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Review article

Symbiotic Interactions between *Nostoc punctiforme*, a Multicellular Cyanobacterium, and the Hornwort *Anthoceros punctatus*

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Abstract

The filamentous, nitrogen-fixing cyanobacterium *Nostoc punctiforme* forms a symbiotic association with representatives of three of the major phylogenetic groups of terrestrial plants; bryophyte, gymnosperm and angiosperm. The association with *N. punctiforme* and the bryophyte hornwort *Anthoceros punctatus* has been used as a model experimental system to define the continuum of interactions between the partners leading to a stable nitrogen-fixing symbiosis. The symbiotic interactions can be experimentally categorized into an infection stage that involves the differentiation and behavior of motile filaments called hormogonia and a function stage that involves the differentiation and behavior of nitrogen fixing cells called heterocysts. The physiological, biochemical and genetic data that support the stages of interaction are described in this review.

Keywords: *Anthoceros punctatus*, cellular differentiation, genetic analysis, genome analysis, nitrogen fixation, *Nostoc punctiforme*, symbiosis

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1. Introduction

The nitrogen-fixing cyanobacterium *Nostoc punctiforme* has an exceptionally large number of phenotypic traits. It expresses the nutritionally independent oxygenic photoautotrophic life style that characterizes all cyanobacteria, requiring only light, water and a few inorganic nutrients for growth. *N. punctiforme* can alter its photosynthetic pigment complement, produce UV light-absorbing compounds, and assimilate ammonium, urea, nitrate, or dinitrogen; these traits enhance its photosynthetic competence in changing environments (Meeks et al., 2001). The vegetative cells of *N. punctiforme* can develop into three distinctly different cell types: terminally differentiated, microoxic heterocysts, which are specialized for nitrogen fixation in an oxic biosphere; spore-like cells called akinetes, which are more resistant than vegetative cells to cold; and motile filaments called hormogonia that respond to chemicals and light, while functioning as short distance dispersal units (Meeks et al., 2002). *N. punctiforme* can also grow well in the dark as a heterotroph (Summers et al., 1995). These traits contribute to its competitive survival in the terrestrial habitats that it occupies. Despite, or perhaps because of, these characteristics of an independent existence, *N. punctiforme* is prized as a symbiotic partner by representatives of three phylogenetic groups of terrestrial plants. *N. punctiforme* strain PCC 73102 (ATCC 29133) was isolated from symbiotic association in a coralloid root of the gymnosperm cycad *Macrozamia* sp. (Rippka et al., 1979). It can reconstitute an association in the laboratory with, and support dinitrogen-dependent growth of, the angiosperm *Gunnera manicata* (Johansson and Bergman, 1994) and the bryophyte hornwort *Anthoceros punctatus* (Enderlin and Meeks, 1983). We developed the *Nostoc* spp.-*A. punctatus* association as an experimental system to study the interactions between partners in cyanobacteria-plant symbioses (Meeks, 1988).

A substantial database on the physiological and biochemical adaptation of *Nostoc* spp. to symbiotic growth in association with *A. punctatus* has been generated (Meeks, 1998). In the course of those studies, encouraged by the initial observations of Flores and Wolk (1985), protocols and materials for genetic analysis of *N. punctiforme* strain ATCC 29133 were established (Cohen et al., 1994; Campbell et al., 1998; Hagen and Meeks, 1999). Mutants generated and characterized in free-living culture are also characterized for symbiotic phenotype in the *A. punctatus* association. The objective of this review is to summarize the results that contribute to a working model of this symbiotic association.

The extensive phenotypic traits, symbiotic competence, and genetic tractability of *N. punctiforme* contributed to its selection as an organism whose genome was sequenced by the Joint Genome Institute, under sponsorship of the US Department of Energy. The shotgun sequencing phase has been completed, an

automated annotated database is publicly available (http://www.jgi.doe.gov), a preliminary analysis published (Meeks et al., 2001) and finishing of the complete sequence is projected to occur in 2003. At approximately 9.5 Mb, with more than 7,500 open reading frames, the genome of *N. punctiforme* is amongst the largest currently known in the bacterial world. Preliminary analyses indicate a genome complexity that is consistent with its phenotypic complexity, including more than 1,600 unique open reading frames with no sequence similarity to any organism in the current database, inclusive of closely related cyanobacteria. While the purpose of this review is not to describe the *N. punctiforme* genome, some observations are relevant. *N. punctiforme* has an extraordinary capacity to interact with its environment, in both a regulatory and metabolic context. This capacity is reflected in the approximately 150 sensor histidine kinases, 103 response regulators (61 with DNA binding domains), 46 additional transcriptional regulators, 14 sigma subunits of RNA polymerase, 55 serine/threonine protein kinases, 52 putative protein-modification proteins and 7 adenylate cyclases. There is evidence for robust amino acid transport and metabolism, organic carbon transport, and inorganic ion transport; as well as 21 homologues of chemotaxis genes. What is not obvious in the genome are the identities of genes encoding regulatory and structural proteins that might be involved in cellular differentiation (i.e. heterocysts, hormogonia and akinetes) and symbiotic association, apart from those previously described. In contrast to the conservation of metabolic pathways, which allow one to identify basic structural motifs and models that can be used to infer similarity in function, there are apparently no universally applicable models of bacterial development cascades. Similarly, the emerging models of the exquisitely complex rhizobia-legume interactions leading to the root nodule (Downie and Walker, 1999; Perret et al., 2000), do not provide a basis for predicting protein function from gene sequence in *N. punctiforme*-plant associations. Therefore, functional genome and proteome analyses will be essential in defining the regulatory cascades of cellular differentiation and symbiotic interactions of *N. punctiforme*. A physiological and biochemical database relative to these processes will contribute to planning, evaluating and modeling the results of global assays.

2. Symbiotic Interactions

Since *N. punctiforme* PCC 73102 (ATCC 29133), and related *Nostoc* strains or species (e.g. *Nostoc* sp. strain UCD 7801 which associates with *A. punctatus* [Enderlin and Meeks, 1983] and *G. manicata* [Johansson and Bergman, 1994]), show broad symbiotic competence within the phylogenetic spectrum of plants, it is difficult to imagine that *Nostoc* species have evolved specific adaptive

processes for each plant. Rather, it is logical to hypothesize that the different plants must have independently evolved strategies to control key regulatory and metabolic pathways of the cyanobacteria that are normally expressed in free-living growth. Moreover, the *Nostoc* species colonize structures or areas in the plant tissues that are present at all times (Adams, 2000; Rai et al., 2000; Meeks and Elhai, 2002); there may be some modifications by the plant in response to the presence of the cyanobacterium, but they are minor relative to the formation of a legume nodule. These observations contribute to the conclusion that the interactions in cyanobacterial symbioses are primarily unidirectional from plant to cyanobacterium (Meeks, 1998). While these interactions most likely occur in a continuum, they can be experimentally defined in two sequential stages in the *Nostoc* spp.-*A. punctatus* association. The continuum and individual steps are depicted in the schematic of Fig. 1. The initial stage is establishment of the association and involves the differentiation and behavior of hormogonium filaments that act as infective units. The second stage is development of a functional association involving the differentiation and behavior of heterocysts and metabolic alteration of vegetative cells. The two stages of interaction can be considered as a simplified analogy to the rhizobial infection (infectiveness) and differentiation (effectiveness) stages of the legume associations.

Infection – establishment of the association

The infection stage can be viewed as three substages: induction of hormogonia, the infective units; control of the direction of hormogonium gliding to insure infection; and repression of hormogonium differentiation following colonization.

Induction – evidence for a hormogonium inducing factor

The differentiation of *Nostoc* spp. hormogonia is induced by a number of environmental changes that are positive and negative for growth (summarized in Campbell and Meeks, 1989). In response to the environmental signal, the vegetative cells cease net macromolecular synthesis, including DNA replication (Herdman and Rippka, 1988), initiate one or more rounds of cell division, and the filaments fragment at the junctions between vegetative cells and heterocysts (Campbell and Meeks, 1989). Thus, hormogonium filaments do not fix nitrogen. The released small-celled hormogonium filaments initiate gliding and remain motile for 48 to 72 hours; they then cease to glide, begin to differentiate heterocysts at the ends of the filaments and reinitiate net macromolecular synthesis. This series of events defines the hormogonium developmental cycle. The 48 to 72 h gliding period encompasses the infection window for the *A. punctatus* association.

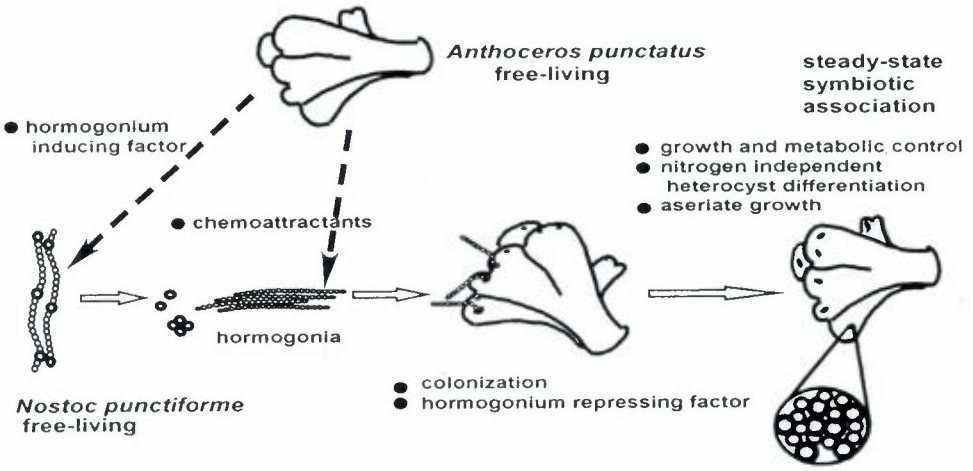

Figure 1. Schematic of the continuum of interactions between *A. punctatus* and *N. punctiforme* leading to a nitrogen-fixing symbiotic association. The interactions are depicted as primarily unidirectional from *A. punctatus* to *N. punctiforme*. There is experimental evidence for plant production of hormogonium-inducing factors, chemoattractants and hormogonium repressing factors in establishment of an association. Plant factors have yet to be detected that influence the distinct *N. punctiforme* symbiotic physiological state of slow growth, heterotrophic metabolism and low ammonium assimilation, and the morphological state of enlarged vegetative cells with weak cell-cell connections (aseriate) and high heterocyst frequencies.

There is evidence for production of a hormogonium-inducing factor (HIF) by *A. punctatus* (Campbell and Meeks, 1989), the cycad *Zamia furfuracea* (Ow et al., 1999) and *G. manicata* (Rasmussen et al., 1994). Based on dialysis experiments in the above systems, HIF appears to be a small molecule with a molecular mass of between 0.5 and 12 kDa, but its identity has yet to be determined. A significant problem in purification of HIF is caused by the induction of hormogonium differentiation (the bioassay) by solvents and materials used in fractionation steps. The results in Table 1 support the extracellular production of a HIF by *A. punctatus* (Campbell and Meeks, 1989). Medium conditioned by incubation of *A. punctatus* in the absence of combined nitrogen consistently induced a high frequency of hormogonium differentiation, whereas fresh medium was ineffective. The key experiments that established a specific process, however, were: (i) conditioning of the medium by *A. punctatus* in the presence of ammonium, which did not markedly induce hormogonium formation; (ii) while adding ammonium to medium conditioned in

its absence yielded the same high frequency of hormogonia as medium conditioned in its absence with no subsequent added ammonium. Moreover, *A. punctatus* conditioned medium induced hormogonium differentiation in the dark and at low pH, conditions that retard differentiation in the free-living state (Campbell and Meeks, 1989). While these results provide indirect evidence for a HIF, its purification and identification are clearly a priority.

Infection – evidence for chemoattractants

Differentiation of hormogonia is essential, but not singularly sufficient, for infection of the symbiotic cavities. For example, *Nostoc* sp. strain ATCC 27896 converted nearly 100% of its vegetative filaments into hormogonia in response to HIF (Campbell and Meeks, 1989), but only rarely did the hormogonia infect *A. punctatus* (Meeks, 1998). There was no selection in the case of rare infections for a spontaneous mutant capable of significant infection. Rather, random infection apparently can occur, but *Nostoc* sp. strain ATCC 27896 appears to lack genetic information for a specific response, such as taxis to chemoattractants. Knight and Adams (1996) clearly established, using capillary assays, that the bryophyte liverwort, *Blasia pusilla*, produced into growth conditioned medium, substances that attract hormogonia of symbiotically competent *Nostoc* sp. Identical experiments have not yet been conducted with other plant partners. Based on the above evidence, and because an attraction mechanism would be required for efficient colonization by the low abundance of *Nostoc* spp. in soils (Meeks and Elhai, 2002), a chemotactic response is included in the model in Fig. 1.

The *N. punctiforme* genome has multiple copies of genes encoding putative chemotaxis proteins (Meeks et al., 2001). Mechanisms for regulation of their synthesis and activity are unknown. However, mutation of the global transcriptional regulator, NtcA (Herrero et al., 2001), resulted in a *N. punctiforme* strain that was less responsive to HIF in differentiation of hormogonia, and the hormogonia that were formed failed to infect *A. punctatus* (Table 2) (Wong and Meeks, 2002). Conversely, a *N. punctiforme* mutant with an insertion in gene (*sigH*) encoding an alternative sigma subunit of RNA polymerase, formed HIF-dependent hormogonia at a similar frequency as the wild type, but there was a 3 to 5 fold increase in the frequency of infection of *A. punctatus* (Table 2) (Campbell et al., 1998). The hormogonium related genes that are transcriptionally regulated by NtcA and SigH are unknown. These results are consistent with genetic control over the behavior of hormogonia; therefore, the behavior is amenable to genetic analysis

Colonization – evidence for repression of hormogonium differentiation

The results in Table 1 demonstrate that the reconstituted *Nostoc* sp.-*A. punctatus* tissue produced HIF during steady state culture with N_2 as the

Table 1. Evidence for a hormogonium inducing activity produced into conditioned medium by *A. punctatus* (derived from Campbell and Meeks, 1989)

Incubation medium[a]	Addition at time 0	% Hormogonia at 24 h[b]
Fresh	None	ca. 10
Conditioned –N	None	ca. 90
Conditioned +N	None	ca. 10
Conditioned –N	2.5 mM NH_4^+	ca. 90
Conditioned, symbiotic	None	ca. 90

[a]Fresh refers to *A. punctatus* growth medium without or with combined nitrogen to which the *Nostoc* sp. was added directly. Conditioned refers to the same medium incubated with *A. punctatus* for 2 d in the absence (–N) or presence (+N) of combined nitrogen, or incubated with reconstituted *Nostoc* sp.-*A. punctatus* (symbiotic) in the absence of combined nitrogen, each of to which the *Nostoc* sp. was added after removal of the *A. punctatus* tissue. [b]The % of hormogonium filaments in the population was determined microscopically 24 h after exposure to the incubation medium.

Table 2. Phenotypes of selected *N. punctiforme* mutants altered in symbiotic infection or function

Strain	Infection frequency[a]	Acetylene reduction activity[b]	Gene induced by[c]	Stage affected
ATCC 29133 (WT)[d]	0.21±0.04	6.3±1.2	–	–
UCD 398 (*sigH*)[d]	1.2±0.2	8.0±3.9	HIF	Infection
UCD 328 (*hrmA*)[e]	1.6±0.1	6.1±1.0	HRF	Infection
UCD 444 (*ntcA*)[f]	0	0	–	Infection
UCD 416 (*hetF*)[f]	0.26±0.06	0	–	Function
UCD 464 (*hetR*)[f]	0.36±0.04	0	–	Function

[a]Number of symbiotic *Nostoc* colonies per mg dry wt of *A. punctatus* tissue per unit of *N. punctiforme* cells added, scored after 2 weeks of co-culture. [b]Acetylene reduction activity is nmol ethylene formed per min per g fresh wt of *A. punctatus* tissue. [c]HIF is hormogonium-inducing factor; HRF is hormogonium-repressing factor. [d]Campbell et al., 1998. [e]Cohen and Meeks, 1997. [f]Wong and Meeks, 2002.

nitrogen source. This result presents a dilemma; if hormogonium filaments immediately reenter the hormogonium cycle, the individual cells will get successively smaller because a period of vegetative growth is necessary to

regain the biomass and chromosome copy number of the normal vegetative cells. Continued reentry into the cycle is, therefore, lethal by extinction. What then stops continued hormogonium differentiation once the filaments have colonized the symbiotic cavity? An autogenic hormogonium repressing activity has been observed in cultures of *Nostoc* species (Herdman and Rippka, 1988). If expressed at the appropriate time, such an activity could provide an "immunity" period for recovery from an initial round of hormogonium differentiation. A similar activity has been identified in *N. punctiforme*. A transposon-induced mutant was isolated that appeared to repeatedly produce hormogonia during co-culture with *A. punctatus*, resulting in a high frequency of infection, possibly by extending the time-dependent infection window (Table 2) (Cohen and Meeks, 1997). In contrast to the wild type, the mutant filaments were rapidly cleared from the medium of the co-culture, consistent with an extinction event. The transposon had inserted into a gene (*hrmA*) that is clustered in a locus (*hrm* locus) with the following structure: 3'-*hrmE*-5'*orfU1*-3'-*orfU2*-5'-3'-*hrmK*-5'-*hrmR-hrmI-hrmU-hrmA*-3'. The gene organization and gene products have similarity to those involved in hexuronic acid metabolism in heterotrophic bacteria (Campbell et al., 2003); HrmE has similarity to an aldehyde reductase, HrmK to gluconate kinase, HrmR to a glucuronate operon transcriptional repressor in the sugar-binding LacI/GalR family, Hrm I to glucuronate isomerase, and HrmU to mannonate oxidoreductase. HrmA has no significant sequence similarity and the open reading frames (*orf*) *orfU1* and *orfU2* encode hypothetical proteins of unknown function in hormogonium differentiation or any other process.

In vivo expression assays established that *hrmA* was poorly, if at all, transcribed under vegetative or hormogonium-inducting conditions, but was induced by an aqueous extract of *A. punctatus* (Cohen and Meeks, 1997). The aqueous extract was termed a hormogonium-repressing factor (HRF) as its presence repressed hormogonium induction in wild type cells, even in the presence of HIF. In contrast, the *hrmA* mutant continued to differentiate hormogonia in the presence of both HRF and HIF. Thus, the HRF exerted its effect, in part, through *hrmA*. Subsequently, the plant flavenoid, naringin, was shown to be a specific extracellular inducer of *hrmA* transcription (Cohen and Yamasaki, 2000). Recent studies have established that HrmR is, indeed, a sugar binding (galacturonate) transcriptional repressor (Campbell at al., 2003). However, the HrmR targets are itself and *hrmE*, and not *hrmK*, *hrmI*, *hrmU* or *hrmA*, as would be expected based on regulon models of the homologous genes in heterotrophic bacteria. Whereas HRF-induced cell extracts of *N. punctiforme* prevented *in vitro* binding of HrmR to its operator regions in the *hrmE* and *hrmR* promoters, naringin-induced and uninduced cell extracts did not (Campbell et al., 2003). Thus, HRF contains more than one factor that influences what is apparently complex transcription in the *hrm* locus.

What is the role of the gene products of the *hrm* locus? Galacturonate, glucuronate and gluconate did not support dark heterotrophic growth of *N. punctiforme*, the low level of gene transcription in the hrm locus is not consistent with a catabolic function, and the presence of hexuronates did not increase or decrease the infection frequency of *A. punctatus* by wild type or mutant strains of *N. punctiforme* (Campbell et al., 2003). We hypothesize that the gene products are part of a complex metabolic pathway that synthesizes a metabolite inhibitor of hormogonium differentiation. The substrates and end products need not be the same as those predicted by the sequence similarities. Hormogonium and heterocyst differentiation are mutually exclusive processes (Meeks et al., 2002). The hypothetical purpose of the inhibitor is to prevent continued differentiation of hormogonia once these filaments have colonized a symbiotic cavity, thereby allowing the differentiation of heterocysts and subsequent expression of nitrogen fixation activity (Cohen and Meeks, 1997).

Development of a functional nitrogen-fixing association

The functional symbiotic state of a *Nostoc* species in association with *A. punctatus*, relative to the free-living growth state, is characterized by a decrease in the rates of growth, photosynthesis and ammonium assimilation, coupled with an increase in heterocyst frequency and in the rate of nitrogen fixation (Meeks, 1998). This functional stage can be arbitrarily divided into the two substages of growth and metabolic regulation, and regulation of heterocyst differentiation, leading to nitrogen fixation and the provision of a nitrogen source for growth of the plant partner.

Growth control and evidence for metabolic regulation

In submerged liquid medium, *A. punctatus* can double approximately every 5 d when supplemented with ammonium nitrate and every 10 d under nitrogen-fixing, symbiotic conditions (Enderlin and Meeks, 1983). While a *Nostoc* species in laboratory culture can double within 24 to 48 h while fixing nitrogen, the growth rate of an associated *Nostoc* species is proportional to that of *A. punctatus* under symbiotic conditions (Enderlin and Meeks, 1983). The symbiotic growth rate of *Nostoc* spp. is, thus, 5 to 10-fold slower than that of free-living laboratory cultures under optimal conditions (Table 3).

In a growth context, the dimensional size of the *Nostoc* sp. colony in *A. punctatus* tissue can vary depending on the experimental conditions. Under steady state laboratory conditions, the colonies mature to an approximate 1 mm in diameter (Meeks, 1998) with an average of 0.55 μg protein per colony and a constant rate of nitrogen fixation per unit biomass of *A. punctatus* tissue (Steinberg and Meeks, 1991). Treatment of the co-culture with penicillin prevents the new *Nostoc* sp. infections at the growing tissue margin that sustain

Table 3. Growth, inorganic carbon and nitrogen metabolic, and enzymological characteristics of *Nostoc* sp. in free-living and symbiotic culture with *A. punctatus* (derived from Meeks, 1998)

Metabolic system/enzyme[a]	Free-living growth		Symbiotic growth	
	Activity	Protein	Activity	Protein
Growth	24–48 h		~240 h	
Photosynthetic CO_2 fixation	128.0		15	
Rubisco	215.0	52	25	43
Ammonium assimilation	13.9		2.9	
GS	130.0	7.1	50.0	6.1
Nitrogenase	6.3		23.5	

[a]Growth rate is doubling time in hours. Photosynthetic CO_2 fixation is light-dependent incorporation of $^{14}CO_2$ into whole cells; reported as nmol/min/mg protein. Rubisco is ribulose bisphosphate carboxylase/oxygenase; *in vitro* activity reported as nmol/min/mg cell protein and protein as µg/mg total cell protein. Ammonium assimilation is light-dependent incorporation of $^{13}NH_4^+$ into the total amino acid pool; reported as % ^{13}N cpm incorporated/$^{13}NH_4^+$ cpm added/5 min/mg protein. GS is glutamine synthetase; *in vitro* activity is reported as the biosynthetic reaction as nmol/min/mg cell protein and protein as µg/mg total cell protein. Nitrogenase activity is acetylene reduction to ethylene; reported as nmol/min/mg cell protein.

the N_2-dependent steady state growth. To maintain a constant rate of nitrogen fixation per unit of *A. punctatus* tissue, the system responded to penicillin treatment by a progressive increase in the size of the existing colonies and an increase in the rate of nitrogen fixation per colony (Enderlin and Meeks, 1983). Conversely, culture of the *Nostoc* sp.-*A. punctatus* association in high light (ca. one-half sunlight) and enriched CO_2 resulted in a decrease in both the number of *Nostoc* sp. colonies per unit of plant tissue and the size of each colony (Steinberg and Meeks, 1991). A rate of nitrogen fixation per unit of plant tissue that is comparable to low light grown tissue was maintained in high light, but the rate per colony doubled (Steinberg and Meeks, 1991). Even mutant strains of *N. punctiforme* with high infection frequencies, express rates of nitrogen fixation per unit of *A. punctatus* biomass that are similar to the wild type (Table 2). These collective results indicated that *A. punctatus* has the capacity to positively and negatively regulate the rate of growth and nitrogen fixation of associated *Nostoc* sp., while maintaining a constant rate of nitrogen fixation per unit of plant tissue; the regulatory mechanisms remain undefined.

The rates of inorganic carbon and nitrogen assimilation by intact *Nostoc* sp. cells associated with *A. punctatus*, are depressed in proportion to the lower growth rate (Table 3). Decreased rates of photosynthesis and nitrogen assimilation are characteristic of the symbiotic *Nostoc* spp. growth state in other plant associations (Meeks, 1998; Adams, 2000; Rai et al., 2000). However, the mechanisms for regulation of the *Nostoc* sp. activities appear to vary in the different associations. In the *A. punctatus* association, one mechanism for regulation of CO_2 and NH_4^+ assimilation is by irreversible inactivation of the catalytic activity of the primary assimilatory enzymes, ribulose bisphosphate carboxylase/oygenase (rubisco) (Steinberg and Meeks, 1989) and glutamine synthetase (GS) (Joseph and Meeks, 1987), respectively (Table 3). In contrast, *Nostoc* spp. in association with *Gunnera magellanica* (Söderbäck and Bergman, 1993) and *Cycas revoluta* (Lindblad et al., 1987) have low rates of photosynthetic CO_2 fixation, but high rates of *in vitro* rubisco activity. Therefore, the mechanism(s) of photosynthetic control in these systems differs from the *A. punctatus* association. Similarly, *Nostoc* spp. GS activity and protein levels in the *C. revoluta* (Lindblad and Bergman, 1986) and *G. magellanica* (Bergman et al., 1992) associations are near to those of free-living cultures. *Nostoc* spp. associated with different cycads appear to make citrulline or glutamine available to the plant partner (Pate et al., 1988), so high GS-dependent ammonium assimilating activity is consistent with the nitrogenous end product. *Nostoc* spp. in association with *A. punctatus* (Meeks et al., 1985) and *G. magellanica* (Silvester et al., 1996), release 80 to 90% of their N_2-derived NH_4^+ for growth of the plant partner. While NH_4^+ release is not consistent with *Nostoc* spp. GS activity in the *Gunnera* spp. symbioses, it appears to be so in the *A. punctatus* association. Nevertheless, even in the *A. punctatus* symbiosis, there is sufficient *Nostoc* sp. GS activity to assimilate all of its N_2-derived NH_4^+ (Meeks and Elhai, 2002). The situation is, therefore, more complicated that it superficially appears. There is insufficient information to propose a universal working model of metabolic regulation of *Nostoc* species in plant symbioses or to suggest any causal relationship between the lower rates of inorganic carbon and nitrogen assimilation, and of growth.

Nitrogenase requires substantial ATP and reductant for activity (Christiansen et al., 2001). Based on the low photosynthetic activities of symbiotically associated *Nostoc* spp., it has been hypothesized that the *Nostoc* spp. assume a largely heterotrophic metabolic mode to support the high rate of nitrogen fixation. This hypothesis was verified *in planta* by using *Nostoc* sp. mutants resistant to a photosynthetic inhibitor to distinguish *Nostoc* sp. photosynthetic versus plant-derived heterotrophic reductant supply for nitrogen fixation (acetylene reduction) in the *A. punctatus* association (Steinberg and Meeks, 1991). *Nostoc* sp. specific photosynthetic activity supported about 30% of the maximum rate of symbiotic nitrogenase activity in

short-term experiments, but the maximum steady state rate was dependent on exogenous sucrose, fructose or glucose, metabolized by *Nostoc* sp. through photoheterotrophic or dark heterotrophic reactions, with the reduced carbon supplied by the plant partner. These results imply that when two organisms existing on the same photosynthetic trophic level establish a symbiosis, one partner differs its photoautotrophic potential; in all *Nostoc*-plant associations, it is apparently the *Nostoc* spp. that differ and assume an energetically dependent heterotrophic mode.

Enhanced symbiotic heterocyst frequency – evidence for nitrogen-independent initiation of differentiation

Physiologically, heterocysts are characterized as sinks for reduced carbon and sources of fixed nitrogen in a multicellular organism (Meeks and Elhai, 2002). Heterocysts of free-living cultures are typically found at a frequency of 3–10% of the cells in a filament, each at a single site and in a nonrandom spacing pattern. The spacing pattern can be considered in the context of four separate phenomena: (i) absence of heterocysts and, therefore, of any pattern when grown in the presence of a combined nitrogen source, such as ammonium or nitrate; (ii) establishment of a nonrandom spacing pattern by induction of heterocyst differentiation upon depletion of the combined nitrogen; (iii) maintenance of the spacing pattern during steady state growth with N_2 as the nitrogen source; and (iv) disruption of the pattern through enhanced heterocyst differentiation during symbiotic growth. The frequency of heterocysts of *Nostoc* spp. in symbiotic association with a photosynthetic eukaryotic partner range from 25 to more than 60% of the cells (Meeks, 1998; Adams, 2000; Rai et al., 2000). The mechanistic questions to ask are: what is the environmental signal that leads to the enhanced symbiotic heterocyst frequency, and does the spacing pattern reflect alterations in the establishment or maintenance of pattern?

The signal for initiation of heterocyst differentiation in free-living cultures is clearly deprivation of combined nitrogen (Meeks and Elhai, 2002). Several lines of evidence imply that nitrogen limitation is not the primary signal in the symbiotic growth state. First, in the *A. punctatus* (Meeks et al., 1985) and *G. magellanica* (Silvester et al., 1996) associations the symbiotic *Nostoc* spp. release 80 to 90% of their N_2-derived NH_4^+. Based on published rates of N_2 fixation (Steinberg and Meeks, 1991), NH_4^+ assimilation (Meeks et al., 1985) and *Nostoc* sp. symbiotic colony dimensions (converted to volume) in the *A. punctatus* association, the steady state NH_4^+ concentration is calculated to be at least 550 μM (J. Meeks, unpublished). In free-living cyanobacteria, 6 to 15 μM NH_4^+ is sufficient for 99% repression of heterocyst differentiation (Meeks et al., 1983). Second, the symbiotic *Nostoc* spp. vegetative cells are replete in nitrogen storage compounds such as cyanophycin, phycobiliproteins and

carboxysomes (Bergman et al., 1992; Meeks, 1998; Adams, 2000). These structures are catabolized to varying degrees in nitrogen starved cells; therefore, symbiotically associated *Nostoc* spp. vegetative cells show no cytological signs of nitrogen limitation, while some continue to differentiate into heterocysts. Third, although high concentrations of exogenous combined nitrogen repress heterocyst differentiation and nitrogen fixation by *Nostoc* sp. in the *A. punctatus* association (Enderlin and Meeks, 1983), the signal seems to be processed through the plant rather than directly by the *Nostoc* sp. This conclusion is based on the response of a *N. punctiforme* mutant defective in assimilation of nitrate (Campbell and Meeks, 1992). In free-living culture, the mutant failed to grow with nitrate, and nitrate failed to repress heterocyst differentiation and nitrogen fixation. However, when the wild type and mutant were separately reconstituted into the *A. punctatus* association, nitrate repressed nitrogen fixation with similar kinetics in both associations. Control experiments and assays eliminated genetic reversion and NH_4^+ accumulation as explanations for the similar mutant and wild type response, leading to the conclusion of plant mediated repression. We, therefore, hypothesize that a plant produced signal(s) initiates heterocyst differentiation in the symbiotic growth state, independent of the nitrogen status of the associated *Nostoc* spp.

What are the genetic targets of a plant produced signal? The global nitrogen regulator, NtcA, is the first gene/gene product known to be activated when cells sense nitrogen limitation (Herrero et al., 2001). NtcA activates transcription of *hetR*, the initial heterocyst specific positive regulatory gene/gene product known to date. Transcription of *hetR* occurs in specific cell clusters (Buikema and Haselkorn, 2001) and HetR accumulates in heterocysts (Wong and Meeks, 2001). HetR accumulation is complex as it depends on its autodegradation (Zhou et al., 1998), activity of an ancillary protein, HetF, (Wong and Meeks, 2001) and its interactions with PatS (Yoon and Golden, 1998), a primary inhibitor of differentiation synthesized in the differentiating cells. Mutant analyses have shown that functional HetR and HetF are required for symbiotic heterocyst differentiation, indicating a common symbiotic and free-living developmental cascade downstream of HetR (Wong and Meeks, 2002). Because NtcA is required for transcription of genes expressed late in heterocyst development (Herrero et al., 2001), we hypothesize that a nitrogen-independent plant signal would enter the symbiotic heterocyst developmental cascade at or before activation of NtcA (Wong and Meeks, 2002).

Even if independent of combined nitrogen, the mechanisms for initiation of differentiation to establish the pattern in free-living cultures may not determine the spacing pattern of heterocysts in symbiosis. Only with the cyanobacterium associated with the water fern *Azolla* spp. can heterocysts be distinctly identified and a symbiotic spacing pattern be seen by light microscopy (Peters and Mayne, 1974). In all other associations, the *Nostoc* spp.

vegetative cells are large and distorted, difficult to distinguish from heterocysts and with little evidence of a filament organization (aseriate in Fig. 1). In the *Azolla* association, the maximal heterocyst frequency in leaf cavities is approximately 26% and the heterocysts are found singly in the filaments with a 3.0 vegetative cell interval (Meeks and Elhai, 2002). A similar frequency and vegetative cell interval was derived from electron micrograph collages of *Nostoc* sp. in association with *A. punctatus* (F. Wong, cited in Meeks and Elhai, 2002). However, in the *Azolla*, cycad and *Gunnera* associations the analyses are complicated by an observable gradient of heterocyst frequencies from the growing stem or root tip to the more distal regions (Rai et al., 2000; Meeks and Elhai, 2002). In the tip regions, heterocysts range from 0 (*Azolla*) to 15% (cycads) of the total cells and are present at single sites nonrandomly spaced along the filaments. Heterocyst frequencies and rates of nitrogenase activity increase as a function of distance from the root or stem tip; maximal nitrogenase activity correlates with heterocyst frequencies between 25 and 40%. In the cycad (Lindblad et al., 1985) and Gunnera (Söderbäck et al., 1990) associations, clusters of 2 or more heterocysts at a site in the filament are apparent in the more distal regions and correlate with declining rates of nitrogenase activity. A similar gradient may occur in the *A. punctatus* association, but there have been no specific studies.

We interpret the presence of a symbiotic developmental gradient from a low to a high frequency of singlet heterocysts, and then to a high frequency of heterocysts in clusters to indicate that heterocysts accumulate in the *Nostoc* filaments as a function of time. If so, this process is not the same as the initiation of heterocyst differentiation and establishment of pattern when free-living cultures are deprived of a source of combined nitrogen. Rather, the symbiotic pattern could be more of a consequence of an alteration in the mechanisms that maintain the spacing pattern during steady state growth on N_2 in the free-living state. The vast majority of studies in free-living cultures focus on initiation of heterocyst differentiation and establishment of the pattern. There is no information as to whether the mechanisms involved in establishment of the pattern are the same as those involved in maintenance of the pattern, or whether symbiotic disruption of pattern by decreasing the vegetative cell interval between heterocysts involves mechanisms associated with either phenomena.

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