad had similar sensitivities to UV. Studies with additional strains are needed to determine whether this is a general trend.
- 3) Sensitivity appeared to be less in stationary phase cells than logarithmic cells. Whether transcription from protective genes is involved requires study.
- 4) Mutants altered in enzymes concerned in protection against oxidative stress were required also for protection against UV exposure. In yeast UV-protection involves both cytochrome- P-450 and catalase activities. Catalase activity not only increases in the presence of irradiation, but when cytochrome P-450 is inhibited, the cells become more sensitive to H₂O₂ (Morichetti, et al. 1998).

UvrA Cloning

Once we found that *Pss* was sensitive to UV-irradiation, we initiated cloning of a *uvrA* homolog in *Pss* (Fig. 5A and 5B) Bands that hybridized to the *uvrA* probe from *Pp* were observed in analysis when different restriction enzymes were used to restrict the DNA from *Pss*. Further subcloning is needed to facilitate the sequencing of the *uvrA* gene from *Pss*.

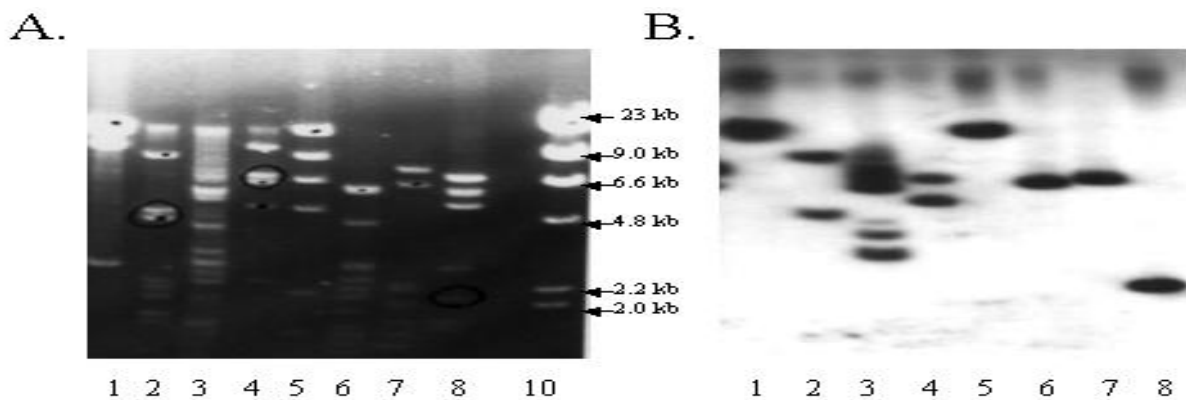


Figure 5. Agarose gel (A) and Southern analysis (B) of *P. syringae* genomic clones UV5 and UV8. (A) The genomic clones UV5 (lanes 1-4) and UV8 (lanes 5-8) were digested with *Cla*I (lanes 1 and 5), *Sa*I (lanes 2 and 6), *Ava*I (lanes 3 and 7), and *Hind*III (lanes 4 and 8). (B) Southern analysis of the agarose gel was transferred to a nylon membrane and probed with the *P. syringae uvrA* gene. Bands of hybridization on the autoradiogram are identified by black bands.

5) A *uvrA* gene appears to be present in *Pss* but until this gene is sequenced its identity will not be verified

6) In comparison to UV-irradiation sensitivity studies with fungal spores of *Metarhizium anisopliae*, bacteria are more sensitive to UV damage. Spores of *Metarhizium* have a LD 50 of 6.5.h (Braga, et al., unpublished), whereas we predict that the pseudomonads would be totally eradicated by this time period.

Questions that we would address in the future would be: are there *uvrB* and *C* genes in *Pss*; if so, do they work independent of each other; are mutants in these genes altered in their survival to UV exposure; are these genes continuously expressed, even in conditions of darkness such as night time, or indirect light on the underside of leaves; are the genes expressed in organisms that inhabit soil or root systems; is the expression of the genes affected by other genes such as *rpoS*, *cat*, and *sods*?

In conclusion, we need to know more about how *uvr* genes relate to oxidative stress in bacteria and fungi. Also, in the future we could explore the effects of transformation of foreign genes into other isolates to determine whether we can engineer enhanced resistance to UV exposure.

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