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THE MICROENVIRONMENT AND PHOTOSYNTHETIC PERFORMANCE OF *PROCHLORON* SP. IN SYMBIOSIS WITH DIDEMNID ASCIDIANS

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1. Introduction

Prochloron spp. (with the type species initially called *Synechocystis didemni* and later renamed *Prochloron didemni* (Lewin 1977)) are oxygenic photosynthetic prokaryotes, i.e. oxyphotobacteria, living in symbiosis almost exclusively with didemnid ascidians in tropical waters (see Lewin and Cheng 1989 for a comprehensive overview). The presence of phototrophic microorganisms (Maurice 1888; Smith 1935) and oxygen production in ascidians (Tokioka 1942) has been known for a long time. However, it was the discovery of *Prochloron* (Lewin 1975; Newcomb and Pugh 1975), and especially its, among prokaryotes unique, pigment composition with chlorophylls *a* and *b* but absence of phycobilins (Lewin and Withers 1975), that triggered more intensive studies of the symbiosis in didemnid ascidians. Thereby, also other prokaryotic symbionts, like cyanobacteria (Lafargue and Duclaux 1979; Larkum *et al.* 1987) and the conspicuous chl *d* containing oxyphotobacterium, *Acaryochloris marina* (Miyashita *et al.* 1996), were discovered in didemnid ascidians. Furthermore, two different free-living prochlorophytes were found (Burger-Wiersma *et al.* 1986; Chisholm *et al.* 1988).

Part of the great interest in *Prochloron* is due to the implications of its photosynthetic apparatus and ultrastructure for our view of the evolution of oxygenic photosynthesis in eukaryotes. Initially the possession of chl *b* and appressed thylakoids led to the suggestion that *Prochloron* was descended from a prokaryotic oxyphotobacterium which gave rise to the green plastids of algae and higher plants (and inspired the name *Prochloron* as well as the general term prochlorophyte (see Lewin 1984). This claim was strengthened by the discovery of the free-living prochlorophytes. Later it was shown that chl *b* was bound to a protein, which is unrelated to the Cab protein from plastids of green algae and higher plants (La Roche *et al.* 1996). Furthermore, SSU-rRNA analysis has established that the three known prochlorophytes all lie within the cyanobacterial radiation, but each on a different branch (Turner 1997). Thus the idea of a direct connection between prochlorophytes and green plastids can no longer be entertained. Nevertheless, the similar pigment composition and structural similarity (such as the presence of appressed thylakoids) makes a comparison of the photosynthetic properties between prochlorophytes and green plastids of considerable

interest, since the prochlorophytes lack the phycobilisome system that typifies cyanobacteria, and gives cyanobacteria photosynthetic characteristics rather different to green plastids (see below).

Despite intensive studies of *Prochloron* cells and of intact ascidians with *Prochloron*, all attempts to cultivate *Prochloron* have failed so far, and a report of partial isolation success (Patterson and Withers 1982) remains unconfirmed. However, literally nothing is known about the microenvironmental conditions that *Prochloron* experiences in its ascidian hosts, and this may in part explain failure to obtain isolates. In this chapter we review recent studies on the ecophysiology of *Prochloron* with emphasis on the microenvironmental characteristics that one can deduce from such studies or, in some cases, directly measure in intact *Prochloron*-ascidian associations.

2. Distribution and photosynthetic characteristics of *Prochloron*

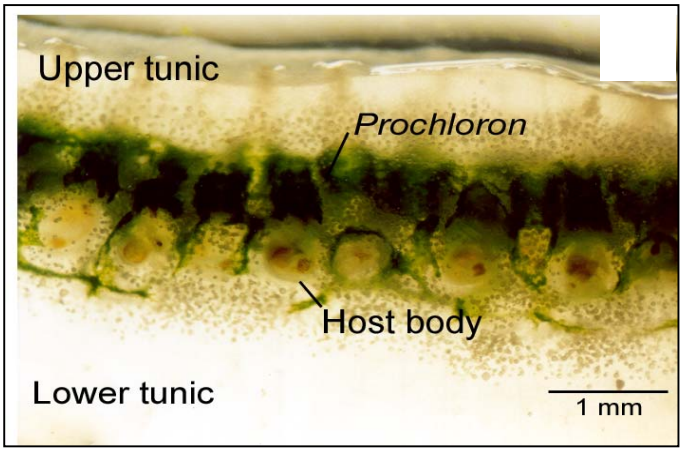
2.1 DISTRIBUTION, CARBON AND NITROGEN TRANSFER IN THE HOST

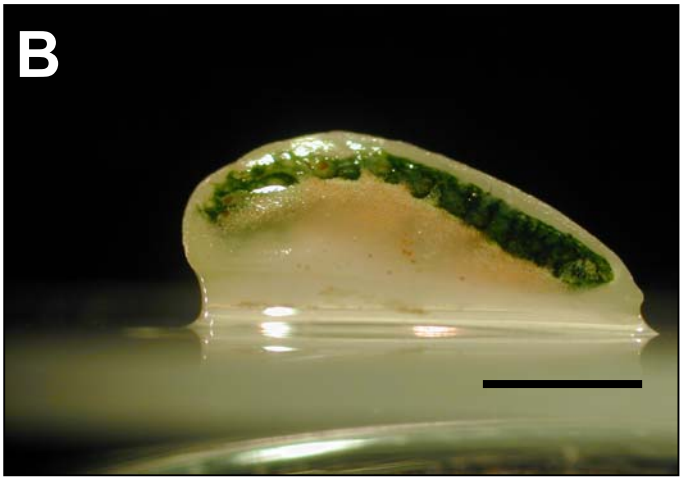
2.1.1 *Distribution in the host*

Prochloron cells line the common cloacae or are embedded in folds of the gelatinous test matrix of colonial didemnid ascidians (Smith 1935; Pardy and Royce 1992) (Fig. 1A,B). *Prochloron* has also forms biofilms on other ascidians (Lewin 1975), sponges (Parry 1986), and holothurians (Cheng and Lewin 1984). In most cases *Prochloron* occurs embedded in a mucilaginous matrix extracellular to the host cells (see e.g. Thinh and Griffiths 1977, Fig. 1C), but intracellular occurrence in phagocytes within the ascidian test has also been reported (Hirose *et al*. 1996, 1998; Lewin and Cheng 1989).

The test is composed of a transparent or slightly opaque gelatinous (in some species more fibrous or even cartilaginous) matrix of protein and cellulose-like carbohydrates, and with a thin tough cuticle composed almost entirely of protein (Goodbody 1974). In many, but not all, didemnids the test also contains calcareous spiculosphaeres. The test is interwoven by blood vessels, microfibrils and host cells like vanadocytes and amoebocytes. Host cells in the test contain highly acidic (pH<2-3) vacuoles of sulfuric acid, and can contain high concentrations of reduced organo-metallic complexes, especially with vanadium and iron (Carlisle 1968). A survey of Stoecker (1980) showed that didemnid ascidians from Bermuda especially accumulated iron and less vanadium, but if this is a general property of didemnids is to our knowledge unknown. *Prochloron* can also accumulate high levels of phenolic compounds (Barclay *et al.* 1987). Acidity and high concentrations of reduced metal compounds and phenolics help protect ascidians against overgrowth and grazing (Stoecker 1980). The test is thus a light transparent living tissue providing a structured and protected environment with a large internal surface area for colonization by *Prochloron*.

Figure 1. *Prochloron* and its distribution in tissues of didemnid ascidians. A. Thin section through the ascidian *Lissoclinum patella*, showing the presence of green *Prochloron* cells in the cloacal cavities under the upper tunic/test of the ascidian (modified from Dionisio-Sese *et al.* 1997, with permission of Springer Verlag and Dr. Tadashi Muruyama). B. Vertical cut through *Diplosoma virens*. *Prochloron* cause the green coloration below the transparent upper tunic/test. C. *Prochloron* cells from *Diplosoma virens* embedded in mucous slime matrix. Note the characteristic peripheral arrangement of the thylakoids. The physical dimension is indicated by scale bars in each picture, i.e. 1 mm in panel A and B, and 0.1 mm in panel C.


Upper tunic
Prochloron
Host body
Lower tunic
1 mm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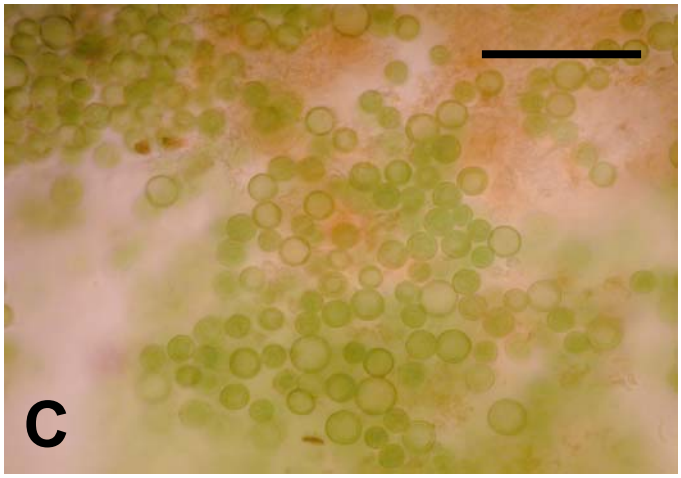
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2.1.2 *Carbon transfer to the host*

Several studies (since the first report by Tokioka 1942) have demonstrated net oxygen production from illuminated intact ascidians with *Prochloron* (Thinh and Griffiths 1977; Pardy 1984; Olson 1986; Alberte 1987; Griffiths and Thinh 1987). Ratios of net oxygen evolution to total respiration range from ~0.6 to ~9 (reviewed in Alberte 1989). The presence of photosynthetic *Prochloron* can significantly enhance the host respiration (Pardy 1984) and the growth rate of the ascidian host in light (Olson 1986). Engulfment of *Prochloron* by host amoebocytes has been suggested as a way of, albeit slow, carbon transfer (Cox 1983). A fast transfer of photosynthates to the host has been demonstrated (Pardy and Lewin 1981; Griffiths and Thinh 1983) involving solute exchange of a range of early products of photosynthesis (Kremer *et al.* 1982). The mechanisms of the fast exchange remain unsolved, but the transfer of photosynthate from *Prochloron* to the host has been estimated to contribute up to ~60% of the hosts carbon demand (Alberte 1987). However, the contribution of *Prochloron* to the carbon demand of their host's differs among different species (Koike and Suzuki 1996). In ascidians like *Didemnum molle*, the carbon demand cannot be covered by the symbionts and must be supplemented by external carbon uptake of the host, while in *Lissoclinum voeltzkowi* the host's carbon demand can be fully met by *Prochloron* (Koike *et al.* 1993).

2.1.3 *Nitrogen exchange and fixation*

Ammonium is the major nitrogenous waste product of the ascidian host (Goodbody 1974) and is effectively taken up by *Prochloron* (Parry 1985). Whether the symbionts are nitrogen limited or not is, however, still a mater of debate (see Alberte 1989). A recent report indicates that nitrogen is efficiently recycled within the *Prochloron*-ascidian association (Koike *et al.* 1993). Nitrogen fixation in light was reported in *Lissoclinum patella* (Paerl 1984) but only in intact *Prochloron*/ascidian associations, and neither isolated *Prochloron* cells nor several other ascidians showed significant nitrogenase activity. Another study showed N_2-fixing activity in encrusting *Prochloron*/ascidian associations, which was, however, not associated with *Prochloron* itself (Odintsov 1991). In contrast, stable nitrogen isotope signatures of isolated *Prochloron* cells were interpreted as evidence of facultative N_2 fixation by Prochloron (Kline and Lewin 1999).

It was speculated (Paerl 1984), that N_2 fixation in light was due to a low oxygen microenvironment of *Prochloron* inside the host effectuated by intense host respiration as well as strong oxygen binding by vanadium-sulfuric acid complexes in the vanadocytes of the host. The role of vanadocytes was, however, disputed by Parry (1985). Aerobic N_2 fixing cyanobacteria have been described (Bergman *et al.* 1997), but the exact mechanisms protecting nitrogenase activity under aerobic conditions remain to be identified. Further studies of nitrogen turnover combined with measurements of the oxygen conditions within didemnid ascidians are needed.

2.2 THE PHOTOSYNTHETIC APPARATUS OF *PROCHLORON*

While there is still much to be discovered about the photosynthetic machinery of prochlorophytes, the evidence so far indicates that it is similar in many ways to that of

thylakoids of green plastids. The thylakoids in *Prochloron* lie in the cytoplasm near the periphery of the cells (Fig. 1C), forming compact undulating parallel layers and often surrounding a central clear region with the nucleoid and various cytoplasmic inclusions (Cox 1986; Swift 1989). Christen *et al.* (1999) obtained highly active thylakoids from *Prochloron didemni*. Further fractionation after passage through a *Yeda* press indicated that no fraction was enriched in PSII (in contradiction to higher plant thylakoids), indicating a fairly homogenous distribution of PSI and PSII in the thylakoid membrane. $P680^+$ reduction kinetics indicated that the reactions were typical for both cyanobacteria and higher plants, suggesting that there is high conservation of the water-oxidizing complex in all the known organisms that perform oxygenic photosynthesis. This agrees with the evidence, from immunolabeling with antibodies directed against *Prochlorothrix hollandica* antenna protein, showing that the light-harvesting complexes are fairly homogeneously arranged in that prochlorophyte (Bullerjahn *et al.* 1990). D2 protein of PSII and PSI has also been shown to be homogeneously distributed (Lichtlé *et al* 1995). Nevertheless different regions of thylakoids have been observed, based on freeze-fracture particle distribution (corresponding to appressed and non-appressed regions of thylakoids) (Miller *et al.* 1988). Also, recent evidence has shown that in some prochlorophytes at least (*Prochlorococcus*) there are small differences in the structural components of PSI and in the light-harvesting arrangements of PSI (Van der Staay *et al.* 1999; Garczarek 2000).

The light-harvesting antenna of *Prochloron* appears to be similar to that of other prochlorophytes (La Roche *et al.* 1996). The ca 35 kDa Pcb protein is related to the isiA protein of some cyanobacteria and more to the CP43 and CP47 (psbC/D) products. In *Prochlorothrix hollandica* three Pcb's (A, B & C) have been found; however in *Prochloron* only one Pcb gene has been found (allied to PcbA/B), along with an isiA protein (La Roche *et al.* 1996). In *Prochloron* the Pcb protein binds [3,8-divinyl]-protochlorophyllide (Mg-2, 4-divinyl phaeoporphyrin A_5 monomethyl ester), as well as chl's *a* and *b*, in a light-harvesting capacity (Larkum *et al.* 1994; Helfrich *et al.* 1999).

The existence of state transitions in *Prochloron* is not well established. Whether Pcb moves between the photosystems and thereby redistributes energy between PSI and PSII is not fully established, although evidence to that effect was presented (Post *et al.* 1993): the evidence could be interpreted as the dissociation of Pcb form PSII without its re-association with PSI. Schuster *et al.* (1985) established that the light-harvesting protein in *Prochloron* is permanently phosphorylated in the light. This makes unlikely the system of redistribution found in green algae and higher plants, where control is effected by the redox state of plastoquinone, Furthermore it is not known whether Pcb is actually associated with PSI. Hiller *et al.* (1985) found chl *b* associated with PSI but in the absence of the 35 kDa lhc (Pcb). Thus it is possible that chl *b* is attached to one or more of the core proteins. In *P hollandica,* van der Staay and Staehelin (1994) found that de-phosphorylation of Pcb (probably PcbC) occurs in the dark, but after much longer periods than in green plastids. They also found evidence for some heterogeneity of PSI and PSII on a microscale.

Thus, there are clear differences between the structure of thylakoids between prochlorophytes and green plastids. Nevertheless, the existence of an intrinsic light harvesting protein in prochlorophytes, in contrast to the extrinsic system in cyanobacteria (based on phycobilisomes), may mean that the photosynthetic reactions

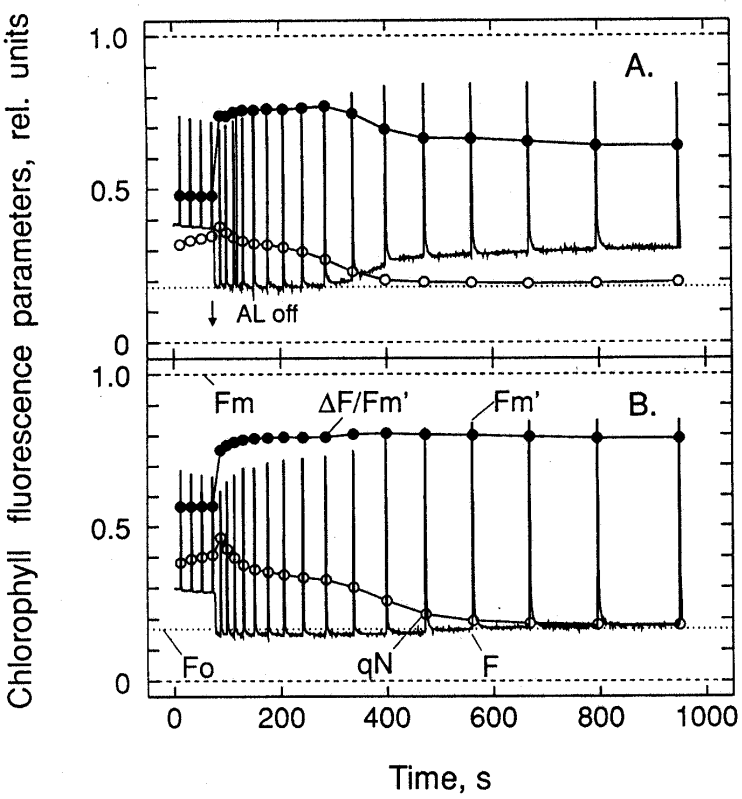

Figure 2. Light-dark relaxation kinetics of fluorescence yield and on-line calculated fluorescence parameters of *Prochloron* in *Lissoclinum patella*. Actinic light (of 170 µmol photons $m^{-2}s^{-1}$) was turned off as indicated (AL off). A. Following AL off, the sample was fully darkened. B. Simultaneously with AL off, continuous far-red light at an intensity of 10 W m^{-2} was applied. The notations of the characteristic fluorescence levels and of on-line calculated fluorescence parameters are given in panel B. Two different samples were used in the experiments of Figs. 3A and B. (from Schreiber *et al.* 1997, with permission of the publisher).

of prochlorophytes show similarities to those of green plastids, where the Cab light-harvesting protein is also intrinsic. In terms of chlorophyll fluorescence the photosynthetic characteristics of *Prochloron* cells are similar to those of higher plants (high PSII yields of ~0.8, and rapidly entrained non-photochemical quenching) and markedly different from those found in cyanobacteria (Schreiber *et al.* 1997). However, there is evidence for the operation of chlororespiration; i.e. that endogenous reductants feed electrons into an intersystem electron transport chain, probably at the plastoquinone level, driven by a cytoplasmic NADPH/NADP reductase. In the dark, oxygen concentration controls the reduction level, keeping PSII acceptors more reduced under low O_2 (Schreiber *et al.* 1997).

Figure 2 (from Schreiber *et al.* 1997) shows the chlorophyll fluorescence kinetics of *Prochloron* cells *in hospite* following a light-dark shift (after 5 min in the light). Only a small part of non-photochemical quenching (qN) relaxes in the first minute after darkening (in contrast to typical responses of green leaves), and the much slower relaxation of qN is closely followed by an increase in steady-state fluorescence yield, F. These results led to the conclusion that chlororespiration is active in thylakoids, but only while oxygen is present in the tissue, and as soon as O_2 becomes depleted (within ~5 min) F rises (Fig. 2A). However, when far-red light is used as background light to activate PSI the plastoquinone pool is no longer over-reduced at low oxygen concentration. These results are consistent with recent observations that the *Prochloron* containing part of the ascidian goes anaerobic after ~10 min (see Section 3.1).

2.3 IRRADIANCE EFFECTS ON *PROCHLORON* PHOTOSYNTHESIS

Didemnid ascidians with *Prochloron* inhabit a wide range of irradiance regimes. Some encrusting forms like *Lissoclinum voeltzkowi* thrive on surfaces exposed to high irradiance environments, while species like *Diplosoma similis* and *Lissoclinum punctatum* are confined to shaded environments. Other didemnids like *Lissoclinum patella* and the motile *Diplosoma virens* can inhabit both low and high irradiance environments. *Lissoclinum voeltzkowi* and *D. virens* even survive periods of air-exposure during low tide. In high irradiance environments, UV-radiation and photooxidation are primary stress factors for *Prochloron* photosynthesis, while shaded environments call for efficient use of available irradiance and minimization of self-shading. Especially, species inhabiting varying light regimes exhibit a phenotypic plasticity in their response to light and optimization of symbiont photosynthesis (Alberte 1989).

2.3.1 *Photosynthesis and respiration as a function of irradiance*

Isolated *Prochloron* cells exhibit high rates of photosynthesis, which can be even higher than rates in free-living cyanobacteria and microalgae (e.g. Critchly and Andrews 1984; Alberte *et al.* 1986; Griffiths and Thinh 1987). Ratios of net photosynthesis to respiration are >5 in high light adapted *Prochloron* cells and can be as high as 16 in cells isolated from shade-adapted colonies (Alberte 1989). Respiration rates of *Prochloron* are higher than in most free-living photosynthetic microorganisms (Alberte *et al.* 1986)), and are ~10 times higher in high-light adapted cells than in cells isolated from shade adapted ascidians. The high respiration results in high compensation irradiances when net photosynthesis is plotted vs. irradiance for isolated *Prochloron* cells (Fig. 3A; Alberte *et al.* 1986). Besides lower respiration and photosynthesis rates as well as lower compensation irradiance, *Prochloron* cells from shade adapted ascidians exhibited a lower chl *a*/ chl *b* ratio, and larger PSI and PSII size than in high light adapted cells. Cells from high light adapted ascidians show no photoinhibition even at 2000 µmol photons m^{-2} s^{-1}, while *Prochloron* from shade adapted ascidians are inhibited at higher irradiances (Fig. 3A; Alberte *et al.* 1986). In a more recent study Dionisio-Sese et al. (2001) found slight photoinhibition of isolated *Prochloron* cells above 1500 µmol photons m^{-2} s^{-1}.

Intact *Prochloron*/ascidian associations, whether high or low light adapted, generally show no photoinhibition up to the highest ambient irradiances encountered in their habitat (~2500 µmol photons m^{-2} s^{-1}) (Fig. 3B; Alberte *et al.* 1987). Apparently, the ascidian host is able to protect its symbionts against excessive irradiance (see also 2.3.2 and 3.2). However, significantly higher compensation irradiances and lower photosynthetic rates (per unit chl) are observed in intact *Prochloron*/ascidian associations as compared to isolated symbionts (Alberte *et al.* 1986, 1987; Griffiths and Thinh 1987). Alberte (1987) attributed the lower rate to self-shading effects in the densely packed *Prochloron* containing zone of the ascidians, while Griffiths and Thin (1987) speculated that host restriction of symbiont photosynthesis may control symbiont proliferation to match the growth rates of the host.

We speculate that other factors may also limit *Prochloron* photosynthesis *in hospite*. While isolated *Prochloron* are investigated in free suspension, the symbionts are densely packed and imbedded in a mucous matrix (or intracellular) within the ascidian, which will affect the light microenvironment and solute transport via diffusion. Like in other photosynthetic biofilms (e.g. Kühl *et al.* 1996) steep light gradients and build-up of high pH and O_2 levels within the *Prochloron* layer may take place in light, while supply of e.g. inorganic carbon for photosynthesis becomes limited by diffusion. We have recently obtained first experimental evidence that such microenvironmental conditions are indeed present in intact *Prochloron*/ascidian associations (see 3.1 and 3.2).

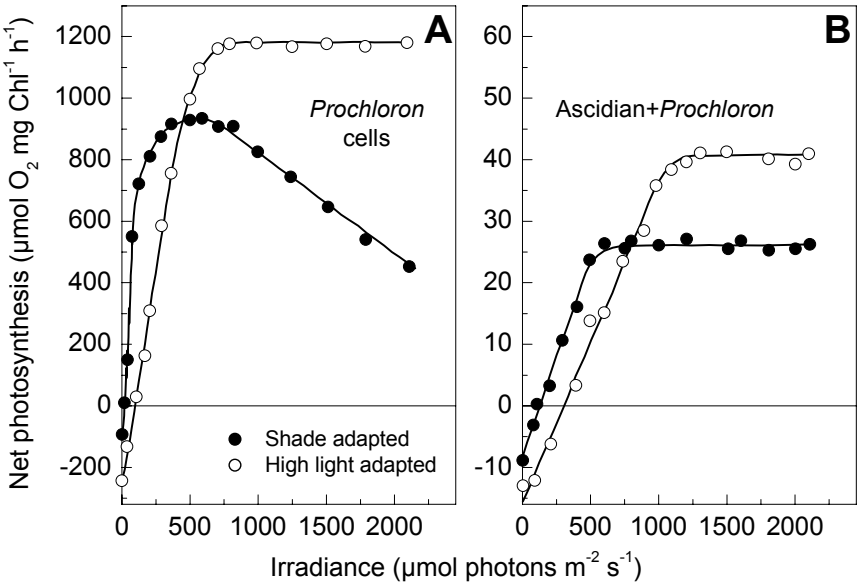

Figure 3. Photosynthesis vs. irradiance of high light (○) and low light (●) adapted *Prochloron* cells (A) as well as of intact *Prochloron*-ascidian associations (B) in *Lissoclinum patella*. (Redrawn from Alberte *et al.* 1986, 1987, with permission of the publisher).

2.3.2. *Protection against UV-radiation and photooxidation*

Ecophysiological studies indicate that the ascidian host provides protection against excessive radiation (Dionisio-Sese *et al.* 1997, 2001; Alberte *et al.* 1987), and *Prochloron* most probably has adapted to the light regime inside the host tissue. Isolated *Prochloron* cells from *Lissoclinum patella* were inhibited by excessive UV-B radiation, whereas cells in intact ascidians showed no photoinhibition by UV-B (Dionisio-Sese *et al.* 1997, 2001). The same authors demonstrated that the outer test is strongly UV absorbing (Fig. 4) and contains water-soluble mycosporine-like amino acids, i.e. mycosporine-glycine, palythine, and shinorine, acting as UV sunscreens. Shinorine, albeit in low concentrations, was also found in *Prochloron* and in the basal tunic of ascidians. *In vivo* spectra of *Prochloron* exhibited a high absorbance below 360 nm (Thinh and Griffiths 1983), which may be due to presence of MAA's. It is not known whether the MAA's are of host or symbiont origin, neither is it known where the water-soluble MAA's are localized and retained in the test matrix.

High irradiance in combination with the high density of pigmented *Prochloron* in the ascidian tissue may lead to photosensitizing processes producing significant amounts of reactive oxygen species, i.e. superoxide radicals, hydrogen peroxide and hydroxyl radicals. The production of these highly reactive compounds is directly proportional to the oxygen level (Jamieson *et al.* 1986), which in photosynthetic systems normally increases with increasing irradiance. Ascidians with *Prochloron* show an intense oxygen production in light (Tokioka 1942, and several studies thereafter) indicating oxygen supersaturation within the symbiont containing ascidian tissue under high irradiance, which may cause photooxidation (see also 3.1).

Lesser and Stochaj (1990) studied protection against photooxidation in *Prochloron* sp. and *Lissoclinum patella* and demonstrated the presence of the antioxidant enzymes superoxide dismutase, ascorbate peroxidase and catalase. The protein activity was directly proportional to irradiance, while pigment content was inversely proportional to irradiance. Furthermore, the high respiration activity of *Prochloron* and its host, which increases with irradiance (Alberte 1986, 1987), may also help moderating local oxygen supersaturation. *Prochloron* within its ascidian host thus seems well protected against photooxidation as well as UV-damage, and this explains why intact *Prochloron*/ascidian associations generally exhibit no photoinhibition even at highest natural irradiances (Alberte 1987; Dionisio-Sese *et al.* 2001).

2.3.4 *Regulation of Prochloron photosynthesis by host behavior*

Another feature of some didemnid ascidians, which may modulate the photosynthesis of their symbionts, is the ability to move with phototaxis or to change the morphology of the colony. *Diplosoma virens* colonies can thus move several mm per day (Birkeland *et al.* 1981; Thinh *et al.* 1981). Furthermore, *Diplosoma virens* can expand its surface area by 60-70% during daytime, which is likely to optimize exposure of *Prochloron* to favorable light conditions (Ryland 1990). Interestingly, the expansion was not directly triggered by irradiance but exhibited a circadian rhythm. The growth pattern and motility of *Lissoclinum patella* was also found to provide optimal growth of its symbionts (Swift and Robertson 1991).

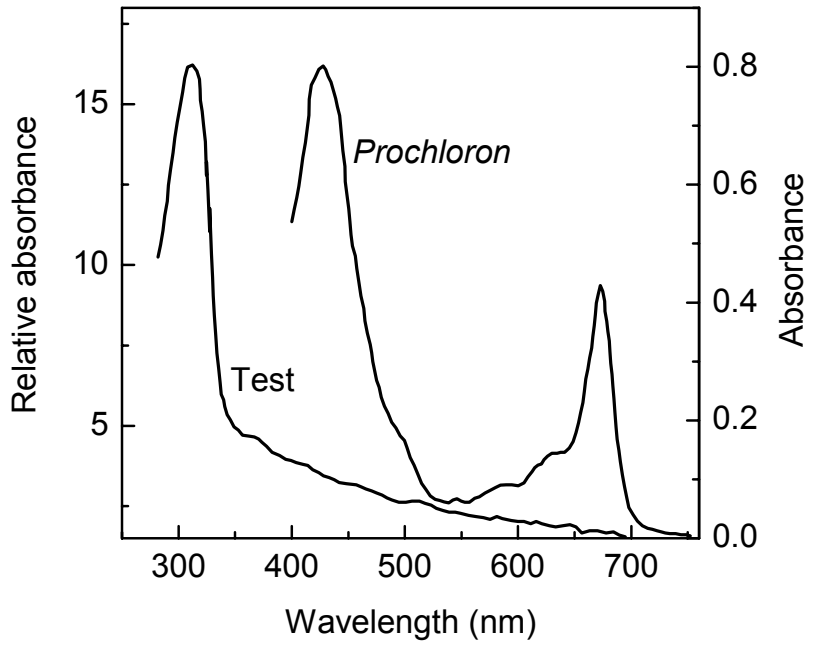

Figure 4. In vivo spectral absorbance of *Prochloron* cells in suspension, and relative absorbance of the outer test of *Lissoclinum patella* (the latter was redrawn from Dionisio-Sese *et al.* 1997, with permission of the publisher).

2.4 EFFECTS OF OTHER ENVIRONMENTAL VARIABLES

2.4.1 *pH effects*

Isolated *Prochloron* cells are photosynthetically competent from pH 6.8 to 9.5, with a peak in photosynthetic rate at the pH of seawater (~pH 8.0-8.2) (Dionisio-Sese *et al.* 2001). The observed peak was, however, rather broad and high rates of photosynthesis, i.e. >50% of maximum photosynthesis, were observed even at pH 9.5, which is significantly above ambient seawater pH. Apparently, *Prochloron* is well adapted to high pH levels, which may build up in the *Prochloron* containing zone of the ascidian during periods of intense photosynthetic carbon fixation. High pH may impose CO_2 limitation, which can, however, be alleviated by host respiration or by inorganic carbon concentrating mechanisms and the enzyme carbonic anhydrase present in *Prochloron* (Dionisio-Sese *et al.* 1993).

Acid release from vanadocytes and/or other host cells in response to strong physical disturbance of the ascidian host can lower the pH to <3-5, and this has lethal effects on *Prochloron* (Lewin and Cheng 1989; Thinh and Griffiths 1977). Consequently,

separation of viable *Prochloron* from the host tissue should always be done in buffered medium

2.4.2 *Temperature effects*
Maximal photosynthesis of both isolated *Prochloron* cells and intact ascidian/*Prochloron* associations is found at 28-35°C (Thinh and Griffiths 1977; Alberte *et al.* 1986; Dionisio-Sese *et al.* 2001). A steep decline in activity occurs above 40°C, which seems to be the upper temperature limit of *Prochloron*. Ambient temperatures experienced by didemnid ascidians with *Prochloron* typically range from 25-30°C, but even higher local temperatures may be experienced in shallow protected waters.

While *Prochloron* is well adapted to its ambient temperature range (and even to temperatures almost 10°C above it), it is very sensitive to decreasing temperature and photosynthetic activity ceases below 20°C. Alberte *et al.* (1986,1987) found that *Prochloron* cells and intact ascidian/*Prochloron* associations were twice as sensitive to temperature changes below ambient (Q_{10} of ~3.5) as compared to temperature changes above ambient temperature (Q_{10} of ~1.5). The temperature dependence of *Prochloron* respiration (Q_{10} of ~1.7) as well as the combined ascidian/*Prochloron* colony respiration (Q_{10} of ~2) was constant from 15-45°C. The low temperature sensitivity of *Prochloron* photosynthesis seems a prime factor limiting its distribution to tropical waters. We speculate that other photosynthetic symbionts of ascidians may exhibit the same temperature relation, which would explain the apparent absence of symbionts in didemnid ascidians of colder waters (e.g. Sanamyan 1999).

3. Microenvironment of *Prochloron*

Existing information on the distribution and photosynthetic performance of *Prochloron* (see above) indicates that the symbionts are growing under special microenvironmental conditions with respect to light, oxygen, pH, nutrients and inorganic carbon inside their ascidian hosts. Yet, the microenvironment of *Prochloron* remains virtually unstudied. Use of minimally invasive microsensor techniques (reviewed in Kühl and Revsbech 2001) has given new insights to microenvironmental controls in the symbioses of microalgae with corals (Kühl *et al.* 1995; De Beer *et al.* 2000, Salih *et al.* 2000), benthic foraminifera (Köhler-Rink and Kühl 2000), and sponges (Sand-Jensen and Pedersen 1994). Recently, we have used a similar approach to study *Prochloron*-ascidian associations (Kühl and Larkum, 2001). Here we present some preliminary examples of our microsensor data (Fig. 5), which give experimental evidence of some of the earlier mentioned speculations on the microenvironment of *Prochloron*.

3.1 OXYGEN MICROENVIROMENT OF *PROCHLORON*

A characteristic feature of *Prochloron* is that it grows attached in a mucous polymer matrix lining the cloacae or external surfaces of its hosts (Lewin and Cheng 1989). The symbionts thus form a photosynthetic biofilm closely associated with the host tissue. In such biofilms very dynamic oxygen conditions are to be expected (Kühl *et al.* 1996).

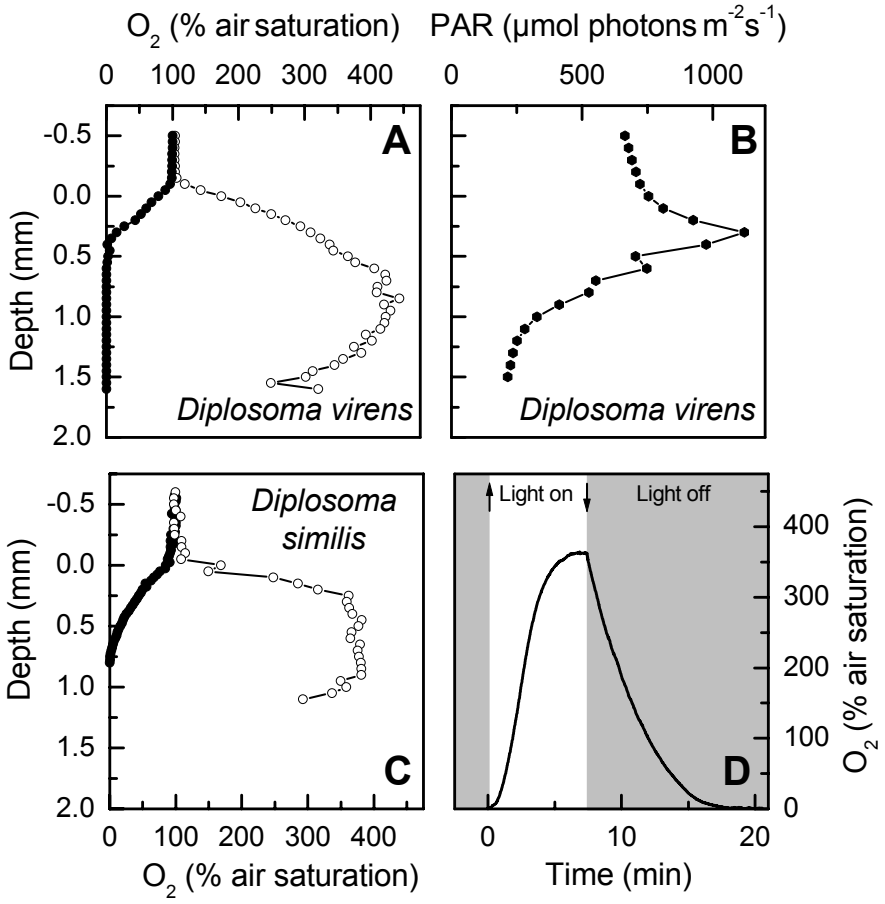

Figure 5. Oxygen microenvironment and light regime of *Prochloron in hospite*. Oxygen concentration profile measured with an electrochemical oxygen microsensor in *Diplosoma virens* (A) and *Diplosoma similis* (C) in light (○, ~300 µmol photons m^{-2} s^{-1}) and darkness (●). B. Depth distribution of PAR (⬢) in *Diplosoma virens*. D. Continuous measurement of O_2 concentration within the *Prochloron* containing zone of *Diplosoma virens* during an experimental light-dark shift. The outer test of the ascidians was 0.2-0.5 mm thick. The *Prochloron* containing zone below was ~0.5-1 mm thick.

First microsensor measurements in *Diplosoma virens* and *Diplosoma similis* indeed showed a very dynamic change in internal oxygen levels as a function of irradiance (Fig. 5). In the light, oxygen levels in the ascidian show a pronounced supersaturation of up to 4 times the O_2 concentration in air saturated water. In darkness, strong oxygen depletion created anoxia in the ascidian below the outermost 0.5-1 mm of the test. Internal oxygen levels were determined by intensive *Prochloron* photosynthesis in light.

Only ~8 minutes after the start of illumination, the photosynthetic activity had changed the internal O_2 levels from anoxia to supersaturation (Fig. 5D). Conversely, a strong oxygen consumption by host and symbionts restored anoxia within ~10 minutes after onset of darkness.

Our data give the first experimental evidence that *Prochloron* lives in an environment exhibiting extreme variations in O_2 as a function of available irradiance for photosynthesis. While respiration is clearly O_2 limited in darkness, the O_2 supersaturation observed in light stimulates host respiration, which is in line with observations by Alberte *et al.* (1987). The accumulation and rapid depletion of O_2 measured within ascidians (exposed to flowing water), and the shape of steady state O_2 profiles indicate that rapid diffusion is a major mode of mass transfer between symbionts and host. Whether advective transport due to pumping of host cells and circulation of test fluids within the ascidian are of significance for internal O_2 levels remains to be investigated. Our data also indicate a potential for various anaerobic reactions like e.g. N_2 fixation within the ascidian tissue under low light or dark conditions (see 2.1.3). Even microaerophilic or anaerobic microbes may be able to proliferate within the ascidians, and an inventory of the bacterial diversity present within didemnid ascidians should be very interesting to undertake.

3.2 LIGHT REGIME OF *PROCHLORON IN HOSPITE*

A major conclusion of earlier studies was that photosynthesis-irradiance characteristics of *Prochloron* reflect adaptation to light regimes *in hospite* and not the irradiance incident on the ascidian surface (Alberte 1989). The optical properties of the ascidian test modify the internal light regime of *Prochloron in hospite*. The test can screen out UV radiation efficiently due to presence of MAA's (see 2.3.2), while PAR (400-700 nm) absorption in the semitransparent test matrix is very weak and 4-8 times lower than for UV-radiation (Fig. 4). Light attenuation in the test is due to both absorption and scattering, which leads to a diffuse light field. Measurements of PAR with a scalar irradiance microprobe (Kühl *et al.* 1997) showed the presence of a peak of PAR in the outer ~0.2-0.5 mm thick test of the ascidian *Diplosoma virens* followed by an exponential decrease of PAR in the *Prochloron* containing zone of the ascidian (Fig. 5B).

The apparent light trapping in the outer test is due to a high scattering to absorption ratio for visible light in combination with a higher refractive index of the test matrix as compared to the overlaying water (Kühl and Jørgensen 1994; Grunwald *et al.* unpublished data). Like in other multiple scattering tissues (Vogelmann *et al.* 1996; Motamedi *et al.* 1989) the light trapping results in an increased pathlength of photons per vertical depth interval transversed, which again enhances the probability of absorption. Therefore, even the presence of relatively low amounts of UV-screening compounds in the outer test can lead to efficient screening of UV-radiation, while PAR is propagating almost unaltered to the underlying *Prochloron*. Our data obtained in *Diplosoma virens* (Fig. 5B) indicate no major light limitation of *Prochloron* due to self-shading (see also 2.3.1)

In some ascidians like *Lissoclinum patella* the test is more optically dense and host pigments in the test may also alter the spectral characteristics of visible light transmitted

to *Prochloron*. Alberte (1989) reported the presence of compounds in the test with strong absorption of green and orange light. The same author hypothesized that the light regime of *Prochloron*, as determined by host pigmentation as well as the ambient light climate of the ascidian in their habitat, is more like terrestrial habitats putting a selective pressure for development of light-harvesting systems with chlorophyll *a* and *b*.

4. Summary and outlook

To isolate and maintain strains of *Prochloron* sp. remains a major task in the study of these fascinating oxygenic prokaryotic phototrophs. However, the microenvironmental conditions under which *Prochloron* sp. and other oxyphotobacteria thrive in symbiosis with didemnid ascidians, are just beginning to be investigated with microsensors and other techniques that allow studies of *Prochloron in hospite*. Such studies will help identify critical boundary conditions for future isolation attempts. The first data on the light and oxygen microenvironment of *Prochloron* within its ascidian host demonstrate that it shares many characteristics with photosynthetic biofilms. *Prochloron* lives in a biofilm within strong gradients of light (largely defined by the optical properties of the test matrix) and oxygen. Oxygen concentration *in hospite* is very dynamic, mainly as a function of irradiance, and O_2 levels change from supersaturation to anoxia or *vice versa* within a few minutes after light-dark or dark-light shifts. Given that *Prochloron* isolation attempts have been based on liquid cultures and suspended cells, it is not surprising that such attempts have failed. It is now time to consider isolation attempts, where the biofilm mode of *Prochloron* life and its physical-chemical microenvironment is acknowledged

Other recent discoveries also may influence future *Prochloron* research. Another oxyphotobacterium, *Acaryochloris marina* (Miyashita *et al.* 1996) has been found in didemnid ascidians. *Acaryochloris* is using chl *d*, which absorbs maximally around 715 nm, as the major photopigment. *Prochloron* and *Acaryochloris* thus have rather complementary light absorption characteristics, which may allow them to co-exist in close proximity within the ascidian host. Preliminary observations (Kühl and Larkum unpublished results) of the spatial organization of *Prochloron* in the didemnid *Diplosoma virens* thus indicate that *Prochloron* is imbedded in a mucous matrix of small Chl *d* containing microorganisms. Further microscopic investigations are now underway to confirm this spatial organization. Cyanobacteria with special phycobilin pigmentation have also been found in didemnid ascidians (Cox *et al.* 1985; Larkum *et al.* 1987) but their interaction with the host and other symbionts remain unstudied. Interactions with oxyphotobacteria and other bacteria *in hospite* may therefore be another important aspect of *Prochloron* ecophysiology yet to be studied in detail.

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