Photo-oxidative stress in *Rhodobacter sphaeroides*: protective role of carotenoids and expression of selected genes

Jens Glaeser and Gabriele Klug

In *Rhodobacter sphaeroides*, carotenoids are essential constituents of the photosynthetic apparatus and are assumed to prevent the formation of singlet oxygen by quenching of triplet bacteriochlorophyll a (BChl a) *in vivo*. It was shown that small amounts of singlet oxygen are generated *in vivo* by incubation of *R. sphaeroides* under high light conditions. However, growth and survival rates were not affected. Higher amounts of singlet oxygen were generated by BChl a in a carotenoid-deficient strain and led to a decrease in growth and survival rates. The data support earlier results on the pivotal role of carotenoids in the defense against stress caused by singlet oxygen. Results obtained under photo-oxidative stress conditions with strains impaired in carotenoid synthesis suggest that sphaeroidene and neurosporene provide less protection against methylene-blue-generated singlet oxygen than sphaeroidenone *in vivo*. Despite their protective function against singlet oxygen, relative amounts of carotenoids did not accumulate in *R. sphaeroides* wild-type cultures under photo-oxidative stress, and relative mRNA levels of phytoene dehydrogenase and sphaeroidene monooxygenase did not increase. In contrast, singlet oxygen specifically induced the expression of glutathione peroxidase and a putative Zn-dependent hydrolase, but mRNA levels of hydrogen-peroxide-degrading catalase E were not significantly affected by photo-oxidative stress. Based on these results, it is suggested that singlet oxygen acts as a specific signal for gene expression in *R. sphaeroides*. Presumably transcriptional regulators exist to specifically induce the expression of genes involved in the response to stress caused by singlet oxygen.

INTRODUCTION

The genus *Rhodobacter* includes highly metabolically versatile *ß*-Proteobacteria, which grow by aerobic respiration, anaerobic respiration or anoxygenic photosynthesis under anoxic conditions in the light. The environmental stimuli that affect the formation of photosynthetic complexes in *Rhodobacter sphaeroides* are oxygen tension and light intensity, and these have been investigated in detail (Bauer et al., 1999; Gregor & Klug, 1999, 2002; Zeilstra-Ryalls & Kaplan, 2004). Only recently has the effect of the reactive oxygen species hydrogen peroxide and superoxide anion on growth, survival and gene regulation been investigated in *Rhodobacter* (Li et al., 2003a, b, 2004; Zeller & Klug, 2004). In contrast, photo-oxidative stress and the protective role of carotenoids against the generation of singlet oxygen in *R. sphaeroides* were recognized 50 years ago (Griffiths et al., 1955). In this study it was reported that a carotenoid-deficient mutant of *R. sphaeroides* is rapidly killed when light and oxygen are present simultaneously. Decades later, it was shown that singlet oxygen is generated by triplet bacteriochlorophyll a (BChl a) in the presence of molecular oxygen (Borland et al., 1987). Evidence for the generation of singlet oxygen by BChl a in intact reaction centres (RCs) and light-harvesting (LH) complexes was obtained later. Protein subunits of carotenoid-free RCs are degraded quickly in the presence of light and oxygen (Tandori et al., 2001) and carotenoid-free LH complexes generate triplet and cation-radical BChl a upon illumination, which give rise to singlet oxygen formation in the presence of oxygen (Cogdell et al., 2000; Limantara et al., 1998).

Carotenoids represent a structurally very diverse class of isoprenoid pigments, which occur in all photosynthetic organisms. In photosynthetic purple bacteria, they are essential constituents of LH antennae (Lang et al., 1995; Zurdo et al., 1993), important in harvesting light energy and exciton transfer (Cogdell et al., 1999), and essential for the...
protection of the photosynthetic apparatus under photodiffuse stress (Cogdell & Frank, 1987; Cogdell et al., 2000). Carotenoids prevent the harmful effects of singlet oxygen by quenching (Foote & Denny, 1968), reacting with singlet oxygen to form oxidized forms of carotenoids (Fiedor et al., 2001, 2002) or quenching the triplet state of BChl a (Borland et al., 1989). In vivo, quenching of triplet BChl a by carotenoids is assumed to be the major process by which the generation of singlet oxygen is prevented in photosynthetic purple bacteria (Cogdell & Frank, 1987; Cogdell et al., 2000; Limantara et al., 1998).

Singlet oxygen has been shown to rapidly kill cells in many biological systems (Foote, 1976; Krinsky, 1978). Possible targets of damage by singlet oxygen include a large variety of biological molecules, such as DNA, proteins and lipids (Briviba et al., 1997). Singlet oxygen has been proven to be genotoxic (Epe, 1991) and mutagenic (Ouchane et al., 1997). Its half-life ranges from 4 μs in water (Foote & Clennan, 1995) to 200 ns in living cells (Gorman & Rodgers, 1992). Diffusion of singlet oxygen is limited to a range of 10 nm in vivo (Sies & Menck, 1992) and 100 nm in aqueous solutions (Kochevar & Redmond, 2000). The efficiency of singlet oxygen quenching varies between structurally different carotenoids and depends in vitro mainly on their concentration (Foote & Denny, 1968), the length of the conjugated system (Foote et al., 1970) and functional groups (Di-Mascio et al., 1989; Foote & Denny, 1968; Hirayama et al., 1994).

Singlet oxygen may elicit stress responses by affecting gene regulation. It has been shown that in response to singlet oxygen the expression of several genes is up-regulated in Chlamydomonas and Arabidopsis species (Leisinger et al., 2001; op-den-Camp et al., 2003). A light-dependent induction of carotenoid synthesis was observed in Myxococcus xanthus, which presumably is mediated by singlet oxygen (Hodgson & Murillo, 1993). The induction of carotenoid biosynthesis by singlet oxygen has also been shown in Pfaffia rhodozyma (Schroeder & Johnson, 1995). The genetic basis of the response to stress induced by singlet oxygen has been recently unravelled in Arabidopsis (Wagner et al., 2004).

However, the mechanisms underlying gene regulation and signal transduction induced by singlet oxygen are so far mostly unclear (Kochevar, 2004; Krieger-Liszkay, 2004).

In the present study, we have investigated the capacity of different carotenoids to protect R. sphaeroides under conditions of photo-oxidative stress and address the question whether genes involved in the defence against photo-oxidative and oxidative stress are induced by singlet oxygen.

METHODS

Bacterial strains and growth conditions. Rhodobacter strains were cultivated at 32 °C in minimal salt medium containing malate as carbon source (Drews, 1983). Semi-aerobic cultures were grown in Erlenmeyer flasks with continuous shaking at 140 r.p.m., resulting in a dissolved oxygen concentration of approximately 25 μM. For aerobic growth, cultures were gassed with air and stirred simultaneously, resulting in a dissolved oxygen concentration of approximately 180 μM. Strains used in this work are listed in Table 1 and have been characterized previously (Lang & Hunter, 1994; Lang et al., 1994, 1995). Kanamycin was added to cultures of strains impaired in carotenoid biosynthesis at a final concentration of 25 μg ml⁻¹. For solid media, 1.5% agar was added to the mineral salts medium.

High light and photo-oxidative stress conditions. Conditions of high light (800 W m⁻²) were generated with a white light halogen bulb (500 W; Osram) in cultures incubated in flat glass bottles. This chosen light intensity represents the maximum light intensity measured at noon under a clear sky on July 21, 2003 in Giessen, Germany (longitude: 8° 40’ 17”, latitude: 50° 35’ 21”) with an LI-250 light meter plus an LI-200 pyranometer sensor with a sensitivity range of 400–1100 nm (Li Cor). In laboratory experiments, light intensities in front of (I₁) and behind (I₂) the cultures were measured and mean values of light intensity within the bacterial cultures (I) were calculated as:

\[ I = \frac{(I_1 - I_2) \ln I_1}{I_2} \]  

(Van Liere & Walsby, 1982).

Light intensity was adjusted throughout the experiments to compensate for changes in turbidity due to bacterial growth. Conditions of photo-oxidative stress were generated by the addition of methylene blue (Sigma–Aldrich) at a final concentration of 0.2 μM to aerobically growing cultures incubated under high light. Methylene blue specifically generates singlet oxygen in the presence of light and oxygen. Bleaching of methylene blue was checked by recording UV/VIS

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Table 1. R. sphaeroides strains and pigment composition under semiaerobic conditions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Pigment composition</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSM158 (2.4.1)</td>
<td>Wild-type</td>
<td>BChl a, sphaeroidenone, traces of sphaeroidenone, neurosporene, hydroxysphaeroidenone</td>
<td>Imhoff et al. (1984); van Niel (1941)</td>
</tr>
<tr>
<td>TC23</td>
<td>Tn5 insertion just upstream of crtE</td>
<td>BChl a, low amounts of sphaeroidenone</td>
<td>Lang et al. (1995)</td>
</tr>
<tr>
<td>TC40</td>
<td>Tn5 insertion into crtC</td>
<td>BChl a, neurosporene</td>
<td>Lang et al. (1995)</td>
</tr>
<tr>
<td>TC52</td>
<td>Tn5 insertion into crtA</td>
<td>BChl a, sphaeroidenone, traces of hydroxysphaeroidenone</td>
<td>Lang et al. (1995)</td>
</tr>
<tr>
<td>TC67</td>
<td>Tn5 insertion into crtI</td>
<td>BChl a, phytoene</td>
<td>Lang et al. (1995)</td>
</tr>
</tbody>
</table>

*Semiaerobic cultures (Lang et al., 1995).
absorption spectra between 400 and 600 nm (Lambda12; Perkin Elmer) in culture medium without cells. Maximum absorption of methylene blue decreased during incubation under high light to 74 and 69% after 90 and 150 min, respectively.

Detection of singlet oxygen. Singlet oxygen was detected by the reaction with DanePy [3-(N-diethylaminoethyl)-N-dansyl-aminomethyl-2,5-dihydro-2,2,5,5-tetramethyl-1H-pyrrrole], yielding the nitroxide radical DanePyO [3-(N-diethylaminoethyl)-N-dansyl-aminomethyl-2,5-dihydro-2,2,5,5-tetramethyl-1H-pyrrrole-1-ylxoyl], which exhibits decreased fluorescence (Kárai et al., 1998). The change in relative DanePy fluorescence (ΔF/Fo) indicates the relative amount of singlet oxygen generated and was calculated as described by Hideg et al. (2000). Fluorescence of DanePy in Rhodobacter cultures was measured by excitation of samples at 337 nm and recording the emission at 545 nm with a Kontron SFM-25 spectrophotofluorimeter. For fluorescence measurements, a slit width of 10 nm and a quartz cuvette with a path length of 10 mm were used. To obtain data on singlet oxygen generation, a final concentration of 20 μM DanePy was added to exponentially growing cultures and then incubated under conditions of high light. For normalization of raw fluorescence data in time series experiments, initial fluorescence values at 0 min were used. Prior to normalization, fluorescence values obtained from dark-incubated control cultures were subtracted from values obtained from cultures incubated under high light.

Survival assays under photo-oxidative stress. The survival of Rhodobacter was determined after exposure to photo-oxidative stress for 90 min by plating aliquots of serial dilutions obtained from liquid cultures. Control cultures were incubated in the presence of high light (800 W m⁻²) and were kept in the dark.

Analysis of bacterial pigments. To gain absorption spectra, samples of cultures from directly before the shift to high light or photo-oxidative stress conditions were used. The optical density of all cultures was 0.4 at 660 nm and 30 ml was harvested at 10,000 g for 10 min. Pigments were extracted from cell pellets with a mixture of acetone and methanol (7/2, v/v) and analysed by UV/VIS spectroscopy (Lambda12). BChl a concentrations were calculated from the absorption at 772 nm with an extinction coefficient of 76 mM⁻¹ cm⁻¹ (Clayton, 1966). Carotenoids were analysed from the same extracts by HPLC (HP1100 Series; Hewlett Packard) on a C18 silica column (Muliphly ODS 5 μm, 250 × 4.6 mm; CS-Chromatographic Service, Langenwehe, Germany) with a mixture of methanol and acetone (9/1, v/v) and a flow rate of 1 ml min⁻¹ (Permentier et al., 2001). Identification of carotenoids was performed by their retention time and the absorption spectra were recorded between 300 and 800 nm with a diode array spectrophotometer (HP1100 Series, model G1324A). Extinction coefficients used for quantification of carotenoids were E1%1 cm⁻¹ = 2500 for sphaeroidene, hydroxysphaeroidene, sphaeroidene and hydroxysphaeroi
dene and E1%1 cm⁻¹ = 2700 for neurosporene (Züllig, 1985). For the preparation of carotenoid standards, acetone extracts of R. sphaeroides strains were separated on silica gel plates (Polygram Sil G; Macherey-Nagel) with a mixture of petroleum ether and acetone (6/1, 2, v/v). Coloured bands containing carotenoid bands were cut out and resolved in acetone, and concentrations were determined by UV/VIS spectroscopy (Lambda12).

RNA extraction and quantitative real-time RT-PCR. Samples from growth experiments with Rhodobacter cultures were obtained at 0, 5, 10, 20, 40 and 90 min after the shift of dark-incubated cultures to high light or to photo-oxidative stress. Samples were rapidly cooled in ice and pelleted by centrifugation at 10,000 g. In all cases, shift experiments for RNA isolation were started at an optical density of 0.4 at 660 nm. Total RNA was isolated by the hot phenol method and quantified by photometric analysis at 260 nm. Samples were treated with RQ1 RNase-free DNase I (Promega) to remove contaminating DNA. Absence of genomic DNA contamination was checked by PCR amplification of RNA samples. A final concentration of 4 ng total RNA µl⁻¹ was used and the following primers were synthesized to quantify relative gene expression by quantitative real-time RT-PCR: crtA, crtA-A (5′-GAATGGCCCATGATCTACC-3′) and crtA-B (5′-GGCGCTTCCAAAGCTTAC-3′); crtI, ccrA (5′-CAACGTACCTCGATGTA-3′) and ccrB (5′-GGAGCCTCGAT-3′); kate, kate-A (5′-CTATCCCGCTGATCGGTT-3′) and kate-B (5′-GTCGGCATAGGAAGAG-3′) (Zeller & Klug, 2004); rpoZ, 2.4.1 ropZ-A (5′-ATGCCTGGAAGAGCCAGCAGG-3′) and 2.4.1 ropZ-B (5′-GAGCCGCGATCCATCTGTC-3′) (Zeller & Klug, 2004); RSP0799, RSP0799-A (5′-GAAATAATACCCCTTCTTCTC-3′) and RSP0799-B (5′-CATCGCTGTGATGCTC-3′); RSP2389, RSP2389-A (5′-CCGCAATTAGACGACATCTT-3′) and RSP2389-B (5′-CCGAGTGTGATGTGTCAT-3′). Normalization of mRNA levels was performed with the rpoZ gene, which encodes the σ subunit of the R. sphaeroides RNA polymerase (Pappas et al., 2004). The one-step RT-PCR kit (Qiagen) was used for reverse transcription followed by PCR as described in the manufacturer’s manual, except SYBR Green (Sigma–Aldrich) was added at a final dilution of 1:50000 to the master final mix. Master mix and RNA solution except were mixed in a final volume of 10 μl for relative quantification of mRNA transcripts in a Rotor-Gene 3000 real-time PCR cycler (Corbett Research). For analysis slope correction and dynamic tube normalization options were applied in the rotor-gene software version 6.0 (Corbett Research). Crossing point (Cp) values representing the number of cycles where fluorescence signals started to increase in real-time RT-PCR was determined for all genes with a fluorescence threshold of 0.002 and relative expression of crtA, ccrA, kate, RSP0799 and RSP2389 mRNA was calculated relative to the expression of untreated samples and relative to rpoZ, according to the method of Pfaffl (2001). Real-time PCR efficiencies were determined by applying serial dilutions of mRNA between final concentrations of 8 and 0.2 ng µl⁻¹ as 1.89 for ccrA, 1.709 for ccrI, 1.96 for kate, 2.02 for rpoZ, 2.31 for RSP0799 and 2.04 for RSP2389.

Statistical analysis. Statistical analysis for comparison of Cp values for the above-mentioned genes under different physiological conditions was performed with Student’s t-test using Microsoft Excel. In all cases, significance was assumed if P<0.05.

RESULTS

Growth and survival of R. sphaeroides under high light and photo-oxidative stress conditions

Photo-oxidative stress occurs in phototrophic organisms in the presence of high light intensities and saturating concentrations of oxygen (Krieger-Liszky, 2004). To investigate the effect of daylight light intensities on growth and survival of R. sphaeroides, pigmented cultures were incubated under a light intensity of 800 W m⁻² and saturating oxygen tensions. Hereafter, these conditions will be referred to as ‘high light’. Exponentially growing semiaerobic cultures were diluted to an OD₆₆₀ of 0.2 and incubated under saturating oxygen concentrations in the dark. The cultures were then shifted to high light after an OD₆₆₀ of 0.4 was reached. Surprisingly, the shift to high light led to a slight increase in growth rate of R. sphaeroides compared to dark controls (Fig. 1a). To create photo-oxidative stress conditions, cultures containing a final concentration of 0.2 µM methylene blue were shifted to high light. Under these conditions a decrease in growth rate was observed and...
In comparison to wild-type cultures, we tested the effect of light on a mutant deficient in carotenoid synthesis (Fig. 1b). Light intensities of 13 W m$^{-2}$ significantly decreased the growth of strain TC67 after 25 min incubation and light intensities of 28 W m$^{-2}$ and higher stopped growth (Fig. 1b). Strain TC67 partially recovered after 180 min incubation at 13 W m$^{-2}$ and a growth rate slightly lower than the dark control was observed. Cultures of strain TC67 incubated at light intensities up to 96 W m$^{-2}$ also partially recovered, but to a lesser extent than the cultures incubated at 13 W m$^{-2}$ (Fig. 1b). Obviously, low light intensities generated photo-oxidative stress in the carotenoid-deficient strain TC67 whereas in wild-type cultures the addition of methylene blue and high light intensities were necessary to generate photo-oxidative stress.

To investigate in more detail the role of carotenoid composition and abundance, *R. sphaeroides* wild-type cultures and mutants impaired in carotenoid synthesis were tested for their survival after incubation under high light and photo-oxidative stress conditions. The survival rate of *R. sphaeroides* wild-type cultures obtained under photo-oxidative stress was not significantly different from high-light- and dark-incubated controls (Fig. 2). As determined by HPLC, wild-type cultures contained 76-6% sphaeroidene, 20-5% neurosporene and 2-6% hydroxysphaeroidene as major carotenoids (Table 2). The molar ratio indicated that four times more BChl a was present than carotenoids (Table 2). These results support the earlier finding that the carotenoid content and composition of

![Figure 1](image1.png)

**Fig. 1.** Growth of *R. sphaeroides* 2.4.1 and TC67 cultures under photo-oxidative stress. (a) Growth of *R. sphaeroides* 2.4.1 in the dark (black circles) and in the dark with 0-2 μM methylene blue (black squares), and under 800 W m$^{-2}$ (white circles) and 800 W m$^{-2}$ white light with 0-2 μM methylene blue (white squares). (b) Growth of *R. sphaeroides* TC67 under increasing light intensities: dark control (diamonds), 13 (black squares), 28 (white squares), 56 (black circles) and 96 W m$^{-2}$ (white circles).

![Figure 2](image2.png)

**Fig. 2.** Survival of *R. sphaeroides* 2.4.1 and mutants impaired in carotenoid synthesis under conditions of photo-oxidative stress. The percentage survival rate represents c.f.u. normalized to OD$_{660}$ and dark controls after 90 min of incubation. Grey bars, high light; black bars, photo-oxidative stress; white bars, dark controls. Survival values were normalized to dark controls. Error bars represent SD calculated from three independent experiments.

will hereafter be referred to as ‘photo-oxidative stress’ (Fig. 1a). The strong decrease in growth rate after 40 min incubation under photo-oxidative stress was only transient. Cultures of *R. sphaeroides* recovered after 120 min of incubation to a growth rate slightly lower than observed in the dark controls (Fig. 1a). Growth rates in dark controls did not change upon addition of methylene blue, indicating that methylene blue in the absence of light was not eliciting stress conditions.
Table 2. Composition and content of major pigments in *R. sphaeroides* strains

Pigments extracts were obtained from cultures directly before the shift to high light conditions. SO, Sphaeroidenone; SE, sphaeroidene; ND, not detected.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pigment [pmol (OD$_{660}$)$^{-1}$ ml$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BChl <em>a</em></td>
</tr>
<tr>
<td>2.4.1</td>
<td>717-9</td>
</tr>
<tr>
<td>TC23</td>
<td>118</td>
</tr>
<tr>
<td>TC40</td>
<td>223-7</td>
</tr>
<tr>
<td>TC52</td>
<td>113-3</td>
</tr>
<tr>
<td>TC67</td>
<td>143-8</td>
</tr>
</tbody>
</table>

*R. sphaeroides* efficiently prevent the generation of singlet oxygen in the light.

In contrast, a decrease in survival rate to 3% under high light and 1-8% under photo-oxidative stress was observed in the carotenoid-deficient mutant TC67 (Fig. 2). Cultures of strain TC67 contained fivefold lower amounts of BChl *a* than wild-type cultures (Fig. 3, Table 2). This indicates that even low amounts of BChl *a* can generate toxic amounts of singlet oxygen *in vivo*. Phytoene, the only carotenoid present in strain TC67 (Lang *et al.*, 1995) is known to be incapable in quenching singlet oxygen or triplet BChl *a* due to a short conjugated polyene chain (Foote *et al.*, 1970). Our results support the earlier finding that quenching of singlet oxygen by carotenoids is essential for the survival of *R. sphaeroides* in the presence of oxygen and light (Griffiths *et al.*, 1955).

The survival rate of strain TC23 decreased to 36% under photo-oxidative stress, but was not changed under high light conditions in comparison to wild-type cultures. This suggests that an amount of carotenoids 21-fold lower than in wild-type cultures and a carotenoid to BChl *a* ratio of 0-07 is sufficient to quench BChl *a*-generated singlet oxygen efficiently. Presumably, the quenching is mainly due to the presence of sphaeroidenone and hydroxysphaeroidenone, which accounted for 56-1% and 25-6% of the total carotenoids, respectively. Relative amounts of sphaeroidenone (6-1%) and neurosporene (12-2%) were much smaller (Table 2). However, the amount of carotenoids in strain TC23 compared to wild-type cultures was too low to prevent damage caused by singlet oxygen generated under photo-oxidative stress (Fig. 2), which underlines the importance of a high carotenoid content for survival of *R. sphaeroides* in the presence of singlet oxygen.

In cultures of strain TC40 only 81% of the cells survived incubation under photo-oxidative stress, but under high light no change was observed compared to dark controls (Fig. 2). Neurosporene is the most abundant carotenoid in strain TC40 and accounts for 97% of total carotenoids (Fig. 3, Table 2), whereas sphaeroidene and hydroxysphaeroidene comprise only 3% and sphaeroidenone was absent. The total amount of carotenoids was only 11-fold higher in strain TC40 compared to strain TC23 (Table 2), which explains its higher survival rate (Fig. 2). The results suggest that neurosporene is less efficient in quenching singlet oxygen than sphaeroidenone *in vivo*, which is mainly present in the wild-type and in strain TC23, because survival decreased despite a higher carotenoid to BChl *a* ratio compared to the wild-type. However, the decrease in survival rate of strain TC40 under photo-oxidative stress was not statistically significant when compared to high-light- and dark-incubated cultures as indicated by the standard deviations (Fig. 2).

The survival rate of strain TC52, which contained mainly sphaeroidene (93-3%), small amounts of hydroxysphaeroidene (5%) and neurosporene (1-7%) decreased to 76 and 63% under high light conditions and photo-oxidative stress, respectively (Figs 2 and 3). Compared to strain TC40, survival rates under both high light and photo-oxidative stress were lower in strain TC52, although similar amounts

![Fig. 3. Absorption spectra of *R. sphaeroides* 2.4.1 and mutants impaired in carotenoid synthesis. Samples for extraction of bacterial pigments were obtained directly before shift to high light or photo-oxidative stress from cultures with an OD$_{660}$ of 0.4. Relative absorption in all spectra except for the wild-type were increased by a factor of 2 for clarity, because strains impaired in carotenoid synthesis contained much smaller amounts of pigments than the wild-type (Table 2).](http://mic.sgmjournals.org)
of total carotenoids are present in both strains and the ratio of carotenoids to BChl \( \text{a} \) is larger in strain TC52 (Fig. 3, Table 2). Therefore, it is very likely that sphaeroidene is less efficient in quenching singlet oxygen than neurosporene \textit{in vivo}.

In conclusion, the abundance of total carotenoids and the type of carotenoid present \textit{in vivo} are important factors for the survival of \textit{R. sphaeroides} under high light and photo-oxidative stress.

Detection of singlet oxygen generated \textit{in vivo}

To investigate if singlet oxygen is generated in \textit{R. sphaeroides} cultures under physiological conditions, we measured singlet oxygen during exponential growth under high light conditions. Relative amounts of singlet oxygen generated were determined by the decrease in fluorescence of DanePy, a trap molecule that specifically reacts with singlet oxygen (Kállai \textit{et al.}, 1998). Only small amounts of singlet oxygen were generated by \textit{R. sphaeroides} wild-type cultures incubated at 800 W m\(^{-2}\) as indicated by the small decrease in relative DanePy fluorescence (Fig. 4). However, singlet oxygen was clearly detected after 120 min incubation. A much larger decrease in DanePy fluorescence was observed when strain TC67 was incubated at light intensities as low as 20 W m\(^{-2}\). In comparison, the relative DanePy fluorescence in strain TC67 was fourfold lower than in wild-type cultures (Fig. 4) even though TC67 cultures were incubated under a 40-fold lower light intensity and contained fivefold lower levels of BChl \( \text{a} \) (Table 2). Obviously, low amounts of BChl \( \text{a} \) are sufficient to generate toxic levels of singlet oxygen under low light intensities. Interestingly, singlet oxygen was also generated under high light in wild-type cultures. However, cellular damage was not observed by a decrease in growth or survival rate (Figs 1 and 2).

Lack of carotenoid accumulation under photo-oxidative stress

Certain carotenoids are known to accumulate under photo-oxidative stress, e.g. astaxanthin in \textit{P. rhodozyma} (Schroeder & Johnson, 1995) lycopene in \textit{M. xanthus} (Hodgson & Murillo, 1993) and zeaxanthin in plants (Demmig-Adams \textit{et al.}, 1999; Foyer & Jeremy, 1999). To investigate if carotenoids accumulate in \textit{R. sphaeroides} wild-type cultures under photo-oxidative stress, reverse-phase HPLC was used to quantify carotenoid abundance after 150 min incubation. Only sphaeroidenone, hydroxysphaeroidenone and neurosporene were detectable in considerable amounts in wild-type cultures under aerobic conditions (Table 2). In comparison to dark controls the ratio of sphaeroidenone to BChl \( \text{a} \) and the sphaeroidenone content normalized to optical density did not increase after 150 min incubation under photo-oxidative stress or incubation under high light (Fig. 5). To control for changes in relative pigment contents, BChl \( \text{a} \) amounts were normalized to optical density. The resulting relative BChl \( \text{a} \) content was similar under all three conditions (data not shown). In conclusion, the relative content of sphaeroidenone was not increased in response to the exposure to photo-oxidative stress or high light under aerobic growth conditions. Similar results were obtained for neurosporene (data not shown).

\[ \text{Relative change in fluorescence} = \frac{F(t) - F(0)}{F(0)} \]

\[ \text{Relative value} = \frac{\text{Amount of carotenoid}}{\text{OD}_{660}} \times 100 \]

Fig. 4. Singlet oxygen as detected by the change of DanePy fluorescence. The change in fluorescence of DanePy (\( \Delta F/F_{545} \)) indicates the relative amount of singlet oxygen generated in \textit{Rhodobacter} cultures. Dark circles, \textit{R. sphaeroides} 2.4.1; white squares, \textit{R. sphaeroides} TC67. \textit{R. sphaeroides} 2.4.1 cultures were incubated at 800 W m\(^{-2}\) and TC67 cultures at 20 W m\(^{-2}\). Error bars represent SD of four independent measurements.

Fig. 5. Relative change in the sphaeroidenone levels in \textit{R. sphaeroides} 2.4.1 cultures. Amounts of sphaeroidenone measured by reverse phase HPLC were normalized to optical density. Relative values represent the percentage change with reference to dark controls. Each bar represents the mean of three independent experiments and error bars represent SD. Grey bars, high light; black bars, photo-oxidative stress; white bars, dark controls; SO, sphaeroidenone.
Levels of phytoene dehydrogenase and sphaeroidene monooxygenase mRNA were not increased by photo-oxidative stress

Carotenoids may undergo a high turnover under photo-oxidative stress due to chemical quenching of singlet oxygen (Edge & Truscott, 1999). To investigate if carotenoid synthesis is stimulated by photo-oxidative stress the relative expression of *crtI* and *crtA* was investigated. Phytoene dehydrogenase (*CrtI*) is involved in the synthesis of neurosporene from phytoene, and sphaeroidene monooxygenase (*CrtA*) converts sphaeroidene into sphaeroidenone (Takaichi, 1999). In a time-course experiment, relative levels of *crtI* and *crtA* mRNA decreased to 0.59 and 0.29, respectively, when normalized to values at 0 min (Fig. 6). The relative mRNA level after 20 min exposure to photo-oxidative stress was confirmed for both genes by three independent experiments (Fig. 7, Table 3). On average, the relative levels of *crtA* and *crtI* mRNA were decreased 1.9- and 1.7-fold after 20 min incubation under photo-oxidative stress, respectively, when normalized to the expression of *rpoZ* and dark controls (Fig. 7). Statistical analysis of raw Cp values showed that the decrease in *crtA* and *crtI* mRNA levels under photo-oxidative stress and high light was significant (Table 2). Relative expression of *crtA* and *crtI* was not affected after the addition of 1 mM hydrogen peroxide using the same time points for obtaining mRNA samples as depicted in Fig. 6 (data not shown). The results indicate that singlet oxygen generated by methylene blue under photo-oxidative stress did not stimulate the expression of genes involved in the biosynthesis of carotenoids.

![Fig. 6. Time-course of relative gene expression under photo-oxidative stress. Levels of mRNA were determined by quantitative real-time RT-PCR for *crtA* (white circles), *crtI* (black circles), *katE* (white squares), RSP0799 (black squares), RSP2389 (white diamonds) and *rpoZ* (black diamonds) and normalized to values obtained at 0 min. All data points represent double amplifications of a representative time-course experiment. Variations in Cp values were below 5% in all cases.](http://mic.sgmjournals.org)

Specific induction of gene expression by photosensitizer-generated singlet oxygen

The expression of glutathione peroxidase was induced specifically by singlet oxygen in *Chlamydomonas* (Leisinger et al., 2001) and was found to degrade singlet-oxygen-generated peptide peroxides rapidly *in vitro* (Morgan et al., 2004). Therefore, we investigated if mRNA levels of the putative glutathione peroxidase gene RSP2389 were increased under photo-oxidative stress in *R. sphaeroides*. In a time-course experiment, the relative expression of RSP2389 increased 16-fold after 40 min incubation under photo-oxidative stress when compared to the non-treated control at 0 min (Fig. 6). Mean mRNA levels from three independent experiments also showed an increase after 20 min exposure to photo-oxidative stress when raw data were normalized to *rpoZ* and dark controls (Fig. 7). The statistical significance of this increase was demonstrated by the comparison of raw Cp values (Table 3). In contrast to photo-oxidative stress, relative levels of RSP2389 were not increased under high light (Fig. 7, Table 3). Glutathione peroxidase very likely has a similar function in *R. sphaeroides* as in *Chlamydomonas*, because its expression is induced by the same stress factor, singlet oxygen.

A putative Zn-dependent hydrolase encoded by gene

![Fig. 7. Relative expression of genes after exposure to high light and photo-oxidative stress. Real-time RT-PCR was used to investigate the relative expression of *crtA*, *crtI*, *katE*, RSP2389 and RSP799 in *R. sphaeroides* 2.4.1 after 20 min incubation under photo-oxidative stress. Relative expression values were normalized to *rpoZ* and to the dark controls. HLMB, Cultures incubated under photo-oxidative stress; HL, cultures incubated under high light.](http://mic.sgmjournals.org)
RSP0799 increased in relative abundance under photo-oxidative stress, determined by the comparison of protein patterns generated by 2D gel electrophoresis (unpublished data). Similar to glutathione peroxidase, the expression of RSP0799 was highest after 40 min exposure to photo-oxidative stress (Fig. 6). Mean mRNA levels of RSP0799 increased 15-fold after 20 min exposure to photo-oxidative stress in three independent experiments when normalized to rpoZ and dark controls (Fig. 7). The increase of RSP0799 expression was statistically significant under photo-oxidative stress when raw Cp values were compared to dark controls (Table 3). The same analysis showed that relative expression of RSP0799 was not significantly increased under high light (Fig. 7, Table 3).

Relative mRNA levels of RSP0799 and RSP2389 did not increase after the addition of 1 mM hydrogen peroxide using the same time points as depicted in Fig. 6 (data not shown). Both genes showed very similar expression in the carotenoid-deficient strain TC67 under a light intensity of 15 W m\(^{-2}\) in comparison to photo-oxidative stress in wild-type cultures (data not shown). In conclusion, RSP0799 and RSP2389 were induced in wild-type cultures only if singlet oxygen was generated by methylene blue. Taking into account that glutathione peroxidase degrades protein peroxides generated by singlet oxygen, it is very likely that singlet oxygen or damage caused by singlet oxygen is a specific signal for gene regulation in *R. sphaeroides*.

### DISCUSSION

**Carotenoids efficiently quench internally and externally generated singlet oxygen**

Several studies have implied that carotenoids mainly prevent the generation of singlet oxygen by quenching triplet BChl \(a\) (Cogdell *et al.*, 2000; Limantara *et al.*, 1998). Carotenoids and BChl \(a\) are in close proximity in the photosynthetic apparatus of photosynthetic purple bacteria (Cogdell *et al.*, 1999; Fraser *et al.*, 2001) and kinetic requirements for triplet energy transfer from BChl \(a\) to carotenoids are fulfilled (Cogdell *et al.*, 2000; Limantara *et al.*, 1998). Since the reaction of BChl \(a\) with molecular oxygen is three orders of magnitude slower than with carotenoids, it has been speculated that singlet oxygen is not generated under physiological conditions in photosynthetic purple bacteria (Cogdell *et al.*, 2000; Fraser *et al.*, 2001). In contrast, our results show that singlet oxygen is generated *in vivo* under high light conditions (Fig. 4), but growth and survival of *R. sphaeroides* were not decreased.
(Fig. 2). Therefore, the amounts of singlet oxygen generated in vivo are not inhibitory for \textit{R. sphaeroides}. Growth experiments with strain TC67 showed that BCHl \textit{a}-mediated generation of high levels of singlet oxygen causes strong damage even under low light intensities (Fig. 1b). Earlier observations from experiments with carotenoid-deficient \textit{R. sphaeroides} mutant R26 (Griffiths et al., 1955) and with a carotenoid-free mutant of \textit{Rubrivivax gelatinosus} (Ouchane et al., 1997) were in agreement with our results.

**Protective role of carotenoids: high light conditions**

Experiments providing evidence for a fast triplet energy transfer in from BCHl \textit{a} to carotenoids in RC and LHII suggested that singlet oxygen could only be generated in \textit{R. sphaeroides} when carotenoids are absent (Cogdell et al., 2000; Limantara et al., 1998). Since mostly isolated RC and LHII complexes have been investigated so far to study the protection against singlet oxygen formation by carotenoids, the question remains, which carotenoids prevent the formation of singlet oxygen in vivo most efficiently? \textit{R. sphaeroides} strains containing mainly neurosporene (TC40) or low amounts of sphaeroidenone and hydroxysphaeroidenone (TC23) survived 90 min of high light at the same rate as the wild-type, but a strain containing mainly sphaeroidene and traces of hydroxysphaeroidene (TC32) showed a lower survival rate (Fig. 2). In contrast, neurosporene was least efficient in singlet oxygen quenching among \textit{R. sphaeroides} carotenoids as determined by the oxidation of linolic acid in a mixture of n-hexane and methanol (Hirayama et al., 1994). According to this study, carotenoids found in \textit{R. sphaeroides} can be sorted by decreasing efficiency in singlet oxygen quenching in the following order: hydroxysphaeroidenone, sphaeroidenone, hydroxysphaeroidene, sphaeroidene, neurosporene. Despite this finding, a neurosporene-containing strain (TC40) was not harmed, but a sphaeroidene-containing strain was harmed by high light conditions. This result cannot be explained by different amounts of carotenoids, which are similar in both strains (Table 2). Presumably, neurosporene is less efficient in quenching triplet BCHl \textit{a} in vivo than neurosporene. However, the lack of sphaeroidenone monooxygenase, which is missing in strain TC52, could also explain a decreased rate of survival (Table 1). It has been suggested that sphaeroidene monooxygenase is involved in photoprotection (Yeliseev & Kaplan, 1997). In this photoprotection mechanism sphaeroidene acts as a sink for singlet oxygen via chemical quenching as mediated by sphaeroidenone monooxygenase. The results on relative mRNA levels of \textit{crtA} were not increased under photo-oxidative stress and, hence, do not support a role for \textit{crtA} in protection against singlet-oxygen-generated damage, because an increase in relative mRNA levels should then be expected. Since post-transcriptional regulation has been observed for \textit{crtA} expression (O’Gara & Kaplan, 1997), its role in photoprotection under photo-oxidative stress remains an open question.

In comparison to the carotenoid-deficient strain TC67, all other strains tested had much higher survival rates under high light and photo-oxidative stress (Fig. 2). These results were supported by an earlier study performed with \textit{Rubrivivax gelatinosus}. Although the carotenoid composition is different in \textit{Rubrivivax gelatinosus}, a similar general conclusion was drawn: mutants containing carotenoids survived under high light conditions, whereas survival decreased drastically in carotenoid-free mutants (Ouchane et al., 1997).

**Protective role of carotenoids: photo-oxidative stress**

Not only does the prevention of singlet oxygen generation play a pivotal role in organisms, but so does the quenching of singlet oxygen (Di-Mascio et al., 1989; Foote & Denny, 1968). Prosthetic groups and the extension of the conjugated electron system, i.e. the structural features of carotenoids, largely influence their efficiency to quench singlet oxygen (Foote et al., 1970; Hirayama et al., 1994). Taking into account this information, the question arose whether the carotenoids found in \textit{R. sphaeroides} are able to prevent damage from externally generated singlet oxygen. Water-soluble photosensitizers have been frequently used to increase the amount of singlet oxygen generated in biological systems (Kochevar & Redmond, 2000). The survival rate of all strains impaired in carotenoid biosynthesis decreased under conditions of photo-oxidative stress (Fig. 2), indicating that quenching of singlet oxygen generated by methylene blue depends on the specific amount of carotenoid present \textit{in vivo} (Foote et al., 1970). This theory is supported by the amount of carotenoids found and the survival rate observed in strains impaired in carotenoid synthesis and wild-type cultures. Clearly, survival and carotenoid content increased in the order TC67, TC23, TC40/TC52 and wild-type cultures (Fig. 3, Table 2). The importance of the type of carotenoid present \textit{in vivo} becomes clear from the comparison of strains TC40 and TC52. Although similar amounts of carotenoids were observed, the neurosporene-containing strain (TC40) exhibited higher survival rates than the sphaeroidene-containing strain (TC52). Hence, our \textit{in vivo} data are not in agreement with results obtained from \textit{in vitro} systems where it was clearly observed that neurosporene is less efficient in singlet oxygen quenching than sphaeroidene (Hirayama et al., 1994).

**Evidence for the cellular adaptation to photo-oxidative stress**

In our growth experiments, cultures of strain TC67 and wild-type cultures recovered from low growth rates during prolonged incubation under low light and photo-oxidative stress, respectively (Fig. 1). This suggests that an adaptation to the stress conditions has occurred. However, the spontaneous generation of mutants more resistant to photo-oxidative stress may also explain this phenomenon. In a previous study with carotenoid-deficient mutants of
Rubrivivax gelatinosus, a frequency of $10^{-3}$ mutants with a higher resistance to photo-oxidative stress compared to carotenoid-deficient mutants was observed (Ouchane et al., 1997). Therefore, generation of mutations can be ruled out as a reason for increased resistance, but a frequency of $10^{-3}$ mutations would only have generated a higher resistance in 0.1% of the cellular population.

Singlet oxygen induces carotenoid biosynthesis in Pfaffia rhodozyma (Schroeder & Johnson, 1995) and very likely mediates the light-dependent induction of carotenoid biosynthesis in Myxococcus xanthus (Hodgson & Murillo, 1993). In plants, zeaxanthin accumulates only under photo-oxidative stress and plays an essential role in protection of the photosynthetic apparatus via the xanthophyll cycle (Demmig-Adams et al., 1999). Therefore, we expected an increase of relative carotenoid content in R. sphaeroides under photo-oxidative stress. However, neither an increase in carotenoid contents nor increased expression of carotenoid synthesis genes was observed in R. sphaeroides (Figs 4 and 7). Therefore, other factors must exist in R. sphaeroides which are expressed in response to photo-oxidative stress to allow adaptation to higher singlet oxygen levels.

**Induction of putative cellular defence systems**

Induction of gene expression by singlet oxygen has been observed in bacteria, yeast and plants (Hodgson & Murillo, 1993; Leisinger et al., 2001; op-den-Camp et al., 2003). As an example, glutathione peroxidase significantly increased upon incubation under photo-oxidative stress in Chlamydomonas (Leisinger et al., 2001) and is also involved in the degradation of peptide peroxides generated by singlet oxygen exposure (Morgan et al., 2004). As in Chlamydomonas the increase of glutathione peroxidase in R. sphaeroides was specifically induced under photo-oxidative stress. Assuming the same function for glutathione peroxidase in R. sphaeroides, its induction indicates a specific response to stress caused by singlet oxygen. Further evidence for the induction of gene expression by singlet oxygen in R. sphaeroides was obtained by the increase of mRNA levels for a putative Zn-dependent hydrolase in response to photo-oxidative stress. These findings and the lack of katE expression by photo-oxidative stress clearly suggested that putative transcriptional regulators for the induction of genes in response to singlet oxygen must exist in R. sphaeroides. More than these factors are different from those involved in the regulation of genes detoxifying hydrogen peroxide, such as katE. The identification of the regulatory factors that mediate the singlet-oxygen-specific response in R. sphaeroides will be the subject of future investigations. Only recently has the genetic basis for the response to singlet oxygen been investigated in Arabidopsis and regulation factors responding specifically to singlet oxygen have been found (Wagner et al., 2004).

Although R. sphaeroides wild-type cultures do not seem to encounter stress by BChl a-mediated generation of singlet oxygen, the presence of a defence system against singlet oxygen generated by an extracellular photosensitizer would potentially be very important under environmental conditions. Extracellular production of singlet oxygen by humic acids has been reported recently (Paul et al., 2004). Humic acids are widespread in environments with high nutrient loads in aquatic and terrestrial habitats. Different efficiencies of singlet oxygen generation by humic acids have been reported to be dependent on an aquatic or terrestrial origin. In the case of aquatic origin, the amounts of singlet oxygen generated depend on the depth of the water column and the season of sampling (Paul et al., 2004). Extracellular sources of singlet oxygen might be important under natural conditions. Carotenoids are potentially insufficient to prevent damage by singlet oxygen generated through humic acids or other extracellular photosensitzers under natural conditions. Therefore, singlet oxygen generated in the environment may affect growth and survival of Rhodobacter under natural conditions, which shows the potentially pivotal role of a defence system expressed in response to high levels of singlet oxygen.

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