Evidence for plant-mediated regulation of nitrogenase expression in the *Anthoceros*–*Nostoc* symbiotic association

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A pleiotropic dinitrogen and nitrate assimilation mutant was obtained by mutagenesis of *Nostoc* sp. strain ATCC 29133 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine followed by penicillin counterselection in the presence of NO_3^- and N_2 . Mutant strain UCD 223 was capable of reducing acetylene in the free-living growth state only under anaerobic conditions, or under atmospheric conditions when in symbiotic association with *Anthoceros punctatus*. Heterocysts of strain UCD 223 were noticeably lacking the cohesive outer polysaccharide layer of wild-type heterocysts. Oxygen microelectrode profiles of symbiotic *Anthoceros*–*Nostoc* tissue revealed an anaerobic environment in the symbiotic cavities containing *Nostoc*. The acetylene-reducing activities of strain UCD 223, and of its spontaneously-arising Fix^+ revertant strain UCD 236, were not repressed by the presence of 10 mM- NO_3^- when in the free-living growth state, in contrast to wild-type *Nostoc* ATCC 29133. However, *in situ* activities of acetylene reduction by symbiotically associated *Nostoc* ATCC 29133 and strains UCD 223 and UCD 236 were repressed by the presence of 10 mM- NO_3^- . It appears that the symbiotic cavities of *Anthoceros punctatus* can physiologically replace the function of the heterocyst outer wall and that the repression of nitrogenase activity in symbiotic *Nostoc* in response to the presence of NO_3^- , and probably NH_4^+ , is mediated by *Anthoceros*.

Introduction

Many cyanobacteria can grow utilizing nitrogen in different oxidation states from the environment. When ammonium is available, it is used preferentially. However, nitrate and nitrite can also be used via assimilatory nitrate and nitrite reduction to ammonium. The most complex system is that of reduction of dinitrogen gas by the oxygen-labile nitrogenase enzyme complex. In filamentous cyanobacteria classified within Sections IV and V (Rippka *et al.*, 1979), nitrogenase is active in terminally differentiated cells called heterocysts. Heterocysts are enclosed by a unique glycolipid layer surrounded by polysaccharides which together impede the entrance of oxygen (Wolk, 1982; Murry & Wolk, 1989). In the presence of combined nitrogen sources, the frequency of heterocysts in the filaments decreases (Fogg, 1949).

Some heterocyst-forming cyanobacteria, especially of the genus *Nostoc*, form symbiotic relationships with

fungi and representative groups in the phylogenetic spectrum of plants (Rai, 1990). When the eukaryotic partner is photosynthetic, the heterocyst frequency of the symbiotic *Nostoc* increases to 30 to 60% of the total cells, compared to 3 to 8% in the free-living growth state (see Rai, 1990). These symbiotic associations are analogous to those between rhizobia and leguminous plants in that the eubacteria function in providing fixed nitrogen for the growth of the associated tissue (Stewart *et al.*, 1980).

The bryophyte *Anthoceros punctatus* L. will accept as symbiotic partners a variety of *Nostoc* strains, which form discrete colonies within preformed cavities in the gametophyte tissue (Enderlin & Meeks, 1983). Specific *Nostoc* mutant strains have been employed in defining aspects of the initial establishment and physiology of the *Anthoceros*–*Nostoc* association. Analogue-resistant mutants were used to examine competition between *Nostoc* strains in infection of *Anthoceros* tissue (Enderlin & Meeks, 1983). *In situ* ^{13}N tracer experiments using mutants of *Nostoc* sp. strain UCD 7801, resistant to the glutamine synthetase inhibitor L-methionine-DL-sulphoximine, established that symbiotic *Nostoc* releases 80 to 90% of its fixed nitrogen as ammonium to support growth of *Anthoceros* tissue (Meeks *et al.*, 1985). Studies utilizing mutants of *Nostoc* UCD 7801, resistant to the

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Abbreviations: Chl *a*, chlorophyll *a*; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FW, fresh weight; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

photosynthetic inhibitor 3-(3,4-dichlorophenol)-1,1-dimethylurea (DCMU), provided unequivocal evidence that the *Nostoc* is capable of complete photosynthesis when in association with *Anthoceros*, albeit at a low rate (Steinberg & Meeks, 1989), and can use its immediate photosynthate to support nitrogenase activity (Steinberg & Meeks, 1991).

The size of the *Nostoc* colony in *Anthoceros* tissue, its rate of nitrogen fixation and frequency of heterocysts are influenced by the growth conditions of the *Anthoceros*–*Nostoc* association (Enderlin & Meeks, 1983; Steinberg & Meeks, 1991). In particular, reconstitution or growth of *Anthoceros*–*Nostoc* tissue in the presence of ammonium results in *Nostoc* colonies with very low to negligible rates of acetylene reduction (Enderlin & Meeks, 1983). We are interested in the chemical interactions between partners of the *Anthoceros*–*Nostoc* association that regulate *Nostoc* gene expression and cellular differentiation. Our working hypothesis is that the currently undefined nitrogen regulatory mechanism controlling *Nostoc* heterocyst differentiation in the free-living state (Fogg, 1949) is superseded by a symbiotic control mechanism when in association with *Anthoceros*. To test this hypothesis, we have isolated a mutant of the symbiotically-competent *Nostoc* sp. strain ATCC 29133 that has defects in aerobic nitrogen fixation (Fix^-) and is also unable to assimilate exogenously supplied nitrate (assimilatory nitrate metabolism; Anr^-). In this paper we describe the phenotypic characteristics of the mutant in both free-living and symbiotic growth states.

Methods

Culture and mutagenesis of *Nostoc*. Dinitrogen-grown cultures of *Nostoc* sp. strain ATCC 29133 (PCC 73102) (1.4×10^9 cells total) were mutagenized using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) at $250 \mu\text{g ml}^{-1}$ for 4 h as previously described (Chapman & Meeks, 1987). After mutagenesis, cultures were grown for approximately seven generations (14 d) at 28°C with continuous lighting (7 W m^{-2}) in a fourfold dilution of the minimal medium of Allen & Arnon (1955), supplemented with 5.0 mM-MOPS/NaOH (pH 7.8) and 2.5 mM- NH_4Cl . This growth period allowed for segregation and expression of mutant genomes. All media, liquid and solid, used for maintenance of mutagenized cultures were buffered with 5.0 mM-MOPS/NaOH. Unless otherwise stated, all cultures were maintained under atmospheric conditions.

Prior to counterselection, cultures were harvested by centrifugation at 1000 g for 5 min and suspended in 25 ml of medium contained in a 125 ml flask. The flasks were then placed in a sonic cleaning bath (Branson, model 12) and subjected to sonic cavitation for sufficient time to reduce the filaments to an average length of 1.4 cells. This step was taken to minimize the amount of metabolite cross-feeding in the filaments. The fragmented cultures were twice washed with fresh medium, suspended in liquid medium supplemented with 5.0 mM- NO_3^- (equimolar sodium and potassium salts) and 0.1 mM- NH_4Cl , and cultured for 48 h to recover from the shock of sonication. The cultures were then incubated for an additional 48 h in liquid medium

supplemented only with 5.0 mM- NO_3^- to induce the dinitrogen fixation and nitrate utilization systems. Mutants lacking only the aerobic N_2 fixation system (Fix^-) should be able to grow in the presence of NO_3^- . Likewise, in mutants lacking the ability to assimilate NO_3^- (Anr^-), there should be no repression of the N_2 fixation system in the presence of NO_3^- . Penicillin was added to a final concentration of 250 units ml^{-1} to enrich for those mutants lacking the ability to utilize both NO_3^- and N_2 as nitrogen sources for growth. After 48 h of incubation in penicillin, cultures were washed once and suspended in fresh medium supplemented with 2.5 mM- NH_4Cl . The counterselected culture was again reduced to 1–2 cell filament length by cavitation as described above and then plated onto solidified (1%, w/v, agar, purified using the methods of Braun & Wood, 1962) minimal medium supplemented with NH_4Cl as before.

Screenings. Isolated colonies obtained following MNNG mutagenesis and penicillin counterselection were individually picked and screened for growth on 5.0 mM- NO_3^- , N_2 or 2.5 mM- NH_4Cl . All plates were incubated at 28°C with continuous lighting. The wild-type strain was included as a control on all plates. Those isolates that showed no growth, or less growth, relative to the wild-type control on the NO_3^- plus N_2 plates were retested twice by streaking from the original NH_4Cl -supplemented plate on which the counterselected culture was grown.

Culture of *Anthoceros*. Pure cultures of gametophyte tissue of *Anthoceros punctatus* L. are maintained in this laboratory (Meeks, 1988). The symbiotic association with wild-type or mutant strains of *Nostoc* ATCC 29133 was reconstituted as described previously (Meeks, 1988). Symbiotically associated tissues were used 5–6 weeks after reconstitution.

Phenotypic characterizations. One *Nostoc* mutant, strain UCD 223, was chosen for further analysis. Symbiotic competence with *A. punctatus* was determined by the presence or absence of symbiotic cyanobacterial colonies and N_2 -dependent growth of *Anthoceros* tissue following co-culture of *Anthoceros* and the *Nostoc* strain in basal medium lacking combined nitrogen (Meeks, 1988). Acetylene reduction assays of symbiotic tissue and free-living cultures were performed as previously described (Enderlin & Meeks, 1983). When specified, 10 mM- NO_3^- (equimolar sodium and potassium salts) was added to the basal medium. Symbiotic cultures were washed and transferred every 3–5 d into fresh medium when grown with NO_3^- in order to maintain the level of NO_3^- in the medium and to control the epiphytic growth of cyanobacteria. Parallel N_2 -grown cultures were also transferred every 3–5 d.

Induction of anaerobic nitrogenase activity in free-living cultures of strain UCD 223 was performed as described by Murry & Wolk (1989) in two steps: (1) 72 h of incubation under atmospheric conditions without nitrogen supplementation in order to promote glycogen accumulation, which subsequently serves as a carbon source and as reductant supply for nitrogenase, followed by (2) addition of 20 μM -DCMU (to inhibit photosynthetic O_2 evolution), and incubation under argon for 12–16 h to allow for synthesis of the oxygen-labile nitrogenase enzyme complex. Calcium-carbide-generated acetylene gas was then added to each culture to a final concentration of 10% (v/v) and the gas phase analysed periodically for up to 200 min to determine the linear rate of ethylene production. Chlorophyll *a* (Chl *a*) in methanolic extracts was quantified as previously described (Meeks *et al.*, 1983).

To determine the O_2 content of *Anthoceros*–*Nostoc* tissues, the tissues were secured onto 2% (w/v) purified agar plates (buffered with 5 mM-MES/NaOH, pH 6.4) using pulled-out capillary tubes that were inserted through the periphery of the gametophyte tissue and into the agar. The plate was then flooded with fresh bryophyte growth medium with atmospheric oxygen content. Tungsten illumination was supplied at 10 inches (25 cm) from the tissue at an intensity of 10 W m^{-2} at the

surface of the liquid. A standard Clark-type O₂ microelectrode mounted in a micromanipulator (Revsbeck & Jorgensen, 1986) was utilized to measure the O₂ content within the symbiotic *Nostoc* colonies and adjacent *Anthoceros* tissue.

The ammonium content of *Anthoceros* and *Anthoceros-Nostoc* tissues, grown with NH₄NO₃, NO₃⁻ or N₂ (Enderlin & Meeks, 1983) was determined by fluorimetry after *o*-phthalaldehyde derivatization of cell extracts (Goyal *et al.*, 1988). Approximately 0.5 g fresh weight (FW) of gametophyte tissue was homogenized in 2.0 ml 10 mM-potassium phosphate, pH 6.8. The extracts were clarified by centrifugation (1000 g, 5 min) and passed through 0.2 µm pore size nylon filters prior to derivatization and separation by HPLC (Goyal *et al.*, 1988). The values are reported as means ± SE of four determinations.

In preparation for electron microscopy, symbiotic *Nostoc* colonies were manually excised from plant tissue with watchmaker's forceps and the aid of a stereomicroscope. Heterocysts were induced in free-living cultures by incubation in medium without combined nitrogen (48 h for strain UCD 223) under atmospheric conditions. Symbiotic *Nostoc* colonies and free-living *Nostoc* cells were then concentrated by centrifugation at 13000 g and fixed in 3% (v/v) glutaraldehyde in 0.2 M-potassium phosphate (pH 7.0). Samples were post-fixed in 1% (w/v) osmium tetroxide for 2 h and then dehydrated in ethyl alcohol followed by a graded propylene oxide series. The samples were embedded in Medcast (Ted Pella), sectioned, and stained with uranyl acetate and lead citrate. Thin sections were examined with a Zeiss EM109 transmission electron microscope under contract with the Facility for Advanced Instrumentation, UC, Davis.

Spontaneous revertants of strain UCD 223 that had regained aerobic N₂ fixation were obtained by plating NH₄Cl-grown cells onto solid medium with no added nitrogen source and incubation under atmospheric conditions. These Fix⁺ revertants were screened for the ability to grow with NO₃⁻ as the sole nitrogen source by inoculation onto solid medium supplemented with NO₃⁻ and incubated at 10 W m⁻² of illumination in a sealed clear container that was flushed continuously with 99% argon/1% CO₂ (v/v). All revertants and wild-type *Nostoc* ATCC 29133 were also plated with no added nitrogen source and incubated under the same conditions as controls. All revertants and *Nostoc* ATCC 29133, on an NH₄Cl-supplemented plate and incubated under atmospheric conditions, were included in parallel for verification of viability. Growth relative to the wild-type strain under all three conditions was noted following 2 weeks of incubation.

Results

Isolation and characteristics of a Fix⁻ Anr⁻ mutant of *Nostoc* sp. strain ATCC 29133

Following mutagenesis and counterselection, 1150 isolates were subjected to three successive screenings and yielded one clone, *Nostoc* strain UCD 223, with a stable pleiotropic aerobic Fix⁻ and Anr⁻ phenotype. All other 191 putative mutants from the initial screening displayed an ability to grow with N₂ and/or NO₃⁻ in subsequent screenings. Strain UCD 236 is a spontaneous aerobic Fix⁺ revertant of strain UCD 223; such spontaneous reversion to Fix⁺ occurred at a frequency of approximately 10⁻⁹. Strain UCD 236 was unable to grow on NO₃⁻ in a 99% argon/1% CO₂ atmosphere, whereas *Nostoc* ATCC 29133 showed extensive growth following 2 weeks of incubation under the same conditions. *Nostoc* ATCC 29133, however, displayed little or no growth

Table 1. Acetylene reduction activity of *Nostoc* ATCC 29133 and its derivative strains

All values are means ± SE; *n* is the number of trials. No acetylene reduction was observed with any of the three strains in the free-living state with 2.5 mM-NH₄⁺ as the nitrogen source (acetylene reduction was not determined for the symbiotic association under these conditions).

Strain*	Acetylene reduction activity	
	10 mM-NO ₃ ⁻	N ₂
Free-living <i>Nostoc</i> [nmol min ⁻¹ (mg Chl <i>a</i>) ⁻¹]		
29133	19.8 ± 9 (<i>n</i> = 4)	132 ± 24 (<i>n</i> = 2)
223†	45.0 (<i>n</i> = 1)	54 ± 26 (<i>n</i> = 4)
236	120.0 ± 18 (<i>n</i> = 3)	109 ± 18 (<i>n</i> = 3)
<i>Anthoceros-Nostoc</i> association (pmol min ⁻¹ per colony)		
29133	12.7 ± 1 (<i>n</i> = 3)	27.9 ± 3 (<i>n</i> = 8)
223	24 (<i>n</i> = 1)	97 ± 6 (<i>n</i> = 5)
236	7.0 ± 1.0 (<i>n</i> = 2)	27.0 ± 1 (<i>n</i> = 18)

* Strain ATCC 29133 is the wild-type, strain UCD 223 is a Fix⁻ Anr⁻ mutant derived from ATCC 29133, strain UCD 236 is a Fix⁺ Anr⁻ revertant of strain UCD 223.

† Activities were measured in the presence of 20 µM-DCMU and argon (see Methods).

under the argon/CO₂ atmosphere when plated on medium containing no nitrogen source.

Nostoc UCD 223 was able to establish an initially functional symbiotic association with *Anthoceros*. The rate of acetylene reduction, normalized to the number of symbiotic colonies, was about three fold higher than that of the association with *Nostoc* ATCC 29133 (Table 1). However, when normalized to g FW of *Anthoceros* tissue, the rate with strain UCD 223 was approximately five-fold lower than that with ATCC 29133 (data not shown). Since *Anthoceros* tissue associated with strains UCD 223 and ATCC 29133 contained an average of 23 ± 4 (*n* = 9) and 1176 ± 153 (*n* = 9) colonies per g FW, respectively, the lower rate of acetylene reduction by strain UCD 223, normalized to FW, was apparently a consequence of a lower infection frequency. The association with strain UCD 223 eventually ceased to grow with N₂ as the nitrogen source due to a lack of the reinfection of the marginal meristematic region of *Anthoceros* tissue that occurs in associations with wild-type *Nostoc* strains.

Electron micrographs show that the heterocysts of strain UCD 223 in the free-living and symbiotic growth states (Fig. 1*a, d*) lack the complete outer polysaccharide layer characteristic of heterocysts in the wild-type strain

Fig. 1. (a, b) Transmission electron micrographs of *Nostoc* UCD 223 in symbiotic association with *Anthoceros punctatus* (a) and in the free-living growth state (b). (c, d) *Nostoc* ATCC 29133, symbiotically associated (c) and free-living (d). Bars, 1 µm. Arrowheads in (a) and (b) indicate the abnormal outer polysaccharide layer of mutant heterocysts. H, heterocyst.

(Fig. 1c, d). The residual polysaccharide layer of mutant heterocysts was most apparent at adjacent cell junctions (see arrowheads in Fig. 1a, b). Heterocysts of strain UCD 223 had a less distorted shape and a more cohesive cytoplasm in the symbiotic, compared to the free-living, growth state, perhaps as a consequence of osmotic

stabilization. The distorted shapes of the heterocysts in free-living cultures of strain UCD 223 were clearly visible in the light microscope (data not shown).

Free-living cultures of strain UCD 223 were able to reduce acetylene under anaerobic incubation conditions at a rate that was less than 50% that of *Nostoc*

ATCC 29133 incubated under atmospheric conditions (Table 1). Consistent results were obtained only after cultures of strain UCD 223 had been starved for NH_4^+ in the light under atmospheric conditions to supplement internal pools of stored photosynthate prior to anaerobic induction of nitrogenase (see Murry & Wolk, 1989). Low and variable rates of acetylene reduction were seen if the cultures were starved for NH_4^+ under anaerobic conditions in the presence of $20\text{ }\mu\text{M}$ -DCMU and 10 mM fructose (data not shown).

Oxygen tension in symbiotic *Nostoc* colonies

The observations that strain UCD 223 was able to reduce acetylene only under anaerobic conditions in the free-living growth state, but under normal atmospheric conditions when in symbiotic association, implied that the oxygen tension could be low in the symbiotic *Nostoc* colony. The O_2 concentration profile of *Anthoceros*–*Nostoc* tissue, as measured with a microelectrode, is shown in Fig. 2. The dissolved O_2 content of the medium at the surface of *Anthoceros* tissue was $259\text{ }\mu\text{M}$ as determined with the microelectrode. The *Anthoceros* tissue at approximately $80\text{--}950\text{ }\mu\text{m}$ internal from the surface of the tissue and $600\text{--}3000\text{ }\mu\text{m}$ from the nearest symbiotic *Nostoc* colony contained an average of $157 \pm 12\text{ }\mu\text{M}$ O_2 ($n = 15$), or 60% of the O_2 content of the medium. The *Anthoceros* cavity housing symbiotic *Nostoc* (strain ATCC 29133 or UDC 223) contained no measurable O_2 . Symbiotic tissue with wild-type *Nostoc* sp. strains UCD 7801 or ATCC 27896 displayed the same decrease in O_2 in the adjacent *Anthoceros* tissue leading to no measurable O_2 within the symbiotic cavity (data not shown).

The symbiotic colonies of *Nostoc* strain UCD 223 were larger than those of strain ATCC 29133 of the same age. As shown in Fig. 2, the strain UCD 223 symbiotic colony after 6 weeks of co-culture with *Anthoceros* was approximately $780\text{ }\mu\text{m}$ in depth, whereas symbiotic *Nostoc* ATCC 29133 colonies of the same age were approximately $600\text{ }\mu\text{m}$ in depth; similar results were observed in other randomly selected colonies. The larger colonies of strain UCD 223 are consistent with its higher rate of symbiotic acetylene reduction when normalized to number of colonies (Table 1). When reisolated from symbiotic association, strain UCD 223 retained its aerobic Fix^- and Anr^- phenotype.

Effect of nitrate on free-living and symbiotic nitrogenase expression by wild-type and *Anr^-* strains

Acetylene-reducing activities of strain UCD 223 in the presence of argon and DCMU were similar with or without NO_3^- in the medium (Table 1). The kinetics of

Fig. 2. Oxygen concentration profile of *Anthoceros* gametophyte tissue and associated colonies of *Nostoc* ATCC 29133 (a) and UCD 223 (b), measured after 6 weeks of co-culture. Each profile is a representative of three trials.

induction of nitrogenase in the absence or presence of combined nitrogen were examined in an aerobic Fix^+ revertant of strain UCD 223. Strain UCD 236 (Fix^+ Anr^-) was used to avoid physiological variability and the experimental complications of maintaining an anaerobic environment during incubation of these *Nostoc* strains, which grow preferentially as oxygenic photoautotrophs. Acetylene-reducing activity of strain UCD 236 showed the same induction kinetics following transfer from NH_4^+ into the absence or presence of NO_3^- (Fig. 3b). The activity of the wild-type strain, however, was inhibited 85% in the presence of NO_3^- relative to the fully induced rate after 4 d of incubation (Table 1, Fig. 3a). When in symbiotic association with *Anthoceros*, the acetylene-reducing activity of all three strains (ATCC 29133, UCD 223 and UCD 236) was repressed in the presence of 10 mM - NO_3^- when compared with the parallel N_2 -grown cultures (54%, 75% and 74% repression, respectively; Table 1, Fig. 4). Strains UCD 223 and UCD 236,

Fig. 3. Acetylene reduction rates of NH₄⁺-grown free-living *Nostoc* cultures transferred at $t = 0$ to medium with: no nitrogen supplement (▲), 10 mM-NO₃⁻ (■), or 2.5 mM-NH₄⁺ (●). (a) Strain ATCC 29133; (b) strain UCD 236. Each datum point represents the mean of 2–4 trials $\pm$ SE.

Fig. 4. *In situ* acetylene reduction rates following the addition of 10 mM-NO₃⁻ to *Anthoceros* tissue previously reconstituted with *Nostoc* ATCC 29133 (●) or UCD 236 (■). NO₃⁻ was added at $t = 0$ in both cases. $n = 1$ for strain ATCC 29133; $n = 2 \pm$ SE for strain UCD 236.

reisolated from NO₃⁻-grown symbiotic associations and cultured in the free-living state, did not grow with NO₃⁻ as sole nitrogen source.

The NH₄⁺ content of symbiont-free *Anthoceros* tissue, grown with NH₄NO₃, was $2.35 \pm 0.17 \mu\text{mol (g FW)}^{-1}$.

Symbiont-free *Anthoceros* and *Anthoceros*–*Nostoc* UCD 236 tissues cultured with 10 mM-NO₃⁻ for more than 14 d (transferred every 3–5 d) contained 0.12 ± 0.01 and $0.14 \pm 0.01 \mu\text{mol NH}_4^+ \text{ (g FW)}^{-1}$. These values are very similar to those found in N₂-grown *Anthoceros*–*Nostoc* ATCC 29133 (0.12 ± 0.01).

Discussion

MNNG mutagenesis followed by penicillin counterselection in the presence of NO₃⁻ and N₂ was an effective method to obtain a mutant defective in two nitrogen assimilatory functions. There are three possible explanations for this pleiotropic phenotype. First, there could be a mutation in a common regulatory gene. The existence of a common nitrogen regulatory gene has been reported in the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942 (Vega-Palas *et al.*, 1990) and global regulatory proteins have now been identified in cyanobacteria (Tsinoremas *et al.*, 1991). Second, there could be a mutation in a common cofactor for activity, such as molybdate processing (ChlD⁻ phenotype; Shah *et al.*, 1984). Third, the strain could carry multiple mutations in structural genes. Strain UCD 223 appears to be representative of the latter category. Elevation of the molybdenum concentration in the medium did not restore growth of strain UCD 223 on NO₃⁻ or N₂ (data not shown), as occurs in molybdate-processing mutants (Shah *et al.*, 1984). Spontaneous reversion to both N₂ and NO₃⁻ prototrophy has not been obtained after plating more than 10¹² cells and reversion to NO₃⁻ prototrophy alone occurs at a frequency approximately fivefold higher than reversion to N₂ prototrophy. We have no specific information about the mutation leading to the Anr⁻ phenotype. However, electron micrographs showing incomplete formation of the polysaccharide layer (Fig. 1) indicate that strain UCD 223 carries mutations in a gene(s) encoding synthesis of a component(s) of the heterocyst envelope. The fact that strain UCD 223 could reduce acetylene under anaerobic incubation conditions is evidence that the mutation results in loss of O₂ protection for either synthesis or catalytic activity of nitrogenase.

Normalized rates of acetylene reduction by *Nostoc* strain UCD 223 in symbiosis were greater than those by strain ATCC 29133 (Table 1), indicating that the O₂ protection function of the outer heterocyst wall could be substituted by the environment within the symbiotic cavity of *Anthoceros*. Such a micro-oxic environment was verified by direct microelectrode measurements (Fig. 2). *Nostoc* UCD 7801 in association with *Anthoceros* is capable of complete photosynthesis at a rate approximately 15% of that expressed in the free-living growth state (Steinberg & Meeks, 1989). Thus, a micro-oxic environ-

ment within the tissue of a symbiotic association involving two O_2 -evolving photosynthetic partners was not expected. Ultrastructural analyses of *Anthoceros*–*Nostoc* tissues have provided no evidence for the presence of a structural barrier to O_2 diffusion within symbiotic cavities (Rodgers & Stewart, 1977; Duckett *et al.*, 1977). A similar low O_2 tension in cyanobacterial-inhabited coralloid roots of cycads has been inferred from assays of nitrogenase activity, but there have been no direct measurements (Lindblad *et al.*, 1991). We suggest the low O_2 tension in the *Anthoceros*–*Nostoc* symbiotic cavity, and perhaps cycad roots, is a consequence of the respiratory activities of both partners. The fact that *Anthoceros* gametophyte tissue had a lower than ambient O_2 content (Fig. 2) implies that the overall rate of photosynthetic O_2 production did not greatly exceed the rate of respiratory O_2 consumption under the specific culture conditions.

Since strain UCD 223 is unable to fix nitrogen in an aerobic environment, it is unable to grow outside of the symbiotic cavity in medium lacking combined nitrogen. This limitation may contribute to its inability to reinfect marginal meristematic regions of the plant and establish new symbiotic colonies; Fix^+ revertants of strain UCD 223 do reinfect *Anthoceros* tissue. The relatively large size of existing symbiotic colonies of strain UCD 223, and accompanying high rate of acetylene reduction, may occur in response to the lack of new infections, whereby the existing colonies provide more fixed nitrogen in an attempt to support the growing bryophyte tissue. A similar response was observed after *Anthoceros*–*Nostoc* tissues had been treated with penicillin to remove epiphytic growth of *Nostoc* (Enderlin & Meeks, 1983).

The presence of NH_4^+ or NO_3^- repressed nitrogenase expression in *Nostoc* ATCC 29133 (Table 1, Fig. 3). Nitrate repression is thought to be mediated through its reduction to NH_4^+ by the assimilatory nitrate and nitrite reductases and subsequent assimilation of the NH_4^+ into organic molecules (Van Baalen, 1987). The mechanism by which assimilated NH_4^+ is sensed and represses heterocyst differentiation and nitrogenase expression is unknown. A protein homologous to the PII sensing protein of the nitrogen regulatory system in enteric bacteria (Bueno *et al.*, 1985) and nucleotide sequences homologous to the gene encoding a bifunctional protein kinase–phosphoprotein phosphatase (*ntrB*; Keener & Kustu, 1988) were recently identified in cyanobacteria (Tsinoremas *et al.*, 1991). These observations suggest the presence of an analogous nitrogen regulatory system in cyanobacteria. As predicted by their Anr^- phenotype, free-living cultures of strains UCD 223 and UCD 236 induced and maintained the same level of nitrogenase activity in the presence or absence of exogenous NO_3^-

(Table 1, Fig. 3). Nevertheless, the presence of nitrate repressed acetylene-reducing activity to a similar extent in *Anthoceros* associations with *Nostoc* ATCC 29133 and with strains UCD 223 and UCD 236 (Table 1, Fig. 4). It is possible that the NO_3^- was reduced to NH_4^+ by *Anthoceros* and excess NH_4^+ released into the symbiotic cavity where it functioned to repress nitrogenase in symbiotic *Nostoc*. The pools of NH_4^+ in tissues of NO_3^- -grown *Anthoceros* or *Anthoceros*–*Nostoc*, however, were identical to those present in N_2 -grown *Anthoceros*–*Nostoc*. The resulting decrease in nitrogenase activity in the presence of exogenous NO_3^- must have been mediated by a change in extracellular signals, other than NH_4^+ , transferred from *Anthoceros* to *Nostoc*.

We conclude that the mechanism(s) regulating the high level of symbiotic heterocyst differentiation and nitrogenase expression of *Nostoc* in association with *Anthoceros* is distinctly different from that involved in the free-living growth state. The apparent lack of direct nitrogen repression and the existence of a microaerobic to anaerobic environment are strikingly similar to the conditions involved in bacteroid differentiation and nitrogenase expression by rhizobia in association with leguminous plants (Long, 1989).

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