APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1990, p. 1530–1535
0099-2240/90/061530-06$02.00/0

Vol. 56, No. 6

Photoadaptation and Protection against Active Forms of Oxygen in the Symbiotic Procaryote *Prochloron* sp. and Its Ascidian Host

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Received 5 December 1989/Accepted 26 February 1990

Superoxide dismutase, ascorbate peroxidase, and catalase activities were studied in the symbiotic photosynthetic procaryote *Prochloron* sp. and its ascidian host *Lissoclinum patella*. The protein-specific activities of these antioxidant enzymes in the *Prochloron* sp. and *L. patella* collected at different depths from the Great Barrier Reef, Australia, were directly proportional to irradiance, whereas the pigment concentrations in the *Prochloron* sp. were inversely proportional to irradiance. The presence of a cyanide-sensitive superoxide dismutase, presumably a Cu-Zn metalloprotein, in the *Prochloron* sp. extends the possible phylogenetic distribution of this protein. The concentration of UV-absorbing mycosporine-like amino acids is inversely proportional to irradiance in both the host and symbiont, suggesting that these compounds may not provide sufficient protection against UV radiation in high-irradiance environments. The significant differences in the specific activities of these antioxidant enzymes, cellular photosynthetic pigment concentrations, and UV-absorbing compounds from high- and low-irradiance habitats constitute an adaptive response to different photic environments. These photoadaptive responses are essential to prevent inhibition of photosynthesis by high fluxes of visible and UV radiation.

Prochloron spp. (*Prochlorales*), oxygenic-photosynthetic symbionts of colonial ascidians (*Urochordata*), were initially thought to be cyanobacteria (37). It is now known that these procaryotes lack the typical phycobiliproteins of cyanobacteria and instead contain chlorophyll *b* as an accessory pigment for photosynthesis (59). Although this accessory pigment is characteristic of eucaryotic green plants (59), the available ultrastructural, biochemical, and molecular data to date unequivocally support the supposition that these photoautotrophs are procaryotic (36).

Prochloron spp. are found on or within several species of didemnid ascidians (33) and successfully occupy habitats with considerable variability in their photic regime (2, 33). These symbioses are found in habitats with maximum photon-flux densities (photosynthetically active radiation, 400 to 700 nm) as high as 2,000 μmol m^{-2} s^{-1} and as low as 50 μmol m^{-2} s^{-1} (2). Several studies have characterized the photosynthetic and photoadaptive features of *Prochloron* spp. that enable the whole symbiosis to occupy different photic environments (1–3).

One photoadaptive feature that has not been investigated is protection against the toxicity associated with active forms of oxygen, which are formed by all organisms during metabolic and photosensitizing processes (6, 21, 23). As a consequence of using water as the primary electron donor for photosynthesis, molecular oxygen is formed in the intrathylakoid space. In the presence of sunlight (visible and UV wavelengths) and potent photosensitizing agents (e.g., chlorophyll), molecular, or ground state, oxygen preferentially undergoes univalent reductions. This is a result of spin restrictions on its unpaired valence electrons (11). The products of these univalent reductions are superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO·) (6, 11, 27). The production of these active species of oxygen is directly proportional to the partial pressure of molecular oxygen (29) and can cause varying degrees of damage to proteins, nucleic acids, and lipids in biological systems (6, 23). Because superoxide radicals are the primary product of photoreduced molecular oxygen within the thylakoids (6), endogenous mechanisms of protection are essential to the maintenance of photosynthetic processes (4, 6, 27). The superoxide theory of oxygen toxicity states that O_2^- is the primary agent, directly or indirectly mediating oxygen toxicity. By preventing its accumulation, the enzyme superoxide dismutase (SOD) is the primary defense against this radical (17, 22, 23). Photosynthetic organisms are known to have SOD within their chloroplasts (6, 27), as well as a high-affinity ascorbate peroxidase to remove the H_2O_2 produced by the dismutation of O_2^- (6, 43).

Another possible mechanism of protection from the detrimental effects of active oxygen is to screen out UV radiation with protective compounds. Shibata (51) discovered several substances having absorption maxima between 315 and 323 nm in the hermatypic corals *Acropora* sp. and *Pocillopora* sp. and in an unidentified cyanobacterium which were referred to as S-320 compounds. Similar substances have been found in a variety of marine organisms, including ascidians, echinoderms, decapod crustaceans, mollusks, corals, alcyonarians, and algae (42). The molecular structures of these compounds have been elucidated and compose a family of compounds called mycosporine-like amino acids, with absorption maxima ranging from 310 to 360 nm (42). The protective function of these compounds has been inferred from both their absorption spectra and decrease in concentration with depth; e.g., shallow water corals contain significantly higher concentrations of UV-absorbing compounds than conspecifics living at greater depths (17).

UV-absorbing compounds and antioxidant enzymes have been demonstrated in both the hosts and symbionts of algal-invertebrate symbioses (17–19, 32, 33, 48). Also, the specific activities of antioxidant enzymes show a direct relationship with irradiance or PO_2, suggesting that these

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enzymes are produced in response to active forms of oxygen (18, 19, 34, 35). This study demonstrates the presence of SOD, ascorbate peroxidase, and catalase in the symbiotic procaryote, a *Prochloron* sp. It also suggests that, as in other photoautotrophic-invertebrate symbioses, protection against active forms of oxygen is an essential component of the photoadaptive response.

MATERIALS AND METHODS

Collection and isolation of *Prochloron* sp. Colonies of *Lissoclinum patella* were collected at depths of 3 and 17 m from Bowl Reef, Great Barrier Reef, Australia, in April 1989, using SCUBA. The irradiances at these depths were calculated from an irradiance (photosynthetically active radiation) extinction curve for a similar outer-shelf reef during the same time of the year and weather conditions (B. E. Chalker, unpublished data): 3 m, 1,230 μmol m^{-2} s^{-1}; 17 m, 525 μmol m^{-2} s^{-1}. Pieces of five individual colonies from each depth, approximately 12 cm^2, were used to obtain the *Prochloron* sp. The symbionts were extracted by squeezing the colonies and expressing the cells through the excurrent canals of the hosts into 100 mM phosphate buffer (pH 7.5). This cell suspension was centrifuged at 500 × *g* for 10 min, and the supernatant was discarded. The remaining pellet was washed with 100 mM phosphate buffer (pH 7.5) with 0.02% (wt/vol) sodium dodecyl sulfate, modified as recommended by McAuley (39) to obtain intact cells uncontaminated by host tissue. Subsequently, the cells were washed three times with 100 mM phosphate buffer (pH 7.5) and centrifuged at 500 × *g* for 20 min; the supernatants were discarded each time. Pieces of the symbiont-free colonies were homogenized in 100 mM phosphate buffer (pH 7.5) and centrifuged at 500 × *g* for 20 min. The supernatant was separated and processed for protein, SOD, and catalase as described below. Also, random samples were processed as described for chlorophyll to check for contamination by any remaining symbionts.

Protein and pigment analyses. Freshly isolated cells were collected as described above and washed in 100 mM phosphate buffer (pH 7.5). After the final wash, the pellet was suspended in 1.5 ml of the same buffer. A 250-μl sample was removed and centrifuged (500 × *g*), and the supernatant was discarded. A 0.4-ml volume of a $MgCO_3$ slurry was added as a buffer, and the mixture was sonicated for 30 s at 4°C. Subsequently, 3.6 ml of acetone (100%) was added and the chlorophylls were extracted overnight in the dark at 4°C. Chlorophyll extracts were centrifuged (500 × *g*) and read against acetone blanks on a spectrophotometer at 647 and 664 nm. The equations of Jeffrey and Humphrey (30) were used to calculate the concentrations of chlorophyll *a* and chlorophyll *b*. A 100-μl sample was removed and diluted with 9 parts of 10% Formalin in filtered (0.45 μm) seawater for replicate cell counts (n = 3 for each sample), using a hemacytometer.

The remainder of the resuspended pellet was sonicated for 1 min at 4°C, using a sonicator (Branson) with a microtip set at 35 W. The sonicated cell suspension was centrifuged for 30 min at 10,000 × *g*. Two 250-μl aliquots from the cell extract were withdrawn for protein analysis by the Bradford procedure (Bio-Rad Laboratories), using bovine gamma globulin standards. It should be noted that this assay does underestimate protein content in phytoplankton samples (14). The remaining cell extract was used for enzyme assays.

Enzyme assays. SOD activity was assayed spectrophotometrically as described by Elstner and Heupel (20) and Oyanagui (45). Standards were prepared by using bovine erythrocyte SOD (Sigma Chemical Co.) for each set of samples. Duplicate assays were run to differentiate between cyanide-sensitive (Cu-Zn) and cyanide-insensitive (Mn or Fe) SOD metalloenzymes by adding KCN to a final concentration of 2.0 mM (7). The presence of the Cu-Zn metalloprotein was confirmed by polyacrylamide gel electrophoresis as described by Lesser and Shick (35).

Catalase activity was assayed spectrophotometrically by following the depletion of H_2O_2 at 240 nm (8), while ascorbate peroxidase activity was assayed by the method of Nakano and Asada (43) by following ascorbate oxidation at 290 nm.

All assays were conducted at 25°C, and results are expressed as units of enzyme activity (1.0 U = 1 μmol of substrate converted min^{-1}) per milligram of protein.

UV-absorbing compounds. Specimens of the *Prochloron* sp. and *L. patella* were collected and separated as described above. The symbionts and host from each specimen were extracted in 5 ml of 100% methanol for 1 h. Three additional 1-h extractions on isolated symbionts and hosts were performed to calculate the extraction efficiencies by the method of Dunlap and Chalker (16). The visible and UV spectra were recorded with a scanning spectrophotometer (Hitachi), and all nonsoluble tissue was frozen for protein determination as described above. *Prochloron* sp. extracts were filtered with a Sep-Pak cartridge to remove chlorophyll prior to analysis by high-performance liquid chromatography.

UV-absorbing compounds were separated and quantified by high-performance liquid chromatography, using the method of Dunlap and Chalker (16). The compounds were separated on a Brownlee RP-8 (Speri-5; 4.6-mm inside diameter by 25 cm) protected with an RP-8 guard (Spheri-5; 4.6-mm inside diameter by 5 cm) with an aqueous mobile phase of 0.1% acetic acid–25% MeOH (by volume) and a flow rate of 0.7 ml min^{-1}. Peaks were detected at 313 and 340 nm and quantified with a Spectraphysics integrator. Individual UV-absorbing compounds were identified by cochromatography, using standards obtained from extracts of the zoanthid *Palythoa tuberculosa*.

Statistical analysis. All biomass, enzyme, and UV-absorbing compound results were evaluated for treatment effects by Student's unpaired, one-tailed t test at a significance level of 5%. No unequal variances were detected by the F_{max} test (53).

RESULTS

Pigment and protein concentration. For the *Prochloron* sp., the concentrations of chlorophylls *a* and *b* per cell were inversely related to irradiance for samples collected in bright and dim habitats (Table 1). The effect of depth, and therefore irradiance, on total chlorophyll content, chlorophyll *a*, and chlorophyll *b* was significant, with cells from 3 m having less chlorophyll than those from 17 m (t test for total chlorophyll, $P = 0.004$; for chlorophyll *a*, $P = 0.015$; for chlorophyll *b*, $P = 0.002$; for chlorophyll *a*/*b* ratio, $P = 0.031$). There were no effects of depth on the protein content per cell ($P = 0.87$; Table 1). No chlorophyll was detected in the supernatants of *L. patella*.

Enzyme activities. The protein-specific activities of SOD, ascorbate peroxidase, and catalase all showed a similar pattern of increasing activities with irradiance (Fig. 1a and b). The enzyme SOD within the *Prochloron* sp. showed a significant pattern of increased specific activities at the higher irradiance (Fig. 1a). Also, a cyanide-sensitive metal-

TABLE 1. Bulk measurements of chlorophyll and soluble protein from freshly isolated *Prochloron* sp.

Irradiance[a]	Total chlorophyll[b]	Chlorophyll *a*[b]	Chlorophyll *b*[b]	Chlorophyll *a/b*	Protein[b]
Bright	3.95 ± 0.50	2.64 ± 0.31	1.31 ± 0.28	2.09 ± 0.44	27.0 ± 8.0
Dim	5.30 ± 0.70	3.29 ± 0.45	2.01 ± 0.28	1.64 ± 0.15	28.0 ± 6.0

[a] Bright = 1,230 μmol m^{-2} s^{-1}; dim = 525 μmol m^{-2} s^{-1}.
[b] pg $cell^{-1}$, mean ± SD (n = 5).

loprotein was detected spectrophotometrically and by polyacrylamide gel electrophoresis (data not shown), indicating the presence of a Cu-Zn form of SOD. This cyanide-sensitive activity was approximately 73 to 77% of the total activity. The specific activities of catalase and ascorbate peroxidase also showed a pattern similar to that of SOD. However, while the differences in specific activities were significant for ascorbate peroxidase, they were not for catalase (Fig. 1b). In the host, *L. patella*, the activities of catalase and total SOD increased with irradiance but were not significant (Fig. 2).

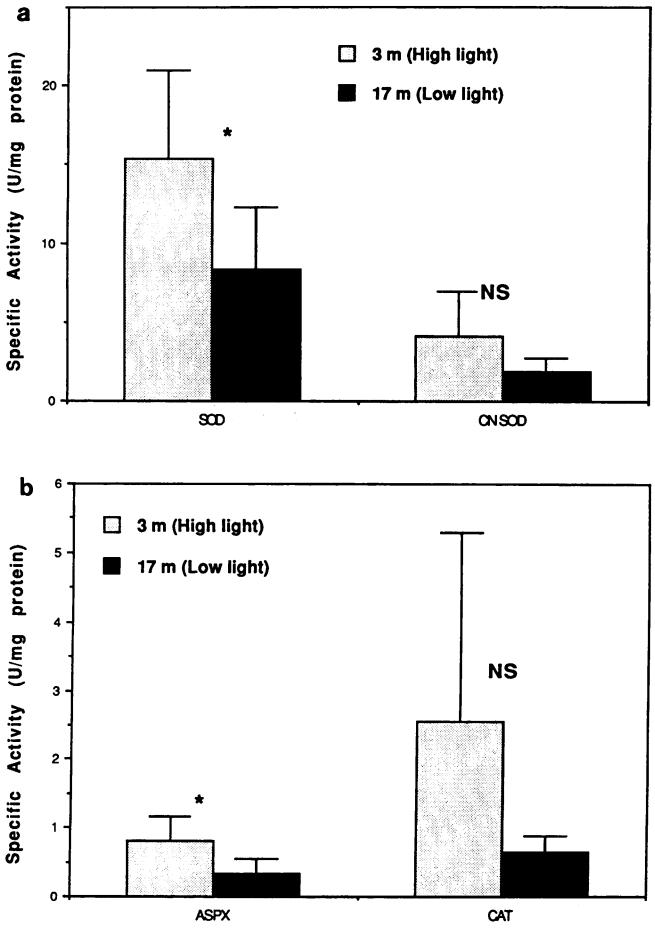

FIG. 1. (a) Total SOD and cyanide-insensitive (CN) SOD activities of freshly isolated *Prochloron* sp., mean ± standard deviation (n = 5). Lightly stippled bars represent bright (3-m) specimens; dark bars represent dim (17-m) specimens. *, $P < 0.05$ (Student's t test). (b) Ascorbate peroxidase (ASPX) and catalase (CAT) activities of freshly isolated *Prochloron* sp., mean ± standard deviation (n = 5). White bars represent bright (3-m) specimens; black bars represent dim (17-m) specimens. *$P < 0.05$; NS, not significant (Student's t test).

However, the cyanide-insensitive SOD (Mg-SOD) of *L. patella* did show a significant effect of irradiance (Fig. 2).

UV-absorbing compounds. Only the major UV-absorbing compound, mycosporine-glycine, was quantified by high-performance liquid chromatography in both the *Prochloron* sp. and *L. patella*. The protein-specific concentrations of mycosporine-glycine, although not statistically different, always showed an inverse relationship with irradiance (Fig. 3).

DISCUSSION

Freshly isolated *Prochloron* sp. from *L. patella* shows an inverse relationship between cell pigmentation and irradiance as described previously for phytoplankton, symbiotic dinoflagellates, and *Prochloron* spp. associated with other hosts (1, 12, 35, 44, 49). The higher chlorophyll *a/b* ratio in shallow water is consistent with smaller photosynthetic unit size (49), suggesting that a change in photosynthetic unit size is one mechanism of photoadaptation. Alberte et al. (3) have demonstrated similar changes in pigment concentration and ratios in other *Prochlorales*-ascidian symbioses. However, recent studies have demonstrated an increase in chlorophyll *a/b* ratios in *Prochlorothrix hollandica*, a free-living member of the *Prochlorales*, when grown under low irradiance (10).

We have demonstrated higher activities of SOD, ascorbate peroxidase, and catalase under high irradiance in *Prochloron* sp. Within the depth range of the specimens examined here, an important factor potentially affecting the relative enzyme activities is the presence of UV radiation (300 to 400 nm), which can penetrate up to 20 m in clear oceanic water (31). Previous laboratory and field studies on the endosymbiotic dinoflagellates of cnidarians have shown an increase in the activities of antioxidant enzymes when exposed to UV radiation (18, 34, 35). It is not known whether UV radiation can penetrate the ascidian host tissues, but the ambient visible radiation reaching the symbionts in vivo is decreased 60 to 80% by the host tissues (2). The host, *L. patella*, does contain UV-absorbing compounds but shows no significant differences between depths in the protein-specific concentrations of these compounds. The lower concentrations of UV-absorbing compounds observed at higher irradiances suggests that these compounds may have some photolability. This could potentially result in an increase in UV penetration within the symbiosis and in higher activities of antioxidant enzymes. Understanding the relationship between the activities of antioxidant enzymes and UV-absorbing compounds is hampered by a lack of knowledge about the kinetics of biosynthesis and destruction of UV-absorbing compounds under different photic regimes in these organisms.

The specific activities of SOD and catalase in the ascidian host *L. patella* also show a significant increase with irradiance. It has also been shown that net oxygen flux, normalized to chlorophyll, is highest in shallow colonies exposed to higher irradiances (1, 3). This could explain the higher activities of cyanide-insensitive Mg-SOD, a mitochondrial

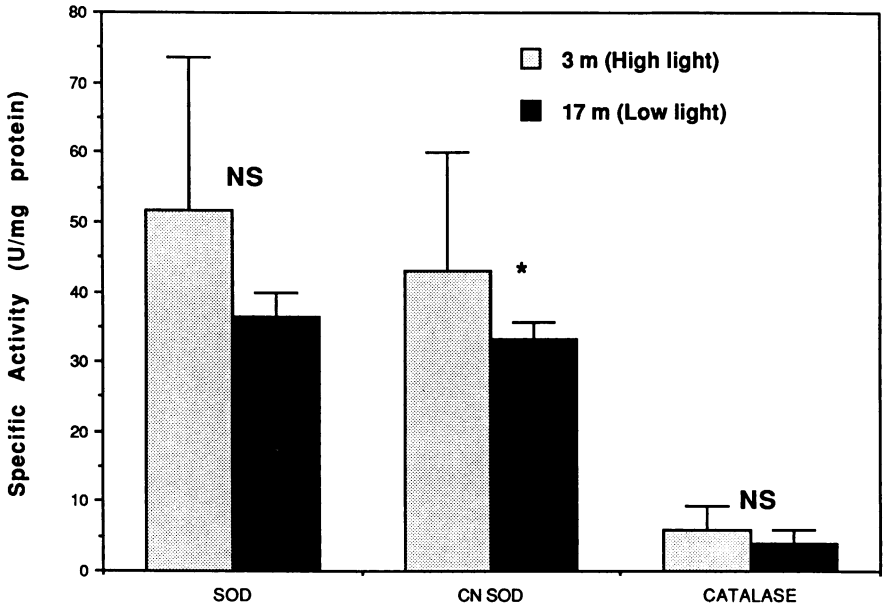

FIG. 2. Total SOD, cyanide-insensitive (CN) SOD, and catalase (CAT) activities of *L. patella*, mean ± standard deviation ($n = 5$). Lightly stippled bars represent bright (3-m) specimens; dark bars represent dim (17-m) specimens. *, $P < 0.05$; NS, not significant (Student's t test).

enzyme, in *L. patella*. This also suggests that, despite the extracellular location of *Prochloron* spp. in these symbioses, the animal host experiences varying PO_2's and maintains specific activities of its antioxidant enzymes that are in proportion to the PO_2 resulting from the photosynthesis of its symbionts.

In procaryotes, as well as other organisms, higher PO_2's, which occur during photosynthesis, result in an increase in the synthesis of SOD and other antioxidant enzymes (19, 25, 28). The induction of SOD has been demonstrated in both laboratory (15, 25, 26, 38), and field (40) conditions, where high PO_2's are positively correlated with higher antioxidant enzyme activities. The evidence presented here also suggests that an increase in the production of O_2^- within the *Prochloron* sp. is probably due to an increase in photosynthetic electron flow (with its inherent increase in PO_2) (6). Measurements of photosynthesis on *Prochloron* sp. show a higher net oxygen flux in cells isolated from colonies exposed to high irradiances (2).

Within the *Prochloron* sp., catalase activities, although not statistically distinguishable, paralleled the increase in SOD activities. With its high apparent K_m (6), catalase could not prevent oxidative damage as effectively as the high-affinity ascorbate peroxidase found in higher plants (43),

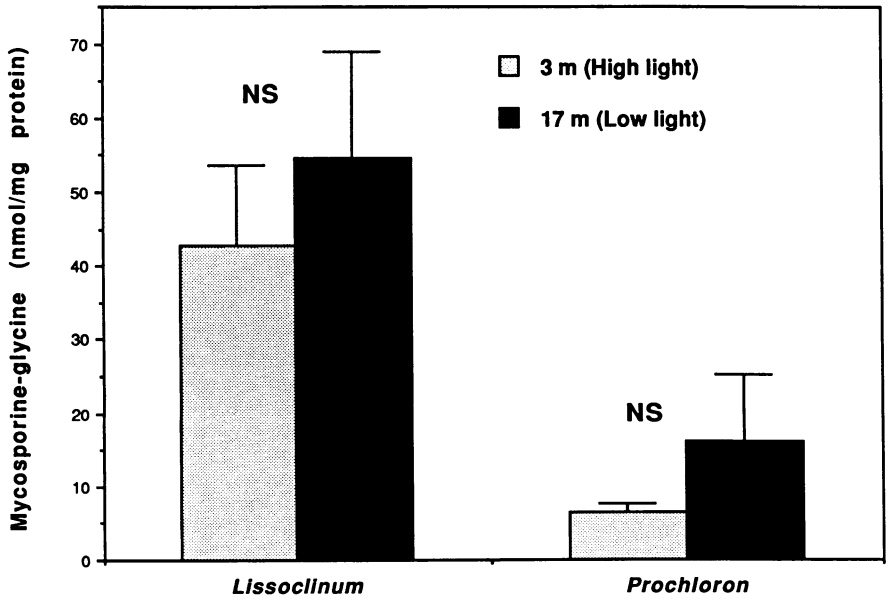

FIG. 3. UV-absorbing compounds (mycosporine-glycine) of *L. patella* and *Prochloron* sp., mean ± standard deviation ($n = 3$). Lightly stippled bars represent bright (3-m) specimens; dark bars represent dim (17-m) specimens. NS, Not significant (Student's t test).

dinoflagellates (35), and cyanobacteria (55), except under photooxidative conditions when a low K_m and high V_{max} could effectively remove the higher fluxes of peroxides. As shown in cyanobacteria (55), the ascorbate peroxidase activity demonstrated in *Prochloron* sp. might be more effective at removing H_2O_2 at the low concentrations continually produced during photosynthesis (6). The removal of H_2O_2 would be especially important, both in preventing the inactivation of ribulose-1,5-bisphosphate carboxylase/oxygenase, the primary CO_2-fixing enzyme in *Prochloron* spp. (58), and in protecting the Cu-Zn SOD, both of which are sensitive to H_2O_2 (6, 7).

Another enzyme whose activity and synthesis are known to be sensitive to active forms of oxygen is nitrogenase (24). Nitrogenase activity has been detected in the *Prochloron* sp.-*L. patella* symbiosis (46), although the presence of nitrogenase is disputed by Parry (48) and awaits immunocytochemical confirmation. The increase in ascidian host respiration with photosynthesis (3), unusually high rates of respiration, when compared with cyanobacteria, in *Prochloron* sp. (1), and the high specific activities of antioxidant enzymes reported in this study could be adaptive in protecting the nitrogenase enzyme from inactivation by molecular and active forms of oxygen (24). The diazotroph *Azotobacter vinelandii* also exhibits high respiration rates and activities of SOD when grown under increasing PO_2's in continuous cultures (15).

The phylogenetic distribution of the various SOD metalloproteins is of interest as it relates to the evolution of higher plant chloroplasts. The position of the *Prochlorales* in regard to the phylogeny of chloroplasts is proving to be a difficult and controversial question to answer (50, 57), due in large part to the present inability to culture these symbionts (1). The discovery of free-living species of *Prochlorales* (9, 13) and recent molecular data from these species have not clarified whether the *Prochlorales* are derived from the cyanobacteria or are more closely aligned with green algal and higher-plant chloroplasts (41, 54, 56). The ability to culture these species (9, 10) should provide sufficient material to resolve these questions.

Interestingly, it has recently been confirmed that the SOD metalloprotein of higher-plant chloroplast lamellae is a Cu-Zn form (47). The recent demonstration and electrophoretic confirmation of Cu-Zn SOD in symbiotic dinoflagellates (18, 35), and in the chlorophyte *Spirogyra* sp. (32), suggest a longer evolutionary history for the Cu-Zn SOD metalloprotein than previously believed (5, 6, 7). Also, Cu-Zn SODs have been reported in several nonphotosynthetic procaryotes (7). The results presented here, confirmed by polyacrylamide electrophoresis, also suggest an older phylogeny for Cu-Zn SOD.

ACKNOWLEDGMENTS

We are grateful to B. Chalker, W. Dunlap, and J. M. Shick for the invitation to participate in this research cruise. The manuscript was greatly improved by comments from Marianne Fisher, John Cullen, and two anonymous reviewers.

The Australian Institute of Marine Sciences and the Center for Marine Studies, University of Maine provided facilities and travel support. This research was partially supported by NSF grant DCB-8509487 (Regulatory Biology) and a National Geographic Society grant to J. M. Shick.

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