THE EFFECTS OF MULTIPLE ENVIRONMENTAL STRESSES ON THE INDUCTION OF SCYTONEMIN IN CYANOBACTERIA

by

CECELIA MARIKO TATSUMI

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Dr. Richard W. Castenholz

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It is known that some cyanobacteria produce scytonemin, a photo-protective pigment, upon exposure to high light and UV radiation. However, the role of other environmental stresses, such as osmotic stress, on scytonemin synthesis have not been investigated until this point. *Gloeocapsa M88-vd-g(1)* is a cyanobacterium that is exposed to the environmental stresses of high light, high temperature and periods of desiccation in its natural environment. *Gloeocapsa* cells were exposed to different salt concentrations (0, 5, 10, 20 g/L) and UVA (+/-) treatments. It was found that cells exposed to salt and/or UVA produced more scytonemin per protein than cells with no exposure to salt or UVA radiation. Scytonemin was induced in cells exposed to > 5 g/L NaCl without UVA, but scytonemin was not produced at as high a level as when UVA was present. Findings indicate that scytonemin production may be correlated with a more general stress response which is triggered by osmotic stress and/or high light.

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To my brother,

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INTRODUCTION

Depleted ozone levels in regions around the world have resulted in an increase in UVB (280-320 nm) fluxes that reach the earth [1]. This directly affects the well being of a variety of organisms due to the detrimental effects of this waveband of ultraviolet radiation on DNA, RNA and proteins [2]. UV radiation also has indirect negative effects on growth, motility, photosynthesis, and a variety of other processes [2]. Photoautotrophs obtain energy from solar radiation which, while providing light for metabolism, also contains harmful UV wavelengths. Cyanobacteria, which are photosynthetic members of the domain Bacteria, have probably lived on the earth for over 3.5 billion years and existed before a time when oxygen, and thus ozone, was present in the atmosphere [3]. They were responsible for the production of an oxygenated atmosphere and have evolved various methods to protect themselves against the different wavelengths of harmful ultraviolet radiation. For example, some cyanobacteria are able to avoid UV radiation by moving away from high solar irradiance, while others are able to synthesize sunscreens, such as scytonemin and mycosporine-like amino acid derivatives (MAAs), which absorb UV radiation [2].

The cyanobacteria which I study produce a photo-protective pigment, scytonemin, upon exposure to high light and UVA conditions. Scytonemin is an extracellular sheath pigment which absorbs strongly between the wavelengths of 325-425 nm (UVA-violet blue) and also has major absorption in the UVC and UVB wavelengths [4]. It serves as a passive screen which protects the cyanobacteria against UV radiation. The characteristic color of the pigment is yellow-brown and once it is synthesized it remains very stable within the extracellular envelope [5].

There has been a substantial amount of research done on the ultraviolet sunscreen role of scytonemin and its effects on laboratory and natural populations of cyanobacteria [5,

6]. However, nothing is known about the effect of other environmental factors, such as osmotic stress, on the synthesis of scytonemin. It is known that general stress responses are induced in bacteria by environmental signals such as salt stress [7]. Many stresses suggest a common response pathway in which certain general stress proteins are produced in addition to proteins specific to a particular stress which act to protect and repair damage caused by the stress [7].

For example, osmotic stress can be induced by increasing salt concentrations outside of a cell. This puts pressure on a cell and causes a net movement of water outward across the plasma membrane. The result is dehydration of the cell which mimics desiccation (drying out). Some cyanobacteria survive in habitats that are periodically desiccated. Thus, during these times of desiccation, stress proteins and osmotic solutes are produced within cells to help maintain turgor pressure as the amount of water in the environment decreases [8, 9, 10]. It is possible that a common stress response pathway may be present in cyanobacteria which are exposed to osmotic stress and UV radiation.

Exposure to ultraviolet radiation is stressful to cyanobacteria and some species are able to respond to UVR through the induction of scytonemin. It is possible that stresses like desiccation or salinity may affect the synthesis of scytonemin if scytonemin is part of a general stress response. Although scytonemin is not a protein, its synthesis and/or transport is probably mediated by structural proteins and enzymes at some level. Thus, it is possible that UV-induced proteins aid in these processes and scytonemin induction may be part of a general stress response [11]. My hypothesis is that salt stress enhances scytonemin production. UVA radiation may still be needed to induce scytonemin, but in combination with salt stress a greater amount of scytonemin may be produced or the rate of synthesis may be affected. The alternative hypothesis is that salt stress does not affect scytonemin synthesis.

For experimental purposes I am using a strain of cyanobacteria known as

Gloeocapsa M88-vd-g(1). This unicellular cyanobacterium was isolated in liquid culture from a desert crust growing on a desert rock in Baja California N., Mexico and is able to produce scytonemin under high light and UVA. In its normal habitat *Gloeocapsa M88-vd-g(1)* is exposed to high amounts of ultraviolet radiation and periods of desiccation. Some cyanobacteria are able to grow in hypersaline habitats with salinities up to 250 g/L but in my experiments the highest salinity the cyanobacteria are exposed to is 20 g/L NaCl [12]. It is logical to use this strain in this series of experiments since it is probable that it can tolerate such conditions in its natural state. Preliminary experiments showed that these cyanobacteria could tolerate growing in 20 g/L NaCl D medium but not in 30 g/L NaCl D medium.

MATERIALS AND METHODS

Strain and culture. The cyanobacterium *Gloeocapsa M88-vd-g(1)* was used for all experiments. It is a clonal isolate from a desert crust growing on a desert rock in Baja California N., Mexico. The cells were grown at 23°C in liquid D medium [13] under coolwhite fluorescent light (Sylvania™, F96T12/CW) to obtain starting biomass for experiments. The cells grew in aggregates, so for experimental purposes samples were homogenized in a Waring blender (Waring, New York, NY) on high for thirty seconds. Cells were then inoculated into appropriate experimental flasks.

Experimental setup for the osmotic stress experiments. Exponentially growing *Gloeocapsa* cells were exposed to osmotic conditions (as stated below) at the beginning of the experiment at 23°C constant temperature. Visible light (~17 W m^{-2}) illuminated flasks from below and low levels of UVA (~2 W m^{-2}) radiated downward from above the flasks. Control flasks with exposure to only visible light were shielded from the UVA by a K-Lite styrene filter (Multicraft Plastics, Eugene, OR). K-Lite is able to screen out UVB radiation and approximately 98% of the UVA.

500 mL Erlenmeyer flasks of D medium with salt (NaCl) added to 0, 5, 10 and 20 g/L were inoculated with cells to a similar optical density at 750nm. Four flasks of each salt concentration were exposed to UVA and visible light, and four control flasks were exposed only to visible light. Samples for protein and pigments were taken every two days for ten days as described below. Protein was used to normalize scytonemin content across all samples. Flasks were rotated daily to help control for variation in light environment. Table 1 lists specific light conditions for each experiment.

General experimental methods. For all experiments, optical density readings of cell suspensions were measured at 750 nm using a Beckman DU 640 recording

spectrophotometer. This was used to obtain essentially equal amounts of starting material for experimental flasks.

Sampling. For all experiments each flask was sampled with a sterile syringe under sterile conditions. Cells were first resuspended from the bottom of the flask and then the flask material was homogenized by pumping with a syringe before sampling. Triplicate 10 mL samples were taken for protein and pigments in all experiments.

Determination of pigments. 10 mL cell samples were filtered onto 25 mm diameter Whatman GF/F glass fiber filters, rinsed with double distilled water (ddH_2O) and then placed filter cell side up in the bottom of a 20 mL scintillation vial loaded with 3 mL acetone. These filters were then incubated at 4°C in the dark overnight. Filters were homogenized using a hand-operated glass tissue grinder and then the acetone-pigment extract was pipetted into Fisherbrand 1.5 mL polypropylene microcentrifuge tubes. These extracts were centrifuged in an Eppendorf centrifuge 5412 for 1 minute. Absorption of the extract supernatant at wavelengths 384, 490, 663 and 750 nm were obtained using a Beckman DU 640 recording spectrophotometer. Pigment content was determined using the trichromatic equations of Garcia-Pichel and Castenholz [14]. Extinction coefficients were used as in reference [5].

Protein quantification. 10 mL cell suspensions were placed into 15 mL centrifuge tubes (Corning, New York) and spun down in a clinical tabletop centrifuge (International Clinical Centrifuge, Boston, MA). The supernatant was removed and the cells were resuspended in 1 mL ddH_2O. Proteins were extracted as in methods used by Wingard *et al.* [15].

Statistical analysis. All statistical tests were made using the computer program SuperANOVA® [16]. Pairwise comparisons of the means of scytonemin content per unit protein, where necessary, were based on the results of Student-Newman-Keuls and Turkey Compromise *post hoc* tests. Figures were produced using SigmaPlot®[17].

Osmotic stress experiments

Figure 1 shows the result of the combined UVA/salt stress experiment. There was a significant UVA treatment effect ($P = .0001$) with the UVA (+) treatments having much higher scytonemin production than the UVA (-) treatments. There was also a significant effect of salt treatment ($P = .0001$) within both UVA treatments (+/-) over the experimental period. In the UVA (+) treatment, there was an increase in the rate of scytonemin induction for cells growing in medium with added salt compared to the commonly observed lag in scytonemin production in flasks with no salt added. Up to day 4, the cells growing in medium with salt added produced similarly increasing amounts of scytonemin (μg) per mg protein (scytonemin) while the cells in the flasks with no salt showed slower synthesis. By day 8 the cells in the 0 g/L salt treatment reached similar levels as the 5 g/L salt treatment in scytonemin, but a significantly greater amount of scytonemin ($P < .05$) was produced in the 10 and 20 g/L salt treatments. After eight days of relatively continuous increase in scytonemin production in all of the salt treatments, there was a drop in scytonemin content in the 10 and 20 g/L salt treatments and a leveling off of scytonemin in the 0 and 5 g/L salt treatments. This indicates that scytonemin production was slowing down relative to cell growth rate which was still substantial, as may be seen in Figure 2. The estimated doubling time of cells in exponential phase growing in the UVA (-) and UVA (+) treatments with exposure to 0, 5, 10 and 20 g/L NaCl is respectively as follows: 6.4, 2.9, 3.4 and 3.4 days for UVA (-) and 4.1, 4.0, 3.8 and 4.7 days for cells in the UVA (+) treatment.

The cells in the UVA (-) treatment showed a similar trend in scytonemin production as in the UVA (+) treatment, as there was an increase in the amount of scytonemin produced as the salt concentration increased. Exposed only to visible light and salt stress,

scytonemin levels initially stayed fairly constant but after one week the 10 and 20 g/L salt treatment cells produced higher amounts of scytonemin than the 0 and 5 g/L salt treatments. In this short term UVA (-) experiment, the 0 and 5 g/L salt treated cells did not have a significant difference in scytonemin production but there was significant difference ($P < .05$) between all the other salt treatments as determined by *post hoc* analysis of the ANOVA data. The cells in the 20 g/L salt treatment produced more scytonemin than in the 10 g/L salt treatment, which in turn produced a greater amount than the cells in the 0 and 5 g/L salt treatments. There also was a significant salt x day interaction term in the UV (-) treatment ($P = .0001$), which means that there was a significant difference, over a period of 10 days, in the amount of scytonemin produced by cells exposed to different salt treatments. The salt effect changed with time. We also found a significant three way interaction among salt concentration, UVA treatment and day ($P = .0001$). This means that there was a significant difference in the amount of scytonemin produced by cells exposed to different UVA (+/-) and salt treatments (0, 5, 10 and 20 g/L NaCl) over a ten day period.

A long term UVA (-) salt stress experiment was run to examine the change in production of scytonemin over a longer period of time. This experiment also showed a significant salt treatment x day interaction ($P = .0001$). The results of this experiment (Fig. 3) supported the short term experimental results and showed a similar decline in scytonemin at day 15 as seen in the UVA (+) experiment on day 8. By day 15, the cells growing in the 20 g/L salt treatment produced the most scytonemin per protein (Mean = 20.2 ug scyt./mg protein, SE = .39) while the cells in the 10 g/L salt treatment produced the second highest amount (Mean = 4.6 ug scyt./mg protein, SE = .20, *post hoc* tests show a significant difference in the above comparison). As seen in the short term UVA (-) experiment, there was not a significant difference ($P > .05$) between the cells in the 0 and 5 g/L salt treatments, but there were significant differences in scytonemin production among the other salt concentrations ($P < .05$). Following day 15

there was a steady decrease in scytonemin in the 20 g/L salt treatment and a slight decrease and leveling off of scytonemin in the 10 g/L salt treatment. Although scytonemin was produced in the UVA (-) salt stressed cells, the synthesis of scytonemin per protein did not reach the same levels as in the UVA (+) experiment, even after 30 days. This indicates that salt stress is not sufficient for full induction of scytonemin.

The results from the osmotic stress experiments show that more scytonemin was produced by *Gloeocapsa* cells growing in D medium with salt > 5 g/L as compared to flasks with no salt added. This occurred in both UVA (+) and UVA (-) treatments. These findings indicate that scytonemin production may be part of a more general stress response. In addition, the salt stressed *Gloeocapsa* cells in the UVA (+) treatments produced more scytonemin than the salt stressed cells in the UVA (-) treatment. Simultaneous exposure to multiple stresses such as osmotic and UV stress are common for this cyanobacterium in its natural desert crust environment. Thus a correlated response may be seen as adaptive.

The *Gloeocapsa* cells growing in the UVA (+) treatment with no salt added experienced a 4 day lag period in scytonemin production over the experimental period of 10 days. This lag period was shorter in cells grown in flasks containing added salt. The lag period in the flask with no salt added suggests that salt stress enhances not only the amount, but also the rate of scytonemin production under UVA exposure. These cyanobacteria may trigger stress responses in order to help cope with the concomitant environmental conditions. It is possible that scytonemin is produced during periods of salt stress in order to prepare for UV stress which could occur during a dormant state when other methods of countering this stress, such as active DNA repair or synthesis of damaged target proteins [2], cannot take place.

Among all of the osmotic stress experiments there was not a significant difference in scytonemin production and no scytonemin produced by cells in the 0 and 5 g/L salt treatments (Figure 1). This may indicate that 5 g/L NaCl was not stressful to the cells. However, there was a significant difference between the other salt treatments. In the UVA (+) treatment, the cells in the 10 and 20 g/L salt treatment produced the most scytonemin and in the UVA (-) treatment, the cells in the 20 g/L salt treatment produced more scytonemin than all the other UVA (-) treatments.

In the long term UVA (-) experiment the amount of scytonemin peaked on day 15 in the 20 g/L salt treatment (Figure 3). However, this high content of scytonemin per unit protein was not maintained within the cells. After day 15, there was a drop in scytonemin content. The cells growing in the 10 g/L salt flask also had a peak production of scytonemin on day 15, but the level of scytonemin plateaued instead of declining on day 30 as in the 20 g/L salt flask. The concentration of salt, whether it be 10 or 20 g/L, affects the synthesis of scytonemin over time.

Previous experiments (data not shown) have shown *Gloeocapsa M88-vd-g(1)* cell growth at 30 g/L NaCl D medium to be lethal. Although there was only a UVA effect on growth rate, we know the range of salt concentration at which these cyanobacteria can grow. The observation that salt has no effect on growth rate might be due to the habitat of this strain of cyanobacteria, where fluctuations in water potential are common. *Gloeocapsa M88-vd-g(1)* most likely has a broad tolerance to salt. We can assume that the cells are very near lethal salt concentrations when growing in 20 g/L NaCl D medium. The osmotic stress experiments have shown that salt stress > 5 g/L enhances scytonemin production under UVA and with only visible light in *Gloeocapsa M88-vd-g(1)*. UVA radiation is not needed to induce scytonemin in salt stressed cells. However, when UVA and salt stress are combined, a greater amount of scytonemin is produced than with cells only exposed to salt stress or UVA alone. UVA is more effective in inducing scytonemin than salt, but salt stress affects scytonemin synthesis and it is likely that scytonemin is part of a general stress response.

Table 1. Light conditions for osmotic stress experiments.

	UVA (+/-) 10 Day Experiment #1	UVA (+/-) 10 Day Experiment #2	Long Term UVA (-) Experiment #3
Visible Light under K-Lite	17.8-18.6 W m^{-2}	17.6-18.3 W m^{-2}	14.643-15.165 W m^{-2}
UVA under K-Lite	.0357-.0400 W m^{-2}	.030-.039 W m^{-2}	NA
Visible Light	18-19 W m^{-2}	17.5-18.7 W m^{-2}	NA
UVA	1.85-2.03 W m^{-2}	1.8-2.0 W m^{-2}	NA

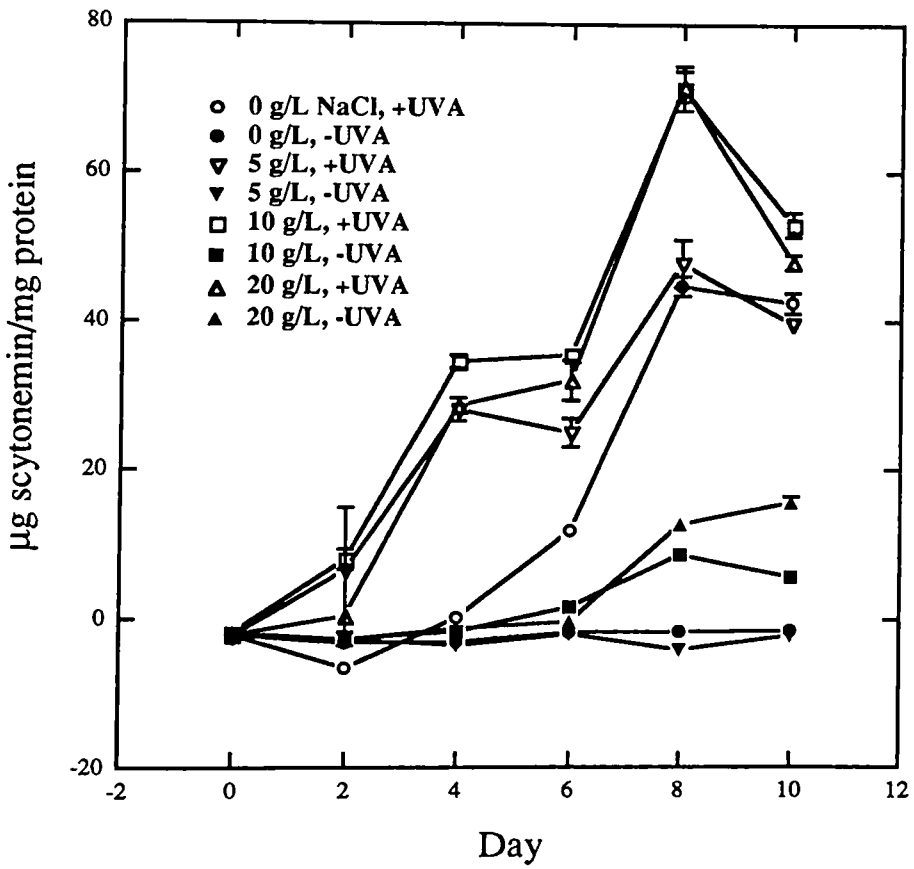

Figure 1. Experiment #1. *Gloeocapsa M88-vd-g(1)* cells were grown under visible light (~18 W m-2) and UVA radiation (~1.9 W m-2) in flasks of D medium with NaCl added to the following concentrations: 0, 5, 10 and 20 g/L. Control flasks with the same salt treatments but with exposure to only visible light were also present. The amount of scytonemin (µg) produced per protein (mg), separated into unique salt and UVA treatments, over a period of ten days is shown in the graph above.

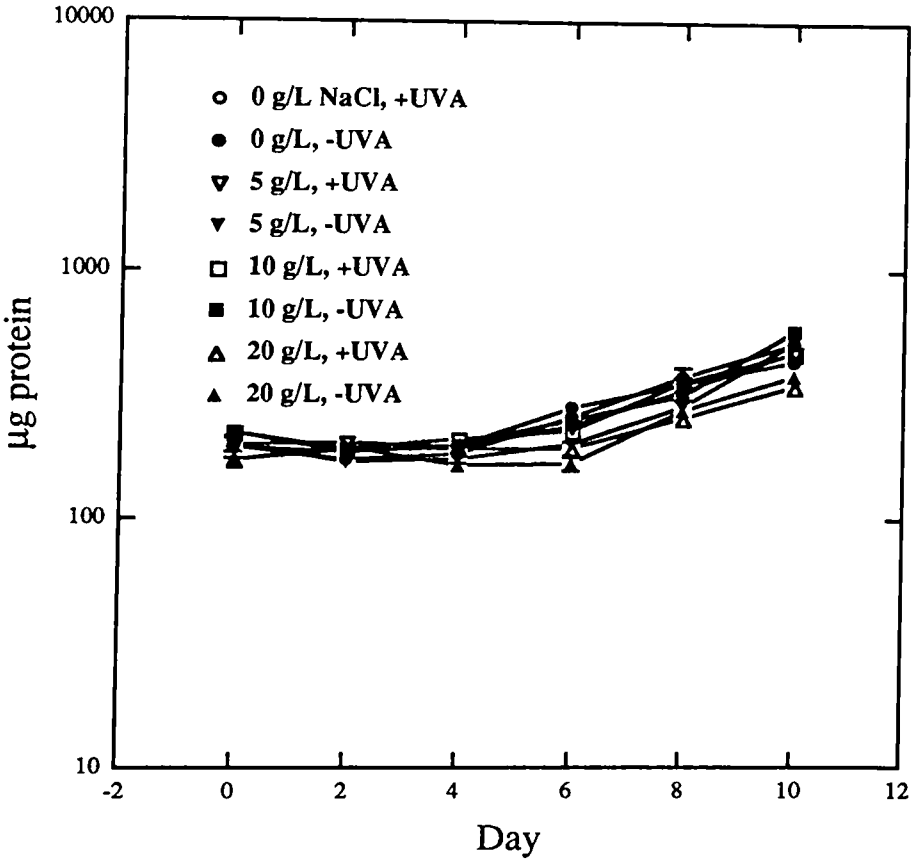

Figure 2. Experiment #2. *Gloeocapsa M88-vd-g(1)* cells were grown under visible light (~18 W m-2) and UVA radiation (~1.9 W m-2) in flasks of D medium with NaCl added to the following concentrations: 0, 5, 10 and 20 g/L. Control flasks with the same salt treatments but with exposure to only visible light were also present. The amount of protein (µg) produced over a period of 10 days is shown above. This demonstrates the growth of cells during the experiment.

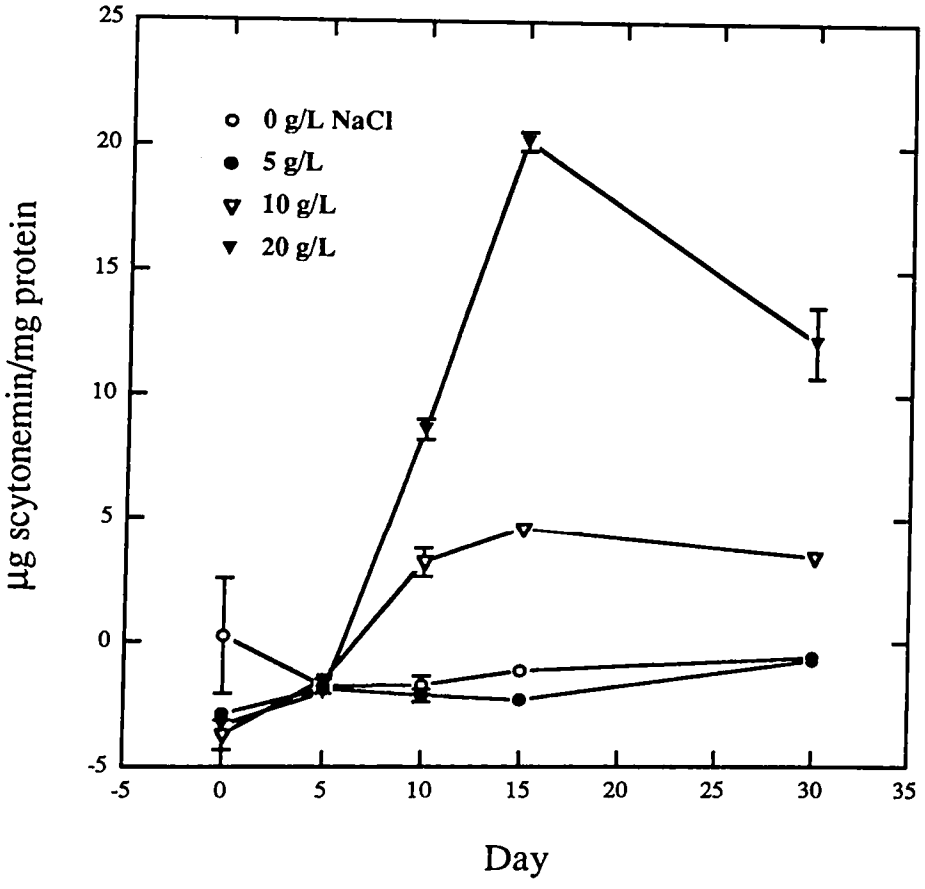

Figure 3. Experiment #3. *Gloeocapsa M88-vd-g(1)* cells were grown under visible light (~15 W m-2, without UVA) in Erlenmeyer flasks of D medium with NaCl added to the following concentrations: 0, 5, 10 and 20 g/L. The amount of scytonemin (µg) produced per protein (mg), separated into unique salt treatments over a period of 30 days, is shown in the graph above. Note the different scale of the figure as compared to Figure 1.

GLOSSARY OF TERMS

Aliquot-	A repeated sample of a given volume.
Cyanobacteria-	Photosynthetic bacteria (photoautotrophs) which have existed on earth for over 2.5 billion years.
D Medium-	A type of nutrient medium containing nitrogen, in the form of nitrate, which supports the growth of cyanobacteria and algae.
Gloeocapsa M88-vd-g(1)-	A strain of cyanobacteria which was isolated from a desert crust in Baja, Mexico. Is able to produce scytonemin under high light.
Osmotic solutes-	Solutes which are produced within bacteria cells during times of increased osmotic concentration in the habitat. Helps to maintain turgor pressure.
Osmotic stress-	Pressure placed on a cell due to increasing salt concentrations outside of a cell. Mimics drying out of a cell since water will move out of a cell to compensate for the high concentration of salts located outside the cell.
Scytonemin-	Photo-protective pigment found in the sheaths of a variety of cyanobacteria. Absorbs light strongly in the UVA and violet blue regions (325-425 nm) and also in the UVC (250 nm) and UVB (280-320 nm) regions.
Stress response-	A type of reaction which takes place due to exposure to an unfavorable environmental condition.

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