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Symbiotic Ciliates Receive Protection Against UV Damage from their Algae: A Test with *Paramecium bursaria* and *Chlorella*

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We assessed the photoprotective role of symbiotic *Chlorella* in the ciliate *Paramecium bursaria* by comparing their sensitivity to UV radiation (UVR) with *Chlorella*-reduced and *Chlorella*-free (aposymbiotic) cell lines of the same species. Aposymbiotic *P. bursaria* had significantly higher mortality than the symbiotic cell lines when exposed to UVR. To elucidate the protection mechanism, we assessed the algal distribution within the ciliate using thin-sections and transmission electron microscopy and estimated the screening factor by *Chlorella* based on an optical model. These analyses evidenced a substantial screening factor ranging, from 59.2% to 93.2% (320 nm) for regular algal distribution. This screening efficiency reached up to ~100% when *Chlorella* algae were dislocated to the posterior region of the ciliate. The dislocation was observed in symbiotic ciliates only under exposure to UV plus photosynthetically active radiation (PAR) or to high PAR levels. Moreover, under exposure to UVB radiation and high PAR, symbiotic *P. bursaria* aggregated into dense spots. This behavior could represent an efficient avoidance strategy not yet described for ciliates. Analyses of the intact symbiosis and their algal symbionts for UV-screening compounds (mycosporine-like amino acids and sporopollenin) proved negative. Overall, our results show that photoprotection in this ciliate symbiosis represents an additional advantage to the hitherto postulated nutritional benefits.

Key words: ciliates; green algae; mutual benefit; symbiosis; UV radiation.

Introduction

Endosymbiotic mutualistic associations of ciliates with green algae are a common trophic relationship known to provide a close nutritional coupling, with inorganic nutrients passing from hosts to algae and photosynthate (e.g., maltose) from algae to hosts (Reisser 1992). Undoubtedly, the best studied system among stable ciliate—algae associations is that of *Paramecium bursaria* (Ehrenberg, 1831) Focke, 1836 (Hymenostomatia) with the unicellular green alga *Chlorella* (Trebouxiophyceae) (Reisser 1986). Such protist symbioses are often interpreted as a result of nutritional adaptation to oligotrophic conditions (Dolan 1992). Their habitats, however, are often also highly transparent to solar UV radiation (UVR, 280—400 nm), which is damaging to protists (Giese 1945; Sanders et al. 2005; Sommaruga

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and Buma 2000; Sommaruga et al. 1996), as well as to other aquatic organisms.

Particularly, UVR damages several cell targets such as DNA, proteins, pigments, and lipids. Damage to DNA and other cell targets may be caused by direct absorption of UVB radiation (280—315 nm) or indirectly through the generation of reactive oxygen species (ROS) induced mainly by UVA radiation (315—400 nm; Vincent and Roy 1993) and high light intensities (Sinha and Häder 2002). In phytoplankton, inhibition of photosynthesis and growth, and cell death through DNA damage are the predominant negative effects caused by excessive UVB radiation (Buma et al. 1997; Sinha and Häder 2002).

Aquatic organisms minimize damage caused by high light and UVR through several usually combined strategies including avoidance, different repair mechanisms, chromatic adaptation, production of quenching substances of ROS such as carotenoids and various enzymes, and production/accumulation of direct photoprotective compounds such as scytonemin and mycosporine-like amino acids (MAAs) (Roy 2000; Sinha and Häder 2002; Sommaruga 2001).

Most of our knowledge on the interaction between UVR and mutualistic associations is based on studies on invertebrate—algal symbiosis, particularly on scleractinian corals and their 'zooxanthellae' of the genus *Symbiodinium* (Gleason 2001; Shick et al. 1996; Shick and Dunlap 2002). The different sensitivity to UVR between free and in hospite forms in this symbiosis appears to be related to protection given by the host through the accumulation of MAAs in their tissue. MAAs are intracellular, colourless water-soluble compounds having their maximum absorption between 309 and 362 nm, i.e., in the range of the damaging UVB and UVA wavelengths (Dunlap and Shick 1998; Karentz et al. 1991).

In *P. bursaria*, the *Chlorella* cells are known to be mainly distributed in the peripheral cytoplasm but also close to the macronucleus of the host (Karakashian et al. 1968). Moreover, some *Chlorella* species have a layer of sporopollenin in the external side of the cell wall (Atkinson et al. 1972) that screens UV wavelengths particularly in the UVB region (Xiong et al. 1997).

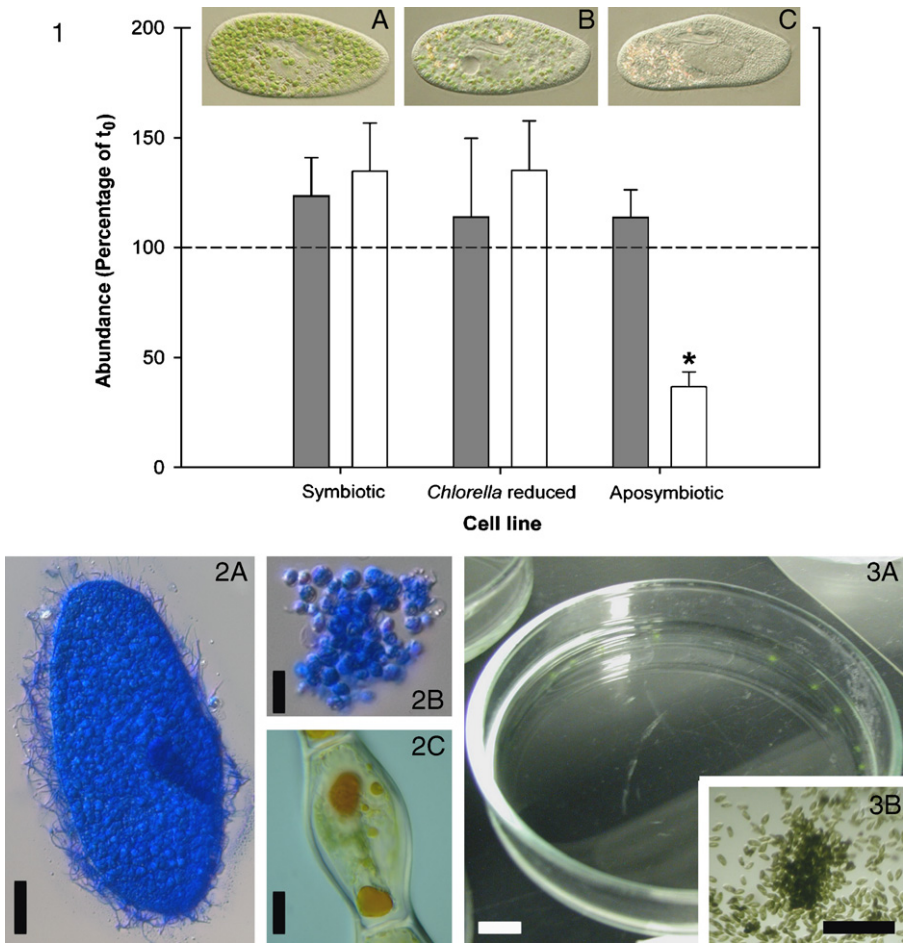
In this study, we tested the hypothesis that in hospite *Chlorella* confer protection to *P. bursaria* against the damaging effects of UVR. We compared the sensitivity to UVR of the symbiotic form with *Chlorella*-reduced and *Chlorella*-free (aposymbiotic) cell lines of the same species and assessed what mechanisms are involved in photoprotection. These experiments showed a significant reduction in mortality of the symbiotic ciliate cell line by *Chlorella* self-shading and revealed new behavior responses of this symbiosis when exposed to UVR.

Results

UV Sensitivity of Symbiotic and Aposymbiotic Ciliates

After 5 h of exposure to artificial UVR, neither visible damage nor reduction of cell densities was observed in the original symbiotic *P. bursaria* KM2 and in the *Chlorella*-reduced cell line (Fig. 1A, B). By contrast, aposymbiotic *P. bursaria* KM2w were highly sensitive to UVR and at the end of the experiment, cell numbers were on average 63% lower than in the control. Already after 2 h of exposure, damaged cells and motionless ciliates were observed. The ANOVA and pairwise multiple comparison procedures (Holm—Sidak post-hoc test; overall significance level = 0.05) for the time factor showed that the cell numbers of the aposymbiotic cell line *P. bursaria* KM2w declined significantly (unadjusted $p = 0.015$) after 5 h of UV exposure (T_5), whereas the dark controls of *P. bursaria* KM2w did not display significant changes ($p = 0.376$) within the same time. Cell numbers of the symbiotic *P. bursaria* KM2, as well as of the *Chlorella*-reduced cell line even increased within 5 h. At the end point of the experiment, the cell numbers in the dark control of green paramecia KM2 and *Chlorella*-reduced cell line of KM2 did not significantly differ from the respective UV-treated samples, whereas in the aposymbiotic cell line KM2w, the reduction in cell numbers under UV exposure was highly significant ($p = 0.001$) compared to the dark control.

The pairwise multiple comparison procedures (Holm—Sidak post-hoc test; overall significance level = 0.05) showed that when comparing the UV treatment with the dark control within the three cell lines at T_5, only the aposymbiotic *P. bursaria* KM2w had significantly lower cell numbers in the UV treatment ($p = 0.015$), whereas the cell lines with symbionts (i.e., KM2 and *Chlorella*-reduced KM2) showed no significant differences (Fig. 1). Within the dark controls, the changes in cell numbers were not significantly different in all three cell lines at T_5, whereas within the UV-treated samples, the aposymbiotic KM2w showed significant relative abundance changes after 5 h compared to KM2 ($p = 0.002$) and *Chlorella*-reduced KM2 ($p = 0.001$).



Figures 1—3. (**1**) Changes in relative abundance (percentage of T_0) of symbiotic *P. bursaria* (**A**), of the cell line with reduced symbiotic *Chlorella* (**B**), and of the aposymbiotic cell line (**C**) after 5 h of exposure to simulated UVR+PAR; dark bars (left) indicate the relative abundance in the control (i.e., darkness) and light bars (right) in the UV treatment; results are reported as the mean ± 1 standard deviation for three independent experiments; asterisk indicates a significant difference between control and treatment ($p < 0.05$). (**2**) Micrographs of *P. bursaria* (**A**) and of isolated *Chlorella* symbionts (**B**) stained deep blue with lactophenol cotton blue indicating the absence of sporopollenin in their cell walls; cells of *Trentepohlia annulata* that were used as sporopollenin-positive control are unstained (**C**); scale bar in **2A**: 20 μm, **2B**: 5 μm, and **2C**: 10 μm. (**3**) Aggregation of green *P. bursaria* into dense spots at the edge of a petri dish after 2 h exposure to UVR (**A**); the inset (**B**) is a magnification of a spot; scale bar in **3A**: 1 cm and in **3B**: 1 mm.

Mycosporine-like Amino Acids

Detection of these UV protecting compounds proved negative for *P. bursaria* and their *Chlorella* symbionts (data not shown).

Test for the Presence of Sporopollenin in the Cell Wall of *Chlorella* Symbionts

The use of lactophenol cotton blue (aniline blue W.S.) resulted in deep blue-stained *Chlorella* both inside *P. bursaria* and freshly isolated (Fig. 2A, B), indicating the absence of sporopollenin in their cell wall. By contrast, the green alga *Trentepohlia annulata* used as positive control, was sporopollenin positive as indicated by the lack of staining (Fig. 2C).

Behavior

Typically, symbiotic *P. bursaria* aggregated into dense spots of 1—3 mm in diameter (Fig. 3) while aposymbiotic ciliates did not. In the first experiment, where different cell densities were used, we found that under UVR+PAR (PAR — photosynthetically active radiation), as well as under high PAR

Table 1. Aggregation response of *Paramecium bursaria* after 2 h under different exposure conditions and cell densities.

Culture age (days)	Ciliate density (cells ml^{-1})	PAR+UVA+UVB	PAR+UVA	PAR	High PAR
10	50	–	–	–	–
10	100	–	–	–	–
10	500	+	–	–	+
10	1000	+	–	–	+
1*	<200*	–	–	–	–
4	500	+	–	–	–
7	500	+	–	–	+
10	500	+	–	–	+
13	500	–	–	–	+
16	500	–	–	–	–

All experiments were performed in triplicates. PAR = photosynthetically active radiation; (–)…no aggregation; (+)…aggregation into spots.
*Very low cell numbers in *P. bursaria* cultures (<200 cells ml^{-1}).

exposure, ciliates aggregated when *Paramecium* density was ⩾500 cells ml^{-1} (Table 1). In the second experiment, we observed aggregations in the exponential (day 4 and day 7) and early stationary growth phases (day 10) exposed to UVR+PAR and from day 7 to day 13 exposed to high PAR (Table 1). Cultures at day 16 did not show such aggregations.

Thin-Sections, Transmission Electron Microscopy

The *Chlorella* symbionts of *P. bursaria* were individually enclosed into perialgal vacuoles, had a noticeably thick cell wall, and their horseshoe-shaped chloroplast surrounded the nucleus. Under culture conditions without UVR, the symbiotic algae were located in one layer close to the cell surface of *P. bursaria* (Fig. 4A, B). By contrast, under exposure to UVR+PAR, we observed a relocation of the algal symbionts resulting in a dense *Chlorella* cluster in the posterior region of the ciliate cell (Fig. 4C, D). The observed intracellular distribution of *Chlorella* symbionts under exposure to high PAR was identical to that shown in Figure 4C. This dislocation of *Chlorella* was reversible, so that after placing the ciliates back to usual culture conditions, the original distribution of the symbionts was re-established within ~15 min.

UV Experiments under Selective Inhibition of Algal and Host Protein Synthesis by Cycloheximide and Puromycin

P. bursaria cultures in the exponential growth phase and with cell densities >500 ml^{-1} supplemented with cycloheximide or puromycin showed identical aggregation into dense spots under UV exposure, as well as in the respective controls (i.e., without the inhibitor). We did not find differences in this behavior whether the inhibitors of protein synthesis were added directly before UV exposure or hours, even days in advance (due to deleterious effects long-term treatment was performed only with 100 μg ml^{-1} cycloheximide and 75 μg ml^{-1} puromycin). Concerning the displacement of algal symbionts within the ciliate cell, the addition of cycloheximide (20—200 μg ml^{-1}), which inhibits the protein synthesis in *Chlorella*, but not in the host cell, had no effect compared to the untreated control cultures. However, the addition of puromycin (75 or 150 μg ml^{-1}) that inhibits additionally the ciliate protein synthesis, totally hindered the shift of *Chlorella* cells into the posterior part of the ciliate. Reproducibility of these results was confirmed at least three times.

Transmittance and Self-Shading of *Chlorella* Symbiont

Calculation of UV-transmittance and self-shading capacities of the algal symbionts resulted in the values listed in Table 2. Self-shading by a single *Chlorella* cell layer was estimated to be 34.6% of the incident radiation at 320 nm (Table 2). According to these calculations, 83.3% of the incident radiation will be reduced by two *Chlorella* cell layers. Considering eight layers of *Chlorella* cells as observed when they were dislocated and rearranged (Fig. 4C, D), ~100% protection from UVR at 320 nm would be obtained.

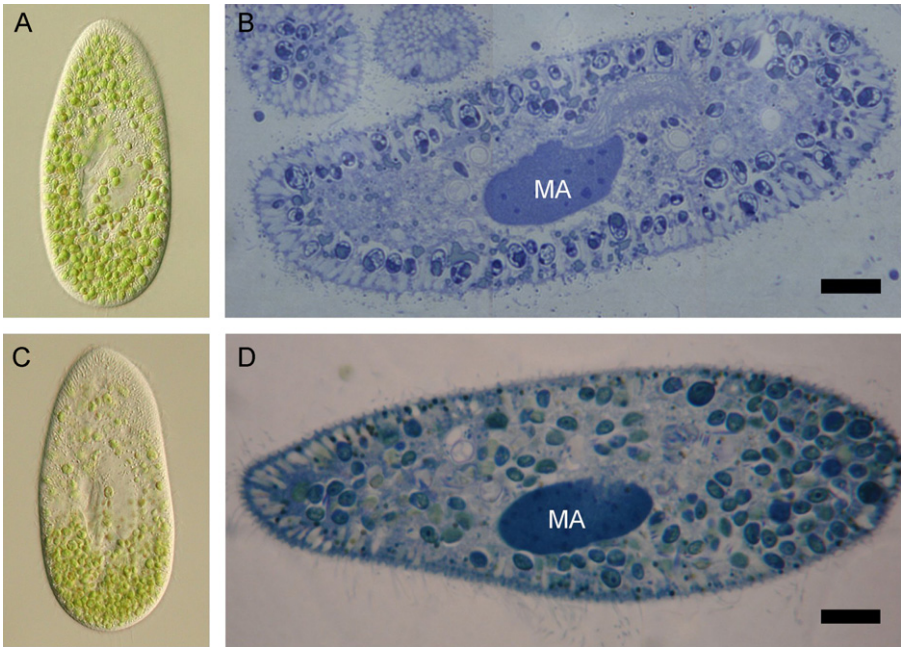


Figure 4. Intracellular distribution of *Chlorella*-symbionts before (**A** and **B**) and after 2 h exposure to UVR (**C** and **D**). Photos on the left (**A**, **C**) are live photos of the ciliate as observed by light microscopy, whereas those on the right show thin-sections (**B**: 0.5 μm; **D**: 0.35 μm) of *P. bursaria* stained with methylene blue-azur II as observed by TEM. MA = macronucleus. Scale bar in **B** and **D**: 10 μm.

Table 2. Transmittance and self-shading at 320 nm for different numbers of *Chlorella* cell layers within its host *Paramecium bursaria*.

Number of cell layers of *Chlorella*	Transmittance (%)	Radiation reaching cell targets of *Chlorella* cells in layer, *n* (%)	*Chlorella* distribution
1	40.81	65.38	Regular
2	16.65	26.68	Regular
3	6.80	10.89	Regular
4	2.77	4.44	Dislocated
5	1.13	1.81	Dislocated
6	0.46	0.74	Dislocated
8	0.08	0.12	Dislocated
10	0.01	0.02	Dislocated

UV Resistance of Single Symbiotic *P. bursaria* Cells

In these experiments where aggregation was hindered by exposing single cells to UVR, the symbiotic cells were still more tolerant than the aposymbiotic ones. On average, 82% of the symbiotic *P. bursaria* survived after 4.5 h of exposure to UVR, whereas only 20.8% and 7.3% of the aposymbiotic *P. bursaria* survived after 3 and 4.5 h of exposure, respectively (data not shown).

Discussion

In this study, we found evidence for a UV photoprotective effect of *Chlorella* living in symbiosis with *P. bursaria* (Fig. 1). Although *P. bursaria* is usually prevalent on periphyton and detritus,

where exposure to UVR is probably reduced, they are also found in the plankton of stagnant (i.e., pools, littoral zone of lakes) and slowly running waters (Foissner et al. 1994) where they are exposed to UVR.

The resistance of green *P. bursaria* against UVR was not associated with the presence of UV screening compounds such as MAAs. These secondary metabolites are known to efficiently protect aquatic organisms from UV damage (Shick and Dunlap 2002) and have recently been reported for the marine *Symbiodinium*-bearing ciliate *Maristentor dinoferus* (Sommaruga et al. 2006) and five planktonic *Chlorella*-bearing freshwater ciliate species (Sonntag et al. 2007). Further, sporopollenin, which is a constitutive component of the cell wall of some green algae (including *Chlorella* strains) and provides protection by screening UVR (Xiong et al. 1997) was not found in the *Chlorella* symbionts of *P. bursaria* (Fig. 2). Thus, the photoprotective mechanism in *P. bursaria* must be based on a different strategy.

The spot aggregation behavior of *Chlorella*-bearing *P. bursaria* (Fig. 3) observed when exposed to high PAR and UVR (Table 1) was not found in the aposymbiotic cell line *P. bursaria* KM2w. Thus, we argue that the presence of *Chlorella* is involved in this behavior. Photomovements are well known in protists (Häder 1987; Kuhlmann 1998; Lenci et al. 1997; Marangoni et al. 2000; Tomaru et al. 2001). For example, the *Chlorella*-bearing ciliates *P. bursaria* and *Euplotes daidaleos* are able to photoaggregate (i.e., gather in a light field) by photokinetic and/or photophobic responses (Machemer and Teunis 1996 and ref. therein, Nultsch and Häder 1988). Reisser and Häder (1984) studying the photobehavior of different symbiotic and aposymbiotic ciliates, found that *P. bursaria* display step-up (out-of-the-light) and step-down (into-the-light) photophobic responses. Photoaccumulation of *P. bursaria* in a light field is considered to be based on repetitive step-down photophobic responses hindering them to exit the light field. This response is supposed to be driven by their *Chlorella* symbionts, because it is not observed in aposymbiotic forms (Reisser and Häder 1984). By contrast, the step-up photophobic response is considered as an intrinsic reaction of the ciliate itself, but which nevertheless is raised by the presence of symbiotic algae (Reisser and Häder 1984).

Johnson et al. (1989), however, described a circadian rhythm of photoaccumulation in symbiotic and aposymbiotic *P. bursaria* probably involving a rhodopsin as the intrinsic photoreceptor (Nakaoka et al. 1991). Yet, despite numerous studies on photobehavior of ciliates, such a spot aggregation of *P. bursaria* under UVR and high PAR conditions (Fig. 3) has not been described before. Nakajima and Nakaoka (1989) analyzing the reactions of *P. bursaria* to visible light (550 nm) found that the ciliary movement of paramecia in light fields decreased when light intensity increased, resulting in accumulation of *P. bursaria* cells in the light. Though, when the light intensity reached a level higher than 1 W m^{-2}, locomotion increased again and a light-avoidance reaction started (Nakajima and Nakaoka 1989). We could not elucidate the mechanism responsible for the observed spot aggregation of symbiotic *P. bursaria*, but based on the results that this phenomenon was cell cycle- as well as density-dependent (Tables 1 and 2), and that it was not affected by the inhibition of protein synthesis of the whole symbiosis, we suggest that chemical signaling or physical interaction (i.e., contact frequency) were involved. For example, in *Dictyostelium discoideum*, an escape response from UV radiation (UVC at 254 nm) was described by Yasukawa (2004). This study also evidenced the release of a factor by irradiated *D. discoideum* into the culture medium. This "conditioned" medium enhanced the motility of non-irradiated cells and induced the expression of DNA repair enzymes in those cells without having direct contact to irradiated *D. discoideum* (Yasukawa 2004).

In addition to the spot aggregation of *P. bursaria*, we observed a dislocation of the algal symbionts within the ciliate cell (Fig. 4C, D). Such a rapid dislocation of *Chlorella* cells within the ciliate might be associated to a novel protection mechanism. According to the model of Garcia-Pichel (1994), free-living *Chlorella* are probably too small to benefit from efficient self-shading. However, *Chlorella* in symbiosis can enhance the protection efficiency by increasing the pathlength where cell matters absorb UVR. Under low PAR conditions, the peripheric distribution of *Chlorella* within *P. bursaria* is probably optimized for an efficient photosynthesis. This distribution would be insufficient for UV protection of symbionts by self-shading, but certain shading for the ciliate cell sensitive targets such as the nuclei would be provided (Table 2). Under high UVR or PAR levels, an aggregation of the symbionts to dense algal packages obviously provides sufficient protection by self-shading for important cell targets not only for the ciliate but probably also for the algae.

It is unclear, whether the displacement of the *Chlorella* symbionts was controlled by themselves

or by the host. The observation that the inhibition of algal protein synthesis alone (cycloheximide treatment) did not affect the displacement of the *Chlorella* symbionts, whereas an additional inhibition of the host protein synthesis (puromycin treatment) hindered it, suggests that some action of the host requiring protein synthesis enables this phenomenon. However, we cannot rule out that the algal symbionts somehow induce the host's action without the need of its own protein synthesis. In any case, the cytoplasmic transport system of *P. bursaria* has to be involved. Cytoplasmic streaming, a process leading to a rotational movement of vacuoles, organelles, and other cell content in a constant direction is well known in *Paramecium* (Sikora 1981). Nishihara et al. (1999) found that the cytoplasmic movement in *P. bursaria* is dependent on microtubuli and not on actin filaments. These authors proposed that dynein and maybe also kinesin act as a motor. Takahashi et al. (2007) observed an arrest of cytoplasmic streaming during cell division of *P. bursaria*, which was accompanied by the proliferation of endosymbiotic algae. As proliferation was also induced when cytoplasmic streaming was inhibited artificially by pressure or drugs, they argued that the arrest of cytoplasmic streaming itself triggered the cell division of algal symbionts. In our study, the assessment of cytoplasmic streaming directly under the UV lamps was not possible, but observations of *P. bursaria* cells with dislocated symbionts immediately after UV exposure did not reveal changes in direction or velocity of the cytoplasm at least at the magnification used. We were not able to investigate cytoplasmic streaming at high magnifications ($600\times$ —$1000\times$) because arresting of living *P. bursaria* cells without disturbing their cytoplasmic movements was not possible.

A dislocation of *Chlorella* symbionts has also been described for the ciliate *Pelagodileptus trachelioides* (Butkay 2004). This author described this phenomenon as a stress reaction followed by cytolysis. However, in our study *P. bursaria* did not die after symbiont dislocation and the process was reversible within minutes. The aggregation phenomenon of *P. bursaria* combined with the intracellular *Chlorella* dislocation are probably the most plausible explanations for the higher UV resistance of this symbiosis (Table 2). However, even when aggregation of symbiotic *P. bursaria* into spots was hindered, they were more resistant to UVR than the aposymbiotic cell line was. Further, the finding that *P. bursaria* with about half of the normal *Chlorella* density was not sensitive to UVR (Fig. 1B) raises several questions. Certainly, the method we used to assess the UV effect is unable to identify sublethal effects. However, those *Chlorella*-reduced *P. bursaria* were able to proliferate and establish normal cultures after UV exposure (M. Summerer pers. obs.). Thus, the number of *Chlorella* symbionts was probably still sufficient to enable protection by physical self-shading. Nevertheless, other protecting mechanisms may be involved because photoautotrophic organisms by their very nature have to cope with elevated levels of photo-oxidative stress during photosynthesis. Thus, they have evolved a powerful scavenging machinery of antioxidant enzymes (Butow et al. 1997; Lesser and Shick 1989). In a study of the free-living *Chlorella stigmatophora*, a significant free-radical scavenging activity of its hydro-soluble extract was demonstrated (Guzmán et al. 2001).

Kawano et al. (2004) found that alga-free cells of *P. bursaria* showed similar viability profiles to the naturally green cells when exposed to hydrogen peroxide. Recently, Hörtnagl and Sommaruga (2007) detected lower photo-oxidative stress in symbiotic *P. bursaria* KM2 compared to its aposymbiotic cell line KM2w, and proposed that this was a result of the scavenging activity of antioxidant enzymes originating from the algae. Similarly, Regoli et al. (2000) found the demosponge *Petrosia ficiformis* to have higher antioxidant defenses in symbiotic than in aposymbiotic individuals. Assuming that photo-oxidative stress accounted for a substantial proportion of the damage in aposymbiotic *P. bursaria*, the mutualistic relation with *Chlorella* might have been a major evolutionary benefit for *P. bursaria* and other mixotrophic ciliates to cope with oxidative stress.

In summary, the results of this study provide evidence that *P. bursaria* benefit from their symbionts in terms of UV resistance. We propose that protection was achieved mainly through shading of sensitive cell compartments by a specific arrangement of the algal symbionts within *P. bursaria*, depending on incident visible light and UV radiation combined with a particular behavior pattern of the host. Overall, our results show that photoprotection in this ciliate symbiosis represents an additional advantage to the hitherto postulated nutritional benefits.

Methods

***Paramecium* cultures**: *Paramecium bursaria* strain KM2 was obtained from Dr. I. Miwa, Ibaraki University, Japan, and

cultured in modified Woods Hole MBL (or WC) medium (Guillard and Lorenzen 1972; Nichols 1973) enriched with 30 mg l^{-1} yeast extract and kept in light/temperature controlled chambers under a 16:8 h light:dark cycle (80 μmol m^{-2} s^{-1} PAR and 0.10 W m^{-2} UVA) at 20 °C. The symbionts were characterized as *Chlorella* PbKM2 by partial 18S rRNA gene (NCBI accession number EF030567), the ITS-1 region (EF030584) and partial chloroplast 16S rRNA gene (EF030599) in a previous study (Summerer et al. 2008). We obtained *Chlorella*-reduced (about half symbiont density) and *Chlorella*-free cell lines from *P. bursaria* KM2 (KM2w) by growing the ciliates in permanent darkness for several weeks at 20 °C on a bacterial diet supported by a lettuce extract amended with 1.5% Chalkley's medium (Thompson et al. 1988). The lettuce extract was prepared by boiling 150 g lettuce leafs in 1 l deionized water. Then, the extract was filtered through a glass fiber filter GF/C (Whatman) and autoclaved.

After successful removal of symbionts, the aposymbiotic cell line KM2w was grown in the light under the same culture conditions as mentioned above for *P. bursaria* KM2 except for that it was cultivated in the amended lettuce extract. *Paramecium bursaria* (KM2w) was grown for at least two months previous to the experiments. The permanent absence of symbiotic algae was regularly checked in an Olympus microscope (BX50).

Sensitivity test of symbiotic and aposymbiotic ciliates to UVR: Short-term exposure (5 h) of *P. bursaria* KM2, the *Chlorella*-reduced, and the aposymbiotic cell lines to simulated UVR were done at the beginning of their stationary phase in a temperature-controlled walk-in chamber at 20 °C. Cultures (20 ml) were placed in petri dishes without lid and exposed under four UVA-340 (Q-Panel, Bolton, UK) fluorescent lamps (maximum emission between 340 and 345 nm, no radiation $<$285 nm, 8.60 W m^{-2} UVA and 2.47 W m^{-2} UVB) and two visible fluorescent tubes (True Lite T12, 40 W, 80 μmol m^{-2} s^{-1} PAR). The spectrum emitted by the lamps used in this set-up is found elsewhere (Sommaruga et al. 1996). Previous experiments showed that PAR delivered in this setup did not cause ciliate mortality or other negative effects (data not shown). The distance from the lamps to the liquid surface was 25 cm so that the organisms were exposed to an estimated biological effective dose (Setlow DNA action spectrum normalized at 300 nm) of 2.11 kJ m^{-2} in 5 h (Sommaruga et al. 1996). Every 30 min, the cell abundance in the UV treatment and the dark control was determined in triplicates by sampling 10 aliquots (10—20 μl, depending on cell density) each on microscopic slides for direct counts under an Olympus SZ40 microscope. Three independent experiments were conducted.

Analysis of mycosporine-like amino acids: We followed the protocol described by Sommaruga et al. (2006) and Sonntag et al. (2007). Briefly, *P. bursaria* cells were cleaned and transferred cell by cell into Eppendorf vials, whereas cultured *Chlorella* from this strain were concentrated by centrifugation. MAAs were extracted with 25% MeOH (v:v) at 45 °C for 2 h, then separated by HPLC under isocratic reverse-phase conditions and detected with a diode array detector.

Screening for sporopollenin in the cell walls of symbiotic *Chlorella*: We followed the method described by Syrett and Thomas (1973) using a lactophenol cotton blue solution (0.05% aniline blue W.S., Fluka, Switzerland) to identify sporopollenin-like structures in algal cell walls. *Chlorella* symbionts were freshly isolated from ciliate cells, suspended in a drop of lactophenol blue solution on a slide and covered with a cover slip for microscopical examination. In addition, whole symbiotic *P. bursaria* were stained as well. As sporopollenin-positive control, the green alga *Trentepohlia annulata* SAG 20.94 was used.

Behavior experiments: During the experiments, we observed an unusual aggregation of the symbiotic *P. bursaria* KM2 cultures. Thus, we tested first, whether this aggregation was ciliate density dependent. At the beginning of the stationary phase, *P. bursaria* at densities of 50, 100, 500, and 1000 cells ml^{-1} were exposed under (a) full UVR and PAR, (b) UVA radiation and PAR (exclusion of UVB radiation by a Mylar-D foil with 50% transmittance at 320 nm), (c) PAR only (exclusion with a Makrolon acrylic plate, Röhm, Germany), and (d) double PAR intensity (160 μmol m^{-2} s^{-1}, hereafter 'high PAR'). In a second experiment, equal cell densities (500 ciliates ml^{-1}) were exposed to the above mentioned conditions and treatments. From day one of inoculation over day four, seven, ten, thirteen to day sixteen, the aggregation behavior was recorded photographically. At each sampling date, we took aliquots of the cultures growing under standard cultivation conditions (see above), so that there was no pre-adaptation to UV or high PAR. All experiments were performed in triplicates and included a control (organisms kept in the dark).

UV experiments under selective inhibition of algal and host protein synthesis: To inhibit protein synthesis of the algal symbionts (Ayala and Weis 1987; Kodama and Fujishima 2008; Kodama et al. 2007), *P. bursaria* cultures in the exponential growth phase and cell densities $>$500 ml^{-1} were supplemented with cycloheximide (Sigma-Aldrich, Austria) in the following concentrations: 20, 100 and 200 μg ml^{-1}. In addition, 75 or 150 μg ml^{-1} of puromycin (Sigma-Aldrich, Austria) was added to the cultures to inhibit protein synthesis of the ciliate and the algal symbionts (Ayala and Weis 1987; Kodama et al. 2007). Experiments under full UVR and PAR were performed in petri dishes as mentioned above. After 2—3 h, spot-aggregation behavior and algal displacement within the ciliate cells were recorded. All experiments were performed in triplicates and included a control (untreated cultures).

UV resistance of single symbiotic *P. bursaria* cells: To test the UV resistance of the symbiotic cell line of *P. bursaria* KM2 compared with its aposymbiotic cell line without having the possibility to form cell aggregates (spots), single *Paramecium* cells were transferred into the wells of a 96-well microtiter plate, each filled with 100 μl of modified Woods Hole MBL medium. Half of the plate (48 wells) was filled with symbiotic cells and the other half with aposymbiotic cells. The plates (two replicates) were then exposed to UVR without cover under the same conditions as mentioned above (only full UVR treatment). The plates were examined with a dissection microscope after 1.5, 3, and 4.5 h for surviving ciliates. To ensure that all remaining ciliate cells were accounted for after 4.5 h, the total well content was transferred onto microscope slides with a micropipette and re-examined for possibly overseen ciliate cells.

Thin and ultrathin sections and transmission electron microscopy: To study the ultrastructure of the algal symbionts and their distribution within *P. bursaria*, ciliate and algal cells were concentrated in 2-ml reaction tubes by centrifugation (2000*g*, 5 min). After fixation with glutaraldehyde and osmium tetraoxide following the procedure described by Shigenaka et al. (1973), samples were dehydrated in a graded series of acetone and embedded into low viscosity resin after Spurr (1969). Thin-sections (0.35—0.5 μm) were stained with methylene blue-azur II (Richardson et al. 1960), embedded

into cedar wood oil, and observed with an Olympus microscope (BX50). Ultrathin-sections (70 nm) were mounted on Formvar-coated grids and contrasted with sterile uranyl acetate for 15 min and sterile lead citrate for 10 min (Reynolds 1963). Sectioned material was observed and photographed with a Zeiss TEM 902 electron microscope.

Calculation of self-shading and transmittance of *Chlorella*: We used the formulas developed by Garcia-Pichel (1994) for the calculation of self-shading and UV transmittance of the algal symbionts:

(1) internal self-shading:

$$J = 1 - \frac{1}{aR} - \frac{\exp(-2aR) - 1}{2(aR)^2}$$

(2) absorption:

$$Q = 1 + \frac{\exp(-2aR)}{aR} + \frac{\exp(-2aR) - 1}{2(aR)^2}$$

Based on those equations we defined

(3) transmission:

$$T_n = (1 - Q)^n$$

(4) Percentage of radiation reaching cell targets of *Chlorella* of the *n*-th layer, considering self-shading:

$$I_n = (1 - Q)^{(n-1)}(1 - J) = T_{n-1}(1 - J)$$

where J is the efficiency factor for internal self-shading, Q the efficiency factor for absorption, a the specific cell matter absorption coefficient (*Chlorella* 0.41 at 320 nm, Garcia-Pichel 1994), R the cell radius (*Chlorella* 1.75 μm), T_n the transmission through n *Chlorella* cells, and I_n the radiation impact on *Chlorella* of the n-th layer.

Data treatment: Statistical analyses were performed using the Sigma-Stat 3.5 software package (SPSS Inc.). Analysis of variance (ANOVA) was used for the normally distributed data (Two-way ANOVA, Holm—Sidak post-hoc test) to test for differences in mortality between the three different *P. bursaria* cell lines over 5 h of UV exposure and dark controls. The overall level of significance was set to $p < 0.05$. To correct for different initial cell densities of the three cell lines at the beginning of the experiments (T_0), the cell densities after 5 h (T_5) were expressed as percentage of T_0. A two-way ANOVA with a post-hoc Holm—Sidak analysis was run to test for differences between UV exposure and dark controls, as well as between the different cell lines at the end point of the experiment.

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