2000

UV screening mechanisms that protect *Haloarcula* sp. from the space environment

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DOI: <https://doi.org/10.31979/etd.6xcs-4jrv>

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**UV SCREENING MECHANISMS THAT PROTECT *HALOARCULA SP.*
FROM THE SPACE ENVIRONMENT**

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Christine Noel Sawyer

May 2000

UMI Number: 1399817

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ABSTRACT

UV SCREENING MECHANISMS THAT PROTECT *HALOARCULA SP.* FROM THE SPACE ENVIRONMENT

by Christine Noel Sawyer

The objective of this study was to assess the effect of high levels of salt, and carotenoid pigments and their precursors, in protecting *Haloarcula G* from UV radiation and desiccation. It was found that NaCl, which may function to absorb UV light, had no protective role. Growth of *Haloarcula G* in diphenylamine inhibited the synthesis of the modified carotenoids, bacterioruberins, with a concomitant increase in a precursor that resulted in increased cell survival when exposed to UV irradiation. UV/Vis spectroscopy of total cellular lipid extracts and thin layer chromatography of neutral/non-polar lipids and polar lipids showed the absence of bacterioruberins and the accumulation of entirely different lipid components in diphenylamine treated cells compared to untreated cells. These data suggest that the carotenoid precursor compounds, rather than N-stage carotenoids such as the bacterioruberins, have a protective role against UV radiation.

ACKNOWLEDGMENTS

I would like to thank my friends and family for all their encouragement. To my grandmother, Melva, and my mother, Betty, thanks for all your patience through the seemingly endless thesis process. To my father and to Thea, thanks for all the coffee breaks. To Dr. Rose Grymes, who inspired me to enter the field of molecular biology. Her strong character, leadership, and ability to delegate more responsibility was a critical source of my development as a scientist. To Babak Alizadeh and Michelle Ogden, your friendship and encouragement will always be appreciated. To my thesis committee, especially Rocco Mancinelli and Steven White, both of your guidance and input were invaluable to the project. To my boyfriend, Matt Buchanan, thanks for all your love and support.

TABLE OF CONTENTS

	Page
LIST OF FIGURES	vii
INTRODUCTION	1
MATERIALS AND METHODS	5
Cell Culture	5
UV Irradiation	6
Cell-Cell Shading	6
Effect of NaCl in UV Protection	6
Determination of Cell Numbers	7
Carotenoid Pigment Inhibition	8
Lipid Extraction	9
Thin Layer Chromatography	10
RESULTS	11
Output of the Deuterium Lamp	11
Cell-Cell Shading	12
Protection from UV Irradiation by NaCl	14
Carotenoid Inhibition	17
Lipid Analysis	21
DISCUSSION	25
REFERENCES	29

LIST OF FIGURES

Figure	Page
1. Emission Spectra of Deuterium Lamp.....	11
2a. The Effect of Cell-cell Shading on Survival of <i>Haloarcula</i> G to UV	13
Irradiation.	
2b. “	13
3. The Protection from UV Irradiation Provided by 120 ul 25% NaCl	15
Solution.	
4. The Protection from UV Irradiation Provided by 160 ul 25% NaCl	16
Solution.	
5. The Protective Effect of Salt without Rehydration/drying Cycles.....	17
6. UV/Vis Absorbance Spectra of Total Lipid Extracts of <i>Haloarcula</i> G ..	18
with and without the Presence of 1.0 ug/ml Diphenylamine.	
7. Survival of <i>Haloarcula</i> G after Inhibition of Carotenoid Synthesis.....	19
by 0.45 ug/ml Diphenylamine.	
8. Survival of <i>Haloarcula</i> G after Inhibition of Carotenoid Synthesis.....	19
by 1.0 ug/ml Diphenylamine.	
9. Survival of <i>Haloferax denitrificans</i> after Inhibition of Carotenoid.....	20
Synthesis by Growth in the Absence of Oxygen.	
10. The Fractionation of Neutral/non-polar and Polar Lipids on a	22
Silica-G Plate.	
11. The Fractionation of Neutral/non-polar and Polar Lipids on	23
an Alumina Plate.	

INTRODUCTION

Early Earth had a high UV radiation flux at its surface due to a lack of O₂ and O₃ in the atmosphere. Evolution of the first organisms on Earth had to survive this high UV flux. Members of the domain Archaea are known not only to survive in, but often to require extremely hot, salty, cold, acidic, or alkaline environments, suggesting that the range of environmental conditions that can support life is much more permissive than previously thought (Woese & Wolfe, 1985). This adaptability of life suggests that microbes may be able to evolve even in environments previously thought unsustainable to life. If life could arise in extreme and inhospitable conditions on Earth, it is possible that life may also have arisen on Mars, as it is probable that Mars had similar composition, temperature and atmospheric conditions as early Earth (Horneck & Brack, 1992). The discovery of potential remnants of extraterrestrial life in the Mars meteorite ALH84001 raised the possibility not only that life may have existed on Mars, but that it could have been transported in a meteorite from Mars to Earth or vice versa (McKay et al., 1996). The arrival of extraterrestrial material either by comets, meteorites, or interplanetary dust particles may have had an important role in the origin of biogenic organics on Earth. Geologic evidence shows that approximately 3.9 billion years ago there was a showering of meteorites to the Earth that was a 10⁴ times greater rate than that of today (Horneck & Brack, 1992). These meteorites have been shown to contain up to 5% organic material, and some contain up to 17 different amino acids (Lawless & Peterson, 1975; Cronin, 1976; Cronin, Pizzarello & Cruikshank, 1988; Stoks &

Schwartz, 1981; Hayatsu & Anders, 1981). It is possible that extraterrestrial material may have been an important input of nitrogen containing organics to early Earth. With regard to the transfer of whole organisms, some present day organisms are surprisingly resistant to the extreme conditions of solar radiation, vacuum, and cold temperatures of the space environment.

A long term-duration flight tested the survival of *Bacillus subtilis* spores and found that if spores were coated with a thin layer of glucose, they retained 80% viability after 6 years of exposure to the space environment (Horneck, Bucker, & Reitz, 1994). However, if a monolayer of *B. subtilis* spores were exposed to solar radiation without a layer of glucose, none survived (Horneck et al., 1994). Multiple experiments exposing a variety of organisms, including *Deinococcus radiodurans* and conidia of *Aspergillus*, to the space environment demonstrate that while some organisms can survive the vacuum of space which causes dehydration, few can survive the combination of vacuum and solar radiation (Dose et al., 1995). In fact, the addition of vacuum compounds the lethality of solar radiation (Horneck, 1993).

On Biopan, a European satellite designed to expose samples to the space environment, *Haloarcula* G was chosen for flight because in its normal habitat of ocean evaporitic ponds, it is exposed to both desiccation and UV radiation on a regular basis. *Haloarcula* G cultures have survived 15 days of full exposure to the space environment accumulating a total dose of 10,000 KJ/m² on the Biopan I flight (Mancinelli, White, & Rothschild, 1998). The implications of this survival are several-fold. It is the first

example of a vegetative cell surviving the typically lethal space environment.

Understanding which cellular attributes protect *Haloarcula* G will give us a better idea of which types of organisms could have survived the high levels of UV irradiating early Earth. Of equal importance is understanding what types of organisms may have the potential to survive the transfer to other planetary bodies by spacecraft or meteorites. Finally, studies of these extremophiles may help focus our search for where on Mars we may find remnants of extinct life.

When *Haloarcula* G was flown on Biopan I, it was dried in saline solution which placed it in a dormant state without available nutrients (Mancinelli et al., 1998). This restricted the cell's ability to activate metabolic processes while being exposed to the space environment. *Haloarcula* G either had to have compounds in the cell at the time of drying that protected the organism from space, or the post-rehydration cellular repair processes activated after rehydration in medium are responsible for permitting the organism to survive. This study aims to evaluate the effectiveness of compounds present in the cell at the time of drying that may assist survival in the space environment.

Haloarcula G, members of the Archea domain, are extreme halophiles that require 3 to 4 M NaCl (Larsen, 1962). Such levels of salt are approximately 100X the concentrations of salt typically found in the environment of most microbes. Since *Haloarcula* G are found embedded in salt crystals, it normally exists in a desiccated environment. Like all other life forms on Earth, *Haloarcula* maintains a

sodium/potassium gradient across the cell membrane. With a 3 to 4 M NaCl concentration outside the cell, this gradient requires 4 to 5 M potassium chloride inside the cell. Solid crystals, such as the solid salt crystals in which *Haloarcula* are embedded, may diffract light away from the cells which accumulate between the planes of salt crystals. The hypothesis of this study is that high levels of extracellular salt protect the organism from UV radiation, and that the carotenoid pigments act as an additional UV shield.

Haloarcula G synthesizes a group of C50 modified cyclic carotenoids called bacterioruberins (Kushwaha, Kramer, & Kates, 1975). Cyclic carotenoids, as opposed to acyclic carotenoids, have been shown to protect organisms from UV radiation by quenching triplet state photosensitizers and singlet oxygen, but not from hydroxyl radicals produced during photosynthesis and metabolism (Tuveson & Sandman, 1993). There is conflicting data with respect to carotenoids acting as a UV shield, possibly depending upon what wavelength of UV light was used. Hescox and Carlberg (1972) for example, found no UV shielding by carotenoids in *Halobacterium cutirubrum*, but found enhanced photolyase activity in the presence of carotenoids, although it is not clear how much of the far UV spectrum was involved in those experiments. Tuveson, Larson and Kagan (1988) found that carotenoids protect cells in the near UV region, but have no effect in the far UV region. This study aims to test if in addition to salt, the carotenoids can act as a passive screen, absorbing UV radiation in the far UV region.

MATERIALS AND METHODS

Cell Culture

A pure culture of *Haloarcula*, designated isolate G, which was isolated from a salt crystal obtained from an evaporation pond at Exportadora del Sal, S. A., Guerrero Negro, Mexico was used for these experiments. It was grown in casein medium (250 g/L NaCl, 5.0 g/L hydrolysed casein, 5.0 g/L yeast extract, 20 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2 g/L KCl, 0.2 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, pH 7.4) at 37° C in a rotary shaker at 160 rpm until it reached mid-log phase of growth. *Haloferax denitrificans*, S1 (ATCC #35960, Manassas, VA) was grown in Hd medium (176 g/L NaCl, 2.0 g/L hydrolysed casein, 5.0 g/L yeast extract, 20 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2.0 g/L KCl, 0.091 g/L CaCl_2 , 12.0 g/L HEPES, 5.0 g/L KNO_3 , pH 6.7), at 37° C in either sealed flasks sparged for 30 minutes with nitrogen gas and kept stationary, or aerated in a rotary shaker at 160 rpm. The cells were washed five times in sterile 25% (wt/vol) NaCl solution to remove medium, extracellular protein, and sugars that might protect cells from radiation, and were then serially diluted to a concentration of 2.5×10^8 cells/ml. 1.0×10^7 cells were pipetted onto sterile 7 mm quartz disks and allowed to dry in a forced air incubator at 30° C for 45 minutes. Based on the surface area of the cell and that of the disk, this cell number should achieve a theoretical monolayer of cells. Cultures for each experiment were initiated from slants established for *Haloarcula* G and stored at 4° C. For each experiment a new slant was used to begin a starter culture which was used at a 1% concentration to inoculate medium used in experiments.

UV Irradiation

Cells were exposed to 2.91×10^{-6} Watts/cm²nm at 250 nm emitted from a deuterium lamp (Oriel Model #68840, Stratford, CT) for varying amounts of time at a distance of 2.5 cm from the lamp. The spectral output of the deuterium lamp as indicated in manufacturer specifications is strongest from 200 nm to 360 nm. Real time output was measured by an OL 752 spectroradiometer (Optronics, Chelmsford, MA) calibrated from 250-800 nm. During irradiation, cell monolayers were enclosed in a black felt lined box to block stray light. A vacuum line was fed into the enclosure to remove ozone produced by the deuterium lamp.

Cell-Cell Shading

To test if plating 1.0×10^7 cells/ 7 mm disk achieved a theoretical monolayer of cells, ½ the standard volume was plated onto disks and allowed to dry followed by another ½ volume plated on top of the previous layer in order to create a double layer of cells. This procedure should exhibit cell-cell shading effects if the total number of cells plated is too high. Samples were also examined by microscopy.

Effect of NaCl in UV Protection

Three experiments tested the role of salt in shielding cells from UV radiation by light absorption. Two tests consisted of layering salt on top of cell monolayers by pipetting a 10 ul aliquot of a 25% aqueous NaCl solution onto the cell monolayer and

allowing it to dry. This was repeated for a total of 12 and 16 layers, constituting a cumulative salt layer of approximately 1.5 mm and 2.0 mm thick, respectively.

Experiments were conducted in sextuplicate for each condition and time point. The third test was designed to exclude rehydration and drying cycles that occur when overlaying 25% aqueous salt solution on top of the cell monolayers. Twelve layers of 10 ul 25% salt were pipetted into the wells of a 96-well quartz plate. Cells were then pipetted on top of the salt layer and allowed to dry. UV radiation was directed from the bottom of the plate to the top. Control cells were pipetted directly into the quartz plate with no intervening salt layer. The distance from the plane of the monolayer to the UV lamp was 2.5 cm and was kept constant for the cells with and without an intervening layer of salt. The experiment was conducted in replicates of 25 for each condition.

Determination of Cell Numbers

Because *Haloarcula* G grows erratically on solid medium, viable cell numbers were determined in liquid culture by using the most probable number method (Alexander, 1982). The most probable number method is a statistical method used to estimate the number of viable organisms in liquid culture. Briefly, the sample is diluted until statistically less than one organism is present in the solution. Multiple aliquots for the diluted solution are inoculated into fresh medium and the percentage of aliquots with positive growth is determined. The original cell number can be determined mathematically from the information derived by the dilution that exhibits between 12 - 40% sterile samples. In these experiments, dry cell monolayers were dissolved into 1.0

ml of a 20% NaCl solution. This concentration of salt permits solubilization of the dry monolayer/solid salt sample without causing cell lysis. The 1.0 ml cell suspension was serially diluted 10-fold to 1.0×10^{-12} and 20 μ l of each dilution inoculated into 180 μ l casein medium in 96-well plates. Between 8 - 32 samples from each dilution were inoculated and incubated at 30° C for 4 - 5 weeks. Positive growth was determined by turbidity and by the presence of pink pigmentation due to carotenoid production.

Carotenoid Pigment Inhibition

In order to inhibit the production of bacterioruberins, *Haloarcula* G was incubated at 32° C in the presence of 1 μ g/ml diphenylamine (DPA) for 9 days in a rotary shaker at 100 rpm in one liter shake flasks, or for 2 days in 0.45 μ g/ml DPA in 0.5 liter shake flasks. The DPA solution was freshly made for each experiment at 1 mg/ml in 100% ethyl alcohol. Control cultures received an equal volume of 100% ethanol as the DPA treated cultures. Cells were prepared for UV treatment by five washes in 25% (wt/vol) saline followed by drying at a concentration of 1.0×10^7 cells on quartz disks as described previously. Samples treated with 0.45 μ g/ml DPA were assayed with replicates of 24 for each condition and time point. Cultures receiving 1.0 μ g/ml DPA were assayed with replicates of 12 for each condition and time point.

Lipid Extraction

Lipids were extracted by the modified Bligh and Dyer method (Bligh & Dyer, 1959; Kates, & Kushwaha, 1995). Briefly, cells were harvested by centrifugation at 4,000 x g for 10 minutes. The supernatant was decanted and cell pellet resuspended in 25% saline. The cells were pelleted at 4,000 x g for 10 minutes, supernatant decanted, and the wet weight determined. The cell pellet was resuspended in 25% saline and extracted twice with 3.75 volumes of methanol: chloroform (2:1). The organic phase was removed, 0.5 volume chloroform added, and followed by addition of an equal volume of water. The suspension was centrifuged at 5,000 x g for 10 minutes to break the emulsion. The organic phase was removed and dried under a stream of nitrogen gas at 40° C. The total lipid fraction was resuspended in 1.0 ml HPLC grade methanol and absorbance measured by UV-Vis spectroscopy. For thin layer chromatography analysis, total lipids were further fractionated into polar lipids and neutral/non-polar lipids by resuspending dry total lipids in 10 ml chloroform: methanol (1:1). Any insoluble material was pelleted at 7,000 x g for 10 minutes and the supernatant removed to a new tube and dried under a stream of nitrogen gas at 40° C. Dried lipids were resuspended in 1.0 ml chloroform: methanol (1:1) followed by addition of 10 volumes of -20° C acetone. The polar lipids were allowed to precipitate by incubating at -20° C overnight and were pelleted at 5,000 x g for 20 minutes. The acetone soluble fraction was removed and dried at 40° C under a stream of nitrogen gas. The polar lipid fraction was washed 3 times with acetone, cooled to -20° C, and dried in a vacuum desiccator in the

dark. Dry weights were determined. Polar lipids were resuspended in chloroform, and neutral/non-polar lipids resuspended in acetone.

Thin Layer Chromatography

Neutral/non-polar lipids and polar lipids were analyzed on silica G plates (Alltech, Deerfield, IL) using 1% ethyl ether in hexane as a solvent and on alumina plates (Alltech) using 7% methanol in chloroform as a solvent (Kates & Kushwaha, 1995). Briefly, the plates were washed twice in chloroform: methanol (1:1) by allowing the solvent front to ascend the plates by capillary action. The plates were allowed to dry for 5 minutes between each wash. The plates were then baked at 120° C for 2 hours prior to loading with lipids. Lipids were loaded and run in a humidified tank for 20 - 30 minutes. The plates were allowed to dry for 5 minutes, and stained by immersion in 0.0006% Rhodamine G solution for 5 minutes and viewed with UV light. All lipid components were then visualized permanently by charring with 30% aqueous sulfuric acid followed by heat (Kates, 1986).

RESULTS

Output of the Deuterium Lamp

To ensure reproducibility of UV exposure and to more accurately determine the energy output of the UV lamp, thus providing estimates of cumulative UV exposure, the spectral output of the lamp was measured from 250 - 800 nm (Figure 1). The spectral output remained constant throughout the course of these experiments.

Figure 1. Emission Spectra of Deuterium Lamp. Spectra of lamp used to irradiate samples as measured with an OL-752 spectroradiometer (Optronics, Chelmsford, MA) at a distance of 2.5 cm from the lamp.

Cell-Cell Shading

Cell-cell shading has been shown to protect organisms from UV radiation (Kruschel & Castenholz, 1998; Brenowitz & Castenholz, 1997). Cell-cell shading occurs when organisms in upper layers of a multi-layered bacterial mat attenuate UV light and protect organisms in the lower layers. Since the aim of these studies was to understand what components of the cells could provide shielding from UV radiation, these experiments were designed to minimize cell-cell shading effects. As shown in Figure 2, by attempting to produce cell-cell shading by plating cells directly on top a previous layer of cells, there was no significant difference in survival from cells that were plated in a single layer.

Figure 2a. The Effect of Cell-cell Shading on Survival of *Haloarcula G* to UV Irradiation. Error bars show standard deviation. N/N_0 is the fraction of cells surviving after drying and UV exposure. Replicates were 6 per group and time point.

Figure 2b. The Effect of Cell-cell Shading on Survival of *Haloarcula G* to UV Irradiation. First three time points shown in Figure 2a.

If shadowing was occurring, one would expect an increase in the survival fraction. Since there was no significant difference in the survival fraction, one of two

conditions exist. Either there was no significant degree of cell-cell shading or shading was already maximal in the single layer samples and thus could not be increased by adding yet another cell layer. Given the initial cell number applied to the 7 mm disks and the lack of obvious cell overlap as determined by microscopy, the former condition of no significant shading is operant, thus confirming that cells are plated at a density low enough that cell-cell shading is not involved in this experimental system. Figure 2 also shows that there is a clear exposure-dependent decrease in the surviving fraction of *Haloarcula* G. Also clear is the sensitivity of *Haloarcula* G to the dry-down and/or rehydration procedure since only about 10% of the original population could be recovered even in the absence of UV exposure (Figure 2b).

Protection from UV Irradiation by NaCl

Reasoning that salt itself might attenuate incident UV radiation and thus provide a protective effect, the survival fraction of *Haloarcula* G monolayers overlaid with additional layers of salt was compared to cultures without additional layers. As shown in Figure 3, there was no benefit gained by the additional layers of extracellular salt.

Figure 3. The Protection from UV Irradiation Provided by 120 ul 25% NaCl Solution. *Haloarcula G* was overlaid with 120 ul of 25% NaCl solution constituting a ~1.5 mm barrier of salt. Error bars show standard deviation. N/N_0 is the fraction of cells surviving after drying and UV exposure. Replicates were 6 per group and time point.

To confirm results shown in Figure 3, the experiment was repeated with 33% more salt layered onto samples than in the previous experiment. As shown in Figure 4, fewer cells survived, overall, in samples with extra salt, even at the 0 time point where no UV irradiation was administered. This curious drop in cell numbers was thought to be due to either additional drying and rehydration cycles that occur as each layer of salt is added to the cell monolayer, or due simply to a general growth inhibitory effect of salt.

Figure 4. The Protection from UV Irradiation Provided by 160 μ l 25% NaCl Solution. *Haloarcula G* was overlaid with 160 μ l of 25% NaCl solution constituting a $\sim$ 2.0 mm barrier of salt. Error bars show standard deviation. N/N₀ is the fraction of cells surviving after drying and UV exposure. Replicates were 6 per group and time point.

The experiment shown in Figure 5 avoids the cycles of drying and rehydration by applying salt layers to a quartz 96-well plate, overlaying cells on top of the salt, and irradiating samples from below the plate. This ensures that all cells experience the dry-down/rehydration procedure only once. Figure 5 again demonstrates fewer cell numbers in the samples with extra salt, however the difference is not statistically significant. Therefore, no conclusions can be drawn regarding general growth inhibition by salt. The rate of cell death is the same for samples both with and without extra salt. This clearly demonstrates that extracellular salt provided no additional protection to cells from UV irradiation.

Figure 5. The Protective Effect of Salt without Rehydration/drying Cycles. A 1.5 mm salt layer was established on a quartz multiwell plate. *Haloarcula* G was overlaid on the salt layer and irradiated from below the plate. Error bars show standard deviation. N/N_0 is the fraction of cells surviving after drying and UV exposure. Replicates were 24 per group and time point.

Taken together, Figures 3, 4, and 5 clearly show there is no advantage to the unique state of extreme halophiles in which they are embedded in solid, crystalline salt.

Carotenoid Inhibition

In addition to serving as a quenching agent of radical species and as a light harvesting molecule, it is also possible that carotenoids function as a UV screen. To test this hypothesis, carotenoid production was inhibited by growth in diphenylamine (DPA). As shown in Figure 6, the carotenoid absorption peaks between 450 - 550 nm are missing in DPA treated cultures. Also noteworthy, is the accumulation of a lipid component absorbing at 278 nm in DPA treated cells (Figure 6).

Figure 6. UV/Vis Absorbance Spectra of Total Lipid Extracts of *Haloarcula G* with and without the Presence of 1.0 ug/ml Diphenylamine.

Surprisingly, Figure 7 appears to show that cultures which fail to produce carotenoids by treatment with DPA, may survive UV irradiation better than untreated controls. The initial surviving fraction in DPA treated cells is much lower than the control, and yet upon exposure to UV, the surviving fraction does not decrease significantly. The controls, however, demonstrate a sharp decline in surviving fraction upon exposure to UV irradiation (Figure 7). To clarify this issue, the experiment was repeated with a greater concentration of DPA. Figure 8 demonstrates more clearly that DPA treated cells are more protected from UV irradiation than untreated cells (Figure 8).

Figure 7. Survival of *Haloarcula* G after Inhibition of Carotenoid Synthesis by 0.45 µg/ml Diphenylamine. Error bars show standard deviation. N/No is the fraction of cells surviving after drying and UV exposure. Replicates were 24 per group and time point.

Figure 8. Survival of *Haloarcula* G after Inhibition of Carotenoid Synthesis by 1.0 µg/ml Diphenylamine. Error bars show standard deviation. N/No is the fraction of cells surviving after drying and UV exposure. Replicates were 12 per group and time point.

Since DPA inhibits the enzyme phytoene desaturase which converts phytoene to ζ -carotene (Goodwin, 1993) which is later converted to more complex carotenoids, treatment by DPA may cause the accumulation of carotenoid precursors and block the production of the more complex C50 carotenoids. To assess if any carotenoid component, precursor or other, may be the agent that protects against UV irradiation, the entire carotenoid pathway was inhibited in a similar species of archaebacteria, *Haloferax denitrificans*. The entire carotenoid biosynthesis pathway can be blocked in *H. denitrificans* by culture under anoxic conditions. As shown in Figure 9, blocking all components of the carotenoid synthesis pathway by growth in N_2 did not provide any protection from UV irradiation.

Figure 9. Survival of *Haloferax denitrificans* after Inhibition of Carotenoid Synthesis by Growth in the Absence of Oxygen. Error bars show standard deviation. N/N_0 is the fraction of cells surviving after drying and UV exposure. Replicates were 6 per group and time point.

Haloarcula G, in the presence of DPA, not only failed to synthesize the modified C50 carotenoids that absorb at 490 nm (Figure 6), but exhibited an accumulation of a lipid that absorbs at 278 nm, which is a favorable absorbance range for protecting DNA and protein from UV irradiation. Although the peak appears to be fairly minor, the absorbance is based up the isolation of total lipids from approximately one-tenth the total number of cells compared to the control (data not shown). Therefore, this small peak represents a significant accumulation of lipid in *Haloarcula* G on a per cell basis.

Lipid Analysis

To further identify the lipid component that absorbed at 278 nm, thin layer chromatography (TLC) of the neutral/non-polar and polar lipids of DPA treated and control cells was conducted. The TLC plates confirm the disappearance of lipids that correspond in RF values to the bacterioruberins, and show an accumulation of lipids with RF values that correspond to bacterioruberin precursors. Specifically, the silica G plate run in 7% methanol in chloroform shows two lipids with an RF of 22 and 15 in control cells that are missing in the DPA treated cells. These RF values are very close to the reported values of bacterioruberin R₂ (RF 21) and neo-bacterioruberin R₁ (RF 14) (Kates & Kushwaha, 1995). The alumina plate in Figure 11 shows accumulation, in the DPA treated cells, of lipids having RF values of 9.5 and 16.8, which are very close to the reported RF values of bacterioruberin precursors, trans-phytofluene (RF 11) and cis-phytofluene (RF 17) respectively (Kates & Kushwaha, 1995).

Figure 10. The Fractionation of Neutral/non-polar and Polar Lipids on a Silica-G Plate. The plate was run in 7% methanol in chloroform, stained with Rhodamine G and then charred by 30% sulfuric acid and heat. a. Control cells, Neutral/Non-polar lipids, b. Control cells, Polar lipids, c. DPA treated cells, Neutral/Non-polar lipids, d. DPA treated cells, Polar lipids, e. Carotenoid complex. $RF = R_f \times 100$.

Figure 11. The Fractionation of Neutral/non-polar and Polar Lipids on an Alumina Plate. The plate was run in 1% ethyl ether in hexane, stained with Rhodamine G and then charred by 30% sulfuric acid and heat. a. Control cells, Neutral/Non-polar lipids, b. Control cells, Polar lipids, c. DPA treated cells, Neutral/Non-polar lipids, d. DPA treated cells, Polar lipids, e. Carotenoid complex. RF = Rf * 100.

As shown in Figures 10 and 11, the picture is much more complex. In sum, there are a total of 10 different lipids that appear in DPA treated cells that are not present in controls. The RF values for these bands are as follows in ascending order: 2.7, 3.7, 6.3, 9.5, 13.9, 15, 16.8, 20, 62, and 75. In addition there is a greater abundance in the DPA treated cells of lipids migrating with an RF of 68, 71, and 77. These values are fairly close to that of squalene (RF 67), dihydrosqualene (RF 73), and tetrahydrosqualene (RF 79) respectively, which should comprise the bulk of the neutral/non-polar lipids (Kates & Kushwaha, 1995). In addition, the silica G plate shown in Figure 10 demonstrates the accumulation of a lipid in the DPA treated cells that has an RF of 31, which does not correspond to any values reported in the literature. In contrast, there were markedly few differences in lipid composition between DPA treated and control cells for the polar lipid fraction as shown in both Figures 10 and 11.

DISCUSSION

There have been reports of cell-cell shading having an important role in providing organisms in lower levels of biomass significant protection from UV radiation (Kruschel & Castenholz, 1998; Brenowitz & Castenholz, 1997). These studies do not attempt to test this suggestion, but rather to understand what specific sub-cellular components may also provide shielding from UV radiation that help explain why some strains are more UV resistant than others. Therefore, these experiments were designed to minimize cell-cell shading effects. Figure 2 confirms that cell-cell shading is not operable in this experimental design.

Despite the fact that NaCl absorbs UV light (Mancinelli, personal communication, January 11, 1996), and despite the unusual characteristics of extreme halophiles being embedded in solid salt, there is no evidence for a significant role for high levels of extracellular salt in protecting *Haloarcula* G from UV radiation in these experiments. The limitation of this approach, of course, is that the concentration of internal osmotica, principally KCl, which is at an even higher concentration of 5 M inside the cell cannot be altered without killing the cells. Although the possibility exists that high levels of internal salt or osmotica can attenuate UV light and protect the organisms, it seems unlikely given the absolute failure of additional extracellular salt to provide any protection. Based on these studies, one can conclude that there is no UV protection acquired by *Haloarcula* G from being embedded in solid NaCl.

The experiment shown in Figure 5 was conceived after noting that samples with additional salt layers exhibited a drop in cell numbers compared to controls even without exposure to UV (See 0 time point, Figure 4). It was hypothesized that the procedure of applying extra layers of salt to samples caused additional cycles of drying and rehydration that incurred additional cell death, similar to the massive cell death (90% of the population) that occurs by the initial dry-down procedure. The experiment shown in Figure 5 avoided this problem, by applying salt uniformly to a quartz 96-well plate, adding cell suspension, and irradiating samples from below the plate. By this method, all cells were dried down only once. Interestingly, the drop in cell numbers repeated, demonstrating that the rehydration drying cycles are not the cause. As far as extra salt inhibiting cell growth in general, this conclusion cannot be drawn from this data because the difference is not statistically significant.

DPA inhibits the enzyme phytoene desaturase which converts phytoene to ζ -carotene which is later converted to a diverse array of carotenoids (Goodwin, 1993). For most organisms that produce carotenoids, DPA causes the accumulation of colorless phytoene and phytofluene and the loss of the more complex colored carotenoids such as α -, β -carotene and lycopene (Lee et al., 1975). The action of DPA in *Haloarcula* may be more complex, possibly causing an increased synthesis of phytoene concomitant with inhibited synthesis of ζ -carotene, which has been evident in other organisms (Lee et al., 1975). This should cause a massive accumulation of both phytoene and phytofluene, the precursors of ζ -carotene. The UV/Vis spectra shows the

absorbance of a lipid component at 278 nm. This corresponds exactly to the absorption peak of phytoene (Kushwaha, Pugh, Kramer, & Kates, 1972). However, there was only evidence for the cis- and trans- phytofluenes accumulating and no evidence of phytoene, which has a reported RF of 46 on the alumina thin layer plate analysis run in 1% ethyl ether in hexane.

Whereas there are few changes in the polar lipid fraction of DPA treated cells and controls, there are marked differences in the neutral/non-polar fraction including the accumulation of at least 9 different lipid components in DPA treated cells and the lack of two lipids normally produced in controls. Specifically, it is likely that cis- and trans- phytofluene accumulate and that the bacterioruberins, such as bacterioruberin R₂ and neo-bacterioruberin R₁, are not synthesized. The results of these experiments implicate the accumulation of phytoene and cis- and trans- phytofluene along with the dearth of bacterioruberins that coincide with enhanced survival to UV irradiation. In this light, it is notable that *H. denitrificans*, when inhibited from producing carotenoids by anoxic conditions, fared less well than the carotenoid producing controls. The difference between this method of carotenoid inhibition and that of DPA is that DPA blocks a step within the carotenoid biosynthesis pathway, whereas, under anoxic conditions, the entire carotenoid pathway is blocked. These data then support the hypothesis that the precursors phytoene and phytofluene are the important attenuators of UV radiation and that in their complete absence cells fared less well than when the pathway is active and at least some level of precursor is present. The notion that

precursors are responsible for UV shielding may explain why cultures with enhanced carotenoid production are more UV resistant, presumably if the carotenoids are being actively produced, then precursor is also present. Taken together, these results suggest that carotenoid precursors are more powerful attenuators of UV radiation, even in the lethal far UV spectra of 200 - 300 nm, than the N-stage carotenoids.

These results, in light of the ubiquity of the carotenoid biosynthesis pathway in all kingdoms of life extending from bacteria to fungi to plants and animals, are very intriguing. The vast variety of modifications of carotenoid molecules supports the notion that this pathway may have been present in early organisms and had plenty of evolutionary time to elaborate the diverse array of molecules seen today. In this respect, the genes involved in the carotenoid biosynthesis pathway may contain data important to understanding early evolutionary relationships. These precursor molecules may have been critical when life first arose on early Earth, given the high levels of UV flux that reached the Earth's surface. As the biosphere evolved and ozone was produced in a high enough concentration, biological protection from far UV radiation was not as critical and organisms were permitted to modify this ancient and prevalent biological pathway to produce the over 600 different carotenoid products that serve the wide range of cellular functions such as light collection, photo-protection, oxidative damage, cell signalling, and development seen today (Armstrong, 1994).

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