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Physiological Adaptations in Nitrogen-fixing *Nostoc*–Plant Symbiotic Associations

John C. Meeks

Section of Microbiology, University of California, Davis, CA 95616, USA
jcmeeks@ucdavis.edu

Abstract *Nostoc* species establish nitrogen-fixing symbiotic associations with representatives of the four main lineages of terrestrial plants: bryophyte hornworts and liverworts, the pteridophyte fern *Azolla*, gymnosperm cycads, and the angiosperm genus *Gunnera*. However, the plant partners represent only narrowly selected groups within these lineages. The plant partner benefits by the acquisition of fixed nitrogen, but the benefits to the *Nostoc* partner are unclear. Thus, the associations are considered a commensal form of symbiosis. A working hypothesis of this chapter is that these associations evolved as the lineage of the plant partner emerged. Inherent in this hypothesis is that the plant partners may have evolved different regulatory signals and targets in control of the *Nostoc* partner. The physiological interactions between the two partners can be modeled as a two-step process. First is the establishment of an association and involves the differentiation and behavior of motile hormogonium filaments, the infective units. Hormogonium formation is essential, but not singularly sufficient for establishment of an association. Second is the development of a functional nitrogen-fixing association involving the differentiation and behavior of heterocysts, the functional units. Heterocysts are the cellular sites of nitrogen fixation, protecting nitrogenase from inactivation by oxygen. The symbiotic growth state of *Nostoc* spp. is characterized by a reduced rate of growth, depressed carbon dioxide and ammonium assimilation, transition to a heterotrophic metabolic

mode, an elevated heterocyst frequency, and an enhanced rate of nitrogen fixation. In all but one association (cycads), dinitrogen-derived ammonium is made available to the plant partner. Physiological measurements indicate that different reactions of *Nostoc* photosynthetic carbon dioxide and ammonium assimilation are modulated by the various plant partners. These results appear to support the working hypothesis and indicate that different mechanisms may be operational, allowing for manipulation of different strategies in engineering new plant partners for symbiotic nitrogen fixation.

1 Introduction

Some filamentous cyanobacterial species or strains of the genus *Nostoc*, order Nostocales, have the unusual property of fixing nitrogen in free-living and plant-associated symbiotic growth states (Meeks 1998). The terrestrial symbiotic plant partners include the spore-producing non-vascular bryophytes, the vascularized spore-producing pteridophyte aquatic fern family Azollaceae, the seed producing gymnosperm order Cycadales, and the angiosperm family Gunneraceae (Adams 2000; Bergman et al. 1996; Meeks 1998; Rai et al. 2000). *Nostoc* species also establish lichenized (Paulsrud et al. 1998) and non-lichenized (Mollenhauer et al. 1996) associations with fungi, but these will not be considered here. A question to be asked is, are there physiological adaptations of the *Nostoc* species that may reflect on the mechanisms of symbiotic interaction with the diverse plant partners?

Cyanobacteria are characterized by the phenotypic traits of oxygen-evolving photosynthesis and a prokaryotic cell structure, which present a dilemma with regard to nitrogen fixation. The nitrogenase enzyme complex is highly sensitive to inactivation by oxygen; as a consequence, cyanobacteria have evolved two strategies to protect nitrogenase from excess oxygen. The behavioral strategy is temporal segregation of photosynthesis to the daylight hours and fixation of nitrogen during the night when oxygen is no longer produced and aerobic respiration can lower the intracellular oxygen tension (Fay 1992; Gallon 1992). This strategy is practiced by both unicellular and non-differentiating filamentous cyanobacteria. Nitrogen-fixing associations with these cyanobacteria have not been documented.

The morphological strategy is to confine oxygenic photosynthesis and nitrogen fixation into separate cells as compartments, such that the two processes can occur simultaneously in the filaments, leading to greater growth efficiency. Under conditions of combined nitrogen limitation, vegetative cells differentiate into microoxic cells, heterocysts, which are specialized for nitrogen fixation. Heterocyst differentiation is terminal and occurs only in, and in part defines, two filamentous cyanobacteria orders: the Nostocales and the Stigonematales (Castenholz 2001). In the free-living growth state, heterocysts appear singly in a non-random spacing pattern in the filament and constitute 5–10% of the cells. The microoxic state of heterocysts is achieved by a cessa-

tion of the oxygen-evolving reaction of photosynthesis, the deposition of a gas and solute impermeable extra wall layer of polysaccharide and glycolipid, and an increased rate of aerobic respiration to consume any oxygen that may enter at the cell pole connections to adjacent vegetative cells (Wolk et al. 1994). The loss of complete photosynthesis dictates a shift from an autotrophic to a heterotrophic metabolic mode in heterocysts. Heterocysts and vegetative cells, therefore, establish a reciprocal source-sink relationship; vegetative cells provide heterocysts with reductant in the form of carbohydrate and heterocysts provide vegetative cells with reduced nitrogen in the form of glutamine (Wolk et al. 1994). This metabolic relationship is characteristic of a multicellular organism, of which the heterocyst-forming cyanobacteria clearly represent. The differentiation of heterocysts and the cellular protection of nitrogenase from oxygen inactivation may be a basis of the recruitment of *Nostoc* species into symbiotic associations with plants, in which the plants do not need to provide an additional mechanism for oxygen protection. Three aspects of the heterocyst spacing pattern, which are relevant also to symbiotic growth, are typically considered: establishment of pattern, maintenance of pattern, and disruption of pattern (Meeks and Elhai 2002).

The cyanobacteria are an ancient and phylogenetically cohesive group of prokaryotes dating back to at least 2500, and perhaps 3500, million years ago (Ma) (Schopf 2000). However, ancestors to the extant Nostocales are thought to be amongst the latest to have emerged in the cyanobacterial radiation, with the heterocyst-formers estimated at around 1500 Ma (Tomitani et al. 2006). Based on the fossil record, the terrestrial plants emerged sequentially starting with the bryophytes at 480–510 Ma, pteridophytes at 420 Ma, but with *Azolla* fossils appearing around 120 Ma, Cycadales at 250 Ma and Gunneraceae about 70–90 Ma (Raven 2002). A fundamental question is whether the cyanobacterial symbiotic associations evolved near to the times when the different plant lineages emerged, or did the associations form within a similar period of time after emergence of the Gunneraceae? There is no fossil specimen depicting any cyanobacterial symbiont within a plant partner. The significance of the question is rooted in the need to understand mechanisms of interactions between the partners in any attempts to exploit cyanobacterial symbiotic nitrogen fixation in agricultural applications. If the associations developed sequentially, different mechanisms could have evolved in stabilization of each association in the establishment of a nitrogen-fixing factory for the plant partner. Different mechanism would present alternative strategies in engineering plant partners for symbiotic association. Two approaches to initial insight into the mechanisms of symbiotic interaction are to define the specificity of partners in various associations and to determine the physiological characteristics of the partners during establishment of the associations. Essentially all of the experimental effort in the latter approach has been directed toward the *Nostoc* partner, with very little information accruing about the plant partners.

2
Specificity of the Associations

Specificity between partners has implications on the variety of mechanisms that may need to be involved in stabilization of the symbiotic interaction. Broadly specific associations are considered to be flexible and, perhaps, less highly evolved.

2.1
Plant Partners

Although the plant partners in cyanobacterial associations span the phylogenetic spectrum of terrestrial plants, they are representative of only a narrow range of partners within each group (Adams 2000; Bergman et al. 2002; Meeks 1998; Rai et al. 2000; see also Rai et al. 2002). Four out of six of the extant bryophyte hornwort genera have a cyanobacterial partner, but only two liverworts form endophytic associations, and moss associations are rare and of unresolved nutritional benefit. The pteridophyte fern associations are confined to the single genus, *Azolla*, in the family Azollaceae. All examined cycads have a symbiotic *Nostoc* partner, but cyanobacterial associations are lacking in all other gymnosperms. A similar distribution holds for the angiosperms, with the Gunneraceae being the only representative. Except for the cycads, the other plant partners share a common ecological distribution of growth in high water content habitats. Cycads have a dry and a rain forest, as well as a grassland, distribution (Costa and Lindblad 2002). Excepting *Azolla*, which can form extensive and uncontrollable surface blooms in eutrophic lakes and rivers (Carrapico et al. 1996), none of the associations represent the dominant vegetation in any ecosystem.

In all associations, the cyanobacterial partner is confined to a specific location in the plant tissue, commonly referred to as a symbiotic cavity or gland. The cavity is formed in all plants irrespective of the presence of a compatible cyanobacterium. In hornworts and liverworts, the cyanobacteria are in a slime-filled cavity that opens on the ventral surface of the gametophyte thallus. The cyanobacteria are present in a cavity in the dorsal leaves of floating *Azolla*. Consistent with increasing structural complexity, the cyanobacteria are found in specialized secondary roots in cycads, called coralloid roots, and are confined to a zone between the inner and outer cortex. Only in the Gunneraceae are the cyanobacteria intracellular, within special mucus secreting stem gland cells at the base of each petiole.

Thus, there is a clear degree of plant specificity, the genetic basis of which is unknown. The most common ecological parameter is a moist habitat, but even that is not exclusive. The most common physiological feature is production of mucus or slime by the plant partner. One gets the impression of an evolutionary experiment that provided a limited competitive advan-

tage for the plant partner, but that did not lead to a dominant role in any ecosystem.

2.2 Cyanobacterial Partners

In the most part, cyanobacterial partners have been identified based on the morphological characteristics of free-living representatives following isolation and culture. The almost exclusive isolate from all associations is one or more species or strains of *Nostoc*. Occasionally, related *Calothrix* sp. (Nostocales) and *Chlorogloeopsis* sp. (Stigonematales) have been cultured from hornwort and cycad associations (Adams 2000). One limitation here, of course, is the requirement for growth under laboratory conditions. The primary symbiotic cyanobacterium in *Azolla* appears to be recalcitrant to culture in the laboratory (Tang et al. 1990). Cyanobacteria, identified as either *Nostoc* or *Anabaena*, have been cultured from *Azolla* associations, but these are thought to be secondary symbionts that contribute little or no nutritional advantage to the plant partner (Peters and Meeks 1989). A major phenotypic difference between the genera *Nostoc* and *Anabaena* in culture is that some *Anabaena* vegetative filaments are motile by gliding, while all *Nostoc* vegetative filaments are sessile. *Nostoc* spp. gain gliding motility by the differentiation of vegetative filaments into morphologically distinct, heterocyst-free, filaments called hormogonia (Rippka et al. 1979). Based on morphological characteristics, the primary uncultured symbiont of *Azolla* has historically been assigned to the genus *Anabaena*, although hormogonium-like filaments have been observed near the plant apical meristem (Peters and Meeks 1989).

Speciation in cyanobacteria is highly problematic (Castenholz 2001). Many of the morphological features used in classical taxonomy of field samples, such a cell length and width, extent of sheath and/or slime production, pigmentation, and cellular differentiation are very plastic, depending on growth conditions and unknown environmental signals. Thus, specificity has been tested experimentally by cross-infection studies. The associations are readily reconstituted in the laboratory with the bryophyte (Enderlin and Meeks 1983; Adams 2000) and *Gunnera* (Johansson and Bergman 1994) partners, but less so with cycads (Ow et al. 1999). Reconstitution experiments indicate broad specificity of *Nostoc* isolates from all associations, including lichens, with bryophyte (Enderlin and Meeks 1983; West and Adams 1997) or *Gunnera* (Johansson and Bergman 1994) partners. This is particularly true of an isolate from the cycad *Macrozamia* sp., identified as *Nostoc punctiforme* strain PCC 73102 (synonym strain ATCC 29133) and defined as the type strain of *Nostoc* Cluster 1 (Rippka and Herdman 1992). *N. punctiforme* strain PCC 73102 reconstitutes associations with the hornwort *Anthoceros punctatus* (Enderlin and Meeks 1983; Meeks 2003) and *Gunnera* spp. (Johansson and Bergman 1994). *N. punctiforme* is amenable to genetic manipulation (Cohen

et al. 1994; Summers et al. 1995) and the genome of strain ATCC 29133 has been completely sequenced (Meeks 2005b; Meeks et al. 2001). *N. punctiforme* has emerged as a model organism for studies of symbiotic interaction (Meeks 2003) and *Nostoc* cellular developmental alternatives (Meeks et al. 2002).

More definitive studies to identify the cyanobacterial symbionts in plant associations have utilized molecular genetic approaches of restriction fragment length polymorphism and sequence analysis of conserved genes and/or intergenic regions (Costa et al. 2001; Rasmussen and Svenning 2001; West and Adams 1997). Due to limitations in size of the database and speciation problems in general, these studies do not lead to absolute identities, but do allow for estimates of diversity. The results of such studies indicate considerable diversity in the *Nostoc* symbionts of bryophytes, both geographically and in a single gametophyte thallus, in cycad coralloid roots from both greenhouse and natural field samples, and naturally grown *Gunnera* species (Rasmussen and Nilsson 2002).

While it appears that differentiation of hormogonia is necessary for symbiotic interaction, it is not singularly sufficient. Many hormogonia-forming *Nostoc* strains are not competent to establish a symbiotic association in the laboratory (Enderlin and Meeks 1983; Johansson and Bergman 1994). Other factors, such as chemotactic attraction (see below) may be required. An unresolved question is why other hormogonia-forming genera are not more commonly found in symbiotic association. Perhaps only strains in the genus *Nostoc* have the genetic capacity to establish associations with plants, and the other documented genera of symbiotic isolates were either only casually associated or infected by chance and did not contribute to the function of an association.

3 Physiological Adaptation in the Associations

The symbiotic *Nostoc*–plant associations have been characterized as a largely (but not exclusive) unidirectional flow of signals from plant to cyanobacterium (Meeks 1998). This characterization is based on the extensive physiological and morphological changes that occur in the symbiotic growth state of *Nostoc*, compared to the relatively minor changes that appear in the plant partner. Moreover, the selective advantage of *Nostoc* spp. in symbiotic association is not obvious. Thus, we have characterized these associations as a commensal form of symbiosis, where the plant partner clearly benefits by the provision of fixed nitrogen and the *Nostoc* partner neither benefits nor is harmed (Meeks 2005a; Meeks and Elhai 2002).

The symbiotic competence of a *Nostoc* strain is distinguished by responses to plant control over two developmental states, those of hormogonia and heterocysts, as well as over growth and metabolism. The interactions mostly likely

occur as a continuum, but they can be modeled as a two-stage process. The first stage is infection involving the differentiation and behavior of hormogonia, which serve as the infective units. The second stage is development of a functional association and involves growth, and the differentiation and behavior of heterocysts.

3.1 Establishment of a Symbiotic Association

The establishment of a symbiotic association can be subdivided into the three substages of induction of hormogonium differentiation, control of the direction of hormogonium gliding, and infection of the symbiotic cavity followed by repression of hormogonium differentiation.

3.1.1 Induction of Hormogonium Differentiation

In any physical interaction, such as symbiotic association, the partners must come together, either by random chance or by directed movement. In the *Nostoc*–plant associations, *Nostoc* is the motile partner through the differentiation of hormogonium filaments. Hormogonia are motile by a gliding mechanism, which requires contact with a substratum; the ultimate substratum is the plant tissue. A multiplicity of physicochemical factors induces the differentiation of hormogonia (Meeks and Elhai 2002; Tandeau de Marsac 1994). Hormogonia are a non-growth state, thus, their development proceeds as a cycle (Campbell and Meeks 1989). Upon induction, there is a cessation of net macromolecular synthesis, including DNA replication (Herdman and Rippka 1988), but cell division continues for a period resulting in smaller and differently shaped cells in the filaments. The filaments fragment at the vegetative cell–heterocyst connections, resulting in a loss of the capacity for nitrogen fixation. Gliding motility is initiated by 18–24 h after induction and the filaments remain motile for another 48–60 h; this interval defines the infection window. The filaments then become sessile, and they re-enter the vegetative cell cycle of growth and division; biomass components increase in an undefined sequence and heterocysts differentiate, first at the ends of the filaments.

To initiate a symbiotic association, the plant partners release a chemical signal that induces the differentiation of hormogonia. The signal has been collectively called a hormogonium-inducing factor (HIF). HIF is produced by gametophyte tissue of bryophytes (referred to as exudate) (Campbell and Meeks 1989; Knight and Adams 1966), the coralloid roots of the cycad *Zamia* sp. (Ow et al. 1999) and is in the mucilage of the stem glands of *Gunnera* spp. (Johansson and Bergman 1992). Even roots of non-symbiotic wheat seedlings release substances that induce hormogonia in *Nostoc* sp. (Gantar

et al. 1993). Production and/or release of HIF are enhanced by nitrogen starvation of hornwort tissue (Campbell and Meeks 1989). The chemical identity of HIF is not known. The factor(s) from *A. punctatus* and *Gunnera* spp. is a small molecule of between 0.5 and 12 kDa and is inactivated by heat. The activity in *A. punctatus* is complexed by polyvinylpyrrolidone (Campbell and Meeks 1989), while that of *Gunnera* spp. is inactivated by protease treatment (Rasmussen et al. 1994). The HIF from wheat appears to be larger than 12 kDa (Gantar et al. 1993). These various properties imply different chemical identities for the HIF activity, but this will not be known until the factor(s) from each plant partner is purified and characterized in detail.

The targets of HIF in *Nostoc* spp. are also unknown. We have now determined that the formation of hormogonia from vegetative filaments involves the differential transcription of more than 1820 genes, 52% of which are up-regulated (Campbell et al. 2007). While these transcriptome experiments have not yet identified HIF targets, they set the stage for working backwards from regulated genes to the regulators and signal transduction systems.

In the *Azolla* associations, the *Anabaena*/*Nostoc* is retained throughout the life cycle and passed to subsequent generations in the spore. Thus, this association has likely not been reconstituted with an environmental source of *Anabaena*/*Nostoc* for some time; it is also recalcitrant to reconstitution with any symbiotically competent *Nostoc* isolate (Peters and Meeks 1989). The *Anabaena*/*Nostoc* population in the fern apical meristem consists of undifferentiated filaments, morphologically similar to hormogonia (Peters and Meeks 1989). Whether formation of these undifferentiated filaments is regulated is unknown. Since the *Anabaena*/*Nostoc* filaments are pulled into the symbiotic cavity, via *Azolla* hair cells, by growth and morphogenesis (Peters and Meeks 1989), the filaments need not be motile.

3.1.2 Control of Hormogonium Movement

It is reasonable to assume that chemotaxis of *Nostoc* spp. to a plant partner is important in efficient formation of an association by the low population sizes of *Nostoc* in a habitat. Recent experiments have established that hormogonia of *Nostoc* spp. are chemotactic to the same exudate from bryophytes (Knight and Adams 1996) and mucilage from *Gunnera* spp. (Nilsson et al. 2006) that induce the differentiation of hormogonia. Hormogonia are known to be phototactic (Tandeau de Marsac 1994). Thus, a chemotactic response that is dominant over phototaxis could be instrumental in colonization of *Gunnera* stem gland cells where the hormogonia must migrate away from light into the mucilage filled channels (Johansson and Bergman 1992).

More rigorous evidence of the role of chemotaxis in establishment of an association needs to be generated by multiple approaches, including genetic

analyses. For example, identification and subsequent removal or complexing of the attractant(s) should lead to failure to infect the plant partner. Moreover, the genome of *N. punctiforme* contains five loci of genes encoding chemotaxis-like proteins that could constitute complete signal transduction systems, one or more of which could be responsible for a chemotactic response to plant signals (Meeks 2005a; Meeks et al. 2001). Specific genes in four of the loci are transcribed primarily or exclusively in hormogonia (Campbell et al. 2007).

3.1.3 Colonization and Repression of Hormogonium Differentiation

Motile hormogonia have been observed to enter the symbiotic cavities of the liverwort *Blasia pustilla* (Adams 2000; Kimura and Nakano 1990). Similar observations have not been recorded for hornworts or cycads. A detailed light and electron microscopic study has well defined the colonization process in *Gunnera* spp., from hormogonium induction, migration in the channel, to intracellular localization into achlorophyllous gland cells (Johansson and Bergman 1992).

In at least the hornwort association, symbiotically associated tissue produces HIF (Campbell and Meeks 1989). Thus, *Nostoc* filaments in the symbiotic cavity, which is open to the environment, continue to be exposed to the signal to differentiate hormogonia. Continued hormogonium formation is potentially lethal by extinction and counter-productive to differentiation of heterocysts and fixation of nitrogen. *A. punctatus* produces a hormogonium repressing factor (HRF) that is dominant over HIF (Cohen and Meeks 1996). Physiological and genetic analyses imply that the HRF consists of more than one component (Campbell et al. 2003; Cohen and Yamasaki 2000). The factors target a genomic locus in *N. punctiforme* that appears to synthesize a metabolite that functions as a repressor of hormogonium differentiation (Campbell et al. 2003).

3.2 Development of a Functional Association

The symbiotic growth state of a *Nostoc* sp. is broadly characterized by a reduced relative growth rate, a shift to a heterotrophic metabolic mode, an increased heterocyst frequency and the release of fixed nitrogen to the plant partner. In addition to the increased heterocyst frequency, the vegetative cells are markedly larger than those in free-living cultures and the cell–cell connections are very weak, such that manually excised symbiotic colonies, mounted on a microscope slide and dispersed by pressure on the cover slip, appear as short two to three cell filaments or unicells in the microscope (Meeks 1990). The life cycle of *Nostoc* species includes macroscopic globular

forms (*Nostoc* balls) where the filaments are confined by a somewhat rigid sheath; the confined filaments lose their filamentous nature and give the appearance of unicells (aseriate). To some extent, the symbiotic growth morphology reflects this aseriate growth stage. In some *Nostoc* species, the aseriate growth morphology is enhanced by green light (Lazaroff 1973). Green light could influence growth morphology of *Nostoc* spp. in bryophyte and *Azolla* associations that are exposed to light, but should have little impact in the darkened cycad or *Gunnera* tissues.

3.2.1 Growth and Metabolism

Nostoc species, even in the natural habitat, can grow considerably faster than the plant partners, excepting, perhaps, for *Azolla*. Moreover, annual bryophytes grow much faster than the perennial cycads and *Gunnera*. Thus, in a stable association, growth of the *Nostoc* partner, such as *N. punctiforme*, must be slowed dramatically in concert with its plant partner. There are no indications as to whether growth is limited in response to metabolic changes or vice versa, or even if physical confinement to a minimal expanding symbiotic compartment is a limiting factor.

Growth control in the *Azolla* spp. association is unique and possibly unprecedented. The *Azolla* associations can double their biomass in about 2 days (Peters et al. 1980), which is comparable to laboratory culture of a recently isolated *Nostoc* symbiont (Enderlin and Meeks 1983). This observation would imply minimal growth control of the primary *Anabaena*/*Nostoc* symbiont under optimal *Azolla* growth conditions. The secondary symbionts, however, cannot be detected in cyanobacterial purifications from crushed whole plant tissues using molecular probes, while the primary symbiont is readily detectable (Meeks et al. 1988). This negative result indicates the secondary symbionts are present at orders of magnitude lower concentration than the primary symbiont. Conversely, doubling times of the isolated secondary symbionts in the laboratory are on the order of 48 h or less. When crushed *Azolla* preparations are plated for enrichment of nitrogen-fixing cyanobacteria, it takes an extraordinarily longer period of time for colonies to emerge compared to plating free-living cultures. These observations indicate highly stringent growth control over the secondary symbionts that takes a prolonged incubation period apart from the plant partner before they recover and can initiate growth. This growth control, however, appears not to be imposed on the primary symbiont and may be an important aspect of the evolutionary changes in this apparent obligate symbiont.

Metabolic studies have focused on carbon and nitrogen metabolism; they are summarized in Tables 1 and 2.

Photosynthetic Carbon Metabolism

Because there are few distinguishing characteristics, other than pigmentation, there have been no direct studies of photosynthetic activity of the *Nostoc* symbiont within the plant tissue. In *Azolla caroliniana*, indirect measurements indicate that the *Anabaena*/*Nostoc* partner contributes to less than 5% of the CO_2 fixed by the association, even though the cyanobacterium is estimated to contribute approximately 16% to the total chlorophyll and protein (Kaplan and Peters 1988). By utilization of *Nostoc* mutants resistant to photosynthetic inhibitors, Steinberg and Meeks (1991), determined that the *Nostoc* symbiont of *A. punctatus* does photosynthesize and could contribute, at most, 30% of the photosynthetically generated reductant required for nitrogen fixation in the association on a short term basis. These studies reinforce the largely heterotrophic nature of the *Nostoc* in symbiotic association.

Table 1 Photosynthetic characteristics of *Nostoc* in symbiotic association with terrestrial plants (derived from Meeks 1998)

Association	Photosynthetic CO_2 fixation in planta	Photosynthetic CO_2 fixation ex planta	Rubisco activity in vitro	Rubisco protein
Free-living *Nostoc* spp.	ND	128 nmol/min/mg protein	215–321 nmol/min/mg protein	52 mg/g cell protein
Hornwort, *Anthoceros punctatus*[1]	Positive, < 30%	12%	15%	100%
Fern, *Azolla* spp.[2]	Positive, < 5%	85%	ND	mRNA, < 10%
Cycad, *Cycas*, *Zamina*, *Macrozamia* spp.[3]	ND	ND	100%	ND
Angiosperm, *Gunnera* spp.[4]	ND	< 1%	100%	100%

Free-living *Nostoc* values were compiled from control experiments in the cited references. Symbiotic values are are given as a percentage of free-living values. Some of the symbiotic values were converted from qualitative data

References: 1. Steinberg and Meeks (1989, 1991), Meeks (1990). 2. Ray et al. (1979), Kaplan et al. (1986), Nierzwicki-Bauer and Haselkorn (1986). 3. Lindblad et al. (1987), Lindblad and Bergman (1986). 4. Soderbäck and Bergman (1992, 1993)

ND not determined

The capacity of the immediately isolated *Nostoc* symbiont to carry out complete photosynthetic CO_2 fixation ex planta varies from essentially no activity in *Nostoc* isolated from *Gunnera* and cycads, to 12% and 85% of the free-living rate in bryophyte and *Azolla* associations, respectively (Table 1). Examination of in vitro ribulose bisphosphate carboxylase/oxygenase (Ru-

bisco) activity reveals a correlation with ex planta CO_2 fixation only in the *Nostoc* associated with bryophytes. In this association, however, the low Rubsico activity does not correlate with the high amount of Rubsico protein present. These results imply that photosynthetic CO_2 fixation is modulated, in a large part, by an irreversible inhibition of the catalytic activity of Rubisco in the *Nostoc* associated with bryophytes.

The in vitro Rubisco activity and protein content that are essentially the same as free-living cultures do not correlate with the ex planta lack of photosynthetic CO_2 fixation in the cycad and *Gunnera* associations (Table 1). In *G. tinctoria*, photosystem II activity of the *Nostoc* symbiont is down-regulated by modification of the D1 protein (Black and Osborne 2004). This is the only association in which photosynthetic electron transport in the *Nostoc* partner has been examined and more of these essential studies need to be done. The discrepancy in the cycad associations has not yet been addressed.

The results with *Anabaena*/*Nostoc* from *Azolla* are particularly confusing. Estimates of Rubisco gene expression indicate that only about 10% of the mRNA is present in the symbiont compared to typical free-living cultures (Nierzwicki-Bauer and Haslekorn 1986). This result is not consistent with the relatively high rates of photosynthetic CO_2 fixation ex planta. Unfortunately, the in vitro Rubisco catalytic activity and amount of protein have not been examined in the *Anabaena*/*Nostoc* symbiont to resolve the inconsistencies. In addition, as noted above, the *Anabaena*/*Nostoc* contributes little photosynthate to the association and appears to be an obligate symbiont, unable to grow apart from the plant partner. What then is the selective pressure to retain the potential for a high rate of photosynthesis if it cannot be utilized?

Since most of these studies have been done using different *Nostoc* isolates, it is problematic to draw broad conclusions regarding different mechanisms for modulating the photosynthetic activities of the symbiont. A more optimal situation would employ the same symbiont in association with both the hornwort *A. punctatus* and *G. tinctoria*, for example *N. punctiforme* PCC 73102, to determine if Rubisco and photosystem II activities are down-regulated only in the hornwort and *Gunnera* association, respectively.

Nitrogen Metabolism

Symbiotically associated *Nostoc* spp. fix nitrogen at a higher rate than free-living cultures, consistent with a higher heterocyst frequency (Table 2). Since the symbiotic *Nostoc* grow slower than free-living cultures, the nitrogen fixed in excess of that required for their growth is likely lost as a metabolic waste product and made available to the plant partner. A questions is, how much and in what form? In all but the cycad associations, ammonium is released by the symbiotic *Nostoc* spp. and the amounts vary from 40% to 90% of the fixed nitrogen (Table 2). Cyanobacteria assimilate NH_4^+, obtained from the envi-

ronment or derived from nitrate/nitrite, dinitrogen, urea or organic nitrogen, by the sequential activities of glutamine synthetase (GS) and glutamate synthase; glutamate dehydrogenase has no significant role (Flores and Herrero 1994). Thus, studies have focused on modulation of GS (encoded by *glnA*) activity or synthesis as a cause of NH_4^+ release.

Table 2 Nitrogen fixation and assimilation characteristics of *Nostoc* in symbiotic association with terrestrial plants (derived from Meeks 1998)

Association	Heterocyst frequency (%)	Nitrogenase specific activity	Nitrogen release as % of fixed	Glutamine synthetase activity	Glutamine synthetase protein
Free-living *Nostoc* spp.	5–10	2.7–6.3 nmol/min/mg protein	< 10 as organic N	0.85–1.8 nmol/min/mg protein	6.8–7.6 mg/g cell protein
Hornwort, *Anthoceros punctatus*[1]	25–45	23.5	80 as NH_4^+	15%	100%
Fern, *Azolla* spp.[2]	26–30	6.2	40 as NH_4^+	30%	38%
Cycad, *Cycas*, *Zamina*, *Macrozamia* spp.[3]	17–46	26.7	unknown as organic N	100%	100%
Angiosperm, *Gunnera* spp.[4]	20–60	24.8	90 as NH_4^+	70%	100%

Free-living *Nostoc* values were compiled from control experiments in the cited references. Nitrogenase activity is acetylene reduction. Glutamine synthetase symbiotic activity and protein values are given as a percent of free-living values. Activity is of the transferase reaction. In some cases, the original data were normalized to units of chlorophyll *a*; these values were converted to protein assuming that chlorophyll makes up 4% of the total cellular protein

References: 1. Stewart and Rodgers (1977), Joseph and Meeks (1987), Steinberg and Meeks (1991). 2. Hill (1975), Ray et al. (1978), Meeks et al. (1987), Lee et al. (1988). 3. Lindblad et al. (1985), Lindblad and Bergman (1986), Pate et al. (1988). 4. Söderbäck et al. (1990), Bergman et al. (1992), Silvester et al. (1996)

Symbiotic *Nostoc* sp., immediately isolated from association with *A. punctatus*, has 20% of the capacity to assimilate exogenous NH_4^+ as does a free-living culture and releases approximately 80% of its fixed nitrogen as NH_4^+ in the intact association (Meeks et al. 1985). The specific in vitro activity of GS in the symbiotic *Nostoc* is about 15% that of its free-living culture, but the amount of GS protein is comparable in both growth states (Joseph and Meeks 1987). These results imply irreversible inhibition of GS catalytic activity, similar to Rubisco, and are superficially consistent with impaired GS as a cause of NH_4^+ release. Such a casual relationship can be examined by a simple comparison. The rate of symbiotic nitrogen fixation in this associ-

ation is 12.5 nmol of NH_4^+/min/mg *Nostoc* protein and the in vitro specific biosynthetic activity of GS is 19.8 nmol of NH_4^+ min/mg protein (Meeks 2003). Thus, there would appear to be sufficient NH_4^+ assimilation activity to sequester all of the N_2-derived NH_4^+. This conclusion could be negated by two conditions: substrates for GS are not at saturating concentrations in the symbiotic *Nostoc*; and the N_2-derived NH_4^+ is assimilated only in the heterocyst and, if not assimilated, may directly diffuse out the heterocyst and into the symbiotic cavity. Metabolite concentrations are unknown in symbiotic or free-living *Nostoc* spp., therefore, the first possibility cannot be adequately analyzed. However, even operating at half-saturation values (ca. 10 nmol/min/mg protein), the catalytic activity is not consistent with the assimilation of only 2.5 nmol of NH_4^+ (20% of the fixed NH_4^+). Walsby (2007) calculated that the heterocyst envelope is impermeable to gasses, and therefore solutes, except at the pole junctions with adjacent vegetative cells. Thus, N_2-derived NH_4^+ must be transferred to adjacent vegetative cells before it could be released into the symbiotic cavity. Therefore, assumptions that reduced GS activity in heterocysts per se could be responsible for NH_4^+ release do not take into account the assimilation of NH_4^+ in vegetative cells. Of course, any release could be a combination of factors.

A situation similar to that of the hornwort, with some variation, holds for *Anabaena*/*Nostoc* associated with *Azolla* (Table 2). Here 40% of the N_2-derived NH_4^+ is released (Meeks et al. 1987), in vitro GS activity is 30%, GS protein is about 38% (Lee et al. 1988) and the *glnA* mRNA concentration is about 10% (Nierzwicki-Bauer and Haselkorn 1986) of free-living cultures. These results imply that GS activity is modulated by synthesis of the protein, rather than inhibition of catalytic activity. Whether the lower amount of GS mRNA is a consequence of regulated transcription or simply of a less efficient promoter structure is unknown. A comparison of catalytic rates similar to that in the hornwort association yields an even greater potential for assimilation of all of the N_2-derived NH_4^+, implying that modulation of GS activity and level may not be the complete story of NH_4^+ release in *Azolla* spp.

There is a notable disconnection between release of N_2-derived NH_4^+ and in vitro GS activity in the *Gunnera* association. In this association, *Nostoc* sp. in vitro GS activity and protein are 70–100% of free-living cultures (Bergman et al. 1992), while 90% of the N_2-derived NH_4^+ is released (Silvester et al. 1996). The most direct explanation for NH_4^+ release in this case would be depletion of substrates and reactants for GS activity. Conversely, the cycad associations are documented to release organic nitrogen to the plant partner (Pate et al. 1988). The *Nostoc* in association with cycads expressed essentially 100% of the in vitro GS activity and protein as a free-living culture (Table 2). The conclusions one can draw from these studies are that the mechanisms of release of fixed nitrogen in symbiotic associations may well vary with the plant partner, but there is insufficient information to currently pro-

pose models. As in CO_2 fixation, it would be of interest to compare the same *Nostoc* sp. in the three associations that can be reconstituted, in which GS activity is and is not irreversibly modulated.

3.2.2 Heterocyst Differentiation

Apart from the aseriate growth form, the most dramatic morphological change in symbiotically associated *Nostoc* spp. is the increase in frequency of heterocysts in the filaments (Table 2). There are two aspects of symbiotic heterocyst differentiation to be considered: what is the signal for induction of differentiation; and what is the spacing pattern and will the pattern(s) reflect on regulatory aspects of differentiation.

Signal for Differentiation

The environmental signal for heterocyst differentiation in free-living cultures is limitation for combined nitrogen (Meeks and Elhai 2002). The limitation signal is thought to be perceived by an elevated cellular level of 2-oxoglutarate (Muro-Pastor et al. 2001; Vazquez-Bermudez et al. 2002). 2-Oxoglutarate activates a transcriptional regulator, NtcA, that modulates transcription of genes encoding proteins for the acquisition of sources of nitrogen alternative to NH_4^+ (Herrero et al. 2001). If no source of combined nitrogen is available, activated NtcA, directly or indirectly, stimulates transcription of regulators of heterocyst differentiation and subsequent fixation of N_2. These include the positive regulators HetR and HetF and the negative regulators PatN and PatS (Fig. 1) (Golden and Yoon 2003; Herrero et al. 2004; Meeks and Elhai 2002; Zhang et al. 2006).

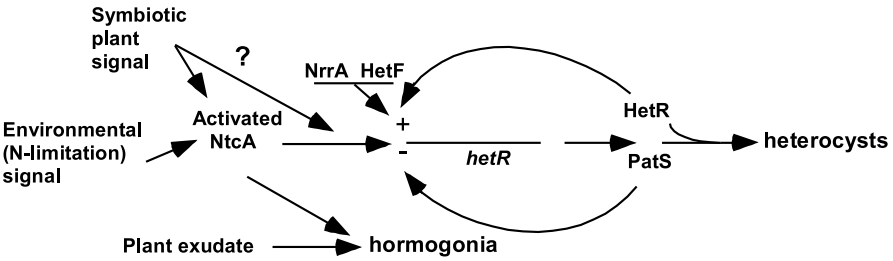

Fig. 1 Schematic of possible regulatory elements and pathways in free-living and plant partner induction of heterocyst and hormogonium differentiation

There are at least two lines of evidence that the same nitrogen limitation signal does not regulate symbiotic heterocyst differentiation. First, the symbiotic *Nostoc* colonies are immersed in a pool of N_2-derived NH_4^+ in

the bryophyte, *Azolla* and *Gunnera* associations, yet heterocyst differentiation continues in these colonies. The concentration of NH_4^+ in the hornwort *A. punctatus* symbiotic cavity is calculated to be about 0.55 mM (Meeks 2003) and that in *Azolla* 0.8–6.0 mM (Canini et al. 1990). In free-living cultures, heterocyst differentiation is repressed by about 0.010 mM NH_4^+ (Meeks et al. 1982). Second, symbiotic *Nostoc* vegetative cells that differentiate into heterocysts do not show morphological signs of nitrogen limitation. Cyanobacteria store nitrogen as the multi-L-arginyl-poly(L-aspartic acid) non-protein polymer (cyanophycin), and as amino acids in phycobilisomes and carboxysomes (Rubisco) (Allen 1984). These polymers are catabolized in nitrogen-starved cells. All of these structures can be seen in symbiotic *Nostoc* vegetative cells, as well as cyanophycin and phycobilisomes in some heterocysts (Meeks 1990). These observations verify that the symbiotic *Nostoc* species assimilate sufficient N_2-derived NH_4^+ to supply their own metabolic needs.

The conclusion from these observations is that the plant partner produces an environment or chemical signal(s) that supersedes the nitrogen limitation signal. Genetic analyses of *N. punctiforme* in association with *A. punctatus* have established that the signal transduction pathway for symbiotic heterocyst differentiation includes functional HetR and HetF proteins, similar to the free-living state (Wong and Meeks 2002). Therefore, the symbiotic signal must enter the signal transduction pathway upstream from HetR/HetF (Fig. 1). NtcA and the response regulator NrrA are the only transcription factors currently known to operate upstream of HetR (Ehira and Ohmori 2006). Elevated transcription of *nrrA* alone does not lead to heterocyst differentiation, at least in filaments committed to hormogonium differentiation (Campbell et al. 2007). A mutation in *ntcA* in *N. punctiforme* leads to a hormogonium defective phenotype, which is also defective in symbiotic infection. Consequently, it cannot be determined whether a plant signal for symbiotic heterocyst differentiation bypasses NtcA. Characteristics of these *N. punctiforme* mutants in other plant associations have not been determined. The possibility of enhanced accumulation of 2-oxoglutarate in the heterotrophic symbiotic vegetative cells does not appear to be reasonable as an aberrant signal for nitrogen limitation. If this were to occur, the cells should respond as if they were nitrogen-limited and subsequently mobilize their nitrogen reserve polymers; symbiotic vegetative cells that differentiate into heterocysts do not appear to be nitrogen-limited.

Pattern of Spacing

The increase in heterocyst frequency leads to a disruption in the free-living spacing pattern of heterocysts in the filaments. The free-living pattern can be disrupted in two ways: (i) additional new heterocysts can appear in the vegetative cell interval between adjacent heterocysts, thereby shortening the interval (multiple singular heterocysts, Msh); or (ii) they may appear adja-

cent to existing heterocysts, yielding a cluster of heterocysts at specific sites in the filament (multiple contiguous heterocysts, Mch). A Mch pattern is commonly observed in mutants or genetic constructs during the establishment of pattern; *patS* (Yoon and Golden 1998, 2001) and *patU* (Meeks et al. 2002) deletions, and over expression of *hetR* (Buikema and Haselkorn 2001) and *hetF* (Wong and Meeks 2001), yield Mch. Mutation of *hetN* (Callahan and Buikema 2001) and *patB* (Jones et al. 2003) results in a Mch pattern, but the pattern appears in the second round of heterocyst differentiation, after the initial establishment of pattern. HetN and PatB are modeled as essential elements in the maintenance of pattern (Callahan and Buikema 2001; Jones et al. 2003).The Msh pattern of the *patN* mutant appears during establishment of pattern (Meeks et al. 2002); this is the only report of a Msh phenotype.

Due to the fragile nature of the filamentous growth pattern, the distorted size of vegetative cells and the frequent presence of phycobiliprotein-induced chlorophyll fluorescence in heterocysts, it is often difficult identify heterocysts and their spacing pattern in symbiosis (Meeks 1990, 1998). Nevertheless, both Msh and Mch patterns can be observed in light and electron micrographs (Meeks and Elhai 2002). The most persuasive light micrographs are of *Anabaena/Nostoc* in the *Azolla* association, taken by G. A. Peters, which depict a Msh pattern (Meeks and Elhai 2002). Examination of collages of electron micrographs of *Nostoc* in the *A. punctatus* association, taken within two months after reconstitution, yield an overwhelmingly Msh pattern as well (Meeks and Elhai 2002). However, the most definitive results come from analysis of experiments that compare the rates of nitrogen fixation and heterocyst frequency along the developmental gradient from plant meristematic to mature tissue in *Azolla* (Hill 1975), cycad (Lindblad et al. 1985) and *Gunnera* (Söderbäck et al. 1990). At the meristematic tip of the *Azolla* leaf, coralloid roots and *Gunnera* stems, the *Nostoc* filaments have a low to no frequency of heterocysts and nitrogen fixation is correspondingly low to absent. Progression away from the tip results in an essentially linear increase in both heterocyst frequency and rates of nitrogen fixation, and the heterocyst spacing reflects a Msh pattern. A distance is reached beyond which an inverse correlation appears between increasing heterocyst frequency and decreasing rates of nitrogen fixation. At this distance, and beyond, the Mch pattern becomes more prevalent. Heterocysts are terminally differentiated with a finite, but undefined, life span. The life span may well be longer in symbiosis compared to free-living growth and variable with respect to the life span of the plant partner.

We suggest that the symbiotic Mch pattern arises as a consequence of new heterocysts differentiating adjacent to a functionally dead heterocyst; confinement in the symbiotic cavity results in the non-functional heterocyst remaining in place rather than being detached and lost from the filament

(Meeks and Elhai 2002). If this analysis is correct, then the Msh pattern arises as a function of time and may be more a consequence of disruption of the maintenance, than of the establishment, of the spacing pattern. Maintenance of the pattern is the more difficult process to study in a randomly dividing population of filaments. Since alteration of the maintenance assigned genes *hetN* and *patB* result in Mch, the symbiotic pattern must be a consequence of the symbiotic manipulation of other, as yet unknown, genes and gene products involved in maintenance of the free-living pattern. Although *patN* is a possible candidate, its spacing pattern in free-living culture emerges during the establishment of pattern.

4
Conclusions and Future Perspectives

What conclusions can be drawn from the specificity and physiological adaptation results with respect to providing a foundation for future studies?

4.1
Specificity

The studies collectively indicate that a large (but how large is unknown) number of *Nostoc* species or strains establish symbiotic associations with phylogenetically and morphologically distinct plant partners. It is possible that some strains are specific for a plant partner, but most appear to be non-specific. Conversely, some free-living *Nostoc* strains do not form symbiotic associations, at least under benign laboratory culture conditions (Meeks 1990). Although symbiotic competence does not appear to be a universal characteristic of the genus *Nostoc*, there must be a genetic basis for expression of such competence. It is difficult, however, to imagine that each symbiotically competent *Nostoc* species or strain contains genetic information that allows it to interact uniquely with the various plant partners.

There is no reason to assume that bryophytes waited for at least 400 million years until emergence of the Gunneraceae to take advantage of the nitrogen fixation activity of heterocyst-forming cyanobacteria, unless other selective pressures are involved in establishment of the association. Because nitrogen-fixing cyanobacterial associations are actually narrowly distributed in the four major terrestrial plant lineages, it also does not seem reasonable that the genetic information of symbiotic competence was linearly transferred from hornworts through ferns and cycads to *Gunnera*. Therefore, one may logically deduce that each plant group learned how to manipulate properties of susceptible *Nostoc* spp. in the adjacent wet soil and that the regulatory signals targeting a specific *Nostoc* physiological characteristic need not be the same in each association.

4.2 Physiological Adaptation in the Associations

All plant partners appear to control the infection process by producing hormogonium-inducing factors and perhaps chemoattractants. However, details on the identities of the factors and attractants, and, thus, the variety of signal receptors and mechanistic responses are unknown. The act of infection appears to vary depending on the plant structures to be colonized. Once colonized, the symbiotic cavities in the *Azolla*, cycad, and *Gunnera* partners appear to be closed off from the external environment, including the presence of HIF. Therefore, perhaps only the bryophyte partners may need to produce hormogonium-repressing factors. If this is so, it is of interest that *N. punctiforme* ATCC 29133, isolated from the cycad *Macrozamia* sp, retains the genetic information to synthesize a hormogonium-repressing metabolite. This observation verifies the broad symbiotic competence of *N. punctiforme* and indicates the presence of an unknown selective pressure to retain that information.

Although growth control of *Nostoc* appears to be an essential aspect of the plant associations, nothing is known how it is achieved. Assimilatory phototrophic carbon and nitrogen metabolism by the *Nostoc* partner are depressed in all associations, but they appear to be depressed in proportion to a decreased growth rate. The different plant partners appear to target different reactions in at least the control of photosynthetic CO_2 fixation. Thus, plants may employ different mechanisms to achieve a similar physiological state of the same *Nostoc* in various associations. A causal relationship between growth and metabolism has not been established. Heterocyst differentiation is enhanced in the symbiotic growth state leading to higher rates of nitrogen fixation and release of fixed nitrogen as NH_4^+ in all but one association; the nitrogen fixation is primarily fueled by photosynthate from the plant partner. The enhanced heterocyst frequency yields a Msh spacing pattern in the most highly functional tissues of the plant partner. The Msh pattern may result from an alternation in the mechanisms that regulate the maintenance, rather than the establishment, of the heterocyst spacing pattern in the free-living growth state. Thus, knowledge of the maintenance mechanisms is essential in understanding plant-dependent enhancement of heterocyst differentiation.

4.3 Perspectives

Nitrogen-fixing *Nostoc* spp. offer the potential for genetic engineering of crop plants for symbiotic nitrogen fixation. *Nostoc* spp. carry their own oxygen protective mechanisms and, thus, inflict less of a burden on a plant partner to develop specialized structures in formation of a functional association. Con-

siderable progress has been made in defining the physiological characteristic of the *Nostoc* spp. as they establish a functional association with their evolved plant partners. Ambiguities in physiological adaptation mechanisms could be resolved by using the same *Nostoc* isolate in various laboratory reconstituted associations. Advances in this area will also be facilitated by knowing the genome sequence of a genetically tractable symbiotic strain, *N. punctiforme* ATCC 29133. I hypothesize that the plant partners have, in fact, evolved different mechanisms of growth and metabolic control over the *Nostoc* symbiont to achieve a stable commensal relationship. Therefore, more effort needs to be expended in characterization of the plant partners. *Gunnera* species have now been genetically transformed (Wan-Ling Chiu, Virginia Commonwealth University, personal communication) and this could open exciting new avenues of research in identifying plant signals and in engineering new plant–*Nostoc* nitrogen-fixing associations.

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