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Organization of the *nif* genes in cyanobacteria in symbiotic association with *Azolla* and *Anthoceros*

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Abstract. The sizes of endonuclease digestion fragments of DNA from cyanobacteria in symbiotic association with *Azolla caroliniana* or *Anthoceros punctatus*, or in free-living culture, were compared by Southern hybridization using cloned nitrogenase (*nif*) genes from *Anabaena* sp. PCC 7120 as probes. The restriction fragment pattern produced by cyanobacteria isolated from *A. caroliniana* by culture through symbiotic association with *Anthoceros* differed from that of the major symbiotic cyanobacterium freshly separated from *A. caroliniana*. The results indicate that minor cyanobacterial symbionts occur in association with *Azolla* and that the dominant symbiont was not cultured in the free-living state. Both the absence of hybridization to an *xis*A gene probe and the mapping of restriction fragments indicated a contiguous *nif*HDK organization in all cells of the symbiont in association with *Azolla*. On the other hand, in the cultured isolate from *Azolla* and in *Nostoc* sp. 7801, the *nif*D and *nif*K genes are nominally separated by an interval of unknown length, compatible with the interruption of the *nif*HDK operon by a DNA element as observed in *Anabaena* sp. PCC 7120. In the above cultured strains, restriction fragments consistent with a contiguous *nif*HDK operon were also present at varying hybridization intensities, especially in *Nostoc* sp. 7801 grown in association with *Anthoceros*, presumably due to gene rearrangement in a fraction of the cells.

Key words: *Anthoceros* – *Azolla* – DNA isolation and restriction – *nif* gene organization – *Nostoc* – Symbiotic cyanobacteria

The free-floating water fern *Azolla* occurs in symbiotic association with a nitrogen-fixing cyanobacterium. The cyanobacterium, generally referred to as *Anabaena azollae* (Moore 1969), provides fixed nitrogen as ammonium (Meeks et al. 1985b; Peters et al. 1980) for growth of the *Azolla* (Peters and Mayne 1974). Differences in cellular morphology of the cyanobacterial symbiont are seen along the developmental gradient of the *Azolla* sporophyte; filaments lacking heterocysts are found in the region of the apical meristem, but the frequency of heterocysts in filaments increases in parallel with emergence and maturation of the leaves (Hill 1975; Peters and Calvert 1983). The nitrogen fixation activity of the symbiont increases along with heterocyst frequency and leaf maturation before declining as leaves senesce (Hill 1975; Kaplan and Peters 1981). The *Azolla* association is unique in that the symbiont is also found in *Azolla* spores; these cyanobacterial cells are presumed to serve as a continuum of internal inoculum for the germinating sporophyte (Peters and Calvert 1983).

Azolla can be cured of symbiotic cyanobacteria and subsequently cultured in the presence of a source of combined nitrogen (Peters and Mayne 1974). Isolation and culture of the symbiont from several species of *Azolla* has been reported (Gates et al. 1980; Newton and Herman 1979; Tel-Or and Sandovsky 1982). However, it is not yet possible to reconstitute the association from the separately cultured partners; thus, conclusive evidence of the symbiotic origin of any cyanobacterial isolate has not been established. Immunological analysis of antigenic determinants of the cell envelope showed similarities in preparations of cyanobacteria immediately separated from several *Azolla* species, but distinct differences were evident between the freshly separated and cultured preparations (Gates et al. 1980; Ladha and Watanabe 1982). The differences were explained, in part, as a consequence of changes in the determinants in the transition between symbiotic and free-living growth.

Franche and Cohen-Bazire (1985) extracted DNA from the symbionts of several *Azolla* species and used Southern hybridization to compare the fragment sizes after endonuclease restriction; portions of the genes coding for the subunit polypeptides of nitrogenase (*nif*) from *Anabaena* sp. PCC 7120 were used as the probes. The results showed that the symbionts from four *Azolla* species had nearly identical DNA restriction patterns with respect to the *nif* genes, but a putative symbiont cultured from *Azolla filiculoides* (Tel-Or and Sandovsky 1982) had a different restriction pattern. Franche and Cohen-Bazire (1985) concluded that the organism in free-living culture was not the principle symbiont of *A. filiculoides*.

Similar comparisons of DNA restriction fragment length were made between the freshly isolated symbiont of *A. caroliniana* and the putative *Anabaena azollae* cultured by Newton and Herman (1979). In this case a number of probes

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Non-standard abbreviations: bp, base pairs; kb, kilobase pairs; kd, kilodaltons

were used: *nif*H (dinitrogenase reductase), *gln*A (glutamine synthetase), *rbc*S (small subunit of ribulose bisphosphate carboxylase/oxygenase) and *psb*A (32 kd quinone-binding protein of photosystem II). The resulting hybridization patterns with each probe were distinctive to the source of DNA, indicating that the major symbiont of *A. caroliniana* and the free-living isolate of Newton and Herman are unrelated (Nierzwicki-Bauer and Haselkorn 1986).

The DNA restriction patterns of the same cyanobacterium in free-living and symbiotic growth states should be compared to test whether, in general, large scale gene rearrangements occur during the transition between symbiotic and free-living growth. The symbiotic association between the bryophyte *Anthoceros punctatus* and isolates of *Nostoc* spp. from various cyanobacterial symbiotic associations can be reconstituted in culture (Enderlin and Meeks 1983). Therefore, we compare the restriction patterns in this study using DNA from *Nostoc* sp. strain 7801 in association with *Anthoceros* or in free-living culture. We have further used *Anthoceros* as a host to selectively isolate symbiotic cyanobacteria from crushed preparations of associated *Azolla caroliniana*. The DNA restriction patterns of these cultured isolates are compared to that of the symbiont freshly separated from *A. caroliniana*. Subclones of the genes *nif*H (dinitrogenase reductase), *nif*D (α-subunit of dinitrogenase), *nif*K (β-subunit of dinitrogenase) and *xis*A (excision activity resulting in genomic rearrangement between, and subsequent linking of, the *nif*D and *nif*K genes; Lammers et al. 1986), all from *Anabaena* sp. PCC 7120, were used as probes.

Materials and methods

Cultures. Nostoc sp. strain 7801, originally isolated from *Anthoceros punctatus*, was cultured phototrophically in the absence or presence of 2.5 mM NH_4^+ as previously described (Enderlin and Meeks 1983; Joseph and Meeks 1987).

Pure cultures of *A. punctatus* gametophyte tissue were maintained, and the symbiotic association reconstituted with *Nostoc* sp. strain 7801, as described (Enderlin and Meeks 1983). To obtain NH_4^+-treated samples of symbiotic *Nostoc*, the *Anthoceros-Nostoc* growth medium was supplemented with 2.5 mM NH_4^+ and maintained above 1 mM by transfer every 3–5 days (Enderlin and Meeks 1983); such cultures were used between 14 and 18 days after the initial treatment with NH_4^+.

Symbiotic *Nostoc* was isolated from N_2-grown or NH_4^+-treated *Anthoceros* associations by the basic procedure of Meeks et al. (1985a), as modified by Joseph and Meeks (1987). The symbiotic *Nostoc* preparations were suspended in 50 mM Tris, pH 7.5, plus 50 mM EDTA (50 TE), frozen as whole cells and stored at −80° C until use.

Azolla caroliniana containing symbiotic cyanobacteria was originally obtained from G.A. Peters, Battelle-C.F. Kettering Research Laboratory, Yellow Springs, OH, as epiphyte-free plants and has been maintained by subculture since 1980 as described (Meeks et al. 1985b). Symbiotic cyanobacteria were released from *Azolla* plants by the "gentle roller" method and enriched by filtration and differential centrifugation (Peters and Mayne 1974). If they were to be used for DNA extraction, the symbiotic cyanobacteria preparations were suspended in 50 TE and stored frozen at −80° C. In selective isolation of organisms competent to form an association with *Anthoceros*, the enriched cyanobacteria preparations were added to cultures of symbiont-free *Anthoceros* in medium free of combined nitrogen; these cultures were incubated under *Anthoceros* growth conditions for 8 weeks before establishment of a symbiotic association was visible. The colonies of symbiotic cyanobacteria were manually excised from *Anthoceros* gametophyte tissue, cultured and cloned on solidified media, under the same culture conditions used to grow *Nostoc*. The selective isolation experiments were done twice, approximately 3 months apart; two isolates, N1 and A1, were obtained in the first experiment and one, A2, in the second.

DNA extraction and purification. All solutions and basic procedures for in vitro DNA manipulation were according to Maniatis et al. (1982). The free-living and symbiotic cyanobacterial vegetative cells and heterocysts were lysed and the DNA purified by modification of the procedure of Golden et al. (1985). The thawed cells (ca. 0.5 g fresh wt.) in about 1.25 ml volumes were transferred to acid-washed and baked 12 ml screwcapped conical glass centrifuge tubes. Approximately 0.6 ml of acid washed, baked 0.45 μm diameter glass beads (Type V; Sigma Chemical Co., St. Louis, MO, USA), 0.25 ml of 20% (w/v) sodium lauryl sulfate and 1.25 ml of phenol:chloroform (1:1, v/v) were added to the cell paste. The suspension was agitated on a micromixer at full power for 10 × 1 min intervals, the tubes cooled in an ice-water bath between mixings. The broken cell suspension and glass beads were centrifuged at 1,400 × *g* for 10 min. The aqueous fraction was removed and extracted twice with an equal volume of phenol:chloroform and then once with an equal volume of chloroform; the phases were established by centrifugation at 1,400 × *g* for 10 min. The final aqueous phase was made 0.3 M in sodium acetate, pH 5.2, and 70% in ethanol; precipitated nucleic acids were harvested by centrifugation at 16,500 × *g* and 5° C for 10 min in a horizontal rotor. The nucleic acid pellet was vacuum dried and suspended in 6 ml of 10 mM Tris, pH 8.0, and 1 mM EDTA (TE), to which 3.0 g CsCl was then added. This suspension was layered over a 2.5 ml pad of 5.7 M CsCl and centrifuged in a SW-41 rotor for 12–16 h at 35,000 rpm and 25° C. (Note: we have been advised that centrifugation of suspensions of 5.7 M CsCl is not recommended at speeds greater than 30,000 rpm.) The DNA was collected from the CsCl interface and pad (ca. 4 ml total volume), leaving the RNA pellet. The CsCl solution was diluted 2-fold with sterile distilled water, the DNA precipitated with 0.3 M sodium acetate and ethanol and collected as above. The pellet of DNA was washed with 70% ethanol, dried, suspended in TE and stored at 4° C or reprecipitated several times before storage.

DNA isolated by the above procedure from symbiotically-grown *Nostoc* was resistant to complete restriction by several endonucleases. However, the resistance was eliminated by subsequent purification of the preparations by ion exchange chromatography using a commercial matrix (NACS PREPAC; Bethesda Research Laboratories, Gaithersburg, MD, USA). For uniformity, all DNA preparations were purified using the NACS system.

DNA restriction, electrophoresis and hybridization. The restriction endonucleases *Eco*RI, *Hinc*II and *Hind*III were used as recommended by the manufacturers (Bethesda Research Laboratories; Boehringer Mannheim, Indianapolis,

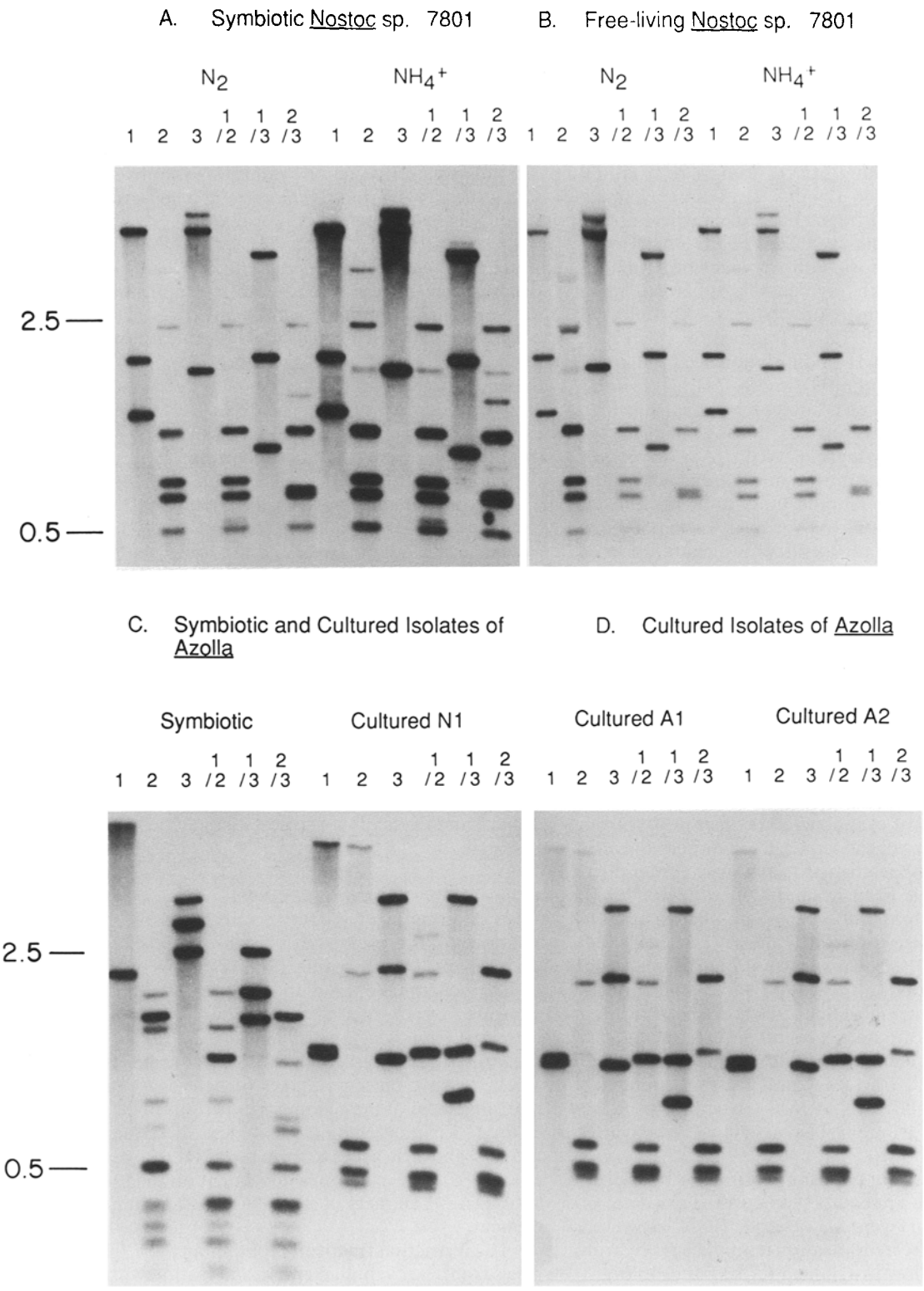


Fig. 1A–D. Autoradiogram of *Anabaena* sp. PCC 7120 pAn154.3 *nif*H ^{32}P-labeled probe hybridized to endonuclease digested DNA extracted from: **A** *Nostoc* sp. 7801 grown in symbiotic association with *Anthoceros* with N_2 or NH_4^+ as nitrogen sources; **B** *Nostoc* sp. 7801 grown in free-living culture with N_2 or NH_4^+ as nitrogen sources; **C** cyanobacterial symbiont freshly separated from N_2-grown *Azolla caroliniana* and N_2-grown free-living isolate N1 cultured from *Azolla* via *Anthoceros*; **D** N_2-grown free-living isolates A1 and A2 cultured from *Azolla* via *Anthoceros*. The DNA was digested singly with the endonucleases *Eco*RI (1), *Hinc*II (2), *Hind*III (3) or in combinations with *Eco*RI and *Hinc*II (1/2), *Eco*RI and *Hind*III (1/3), and *Hinc*II and *Hind*III (2/3). The numbers on the side refer to size, in kb, of fragments migrating the distance shown as determined by digestion of lambda phage DNA with *Bst*EII

IN, USA) to digest the DNA to completion. Restriction fragments were separated by electrophoresis in 0.7% (w/v) agarose and transferred to nylon membranes (Gene Screen Plus; DuPont NEN Products, Boston, MA, USA) by alkaline capillary blot. Hybridization was under stringent conditions as suggested by the manufacturer.

Hybridization probes were prepared by nick translation (Bethesda Research Laboratories) using (α^{32}P)dCTP (Amersham Corp., Arlington Heights, IL, USA). The probes were isolated as *Hind*III fragments (except where noted) from subclones of *Anabaena* sp. PCC 7120 DNA (Rice et al. 1982) and included *nif*K (0.7 kb fragment of pAn207.8 internal to *nif*K), *nif*D (2.9 kb fragment of pAn256 extending approximately 1.4 kb downstream of the 1.4 kb unlinked *nif*D coding sequence), *nif*H (1.8 kb fragment of pAn154.3, including 900 bp of the *nif*H coding sequence, 100 bp of *nif*D and 800 bp upstream of *nif*H), and X-7 (1.65 kb *Bal*31 derivative of pAn207.65 which is an *Eco*RI to *Kpn*I subclone of the 17 kb *Eco*RI generated pAn207; X-7 was made by W. Buikema and contains almost exclusively *xis*A; Lammers et al. 1986). Autoradiograms were obtained by exposing the hybridized and washed membrane blots to X-ray film at room temperature or −80° C for various periods of time. The membrane blots were regenerated by denaturation of the DNA hybrids as suggested by the manufacturer and, after background check by exposure to X-ray film, reused in subsequent hybridizations.

Results

Restriction pattern of the nifH gene in DNA from symbiotic and free-living Nostoc sp. 7801. The suggestion of large scale genomic rearrangements in the transition between symbiotic and free-living growth of cyanobacteria comes in part from the observed differences of the sequences in or near the *nif*H gene of DNA in cyanobacteria associated with, or cultured from, *Azolla* (Fig. 2 in Franche and Cohen-Bazire 1985). There are no apparent differences in the restriction fragments that hybridize to the *nif*H probe (pAn154.3) between *Nostoc* sp. 7801 grown in symbiotic or free-living states, with or without NH_4^+ (Fig. 1 A, B). We conclude that large scale rearrangements of the DNA in the transition between growth states are not a general occurrence, at least with respect to the sequences surrounding the *nif* structural genes, as well as *gln*A (the structural gene of glutamine synthetase, data not shown) and *rbc*S (structural gene for the small subunit of ribulose-bisphosphate carboxylase-oxygenase, data not shown).

The restriction fragment patterns obtained by hybridization with a *nif*H probe are complex due to the existence of extra copies of the *nif*H gene (Rice et al. 1982). However, comparisons of the *nif*H hybridization with the *nif*D hybridization (Fig. 2 A, B) allow assignment of the bands in Fig. 1 that identify the *nif*H gene linked to *nif*D. These are indicated in Table 1 by an asterisk.

Restriction patterns of DNA from cyanobacteria in symbiotic association with Azolla and isolates cultured from Azolla via Anthoceros. Comparisons of the pattern of *nif*H hybridization to restriction fragments of DNA isolated from the cyanobacteria in association with *Azolla* and from the three cultured isolates (Fig. 1 C, D) indicate that there are extensive differences between the isolates and the associated organism. However, the three cultured isolates have an identical restriction pattern, implying that they are the same organism although they were morphologically different when reisolated from *Anthoceros* tissue.

As shown below, the complete restriction map of the *nif* region also differs in the *Azolla*-associated cyanobacteria relative to the cultured isolates. Thus, it is apparent that we did not isolate the major symbiotic cyanobacterium from *Azolla*, even when using *Anthoceros* as a host system to select symbiotically-competent organisms.

Restriction patterns obtained with nifD and nifK probes. Comparisons of restriction fragment lengths observed with *nif*D and *nif*K probes are shown in Fig. 2. The DNA from *Nostoc* sp. 7801 yields nearly identical patterns, with each probe (Fig. 2 A, C), when the DNA is prepared from free-living cells grown on NH_4^+, or from cells in association with *Anthoceros* grown on N_2. The departures from identity are extra bands, present in the DNA from N_2-grown cells (marked r in the Figs.), due to rearrangement of the *nif*DK region in a fraction of the cells (see Discussion).

In panels B and D of Fig. 2, the *nif*D and *nif*K probes, respectively, are shown hybridized to DNA from the freshly isolated symbiont of *Azolla* and to DNA from the organism (A1 or N1) cultured from *Azolla* through association with *Anthoceros*. In the cases of both probes, the resulting patterns are entirely different. As described below, the patterns observed for the freshly isolated symbiont are consistent only with a contiguous *nif*DK organization, while the patterns observed for the free-living isolate are consistent with an interrupted *nif*DK organization. The distribution of restriction sites not affected by *nif* gene rearrangement differs considerably for the two preparations, as was the case for *nif*H. The actual fragment lengths are listed in Table 1 and the maps constructed from these data are shown in Fig. 4.

Organization of the nif genes in the symbiotic cyanobacteria. Restriction maps of the *nif* region, based on the Southern hybridizations presented in Figs. 1 – 3, are shown in Fig. 4. These maps depict organization of the *nif*HDK genes both as a polycistronic operon and with the *nif*D and *nif*K genes separated by an interval of unknown length. In the DNA extracted from vegetative cells of *Anabaena* sp. PCC 7120, the *nif*D gene is interrupted by an 11 kb segment (Rice et al. 1982). During the later stages of heterocyst maturation a rearrangement of the genome occurs resulting in the excision of the 11 kb intervening sequence and subsequent linking of the *nif*D and *nif*K genes to form the *nif*HDK operon (Golden et al. 1985). The excision is dependent on the product of the *xis*A gene (Lammers et al. 1986), found within the 11 kb element.

The restriction fragments containing the *nif*HD and *nif*K genes from the cyanobacteria in association with *Azolla* best fit an organization in which the genes are contiguous. The complete absence of any hybridization of the *xis*A probe to DNA from the symbiont in *Azolla* (Fig. 3 B) is also consistent with a contiguous *nif* gene organization. In *Anabaena* sp. PCC 7120, *xis*A is adjacent to *nif*K in the interval between *nif*K and *nif*D and is lost from the chromosome after rearrangement (Golden et al. 1985); such a proximity of *xis*A to *nif*K also appears in *Nostoc* sp. 7801 and the cultured isolate obtained from *A. caroliniana* via association with *Anthoceros* (Fig. 4).

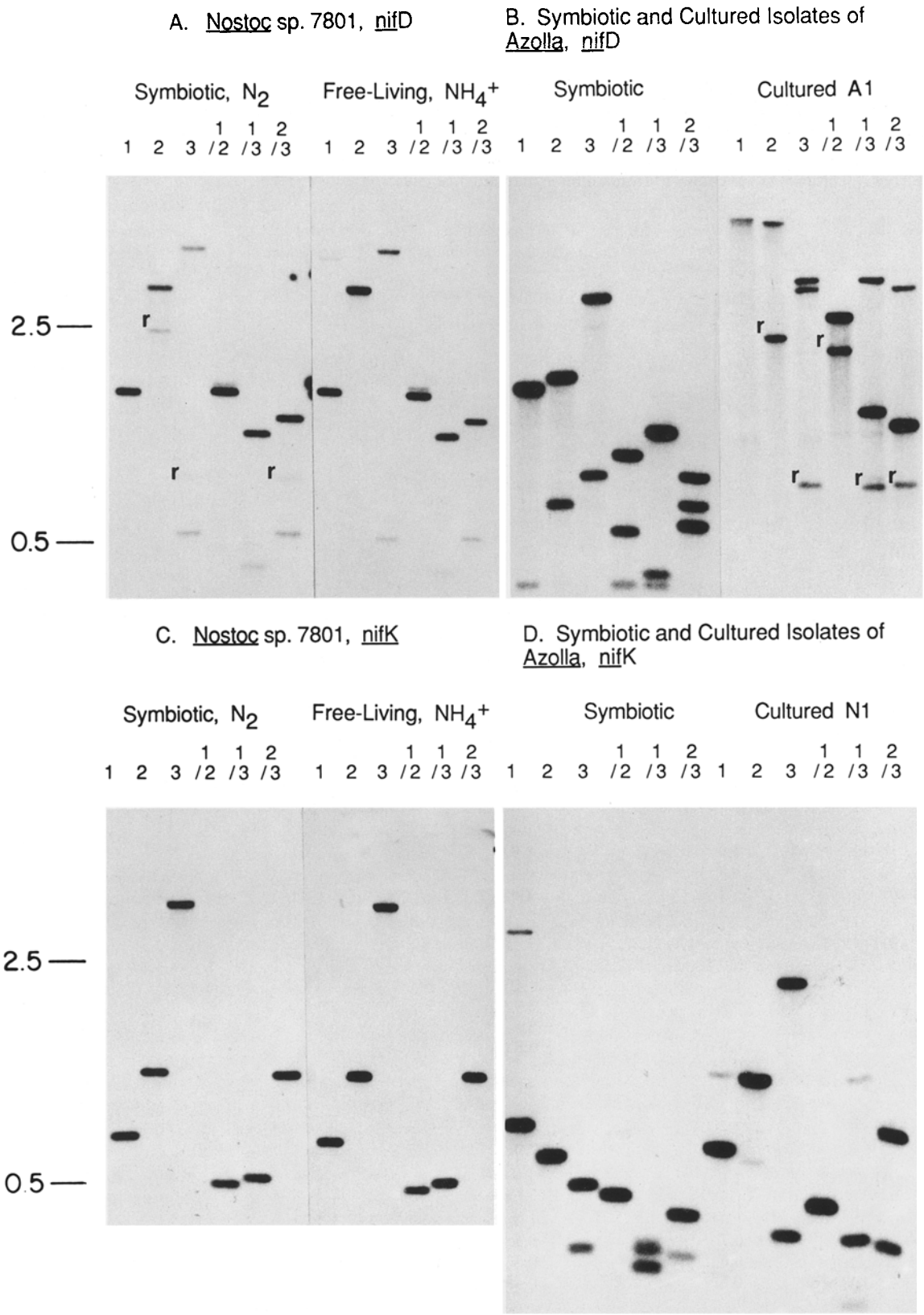


Fig. 2A–D. Autoradiogram of *Anabaena* sp. PCC 7120 pAn256 *nif*D and pAn207.8 *nif*K ^{32}P-labeled probes hybridized to endonuclease restricted DNA of the following samples: **A** *nif*D hybridization to *Nostoc* sp. 7801 grown with N_2 in association with *Anthoceros* or grown with NH_4^+ in free-living culture; **B** *nif*D hybridization to the cyanobacterial symbiont in *Azolla* or to N_2-grown free-living isolate A1 cultured from *Azolla* via *Anthoceros*; **C** *nif*K hybridization to *Nostoc* sp. 7801 grown in the two conditions as in A; **D** *nif*K hybridization to the cyanobacterial symbiont in *Azolla* and to the free-living isolate N1 cultured from *Azolla* via *Anthoceros*. The endonuclease digests and size markers are as in Fig. 1; r refers to bands that appear after rearrangement to form a contiguous *nif*HDK operon

Table 1. Sizes in kb of endonuclease restriction fragments of DNA from *Nostoc* sp. strain 7801, a free-living isolate from *Azolla caroliniana* cultured through association with *Anthoceros punctatus*, and the symbiont in *A. caroliniana* that hybridize to *nif*H, *nif*D, *nif*K and *xis*A probes from *Anabaena* sp. strain PCC 7120. The probes are described in Materials and methods and shown in Fig. 4D. Also included are fragment sizes of the respective genes in *Anabaena* sp. strain 7120, based on sequence analyses (Haselkorn 1986). *Nif*H fragments denoted by asterisks are linked to *nif*D; the other *nif*H fragments are from an extra copy or copies of the *nif*H gene. Fragments in bold type are present in both *Nostoc* sp. strain 7801 and the isolate cultured from *A. caroliniana* through association with *Anthoceros*. Fragments marked with (r) are present in less than molar amounts with respect to other fragments; these are presumably from rearranged DNA in heterocysts. The results for *Nostoc* sp. strain 7801 are compiled from free-living or symbiotic growth, with or without combined nitrogen; the results for the cultured isolate from *A. caroliniana* are compiled from all three individual isolates

Restriction endonuclease	Probe	*Nostoc* sp. 7801	Cultured isolate from *A. caroliniana*	Symbiont in *A. caroliniana*	*Anabaena* sp. 7120
*Eco*RI	*nif*H	5.2	>10.0*	>20.0	19
		1.7*	1.2	2.1	10.5*
		1.1	**1.1**		6 (r)
	*nif*D	4.7	>10.0	1.55	17
					10.5
		1.5		0.35	6 (r)
	*xis*A		>10.0	none	17
		1.3	**1.35**		
		0.55			
		0.5			
	*nif*K	**1.1**	**1.1**	3.2	17
		0.7	**0.65**	0.75	6 (r)
*Hinc*II	*nif*H	3.5	>10.0	1.75	5 fragments
		2.1	2.05*	1.6	<400 bp*
		1.5	1.25	1.4	
		1.0*		0.8	
		0.7	**0.6**	0.7*	
		0.6	**0.5**	0.5*	
		0.5	**0.45**	0.4	
				0.35	
				0.3	
				0.25*	
	*nif*D	3.5	>10.0	1.6	2.4
		2.4 (r)	**2.3** (r)	0.7	2.2 (r)
					2.0
	*xis*A	3.2 (r)		none	5.3
		1.9	**1.9**		
		0.7	**0.6**		
	*nif*K	**1.1**	**1.05**	0.65	0.7
					0.5
*Hind*III	*nif*H	6	3.95*	3.9	2.7
		5.0*	2.1	3.1*	1.8*
		1.5	1.15	2.4	
	*nif*D	5.3	3.9	3.1	2.9
			3.5	0.9	1.8 (r)
		0.8 (r)	**0.8** (r)		
		0.55			
	*xis*A	2.6	5.0	none	1.05
		2.2 (r)			0.95
	*nif*K	3.7	2.2	0.55	0.7
			0.4	0.35	
*Eco*RI/*Hinc*II	*nif*H	2.1	2.7	1.8	5 fragments
		1.5	2.05*	1.4	<400 bp*
		1.0*	1.2	1.15	
		0.7	**0.6**	0.8	
		0.6	**0.5**	0.5*	
		0.5	**0.45**	0.4	
				0.35	
				0.3	
				0.25*	
	*nif*D	1.6	2.8	1.0	2.0
		1.5	2.2 (r)	0.6	1.5
				0.35	0.9
					0.7 (r)

Table 1 (continued)

Restriction endonuclease	Probe	*Nostoc* sp. 7801	Cultured isolate from *A. caroliniana*	Symbiont in *A. caroliniana*	*Anabaena* sp. 7120
	*xis*A	2.5 (r)		none	5.3
		1.3	**1.35**		
		0.55	**0.6**		
		0.40	**0.4**		
	*nif*K	**0.50**	**0.5**	0.5	0.7
					0.5
*Eco*RI/*Hind*III	*nif*H	4.5	3.9*	2.1	1.8
		3.7		1.8*	1.8*
		1.7*	1.2	1.5	
		0.9	**0.9**	1.2	
	*nif*D	1.15	3.9	1.15	1.8
		0.45	1.4	0.45	1.1
			0.8 (r)	0.35	0.7 (r)
	*xis*A	**1.3**	3.5	none	
		1.0 (r)	**1.35**		1.05
		0.55			0.95
		0.50			
	*nif*K	**1.1**	**1.1**	0.35	0.7
		0.65	0.4	0.3	
*Hinc*II/*Hind*III	*nif*H	2.1	2.05*	1.6	7 fragments
		1.5	1.3	1.1	<400 bp*
		1.0*		0.75	
				0.7*	
		0.7	**0.6**	0.5*	
		0.6	**0.5**	0.4	
		0.5	**0.45**	0.35	
				0.3	
				0.25*	
	*nif*D		3.5		
		1.3	**1.3**	0.85	2.0
		0.8 (r)	**0.8** (r)	0.7	1.8 (r)
		0.55		0.6	0.55
	*xis*A	**1.8**	**1.7**	none	1.05
		1.2 (r)			0.95
		0.7	**0.6**		
	*nif*K	1.1	0.8	0.45	0.50
			0.4	0.3	0.13

Discussion

The restriction pattern of *nif*H (pAn154.3) hybridization for the cyanobacterial isolate cultured from *Azolla caroliniana* via *Anthoceros* in this study (Fig. 1) differs from the pattern found for a putative symbiotic isolate from *A. filiculoides* (Franche and Cohen-Bazire 1985) (isolate of Tel-Or and Sandovsky 1982) and an isolate (Newton and Herman 1979) from *A. caroliniana* (Nierzwicki-Bauer and Haselkorn 1986); the *nif*H pattern in the latter two isolates further differ from one another. We conclude from these data that *Azolla* species harbor minor symbionts in addition to the dominant symbiotic strain. It would appear that these minor symbionts vary, perhaps depending on the geographical area where the *Azolla* was obtained. Although the isolate of Tel-Or and Sandovsky (1982) has not been tested for symbiotic competence in the *Anthoceros* association, the Newton and Herman isolate from *A. caroliniana* is non-symbiotic with *Anthoceros* (Enderlin and Meeks 1983). By virtue of the selection process, the cyanobacterial isolate obtained from *A. caroliniana* in this study has broad symbiotic competence.

The cultured isolates have biologically interesting properties, especially with respect to control of their growth by *Azolla*. The *Azolla caroliniana* association has been maintained by subculture in this laboratory for more than 6 years, after the culture had been established as epiphyte-free in 1973 by Peters and Mayne (1974); at no time, even during delayed subculturing, have we seen evidence of epiphytic outgrowth of any phototrophic organism as reported by others (Habte 1986). Nevertheless, in two instances, separated by 2 subcultures, what appears to be the same organism was isolated by association with *Anthoceros*. The cultured organism apparently occurred in *Azolla* in symbiotic densities too low to be detected by the cellular separation, DNA isolation and hybridization procedures.

Franche and Cohen-Bazire (1987) recently reported lack of hybridization between DNA of cyanobacteria immediately separated from various *Azolla* species and a probe (pAn207.3) that is localized near the *nif*D gene in the 11 kb excised region of *Anabaena* sp. PCC 7120. We observed a similar lack of hybridization under stringent conditions between pAn207.3 and DNA from the symbiont of *A. caroliniana* (data not shown). The probe pAn207.3 does hybridize with DNA from all of the free-living cyanobacteria used in this study, except for an organism apparently lacking *xis*A (data not shown); this hybridization is in agreement

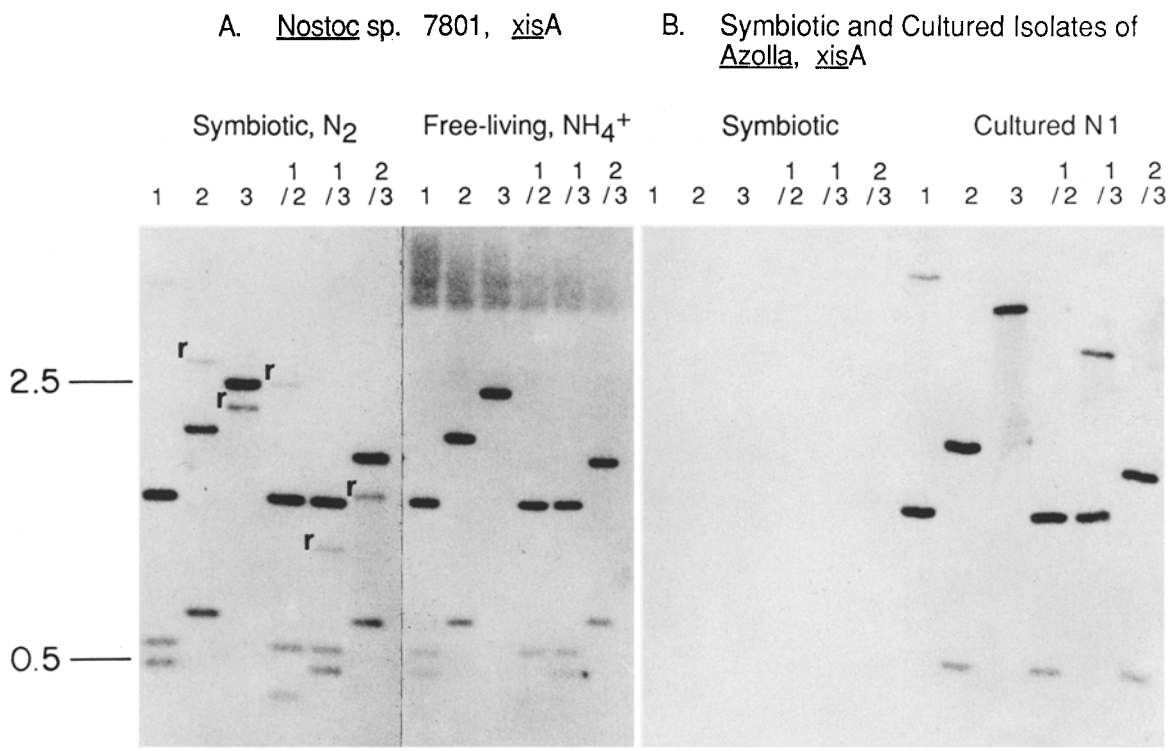


Fig. 3A, B. Autoradiogram of *Anabaena* sp. PCC 7120 X-7 *xis*A ^{32}P-labeled probe hybridized to endonuclease digested DNA from: **A** *Nostoc* sp. 7801 grown in N_2-fixing association with *Anthoceros* or grown in free-living culture with NH_4^+ as the nitrogen source; **B** the cyanobacterial symbiont freshly isolated from *Azolla* and isolate N1 cultured from *Azolla* via *Anthoceros*. Endonuclease digestion, size markers and symbols are as in Figs. 1 and 2. Note in B the complete lack of hybridization of *xis*A to DNA from the major symbiont in *Azolla*

with similar observations of Franche and Cohen-Bazire (1987), who examined seven recently isolated cyanobacterial strains from Senegal.

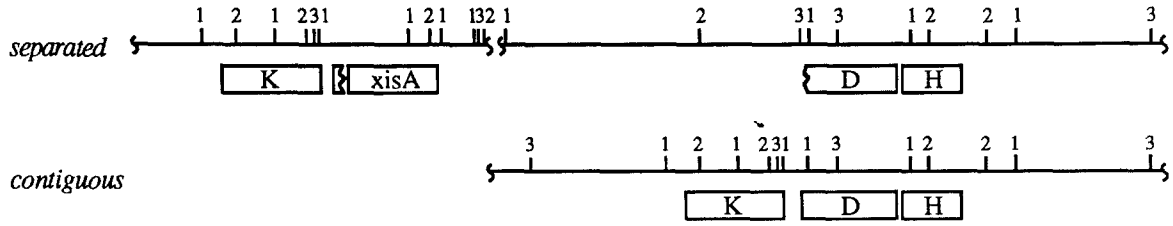
Assuming that *xis*A is always located in the intervening sequence in vegegative cells of heterocyst-forming cyanobacteria, its absence as well as that of pAn207.3 from the associated cyanobacteria implies that the entire intervening sequence is also lacking in all cells. The absence of sequences hybridizing to the *xis*A gene and to pAn207.3 serves as a characteristic marker of the symbiont in *Azolla* in addition to the DNA restriction pattern. With the advantage of *Hinc*II fragments to orient the *Eco*RI and *Hind*III restriction sites and a probe extending beyond the end of the *nif*D gene [2.9 kb pAn256 compared to the 1.1 kb pAn256.Δ1 used by Franche and Cohen-Bazire (1985)], the restriction map of the *nif* gene region in the symbiont of *Azolla* given in Fig. 4 differs from the map presented by Franche and Cohen-Bazire (1985). However, the mirror image of their map of the *nif*D region can be superimposed on the one in Fig. 4.

The thrust of the studies of Franche and Cohen-Bazire (1985, 1987) was a comparison of the *nif* gene organization in the symbionts freshly separated from a number of *Euazollae* and *Rhizosperma* species. In general, they found that the symbionts in five *Rhizosperma* are closely related, as are those in four *Euazollae*, but that the symbionts in the two subgenera are more distantly related. Since there are differences in strain and culture history, it is worth noting that the restriction fragment patterns we observed for the symbiont from *A. caroliniana* (summarized in Table 1) are consistent with the results of Franche and Cohen-Bazire (1985, 1987) for the same species. In some cases direct comparisons cannot be made because we used fewer enzymes and did more double digests; in other cases fragments less than 1.1 kb in size, which are important for mapping, were not measured by Franche and Cohen-Bazire (1985, 1987).

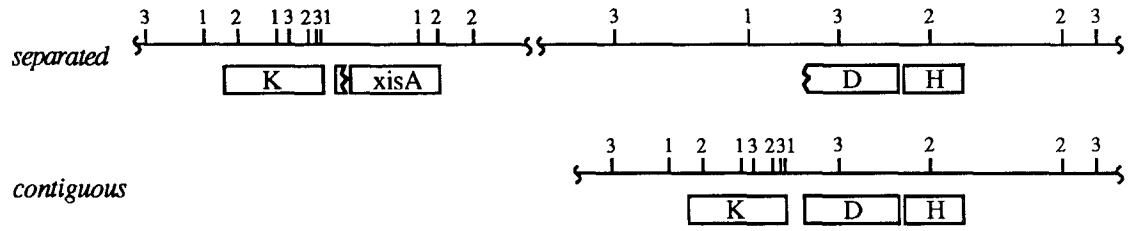
Since the dominant symbiotic cyanobacterium in *Azolla* apparently has not been cultured (at least in the three instances cited above), it could be argued that the organism is an obligate symbiont, perhaps as a consequence of not having the DNA sequences between the *nif*D and *nif*K genes in vegetative cells. However, we have also observed an absence of *xis*A (and no hybridization to pAn207.3) and contiguous *nif*HDK organization in all cells of a free-living, N_2-fixing and heterocyst-forming cyanobacterium (Meeks and Haselkorn, unpublished observations). Thus, the possibility of obligate symbiosis with respect to the dominant cyanobacterium in *Azolla* must have another basis.

Although we did not specifically isolate heterocysts, hybridization bands representing contiguous or separated *nif*D and *nif*K organization can be deduced by comparing preparations from cultures lacking heterocysts (NH_4^+-grown free-living *Nostoc* sp. 7801) to those with high heterocyst frequency (N_2-grown symbiotic *Nostoc* sp. 7801) as well as from the symbiont in *Azolla*. For example, in all three strains shown in Fig. 2, a 0.8–0.9 kb *Hind*III fragment, in single or *Hinc*II double digestions, hybridizes to the *nif*D pAn256 probe when the genes are contiguous (noted as r in Fig. 2). The intensity of hybridization to this band depends on the extent of rearrangement (or heterocyst breakage), and it is absent from NH_4^+-grown free-living *Nostoc* sp. 7801

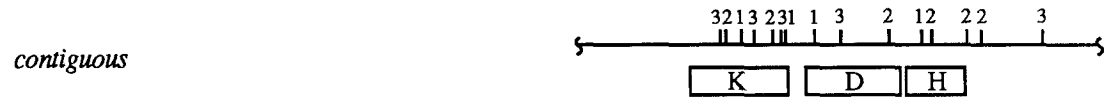
A. Nostoc sp. 7801



B. Cultured isolate from Azolla



C. Symbiont in Azolla



D. Anabaena sp. 7120

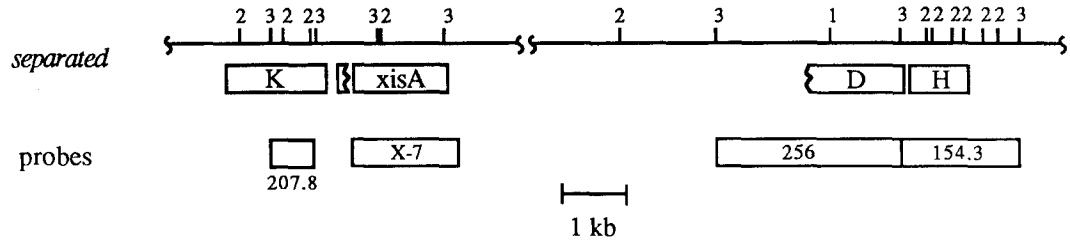


Fig. 4A–D. Physical map of the *nif* gene regions of free-living and symbiotic cyanobacteria. The maps shown are based on the data of Figs. 1–3 as summarized in Table 1. The probes are described in detail in Materials and methods. The physical map of *Anabaena* sp. 7120 is based on the complete nucleotide sequences of the regions shown. The arrangement labeled "separated" is presumably found in vegetative cells of free-living cultures and *Nostoc* in association with *Anthoceros*; the "contiguous" arrangement is presumably found in all heterocysts and in all cells of the freshly isolated symbiont of *Azolla*. The small segment of a gene to the left of *xis*A in A, B and D corresponds to the C-terminal 44 amino acids of the *nif*D gene. Since the product of the *xis*A gene is a site-specific recombinase whose target is located within the *nif*D gene of *Anabaena* sp. 7120, the existence of a homologous *xis*A region in *Nostoc* sp. 7801 and in the cultured isolate from *Azolla* indicates that the *nif*D genes in these organisms are interrupted in a similar way. For that reason, the C-terminal *nif*D gene fragment is shown in A and B as well. The actual length of the interrupting element is known only for *Anabaena* sp. 7120, in which the element is 11 kb. Symbols on the maps: 1 = *Eco*RI, 2 = *Hinc*II, 3 = *Hind*III

(Fig. 2A). However, when *nif*D and *nif*K are separated, the probe hybridizes to a *Hind*III fragment that varies from 0.55 kb in *Nostoc* sp. 7801 to 3.5 kb in the cultured isolate from *Azolla*. Moreover, in digests of symbiotically associated N_2-grown *Nostoc* sp. 7801 an additional 2.4 kb *Hinc*II fragment (noted as r) is present that is absent in NH_4^+-grown free-living cultures.

A similar variation in hybridization bands can be seen in comparisons between symbiotic and free-living *Nostoc* sp. 7801 when *xis*A is the probe (Fig. 3). In NH_4^+-grown cells, where *nif*K and *nif*D are separated, *xis*A can be linked to *nif*K by overlapping *Hinc*II (1.9 kb) and *Hind*III (2.4 kb) fragments. However, in symbiotic *Nostoc* sp. 7801 additional 3.2 kb *Hinc*II and 2.2 kb *Hind*III fragments (noted as r in Fig. 3) are present that represent the *xis*A gene separated from *nif*K. Whether, after the *nif*DK rearrangement in *Nostoc* sp. 7801, the *xis*A gene and accompanying interval assumes a circular form as in *Anabaena* sp. PCC 7120 (Golden et al. 1985) is not known. Comparison of the maps in Fig. 4 shows that the restriction pattern of the symbiont in *Azolla* (Fig. 4C) cannot be obtained by excision of DNA from the free-living isolate (Fig. 4B). Even in the contiguous *nif*HDK organization, the free-living isolate contains only seven of the fifteen restriction sites mapped in the symbiont, while having five sites not found in the symbiont. Thus, there can be no doubt that these are different organisms.

Based on the limited mapping we were able to do in the *nif*H region that is contiguous with *nif*D, and the numerous *Hinc*II restriction fragments hybridizing to the pAn154.3 probe, it appears that multiple copies of *nif*H are present in all of the strains examined (Fig. 1). Multiple copies of *nif*H are found in a variety of organisms, including *Anabaena* sp. PCC 7120 (Haselkorn 1986).

According to the maps presented in Fig. 4, there are several conserved restriction sites within the *nif*D and *nif*K genes that were also reported by Kallas et al. (1985) as present in *Nostoc* sp. PCC 7121 and *Nostoc* sp. PCC 7906. These include a *Hind*III site approximately 500 bp from the C-terminus of the *nif*D gene and *Eco*RI and *Hind*III sites within the first 100 bp of the *nif*K gene. An *Eco*RI site in the *nif*D gene, upstream from the presumed region of genomic rearrangement, is missing only in the cultured isolate from *Azolla*. Additional conserved regions in the organisms examined here include a *Hinc*II site in the *nif*K gene clustered with the initial *Eco*RI and *Hind*III sites, and another in *nif*H about 300–400 bp upstream from its C-terminus. Apart from these conserved regions, there is considerable variation in restriction sites, both within and outside of the coding sequences.

The conserved *Hind*III and *Eco*RI sites in the *nif*K and *nif*D genes of the organisms presented here (Fig. 4) and by Kallas et al. (1985) are not apparent in restriction maps of *Anabaena* sp. PCC 7120 (Rice et al. 1982; Golden et al. 1985; cf. Fig. 4D) or *Anabaena variabilis* (ATCC 29413; Herrero and Wolk 1986). The sites are present in three additional *Nostoc* strains: MAC, ATCC 29133 and ATCC 27896 (Meeks and Haselkorn, unpublished observations). Kallas et al. (1985) suggested that restriction sites in the *nif* region could be used in taxonomic applications. We concur and suggest that the conserved *Eco*RI, *Hind*III and *Hinc*II sites in the *nif* region may be used to distinguish the genus *Nostoc* from *Anabaena*. If so, this would imply that the dominant symbiotic organism in association with *Azolla* is a species of *Nostoc* rather than *Anabaena*. Moore (1969) discussed the etymology of the symbiont taxonomy in *Azolla* and suggested that the authority, Strasburger, in fact delineated it as a *Nostoc*.

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