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UVB Photoprotective Role of Mycosporines in Yeast: Photostability and Antioxidant Activity of Mycosporine-Glutaminol-Glucoside

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Several yeast species are able to synthesize and accumulate UV-radiation-absorbing microsporine metabolites that are of unclear physiological function. In this work we analyzed the relationship between mycosporine-glutaminol glucoside (MGG) production, cell survival after UVB irradiation, and formation of cyclobutane pyrimidine dimers (CPDs). We also assessed the photostability and singlet oxygen quenching activity of MGG. A set of nine isolates of the basidiomycetous yeast *Cryptococcus steppossus* cultured in both dark and light conditions was used for the studies. Survival of the UVB-irradiated isolates and MGG concentration had a linear relationship when the concentration was over 2.5 mg g^{-1}. CPD accumulation and MGG accumulation were inversely related. MGG in aqueous solution was photostable with a photodecomposition quantum yield of 1.16×10^{-5}. MGG quenching of singlet oxygen was also observed, and the rate constant for the process in D_2O was 5.9×10^7 M^{-1} s^{-1}. Our results support the idea that MGG plays an important role as a UVB photoprotective metabolite in yeasts by protecting against direct damage on DNA and probably against indirect damage by singlet oxygen quenching. © 2011 by Radiation Research Society

INTRODUCTION

Living organisms have developed several protective strategies against UV radiation. Some cellular metabolites like melanin protect the cells against direct UV-radiation injury by absorbing the radiation and avoiding accumulation of damage in DNA (*1*), while others such as carotenoid pigments provide indirect protection to the cells by quenching the reactive oxygen species (ROS) produced by the radiation (*2*). A group of cellular metabolites collectively known as mycosporines and mycosporine-like amino acids (MAAs) have also been proposed as photoprotective compounds (*3*). Mycosporines (*sensu-stricto*) are water-soluble molecules formed by a distinctive cyclohexenone unit and an amino acid or amino alcohol conjugated subunit. The first mycosporine molecule was detected in the 1960s. Originally, it was thought to be associated with some filamentous fungi sporulation process (*4–6*). Recently, however, Leite and Nicholson (*7*) found that mycosporine-alanine inhibits germination of the *Colletotrichum graminicola* conidia. The ability of mycosporines to absorb UV radiation, with a maximum peak at 310 nm (*8*), has led to the hypothesis that they may protect fungi by acting as a sunscreen (*9*). However, experimental evidence is lacking to support this hypothesis, and most present theories are based on the chemical similarities between mycosporines and the MAAs.

MAAs are imine derivates of mycosporines with UV absorption maxima between 310 and 360 nm. They are considered to be multipurpose metabolites with a variety of functions including antioxidant capacity, accessory pigments in photosynthesis, nitrogen storage, thermal protection and osmotic stress protection; they have also been widely accepted as UVA photoprotection agents (*10*). In most cases the evidence supporting these functions is indirect and is based either on high stimulation of MAA synthesis under stress conditions (*10*) like UV irradiation (*11*) or on theoretical models (*12*). Mycosporines and MAAs synthesis appear to be related through the shikimate pathway, although this hypothesis is still unproven (*3*). Both types of compounds have been found in a variety of organisms such as cyanobacteria, algae and fungi. Certain yeast species

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are able to produce a single type of mycosporine identified as mycosporine-glutaminol-glucoside (MGG) (*13*) with unknown function in these microorganisms. Previous work revealed that the production of MGG in yeasts is stimulated by photosynthetically active radiation and UV radiation, suggesting a photoprotective function for this molecule (*14*). The main objective of the present study was to investigate the photoprotective functions of MGG using yeast as a model eukaryotic organism. Characterization of the photochemical and photophysical properties of a natural sunscreen is essential to support the hypothetical photoprotective role. *In vitro* photostability of MAAs has been reported (*15–17*), but mycosporines have not been studied in this regard. Other studies indicated that some MAAs (*18*), the gadusol (*19*) and the mycosporine-glycine (*20*), exhibit antioxidant properties. Whether the antioxidant capability is a typical property of mycosporines/MAAs needs to be determined experimentally with other members of the family of molecules.

In this work, we studied the production of MGG in the yeast *Cryptococcus stepposus* cultured under both light and dark conditions and the relationship between MGG concentration, UVB-radiation survival, and DNA damage by measuring cyclobutane pyrimidine dimer (CPD) accumulation. We also assessed the photostability of MGG and singlet oxygen quenching activity.

MATERIALS AND METHODS

Yeast

Nine natural environment isolates of the basidiomycetous yeast *Cryptococcus stepposus* were used. Yeast isolates were collected from Tronador Mountain Frias Glacier meltwaters (*21*). They belong to the Centro Regional Universitario Bariloche-INIBIOMA Culture Collection and were selected for their ability to differentially accumulate MGG when grown under photosynthetically active irradiation.

Culture Medium and Conditions

Yeasts were cultured in modified MMS agar [10 g liter^{-1} glucose, 2.5 g liter^{-1} Bacto-Peptone®, 2.5 g liter^{-1} $KH_2(PO_4)$, 0.5 g liter^{-1} $MgSO_4.7H_2O$, 1 g liter^{-1}, 1.5 g liter^{-1}Agar-Agar). Petri dishes with each isolate were incubated under photosynthetically active irradiation and dark conditions at 18°C for 48 h in an environmental test chamber (Sanyo MLR 350). Light was provided by 10 white light fluorescent tubes (Sanyo, 40 W). Identical conditions were used for the dark treatments but dishes were wrapped in aluminum foil. Cells were suspended in distilled water and transferred to quartz tubes at 2 $\times$ 10^5 cell ml^{-1} final concentration. Additional samples were collected for quantitative analysis of MGG.

UVB-Irradiation Conditions and Yeast Survival

Quartz tubes containing each isolate suspension, were exposed (20 cm) to a Spectroline XX15- B UVB lamp, covered with a preburned (120 min exposure to the UVB lamp) cellulose acetate filter, and placed in a dark chamber for 120 min. The final UVB irradiation and intensity patterns have been reported previously (*22*). Cell survival was determined by a colony formation assay. Radiation experiments were run in triplicate; each point in the regression model represents the median survival and median MGG content.

Quantification of CPDs

The concentration of CPDs was determined in the nine isolates cultured under photosynthetically active irradiation. DNA extraction was performed immediately after exposure to UVB radiation, and extracts were kept at −20°C until processed as described previously (*22*). CPDs were quantified by comparing sample DNA with a dilution series (1280 to 20 CPDs/MB) of damaged standard DNA (Promega). The amount of CPDs in the standard DNA was determined previously by Dr. Anita Buma (*23*).

Quantification of MGG

MGG was extracted with 1 ml of methanol (20% v/v) and placed in a water bath at 80°C for 4 h. Quantitative analysis was carried out at 310 nm using the mycosporine-glutaminol molar extinction coefficient, ε = 25,000 (*24*).

Purification of MGG for Chemical Characterization

MGG was first passed through ion exchange columns prepared with Dowex 50W H^+ 200 mesh and Dowex 1×8 Ac^- 200–400 mesh resins. Each eluted fraction with absorption at 310 nm was purified by reverse-phase isocratic high-performance liquid chromatography (HPLC) on a Shimadzu (LC-10A) system. A C-18 Merck® column (5 μm, 4.6 mm × 250 mm) and 25% v/v methanol 0.05% v/v acetic acid in water as mobile phase were used with 1 ml min^{-1} flux. The UV absorbance detection point was set at 310 nm. Fractions showing a 4.2-min retention time were collected. The collected elute was purified in a new HPLC cycle.

Continuous Photolysis and Actinometry

The stationary photolysis experiment was carried out by illuminating an MGG solution in a spectrophotometric quartz cell of 1 cm path length. The light source was a high-pressure 1000-W Hg-Xe lamp (Hanovia) coupled to a Czerny-Turner monochromator for high intensities (Schoeffel-Kratos) set at 310 nm ± 10 nm. A 2.6 × 10^{-5} *M* MGG solution was irradiated under continuous stirring at 21°C for 6 h. A solution of phenylglyoxylic acid about 2.5 3 × 10^{-2} *M* in acetonitrile:water 3:1 with saturated absorbance at 310 nm (*25*) (Aldrich, recristallyzed from Cl_4C), was used as a chemical actinometer, and irradiated for 15 min. The decrease in absorbance was monitored at 310 nm for the MGG and at 380 nm for the actinometer with a Shimadzu UV-2101PC spectrophotometer. Samples of MGG at the different times were collected and analyzed by HPLC using a Phenomenex C-8 column (5 μm, 4.6 mm × 250 mm) under the conditions described above.

Determination of Singlet Oxygen Quenching

Singlet oxygen was generated in air-saturated D_2O (90% Aldrich) solutions using rose bengal (Sigma) as a sensitizer. The emission from a Nd:YAG Laser (Spectron) at 532 nm was used as the pulsed-excitation source. The decay of the phosphorescence emission from singlet oxygen after the laser shot was registered at 1270 nm by means of a Ge amplified detector (Judson J16/8Sp) after passing the emitted radiation through a cutoff filter (<1000 nm) plus an interference filter (1270 nm). This detector is able to readily follow the emission for about 6 ⌠s after the laser pulse. The decays after this blind gap were averaged for three to five runs. The traces were fitted respectively to a monoexponential function of time, characterized by |, the exponential time constant or lifetime. The total rate constant for the quenching of singlet oxygen by MGG (k_t) was determined by

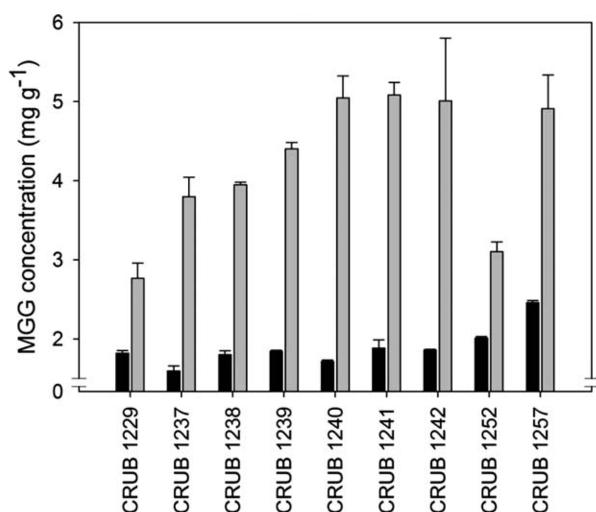


FIG. 1. MGG accumulation of nine isolates of *C. stepposus* cultured in the dark (black bars) and light (gray bars).

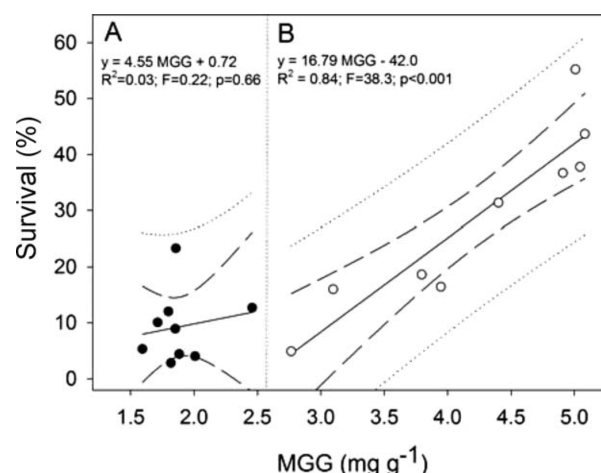
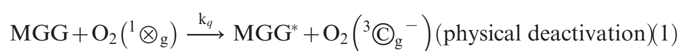


FIG. 2. Relationship between MGG concentration and survival after UVB-radiation exposure in nine *C. stepposus* isolates. Black dots: isolates cultured in the dark; white dots: isolates cultured under photosynthetically active irradiation.

following a simple Stern-Volmer treatment (*26, 27*). The mechanism of singlet oxygen quenching includes both physical and chemical processes, respectively described by Es. (1) and (2). In the equations, P denotes the product(s) of the oxidation reaction and MGG* is an electronically excited MGG.



$$MGG + O_2(^1\otimes_g) \xrightarrow{k_q} MGG^* + O_2(^3©_g^-)\text{(physical deactivation)} \quad (1)$$

$$MGG + O_2(^1\otimes_g) \xrightarrow{k_q} P\text{(chemical deactivation)} \quad (2)$$

Therefore, the total rate constant for the quenching k_t is expressed by the sum of k_q and k_r. The value of k_t was determined as the slope of a plot of the inverse of the lifetime of $O_2(^1\otimes_g)$ as a function of the MGG concentration, according to Eq. (3).

$$\tau^{-1} = \tau_0{}^{-1} + {}_0k_t \times [MGG], \quad (3)$$

where τ_0 and τ correspond, respectively, to the lifetimes of $O_2(^1\otimes_g)$ in the absence and presence of the putative quencher MGG (2.5×10^{-4} and 5×10^{-5} *M*).

The susceptibility of *E. coli* to photodynamic inhibition was tested by the filter paper disk method (*28*). Agar culture plates with nutritive medium were inoculated with 100 µl of the 24-h liquid cultures of *E. coli* ATCC 8739, filter paper disks were soaked with rose bengal alone (0.1 m*M*), rose bengal plus 0.1 mg of MGG, and rose bengal plus 0.25 mg citric acid. Disks were placed on the agar plates and cultured for 24 h at 36°C under white light.

Statistical Analysis

The effects of MGG concentration on survival and CPD accumulation were tested using linear regression model analysis. Normality and homoscedasticity were tested using Shapiro-Wilk and Levene tests. The Mann-Whitney test was used to assess differences in MGG accumulation between treatments.

Results

Photosynthetically active radiation-induced MGG production in *C. stepposus* ($P < 0.001$; Fig. 1). When yeast was cultured in the dark, all isolates produced very similar concentrations of MGG (average 1.88 ± 0.23 mg g^{-1}); however, each isolate accumulated MGG differently when cultured under photosynthetically active irradiation (average 4.23 ± 0.88 mg g^{-1}).

For isolates cultured in the dark (Fig. 2A), regression analysis between MGG production and UVB-radiation survival was not significant ($R^2 = 0.03$; $P = 0.66$). In contrast, for isolates cultured under photosynthetically active irradiation (Fig. 2B), regression analysis between production of MGG and survival was positive and significant ($R^2 = 0.84$; $P < 0.001$). Isolate CRUB 1229 had the lowest increase in MGG production (152%), and its survival remained unchanged ($P = 0.20$). In contrast, isolates CRUB 1240, 1241 and 1242 had the highest increase in MGG accumulation (~278% average) and the highest survival after UVB irradiation (37.8 ± 8.1, 43.7 ± 5.3 and 55.2 ± 6.8, respectively). When the data were analyzed together (isolates cultured in the dark and under photosynthetically active irradiation), the coefficient of determination of the model was lower ($R^2 = 0.76$) but was still statistically significant ($P < 0.001$).

The regression analysis between accumulation of CPDs and MGG in the different isolates exposed to UVB radiation was negative and significant ($R^2 = 0.69$, $P = 0.005$; Fig 3), as were survival and CPDs ($R^2 = 0.57$, $P = 0.018$; data not shown). Isolate CRUB 1229 had the highest CPD/MB accumulation (1129.9 ± 67.4) while the isolates

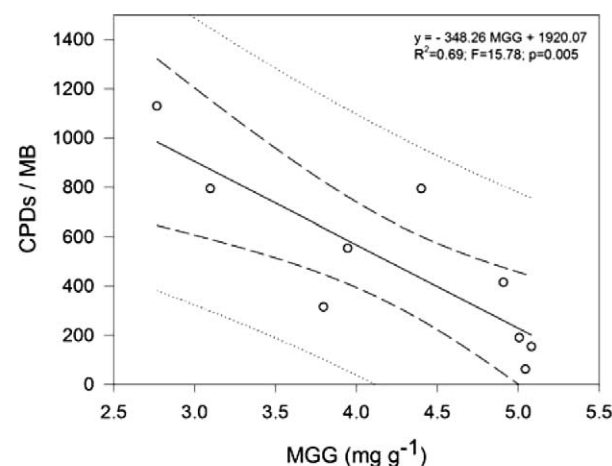


FIG. 3. CPD accumulation in isolates of *C. stepposus* (cultured under photosynthetically active radiation) after UVB irradiation and their relationship to MGG accumulation.

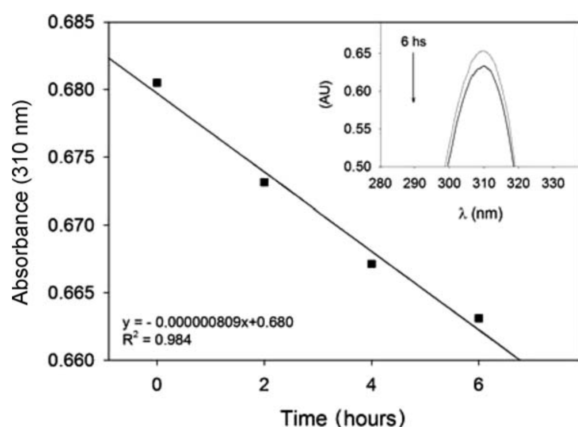


FIG. 4. MGG absorbance at 310 ± 10 nm as a function of the irradiation time. The inset shows a detail of the absorbance decay at the beginning and at the end of photolysis period.

CRUB 1240, 1241 and 1242 had lower rates of CPD/MB (48.9 ± 69.2, 154.2 ± 61.4 and 31.2 ± 44.1, respectively).

The photostability of MGG in an aqueous solution was estimated by following the decay of the MGG concentration after UVB irradiation. The results of quantitative analyses by HPLC were in agreement with absorbance measurements and confirmed the absence of photoproducts absorbing in the range of MGG absorption spectra. The intensity of the incident light could be measured from the phenylglyoxylic acid (actinometer) photodecomposition rate, yielding $I_o = 1.25 \times 10^{-8}$ einsteins s^{-1}. A plot of the maximal absorbance of MGG at 310 nm as a function of irradiation time led to the estimation of the initial photolysis rate (Fig. 4). Thus the quantum yield for photodecomposition of MGG (Φ_R) was calculated as the ratio of the initial rate of photolysis and the initial absorbed intensity $I_a = I_o\,(1 - 10^{-A})$, where A is the absorbance at 310 nm averaged over the photolysis period. It results in $\Phi_R = (1.16 \pm 0.2) \times 10^{-5}$.

To assess the antioxidant potential of MGG, we evaluated the deactivation of electronically excited molecular oxygen $O_2(^1\otimes_g)$ (singlet oxygen) by its phosphorescence emission in the absence and presence of MGG using time-resolved phosphorescence detection (TRPD). The results of the TRPD measurements are shown in Fig. 5. As can be seen in the inset, the $O_2(^1\otimes_g)$ phosphorescence was shortened in the presence of MGG. The fit to a straight line of the inverse of the lifetime as a function of MGG concentration yielded a slope of $(5.9 \pm 0.3) \times 10^7$ M^{-1} s^{-1} ($R^2 = 0.998$) that corresponded, according to Eq. (3), to the k_t value.

Growth of *E. coli* was inhibited by rose bengal under photosynthetically active irradiation, as indicated by the presence of a clear halo without bacterial colonies surrounding the filter paper disks. The addition of MGG as well as citric acid (positive control) reversed the effect of inhibition and allowed normal growth.

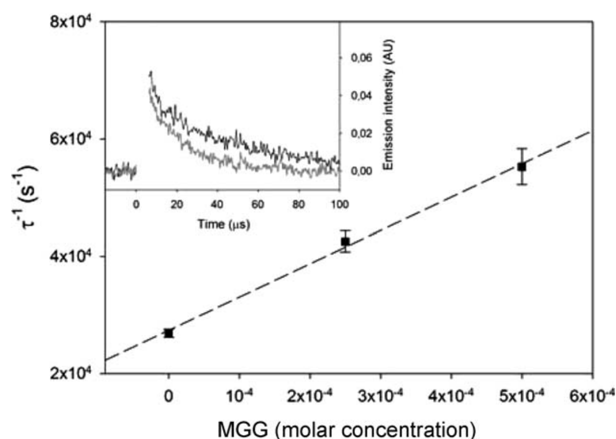


FIG. 5. Stern-Volmer plot for the quenching of $O_2(^1\otimes_g)$ by MGG. Experimental data were fitted to a straight line (dashed line). Vertical bars represent the experimental error in $|^{-1}$. Inset shows two decays of the emission signals from $O_2(^1\otimes_g)$ without (dark line) and with (gray line) 2.5×10^{-4} M MGG.

DISCUSSION

Previous studies showed that the nine yeast isolates we used were highly similar, either phenotypically in sugar assimilation tests or genetically in micro/minisatellite-primed PCR analysis (*21*). However, their patterns of MGG production are different, suggesting that the production of these molecules as a response to light may be affected by the specific characteristics of each isolate.

This is the first report examining the ability of *C. stepposus* to produce MGG, and the accumulation of MGG in the selected yeast species is clearly related to light exposure. The positive effect of light in the accumulation of mycosporines has been observed in other yeast species (*14*) and has also been found in organisms that accumulate MAAs (*29–32*). Light induces shikimate pathway enzymes (*33, 34*), and mycosporine/MAAs synthesis is related to this metabolic path (*35, 36*). Thus it is difficult to say whether the higher MGG concentration under light exposure is a result of a specific photostimulation mechanism or a consequence of a general induction mechanism involving all or the whole shikimate pathway products. Nevertheless, the greater MGG accumulation under light supports the idea of a photoprotective function of their molecule. Brook *et al.* (*37*) were the first to attribute a photoprotective role to mycosporines. They based this assumption on the fact that spores produced in light had higher survival and mycosporine concentrations than those of spores produced in the dark. However, light could also affect other unknown photoprotective mechanisms. In the present study, we found that small changes in MGG accumulation clearly affected the survival of different *C. stepposus* isolates cultured in the same conditions (photosynthetically active radiation). Linear regression analyses revealed a high correlation between survival and MGG concentration when isolates were cultured under light (MGG production greater than 2.5 mg g^{-1}); in particular, isolates with more than 4 mg g^{-1} had significantly higher survival than the isolates cultured in the dark or those cultured under photosynthetically active irradiation with a lower MGG content. In contrast, in the dark (MGG lower than 2.5 mg g^{-1}), no relationship was observed. Our results showed that in *C. stepposus* UV-radiation tolerance increases only above a certain MGG threshold. Although the linear model explains the survival for isolates in the photosynthetically active radiation

treatment satisfactorily, MGG accumulation is restricted to a small range. In this context and considering the results for the dark treatment, it is probable that other models (e.g. exponential) could better explain the survival after UVB irradiation.

The production of MGG in yeast cultured in the dark is below the level required for effective photoprotection. Other yeast species have been found to produce MGG when cultured in the light (*14*). However, the basal MGG accumulation produced by *C. stepposus* in the dark is conspicuous and might be related to unknown functions.

Given that MGG absorbs UVB radiation, we hypothesize that MGG acts as a sunscreen for yeasts cells by protecting against direct damage of DNA. Here we confirmed that the CPD accumulation observed after UVB irradiation was negatively related to MGG accumulation. This result leads us to conclude that the mechanism of photoprotection by MGG involves, at least in part, a reduction in DNA damage caused by UVB radiation. However, it is important to note that the coefficient of determination between CPDs and survival or CPDs and MGG concentration is lower than that observed between MGG concentration and survival. This suggests that MGG includes additional mechanisms of protection, probably by reducing damage in other important cell targets or, as we discuss below, by reducing the oxidative stress generated by UV radiation. Regarding photochemical characterization, MGG is the first mycosporine described and appears to be a highly photostable metabolite. The photostability of MAAs has been described by others (*38, 39*), and the quantum yield of photodecomposition (Φ_R) in aqueous solution was first determined by Conde *et al.* for porphyra-334, shinorine (*15, 16*) and recently for palythine (*17*). The photodecomposition quantum yield for MGG (Φ_R) is comparable to that of palythine and is one order of magnitude lower than the others. In this sense, MGG is more stable than other MAAs studied so far. In summary, MGG efficiently absorbs UVB radiation and is highly stable and thus could act as a natural sunscreen in these microorganisms.

Finally, our results reveal that MGG is able to deactivate $O_2(^1\otimes_g)$ in an aqueous solution. Nonenzymatic antioxidants such as ascorbic acid, <-tocopherol and ®-carotene base their action on the inhibition of ROS such as $O_2^{\cdot -}$, H_2O_2, ROO$^\cdot$, OH$^\cdot$ and $O_2(^1\otimes_g)$. Thus the ability of a molecule to scavenge or quench any of these species is considered to contribute to its antioxidant activity (*40*). Therefore, the ability of MGG to act as a quencher of singlet oxygen can be considered as a measure of the contribution of this mechanism to its antioxidant activity. The rate constant for the process, $k_t = 5.9 \times 10^7\ M^{-1}\ s^{-1}$ is lower than those of other antioxidants such as ascorbate ($1.6 \times 10^8\ M^{-1}\ s^{-1}$) or α-tocopherol ($6.4 \times 10^8\ M^{-1}\ s^{-1}$) in the same medium (*26*). However, it is comparable to that obtained by Suh *et al.* (*20*) for mycosporine-glycine in the organic solvent mixture methanol/chloroform. The large accumulation of MGG in *C. stepposus* (1.6 to 5.1 mg g^{-1} dry weight) probably compensates for the lower quenching rate constant.

The antioxidant activity of mycosporine-glycine has been reported in marine organisms (*41, 42*), and it is likely that MGG has a similar function in yeast. In this sense, the *E. coli* susceptibility test results provide the first evidence of protection against $O_2(^1\otimes_g)$ mediated by MGG. Although UVB is typically associated with direct damage, it has been demonstrated that UVB radiation induces the *in vivo* formation of ROS (*43*) in the cyanobacterium *Anabaena* sp. Furthermore, we recently found that torularhodin, a potent antioxidant pigment, protects yeast against UVB radiation by an indirect mechanism, probably by quenching the ROS species produced by the radiation (*22*). The photoprotective mechanism of MGG may include not only a reduction of UVB levels reaching important cell targets but also oxidant species deactivation, in particular of $O_2(^1\otimes_g)$. However, additional experimental evidence is needed to evaluate other mechanisms by which MGG may exert its antioxidant effect as well as the biological implications of these mechanisms.

CONCLUSIONS

The high accumulation of MGG under photosynthetically active irradiation, the relationship between MGG concentration and survival, the reduction of DNA damage, the high photostability, and the $O_2(^1\otimes_g)$ quenching capacity of MGG reported here indicate that MGG is a UVB photoprotective compound in *C. stepposus* and probably in other yeast species as well.

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