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Regulation of Cellular Differentiation in Filamentous Cyanobacteria in Free-Living and Plant-Associated Symbiotic Growth States

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INTRODUCTION

The word "bacteria" generally conjures up images of individual rods or spheres swimming around, every bug for itself. In fact, however, the solitary life is rare in the microbial world; bacteria interact with other microbes and macroorganisms in the environment and with their own siblings, and these interactions often result in complex social interactions. Many bacterial species exchange signals amongst themselves in order to assess cell density and to respond appropriately (62, 71). Myxobacteria take signaling a step further and use it to direct their differentiation into complex multicellular structures, enabling them to survive periods of starvation (47). Rhizobia communicate outside of their species. They respond to signals from leguminous plants (e.g., beans) to differentiate into nitrogen-fixing cells. At the same time, they produce signals that induce the plants to form specialized nodules, microoxic cavities required for the O_2-sensitive process of N_2 fixation (198).

Like myxobacteria, certain N_2-fixing species of filamentous cyanobacteria generate signals to direct their own multicellular development, and, like rhizobia, they respond to signals from plants, initiating or altering the extent of their cellular differentiation. A main objective of this review is to consider the degree to which signals used by cyanobacteria to control their own differentiation are appropriated by plants to achieve stable symbioses. General reviews on the biology of symbioses between cyanobacteria and plants have recently appeared (3, 151).

Symbiosis of Cyanobacteria with Plants

What makes cyanobacteria valuable partners in symbioses? Cyanobacteria are an ancient and diverse class of bacteria characterized by their ability to use light energy to split water into oxygen and reductant, which is subsequently consumed in photosynthesis. Some cyanobacteria are also able reduce atmospheric dinitrogen gas to ammonium (N_2 fixation), giving them the simplest of nutritional requirements: air, water, a few inorganic nutrients, and light. Nitrogen fixation and oxygenic photosynthesis, however, are intrinsically incompatible, because nitrogenase, the enzyme responsible for reduction of N_2, is inactivated by minute concentrations of oxygen (52). Certain multicellular cyanobacteria, e.g., the genera *Anabaena* and *Nostoc* in the order *Nostocales*, overcome this incompatibility by differentiating specialized, nearly anoxic cells at intervals within chains of other cells, like isolated pearls in a string of beads (Fig. 1). Nitrogen fixation takes place in these specialized terminally differentiated cells (called heterocysts), and photosynthesis takes place in the other cells (vegetative cells) (214).

The ability of these cyanobacteria to fix N_2 is evidently prized by other organisms, because N_2-fixing cyanobacteria, primarily in the genus *Nostoc*, form symbioses with a wide variety of fungi, plants (Table 1), and other organisms (148, 165). Cyanobacterial symbioses occur with representatives of all four of the major phylogenetic divisions of terrestrial plants: primitive spore-producing bryophytes (hornworts and liverworts) and ferns (*Azolla*) and the two classes of seed-producing plants, gymnosperms (cycads), and angiosperms (*Gunnera*). By contrast, rhizobia form associations only with angiosperm le-
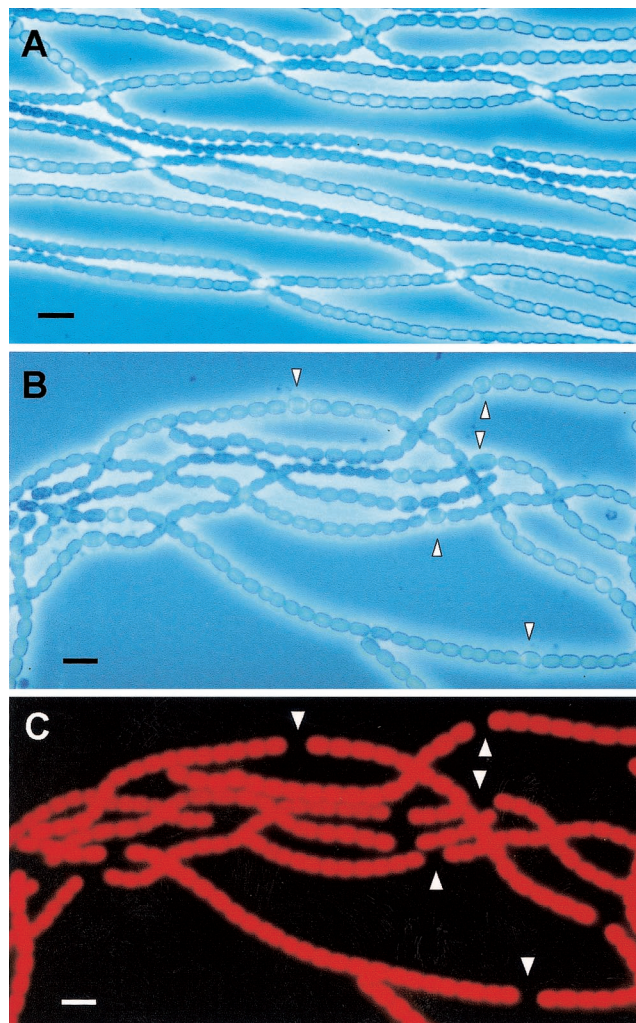
FIG. 1. Photomicrographs of vegetative and heterocyst-containing filaments of the cyanobacterium *N. punctiforme*. (A) Phase-contrast image of vegetative filaments grown in the presence of ammonium. No heterocysts are visible. (B) Phase-contrast image of filaments grown in the absence of any combined nitrogen source in the culture medium. Heterocysts, identified by arrowheads, are present at well-spaced intervals. (C) Epifluorescence image of the same filaments as in panel B. Excitation was at 510 to 560 nm (green), exciting phycoerythrin, and emission was greater than 600 nm. Heterocysts have negligible fluorescence, while vegetative cells have intense combined fluorescence from phycobiliproteins and chlorophyll *a*. Bar, 10 μm.

gumes (43) and one genus, *Parasponia*, of nonleguminous trees (14). Various N_2-fixing and non-N_2-fixing cyanobacteria also associate with fungi (to establish lichens) and with marine organisms (171). This review will focus on cyanobacterial associations with terrestrial plants.

In such associations, fixed nitrogen provided by heterocyst-forming cyanobacteria is able to support the nitrogen needs of the plant partner (148). Even free-living heterocyst-forming cyanobacteria can supply all of the nitrogen needed by nonsymbiotic plants under laboratory conditions (64, 134, 175). Plant partners do not, therefore, need to provide a specialized environment for cyanobacterial partners, as do legumes for

TABLE 1. Plants that form symbioses with nitrogen-fixing cyanobacteria

Taxonomic division of plant partner (references)[a]	Plant genus	Tissue localization of cyanobacterial symbiont	Heterocyst frequency (%)[b]
Angiospermae (16, 85, 174)	*Gunnera* (1 genus)	Intracellular, within cells of the stem gland	1–60
Gymnospermae (105, 135, 106)	Cycads (11 genera)	Extracellular, within specialized coralloid roots	5–45
Pteridophyta (ferns) (101, 119 and 185, 78)[c]	*Azolla* (1 genus)	Extracellular, within cavities of the dorsal leaves	0–30
Bryophyta (116, 49, 163)	Liverworts (2 genera), hornworts (4–6 genera)	Extracellular, within cavities of the gametophyte	25–45
Fungi (149, 8, 149)[d]	Lichens (perhaps 300–350 genera)	Extracellular, localized in cephadolia or evenly distributed in the thallus	2–6 / 10–30

[a] For each symbiosis, the first reference is to a general review, the second describes reconstitution of the symbiosis, and the third reports heterocyst frequency.
[b] Fraction of the total cyanobacterial cells that are heterocysts, which may vary by position within the plant. For fungi, the first range is for bipartite lichens (cyanobacterium plus fungus) and the second range is for tripartite lichens (cyanobacterium plus green alga plus fungus).
[c] Cyanobacterial symbiont has yet to be cultured according to references 119 and 185 (while historically identified as *Anabaena*, it is likely to be in the genus *Nostoc*; we refer to it as *Nostoc/Anabaena*).
[d] Reconstitution has been attained but is rare (8).

rhizobia. A haven safe from competitors and predators may be sufficient.

What do plants bring to the symbiosis? The self-sufficiency of heterocyst-forming cyanobacteria with respect to N_2 fixation may account for the relatively simple plant structures that house symbiotic cyanobacteria, in contrast to those that house rhizobia. Infecting rhizobia induce plants to differentiate nodules, which have been likened in their complexity to new plant organs (201). Nodules differ from surrounding root tissue in several regards, many of them related to the control of O_2 tension. One accommodation is the production of plant- and bacterium-encoded leghemoglobin, a protein that binds O_2, keeping it at a concentration low enough to permit N_2 fixation but high enough to permit rhizobial respiration.

In contrast, the plant structures inhabited by cyanobacteria exist even in the absence of the cyanobacterial symbiont, at sites that differ from plant to plant (Fig. 2). *Gunnera* possesses a unique gland on its stem that attracts cyanobacteria. Cycads have specialized lateral roots (coralloid roots) in which a layer has been prepared for infecting cyanobacteria. Bryophytes come with cavities in the gametophyte tissue that are infected by cyanobacteria. Cyanobacteria associated with *Azolla* in cavities of the dorsal leaves are unique in that they appear to be obligate symbionts (141), but *Azolla* cured of cyanobacteria by treatment with antibiotics continues to grow in the presence of combined nitrogen with empty leaf cavities (60). These different plant structures will be referred to collectively as symbiotic cavities, and the cyanobacterial populations within them will be referred to as symbiotic colonies.

Although the symbiotic cavities exist without symbiosis, the environment within the cavities is clearly specialized for the symbiotic state. A feature that may be common to all symbiotic cavities is the presence of mucilage, a complex polysaccharide (133, 143, 155, 157, 168, 196). The cavities are also relatively axenic, except for the cyanobacterium itself (84). Cavities of *Azolla* are exceptional in supporting other bacteria (26) that may play an important role in the symbiosis (60). Finally, the symbiotic cavities respond to the presence of cyanobacteria by elaborating long, finger-like cells (103, 163) that may serve to increase the surface area for nutrient exchange.

It is a matter of controversy whether all symbiotic cavities, like the nodules of legumes, have O_2 tensions much lower than ambient. Microelectrode measurements have indicated rela-

tively high O_2 tension within coralloid roots of cycads (35), slightly lower than ambient (83%) in symbiotic cavities of *Azolla* (68), and very low (near to anoxic) in the bryophyte hornwort *Anthoceros* (34). No plant-derived hemoglobin-like protein has been reported. Cyanoglobin, a heme protein with high affinity for oxygen, is found in many *Nostoc* strains, but some strains, including a few symbiotic strains, lack the protein (79). Free-living cyanobacteria can maintain high nitrogenase activities while growing under atmospheres ranging from 1 to 50% O_2 (131).

How specific are associations between plants and cyanobacteria? Strains or species of the genus *Nostoc* are the dominant but not exclusive symbiotic cyanobacteria in plant associations. The relatively low requirements of plant-cyanobacterium symbioses are illustrated by the lack of strain specificity between the partners in reconstitution experiments (22, 49, 85). Of 10 strains of *Nostoc* isolated from symbioses with *Gunnera*, 6 achieved an intracellular association with a different species of *Gunnera*. Of four strains of *Nostoc* isolated from symbioses with cycads, *Anthoceros*, or *Peltigera* (a lichenized fungus), three successfully infected *Gunnera*. Only one of nine *Nostoc* strains isolated from various plant or lichen symbioses failed to infect *Anthoceros*. Free-living isolates have generally not established symbioses, although exceptions have been noted (49, 135). DNA-fingerprinting techniques revealed a wide cluster of symbiotic and free-living isolates from a single field site associated with the hornwort *Phaeoceros* (202). The isolates included primarily 65 *Nostoc* strains, but three each of *Calothrix* and *Chlorogloeopsis* were also identified illustrating some flexibility in cyanobacterial partners. Except for one *Nostoc* isolate, all reconstituted a symbiotic association with *Phaeoceros* or the liverwort *Blasia*.

DEVELOPMENTAL REPERTOIRE OF FREE-LIVING CYANOBACTERIA

The relative lack of discrimination by plants with regard to their *Nostoc* partners indicates that the properties required for a wide variety of associations are found in most strains. It is difficult to imagine that cyanobacteria, unlike rhizobia, have evolved to exchange signals with multiple partners spanning nearly the entire plant kingdom. The alternative is that different plants have independently learned how to exploit the ca-
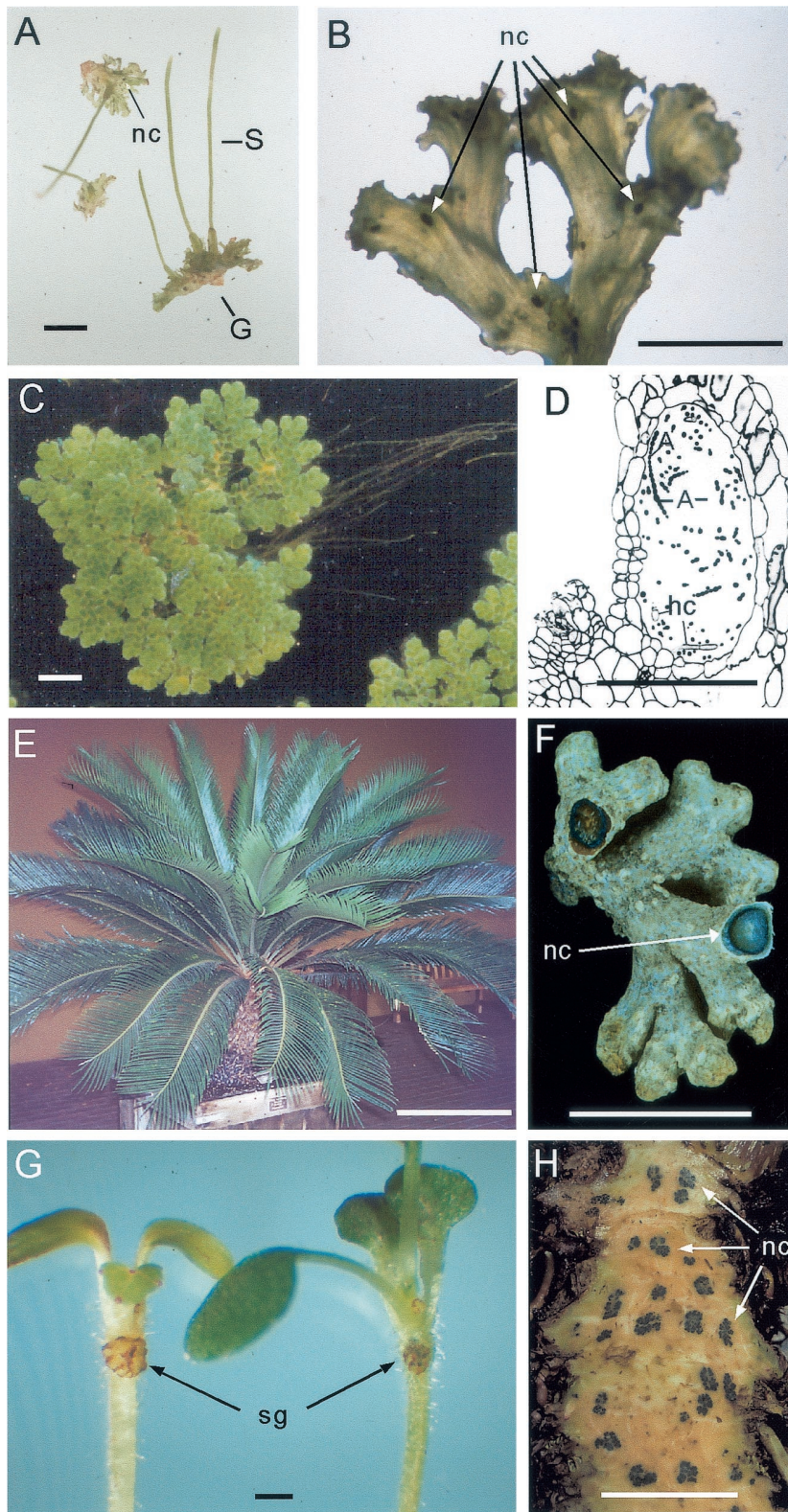
FIG. 2. Photographs of plant partners showing the location of symbiotic cavities housing associated cyanobacteria. (A and B) Sporophyte (S) and gametophyte (G) generations of the bryophyte *Anthoceros punctatus* (A) and *Nostoc* colonies (nc) (B) within the gametophyte tissue. Bar, 1.0 cm. (C and D) *Azolla caroliniana* floating sporophyte leaves and trailing submerged roots (C). The cavity of a dorsal leaf housing symbiotic *Nostoc*/*Anabaena* is shown in a vertical position. A, *Nostoc*/*Anabaena* filaments; hc, *Azolla* hair cells (D). Photographs copyright Gerald Peters. Panel D reprinted from reference 142 with permission of the author. Bar, 0.25 cm and 0.25 mm in panels C and D, respectively. (E and F) Stem and leaves of the cycad *Cycas taiwaniana* (E) and ventral view and cross section of a coralloid root cluster from *C. taiwaniana* (F). *Nostoc* is present in a green annular ring (nc) within the root. Bar, 0.5 m and 0.5 cm in panels E and F, respectively. (G and H) Seedling of stoloniferous *Gunnera manicata* showing the location of the stem gland (sg) (G) and tangential stem section of a giant *Gunnera chilensis* showing location of the *Nostoc* colonies (nc) (H). Photographs copyright Warwick Sylvester. Panel G reprinted from reference 22 with permission of the publisher. Bar, 1.0 cm.

pabilities common to free-living *Nostoc*. In all resulting symbioses, plants influence two developmental responses of *Nostoc*—the formation of hormogonia and the differentiation of heterocysts. Vegetative cells of *Nostoc* have a third developmental alternative in the differentiation into perennating spore-like cells called akinetes (6). Akinetes are hypothesized to be evolutionary precursors of heterocysts (214). Heterocysts and akinetes may (99) or may not (217) have common developmental regulatory elements. Akinetes are present in large numbers in older *Azolla* leaves and in the developing sporocarp, where they presumably play a role in generational continuity (141). However, akinetes have not consistently been observed in all symbioses, and there have been no studies on their symbiotically associated differentiation or germination.

In this section, information from studies of free-living cyanobacterial strains on heterocyst and hormogonium developmental responses is considered. In subsequent sections, we will examine possible mechanisms by which plants may draw on the free-living signal transduction systems to assert symbiotic control.

Patterned Differentiation to Heterocysts, Well-Spaced Nitrogen-Fixing Cells

Filaments of *Anabaena* or *Nostoc* grown in the presence of a combined nitrogen source, such as NH_4^+ or NO_3^-, may consist solely of vegetative cells, morphologically indistinguishable from one another. When the source of nitrogen is removed (nitrogen step-down) or exhausted, heterocysts appear within one to two cell generations at nearly regular intervals along the filament (Fig. 1). Plants appear to alter both the number and the pattern of heterocyst spacing, perhaps to maximize the rate of N_2 fixation at the expense of cyanobacterial growth.

What are heterocysts, and how do they work? The major function of heterocysts is to provide a microoxic environment necessary for the production and proper functioning of nitrogenase and other proteins related to N_2 fixation (214). In essentially all heterocyst-forming cyanobacteria, heterocysts appear to be the only sites for N_2 fixation within the filament (48, 188). The exception is *Anabaena variabilis* strain ATCC 29413 (and a few closely related strains), which expresses a vegetative cell-specific nitrogenase under anoxic incubation conditions (188). The heterocyst achieves a near anoxic state by at least three means. First, photosystem II, the O_2-producing end of the photosynthetic electron transport chain, is dismantled during heterocyst differentiation (186), so that the heterocyst need contend only against O_2 produced by neighboring vegetative cells and that dissolved in the environment. Second, heterocysts are invested with a specialized envelope (214) that limits the influx of gases (199). Two layers within the envelope have been implicated in O_2 protection (130): an inner layer composed of a hydroxylated glycolipid and an outer layer of polysaccharide. Neither layer is found in vegetative cells. Third, much of the O_2 that overcomes these barriers is consumed by the high oxidase activity associated with heterocysts (144).

Abandoning complete photosynthesis allows the recycling of the amino acids contained in phycobiliproteins, light-harvesting pigments associated primarily with photosystem II that may account for up to 50% of the soluble protein within the cya-

nobacterial cell (21). Nitrogen deprivation elicits a general increase in proteolysis and degradation of phycobiliproteins (187, 218). At the onset of nitrogenase activity, phycobiliproteins return to their original levels in vegetative cells (23). The new heterocysts themselves, however, are grossly deficient in fluorescence associated with phycobiliproteins (197), as shown in Fig. 1C. After several days, heterocysts in some strains may regain this fluorescence (145, 190), but filaments tend to liberate aged heterocysts as single fluorescent cells. Loss of fluorescence in specific cells can thus be used as a diagnosis of differentiation; even in mutants that cannot complete differentiation, a pattern of well-spaced nonfluorescent cells is indicative of at least initiation of differentiation (48).

In the absence of complete photosynthesis, the heterocyst becomes dependent on adjacent vegetative cells for reduced carbon, just as the vegetative cells are dependent on heterocysts for reduced nitrogen. Nitrogen fixed within the heterocyst as ammonium is first converted to glutamine and then passes as amino acids to adjacent vegetative cells (191). In return, fixed carbon, probably sucrose (166), flows from vegetative cells to heterocysts (208). Sucrose synthase, or perhaps sucrose phosphate synthase (146), is present in high activity specifically in vegetative cells, while alkaline invertase, which converts sucrose to glucose and fructose, is confined largely to heterocysts (166). The resulting hexoses are catabolized in the heterocyst via the oxidative pentose phosphate cycle, providing reductant for nitrogenase and respiratory activities (181).

Heterocyst-forming cyanobacteria have made a number of adaptations in their nitrogen metabolism, which are important in our understanding of the basis of heterocyst differentiation and interactions with vegetative cells (Fig. 3). Like other cyanobacteria and, so far as we know, unlike any other organism on Earth, heterocyst-forming strains store amino nitrogen in a nonprotein polymeric form, as a branched polypeptide called cyanophycin (10, 111). Cyanophycin is a copolymer of aspartate and arginine and is present in both vegetative cells and mature heterocysts. Just as phycobiliproteins may serve as a source of amino nitrogen during differentiation, so may cyanophycin.

There is a partitioning in the enzymes for assimilation of N_2-derived NH_4^+ between vegetative cells and heterocysts (113, 191). In cyanobacteria, the glutamine synthetase (GS)-glutamate synthase (GOGAT) cycle is the route of NH_4^+ assimilation for growth (56). GS, which incorporates NH_4^+ into glutamate to form glutamine, is present in both cell types, but most, if not all, of the assimilation of N_2-derived NH_4^+ occurs in heterocysts. The bulk of the glutamate required by GS, however, must come from vegetative cells, because GOGAT is present at negligible levels in heterocysts (113, 191). GOGAT catalyzes the transfer of the amide nitrogen in glutamine to α-ketoglutarate.

Like glutamine, α-ketoglutarate may be made primarily in heterocysts; under N_2-fixing conditions, in vitro activity of isocitrate dehydrogenase (IDH) appears to be largely absent from vegetative cell extracts (136), although, based on immunological assays, the IDH protein is present in equal amounts in vegetative cells and heterocysts (113). IDH is highly active in heterocyst extracts, which appears anomalous, since the primary metabolic role of α-ketoglutarate is to serve as precursor to glutamate (α-ketoglutarate dehydrogenase is absent in cya-

FIG. 3. Metabolic interactions between heterocysts and vegetative cells. A lighter vegetative cell exchanges metabolites (thin lines) with a darker heterocyst bound by its characteristic thick envelope. The heterocyst has polar plugs at either end. Thick lines indicate metabolic pathways. The dotted line indicates a pathway whose existence is uncertain. Carbon dioxide is fixed in vegetative cells through the dark reactions of photosynthesis (PS), and the resulting triose is metabolized to pyruvate through the partial tricarboxylic acid cycle to isocitrate and then via IDH to α-ketoglutarate (αKG). The α-ketoglutarate combines with glutamine (Gln) via glutamate synthase (GOGAT) to form glutamate (Glu). In heterocysts, carbohydrate from vegetative cells enters the oxidative pentose phosphate (OPP) pathway to produce reductant (H:) used to support the activity of nitrogenase (Nase) to produce ammonium and concurrently yield α-ketoglutarate. Ammonium combines with glutamate, derived from the vegetative cell, through a reaction catalyzed by GS to form Gln. These reactions (if confirmed) should serve to reduce the level of α-ketoglutarate in the vegetative cell (small type) and increase it in heterocysts (large type). See the text for a discussion of the proposed cellular levels of α-ketoglutarate.

nobacteria, which thus lack a complete citric acid cycle [170]). However, IDH-generated NADPH may also contribute to the reductant pool for nitrogenase and respiration in heterocysts (109). In brief, glutamine is made in heterocysts, using glutamate transferred from vegetative cells, derived from α-ketoglutarate, which may arise in heterocysts. A possible rationale for this state of affairs in which α-ketoglutarate also serves as a signaling molecule will be offered in a subsequent section.

What are the regulatory circuits in heterocyst differentiation? Much of what we know about heterocyst differentiation comes from the study of the nonsymbiotic laboratory strain *Anabaena* sp. strain PCC 7120. Based on DNA-DNA hybridization and sequence analysis of DNA encoding small-subunit rRNA, this strain is closer to those in the genus *Nostoc* (160), but since the name is entrenched in the literature we will continue to refer to it as *Anabaena* strain PCC 7120. Within one or two generations (about 18 to 36 h under standard conditions), well-spaced vegetative cells suddenly deprived of a source of combined nitrogen differentiate into N_2-fixing heterocysts. The program of development begins with the sensation of nitrogen deprivation and culminates in the expression of the N_2 fixation apparatus in the mature heterocyst. In between, there is an ordered sequence of events controlled to a great degree at the level of transcription.

Heterocyst differentiation is inhibited by the presence of a usable source of nitrogen, such as NH_4^+ or NO_3^-. Even 3 to 7 μM NH_4^+ is sufficient to repress the appearance of heterocysts in *Anabaena* strain PCC 7120 (122). Since heterocysts persist in *Nostoc* within symbiotic cavities despite a level of ammonium that is much higher than this (35, 36, 118, 120, 169), it is imperative to understand the basis of the repression. The following discussion will not detail all genes now known to

influence heterocyst formation and function; a more thorough listing can be found in references 211 and 212.

(i) Earliest signals in heterocyst differentiation. In *Escherichia coli* and other proteobacteria, nitrogen starvation is signaled in part by the *glnB*-encoded protein P_{II}, which interacts with GlnE to modify GS activity by adenylylation and interacts with NtrB to modify the regulatory activity of NtrC by phosphorylation (124). NtrC-P enhances the transcription of several genes that respond to nitrogen conditions, including *glnA*, the structural gene of GS. The metabolic status of the cell is sensed directly by P_{II} through its binding to α-ketoglutarate and ATP and through a uridylyltransferase (encoded by *glnD*) that modifies P_{II} in response to the levels of glutamine.

P_{II} is the only component of this regulatory web that has been found in cyanobacteria (65, 70, 108, 192). In unicellular cyanobacteria, the P_{II} protein is modified by phosphorylation, and not uridylylation as in *E. coli* (58), in response to the level of α-ketoglutarate (83). Unmodified P_{II} appears to regulate nitrate assimilation in unicellular *Synechococcus* sp. strain PCC 7942 (96). While a mutant strain of *Synechococcus* strain PCC 7942 in which *glnB* has been insertionally inactivated is minimally affected in nitrogen-related functions (59), it has proven impossible to obtain a similar mutant in *N. punctiforme*, implying that the *glnB* gene is essential in heterocyst-forming cyanobacteria (70). It should be noted that cyanobacteria, unlike many bacteria, possess only one GlnB-like protein (11) (see http://www.jgi.doe.gov/ and http://www.kuzusa.or.jp/cyano/).

The earliest documented step in the response of cyanobacteria to nitrogen deprivation is activation of the DNA binding protein NtcA (77, 110). Mutants lacking functional *ntcA* are unable to respond to nitrogen deprivation; they fail to increase levels of GS, to respond to alternate nitrogen sources (such as

NO_3^-), to make heterocysts, or to induce nitrogenase expression (61, 200). Many genes whose transcription is altered by nitrogen deprivation (61) are preceded by a consensus sequence (GTAnCaannnnTAC in *Anabaena* strain PCC 7120 [77]) to which NtcA binds (153). One such gene is the NtcA-regulated gene *glnB* from the non-heterocyst-forming cyanobacterium *Synechococcus* strain PCC 7942 (97). Transcription of *glnB* from *N. punctiforme* is also induced by nitrogen limitation (70). NtcA binding activity is present in both vegetative cells and heterocysts (153). Although the transcription of *ntcA* is subject to variation over the course of heterocyst differentiation (200), perhaps owing to a NtcA binding site 5′ to the gene (154), the means by which NtcA controls heterocyst differentiation remains unknown.

An unresolved question is the mechanism by which NtcA directly or indirectly senses nitrogen status or, even more generally, how nitrogen status is translated into the decision of a cell to differentiate. It would be convenient if the known ability of P_{II} to sense levels of α-ketoglutarate could account for that decision, but if so, the role of the protein in heterocyst-forming cyanobacteria would be significantly different from that in unicellular strains. The regulation of transcription of nitrogen-regulated genes in unicellular *Synechococcus* strain PCC 7942 is little affected by the absence or modification of P_{II} (59), although the state of the protein is critical in mediating the repression of nitrate/nitrite transport by ammonium (96). The nitrogen status in cyanobacteria thus appears to be sensed in two different ways: by P_{II}, leading to the alteration of enzyme activity, and (perhaps indirectly) by NtcA, leading to the alteration of gene transcription.

The ability of ammonium to suppress heterocyst differentiation is widely believed to be indirect, requiring the activity of GS. When ammonium assimilation in *A. cylindrica* (164) and other cyanobacteria (210) is blocked by the presence of methionine-DL-sulfoximine, an inhibitor of GS, heterocysts differentiate in the typical spacing pattern in the presence of ammonium. This result implies that the suppressor signal must be a consequence of ammonium assimilation, such as increased glutamine or decreased α-ketoglutarate levels. This conclusion has been questioned as the result of experiments exploiting the ability of *A. variabilis* to express an alternative nitrogenase in vegetative cells under anoxic growth conditions (189). Under these conditions, this strain can grow on N_2, even when the heterocyst-specific nitrogenase is inactivated by mutation. Despite the production of ammonia by the alternative nitrogenase, expressed uniformly in all vegetative cells, heterocysts persist and with the usual spacing. The normal appearance of heterocysts has been taken as evidence that exogenous ammonium is metabolized differently from that produced by endogenous nitrogenase and that only the former inhibits differentiation (189). Alternatively, the minimal level of fixed nitrogen required to suppress differentiation may be markedly higher than the minimal level required to support growth. This is a reasonable supposition, since it is consistent with the behavior of *A. cylindrica* in more natural circumstances, where the cyanobacterium responds to gradually diminishing supplies of fixed nitrogen well before it is exhausted (111).

Fragmentary evidence points to a crucial role of DNA replication early in heterocyst differentiation. The DNA-damaging agent mitomycin C (5) and the DNA gyrase inhibitor nalidixic acid (M. Gantar and J. Elhai, unpublished results) block heterocyst differentiation when individually applied during the first hour after nitrogen deprivation. Mitomycin C at the effective level has no discernible effect on new RNA synthesis. A mutant of *Anabaena* strain PCC 7120 defective in a gene, *hanA*, which encodes a histone-like protein (HU) is blocked in the earliest known stage of heterocyst differentiation (89). HU protein from *Anabaena* strain PCC 7120 is able to substitute for *E. coli* HU protein to promote the initiation of DNA synthesis in vitro (132). *hanA* is preceded by a NtcA binding site. HU protein is by far the most abundant DNA binding protein in vegetative cells, based on autoradiography of acrylamide gels, The vegetative cell band is replaced by a slightly larger DNA binding protein on gels of heterocyst proteins (132), but the protein has not yet been characterized.

Heterocyst differentiation is also blocked by ethionine (93), an analogue of methionine. At the micromolar levels that are effective, ethionine has no effect on bulk protein, DNA, or RNA synthesis or on growth, but it reportedly prevents the rise in *hetR* expression (P. S. Duggan and D. G. Adams, personal communication) that normally accompanies the earliest stage of differentiation. Ethionine may act by affecting *S*-adenosyl-methionine-dependent methylation of DNA. Such a mechanism has been demonstrated to explain the induction by ethionine of spores of *Bacillus subtilis* (9).

(ii) Signals specific to early differentiating cells. Several genes have been found that are required for nitrogen deprivation to evoke any visible sign of heterocyst differentiation. One of these, *hetR*, clearly plays a central role in the decision of a nitrogen-starved cell to differentiate. Mutants with mutations in *hetR*, unlike those with mutations in *ntcA*, are able to grow normally in NO_3^- (unaffected in a general response to nitrogen deprivation), but they do not differentiate. On the other hand, strains in which wild-type *hetR* is present on a multicopy plasmid (94) form heterocysts even in the presence of NO_3^- (27), and the fraction of cells that become heterocysts when *hetR* is overexpressed in the absence of combined nitrogen can reach 29% (28).

The expression of *hetR* also is consistent with a role for the protein as an important molecular switch. Transcription of *hetR*, which is low in vegetative cells, begins to increase as little as 0.5 h after nitrogen deprivation (20, 27, 28, 216). By 3.5 h after deprivation, expression (P_{hetR}::*luxAB*) is confined largely to a small fraction of cells, with a spacing in the filaments similar to that of heterocysts, even though morphological differentiation is not evident until many hours later. Presuming that all the increase in *hetR* expression is confined to cells with high expression, the increase per cell can be estimated to be 20-fold, relative to expression under nitrogen-replete conditions (20). The starvation-dependent increase requires functional *ntcA* (61, 216) and *hanA* (89). The increase also requires the function of HetR, positively regulating its own expression (20). A positive autoregulatory switch, which magnifies small differences in expression and locks expression into one of two states, is known to stabilize many developmental decisions: lysogeny by coliphage lambda (147), cell type determination by *Bacillus* (92, 182), and commitment to different developmental fates by eukaryotic cells (7, 123).

The predicted amino acid sequence of HetR gives no clues to its function. For example, it possesses none of the sequence

motifs typical of DNA binding proteins (27). Only one function of the protein, autoproteolytic activity, has been detected, evidently stimulated in vivo by the presence of a nitrogen source (223). The active site for proteolysis is required for heterocyst differentiation although not for the HetR-dependent induction of *hetR* (44). The protein appears to be modified in vivo, since HetR isolated under nitrogen-depleted conditions is considerably more acidic than the protein isolated from nitrogen-replete cells or than the recombinant protein isolated from *E. coli* (222).

Two genes, *hetF* and *patA*, appear to positively influence heterocyst differentiation by modulating the expression or activity of HetR. The *hetF* gene, best characterized in *N. punctiforme* (216), encodes a constitutively expressed protein that is essential for heterocyst differentiation; *hetF* mutants do not initiate even the earliest stages of heterocyst differentiation. The absence of HetF does not alter NtcA-dependent *hetR* transcription, but its absence eliminates HetR-dependent *hetR* transcription and the subsequent accumulation of HetR in differentiating cells. HetF bears no obvious similarity to any known protein. A second protein, PatA, has multiple domains, the carboxyl-terminal of which has similarity to the receiver domain of response regulators (100). Mutants defective in *patA* form heterocysts only at the ends of filaments and are consequently unable to grow well on N_2 (100). The multiple contiguous heterocyst phenotype of strains overexpressing *hetR* is suppressed in *patA* mutants (28). These results imply that PatA is in the same regulatory circuit as HetR and may function downstream of *hetR* transcription to modulate HetR activity. Since HetR appears to be modified to a more acidic form in nitrogen-limited cultures, it is tempting to speculate that PatA is part of a phosphorelay signal transduction pathway that activates HetR by phosphorylation (28).

Another gene, *hetC*, encodes a product important in early heterocyst differentiation. Mutants insertionally inactivated in *hetC* fail to show any morphological signs of differentiation, but, as in wild-type filaments, mutant filaments lose fluorescent pigments in cells spaced as one would expect of differentiating heterocysts (90). Like *hetR*, *hetC* is induced early in the course of nitrogen starvation, is dependent on NtcA (128), and requires its own function for full expression.

HetC shows considerable sequence similarity to members of the superfamily of ATP binding cassette (ABC) proteins (90), which couple the cleavage of ATP to the transport of molecules across membranes (51). This similarity, the propensity of *hetC* mutants to exhibit pairs of nonfluorescent (partially differentiated) cells, and the confinement of expression of *hetC* to proheterocysts raise the possibility that HetC participates not in the differentiation of heterocysts but, rather, in the export of an inhibitor of differentiation (90).

Other heterocyst-specific genes have been identified that appear to play a role in later heterocyst differentiation (53, 211, 212). Most, like *hepA* (formerly *hetA* [82]) and *devBCA* (54, 112) are needed in the formation of the heterocyst-specific envelope, and so it is not surprising that they are expressed specifically in well-spaced and presumably differentiating cells (112, 213). The epistatic relationships between the genes required for heterocyst differentiation are just beginning to be worked out (28, 29).

The diversity in times after nitrogen step-down at which genes become active implies that there may be a hierarchy of transcriptional regulators, each controlling the expression of a set of genes at a characteristic time. Such ideas are inspired by the interlocking tiers of regulators and their modifiers that govern differentiation in *B. subtilis* (180) and other bacteria (161).

Much attention has been given to the role of sigma factors, modular subunits of RNA polymerase that determine promoter specificity in bacteria, in defining the course of differentiation. While alternative sigma factors have been identified in filamentous cyanobacteria (25, 32), none have so far been found to markedly influence the course of heterocyst differentiation. In fact, of the eight group 2 sigma factors that can be readily discerned from the sequence of *Anabaena* strain PCC 7120, all except the essential main sigma factor (24) are dispensable for heterocyst differentiation (88a; I. Khudyakov and J. Golden, personal communication).

Nonetheless, it is very likely that some such differentiation-specific sigma factors or other proteins regulating transcription initiation exist. Several genes are now known that are expressed in both vegetative cells and heterocysts. In such cases, transcriptional start sites have proven to be different under nitrogen-replete and N_2 growth conditions (24, 55, 193). Sites active in nitrogen-replete cultures are preceded by sequences similar to the promoter sequence recognized by the principal *E. coli* sigma factor, while sites active under N_2 growth conditions are preceded by quite different sequences (41). The principal sigma factor, attached to RNA polymerase from *Anabaena* strain PCC 7120, binds and transcribes only at sites presumptively active in vegetative cells (152, 167). Taken together, these results are consistent with a few possibilities: (i) combinations of redundant sigma factors are required for differentiation-specific transcription; (ii) *Anabaena* strain PCC 7120 possesses sigma factors essential for differentiation that cannot be recognized by their sequences; or (iii) proteins distinct from canonical sigma factors significantly alter the specificity of RNA polymerase during differentiation. Regardless of which possibility, if any, is true, an increased understanding of transcriptional regulation during heterocyst differentiation promises to teach us something new about how bacteria regulate their behavior.

(iii) Signals specific to mature differentiated cells. A large suite of genes begins to be expressed at about the same time after the emergence of immature heterocysts (proheterocysts), an event that takes place at about 12 h after the nitrogen step-down. Some of the new gene expression presumably relates to the increase in respiratory capacity that occurs in proheterocysts at about this time. The resulting drop in the intracellular O_2 concentration is required for expression of genes encoding nitrogenase (73), but low O_2 content and nitrogen deprivation are not sufficient for nitrogenase expression (48). Late gene expression is accompanied by at least three DNA rearrangements (72), removing insertions within genes encoding nitrogenase (67); a heterocyst-specific electron donor, ferredoxin (66); and a hydrogenase (37, 114). While ammonium has been reported several times to inhibit nitrogenase activity in heterocysts, this effect appears to be indirect, at least in *A. variabilis* ATCC 29413, acting by alteration of oxygen levels (50). At present, it is not clear whether nitrogenase

A

B

Probability of (HH) = a = a

Probability of (HVH) = (1-a) * a = a*(1-a)

Probability of (HVVH) = (1-a)*(1-a) * a = $a*(1-a)^2$

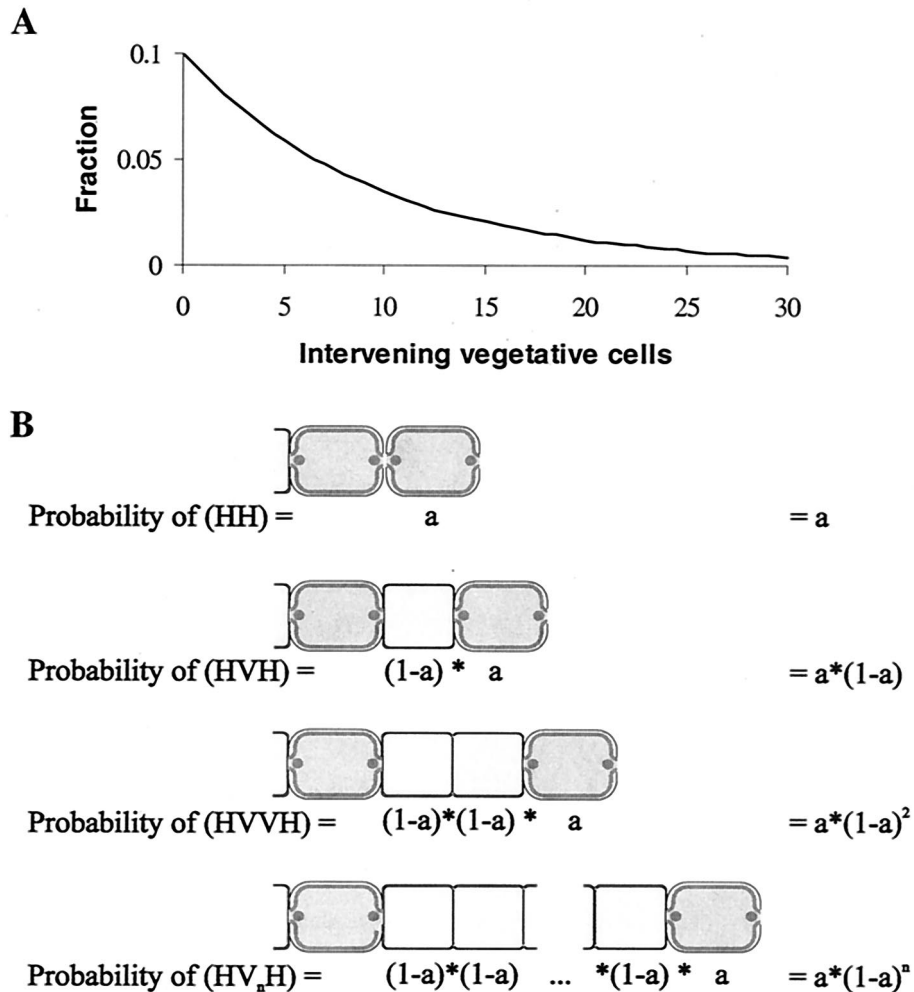Probability of (HV$_n$H) = (1-a)*(1-a) ... *(1-a) * a = $a*(1-a)^n$

FIG. 4. Distribution of randomly spaced heterocysts. (A) Calculated distribution of heterocysts if each cell differentiates with a probability of 0.1 (inspired by references 207 and 209). The graph shows the fraction of intervals with a given number of vegetative cells intervening between two heterocysts, according to the formula $P_n = a(1 - a)^n$, where n represents the number of intervening vegetative cells and a is the probability that a specific cell differentiates. The average heterocyst spacing is $(1 - a)/a$, which in this case is one heterocyst every 9 vegetative cells. (B) Graphical derivation of the formula that predicts the distribution of heterocysts given random differentiation.

synthesis and activity within mature heterocysts in general is directly repressed by nitrogen in the environment.

How is the spacing of heterocysts determined? From the perspective of a developmental biologist, the most striking behavior of *Anabaena* and *Nostoc* is the elaboration of spaced heterocysts at nearly regular intervals along their filaments (Fig. 1B). The nonrandomness of heterocyst spacing in wild-type *Nostoc* is obvious by inspection, but for purposes of comparison, it is important to have a clear picture in mind of what a truly random distribution of heterocysts would look like. Figure 4A shows a random distribution of heterocysts, calculated on the assumption that each cell differentiates, or not, independently of other cells in the filament.

The monotonic decline in probability as the heterocyst distance increases may be surprising at first glance, since many feel intuitively that the peak probability should coincide with the average heterocyst spacing. This is not the case, for reasons illustrated in Fig. 4B. Suppose that each cell differentiates with a fixed probability, say 10% (given as **a** in Fig. 4B). If one

wanted to find an interval of a specific length, say a given heterocyst separated from another heterocyst by 9 vegetative cells, then certainly the cell 9 vegetative cells away from the first heterocyst would need to differentiate. This, by hypothesis, would occur 10% of the time. However, this is not enough. Even if the cell differentiates, the interval would still be less than 9 vegetative cells any time an intermediate cell also differentiates. Therefore, the probability of an interval with of exactly 9 vegetative cells from a given heterocyst is reduced by the probability that each intervening cell does not differentiate. Seen this way, the probability of a shorter interval is always greater than the probability of a longer interval, because fewer intervening cells are required not to differentiate.

In fact, the peakless function predicted by random differentiation bears little resemblance to the actual distribution of intervals (Fig. 5A), and so heterocyst spacing is demonstrably nonrandom. The bias toward even-numbered intervals is expected if cells destined to vegetative cells status, but not those marked for differentiation, divide precisely once prior to the
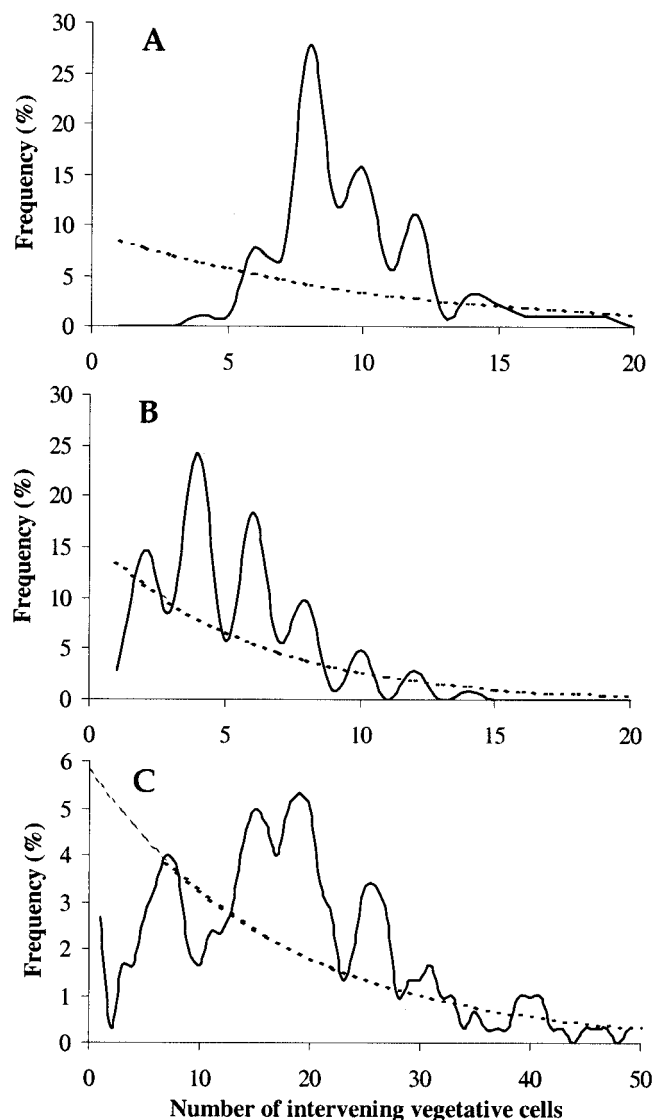
FIG. 5. Spacing of heterocysts. Distances between heterocysts, measured as the number of intervening vegetative cells, are given, and their relative frequencies by actual count (solid line) and by calculation presuming no influence of one cell on another (dashed line) (see Fig. 4 for the equation defining *P*, the probable heterocyst frequency). The value of *a*, the probability that a cell differentiates, was taken to be the heterocyst density (heterocysts per total cells) from the given data. Graphs inspired by reference 215, using data taken from reference 221. The frequencies of interheterocyst intervals of length zero are not shown in the first two panels. (A) Wild-type *Anabaena* strain PCC 7120 grown on nitrate and shifted to no fixed nitrogen for 24 h (*P* = 9.5%). (B) *patS Anabaena* strain PCC 7120 mutant grown on nitrate and shifted to no fixed nitrogen for 24 h (*P* = 16.1%). (C) *patS Anabaena* strain PCC 7120 mutant grown on ammonium and shifted to nitrate for 96 h (*P* = 5.6%). Owing to the dispersed nature of the data under the conditions in panel C, the frequencies have been displayed as a rolling average, averaging three intervals at a time centered about the given number.

time of observation. A model that accounts for all spatial influences on heterocyst differentiation should be able to predict the distribution in Fig. 5A. Furthermore, if all the influences comprising the model can be removed (e.g. by muta-

tion), the model predicts that heterocyst spacing should then be random, i.e., as shown in Fig. 4A.

We have found it useful to consider first a minimal set of assumptions (the one-stage model) that can explain the observed pattern of heterocyst differentiation. To accommodate inconsistencies in this model, as well as more recent observations and concepts, we shall then modify the first model into what we will call the two-stage model.

(i) Characteristics of the one-stage model. Wolk and Quine (215) showed by computer simulation that two assumptions were sufficient to reproduce a pattern of spaced heterocysts indistinguishable from that actually observed in filaments of *A. cylindrica*. First, they assumed that any cell is competent to differentiate at the moment when nitrogen is removed from the environment and that the choice of cells that initiate differentiation is random. Second, they postulated the existence of a diffusible inhibitor made by heterocysts and differentiating cells and consumed by nondifferentiating cells, as predicted by experimental data (127, 204, 205, 207, 210).

The model is attractive for its simplicity and because its elements may be interpreted in ways that are physically appealing. Figure 6 illustrates one interpretation of the one-stage model, in which differentiation is initiated in cells that are randomly distributed through the filament and have attained a critical level of starvation for nitrogen. These cells respond both by synthesizing the components particular to heterocysts and by releasing a compound that inhibits adjacent cells from differentiating. The compound may pass from cell to cell through junctions or through the periplasm, the region between the cytoplasmic membrane and the outer membrane of gram-negative bacteria that includes the peptidoglycan polymer. Because the outer membrane is contiguous in filamentous cyanobacteria and does not envelop individual cells (46), the periplasm is thought to be common to all cells in the filament, at least in the interval between adjacent heterocysts.

Why is it that the inhibitor made by a differentiating cell, according to the model, does not inhibit the cell's own differentiation? First, the cell may have rendered itself oblivious to the metabolic effects of the signal. For example, if, early in differentiation, the cell turns off the synthesis of GOGAT and turns on the synthesis of IDH (129), then the concentration of α-ketoglutarate may be set at a high level. If α-ketoglutarate acts downstream from the target of the putative diffusible inhibitor, perhaps by blocking the modification of the P_{II} protein, as it does in *Synechococcus* strain PCC 7942 (83), then the effect of the inhibitor may be diminished.

Alternatively, the cell may have committed itself genetically to differentiation through a positive-feedback switch. The model of Wilcox et al. (204), similar to the ideas of Turing (194), included not only a diffusible inhibitor, Y, but also a nondiffusible component, X, that acts to increase its own synthesis, to increase the synthesis of inhibitor, and to promote differentiation. Once X has reached a level sufficient to feedback on itself, the cell may be irreversibly locked into a state of differentiation. Reasons to equate HetR with X have already been discussed.

Finally, the inhibitor may be made only after its export from the cell. One can imagine that PatS protein (see below) may be inactive until cleaved outside the differentiating cell (2).

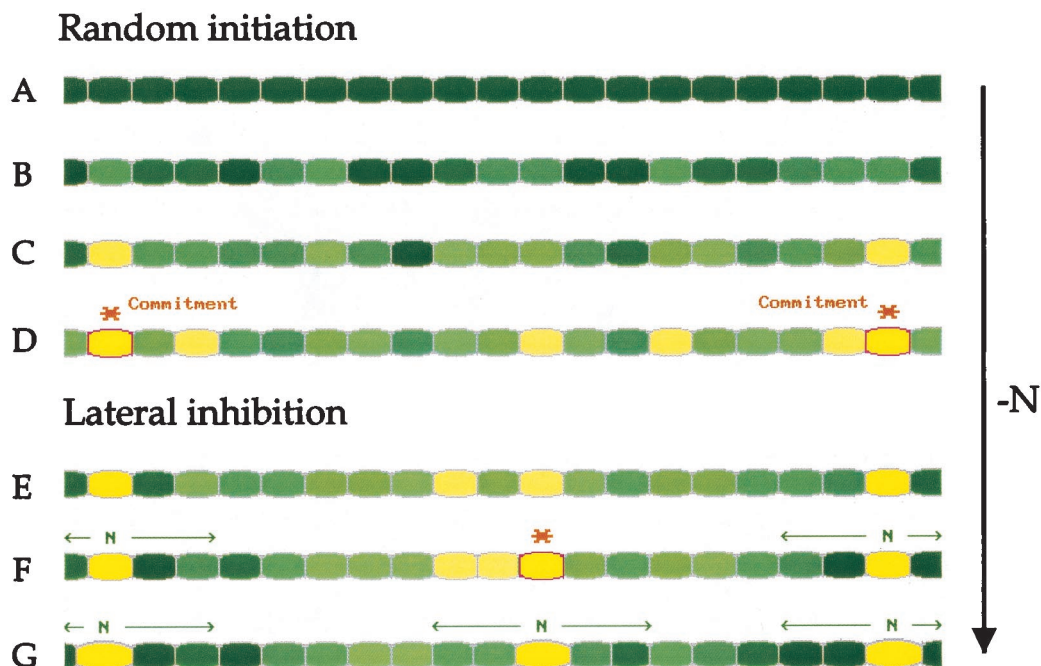## Random initiation

Lateral inhibition

FIG. 6. Physical interpretation of the one-stage model of heterocyst spacing: Each line represents a filament consisting of many cyanobacterial cells. The color within each cell represents its nitrogen status: the darker the color, the greater the amount of available nitrogen. (A to D) A filament is suddenly starved for nitrogen. Each cell draws on nitrogen reserves, postulated to be available in different cells to different degrees. When a cell has depleted its reserves to the extent that a critical level of starvation is reached (∗), it becomes committed to heterocyst differentiation. (E to G) Commitment is postulated to have two effects. The committed cell releases a signal (N) that diffuses to adjacent cells and prevents them from differentiating. In this interpretation, the signal is postulated to be a nitrogenous substance that feeds adjacent cells (symbolized by a darkening of their color). In addition, commitment prevents the committed cell from responding to its own inhibitor. Cells distant from the committed cells continue to starve, until one reaches the critical level. The position of the first cells that initiate differentiation is not critical to spacing. Spacing is determined by lateral inhibition.

(ii) Multiple contiguous heterocysts. The distinctive attribute of the one-stage model is that the pattern of heterocyst spacing in the filament is an outcome solely of lateral inhibition by differentiating cells. The pattern does not rely on the choice of cells initially selected to differentiate. In its basic form, the one-stage model postulates that initiation is random (215), but this need not be the case and evidently is not so. Wilcox et al. (204) reported seeing strings of adjacent differentiating cells in *A. catenula* that eventually resolved to single heterocysts, and they interpreted them as normal intermediates of pattern formation. Such intermediates would imply that initiation of differentiation is not random but, rather, takes place in clusters of contiguous cells along the filament.

Apart from the observations of Wilcox et al. (204), strings of differentiating cells have rarely been seen with wild-type strains of cyanobacteria, including *Anabaena* strain PCC 7120 (212); however, they are common results of chemical and genetic manipulations (Table 2) (for a more extensive discussion, see reference 214). Exceptionally high light intensity (4) and several chemicals often produce strings of two or more heterocysts where single heterocysts would otherwise be expected. Among the chemicals that produce strings of heterocysts are 7-azatryptophan (1, 38, 126), cyclic AMP (172), and rifampin (215), an inhibitor of RNA polymerase. Whether the strings produced chemically occur at random, suggestive of random initiation, or in a pattern, suggestive of a separate pattern-generating mechanism, has to our knowledge never been addressed. Mutant

strains that show contiguous heterocysts have been reported (19, 206, 216, 220).

HetR is connected with two examples of contiguous differentiating cells. First, when *hetR* is present on a multicopy plasmid in *Anabaena* strain PCC 7120 (27) and *N. punctiforme* (216), removal of nitrogen from the medium leads to the appearance of contiguous heterocysts. Second, hours before the first sign of morphological differentiation, there is a moderate increase in *hetR* expression (judged by fusion of the *hetR* promoter to green fluorescent protein), sometimes seen in groups of two or four contiguous cells in *Anabaena* strain PCC 7120 28; B. Buikema, personal communication). Under these conditions, only single heterocysts are ultimately observed, so that the initial distribution of *hetR* expression may represent a normally invisible coarse pattern that resolves to well-spaced individual heterocysts. Only hours later is *hetR* fully induced, and then only in well-separated, differentiating cells. *patS* expression provides a better studied case in which gene expression in contiguous cells precedes expression confined to heterocysts (221).

(iii) Apparent inconsistencies with the one-stage model. The one-stage model makes a few predictions that have sometimes not been fully borne out. Chief among these is the existence and behavior of an inhibitory signal emanating from differentiating cells. The one-stage model predicts that abolishing this signal would lead to random spacing of heterocysts while overproducing it might suppress heterocyst formation altogether.

TABLE 2. Conditions that markedly affect heterocyst differentiation and spacing

Gene or treatment (reference)	No heterocysts	Contiguous heterocysts	Function and interpretation
Positively acting systems			
ntcA (61, 200)	Mutant		Initiation: mediates sensing of nitrogen deprivation
hanA (89)	Mutant		Initiation?: HU histone-like protein
Ethionine[a]	1 μM		Initiation?: blocks DNA methylation
hetR (27)	Mutant	Multicopy	Resolution: master regulator of heterocyst differentiation
hetF (216)	Mutant	Multicopy	Resolution: regulates transcription of *hetR*
hetC (90)	Mutant[b]		Resolution: ABC transporter (of diffusible inhibitor?)
Negatively acting systems			
patS (220)	Multicopy	Mutant	Resolution: peptide signal
hetN (13, 19)	Multicopy[c]	Mutant	Resolution: unknown signal
7-Azatryptophan (38)		10 μM[d]	Resolution: unknown synthesis

[a] P. Duggan and D. Adams, personal communication.
[b] Pattern of nonfluorescent cells still present.
[c] Inhibits only secondary heterocysts; no effect on heterocyst differentiation within 24 h of nitrogen step-down.
[d] Effect is strain dependent.

Bauer et al. (13) found two regions of the chromosome that could suppress heterocyst formation when present in *Anabaena* strain PCC 7120 on a multicopy vector. One of the effective regions was narrowed to *patS*, a small open reading frame capable of encoding a 13- or perhaps 17-amino-acid polypeptide (220). Controlled overexpression of *patS* also led to suppression of heterocyst differentiation. Moreover, suppression was observed by exogenous application of a chemically synthesized peptide corresponding to the five C-terminal amino acids of PatS but not by other similar peptides or by the component amino acids. These results indicate that a secreted peptide encoded by *patS* might serve as the signal mediating lateral inhibition of differentiation.

Deletion of *patS* resulted in a mutant *Anabaena* strain PCC 7120 that made heterocysts even in the presence of nitrate and produced strings of heterocysts in the absence of a combined nitrogen source (220, 221). In the latter case, heterocyst clusters are spaced nonrandomly, as judged by the absence of a monotonic decline in frequency as the interheterocyst distance increases (Fig. 5B). Indeed, if one accounts for the fact that contiguous heterocysts diminish the distance between clusters by decreasing the number of dividing, nondifferentiating cells, then the distribution of interheterocyst distances of the *patS* mutant is much closer to that of the wild-type strain than is at first apparent. The PatS polypeptide, thus, cannot by itself be the signal called for by the one-stage model, since its loss does not lead to random differentiation. Rather, the polypeptide is evidently required to prevent contiguous differentiating cells from producing strings of heterocysts.

The absence of randomly distributed heterocysts in a *patS* mutant grown on nitrate (221) is equally informative. The presence of the nitrogen source should inhibit the production of the *hetN*-associated signal (see below), and one might not expect a gradient of nitrogenous compounds emanating from the nitrogenase-deficient heterocysts that appear in the *patS* mutant. Still, the distribution of interheterocyst distances is not random but, instead, is biased against short intervals; this can be seen by comparing Fig. 5C to the predicted random distribution of heterocysts in Fig. 4A. Thus, there is evidently another pattern-producing signal besides those associated with *patS* and *hetN*. The signal may be related to the position of cells

in their cell cycles, as discussed below, or the result of heterocysts serving as a sink for glutamate or a source of α-ketoglutarate, glutamine, or some other amino acid produced from glutamine (121). The presence of an exogenous nitrogen source does not necessarily imply the absence of a gradient of nitrogenous compounds.

The second heterocyst-suppressing region (13, 19), *hetN*, encodes a protein with similarity to NAD(P)H-dependent oxidoreductases involved in fatty acid biosynthesis. Mutants with insertions *hetN* exhibit an unstable phenotype that is initially similar to that of *patS* mutants but then changes to a total inability to differentiate heterocysts (19), presumably the result of secondary mutations (19,30). HetN is expressed exclusively in heterocysts and only after *hetR* is induced during nitrogen step-down experiments (13, 30). It therefore plays no role in the initial pattern of heterocyst differentiation. However, conditional *hetN* mutants exhibit, under nonpermissive conditions, multiple contiguous heterocysts from secondary rounds of differentiation (30).

Mutants that produce nonrandomly placed strings of heterocysts are therefore readily obtained. In contrast, no mutant has been reported in which heterocysts are spaced at random, contrary to what one would expect if pattern were produced by a single mechanism. We take from this the lesson that there is a complicated and dispensable machinery that resolves strings of differentiating cells but that the fundamental mechanism that produces well-spaced heterocysts is embedded in basic cellular functions (and thus immutable) or is the result of multiple redundant processes.

Mitchison et al. (127) observed that differentiation is apparent only in cells where 6 to 8 h have elapsed since cell division in *A. catenula*. This raises the possibility that the position of a cell within the cell cycle may govern whether it is competent to differentiate (5). Consistent with this idea, synchronization of a mutant of *Anabaena* strain PCC 7120 that grows as single cells gives a culture that rises and falls twofold in competence to differentiate over the course of a generation time (Gantar and Elhai, unpublished). If competence were tied to position within the cell cycle, then any preexisting pattern in filaments with respect to the cycle would contribute to the ultimate pattern of heterocysts. Such a pattern exists within filaments of *Anabaena*
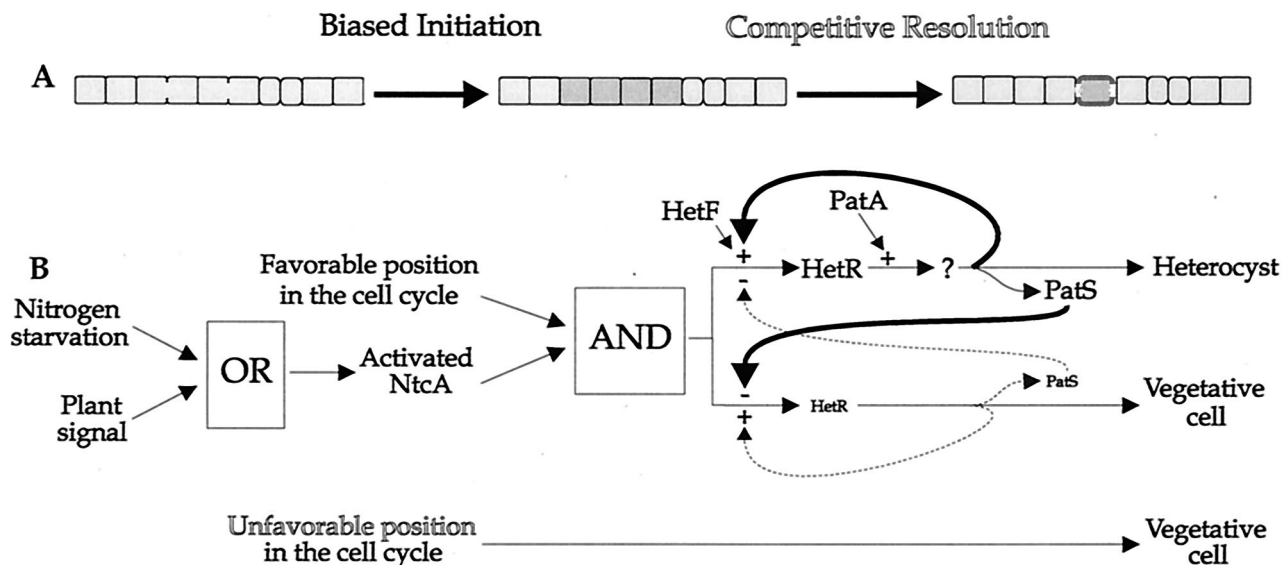
FIG. 7. Two-stage model of heterocyst spacing. (A) In response to nitrogen deprivation, four contiguous cells at similar stages in their cell cycles (shown here beginning cell division) initiate differentiation. The string of differentiating cells (dark) is resolved by competitive interaction to a single heterocyst (thick envelope). (B) Either nitrogen deprivation or the presence of a plant signal activates NtcA protein in all cells. The presence of activated NtcA protein and passage of the cell through a critical stage in the cell cycle induce *hetR* to a middle level of expression, mediated through HetF. Some initiate cells attain a PatA-dependent state (?; perhaps activation of HetR), characterized by a further induction of *hetR* expression. In these cells, PatS is highly expressed and diffuses to adjacent cells, where it is taken up and inhibits HetR expression. Cells where the positive intracellular HetR feedback loop (thick line) dominates become heterocysts. Cells where the negative intercellular PatS effect dominates revert to vegetative status. As they do, their own positive-feedback loop weakens as PatS inhibits HetR expression and those cells produce less PatS (dotted lines). Cells that are not passing through the critical stage of the cell cycle when nitrogen deprivation takes hold do not initiate differentiation and remain vegetative cells.

strain PCC 7120 grown on nitrate (R. Bucheimer, A. Meng, and J. Elhai, unpublished results). On average, neighboring cells tend to divide at similar times while cells separated by six or seven positions along the filament tend to divide about 180° out of phase.

(iv) Two-stage model of pattern formation. The one-stage model places the entire burden of pattern formation on lateral inhibition. The common appearance of contiguous differentiating cells in genetically or chemically perturbed strains raises the possibility that the pattern emerges in two stages: first, the development of a crude pattern consisting of regions of differentiating cells, and second, the resolution of these regions into single heterocysts. Influenced by the requirement of heterocyst differentiation for DNA replication (5, 6) and other results already discussed, we present a two-stage model in which one stage of pattern development is dependent on the position of a cell in the cell cycle (Fig. 7), but it is possible, of course, that the crude pattern may be governed by other influences.

(a) Stage 1 (initiation). All cells are postulated to experience the early effects of nitrogen starvation, presumably sensed directly or indirectly by NtcA. Only a fraction of the cells, possibly those in a critical stage of the cell cycle, are competent to initiate differentiation, and only in these cells is the transcription of *hetR* induced to the first, moderate level. The initiate cells are generally contiguous, since sibling cells are typically at the same stage in the cell cycle.

(b) Stage 2 (resolution). Expression of *hetR* prompts the synthesis and release of an inhibitory signal, presumably PatS (and, after long periods of starvation, perhaps a HetN-related product). Each of the cells that have initiated differentiation

must choose one of two directions away from their unstable state. A relatively high intracellular level of HetR would, through positive feedback, tend to increase the expression of HetR, committing the cell to differentiation. Conversely, a high level of PatS transported from adjacent initiate cells would tend to decrease the level of *hetR* expression, leading to regression.

The existence of genes whose mutation leads to the failure to initiate heterocyst differentiation when mutated and whose overexpression leads to multiple contiguous heterocysts, or vice versa (Table 2), is explicable by their roles in resolution: an overactive resolution stage eliminates all heterocysts, while an underactive resolution stage leaves the intermediate stage intact. By the same token, the many treatments that lead to multiple contiguous heterocysts also can be seen as interfering with resolution. Mutations in some genes are proposed to act on initiation; these include *ntcA*, through the requirement for nitrogen deprivation, and perhaps *hanA*, through the requirement for DNA replication.

Some observations do not fit readily into this simple model. A *patA* mutant produces heterocysts only at the ends of filaments (100), similar to the behavior of cyanobacteria of the genus *Cylindrospermum* grown continuously on N_2 (215). A *patB* mutant of *Anabaena* strain PCC 7120 (101) and a *patN* mutant of *N. punctiforme* (F. C. Wong and J. C Meeks, unpublished results) both exhibit a higher frequency of heterocysts than normal, but not as multiple contiguous heterocysts. However, an increased frequency is also seen in wild-type *A. cylindrica* exposed to high-intensity light (4). It is conceivable that a metabolic deficiency affecting cell division, such as that de-
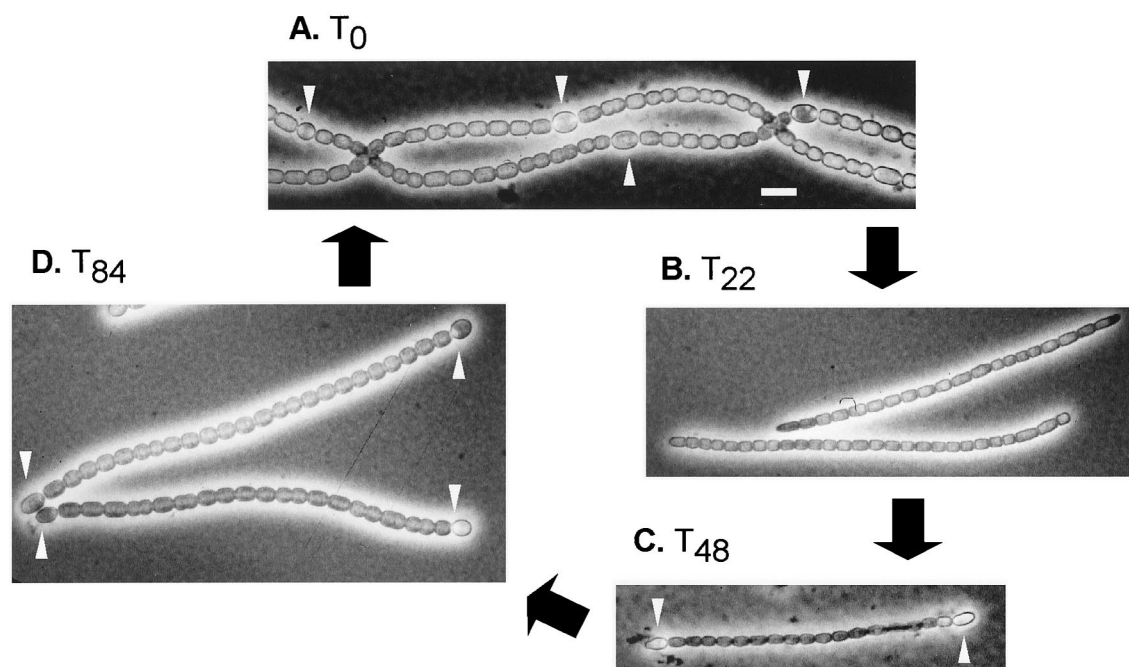
FIG. 8. Photomicrograph of filaments of *N. punctiforme* in the N_2-dependent vegetative growth state and at different stages of the hormogonium cycle. Phase-contrast images of vegetative filaments (A) and hormogonia (B to D) are shown. Note the process of heterocyst differentiation by the end cells of the hormogonium filaments starting at T_{48}(C), with the appearance of mature heterocysts and an increase in size of vegetative cells by T_{84}(D); this is followed by vegetative cell growth and division and eventually by differentiation of intercalary heterocysts. Bar, 10 μm.

scribed in the *patB* mutant, might account for the phenotype. Mitchison and Wilcox (125) observed that cell division in *A. catenula* is asymmetric and that heterocysts arise only from the smaller of the two daughter cells. While asymmetric division has been reported in only one other heterocyst-forming strain (4), asymmetry not apparent by light microscopy is still possible. The rules found by Mitchison and Wilcox (125) make the strong prediction that if adjacent cells differentiate (as in a *patS* mutant), they will be no closer than three generations distant (i.e., they will share the same greatgrandparent cell). The two-stage model predicts instead that the adjacent cells are most likely to be siblings.

Within the context of the two-stage model, it is possible to see that plants could influence the number of heterocysts by increasing initiation, perhaps by altering the cell's perception of its nitrogen status or by modulating resolution, or both.

Global Differentiation to Motile Filaments: the Hormogonium Cycle

What are hormogonia, and what is their role in the cyanobacterial life cycle? Hormogonia are short filaments that are released from parental filaments of a variety of heterocyst-forming and non-heterocyst-forming cyanobacteria (75, 184). They are distinguished from vegetative filaments primarily by their gliding motility, the small size of their cells (Fig. 8), and the absence of heterocysts. In some species, hormogonia cells contain gas vacuoles, which control buoyancy.

Hormogonia play a major role in short-distance dispersal of filamentous cyanobacteria, through their gliding motility when in contact with a solid substratum or their buoyancy in the

water column. Hormogonia of many strains display positive phototaxis (95), which is important in the colonization of illuminated portions of the habitat by these photoautotrophic organisms. While there is evidence of chemotaxis by a *Nostoc* species to extracellular products of a symbiotic plant partner (see below), there is none for chemotaxis by hormogonia of any species to inorganic or organic nutrients.

The mechanism by which hormogonia glide is unknown. A protein termed oscillin, which may be essential for gliding, has been identified in vegetative filaments of non-heterocyst-forming *Phormidium uncinatum* (81). Oscillin forms as fibrils external to the outer membrane of the cell wall and is proposed to passively direct the flow of mucilage between the filament and substratum. A similar mechanism may be involved in hormogonium motility.

The acquisition of motility comes at a price. Hormogonia are unable to fix nitrogen or grow. Phycobiliprotein synthesis ceases (42), leading through attrition to an accompanying decrease in fluorescence. Cells of hormogonia continue to photosynthesize and assimilate exogenous ammonium if it is available, although the rates of CO_2 fixation and NH_4^+ incorporation are only 70 and 62%, respectively, of those of vegetative filaments (33). The metabolic fates of the photosynthate and newly synthesized or regenerated amino acids in the non-growth hormogonium state are not known. However, much of the metabolic output is probably devoted to the production of the proton motive force (80) and the synthesis and secretion of mucilage (81) thought to be required for gliding motility.

Since cells within hormogonia do not grow, the state by necessity is transient. One can therefore describe a hormogonium cycle, starting with the induction of hormogonia and

ending with the return of hormogonia to the vegetative state within the equivalent of two or three generation times (Fig. 8). On induction, cell division begins at a nearly simultaneous rate in all cells of a filament, but not necessarily at the same time in all filaments. The result is that except for a very short period, filaments at a given moment consist of all newly divided cells (reflecting global differentiation) or virtually no newly divided cells (33, 42). There is no significant net synthesis of DNA or protein during hormogonium differentiation (75), although some protein synthesis is still required by the process (33); therefore, the smaller hormogonium cells result from multiple cell divisions in the absence of cell growth. Cell division without DNA synthesis is possible because cyanobacteria, in general, contain multiple copies of their genomes (74). Even in the absence of DNA replication, hormogonium cells are thus likely to receive at least one copy of the genome following multiple septations of the parental cell. Shortly after cell divisions, the filaments begin to fragment in a random manner. In filaments containing heterocysts, fragmentation takes place preferentially between heterocysts and their adjacent vegetative cells (33). The detachment of heterocysts from vegetative cells results in an interruption in the flow of reduced carbon from vegetative cells that supports both nitrogenase activity and respiration, and O_2 protection is lost. Detached heterocysts therefore cannot fix N_2, and nor can hormogonia.

The hormogonia remain in the gliding state for about 36 to 48 h, after which they cease to move. Only at this stage, in the absence of combined nitrogen, do heterocysts differentiate, first at the ends of the filaments and later, as the vegetative cells grow, with the typical spacing pattern within the filaments. Based on the resumption of pigment and total protein synthesis, the conversion of hormogonia back to vegetative filaments is complete within 96 h after their induction. Preliminary experiments indicate that cultures of *Nostoc* (E. L. Campbell and J. C. Meeks, unpublished data) and *Calothrix* (184) that have extensively differentiated hormogonia require a vegetative growth period before they are again able to differentiate a substantial number of hormogonia.

What induces the differentiation of hormogonia? Combined nitrogen deprivation is sufficient to induce heterocyst differentiation in all capable free-living cyanobacteria. In contrast, no single environmental factor has been identified that will induce hormogonium differentiation in all capable strains. The one common factor in initiation of hormogonium differentiation appears to be a change in some environmental parameter: an increase or decrease of a nutrient or a change in the quantity or quality of light. The most frequently used inducer of hormogonium differentiation in the laboratory is the transfer of a dense culture, near stationary growth phase, to fresh medium (159). This procedure has the possibility of multiple environmental changes, including the dilution of an endogenous repressor of hormogonium differentiation (63, 75), increased light intensity, and replete nutrients. We consider these changes to signal either a positive or negative stress condition for growth. We attempt below to find some order in a complicated set of observations.

(i) Hormogonium differentiation in response to changes in the chemical environment. When certain strains of either *Calothrix* or *Nostoc* (including symbiotic *N. punctiforme*), grown in the presence of combined nitrogen, are transferred to hor-

mogonium-inducing conditions in the absence of combined nitrogen, the majority of filaments enter into the hormogonium cycle rather than differentiate heterocysts (42, 64, 75). The extent of hormogonium induction after such a shift is variable and may depend on the position of the culture in the growth curve. Under these conditions, hormogonium differentiation supersedes nitrogen-dependent differentiation of heterocysts. Heterocysts appear in these cultures only as the hormogonium filaments reenter the vegetative growth state. These observations also establish that hormogonium differentiation and heterocyst differentiation are mutually exclusive processes in any one filament.

In addition to nitrogen-limited stress signaling, supplementation with excess nutrients, such as iron (45) or phosphate (219), induces hormogonium differentiation in certain strains. Osmolarity may also serve as a stimulus, as illustrated by a symbiotic *Nostoc* strain that consistently forms hormogonia following an immediate decrease in osmolarity previously established by glucose or salt (91). With some other strains of *Nostoc*, a drop in ionic strength triggers hormogonium formation (12, 63). The mat-forming thermophilic cyanobacterium *Mastigocladus laminosus* (distantly related to *Nostoc* and *Calothrix*) produces hormogonia when liquid suspension cultures are transferred to agar-solidified plate medium, perhaps in response to water stress (76).

(ii) Hormogonium differentiation in response to changes in light quality. Many *Nostoc* and *Calothrix* strains grown in green or white light differentiate hormogonia when the cultures are switched to growth in red light (184). This manipulation may seem at first no more than a laboratory curiosity, but it reflects the ambient conditions faced by a cyanobacterium when shielded by plant tissue or when buried deep in the water column. The induction of hormogonia by red light can be reversed by subsequent exposure to green light. This photoreversible response bears similarities to the process of complementary chromatic adaptation (CCA), in which certain cyanobacteria alter their pigmentation in order to use most efficiently the wavelengths of incident light (69). However, the two processes are not linked (184). CCA occurs in strains that do not form hormogonia, and many hormogonium-producing strains are incapable of CCA.

Photoreversible hormogonium induction in CCA-responsive *Calothrix* appears to be a consequence of an imbalance in electron flow through the photosynthetic electron transport chain rather than the direct action of a morphologically active pigment (31). In cyanobacteria, short-term exposure to red light tends to oxidize the electron transport components in the chain between photosystems II and I, while green light tends to reduce those components. Inhibitors that block the reduction of the quinone pool of the electron transport chain mimic red light and induce hormogonium formation while blocking heterocyst differentiation. Conversely, inhibitors that block oxidation of the quinone pool mimic green light and inhibit hormogonium formation while allowing heterocyst differentiation (31).

The mechanisms by which an imbalanced electron transport signal (i.e., the oxidation-reduction state of the quinone pool) could be integrated into the developmental response are unclear. It has been suggested that the P_{II} protein, described earlier as a sensor of carbon and nitrogen status, also acts as a

sensor of the oxidation state of the quinone pool (184). However, there is currently no direct evidence for an essential role of the P_{II} protein in hormogonium differentiation.

(iii) Differential gene expression in the hormogonium cycle. It is not clear how much specific gene expression occurs during the hormogonium cell cycle or if the major response is the activation or inactivation of constitutively expressed proteins. To date, only genes encoding gas vesicles in *Calothrix* (42) and an alternative sigma subunit in *N. punctiforme* (32) have been shown to be specifically transcribed in hormogonia. The transcription of other genes may be enhanced from a constitutive level in vegetative cells.

PLANT CONTROL OVER THE INFECTION PROCESS

All symbiotically competent filamentous cyanobacteria differentiate hormogonia, and these motile filaments function as the infective units in the formation of a symbiotic association with plants (16, 115, 116). For any individual hormogonium, the time window for infection is that of active gliding, or the period of 12 to 60 h after induction. Plants appear to send out chemical signals that influence at least three characteristics of the hormogonium cycle in relation to the infection process: (i) induction of hormogonium differentiation (a higher frequency of hormogonium differentiation in the cyanobacterial population will increase the probability of an infection event); (ii) control of hormogonium behavior (use of chemoattractants to influence the direction and perhaps speed of gliding in colonization of the symbiotic cavities); and (iii) regulation of the period between exit from and reentry into the hormogonium cycle (this response prevents continual entry into a nongrowth state in the presence of a stimulus and allows the differentiation of heterocysts).

Plant Induction of Hormogonium Differentiation

There is evidence for the extracellular production of one or more hormogonium-inducing factors (HIF) by four plant partners, the hornwort *Anthoceros* (33), the liverwort *Blasia* (91), the cycad *Zamia* (135), and the angiosperm *Gunnera* (155), as well as a plant not known to participate in symbioses, wheat (63). *Anthoceros* and *Blasia* produce the HIF activity in the suspension medium of liquid cultures (conditioned medium). In *Zamia*, the activity appears to be present in an aqueous extract of seeds and to be secreted from the specialized coralloid roots of the plant. In *Gunnera*, the HIF activity is present in the acidic mucilage that is excreted onto the surface of the stem by the stem glands that are colonized by the *Nostoc* organisms. The HIF activity is produced by the roots of wheat seedlings and exuded into the medium of hydroponic cultures.

The HIF has yet to be isolated and characterized from any plant. However, the activity from both *Anthoceros* and *Gunnera* exudates is more easily interpreted as a direct effect of the plant-derived substance on cyanobacteria rather than as a secondary effect acting through the chemical environment. For examples, low pH (5.0) and darkness tend to inhibit hormogonium differentiation, but the presence of exudate from *Gunnera* and *Anthoceros* overrides the inhibition inducing hormogonia without changing the bulk pH. The fact that seed rinses and aqueous extracts of leaves, stems (apart from the gland area),

and roots of *Gunnera* do not induce hormogonia indicates that the activity in the gland mucilage is tissue specific and not a nonspecific plant metabolite. Moreover, in *Anthoceros*, the HIF activity is produced primarily by nitrogen-limited tissue (33). The activity therefore appears only when N_2-fixing cyanobacteria would be useful to the plant.

It is not clear whether the HIF activities prepared from different plants represent similar or different substances. HIF activity from both *Anthoceros* (33) and *Gunnera* (155) is inactivated by heat (autoclaving) and, based on dialysis experiments, correlates with a small molecule of between 0.5 and 12 to 14 kDa. In contrast, HIF from wheat was retained by dialysis tubing with a 12- to 14-kDa cutoff (63). The HIF activity was lost in *Gunnera* mucilage treated with proteinase K, indicating that the HIF could be a small polypeptide or a ligand-modified polypeptide. On the other hand, HIF activity from *Anthoceros* was complexed by polyvinylpyrrolidone, which characteristically binds polyphenol-like molecules. Very different stabilities have been reported for HIF activities from different plants (39, 91, 155), but the conditions were too dissimilar to permit comparison.

Behavioral Characteristics of Hormogonia during Infection

There exist *Nostoc* strains that differentiate a high frequency of motile hormogonia in response to HIF yet either do not infect *Gunnera* (85) or *Anthoceros* (49) tissues or cause a delayed, low level of *Anthoceros* infection (116). This implies that an additional specific response, such as chemotaxis by the *Nostoc* strain to the plant tissue, is required for efficient infection. Indeed, since N_2-fixing cyanobacteria are in low abundance in soils, it is difficult to imagine how new plant-cyanobacterium associations might arise without chemotaxis.

Hormogonia of *Nostoc* are positively phototactic (95), yet they migrate away from light in the colonization of interior gland cells in the *Gunnera* association. Based on this observation, Johansson and Bergman (84) suggested that chemotaxis must be involved in the infection of the gland cells. However, a chemotactic response could not be observed in specific plate assays using the gland mucilage (155). Knight and Adams (91) unequivocally demonstrated chemotaxis of a symbiotic *Nostoc* strain toward exudate from *Blasia*, using preinduced hormogonia to separate the phenomena of chemotaxis from hormogonium induction.

Evidence of specific cyanobacterial responses beyond hormogonium induction is emerging from studies of the infection of *Anthoceros* by genetically altered *Nostoc* mutants. Mutation of an alternative sigma factor (SigH) of RNA polymerase in *N. punctiforme* results in a sixfold-higher initial infection of *Anthoceros* compared to that by the parental strain (32). Relative to the parental culture, the mutant does not differentiate more hormogonia, nor do the hormogonia remain in a motile state for longer or reenter the hormogonium cycle more frequently. Therefore, it is the behavior of the hormogonia that appears to be altered in the mutant. *sigH* is not transcribed under various stress or vegetative growth conditions, with or without combined nitrogen, but it is specifically induced in a burst pattern between 1.5 and 6 h following exposure to *Anthoceros* conditioned medium containing HIF. The genes whose transcription

is dependent on SigH are not known, but they clearly modulate the infection process in some manner.

Conversely, mutation of *ntcA* in *N. punctiforme* yields a non-infective phenotype in the *Anthoceros* association (217). While the *ntcA* mutant forms hormogonia following shifts in growth conditions, its response to HIF is markedly less strong than that of wild-type cultures and the hormogonia formed by either method fail to infect *Anthoceros* tissue. Since mutants unable to fix N$_2$ infect *Anthoceros* at a rate similar to that of wild-type cultures, the symbiotic defect in the *ntcA* mutant is not related to the inability of the mutant to fix nitrogen. These observations indicate that NtcA may have a wider role than response to nitrogen status in cyanobacteria.

Plant Regulation of the Hormogonium Cycle

Hormogonia are a nongrowing developmental state; therefore, the continual entry into the hormogonium cycle is presumably lethal by extinction. When in prolonged coculture with *Anthoceros*, very few of the *Nostoc* filaments, both within and outside of the symbiotic cavities and in the suspension medium, are in the hormogonium state (49), yet HIF is continually present (33). Similarly, *Nostoc* appears to show a growth stimulation during prolonged incubation on plates with gland mucilage from *Gunnera*, which also contains HIF (155). These observations imply that *Nostoc* must have a mechanism to block either the sensing of or the response to the presence of HIF. Various *Nostoc* strains produce an unidentified autogenic repressor of hormogonium differentiation into the culture medium (63, 160, 162). It is conceivable that plants also produce an activator of the synthesis of this or a similar repressor.

The benefit of such a repressor to the plant would not be in blocking hormogonium formation in the immediate surrounding (i.e., the medium) but in the symbiotic cavities after infection has occurred. Hormogonium formation is clearly counterproductive to nitrogen fixation, and the provision of fixed nitrogen by the cyanobacterium for the plant is presumably the selective pressure in the establishment of these symbiotic associations; therefore, a repressor of hormogonium formation in the symbiotic cavities could also shift the developmental direction of associated *Nostoc* toward heterocyst differentiation and N$_2$ fixation.

A plant-induced hormogonium-regulating locus (*hrm*) was identified in *N. punctiforme* by analysis of a transposition mutant that is 8- to 10-fold more infective of *Anthoceros* than is the wild-type parental strain (39, 117) and produces 2- to 3-fold more hormogonia when challenged with HIF. The mutant strain UCD 328 grows identically to the wild-type in the free-living state, in the presence or absence of combined nitrogen, but responds quite differently in coculture with *Anthoceros*. Within one week following inoculation, short hormogonium-like filaments and clusters of detached heterocysts are present in the cocultures with strain UCD 323, in contrast to the predominantly vegetative filaments in cocultures with wild-type *Nostoc*. Within 2 weeks the mutant had died, while the wild-type accumulated biomass in the medium and epiphytically on the gametophyte tissue. These observations indicate that the mutant may have emerged from the initially induced hormogonium state and differentiated heterocysts but that the

filaments apparently immediately reentered the hormogonium cycle. The loss of the mutant *Nostoc* from the coculture medium is consistent with an extinction effect of continual entry into the hormogonium cycle.

The transposon had inserted into a gene termed *hrmA* (39). The sequence of the region around *hrmA* revealed a five-gene locus (117) that shows a remarkable similarity in gene sequence and genomic organization to the *uxu* and *exu* operons of *E. coli*, encoding enzymes responsible for glucuronic and galacturonic acid catabolism, as well as a transcriptional repressor (102). However, inducers of these operons, sodium glucuronate, sodium galacturonate, and sodium gluconate, failed to induce *luxAB* expression in a *hrmA*::*luxAB* mutant, nor did they alter infection by wild-type *Nostoc* (E. L. Campbell, F. C. Wong, and J. C. Meeks, unpublished data). The substrates metabolized by gene products of the *hrm* locus are currently unknown and need not be glucuronic or galacturonic acids. Transcription of genes in the locus is induced by exposure to an aqueous extract of the *Anthoceoros* gametophyte tissue that also blocks hormogonium formation in the wild-type strain (39). The factor in the extract was termed a hormogonium-repressing factor (HRF), since it appeared to prevent the entry of vegetative filaments containing an intact copy of *hrmA* into the HIF-induced hormogonium cycle, in part by upregulation of *hrmA*.

It is unlikely that enzymes of the *hrm* locus produce the autogenic repressor of hormogonium differentiation postulated to exist in dense cultures (75). The mixing of mutant and wild-type *Nostoc* cultures in reconstitution of the association with *Anthoceros* does not repress the high-frequency infection by the mutant (39). It would appear that if the *hrm*-encoded metabolic pathway does produce a repressor, it is not released into the medium at an effective level by wild-type cells.

Although *sigH* and *hrmA* null mutants yield a high infection phenotype, the phenotype is not the consequence of SigH-dependent transcription of the *hrm* locus. Two results lead to this conclusion: (i) *sigH* is induced by HIF while *hrmA* is not, and (ii) *hrmA* is induced by HRF in a *sigH* mutant background (31). The similarity in phenotype is probably a consequence of at least two unlinked metabolic systems under control by the plant that influence hormogonium differentiation (*hrm*) and behavior (*sigH*). One can conclude from these studies that plants clearly exert both positive and negative regulatory control over hormogonium differentiation in *Nostoc*. The mechanisms and signal molecules remain to be defined.

PLANT CONTROL OVER THE CYANOBACTERIAL SYMBIOTIC GROWTH STATE

The cyanobacteria are the passive partners in all associations with plants. The extent to which this is true might be appreciated by comparing plant-cyanobacterium associations with the carefully orchestrated dance of legumes and rhizobia. Interactions of rhizobia with roots elicits gross morphological transformations in the plant: the curling of young root hairs, the formation of the infection thread, and the differentiation of new nodules. Likewise, the plant induces rhizobia to differentiate into bacteroids, a form that does not exist outside the plant.

In contrast, when cyanobacteria enter into a symbiotic asso-

ciation with plants, only the cyanobacterial partner undergoes dramatic physiological and morphological changes, but no change represents a behavior outside of its normal repertoire of the free-living growth state. Although the plants may increase the size of the preexisting symbiotic cavities and elaborate transfer cells for metabolite exchange in response to the presence of the cyanobacteria, these changes are relatively minor. Plants have thus learned how to activate regulatory pathways that function normally in free-living cyanobacteria, but cyanobacteria scarcely affect the plant in return, except, of course, to supply it with combined nitrogen. In this section, we focus on the cyanobacterial behavior modified by plants in the symbiotic cavities.

Physiological Characteristics of Cyanobacteria in the Symbiotic Growth State

A decrease in the rates of three physiological processes have been documented when cyanobacteria enter the symbiotic state; these processes are growth, assimilation of CO_2, and assimilation of NH_4^+. Conversely, there is an increase in the rate of N_2 fixation that accompanies the increased frequency of heterocyst differentiation. During balanced growth in the free-living state, these processes are regulated such that photosynthetically reduced carbon is provided as required for growth and N_2 fixation while the amount of nitrogen that is fixed and assimilated is proportional to the organic nitrogen needs for growth. The uncoupling of N_2 fixation and NH_4^+ assimilation reflects an unbalanced physiological state in symbiosis, but it is similar to the physiology of rhizobial bacteroids and consistent with the cyanobacteria providing N_2-derived NH_4^+ (or a derivative) for growth of the plant partner.

Do plants control the growth of symbiotically associated *Nostoc*? If *Nostoc* grows too slowly within the plant, the cyanobacterial symbiont will gradually be lost. If it grows too fast, it will outgrow the plant and compete with it for light and nutrients. The growth rate of cyanobacteria in symbiosis has been shown to be proportional to that of the plant partner in the *Anthoceros* association (49), as it should, and a similar proportionality is evident in other associations. Whereas free-living *Nostoc* strains may have doubling times that range from 15 to 48 h in nitrogen-fixing culture, *Anthoceros* doubles in biomass under optimal laboratory conditions within 5 days when supplied with combined nitrogen or 10 days when dependent on N_2-derived nitrogen from *Nostoc* (49). Thus, in the maintenance of a stable association, the growth of the cyanobacterium must be slowed in some manner.

It appears that *Anthoceros* regulates both the size of the symbiotic *Nostoc* colony and its nitrogenase specific activity. This assertion is based on the results of two experiments. First, under steady-state culture conditions of relatively low light intensity in medium supplemented with glucose (49), new symbiotic *Nostoc* colonies emerge at the margins of the growing gametophyte tissue and enlarge up to a relatively uniform size and density. The new infections are dependent on a low level of hormogonia produced from the existing *Nostoc* colonies. The immature and mature *Nostoc* colonies provide a constant rate of fixed nitrogen per colony and per unit of gametophyte tissue. New infections can be prevented by transient treatment of the culture with penicillin, which lyses *Nostoc* filaments in

the medium. One might anticipate that this intervention would result in fewer colonies and lower nitrogen production. Instead, while new colonies are prevented, preexisting symbiotic colonies increase in size, with a proportional increase in the rate of N_2 fixation per colony (49). The symbiosis maintains a constant rate of N_2 fixation per unit of the growing gametophyte tissue through an increase in cyanobacterial growth without a change in nitrogenase specific activity.

Second, when the *Anthoceros-Nostoc* association is grown under high light intensity without glucose, the growth rate of the associated tissue and that of N_2 fixation per unit of gametophyte tissue is identical to that of tissue grown under low light intensity in glucose-supplemented medium (177). However, both the number and the biomass of individual high-light-intensity-grown *Nostoc* colonies are approximately half those of low-light-intensity-grown colonies, and the specific activity of nitrogenase is fivefold higher. Here, the symbiosis maintains a constant rate of N_2 fixation through a decrease in growth response and increase in nitrogenase specific activity. The mechanisms regulating these opposite responses have not been examined, but they clearly illustrate plant-mediated control over growth and specific protein synthesis of the associated *Nostoc* in order for the plant partner to maintain a constant growth rate.

What causes impaired photosynthesis in symbiotically associated *Nostoc*? Complete photosynthesis, culminating in the reduction of CO_2 to organic carbon, is highly depressed in cyanobacteria associated with a eukaryotic photosynthetic partner (116). The rate of light-dependent CO_2 fixation by freshly separated symbiotic *Nostoc* is generally low relative to that in free-living cultures: undetectable from cycad (107), 1% from *Gunnera* (173), and 12% from *Anthoceros* (176) associations. An exception is the cyanobacterium associated with *Azolla*; when immediately separated from the fern, it has 85% of the rate of typical free-living *Nostoc/Anabaena* cultures (156). However, $^{14}CO_2$ pulse-chase experiments indicate that the associated cyanobacterium fixes little or no CO_2 in the intact *Azolla* association (88). Therefore, the cyanobacterial partner in all associations defers its independent photosynthetic potential and assumes a dependent heterotrophic role, exchanging fixed nitrogen for fixed carbon.

Heterocyst differentiation is accompanied by the loss of photosynthetic antenna biliproteins, but the depressed rates of light-dependent CO_2 fixation by the symbiotic *Nostoc* cannot be explained by the loss of these pigments. Epifluorescence light microscopic and immunoelectron microscopic analyses have shown that the light-harvesting phycobiliproteins are present in vegetative cells of *Nostoc* in all symbiotic associations at concentrations similar to those in free-living cultures (Fig. 9) (116). Symbiotic *Nostoc* strains therefore retain phycobiliproteins even though they may be of little functional value, just as free-living cyanobacteria retain phycobiliproteins even during prolonged dark heterotrophic culture (158, 203). Even heterocysts retain phycobiliproteins and their attendant fluorescence in association with some plants (Fig. 9) (87, 115; but see also reference 150), in stark contrast to the loss of fluorescence within heterocysts of free-living *Nostoc* strains (Fig. 1C). The phycobiliproteins in heterocysts of the *Azolla* symbiont are functional, as judged by action spectra for nitrogenase activity (195).
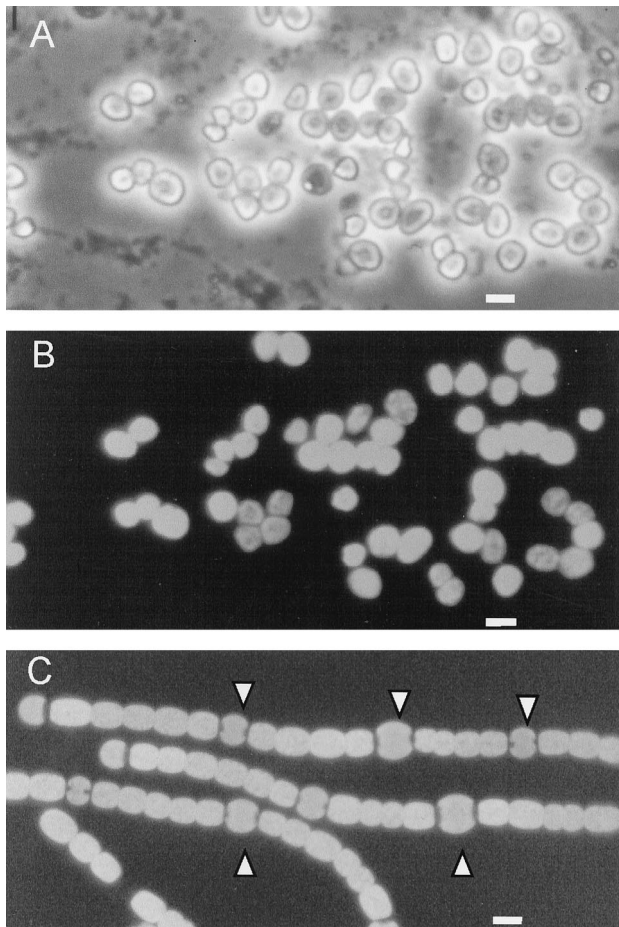
FIG. 9. Photomicrographs of symbiotically associated cyanobacteria. (A and B) *Nostoc* associated with *Anthoceros*; (C) *Nostoc/Anabaena* associated with *Azolla*. Panel A is a phase-contrast image and panels B and C are epifluorescence images with excitation light near 510 to 560 nm and emission greater than 600 nm. Arrowheads point to heterocysts in panel C. Bar, 10μm. Compare the cell sizes to those in Fig. 1 and 8. Panels A and B reprinted from reference 116 with permission of the publisher. Panel C reprodinted from reference 195 with permission of the publisher (kindly provided by Gerald Peters).

Another possibility to explain the loss of photosynthetic capacity is a decrease in the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the primary carboxylating enzyme of the carbon-assimilating Calvin-Benson-Bassham cycle (183), but here the situation is more complicated. Immunological titrations have demonstrated that the Rubisco protein accumulates to cellular levels that are similar to those in free-living cultures in the *Nostoc* strains of all associations examined (116). Rubisco activity is another matter. *Nostoc* cultures freshly separated from *Anthoceros* have only 12% of the in vitro Rubisco activity of corresponding free-living cultures, similar to the in vivo rate of symbiotic light-dependent CO_2 assimilation (176). In contrast, the in vitro Rubisco activities in *Nostoc* from both the cycad (107) and *Gunnera* (173) associations are equivalent to those in free-living cultures. It may be that the low rate of in vivo CO_2 fixation by *Nostoc* in association with cycads or *Gunnera* is attributable to a revers-

ible inhibitor of Rubisco, which is lost by dilution in the in vitro assay.

Collectively, these results indicate that the mechanisms resulting in the common phenotype of impaired complete photosynthesis in *Nostoc* varies in the different associations, perhaps as a consequence of unique plant signals affecting different key reactions of the metabolic system.

What are the causes and consequences of uncoupled nitrogen fixation and ammonium assimilation in symbiotically associated *Nostoc*? Nitrogen metabolism is profoundly different in symbiotically associated *Nostoc* strains from that in strains grown outside the plants. First, the specific activity of nitrogenase in *Nostoc* associated with *Anthoceros* (177), cycads (106), and *Gunnera* (174) is three- to fivefold higher than that in free-living cultures. Second, much of the nitrogen that is fixed is released and made available to the plant. The amounts of N_2-derived NH_4^+ released by symbionts in associations grown under controlled conditions in the laboratory are 40% in *Azolla* (120), 80% in *Anthoceros* (118), and 90% in *Gunnera* (169). These values are in contrast to 6% (57) to 20% (178) of NH_4^+ and organic nitrogen combined by free-living N_2-fixing cyanobacteria under variable conditions in the natural habitat. An undefined amount of cell lysis undoubtedly contributes to the measured nitrogen release in the habitat. These results point to an imbalance in cyanobacterial metabolism associated with the symbiotic state and raise several important questions addressed below. At the root is the question why *Nostoc* associated with plants adopts a developmental profile appropriate for extreme nitrogen deprivation when in fact the cyanobacterium is immersed in the products of N_2 fixation.

(i) Sources of reductant for symbiotic nitrogen fixation. *Nostoc* in the free-living state produces enough reductant through photosynthesis to provide exactly for N_2 fixation and other metabolic needs. What is the case with symbiotically associated *Nostoc*? Steinberg and Meeks (177) addressed this question by performing experiments with a *Nostoc* mutant that is resistant to 3-(3,4-dichlorophenol)-1,1-dimethylurea (DCMU), an inhibitor of electron transport out of the photosystem II reaction center. The addition of DCMU thus serves to separate photosynthesis by resistant *Nostoc* from that by sensitive *Anthoceros*. Associations with the wild-type (DCMU-sensitive) *Nostoc*, when sufficiently depleted of internal stores of reduced carbon, showed no nitrogenase activity in the dark or in the light in the presence of DCMU, as expected. Associations with the DCMU-resistant *Nostoc*, incubated in the light and in the presence of DCMU to inhibit plant photosynthesis, had 30% of the uninhibited light-dependent rate of nitrogenase activity. This result verified that the *Nostoc* is capable of complete photosynthesis when in the *Anthoceros* association, albeit at a low rate, and that maximal nitrogenase activity is maintained primarily by photosynthate from the plant.

Full nitrogenase activity can be restored to light grown, DCMU inhibited associations by the addition of sucrose, fructose, or glucose but not the dicarboxylic acids favored by rhizobia in the legume associations (201). The few effective carbohydrates are the same as those able to support heterotrophic growth in free-living *Nostoc* cultures. Of these hexoses, sucrose appears to be the natural currency between the plant and the cyanobacterial symbiont, at least in the *Azolla* association (88).

One can conclude from these results that steady-state N_2 fixation by symbiotic *Nostoc* probably relies on plant-derived hexoses. The symbiotic state does not change the types of exogenous organic compounds that *Nostoc* can metabolize but, rather, alters the usual connection between cyanobacterial photosynthesis and N_2 fixation.

(ii) Routes of carbon catabolism by symbiotic *Nostoc*. Carbohydrate translocated into heterocysts is catabolized by the initial dehydrogenases of oxidative pentose phosphate metabolism to generate the reductant required for both nitrogenase and oxidative respiration. Oxidative pentose phosphate metabolism in vegetative cells or heterocysts can proceed in either a cyclic or a linear manner. In the purely cyclic mode, glucose-6-phosphate is ultimately catabolized to CO_2 while generating reductant, whereas in the linear mode the intermediates can be metabolized to pyruvate and ultimately to α-ketoglutarate (181). Free-living *Nostoc* growing on sugar must divert a substantial amount of carbon through the linear pathway to generate biosynthetic precursors, including the α-ketoglutarate essential for net assimilation of the N_2-derived NH_4^+ into glutamate via the GS-GOGAT cycle. However, in the symbiotic growth state, where an elevated demand for reductant derived from glucose-6-phosphate is not paralleled by an elevated demand for carbon skeletons, *Nostoc* should favor flux through the cyclic oxidative pentose phosphate metabolic route to avoid the detrimental accumulation of tricarboxylic acid cycle intermediates, such as α-ketoglutarate. This predicted change in metabolism evoked by the transition from free-living to symbiotic states has not yet been identified, nor has any regulatory mechanism susceptible to exploitation by the plant.

(iii) Regulation of ammonium assimilation in symbiotic *Nostoc*. Free-living cyanobacteria growing on N_2 typically release very little fixed nitrogen (57). In contrast, *Nostoc* in the symbiotic state releases much or most of the nitrogen it fixes, in the form of NH_4^+ in its symbioses with *Anthoceros* (118), *Azolla* (120), and *Gunnera* (169) or organic nitrogen in its symbiosis with the cycad *Zamia* (137). To explain this behavior, studies have focused on the presence and activities of GS, the first enzyme in the pathway of NH_4^+ assimilation. It may be instructive to consider the associations with *Anthoceros*, cycads, and *Gunnera*, for which it has proven possible to compare the same strain of *Nostoc* in its symbiotic and free-living states. These comparisons are complicated by the fact that heterocysts in free-living cultures have twice as much GS activity (191) and GS antigen (17) as do vegetative cells. Since it is not technically possible to separate heterocysts from vegetative cells in *Nostoc* associated with plants, activity measurements are necessarily averaged over both cell types, and cell-type-specific effects brought on by the symbiotic state could be obscured.

GS is greatly affected by the transition of *Nostoc* from the free-living state to association with *Anthoceros*. While there is no significant difference in the overall concentration of GS protein, the in vitro GS catalytic activity of symbiotic *Nostoc* is only 15% that of free-living cultures (86). Immunoelectronmicroscopic analyses indicated that the accumulation of GS protein in the heterocysts of symbiotic *Nostoc* in the *Anthoceros* association was lower than that in the heterocysts in the free-living growth state and equal to that in the heterocysts in vegetative cells (150). In summary, the symbiotic state with *Anthoceros* may lead to small differences in the level of GS protein but large differences in activity, perhaps the result of modification of the enzyme.

The lower GS activity in *Nostoc* in symbiosis with *Anthoceros* does not, however, appear to be sufficient to account for the amount of N_2-derived NH_4^+ released. To illustrate, an acetylene reduction rate of approximately 25 nmol per min per mg of protein, as reported for the *Anthoceros*, cycad, and *Gunnera* associations (summarized in reference 116), using a 4:1 ratio of acetylene reduced to N_2 reduced (179), leads to a value of 12.5 nmol of NH_4^+ produced per min per mg of protein. The GS specific biosynthetic activity of 19.8 nmol of NH_4^+ assimilated per min per mg of protein for *Nostoc* in the *Anthoceros* association (98) would be sufficient to assimilate all of the N_2-derived NH_4^+, and yet 80% of the ammonium is released.

The *Nostoc* in association with cycads has a GS specific activity similar to that of free-living cultures (104), but this activity is consistent with a report that the *Nostoc* in this association releases organic nitrogen for use by the plant partner (137).

The release by *Nostoc* in association with *Gunnera* of 90% of its N_2-derived NH_4^+ (169) cannot be ascribed to low GS capacity, since the symbiotic *Nostoc* is reported to have as much as 70% of the in vitro GS activity of free-living cultures (18). It is possible, of course, that metabolic flux through GS in vivo is low owing to substrate concentration or allosteric inhibition.

Collectively, these observations indicate that the release of fixed nitrogen by symbiotic *Nostoc*, depending on the association, may or may not be due in part to the low activity of GS. The crucial question arises: is it the specific *Nostoc* strain or the plant partner that determines the response of GS to a particular association? The only information on the subject is the observation that when *N. punctiforme* ATCC 29133, isolated from the cycad *Macrozamina* (159), is reconstituted in the *Anthoceros* association, its GS is now subject to the irreversible inhibition that is characteristic of the *Anthoceros* association (K.-Y. Lee and J. C. Meeks, unpublished results). It is unknown if *Nostoc* in *Macrozamina*, as in *Zamia*, has normal GS activity, but perhaps it is the plant that controls the output of fixed nitrogen. Even in *Anthoceros*, the enzymatic capacity of GS is only part of the story; there must be other mechanisms that contribute to the release of fixed nitrogen by *Nostoc* to sustain the symbiotic relationship.

Morphological Characteristics of Cyanobacteria in the Symbiotic Growth State

Rhizobia within legume root nodules differentiate into a form, bacteroids, that lies beyond the repertoire of rhizobia in their growth environment outside the plant. The morphology of *Nostoc* cells is also affected by associations with plants, but, unlike the case with rhizobial associations, cyanobacterial associations are typified by differences in degree rather than kind. The morphology of vegetative cells within associations is also seen outside them on occasion. Heterocysts exist, of course, in free-living cyanobacteria, but to nowhere near the frequency at which they are found in cyanobacteria associated with plants.

Morphological alteration of vegetative cells. Vegetative cells of *Nostoc* within mature symbiotic cavities of *Azolla* (15) and
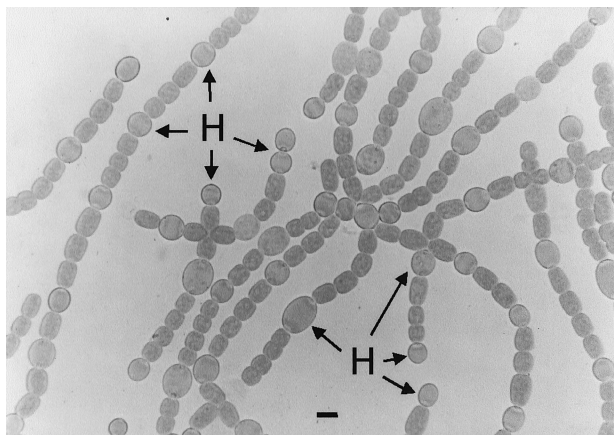
FIG. 10. Photomicrograph of *Nostoc/Anabaena* in association with *Azolla*. The bright-field image shows intact filaments, heterocyst frequency, and vegetative cell intervals. Bar, 10 μm. Reprinted from reference 140 with permission of the publisher (kindly provided by Gerald Peters).
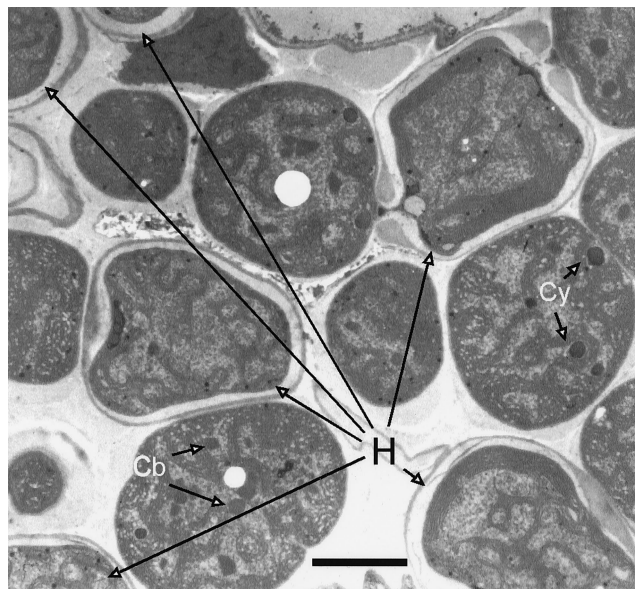

FIG. 11. Electron micrograph of a portion of a *Nostoc* colony in association with *Anthoceros*. There are six heterocysts (H) distinguishable from nine vegetative cells in this field of view. Cells contain carboxysomes (Cb), where Rubisco is localized, and cyanophycin granules (Cy). Bar, 10 μm. Reprinted from reference 115 with permission of the publisher (kindly provided by Yvonne Weeden-Garcia).

other plants (116) are up to fourfold larger than the vegetative cells of free-living cyanobacteria, but the heterocysts that differentiate from these vegetative cells are also proportionally larger (Fig. 9 and 10). Moreover, the cell-cell connections of *Nostoc* in associations with bryophytes, cycads, and *Gunnera* are very fragile. The characteristic of enlarged vegetative cells and weak cell-cell connections are seen also in the aseriate stage of the *Nostoc* life cycle (95). Filaments enter the aseriate stage through cell divisions that occur at various angles and that depart from the transverse plane of the filament. Confinement of the filament within the semirigid sheath typical of *Nostoc* results in the loss of obvious filamentous structure, hence the classical reference to cells not being in a series. The degree of communication among cells in this aseriate stage is unknown. The aseriate stage is one of slow growth. The regulatory mechanisms for entry into and exit from the aseriate stage have not been studied, because this morphological form is rapidly lost during laboratory culture. Based on the similarities in growth and filamentation, it is conceivable that the symbiotic growth state is equivalent to the aseriate growth stage, with the plant partners appropriating the regulatory signals that control entry into and maintenance of this state.

Nothing is known of the function, if any, of this morphological change. Nevertheless, it clearly reflects an alteration in peptidoglycan organization of the *Nostoc* strains growing within the plant.

What are the heterocyst frequencies of symbiotically associated *Nostoc*? It has proven difficult to distinguish heterocysts from vegetative cells in most symbiotically associated *Nostoc*, whether by bright-field or fluorescence microscopy (Fig. 9). Heterocysts can clearly be distinguished from vegetative cells by electron microscopy (EM), based on the extra wall layers and in many cases by organization of the cytoplasm in heterocysts (Fig. 11). Heterocyst frequencies derived from EM studies range from 25 to 80% of the total cells, depending on the association and maturity of the symbiotic colony (Table 3) (148). The major limitation in EM studies is visualization of a continuous filament. The aseriate morphology of the cya-

nobacteria in all but the *Azolla* association yields a filament with cells that pass into and out of a sectional plane. This limitation makes it difficult to analyze the pattern of heterocyst spacing in the symbiotic growth state; doublet heterocysts can be recognized, but identification of higher orders of contiguous heterocysts and of the numbers of vegetative cells between heterocysts is equivocal in the absence of serial sectioning.

Heterocyst frequencies in the *Azolla* (15, 78), *Zamia* (103), and *Gunnera* (16, 174) associations are a function of the position of, or in, the cyanobacterial colony. A developmental gradient from low to high heterocyst frequencies is clearly seen in the stem axis of *Azolla* and *Gunnera* and in the tip to the base of coralloid roots of *Zamia*. In *Anthoceros*, new symbiotic colonies form near the marginal meristem of the growing gametophyte tissue, with mature and degenerating colonies at successive distances from the margin. No specific morphological or physiological comparisons have been reported between young marginal colonies and those deep in the *Anthoceros* gametophyte tissue. The stem-apical meristem of *Azolla* contains filaments of undifferentiated *Nostoc/Anabaena* (our taxonomic equivocation of the cyanobacterial symbiont is given in Table 1); heterocysts appear as the filaments are enclosed within the symbiotic cavity in leaf 1 and increase in frequency with successive leaves, reaching a maximum frequency of 25 to 45% by leaves 12 to 15 (15, 78). Absolute values vary with the *Azolla* species and growth conditions. Doublet and triplet or higher numbered contiguous heterocysts can be observed, but they are as rare in the *Azolla* associations as they are in free-living cultures. Heterocyst frequencies increase from 15% at the tip of the *Zamia* coralloid root to 45% within 7 to 10 mm back from the tip (106). In the association with a small stoloniferous *Gunnera* species, colonies in glands near the stem apex

TABLE 3. Parameters of heterocyst spacing in free-living and plant-associated cyanobacteria[a]

Sample	Heterocyst frequency (H_T)[b]	Single heterocysts (h_1)[c]	Doublet heterocysts (h_2)[c]	Contiguity (C)[d]	Foci (F)[e]	Average interval (I)[f]
Anabaena strain PCC 7120[g]	*0.098*	1.0	0	1.0	*0.098*	9.2
Anabaena strain PCC 7120 *patS*[g]	0.29	0.39	0.55	*1.79*	*0.20*	*3.6*
N. punctiforme[h]	0.087	0.89	0.06	*1.19*	*0.081*	*11.1*
N. punctiforme patN[h]	0.26	0.97	0.04	*1.03*	*0.255*	*2.90*
Nostoc in *Anthoceros*[h]	0.25	0.86	0.13	*1.17*	*0.23*	*3.2*
Nostoc/Anabaena in *Azolla*[i]	0.26	0.96	0.04	*1.04*	*0.25*	*3.0*
Nostoc in *Zamia* (section 1)[j]	0.167	0.856	0.14	*1.14*	*0.15*	*5.4*
Nostoc in *Zamia* (section 2)[j]	0.30	0.62	0.34	*1.3*	*0.24*	*2.9*
Nostoc in *Zamia* (section 3)[j]	0.38	0.49	0.37	*1.59*	*0.27*	*2.3*
Nostoc in *Zamia* (section 7)[j]	0.45	0.32	0.46	*1.92*	*0.28*	*2.0*

[a] Numbers in normal font are derived from data presented in the indicated sources. Numbers in italics are calculated from the data. For a conceptual visualization, see Fig. 12.

[b] H_T = fraction of total cells that are heterocysts.

[c] h_n = fraction of total heterocysts that are part of a string of $n = 1$ or more than 1 contiguous heterocysts (e.g., $h_1 = H_1/H_T$).

[d] C = contiguity, i.e., the average number of heterocysts per string = $\Sigma(n \cdot h_n)$, where n is the number of contiguous heterocysts.

[e] F = foci, i.e., the fraction of cells that are heterocysts strings, counting each string as a single entity = $\Sigma(H_n/n)$, where n is the number of contiguous heterocysts. When there are only single heterocysts, $F = H_T$.

[f] I = ratio of vegetative cells to foci of differentiating cells = $V/F = (1 - H_T)/F$, where V is the fraction of total cells that are vegetative cells. As the length of the filament increases, I approaches the average interval of vegetative cells intervening between heterocyst strings.

[g] From an analysis of at least 1,000 *patS* cells and a unstated number of wild-type cells (data from reference 220).

[h] From an analysis of 1,258 cells of free-living *N. punctiforme*, 499 cells of the *patN* mutant, and 761 cells of *Nostoc* in association with *Anthoceros* (from light micrographs and collages of electron micrographs, [F. C. Wong, Y. Weeden-Garcia, and J. C. Meeks, unpublished data]).

[i] From an analysis of 172 cells of *Nostoc/Anabaena* in *Azolla* (taken from Fig. 10, which is Fig. 3 of reference 140).

[j] From an analysis of at least 4,750 cells taken from a coralloid root of the cycad *Zamia*. Section 1 was closest to the tip of the root and represented the youngest part. Sections are increasingly more distant from the tip (data from reference 106).

contain 19% heterocysts, which increases to an average of 55% by 11 to 20 mm from the apex (16). Doublet, triplet, and higher numbered contiguous heterocysts are routinely seen in the *Zamia* and *Gunnera* associations.
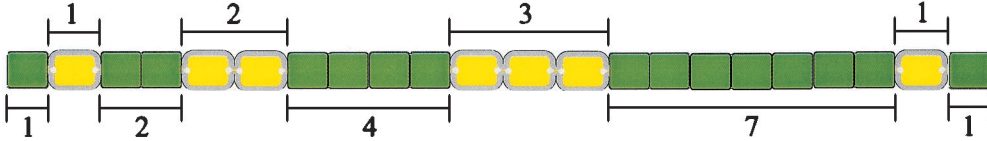
Increasing nitrogenase activity parallels increasing heterocyst frequency only during the early portions of the developmental gradient in associations with *Azolla* (15, 78, 87), *Zamia* (106), and *Gunnera* (16, 174). The peak of nitrogenase activity consistently correlates with heterocyst frequencies between 25 and 35% in these three associations and declines as a function of distance from the stem apex or root tip, while heterocyst frequencies increase in the *Zamia* and *Gunnera* associations. At the lower heterocyst frequencies, doublets and higher-order multiple contiguous heterocysts are rare, but they increase in frequency with distance from the tip or apex conversely with the decline in nitrogenase activity until they may comprise 60% or more of the total heterocysts (174). Ultrastructure analysis in the *Anthoceros* association indicates that approximately 40% of the heterocysts have a degenerative cytoplasmic organization and are likely to be nonfunctional (Y. Weeden-Garcia and J. C. Meeks, unpublished results); therefore, maximal nitrogenase activity may correlate with a functional heterocyst frequency of 25 to 30% in the *Anthoceros* association.

The correlation of maximal nitrogenase activity with a heterocyst frequency of approximately 30% in all of the associations may represent a physiological optimum. The unique bilayered polysaccharide and glycolipid wall of the heterocyst retards the translocation of chemical substrates as well as gases; the region of transport into and out of heterocysts is the relatively small junction with adjacent vegetative cells. In the free-living state, photosynthate generated in vegetative cells is transported into heterocysts via this junction and catabolized to provide reductant (214). If reductant for heterocyst function in symbiosis is ultimately derived from the photosynthate of the plant partner, vegetative cells would provide a greater surface area to accumulate that photosynthate and perhaps be required to convert it into a form that is translocated into heterocysts. Based on source-sink considerations, in both the free-living and symbiotic growth states a heterocyst that is attached to a vegetative cell on each pole has a probability of greater reductant supply than one attached to a single vegetative cell, as is the case for doublet heterocysts and heterocysts at the ends of a filament. On average, a 30% heterocyst frequency would allow both poles of each heterocyst to be associated with a vegetative cell (e.g., two vegetative cells specifically supplying each heterocyst). The activity of heterocysts located at the center of a cluster of multiple contiguous heterocysts would be considerably lower than that of a singlet heterocyst, because it would be dependent both on the length of the diffusion pathway and on the utilization of substrate by heterocysts between it and the nearest vegetative cell.

How Plants Overcome the Normal Controls over Heterocyst Differentiation

Does the high frequency of heterocysts in plant-associated cyanobacteria result from an increased frequency of initiation at discrete sites or a lack of resolution of multiple contiguous heterocysts? In the context of the proposed two-stage model of heterocyst differentiation and pattern formation, the high heterocyst frequencies in symbiotic associations could be the consequence of increased sites of initiation of differentiation, a decrease in the resolution of multiple contiguous heterocysts differentiating at a single site, or both. If failure to resolve strings of heterocysts is responsible, one would expect to see an increase in the average number of heterocysts per string. If there is increased initiation, one would expect to see an increased number of sites or focal points of differentiation.

$$H_T = \frac{1+2+3+1+\ldots}{1+2+3+1+\ldots+ \text{veg. cells}} \qquad C = \frac{1+2+3+1+\ldots}{1+1+1+1+\ldots}$$

$$F = \frac{1+1+1+1+\ldots}{1+1+1+1+\ldots+ \text{veg. cells}} \qquad I = \frac{1+2+4+7+1+\ldots}{1+1+1+1+1+\ldots}$$

FIG. 12. Model illustrating heterocyst strings (C), differentiation sites (F), and vegetative cell intervals (I). The filament is shown, where green cells represent vegetative cells and yellow cells represent heterocysts. A sample calculation for each of four parameters is explained further in the text. H_T is the fraction of total cells that are heterocysts (7/22 in this example). C is the contiguity, i.e., the average number of heterocysts per string (7/4 in this example). F is the fraction of cells that are heterocyst strings or foci (4/19 in this example). I is the ratio of vegetative cells to foci of differentiating cells (15/4 in this example).

An analysis of heterocyst frequency and spacing for free-living *Anabaena* strain PCC 7120 and *N. punctiforme*, both wild-type and mutant strains, and *Nostoc* in symbiotic associations is summarized in Table 3 and modeled in Fig. 12. Wild-type *Anabaena* strain PCC 7120 produces only singlet heterocysts, and so the contiguity factor (C; heterocysts per string) is 1.0 and the frequency of focal points (F) is the same as the frequency of heterocysts, about 0.10 (Table 3). Mutation of *patS* results in much higher values of both contiguity of heterocysts ($C = 1.79$) and of frequency of foci ($F = 0.20$). *N. punctiforme* grown apart from the plant behaves similar to *Anabaena* strain PCC 7120, with a slightly higher contiguity ($C = 1.19$). The *N. punctiforme patN* mutant has a higher frequency of foci ($F = 0.255$) than the *patS* mutant, coupled with an essentially unaltered contiguity factor.

Analyses of the symbiotically associated *Nostoc* show that the *F* value increases in all associations, approximately in proportion to the general increase in heterocyst frequency, up to a certain limit. However, in the *Anthoceros* and *Azolla* associations and near the tip of the *Zamia* coralloid root, the contiguity factor resembles that of wild-type cells and the *patN* mutant, but not the *patS* mutant with its distinctive multiple contiguous heterocysts. The multiple singular heterocyst pattern in symbiosis with low *C* and high *F*, yielding a vegetative-cell average interval of 3.0, can be visualized in the photomicrograph of the *Nostoc/Anabaena* in association with *Azolla* in Fig. 10. In the more distal and mature portions of the *Zamia* coralloid root, *C* increases reflecting the presence of multiple contiguous heterocysts.

The high *C* value in more mature regions of the cycad coralloid root, as well as *Gunnera* stems, probably does not represent a failure to resolve strings of heterocysts. If resolution were completely defective, then the contiguous heterocysts would differentiate simultaneously. Instead, the frequency of multiple heterocysts increases with distance (time) from the growing tip. Since nitrogenase activity does not follow the increase in heterocyst number and ultimately decreases, it is likely that many of the heterocysts are nonfunctional, informationally separated from the filament. Heterocysts lose func-

tion after several generations of growth in free-living culture (214), but they pinch off and separate from the filament and therefore do not appear in the usual accounting. Heterocysts in symbiotic cavities may be prevented by the matrix from separating fully from their parent filaments. A mutant *A. variabilis* strain has been described that produces strings of heterocysts in a sequential manner, none of which are able to fix nitrogen (40, 214).

The *N. punctiforme patN* mutant with a high *F* and unaltered *C* is currently being characterized, but we know that it has no significant growth defect in medium containing combined nitrogen. Therefore, we provisionally conclude that the mutation is unlikely to be in an essential cell cycle gene and that the multiple singular heterocyst phenotype may be a consequence of aberrant resolution of an initial cluster, rather than increased sites of initiation. The current evidence therefore does not distinguish between an increase in initiation of heterocyst differentiation within symbiotic cavities, relative to that seen in free-living cultures, and an incomplete resolution of an initial cluster of differentiating cells similar to the situation, perhaps, in the *patN* mutant. It is of clear interest to determine how association with the plant modifies cyanobacterial behavior in either way.

What is the signal(s) initiating heterocyst differentiation in the symbiotic growth state? We have argued that initiation of heterocyst differentiation in the free-living growth state occurs when NtcA receives a signal of nitrogen limitation and elicits a sequence of transcriptional activation events in clusters of cells able to respond to the signal. We have further noted that the nature of the nitrogen status signaling system is unresolved in filamentous cyanobacteria. Hence, the way such a signaling and response system could be modified in symbiosis to yield the markedly higher heterocyst frequencies cannot be modeled. However, three lines of evidence, largely drawn from the physiological characteristics presented above, imply that the signal initiating the developmental cascade in symbiosis is independent of the nitrogen status of the *Nostoc* cells and may involve a symbiosis-specific signaling and sensing system (Fig. 7).

First, vegetative cells of symbiotic *Nostoc* in all associations exhibit the characteristics of cells growing under nitrogen excess and not nitrogen-limited conditions. Electron micrographs show the presence of carboxysomes, cyanophycin granules (Fig. 11), and phycobilisomes (150), structures that in free-living cultures grown under nitrogen starvation are consumed to provide amino acids for the synthesis of new proteins (10). Cyanophycin granules re-form in heterocysts, but phycobiliproteins and Rubisco typically remain absent. Thus, the vegetative cells that differentiate into heterocysts in symbiosis appear not to reflect a nitrogen-limited physiology. This idea is supported by the observation that heterocysts in cyanobacteria associated with *Azolla* (Fig. 9) (195) and perhaps *Anthoceros* (Fig. 9) retain phycobiliproteins. The presence of phycobiliproteins in heterocysts implies that they are not degraded in the precursor vegetative cells as they differentiate into heterocysts, perhaps because the cells do not receive a signal of nitrogen limitation.

Second, N_2 fixation is uncoupled from NH_4^+ assimilation, indicating that heterocysts in plant-associated cyanobacteria may result from a different developmental program. The heterocysts are physiologically distinguished from those of free-living cyanobacteria by their high demand for reductant in the form of carbohydrate and their low demand for carbon skeletons, such as α-ketoglutarate, for the assimilation of NH_4^+. Consistent with this, levels of GS may remain equivalent to those in vegetative cells in heterocysts of symbiotically associated cyanobacteria due to the lack of a nitrogen starvation signal.

Third, high concentrations of combined nitrogen do not appear to directly repress heterocyst differentiation in symbiosis. Ammonium is excreted from the *Nostoc* vegetative cells in the *Azolla, Anthoceros*, and *Gunnera* associations and accumulates in the symbiotic cavities. Due to the limited permeability of the heterocyst envelope, the excess N_2-derived NH_4^+ must be translocated from heterocysts to vegetative cells and then extracellularly into the symbiotic cavity. Thus, the vegetative cells are exposed to relatively high concentrations of both internal and external NH_4^+, and yet they continue to differentiate into heterocysts. Why is it, then, that exogenously supplied NH_4^+ or NO_3^+ represses heterocyst differentiation and nitrogenase expression in cyanobacteria associated with *Anthoceros* (49) and, to a lesser extent, *Azolla* (138, 139)? Analysis of a *N. punctiforme* mutant unable to assimilate NO_3^- supports the idea that repression requires the mediation of the plant partner (34). Nitrate neither permitted growth of the mutant in the free-living state nor repressed heterocyst differentiation and nitrogenase expression. When the mutant was reconstituted into the *Anthoceros* symbiotic association, however, NO_3^- repressed nitrogenase activity in a kinetic pattern similar to associations with the wild-type *N. punctiforme*. Control experiments and analyses eliminated reversion of the mutant to NO_3^- prototrophy and accumulation of NO_3^--derived NH_4^+ as contributors to the repression.

Taken together, these results are consistent with the existence of a signal produced by the host plant that regulates the differentiation of heterocysts. The nature of the plant-derived signal and where it might interact with the developmental cascade of heterocyst differentiation is unknown. Results of analysis of *N. punctiforme* mutants in the *Anthoceros* associa-

tion have established that *hetR* is essential for symbiotic heterocyst differentiation; therefore, the signal must enter the developmental pathway prior to *hetR* (217). It is likely that the symbiotic signal enters the pathway prior to activation of NtcA (Fig. 7), because NtcA appears to be essential for the transcription of genes expressed late in heterocyst maturation and for nitrogenase function (e.g., excision of the *nifD* element [200]). Interestingly, mutants of *N. punctiforme* defective in *ntcA* fail to infect *Anthoceros* tissue; therefore, their ability to respond to plant signals influencing symbiotic heterocyst differentiation cannot readily be determined (217).

CONCLUSIONS AND FUTURE DIRECTIONS

Many societies have learned to domesticate the cow. This is because it is necessary only to learn the ways of the cow, what it needs, and how to control its behavior. It is not necessary for the cow to learn each society's idiosyncrasies. We have drawn a picture of certain filamentous cyanobacteria as a cow that has been domesticated repeatedly by diverse plants. The cyanobacteria that enter into symbiotic relationships are not highly specialized for the task. Rather, the plant exploits behaviors that the cyanobacteria otherwise exhibit by themselves.

Plants have learned how to induce motility in cyanobacteria, direct the motile cyanobacteria to symbiotic cavities, and shut off the response when the filaments have achieved their final destination. Plants have also gained control over cyanobacterial growth and metabolism within the symbiotic cavities in the process of converting the cyanobacteria into NH_4^+-producing factories.

We are only just beginning to figure out how plants achieve their control over cyanobacteria. If we understand how they do it, we may then understand the fundamental mechanisms governing cyanobacterial differentiation. We may also perceive how to extend the abilities possessed by plants capable of symbiosis to crop plants that are not capable of symbiosis. Identification of the regulatory circuits in *Anabaena* and *Nostoc* that are involved in cellular differentiation and those that are appropriated by the plant partners will soon be approachable by global genomic and proteomic analyses, following completion of the genomic sequences of *N. punctiforme* ATCC 29133 (accessible at http://www.jgi.doe.gov/) and *Anabaena* strain PCC 7120 (accessible at http://www.kazusa.or.jp/cyano).

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