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Natural $^{15}N/^{14}N$ Abundance as Evidence for N_2 Fixation by *Prochloron* (Prochlorophyta) Endosymbiotic with Didemnid Ascidians

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Abstract

Prochloron is a symbiotic unicellular alga normally associated with didemnid ascidians in coral reef areas. We compared the natural abundance of $^{15}N/^{14}N$ in isolated cells of these algae and their hosts with those of other primary producers and symbiotic systems from Palau, Western Caroline Islands. Isolated *Prochloron* cells had $\delta^{15}N$ values of 1.1±0.5‰ (SD, n = 22; see Methods for definition of delta notation). When compared with local nitrogen fixers and non-fixers, the lower observed values approached those of nitrogen fixers (0‰), while the higher values (around 2.0‰) indicated that about 30% of the nitrogen was derived by fixation of dissolved N_2. Host ascidians also had $\delta^{15}N$ values lower than tissues of coral species containing non-fixing dinoflagellate symbionts. These $\delta^{15}N$ data support an earlier report that *Prochloron* may be facultatively capable of nitrogen fixation, and that these symbiotic algae, or possibly some of the less evident bacteria associated with their host tissues, provide a source of fixed nitrogen to the symbiotic *Lissoclinum* colonies.

Keywords: *Prochloron*, *Lissoclinum*, $^{15}N/^{14}N$, $\delta^{15}N$, nitrogen fixation, Palau

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1. Introduction

Prochloron is a symbiotic unicellular alga normally associated with didemnid ascidians in coral reef areas. Its cells resemble those of a blue-green alga, *Synechocystis*, but lack bilin pigments. Instead, like green plants, it has chlorophylls *a* and *b*. Evidence from nucleotide sequence studies suggests that *Prochloron* is related to cyanophytes (Swift and Palenik 1993). It is therefore of interest to determine whether, like other symbiotic prokaryotes (e.g., in legume root nodules), it can fix molecular N_2.

Paerl (1984) examined this possibility by testing for an enzyme system analogous to nitrogenase, i.e., one that can reduce acetylene to ethylene. He conducted most of his experiments in Palau, Western Caroline Islands, using local species. Although he was able to demonstrate acetylene reduction with the intact host tunicate, *Lissoclinum patella* full of symbiotic *Prochloron* cells, he obtained negative results using *Prochloron* cells isolated from their hosts. Paerl's positive results were challenged by Odintsov (1991), who was unable to achieve similar results and attributed the acetylene reduction to contaminant bacteria.

We therefore employed a different test for N_2 fixation. The ratio of $^{15}N/^{14}N$ can be used as a natural indicator of N_2 fixation since there is relatively little isotopic fractionation by N_2-fixing prokaryotes, which therefore have a ratio similar to N_2 in air (in conventional notation, $\delta^{15}N = 0‰$, see Methods; reviewed by Goericke et al., 1994). Other forms of inorganic nitrogen available for primary producers are typically enriched in ^{15}N; for example, sea-water nitrate values range from 4 to 19‰ greater than atmospheric N_2 (Michener and Schell, 1994). Thus, typically higher $^{15}N/^{14}N$ values are found in cells which have obtained nitrogen by means other than fixation of N_2 (reviewed by Handley and Raven, 1992).

When N_2 is fixed (reduced and incorporated) by other symbiotic prokaryotes, such as those in legume root nodules, the intracellular $^{15}N/^{14}N$ ratio remains approximately unchanged (Bergerson et al., 1988; Shearer and Kohl, 1989; Goericke et al., 1994). To test for N_2 fixation by using the $^{15}N/^{14}N$ natural abundance method, it is necessary to show that low values consistent with diazotrophy are not a result of an alternative ^{15}N-depleted source by making comparisons with non-fixing reference organisms (Virgina and Delwich, 1982; Handley and Raven, 1992; Gu and Alexander, 1993). In this study we compared the natural $^{15}N/^{14}N$ ratios of *Prochloron* and its symbiotic host with those of co-occurring biota from Palau, Western Caroline Islands.

Table 1. Nitrogen isotopic data for several plants, prokaryote taxa and symbiont hosts from Palau, Western Caroline Islands.

Sampling date	Taxon		$\delta^{15}N$ (‰)	
			Repl. 1	Repl. 2
Prochloron cells isolated from *Lissoclinum patella*				
September 1979			2.1	2.1
September 1979			1.0	0.8
June 1981			0.7	0.6
June 1981			0.5	0.4
July 1994			1.1	0.9
July 1994§			1.4	1.2
July 1994			0.9	0.7
July 1994			1.0	0.8
July 1995			1.7	1.6
July 1995			1.4	1.2
July 1995			1.2	1.2
		Mean, S.D.:	1.10	0.46
Host ascidians with *Prochloron* cells				
March 1983	*Lissoclinum voeltzkowi*		2.5	2.5
March 1983	*Diplosoma virens*		3.0	2.3
July 1994	*Lissoclinum punctatum*		3.4	3.6
		Mean, S.D.:	2.88	0.52
Host ascidians, *Prochloron* cells removed				
September 1979	*Lissoclinum patella*		1.9	2.4
March 1983	*Lissoclinum voeltzkowi*		2.8	2.8
		Mean, S.D.:	2.48	0.41
Reference samples				
Primary producers				
July 1995	*Halymenia dilatata*		2.2	2.6
July 1995	*Halymenia dilatata*		3.1	3.0
		Mean, S.D.:	2.72	0.42
March 1992	Blue green mat*		0.0	
March 1992	*Thalassia* spp.*		2.2	
Consumers				
March 1992	Zooplankton*	Mean, S.D.:	9.2	1.7
March 1992	*Porites lutea*†*	Mean, S.D.:	4.5	0.5
March 1992	*Porites rus*†*	Mean, S.D.:	5.5	0.5
March 1992	*Acropora nasuta*†*	Mean, S.D.:	4.7	0.0

§Sample collected at ca. 7 m; *Data from Yamamura et al., 1995; †Coral tissues.

2. Materials and Methods

Field sampling

All colonies of didemnids and algae were collected at about 1 m below MLW (except one, as indicated in Table 1). Samples of *Prochloron* cells were hand-pressed from colonies of their symbiotic didemnid host, *Lissoclinum patella*, collected from inshore waters of Palau, Western Caroline Islands in 1979, 1981, 1994, and 1995. The algal cells were washed twice in buffered seawater (10 mM $NaHCO_3$), and freeze-dried for later examination. For comparison, samples of a red alga (provisionally identified as *Halymenia dilatata* Zanardini) were collected at the same site in 1995 and freeze-dried.

The freeze-dried samples were ground to a fine powder, and shipped in LSC vials to the Stable Isotope Facility at the University of Alaska Fairbanks, where replicate aliquots of about 1.5 mg (weighed to the nearest μg) were loaded into combustion boats for mass-spectrometric analysis.

Isotopic determination

A Europa Scientific model 20/20 stable-isotope analyzer equipped with a Europa Scientific Roboprep sample preparation and purification unit was used. Analytical results are expressed as stable $^{15}N/^{14}N$ ratios in standard delta units, $\delta^{15}N$, relative to international standards (air for N) defined by the following expression:

$$\delta^{15}N = \left(\frac{R_{sample}}{R_{standard}} - 1 \right) \times 1000‰$$

where $R = {}^{15}N/{}^{14}N$. Isotope standards for atmospheric N_2 have delta values of 0 by definition, i.e. $\delta^{15}N = 0‰$. Naturally occurring ($\delta^{15}N$ values in cells and tissues of associated biota range from ~0 to ~20‰.

3. Results and Discussion

Isolated *Prochloron* cells had $\delta^{15}N$ values of 1.1 ± 0.5‰ (SD), a value much lower than that of other, non-fixing, primary producers from the same locale, namely, non-calcareous red algae and the sea-grass *Thalassia* (Table 1). Host ascidians with *Prochloron* cells had a mean $\delta^{15}N$ value of 2.9‰, contrasting with tissues of coral species containing symbiotic non-N-fixing symbionts which had $\delta^{15}N \geq 4.5‰$ (Table 1). Although the symbiotic ascidian $\delta^{15}N$ values exceed those obtained by Yamamuro et al. (1995) for a blue-green algal mat (Table 1), they are nevertheless consistent with some N_2 fixation, since

$\delta^{15}N$ values for N-fixers and hosts of N-fixers are typically 1 to 6‰ lower than those for non-fixing plants (Shearer and Kohl, 1989, 1993).

The low $\delta^{15}N$ values therefore indicate that some of the N was obtained by diazotrophy; the fraction may be deduced by considering an isotope-mixing model (e.g., Kline et al., 1993), using a delta value of 0 for 100% diazotrophy and a value of ~3‰ for N otherwise obtained (suggested by non-fixing Palauan primary producers, Table 1). Thus the *Prochloron* cells, which ranged in $\delta^{15}N$ from 0 to 2‰ (mean $\delta^{15}N$ value ± 2 SD), had apparently obtained some 30 to 100% of their N by diazotrophy.

The suggested facultative nature of diazotrophy, if dependent on low ambient dissolved inorganic nitrogen (DIN) concentrations, could be tested by using an experimental approach similar to that used by Peterson et al. (1997). Captive ascidians containing *Prochloron* could be maintained in media supplemented with various concentrations of substrates of known enriched $\delta^{15}N$ values. Following incubation, the *Prochloron* cells could be extracted for $\delta^{15}N$ analysis. The close correlation, if any, between the $\delta^{15}N$ of *Prochloron* and that of the DIN would confirm diazotrophy. Potentially, the $\delta^{15}N$ of *Prochloron* could also be an indicator of localized DIN levels.

However, we must also envisage the possibility that molecular nitrogen is being fixed by heterotrophic bacteria, and that the fixed nitrogen is then transferred to the didemnid and the algal cells. Admittedly large numbers of bacteria are normally associated with colonies of *Lissoclinum patella* (Swift and Robertson, 1991). However, most are firmly embedded in the matrix, and are not readily dislodged by the squeezing process whereby symbiotic *Prochloron* cells can be easily expelled. Microscopic examination of such algal cell suspensions reveals very few free bacteria, even fewer after the algae have been rinsed once or twice in buffered and filtered sea-water. Even if some of the bacteria were capable of fixing nitrogen, with consequent low delta values for whole *Lissoclinum* colonies, this could not explain the lower values obtained for the expressed *Prochloron* cells. (If they could not fix nitrogen by themselves, but obtained fixed nitrogen from the host, then the delta values for the algae would be higher, not lower, than those of the animal host.) Unless and until one can test axenic cultures of *Prochloron*, which have not yet been obtained, this possibility cannot be excluded. Nevertheless, the evidence we present strongly indicates that *Prochloron* can fix molecular nitrogen.

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